

**Effects of Mineral Nutrition and Iron Supply on Growth and
Physiological Responses of Selected Boreal Plant Species to
Root Zone pH**

by
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Abstract

High soil pH can aggravate the effects of water deficit stress, inhibit root growth, and reduce the availability of essential elements to plants, especially iron. Iron deficiency can severely decrease plant growth and yield and result in plant mortality. In my study, I examined the effects of root zone pH and the supply of iron and other essential mineral nutrients on several species of boreal forest plants including trembling aspen (*Populus tremuloides*), red osier dogwood (*Cornus sericea*), jack pine (*Pinus banksiana*), white spruce (*Picea glauca*), black spruce (*Picea mariana*), tamarack (*Larix laricina*), paper birch (*Betula papyrifera*), green alder (*Alnus viridis*), blueberry (*Vaccinium myrtilloides*), and bearberry (*Arctostaphylos uva-ursi*). Several studies were carried out through the controlled-environment experiments in sand culture and hydroponics. These studies were aimed at helping to understand the processes in plants that contribute to high soil pH tolerance and improve the revegetation success of oil sands reclamation areas. The results of Chapter 2 demonstrated that the responses to high pH varied between studied plant species, likely due to their different nutrient demands. In high-pH sensitive plants, the high root zone pH reduced the plant biomass, net photosynthetic rates, transpiration rates, leaf chlorophyll concentrations, chlorophyll a to b ratios, and tissue concentrations of essential elements. The increased supply of essential mineral nutrients had a beneficial impact, but only on the total dry weights in trembling aspen and when added at the lower pH levels. In Chapter 3, I found that the positive impact of increased iron supply was effective only when the pH of the growth medium was neutral (pH 7). Therefore, the increased supply of mineral nutrients can be effective in overcoming high soil pH stress when the conditions for nutrient uptake and utilization are maintained. In the split-root experiment of Chapter

4, the high pH stress could be alleviated when part of the root system was exposed to lower pH. This improvement may be attributed to acidified localized areas that increase the solubility and uptake of micronutrients. The results also demonstrated that high pH induced stress responses in plants through the part of the root system that was exposed to high pH and affected the responses of the other part of the root system that was exposed to low pH. Among the studied plant species, white spruce was found to be relatively tolerant to high pH and its tolerance mechanisms may be related partly to higher biomass allocation to the roots. Trembling aspen and dogwood also showed some degree of high pH tolerance and the results suggested that dogwood may have higher Fe utilization efficiency and trembling aspen may be able to allocate more root biomass to the part of the roots exposed to high pH conditions when supplied with high levels of mineral nutrients. These plants could be considered as desirable candidate species for the revegetation of oil sands reclamation sites.

Preface

This document presents three studies (Chapters 2, 3 and 4) intended for publication. All the work in the thesis was done by myself including experiments implementation, data collection and analyses, literature review, and writing throughout this document. Dr. Janusz Zwiazek was involved in concept formation and manuscript review throughout the work.

The sand culture pH control and watering system mentioned in chapter 2 was designed by myself, with the assistance from Dr. Mónica Calvo-Polanco. It took me about half a year to develop the sand culture protocol. The hydroponic pH control system mentioned in chapter 3 was designed by Dr. Mónica Calvo-Polanco. The split-root hydroponic system mentioned in chapter 4 was designed by Dr. Wenqing Zhang and me together.

The study of effects of ectomycorrhizal associations on pH tolerance in boreal forest plants was also completed, but it is not included in this thesis since there were no significant differences in responses of control and treatments applied plants. The reasons need to be figured out and then this study need to be tried again in the future because it is a good idea to find out a way to make the plant better tolerate high pH stress with the help of ectomycorrhizal associations.

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Never give up!

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List of Abbreviations

AQP	aquaporin
ANOVA	analysis of variance
BPDS	bathophenanthrolinedisulfonic acid
CDC	critical deficiency concentrations
Chl a/b	chlorophyll a to b ratios
ChlO	chlorophyll concentration in old leaves (needles)
ChlY	chlorophyll concentration in young leaves (needles)
DMSO	dimethyl sulfoxide
DW	dry weight
E	transpiration rate
EC	electric conductivity
EPEA	Environmental Protection and Enhancement Act
FCR	ferric chelate reductase
Fe-S	iron-sulphur protein
GHG	greenhouse gas
NA	non-proteinogenic amino acid
ICP-MS	inductively coupled plasma mass spectrometry
Pn	net photosynthetic rate
PPFD	photosynthetic photon flux density
PVC	polyvinyl chloride
SAGD	steam assisted gravity drainage
s/r ratio	shoot to root dry weight ratio

Chapter 1

Introduction and literature review

1.1 Introduction

High soil pH conditions profoundly affect plant productivity, largely through the effect on nutrient availability. More than 30% of the world soils have a high pH problem (Chen and Barak 1982) and are classified as calcareous, saline, or sodic soils. Human activities causing land disturbance, such as oil sands mining in northeastern Alberta, can locally aggravate the soil pH problems. Oil sands mining causes severe disturbance to boreal ecosystems, which involves a complete removal and reconstruction of landforms and production of large volumes of high pH tailings from oil extraction processes. In the oil sands reclamation areas, soil pH values frequently exceed 8.0 while the pH of undisturbed soils in the nearby boreal forest is typically below 6.0 (Howat 2000). This high soil pH may be of particular concern to the acid-loving plant species in the forest understory. However, little research has been conducted to understand the effects of pH on the growth and physiology of forest plants in the absence of other confounding factors, such as salinity (Kopittke and Menzies 2005, Yousfi et al. 2007). This knowledge is essential to improving the revegetation success of oil sands reclamation areas that are affected by high soil pH.

High soil pH is detrimental to growth and yield of many species of plants. High pH soils are usually associated with low availability of several essential mineral nutrients including Fe, Mn, P, and Zn (Lindsay 1984, Yang et al. 1994, Valentine et al. 2006), and sometimes

B and N (Marschner 2012). Deficiencies of these nutrients have a profound effect on many physiological processes in plants (Marschner 2012). High pH can also aggravate the effects of water deficit stress, and result in reductions of stomatal conductance (Tang and Turner 1999, Kamaluddin and Zwiazek 2004), shoot water potential (Tang et al. 1993b) and root water flux (Kamaluddin and Zwiazek 2004). This may be related to changes in the aquaporin (AQP) activities, which mediate the cell-to-cell pathway for root water flux (Voicu and Zwiazek 2004, Aroca et al. 2006). A direct effect of high pH on plant root growth may be due to the plant's inability to maintain low apoplastic pH that is required for cell wall loosening and cell expansion (Zhang et al. 2015).

Among the mineral nutrients that may be deficient in high pH soils, Fe plays a significant role in many metabolic processes such as respiration, photosynthesis, and chlorophyll synthesis. Plants subjected to Fe deficiency may form more root hairs (Römheld and Marschner 1981), increase the release of protons to the soil (Landsberg 1981, Römheld et al. 1984, de Vos et al. 1986), reduce more Fe^{3+} to Fe^{2+} (Bienfait et al. 1983) and increase organic acid synthesis (Landsberg 1981). These mechanisms help enhance Fe solubility in soil and Fe uptake by roots (Bienfait 1988).

In my thesis research, I focused on the effects of high pH, mineral nutrition, and Fe supply on the growth and physiological processes of selected boreal forest plant species that are commonly used for oil sands reclamation. I carried out all studies under controlled-environment conditions with plants growing in hydroponics and sand culture. The main objective of the studies was to develop better understanding of the processes contributing

to high pH tolerance in forest plants by:

- 1) Examining the effects of root zone pH and mineral nutrient supply on the growth and physiological responses in different boreal forest plant species.
- 2) Determining the effectiveness of supplemental Fe on plant responses to elevated pH.
- 3) Investigating plant responses to heterogeneous root zone pH and Fe levels.

I have examined the following hypotheses:

- 1) Boreal forest plants vary in their sensitivity to high root zone pH, and in their abilities to absorb mineral nutrients at high pH.
- 2) Fe deficiency is the main detrimental factor affecting plants exposed to high pH and the effects of high pH can be alleviated by increasing Fe supply.
- 3) High pH effects on plants can be alleviated by exposing part of the root system to lower pH conditions to facilitate nutrient uptake.

The thesis consists of the following five chapters:

Chapter 1: Provides an overview of oil sands mining and reclamation, effects of high pH and Fe on plants, and the biology of studied plant species.

Chapter 2: Describes the research investigating the effects of root zone pH and two mineral nutrition levels on growth and physiological responses of trembling aspen, jack pine,

and white spruce in sand culture.

Chapter 3: Reports results of the study that examined how Fe availability affects responses of paper birch, white spruce, green alder, and tamarack to high root zone pH.

Chapter 4: Describes the study that was carried out in a split-root hydroponic system to examine the growth and physiological responses of paper birch, trembling aspen, and red osier dogwood to Fe deficiency at different root zone pH levels.

Chapter 5: Provides general conclusions and suggestions for future research.

1.2 Literature review

1.2.1 Oil sands mining and reclamation

Oil sands mining plays a significant role in Alberta's and Canadian economy. It has been estimated that there are about 170 billion barrels of bitumen in Alberta's oil sands deposits (Alberta Energy 2015), and the recovery of these oil reserves has had a significant economic impact and created many job opportunities. However, oil sands developments also have been criticized for being environmentally unfriendly and for causing severe disturbance to boreal ecosystems. Mitigation of this disturbance through successful reclamation is crucial to sustainable development of oil sands.

1.2.1.1 Oil sands mining processes

Alberta's oil sands underlie 142,200 square kilometers (km²) of land (Alberta Energy 2015), primarily in the Athabasca, Cold Lake, and Peace River areas in the northern Alberta. They are usually present from 50 to 200 m below the surface (Natural Resources Canada 2017)

and need to be exploited by different techniques depending on their depth. Twenty percent of the oil deposit, which covers 4,802 km² is suitable for open-pit mining, while the other 80% of the deposit, which occupies about 97% of the oil sands area, must be mined by the in-situ methods because they are too deep (usually deeper than 75 m) for the open-pit mining (Alberta Chamber of Resources 2015).

The open-pit mining consists of three main processes: ore collection and crushing, extraction, and upgrading. Prior to open-pit mining, the wetlands are drained, trees in the forest are logged, and all other vegetation is removed. Organic soils (peat, muskeg, LFH layers) are stripped and either placed directly on the sites that are ready to be reclaimed or stockpiled for future reclamation purposes (Fung et al. 2000). Below the soil and above the oil sands is the overburden material, which is also removed and used for later site reclamation.

After being collected and crushed, the oil sand ore is prepared by adding hot water and chemicals and then transported to the extraction plant through the hydrotransport pipelines. Then, the conditioned oil sands slurry goes into a large separation vessel and more hot water and alkaline chemicals are added to recover the bitumen. Na₂CO₃, Na₂SiO₃ as well as NaOH are used to make a weak alkaline environment and ionize carboxylic acids in the bitumen to produce surfactants (Sanford 1983). For maximum bitumen recovery, the optimal amount of NaOH needs to be calculated (Schramm and Smith 1989). The slurry components separate into three layers in the separation vessel, the bitumen froth on the surface, the middle layer for further bitumen recovery, and sediments (rocks, clay, and sand)

settle to the bottom.

At the end of the process, the recovered bitumen is sent to the upgraders or refineries to produce synthetic crude oil. The tailings, which contain clays, water, chemicals and traces of bitumen as the main byproduct of the extraction process, are pumped to the tailings ponds. The water from the top three meters in the tailing pond is recycled back to the extraction plant, and the tailings sand material is used as a reconstruction substrate in reclamation (CAPP 2017). For the in-situ extraction, steam assisted gravity drainage (SAGD) is the most popular technique and about 50-60% of bitumen is recovered by this method (Natural Resources Canada 2017). In the SAGD, two parallel horizontal wells are drilled into oil sands deposits, and one is slightly lower than the other. High-pressure steam is injected through the upper well (injection well) to heat the bitumen and make it more fluid. The bitumen then flows to the lower well (production well) due to gravity and is finally pumped to the surface (Dyer and Huot 2010).

1.2.1.2 Environmental impact of oil sands mining

Both methods of oil sands mining have severe detrimental impacts on the environment including land disturbance, high water consumption, and air pollution. Although land disturbance impacted by in-situ operations is less severe than that by open-pit mining, considering the large in-situ area, the cumulative environmental impacts from in-situ operations are greater than those from open-pit mining (Dyer and Huot 2010).

For the land disturbance, aside from deforestation, habitat fragmentation, and species loss, the biggest problem is tailings management. In 2013, there were more than 976 million m³ of tailings stored in large tailing ponds which cover over 220 km³ in the Athabasca oil sands area including dikes, berms, beaches, and in-pit ponds (Alberta Energy Regulator 2014). Due to the use of chemicals, especially NaOH, in the extraction process and water recirculation, tailings commonly have high pH values as well as elevated salinity and sodicity levels. Additionally, in order to accelerate the consolidation process in the tailings ponds, gypsum (CaSO₄·2H₂O) may be added as a densification agent (Chalaturnyk et al. 2002), which aggravates the salinity problem by releasing Na from clay particles. Another concern with tailings ponds is leakage of tailings into the surrounding soil and surface water through the groundwater system (National Energy Board 2015). The waste water from in-situ mining process does not go to tailings ponds but is injected into deep aquifers on site.

During the oil sand extraction process, a large volume of water is used. On average, it takes two tons of water to produce one barrel of bitumen in open-pit mining operations (Kasperski 1992), and about two to four barrels of fresh water after taking water recycling into account (Mikula et al. 2008). In the in-situ operations, approximately 0.9 barrels of water are used to extract one barrel of bitumen. However, this is still 2-3 times higher than the amount of water used by conventional oil exploitation (CERA 2009). Every year, more than 590 million m³ of water is withdrawn from the Athabasca River, which is equal to the usage of a city of about 3 million people. This water cannot be released back to the river because of its contamination. At the low flow periods (winter), the large water consumption

poses a risk to aquatic life.

The extraction and upgrade of oil require energy by burning natural gas, which is a major source of CO₂ emissions. The production of bitumen is the fastest-growing source of greenhouse gas (GHG) emissions in Canada, which contributes to climate change (Dyer and Huot 2010). The oil sands contributed 7.8% of Canada's total GHG emissions in 2011, which is equal to approximately 0.1% of global emissions (Environment Canada 2012). Canada is one of the 39 industrialized countries which signed the Kyoto Protocol in 2002 to reduce its national greenhouse gas emissions. From 1990 to 2011, oil sands GHG emissions per barrel decreased by 26% (Government of Canada 2013). The level of emissions may be further reduced in the future by adopting more efficient and cleaner technologies.

1.2.1.3 Requirements and processes of reclamation

In order to reduce negative environmental impact and keep oil sands mining sustainable, Alberta enacted in 1993 the statute of Environmental Protection and Enhancement Act (EPEA). According to EPEA, “the objective of conservation and reclamation of specified land is to return it to an equivalent land capability”. An oil sands company can get the right to extract bitumen from the oil sands existing within the new specified lease area only if it can prove that the reclamation meets the environmental standards and the reclaimed area can be certified and returned to the Crown.

Oil sands reclamation is a multistep process that includes: 1. Surveying the pre-disturbed soils to delineate suitable reclamation materials, 2. Salvage of reclamation material, 3. Replacement of the reclamation material, 4. Revegetation, 5. Monitoring, and 6. Certification (Fung et al. 2000).

Reconstruction of functional soil is an important step in the reclamation process (Macdonald et al. 2012) that provides anchorage support, water and nutrient storage and supply for plants, and habitat for microbial and faunal diversity (Dominati et al. 2010). In the oil sands region, overburden materials, as well as tailings sand, are used as substrates to fill the mined out pits (Alberta Environment 2010). The overburden which comes from the marine clayshales near Clearwater or Ft. McMurray is high in salts and sodium, and it is referred to as the saline-sodic overburden. Due to the properties of the substrate, a thicker soil cover is required to increase the nutrient availability and water-holding capacity before revegetation. A typically reclaimed soil profile contains three “horizons” that include topsoil (0 to 20 cm depth), subsoil (20 to 50 cm depth) and lower subsoil (50 to 100 cm depth), for a total of 1 m (Leskiw 1998). Either a “one-lift” or “two-lift” soil replacement technique is used by mixing different proportions of peat and sandy material with cover soil depending on the quality of the mineral component of the mixture (Alberta Energy Regulator 2013). While the pH of undisturbed soils in the boreal forest near Fort McMurray is typically below 6.0, the soil pH values in oil sands reclamation areas frequently exceed 8.0 (Naeth et al. 1999, Howat 2000). The overburden material at the mining side of Syncrude is high in salts and its electrical conductivity (EC) is typically between 4 and 11 dS m⁻¹, while the EC of undisturbed soils nearby ranges between 0.32 and 1.03 dS m⁻¹ in

the surface organic layers and from 0.06 to 0.33 dS m⁻¹ in the top 10 cm of the mineral soil (Howat 2000).

After the reconstruction of landscapes, barley or oats are often sown to add vegetation cover and prevent erosion (Alberta Environment 2010). Then, native tree species are planted according to the guidelines for reclamation in the Athabasca oil sands region (Alberta Environment 2010). For the upland sites, the dominant tree species are trembling aspen (*Populus tremuloides*), white spruce (*Picea glauca*), and balsam poplar (*Populus balsamifera*). For the sandy sites, jack pine (*Pinus banksiana*) is the dominant species. And for the lowlands, black spruce (*Picea mariana*) and tamarack (*Larix laricina*) are the dominant tree species. The understory shrub communities usually consist of nitrogen-fixing shrubs such as green alder (*Alnus viridis*) and other shrubs including red osier dogwood (*Cornus stolonifera*) (Fung et al. 2000). Woody tree species are planted at an average density of 2,000 stems/ha and shrubs at a density of 500 stems/ha (Fung et al. 2000).

In March 2008, Syncrude's Gateway Hill became the first successful reclaimed area to achieve a Certificate of reclamation from the Alberta Government. Wetland reclamation has been even less successful largely due to unsophisticated reclamation techniques, gaps in reclamation knowledge, and high costs (Grant et al. 2008).

1.2.2 High pH and plants

1.2.2.1 Introduction

Soil pH is a critical parameter that affects plant distribution and growth. More than one-third of the earth terrestrial areas contain high pH soils (Marschner 2012). For most plants, the optimum pH range is slightly acidic to neutral. Therefore, the high soil pH can adversely affect plant growth, reduce water uptake, and decrease nutrient availability. Natural selection under high pH has resulted in the evolution of high pH tolerance mechanisms in some plant species including increased extrusion of H^+ to the rhizosphere. However, there is generally little information concerning soil pH tolerance of plants in the boreal forests, especially for the understory species. Additionally, previous studies of high-pH tolerance mechanisms have often failed to separate the growth inhibition induced by the direct effects of pH from those that often accompany high soil pH including salinity.

In plants, both apoplastic and vacuole pH is approximately 5.5 whereas the pH of the cytoplasm is approximately 7.2-7.5 (Epstein and Bloom 2005). Plants use a pH-stat system to maintain H^+ homeostasis. When encountering environmental stresses, the changes of both apoplast and symplast pH could be a cellular signal showing an ongoing or preceding process in response to these stresses (Felle 2001).

1.2.2.2 Effects of high pH on plant root growth

The high soil pH profoundly impairs root growth, and the extent of this decrease varies between plant species. For alkalinity-sensitive species such as *Lupinus angustifolius* L., the reductions of overall root growth and root surface area were reported. These reductions

were explained by the decrease of cell elongation rather than cell division (Tang et al. 1993b). The development of lateral roots and root hairs was also inhibited by high pH (Tang et al. 1993a). However, in the alkalinity tolerant plant *Pisum sativum*, the roots were less affected by high pH (Tang et al. 1992, Tang et al. 1993b). The decreased root growth at high pH is thought to be the result of the plant's inability to maintain low apoplastic pH, instead of a decreased membrane potential, the increased permeability of cortical cells to Na^+ and K^+ , or low net H^+ efflux in both intact and excised roots (Tang et al. 1996). Bicarbonate (HCO_3^-) and Ca have often been used as the means of producing high pH conditions and to examine high pH tolerance in plants. In white lupin (*Lupinus albus*), HCO_3^- resulted in specific stress effects such as decreased shoot growth, taproot death and in decreased number and density of determinate roots while high levels of Ca affected the whole root system, reducing their growth and lateral branching (Kerley and Huyghe 2002). In some plant species, even a relatively small increase in pH can be deleterious. In *Pinus pinaster*, an increase in pH from 5.5 to pH 6.5 resulted in a decrease in the elongation rate of the main root, root thickness, as well as the lateral root growth after a 4-week treatment (Arduini et al. 1998).

1.2.2.3 Effects of pH on water relations

High soil pH can aggravate the effects of water deficit stress. In paper birch (*Betula papyrifera*), root hydraulic conductivity and root water flow at pH 8 were found to be reduced compared with pH 6 (Kamaluddin and Zwiazek 2004). This reduction may be related to changes in AQP activities which regulate root water flux (Voicu and Zwiazek

2004, Aroca et al. 2006). The water flows from the root surface to the xylem through apoplastic (through cell walls and intercellular spaces), symplastic (through plasmodesmata) and transmembrane pathways (Steudle and Peterson 1998). In the transmembrane pathway, water is transported across cell membranes predominantly through AQP (Maurel 1997, Tyerman et al. 1999), and the flow rate is regulated mainly by changes in the density and activity of AQP (Steudle and Henzler 1995, Johansson et al. 1998). AQP activity is known to be affected by pH (Tournaire-Roux et al. 2003, Kamaluddin and Zwiazek 2004, Vander Willigen et al. 2004, Törnroth-Horsefield et al. 2006). In fact, pH may be an effective short-term regulation signal that provides fast fine-tuning of AQP activity by controlling AQP gating (Tournaire-Roux et al. 2003). A histidine residue may be associated with the pH regulation of AQPs (Tournaire-Roux et al. 2003, Törnroth-Horsefield et al. 2006). Additionally, high pH may decrease water uptake indirectly by inhibiting root growth, and, consequently, reducing the surface area of young roots which are largely responsible for water absorption (Tang et al. 1992, Tang et al. 1993b). The reduction of water uptake and root water flow rates negatively affects stomatal conductance, photosynthetic rates, and shoot growth (Tang and Turner 1999).

1.2.2.4 Nutrient deficiencies in high pH

Rhizosphere pH plays an important role in nutrient uptake since it affects the solubility and mobility of mineral nutrients (Comerford 2005). High pH soils are usually associated with the low availability of Fe, Mn, P and Zn (Lindsay 1984, Yang et al. 1994, Iles 2001, Valentine et al. 2006), and to some extent also B and N (Marschner 2012). Therefore,

preventing deficiencies of these nutrients may be part of the plant strategies that contribute to tolerance of high pH.

Fe^{3+} has low solubility in neutral and high pH, which can result in leaf chlorosis and poor plant growth (Kosegarten et al. 2001, Tang et al. 2006). In alkaline or calcareous soils, Fe^{3+} precipitates in several oxide forms such as Fe_2O_3 , $\text{Fe}(\text{OH})_2^+$, and $\text{Fe}(\text{OH})_3$. In the pH range (7.4 to 8.5) of minimum iron solubility, $\text{Fe}(\text{OH})_3$ is the major soluble species at about 10^{-10} M, making the availability of Fe inadequate to plants (Lindsay 1984). However, some plants can cope with low Fe solubility in high pH soils. Six arid-zone shrubs were found to extrude more H^+ and chemical reductants from roots, as well as reduce Fe at the root tips to improve Fe uptake (Nelson 1992). *Eucalyptus halophila* is capable of sequestering more Fe, and thereby maintains higher leaf Fe concentrations under high pH conditions with low Fe availability (James et al. 2002). Barley (*Hordeum vulgare* L.) exposed to high pH and low Fe availability conditions developed more lateral roots with a highly stimulated phytosiderophore release (Yousfi et al. 2007).

Another microelement which is associated with photosynthesis and may be deficient in plants growing in high pH soils is Mn. This element is a structural component of the chloroplasts and an enzyme activator during chlorophyll production. Mn deficiency is also associated with neutral to high soil pH especially at pH 6.7 or higher (Stone 1968). In soil pH ranging from 6 to 8, Mn is ready to be oxidized to insoluble MnO_2 (Hewitt et al. 1974), adsorb on CaCO_3 , and precipitate as manganese calcite $[(\text{Ca}, \text{Mn})\text{CO}_3]$ (Jauregui and Reisenauer 1982).

Of the essential macronutrients, P uptake may be among the most affected by high pH. The maximum solubility of P is at pH 6.5, and the solubility is reduced by both lower and higher pH (Valentine et al. 2006). P deficiency was reported for sweet potato grown at pH 8 (Ila'ava et al. 1999). However, it has also been demonstrated that some plant species are tolerant of low P availability at high pH. Chickpea (*Cicer arietinum* L.) was reported to have an increased activity of PEPcase (phosphoenolpyruvate carboxylase) in shoots and a greater extent with more branching density of roots (Alloush 2003). Green ash (*Fraxinus pennsylvanica* Marsh.) was found to require adequate levels of available P (15-20 ppm) in its first year of growth in alkaline conditions. However, in subsequent years, P nutrition was of less importance (Carter 1980).

Zinc deficiency is common in high pH soils, especially in calcareous soils. For each unit of soil pH rising from 5.5 to 7.0, the equilibrium concentration of Zn may be reduced by 30- to 45-fold (Moraghan and Mascagni 1991). The mechanisms that some plants may use to increase Zn uptake at high pH include increased root growth (Dong et al. 1995), the release of Zn-mobilizing phytosiderophores from roots (Cakmak et al. 1996), and having a higher root capacity of Zn-efficient genotypes for Zn uptake (Rengel and Graham 1996).

Boron also becomes less available in alkaline and calcareous soils (Marschner 2012). Berger and Truog (1945) reported that available B in the soils increases as the pH increases from 4.7 to 6.7 and decreases from pH 7.1 to 8.1. In calcareous soils up to pH 9.0, undissociated boric acid (H_3BO_3) is the predominant species of B in the solution (Peterson

and Newman 1976). Another reason that makes B less available to plants at high pH is the adsorption of B in the soil. In the study of Goldberg and Glaubig (1986), the sorption of B in an calcareous soil sample increased with increases in solution pH from 5.5 to 9.5, and calcite played an important role in B sorption in calcareous soils since B adsorbs to the surfaces of CaCO_3 through ligand exchange with reactive surface hydroxyl groups (Goldberg 1997).

Generally, alkaline-adapted plants (calcicole species) prefer utilizing nitrate, but acid-adapted plants use ammonium preferentially (Marschner 2012). This may be explained by the inability of acid-adapted plants to assimilate nitrate and results in N deficiency at high pH (Merhaut 1993). For example, in blueberries, which typically grow under acidic conditions, leaf and root nitrate reductase activity was low or undetectable at high pH in several cultivars (Merhaut 1993, Claussen and Lenz 1999). With N deficiency in calcareous soil, more assimilation was transported to the roots and results in a lower shoot/root biomass ratio.

1.2.2.5 Root-mediated rhizosphere pH changes

Plants and their rhizosphere pH have a dynamic and complex relationship. Rhizosphere pH can affect plant root growth, water relationships, and nutrient uptake, and in turn, plants can change their rhizosphere pH to respond to the environmental constraints. Root-mediated changes in pH of the rhizosphere may be related to the extrusion of H^+/OH^- and organic anions from roots, respiration which produces CO_2 that may form carbonic acid, and redox-coupled reactions with Fe, Mn, and N (Hinsinger et al. 2003).

ATPases play an important role in linking H^+ and/or OH^- ion excretion with ion uptake.

ATPases are integral membrane proteins located at both the plasma membrane and tonoplast (Briskin 1986). They convert energy from ATP hydrolysis to maintain a pH gradient and a membrane electrical potential difference by pumping protons across the membrane. This process is called the primary active transport, which provides the driving force for transport of various molecules, via specific transport proteins.

Both H^+/OH^- and organic acid fluxes are involved in compensating for the imbalance of electrical charges in plant roots (Tang and Rengel 2003). When plants take up more cations than anions, such as when supplied with a K_2SO_4 solution (Marschner 2012), an equivalent amount of H^+ will be secreted into the rhizosphere, which decreases the apoplast pH and increases the cytosolic pH. In contrast, when plants take up more anions than cations, such as when supplied with $CaCl_2$ in solution (Haynes 1990, Marschner 2012), an equivalent amount of OH^- or HCO_3^- will be extruded into apoplast (or H^+ will be absorbed from the apoplast), which leads to an increased rhizosphere pH and a decreased cytosolic pH. The mechanism of anion absorption is important since the uptake of anions often exceeds the uptake of cations in most of the situations (Cunningham 1964).

Nitrogen is the nutrient in greatest demand during the whole life of the plant, and comprises about 80% of the total cations and anions taken up by plants (Mengel et al. 2001). Therefore, its absorption mechanism is especially important for the maintenance of cation-anion balance. The form of N uptake is an important factor determining the uptake of other

cations and anions, cellular pH regulation, and the rhizosphere pH. There are several studies providing evidence that NO_3^- uptake is linked with rhizosphere alkalization while NH_4^+ uptake induces rhizosphere acidification (Hoffmann et al. 1992, Mengel 1994). To maintain a stable pH with the uptake of NO_3^- and NH_4^+ in nutrient solution, about 80-90% of nitrogen was present in the form of NO_3^- (Trelease and Trelase 1933), for example, modified Hoagland's solution contains 12.5% N as NH_4^+ (Epstein 1972).

Root-induced changes in rhizosphere pH vary spatially along the root axis, and sometimes even exceed two pH units (Marschner et al. 1986a). Marschner and Römheld (1983) found the most distinct rhizosphere pH changes along the main root apex. In a single root, different parts of the root may behave differently. For example, the parts closest to the root apex may release H^+ while the basal parts of the roots extrude OH^- (Plassard et al. 1999, Jaillard et al. 2003). This phenomenon may be due to differences in the uptake of cations and anions in different root zones (Marschner et al. 1986a).

Localized root-induced rhizosphere pH changes can help plants alleviate the adverse nutrient stress, which includes rhizosphere acidification stimulated by Fe and P deficiencies (Römheld and Marschner 1986, Bienfait 1988, Hoffland et al. 1989a, Hoffland et al. 1989b), and the rhizosphere alkalization in the case of Al toxicity (Jens 1988). Fe deficiencies could increase the capacity of H^+ extrusion in all examined dicots and non-grass monocots (Bienfait 1988). This process was confined to the apical root zone and may be attributed to rhizodermal transfer cells which have a strong H^+ -ATPase activity (Römheld and Kramer 1983, Haynes 1990). Additionally, more organic substances acting as ferric reducing

compounds were released, such as phenolic compounds, which may help increase Fe availability. Under P deficiency, similar responses were found as described for Fe deficiency, including acidification of the rhizosphere behind the root apex (Hoffland et al. 1989b) and increased extrusion of organic anions such as malate and citrate (Hoffland et al. 1989a). In a study carried out with white lupin (*Lupinus albus* L.), cluster roots that were produced in response to low P supply extruded most of the citrate and malate into the medium and this was accompanied by some H⁺ release (Neumann and Römheld 1999). In response to Al³⁺ toxicity, Al-tolerant plants tend to absorb more anions than cations from the soil and, therefore, maintain a relatively high soil pH. For instance, Taylor and Foy (1985) found that Al-tolerant cultivars of wheat (*Triticum aestivum* L.) resisted acidification by taking up more NO₃⁻ than NH₄⁺ in mixed solutions where both NO₃⁻ and NH₄⁺ were present.

Redox processes coupled to ion absorption also need to be taken into account in calculating rhizosphere pH changes. Among the redox processes, Fe reduction is demonstrated most clearly. In all strategy I plant species, Fe³⁺ is reduced to Fe²⁺ which is accompanied by H⁺ consumption and thereby increasing rhizosphere pH (Marschner and Römheld 1994). This plasma membrane-bound reduction process occurs behind root tips (Dinkelaker et al. 1993), likely in the same zone where localized acidification takes place (Fischer et al. 1989). Another process is root-induced oxidation in wetlands and during flooding. O₂ is transferred from the shoots through aerenchyma and released from the roots, and then oxidation of Fe²⁺ to Fe³⁺ occurs in the rhizosphere (Ando et al. 1983). This process released H⁺ and dramatically acidified the root zone (Begg et al. 1994). This oxidation involving Fe

was also found for Mn, S, and N (Sposito 2008).

Root respiration inputs a large amount of CO₂ into soils as an important source of carbon in the rhizosphere. It has a significant influence on rhizosphere pH, especially in calcareous soils. For example, with increasing CO₂ concentration, soil pH dropped drastically from 8.3 to 6.7. However, in acidic or neutral soils, this contribution of rhizosphere pH changes is negligible (Nye 1981) since the pK of H₂CO₃ is 6.36 (Lindsay 1979).

In addition to being plant specific, the amount of rhizosphere pH changes depends on the initial soil pH (Youssef and Chino 1989) and soil pH buffering capacity (Schubert et al. 1990). The higher the pH buffering capacity, the smaller the plant-induced pH change. Usually, sandy soils have low pH buffering capacity since they contain little organic matter. Therefore, the effectiveness of H⁺ extrusion by roots in acidifying the rhizosphere to take up elements such as Fe or Mn from high pH root medium will be grossly affected by the buffering capacity of the root medium.

1.2.2.6 H⁺ homeostasis and pH changes in response to environment constraints

The pH responses of enzyme activities are represented by a bell-shaped curve and, therefore, H⁺ homeostasis is the premise to maintain optimum pH conditions in plants. The pH homeostasis is regulated by a so-called pH-stat system consisting of ion transport, H⁺ buffering, and H⁺ consuming and producing reactions. Usually, H⁺ membrane transport is regarded as “physical pH-stat” and H⁺ reactions are regarded as “biochemical pH-stat”

(Davies 1986). The pH inside plants is the summation of intracellular pH and apoplastic pH, and the pH regulation of these parts is not independent of each other. Cytosolic pH is slightly alkaline at about 7.2-7.5 while apoplastic pH is kept acidic at approximately 5-6. ATPases, acting as the H⁺ pumps, are important proteins to carry on biophysical pH-stat in the cytosol, organelle, and apoplastic zone. Buffering is generally considered to be a passive property to curb sudden pH changes by the production of organic acids. Cells produce proportions of strong and weak organic acids by carboxylation/decarboxylation mechanisms as buffering elements (Lüttge et al. 1982, Davies 1986). For example, excess cation uptake could result in a corresponding net release of H⁺ into the medium and thus increasing cellular OH⁻. To buffer the cellular pH change, organic acids will be synthesized and deposited in the root cells. Similarly, the reverse process with organic acids degradation will take place when cellular pH decreases as excess anion uptake (Marschner 2012). In these processes, malate is the dominant balancing organic acid while oxalate and citrate also play an important role in several plant species (Davies 1986). The cytoplasmic buffering capacity ranges from 20 to 80 mM per pH unit and is about one order of magnitude higher than that of the apoplast (Felle and Hanstein 2002).

