Assessing differential expression of genes associated with nickel resistance in jack pine (*Pinus banksiana*) and eastern white pine (*Pinus strobus*): effects of nickel toxicity

By

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Abstract

Eastern white pine (*Pinus strobus*) and jack pine (*P. banksiana*) represent two of the major coniferous species planted in the metal contaminated region of Northern Ontario. Studies on coping mechanisms of these two species to soil metal contamination are limited. The main objectives of the present project were to 1) determine how these two species respond to soil nickel (Ni) contamination and 2) assess the effects of different doses of nickel on the expression of six genes associated with Ni resistance. These genes include high affinity Ni transporter family (AT2G16800), 1-aminocyclopropane-1-carboxylic acid deaminase (ACC deaminase), Natural resistance-associated macrophage proteins (NRAMP3), and serine acetyltransferase (SAT), Nicotianamine synthase (NAS) and Glutathione reductase (GR). Seedlings were grown in soils treated with increasing concentrations of $Ni(NO_3)_2$. Potassium nitrate (KNO_3) was used to control for potential toxicity of nitrate ions. Gene expression was assessed using RT-qPCR. Damage ratings reveal that *P. banksiana* is sensitive to $Ni(NO_3)_2$ doses of 1,600 and 3,200 mg/kg as well as 3,200 mg/kg KNO_3. However, *P. strobus* was tolerant to all treatment. Soil Ni treatments induced a downregulation of NAS in *P. strobus* as well as AT2G16800 and NRAMP3 in both species with the severity increasing at high doses of Ni. An upregulation of ACC in both species consistent with an inverted-U hormetic effect was observed. Ni also triggered a hormetic U-effect downregulation of SAT in *P. strobus* with the lowest dose showing the highest repression. An opposite trend was observed in *P. banksiana* where Ni induced an upregulation of SAT. Moreover, GR induced an inverted-U hormetic upregulation in *P. banksiana*. Knowing the coping mechanisms of these conifers to metal contamination is essential for ensuring their long-term sustainability.

Key Words: Jack pine (*Pinus banksiana*); Eastern white pine (*Pinus strobus*); Nickel toxicity; Gene expression; RT-qPCR.

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Chapter 1: Literature review

1.1 History of mining in Sudbury

Burning coal, smelting and other anthropogenic sources lead to the accumulation of nickel (Ni), copper (Cu), zinc (Zn), cadmium (Cd), lead (Pb), chromium (Cr) and mercury (Hg). These will then seep into the surrounding air, water and soil ultimately posing as environmental toxins (Winterhalder, 1996). The city of Greater Sudbury (CGS) in Northern Ontario is known for Ni, Cu, Co and iron (Fe) deposits (Hutchinson & Symington, 1997). Over the past 100 years, its atmosphere has experienced the release of more than tens of thousands of tonnes of Cu, Ni, and Fe from its roast beds and smelters (Gunn et al., 1995). Consequently, this led to Sudbury becoming a highly ecologically disturbed area with soil erosion, and frequent fire incidents causing the barren landscape observed in the region (Amiro & Courtin, 2011). Sulfur dioxide (SO_2) has been released due to the oxidation of metal ores (Amiro & Courtin, 1981). Smelters were introduced as a more environmentally friendly alternative to open roast yards yet they also negatively impacted the vegetation. In fact, sulfur dioxide fumigation and heavy metal particulates have led to deforestation and decreased vascular plant diversity (Hutchinson & Symington, 1997). While the SO_2 emissions have seen an immense decrease, Ni and Cu emissions still remain high (Gunn et al., 1995). The mycobiont and photobiont in the roast beds have revealed through X-ray spectrometry, the potential for toxic levels of Cu, Ni, Fe and Al (Bačkor & Fahselt, 2004).

These activities as well as the metal-induced stress on nearby vegetation have led to decreased plant growth (Narendrula & Nkongolo, 2015). An increase in the level of bioavailable metals is reported to be in association with decreased nutrient availability (Winterhalder, 1996). Fortunately, many successful initiatives have been taken to improve the terrestrial landscape of

the CGS; reforestation attempts such as 35 years of dolomitic applications have been found to have improved forest diversity, abundance as well as the overall health of the terrestrial ecosystem (Nkongolo et al., 2016).

1.2 Resistance strategies and translocation

The increase in heavy metal concentration in the rhizosphere, results in a high-level accumulation of metals in not only plant tissue, but also in the different organisms growing in these contaminated soils. Consequently, this disturbs the function of ecosystems, limiting the number of species that can use these conditions as a niche and thrive on stressed regions around the Greater Sudbury area (McIlveen & Negusanti, 1994). That is why only certain plants are able to withstand higher doses of heavy metals that would otherwise be lethal to other species (Leitenmaier & Küpper, 2013). These plants can be used in phytoremediation, as they clean up their surrounding environment of these toxic metals by developing strategies to accumulate them (Tiwari, 2021). Root uptake can be via passive diffusion through the cell membrane along with water (Maleki et al., 2017) or by active uptake evolved for acquiring trace metals wherein simultaneously other elements are acquired as well (Silver, 1983). Following the uptake, the metals will move along the concentration gradient through cation channels in cells (Kochian, 1995). Foliar uptake (through stomata and cuticle) is another route of entry that could serve as a reflection of aerosol toxicity in the surroundings (Maleki et al., 2017).

1.2.1 Avoidance strategy

Avoidance refers to strategies employed as a first line of protection to limit the entrance of metals through roots. These include extracellular strategies such as immobilization of metals

via establishing mycorrhizal relationship between roots and fungi, and utilizing root exudates (Dalvi & Bhalerao, 2013). Among plants growing in heavy metal contaminate regions, many use their relationships with ectomycorrhiza and arbuscular mycorrhiza to deal with metal contamination (Leyval et al., 1997). Mycorrhiza keep toxic metals at bay via chelation, absorption and adsorption of the metal ions (Hall, 2002). As for root exudates- a collective term used to refer to water, sugars, organic acids, inorganic ions, mucilage, siderophores, protons and bicarbonate etc… (Marschner, 1995)- they are capable of forming stable complex ligands with the metal ions and rendering them inactive. Some exudates increase pH which in turn precipitates the metal ions and reduces their bioavailability (Dalvi & Bhalerao, 2013).

1.2.2 Tolerance strategies

After avoidance strategies, which deal with extracellular management of heavy metals, as a second line of defence, tolerance comes into play as it consists of the intercellular management of heavy metals. During this process, heavy metals are stored or immobilized via binding to amino acids and peptides (Pál et al., 2006). A few strategies aim to prevent the crossing over of metals past the plasma membrane namely the binding of metals to cell wall pectin or active efflux pumping at the plasma membrane levels via family proteins such as the ABC transporters (Manara, 2012). Once within cytosol, organic acids which are metabolic intermediates bind complexes with metals and keep them inactive (Dalvi & Bhalerao, 2013). Moreover, phytochelatins and metallothioneins also form complexes with metals wherein these complexes are shuttled to the vacuole for storage where they can't exhibit their toxicity (Tong et al., 2004). Other tolerance strategies include the production of stress-related proteins- such as heat shock proteins that function as molecular chaperones and aid in proper protein folding (Dalvi &

Bhalerao, 2013). Moreover, reactive oxygen species (ROS) which are synthesized as a response to metal stress, can induce the antioxidant defense pathway when present in moderate levels (Chen et al., 2009; Freeman et al., 2004). While ROS are toxic in high amounts, moderate amounts help adaptation to stress wherein the defence pathway contributes to the scavenging of these species which in turn mitigates oxidative damage (Rastgoo & Alemzadeh, 2011). Dalvi and Bhalerao (2013) comment on the role of plant hormones such as salicylic, gibberellic and jasmonic acid as well as ethylene among many other plant hormones involved in the mitigation of metal toxicity.

Based on their potential to accumulate and tolerate, plants can be classified as indicator, excluder and hyperaccumulators (Tiwari, 2021). Others are considered hyperaccumulators (Dalvi & Bhalerao, 2013). The indicators, as the name suggests, they indicate the bioavailable amount of metal in soils as they are very sensitive and a direct reflection of their surrounding toxicity (Tiwari, 2021). Most metal tolerant plants however, belong to the excluder category as their strategy is to avoid metal build-up in their cells (Tiwari, 2021). They do so by blocking metal uptake at their roots (Lux et al., 2011) or by actively outflux pumping (Van Hoof et al., 2001). Since the leaves of these plants are more sensitive to metal toxicity, these plants hold on to and detoxify metals in the roots with minimal translocation to shoots (Hall, 2002). Whereas hyperaccumulators are able to translocate to their shoots and not exhibit any symptoms of phytotoxicity despite taking up a large amount of metal from their surrounding soil (R. D. Reeves, 2006).

1.2.3 Hyperaccumulators

Hyperaccumulator species are able to translocate metal to their shoots 100-1000 times the amount seen in non-hyperaccumulator species. Some thresholds suggested for the classification of hyperaccumulator species are; 10000 mg/kg for Zn, 1000 mg/kg for Ni and Cu and 100 mg/kg for non-essential elements like Cd and Pb (Boyd, 2004; Reeves & Baker, 2000). This process starts with root uptake (limited sequestration in root vacuoles), xylem loading, xylem unloading, using chelator molecules for the detoxification of metals, sequestration of metals in vacuoles and other adaptive processes which may include stress protection and homeostasis with other nutrients (Verbruggen et al., 2009). Some transporters involved in enhanced root uptake are from the ZIP family (Zn regulated transporter). Some families involved in translocation from root to shoot are: HMA (heavy metal transporting ATPases), MATE (multidrug/toxic compound efflux membrane protein), and OPT (Oligopeptide transporters including YSL-yellow strip-like). While some families involved in short vacuole sequestration are: CDF (cation diffusion facilitators), HMA, CaCA (Ca2+/cation antiporter), ABC (ATP binding cassette). Other molecules involved in chelation are histidine, nicotianamine, glutathione, phytochelatins, metallothioneins and other organic acids; and lastly some families like NRAMP
(Natural Resistance and Macrophage Protein) and IREG (Iron regulated transporters) (Verbruggen et al., 2009). There is a convergent set of genes coding for ZIP,CDF, HMA and NRAMP and NAS and FRD3 (Verbruggen et al., 2009).

The majority of hyperaccumulators (about 75%) accumulate Ni (Verbruggen et al., 2009) meanwhile Cd is the least preferred metal for hyperaccumulation (Rascio & Navari-Izzo, 2011). Judging how a wide variety of many distantly related families have evolved Ni hyperaccumulation, it can be concluded that this trait was independently evolved, suggesting

polyphyletic origin (Macnair, 2003). While some researchers are looking to study the mechanism involved in metal acquisition and sequestration by these species (Salt, 2001), others are looking into their use as biofortification for crops (Broadley et al., 2007), or in phytoremediation to clean up contaminated sites (McGrath & Zhao, 2003). Anderson et al. (1999) are interested in phytomining, how best to use these species for mining metals (Boyd, 2004). Plants hyperaccumulate for different documented rationales including protection against herbivores that feed on them, drought tolerance, littering the canopy and the soil under them with metal to prevent the growth of less metal tolerant species, accumulating and sequestering in tissue to be later disposed of (Boyd, 2004). The gene regulation of these families of transporters in response to metal toxicity is complex due to the fact that these can be tissue-specific. There are differences in response to metals among species and also populations. Thus further molecular studies of hyperaccumulation need to look at single and global gene levels with respect to tissue (Verbruggen et al., 2009).

1.3 Heavy metals as micronutrient and agents of toxicity

Many heavy metals and metalloids are considered micronutrients, that is a small quantity of them is deemed essential for normal growth. Copper (Cu), nickel (Ni), zinc (Zn), manganese (Mn), and molybdenum (Mo) are the essential heavy metals for higher plants and although iron (Fe) is not often considered a heavy metal, it is another important micronutrient (Alloway, 2013). Other elements such as cadmium (Cd), lead (Pb), arsenic (As) and tin (Sn) may perhaps play important roles at much lower concentrations (Alloway, 2013).

1.3.1 Copper

Copper as an important micronutrient, is at the optimum range when present at 5-30 mg/kg in plant tissue (Wuana & Okieimen, 2011). It plays an essential role as a cofactor for oxidases and oxygenases and other enzymes involved in the regulation of the ROS (Demirevska-Kepova et al., 2004). It is also important for the incorporation of CO2 and ATP synthesis (Thomas et al., 1998). Overall, Cu is integral to many enzymes and transcription factors that participate in maintaining cell integrity, proliferation and signalling processes, oxidation and reduction as well as defense pathways (Kardos et al., 2018). Some signs of Cu deficiency include wilting, white twisted tips and melanism (Alloway, 2013).

Copper in high doses can induce interference in the metabolic pathways, for example, high levels of Cu toxicity have been reported to be harmful to the roots of Rhodes grass, wherein causing root cuticle damage, lowered root hair proliferation as well as significant root structure deformation is observed (Sheldon & Menzies, 2005). Moreover, Cu in toxic doses is found to act as a chlorosis factor such as in stevia leaves and *Thlaspi ochroleucum* where damaged plasma membrane permeability has been observed (Baroni-nezhad et al., 2021). Copper tends to accumulate in roots with minimal translocation to shoots (Anjum et al., 2015). It is important to note the Cu toxicity is more concerning for roots than shoots and it is the rhizotoxicity that is often a good indicator of the level of Cu toxicity above ground. This includes halted growth, abnormal branching, increased thickness and dark coloration in roots (Anjum et al., 2015).

1.3.2 Zinc

Zn is another important metal, and is often reported to be the most common micronutrient deficiently in plants (Broadley et al., 2007). It is important in carbohydrates and protein

synthesis, the integrity of biomembranes, gene regulation, and protection from free radical damage (Alloway, 2013). Zn deficiency is reported to negatively affect the detoxification of O_2- and H_2O_2 in the leaves of bean plants (*Phaseolus vulgaris*) wherein the activity of superoxide dismutase and glutathione reductase were significantly decreased (Cakmak & Marschner, 1993). Although the effects of Zn deficiency are more pronounced on C4 plants (due to their reliance on carbonic anhydrase which utilizes Zn), net photosynthesis is reduced in all plants (Alloway, 2013). Some classic physical signs of deficiency include stunted growth, tree rosetting and violet-pink spots on leaves (Alloway, 2013).

Resistance to Zn can be passed down generations as a heritable trait wherein this ecotypic tolerance is reported via several strategies: Zn exclusion from roots and foliar accumulation (Chaney, 1993). It is suggested that compartmentalizing the metal in the vacuole and strong chelation (by cytoplasmic agents such as malate, citrate and glutathione) are the main mechanisms involved in Zn resistance (Chaney, 1993). Interestingly Zn has been reported in several studies to reverse the toxic effects of other metals. In aquatic duckweed *Spirodela polyrhiza L.*, oxidative stress is marked by higher levels of lipid peroxidation, greater total peroxide, superoxide anion and lipoxygenase activity. Zn interaction with Cu reduced the oxidative damages by stimulating higher activities of antioxidant enzymes (catalase, ascorbate peroxidase and peroxidase) as opposed to treatments with just Cu or Zn (Upadhyay & Panda, 2010). In another study on rice, Cd's toxicity induced increased superoxide dismutase and peroxidase activity which is thought to be a secondary defense mechanism against oxidative stress (Hassan et al., 2005). Zn was found to alleviate Cd toxicity by boosting plant height and biomass, chlorophyll content and photosynthetic rate, as well as reducing malondialdehyde concentration and lowering the activity of anti-oxidative enzymes (Hassan et al., 2005).

The normal level of Zn is between 30–100 mg kg−1 d.w and concentrations above 300 mg Zn kg−1 d.w. are considered phytotoxic (Anjum et al., 2015). Zn in excess due to anthropic contamination such as mines and smelters, especially in acidic soils, induces Fe-deficiency and thus leaf chlorosis and interference in chlorophyll biosynthesis and lowered yield (Chaney, 1993). Zn and other strongly chelated metal ions, can displace Fe resulting in extreme phytotoxicity (Chaney, 1993). Among some of the typical signs of Zn toxicity are interfered meiosis and mitosis, stunted growth, leaf necrosis and chlorosis (Anjum et al., 2015).

1.3.3 Iron

Fe is another essential micronutrient for plants as it's a prosthetic constituent of cytochromes of the electron transport chain and it plays a key role for chlorophyll production and the maintenance of chloroplasts (Rout & Sahoo, 2015). It is also important for plant incorporation of N and S, production of heme-based proteins, some of which break down peroxides, and non-heme proteins such as cytochromes (Alloway, 2013). In young leaves, a classic sign of Fe deficiency is intravenous chlorosis (Alloway, 2013). When compared to other micronutrients, Fe is found in higher concentration in plant tissues (approximately 2.0 μmol g−1 plant d.w.) (Anjum et al., 2015).

In high toxic concentrations however, much like deficiency, Fe can interfere with homeosis and trigger toxic effects such as brown spots on leaves which results in necrosis (Zhang et al., 1999). A general effect of Fe toxicity was lowered chlorophyll content as well as thiol depletion, lipid peroxidation, and K+ leakage wherein there was a loss of glutathione, higher levels of oxidized glutathione and finally higher activity of superoxide dismutase which perhaps the latter is related to higher levels of oxygen-free radicals and tissue damage (Sinha et

al., 1997). Fe-induced oxidative stress through the Fenton pathway when large amounts of Fe are taken up by the roots and translocated to shoots, ultimately increasing ROS formation (Becana et al., 1998). Increased levels of ROS lead to lipid peroxidation as well as damage to proteins and nucleic acids, thus altering plasma membrane structure and permeability as well as enough accumulated damage to result in programmed cell death (Connolly & Guerinot, 2002). A study on young *Eugenia uniflora* reveals iron toxicity to affect the activity of antioxidant enzymes and antioxidant levels (ascorbate and reduced glutathione levels) (de Oliveira Jucoski et al., 2013) Superoxide dismutase (SOD) and glutathione reductase (GR) activity increased with higher doses of Fe and treatment exposure time. Catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX) activities also increased with higher Fe concentration but were reduced by a longer exposure time (de Oliveira Jucoski et al., 2013).

1.3.4 Lead

On the other hand, Pb has not been considered to have significant benefits for plants, in fact, once leaked into the ecosystem due to inefficient mining processes, it can not only be toxic for plants but also the ensuing food chain (Pourrut et al., 2011). Shahid et al. (2012) summarized research findings suggesting that Pb has no biological function and can lead to hindered seed germination and growth, compromised chlorophyll production as well as oxidative stress such as lipid peroxidation and DNA damage. Examples of phytotoxicity include the inhibition of seed germination in rice which might be due to the compromised activity of protease and amylase in the endosperm; moreover, rice seedling growth was reported to be inhibited among other crops (Mukherji & Maitra, 1976). Other effects include inhibited photosynthesis which may result in chlorosis (Sharma & Dubey, 2005). Similarly to other metals, excess Pb can pile on oxidative

stress; *Pisum sativum* roots, expressed higher Pb accumulation, oxidative stress and interference with phytohormones (Dias et al., 2019). In roots, increased stimulation of glutathione reductase and ascorbate peroxidase, catalase and superoxide dismutase were noted. Protein oxidation being the first target, lipid peroxidation and altered membrane permeability were also observed (Dias et al., 2019).

