Effects of Root Zone pH, Calcium and Phosphorus Supply on Selected Boreal Forest Plant Species

by

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Abstract

Soil pH is among the major environmental factors affecting plant growth. In the reclaimed areas following open-pit oil sands mining in northeastern Alberta, Canada, the pH of reclaimed soil is commonly higher than 8.0. The optimum range of soil pH for growth and the tolerance of pH extremes widely varies among plant species, but is generally low for boreal forest plants. High soil pH affects many processes in plants in a complex manner. A common problem associated with high soil pH is reduced availability of certain essential elements including phosphorus, iron, manganese and zinc. High soil pH can also reduce root water flux. Calcium is known to aggravate the detrimental effects of high pH on plants by inhibiting root growth. High calcium levels are of concern in calcareous soils and in some of the oil sands reclamation areas due to the use of gypsum during the tailings consolidation process. The goal of oil sands reclamation is to restore disturbed forest ecosystems to their original state including forest productivity and biodiversity. In this project, I examined the effects of root zone pH, calcium and phosphorus supply on several boreal forest plant species including trembling aspen, white spruce, black spruce, jack pine, tamarack, paper birch, green alder, red osier dogwood, blueberry and bearberry. The studies were conducted through a series of controlled-environment experiments with hydroponically-grown plants. I found that the effects of high root zone pH varied between the different plant species and in sensitive plants high root zone pH and high calcium levels reduced growth, net photosynthesis, transpiration rates, root water flux, leaf chlorophyll concentrations, root cortex cell lengths, and tissue elements concentrations in seedlings. The effects of high pH, including those on leaf chlorophyll concentration, were partly alleviated by exposing a part of the root system to

low pH while the remaining part was exposed to high pH. However, I did not find substantial beneficial effects of increasing phosphorus supply to the plants subjected to high root zone pH conditions. The reasons for greater tolerance to high pH in some of the studied plant species likely included enhanced ability to maintain optimum apoplastic pH, high root hydraulic conductivity, and high ferric chelate reductase activity under high pH conditions. Among the examined species, dogwood, white spruce and black spruce showed greater resistance to high root zone pH and are likely to outperform the other plants following planting in oil sands reclamation sites with elevated pH levels.

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List of Abbreviations

$[Ca^{2+}]$	calcium ion concentration
ψ_w	shoot water potential
ANOVA	analysis of variance
AQP	aquaporin
BPDS	bathophenanthrolinedisulfonic acid
chl	chlorophyll
CRBD	complete randomized block design
DMSO	dimethyl sulfoxide
DW	dry weight
E	transpiration rate
EC	electric conductivity
FCR	ferric chelate reductase
GFP	green fluorescence protein
gs	stomatal conductance
HPFM	high pressure flow meter
ICP-MS	inductively coupled plasma mass spectrometry
Kr	root hydraulic conductance
LPr	root hydraulic conductivity
Pi	inorganic phosphorus
Pn	net photosynthesis rate
PPFD	photosynthetic photon flux density
PVDF	polyvinyl difluoride
RSHG	relative shoot height growth
SE	standard error
SEM	scanning electron microscope
SNK	Student-Newman-Keuls

TBO	Toluidine Blue O
WUE	water use efficiency
w/v	weight/volume
v/v	volume/volume

Chapter 1

Introduction and literature review

1.1 Introduction

High soil pH is a common problem impacting plant growth in many arid and semi-arid areas. Calcareous soils cover approximately one-third of the world land surface, with soil pH usually higher than 7.5 (Wallace and Lunt 1960). In some areas disturbed by human activities which discharge high pH contaminants, soil pH may also increase and negatively affect growth of native plants. In reclamation sites of the oil sands mining areas in Alberta, Canada, the soil pH values are often 8 or higher depending on the site (Howat 2000). Excessive calcium in disturbed soils may create another serious environmental issue in oil sands reclamation sites where mine tailings treated with gypsum were deposited to accelerate tailings consolidation (Ramos-Padrón et al. 2010). Until now, very few studies had been conducted to examine plant responses to high root zone pH in combination with other soil factors, especially in tree species.

One of the major problems associated with high soil pH is limited availability of micronutrients such as iron, manganese and zinc, as well as phosphorus for plants (Marschner 2012). Due to nutrient deficiencies, leaf chlorosis is a common symptom for plants growing in alkaline soils (Mengel and Geurtzen 1986). Root growth is usually inhibited by high root zone pH, and this inhibition effect is mainly on root cell elongation rather than cell division (Tang 1992). Root water transport was also reported to be inhibited by high pH, along with reduced transpiration and stomata conductance (Tang

1993a). Root medium pH can affect the apoplastic pH, and thus indirectly affect the activities of membrane proteins such as aquaporins and ferric chelate reductase (Mengel 1994; Felle and Hanstein 2002; Tournaire-Roux et al. 2003).

In addition to being an important macroelement, calcium may be beneficial to plants by improving their resistance of abiotic stresses including aluminum and salt toxicities (Kinraide and Parker 1987; Cabanero 2004). However, in plant cell elongation, apoplastic acidification is preceded before cell expansion and, in this process, protons are supposed to replace calcium ions at the cell wall as calcium acts as bridge between pectin chains to rigidify the cell wall (Rayle and Cleland 1992; Hepler 2005). Therefore, calcium and protons may have opposing roles in cell elongation processes. Supplying excessive amounts of calcium was reported to inhibit plant cell elongation (Tang 1993b). However, the interactions of calcium with physiological processes in plants are complex and have not been thoroughly investigated under high pH conditions.

In this thesis I studied the effects of pH, calcium and phosphorus on ten major boreal forest plant species, which are often used for oil sands reclamation. The studies were carried out using hydroponic and split-root setups. I measured the growth and physiological parameters to demonstrate responses of different plant species to pH treatments in order to examine how different species of boreal forest plants cope with high pH conditions.

The objectives of the present study were to:

1) Examine the effects of root zone pH on growth and physiological responses of different boreal forest plant species.

2) Determine the growth and physiological responses of tree seedlings to neutral and high root zone pH in the presence of different calcium concentrations.

3) Examine the effects of phosphorus supply and heterogeneous root zone pH on plant responses to high pH and calcium treatment.

4) Investigate mechanisms of high pH tolerance in two boreal forest plant species.

I tested the following hypotheses:

1) Boreal forest plants vary in their sensitivity to high root zone pH.

2) Negative effects of high pH on plants are aggravated by high calcium concentrations.

3) Increasing phosphorus availability and subjecting part of the root system to low pH can alleviate effects of high pH on plants.

4) High pH tolerance of plants is partly determined by their ability to maintain high rates of root water transport, control apoplastic pH and maintain the activity of ferric chelate reductase.

Thesis structure

This thesis includes six chapters. Chapter 1 provides general information of the oil sands mining and reclamation, biology of examined species, and effects of pH, calcium, phosphorus on plants. In Chapter 2, I studied the effects of different root zone pH on growth and physiological responses of trembling aspen, white spruce and tamarack.

In Chapter 3, I examined the responses of jack pine, blueberry, bearberry, red osier dogwood, white spruce, and trembling aspen seedlings to different root zone pH and calcium conditions. In Chapter 4, I used the split-root hydroponic system to study the effects of phosphorus and calcium availability on the responses of paper birch, jack pine, green alder, trembling aspen, black spruce, and white spruce to high pH. In Chapter 5, I investigated possible mechanisms of high pH tolerance in plants by comparing the differences in responses to pH of relatively high pH tolerant dogwood and relatively high pH sensitive paper birch. Chapter 6 provides the final synthesis of these studies and general conclusions and suggestions for future research.