Changes in pH can indicate that the plants confront stress such as light intensity change, drought, the approach of microorganisms, and gravistimulation. Cytoplasmic pH of the light-adapted cells decreased by about 0.3 units in darkness while there was a rapid pH increase in response to light in dark-adapted cells (Felle and Bertl 1986). In response to water deficit, the increased pH in xylem sap can signal drought and this information may go along to shoots and leaves. Since the distribution and production of abscisic acid (ABA)

are pH dependent, under water stressed conditions plants can sense the alkaline xylem sap and produce more ABA to increase water flow and close stomata (Hose et al. 2000, Kamaluddin and Zwiazek 2004). Under hypoxic conditions, the cytosolic pH decreased by 0.5-0.6 units (Felle and Bertl 1986, Fox et al. 1995), which may be due to lactic acid produced as a fermentation product. The cytosolic pH may change in plants affected by various microorganisms. When infected by pathogenic microorganisms, the intracellular pH decreases likely as a result of an oxidative burst for defense reactions (Felle et al. 2000), and the extracellular pH becomes alkaline to decrease the activity of cell wall digesting enzymes. During symbiotic formation, intracellular pH tends to increase (Felle et al. 1996). In response to gravistimulation, Fasano et al. (2001) indicated that the cytosolic pH of the root cap in *Arabidopsis* increased by about 0.4 pH units while the apoplastic pH declined from 5.5 to 4.5. The acidification of apoplastic pH is usually associated with stimulation of cell elongation. In the acid growth theory, the auxins (IAA) stimulate H⁺ pumping into the cell wall, which loosens the connections between cellulose microfibrils and facilitates cell extension (Cleland 1976).

1.2.2.7 Measurements of apoplastic and intracellular pH

As discussed above, measurements of apoplastic and intracellular pH are important to understand the processes such as nutrient uptake, pH regulation, plant growth, plant tolerance to stresses and so on. The intracellular zone is the space inside the plasma membrane (protoplast), while the apoplast is the part outside of the intracellular zone and consists of the cell wall, extracellular space, and xylem-lumen space (Canny 1995). There

are several methods to estimate or directly measure apoplastic and intracellular pH, and each one has its own advantages and limitations.

The apoplastic pH range varies from 3.5 to 8.3 in different plant species, plant components, and experimental conditions measured by different methods. Yu et al. (2000) reviewed the methods of measuring apoplastic pH in plants. Bromocresol purple can be used as the pH indicator to estimate the apoplastic pH of plants grown in agar or beads (Mulkey and Evans 1981). However, the pH value is not the real apoplastic pH but the pH outside of the root surface and this method is relatively time-consuming. Apoplastic pH can also be obtained by measuring apoplastic sap collected by centrifugation (Yu et al. 1999). Water stress (Hartung et al. 1992) and contamination were usually of concern in these measurements, and the apoplastic sap lacked temporal and spatial concentration gradients (Yu et al. 1999). H^+ -selective electrodes can be placed in the solution (Peters et al. 1997, 1998), on the tissue surface or inserted into the apoplast of selected cells (Felle 1998) to measure the apoplastic pH. Although this method is sensitive and can detect the heterogeneity of pH in the apoplast, it requires experience and is time-consuming (Felle 1998). Fluorescence microscopy and confocal laser scanning microscopy are the most advanced techniques to measure apoplastic pH. The principle of these techniques is to measure fluorescence intensity at two different wavelengths (ratio imaging) after fluorescent probes are applied. The fluorescence intensity measured at one wavelength is little affected by pH, while at the other wavelength, the pH dependence of the dye becomes apparent. The ratio of fluorescence excited by two wavelengths is related only to pH. Coumarin, promulin (Edwards et al. 1988) and fluorescein isothiocyanate dextran (FITC-dextran) (Hoffmann and Kosegarten 1995) can

be used as the fluorescent dyes. The problems with this method include a limited choice of suitable fluorescent dyes and the high cost of equipment.

Most of the methods used for apoplastic pH measurements can also be applied to measure intracellular pH including cell sap measurements (Raven and Smith 1980), H⁺-selective microelectrodes (Kurkdjian and Barbier-Brygoo 1983) and fluorescent probes (Han and Burgess 2009). Also, the ³¹P NMR spectrometry technique can be used to measure cytoplasmic and vacuolar pH of plants *in vivo* (Roberts 1984), which makes it a powerful method to study mobile plant metabolites. The limitations of this method include low sensitivity and a requirement for a high amount of plant material. Therefore, this method is rarely used for the apoplastic pH measurements.

1.2.3 Iron and plants

Iron is the fourth most common element in the Earth's crust. It is an essential micronutrient for plants and it is required in many metabolic processes such as respiration, photosynthesis, and chlorophyll synthesis. In these processes, Fe plays a role as a metal cofactor of enzymes and in the forms of Fe-heme groups or Fe-S (iron-sulfur protein) clusters in oxidation-reduction reactions as an electron donor and acceptor. A total soluble concentration of inorganic iron in soil should be 10⁻⁶-10⁻⁵ M required for the optimal amount for plant growth, however, plants usually cannot obtain enough Fe because of low Fe solubility in soil solution (Römheld and Marschner 1986, Guerinot and Yi 1994). Fe deficiency often occurs in high pH and calcareous soils, which makes it a challenge for agriculture. Fe

deficiency can lead to interveinal chlorosis in leaves and a reduction of crop yields. Fe also can be injurious to plants if presents as a free ion. Cells can generate highly reactive hydroxyl radicals through the Fenton reaction (Fe^{2+} is oxidized by H_2O_2 to Fe^{3+}) (Hallowell and Gutteridge 1992), which can cause serious damage to DNA, proteins, lipids, and sugars. In order to maintain Fe homeostasis and overcome these two barriers, plants need to have efficient uptake mechanisms and bind iron by intricate chelation systems in the tissues.

1.2.3.1 Iron and photosynthesis

Most of the Fe (more than 80%) is present in chloroplasts in mesophyll cells (Terry and Abad 1986). Fe deficiency negatively affects both photosynthetic rate and chloroplast structure in higher plants. In thylakoid membranes, Fe is mainly involved in the electron transfer chain of the photosynthetic light reactions. Fe is a constituent of heme and Fe-S in the PSI, PSII and Cyt b6f complex, their main function being to transfer electrons going through the PSII-Cyt b6f-PSI complex to ferredoxin-NADP reductase which reduces NADP^+ to NADPH (Briat et al. 2015).

Fe is also involved in chlorophyll biosynthesis, and Fe deficiency results in the interveinal yellowing of leaves (chlorosis) (Mengel 1994, Ranieri et al. 2001). Protoporphyrin is the common precursor for chlorophyll and heme. One step in protoporphyrin synthesis is catalyzed by Fe-containing enzyme (Ouchane et al. 2004). Additionally, the chlorophyllide a oxygenase, containing a Rieske Fe-S cluster, catalyzes the synthesis of chlorophyll b from chlorophyll a (Eggink et al. 2004).

1.2.3.2 Responses to iron deficiency

Fe availability is determined by the soil redox potential and pH. In aerobic or high pH soil, Fe tends to be oxidized to form insoluble ferric oxides. Fe^{2+} is readily oxidized by atmospheric oxygen and Fe^{3+} precipitates by polymerization bonding with inorganic anions and hydrolyzation as $\text{Fe}(\text{OH})_3$ in alkaline soil. Although the concentration of Fe^{3+} is up to 10^{-6} M at pH 3.3, only 10^{-17} M of Fe^{3+} is available at a pH of 7 (Winkelmann et al. 1987), which is several orders of magnitude lower than the concentration required for optimal plant growth (10^{-6} - 10^{-5} M).

Plant roots respond to Fe deficiency in several ways. Morphologically, more root hairs form and root tips become swollen (Kramer et al. 1980, Römheld and Marschner 1981). Physiologically, two strategies of Fe acquisition in higher plants are induced by a Fe deficiency. All nongraminaceous angiosperm plants carry out a reduction-based Strategy I while grasses use a chelation-based strategy II (Marschner et al. 1986b).

There are three steps for the Strategy I: acidification, Fe^{3+} chelate reduction, and Fe^{2+} transport. First of all, the proton-ATPases of plant roots extrude protons into the rhizosphere to increase the solubility of Fe^{3+} since there is a 1000-fold Fe^{3+} rise for every pH unit drop (Olsen et al. 1981). Then, the root Fe^{3+} chelate reductases reduce Fe^{3+} to more soluble Fe^{2+} , which is the rate-limiting step in iron uptake (Grusak et al. 1990). The FRO family of ferric chelate reductases in Arabidopsis are overexpressed under low Fe growth conditions (Connolly et al. 2003). There are seven sets of FRO proteins which are involved in Fe uptake in different plant tissues (Wu et al. 2005, Mukherjee et al. 2006). Fe^{2+} is

transported by IRT1 (iron-regulated transporter 1) as the main Fe transporter from soil to roots. IRT1, a member of the ZIP metal transporter family, localizes to the plasma membrane of epidermal cells and is expressed under the Fe-deficiency conditions (Vert et al. 2002).

The Strategy II involves secretion of high-affinity ferric iron chelators termed PS to bind Fe^{3+} in the rhizosphere in response to iron deficiency. The tolerance to Fe-deficiency depends on the types and amounts of PS released (Marschner 2012). The Fe^{3+} -PS complexes are then absorbed by the epidermal cells of plant roots via YSL1 transporters. The Gramineae are capable of tolerating more Fe-limiting conditions because they are more efficient than the Strategy I plants in Fe uptake (Mori 1999). Some strategy II plants, such as rice, are also able to take in ferrous Fe via IRT-like transporters (Ishimaru et al. 2006, Cheng et al. 2007).

Apoplastic pH can affect both leaf and root ferric chelate reductase (FCR) activity, and the increased apoplastic pH can significantly decrease the FCR activity (Mengel 1994, Romera et al. 1998). The optimal pH for FCR activity depends on Fe status (sufficient vs deficient conditions) of the tissues and species, and it usually corresponds to the typical pH of the apoplastic space. González-Vallejo et al. (2000) found that the optimal pH of the FCR activity is about 5.5-6 in sugar beet leaves, and when the apoplastic pH increased to 6.5, FCR activity decreased by about 50%. Cohen et al. (1997) reported that the optimum FCR activity of intact pea roots under both Fe-sufficient and Fe-deficient conditions is at pH 5.5 to 6.0. When pH increased to 7.5, FCR activity decreased sharply (Cohen et al. 1997).

Sufficient-Fe supply may enhance root tolerance to a higher pH. The FCR activity of apple roots did not statistically change from pH 5 to 8 with sufficient-Fe supply (90 μ M Fe-DTPA). However, under Fe deficiency, the optimum pH of FCR activity was only between 4 and 6 (Yue Ao et al. 1985).

The mechanism of Fe-deficiency responses involves positive regulation of the genes including *FRO2* and *IRT1* gene expression which encode the root ferric-chelate reductase and the high-affinity iron transporter, respectively. Vert et al. (2003) found that both a local and a shoot-borne signal play an important role in the control of the expression of *IRT1* and *FRO2* in Arabidopsis. Enomoto et al. (2007) also reported that the long-distance signal generated in iron-deficient tissues including roots is a major factor in up-regulation of the expression of *NtIRT1* and *NtFRO1* in tobacco (*Nicotiana tabacum* L.) roots.

Phytoferritin (plant ferritin) in the stroma of plastids plays an important role in iron storage and maintenance of Fe homeostasis. With Fe deficiency, the lamellar Fe concentration increases from about 60% to 80% at the expense of the stroma Fe occurred in the chloroplasts (Terry and Low 1982).

1.2.3.3 Fe transport and allocation

Studies on Fe transport have focused mainly on long-distance Fe transport that takes place in the xylem and phloem, and intracellular Fe transport in the cytoplasm or through different organelles (Fig. 1.1). In these processes, Fe must be bound by chelating

compounds to keep it redox inactive to avoid reactive oxygen generation since, Fe tends to be involved in the redox reactions when it is in a free state (Marschner 2012).

After entering the root, Fe has been reported to be chelated with the NA (non-proteinogenic amino acid) in the cytosol at neutral pH (von Wirén et al. 1999, Rellán-Álvarez et al. 2008). The Fe³⁺-NA complex moves into the xylem along a diffusion gradient and then is transported into the xylem by a Fe-PS (phytosiderophores) transporter. In the xylem, Fe³⁺ is likely chelated with citrate at pH around 5.5-6 (Hell and Stephan 2003) and is transported to shoots and leaves. In the process of Fe release from stele to the leaves, the Strategy I components help Fe cross the plasma membrane of the leaf cells. Several proteins from FRO and ZIP (ZRT, IRT-like proteins) families are expressed in shoots.

Fe is reallocated from older leaves to younger leaves via phloem so that it can be used more efficiently. Seeds and root tips, which cannot get Fe through xylem transport, also need to have Fe delivered by the phloem. In the phloem, Fe is thought to be transported as the Fe³⁺-ITP (Fe transport protein) complex (Krüger et al. 2002). NA also plays an important role in phloem Fe chelation (von Wirén et al. 1999).

Fe can be transported between leaf cells by transporters in the plasma membrane or through the plasmodesmata. Once in the cytoplasm, Fe is transported between the cytosol and different organelles such as the mitochondria, chloroplasts, and vacuoles (Pandey et al. 2014).

The vacuole is a significant organelle in the regulation of cellular Fe homeostasis. On the one hand, it can release Fe into the cytosol by the transporter of Nramp3 (Nramp, the natural resistance associated macrophage proteins) and Nramp4 (Thomine et al. 2000) when Fe is below the optimal supply. On the other hand, it can import the excess Fe by the transporter (e.g. VIT1, Vacuolar Iron Transporter 1 in Arabidopsis) and store Fe, thereby preventing Fe toxicity (Kim et al. 2006). Some members of the *YSL* (Yellow Stripe1-Like) gene family can transport Fe to both sides of the tonoplast.

Mitochondria contain Fe-S as reducing agents. In Arabidopsis, Fe enters the mitochondria through MFL 1 (transporter mitoferritin-like1) and the transporter STA1 (mitochondrial ABC transporter), located on the inner mitochondrial membrane, has been reported to export Fe to the cytosol (Kushnir et al. 2001).

Fe uptake by chloroplasts requires reducing Fe³⁺ to Fe²⁺ by FRO6, whose expression is a light-dependent process (Feng et al. 2006). PIC1 (permease in chloroplasts 1) and MFL1 in Arabidopsis (Duy et al. 2007) and FDR3 (Fe-deficiency related 3) in maize (Han et al. 2009) are identified as the transporters importing Fe into the chloroplast. The excess Fe can be stored in a Fe storage protein of ferritin (Roschttardt et al. 2013) in the stroma of plastids.

1.2.4 Biology of the studied plant species

Plants have a wide soil pH tolerance ranging from 3.5 to 8.5, and the optimum pH for plant growth varies by the species (Iles 2001, Larcher 2003). In my studies, I focused on 10

boreal forest species including seven tree species: trembling aspen (*Populus tremuloides* Michx.), white spruce (*Picea glauca* (Moench) Voss), paper birch (*Betula papyrifera* Marsh.), jack pine (*Pinus banksiana* Lamb.), black spruce (*Picea mariana* (Mill.) B.S.P.), tamarack (*Larix laricina* (Du Roi) K. Koch), and green alder (*Alnus viridis* (Chaix.) D.C.), and three shrub species: red osier dogwood (*Cornus stolonifera* Michx.), blueberry (*Vaccinium myrtilloides* Michx.), and bearberry (*Arctostaphylos uva-ursi* (L.) Spreng.). Below is a description of the biology of plants that I used in my study.

1.2.4.1 Trembling aspen (*Populus tremuloides* Michx.)

Trembling aspen is a deciduous tree that is widely distributed in North America (Zasada and Phipps 1990). There are three major forest cover types in which trembling aspen grows (Eyre et al. 1980): Aspen (Eastern Forest) (Society of American Foresters Type 16), Aspen (Western Forest) (Type 217), and White Spruce-Aspen (Type 251). Trembling aspen grows in a wide range of soils, and the soil pH tolerance of trembling aspen was reported to be 5.3 to 8.4 (Renault et al. 1999). Due to its fast growth rate and high nutrient demand, the optimal soils for trembling aspen are well-drained, loamy, and abundant in organic matter and mineral nutrients (Alban 1982). Trembling aspen is a pioneer tree in disturbed sites, and the pure aspen stands gradually deteriorate and are replaced over time by slower-growing but more shade-tolerant conifers.

Trembling aspen can reproduce by both seeds and vegetatively by root suckers. The flowers on catkins of trembling aspen bloom in April or May (DeByle and Winokur 1985). The

seeds, which can be carried by the wind or water for many kilometers, usually mature in about 4 to 6 weeks after flowering (Strothmann and Zasada 1965). When trembling aspen is older than one year, its roots are ready to sprout suckers, and the mature stands reproduce vigorously by this means (Brinkman and Eugene 1975).

Trembling aspen has a variety of uses. It provides good habitat for wildlife (Patton and John 1977, Ohmann et al. 1978), makes great firebreaks (DeByle and Winokur 1985), and allows more water recharge and stream flow compared with conifer forests (Gifford et al. 1984).

1.2.4.2 White spruce (*Picea glauca* (Moench) Voss)

White spruce has a transcontinental range and grows from sea level to about 1520 m elevation. It can adapt to various conditions of the Northern Coniferous Forest. The trees also tolerate a variety of fertility and pH levels (pH 4.7 - 7.0, Nienstaedt and Zasada 1990). Even though good growth requires moisture, white spruce can also grow on fertile dry sites. The mature northern white spruce stands develop thick moss layers in regions with adequate moisture and the layers can greatly affect the mineral soil.

White spruce produces a large number of seeds starting at age 30 (Nienstaedt and Telch 1972). The great quality of seed may be associated with a hot, dry summer at the time of bud differentiation (Nienstaedt 1981). Layering is a common vegetative reproduction strategy of white spruce at some latitudinal treeline sites in Canada and Alaska (Densmore

1980). It may be because the sexual reproduction is limited or nonexistent in those places due to the climatic limitations.

White spruce usually has a shallow root system ranging from 90 to 120 cm in depth, and the taproots and layered roots develop differently depending on soil conditions, competition, and genetics (Wagg 1967, Strong and Roi 1983). The white spruce trees can form stands quickly after a disturbance. They remain in the understory for 50-70 years because of slow-growth (Nanson and Beach 1977, Walker and Chapin 1986). White spruce is able to tolerate medium shade and is equally or less shade tolerant than black spruce, but more tolerant than trembling aspen and paper birch.

White spruce is an important commercial species which can be used for pulpwood and lumber for general construction. The white spruce forest can maintain soil stability and the trees are often used for shelterbelts.

1.2.4.3 Paper birch (*Betula papyrifera* Marsh.)

Paper birch can grow in most kinds of soils (pH 5.0 - 7.5) and topographic situations because of its genetic diversity. The optimum environment for paper birch is deep, well-drained, nutrient-rich sandy loam on a cool moist site and its favored soil pH is slightly acidic (pH 5.0 - 6.5).

Paper birch is a monoecious species (Brinkman 1974) and starts producing seeds at about

15 years of age. The flowers are aments (catkins) and bloom from mid-April to early June, varying with locality, and the seeds mature in the following early August to mid-September. The majority of light, winged paper birch seeds are dispersed by wind and fall close to the trees which produced them.

Paper birch has a shallow root system and the root depth is usually not greater than 60 cm (Pomerleau and Lorti 1962). The shade-intolerant trait of paper birch makes it easier to be displaced by the more tolerant species after one generation in natural succession (Hutnik and Cunningham 1965). Paper birch is sensitive to nutrients, and it reacts more than trembling aspen when nitrogen, phosphorus, or lime are added to the soil (Schlentner and Cleve 1985).

Paper birch is quite useful. Young seedlings of paper birch are browsed by deer and moose (Shaw 1969, Stocker and Gilbert 1977). Due to its white bark, paper birch is a good choice as an ornamental tree. Paper birch is a pioneer species used for planting in disturbed sites and its wood is often used to produce pulp and fuel.

1.2.4.4 Jack pine (*Pinus banksiana* Lamb.)

Jack pine is a small-to-medium-sized coniferous tree which has a wide distribution in the northern United States and Canada (Benzie 2006). It is also the most broadly spread pine species in Canada. Jack pine can grow on very dry sandy soils and soils covered by limestone. However, the optimum soil site is well drained fertile sand. With the mycorrhizal

association, jack pine can grow on alkaline soil (pH 8.2) (Rudolph and Laidly 1990).

Jack pine is monoecious. The female cones are usually on upper branches and the male cones are usually on the third branches and lower in the crown (Doak 1935). Jack pine is pollinated by the wind and cross-fertilization is common (Fowler 1965, Rudolph et al. 1966). In the next growing season after pollination, fertilization takes place and then the seeds mature late in the growing season. Jack pine usually does not reproduce vegetatively in the natural forest.

Jack pine frequently forms a taproot. During the first and second growing season following germination, the root system grows downward to penetrate the soil, and then lateral roots spread widely and produce a large volume of roots. The trees commonly develop ectomycorrhizal associations (Cayford et al. 1967).

Jack pine is a pioneer species after a fire or other disturbance. It is a relatively shade-intolerant species and tolerates shade just a little more than trembling aspen and tamarack. Jack pine seedling stands need sunlight to keep themselves alive (Benzie 2006). Over time, jack pine may be over taken by more shade-tolerant species, except on the poorest or driest sites.

1.2.4.5 Black spruce (*Picea mariana* (Mill.) B.S.P.)

Black spruce is a widespread, coniferous tree species in Canada and the northern USA. Black spruce usually grows in pure stands or in association with white spruce, jack pine, tamarack, paper birch, and (or) trembling aspen. Black spruce can tolerate cold and moist sites well.

Black spruce often grows on wet organic soils and well-drained sites, and the favored pH may range from slightly acidic to slightly alkaline (Viereck and Johnston 1990). The optimum site for productive stands is nourished organic soil, which usually contains organic and mineral material (Zoltai and Pettapiece 1974). Black spruce has a taproot with lateral fibers at the moss-humus interface. The majority of root biomass is distributed in the upper 20 cm of organic horizons and the young roots may form some adventitious roots in this layer. Due to its shallow-rooted character, black spruce can grow on permafrost.

Black spruce is a monoecious species. Black spruce produces the most cones when 100 to 200 years old. The seeds are dispersed by the wind (Johnston and Thomas 1983), and fires can help to open the cones and accelerate seed release (Wilton 1963). Layering plays a significant role in the vegetative reproduction of black spruce on some sites, particularly where the tree's lower branches are covered by moss (Stanek 1975).

Black spruce is a shade-tolerant species. It grows immediately after a fire and becomes a dominant species on both uplands and peatlands. Black spruce grows quite slowly. Therefore, it must compete with faster-growing shrubs and other trees. On better peatland

sites, black spruce is in the understory and is dominated by trembling aspen, paper birch, and tamarack for many years before it succeeds them.

1.2.4.6 Tamarack (*Larix laricina* (Du Roi) K. Koch)

Tamarack is a small to medium-sized deciduous conifer. It is distributed widely all over North America because it can adapt to variable climate and soil conditions. Tamarack is usually found on wet to moist organic soils, and the optimum sites for its growth are humid but well-drained loamy soils and mineral soils with humus on the top (Nienstaedt and Zasada 1990). Tamarack is a typical peatland species and can tolerate high acidity. It can also grow well on calcareous soils but is not abundant in certain limestone-rich areas (Johnston 1990). Tamarack forms both pure stands and mixed stands with black spruce and is a minor component in mixed forests when it grows together with trembling aspen, white spruce, and paper birch (Renault et al. 1999).

Tamarack is monoecious. The flowers usually bloom from April to May. The cones are usually ripe between August and September, and then the seeds disperse from September to the following spring. However, due to damages by rodents, fungi, and bacteria, the germination rate is only 4%-5%. Layering is the dominant reproductive method in cold places in the north (Gifford et al. 1984, Matthews 1992) while in the south, layering usually occurs when fast-growing sphagnum moss or drifting sand covers the branches.

Tamarack has a shallow and wide-spreading root system. Roots in the sandy upland soils

rarely penetrate 30 cm below the surface or form taproots. On rich sites such as peatlands, roots may grow over a range that is wider than the tree height, but are usually less than 60 cm deep.

Tamarack is quite intolerant of shade. Although it can tolerate some shade during the first several years of growth (Viereck 1970, Matthews 1992), it must become dominant and be in the overstory to survive. Tamarack is a pioneer tree, especially on open unburned bogs and burned organic soil (Renault et al. 1999). Tamarack is fairly well adapted to reproduce successfully on burns (Cayford et al. 1967), so it is one of the common pioneer trees on most sites in the boreal forest immediately after the fire. Because tamarack is very shade-intolerant, it does not become established in its own shade. Consequently, the more tolerant black spruce eventually succeeds tamarack on poor (bog) sites, whereas northern white cedar, balsam fir, and swamp hardwoods succeed tamarack on good (swamp) sites (Nienstaedt and Zasada 1990).

1.2.4.7 Green alder (*Alnus viridis* (Chaix.) D.C.)

Green alder is a deciduous, large, fast-growing shrub of about 3-12 m in height. It can be found on acidic (pH 5.0-6.5), well-drained, moist hillsides or soils with sandy to gravelly or rocky textures across the cooler parts of the Northern Hemisphere (Alberta Environment 2010).

Green alder can reproduce both sexually and asexually. The seeds are dispersed by the wind

and then colonize on the mineral soil of disturbed habitats. This is the dominant way of reproduction. Vegetative propagation includes layering and sprouts originating from the root crown. The type of vegetative reproduction often occurs after natural disturbances, especially fire.

Green alder is a pioneer species. It is considered to be semi-shade tolerant and it cannot grow under a dense overstory (Hardy 1989, Matthews 1992). It can grow fast and well in nitrogen-poor soils since its nodules contain nitrogen-fixing microorganisms. The trees can add about 62 kg/ha of nitrogen per year to the soil (Ewing 1996).

1.2.4.8 Red osier dogwood (*Cornus stolonifera* Michx.)

Red osier dogwood is a multi-stemmed, deciduous shrub which grows 1 to 3 m tall and 3 m wide or more, often developing a loose, broad-spreading dense thicket. Red osier dogwood can adapt to a variety of climatic conditions and has a wide distribution in North America (Johnson et al. 1995). The best site conditions for its growth are rich, moist, and slightly acidic (pH 5.5 - 7.0) soils.

Red osier dogwood reproduces both by seed and vegetatively. The seeds have dormant embryos and hard seed coats, and in this case, they require 1-3 months of cold stratification for germination (Crane 1989). Red osier dogwood can also reproduce by stolons, layering and root suckers (Dirr 1998) to spread rapidly.

Red osier dogwood is an excellent species for rehabilitation of disturbed sites as a mid-successional species. It is useful for oil sand reclamation and soil stabilization because of its relative tolerance to high salinity and tailings (Renault et al. 2001), easy asexual propagation, vigorous growth, and thick, extensive root system (Crane 1989). Red osier dogwood is often one of the first shrubs to invade wet sites after flooding due to its tolerance of fluctuating water tables. Red osier dogwood has a moderate fire tolerance and is a seed-banking species. Red osier dogwood can be used as a landscape plant because of its bright red stems, and it can also be used as wildlife shelter and food source for animals.

1.2.4.9 Blueberry (*Vaccinium myrtilloides* Michx.)

Blueberry is a perennial, 10-50 cm high shrub that grows in small thickets. Blueberry is usually found on acidic soil bogs and rocky areas, and the optimal site conditions are dry and acidic (pH 4.0 - 5.5) soils, especially in sandy loam areas (Carter and St-Pierre 1996) under coniferous trees and wooded hillsides. The plants are common in the boreal forest across North America (Moss and Packer 1983).

Blueberry appears to regenerate both by seed and vegetatively. It flowers from April to July, and when the fruits are ripe, the seeds are eaten and dispersed by black bears, deer, birds, and other animals. Vegetative reproduction is mainly via rhizomes, sprouts, and suckers.

Blueberry forms relatively deep and branched roots with no taproots. It prefers soft shade and moist conditions. Blueberry is associated with ericoid mycorrhizal fungi and

endophytes. Whereas, young plants develop few mycorrhizal associations. It is a fire-tolerant species and is often abundant after moderate disturbance, although it recovers slowly. Blueberry is a commercial species and also an important food source for small mammals and birds.

1.2.4.10 Bearberry (*Arctostaphylos uva-ursi* (L.) Spreng.)

Bearberry is an evergreen shrub which creeps over the ground and the trailing stems can reach more than 1 meter in length. However, they are usually shorter than 20 cm. Bearberry is commonly found across Canada and the northern United States. It is a dominant understory species under jack pine, white spruce, black spruce, paper birch, and trembling aspen.

Bearberry primarily reproduces vegetatively. The adventitious roots which are derived from stem nodes can grow as clones if the stems break from the original plant. Seeds have hard seed coats and dormant embryos and need scarification to germinate.

Bearberry can grow on coarse and medium textured soils and tolerates pH ranging from 5.5 to 8.0 (USDA 2017). It is highly drought tolerant and can tolerate some salinity, but it is shade-intolerant. It recovers well and rapidly after the fire which helps with seed germination. The bearberry's fruit is edible and the leaves of bearberries are used for medicinal purposes.

1.3 References

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1.4 Figure

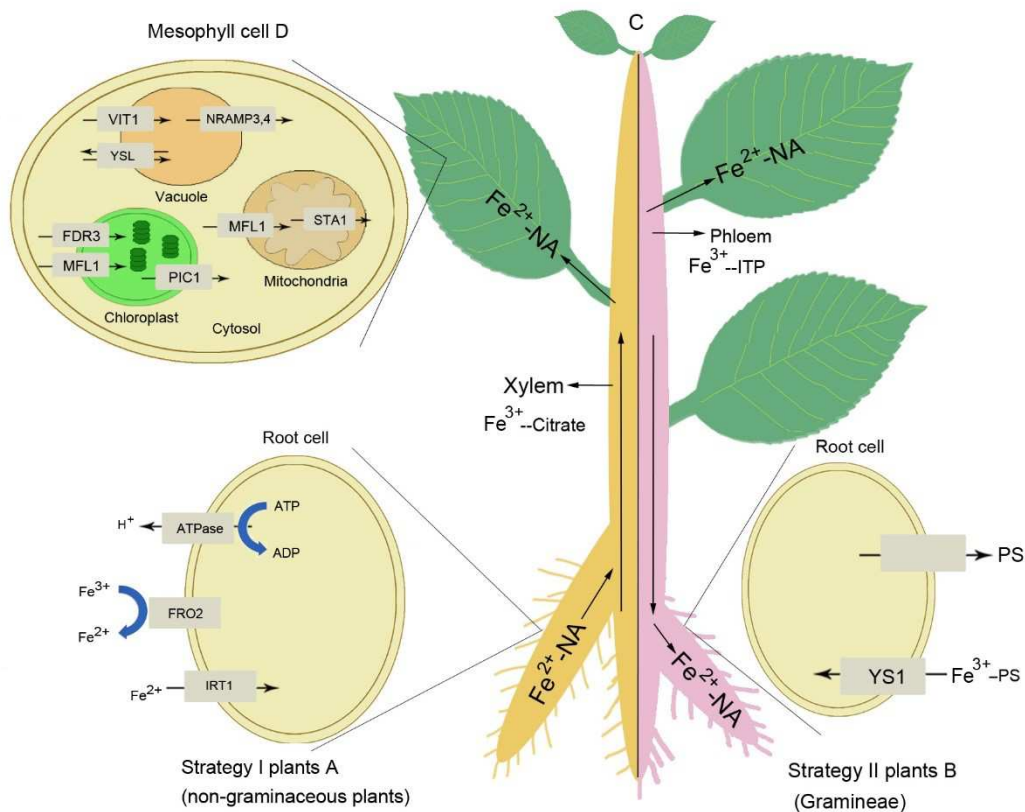


Figure 1. 1 Schematic representation of Fe uptake and transport. Fe uptake according to the Strategy-I (A) and Strategy-II (B) concepts. All nongraminaceous angiosperm plants carry out a reduction-based Strategy I while grasses use a chelation-based strategy II (Marschner et al. 1986b). There are three steps for the Strategy I: acidification, Fe³⁺ chelate reduction, and Fe²⁺ transport. The Strategy II involves secretion of high-affinity ferric iron chelators termed PS to bind Fe³⁺ and this Fe³⁺-PS complexes are then absorbed by the epidermal cells of plant roots via YSL1 transporters. After entering the roots or transport from stele to leaves, Fe has been reported to be chelated with NA. For the long-distance Fe transport (C), Fe³⁺ is likely chelated with citrate in the xylem and is transported as the Fe³⁺-ITP complex in phloem sap. (D) shows that Fe is transported between cytosol and different organelles such as mitochondria, chloroplasts, and vacuoles. Modified from: Kobayashi and Nishizawa (2012) and Pandey et al. (2014).

Chapter 2*

Effects of pH and mineral nutrition on growth and physiological responses of trembling aspen (*Populus tremuloides*), jack pine (*Pinus banksiana*), and white spruce (*Picea glauca*) seedlings in sand culture

2.1 Introduction

Soil pH is an important variable for plant growth since it profoundly affects soil nutrient availability and various plant physiological processes (Rengel 2002). More than 30% of the world's soils have a high pH problem (Chen and Barak 1982) including vast areas of calcareous, saline, and sodic soils. Since the optimum pH for most plant species is acidic to neutral, high soil pH makes it a serious agricultural problem. In addition, some human activities have made the high soil pH problem even worse. In northeastern Alberta, Canada, oil sands mining causes a severe disturbance to boreal ecosystems (National Energy Board 2015). In oil sands reclamation areas, soil pH frequently exceeds 8.0 while the pH of undisturbed soils in the boreal forest nearby is typically below 6.0 (Howat 2000). The high soil pH in reclamation sites may be particularly detrimental for the acid-loving forest

*I also examined dogwood, blueberry, and bearberry in this study. The results of other parameters of the studied species and the results of dogwood, blueberry, and bearberry are in Appendix 1.

understory plant species. For successful revegetation in oil sands reclamation areas, it is essential to understand how high soil pH affects plants and how plants tolerate high soil pH. Additionally, most of the previous studies examined the effects of high pH on plants in the presence of other confounding factors such as salinity (Kopittke and Menzies 2005, Yousfi et al. 2007); high pH tolerance mechanisms in the absence of these confounding factors are little understood.

High soil pH negatively affects plant growth and yield in several ways, and the effects vary among plant species. High rhizosphere pH impairs the root growth of plants. In alkaline-sensitive species such as *Lupinus angustifolius* L., a reduction was observed in overall root growth (Tang et al. 1992, Tang et al. 1993b) and root surface area (Tang et al. 1993a, Tang et al. 1993c). The reduced root growth at high pH may be attributed to the plant's inability to preserve the root cell apoplast acidification (Tang et al. 1996). Additionally, high pH can aggravate the effects of water deficit stress, and result in reductions in stomatal conductance (Tang and Turner 1999, Kamaluddin and Zwiazek 2004), shoot water potential (Tang et al. 1993c), and root water flux (Kamaluddin and Zwiazek 2004). This may be related to changes in aquaporin (AQP) activities (Voicu and Zwiazek 2004, Aroca et al. 2006).

High pH soils are usually associated with nutrient deficiencies including those of Mg, Ca, Fe, Mn, P, and Zn (Marschner 2012), since high pH decreases the availability of these nutrients in soil solution (Brady and Weil 1996). Under nutrient deficient conditions, plant response strategies include low nutrient demand (high nutrient use efficiency) and high

nutrient acquisition (nutrient acquisition efficiency) (Marschner 2012). Plant apoplastic pH is approximately 5.5 while the cytoplasm pH is about 7.2-7.4 (Epstein and Bloom 2005). The high medium pH increases the root apoplastic pH, thereby impairing pH gradient across the plasma membrane, which is essential for nutrient uptake (Felle and Hanstein 2002). Since high soil pH problems cannot be ameliorated easily, high nutrient input may be an approach to increase the tolerance of plants to high pH conditions.