1.3.5 Cadmium

Generally, Cd is thought to have negative impacts on plants and there are no studies that suggest Cd to be fundamentally essential for plant development (even in small doses). Carvalho et al. (2020) report on findings that suggest Cd benefitting certain species despite its accumulation in roots and shoots; such benefits include increased root length, number area and volume as well as transgenerational effects such as improved quality of fruits and germination. The hormesis effect is defined as a concentration dependent phenomenon where a low dose stimulates and a high dose inhibits, this often presents itself as a right-up or upside-down U response curve observed with Cd toxicity (Agathokleous et al., 2019). As for its negative effects, its toxicity is generally thought to lead to an indirect increase in ROS. However, Cd exposure is also thought to directly increase OH radicals (Kuznetsov et al., 2014). Altered gene expression is thought to be the result of Cd^{2+} covalently bonding with nitrogenous bases (Hossain & Huq, 2002).

Cd is thought to be extremely toxic as it resides in agricultural soils for over thousands of years (Sanità di Toppi & Gabbrielli, 1999). Cd concentration in uncontaminated soil is reportedly below 0.5 mg/kg (Vahter et al., 1991). Cd leads to oxidative DNA damage, altered gene expression leading to higher proliferation and lowered apoptosis, altered repair of DNA,

introduction strand breaks in DNA and crosslinks between DNA and protein (Mourón et al., 2004). Cd is reported to cause stomatal closure which leads to lower transpiration, moreover, it inhibits photosynthesis by interfering with chlorophyll metabolism (Nazar et al., 2012) and in rice it inhibits photosynthesis by interfering with key enzymes in the Calvin cycle and electron transport chain (Nwugo & Huerta, 2008). Moreover, it interferes with nutrient uptake by negatively altering the permeability in the plasma membrane causing nutrient leakage (Obata & Umebayashi, 1997).

1.3.6 Nickel

1.3.6.1 Ni as a micronutrient

Ni deficiency can lead to stunted growth, senescence, disruption in nitrogen assimilation and iron uptake, chlorosis in leaves and meristematic necrosis as well as interference with the metabolism of ureides, amino acids, and organic acids (Bai et al., 2006; Wood et al., 2006). Ni was first established as an essential micronutrient by Brown in 1987, when its role in seed germination, embryo, endosperm and roots development as well as dehydrogenase activity was elucidated (Yusuf et al., 2011), it is also considered an important part of many enzymes that aid coordination with S-ligands (those that contain cysteine like hydrogenase) and O-ligands (such as urea) (Marschner, 1995). Yet it is worth noting that in higher plants, the only enzyme that is reported to require Ni as an integral component is urease (Dixon et al., 1980). Ni deficiency interferes with urease activity and in turn disrupts N metabolism leading to a toxic build-up of urea in the shoots (Gerendás & Sattelmacher, 1999). Ni's function cannot be replaced by those of other metals such as Al, Cr, Cd, Pb yet small doses of Ni can mitigate these deficiency symptoms (Eskew et al., 1983).

1.3.6.2 Ni uptake and translocation

Ni's phytoavailability, uptake, accumulation and translocation depend on its concentration in soil, soil pH, dissolved organic materials and the amount of available iron-manganese oxides, wherein acid conditions Ni can become more soluble and mobile (Rooney et al., 2007). Ni is usually taken up at roots and then transported to shoots and leaves (Peralta-Videa et al., 2002). Its transport relies on the formation of Ni ligand complexes with NA, histidine, organic acids (Haydon & Cobbett, 2007). These complexes function as chelators that help transport Ni from roots via xylem to shoots, and without these transporters, translocation of Ni to shoots would not be possible as xylem has high cationic exchange capacity which hinders Ni mobility (Briat & Lebrun, 1999). Most of the taken up Ni (about 80%) is maintained at the vascular level with only a small portion (20%) transported to the cortex which goes to suggest that Ni has good mobility in phloem and xylem (Riesen & Feller, 2005) with Brooks et al. (1981) reporting that most of this Ni is found in high concentrations in cytosol and vacuole with much lower concentrations in mitochondria, chloroplast or ribosomes (Hassan et al., 2019). In cytosol where pH is above 6, Ni has a higher affinity for binding to amino acids and proteins thus organic acids cannot provide protection against Ni toxicity, however in vacuoles and apoplast, where pH is lower than 6, Ni has a higher affinity for organic acids and such as citrate which can provide protection against Ni toxicity by rendering it inactive (Salt & Kramer, 2000).

1.3.6.3 Ni Toxicity

While Ni is considered an essential micronutrient at low doses (0.05-10 mg/kg dry weight), at higher concentrations it becomes toxic (Ragsdale, 1998). Some physical signs of Ni toxicity include leaf chlorosis, necrosis, wilting, compromised photosynthesis, growth and

development as well as lowered product yield (Chen et al., 2009). Ni exerts its toxicity via two main ways: one being induction of oxidative stress similar to other heavy metals, the other being interference with the function of other essential metals (Chen et al., 2009). Due to the similar characteristic of Ni to many elements some including other heavy metals, (ex K, Na, Ca, Mg, Fe, Cu, Zn, Mn) Ni can serve as competition and thus reducing their absorption in result in symptoms relating to the deficiency of these metals (Chen et al., 2009; Marschner, 1995). A study on wheat shoots by Gajewska et al., 2006 reports that Ni in toxic levels decreases the biosynthetic activity of metalloenzymes such as SOD and CAT that have prosthetic component such as Fe, Cu, Mn, Zn, increase the activity of peroxidase (POD) and glutathione s- transferase (GST), reduce chlorophyll and relative water content and result in the accumulation of proline- proline synthesis is a sign of oxidative stress as it serves as a ROS scavenger (Chen et al., 2009). Chen et al., 2009 compiles various studies which suggest at low doses of Ni (about 0.05 mM), antioxidant enzymes such as SOD, POD, GR, and GOPX show enhanced activity in order to strengthen the defense pathway and remove/ scavenge more ROS, however in higher doses, the activity of these enzymes are compromised thus reducing the plants' ability to deal with oxidative stress and scavenge ROS. It's important to note that the activity of these antioxidant enzymes vary with respect to the stage of growth, type of plant, cultivation circumstances, type of plant tissue and exposure time (Chen et al., 2009; Marschner, 1995). Moreover, Ni can damage proteins by binding and altering their functional groups such as SH-group, resulting in the modification of the overall structure of the protein and thus hindering its activity (I. V. Seregin & Kozhevnikova, 2006). High Ni concertation increases protein degradation that may result in an increase in amino acid content, these amino acids are a sign of Ni stress however

they improve plant tolerance and detoxification by binding to Ni for the purpose of translocation from root to shoot (Hall, 2002; Hassan et al., 2019)

1.3.7 Oxidative stress: the common denominator

A common response to heavy metal toxicity is ROS production wherein the oxidative stress leads to lipid peroxidation, macromolecule deterioration, membrane damage, enzyme inactivation, ions leakage and DNA strand breakage (Singh et al., 2006; Tiwari, 2021). As a combat method, plants rely on antioxidant pathways (Singh et al., 2006), some of these antioxidant enzymes involved in these protective pathways are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), glutathione S-transferase, glutathione reductase (GR) and dehydroascorbate reductase (Maleki et al., 2017). Some heavy metals work against each other. In other words, the negative effects of one can be reversed in another. As previously discussed, Zn tends to reverse the effects of Cd (Hassan et al., 2005), selenium is found to provide relief by restoring biomass, pigment and water content in mustard plants through the upregulation of the antioxidant pathway and secondary metabolites (Ahmad et al., 2016).

1.3.8 Other considerations of metal toxicity: proximity, dosage, speciation, bioavailability and ligands

The saturation of ecosystems with metal pollution is a common occurrence particularly around centres of mining, smelting and industrial locations in many countries (Nriagu et al., 1998). Several studies report high levels of metal toxicity in the soil near smelting sites, where a correlation between the distance from the site and the concentration of metal particulates was

recorded (Narendrula & Nkongolo, 2015). Narendrula and Nkongolo (2015) reported a decreased plant population, diversity and abundance near smelting sites, as well as a decreased soil respiration and microbial biomass, a low fungi to bacteria ratio suggesting environmental stress.

Although micronutrients play an essential role in plant development, their concentration needs to fall within the need range of the target plant for higher concentrations may exert toxic effects on their physiology (Baroni-nezhad et al., 2021). Stress introduced by metal toxicity alters plant metabolism, physiology and biomass (Mehes-Smith et al., 2013). As previously discussed, essential trace elements metals yield an inverted U response curve (Alloway, 2013). Similarly, the hormesis effect explains why some metals can be stimulating at low doses and inhibiting at higher doses (Agathokleous et al., 2019).

While speciation looks at identifying and quantifying the different species and stages at which metal arises (relative existence of metal in different chemical forms given different environments), bioavailability investigates the amount of metal that is accessible to species' cellular membrane for uptake. Together the two assesses the damaging impact of metal on a biological system (Cui et al., 2020). It is worth noting that free metal ions tend to be more reactive when they are in a complex form, which is why they are mostly available in their biological form (Cui et al., 2019). Shahid et al. (2012) indicated that the available amount of Pb cations is a better indicator of its uptake and potential phytotoxicity than total concentrations. This would suggest although calculation of total concentration is easier, it might not be an accurate predictor of phytotoxicity thus it is important to consider metal speciation when assessing toxicity risks.

Moreover, depending on the organic ligand solubility of the metal, its uptake will be altered (Shahid et al., 2012). For instance, three types of organic ligands are EDTA (ethylene diamine tetra-acetic acid), low molecular weight organic acids and humic substances (Shahid et al., 2012). Thus in order to break down the environmental behaviour of metals, it is essential to study and link their speciation, mobility, solubility, phytoavailability and phytotoxicity (Shahid et al., 2012).

1.4 Gene expression with respect to metal contamination

Among the recently identified transporter families involved in micronutrient/heavy metal transportation mostly based on model plant *A. thaliana*. (non-accumulator relative to thlaspi) are: Major ZIP (ZRT/IRT like Protein), CDF (Cation Diffusion Facilitator), NRAMP (Natural Resistance associated Macrophage Protein) and HMA (Heavy Metal ATPase) (Freeman et al., 2004; Milner & Kochian, 2008; Mizuno et al., 2005), based on hyperaccumulator genus Thlaspi , ex *Thlaspi goesingense,* Serine acetyl transferase (SAT) and glutathione reductase (GR) (Freeman et al., 2004). Other heavy metal-associated genes include aminocyclopropane-1-carboxylic acid deaminase (ACC) (Stearns et al., 2005), NAS (Mari et al., 2006), Zinc Finger of Arabidopsis Thaliana (ZAT), Thioredoxin family protein (Lemaire et al., 2004), Iron-Regulated Transporter (IREG), and AT2G16800 which is a type of At-NiCo (Theriault et al., 2016).

1.4.1 Nicotianamine synthase

Nicotianamine (NA) as a phytosiderophore, chelates metal cations like Fe^{+2} and Zn^{+2} (Rout & Sahoo, 2015). Moreover, all higher plants synthesize and use nicotianamine for the

internal transportation of iron as well as other metals (Inoue et al., 2003). Masuda et al. (2013) add that in addition to acting as phytosiderophores, nicotianamine synthase (NAS) and nicotianamine are very important for the long-distance transport of iron in rice (Rout & Sahoo, 2015). It is thought that as a response to Ni, nicotianamine (NA) is translocated to roots to chelate Ni to make its transport to shoots easier (Milner & Kochian, 2008).

Evidence for the role of a nicotianamine synthase (*TcNAS1*) in Ni hyperaccumulation is reported in a study where *TcNAS1* was upregulated (overexpressed) in transgenic *A. thaliana* plants (Pianelli et al., 2005) which in turn results in a notable increase in Ni tolerance and accumulation in shoots. These findings further suggest that more than one transporters are involved in moving free and metal-bound NA in plants (Milner & Kochian, 2008). Ni detox response includes increase in the production of NA and its synthase (Weber et al., 2004). YSL gene family has been implicated to code for a nicotianamine-Ni/Fe transporter in the metal hyperaccumulating plant, *Thlaspi caerulescens* (Gendre et al., 2007).

NAS3 gene was upregulated in response to 10 μM NiSO4, in an experiment involving a Ni hyperaccumulator population of *Noccaea caerulescens*, wherein at this concentration the most amount of Ni was translocated. For the non-metallicolous population of the same species, this upregulation of NAS3 and NAS4 was observed at a dose of 100 μM NiSO4 (Visioli et al., 2014).

1.4.2 Serine acetyl transferase

Serine acetyl transferase or SAT is and its product OAS are responsible for sulfur reduction and cysteine biosynthesis which in turn lead to elevated levels of GSH (Freeman et al., 2004; Wirtz & Hell, 2003). GSH is a strong antioxidant capable of directly reducing ROS (Noctor et al., 1996) and thus it plays an important role in protection against oxidative stress

induced by many metals such as Ni, Co and Zn (Freeman & Salt, 2007).

1.4.3 Glutathione reductase

Glutathione reductase or GR maintains high levels of GSH which functions as an intermediate reductant in the antioxidative defense induced by Ni stress. Therefore, high levels of GSH (reduced glutathione) in the hyperaccumulator *Thalspi goesingense,* should play a protective role against oxidative damage. This theory is constant when comparing this hyperaccumulator species with non-accumulator *Aradopsis thaliana* wherein less lipid peroxidation in shoots and less accumulation of ROS (due to Ni stress) is observed in *Thalspi goesingense* (Freeman et al., 2004). The overexpression of SAT in *A. thaliana* was shown to result in the accumulation of OAS, Cys and glutathione and the GSH levels are maintained by GR contributing to the GSH dependant antioxidant pathway. High concentrations of OAS, Cys and GSH correspond with high activity of Sat and GR (Freeman et al., 2004).

In response to heavy metals, the enzymatic activity of Serine acetyl transferase (SAT) and Glutathione reductase may also be enhanced wherein increased activity of GR is reportedly induced by $NiCl_2$ in *Brassica juncea* the Indian mustard plant (Ali et al., 2008; Chen et al., 2009).

1.4.4 1-amino-cyclopropane-1-carboxylic acid deaminase

1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase enzyme is responsible for cleaving ACC (a precursor to the ethylene hormone) which results in lowered ethylene levels that can improve plant growth in presence of heavy metal toxicity (Burd et al., 1998; Stearns et al., 2005). In fact, transgenic tomato plants containing the bacterial gene for ACC deaminase are

able to not only accumulate Cd, Co, Cu, Ni, Pb, and Zn but also experience less of their toxic effects and grow in their presence (Grichko et al., 2000).

In a study looking at different bacterial strains in the rhizosphere hosted in different plants, ACC deaminase enzyme activity was reportedly higher in the presence of heavy metals such as Pb, As, and Cu, whereas in almost all cases, the ACC deaminase activity was reportedly lower in the presence of Ni, Cd, and Mn (Carlos et al., 2016). The only bacterial strain showing positive ACC deaminase activity in the presence of all the aforementioned metals was Serratia K120 (Carlos et al., 2016).

1.4.5 Natural Resistance associated Macrophage Protein

Natural Resistance Associated Macrophage Protein (NRAMP) are a family of integral membrane proteins with homologues identified in bacteria, fungi, plants, and animals and some NRAMP proteins are recently linked to metal transport (Thomine et al., 2000). This family is non-ATP hydrolyzing (Visioli et al., 2014). As with most other metal transporters, NRAMP transporters are said to have low metal specificity; in other words, the same system transports toxic ex Cd and non-toxic metals such as Fe^{2+}, Zn^{2+} and Mn^{2+} and thus it is speculated that Ni transport takes place along with the transportation of other metals in plants (Mizuno et al., 2005).

TjNRAMP4-expressing recombinant yeast expressed higher levels of Ni accumulation compared to controls (Mizuno et al., 2005). At 10 μM $NiSO_4$, Ni hyperaccumulator Brassicaceae *Noccaea caerulescens*, metallicolous population Mt. Prinzera, exhibited its highest level of Ni translocation and it showed an over-expression of NRAMP3 and NRAMP4 genes, meanwhile, its non-metallicolous population exhibited an over-expression of these two NRAMP genes at 100

μM NiSO4 (Visioli et al., 2014)

1.4.6 High-affinity Ni transporter AT2G16800

AT2G16800 is a high-affinity nickel-transport protein and is part of the NiCoT protein family responsible for transporting Ni and Co via proton motive force (Rodionov et al., 2006; Theriault et al., 2016). While the roles of NiCoT in Ni accumulation are well established for prokaryotes, our knowledge of them in eukaryotes is lacking (Theriault et al., 2016).

AT2G16800 is reportedly downregulated at the high $Ni(NO_3)_2$ dose of 1600 mg/kg for white birch which suggests that while Ni affects the expression of AT2G16800, this gene is not involved in Ni resistance for white birch (Theriault et al., 2016).

1.5 Species of interest: *Pinus strobus* (white pine) and *Pinus banksiana* (jack pine)

The Greater Sudbury Region contains a mix of deciduous boreal forest species, the main ones being white pine (*Pinus strobus*), jack pine (*Pinus banksiana*), red oak (*Quercus rubra*) and red maple (*Acer rubrum*) (Hutchinson & Whitby, 1977).

Eastern white pine (*Pinus strobus L.*) also referred to as Weymouth Pine, and Pin du Lord, is a dominant species in North America and regarding the lumber industry and it has been a great source of revenue in Ontario (Syme, 1985; Webb & Flinn, 1991). In Canada eastern white pine stretches out from Newfoundland to Manitoba and in the USA, from Iowa to Atlantic coast states. Reportedly, 12m high white pine trees, had accumulated 20 times more lead (Pb) in their woody surface compared to their foliage despite the fact of their foliage having 10 times the surface area of their woody surface (Heichel & Hankirt, 1976; Watmough & Hutchinson, 2003). Although Pb and Cd can enter white pine woody tissue through the bark, this route of uptake is

not as important as soil uptake (Watmough & Hutchinson, 2003). In a study between white pine, red maple, and Norway spruce, white pine seedlings accumulated the most Cd and Zn and showed higher tolerance against these metals based on observed physical phytotoxicity at only the higher treatment level (Mitchell & Fretz, 1977).

Jack pine (*Pinus banksiana*) also referred to as Hudson Bay pine, Banksian pine or scrub pine is an important coniferous species in Canada and the USA and is the most widely distributed in Canada with various uses such as lumber wood (Rudolph & Laidly, 1990). In Canada, the natural range extends from the Northwest Territories to Cape Breton Island in Nova Scotia (Rudolph & Laidly, 1990). *Pinus banksiana* has the ability to grow on dry sandy conditions as well as run its roots in rocks with little soil at its disposal (Barton, 2016; Cheyney, 1932). A study in the year 2000 in the CGS showed a Ni range of 28.9 to 50.8 mg kg^{-1} in *Pinus banksiana* needles collected within 15 km from the smelters which is 7-10 times more than the control sites (Gratton et al., 2000). Although not enough metal was accumulated to use *Pinus banksiana* for the purposes of phytoremediation, a trace metal analysis of shoot tissue harvested at mine sites in central Manitoba, showed 4-5 times the normal accumulation of both Au and Cu (Renault et al., 2004). In another study in northern Manitoba, with distance from the smelter, the concentration of accumulated Ni and Cu dropped in the cones of *Pinus banksiana* wherein a significant inverse relationship between seedling growth and the amount of Cu/Ni in the upper organic soil layer was also established. Moreover, the accumulated metal in soils within the 5km radius of the mine site prohibited root development of seedlings (Wotton et al., 1987).