1.2 Literature review

1.2.1 Oil sands mining and reclamation

In northeastern Alberta, Canada, an estimated 170 billion barrels of bitumen is present underneath the boreal forest (Government of Alberta 2014a). Oil sands deposits that are close to the surface (less than 100 meters deep) cover an area of almost 4800 km^2 and are feasible for open-pit mining (Government of Alberta 2014b). Prior to surface mining, the wetlands are drained and forests are completely cleared. The organic layer and mineral soil layer are stripped and stockpiled to be later used as cover soils in reclamation strategies (Mackenzie and Naeth 2010). The material overlying the bitumen deposit and underlying the mineral soil is called overburden. The overburden is mined by large shovels and then placed at above ground waste dumps. For mine reclamation, overburden can be used as subsoil in upland forests or to create dams for tailing ponds (Grant et al. 2008). In the oil sands open-pit mining areas near Fort McMurray, the overburden often consists of saline-sodic clay shale from the Clearwater geologic

formation and is termed the saline-sodic overburden (SSOB) (Lazorko 2008). The electric conductivity (EC) of SSOB can be as high as 11 dS/m (Howat 2000).

After the oil sands are mined, the ore is delivered to the extraction plant via conveyor belts or large dump trucks. The bitumen is extracted with hot water and NaOH using the method pioneered by Dr. Karl Clark (Masliyah et al. 2004). The resulting slurry is transferred to large vessels where the bitumen is separated from the sands. A significant by-product of this extraction process is referred to as mine tailings, which includes sand, silt, clay, residual bitumen, and naphtha (Fung and Macyk 2000). The tailings sands are used as subsoil during the reclamation process, while the tailings water is discharged into large tailings ponds. As large amount of NaOH is used to extract bitumen from oil sands, the pH of tailings water can be higher than 8.4 (Howat 2000).

The tailings ponds consist of about 20 to 30% solids, 1 to 3% residual bitumen, and alkaline water on weight basis (Ramos-Padrón et al. 2010). The solids are allowed to settle down and the remaining clear water is reused in the bitumen extraction process. For production of 1 barrel (159 L) of oil, 1 m^3 of oil sands is extracted with 3 m^3 of water, thus producing 4 m^3 of tailings (Ramos-Padrón et al. 2010). Each day, approximately 400,000 m^3 of tailings are released in the oil sands region (Ramos-Padrón et al. 2010). The fine silts and clays in the tailings pond form a stable suspension that requires long time to consolidate. It is estimated that it would take more than 100 years for natural consolidation to form a trafficable surface capable of supporting a productive soil layer (Fung and Macyk 2000). Oil sands operators use different approaches to accelerate the consolidation process. One typical method is through the use of gypsum ($CaSO_4 \cdot 2H_2O$), usually added at 1 kg per m^3 tailings, where Ca^{2+} serves as a densification agent

(Chalaturnyk et al. 2002). The soil Ca content in some reclaimed sites can exceed 400 mg kg^{-1} compared with less than 6 mg kg^{-1} typically found in the surrounding mixedwood forests (Visser 2005).

According to the current reclamation strategies, soil replacement occurs via either a "one-lift" or "two-lift" techniques (see Fig. a1.1) (Lazorko 2008). In the one-lift method, 25 to 50% (by volume) of mineral soils is stripped off and incorporated with peat. This peat-mineral mix is used as a cover soil spreading to a depth of 15 to 50 cm over the prepared site. For reclamation using overburden, the soil amendment of peat is typically mixed with coarse-textured material such as sand and gravel. In the case of tailings sand, the peat is incorporated with fine-textured silt or clay (Lazorko 2008). For the two-lift method, usually a 50 cm capping materials of sandy or clay subsoils are placed over the substrate, and then a 15 to 25 cm peat-mineral mix is used as a cover soil. However, different oil sands operators may have different reclamation prescriptions. For example, Syncrude Canada Ltd (Syncrude) calls for tailings sand surface to be capped with 70 cm (±10 cm) overburden with a minimum 100 cm (±10 cm) of suitable cover soils. In contrast, in some reclaimed sites in Suncor Energy Inc. (Suncor), only 15 cm of reclamation material, made up of >50% organic, is placed on both tailings sand and overburden substrate (Fung and Macyk 2000).

Both the saline sodic overburden and tailings sand contain a large volume of sodium, and the sodium can translocate upwards to the cover soil layer by the capillary phenomenon (Jorenush and Sepaskhah 2003). The sodium can react with bicarbonates to form sodium bicarbonate. When the sodium bicarbonate is hydrolyzed, it significantly increases soil pH and results in alkaline soils (Marschner 2012).In addition, leaking of

tailings ponds can also increase the soil pH in surrounding areas (Tenenbaum 2009). Therefore, in the oil sands reclamation sites, high soil pH and salinity are major challenges for revegetation (Howat 2000; Alberta-Environment 2010). In oil sands reclamation areas, soil pH commonly ranges from 7.0 to 8.5, while pH of the soil in the native boreal ecosystems in these areas is typically lower than 6 (Howat 2000). There have been extensive studies on effects of salinity on plant species native to the boreal forest in northeastern Alberta (Renault et al. 1998; Renault et al. 1999; Renault et al. 2001; Calvo-Polanco et al. 2009b; Calvo-Polanco et al. 2014). However, studies on responses of boreal plants to high soil pH are relatively limited (Calvo-Polanco et al. 2009a; Siemens and Zwiazek 2011). Excessive calcium in reclaimed soils from the gypsum usage when settling down tailing ponds brings another major problem to the revegetation of oil sands (Chalaturnyk et al. 2002).

According to current policy, oil sands operators are required to restore disturbed forest ecosystems to equal or better than its original state after reclamation including forest productivity, biodiversity, and varied geographic landscapes that are similar to those prior to disturbance (Alberta Environment 2010).

1.2.2 Biology of the studied plant species

In this project, I studied seven major boreal forest tree species: trembling aspen (*Populus tremuloides* Michx.), white spruce [*Picea glauca* (Moench) Voss], black spruce (*Picea mariana* (Mill.) B.S.P.), jack pine (*Pinus banksiana* Lamb.), paper birch (*Betula papyrifera* Marsh.), tamarack [*Larix laricina* (Du Roi) K. Koch], green alder (*Alnus viridis* (Chaix.) D.C.), and three shrub species: red osier dogwood (*Cornus stolonifera* Michx, synonym as *Cornus sericea*), blueberry (*Vaccinium myrtilloides* Michx.) and

bearberry (*Arctostaphyllos uva-ursi* (L.) Spreng.). The biology of these species is briefly described below.

1.2.2.1 Trembling Aspen (*Populus tremuloides*)

Trembling aspen is the most widely distributed tree species in North America. It is a small to medium sized, fast-growing, and short-lived tree. Aspen grows on many different soil types, especially sandy and gravelly slopes. Good aspen soils are well drained, loamy, and high in organic matter, calcium, magnesium, potassium, and nitrogen. Aspen has an important role in nutrient cycling due to its rapid growth and high nutrient demand (Boyle 1973; Alban 1982). It often grows poorly on sandy soils because of low moisture and nutrients. The best aspen sites are usually on soils with silt-plus-clay content of 80 percent or more (DeByle 1985). Aspen usually does not grow well on heavy clay soils, due to limited available water and poor aeration (Perala 1977).

Aspen grows with a large number of trees and shrubs over its range. Aspen-white spruce mixed stand is a major forest cover type in North America. The shrub species that associate with trembling aspen in the boreal forest include American green alder (*Alnus viridis*), red-osier dogwood (*Cornus stolonifera*), highbush cranberry (*Viburnum edule*), and bearberry (*Arctostaphylos uva-ursi*) (Perala 1990). Aspen seedlings initially have a short taproot, but on deep and well drained soils a heart root system develops (Perala 1990). Aspen seedlings at one year of age are capable of reproducing by root sprouts (suckers), and by these means the aspen stands can reproduce vigorously (Brinkman 1975). Trembling aspen is classified as very intolerant of shade, and it is quick to pioneer disturbed sites such as burned lands by root suckering (Shepperd 1986; Perala 1990).

Aspen is generally regarded as an early successional species and is able to dominate on a site, until it is gradually replaced by less fire-enduring but more shade tolerant conifers.