In the present study, three boreal forest species, commonly used for oil sands reclamation, were selected including trembling aspen (*Populus tremuloides*), jack pine (*Pinus banksiana*), and white spruce (*Picea glauca*). The main objective of this study was to examine the effects of different root zone pH and nutrient supply to understand the mechanisms of high pH tolerance and assess the suitability of these plant species for the reclamation of oil sands mining areas. Despite their commercial and ecological importance, the soil pH tolerance of these tree species has been rarely studied. Trembling aspen was reported to grow in soil pH ranging from 5.3 to 8.4 in tailings water (Renault et al. 1999), while jack pine and white spruce were found to tolerate pH as high as 8.2 (Rudolph and Laidly 1990, Nienstaedt and Zasada 1990). In controlled-environment studies, white spruce exhibited relatively high tolerance to high root zone pH (Maynard et al. 1997, Zhang et al. 2013). Since nutrient deficiencies are common in high pH soils and inhibit plant growth, I hypothesized that increased nutrient supply can ameliorate the high pH effects on plants. The studies were carried out in sand culture to provide roots with a solid growth medium and a relatively simple culture system that made it possible to control the pH level.

2.2 Materials and methods

2.2.1 Plants and experimental setup

One-year-old dormant seedlings of trembling aspen (*Populus tremuloides*), jack pine (*Pinus banksiana*), and white spruce (*Picea glauca*) were obtained from the Boreal Horticultural Services Ltd., Bonnyville, Alberta, Canada. The seedlings had been grown in the tree nursery from seed in containers (415D styroblocks™, Beaver Plastics, Acheson, AB, Canada) for one year. After the roots of seedlings were washed free of soil and rinsed thoroughly, the seedlings were transplanted into 1-gallon (3.8 L) pots filled with washed sand (20/40 abrasive sand and 20/40 abrasive sand, 3:1 (v/v), Target Products Ltd., Burnaby, BC, Canada). Garden fabric (Spectrum brands Inc., Madison, WI, USA) was placed at the bottom of the pots to prevent leaking of the sand. The plants were grown in a controlled-environment growth room at 22/18°C (day/night) temperature, 65 ± 10% relative humidity, and 16-h photoperiod with 300 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD). They were supplied with 25% Hoagland's mineral solution (Epstein 1972) twice a week for 3 weeks before the commencement of treatments.

An automatic irrigation system was set up for this study to maintain uniform water supply and stable pH (Fig. 2.1). This system consisted of the watering and pH-control components. In the watering system, for each treatment, nutrient solution was placed in a 120 L bucket and delivered to each pot by a water pump (Model 9.5 950GPH, Danner MFG Inc., New York, NY, USA) through a tubing setup. The main part of the tubing setup was the 19 mm polyvinyl chloride (PVC) tubing to which 6 mm PVC tube was connected and attached to

the top of each pot by the 4 x 6 mm support stakes. There were four emitters connected to the 6 mm tube to ensure that the same amount of solution was delivered to each pot. A timer was connected to the water pumps to control the watering time. In the pH-control system, a gel-filled combination pH electrode (Orion 9106 BNWP, Thermo Scientific, Rochester, NY) was placed in the solution and connected to a pH controller (PHCN-70, Omega Engineering Inc., Laval, QC, Canada), which controlled an electronic valve (Model 8260G071 120/60 ASCO Valve, Inc., Florham Park, NJ, USA). The valve opened and closed to adjust the solution pH to the preset level by adding 5% (w/w) KOH or 1% (v/v) H₂SO₄.

2.2.2 Experimental treatments

The seedlings were divided into 4 blocks according to the different location in the growth chamber and subjected to different nutrition and pH treatments for 8 weeks (Fig. 2.1). There were 8 seedlings per treatment for a total of 112 plants for each species. The treatments consisted of two nutrition levels (25% and 100% Hoagland's solution) and seven pH levels (5.0, 6.0, 7.0, 7.5, 8.0, 8.5, and 9.0). The pH of the solution that was required to achieve a desired pH in the sand culture was experimentally determined in preliminary experiments (Table 2.2). The seedlings were watered 3 times per day to maintain the desirable pH value of the sand and provide water to plants. The sand pH was measured with a pH meter (Model IQ170, Hach Company Loveland, CO, USA.) equipped with a stainless steel probe (Model PH77-SS, Hach Company, London, ON, Canada). The pH measurements were carried out twice a week to keep the sand pH fluctuations < 1.0 of

the preset values (Fig. 2.2). The sand was flushed every two weeks to prevent ion accumulation.

2.2.3 Dry weights

At the end of treatments, shoot and root dry weights were measured in eight seedlings ($n = 8$) per treatment and tree species. The seedlings were excised into roots and shoots and dried in an oven at 70°C for 72 h. The leaves for chlorophyll measurements were detached from the stems and immediately placed in a freezer at -80°C for 72 h. The leaves were separated into young leaves (those that sprouted after the start of treatments and were close to the shoot apex) and old leaves (those that expanded fully before the treatments). The sum of the dry weights of stems, old leaves and young leaves from each plant was referred to as the shoot dry weight.

2.2.4 Net photosynthetic (Pn) and transpiration (E) rates

After 8 weeks of treatments, Pn and E were measured in the growth room with eight seedlings ($n = 8$) per treatment for each species. Fully developed leaves with minimal or no necrosis on the uppermost branches were selected for measurement using the infrared gas analyzer (LI-6400, LI-COR, Lincoln, NE, USA). The reference CO_2 concentration was $400\mu\text{mol mol}^{-1}$ and the flow rate was $200\mu\text{mol s}^{-1}$ in the leaf chamber. The leaf chamber temperature was kept at 20°C , and the PPFD was set to $400\mu\text{mol m}^{-2}\text{ s}^{-1}$. The measurements were taken from 8:00 to 14:00. For conifers, about 3-cm distal part of the

uppermost branch in white spruce and about 3-cm distal parts of needles in jack pine were placed in the leaf chamber for measurement. The needles in the leaf chamber were then cut with scissors and scanned to estimate needle areas with the Sigma-scan Pro 5.0 (Systat Software, San Jose, CA, USA).

2.2.5 Leaf (needle) chlorophyll concentrations

Chlorophyll a and chlorophyll b concentrations were determined in old and young leaves (needles) in six randomly-selected seedlings per treatment ($n = 6$) for each species. After freeze-drying, the leaves (needles) were ground with a Thomas Wiley Mini-Mill (Thomas Scientific, NJ, USA). Pulverized leaf samples (10 mg) were extracted with 8 ml dimethylsulfoxide (DMSO) at 65°C for 22 h. Chlorophyll concentrations were measured with DMSO extracts at 648 nm and 665 nm using a spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden). Total chlorophyll concentration was calculated using the Arnon's equation for DMSO (Barnes et al. 1992).

2.2.6 Elemental analysis of young leaves (needles)

Six seedlings ($n = 6$) were randomly selected per treatment for each plant species. Elemental concentrations were analyzed in young leaves (needles) since visible symptoms such as leaf chlorosis attributed to treatments mainly occurred in young leaves (needles). Concentrations of Mg, P, Ca, Fe, Mn, and Zn were determined due to concerns of their possible reduced uptake at high pH (Valentine et al. 2006, Marschner 2012, Zhang et al. 2013). Ground young leaf (needle) samples of 0.3-0.4 g dry weight were digested with 10

ml 70% HNO₃ and heated in a digestion block for 1 h. After complete digestion and cooling, the solution was diluted with Milli-Q water to 40 ml. The extracts were then filtered and analyzed by ICP-MS (inductively coupled plasma mass spectrometry) (Zarcinas et al. 1987) in the Radiogenic Isotope Facility at the University of Alberta, Edmonton, AB, Canada.

2.2.7 Experimental design and statistical analysis

All data were analyzed with SAS (Version 9.3, SAS Institute Inc., Cary, NC) to determine statistically significant differences ($p \leq 0.05$). The model was a randomized complete block design (block = location in a growth chamber, with two seedlings from each species per treatment per block) with a 7 (pH) \times 2 (nutrition level) factorial treatment arrangement. Two-way ANOVA (Analysis of Variance) was used to compare differences between the means. Residuals were checked for normality and homogeneity of variance. The log₁₀ function was used to transform the data if they did not meet the ANOVA assumptions. Comparisons between different treatment means were carried out by Tukey's test.

2.3 Results

2.3.1 Total dry weights and shoot to root (s/r) dry weight ratios

Total dry weights of trembling aspen grown in 100% Hoagland's solution were more like 1.5 times as high compared with those grown in 25% Hoagland's solution at pH 5.0 (Fig. 2.3a). Lower total dry weights were observed at pH 8.5 and 9.0 compared with pH 5.0 in 25% Hoagland's solution in jack pine (Fig. 2.3b) and at pH 7.0 compared with that at pH

5.0 in 100% Hoagland's solution in white spruce (Fig. 2.3c). The interaction effects (nutrition x pH) on the total dry weight were significant in white spruce (Table 2.1).

In trembling aspen, the interaction of pH and nutrient supply on s/r ratios was significant (Table 2.1), and the highest s/r ratio was measured at pH 8.0 in 25% Hoagland's solution (Fig. 2.3d). In jack pine, the s/r ratio at pH 7.5 and 9.0 in 100% Hoagland's solution increased by about 80% compared with 25% Hoagland's solution (Fig. 2.3e). Both the pH and nutrient supply treatments had little effect on s/r ratio in white spruce (Fig. 2.3f).

2.3.2 Gas exchange

There were no statistical interaction effects (nutrition x pH) for both Pn and E in the three examined species (Table 2.1). For trembling aspen, the significant reductions in Pn occurred at pH 8.0-9.0 in both nutrient concentrations (Fig. 2.4a). For the other two species, there were no significant differences in Pn across the treatments (Fig. 2.4b, c). In trembling aspen and white spruce, E decreased at pH 9.0 in 25% Hoagland's solution (Fig. 2.4d, f).

2.3.3 Chlorophyll concentrations

Increasing pH resulted in a decrease in leaf (needle) chlorophyll concentrations of the three species in both nutrient supply levels (Fig. 2.5a, b, c). There were significant interactions between pH and nutrient supply for the chlorophyll concentrations in old leaves (ChlO) of

trembling aspen, and the chlorophyll concentrations in young needles (ChlY) of jack pine and white spruce (Table 2.1). For white spruce, both ChlO and ChlY were drastically reduced at pH 9.0 in the 25% Hoagland's solution, while there was no significant pH effect in the 100% Hoagland's solution (Fig. 2.5c).

2.3.4 Elemental concentrations of young leaves in 25% Hoagland's solution

In trembling aspen, the concentrations of Mg, P, Ca, Mn, and Zn decreased when the roots were exposed to high pH (Fig 2.6a). The concentrations of Mg, P, and Ca in young leaves decreased at pH 8.5 and 9.0 while Zn decreased with increasing pH starting at pH 7.0 (Fig 2.6a). The pH treatments had minor effects on Fe concentrations in trembling aspen (Fig 2.6a).

In jack pine, significant reductions in Mg, P, Ca, Mn, Zn, and Fe concentrations were measured with increasing pH. The pH threshold required to trigger these decreases varied depending on the element. For the concentration of Mg, P, Ca, Fe, and Zn, the decreases started at pH 8.0 and higher. However, the concentration of Mn decreased at and above pH 6.0 (Fig 2.6b).

In white spruce, the concentrations of Mg, P, Ca and Fe decreased at pH 9.0, but the concentrations of Mn decreased from pH 7.0 (Fig 2.6c).

2.4 Discussion

The studies that previously examined high pH tolerance in boreal forest plants were carried out in solution culture due to the difficulty of effective pH control (Zhang et al. 2013, Zhang and Zwiazek 2016, Calvo-Polanco et al. 2017). In the present investigation, sand culture was used to examine physiological responses of trembling aspen, jack pine, and white spruce seedlings to root zone pH as affected by the supply of mineral nutrients. Sand culture is a preferred method to study plant responses to pH because it minimizes the problems of complexity and high buffering capacity of soil, yet provides a more natural environment for roots compared with hydroponics (Chen and Gabelman 1990, Donald and Porter 2004). Therefore, sand culture can better simulate the soil environment and provide good aeration, support for plants and habitat for soil microorganisms (Siemens and Zwiazek 2011). Nevertheless, caution must be taken when interpreting the results of controlled-environment studies, since they may not always represent plant responses in natural variable environments and the complexity of natural soils with diverse physical, chemical or biological characteristics (Calvo-Polanco et al. 2008).

Compared with two earlier studies that examined the same pH treatments in 25% Hoagland's solution as the present study, but were carried out in hydroponic culture (Zhang et al. 2013, 2015), the total dry weights of trembling aspen were relatively less affected by high pH (Zhang et al. 2013). The dry weights of jack pine decreased at pH 8.5 and 9.0 in sand culture, but they decreased at and above pH 7.0 compared with pH 5 in hydroponic culture (Zhang et al. 2015). White spruce showed the same patterns of responses to pH in

sand and hydroponic cultures (Zhang et al. 2013) with little effect of pH on dry weight. Although some of the differences could be due to plant population differences in the two studies, overall, the results indicated that there was relatively less effect of high pH on plants in sand culture compared with the hydroponics. Plant roots can mediate rhizosphere pH in response to environmental constraints (Hinsinger et al. 2003). H⁺-ATPases play an important role in pumping protons across the membrane with ion uptake (Rengel 2002). Interacting with a localized rhizosphere, the apoplastic pH of roots actually could be lower than that of the growth medium. For instance, in lupin (*Lupinus angustifolius* L.), the root apoplastic pH increased by 0.3 units when the external root zone pH increased from 5.2 to 7.5 (Yu et al. 2001). However, in hydroponic culture, the constant circulation of nutrient solution makes it more difficult for plants to maintain a proton gradient compared with the solid growth medium.

With the high nutrition supply, the total dry weight of trembling aspen at pH 5.0 was about twice as high compared with in 25% Hoagland's solution, but P_n was little affected by the increased nutrition supply. It was consistent with the earlier study that showed the total dry weight of trembling aspen to be substantially increased by higher nutrient availability with no increases of P_n (Hemming and Lindroth 1999). With high fertilizer supply, trembling aspen tended to maintain photosynthesis but increased leaf production (Hemming and Lindroth 1999) and *Salix glauca* produced more leaves per shoot than did unfertilized trees instead of increasing P_n (Bowman and Conant 1994). The same phenomenon was also observed in the present study. This could explain why there were no differences in P_n of trembling aspen between the two nutrition levels, but higher leaf areas (higher effective

photosynthesis areas) made the total dry weights higher in 100% Hoagland's solution at pH 5.0. The Pn of trembling aspen was about two-fold compared with jack pine and white spruce at pH between 5.0 and 7.0 in both nutrition levels. The higher Pn reflects higher growth rates of trembling aspen and consequently higher demands for nutrients and water. Since jack pine and white spruce are relatively slow-growing plants compared with trembling aspen (Walker and Chapin 1986), their nutrient and water requirements are lower. This demonstrates that increasing the nutrient supply was beneficial to meet the optimum nutrient requirement of fast-growing species (trembling aspen), especially under low pH conditions, but did not have a major impact on slow-growing species (jack pine and white spruce). However, at the high pH levels, no significant benefits of high nutrition supply on the total dry weights was observed in any of the three species, including trembling aspen, suggesting either that other factor(s) than nutrients were limiting to growth at high pH or that the additional nutrients could not be absorbed and/or utilized at the higher pH levels.

When supplied with 100% Hoagland's solution, the s/r ratios of jack pine significantly increased at pH 7.5 and 9.0 compared with those at 25% nutrient supply, but there was no effect on s/r ratios of white spruce at the neutral to high pH. According to the theory of functional equilibrium of biomass allocation, plants tend to allocate a larger proportion of carbohydrates to shoot growth under better nutrition conditions (Brouwer 1963, Shipley and Meziane 2002). As the relatively slow-growing species, jack pine allocated more biomass to shoots while white spruce partitioned more assimilates to roots under the higher nutrition conditions and high pH. This suggests that white spruce had higher nutrient acquisition efficiency compared with jack pine since more investment to root growth could

facilitate nutrient uptake. The results from analysis of total dry weight and chlorophyll concentration also suggest that white spruce was more tolerant of high pH in this study compared with trembling aspen and jack pine. Allocation of more biomass to roots may be an adaptation mechanism of white spruce to low nutrient availability and high pH.

In trembling aspen, P_n decreased with increasing pH. The P_n at pH 8.0-9.0 was reduced by more than 50% compared with at the lower pH levels in both nutrition levels. The reduction of photosynthesis is usually related to stomatal closure and decreased efficiency of the photosynthetic system. Since a major decrease of E in trembling aspen was only at pH 9.0 in 25% Hoagland's solution, the decrease in P_n with increasing pH was likely mostly due to the non-stomatal factors. Under nutrient deficiency stress, photosynthetic depression was found to be caused by the biochemical, rather than stomatal, limitation (Dang and Zhang 2006). In high pH treatments in 25% Hoagland's solution, the concentrations of foliar nutrients decreased in trembling aspen including Mg, P, and Mn, and these elements are required for photosynthesis and chlorophyll synthesis (Marschner 2012). Although Fe reduction was not observed in trembling aspen in 25% Hoagland's solution, the 'physiological activation' of Fe may be lower in chlorotic leaves under high pH condition (Mengel 1994).

The E of trembling aspen and white spruce decreased at pH 9.0 in 25% Hoagland's solution. Since all of the plants in this study were provided with adequate water, the apparent differences in E at different pH levels likely reflect a reduced ability of absorb and transport

water through the root system. When roots of paper birch (*Betula papyrifera* Marsh.) were exposed to high pH, root hydraulic conductivity and stomatal conductance significantly decreased (Kamaluddin and Zwiazek 2004). The decrease in root water flux may result from the reduced root system size (Tang et al. 1993a, Zhang et al. 2015) and the reduction of root aquaporin activity (Kamaluddin and Zwiazek 2004). According to the acid-growth-theory, high apoplastic pH can decrease cell wall extensibility and inhibit cell growth (Rayle and Cleland 1970). Additionally, AQP activities which mediate the cell-to-cell pathway for root water transport, are sensitive to changes in pH (Tournaire-Roux et al. 2003, Kamaluddin and Zwiazek 2004).

The chlorophyll concentrations decreased with increasing pH in trembling aspen and jack pine, and the ChlY decreased more compared with ChlO. In high pH environments, nutrient deficiencies such as Mg (Baszynski et al. 1980), Fe (Larbi et al. 2006), and Mn (Shenker et al. 2004) can cause decreases in chlorophyll concentration. Lower concentrations of these elements were found in young leaves of trembling aspen and jack pine grown in 25% Hoagland's solution. Since Mn and Fe are immobile or intermediate-mobile elements in plants (Marschner 2012), they are usually not transferred from old to young tissues. Therefore, ChlY was more severely affected by high pH compared with ChlO. However, for white spruce, although Mg, Fe, and Mn also decreased with increasing root zone pH, both ChlY and ChlO were little impacted by these reductions over the study period of 8 weeks. Since white spruce has likely the lowest growth rate among the studied species and, therefore, is less nutrient demanding, however, it cannot be excluded that it would be more impacted by high pH in the longer run.

In conclusion, the responses of the studied plants to pH and nutrition levels in sand culture varied among species and this variation was likely related to the differences in their nutrient demands. White spruce was relatively tolerant of high pH compared with trembling aspen and jack pine, and this tolerance may be partly due to higher biomass allocation to roots which facilitated nutrient uptake and possibly was due to slower growth. Most of the high pH effects on plants observed in the present study could be attributed to mineral deficiencies including Mg, P, Fe, Mn, Ca, and Zn. However, higher nutrition supply had a beneficial impact only on the total dry weights and only at the lower pH levels in trembling aspen. An approach to help mitigate high pH stress should include not only increased nutrient supply but also improved nutrient uptake and utilization.

2.5 References

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2.6 Tables

Table 2. 1 ANOVA table showing effects of pH and nutrition treatments on the measured parameters for trembling aspen, jack pine and white spruce seedlings.

Trembling aspen

p-value	tdw	s/r ratio	Pn	E	ChlO	ChlY
nutri	0.1435	0.5272	0.0002	<.0001	0.8623	0.0015
pH	<.0001	0.0478	<.0001	0.0023	<.0001	<.0001
Nutri x pH	0.2456	0.031	0.7181	0.4015	0.0033	0.0993

p-value	Mg	P	Ca	Fe	Mn	Zn
pH	<.0001	<.0001	0.0011	0.1262	0.0433	<.0001

Jack pine

p-value	tdw	s/r ratio	Pn	E	ChlO	ChlY
nutri	0.1765	<.0001	0.0033	0.0009	0.5763	0.8062
pH	0.0013	0.2198	0.2656	0.0712	<.0001	<.0001
Nutri x pH	0.1107	0.8615	0.8682	0.2563	0.3745	0.0215

p-value	Mg	P	Ca	Fe	Mn	Zn
pH	<.0001	0.0039	<.0001	0.0002	<.0001	0.0061

White spruce

p-value	tdw	s/r ratio	Pn	E	ChlO	ChlY
nutri	0.6937	<.0001	<.0001	<.0001	0.0539	0.2199
pH	0.024	0.3273	0.0002	<.0001	0.0005	0.0001
Nutri x pH	0.0089	0.0763	0.6236	0.8809	0.187	0.0355

p-value	Mg	P	Ca	Fe	Mn	Zn
pH	0.0002	<.0001	0.0003	0.0066	<.0001	0.1072

Abbreviations are: tdw - total dry weight (n = 8); s/r ratio- shoot to root dry weight ratio (n = 8); Pn - net photosynthetic rate (n = 8); E - transpiration rate (n = 8); ChlO - chlorophyll concentrations in old leaves (n = 6); ChlY - chlorophyll concentrations in young leaves (n = 6); element concentrations (n = 6).

Table 2. 2 The pH of 25% and 100% Hoagland's solution that was required to achieve the aimed pH in the sand culture.

Aimed sand pH	Solution pH (25%)	Solution pH (100%)
5.0	3.5	3.8
6.0	5.0	5.0
7.0	9.5	9.0
7.5	10.0	9.5
8.0	10.5	10.0
8.5	11.0	10.5
9.0	11.5	11.0

2.7 Figures

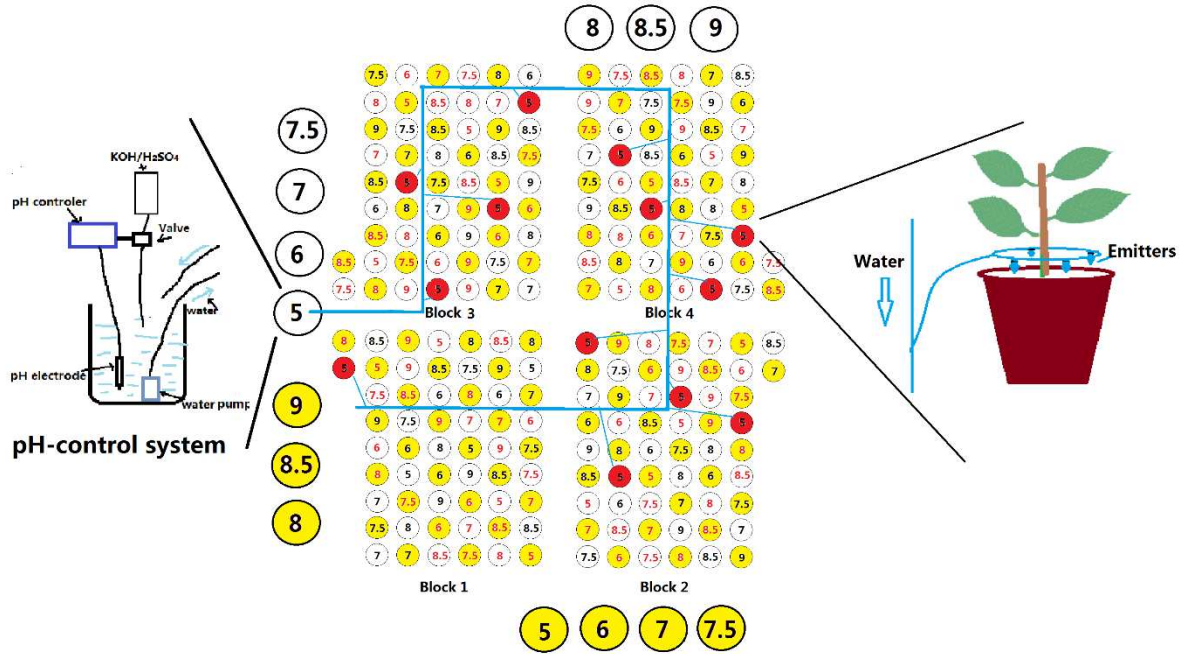


Figure 2. 1 Schematic diagram of the automatic irrigation system. This system consisted of the watering system and pH-control system parts. The main part of the watering system was made of tubing setup which composed the 19mm PVC tubing (thick blue line) to which 6mm PVC tube (thin blue line) was connected and fixed on the top of each pot by the 4 x 6mm support stakes. There were four emitters connected to the 6mm tube to ensure that the same amount of solution was delivered to each pot. In the pH-control system, a gel-filled combination pH electrode was placed in the solution and connected to a pH controller to adjust the solution pH by adding 5 % (w/w) KOH or 1 % (v/v) H₂SO₄ as required to adjust pH to the pre-set level.

The larger cycles denote buckets with treatment solution and the small cycles denote pots with plants. There were water pumps in the buckets to delivery nutrient solution to each pot. Yellow color denotes plants supplied with 25% Hoagland's solution and white color denotes plants supplied with 100% Hoagland's solution. The numbers denote the pH levels. All the plants were placed randomly. Different blocks were according to locations in a growth chamber.

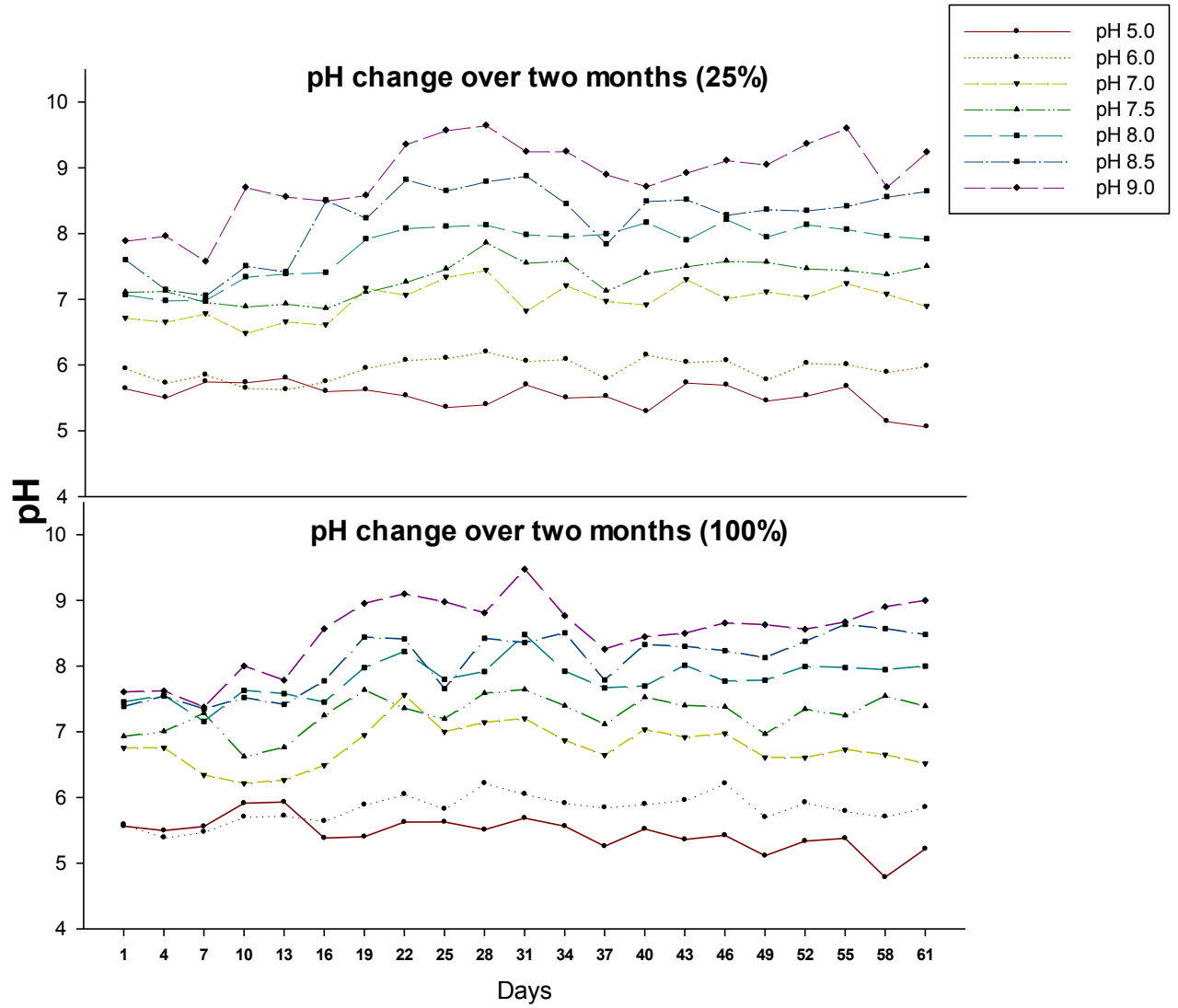


Figure 2. 2 The pH in sand culture during two months, x-axis showed the days since treatment started.

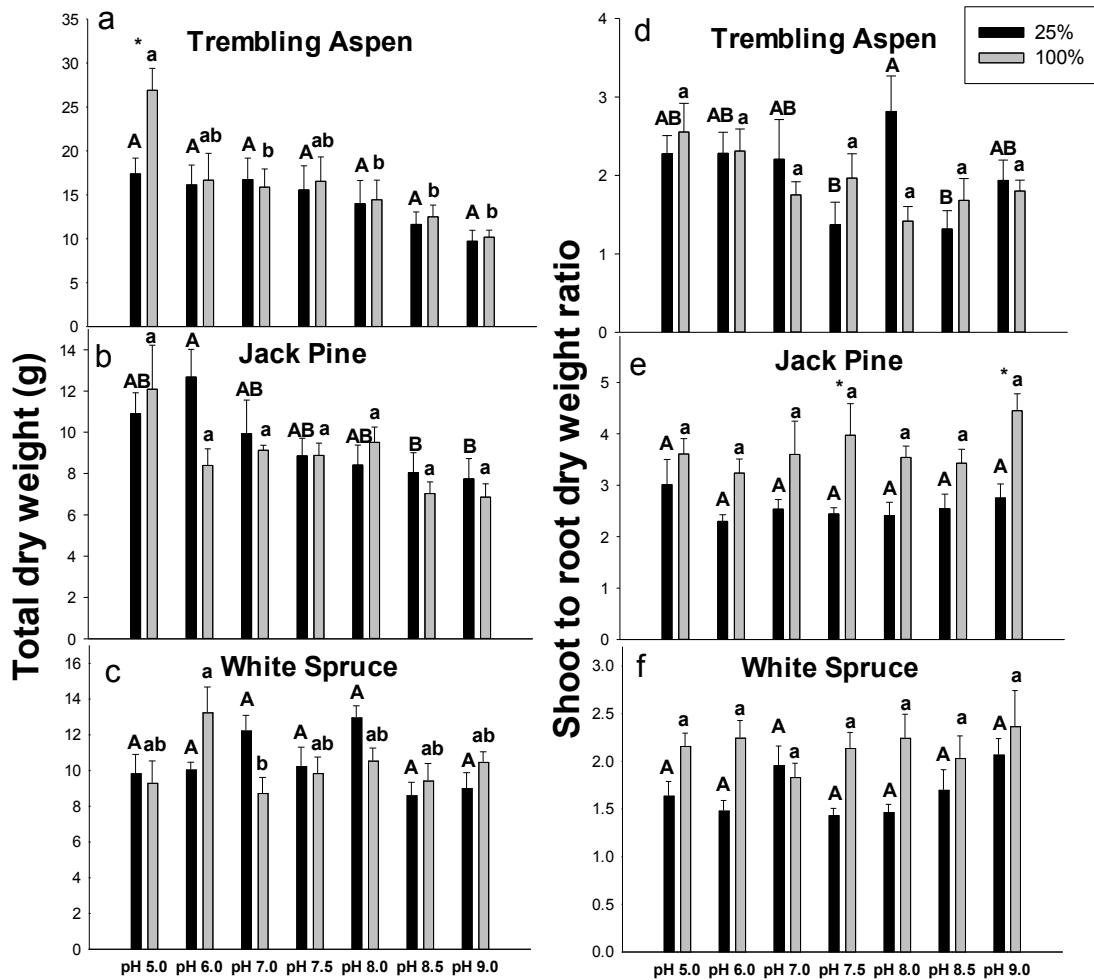


Figure 2. 3 Effects of pH and nutrition level on total dry weights and shoot to root dry weight ratios in trembling aspen, jack pine, and white spruce. Different letters above the bars (uppercase letters for 25% Hoagland's solution and lowercase letters for 100% Hoagland's solution) indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. The asterisk above the bars indicates significant differences ($\alpha = 0.05$) between 25% and 100% Hoagland's solution. Means ($n = 8$) \pm SE are shown.

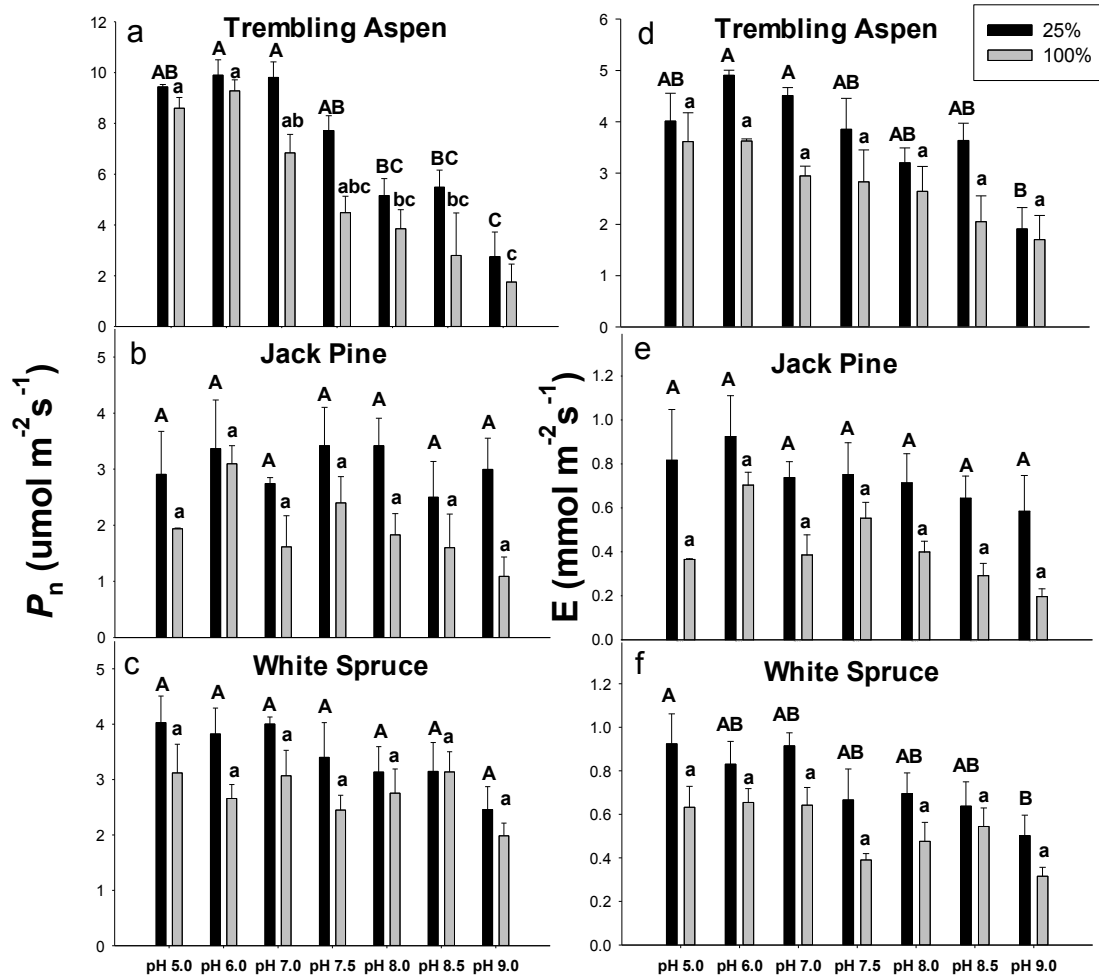


Figure 2. 4 Effects of pH and nutrition level on net photosynthetic (P_n) and transpiration rates (E) in trembling aspen, jack pine, and white spruce. Different letters above the bars (uppercase letters for 25% Hoagland's solution and lowercase letters for 100% Hoagland's solution) indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 8$) \pm SE are shown.