1.6 Rationale and objectives

There have been numerous studies on the importance of plant coping mechanisms against metal toxicity, however information on metal-induced gene expression in coniferous species, especially *Pinus strobus* and *P. banksiana*, is lacking in the literature. There have been no reports on the effects of metal dosage on gene expression in these species. These pine species are important contributors not only to Northern Ontario ecosystems and economy but also to many regions in North America. Considering the history of metal contamination and mining activities in the CGS, the adaptability of these species to metals and their potential use in bioremediation warrant an investigation in molecular response of these species to metal toxicity.

Hence the main objectives of the present study are to determine the effects of increasing doses of Ni on the gene expression of genes associated with metal resistance in *Pinus strobus* and *P. banksiana.*

Chapter 2: Nickel toxicity in white pine (*Pinus strobus*) and *P. banksiana*

2.1 Introduction

While in low dosage, Ni is an essential micronutrient for normal plant development, the toxic effects of Ni are apparent when plants are exposed to high concentrations. Signs of toxicity include leaf chlorosis and necrosis, wilting, compromised photosynthesis, growth, and development as well as lowered product yield (Chen et al., 2009). Ni induces suppression in roots growth and increases shape abnormality (Kopittke et al., 2007; Wong & Bradshaw, 1982; Yang et al., 1996). For instance, Ni toxicity has been seen to suppress lateral root growth in crops such as maize (Seregin et al., 2003). It is thought that these effects are the result of Ni easily passing through the endodermal barrier and accumulating to toxic levels in the pericycle cells which are important for cell division and proliferation (Seregin & Kozhevnikova, 2006). Recently, the toxicity of Ni to *Betula papyrifera* (Theriault et al., 2016), *Acer rubrum (Kalubi et al., 2018), Quercus rubra (Djeukam et al., 2019), Populus tremuloides* (Czajka et al., 2019), *Acer saccharinum* (Nkongolo et al., 2017), and *Picea glauca* (Boyd & Nkongolo, 2020) has been investigated. These species showed different levels of resistance to Ni. Response of pine spp. to nickel toxicity has not been analyzed. Hence, the specific objective of this study is to determine the effects of Ni on jack pine (*Pinus banksiana*) and white pine (*P. strobus*) morphology in growth chamber settings.

2.2 Materials and methods

2.2.1 Treatment set-up and scoring damage

Six-month-old *P. banksiana* and *P. strobus* obtained from College Boreal were transplanted to pots containing a 1:1 mixture of sand: soil and were left to grow in growth

chambers for an additional month. They were watered regularly and fertilized with a 1:1:1 mixture of nitrogen: phosphorus: potassium fertilizer. To determine the effects of Ni on plant morphology, plants were treated with different concentrations of an aqueous solution of commercial nickel nitrate ($Ni(NO_3)_2$). These doses included 150 mg (28 μmol of Ni), 800 mg (151μmol of Ni), 1600 mg (302 μmol of Ni) and 3200 mg (603 μmol of Ni) per 1 kg of dry soil dissolved in distilled water. These correspond to bioavailable, half, total and twice the total concentrations of Ni in metal-contaminated sites in GSR (Nkongolo et al., 2013). In order to control for the potential toxic effects of nitrate ions, potassium nitrate solutions in the same concentrations were used. For the potassium nitrate control series, an aqueous solution of commercial potassium nitrate (KNO_3) at doses of 150 mg (57 μmol of nitrate), 800 mg (302 μmol of nitrate), 1600 mg (603 μmol of nitrate) and 3200 mg (1207 μmol of nitrate) per 1 kg of dry soil dissolved in distilled water were used. Distilled water was used as the main reference control. In total, 100 seedlings of each species were analyzed during this study. The experimental design was a completely randomized design with 10 replications. Prior to treatments, a picture was taken of each group and the physical damage of each seedling was rated based on the 1-9 rating where 1 represents no damage and 9 dead or dying seedlings (Table 1).

Table 1. Damage rating scale and corresponding plant classification based on reaction to nickel and potassium nitrate treatments.

% of Leaf area with chlorosis/necrosis	Damage Rating	Genotype Classification
0-10	1	Resistant (RG)
10-20	2	
20-30	3	
30-40	4	Moderately Susceptible (MSG)
40-50	5	
50-60	6	
60-70	7	Susceptible (SG)
70-80	8	
> 80	9	

2.2.2 Data Analysis

Data were analyzed using SPSS version 20. The Shapiro-Wilks test was used to check for data normality (non-normal if $p < 0.05$). Since data was non-parametric, the Krustal-Wallis test and the post-hoc Games-Howel test were carried out to determine the significant differences among means.

2.3 Results

No damages were observed on *P. strobus* genotypes that were tested at different doses. All white pine groups post-treatment were in excellent conditions, showed no damage and were scored 1 (Table 2 and Figure 1). Therefore *P. strobus* is classified as resistant to both Ni and K solutions (Table 1). *P. banksiana* on the other hand, showed significant variations in damage ratings in response to different treatments. In fact, the damage ratings for the lowest doses of Ni (150 mg /kg and 800 mg/kg) were similar to that of the water control. Significant differences

were observed in the damage for the groups treated with 1,600 and 3,200 mg/kg Ni. *P. banksiana* seedlings were resistant to 150 mg/kg and 800 mg/kg, moderately susceptible to 1,600 mg/kg and highly susceptible 3,200 mg/kg of Nickel nitrate (Table 2 and Figure 3a). The physical damage of these Ni doses can also be seen in Figures 2d and 2e. Fewer damages were observed on the seedlings treated with potassium nitrate. No significant differences were observed on seedlings treated with this salt at the concentrations of 150, 800 and 1,600 mg/kg compared to water. *P. banksiana* seedlings were moderately susceptible to the highest dose of 3,200 mg /kg. Overall, Ni induced more damages to *P. banksiana* than nitrate at high doses. *P. banksiana* was resistant to the lowest doses (150 mg / kg and 800 mg /kg) of potassium nitrate since these doses caused the same level of damage as water whereas the highest dose (3,200 mg/kg) induced significantly more damages than other K treatments (Figure 2i and Figure 3b). When comparing the Ni and their respective potassium control, Ni doses at 1,600 and 3,200 mg/kg caused significantly more damages compared to their control pair (Figure 3c).

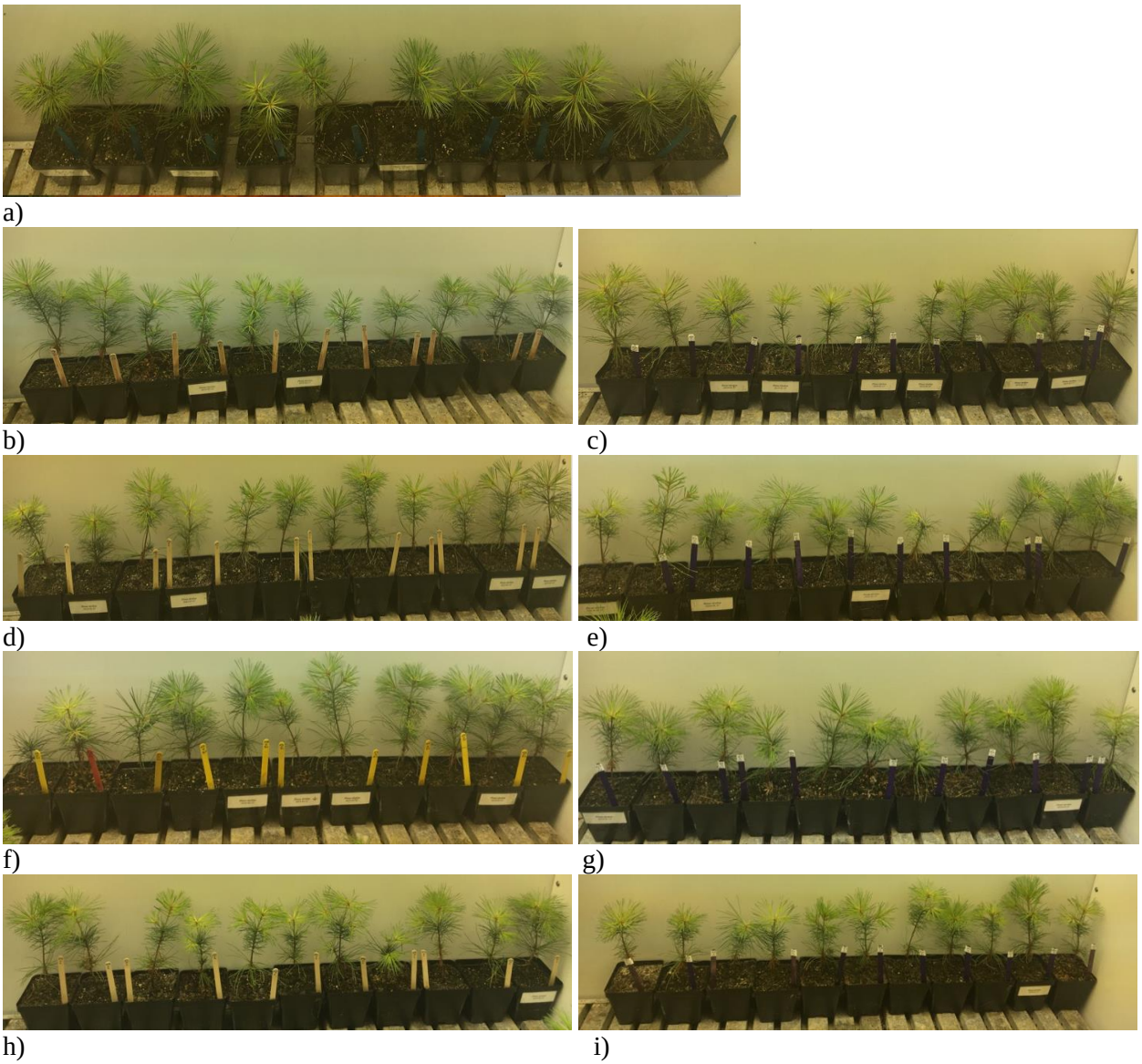

Figure 1. White pine (*Pinus strobus*) seedlings seven days after nickel and potassium and nitrate treatments, post-treatment, prior to harvest. The water, 150 mg/kg, 800 mg/kg, 1600 mg/kg and 3200 mg/kg Ni nitrates are depicted in a, b, c, d, and e. Whereas potassium nitrates 150 mg/kg, 800 mg/kg, 1600 mg/kg and 3200 mg/kg are depicted in f, g, h and i. All seedlings show zero to no damage.

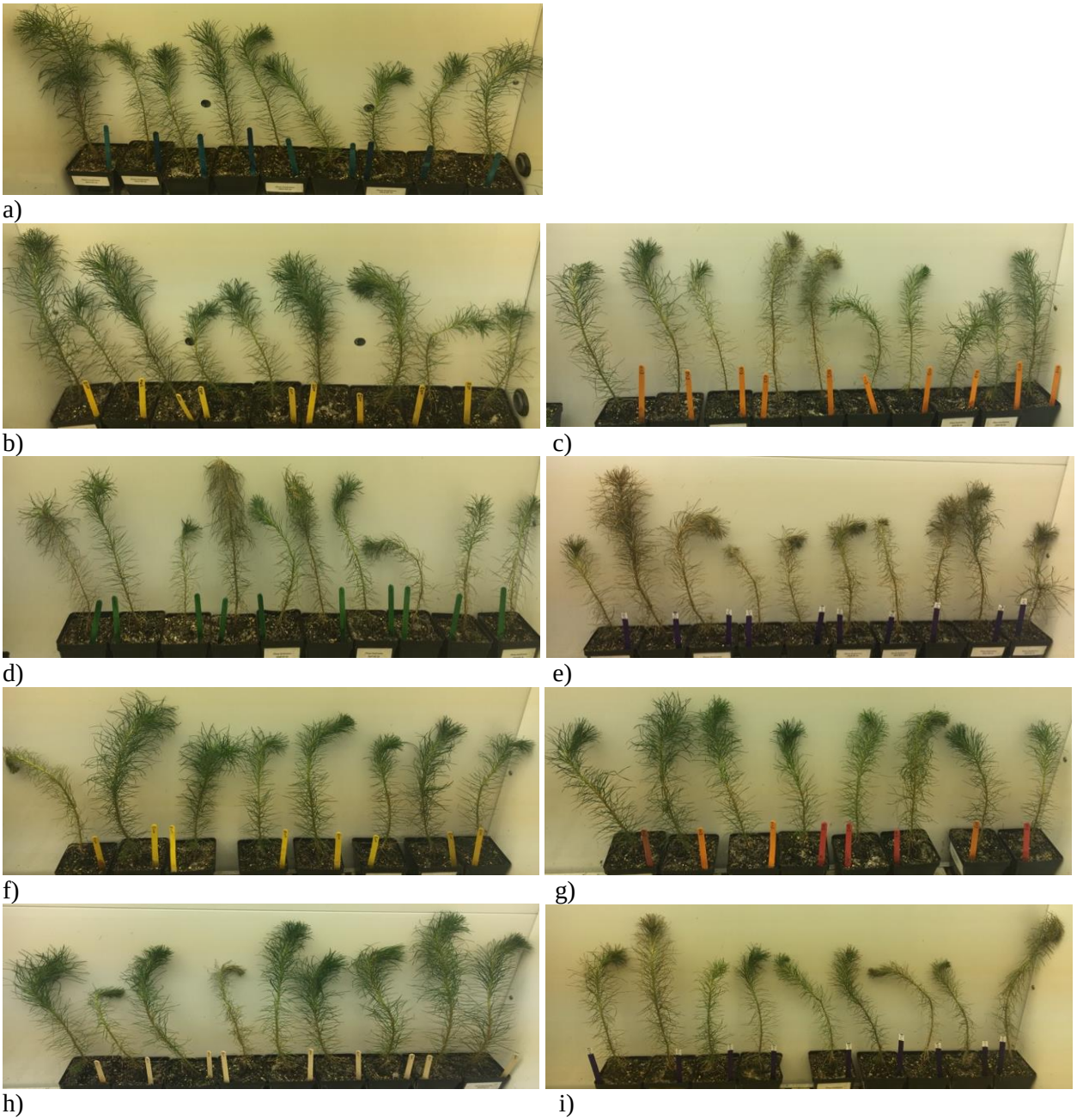

Figure 2. Jack pine (*Pinus banksiana*) seedlings seven days after nickel and potassium nitrate treatments, post treatment, prior to harvest. The water, 150 mg/kg, 800 mg/kg, 1600 mg/kg and 3200 mg/kg Ni nitrates are depicted in a, b, c, d, and e. Whereas potassium nitrates 150 mg/kg, 800 mg/kg, 1600 mg/kg and 3200 mg/kg are depicted in f, g, h and i. Excessive physical damage is noted in 1600 mg/kg and 3200 mg/kg Ni nitrates and 3200 mg/kg potassium nitrate.

Table 2. Damage scores of white pine (*Pinus strobus)* and jack pine *(P. banksiana)* seedlings treated with nickel and potassium nitrates. A score of 1 indicates no damage and 9 indicates severely damaged plants.

White pine			Jack pine		
0 mg/kg	1± 0		0 mg/kg	1.33 ± 0.17	
	$Ni(NO_3)_2$	KNO_3		$Ni(NO_3)_2$	KNO_3
150mg/kg	1± 0	1± 0	150mg/kg	2.22±0.22	1.38 ±0.26
800mg/kg	1± 0	1± 0	800mg/kg	2.4±0.86	2.00 ±0.33
1600mg/kg	1± 0	1± 0	1600mg/kg	5.3±0.73	1.67 ±0.67
3200mg/kg	1± 0	1± 0	3200mg/kg	8.2±0.25	4.56±0.58

± represents standard errors.

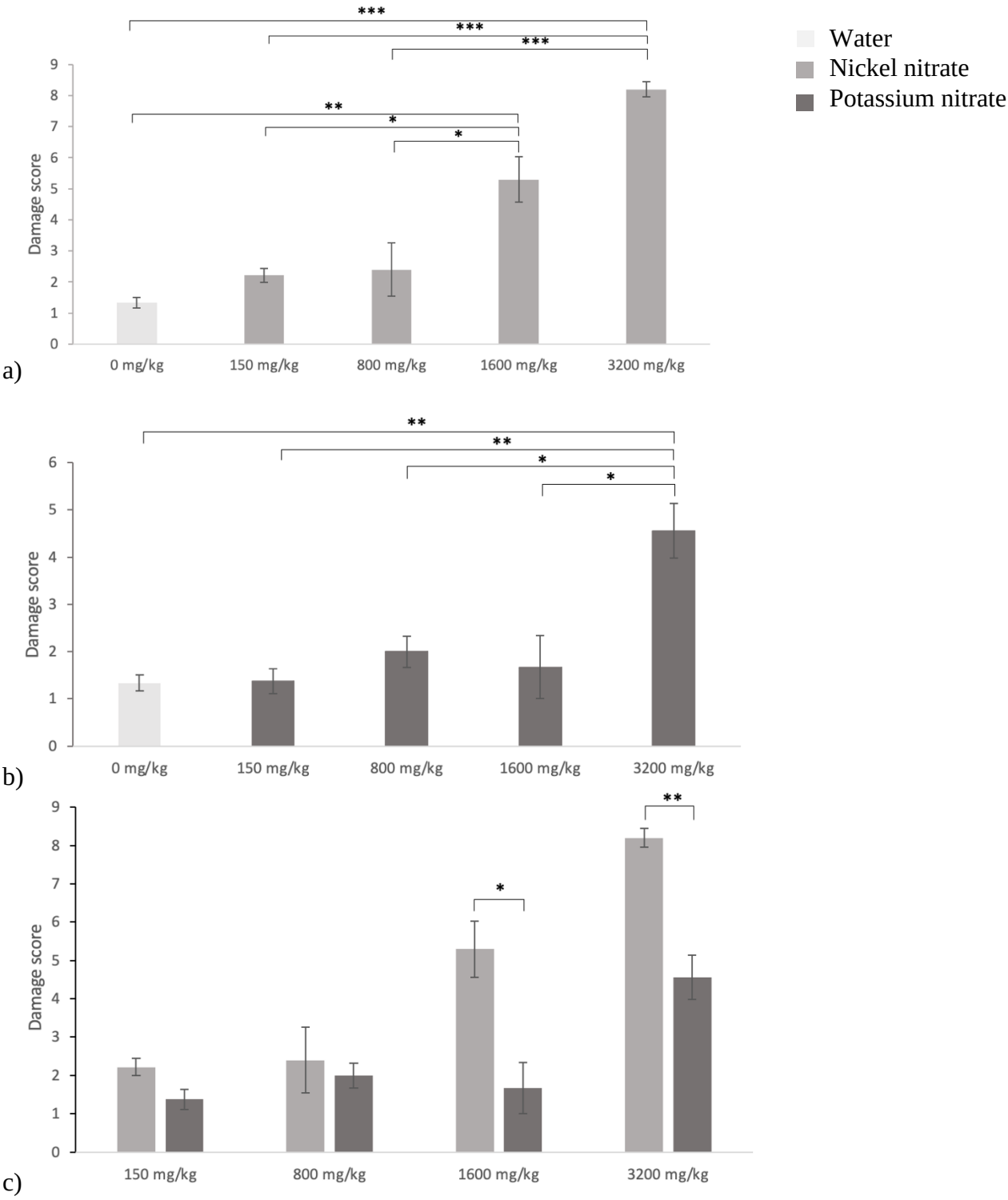

Figure 3. Damage scores in jack pine (*Pinus banksiana*) in a) water and nickel nitrates, b) water and potassium nitrates, c) nickel and potassium nitrate pairs. A damage score of 1 indicates no damage and 9 indicates dying plants with severe damages. Only significant differences are depicted ($p \leq 0.05$ is *, $p \leq 0.01$ is ** and $p \leq 0.001$ is ***). Error bars represent standard error.