1.2.2.2 White spruce (*Picea glauca*)

White spruce is adapted to a wide range of soil and climatic conditions across the boreal forest. At the northern limit of white spruce's range, temperature extremes range from -54℃ in January to 34℃ in July according to the historical climate records (Maini 1966). White spruce grows on both acid and alkaline soils and pH values ranging from 4.7 to 7.0 (Sutton 1969; Brand et al. 1986). It was also reported to grow well in alkaline soils in Prairie Provinces (Stiell 1976). In New York state, most of the white spruce sites are commonly abundant in calcium (Nienstaedt and Zasada 1990). This species also tolerates a range of nutrition levels and moisture conditions. The development of white spruce stands can significantly change forest floor composition and biomass together with soil physical and chemical properties (Nienstaedt and Zasada 1990). The soil pH of a spruce plantation decreased by 1.2 over a 45-year period (Brand 1986).

The species commonly associated with white spruce include black spruce (*Picea mariana*), paper birch (*Betula papyrifera*), quaking aspen (*Populus tremuloides*), balsam fir (*Abies balsamea*), jack pine (*Pinus banksiana*), lodgepole pine (*Pinus contorta*), snowberry (*Symphoricarpos albus*), and red-osier dogwood (*Cornus stolonifera*). White spruce can produce seeds and cones at 4-years of age, and the seeds are primarily dispersed by wind (Sutton 1969). Vegetative reproduction from layering is also common at some sites in Canada (Densmore 1980). White spruce is often characterized as shallow rooted, with a common depth between 90 and 120 cm. However, the taproots and sinker

root can descend to a depth of 3m (Nienstaedt and Zasada 1990). The shade tolerance of white spruce is lower than black spruce, and higher than aspen and paper birch (Nienstaedt and Zasada 1990).

1.2.2.3 Black spruce (*Picea mariana*)

Black spruce, with other common names including bog spruce, swamp spruce, and shortleaf black spruce, is a wide-ranging, abundant conifer in the northern parts of North America. Black spruce grows on various soil types, but most commonly on wet organic soils. In Canada, black spruce commonly grows in peat bogs and swamps, and it also grows in uplands especially in Ontario. The most productive black spruce stands are on dark brown and blackish peats (Viereck and Johnston 1990). Due to its shallow rooting habit, in northern regions, black spruce is best adapted to growing on permafrost soils (Zoltai and Pettipiece 1974). Black spruce can associate with white spruce, balsam fir (*Abies balsamea*), jack pine (*Pinus banksiana*), tamarack (*Larix laricina*), paper birch (*Betula papyrifera*), lodgepole pine (*Pinus contorta*), and trembling aspen (*Populus tremuloides*). However, black spruce grows more slowly than many of its associated trees and shrubs, and it is common as an understory tree in jack pine and lodgepole pine stands on dry sites (Fowells 1965). Black spruce cones can be opened by fire and disperse seed for several years, providing an adequate supply of seeds to reproduce the stand (Haavisto 1975). Fires that completely remove the surface organic layer usually provide good seedbeds for black spruce. At sites where mosses cover lower branches of slow-growing seedlings and saplings, layering is an important means for reproduction of black spruce (Stanek 1975). Black spruce is often a pioneer species after fire on both uplands and peatlands.

1.2.2.4 Jack pine (*Pinus banksiana*)

Jack pine, a small- to medium-sized coniferous tree, is the most widely distributed pine species in Canada (Rudolph and Laidly 1990). Jack pine is usually found on sandy and loamy soils (Cayford et al. 1967; USDA 1975). It does not grow naturally on sites where surface soil is alkaline, but it can grow on soils overlying limestone. If mycorrhizal associations are present, jack pine can grow on calcareous soils with pH 8.2 (Rudolf 1965). The jack pine forest cover type typically originates after forest fires. In boreal forests, the most common species associated with jack pine are trembling aspen, paper birch, balsam fir, and black spruce. Associated species are nearly always subordinate to jack pine except for aspen and paper birch which may be coordinate (Rudolf 1965; Eyre 1980). Jack pine seeds usually germinate after fire, and most seedlings die if the organic layer of the soil is less than 1.3 cm (Cooley 1972). Jack pine seedlings frequently develop taproot and maintain it to maturity (Rudolph and Laidly 1990). This is one of the least shade intolerant species in the boreal forest, least intolerant among all associated pines and slightly more tolerant than aspen, birch and tamarack (Benzie 1977). Jack pine is a pioneer species on burned sites or other exposed lands. In parts of Canada, jack pine is succeeded by black spruce, white spruce, balsam fir and paper birch (Rudolf 1965; Benzie 1977). In west-central Alberta, jack pine hybridizes with lodgepole pine (*P. x murraybanksiana Righter and Stockwell*) (Righter and Palmer1949).

1.2.2.5 Paper birch (*Betula papyrifera*)

Paper birch, also called white birch or silver birch, is a medium-sized, fast-growing and short-lived tree. It is a northern species adapted to cold climates. Paper birch grows on almost all kinds of soil and topographic types ranging from outcrops of

mountains to muskegs in the boreal forest (Safford et al. 1990). However, the best development and growth of paper birch occurs on deeper well-drained to moderately well-drained soils. Paper birch litter, enriched with calcium, potassium, magnesium, phosphorus and born, has a rapid decomposition rate and contributes largely to the forest floor (Tappeiner and Alm 1975). Paper birch can associate with balsam fir, white spruce, black spruce, aspen, blueberry and green alder. Paper birch can regenerate from sprouts after cutting or fire (Hutnik and Frank 1965). The root system of paper birch is generally shallow, with a bulk of roots in the top 60 cm soil, and taproots do not form (Horsley and Brayton 1971). Paper birch is a shade-intolerant species. In natural succession paper birch usually lasts for one generation then is replaced by more shade-tolerant species (Hutnik and Frank 1965). Paper birch is also a nutrient sensitive species, as seedlings, saplings and trees all have quick responses to fertilizer treatment (Safford 1973; Chapin 1983; Safford 1990).

1.2.2.6 Tamarack (*Larix laricina*)

Tamarack, also called American larch, is a small- to medium-sized deciduous conifer. It grows widely in North America under extremely varied climate conditions. Tamarack tolerates a wide range of soil conditions but most commonly grows on wet to moist organic soils. It also grows fairly well on extremely dry and calcareous soils. Tamarack is a representative tree of peatlands, where it is more abundant than trees of surrounding uplands (Johnston 1990). The typical associates of tamarack include black spruce, jack pine, white spruce, balsam fir, balsam poplar, trembling aspen and red-osier dogwood. When light is not limited, tamarack is one of the fastest growing conifers in boreal forest and on peatlands, and it outgrows any other native conifer (Curtis 1959;

Fowells 1965). The survival of tamarack is poor on shallow soils over limestone. Tamarack has a shallow and spreading root system (Johnston 1990). It is very intolerant of shade, and to survive it must dominate. Tamarack is a pioneer tree on open unburned bogs and burnt organic soils after fire in boreal forest (Rowe 1973; Eyre 1980). On poor sites, the more shade tolerant black spruce usually succeeds tamarack (Fowells 1965).

1.2.2.7 Green alder (*Alnus viridis*)

Green alder is a small tree, with a height of 3 to 12 m. It distributes widely across the cooler parts of the Northern Hemisphere, usually found on sand hills, open forests and edges of wetlands and streams. It has a shallow root system, and can be reproduced by root suckers (Wikipedia 2014). Green alder is light-demanding and fast-growing, and usually grows well on coarse textured sandy to gravel soils. Green alder can be used for afforestation on infertile soils which it enriches by means of its nitrogen-fixing nodules. It was reported that in Alaska, green alder could add 55 lbs of nitrogen per acre per year to the soil (Alberta Environment 2010; Wikipedia 2014). Green alder is semi-shade tolerant, and is a pioneer species after fire that establishes well on burnt bare mineral soils (Alberta Environment 2010).