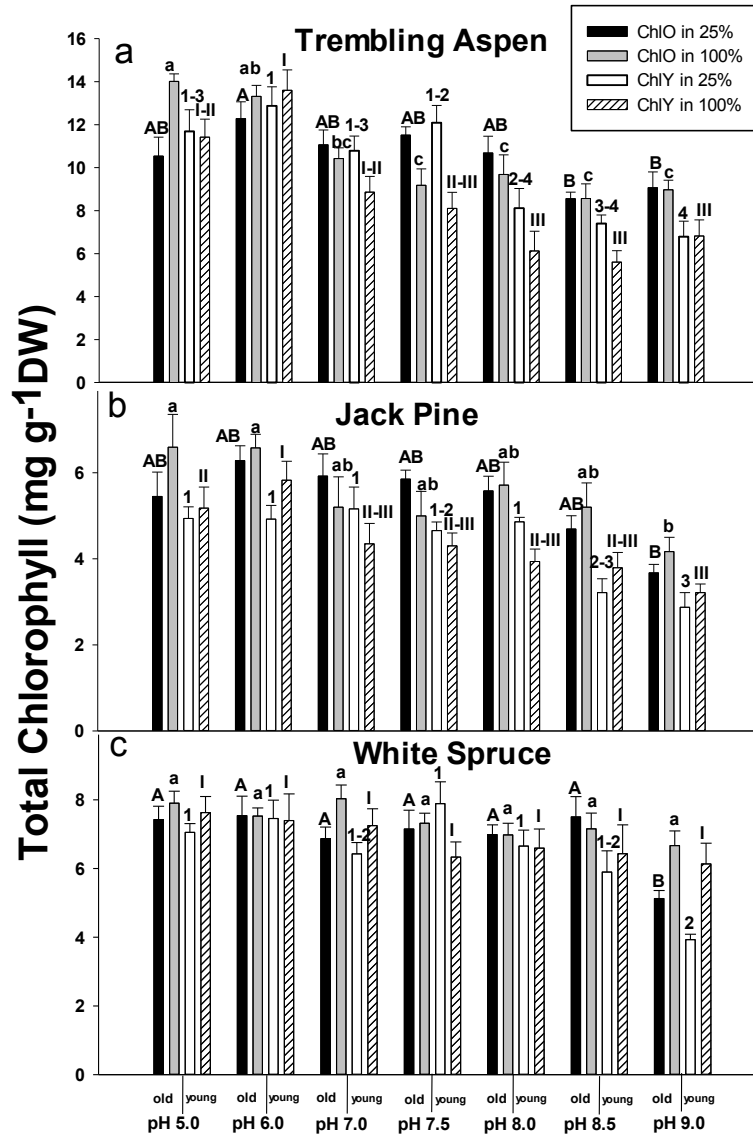


Figure 2. 5 Effects of pH and nutrition level on total chlorophyll concentrations (chlorophyll-a + chlorophyll-b) in old and young leaves of trembling aspen, jack pine, and white spruce. The old leaves were those which expanded fully before the treatments and the young leaves were those that sprouted after the start of treatments and were close to the shoot apex. Different letters above the bars (uppercase letters for chlorophyll in old leaves (ChlO) in 25% Hoagland's solution, lowercase letters for ChlO in 100% Hoagland's solution, numbers for chlorophyll in young leaves (ChlY) in 25% Hoagland's solution, and Roman letters for ChlY in 100% Hoagland's solution) indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 6$) \pm SE are shown.

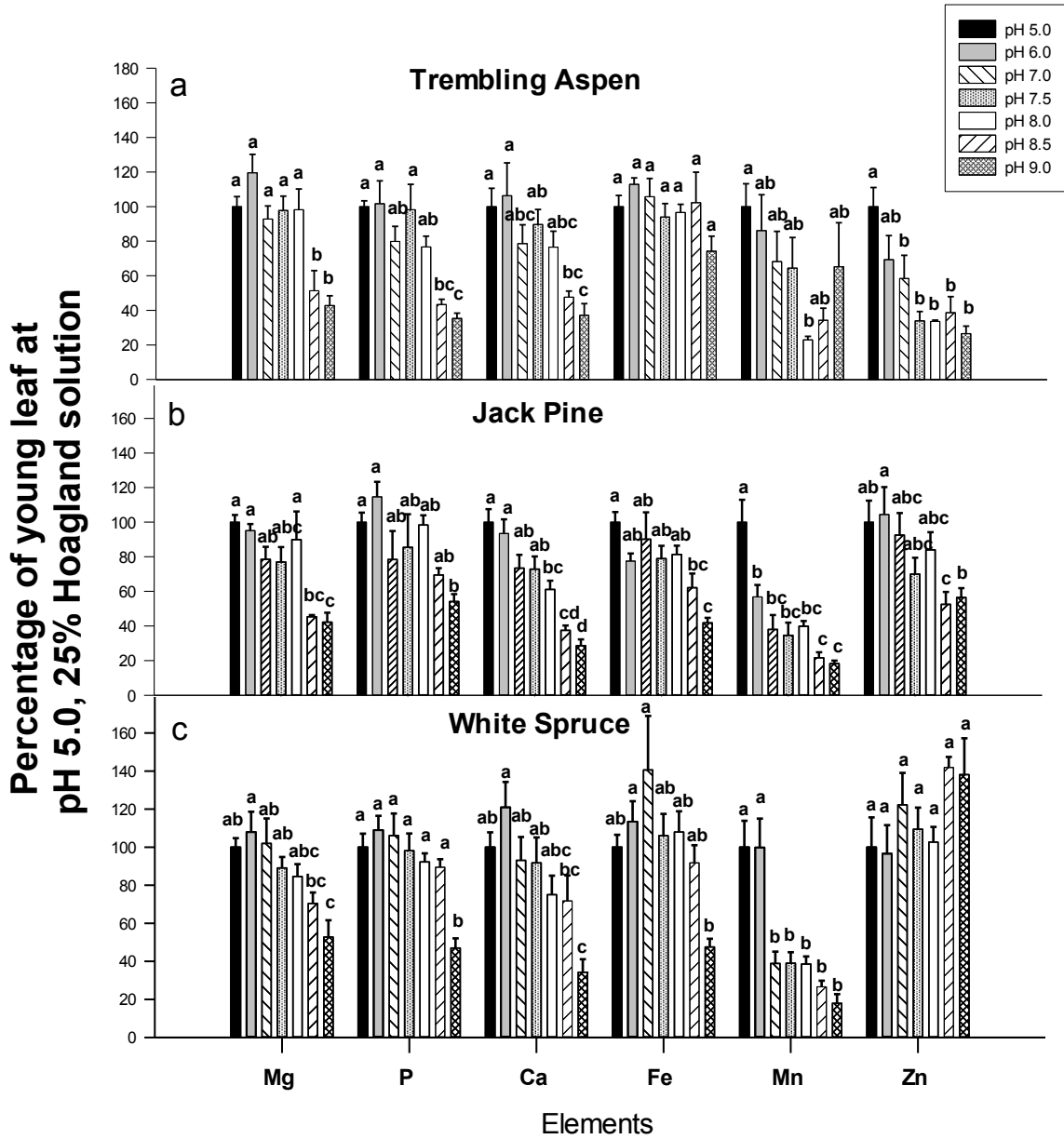


Figure 2. 6 Effects of pH and nutrition level on Mg, P, Ca, Fe, Mn, and Zn concentrations in young leaves of trembling aspen, jack pine, and white spruce seedlings in 25% Hoagland's solution, presented as the percentages of values measured at pH 5.0 in young leaves. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 6$) \pm SE are shown.

Chapter 3*

Effects of iron supply at different solution culture pH on growth and physiological responses of paper birch (*Betula papyrifera*), white spruce (*Picea glauca*), green alder (*Alnus viridis*), and tamarack (*Larix laricina*)

3.1 Introduction

Soil pH is among the most important factors determining nutrient availability in soil (Comerford 2005). Approximately one-third of the world's land surface is covered by high pH soils (Chen and Barak 1982). In high pH soils, the availabilities of elements essential to plants, including P, B, Zn, Fe, Mn, and Cu, are low (Marschner 2012). Among these nutritional constraints, chlorosis induced by Fe deficiency is a severe agricultural problem since Fe deficiency decreases both plant productivity and the quality of their plant products. Although Fe is the fourth most abundant element in the Earth's crust, it is often limiting to plant growth. The solubility of Fe (III) dramatically decreases with increasing pH due to the formation of Fe hydroxides, oxyhydroxides, and oxides, especially in well-aerated alkaline soils (Lemanceau et al. 2009).

Fe deficiency is a limiting factor leading to the reduction of plant productivity. The

*I also examined trembling aspen and black spruce in this study. The results of other parameters of the studied species and those for trembling aspen and black spruce are in Appendix 2.

biomass of phytoplankton (Martin and Fitzwater 1988), *Arabidopsis* (*Arabidopsis thaliana*) (Ravet et al. 2009), and crops, including tomato (*Solanum lycopersicum*) (Jin et al. 2009), spinach (*Spinacia oleracea*) (Jin et al. 2013), and rice (*Oryza sativa*) (Takahashi et al. 2001) have been demonstrated to be affected by Fe availability. These reductions resulted from the inhibition of photosynthesis, which is a common response of plants to Fe deficiency. Chloroplasts are the largest pool of Fe containing more than 80% of the Fe present in cells (Marschner 2012). Fe deficiency affects the structure and function of chloroplasts, which is the main reason for the reductions in photosynthetic rates (Terry and Abada 1986). Fe is an important constituent of the electron transfer chain located in the thylakoid membranes (Terry and Low 1982). It is directly involved in PSI, PSII, and Cyt bf complex in forms of the heme, Fe-S cluster or other Fe-binding sites (Raven et al. 1999). Fe deficiency can also diminish the synthesis of all light-harvesting pigments. Several reports have indicated that several steps of chlorophyll biosynthesis precursors, such as the formation of protochlorophyllide and aminolevulinic acid, are Fe-dependent (Miller 1982, Pushnik 1984, Marschner 2012). The chlorosis of young leaves is usually reversible (Platt-Aloia et al. 1983) and Fe-deficient plants respond to Fe resupply by a rapid increase in photosynthesis (Larbi et al. 2004). Therefore, adding more Fe fertilizer to the soil is the most commonly used treatment for correcting Fe chlorosis.

Plants have developed two principal strategies to improve Fe uptake, the reduction-based strategy I used by the non-graminaceous plants and the chelation-based strategy II used by the graminaceous monocots (Marschner et al. 1986). The strategy I plants respond to Fe deficiency with both morphological and physiological changes, including formation of

more root hairs and transfer cells, excretion of diverse chemical species such as protons, phenolic compounds, and flavins to the rhizosphere, and an increase in the capacity for Fe reduction (Marschner et al. 1986). The reduction of Fe^{3+} at the apoplast/symplast interface for absorbing Fe from the rhizosphere and transporting Fe across the plasma membrane is the critical process for strategy I plants. These processes are pH-dependent since the ferric chelate reductase (FCR) activity is highly pH sensitive. The chlorotic leaves suffering from high pH condition usually are green along the vascular veins, probably due to the inhibition of Fe reduction along the vascular path (Mengel and Bubl 1983). Increased Fe supply was found to enhance the root FCR tolerance of higher pH (Yue Ao et al. 1985). However, these processes are still poorly understood and it is important to investigate plant responses to high pH in the presence of different Fe concentrations to understand how to alleviate pH-induced Fe deficiencies.

In the present study, I examined the growth and physiological responses of the strategy I plants: paper birch (*Betula papyrifera*), white spruce (*Picea glauca*), green alder (*Alnus viridis*), and tamarack (*Larix laricina*) to Fe supply at different root zone pH in hydroponic culture. The study was carried out to shed more light on the role of Fe deficiencies in the plant responses to root zone pH. The experiment included four species that showed relatively higher and lower tolerance to high root zone pH in preliminary experiments. Since Fe is an essential element to plants and its solubility is low under aerobic alkaline conditions, I hypothesized that high pH tolerance in plants is associated with their tolerance to Fe deficiency and can be improved by increasing the supply of Fe.

3.2 Materials and methods

3.2.1 Plants and experimental setup

Dormant seedlings of four species: paper birch (*Betula papyrifera*), white spruce (*Picea glauca*), green alder (*Alnus viridis*), and tamarack (*Larix laricina*) were obtained from Boreal Horticultural Services Ltd., Bonnyville, Alberta, Canada. The seedlings were grown in tree nursery in containers (415D styroblocks™, Beaver Plastics, Acheson, AB, Canada) for one year. For the experiment, the roots of the seedlings were washed free of soil and rinsed thoroughly, and the seedlings were placed in 30 L tubs with aerated 25% Hoagland's solution for ten days in a controlled-environment growth room. Environmental conditions in the growth chamber were maintained at 22/18°C (day/night) temperature, 65 ± 10 % relative humidity, and 16-h photoperiod with 300 ± 50 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD) provided by the full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada).

An Automatically-controlled hydroponic setup (Zhang et al. 2013) as shown in Fig. 3.1 was prepared for the study. For each treatment, three replicated 30 L opaque plastic tubs were connected to a 120 L barrel through the PVC (polyvinyl chloride) tubing. A water pump (Model 9.5 950GPH, Danner MFG Inc., New York, NY, USA) was immersed in the modified Hoagland's solution (Epstein 1972) in the barrel to circulate nutrient solution between the barrel and the tubs. All tubs had spouts installed into their sides with 1-m-long tubing to facilitate nutrient solution circulation. In this way, the solution was constantly circulated and sufficiently aerated (~ 8 mg L⁻¹ O₂). A styrofoam board with twenty holes (each 3.8 cm in diameter) was placed on the top of each tub. The seedling stems were

secured with foam plugs and the roots inserted through holes into the nutrient solution. There were 5 seedlings for each of the 4 species in the tub for the total of 20 seedlings per tub.

3.2.2 Experimental treatments

The plants were supplied with 25% Hoagland's solutions containing different Fe concentrations (1.25, 5, and 20 μM , which were 0.25x, 1x and 4x of the Fe concentration present in the 25% Hoagland's solution) (Epstein 1972) at different pH levels (5, 7, and 9). Solution pH was maintained by a pH-control system as previously described (Zhang et al. 2013). In this system, the pH controller (PHCN-70, Omega Engineering Inc., Laval, QC, Canada) was connected to the pH electrode (Orion 9106 BNWP gel-filled combination pH electrode, Thermo Scientific, Rochester, NY) that was immersed in the nutrient solution in each barrel. The controller automatically opened and closed an electronic valve (Model 8260G071 120/60 ASCO Valve, Inc., Florham Park, NJ, USA) to adjust the solution pH by adding 5 % (w/w) KOH or 1 % (v/v) H_2SO_4 as needed. During the experiments, the variation in solution pH in the containers was maintained within a ± 0.1 range. The treatments lasted for 8 weeks and the nutrient solution was replaced every 2 weeks.

3.2.3 Elemental analysis of nutrient solution

The concentrations of soluble Fe, B, Mg, P, K, Ca, Mn, Cu, and Zn in the solution were measured for all treatments. Three 30 ml samples ($n = 3$) were obtained from each treatment

solution and filtered through the sterile syringe filters (Millex-HV Syringe Filter Unit, 0.45 μm pore size PVDF (hydrophilic polyvinyl difluoride) membrane, EMD Millipore Corporation, Billerica, MA, USA). The elements in the filtered solution were analyzed by the ICP-MS (inductively coupled plasma mass spectrometry) method (Zarcinas et al. 1987) in the Radiogenic Isotope Facility at the University of Alberta, Edmonton, AB, Canada.

3.2.4 Net photosynthetic (Pn) and transpiration (E) rates

After 8 weeks of treatments, Pn and E were measured in six seedlings per treatment for each plant species ($n = 6$). Fully-developed uppermost leaves with minimal or no necrosis were selected for the measurements with an infrared gas analyzer (LI-6400, LI-COR, Lincoln, Nebraska USA). For conifers, about 3-cm distal part of the uppermost branch was placed in the leaf chamber for measurement. The needles in the leaf chamber were then cut with scissors and scanned to estimate needle area with the Sigma-scan Pro 5.0 (Systat Software, San Jose, CA, USA). The reference CO_2 concentration was $400\mu\text{mol mol}^{-1}$ and the flow rate was $200\mu\text{mol s}^{-1}$ in the leaf chamber. The leaf chamber temperature was kept at $20\text{ }^\circ\text{C}$, and the PPFD was set to $400\mu\text{mol m}^{-2}\text{ s}^{-1}$. The measurements were taken from 8:00 to 14:00.

3.2.5 Dry weights

At the end of the treatments, shoot and root dry weights were determined for six seedlings per treatment for each tree species ($n = 6$). The seedlings were separated into roots, stems,

young and old leaves and dried in an oven at 70°C for 72 h. The leaves for chlorophyll measurements were freeze-dried for 72 h. The old leaves were those which expanded fully before the treatments and the young leaves were those that sprouted after the start of treatments and were close to the shoot apex. The combined dry weight of stems, old leaves and young leaves from each plant was referred to as the shoot dry weight.

3.2.6 Leaf chlorophyll concentrations

Chlorophyll a and b concentrations were determined in old and young leaves (needles) of six randomly selected seedlings per treatment for each species (n = 6). Freeze-dried leaves (needles) were ground in a Thomas Wiley Mini-Mill (Thomas Scientific, NJ, USA). Pulverized leaf (needle) samples (10 mg DW each) were extracted with 8 ml of extraction solvent. For paper birch, white spruce, and tamarack, the chlorophyll was extracted with dimethylsulfoxide (DMSO) at 65°C for 22 h. For green alder, the chlorophyll was extracted with 8 ml methanol at 55°C for 24 h since the DMSO extracts of green alder leaves were black, which interfered with chlorophyll analysis. Chlorophyll concentrations were measured in DMSO extracts at 648 nm and 665 nm, and in methanol extracts at 652 nm and 665 nm with a spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden). Chlorophyll a and b concentrations were calculated using the Arnon's equation for DMSO (Barnes et al. 1992) and the MacKinney's equation for methanol extracts (Sestak et al. 1971).

3.2.7 Statistical analysis

All data were analyzed with SAS (Version 9.3, SAS Institute Inc., Cary, NC) to determine statistically significant differences ($p \leq 0.05$). The model is a 3 (pH) x 3 (Fe concentration) factorial treatment arrangement. Two-way ANOVA was used to statistically compare differences between the means. Residuals were checked for normality and homogeneity of variances. The Log10 function was used to transform the data if they did not meet the ANOVA assumptions. Comparisons between different treatment means were conducted by Tukey's test.

3.3 Results

3.3.1 Elemental analysis of nutrient solution

The Fe concentrations decreased only slightly at pH 7 compared with those at pH 5 while they decreased almost by half at pH 9, especially in the higher concentration Fe treatments (1x and 4xFe) (Table 3.1). In the 0.25xFe treatment, the soluble Fe concentration decreased by about 25% ($1.46 \pm 0.08 \mu\text{M}$, mean \pm SE) and 35% ($1.28 \pm 0.241 \mu\text{M}$) at pH 7 and 9, respectively, compared with those at pH 5 ($1.92 \pm 0.252 \mu\text{M}$) (Table 3.1). For the 1xFe treatment, the soluble Fe concentrations decreased at pH 7 and 9 by about 15% ($4.23 \pm 0.148 \mu\text{M}$) and 45% ($2.79 \pm 0.64 \mu\text{M}$), respectively, compared with those at pH 5 ($4.84 \pm 0.124 \mu\text{M}$) (Table 3.1). For the 4xFe treatment, the soluble Fe concentrations at pH 7 and 9 decreased by about 5% ($16.47 \pm 0.238 \mu\text{M}$) and 40% ($9.98 \pm 1.28 \mu\text{M}$), respectively, compared with those at pH 5 ($16.81 \pm 0.072 \mu\text{M}$) (Table 3.1).

The concentrations of B and Mg were not greatly affected by pH (Table 3.1). The

concentrations of P, Ca, Mn, Cu, and Zn were drastically reduced at pH 9 compared with pH 5 and 7 (Table 3.1). Since KOH was added to adjust the solution pH, the concentration of K slightly increased at pH 7 and 9 (Table 3.1).

3.3.2 Net photosynthetic (Pn) and transpiration (E) rates

In paper birch, Pn was higher at pH 7 in the 4xFe treatment compared with the other two Fe treatments (Fig. 3.2a) and the interaction effects of Fe x pH were highly significant ($p < 0.0001$) (Table 3.2). The Pn of white spruce did not significantly change in response to the pH treatments (Fig. 3.2b). In green alder, there were no significant differences in Pn at all pH levels in the 4xFe treatment (Fig. 3.2c). However, in the lower Fe levels (0.25x and 1xFe), Pn decreased with an increase in pH (Fig. 3.2c). In tamarack, the reduction of Pn started at pH 7 compared with that at pH 5 in the 0.25xFe treatment, but it decreased at pH 9 in the 4xFe treatment (Fig. 3.2d).

The E of paper birch severely decreased at pH 9 in all Fe levels (Fig. 3.2e). In white spruce, E had a similar trend as Pn and was not affected by the treatments (Fig. 3.2f). There were significant interactions between Fe and pH on E in green alder and tamarack (Table 3.2). For green alder, E increased with increasing Fe treatment concentration at pH 7 (Fig. 3.2g). However, at the other pH levels, Fe had no effect on E (Fig. 3.2g). In tamarack, E in the higher Fe treatments increased sharply compared with the lower Fe concentration treatments (0.25x and 1xFe) at pH 7 and 9 (Fig. 3.2h).

3.3.3 Total dry weights and shoot to root dry weight ratios

The total dry weights of paper birch sharply decreased at pH 9 in the 4xFe treatment (Fig. 3.3a). For white spruce, the interaction effect (Fe x pH) was significant and there were reductions of the total dry weights at pH 5 and 7 in the 1xFe concentration treatment compared with that at pH 9 (Fig. 3.3b). In green alder, the total dry weights decreased with an increase in pH in all three Fe concentration treatments, but did not significantly differ between the different Fe concentration treatments at the same pH level (Fig. 3.3c). The total dry weights in tamarack were not affected by the pH and Fe treatments (Fig. 3.3d).

In paper birch, the shoot/root dry weight ratios (s/r ratio) were lower at pH 7 and 9 compared with those at pH 5 in the 0.25x and 4xFe treatments (Fig. 3.3e). There were relatively little effects of Fe and pH treatments on the s/r ratio in white spruce (Fig. 3.3f). For green alder, the s/r ratio was higher in 4xFe treatment compared with the lower concentration Fe treatments (0.25x and 1xFe) at pH 7 (Fig. 3.3g). The s/r ratios of tamarack at pH 5 in the two higher Fe concentration treatments (1x and 4xFe) were higher than those in the 0.25xFe treatment (Fig. 3.3h). The interactions of Fe and pH for s/r ratios in green alder and tamarack were significant (Table 3.2).

3.3.4 Chlorophyll concentrations

In the four examined plant species, the reductions of total chlorophyll concentrations with increasing pH were greater in young leaves (ChlY) compared with the old leaves (ChlO) (Fig. 3.4a, b, c, d). The ChlY in the 4xFe treatment was higher compared with the lower

concentration Fe treatments (0.25x and 1xFe) at pH 7 in paper birch, green alder, and tamarack (Fig. 3.4a, c, d). The interaction of Fe x pH was significant for both ChlY and ChlO in white spruce and green alder but it was significant only for the ChlY of paper birch and tamarack (Table 3.2). In paper birch, ChlY in the 4xFe treatment was about three-fold higher compared with the lower Fe concentration treatments (0.25x and 1xFe) at pH 7 (Fig. 3.4a). For white spruce, no consistent effects of pH and Fe treatments on ChlO were found and there was no significant impact of different Fe concentrations on ChlY at each pH level (Fig. 3.4b). The ChlO in green alder was higher in the 1x and 4xFe treatments compared with the 0.25xFe treatment at pH 5 (Fig. 3.4c). In tamarack, both ChlY and ChlO increased in the 4xFe concentration treatment compared with the 0.25x and 1xFe treatments at each pH except for ChlY at pH 9 (Fig. 3.4d).

The chlorophyll a to b ratios (Chl a/b) of paper birch showed a similar trend to the total chlorophyll concentrations and the decreases of Chl a/b with increasing pH were greater in the young leaves compared with the old leaves (Fig. 3.4e). In white spruce, pH and Fe treatments had less effect on Chl a/b in young compared with old needles (Fig. 3.3f). However, in the old needles of white spruce, lower values of Chl a/b were observed at pH 5 in 0.25xFe treatment and at pH 7 in 4xFe treatment compared with the remaining treatments (Fig. 3.4f). There were significant statistical interaction effects of Fe x pH on Chl a/b in young leaves of green alder and young needles of tamarack (Table 3.2). In green alder, there were no significant effects of treatments on Chl a/b ratios in old leaves, but in the young leaves Chl a/b ratios showed a decrease in the 1xFe treatment at pH 7 and 9 compared with pH 5 (Fig. 3.4g). In tamarack, the decrease in Chl a/b ratios in young

needles was observed at pH 9 in the 4xFe treatment, while the reductions in Chl a/b of young needles in the 0.25x and 1xFe treatments occurred at pH 7 and 9 (Fig. 3.4h).

3.4 Discussion

In the present study, growth and physiological responses of paper birch (*Betula papyrifera*), white spruce (*Picea glauca*), green alder (*Alnus viridis*), and tamarack (*Larix laricina*) were examined in response to different concentrations of Fe applied at acidic (pH 5), neutral (pH 7), and alkaline (pH 9) conditions. In soil solutions, the total concentration of inorganic Fe species can be as low as 10^{-10} M under neutral and alkaline conditions (Boukhalfa and Crumbliss 2002), but the concentration of soluble Fe required for optimal growth is about 10^{-6} to 10^{-5} M (Römheld and Marschner 1986). Since high root zone pH can reduce Fe availability and root Fe uptake (Mengel 1994), Fe concentration in the hydroponic solution was increased in the present study with the expectation that the increased supply of Fe could counteract this inhibitory effect. The increased concentration of Fe in the nutrient solution enhanced total chlorophyll concentration, Pn, E, and total dry weight at the neutral pH level, but had relatively rare effect at the high pH level.

Fe was supplied in the nutrient solution in the chelated form of Fe-EDTA that likely maintained a relatively higher level of Fe solubility at high pH compared with Fe in the soil solution (Römheld and Marschner 1986). Fe(III)-EDTA is soluble but the stability constant of Fe(III)-EDTA decreases sharply at pH 9 compared with pH 5 and 7 (Orama et al. 2002). Therefore, the concentrations of soluble Fe changed relatively little at pH 7 compared with pH 5, but dramatically decreased at pH 9 due to Fe(OH)₃ that is formed

through the hydrolysis reaction under alkaline conditions. The concentrations of soluble P, Ca, Mn, Cu, and Zn followed a similar trend. Although the concentration of soluble N was not measured in this study, high pH (pH 9) had little effect on N concentrations in Hoagland's solution (Zhang and Zwiazek 2016). These results indicated that, for the same Fe concentration treatment, the decreases in total dry weight, gas exchange, and chlorophyll concentrations in plants at pH 7, compared with pH 5, were unlikely caused by nutrient availability in the mineral solution. However, at pH 9, the effects on the physiological parameters could, at least partly, be explained by the reduced solubility of some of the essential nutrients in solution culture.

In paper birch, green alder, and tamarack, the ChlY significantly decreased at pH 7 and 9 compared with pH 5 in all Fe supply levels. The linear negative correlation between chlorophyll concentration and leaf apoplast pH was also reported in previous studies (Tagliavini et al. 1995). The decrease in chlorophyll concentrations in young leaves (ChlY) at pH 7 and 9 may be explained by decreased nutrient concentration in the young leaves. The concentration of nutrients related to chlorophyll concentration such as Fe and Mn in young leaves of trembling aspen and white spruce markedly decreased when their roots were exposed to the solution pH 7 and higher (Zhang et al. 2013). It is essential to maintain pH gradient across the plasma membrane for nutrient uptake. The apoplastic pH is usually kept acidic at pH of approximately 5-6, but high root medium pH can increase the root apoplastic pH (Felle and Hanstein 2002) and impair the pH gradient.

At pH 7, the ChlY in paper birch, green alder, and tamarack was about two- to three-fold

higher in the 4xFe treatment compared with the 0.25xFe and 1xFe treatments. Since Fe is involved in the various steps of chlorophyll biosynthesis, it should be expected that a higher Fe concentration in the solution would significantly increase ChlY when the supply of Fe is suboptimal. However, this positive relationship between chlorophyll concentrations and Fe supply could be poor or absent when medium pH is high. Although a higher Fe concentration resulted in higher ChlY at pH 7, at pH 9 adding more Fe to the mineral solution was not effective. Under a higher pH condition, the Fe utilization efficiency in roots and leaves is crucial. The Fe utilization efficiency is associated with the Fe uptake and transport across cell membranes, which involve ferric reduction catalyzed by ferric chelate reductase (FCR). The FCR activity can be severely depressed by high apoplastic pH in both leaves and roots (Mengel 1994, Romera et al. 1998). The optimum pH for FCR activity ranges from 5.5-6.5 (Moog and Brüggemann 1994). Susin et al. (1996) found that FCR activity dramatically dropped with increasing pH under Fe-deficiency conditions. At pH 7, the FCR activity is likely sufficiently high so that with the addition of Fe, plants can receive sufficient Fe. However, since at pH 9 the FCR activity is severely depressed, increasing Fe concentration at the root zone may not be effective in improving Fe nutrition and the FCR activity becomes the limiting factor for Fe uptake and transport to the leaves.

Compared with young leaves, ChlO was relatively less affected by the Fe and pH treatments in the four studied species. Fe is a relatively immobile element (Bukovac and Wittwer 1957) and is not readily translocated from the old to young leaves. Young leaves and meristematic tissues require more nutrients compared with the older leaves and tissues. The critical deficiency concentrations (CDC) of Fe were reported to be 60 to 70 mg kg⁻¹

dry weight in old leaves (Smith et al. 1984), but for the young leaves, the CDC may be as high as 200 mg kg⁻¹ (Haussling et al. 1985). Clearly, satisfying the supply of young growing tissues with Fe can be challenging under elevated pH conditions.

The effects of high pH and Fe supply have different effects on Chl a/b ratios (Chl a/b). The Chl a/b was reported to decrease under high pH conditions (Zhang et al. 2013), but also to increase in response to Fe-deficiency (Morales et al. 1990, 1994, Larbi et al. 2006). This suggests that high pH and Fe deficiency may act independently on the biosynthesis of chlorophyll and chlorophyll a may be more sensitive to high pH while chlorophyll b may be more sensitive to Fe deficiency. In the present study, the Chl a/b in paper birch, green alder, and tamarack decreased in response to the increase of pH. This trend was little changed by the increased supply of Fe. However, in white spruce, the Chl a/b of old needles increased slightly in two Fe treatments (0.25x and 4x Fe) with increasing pH and high pH had less effect on Chl a/b in white spruce compared with the other three studied species. Combined results of chlorophyll concentrations and Chl a/b with Pn, E, and total dry weights point to a greater high pH tolerance of white spruce compared with the other species in this study, which was consistent with the earlier report (Zhang et al. 2013).

In paper birch, high pH dramatically diminished Pn in all Fe concentration treatments. With a higher Fe supply (4x Fe), the Pn of paper birch at pH 7 significantly increased compared with lower Fe supplies. Fe status in plants profoundly influences Pn. Sufficient Fe supply maintains the integrity and functionality of the thylakoid membranes and improves the efficiency of photosynthetic electron transport chain (Marschner 2012). Additionally, a

higher Fe supply could intensify chlorophyll pigment synthesis and contribute to the increase in Pn. As discussed above, the utilization of Fe may be impeded under higher pH conditions, making Fe less available to plants. Therefore, the increased Fe supply could only enhance Pn in the neutral pH level. In the 4xFe treatment, although the chlorophyll concentration decreased at pH 7 compared with pH 5, the Pn at pH 7 had similar values as those at pH 5. Generally, Pn decreases concomitantly with reductions in chlorophyll concentration, since photosynthetic pigment concentrations could limit Pn by decreasing light absorption (Masoni et al. 1996, Abadi 'a et al. 1999). In response to Fe deficiency, light absorption and photosystem efficiency were well coordinated (Larbi et al. 2006). More light energy was absorbed per chlorophyll molecule than required for photosynthesis to optimize the use of the remaining photosynthetic pigments and electron transport carriers (Abadía et al. 1999). This could also explain why the chlorophyll concentrations in green alder and tamarack dramatically decreased in high pH levels (pH 7 and 9), but the Pn only decreased slightly compared with pH 5.

The E of paper birch, green alder, and tamarack decreased with increasing pH. Since plants were freely provided with water from the nutrient solution, these E reductions may be the consequence of a reduced root water flux induced by high pH. The high external pH can enhance root apoplastic pH (Felle and Hanstein 2002) which inhibits aquaporin activity and decreases root water flux (Voicu and Zwiazek 2004, Aroca et al. 2006). Moreover, the higher apoplastic pH can inhibit root growth which also impairs water uptake (Zhang et al. 2015). When the Fe concentrations increased, the Pn also increased leading to more carbon assimilation. Since the stomatal aperture is determined by the capacity of the carbon

fixation (Wong et al. 1979), the stomatal conductance enhanced concomitantly with the Pn increase. Therefore, the increased Fe supply (4x Fe) resulted in the increase of E in paper birch, green alder, and tamarack.

The total dry weights of paper birch and green alder slightly decreased at pH 7 and sharply decreased at pH 9 compared with pH 5 in all Fe treatment concentrations. The total dry weights at pH 5 and 7 in 4xFe concentration had an increasing trend compared with the lower Fe concentrations. Since Pn and E increased with sufficient Fe supply, it could be assumed that this trend would be more apparent in the longer term. The changes of s/r ratios were mainly affected by the pH treatment. Although the effect of Fe supply on dry matter allocation patterns was reported to be relatively small (Ericsson 1995), in the present study, higher Fe concentration to some extent increased the s/r ratio. And the s/r ratio of paper birch, green alder, and tamarack decreased with increasing pH. These results point to a strong link between growth processes and Fe availability at high pH.

In conclusion, high pH profoundly inhibited plant growth and physiological parameters including chlorophyll concentrations, Chl a/b, Pn, E, total dry weight, and s/r ratios in paper birch, white spruce, green alder, and tamarack seedlings. The increase in Fe supply had a positive impact on plant physiological parameters, which confirmed the hypothesis that elevated Fe concentrations can help plants tolerate the high pH. However, this positive impact on plants was only effective when the pH of the growth medium was neutral (pH 7). At pH 9, even the highest Fe concentration treatments resulted only in minor improvements in the measured parameters. Therefore, the remediation of Fe deficiency

requires increasing both Fe availability and Fe utilization. Since Fe cannot be absorbed by leaves when the apoplastic pH is high, spraying acid solution to the chlorotic leaves to lower leaf apoplast pH may be required to improve Fe nutrition (Kosegarten et al. 2001). However, the choice of fertilizer application may depend on the species and soil conditions such as the pH level. Of the four studied species, white spruce seedlings exhibited a relatively greater tolerance to high pH and Fe deficiency and should be considered for the revegetation of areas affected by high soil pH.