2.4 Discussions

The damage scoring was important in revealing insight about variation within and between species. *P. strobus* showed no damage caused by nickel or nitrate salts with no intra or intergroup variation whereas *P. banksiana* behaved differently. Not only there was intragroup variation (shown by error bars in Figure 3) but they also exhibited differences in damage ratings among the treatments evaluated especially between 1,600 and 3,200 mg/kg Ni and 3,200mg/kg K nitrate. This would suggest that the highest concentration of either salt resulted in damages for *P. banksiana* wherein this damage is more pronounced for nickel compared to potassium controls. The damage caused in *P. banksiana* is not only prone to signs of physical damages induced by nickel toxicity but also toxic levels of potassium nitrate. Moreover, it seems the threshold for damage by Ni is at 1,600 mg/kg as no significant change or damage was observed at lower doses of 150 mg/kg and 800mg/kg of Ni.

Potassium nitrate (KNO_3), as a non-toxic common plant fertilizer, offers a soluble source of two very important plant nutrients. It is an excellent source of N and K needed for optimal plant nutrition. Several reports have noted the synergistic effect between K^+ and NO_3^- in these salt fertilizers (*Nutrient Source Specifics: Potassium Nitrate*, 2019). The salt facilitates the uptake of both ions by the plant roots wherein the potassium component helps with building thicker cell walls, with increasing the amount of electrolytes inside the cell and counteracting the harmful effects of sodium. While potassium toxicity in plants does not necessarily exist, nitrate at high doses can have negative effects. However, nitrates are natural components of plants and can be found in large concentrations in numerous vegetables (Wolff & Wasserman, 1972). If present in soil or water, the nitrate ion reduces the uptake of chloride anion in plants. However, nickel nitrate, more specifically the Ni, is quite toxic to different organisms and is highly toxic to plants at high doses (Bhalerao et al., 2015). Visible damage to plants induced by Ni toxicity

includes reduced plant growth and alteration in leaf color (Theriault & Nkongolo, 2016). While many plant species are severely affected by high doses of toxic metals, others have the ability to cope with contamination via minimizing their effects. Most of Ni is insoluble (which remains bound to soil colloids particles) yet the soluble form which is the Ni ion, bonds with ligands like chloride ion Cl^-), sulphate (SO_4^{2-}), nitrate (NO_3^-), and organic ligands (Tewari et al., 2010). Thériault and Nkongolo (2016) report on a reduction in stem growth in *B. papyrifera* treated with 1600 mg/kg of Ni nitrate. In this study, *P. strobus* and *P. banksiana* seedlings were treated with Ni nitrate at different doses including 150 mg /kg, 800 mg /kg, 1600 mg /kg, and 3200 mg/kg. No phenotypic symptoms (chlorosis or necrosis) or growth reduction even at the highest doses were observed in *P. strobus* while *P. banksiana* seedlings were susceptible to high concentrations. The difference between the two species is elusive as there are not many studies commenting on the sensitivity of these two species to salts such as Ni nitrate and potassium nitrate. This suggests that the two species process Ni ions differently once it enter plant cells Another potential explanation for *P. banksiana* increased sensitivity to these salts could rest in their habit evolution, wherein this species as opposed to *P. strobus*, is adapted to grow in harsh dry and rocky soil bed (Barton, 2016; Cheyney, 1932) and perhaps too much access to nutrients is above the threshold tolerance of this species. Yet reports on roadside salt and vegetation classifies jack pine as a salt-tolerant and white pine as a salt-intolerant species (Transportation Association of Canada, 2013).

Likewise, plants can assimilate both nitrate and ammonium and may have different preferences for the two depending on their reductive ability (Li et al., 2013). Since nitrate has to be converted to ammonia before it can be used, if a plant lacks the reductive ability to efficiently use nitrate as their main source of nitrogen it may thus prefer ammonia (Li et al., 2013). If *P.*

strobus and *P. banksiana* process nitrate differently, it may result in different sensibility to nitrate ions if the two species happen to have different preferences for their nitrogen source. A *P. banksiana* study examining the metabolism of nitrate reports lower N incorporation from nitrate compared to N from ammonium. They explained that a portion of nitrate must go through the glutamine synthetase/ glutamate synthase cycle suggesting that nitrate will be reduced in the *P. banksiana* root cells (Vézina et al., 1993).

In conclusion, higher doses of both Ni nitrate and potassium nitrate induce physical stress in *P. banksiana* but had no effect on *P. strobus*. Future studies with other metals such as copper, cobalt, and cadmium with larger populations of these two species could be insightful in revealing the adaptive strategies at play when these plants are pitted against heavy metal toxicity.

Chapter 3. Expression of nickel-resistance-associated genes by increasing doses of nickel nitrate and potassium nitrate in *Pinus strobus and P. banksiana* needle tissue

3.1 Introduction

The amount of nickel in soil changes with locations and the related anthropogenic activities. Once taken up at the roots, nickel and its compounds can be shuttled to the shoots and the leaves via the xylem (Krupa et al., 1993; Peralta-Videa et al., 2002). The bioavailable form of nickel can be translocated within the plant rather easily wherein some proteins can bind to and facilitate Ni transportation (Colpas & Hausinger, 2000; Hausinger, 1997). Moreover, these metal-ligand complexes (such as nicotianamine and histidine complexes) also take part in a regulatory role (Haydon & Cobbett, 2007; Kim et al., 2005; Vacchina et al., 2003). Usually, about half of nickel will be stored within the plant roots system (Cataldo et al., 1988). Some accumulator species (including *Populus tremuloides*) are thought to retain a greater amount of nickel in the shoots and leaves when faced with excess nickel concentrations as a means to prevent its toxic effects from affecting the rest of the plant biomass (Kalubi et al., 2016).

Nickel is a heavy metal that induces its toxicity via oxidative stress wherein metal resistance genes and their coded enzyme function as different lines of defense. Ni has the ability to trigger variation in the gene regulation of plants (Narendrula-Kotha et al., 2020). Expression levels of these genes differ in species.

The specific objective of this study is to compare the effects of Ni on the expression of six targeted genes associated with Ni resistance in *Pinus strobus* and *P. banksiana*. The target genes investigated are high affinity- Ni transporter family (AT2G16800), 1-aminocyclopropane-1-carboxylic acid deaminase (ACC deaminase), Natural resistance-associated macrophage proteins (NRAMP3), and serine acetyltransferase (SAT) for both species while nicotianamine

synthase (NAS) for *Pinus strobus* and glutathione reductase (GR) for *Pinus Banksiana.* The expression of these genes of interest will be normalized with respect to the housekeeping gene Elongation factor 1-alpha (ef1alpha) and the water control. It is hypothesized that these genes will be upregulated or downregulated in response to Ni stress. More specifically based on current literature, it is hypothesized that NAS, SAT, ACC, NRAMP3 and GR will be upregulated in presence of lower doses of Ni and downregulated with higher doses and as for AT2G16800, it is hypothesized to be downregulated.

3.2 Materials and Methods

3.2.1 Treatment set-up

Seedling set up and treatment was carried as outlined in the materials and methods in chapter 2 (2.2). A week after treatments, needles were harvested from all the seedlings. Needles of each individual seedling were picked off of the stem and placed into their corresponding labelled aluminum foil. Each foil was folded and momentarily dipped in liquid nitrogen and stored in a -20 ℃ freezer until RNA extraction.

3.2.2 RNA extraction

For *P. strobus* needles, RNA was extracted from each individual seedling using the manual protocol outlined by Chang et al. with a few modifications (Chang et al., 1993; Theriault et al., 2016). These modifications were, only 0.3 g of needle tissue was ground and added to 1 ml CTAB, 1 ml of phenol-chloroform was added to the 1ml CTAB solution, RNA was precipitated in 100 µl of SDS buffer followed by another 100 µl chloroform extraction step. As for *P. banksiana,* RNA extraction was performed according to the Plant/Fungi Total RNA Purification

Kit by Norgen Biotek. Only 0.05 g of tissues were needed using this protocol. All extracted RNA was stored at – 60 ℃ until use. The integrity of RNA was checked through gel electrophoresis with a 1% agarose gel. The RNA was then quantified using the Qubit® RNA BR assay kit by Life Technologies. Pooled samples were adjusted to make up to a total of 10 μg of total RNA per treatment for each species. These pooled samples were topped off with 1 μl DNase 1 and 1 μl buffer (Life Technologies #EN0521) to make a total of 10 μl. They were incubated at 37 ℃ for an hour. The absence of DNA in these samples was verified by running a 2% agarose gel prior to deactivating the DNase enzyme in the samples. Only RNA without DNA were used after this step for further gene expression analysis. DNAse was then deactivated by adding 1μl of 50 mM EDTA to each pool and incubated at 65℃ for 10 minutes.

3.2.3 Primer design

Ni resistance-associated genes (ACC, SAT, NAS, Nramp3, GR, AT2G16800) and the housekeeping gene (ef1 alpha) were chosen (Table 3 and 4). The sequences of these genes were determined using Congenie.org (https://congenie.org/citation?genelist=enable) database based on *Pinus taeda* as the model species. The accession code of *Pinus taeda* was used to obtain its sequence and this was then inputted as the query sequence into NCBI BLAST (basic local alignment search tool) program more specifically BLASTn for somewhat similar sequences to find corresponding *Pinus strobus* and *P. banksiana* accession codes under the TSA (transcriptome shotgun assembly) database. The TSA database was used since the nucleotide collection database was unsuccessful at recovering any matches. The *Pinus strobus* and *P. banksiana* accession codes with the highest coverage and specificity (lowest number of matches indicated higher specificity which was preferred) were chosen as candidates. These candidate

codes were then used to design primers with the same BLAST program. Potential primer pairs flanking the gene sequences were checked for lack of self and heterodimers, TM mismatch and hairpins using the OligoAnalyzer tool from Integrated DNA Technologies (https://www.idtdna.com/calc/analyzer).

3.2.4 cDNA conversion and RT-qPCR

Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit was used for cDNA conversion, following the kit's protocol described in https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_042557.pdf. A 2% gel was run on the synthesized cDNA to verify its integrity and correct band placement corresponding to each primer pair.

Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) was performed according to the Dynamo HS SYBR Green Kit (Life Technologies) instructions with the addition of 15µl of mineral oil to each well. For each gene of interest, reactions were run twice and in triplicates. For each run, six standards were included to generate a standard curve as well as a No Template Control. Plates were run using the BioRad CFX Connect RT-qPCR Detection System. The program consisted of the following steps: step 1- initial denaturation at 95℃ (10 mins), step 2- denaturation at 95°C (15s), step 3- annealing temperature between 58-64°C corresponding to the optimized temperature for the primer pair for (1min), step 4- plate is read, step 5- steps 2 to 4 are repeated for 41 cycles, step 7- temperature moves up 65°C and plate is read (5s), step 8- melting curve 65 –95°C, every 1°C, held for 10 seconds, step 9- 72°C for 3 minutes.

3.2.5 Data analysis

The CFX Connect program was used to obtain results. For each gene, the Cq data were quantified using the equation of the line of the six standards. This resulted in six data points wherein outliers were excluded from further analysis. Within each gene, these values were then normalized to the housekeeping gene and then the water control group to obtain the ratio of gene expression compared to a value of 1 (gene expression for water =1). Final normalized data points were inputted in the statistical analysis program SPSS 20 to determine if there were any significant differences among means (significant differences if $p < 0.05$). The Shapiro-Wilk test was performed to determine normality (non-normal if $p < 0.05$). Some data sets that were not normal were successfully normalized using log transformation while other non-normal data sets required a non-parametric analysis. All data sets did not have homogeneous variances as they failed the Levene's test (unequal variances if $p < 0.05$). Since a one-way ANOVA would not be an accurate method for assessing mean differences, the Welch test (parametric, does not assume equal variances) was performed for the normal data set (significant differences among groups if $p < 0.05$) and the Krustal-Wallis was performed for non-parametric data set (significant differences among groups if $p < 0.05$). Both the Welch and the Krustal-Wallis revealed there to be significant differences among group means. Games-Howel test was performed on all data sets for the post-hoc analysis to determine which group pairs were significantly different (significant differences among pairs if $p < 0.05$).

Rather than using the standard deviation of the final normalized data inputted into SPSS 20, standard deviation was calculated on original raw data in order to account for the deviation carried forward. For a given gene, this process entailed calculating the standard deviation (SD) of each group and diving it by the SD of the corresponding group in the housekeeping gene (HKG).

The average of each group was divided by the average of the corresponding group in the HKG to yield the average quotient. The coefficient of variation (CV) was then calculated for each group, wherein the CV of the given group was added to the HKG's CV to yield a final CV. All groups' quotient averages are then divided by the water group quotient average to yield a final quotient average with respect to the water control. The final SD is the SD normalized to the water and it is calculated by multiplying the final quotient average by the final CV. These standard deviations are depicted in figures 4-13.

3.3 Results

As described in chapter 2, *P.strobus* was determined to be Ni resistant due to the absence of damages caused by Ni toxicity. Several *P. banksiana* seedlings however seem extremely distressed after nickel nitrate treatments with some scoring up to 8 and 9. Primer pairs that successfully amplified the housekeeping gene (ef1 alpha) and the target genes (SAT, NAS, ACC, NRAMP3, GR and AT2G16800) are outlined in Tables 3 and 4. These were selected for RT-qPCR and further gene expression analysis.

3.3.1 AT2G16800

The AT2G16800 was downregulated in *P. strobus* for all the nickel treatments, with the highest dosage of 3200 mg/kg showing the most gene repression. There was a dip in expression going from water to the first three doses of Ni (exhibiting the same level of expressions) such that collectively they show an expression of about 0.4X of the water. Finally, more repression in the last dose with about 0.15X expression of water (Figure 4a). Potassium nitrate treatments induce slight decreases of AT2G16800 compared to water but no clear dose-dependent effects

were observed (Figure 4b). When comparing the Ni nitrate with its potassium nitrate controls, all the pairs exhibited the same expression with the exception of the 800 mg/kg and 3,200 mg/kg doses wherein the Ni treatments induced AT2G16800 expression levels that were one-third their potassium control counterpart (Figure 4c). The Ni and K series exhibit different patterns of expression.

Like in *P. strobus,* the expression of AT2G16800 *in P. banksiana* followed the same trend. All the Ni nitrate treatments induced a significant repression of this gene compared to water with the strongest downregulation observed with the highest dose of 3,200 mg/kg. There was a dip in expression induced by all the nickel nitrate treatments. The doses of 150 mg /kg, 800 mg/kg and 1,600 mg/Kg of Ni induced the same level of expressions averaging 0.65X of that observed with the water treatment. The highest dose of 3,200 mg /kg induced the most repression of the gene i.e. of 0.3X of the level observed with the water (Figure 5a). Most potassium nitrate treatments caused a repression as well, however no dose-dependent effect was observed (Figure 5b). When Ni nitrate and potassium nitrate treatments were compared, Ni nitrate at the 800 mg/kg induced a higher expression of the AT2G16800 compared to its pair while Ni nitrate at 3,200 mg/kg showed a significantly lower expression than the potassium nitrate (Figure 5c). The Ni and K series exhibit different patterns of expression. Overall, both species show repression for this gene specially for the highest dose of Ni nitrate.

3.3.2 ACC deaminase

Ni nitrate induced a significant upregulation of ACC at the dose of 800 mg /kg. This overexpression is observed at 800 mg/kg of Ni. The overexpression level increased when the dosage was doubled to 1,600 mg/kg to reach 3X the expression achieved with water. But the

gene expression decreased significantly when the dosage was further increased to 3,200 mg /kg reaching 0.4X the level of ACC expression for the water control (Figure 6a). As for its potassium nitrate counterpart, a significant decrease was observed at 150 mg/kg and an upregulation in 800 mg/kg compared to water (Figure 6b). Significant differences were noted between the Ni and K treatments wherein Ni shows higher expression for the 800 mg/kg and 1,600 mg/kg treatments and lower expression for the 3,200 mg/kg compared to K (Figure 6c). The Ni and K treatments induced different patterns of ACC expression.

As for *P. banksiana*, the highest expression is seen at 150 mg/kg Ni (4.2X water) and with increasing dosage, expression is reduced with the lowest at 3,200 mg/kg corresponding to 0.7X of water control (Figure 7a). Potassium nitrate induced an upregulation of ACC at the lowest and highest doses (150 and 3,200 mg/kg doses) representing 1.6X of the level achieved with water (Figure 7b). When Ni nitrate induced a higher expression of ACC compared to its K counterparts for 150, 800 and 1,600 mg/kg Ni. However, a lower expression was observed when compared to K at 3,200 mg/kg dose. The Ni and K series exhibit different patterns of expression (Figure 7c). The starting point at which ACC overexpression is induced is at 800 mg/kg Ni for *P. strobus* and 150 mg/kg for *P. banksiana*. ACC was repressed in both species at the 3,200 mg/kg Ni treatments.

3.3.3 NRAMP3

A downregulation of NRAMP3 was observed in *P. strobus* for the 800 and 3,200 mg/kg Ni dose that was 0.5X of the water-induced level. However, an upregulation of this gene induced by the 1,600 mg/kg Ni resulting in an expression level that was 1.8X that of water was recorded (Figure 8a). Potassium nitrate treatments induced a downregulation of NRAMP3 at the 150

mg/kg dose and an upregulation at the 800 mg /kg (Figure 8b). Significant higher expression was observed in Ni treatments at 150 and 1,600 mg/kg and lower expression at 800 and 3,200 mg/kg compared to its K counterparts. Hence, the Ni and K series exhibited different patterns of expression (Figure 8c).