1.2.2.8 Red osier dogwood (*Cornus stolonifera*)

Red osier dogwood is a medium to tall, fast-growing deciduous shrub that range from the east coast to the west coast of Canada and the northern US states. It can grow on riverbanks and marshes as well as in drier environments such as forest clearings and edges. In boreal forest, red osier dogwood is important as wild life shelter and food source (Davis 2012). Red osier dogwood is usually found on well drained to poorly

drained soils, and grows best on free draining soils with adequate moisture availability (Alberta Environment 2010). For red osier dogwood, high levels of mineral nutrients are needed to maintain vigorous growth. It is tolerant of flooding, and is often one of the first shrubs to invade wet meadows. Red osier dogwood is an early to mid-successional species that is supressed in shade, and is not normally found in the understory of closed canopy forests. Thus, it is usually found in the understory of mixed open forests. It is a semi-fire-tolerant, seed-banking species, and can sprout from surviving roots or stolons and from the base of aerial stems following fire. Red osier dogwood may invade a recently burned area from adjacent unburned areas. The associates of red osier dogwood include paper birch, trembling aspen, and alders (*Alnus* spp.) (Rook 2014a).

1.2.2.9 Blueberry (*Vaccinium myrtilloides*)

Blueberry is a low spreading, deciduous, evergreen shrub growing to 50 cm tall, often in small thickets. This shrub frequently grows in drier, relatively infertile conifer stands, common on acidic soil (pH from 3.0-5.9) in peat bogs, muskegs, peatlands, alpine and mountain meadows, sandy soils in open forests and clearings. It grows best in sandy loam soils, with highest production on well drained acidic soils high in organic matter under ample light conditions (Rogers 1974). Most of blueberry's root system occurs in the top 15 cm of soil profile (Eaton and Patriquin 1988). It is generally tolerant of shade and fire and grows well in open woods. This shrub is often abundant following forest fires or clear-cut logging (Alberta Environment 2010; Rook 2014b). Blueberry is very sensitive to sulfur dioxide pollution. It associates with balsam fir, paper birch, tamarack, white spruce, black spruce and jack pine (Rook 2014b). Blueberry also associates with

the ericoid mycorrhiza fungi (Hambleton et al. 1999; Massicotte et al. 2005) that increase nitrogen uptake in acidic soils (Jans and Vostka 2000).

1.2.2.10 Bearberry (*Arctostaphyllos uva-ursi*)

Bearberry is an evergreen, 5-30-cm-tall shrub. It often forms mats with 50-100 cm long flexible rooting branches (Runesson 2014). The root of bearberry can extend to a depth of 10 to 15 cm (Rook 2014c). It is often a dominant understory species in open pine forests under jack pine, also in the understories of white spruce, black spruce, paper birch, and trembling aspen. Bearberry grows on varied soil textures, although it is commonly found on well-drained soils with relatively low amounts of clay and silt. It frequently grows on sandy soils, shallow, dry and nutrient poor soils. This shrub grows best in high light situations and becomes very rare when shade becomes intense. It is a sprouting species best suited to short fire cycles with low fuel buildup and low fire intensities. Bearberry associates with paper birch, white spruce, black spruce, jack pine and trembling aspen (Rook 2014c).

1.2.2.11 pH tolerance of these species

Many plants can tolerate a wide pH range from 3.5 to 8.5, although the pH optimum for growth is species-dependent (Iles 2001; Larcher 2003). The reported pH tolerance ranges of the species used in the present study are as following:

1) Trembling aspen

    5.3 to 8.4 (Renault et al. 1999)

2) White spruce

    4.7-7.0, possibly higher (Wilde 1966; Sutton 1969; Stiell 1976; Brand et al. 1986; Renault et al. 1998)

6.64 – 10.94 (Maynard et al. 1997)

3) Black spruce

5.26 to 8.41 (Renault and Zwiazek 1997)

4) Jack pine

3.5 (Hardy BBT 1989), 5.3 to 8.4 (Renault et al. 1999)

5.0-7.0 (Wilde 1966)

5) Paper birch

5.0-7.5 (NDSU 2014)

6) Tamarack

4.0-7.5 (South St. Louis Soil and Water Conservation District 2007)

7) Green alder

5-6.5 (Rook 2014d)

4.3 to 6.8 (Alberta Environment 1982), 8.0 (Crocker and Major 1955)

8) Red osier dogwood

C. stolonifera, 5.12 (Renault et al. 1999), 5.3 (Renault et al. 1998), 7.2 - 8.2 (Yarie et al. 1993), 5-7.5 wide variety of soils (Herman et al. 2007)

C. canadensis, 4.5 - 6 (Out Back Nursery 2007)

C. canadensis, 4.3 - 6.8 (Alberta Environment 1982)

9) Blueberry

V. myrtilloides, pH 3-5.9, 4.5 for optimum germination (Rook 2014b)

V. myrtilloides, 4.2 to 5.0 in undisturbed soil (Webster and Innes 1981)

10) Bearberry

Optimum at 4.5-5.5 (Fornari 2014)

1.2.3 High pH and plants

1.2.3.1 Introduction

Soil pH is one of the most important environmental factors affecting plant growth and development. More than 30% of the earth surface is covered by high pH soils (Chen

and Barak 1982) and soils in semi-arid and arid environments have pH > 7 commonly (Marschner 2012). High pH soils can be classed as calcareous and alkaline soils, with pH of calcareous soils ranging from 7.5 to 8.5 and alkaline soils having a pH higher than 8.5 (Marschner 2012). However, some researchers also consider calcareous and alkaline as synonymous. The pH of calcareous soils is determined by the presence of $CaCO_3$, which varies from a few percent to 95% in the upper soil horizons. Typical alkaline soils are common in less arid grassland areas and very dry climates (Chesworth 2008).

Most field crops have a pH optimum of 6.0 - 6.5 (Fageria and Baligar 2003). Plants with optimal growth and survival below and above pH 5 - 7 are named as acidophiles and calciphiles, respectively (Ehrenfeld et al. 2005). High soil pH is capable of directly affecting plant root growth by the presence of high concentrations of [OH^-] (Zieslin and Snir 1989; Kopittke and Menzies 2004), or indirectly affect plant growth by affecting nutrient availability in soil solution (Brady and Weil 1996) and activity of microbial community (Baath et al. 1980; Wang et al. 1985; Nicol et al. 2008). However, most of the earlier research in which pH was a factor focused mainly on the effects of salinity (Renault et al. 1999; Kopittke and Menzies 2005; Youfsi et al. 2007; Calvo-Polanco et al. 2014b), heavy metal toxicity (Bernal and McGrath 1994; Kopittke and Menzies 2004), and nutrient uptake (Dhyr-Jensen and Brix 1996; Jentschke et al. 1998; Kopittke and Menzies 2005).

The extensive effects of pH on plants include ecological, physiological, cellular and molecular levels.

1.2.3.2 Soil pH and plants distribution

Soil pH is an important environmental variable that is related to the distribution and diversity of plant species within terrestrial ecosystem. Differences in plant species distribution along a soil pH gradient are usually referred to as the calcifuges-calcicole gradient (Hayati 1990), as calcifuge species grow on acid soils and calcicole species are mainly found on high pH soils. Roelofs et al. (1996) distinguished three different groups of plant species within a wet heathlands on nutrient-poor, weakly buffered soils in the Netherlands, based on several environmental factors including soil pH. It was also reported that a pygmy forest developed on extremely acidic soils along a narrow coastal land in Northern California (Northup 1995). For a unique pine forest ecosystem in the Rocky Mountains in Colorado, the vegetation is strongly determined by soil pH, as *Vaccinium myrtilus* was found only on acidic soils (pH<5.1) (Ranne 1997). Soil pH is thought to be a significant factor affecting Cerradão (Brazilian woodland savannah) and seasonally dry forest species distribution in the forest-savannah boundary in south-eastern Brazil (Viani et al. 2014). A study in the arctic tundra in Alaska also reported that plant species diversity and composition are directly related to soil pH (Gough 2000). However, it is debatable to what extent the distinction of plant species distribution is simply a matter of soil pH or those of other associated environmental factors that may be associated with pH differences (Rorison 1986; Gough 2000).