3.5 References

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3.6 Tables

Table 3. 1 Concentrations (means \pm SE, n = 3) of selected essential elements remaining soluble in 25% Hoagland's solution containing 0.25x, 1x and 4xFe concentration of the 25% Hoagland's solution at pH 5, 7 and 9.

Treatment	B (μ M)	Mg (mM)	P (mM)	K (mM)	Ca (mM)	Fe (μ M)	Mn (μ M)	Cu (μ M)	Zn (μ M)
0.25*Fe + pH 5	6.21	0.23	0.51	1.49	0.89	1.92	0.47	0.12	0.59
	\pm 0.196	\pm 0.004	\pm 0.004	\pm 0.004	\pm 0.012	\pm 0.252	\pm 0.0006	\pm 0.001	\pm 0.027
0.25*Fe + pH 7	5.88	0.23	0.50	1.81	0.88	1.46	0.47	0.10	0.54
	\pm 0.203	\pm 0.006	\pm 0.004	\pm 0.014	\pm 0.008	\pm 0.08	\pm 0.0026	\pm 0.002	\pm 0.022
0.25*Fe + pH 9	5.93	0.20	0.20	2.43	0.52	1.28	0.02	0.05	0.08
	\pm 0.086	\pm 0.005	\pm 0.001	\pm 0.016	\pm 0.003	\pm 0.241	\pm 0.0009	\pm 0.0005	\pm 0.03
1*Fe + pH 5	6.13	0.22	0.50	1.45	0.87	4.84	0.45	0.11	0.63
	\pm 0.43	\pm 0.002	\pm 0.002	\pm 0.013	\pm 0.008	\pm 0.124	\pm 0.006	\pm 0.0015	\pm 0.025
1*Fe + pH 7	5.99	0.22	0.50	1.81	0.87	4.23	0.45	0.10	0.54
	\pm 0.372	\pm 0.005	\pm 0.005	\pm 0.017	\pm 0.006	\pm 0.148	\pm 0.0069	\pm 0.002	\pm 0.021
1*Fe + pH 9	6.02	0.19	0.18	2.43	0.49	2.79	0.02	0.04	0.10
	\pm 0.261	\pm 0.005	\pm 0.003	\pm 0.027	\pm 0.005	\pm 0.64	\pm 0.0009	\pm 0.0003	\pm 0.027
4*Fe + pH 5	5.93	0.22	0.50	1.46	0.87	16.81	0.47	0.12	0.61
	\pm 0.255	\pm 0.003	\pm 0.003	\pm 0.007	\pm 0.006	\pm 0.072	\pm 0.0035	\pm 0.0013	\pm 0.012
4*Fe + pH 7	5.87	0.22	0.49	1.79	0.87	16.47	0.46	0.11	0.54
	\pm 0.111	\pm 0.006	\pm 0.003	\pm 0.02	\pm 0.004	\pm 0.238	\pm 0.0024	\pm 0.0016	\pm 0.02
4*Fe + pH 9	5.91	0.20	0.17	2.44	0.48	9.98	0.02	0.05	0.12
	\pm 0.212	\pm 0.005	\pm 0.002	\pm 0.025	\pm 0.002	\pm 1.28	\pm 0.0004	\pm 0.0011	\pm 0.023

Table 3. 2 ANOVA table showing effects of Fe and pH treatments on measured parameters in paper birch, white spruce, green alder, and tamarack seedlings (n = 6).

Paper birch

p-value	tdw	s/r ratio	Pn	E	ChlO	Chl a/b old	ChlY	Chl a/b young
Fe	0.331	0.0001	0.004	0.7728	0.7512	0.2892	<.0001	0.0957
pH	<.0001	<.0001	<.0001	<.0001	<.0001	0.0054	<.0001	<.0001
Fe*pH	0.1132	0.3102	<.0001	0.1724	0.3211	0.7474	<.0001	0.7703

White Spruce

p-value	tdw	s/r ratio	Pn	E	ChlO	Chl a/b old	ChlY	Chl a/b young
Fe	0.7856	0.4923	0.4428	0.8148	0.3401	0.0091	0.0179	0.8615
pH	0.1579	0.6848	0.7557	0.2946	0.0315	0.0987	<.0001	0.4368
Fe*pH	0.0297	0.8016	0.4464	0.2259	0.0304	0.1835	0.019	0.9392

Green Alder

p-value	tdw	s/r ratio	Pn	E	ChlO	Chl a/b old	ChlY	Chl a/b young
Fe	0.1393	0.0576	0.0656	0.0183	0.0264	0.7155	0.0001	0.0023
pH	<.0001	<.0001	<.0001	<.0001	<.0001	0.0031	<.0001	0.0006
Fe*pH	0.4408	0.0073	0.3422	0.0006	0.0197	0.6528	0.0002	0.0083

Tamarack

p-value	tdw	s/r ratio	Pn	E	ChlO	Chl a/b old	ChlY	Chl a/b young
Fe	0.026	0.0138	0.1412	<.0001	<.0001	0.0002	<.0001	<.0001
pH	0.0972	<.0001	<.0001	0.0005	<.0001	<.0001	<.0001	<.0001
Fe*pH	0.8131	0.0054	0.2782	0.0001	0.0631	0.1558	<.0001	<.0001

Abbreviations are: tdw - total dry weight; s/r ratio - shoot to root dry weight ratio; Pn - net photosynthetic rate; E - transpiration rate; ChlO - chlorophyll concentrations in old leaves; Chl a/b old - chlorophyll a to b ratios in old leaves; ChlY - chlorophyll concentrations in young leaves; Chl a/b young - chlorophyll a to b ratios in young leaves.

3.7 Figures

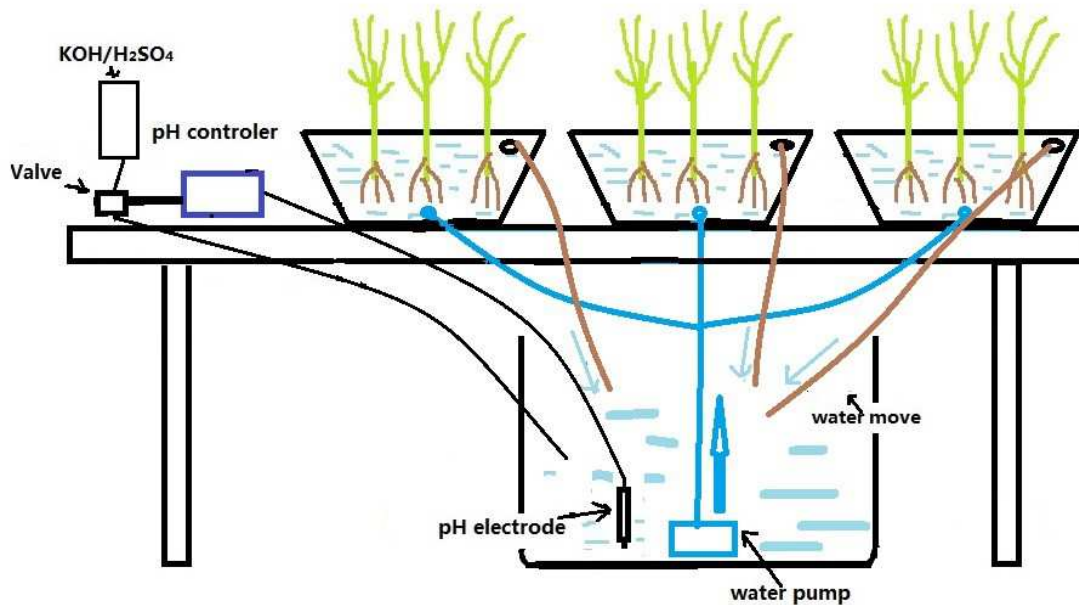


Figure 3. 1 Schematic diagram of the automatically-controlled hydroponic system. Three replicated 30 L opaque plastic tubs were connected to the 120 L barrel. A water pump was immersed in the Hoagland's solution to circulate nutrient solution between the barrel and the tubs. All tubs had spouts installed into their sides with 1-m-long tubing to facilitate nutrient solution circulation. The pH controller automatically opened and closed an electronic valve to adjust the solution pH by adding 5 % (w/w) KOH or 1 % (v/v) H₂SO₄ as needed.

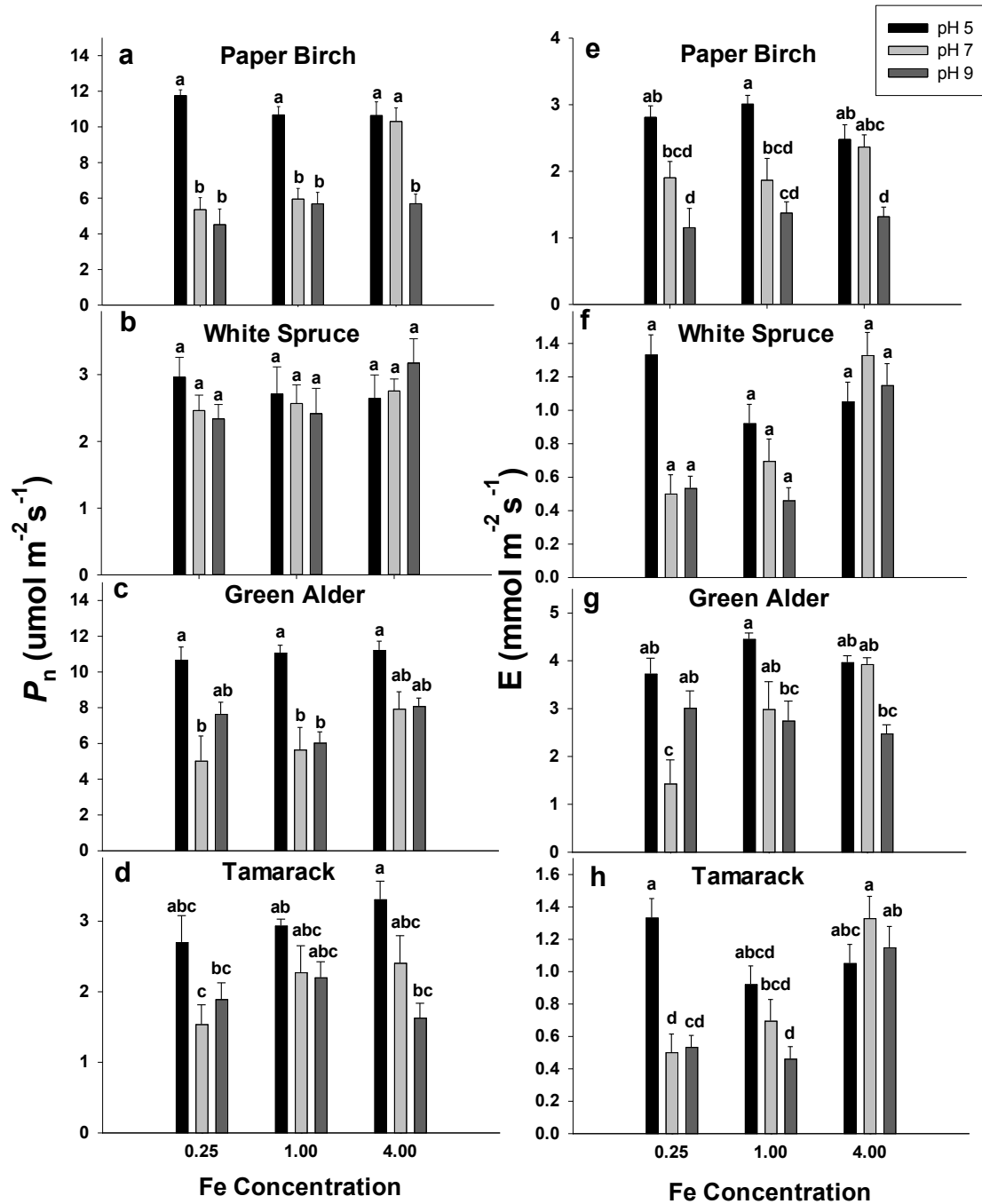


Figure 3. 2 Effects of Fe and pH treatments on net photosynthetic (P_n) and transpiration (E) rates in paper birch, white spruce, green alder and tamarack seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 6$) \pm SE are shown.

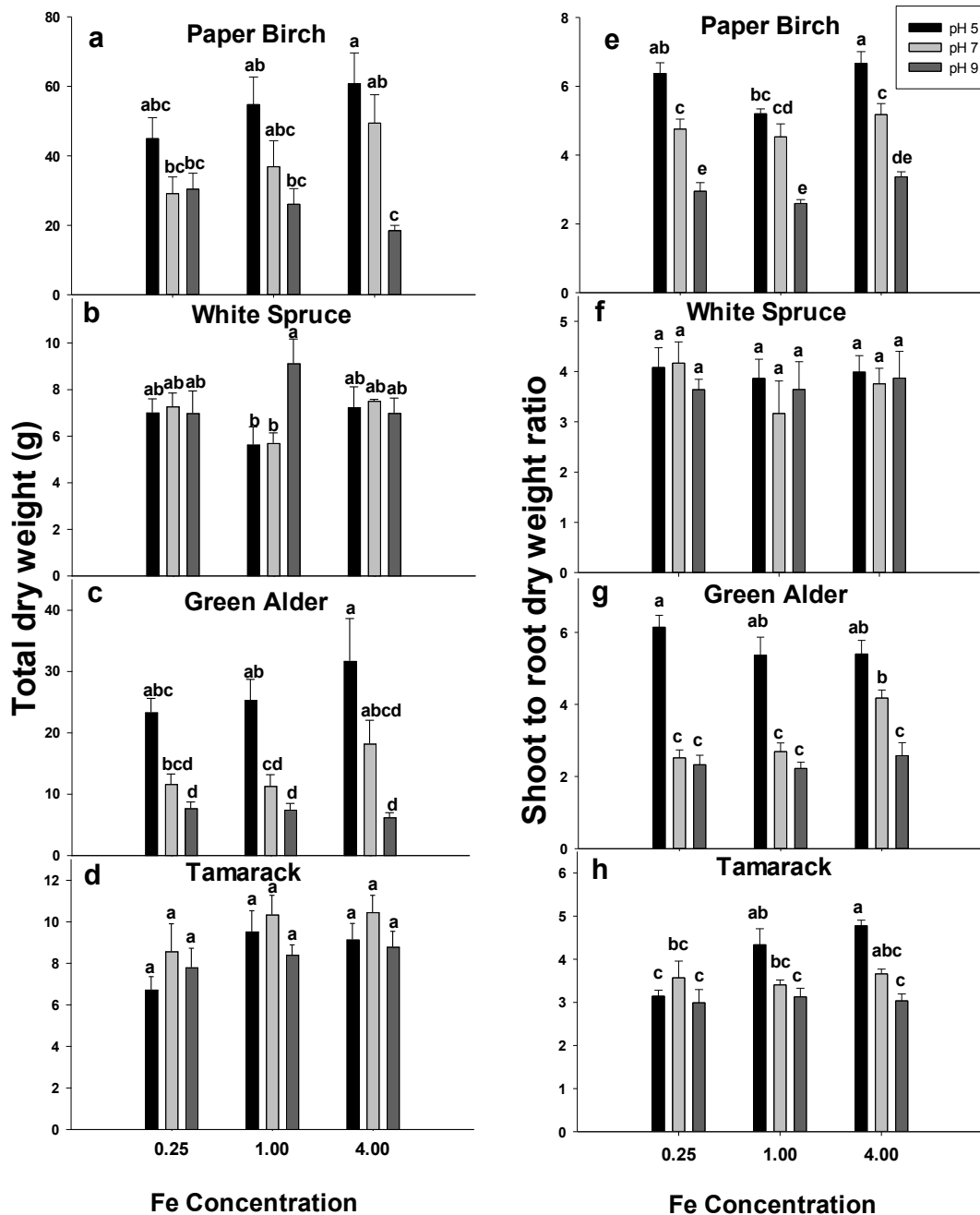


Figure 3. 3 Effects of Fe and pH treatments on total dry weights and shoot to root dry weight ratios in paper birch, white spruce, green alder and tamarack seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 6$) \pm SE are shown.

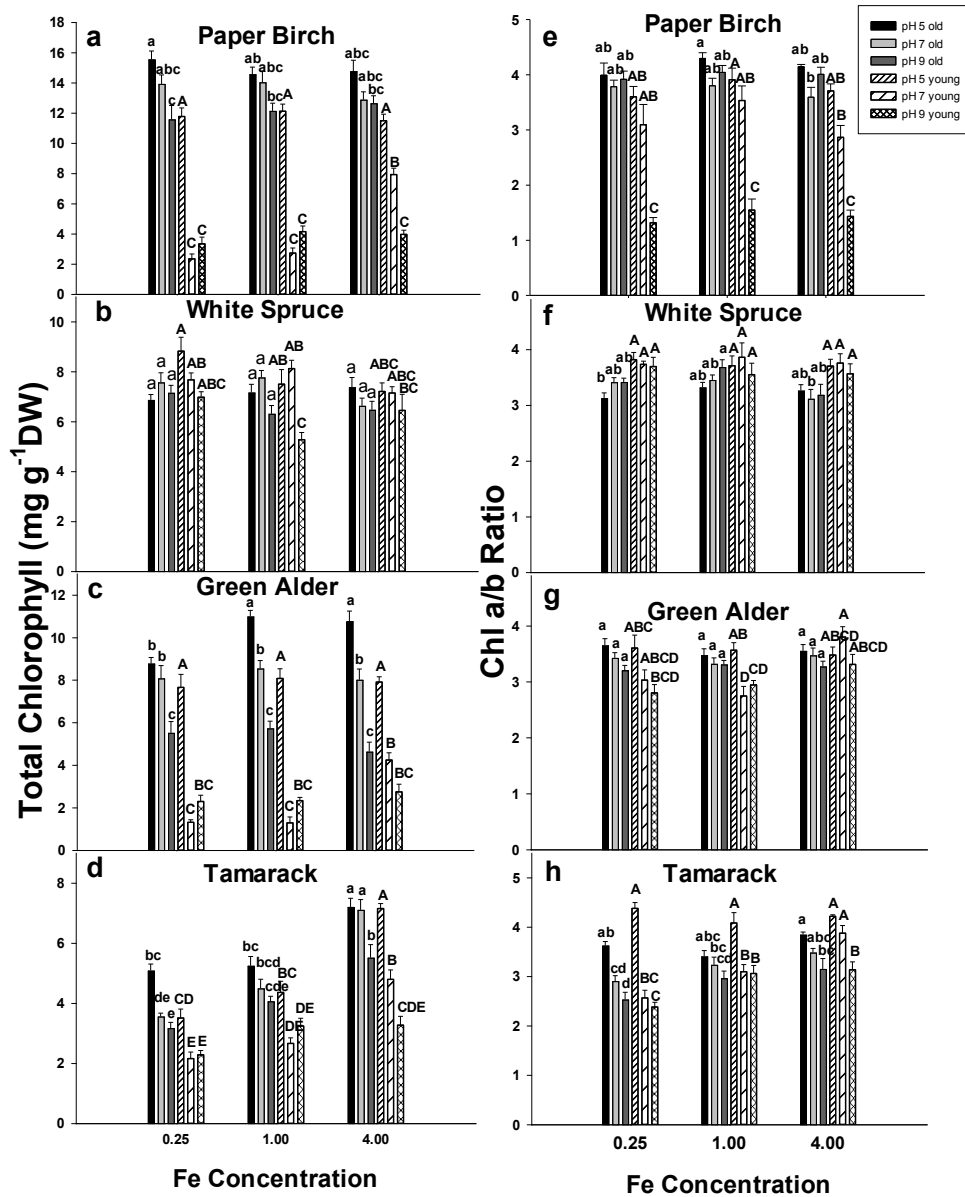


Figure 3. 4 Effects of Fe and pH treatments on total chlorophyll concentrations and ratios of chlorophyll-a to chlorophyll-b in old and young leaves of paper birch, white spruce, green alder, and tamarack. The old leaves were those which expanded fully before the treatments and the young leaves were those which sprouted after the start of treatments and were close to the shoot apex. Different letters above the bars (lowercase letters for old leaves, uppercase letters for young leaves) indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 6$) \pm SE are shown.

Chapter 4*

Effects of Fe at different root zone pH on growth and physiological responses of paper birch (*Betula papyrifera*), trembling aspen (*Populus tremuloides*), and red-osier dogwood (*Cornus stolonifera*) in split-root hydroponic system

4.1 Introduction

High soil pH profoundly inhibits plant growth and productivity. High pH soils can limit root water uptake and reduce stomatal conductance, transpiration, photosynthesis, and shoot growth (Tang and Turner 1999, Kamaluddin and Zwiazek 2004). In high-pH sensitive species such as lupin (*Lupinus angustifolius* L.), root growth, root surface area, root elongation, and root hair formation were among the most sensitive growth processes affected by high pH (Tang et al. 1993). In high pH soil, nutrient availability to plants is reduced due to low solubility and mobility (Comerford 2005). The essential nutrients with commonly reduced availability to plants at high soil pH are Fe, Mn, Cu, and Zn (Lindsay 1984, Yang et al. 1994, Valentine et al. 2006, Iles 2001). The causes of high soil pH can be both natural and man-made. In undisturbed sites, high soil pH is usually associated with calcareous soils that are high in bicarbonate and nitrate (Mengel 1994). Additionally, in reclaimed mining areas, including those following oil sands mining, frequently have soil pH values exceed 8.0 (Howat 2000). In the oil sands reclamation areas, the high pH problem arises from alkaline chemicals such as Na_2CO_3 , Na_2SiO_3 , and NaOH added during

*I also examined green alder in this study. The results of other parameters of the studied species and the results of green alder are in Appendix 2.

the oil extraction processes and the tailings sand material used as a reconstruction substrate in reclamation. Since about one-third of the earth is covered by high pH soils (Guerinot 2007), understanding the mechanisms that plants use to cope with high soil pH is fundamental for the revegetation and improvement of plant productivity in these areas.

Fe deficiency is common in soils with a high pH (Marschner 2012). Fe is an essential micronutrient for plant growth including photosynthesis, respiration, and chlorophyll biosynthesis (Kobayashi and Nishizawa 2012). Fe deficiency can lead to reductions in photosynthetic light-harvesting pigments and electron transport components of PSI, PSII, and Cyt b6f (Raven et al. 1999). Although Fe is abundant in the Earth's crust, it is present mainly in insoluble forms (iron oxides). The optimal concentration of soluble Fe in the soil for plants is about 10^{-6} to 10^{-5} M (Römheld and Marschner 1986). As soil pH increases from 4 to 8, the concentration of soluble Fe^{3+} decreases from 10^{-6} to 10^{-10} M (Römheld and Marschner 1986). Plants have developed two principal strategies for Fe uptake when Fe is deficient. Strategy I is used mainly by non-graminaceous plants and the strategy II is used mainly by graminaceous monocots (Marschner et al. 1986). For strategy I plants, Fe-deficiency stress responses include several morphological and physiological changes. Depending on the Fe-deficiency resistance, different species may show one or more of these reactions. The morphological changes involve root hair development and formation of transfer cells in the rhizodermis (Römheld and Marschner 1981a), which increase the external and internal root surface and facilitate nutrient and water uptake. The physiological changes include enhanced H^+ release, increased root Fe^{3+} -reduction capacity, and greater reductant extrusion (Marschner et al. 1986).

Soil is a complex medium and the rhizosphere pH may vary in different microsites depending on root respiration, cations that accompany proton extrusion when entering the roots, organic acid extrusion, redox reactions, and microorganism actions etc. (Hinsinger et al. 2003). The split-root system was employed here to simulate the different conditions in root zone pH. Wallace and Mueller (1978) reported that small acidified spots of high pH soil may prevent Fe chlorosis in the Fe-deficient cultivar of soybean. Gile and Carrero (1920) showed that the plant could absorb sufficient Fe absorbed by part of the roots in split-root experiments. Since plants only need a relatively small amount of micronutrients and high pH may affect the availability of these nutrients, lowering the pH in part of the root system was expected to provide sufficient amount of micronutrients for the whole plant and partly overcome the effects of high pH on plants.

In the present study, I examined the growth and physiological responses of paper birch (*Betula papyrifera*), trembling aspen (*Populus tremuloides*), and red-osier dogwood (*Cornus stolonifera*) to different pH and Fe nutrition levels. These species are commonly planted in oil sand reclamation areas and belong to the strategy I plant species. In the previous studies, paper birch (*Betula papyrifera*) and red-osier dogwood (*Cornus sericea*) were found to be pH-sensitive plants, while trembling aspen (*Populus tremuloides*) was relatively tolerant to high root zone pH (Calvo-Polanco et al. 2017). The objectives of this study were to investigate the responses of these plant species to heterogeneous root zone pH differing in Fe availability to evaluate the tolerance mechanisms of high pH and Fe deficiency. I examined the hypothesis that a partial exposure of the root system to low pH

conditions would alleviate the effects of high pH conditions applied to the other part of the root system.

4.2 Materials and Methods

4.2.1 Plant material and growth conditions

One-year-old dormant seedlings of paper birch (*Betula papyrifera*), trembling aspen (*Populus tremuloides*), and dogwood (*Cornus stolonifera*) were obtained from Boreal Horticultural Services Ltd., Bonnyville, Alberta, Canada. The roots of seedlings were washed free of the soil and the seedlings were transplanted into aerated 50% modified Hoagland's solution (pH 5.8) (Epstein 1972) in the controlled-environment growth chamber. The conditions were: 22/18°C (day/night) temperature, 65±10% relative humidity, and 16-h photoperiod with $300 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) obtained from full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada).

A semi-automated hydroponic split-root system was used in this study (Fig. 4.1). Two 11 L plastic tubs (31 cm length x 18 cm width x 40 cm height) were glued together to construct as a split-root growth container. For each treatment, five split-root growth containers were connected to two separate 40 L plastic barrels (40.5 cm length x 31 cm width x 62 cm height) by polyvinyl chloride (PVC) tubing. A water pump (Model 9.5 950GPH, Danner MFG Inc., New York, NY, USA) was placed in each barrel to circulate nutrient solution between the split-root growth containers and the barrels. All split-root growth containers

had spouts installed into their sides with 1-m-long tubing to facilitate drainage. This way, the two parts of each split-root growth container were supplied with a different solution, and the solution could circulate constantly and provide sufficient oxygen to plants ($\sim 8 \text{ mg L}^{-1} \text{ O}_2$). The split-root growth container was covered by a Styrofoam lid with four 3.8 cm holes in its center. The roots of the seedlings were inserted into one side of the split-root growth container secured with foam plugs for two weeks. After the roots expanded, they were evenly divided and placed into the two parts of the split-root container. There were 4 seedlings per species in each split-root container.

4.2.2 Experimental treatments

The plants were treated with two pH levels (5 and 9) and two Fe levels (0 and 40 μM , corresponding to no Fe or 4x Fe concentration present in the 50% Hoagland's solution). The 0 μM Fe treatment was applied in 50% modified Hoagland's solution while the 40 μM Fe treatment was applied in Milli-Q water. The combination of treatments is illustrated in Fig 4.1. The pH was adjusted to 5 and 9 with KOH or H_2SO_4 as described below. The solution pH was controlled within ± 0.1 range using the set up presented in Fig. 4.1. In this system, the pH controller (PHCN-70, Omega Engineering Inc., Laval, QC, Canada) connected a pH electrode (Orion 9106 BNWP gel-filled combination pH electrode, Thermo Scientific, Rochester, NY) immersed in the solution to read the solution pH values and then controlled an electronic valve (Model 8260G071 120/60 ASCO Valve, Inc., Florham Park, NJ, USA) to added 5 % (w/w) KOH or 1 % (v/v) H_2SO_4 solution according to the reading automatically. The treatments lasted for 8 weeks and the solution was replaced every 2

weeks.

4.2.3 Net photosynthetic (Pn) and transpiration (E) rates

After 8 weeks of treatment, Pn and E were measured using the infrared gas analyzer (LI-6400, LI-COR, Lincoln, Nebraska USA) at $400 \mu\text{mol m}^{-2}\text{s}^{-1}$ PPFD with five seedlings ($n = 5$) per treatment per species. Fully developed leaves with minimal or no necrosis on the uppermost branches were selected for measurement. The reference CO_2 concentration was $400 \mu\text{mol mol}^{-1}$ and the flow rate was $200 \mu\text{mol s}^{-1}$ in the leaf chamber. The leaf chamber temperature was kept at 20°C . The measurements were taken from 8:00 to 14:00.

4.2.4 Dry weights

At the end of the treatments, shoot and root dry weights were determined in five seedlings ($n=5$) per treatment for each tree species. The seedlings were separated into roots (taken separately from each of the two split containers), stems, and leaves and dried in an oven at 70°C for 72 h. The leaves for chlorophyll measurement were detached from the stems and freeze-dried at -80°C for 72 h. The total dry weights were the sum of the dry weights of stems, roots, and leaves from each plant, and the combined weights for leaves and stems were referred to as the shoot dry weights.

4.2.5 Leaf chlorophyll concentrations

Chlorophyll a and b concentrations in old and young leaves were determined in five seedlings per treatment (n=5) for each species. The old leaves were those that expanded fully before the treatments and the young leaves were those sprouted after the start of treatments and were close to the shoot apex. After freeze-drying, the leaves were ground in the Thomas Wiley Mini-Mill (Thomas Scientific, NJ, USA). Each pulverized leaf sample (10 mg) was extracted with 8 ml of the extraction solvent. Chlorophyll from paper birch and trembling aspen leaves was extracted with dimethylsulfoxide (DMSO) at 65°C for 22 h, and from dogwood with methanol at 55°C for 22 h. Different solvents were used since the DMSO extracts of dogwood leaves were black, which interfered with chlorophyll analysis. Chlorophyll concentrations were measured in the DMSO extracts at 648 nm and 665 nm, and in the methanol extracts at 652 nm and 665 nm with the spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden). Total chlorophyll concentration was calculated using the Arnon's equation for the DMSO extracts (Barnes et al. 1992) and the MacKinney's equation for the methanol extracts (Sestak et al. 1971).

4.2.6 Ferric-chelate reductase (FCR) activity

Root tips (1-cm long) of five seedlings per species (n = 5) were excised from each treatment. Each root sample was rinsed in 0.2 mM CaSO₄ and placed in a test tube filled with 0.2 mM CaSO₄. The test tube with roots was placed in an ice box and taken to the laboratory. To about 0.2 g root FW 10 ml of assay solution was added. The assay solution contained 0.2 mM CaSO₄, 0.1 mM Fe-EDTA, and 0.3 mM BPDS (bathophenanthrolinedisulfonic acid) (Cohen et al. 1997) and adjusted to pH 5 or 9 with 5 % (w/w) KOH or 1 % (v/v) H₂SO₄ as

required. One test tube containing 10 ml assay solution with no roots was used as a control. The tubes were shaken in the dark room temperature. After 1 h of incubation, the absorbance of each solution was measured with the spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden) at 535 nm. The concentration of Fe(II)-BPDS was calculated using the molar extinction coefficient of $22.14 \text{ mM}^{-1}\text{cm}^{-1}$ (Cohen et al. 1997).

4.2.7 Elemental analysis of young leaves

The concentrations of Ca, Cu, Fe, Mg, Mn, P, and Zn were determined in young leaves in five seedlings per treatment per species ($n = 5$) since the availability of these elements tend to be reduced in high pH soils (Marschner 2012). Young leaves were freeze-dried and ground with a Thomas Wiley Mini-Mill. Each sample (0.3-0.4 g dry weight) was digested with 10 ml 70% HNO_3 and heated for 10 minutes at 185°C in a microwave oven (Mars 5 Microwave Accelerated Reaction System, CEM, Matthews, NC, USA). After complete digestion, the solution was diluted with Milli-Q water to 40 ml. The extracts were then filtered and analyzed by ICP-MS (inductively coupled plasma mass spectrometry) (Zarcinas et al., 1987) in the Radiogenic Isotope Facility at the University of Alberta, Edmonton, AB, Canada.

4.2.8 Experimental design and statistical analysis

All data were analyzed with GLM model of SAS (Version 9.3, SAS Institute Inc., Cary, NC) to determine significant differences ($p \leq 0.05$). The data for the root dry weights of the

split roots and chlorophyll concentration of old (ChlO) and young (ChlY) leaves were analyzed using the paired t-test. One-way ANOVA was used to detect significant differences between the means. Residuals were checked for normality and homogeneity of variances. The Log10 function was used to transform the data that did not meet the ANOVA assumptions. Comparisons between different treatment means were conducted by the Tukey's test.

4.3 Results

4.3.1 Total dry weights and shoot to root dry weight ratio (s/r ratio)

Total dry weights of paper birch and trembling aspen in the pH 5:5 and pH 5:9 (${}^5\text{H-}{}^9\text{Fe}$ and ${}^9\text{H-}{}^5\text{Fe}$) treatments were similar (Fig. 4.2a, b); while for dogwood, the total dry weights were higher when subjected to pH 5:5 treatments compared with the other treatments (Fig. 4.2c). The trends for s/r ratios were similar in the three species and the s/r ratios in ${}^9\text{H-}{}^5\text{Fe}$ treatment were lower compared with the other three treatments (Fig. 4.2d, e, f).

4.3.2 Gas exchange

The P_n of the three species with half roots exposed to the low pH (${}^5\text{H-}{}^9\text{Fe}$ and ${}^9\text{H-}{}^5\text{Fe}$ treatments) had a similar value compared with those in the pH 5:5 treatment (Fig. 4.3a, b, c). E followed the same pattern in all the studied species that their lowest values were observed in pH 9:9 treatment (Fig. 4.3d, e, f).

4.3.3 Root dry weights and ferric-chelate reductase (FCR) activity

Paper birch and dogwood showed similar responses to the pH 5:5 treatment that their root dry weights were higher in the Hoagland's-supplied side (Fig. 4.4a, c). When subjected to pH 5H-9Fe treatment, the root dry weights of paper birch, trembling aspen, and dogwood were four-, six-, and six-fold in the low-pH side as those in the high-pH side, respectively (Fig. 4.4a, b, c). However, for 9H-5Fe treatment, trembling aspen responded differently compared with the other two species in that the root dry weights were significantly higher in the Hoagland's-supplied side although pH was 9 (Fig. 4.4b). For the pH 9:9 treatment, the root dry weights of paper birch were higher in the side supplied with Fe, while the root dry weight of dogwood was higher in the side supplied with Hoagland's solution (Fig. 4.4a, c).

The FCR activities in paper birch and trembling aspen were little affected by the treatments (Fig. 4.4d, e). For dogwood, the FCR activity was significantly higher in the Fe-supplied side in the pH 5:5 treatment and in the Hoagland's-supplied side in the 9H-5Fe treatment (Fig. 4.4f).

4.3.4 Chlorophyll concentrations

In the three examined species, the highest chlorophyll concentration in old (ChIO) and young (ChIY) leaves was observed in the pH 5:5 treatment and the concentrations were

similar in young and old leaves (Fig. 4.5a, b, c). In paper birch and trembling aspen, the ChlY dramatically decreased compared with ChlO in the 5H-9Fe treatment (Fig. 4.5a, b), while in dogwood, the ChlY increased compared with ChlO in 5H-9Fe treatment (Fig. 4.5c).

4.3.5 Elemental concentrations in young leaves

In paper birch and trembling aspen, leaf Fe concentrations consistently varied with the soluble Fe concentration in the treatment solutions. For the 5H-5Fe and 9H-5Fe treatments with Fe supplied in the low-pH side, the leaf Fe concentrations were about one to two-fold higher compared with the remaining treatments (Fig. 4.6a, b). In dogwood, the highest leaf Fe concentration was in the pH 5:5 treatments (Fig. 4.6c).