As for *P. banksiana*, an under-expression is noted at 800 mg/kg Ni corresponding to 0.25X of the level of water. It was slightly more repressed for the last two doses (1,600 mg /kg and 3,200 mg /kg) resulting in 0.15X level of expression for water (Figure 9a). In K series, all points are under-expressed, and no dose dependant trend was observed. (Figure 9b). When comparing the pairs, all the treatments are significant with Ni causing an overexpression at 150 and 800 mg/kg and under-expression compared to K at points 1,600 and 3,200mg/kg. The Ni and K series exhibit different patterns of expression (Figure 9c). The starting point at which overexpression is induced is at 1,600 mg/kg Ni for *P. strobus*. The starting point for repression is 800 mg/kg for both species.

3.3.4 SAT

The SAT gene was downregulated in *P. glauca* in all genotypes treated with Ni nitrate compared to water with the 150mg/kg Ni dose causing the the highest repression corresponding to about 0.15X of that of water. A dose-dependent trend is noticed where with increasing doses of Ni, expression increased with the highest expression at 3,200 mg/kg Ni with approximately 0.6X water (Figure 10a). As for the K controls, all doses induced an under expression compared to water except for 800 mg/kg with an overexpression of about 1.6X of water control. However, no dose trend is observed (Figure 10b). When comparing the pairs, Ni doses of 150 and 800

mg/kg are significantly under expressed compared to their K counterpart. The Ni and K series exhibited different patterns of expression (Figure 10c).

For *P. banksiana*, an overexpression was observed compared to water for 150 mg/kg Ni (1.8X), 1600mg/kg Ni (1.5X) and 3200 mg/kg Ni (2.5X) (Figure 11a). The K controls only show overexpression of about 1.4X compared to control for the 800 mg/kg. No dose-dependent trend was observed for these controls (Figure 11b). When comparing the pairs to their controls, Ni was significantly overexpressed compared to K at 150 and 3,200 mg/kg while under expressed compared to its K control at 800 mg/kg. The Ni and K series exhibited different patterns of expression (Figure 11c). Downregulation of SAT was observed in *P. strobus* while the same dose induced an upregulation in *P. banksiana*.

3.3.5 NAS

Repeatable results for the expression of this gene were observed only for the *P. strobus* species. All the Ni doses expressed strong suppression of NAS with levels lower than 0.1X of the water treatment (Figure 12a). But potassium nitrate also induced a significant decrease of expression compared to water with its lowest expression of 0.2X of the water observed with the 3200 mg/kg (Figure 12b). When potassium and Ni nitrates were compared, the K treatments exhibited a similar pattern of repression to Ni. However, the repression was much stronger for the Ni treatments compared to K (Figure 12c). For this gene, the effects of Ni and K in *P. strobus* were observed even at the lowest dose of 150 mg/kg.

3.3.6 GR

Expression of GR was only analyzed for *P. banksiana*. A slight overexpression was noted for both 800 and 1600 mg/kg Ni treatments compared to water as the levels were about 1.2X of water. But the highest dose of 3200 mg/kg induced an expression level that was 0.5X that of water (Figure 13a). The K controls showed an under expression in the first three doses collectively at about 0.6X of water and an overexpression at 3200mg/kg of about 1.6X (Figure 13b). When comparing the pairs, Ni is significantly over-expressed compared to its control at doses 150,800 and 1600mg/kg and is significantly under-expressed compared to K at 3200mg/kg. The Ni and K series exhibit different patterns of expression (Figure 13c). The starting point of overexpression stimulation for jack pine is at 150mg/kg for this gene and the point at which it experiences sensitivity to repression is at 3200mg/kg.

Primers targeting GR for *P. strobus* and NAS for *P. banksiana* did not amplify consistently and other major PCR products were present thus these were excluded from the analysis.

Table 3. Sequences of white pine (*Pinus strobus*) primers used for RT-qPCR

Target	Melting temp (° C)	Primers	Expected amplicon (bp)	PCR product in cDNA (bp)
Elongation factor 1-alpha (EF1 alpha)	F:61.6 R:61.93	F: GGGTTCCATCGCATTTGGCA R: GAAAGCAGCTTCGCCTTCCC	126	126
Urease accessory (AT2G16800)	F:61.76 R:61.92	F: ATATCCCTGTCGTGCCGCAT R: AGTGCCAGATGCCTTCCCTC	100	100
D-cysteine desulfhydrase mitochondrial (ACC deaminase)	F:62.57 R:61.93	F: CCTATGGGTTCGGCGTCGTT R: ATCCAATGGCAGCTCCTGCT	89	89
Metal transporter Nramp3-like (NRAMP3)	F:62.1 R:62.0	F: CATCTGGCGGAACACTTCA R: CACTTCAGGGACATCTGCTAC	102	102
Nicotianamine synthase (NAS)	F:62.11 R:62.4	F: CCCTCCACTCGCTTCCACAA R: GCCTTGCCTCGCCTATCCAT	197	197
Serine acetyltransferase chloroplastic-like (SAT)	F:62.5 R:62.6	F: TGGAGATGGCGTGCTAATTG R: GGCACTGTGGTGAGTACAAC	105	105

Table 4. Sequences of jack pine (*Pinus banksiana*) primers used for RT-qPCR

Target	Melting temp (° C)	Primers	Expected amplicon (bp)	PCR product in cDNA (bp)
Elongation factor 1-alpha (EF1 alpha)	F:62.18 R:61.71	F: GGCTGCGGTCAAGAAGGGTA R: ATGTTTGCATACGCCGAGCC	167	167
Urease accessory (AT2G16800)	F:61.76 R:61.92	F: ATATCCCTGTCGTGCCGCAT R: AGTGCCAGATGCCTTCCCTC	100	100
D-cysteine desulfhydrase mitochondrial (ACC deaminase)	F:62.72 R:61.61	F: TGGGTTCGGCGTCGTTCATT R: TCGCTCTGATGCTCTTGCCT	147	147
Metal transporter Nramp3-like	F:61.24 R:62.09	F: CAGGATTGCAGCGGGACAAA R: CGCATTGGGCAAGTGGTGTT	119	119
Glutathione reductase cytosolic (GR)	F:61.44 R:62.23	F: GCACTGCCGTGTCCTTCAAA R: CAAGTCCAGAGCCGAGGTCC	82	82
Serine acetyltransferase chloroplastic-like (SAT)	F:61.8 R:61.9	F: GTTGGCAATCCAGCACGGTT R: TGCTAATCCCTGGTGGGCAG	148	148

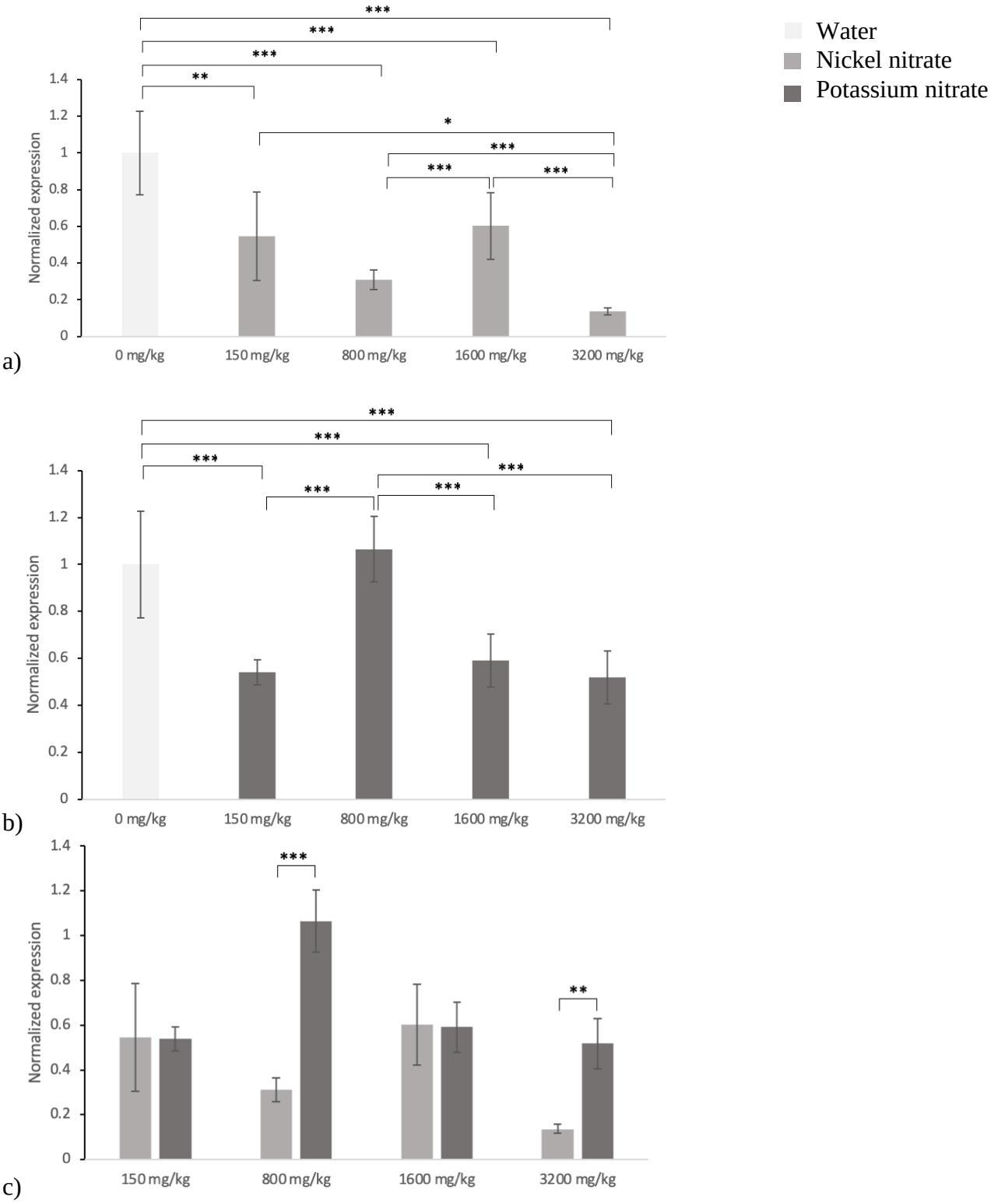

Figure 4. Expression of AT2G16800 gene in white pine (*Pinus strobus*) for a) water and nickel nitrate, b) water and potassium nitrate, c) salt pairs. Only significant differences are depicted ($p \leq 0.05$ is *, $p \leq 0.01$ is ** and $p \leq 0.001$ is ***). Error bars represent standard deviation.

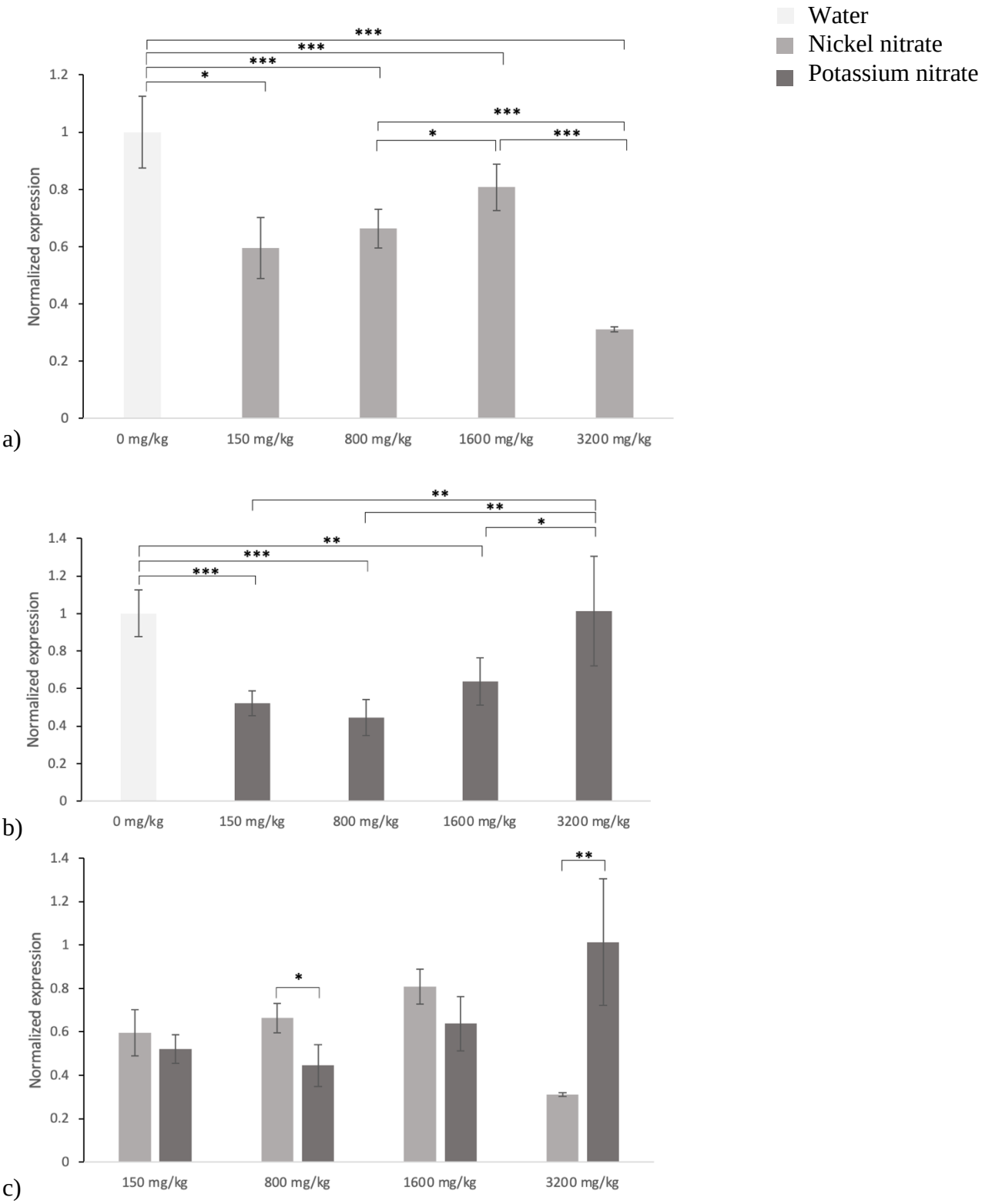

Figure 5. Expression of AT2G16800 gene in jack pine (*Pinus banksiana*) for a) water and nickel nitrate, b) water and potassium nitrate, c) salt pairs. Only significant differences are depicted ($p \leq 0.05$ is *, $p \leq 0.01$ is ** and $p \leq 0.001$ is ***). Error bars represent standard deviation.

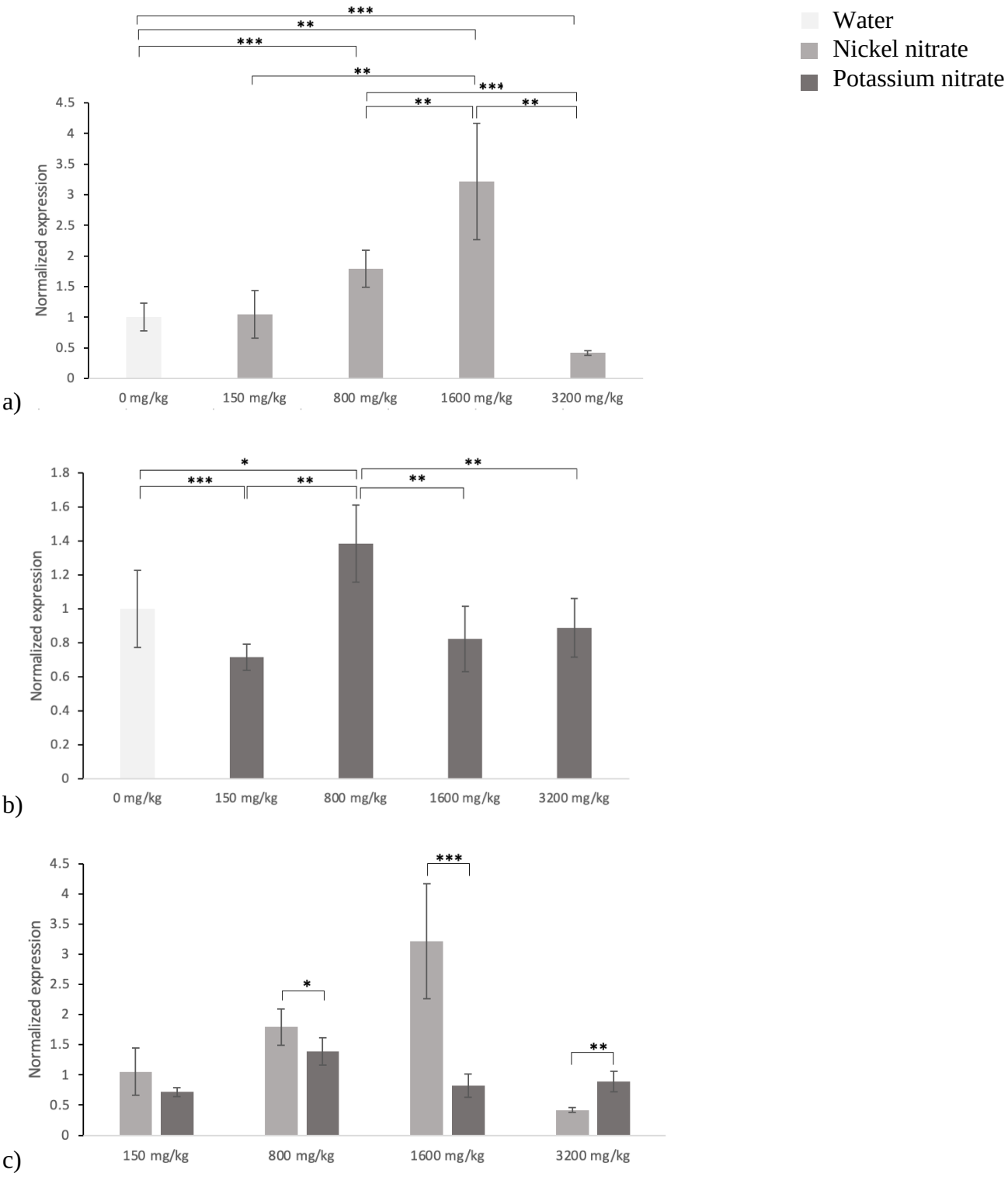

Figure 6. Expression of ACC deaminase gene in white pine (*Pinus strobus*) for a) water and nickel nitrate, b) water and potassium nitrate, c) salt pairs. Only significant differences are depicted ($p \leq 0.05$ is *, $p \leq 0.01$ is ** and $p \leq 0.001$ is ***). Error bars represent standard deviation.