1.2.3.3 High soil pH and plant growth

The inhibition effect of alkaline pH on plant growth is particularly prominent in roots, however, different species may respond differently (Bertoni et al. 1992; Tang et al. 1992; Zhang et al. 2013). In an alkaline-sensitive species of *Lupinus*, a combination of

decreases in overall root growth, root surface area, root elongation, root cell division or cell elongation, and cell volume (in root epidermis, outer cortex layer) was observed, while the alkaline-tolerant species of *Pisum* was less affected (Tang et al. 1992; Tang et al. 1993b). A higher and ecological long-term pH treatment (>5.5) for *Pinus pinaster* resulted in decreased root length and elongation, which slowed down with increasing exposure over a period of 27 days, but root initiation was unaffected (Arduini et al. 1998). The formation of lateral roots and root hairs was reported to be inhibited largely by high root zone pH (Ewens and Leigh 1985; Tang et al. 1993c; Canmore-Neumann et al. 1996). This could be explained as the most suitable pH for root development, which was suggested to be around 4 (Takahashi et al. 2003).

1.2.3.4 High soil pH and nutrient availability

High soil pH is associated with several nutrient disorders in plants, because pH is one of the most important factors determining nutrient sorption and dissolution processes in soil (Comerford 2005). Nitrogen limits growth and production of most crop species growing in alkaline soils, due to the effect of soil pH on mineralization processes of organic nitrogen caused by micro-organisms (Marschner 2012). Iron induced chlorosis is a typical symptom of plants growing in calcareous soils (Schinas and Rowell 1977; Mengel 1994), due to the particularly low solubility of Fe under high pH conditions (Boukhalfa and Crumbliss 2002). *Eucalyptus* capable of sequestering Fe was more tolerant of high pH conditions with low Fe availability (James et al. 2002). Plants that are suffering from Fe deficiency at high pH tend to extrude more H^+ from roots, as well as chemical reductants, and reduce Fe at root tips (Nelson 1992). The solubility of uncomplexed Zn and its diffusion coefficients in alkaline soils are extremely low

compared with acidic soils (Melton et al., 1973; Marschner 2012). In crops, zinc deficiency is probably the most widespread micronutrient deficiency on calcareous soils (Graham et al. 1992; Graham 2008). It is well established that Mn availability to plants decreases with increasing soil pH (Parker and Walker 1986). In calcareous soils, the solubility of Mn decreases due to its adsorption on $CaCO_3$, oxidation on MnO_2 surfaces, and precipitation of Mn calcite (Jauregui and Reisenauer 1982). Phosphorus has a maximum solubility at pH 6.5, and its solubility decreases at both lower and higher soil pH (Valentine et al. 2005). In alkaline soils, due to P-fixation to calcium, only relatively little P is available to plants (Brady and Weil 1996). Adsorption of boron to clay minerals increases strongly at pH 6.5 and is highest at pH 9 (Goldberg 1997). However, in alkaline soils, due to lack of leaching or supplied by irrigation water, boron toxicity is more likely than boron deficiency, particularly in sodic soils (Marschner 2012).

1.2.3.5 Apoplastic pH

The plant apoplast is the space outside the symplast, which includes the cell walls, intercellular and xylem-lumen spaces (Canny 1995). The apoplast significantly affects important cellular processes including nutrient uptake (Schroeder et al. 2009), cell elongation (Rayle and Cleland 1992), and water relations (Steudle 2000). The cytosolic pH of plants is about 7.2 while the apoplastic pH has been reported to be usually between 5 and 6.5 (Grignon and Sentenac 1991; Rengel 2002). The form of nitrogen supply (NO_3^- or NH_4^+) can affect apoplastic pH of leaf cells. It has been suggested that NO_3^- nutrition leads to an alkalization while NH_4^+ induces an acidification in the apoplast (Hoffmann et al. 1992; Mengel et al.1994). High apoplastic pH can induce Fe immobilization in leaf apoplast and Fe related leaf chlorosis in plants growing in calcareous soils (Mengel 1994).

The root zone pH can increase apoplastic pH by 1.5 units and disrupt the pH gradient between cytosol and apoplast (Felle and Hanstein 2002). However, plant apoplast can be acidified by H^+-ATPase and it was argued that under alkaline conditions, apoplastic pH can be up to two units lower than bulk solution (Kurdjian and Guern 1989; Peters and Felle 1999). It has been long known that apoplast is first acidified prior to cell elongation, usually in response to auxin (Rayle and Cleland 1992; Cosgrove 1998), which is usually referred as the "acid growth theory" (Taiz 1984; Rayle and Cleland 1992). However, this "acid growth" hypothesis is mainly based on work with shoot tissues (e.g. excised coleoptiles and epicotyls) (Kutschera 1994). Regarding root tissues, cell wall acidification has been implicated in some species (e.g. maize, Evans et al. 1980; Mulkey and Evans 1981), but not others (e.g. barley, O'Neill and Scott 1983). The inhibition effect of alkaline bulk solution on *Lupinus angustifolius* root cell elongation can occur in a short (one hour) time (Tang et al. 1992).

High apoplastic pH may impair plasma membrane integrity and affect transport processes. In rose plants, the leakage of electrolytes from roots at pH 8 increased significantly compared with pH 6 (Zieslin and Snir 1989). The properties of membrane enzymes may also be affected by apoplastic pH. It has been reported that high soil pH can decrease activity of ferric chelate reductase, which may worsen the Fe deficiency problems of plants growing in alkaline soils (Chaney et al. 1972; Mengel et al. 1994).

1.2.3.6 Effects of pH on plant water relations

The inhibition effects of pH on plant water uptake have been extensively reported. High root zone pH may inhibit shoot and root growth (Atwell, 1991; Bertoni et al. 1992; Tang et al. 1993a), and result in poor water uptake, reductions of stomatal conductance

and shoot water potential (Tang et al. 1993a; Tang and Turner 1999; Kamaluddin and Zwiazek 2004). Alkaline soils can worsen the effects of drought stress, leading to reduced plant growth, nutrient uptake and stomatal conductance compared to soils with adequate moisture (Tang and Turner 1999). Alkaline pH conditions (>7) can reduce root water flux (Tang et al. 1993a; Kamaluddin and Zwiazek 2004). These reductions under alkaline conditions may be due to changes in aquaporin (AQP) activity, which extensively mediates the transmembrane pathway for root water transport (see Fig. a1.2) (Steudle and Peterson 1998; Voicu and Zwiazek 2004; Aroca et al. 2006). Decreased AQP activity may occur via pH-dependent structural changes (Törnroth-Horsefield et al. 2006). It was found that acidification below pH 6.6 resulted in reduced AQP-mediated flow compared with higher cytosolic pH (8.3) in purified membrane vesicles (Alleva et al. 2006).

1.2.3.7 pH as a signalling factor

The increase of xylem pH was suggested to serve as a drought stress signal, communicating to the shoot the fact that the roots are in contact with dry soil (Davies and Zhang 1991; Wilkinson and Davies 1997). When plants are under anoxic conditions, cytosolic pH is lowered by 0.1 units to optimize the cellular metabolism under suboptimal oxygen supply, while an increase of apoplastic pH is usually accompanied with cytosolic acidification (Felle 2001). It was demonstrated that the cytosolic pH of some root cap cells of Arabidopsis increased 0.4 units following gravistimulation (Scott and Allen 1999). Low CO_2 concentration induced stomata opening is accompanied by an increase of apoplastic pH (Hedrich et al. 2001). It was also noted that the cytosolic pH of green cells decreased by about 0.3 units temporarily after dark exposure (Felle and Bertl

1986). The cytosol acidifies when plants are invaded by pathogenic microorganisms (bacteria, fungi) (Kuchitsu et al. 1997; Pugin et al. 1997; Felle et al. 2000), but alkalinize following symbiotic signal exchanges (Felle et al. 1996). In general, the change of cytosolic or apoplastic pH appears to be a universal response for plants under different types of environmental stimuli.