In paper birch, the leaf Ca and Mg concentrations were about a 100% higher in 5H-9Fe treatment compared with the other treatments. The leaf Cu and Mn concentrations were little affected by the applied treatments. The leaf P and Zn concentrations were dramatically higher in 5H-5Fe and 5H-9Fe treatments compared with the remaining treatments (Fig. 4.6a). The responses of the leaf P and Zn concentrations in trembling aspen and dogwood followed the similar trend (Fig. 4.6b, c).

In trembling aspen, there were no significant differences in leaf Ca, Mg, and Mn concentrations among the treatments. Leaf Cu concentration was slightly higher in the 5H-9Fe treatment (Fig. 4.6b).

In dogwood, the lowest leaf Ca, Mg, and Mn concentrations were observed in the 9H-5Fe treatments. There were no significant differences found in the leaf Cu concentrations across all treatments (Fig. 4.6c).

4.4 Discussion

The overall performance of paper birch, trembling aspen, and dogwood, including total dry weights, Pn, and E, was the greatest in the 5H-5Fe treatment and the worst in the 9H-9Fe treatment. For the pH 5:9 treatments (5H-9Fe and 9H-5Fe), with the root partially exposed to the low-pH medium, most of the plant growth and physiological parameters improved compared with the 9H-9Fe treatment. The different side-arrangement of the Hoagland's solution and Fe supply in the pH 5:9 treatment mainly affected the parameters of biomass allocation and elemental concentrations in leaves, but had minor effects on total dry weights, total chlorophyll concentrations, Pn, and E. These results confirm my hypothesis that partial exposure of root system to low pH could alleviate the effects of high pH.

The total dry weights of paper birch and trembling aspen dramatically decreased only in the 9H-9Fe treatment, while in dogwood the total dry weights decreased when high pH was present. This appears to be consistent with a previous study that dogwood showed strong growth reductions when subjected to high pH (Calvo-Polanco et al. 2017). The mean dry weight of dogwood plants was around 120 g, while the total dry weights of paper birch and trembling aspen were about 14 g and 10 g, respectively, after 8 weeks of the 5H-5Fe treatment. Among the studied species, dogwood showed relatively highest growth rates.

The decreased dry weights of dogwood under high pH conditions may be due to inadequate nutrients absorbed to support the high rate of growth.

Plants have different strategies to allocate their root biomass in order to optimize nutrient uptake. In the ${}^5\text{H-}^5\text{Fe}$ and ${}^5\text{H-}^9\text{Fe}$ treatments, paper birch, trembling aspen, and dogwood grew a larger root system in the Hoagland's-supplied side. In the ${}^9\text{H-}^5\text{Fe}$ treatment, although Hoagland's solution was supplied in the high-pH side, it was expected that more roots of the studied species would be present in the low-pH side since a high pH inhibits root growth. Paper birch and dogwood followed this pattern, but trembling aspen had higher root dry weights in the side containing Hoagland's solution regardless of high pH. These results suggest that the root growth of trembling aspen was relatively tolerant of high pH but sensitive to the nutrition status. As a high nutrient demanding species (Alban 1982), this root growth pattern in trembling aspen could facilitate nutrient uptake and support growth processes. Since Hoagland's solution was Fe-free, the Fe-deficiency response of proton extrusion to lower the apoplastic pH may also play a role in this process when the roots were in the high-pH side.

In the strategy I plants, the reduction of Fe^{3+} to Fe^{2+} by FCR is thought to be an obligatory and rate-limiting step in Fe uptake (Grotz and Guerinot 2002, Curie and Briat 2003, Schmidt 2003). In dogwood, the FCR activity was much higher in the Fe-supplied side in the ${}^5\text{H-}^5\text{Fe}$ treatment. These results are in agreement with a study which showed that the reduction activity increased in the Fe-supplied side in a split roots experiment while Fe-free grown roots had lower FCR activity (Schikora and Schmidt 2001). This response

contributed to more Fe uptake under uneven Fe concentrations. For 9H-5Fe treatment, the responses of root the FCR activity were complex since the FCR activity was affected by both high pH and Fe concentration. On the one hand, the Fe³⁺-reduction rate is pH-dependent and the optimal pH for FCR activity *in vivo* is around 5.5 (Moog and Brüggemann 1994). FCR activity may be severely depressed by high root apoplastic pH (Toulon et al. 1992, Mengel 1994). On the other hand, as discussed above, the root Fe³⁺-reduction capacity may increase in the side with sufficient Fe supplied under uneven Fe conditions. Therefore, it was expected that the root FCR activity in the low-pH side with sufficient Fe supplied (5Fe) would be higher compared with the high-pH side with Fe-deprivation (9H). However, for dogwood, the FCR activity was higher in the Hoagland's-supplied side at pH 9 (9H) in 9H-5Fe treatment. The similar pattern also found in trembling aspen and paper birch although they were not significant. It may reflect a general response of plant roots to a nutrient deficiency that when part of the root system was exposed to the 9H treatment, plants tended to increase the enzyme activities in order to absorb more nutrients from the Hoagland's-supplied side. However, more research is needed to clarify the relationship between FCR activity and high pH under Fe deficiency conditions.

In three studied species, both ChlY and ChlO in the treatments involving high pH dramatically decreased compared with the 5H-5Fe treatment. Exposing part of the root system to lower pH had little positive impact on chlorophyll concentrations. This could be due to decreased concentration of some of the leaf elements including Fe and (or) Mn, which are involved in chlorophyll synthesis. Fe deficiency can diminish chlorophyll biosynthesis by affecting Fe-containing enzymes which catalyze the formation of

chlorophyll precursors (Ouchane et al. 2004). The Fe concentration in young leaves was lower in all three studied species in the ${}^5\text{H-}^9\text{Fe}$ treatment compared with that in the ${}^5\text{H-}^5\text{Fe}$ treatment. In the ${}^5\text{H-}^9\text{Fe}$ treatment, the ChlY in paper birch and trembling aspen was significantly lower compared with ChlO, while the ChlY in dogwood was much higher compared with ChlO. Fe is generally considered to be an immobile element in plants, however, under Fe deficiency, the Fe in mature leaves can be remobilized to the young leaves and shoot apex in bean (*Phaseolus vulgaris* L.) (Zhang et al. 1995). The degree of mobility of Fe may vary between plant species. Dogwood was found to better maintain lower root xylem sap pH compared with paper birch when exposed to high pH (Zhang and Zwiazek 2016). Therefore, it can be speculated that dogwood may be able to retranslocate more Fe from the old to the young leaves. This may suggest that the chlorophyll concentrations of dogwood may be less affected by high pH conditions compared with paper birch and trembling aspen.

Although exposure of parts of the roots to low pH did not increase chlorophyll concentrations in the ${}^5\text{H-}^9\text{Fe}$ and ${}^9\text{H-}^5\text{Fe}$ treatments, it had a beneficial effect on Pn in all three species and Pn decreased only in the ${}^9\text{H-}^9\text{Fe}$ treatment compared with ${}^5\text{H-}^5\text{Fe}$ treatment. Photosynthesis can be influenced by stomatal factors and efficiency of the photosynthetic system. The similar patterns of Pn and E reductions indicated that stomatal limitations were likely the main contributor to the decrease of Pn. High pH can reduce root water flow and, consequently, stomatal conductance (Kamaluddin and Zwiazek 2004). Although the efficiency of the photosynthetic system in the ${}^5\text{H-}^9\text{Fe}$ and ${}^9\text{H-}^5\text{Fe}$ treatments may have been reduced due to Fe or other nutrient deficiencies, treatment duration could

have been too short to significantly impact Pn.

Due to the effects of high pH on the uptake of some of the essential nutrients, I expected that the plants would have the best nutrient status in the ${}_5\text{H}-{}_5\text{Fe}$ treatment. However, the elemental analysis of young leaves demonstrated that some of the nutrient concentrations were higher or similar in the ${}_5\text{H}-{}_9\text{Fe}$ treatments compared with those in the ${}_5\text{H}-{}_5\text{Fe}$ treatments. These included Ca, Mg, P, and Zn in paper birch, Cu, P, and Zn in trembling aspen, and Ca, Mg, Mn, P, and Zn in dogwood. The difference between the ${}_5\text{H}-{}_9\text{Fe}$ and ${}_5\text{H}-{}_5\text{Fe}$ treatments was that half of the root system was exposed to high pH in the Fe-supplied side. Therefore, Fe deficiency and high pH stress may both have the possibility to induce stress responses in the ${}_5\text{H}-{}_9\text{Fe}$ treatment. The Fe-deficiency response could improve not only Fe uptake but also that of other nutrients. The uptake of several micronutrients including Mn and Zn in sunflower (*Helianthus annuus* L.) was enhanced by Fe deficiency and the uptake rate was slightly lower than that of Fe (Römheld et al. 1982). Additionally, more chelating substances such as phenolics were likely extruded under Fe deficiency, which could facilitate the transport of cations across the plasma membrane (Römheld and Marschner 1981b). Another possibility may be that high pH stress triggered a signal from the ${}_9\text{Fe}$ -side roots to the ${}_5\text{H}$ -side roots and made the roots increase the activities of enzymes involved in nutrient uptake. However, further studies are needed to test this hypothesis.

In conclusion, partial exposure of the root system to low root zone pH alleviated the high pH stress in the studied plants. The improvement may not only be attributed to acidified spots affecting solubility and uptake of micronutrients under high pH condition but also

that the general stress responses in the high-pH side modified the responses in the low-pH side. Under Fe deficiency, dogwood had higher Fe utilization efficiency compared with paper birch and trembling aspen under high pH stress. The roots of trembling aspen could tolerate high pH well in order to obtain more nutrients.

4.5 References

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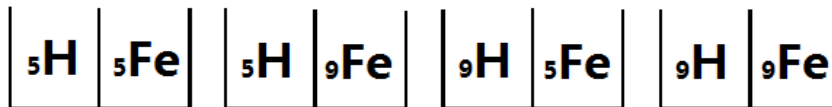
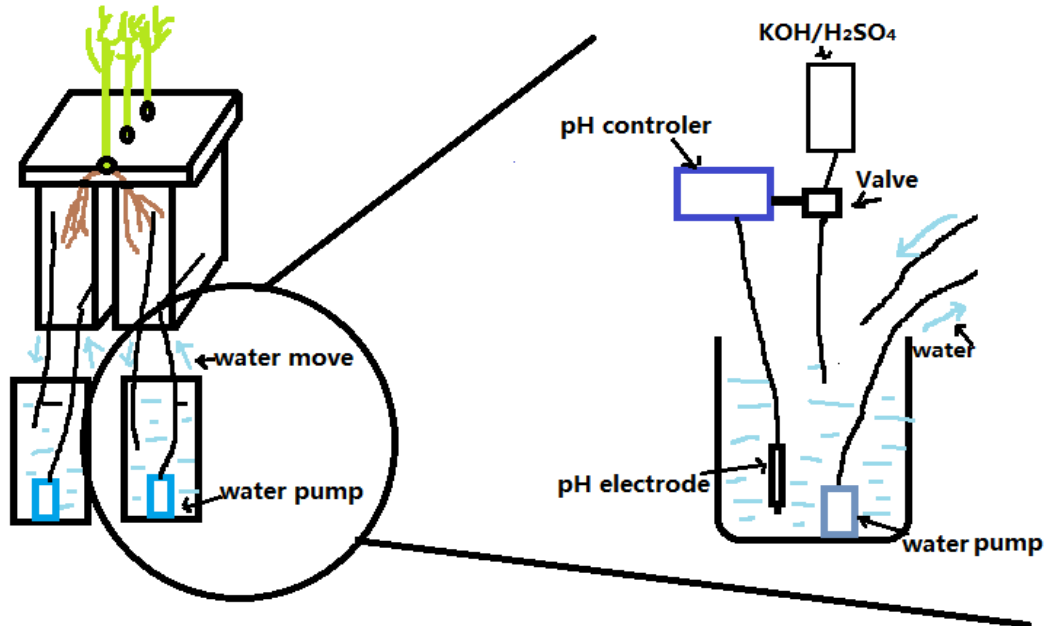
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4.6 Figures



Treatment	pH	Hoagland	Fe
${}_5\text{H}$	5	+	-
${}_5\text{Fe}$	5	-	+
${}_9\text{H}$	9	+	-
${}_9\text{Fe}$	9	-	+

Figure 4. 1 Schematic diagram of the split-root system setup and treatments in the split-root containers. There were four treatments including ${}_5\text{H}$ - ${}_5\text{Fe}$, ${}_5\text{H}$ - ${}_9\text{Fe}$, ${}_9\text{H}$ - ${}_5\text{Fe}$, and ${}_9\text{H}$ - ${}_9\text{Fe}$ treatments. The symbol “H” refers to Hoagland’s solution without Fe and “Fe” refers to Fe

(40 μM) in Milli-Q water. The subscripts are pH levels. In the experiment setup, two 11 L plastic tubs were glued together to construct as a split-root growth container. The roots of each plant were evenly divided and put into the two parts of the split-root growth containers. For each treatment, five split-root growth containers were connected to two separate 40 L plastic barrels which were supplied with two different kind of solution. A water pump was placed in each barrel to circulate nutrient solution between the split-root growth containers and the barrels. All split-root growth containers had spouts installed into their sides with 1-m-long tubing to facilitate drainage. The pH controller automatically opened and closed an electronic valve to adjust the solution pH by adding 5 % (w/w) KOH or 1 % (v/v) H_2SO_4 as needed.

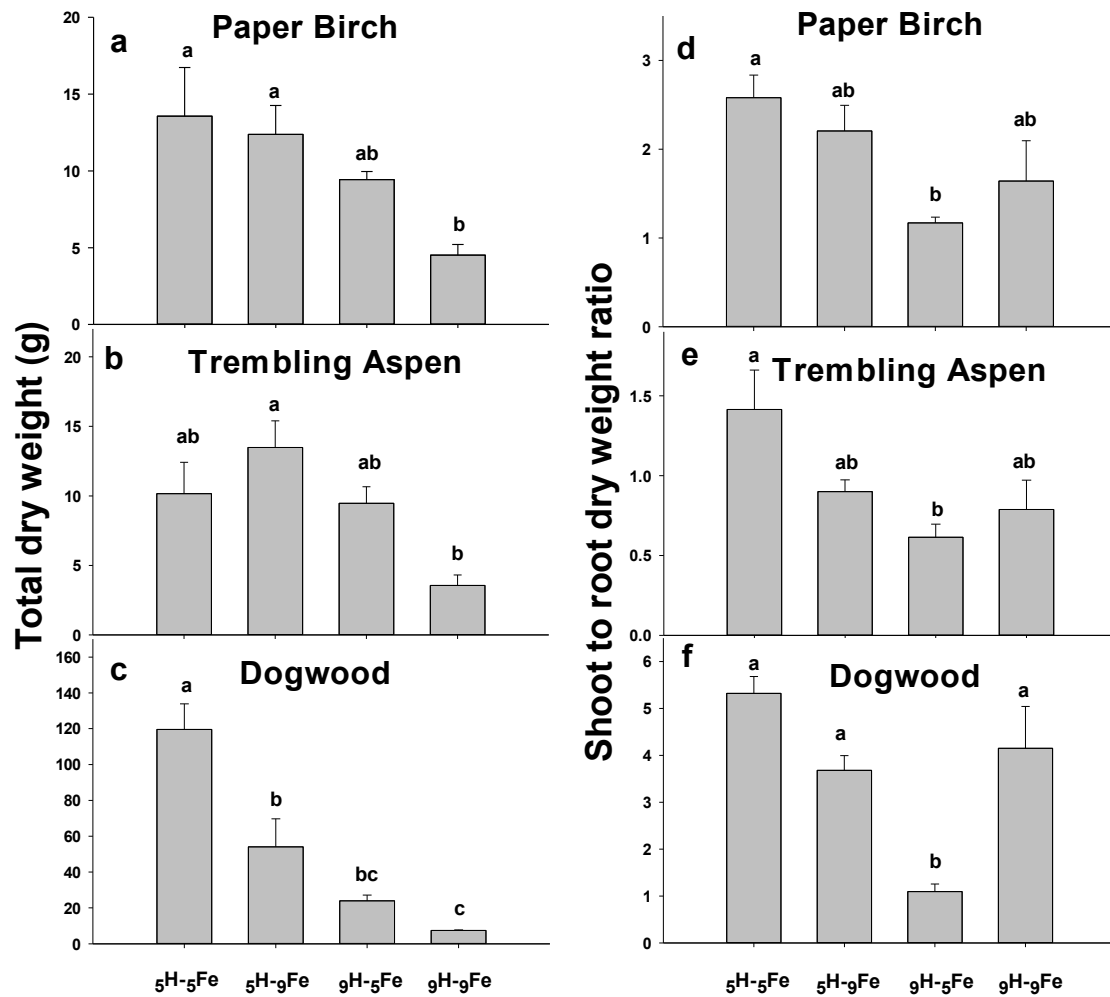


Figure 4. 2 Effects of pH and Fe supply in a split root system on total dry weights and shoot to root dry weight ratios in paper birch, trembling aspen, and dogwood. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 5$) \pm SE are shown. The symbol “H” refers to Hoagland’s solution without Fe and “Fe” refers to Fe (40 μ M) in Milli-Q water. The subscripts are pH levels.

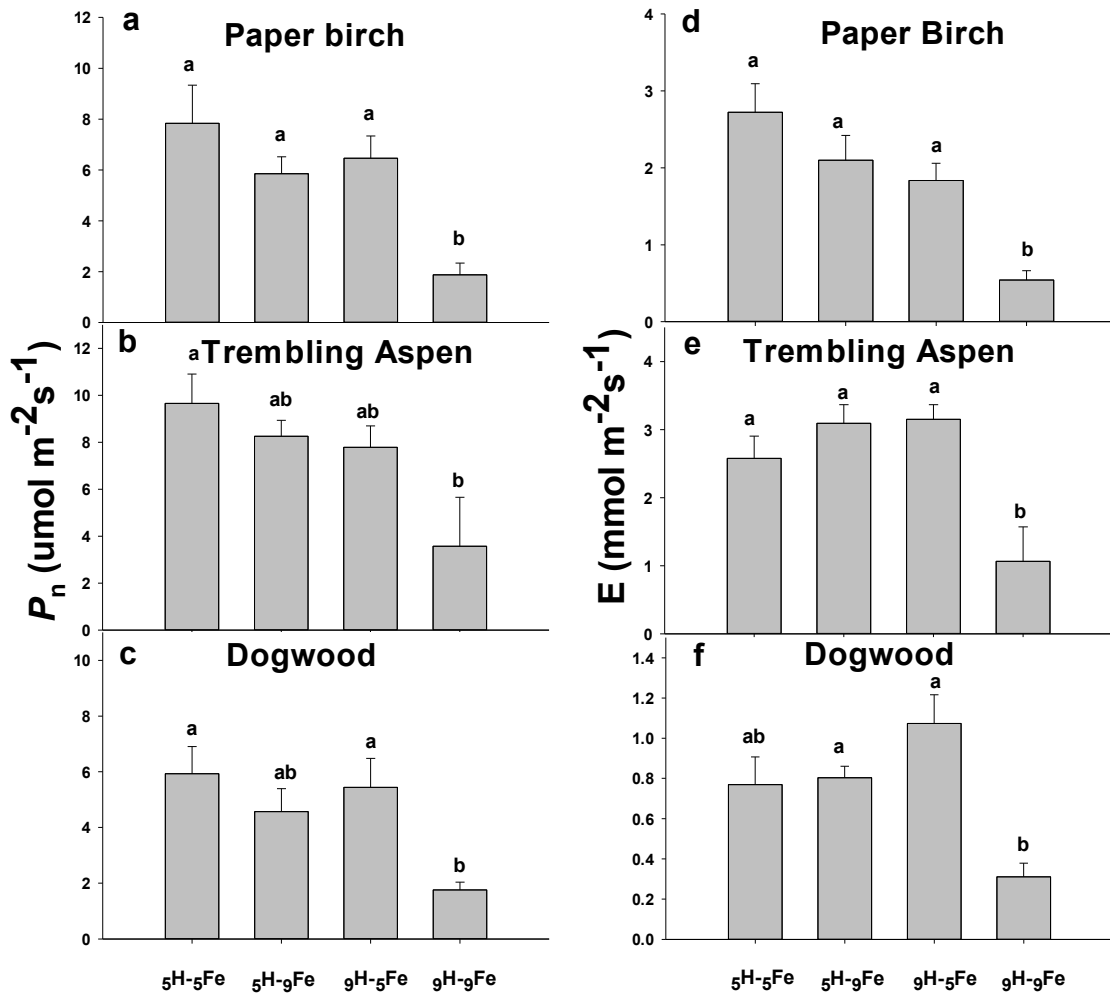


Figure 4.3 Effects of pH and Fe supply in a split root system on net photosynthetic rates (P_n) and transpiration rates (E) in paper birch, trembling aspen, and dogwood seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 5$) \pm SE are shown. The symbol “H” refers to Hoagland’s solution without Fe and “Fe” refers to Fe ($40 \mu\text{M}$) in Milli-Q water. The subscripts are pH levels.

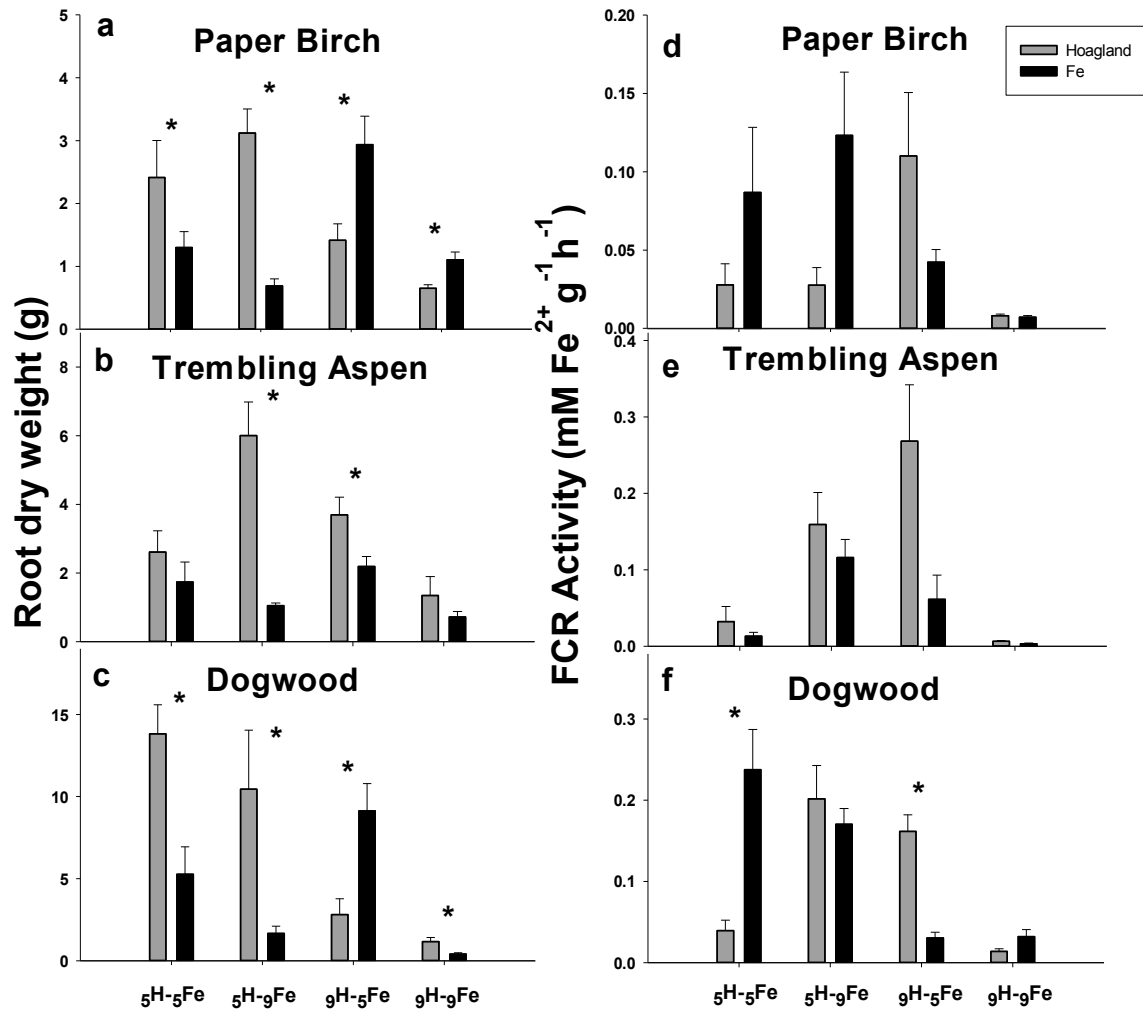


Figure 4. 4 Effects of pH and Fe supply in a split root system on root dry weights and FCR activity in the Hoagland's and Fe sides of paper birch, trembling aspen, and dogwood. The asterisk above the bars indicates significant differences ($\alpha = 0.05$) between two sides of root dry weight and FCR activity determined by the paired t-test. Means ($n = 5$) \pm SE are shown. The symbol "H" refers to Hoagland's solution without Fe and "Fe" refers to Fe ($40 \mu\text{M}$) in Milli-Q water. The subscripts are pH levels.

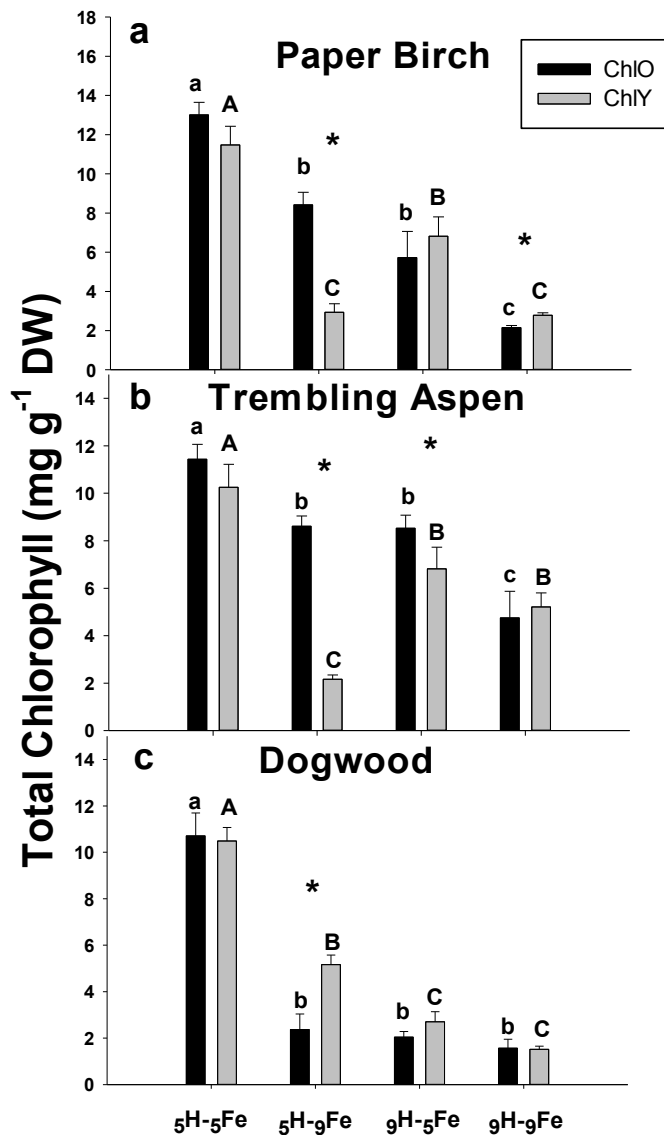


Figure 4.5 Effects of pH and Fe supply in a split root system on total chlorophyll concentrations (chlorophyll-a + chlorophyll-b) in old and young leaves of paper birch, trembling aspen, and dogwood. The old leaves were those expanded fully before the treatments and the young leaves were those sprouted after the start of treatments and were close to the shoot apex. Different letters above the bars (lowercase letters for old leaves, uppercase letters for young leaves) indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 5$) \pm SE are shown. The symbol “H” refers to Hoagland’s solution without Fe and “Fe” refers to Fe (40 μ M) in Milli-Q water. The subscripts are pH levels.

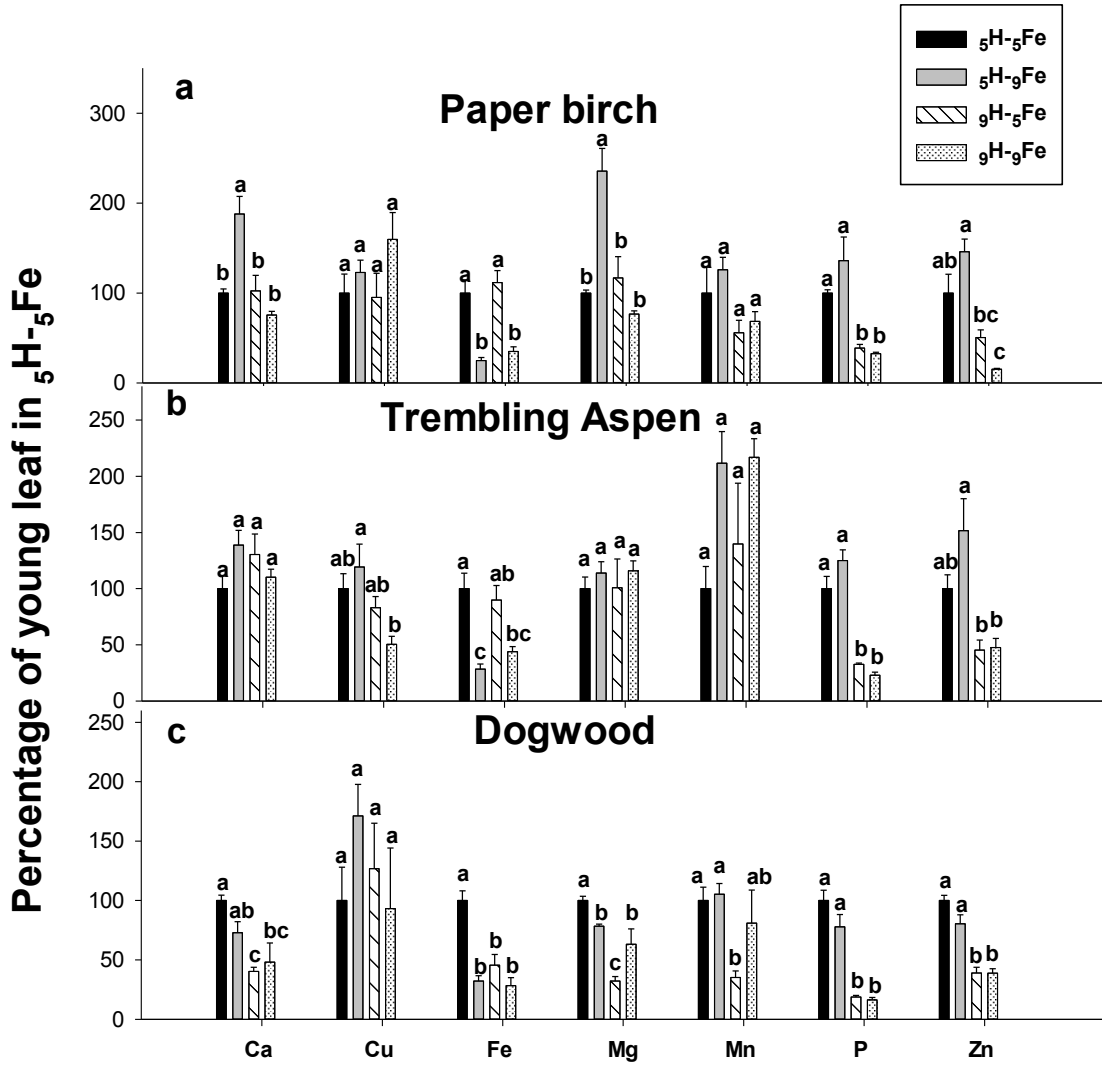


Figure 4. 6 Effects of pH and Fe supply in a split root system on Ca, Cu, Fe, Mg, Mn, P and Zn concentrations in young leaves of paper birch, trembling aspen, and dogwood seedlings, presented as the percentages of values measured at ${}^5\text{H-}{}^5\text{Fe}$ treatment in young leaves. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 5$) \pm SE are shown. The symbol “H” refers to Hoagland’s solution without Fe and “Fe” refers to Fe (40 μM) in Milli-Q water. The subscripts are pH levels.

Chapter 5

General discussion and conclusion

5.1 Summary of findings

High soil pH profoundly affects plant growth and function, largely due to the detrimental effects on nutrient availability and water uptake (Marschner 2012). The Fe deficiency-induced leaf chlorosis in high pH soils decreases plant productivity, making it a severe agricultural and revegetation concern. In this thesis, I focused on the effects of high pH, mineral nutrition, and Fe supply on growth and physiological processes in selected boreal forest species in order to better understand the processes contributing to high pH tolerance in plants and improve the revegetation success of oil sands reclamation areas. I carried out all studies under controlled-environment conditions with all treatments carried out in the sand and hydroponic cultures to exclude environmental variables.

In Chapter 2, I examined the effects of pH and mineral nutrient supply on growth and physiological responses of trembling aspen (*Populus tremuloides*), jack pine (*Pinus banksiana*), and white spruce (*Picea glauca*) seedlings in sand culture. I hypothesized that higher nutrient supply could ameliorate the effects of high pH in plants. Although most of the effects of high pH stress could be attributed to mineral deficiencies including Mg, P, Fe, Mn, Ca, and Zn, increased supply of nutrients was not highly effective in alleviating the effects of high pH stress in the three studied species and had only a beneficial impact on the total dry weights in trembling aspen at the lower pH level. The responses of plants

to pH and nutrient supply varied between species and were likely in part due to the differences in plant nutrition demands. White spruce was relatively resistant to high pH compared with the other examined plant species and its tolerance mechanism of high pH likely involves greater biomass allocation to roots to facilitate nutrient uptake.

Reduced Fe availability to plants in high pH soils often affects photosynthetic efficiency and chlorophyll synthesis (Briat et al. 2015). In Chapter 3, I investigated the effects of Fe supply on growth and physiological responses in paper birch (*Betula papyrifera*), white spruce (*Picea glauca*), green alder (*Alnus viridis*), and tamarack (*Larix laricina*) to different root zone pH levels in hydroponic culture. I examined the hypothesis that the effects of pH on plants can be alleviated by increasing the Fe supply. The results were consistent with this hypothesis and showed that the increased Fe supply had a positive impact on plant physiological parameters. However, the increased supply of Fe was only effective in the neutral pH (pH 7) and had a minor impact on plants at pH 9. Therefore, the remediation of high pH-induced Fe-deficiency requires increasing Fe availability as well as Fe absorption and utilization. Of the four studied species of trees, white spruce exhibited relatively higher tolerance of high pH and Fe deficiency and should be considered for the revegetation of areas affected by high soil pH.

Since a heterogeneous pH is common in the rhizosphere (Hinsinger et al. 2003), in Chapter 4 I used a split-root hydroponic system to expose one part of the root system to high pH while the other part was exposed to low pH with different concentrations of Fe supplied to

both parts. Then, I examined the effects of these treatments on growth and physiological responses of paper birch (*Betula papyrifera*), trembling aspen (*Populus tremuloides*), and red-osier dogwood (*Cornus stolonifera*). I formulated a hypothesis that partial exposure of the root system to low pH conditions would alleviate the effects of high pH conditions. The results confirmed the hypothesis and suggested that the improvement may not only be attributed to the solubility and uptake of micronutrients, but also to the general stress responses in the high-pH side modified the responses in the low-pH side. Dogwood showed higher Fe utilization efficiency in response to Fe deficiency under high pH stress conditions. Interestingly, trembling aspen allocated relatively more biomass to the part of the root system that was exposed to high pH, likely to increase nutrient uptake.