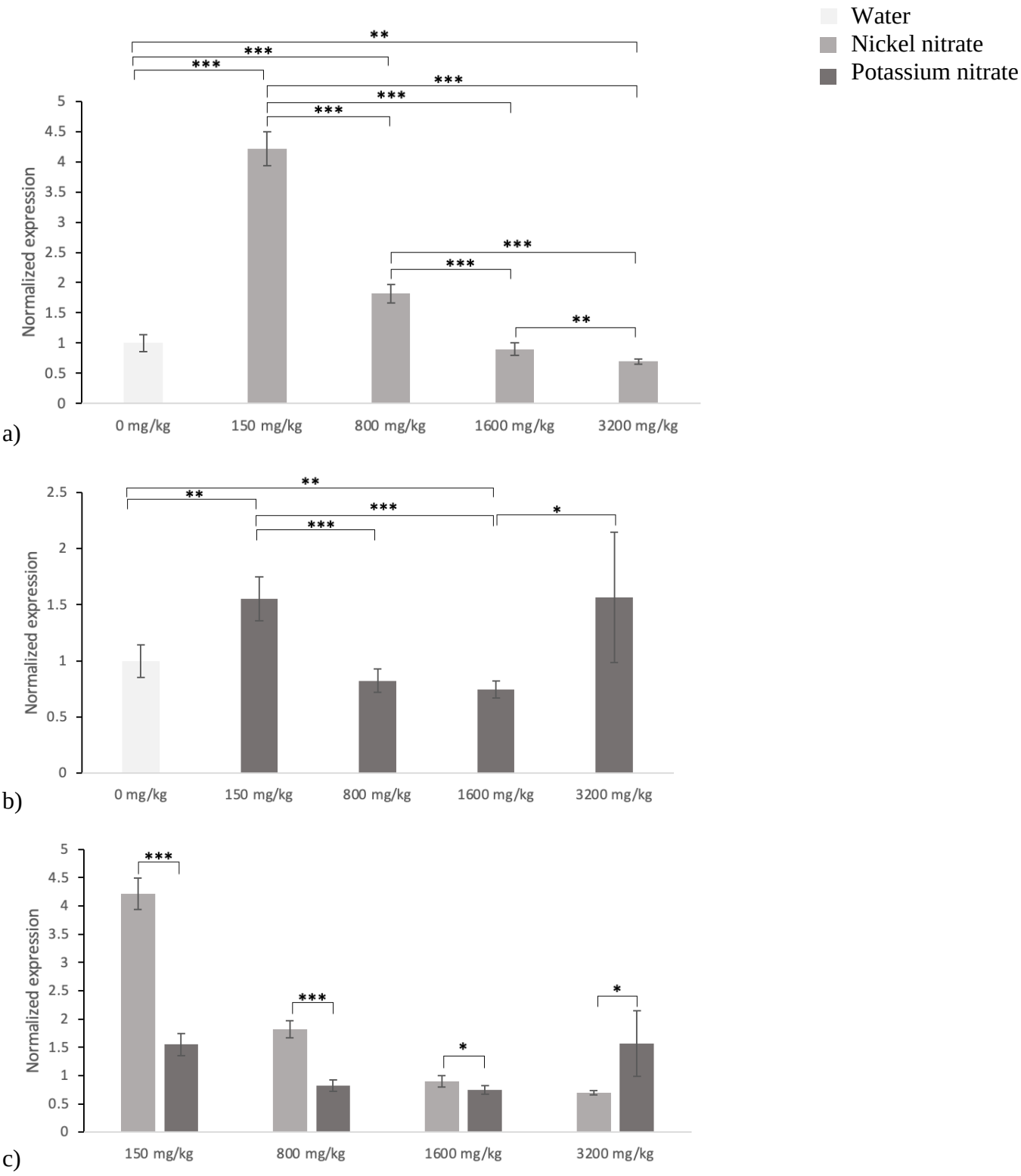

Figure 7. Expression of ACC deaminase gene in jack pine (*Pinus banksiana*) for a) water and nickel nitrate, b) water and potassium nitrate, c) salt pairs. Only significant differences are depicted ($p \leq 0.05$ is *, $p \leq 0.01$ is ** and $p \leq 0.001$ is ***). Error bars represent standard deviation.

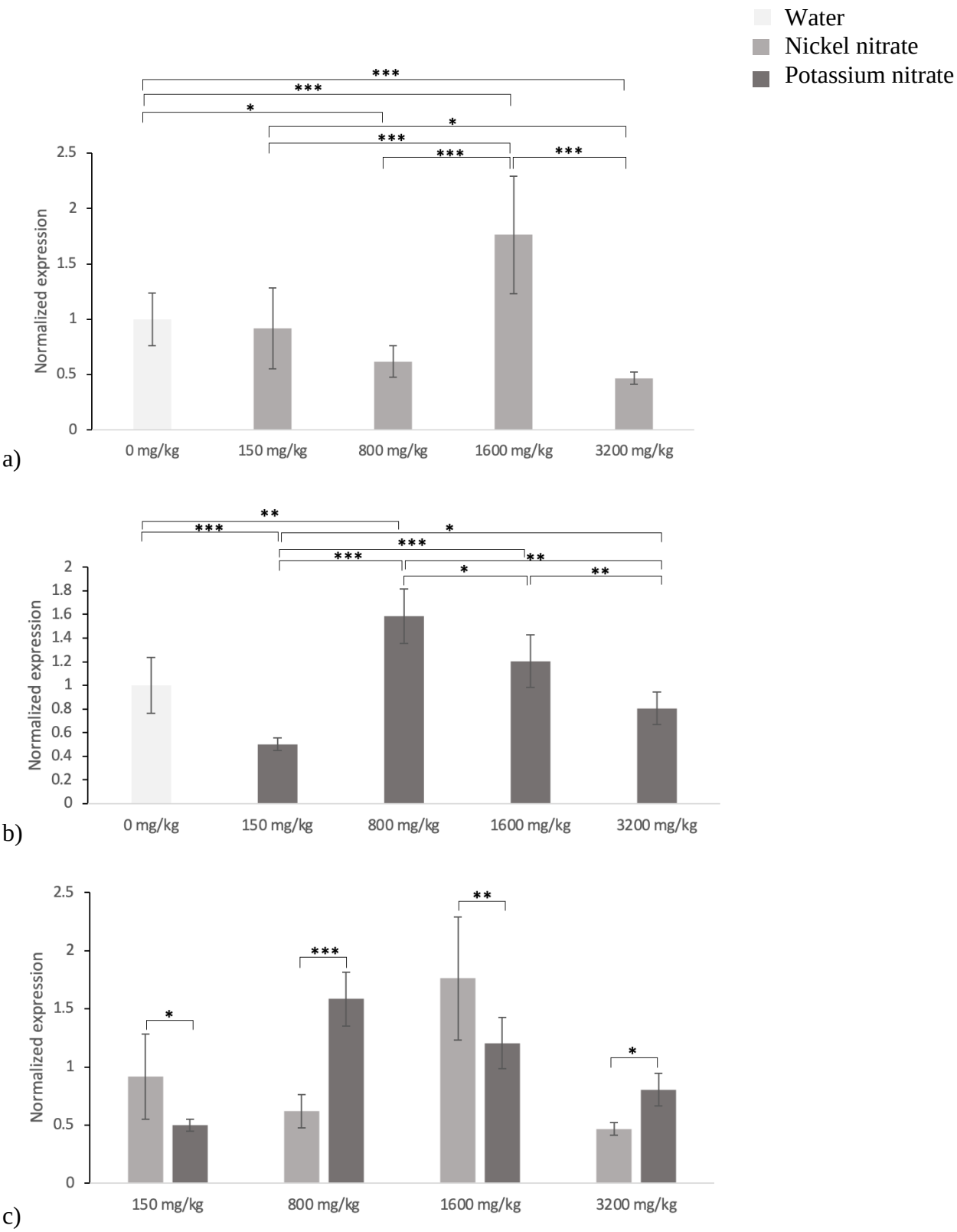

Figure 8. Expression of NRAMP3 gene in white pine (*Pinus strobus* for a) water and nickel nitrate, b) water and potassium nitrate, c) salt pairs. Only significant differences are depicted ($p \leq 0.05$ is *, $p \leq 0.01$ is ** and $p \leq 0.001$ is ***). Error bars represent standard deviation.

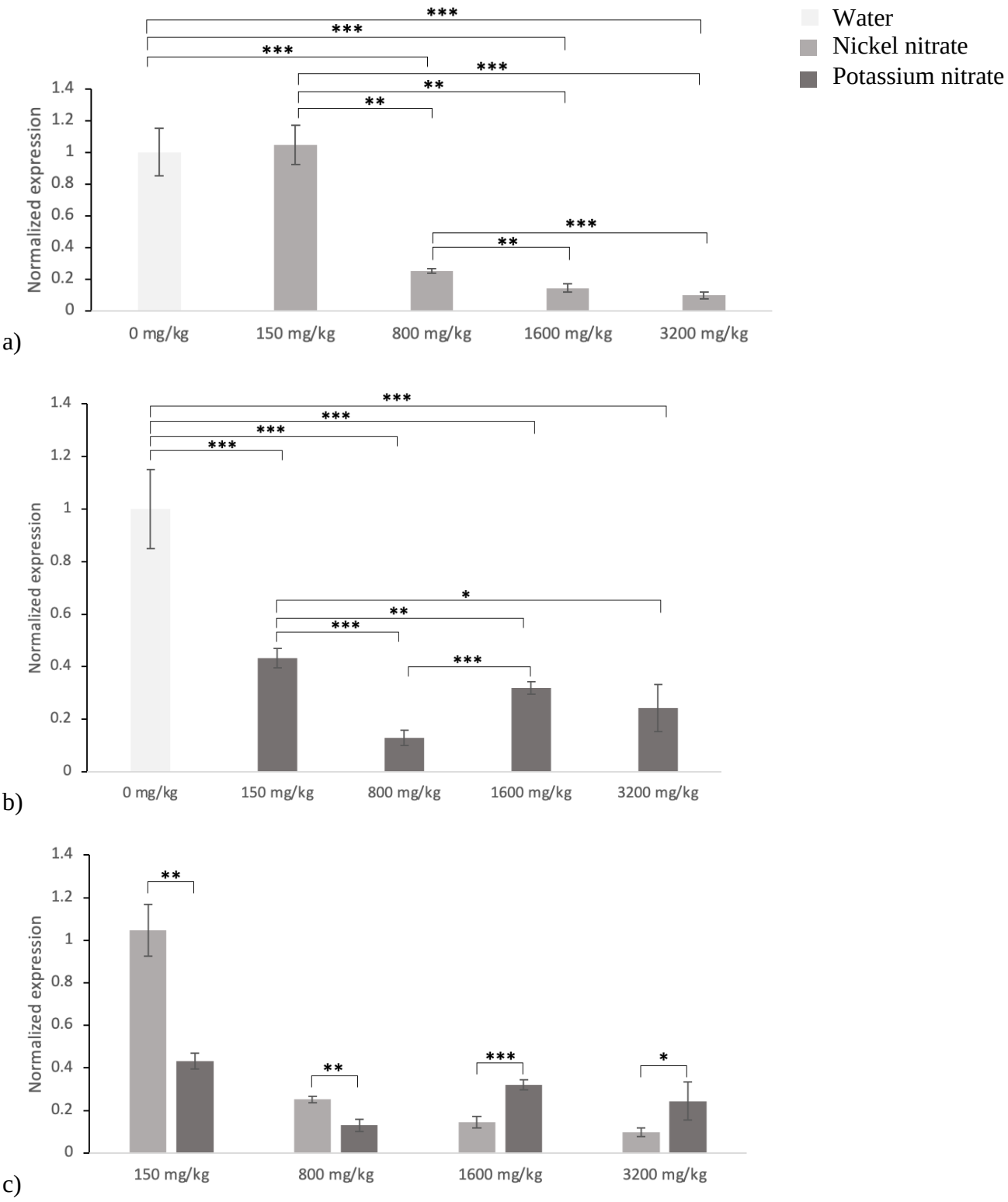

Figure 9. Expression of NRAMP3 gene in jack pine (*Pinus banksiana*) for a) water and nickel nitrate, b) water and potassium nitrate, c) salt pairs. Only significant differences are depicted ($p \leq 0.05$ is *, $p \leq 0.01$ is ** and $p \leq 0.001$ is ***). Error bars represent standard deviation.

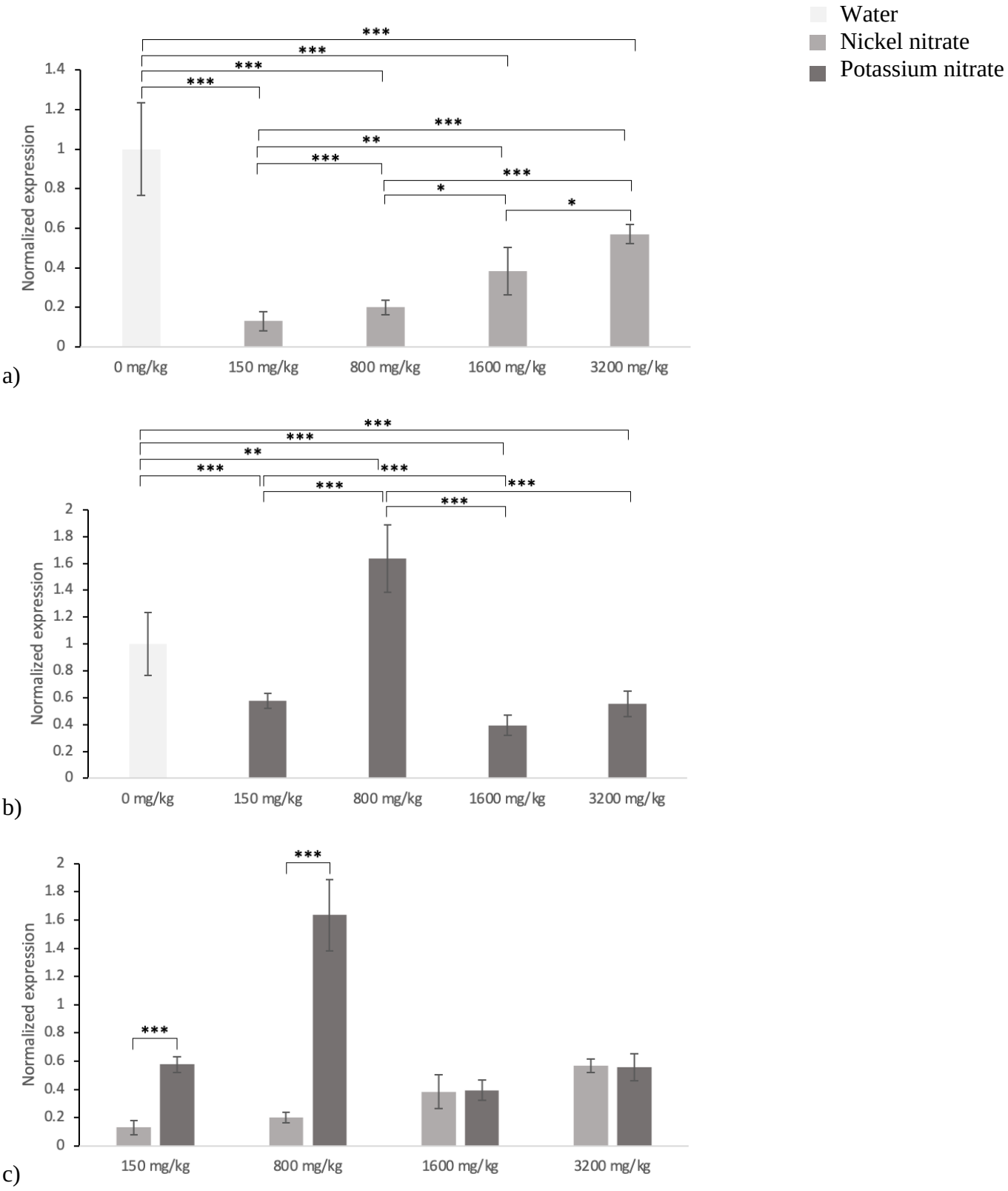

Figure 10. Expression of SAT gene in white pine (*Pinus strobus*) for a) water and nickel nitrate, b) water and potassium nitrate, c) salt pairs. Only significant differences are depicted ($p \leq 0.05$ is *, $p \leq 0.01$ is ** and $p \leq 0.001$ is ***). Error bars represent standard deviation.

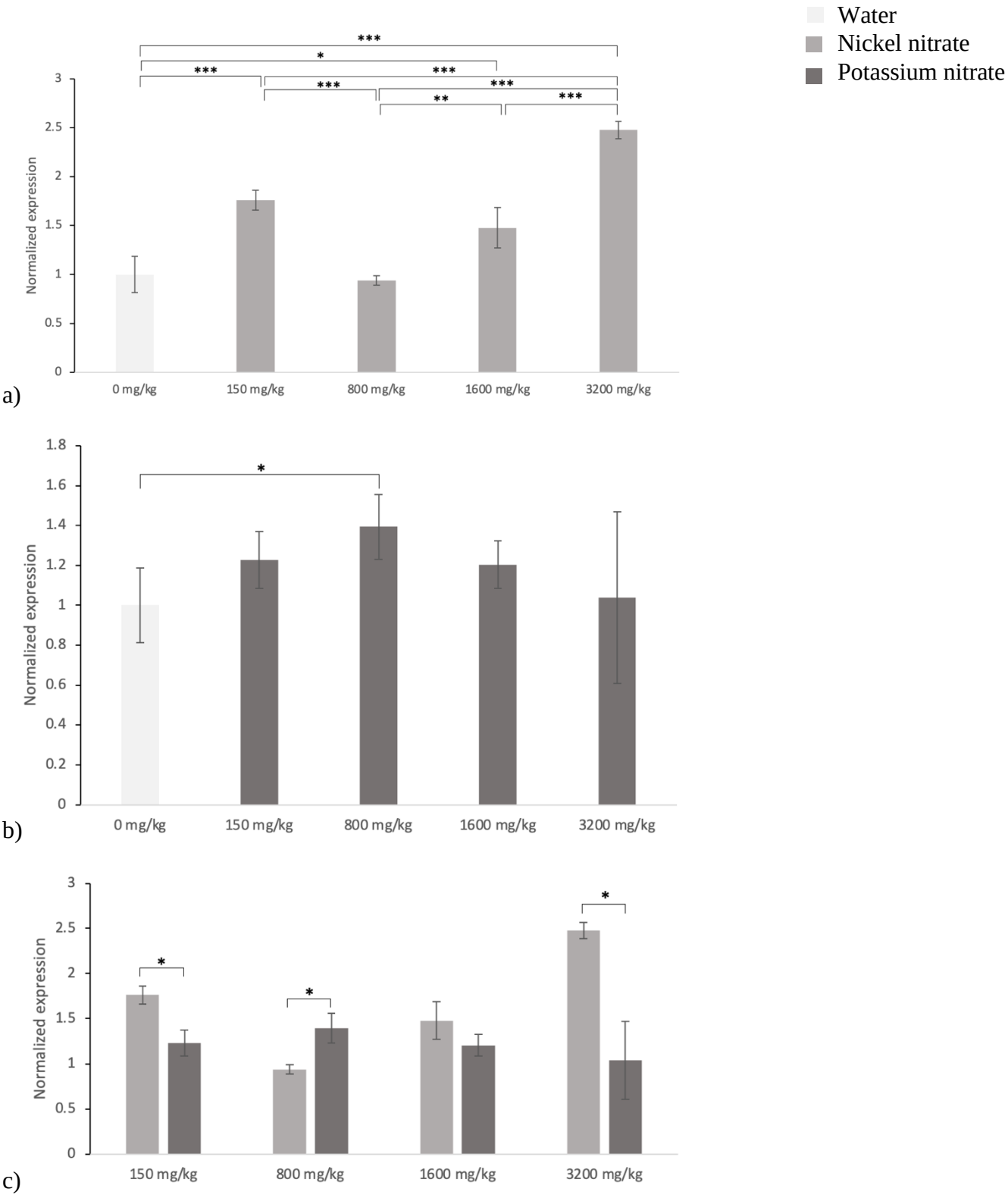

Figure 11. Expression of SAT gene in jack pine (*Pinus banksiana*) for a) water and nickel nitrate, b) water and potassium nitrate c) salt pairs. Only significant differences are depicted ($p \leq 0.05$ is *, $p \leq 0.01$ is ** and $p \leq 0.001$ is ***). Error bars represent standard deviation.