1.2.3.8 Techniques to measure apoplastic pH

Yu et al. (2000) reviewed in detail the measurement methods of apoplastic pH in plants. The apoplastic pH can be directly measured in the apoplastic sap. The apoplastic sap can be obtained by a modified Scholander bomb, perfusion, centrifugation, and infiltration/centrifugation method (for details of these methods see review by Yu et al. 2000). However, the drawbacks for these methods include a small volume of fluid for routine pH measurement, contamination by cytoplasmic fluid, and apoplastic pH being affected by water stress induced by pressure dehydration (Hartung et al. 1988; Hartung et al. 1992). A pH-sensitive microelectrode with tiny blunt tip (2-5 μm) can be inserted into apoplast of leaf and root cells for measuring apoplastic pH (Felle 1998; Hanstein and Felle 1999; Pitann et al. 2009). The problem with this method is fabrication of the microelectrode which is very time-consuming and requires experience, as well as the low rate (10%) of successful insertions (Yu et al. 2000). Another widely reported technique for measuring apoplastic pH is the pH-sensitive florescence probe combined with florescence and confocal laser scanning microscopy (Hoffmann and Kosegarten 1995; Kosegarten et al. 1999; Pitann et al. 2009). Briefly, the principle of this technique is loading fluorescent dye and measuring fluorescence intensity at two different wavelengths. At one wavelength, the fluorescence intensity is dependent on pH and at the

other wavelength, it is independent of pH. The apoplastic pH can be inferred from the ratio of the fluorescence excited by two wavelengths (Yu et al. 2000). In recent years, several genetically encoded fluorescence proteins have been expressed in plant cells to detect pH changes in vivo and non-invasively (Miesenböck et al. 1998; Schulte et al. 2006). In contrast to fluorescent dyes, these biosensors can be expressed and targeted to both cytosol and apoplast. Among these fluorescent proteins pHusion and pHluorin GFP (green fluorescence protein) were both proved to be effective in measuring cell wall pH (Gao et al. 2004; Gjetting et al. 2012).

1.2.3.9 Methods to investigate effects of pH on plants

Little research has been conducted concerning the effects of alkaline pH as a single experimental factor on plant growth and physiological performance. In solution culture experiments, the composition of mineral nutrients needs to be carefully considered as it can significantly change solution pH. Due to the unbalanced uptake of nutrients by plants, solution pH can change rapidly. Nitrogen forms can grossly affect nutrient solution pH as uptake of NO_3^- and NH_4^+ results in alkalinisation and acidification, respectively (Fageria 2005). To maintain stable pH with uptake of NO_3^- and NH_4^+, approximately 80-90% of nitrogen should be in the form of NO_3^- (Trelease and Trelease 1933). A version of modified Hoagland's solution contains 12.5% N as NH_4^+, which tends to make more stable solution pH (Epstein 1972).

The most problematic issue in solution culture experiments is maintaining constant solution pH over time. Previous experiments have used several different methods to adjust pH for maintaining constant solution pH, including manual adjustment (Fageria 2005; Siemens and Zwiazek 2011), use of ion exchange buffers (Lahav et al.

1976; Checkai et al. 1987), and buffers such as MES, Tris, or HEPES (Bugbee and Salisbury 1985). However, buffers can impose unknown effects on plant growth and physiological responses (Clark 1982). Kopittke and Menzies (2004, 2005) have developed an automated pH-titration system used in solution culture for high pH studies, which is more effective in controlling solution pH than manual adjustment or using buffers. This system involves a nutrient solution that is free of Cu, Fe, Mn, and Zn, and aerated with CO_2-depleted air to minimize pH and mineral nutrient concentrations between treatments.

However, under field conditions, the physical, chemical and biological factors are more complicated than in solution culture. The physical soil factors including compaction, particle size, parent material, and the microbial factors, such as community component and fungal association, could all affect plant growth under different stress conditions. In a study on the response of *Lupinus spp.* to alkaline lime and Ca in both solution and soil cultures, it was noted that differences in growth response to the applied treatments were dependent on whether plants were treated in solution or soil (Kerley and Huyghe 2001).

1.2.4 Calcium and plants

About 25-30% of the world's land surface is covered by calcareous soils (Wallace et al. 1966), which are characterized by ample Ca supply and pH of 7.5 - 8.5 (Marschner 2012). Usually, Ca can be supplied at high concentrations to plants and Ca toxicity rarely happens. In some mature leaves Ca concentration can reach 10% of dry weight without any toxicity symptoms or any inhibition of plant growth (Marschner 2012). The excessive Ca can be stored as calcium oxalate crystals in the vacuoles of specialized cells called crystal idioblasts for regulating Ca metabolism and metal detoxification (Nakata

2003). It has been reported that as much as 90% of total Ca can be in the form of crystals (Gallaher 1975; Libert and Franceschi 1987). As a relatively low Ca^{2+} concentration is able to induce callose formation, which can block phloem, the mobility of Ca^{2+} in phloem is quite low, thus Ca is mainly delivered by transpiration flow in xylem (Marschner 2012).

The functions of Ca in higher plants can be divided into four major types: a) effects on cell walls; b) effects on membranes; c) effects on enzymes; d) interaction with phytohormones (Bangerth 1979). However, as the development of Ca studies in plants has progressed in time, the effects on enzymes and interactions with phytohormones are usually presently regarded as signaling factors in plant metabolism (Dodd et al. 2010; Bickerton and Pittman 2012).

1.2.4.1 Calcium and cell wall

Calcium plays a significant role in stabilizing cell walls as it binds as Ca-pectate in the middle lamella and acts as a bridge at cross-linking pectin chains (Hepler 2005; Lee and Woolhouse 2006; Marschner 2012). In the cell elongation process, according to the acid growth theory, plant cell apoplast is first acidified and the Ca ions at the cell walls are replaced by protons. This makes the cell wall more plastic and facilitates cell elongation (Bangerth 1979; Rayle and Cleland 1992; Hepler 2005). Therefore, Ca and protons appear to have opposing roles in cell extension. In fact, the ability of high concentrations of Ca to inhibit plant cell elongation has been known for a long time (Cooil and Bonner 1957; Adamson and Adamson 1958). However, it has been suggested that Ca inhibits plant cell growth by interfering with the biochemical wall-loosening processes, as Ca can only decrease cell wall plasticity and elasticity with the *in vivo*

method, and not *in vitro* (Cleland and Rayle 1977). The cell wall protein expansin might be involved in this process (Cosgrove 1998).

1.2.4.2 Calcium and cell membrane

Calcium plays a significant role in maintaining membrane structure and stability as well as cell integrity. At membrane surfaces, Ca ions bridge phosphate and carboxylate groups of phospholipids and proteins (Legge et al. 1982). Calcium ions link adjacent phosphatidyl-serine head groups, binding the phospholipids together in certain areas and making these areas more rigid than surrounding areas (Grant 1983). The cells of severely Ca-deficient plant tissues show increased leakage of low molecular weight solutes, disintegration of membrane structures and loss of cell compartmentation (Marschner 2012). The effects of Ca on maintaining membrane integrity and stability are highly beneficial for plants under stress conditions. Addition of Ca can ameliorate stress due to low pH and aluminium toxicity in wheat seedlings, probably by preventing influx of toxic ions into the cytosol (Kinraide and Parker 1987). Adequate concentrations of Ca ions in the soil can improve the K^+/Na^+ selectivity by favoring uptake of K^+ at the expense of Na^+ (Shabala et al. 2006). The negative effects of salinity on plant water relations were also prominently mitigated by treatment with Ca and this effect was especially prominent at high temperature (Cabañero et al. 2004).