5.2 General discussion

In my studies, I used hydroponic and sand culture systems since they both offer a more precise control of mineral nutrition and pH levels compared with soil culture. In Chapter 2, I carried out the study in sand culture and found that the same high pH stress that was imposed on plants in sand culture was relatively less effective compared with that reported for hydroponic culture. This could be explained partly by the ability of roots to extrude protons into the rhizosphere which is more effective in sand culture than in the hydroponic culture with circulating mineral solution. However, since soil conditions may be complex and soil has higher buffering capacity compared with sand, the results of the experiments carried out in sand and hydroponic cultures should be interpreted with caution. As an important factor in the soil, microbial activities, especially mycorrhizal associations, could

enhance water and nutrient uptake of the host plants significantly. Although the control of pH is more difficult in the sand culture, this system can better simulate the soil environment than the hydroponic system and provide a habitat for rhizosphere microorganisms (Siemens and Zwiazek 2011). Inoculating mycorrhizae to plants may be an effective method to alleviate the effects of high pH stress on plants and should be further explored in future studies. It also will be important to verify the results obtained from the solution and sand culture studies with field studies, especially if mycorrhizal associations are involved (Nowak and Friend 2006).

High pH significantly affects nutrient availability to plants by impacting nutrient solubility, uptake, and transport. Methods to alleviate the high pH problem can be explored by improving these processes. To enhance nutrient solubility, I attempted increasing nutrient concentrations in the medium (Chapters 2 and 3). However, contrary to my hypothesis, increasing nutrient supply to the root zone had only a minor effect on plants exposed to high pH and the beneficial impact was effective only at low and neutral pH, especially in the faster-growing species. Although the solubility of nutrients was enhanced, high pH stress still decreased the root growth and, thereby, a) reduced the root nutrient uptake area (Tang et al. 1993a, b), b) impaired the pH gradient across the plasma membrane which is essential for nutrient uptake (Felle and Hanstein 2002), c) decreased FCR activity which is involved in Fe uptake and transport, and d) decreased water flow and transpiration rates on which nutrient uptake and transport largely depend. Therefore, the addition of nutrients without increasing nutrient uptake and utilization efficiency may not be sufficient to mitigate high pH stress. The foliar applications of micronutrients have been partly

successful in reducing leaf chlorosis in agriculture (Kosegarten et al. 2001, Dordas 2009). The aerial spraying of fertilizers should also be tested in reclamation practices for high soil pH sites. In Chapter 4, I demonstrated that exposing part of the root system to low pH conditions could ameliorate the high pH stress and improve nutrient uptake. Since plants can modify their rhizosphere pH by extrusion of H^+/OH^- (Hinsinger et al. 2003), the application of fertilizers that could acidify the soil or stimulate root extrusion of H^+ is also an option to alleviate high pH concerns. However, the choice of fertilizer application may depend on the species and soil conditions and needs to be explored further. Finally, another option that should be explored is the selection of plant species with superior tolerance to high pH conditions.

Among the species selected for this thesis research, white spruce was shown to be the most tolerant of high root zone pH. In the three studies, the growth and physiological responses of white spruce were relatively less impacted by the different pH treatments compared with other plants. White spruce has the lowest growth rate of these species and is relatively less nutrient demanding. Additionally, the high pH tolerance mechanism of white spruce may involve the allocation of more biomass to roots as shown in Chapter 2. In this manner, white spruce can absorb more nutrients to support the relatively low rates of shoot growth. Interestingly, the two fast-growing species, dogwood and trembling aspen, also showed some high pH tolerance characteristics, although their physiological responses were significantly affected by high pH. In Chapter 4 of the split-root study, dogwood plants were found to have higher ChIY than ChIO in a split-root system, suggesting higher Fe utilization efficiency and possible retranslocation of Fe from old to young leaves. The roots of

trembling aspen could tolerate high pH well and this species allocated more biomass to the part of the root system exposed to high pH. To restore disturbed ecosystems to their original state and maintain their self-sustainability over time, the degree of biodiversity in reclamation areas is important (Alberta Environment 2010). Therefore, white spruce, dogwood, and trembling aspen could be all considered as desirable candidate species for the revegetation of sites affected by high soil pH. Since in the present study all treatments were of relatively short duration, while the reforestation of oil sands areas may take several decades (Grant et al. 2008), longer-term field studies should be conducted before formulating reclamation strategies.

5.3 Suggestions for future research

Although short-term controlled environment studies have many advantages and were appropriate to meet the objectives of my thesis research, they also have many limitations and should be considered as the first phase of a systematic research effort that is needed to improve growth and survival of plants in high pH soils. There are also several aspects related to the high pH tolerance mechanisms in plants that merit more research including:

- 1) Examining the FCR activity and gene expression patterns in major boreal forest plant species exposed to high pH conditions.

- 2) Investigating apoplastic pH changes in roots as well as in xylem, and leaf tissues in plant species that are tolerant or sensitive to high rhizosphere pH conditions

- 3) Examining the relationship between rhizosphere microorganisms, especially, mycorrhizal associations, and high pH tolerance in plants exposed to a high root zone pH.

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Appendix 1

(Supplementary results of Chapter 2)

Table a2. 1 ANOVA table showing effects of pH and nutrition treatments on measured parameters for trembling aspen, jack pine, white spruce, dogwood, blueberry, and bearberry seedlings.

Trembling aspen

p-value	Chl a/b old	Chl a/b young	Wp
nutri	0.0713	<.0001	0.4568
pH	<.0001	<.0001	0.0668
nutri*pH	0.0004	<.0001	0.3207

p-value	B	K	Cu
pH	0.04	<.0001	<.0001

Jack pine

p-value	Chl a/b old	Chl a/b young
nutri	0.72	0.0012
pH	0.0005	<.0001
nutri*pH	0.5192	0.0077

p-value	B	K	Cu
pH	0.0005	0.0109	0.461

White spruce

p-value	Chl a/b old	Chl a/b young
nutri	0.2441	0.1739
pH	<.0001	<.0001
nutri*pH	0.8161	0.4874

p-value	B	K	Cu
pH	0.8344	<.0001	0.0038

Dogwood

p-value	tdw	s/r	Pn	E	ChlO	Chl a/b old	ChlY	Chl a/b young	wp
nutri	0.2293	0.0345	0.0303	0.0345	0.9266	<.0001	0.5045	0.0008	0.0942
pH	<.0001	0.0117	0.0005	0.0117	<.0001	<.0001	<.0001	<.0001	0.2986
nutri*pH	0.0013	0.1287	0.4207	0.1287	0.0036	0.2989	0.3341	0.1146	0.4066

p-value	B	Mg	P	K	Ca	Fe	Mn	Cu	Zn
pH	0.0435	<.0001	<.0001	<.0001	<.0001	0.0006	<.0001	<.0001	<.0001

Blueberry

p-value	tdw	s/r	Pn	E	ChlO	Chl a/b old	ChlY	Chl a/b young	wp
nutri	0.0201	0.8249	0.0041	<.0001	<.0001	<.0001	<.0001	0.415	0.017
pH	0.0008	0.3007	<.0001	<.0001	<.0001	<.0001	0.0002	<.0001	0.0137
nutri*pH	0.2788	0.2241	0.128	0.0277	0.231	0.2123	0.72	0.6267	0.0402

p-value	B	Mg	P	K	Ca	Fe	Mn	Cu	Zn
pH	0.0512	0.0162	0.0005	0.2641	0.5755	0.0046	0.0055	0.0356	0.2079

Bearberry

p-value	tdw	s/r	Pn	E	ChlO	Chl a/b old	ChlY	Chl a/b young	wp
nutri	0.9356	<.0001	0.0005	<.0001	0.01	<.0001	0.0125	<.0001	0.293
pH	0.0001	<.0001	0.0389	0.0068	<.0001	<.0001	<.0001	<.0001	0.0305
nutri*pH	0.9317	0.8825	0.1907	0.0198	0.5401	0.2234	0.1267	0.7261	0.1137

p-value	B	Mg	P	K	Ca	Fe	Mn	Cu	Zn
pH	0.0674	<.0001	0.0013	<.0001	<.0001	0.0735	0.1689	0.0818	0.0002

Abbreviations are: tdw - total dry weight (n = 8); s/r ratio- shoot to root dry weight ratio (n = 8); Pn - net photosynthetic rate (n = 8); E - transpiration rate (n = 8); ChlO - chlorophyll concentrations in old leaves (n = 6); ChlY - chlorophyll concentrations in young leaves (n = 6); Chl a/b old - chlorophyll a to b ratios in old leaves (n = 6); Chl a/b young - chlorophyll a to b ratios in young leaves (n = 6); wp - water potential (n = 8); element concentrations (n=6).

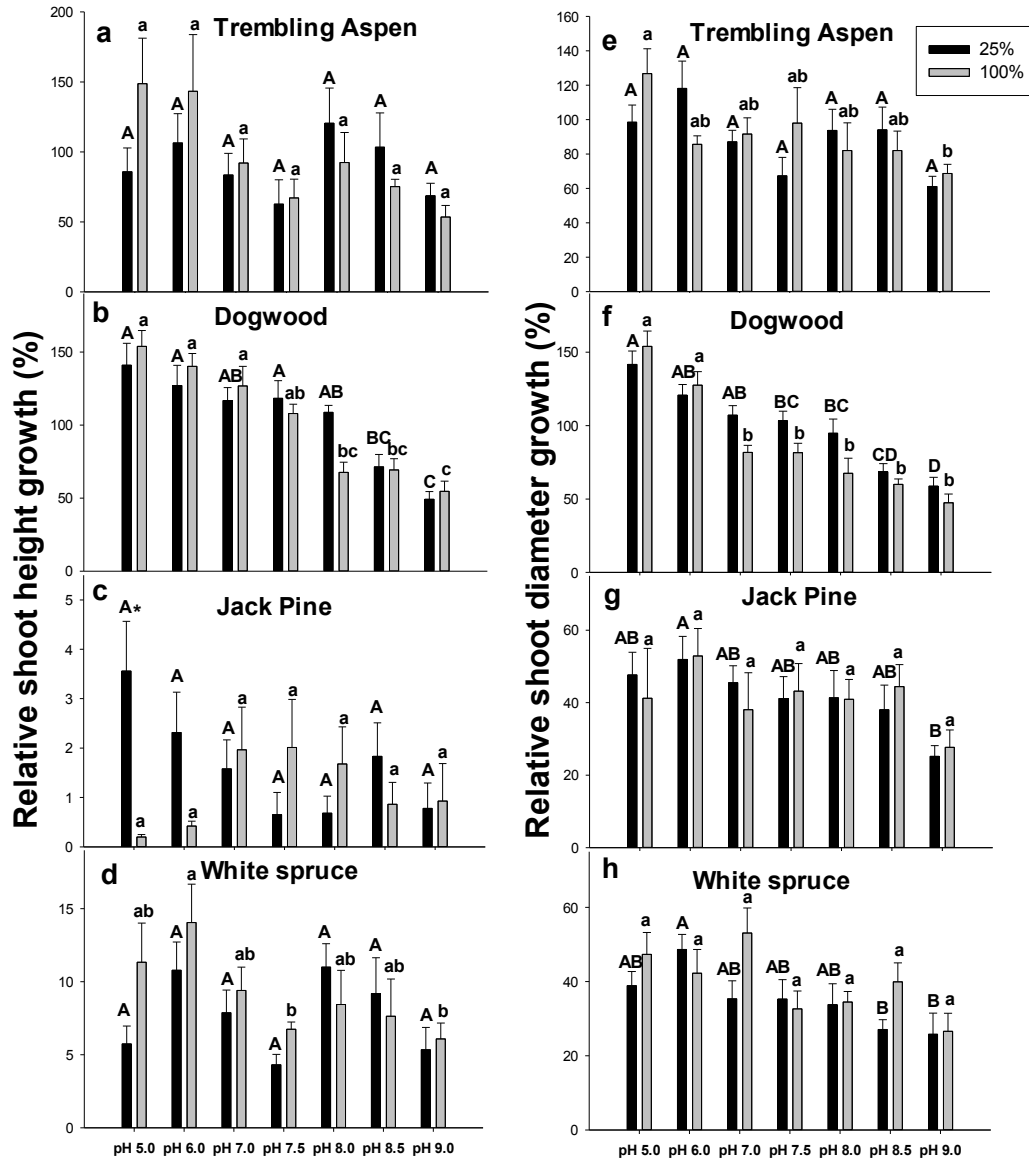


Figure a2.1 Effects of pH and nutrition on relative shoot height growth and relative stem diameter growth* in trembling aspen, dogwood, jack pine, and white spruce seedlings. Different letters above the bars (uppercase letters for 25% Hoagland's solution and lowercase letters for 100% Hoagland's solution) indicate significant differences ($\alpha = 0.05$) between pH treatments within each plant species. The asterisk above the bars indicates significant differences ($\alpha = 0.05$) between 25% and 100% Hoagland's solution. Means ($n = 8$) \pm SE are shown.

* The relative shoot height and stem diameter growth were calculated by dividing the difference in the initial and final values by the initial value.

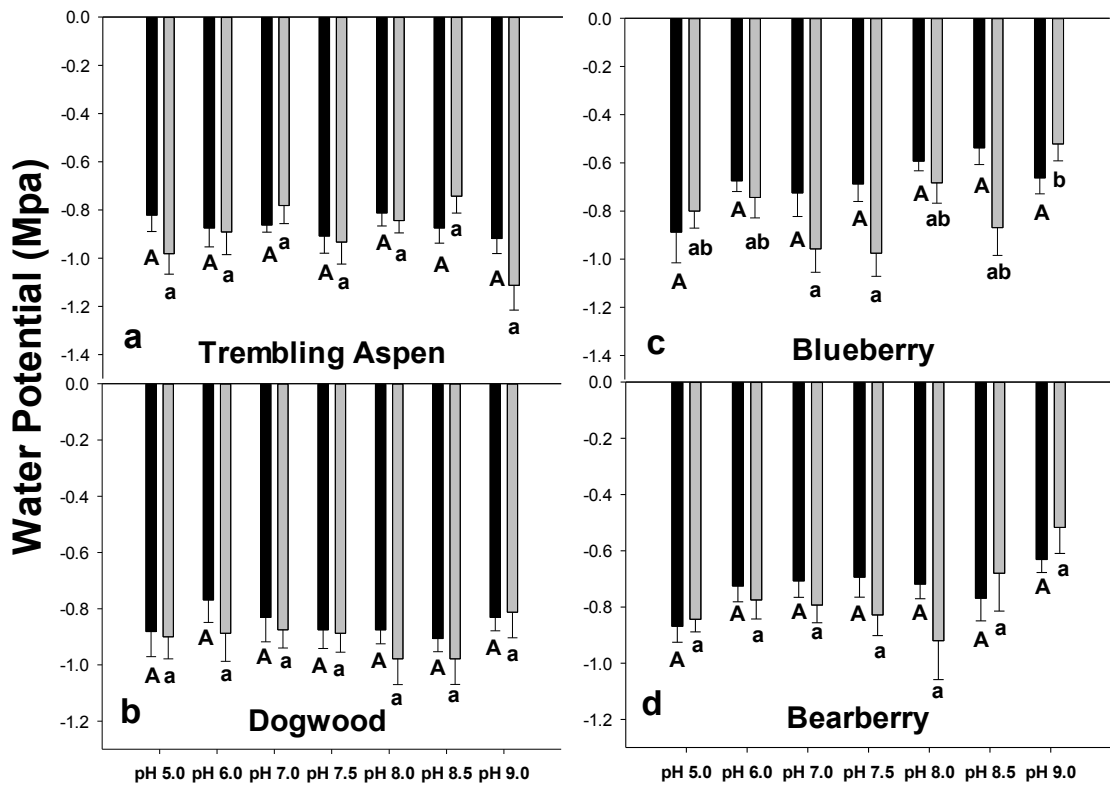


Figure a2. 2 Effects of pH and nutrition on stem water potential in trembling aspen, dogwood, blueberry, and bearberry seedlings. Different letters under the bars indicate significant differences ($\alpha = 0.05$) between pH treatments within each plant species. Means ($n = 8$) \pm SE are shown.

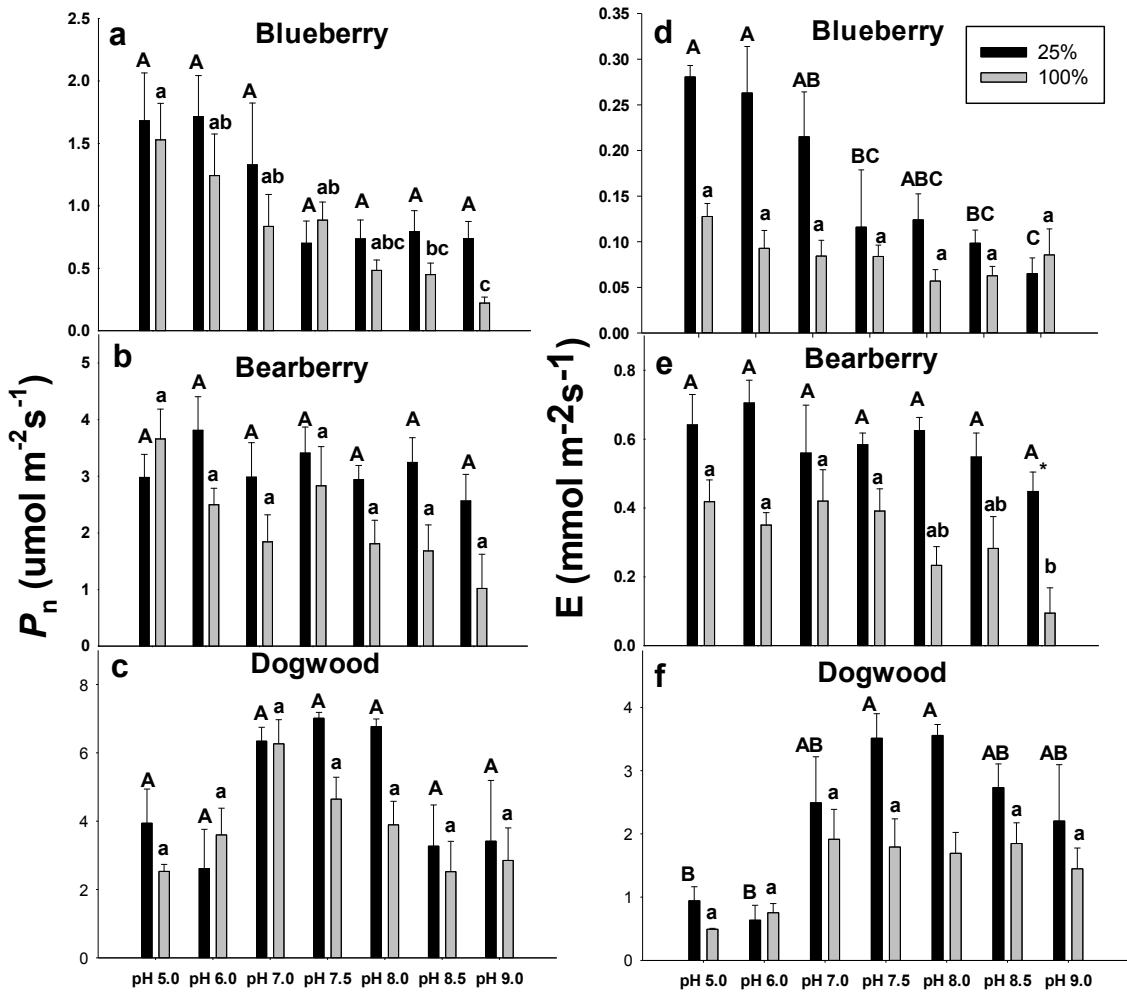


Figure a2.3 Effects of pH and nutrition on net photosynthetic (P_n) and transpiration rates (E) in blueberry, bearberry, and dogwood. Different letters above the bars (uppercase letters for 25% Hoagland's solution and lowercase letters for 100% Hoagland's solution) indicate significant differences ($\alpha = 0.05$) between pH treatments within each plant species. The asterisk above the bars indicates significant differences ($\alpha = 0.05$) between 25% and 100% Hoagland's solution. Means ($n = 8$) \pm SE are shown.

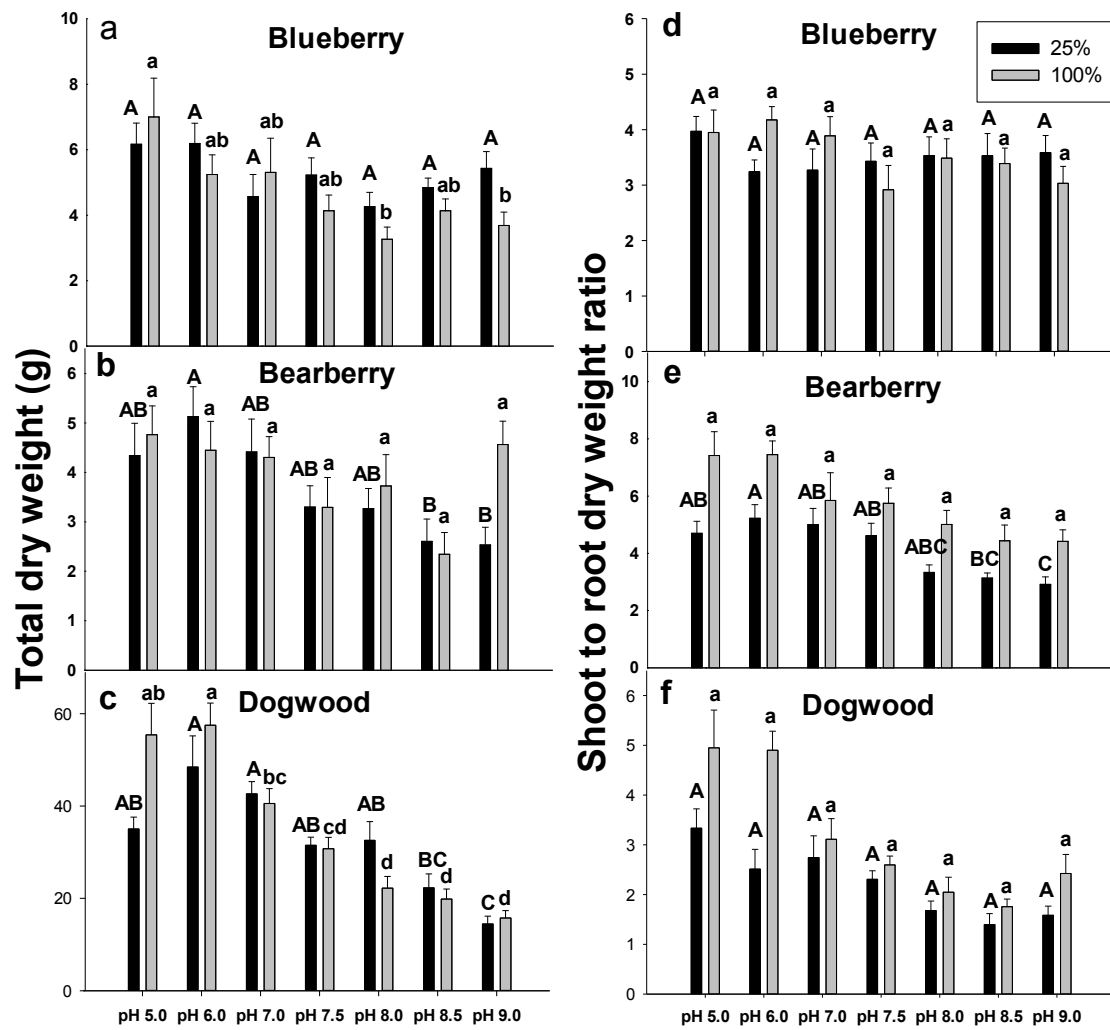


Figure a2. 4 Effects of pH and nutrition on total dry weights and shoot to root dry weight ratios in blueberry, bearberry, and dogwood seedlings. Different letters above the bars (uppercase letters for 25% Hoagland's solution and lowercase letters for 100% Hoagland's solution) indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 8$) \pm SE are shown.

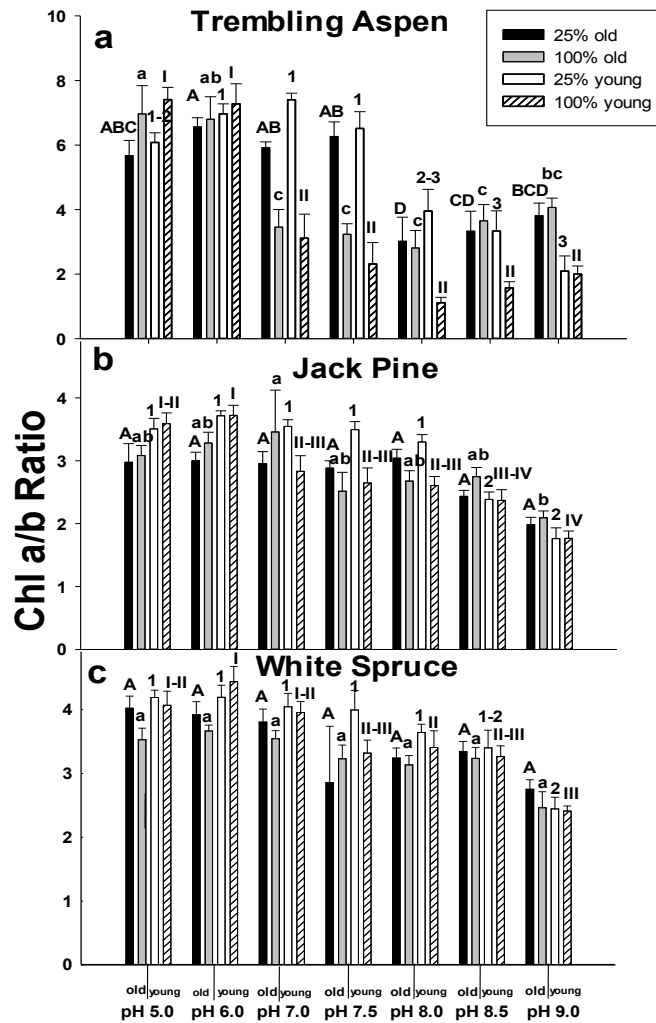


Figure a2. 5 Effects of pH and nutrition on ratios of chlorophyll-a to chlorophyll-b in old and young leaves of trembling aspen, jack pine, and white spruce. The old leaves were those that expanded fully before the treatments and the young leaves were those that sprouted after the start of treatments and were close to the shoot apex. Different letters above the bars (uppercase letters for old leaves in 25% Hoagland's solution, lowercase letters for old leaves in 100% Hoagland's solution, numbers for young leaves in 25% Hoagland's solution and Roman letters for young leaves in 100% Hoagland's solution) indicate significant differences ($\alpha = 0.05$) between pH treatments within each plant species. Means ($n = 6$) \pm SE are shown.

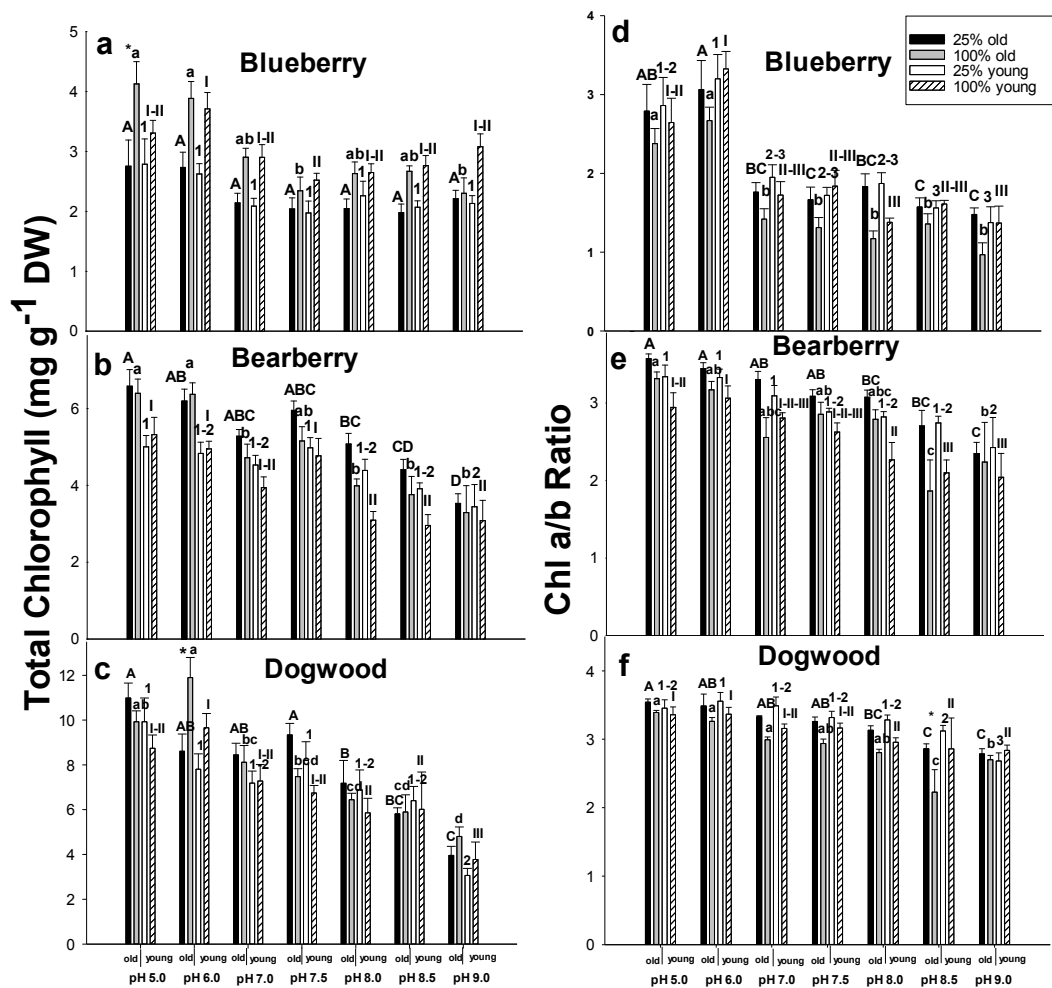


Figure a2.6 Effects of pH and nutrition on total chlorophyll concentrations (chlorophyll-a + chlorophyll-b) and ratios of chlorophyll-a to chlorophyll-b in old and young leaves of blueberry, bearberry, and dogwood. The old leaves were those that expanded fully before the treatments and the young leaves were those that sprouted after the start of treatments and were close to the shoot apex. Different letters above the bars (uppercase letters for old leaves in 25% Hoagland's solution, lowercase letters for old leaves in 100% Hoagland's solution, numbers for young leaves in 25% Hoagland's solution and Roman letters for young leaves in 100% Hoagland's solution) indicate significant differences ($\alpha = 0.05$) between pH treatments within each plant species. The asterisk above the bars indicates significant differences ($\alpha = 0.05$) between 25% and 100% Hoagland's solution. Means ($n = 6$) \pm SE are shown.

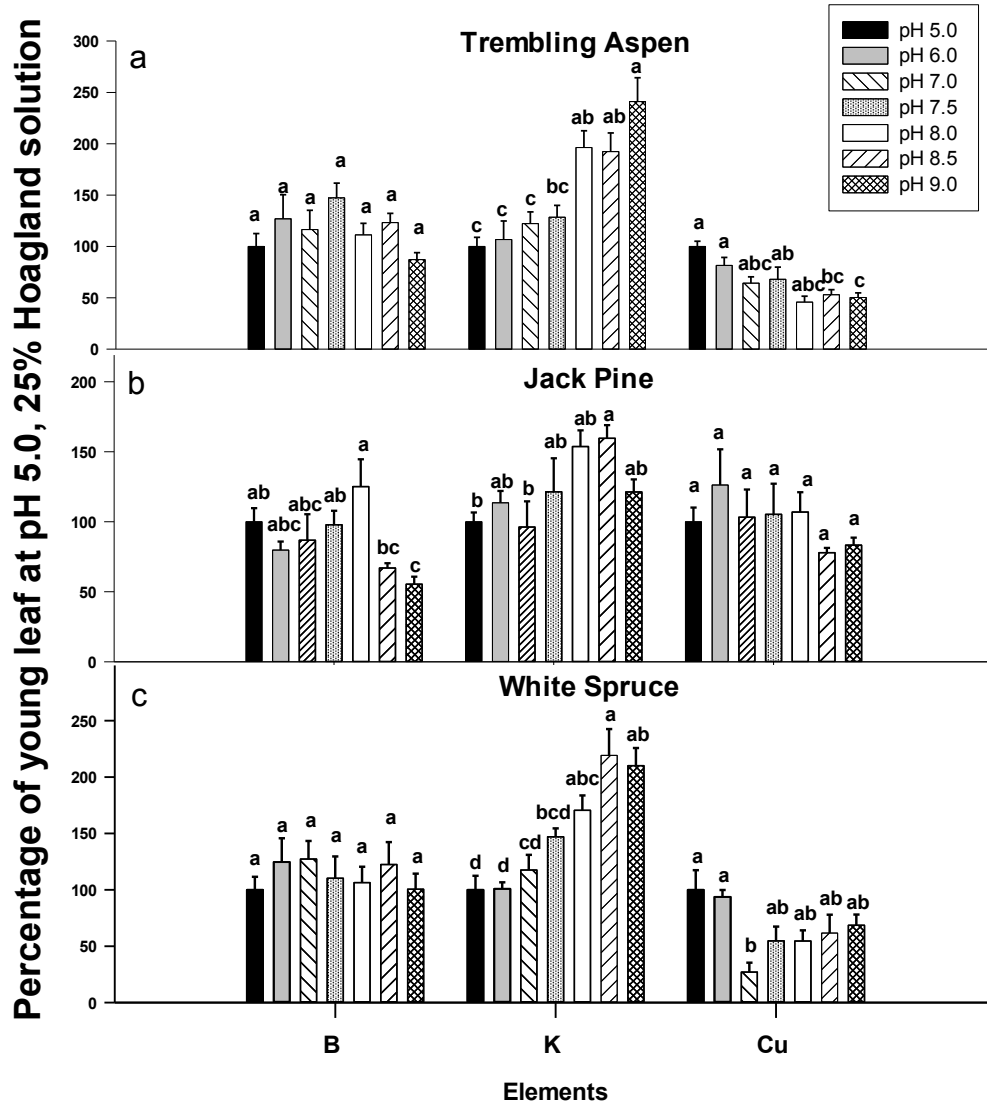


Figure a2. 7 Effects of pH on B, K, and Cu concentrations in young leaves of trembling aspen, jack pine, and white spruce seedlings in 25% Hoagland's solution, presented as the percentages of values measured at pH 5.0 in young leaves. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between pH treatments within each plant species. Means ($n = 6$) \pm SE are shown.