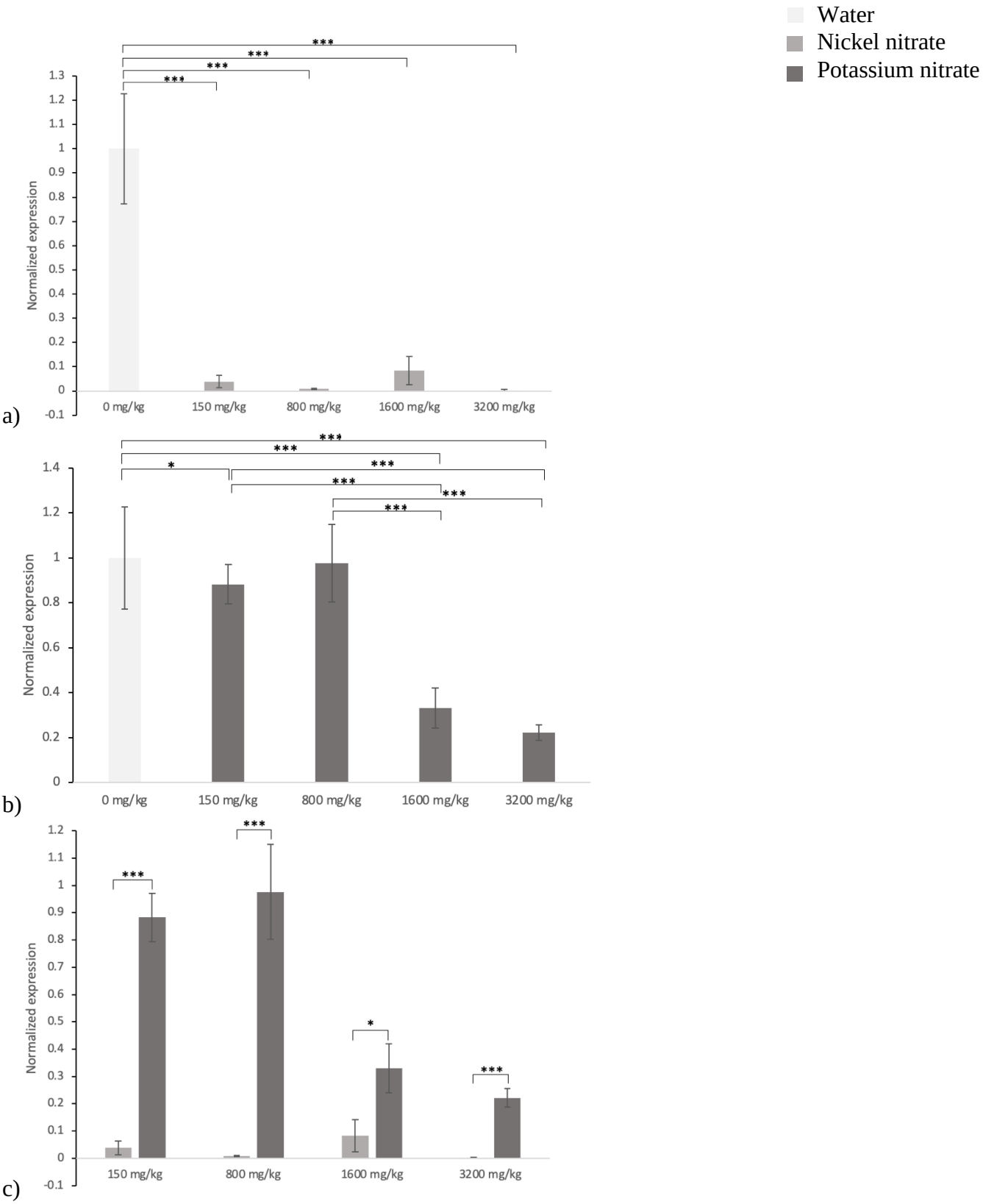

Figure 12. Expression of NAS gene in white pine (*Pinus strobus*) for a) water and nickel nitrate, b) water and potassium nitrate, c) salt pairs. Only significant differences are depicted ($p \leq 0.05$ is *, $p \leq 0.01$ is ** and $p \leq 0.001$ is ***). Error bars represent standard deviation.

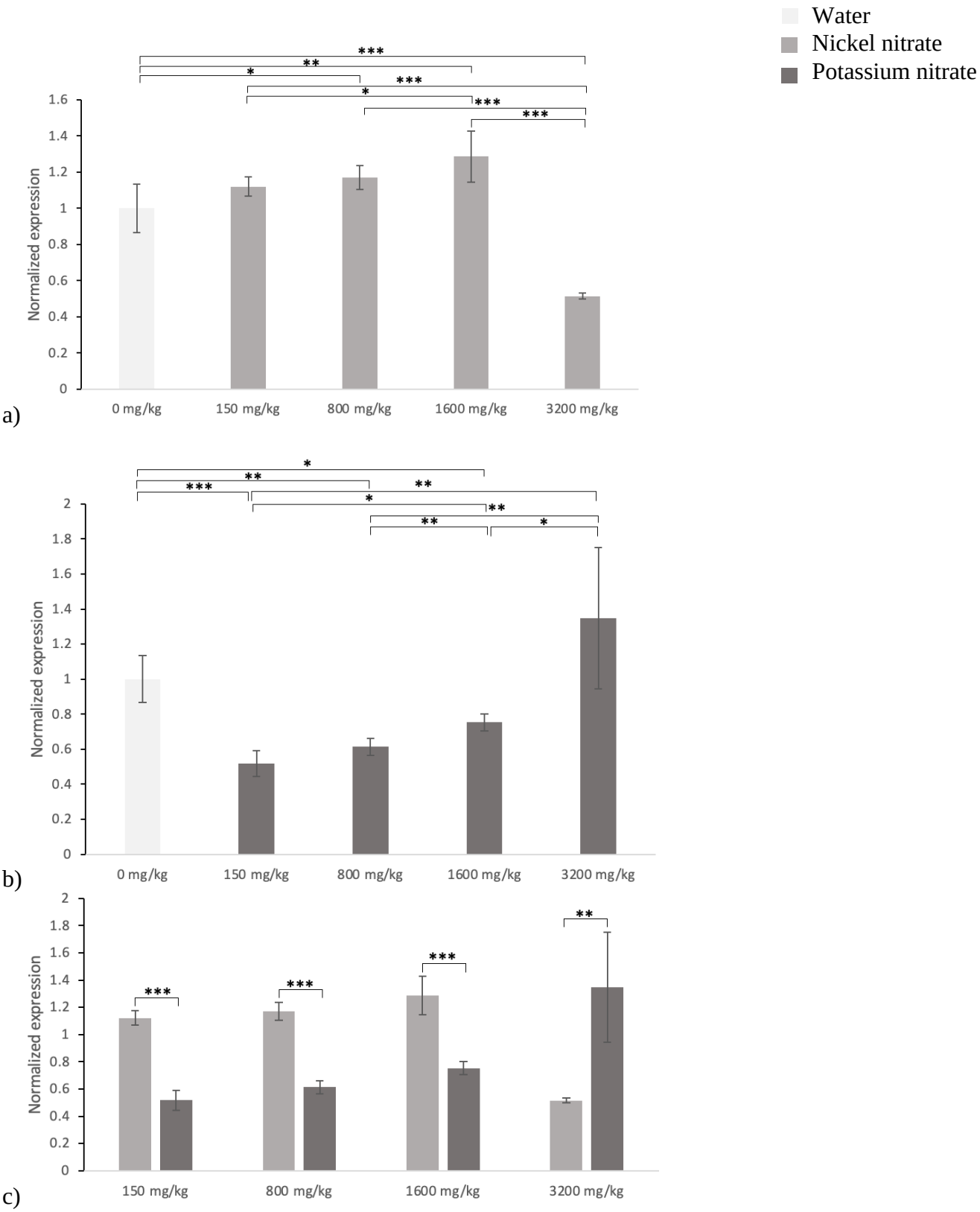

Figure 13. Expression of GR gene in jack pine (*Pinus banksiana*) for a) water and nickel nitrate, b) water and potassium nitrate, c) salt pairs. Only significant differences are depicted ($p \leq 0.05$ is *, $p \leq 0.01$ is ** and $p \leq 0.001$ is ***). Error bars represent standard deviation.

3.4 Discussions

This study aimed to investigate whether genes associated with nickel resistance are affected by different levels of nickel toxicity. These genes studied are involved in chelation and metal transport which are the main modes of resistance.

3.4.1 AT2G16800

AT2G16800 is a Ni^{2+} and Co^{2+} high-affinity transporter (Rodionov et al., 2006; Theriault et al., 2016). This gene was repressed in both species with the highest level of downregulation observed at the high concentration of Ni. This is consistent with data reported by (Theriault et al., 2016) studying Ni toxicity in *Betula papyrifera* (white birch). Since no differences were observed among the *B. papyrifera* susceptible, moderately susceptible and resistant genotypes with regard to AT2G16800 expression, it was concluded that this gene does not play a key role in *B. papyrifera* resistance against nickel toxicity (Theriault et al., 2016). Suppression of AT2G16800 induced by Ni was also observed at the 800 mg/kg Ni dose in *Populus tremuloides* (Czajka et al., 2019). Moreover, in field studies in the CGS, the level of AT2G16800 was lower in *Acer rubrum* trees from metal-contaminated sites compared to metal-uncontaminated sites (Kalubi et al., 2018). The same phenomenon was observed in *Quercus rubra* populations from the CGS (Djeukam & Nkongolo, 2018). The present study also shows some downregulation of AT2G16800 induced by potassium nitrate at high doses. However, both species show significantly more suppression at 3200 mg/kg Ni compared to their potassium counterparts.

3.4.2 ACC deaminase

ACC deaminase lowers levels of ACC and ethylene and confers metal resistance by allowing plants to accumulate heavy metals and function normally (Burd et al., 1998; Grichko et al., 2000; Stearns et al., 2005). Similarly, the benefits of ACC deaminase against Ni toxicity is reported in a study wherein transformed canola plants carrying the ACC deaminase gene showed much higher tolerance to Ni compared to non-transformed plants (Stearns et al., 2005). In this study, both species reveal an upside-down U trend wherein a peak and a downward expression were noted. In both species, the 3200 mg/kg Ni dose had the most suppressed expression of this gene. In *P. strobus* the peak in expression was observed at 1600 mg/kg while in *P. banksiana* it was observed at 150 mg/kg Ni. The effects observed at the peak and the low point can be solely attributed to Ni in *Pinus strobus*. In *P. banksiana* however, while the low point is attributed to Ni since its potassium control has the same level of expression as water, the Ni overexpression at the peak was also observed with the potassium nitrate used as control. Boyd (2020) reported an overexpression of ACC deaminase in *Picea glauca* samples treated with 150, 800 and 1600 mg/kg (Boyd, 2020). A similar inverted U effect is found in the transcription of ACC deaminase in *Quercus rubra* subjected to Ni with overexpression at 800 and 1600mg/kg Ni (Djeukam et al., 2019).

This U pattern observed in the gene expression response suggests a hormetic effect at play. Hormetic effect or hormesis is referred to a dose-dependent response where the low doses treatments result in a stimulatory effect and high doses of metals can have inhibitory effects (Agathokleous et al., 2019; Carvalho et al., 2020). This manifests itself as a right up or an inverted U response curve depending on the variable under study (Agathokleous et al., 2019; Carvalho et al., 2020). Typically 100% response is assigned as the baseline response to an ideal

growth condition (a control like water much like this study), any response higher or lower than 100% will result in a peak or dip respectively (Agathokleous et al., 2019; Carvalho et al., 2020). This response can be anything from biomass, length, to markers of oxidative stress, wherein this study looks at gene expression as a response which are indicators of oxidative stress. For instance, with increasing dosage of Cd, a study on GR activity in *Arabis paniculate* roots expresses a U effect (Qiu et al., 2008). Other studies (Jia et al., 2013) also comment on the hermetic effects of Cd for increased capacity to resists oxidative stress.

3.4.3 NRAMP3

The family of this membrane protein (NRAMP3) are non-specific metal transporters that are involved in the accumulation of heavy metals (Mizuno et al., 2005; Thomine et al., 2000). Visioli et al. (2014) report on the NRMAP3 overexpression at the 10 μM and 100 μM doses of NiSO4 in different populations of *Noccaea caerulescens*. In the present study, we only observed an overexpression in *P. strobus* at dose 1600 mg/kg Ni. This overexpression can be solely attributed to Ni since its potassium control had the same expression as water. The other doses resulted in a suppression in their expression of NRAMP3. In fact, a dose-dependent downward expression with the lowest expression at the 3200mg/kg Ni dose was observed in *P. banksiana*. Both species show a generalized suppression effect with increasing dosage of Ni except for the upregulation at 1600 mg/kg in *P. strobus.* The mechanism in the regulation of this gene remains elusive as another study on Ni toxicity in *P. glauca* reports no changes in NRAMP expression in needle samples compared to the water control (Boyd & Nkongolo, 2020). Another study targeting *Populus tremuloides* found no significant changes in NRAMP 4 expression at any Ni doses tested, yet concluded that the potassium nitrate at 800 and 1600 mg/kg led to

overexpression and thus played a more significant role than Ni in altering this gene expression (Czajka, 2018). Meanwhile, a study on tomatoes subjected to Cd toxicity reports increasing expression of NRAMP3 with increased Cd concentration up to the highest dose of 250 μM of Cd with the most NRMAP3 expression (Meena et al., 2018).

3.4.4. SAT

SAT results in elevated levels of GSH which scavenges ROS and aids the antioxidant response pathway (Freeman et al., 2004; Noctor et al., 1996; Wirtz & Hell, 2003). SAT enzyme activity and GR are thought to be overexpressed in response to heavy metals (Ali et al., 2008; C. Chen et al., 2009). This study shows an under expression of SAT in *P. strobus* however, it takes on a hormetic U effect with all doses still having lower expressions than water. This suppression cannot solely be attributed to Ni as potassium controls induced also lower expressions of SAT compared to water. In *P. banksiana* however, we observe an upregulation in 150, 1600 and 3200mg/kg dose. In another study on SAT, upregulation in 800 and 1600mg/kg Ni is noted in the leaves and roots of *Quercus rubra* (Djeukam et al., 2019). Another study on hyperaccumulating ecotypes (HE) and non-hyperaccumulating ecotypes (NHE) of *Sedum alfredii* Hance, reports on how incrementally increasing Cd concentration up to 100 μmol/L resulted in higher SAT activity in both ecotypes (with a slight plateauing effect at higher doses). While NHE was more sensitive to the dosage, HE had higher SAT activity induced by Cd (Guo et al., 2009).

3.4.5 NAS

Several studies have established the role of NAS in producing nicotianamine, a chelator product involved in the long-distance transportation of heavy metals such as iron (Inoue et al.,

2003). It has been demonstrated that transgenic tobacco plants expressing NAS accumulate more iron and have higher Ni tolerance (Douchkov et al., 2005). While an investigation on different populations of *Noccaea caerulescens* reports on NAS overexpression at doses 10μM and 100μM (Visioli et al., 2014), the present study has found a total suppression in *P. strobus* treated with different doses of Ni doses. Djeukam et al., (2019) reported an under expression of NAS3 for doses 150mg/kg Ni in *Quercus rubra*. They also observed a lower expression of NAS3 in *Quercus rubra* trees growing in metal-contaminated sites compared to genotypes from uncontaminated areas in the CGS (Djeukam & Nkongolo, 2018). Another study on *Aradopsis thaliana* concluded that NAS gene is activated and overexpressed in response to iron and zinc deficiencies (Wintz et al., 2003).

3.4.6 GR

GR, much like SAT, contributes to the build-up of GSH and thus the maintenance of GSH- dependant antioxidative pathway (Freeman et al., 2004). Rao and Reddy compiled a list of studies wherein increased activity of GR is recorded in response to heavy metal toxicity in *Aradopsis thaliana*, sugar cane, potato, radish and soybean (Rao & Reddy, 2008). This study shows a slight overexpression in the shape of a slightly inverted U pattern for the Ni series in *P. banksiana.* Interestingly, the potassium controls show a much more pronounced effect resulting in an upright U pattern. This suggests that the nitrate ions have a stronger effect on this gene. A somewhat similar hormetic effect is observed for GR expression in the leaves of *Arabis paniculata* subjected to increasing Cd toxicity (Qiu et al., 2008). An over-expression of GR in *P. glauca* roots in response to Ni treatments has also been reported (Boyd, 2020). While another study reports downregulation of GR at 1600mg/kg Ni for *Betula papyrifera* (Theriault et al.,

2016).

3.5.6 Effects of nitrate ions

Regarding the expression profile of nitrate controls, both upregulation and downregulation was observed. Nitrate ions have been found to play a role in changing gene expression in the roots of *Aradopsis thaliana* (Alvarez et al., 2019). Regarding nitrate, before it can be used, it must be converted to ammonia and ammonia can be toxic by penetrating the cell membrane and resulting in the elevation of cytoplasmic pH (Li et al., 2013). This may account for the suppression observed due to potassium nitrate controls. On the other hand, nitrate has always been known for its properties to benefit plant growth and development (Coruzzi & Bush, 2001; Marschner, 1995). For instance total leaf chlorophyll concentrations and root ammonium concentration increase in high nitrate doses (7.5 and 10 mM) (Horchani et al., 2010). Nitrate is also able to promote plant restoration by reducing oxidative stress through increasing antioxidant substances and their activity (Li et al., 2013). This may explain the overexpression of some of the target genes studies by the potassium nitrate controls.

In conclusion, hormesis effect was noted for ACC, SAT, GR and an over-expression observed was in genes ACC (both species) SAT (*P. banksiana)* and GR *(P. banksiana),* whereas the AT2G16800, NAS and NRMAP3 were mostly suppressed for both species. Moreover, both species had similar responses to AT2G16800, ACC, SAT and NRAMP3. All gene targets were repressed at the highest dose of Ni (3200mg/kg) except for SAT in both species. The highest dose of Ni is extremely toxic as it is 2X the total reported toxicity in contaminated fields. Nitrate ions affected expression (both suppression and overexpression) and their influence must be considered when accounting for Ni nitrate toxicity.