1.2.4.3 Calcium as a signalling factor

Due to the relatively high concentrations of phosphorus in cytosol, the free cytosolic Ca^{2+} must be maintained at low levels, otherwise precipitation of Ca ions can take place (Sanders et al. 1999). The free cytosolic Ca^{2+} concentration is approximately

0.1 μM, which is much lower than that of apoplast and storage compartments, such as vacuole and endoplasmic reticulum, where the Ca^{2+} concentration ranges 0.1 to 10 mM (Hepler 2005). This submicromolar cytosolic Ca^{2+} concentration is maintained by the Ca^{2+}-ATPase and H^{+}/Ca^{2+}-antiporters (Sze et al. 2000; Hirschi 2001). Environmental stimuli or phytohormones can induce transient changes of cytosolic free Ca^{2+} concentration which acts as a secondary messenger to trigger plant physiological responses (Hepler 2005; Moran 2007; Dodd et al. 2010; Kim et al. 2010). These changes of Ca^{2+} are decoded by different types of Ca^{2+} sensor proteins (e.g., CaM, CMLs, CDPKs, CBL/CIPKs) (Bose et al. 2011), as the conformation and catalytic activity of these proteins change by Ca^{2+} binding (White and Broadley 2003). Actually, the rapid change of cytosolic Ca^{2+} concentration is a universal feature of signaling network in plants under almost all biotic and abiotic stresses, including soil acidity, salinity, anoxia, ozone, drought, osmotic, oxidative, heat, and cold stresses, gaseous pollutants, mechanical cues, light, plant hormones, pathogens, bacterial, and fungal signals (Bose et al. 2011). The opening and closing of aquaporins is also affected by the cytosolic concentration of Ca^{2+} (Cabanero et al. 2006). Due to the versatile signaling features of Ca^{2+} and the steep gradient of cytosolic and apoplastic Ca^{2+} concentration, the integrity of plasma membrane and normal functions of Ca related membrane proteins must be maintained to prevent excessive exogenous Ca^{2+} influx into cytosol.

1.2.5 Phosphorus and plants

Phosphorus is often among the most limiting nutrients to plants in both low and high pH soils (Jing et al. 2010). In low pH soils the low availability of P is mainly due to adsorption by iron and aluminum oxides and hydroxides (Shen et al. 2011). In alkaline

soils, phosphate usually precipitates with Ca, generating dicalcium phosphate (DCP). DCP can be transformed into more stable forms such as octocalcium phosphate and hydroxyapatite (HAP), which are less available to plants (Arai and Sparks 2007). Therefore, the most optimum soil pH for P uptake in most of the land plants is usually around 6.5 (Valentine et al. 2006). In soil solution, the concentrations of available inorganic phosphorus (Pi) seldom exceed 10 μM (Bieleski 1973), which is much lower than in plant tissues where the Pi concentration usually ranges between 5 and 20 mM (Raghothama 1999). In addition, the diffusion rate of Pi in soils is extremely low, with a diffusion coefficient of 10^{-12} to 10^{-15} m s^{-1} (Schachtman et al. 1998). Phosphorus toxicity rarely happens in plants because when the roots are supplied with excessive P, plants can down-regulate their Pi transporters (Dong et al. 1999).

Phosphorus in plants is a component of numerous essential molecules including nucleic acids (DNA and RNA). Phosphorus is also a structural element of phospholipids and plays a significant role in energy conversions in plant cells. Phosphate esters and energy-rich phosphates are intermediates in metabolic pathways of biosynthesis and degradation, and their functions and formation can significantly affect energy metabolism (Marschner 2012). Because of the negative plasma membrane potential created by cytosolic and apoplastic pH gradients, phosphate anions are co-transported with H^+ into cytosol (Ullrich-Eberius et al. 1984).

In P-deficient plants the most obvious effects are reductions of leaf expansion and number of leaves (Fredeen et al. 1989; Lynch et al. 1991). Under P-deficiency, shoot growth is usually more inhibited than root growth, resulting in a decrease of shoot/root ratio (Marschner 2012). It has been suggested that in P-deficient plants a decrease of root

hydraulic conductivity may be caused by reduced expression of aquaporin genes (Clarkson et al. 2000). Plant responses to P deficiency include changing root architecture including root morphology, topology, and distribution patterns (Shen et al. 2011). In some species P deficiency induces root cluster (proteoid root) formation, which exude high amounts of organic acids to acidify soil and chelate metal ions around the roots (Marschner 1995). The root morphological changes upon P starvation are suggested to be an outcome of complex interactions between Pi and other nutrients, especially Fe (Svistoonoff et al. 2007; Ward et al. 2008). In plants, possibly two phosphate transport systems exist, and when P was not deficient in growth medium, the low affinity system was activated (Schachtman et al. 1998). It was reported the H^+-ATPase activity of rice roots increased under P deficiency (Zhang et al. 2011). Rhizosphere acidification caused by plant roots activities may decrease soil pH by 2 to 3 units (Marschner 1995), which can significantly increase P availability (Moorby et al. 1985; Neumann and Römheld 1999). Under P starvation, 90% of the total P acquired by plants might be absorbed by root hairs (Raghothama 1999). Colonization by mycorrhizal fungi is a typical response in plants suffering from P deficiency, which can increases P uptake by 3 to 5 times compared with nonmycorrhizal roots (Smith and Read 2010).

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Chapter 2*♣

Growth and physiological responses of trembling aspen (*Populus tremuloides*), white spruce (*Picea glauca*) and tamarack (*Larix laricina*) seedlings to root zone pH

2.1 Introduction

Soil pH is among the major environmental factors affecting plant growth, largely due to its effect on the availability of essential nutrients and the accumulation of potentially phytotoxic compounds in the soil (Brady and Weil 1999). Although the optimum range of soil pH for growth and the tolerance of pH extremes widely vary among plant species, the pH tolerance mechanisms in plants are poorly understood (Rengel 2002).

In northeastern Alberta, Canada, oil sands mining disturbs vast areas of northern boreal ecosystems which need to be restored after mine closure. In some of the oil sands reconstructed landforms, soil pH is elevated due to the presence of saline-sodic overburden and the effects of the reclaimed mine tailings since NaOH is used during the bitumen extraction process from the oil sands (Howat 2000). In these areas, soil pH

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♣I also examined jack pine in this study. The results of jack pine of this study and study 2 (chapter 3) were combined and published. Parts of the results of this study were used in the published article, and the other results are in appendix 2.

commonly ranges from 7.0 to 8.5, while pH of the soil in the native boreal ecosystems in the area is typically lower than 6 (Howat 2000). The high soil pH poses serious challenges to the revegetation process since only native species from the local genetic sources can be used for mine reclamation and these plants are adapted to lower pH soils (Alberta Environment 2010).

The effects of high pH may be quite complex and involve different processes in plants. Most commonly, high soil pH is associated with reduced availability of Fe, Mn, P, and Zn (Yang et al. 1994; Valentine et al. 2006). High soil pH can also reduce root water flux (Tang et al. 1993; Kamaluddin and Zwiazek 2004; Siemens and Zwiazek 2011). This reduction is likely due to the effect of pH on the function of aquaporins (Tournaire-Roux et al. 2003; Kamaluddin and Zwiazek 2004; Tornroth-Horsefield et al. 2006; Siemens and Zwiazek 2011) and the extent of it may vary among plant species (Calvo-Polanco et al. 2009). Reductions in nutrient and water uptake by high pH may lead to stomatal closure (Tang et al. 1993; Kamaluddin and Zwiazek 2004), decreased shoot water potential (Tang et al. 1993) and, consequently, reduced growth (Bertoni et al. 1992; Tang et al. 1992).

Since plant responses to high root zone pH vary among species, some plants appear to have effective mechanisms helping them function under high pH conditions. This difference in plant responses offers an opportunity to learn about the high pH tolerance mechanisms using controlled-environment studies. The main objective of the present study was to investigate the effects of root zone pH and examine the processes which could help explain the differences in pH responses of the dominant northern boreal forest tree species: trembling aspen (*Populus tremuloides*), white spruce (*Picea glauca*), and tamarack (*Larix laricina*). These tree species are among the dominant trees in the

boreal forest in northern Alberta, Canada, and are commonly used for oil sands revegetation. Earlier reports indicated that white spruce could tolerate high soil pH relatively well and grow in soil with pH ranging from approximately 6.6 to 11 (Maynard et al. 1997). The ranges of soil pH tolerance for trembling aspen and tamarack were reported to be 5.3-8.4 (Renault et al. 1999) and 4.0-7.5 (South St. Louis Soil and Water Conservation District 2007). However, there have been no direct comparisons of the pH tolerance among these tree species using the same parameters and under the same environmental conditions. Since the uptake of mineral nutrients is likely to be among the key factors in plant responses to high pH (Marschner 2012), I hypothesized that the differences in high pH tolerance between white spruce, tamarack and trembling aspen are largely due to different nutrient uptake and allocation mechanisms. To minimize the effects of potentially complex pH interactions, high buffering capacity and other factors in real soils, I used an aerated solution culture system to examine nutrient uptake, plant growth, chlorophyll concentrations, gas exchange, and water relations in tree seedlings grown under controlled-environment conditions and subjected to the root zone pH ranging from 5.0 to 9.0.