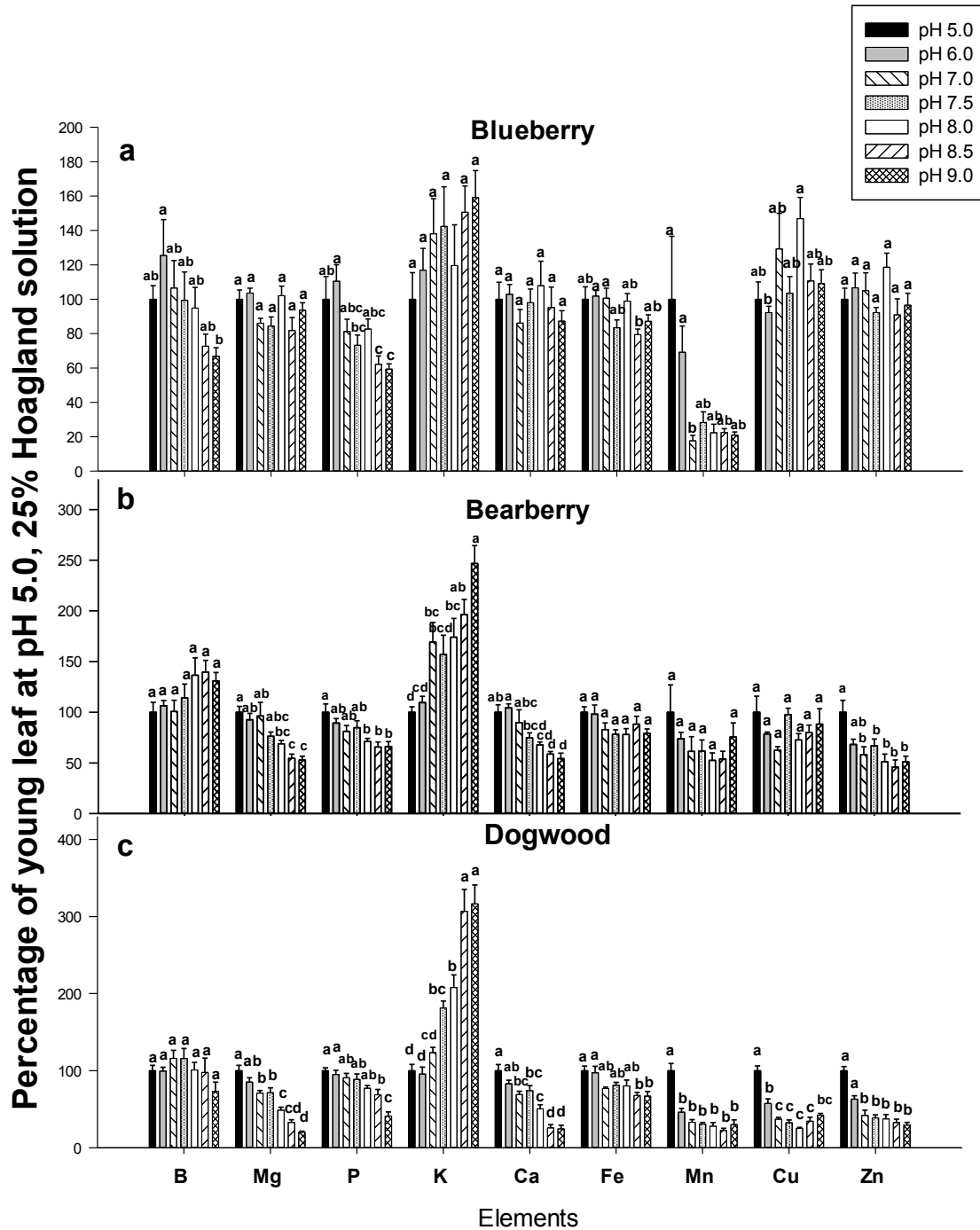


Figure a2. 8 Effects of pH on B, Mg, P, K, Ca, Fe, Mn, Cu and Zn concentrations in young leaves of blueberry, bearberry, and dogwood seedlings in 25% Hoagland's solution, presented as the percentages of values measured at pH 5.0 in young leaves. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between pH treatments within each plant species. Means ($n = 6$) \pm SE are shown.

Appendix 2

(Supplementary results of Chapter 3)

Table a3. 1 ANOVA table showing effects of Fe and pH treatments on measured parameters in paper birch, white spruce, green alder and tamarack seedlings (n = 6).

Species	paper birch		white spruce		green alder		tamarack	
p-value	Wp	Lpr	Wp	Lpr	Wp	Lpr	Wp	Lpr
Fe	0.0366	0.1866	0.7776	<.0001	<.0001	0.411	0.4953	<.0001
pH	0.211	0.0856	0.3702	<.0001	0.7663	0.9311	0.0053	<.0001
Fe*pH	0.0266	0.6909	0.059	<.0001	<.0001	0.3363	0.5256	<.0001

Trembling Aspen

p-value	tdw	s/r ratio	Pn	E	ChlO	Chl a: b old	ChlY	Chl a: b young	Wp
Fe	0.2506	0.7218	0.0566	0.014	0.7018	0.3376	0.1043	0.0064	0.002
pH	0.8603	0.0001	0.0491	0.4293	<.0001	0.0043	<.0001	<.0001	0.2897
Fe*pH	0.2563	0.9052	0.8835	0.1284	0.273	0.2857	0.0409	<.0001	0.0725

Black Spruce

p-value	tdw	s/r ratio	Pn	E	ChlO	Chl a: b old	ChlY	Chl a: b young	Wp	Lpr
Fe	0.0313	0.0041	0.083	0.2114	<.0001	0.0002	<.0001	<.0001	0.3086	<.0001
pH	0.1073	0.3669	0.0818	0.04	<.0001	<.0001	<.0001	<.0001	0.001	<.0001
Fe*pH	0.0347	0.0082	0.7623	0.2856	0.2151	0.002	0.9165	0.1192	0.3853	<.0001

Abbreviations are: tdw – total dry weight; s/r ratio - shoot: root dry weight ratio; Pn - net photosynthetic rate; E - transpiration rate; ChlO - chlorophyll concentrations in old leaves; Chl a/b old - chlorophyll a to b ratios in old leaves; ChlY - chlorophyll concentrations in young leaves; Chl a/b young - chlorophyll a to b ratios in young leaves; Wp - water potential; Lpr - hydraulic conductivity.

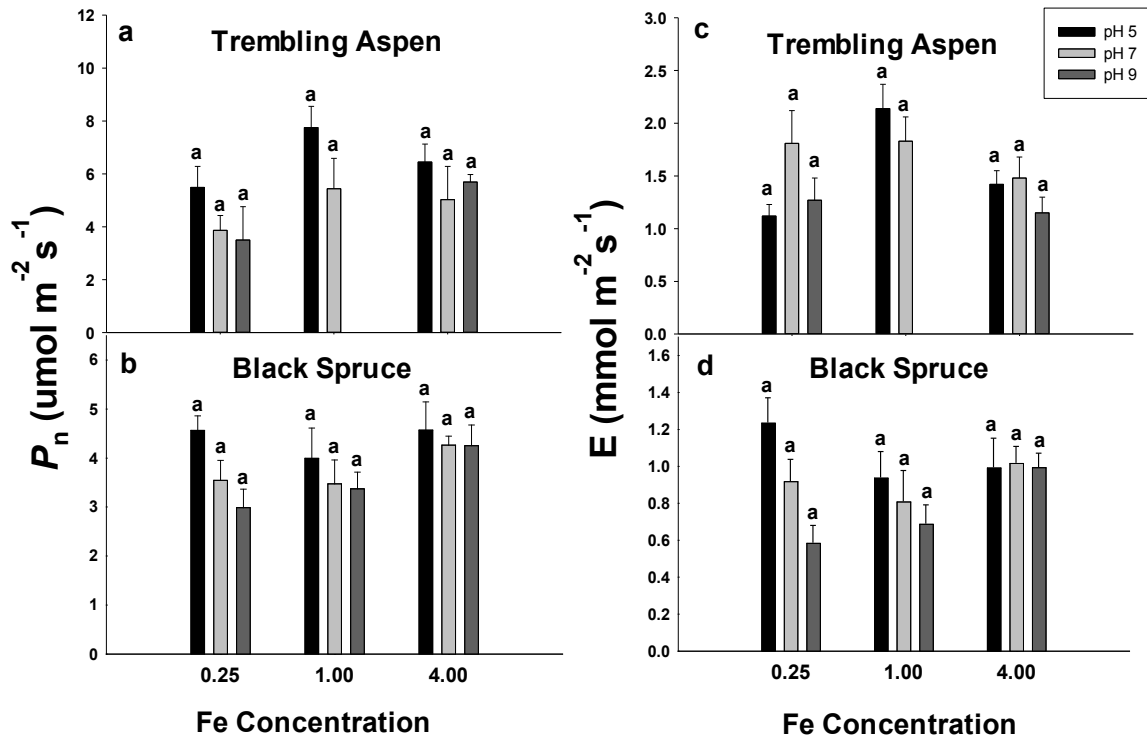


Figure a3. 2 Effects of Fe supplies and pH treatments on net photosynthesis rates (P_n) and transpiration rates (E) in trembling aspen and black spruce. Means ($n = 6$) \pm SE are shown.

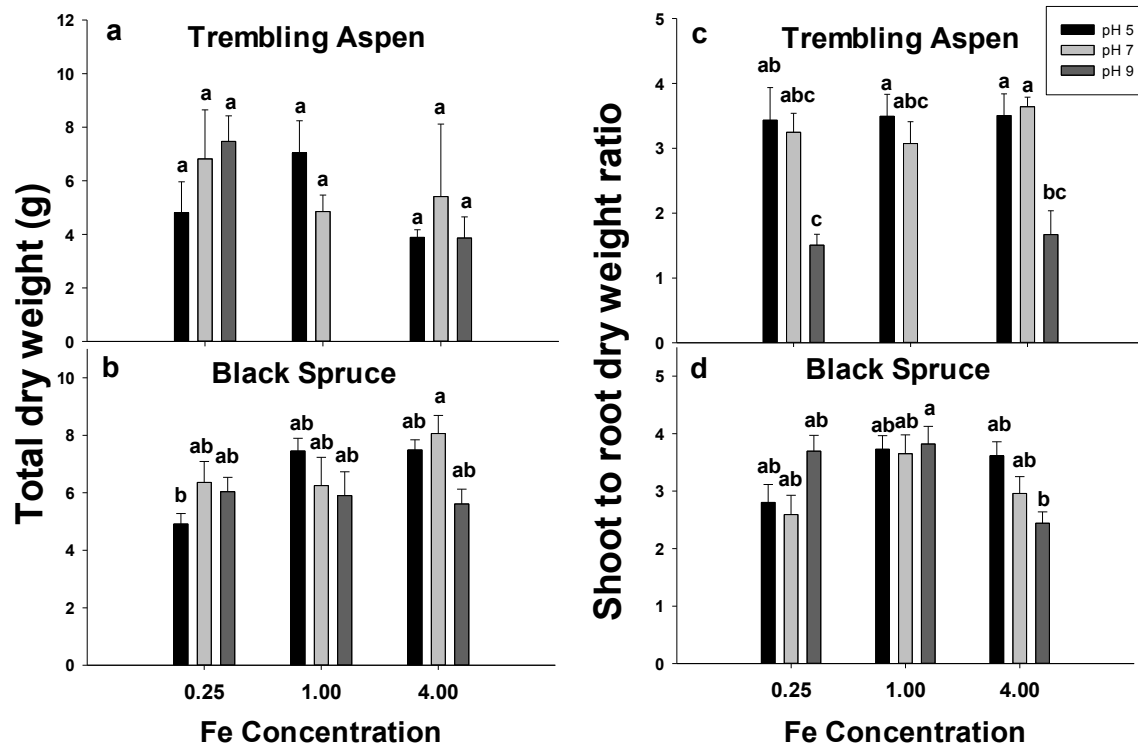


Figure a3. 3 Effects of Fe supplies and pH treatments on total dry weights and shoot to root dry weight ratios in trembling aspen and black spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 6$) \pm SE are shown.

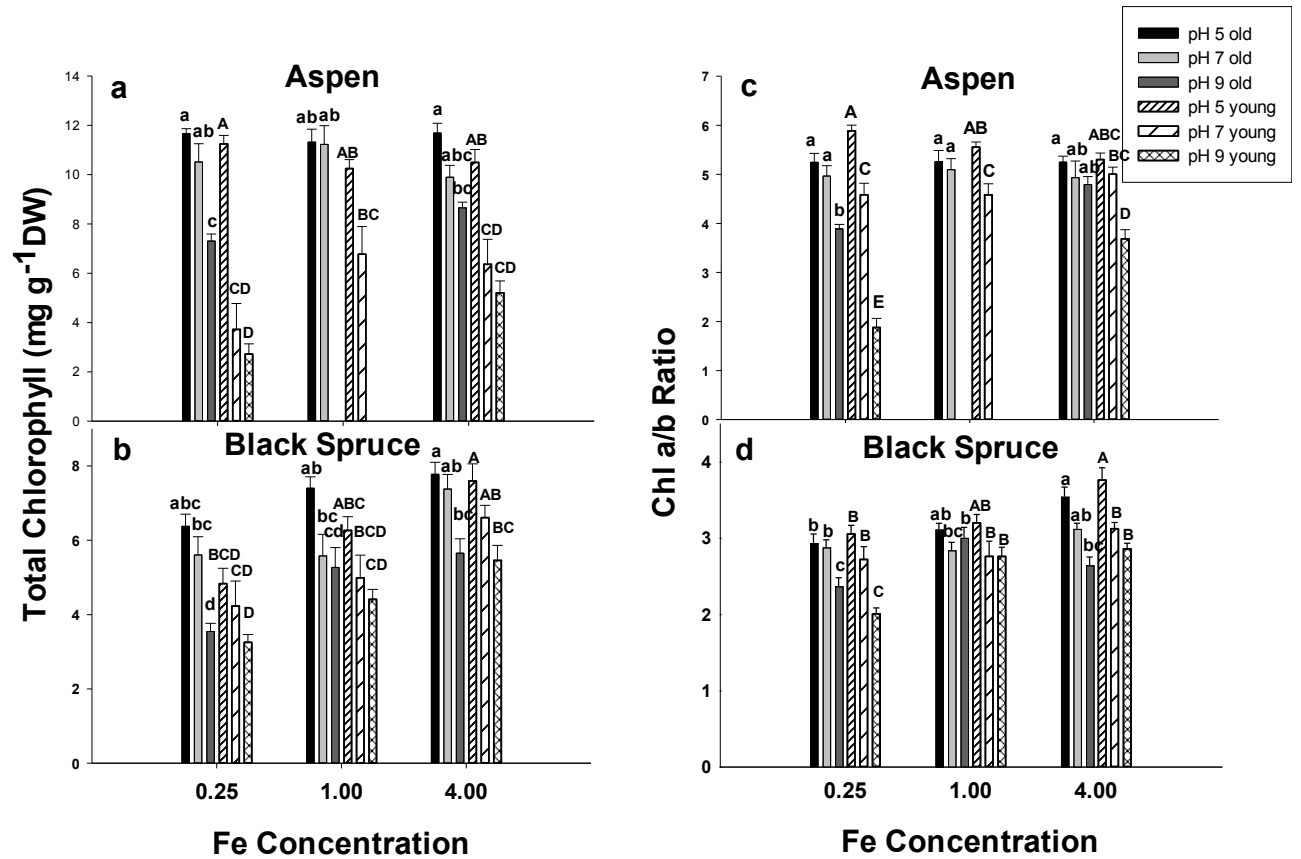


Figure a3.4 Effects of Fe supplies and pH treatments on total chlorophyll concentrations (chlorophyll-a + chlorophyll-b) and ratios of chlorophyll-a to chlorophyll-b in old and young leaves of trembling aspen and black spruce. The old leaves were those that expanded fully before the treatments and the young leaves were those that sprouted after the start of treatments and were close to the shoot apex. Different letters above the bars (lowercase letters for old leaves, uppercase letters for young leaves) indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 6$) \pm SE are shown.

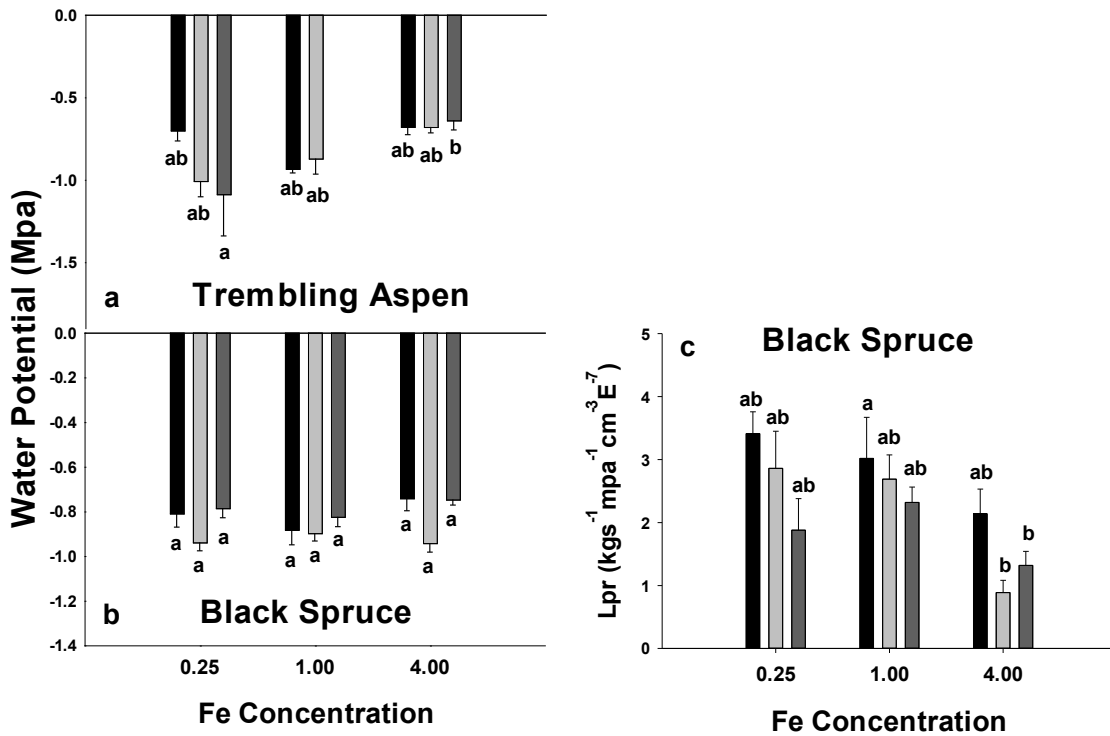


Figure a3.5 Effects of Fe supplies and pH treatments on water potential and root hydraulic conductivity (Lpr) in trembling aspen and black spruce. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 6$) \pm SE are shown. The Lpr of trembling aspen was not measured since high mortality.

Appendix 3

(Supplementary results of Chapter 4)

Figures

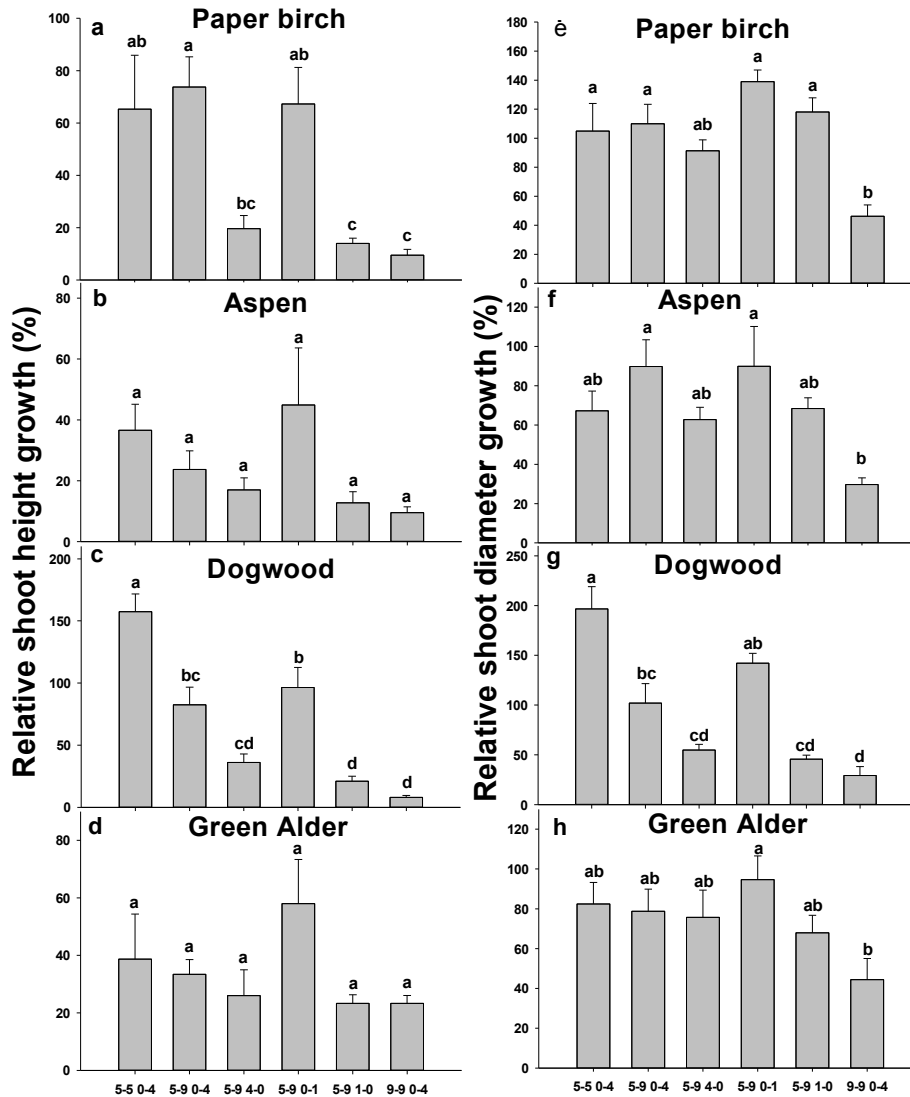


Figure a4.1 Effects of Fe supplies and pH treatments in split-root design on relative height growth and relative shoot diameter growth* in paper birch, trembling aspen dogwood and green alder. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 5$) \pm SE are shown.

* The relative shoot height and stem diameter growth were calculated by dividing the difference in the initial and final values by the initial value.

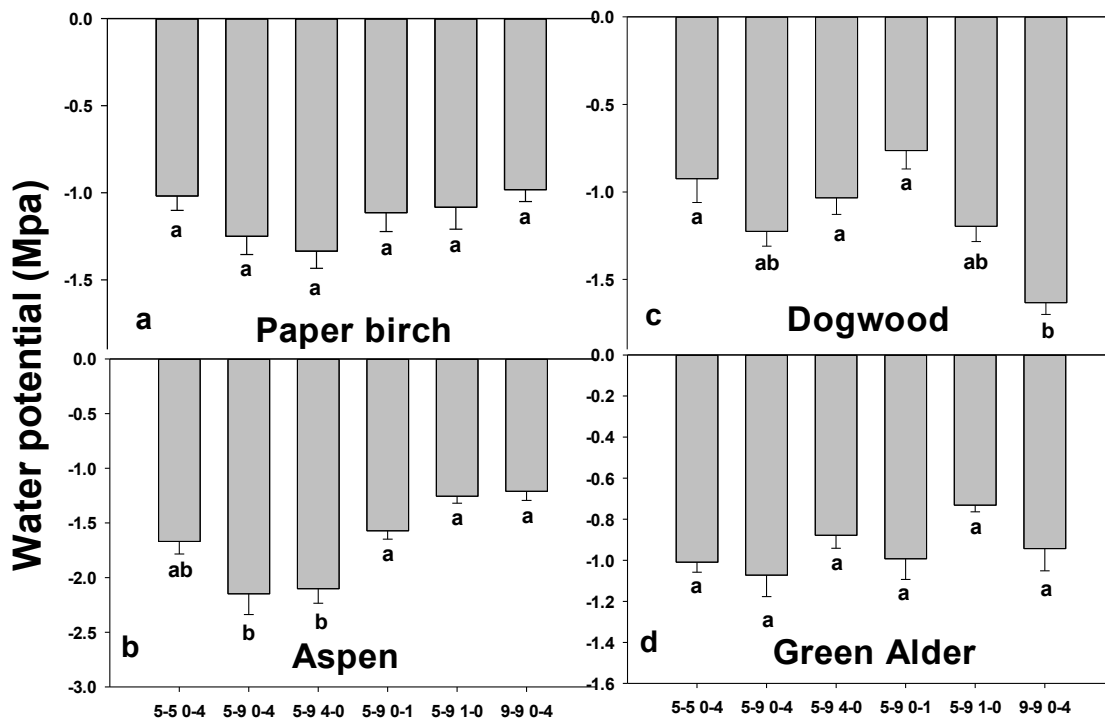


Figure a4. 2 Effects of Fe supplies and pH treatments in split-root design on water potential in paper birch, trembling aspen, dogwood, and green alder. Different letters under the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 5$) \pm SE are shown.

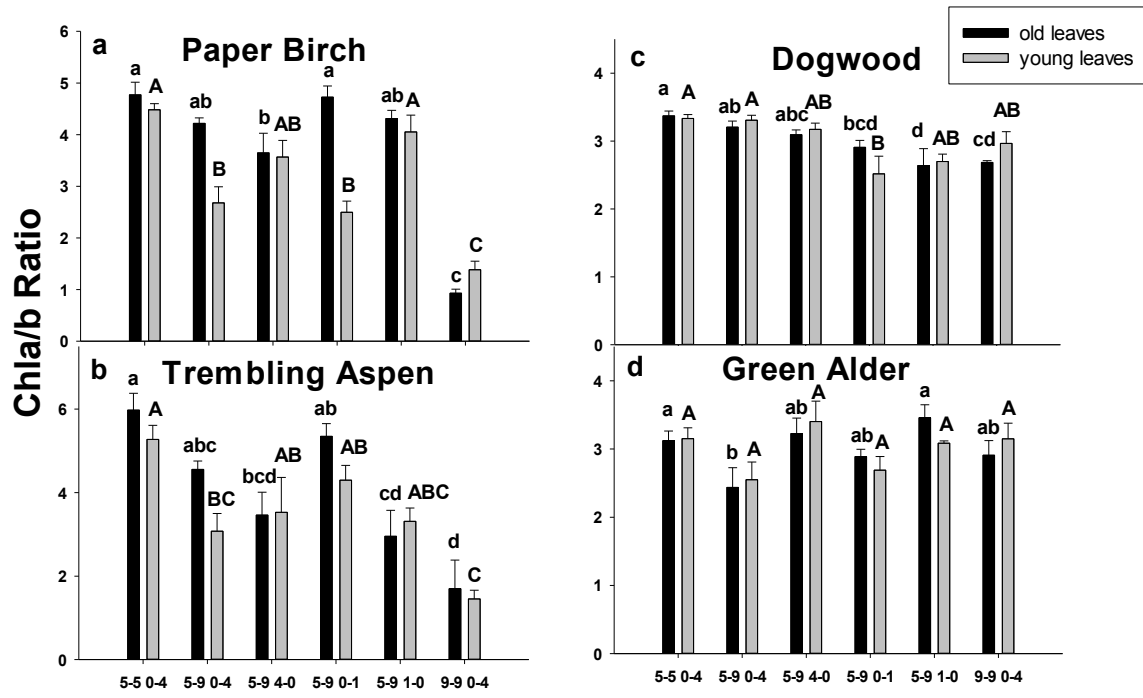


Figure a4. 3 Effects of Fe supplies and pH treatments in split-root design on ratios of chlorophyll-a to chlorophyll-b in old and young leaves in paper birch, trembling aspen, dogwood and green alder. The old leaves were those that expanded fully before the treatments and the young leaves were those that sprouted after the start of treatments and were close to the shoot apex. Different letters above the bars (lowercase letters for old leaves, uppercase letters for young leaves) indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 5$) \pm SE are shown.

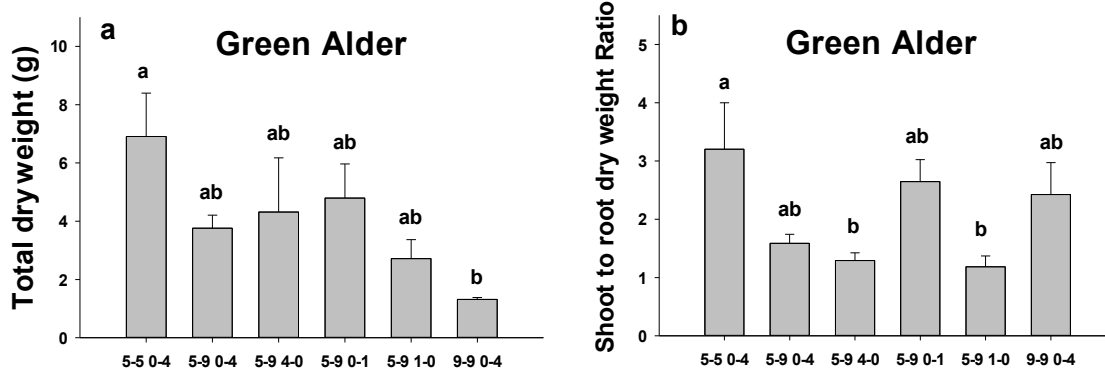


Figure a4. 4 Effects of Fe supplies and pH treatments in split-root design on total dry weights and shoot to root dry weight ratios in green alder. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 5$) \pm SE are shown.

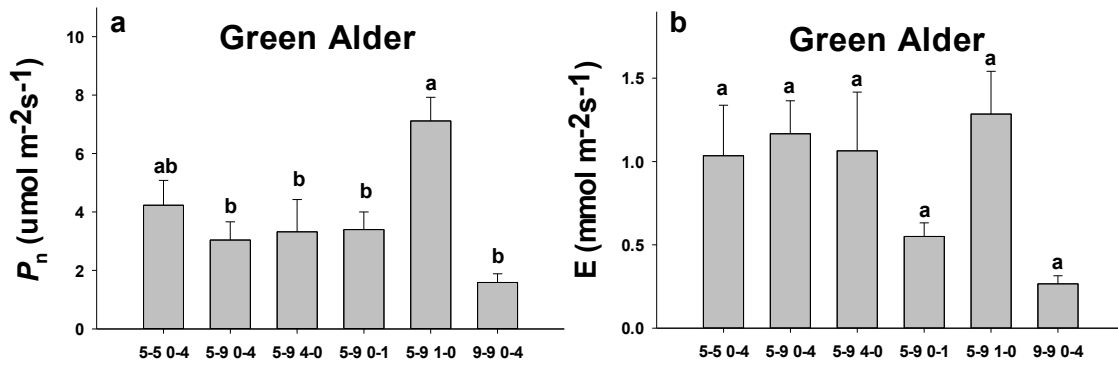


Figure a4. 5 Effects of Fe supplies and pH treatments in split-root design on net photosynthesis rates (P_n) and transpiration rates (E) in green alder seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 5$) \pm SE are shown.

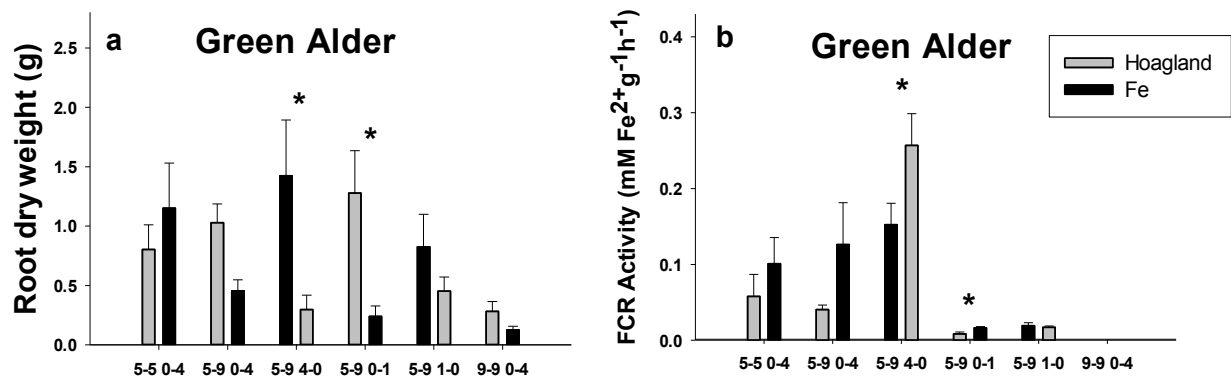


Figure a4. 6 Effects of Fe supplies and pH treatments in split-root design on root dry weight and FCR activity in Hoagland and Fe side of green alder. The asterisk above the bars indicate significant differences ($\alpha = 0.05$) between two sides of root dry weight and FCR activity determined by the paired t-test. Means ($n = 5$) \pm SE are shown.

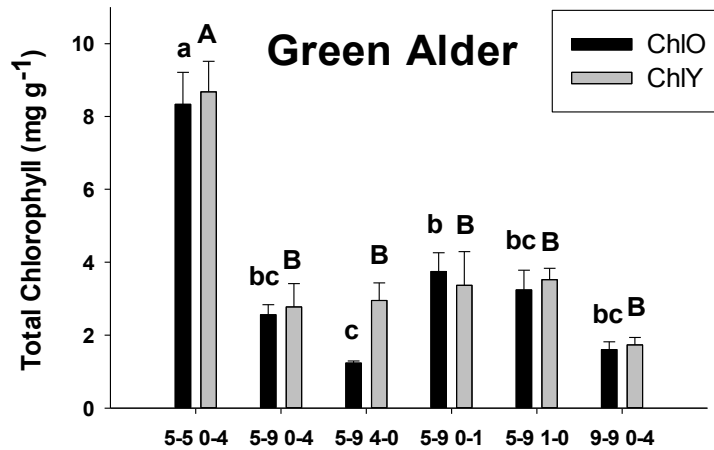


Figure a4. 7 Effects of Fe supplies and pH treatments in split-root design on total chlorophyll concentrations (chlorophyll-a + chlorophyll-b) in old and young leaves of green alder. The old leaves were those that expanded fully before the treatments and the young leaves were those that sprouted after the start of treatments and were close to the shoot apex. Different letters above the bars (lowercase letters for old leaves, uppercase letters for young leaves) indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 5$) \pm SE are shown.

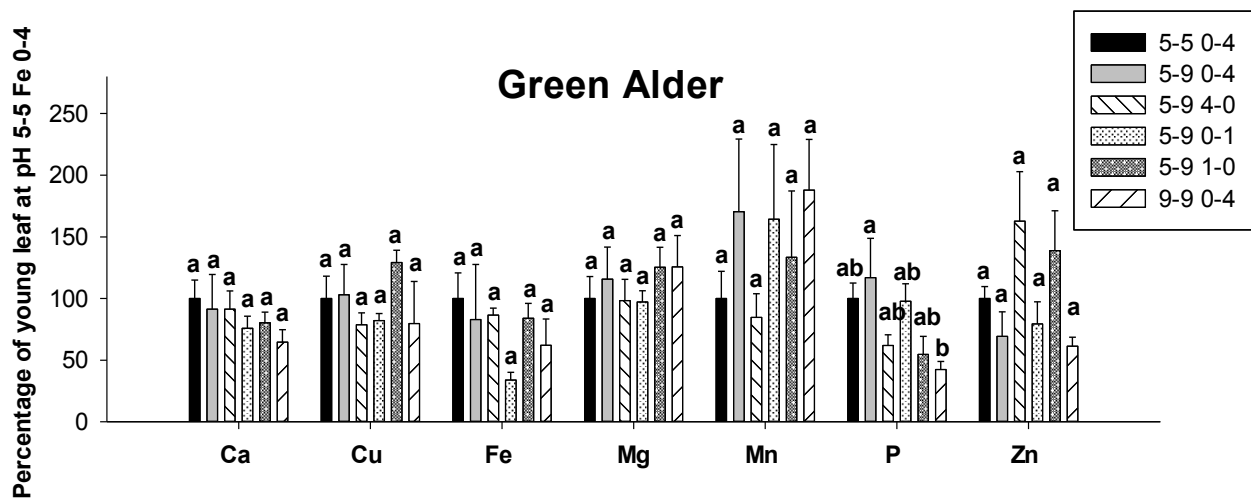


Figure a4. 8 Effects of Fe supplies and pH treatments in split-root design on Ca, Cu, Fe, Mg, Mn, P and Zn concentrations in young leaves of green alder seedlings, presented as the percentages of values measured at pH 5-5 Fe 0-4 treatment in young leaves. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ($n = 5$) \pm SE are shown.