Chapter 4: General conclusions and further studies

4.1 Summary

The objectives of this study were to 1) determine if *P. storbus* or *P. banksiana* are Ni resistant and 2) If Ni associated genes will show differential expression when induced to increasing dose of Ni and its controls. The first part of the component of this study revealed that *P. banksiana* to be more physically distressed than white pine especially in the two doses of Ni and the last dose of the nitrate control. The reason for this may lie in their evolutionary habitat, sensitivity to Ni nitrate and potassium nitrate salts or their differential ability to incorporate nitrate ions efficiently. This study shoed an upregulation (ACC, SAT and GR) and a downregulation (NRAMP3, NAS, AT2G16800) as well as differential expression induced by potassium nitrate controls confirming that nitrate can affect gene expression.

4.1 Future directions

Aside from assessing gene expression, there are other means of assessing the antioxidant pathway activity. Direct identification of short-lived ROS levels in plants is challenging and looking to the changes in the antioxidant system has its own limitations when trying to draw inference on the amount and direction of ROS production since this is a mechanism in response to oxidative stress to subdue the effects of ROS metabolization (Lin et al., 2007). This mechanism is affected by many intervening factors and Lin et al. (2007) suggest this to be the reason why many reports on antioxidant responses to heavy metals such as Cd can be inconsistent. The current study targeted the gene expression which is a few steps upstream of the activity of antioxidant enzymes, hoping that this upstream step would be elucidating as to some of the major changes that can take place on a genetic level. The vast majority of the literature has

focused on the activity of the enzymes or the transgenic expression or overexpression of these genes of interest and subsequently commenting on conferred heavy metal accumulation as a result of the expression of these genes (Kim et al., 2005; Stearns et al., 2005). However, this study is investigating the reverse process; that is whether heavy metals such as Ni can induce overexpression of these genes. This turns into a chicken and egg scenario wherein it could be worthwhile looking into which comes first or which is more significant for a species of interest; the exposure to heavy metals and altered gene overexpression or altered gene overexpression and heavy metal accumulation. These two may also feed into each other and do not have to be mutually exclusive for instance this was true for the overexpressed genes in this study (ACC, SAT and GR) which literature suggested their increased activity. Yet while some studies suggest that the enzyme activities of NAS, NRAMP or AT2G16800 activity will be enhanced, this does not necessarily mean that this will be reflected in their gene expression as was the case for the results of this study.

Epigenetics in metal-induced changes is another area of interest wherein these genetic changes can prove to be transgenic and manifest themselves in their offsprings (Carvalho et al., 2020). Hence epigenetics studies on the topic of heavy metal-induced gene expression, could prove to be very insightful about its long-term effects.

It will be interesting to test the sensitivities of *P. glauca* and *P. banksiana* to different salt candidates for this study, not only will this be insightful for the first part of the study in terms of the damage ratings but also for the second component of the study on differential expression. Similar Ni toxicity studies have used NiCl (Hoegler & Hecht, 2018), NiSO4 (Visioli et al., 2014), Ni acetate (Freeman et al., 2005) instead of the nitrate salt and perhaps another anion candidate will have minimal influence on gene expression.

Studying the effects of other heavy metals such as Zn, Cu, Cd, Co and Fe on the target genes can reveal insight about the specificity or the multi-functionality of the genes for certain heavy metals and whether their hormesis patterns are similar or not. Likewise investigating other heavy metals associated genes such as those traditionally associated with other metals (ex IREG1, ZAT11 (Theriault et al., 2016), MRP4 (Kalubi et al., 2018)) can provide us with other potential targets for Ni toxicity studies in white pine and jack pine.

Depending on tissue types (such as needles/leaves versus shoots), hormesis can manifest differently (Carvalho et al., 2020), therefore plant tissue focused studies such as metal translocation analysis can provide a more comprehensive outlook on not only the location of the metal accumulation but also tissue-specific activation of target genes (Boyd, 2020). Insight in accumulation behaviour and depending on whether a plant is accumulating or the non-accumulating type, its growth can be promoted or inhibited when subjected to metal toxicity as in the example of hyperaccumulating ecotype (HE) *Sedum alfredii* Hance whose growth was promoted when exposed to high concentrations of Cd and its non-hyperaccumulating ecotype (NHE) whose growth was inhibited (Guo et al., 2009). Moreover, the accumulation type can have implications on the sensitivity to metal which can be reflected in the expression levels of target heavy metals associated genes such as SAT (Guo et al., 2009).

When identifying phytoremediation candidates to clean up heavy metals, hormetic effects should be taken into account as suggested by a Cd toxicity and hormesis study in hyperaccumulator species *Lonicera japonica Thunb* (Jia et al., 2013). Investigating other boreal forest species native to the GSR mining sites such as white spruce, red pine, red maple etc. could provide us with potential phytoremediation candidates. Moreover, if in the future, white pine and jack pine are fully sequenced, this eliminates the need to rely on close species such as *Pinus*

taeda, however in the meantime, closely related species are a great resource to design primers for targeting metal associated genes of interest. With a concerted effort to inform our mining policies that concern the health of our plant biota and by taking into account remediation strategies such as stress suppressors, we can begin to bridge the gap in our mining safety knowledge. For instance, adding Si as suggested in a study on Cd toxicity is reported to have diminished the effects of the toxicity of the metal and help with increasing antioxidative enzyme response and reducing ROS production in *Arabidopsis thaliana* (Carneiro et al., 2017). Zn is also reported to alleviate some of the effects of Cd toxicity (Hassan et al., 2005). Perhaps similar strategies can be employed to alleviate Ni toxicity.

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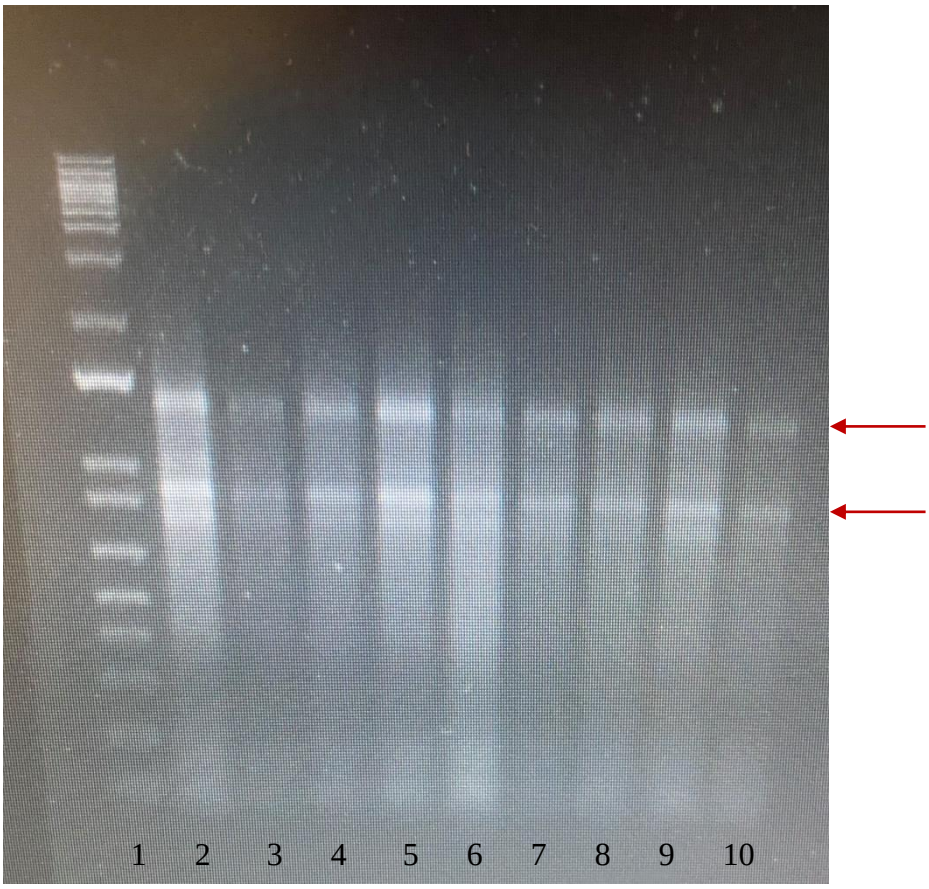
Appendices

Appendix 1. *Pinus strobus* seedlings in growth chamber prior to treatment.

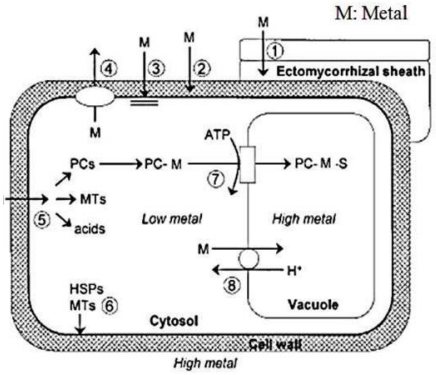
Appendix 2. (a) *Pinus banksiana* labels for the 9 treatment groups (b) *Pinus banksiana* arranged in randomized block design pre and post-treatment.

a) b)

Appendix 3. 1% agarose gel of extracted RNA samples from *Pinus strobus* needles. Arrows depicts intact RNA bands and lane 1 is the 1k-b ladder and lanes 2-10 are the extracted needles from the 9 treatment groups.



Appendix 4. A schematic of avoidance and tolerance strategies a) adapted from (Dalvi & Bhalerao, 2013) originally in (Hall, 2002). b) (Dalvi & Bhalerao, 2013).

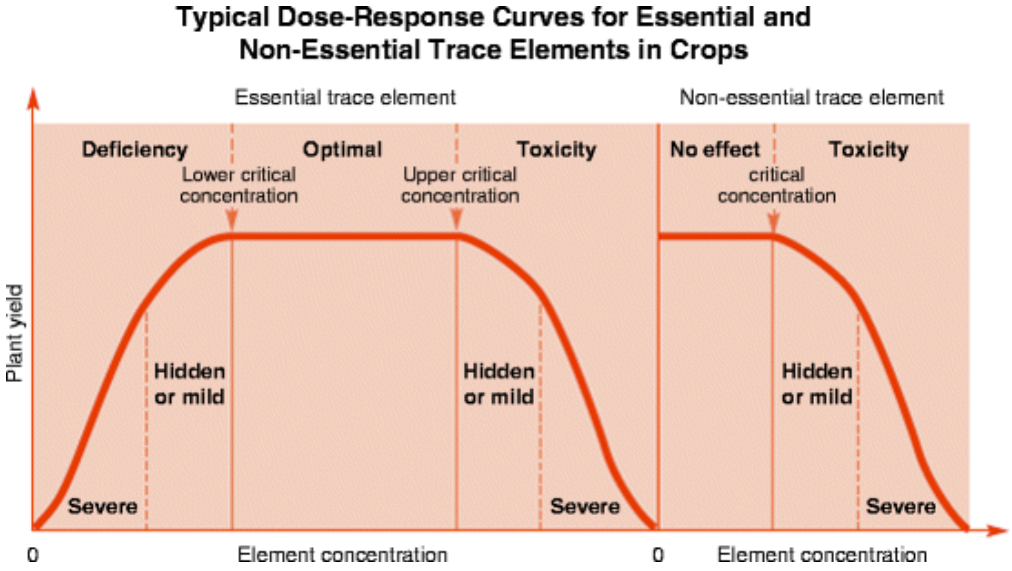


a)

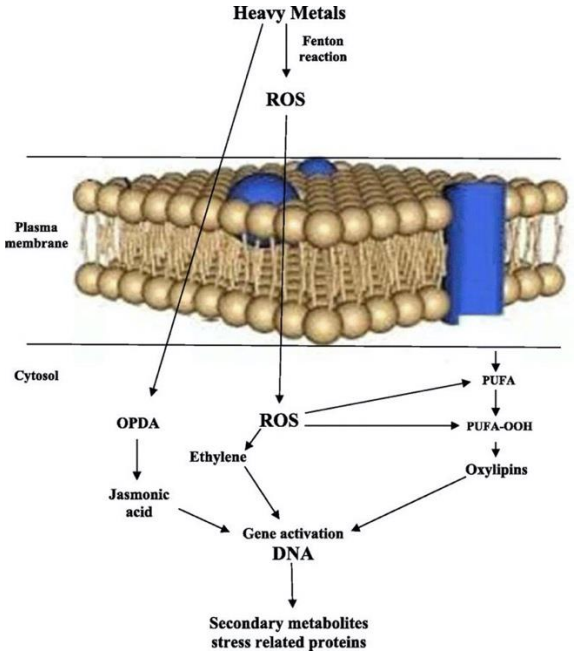
1. Restriction of metal movement to roots by mycorrhizas,
2. Binding to cell wall and root exudates,
3. Reduced influx across plasma membrane,
4. Active efflux into apoplast,
5. Chelation in cytosol by various ligands,
6. Repair and protection of plasma membrane under stress conditions,
7. Transport of PC-M complex into the vacuole,
8. Transport and accumulation of metal in vacuole

b)

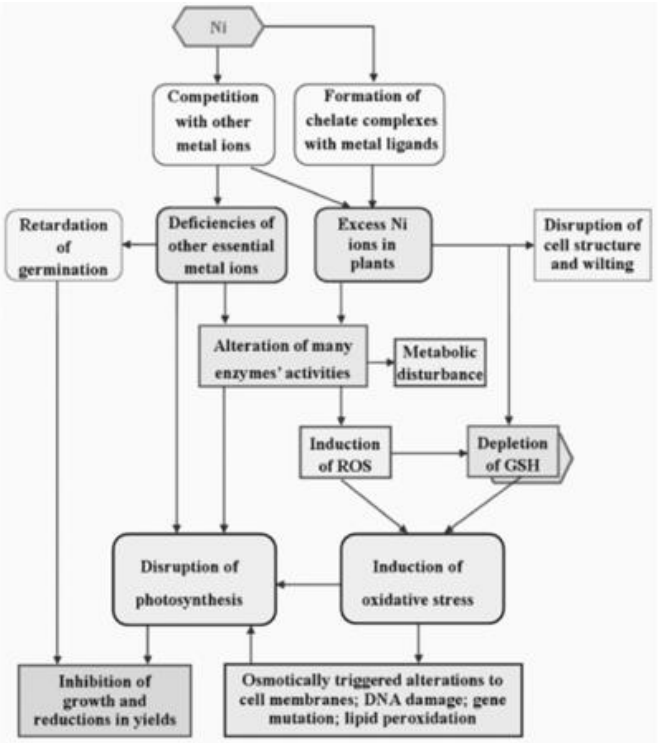
Appendix 5. Dose-dependent response curve for essential and non-essential trace elements in crops (Alloway, 2013).



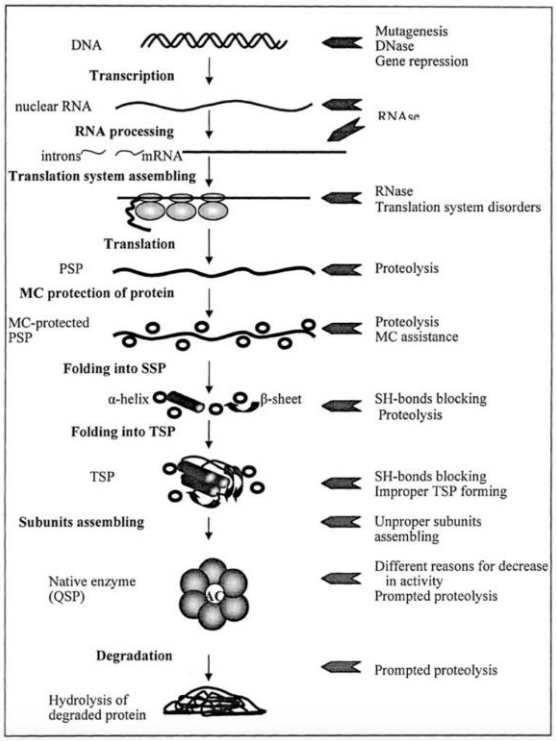
Appendix 6. ROS pathway and gene activation induced by heavy metals (Maleki et al., 2017).



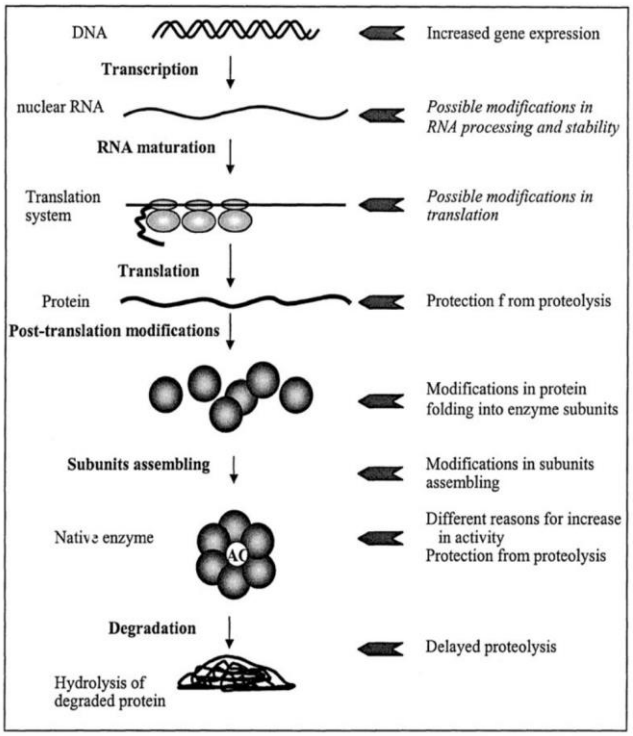
Appendix 7. The mechanism of Ni toxicity; the deficiency of other metals and oxidative stress effects intertwine and result in physical stress such as growth inhibition, yield reduction and wilting (Chen et al., 2009).



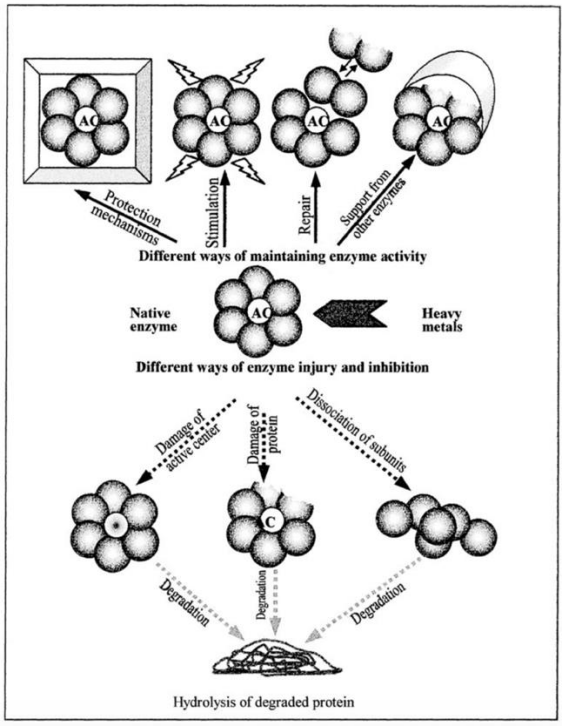
Appendix 8. a) Heavy metal-induced upregulation and maintenance of enzyme b) Heavy metal-induced downregulation and maintenance of enzymes c) An illustration of how enzymatic activity is maintained or inhibited by heavy metals (Siedlecka & Krupa, 2002).



a)



b)



c)