2.2 Materials and methods

2.2.1 Plant material and growth conditions

One-year-old container-grown (415D styroblocks™, Beaver Plastics, Acheson, AB, Canada) trembling aspen (*Populus tremuloides* Michx.), white spruce [*Picea glauca* (Moench) Voss], and tamarack [*Larix laricina* (Du Roi) K. Koch] dormant seedlings were obtained from Boreal Horticultural Services Ltd., Bonnyville, Alberta, Canada. Seedlings were stored for two weeks at 4 ^{o}C in the dark prior to the experiments. For the

experiments, roots of seedlings were washed free of the potting medium and placed in aerated mineral solution culture in a controlled environment growth room. Environmental conditions in the growth room were maintained at 22/18^{o}C (day/night) temperature, 65±10% relative humidity, and 16-h photoperiod with 300 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at the top of the seedlings provided by full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada).

The solution culture set-up consisted of three 30 L opaque plastic tubs with Styrofoam lids. Into each lid, 20 x 3.8 cm holes were cut, so that seedling roots could be slipped into the nutrient solution through the lid. There were 6 seedlings per species in each tub for a total of 18 plants per treatment. Foam plugs were fitted around the stems, and inserted into the holes to hold the stems in place while the roots were immersed in solution, with the stems protruding through the lid. All tubs had spouts installed into their sides to facilitate drainage and circulation of nutrient solution via plastic tubing. Each tub was directly connected through the PVC tubing to a 120 L pail filled with 25% modified Hoagland's solution (Epstein 1972). In each pail, a circulating pump (Model 9.5 950GPH, Danner MFG Inc., New York, USA) and a pH electrode were immersed. The pump continuously circulated the solution between the tubs and the pail and the pH electrode was connected to a pH controller (PHCN-70, Omega Engineering Inc., Laval, QC, Canada) for a continuous control of pH. The concentration of dissolved O_2 in containers with seedlings was monitored with the oxygen electrode (Dissolved oxygen meter YSI 5000, YSI Inc., Yellow Springs, Ohio, USA) and measured no less than 6 mg L^{-1} throughout the experiment.

2.2.2 Treatments

The seedlings had been placed in modified Hoagland's nutrient solution (pH 5.0) for two weeks to break dormancy before the start of pH treatments. The pH of nutrient solution was then adjusted according to the treatment levels at 5.0, 6.0, 7.0, 7.5, 8.0, 8.5 and 9.0. An electronic valve (Model 8260G071 120/60 ASCO Valve, Inc., Florham Park, NJ, USA) was controlled by the pH controller and connected to a 5% (w/v) KOH or 1% (v/v) H_2SO_4 solution container. A plastic ball valve (Model R-01377-84, Cole-Parmer Canada Inc., Montreal, QC, Canada) was connected to the electronic valve to automatically release KOH or H_2SO_4 to the nutrient solution to maintain the preset pH levels. The pH value was continuously measured with the Orion 9106 BNWP gel-filled combination pH electrode (Thermo Scientific, Rochester, NY) and recorded by a computer. The pH treatments lasted for 8 weeks and the solution was replaced every two weeks. Over the entire experiment and in all of the pH treatments, the pH fluctuations were less than ± 0.2 of the preset values.

2.2.3 Elemental analysis of nutrient solution

To determine the effects of pH on the solubility of essential elements, 1 L of 25% modified Hoagland's solution was prepared and adjusted to pH 5.0, 6.0, 7.0, 7.5, 8.0, 8.5 and 9.0 with 5% KOH (w/v) or 1% H_2SO_4 (v/v). For each pH treatment, four nutrient solution samples, at 20 ml each, were filtered by 0.45μm Polyvinyl Difluoride (PVDF) syringe-driven filter unit (EMD Millipore Corporation, Billerica, MA, USA) to determine the concentrations of essential elements that remained soluble. The measurements were carried out using the inductively coupled plasma mass spectrometry (ICP-MS) (Zarcinas et al. 1987) in the Radiogenic Isotope Facility of the University of Alberta.

2.2.4 Dry weights and leaf chlorophyll concentrations

Shoot and root dry weights were determined for all of the seedlings from each pH treatment (n =18). Roots and stems were dried in an oven at 70^{o}C for 72 h after leaves were separated from the stems. The leaves were divided into young and old leaves, as explained below, immediately placed in an ultra-low temperature freezer at -80^{o}C, and freeze-dried for 72 h. To determine shoot dry weights, the dry weights of all leaves and stems from each plant were added.

Leaf chlorophyll-a and chlorophyll-b concentrations were determined in young and old leaves in six randomly selected seedlings per treatment (n = 6). Fully-expanded leaves grown before the onset of pH treatments were regarded as old leaves, while the young leaves were those which started expanding after the onset of pH treatments and were close to the shoot tips. The leaves were frozen, freeze-dried and pulverized with a pestle and mortar. Chlorophyll was extracted from pulverized leaf samples (10 mg dry weight) with 8 ml dimethyl sulfoxide (DMSO) at 65^{o}C for 22 h. After filtering, chlorophyll concentrations were measured in DMSO extracts with a spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden), at 648 nm and 665 nm for chlorophyll-a and chlorophyll-b. Total chlorophyll concentration was calculated using the Arnon's equation (Sestak et al. 1971).

2.2.5 Gas exchange and shoot water potential

After 8 weeks of pH treatments, six seedlings per species (n = 6) were randomly taken from each pH treatment for the measurements of gas exchange and shoot water potentials. Net photosynthesis rate (Pn) and transpiration (E) rates were measured in the upper, fully developed leaves using an infrared gas analyzer (LCA-4, Analytical Development Company Ltd., Hertfordshire, UK) with an auxiliary LED bulb (400 μmol

m^{-2} s^{-1} PPFD) as previously described (Voicu et al. 2008). For conifers, about 5-cm distal part of a branch with needles was inserted in the sample chamber. To determine needle areas in conifer seedlings, the needles were detached from stems and scanned. Needle areas were calculated using the Sigmascan Pro 5.0 computer software (Systat Software, San Jose, CA).

Shoot water potential (ψ_w) measurements were conducted using a Scholander-type pressure chamber (PMS instruments, Corvallis, OR, USA) in the distal 15-cm shoot segments as previously described (Wan et al. 1999).

2.2.6 Elemental concentrations in old and young leaves

Leaf samples (0.2 g dry weight) were digested with 10 ml 70% HNO_3 and diluted with water to 40 ml. The samples were then analyzed by ICP-MS in the Radiogenic Isotope Facility of the University of Alberta for the concentrations of Fe, Mn, Zn, K, Ca, and P (Zarcinas et al. 1987).

2.2.7 Statistical analysis

All data were analyzed by SAS GLM model (Version 9.2, SAS Institute Inc., Cary, NC) to determine statistically significant ($p \leq 0.05$) differences between treatments. The data that did not meet the ANOVA assumptions of normality of distribution and homogeneity of variance were transformed with a log10 function. The transformed means and their standard errors were back transformed for their representation in figures. Comparisons between different treatment means were conducted using Student-Newman-Keuls (SNK) test.

2.3 Results

2.3.1 Elemental analysis of nutrient solution

Increasing the pH of 25% Hoagland's solution resulted in precipitation of several essential elements. Concentrations of Zn were drastically reduced at pH 7.5 and higher and those of Mn at pH 8.0 and higher (Table 2.1). The concentrations of soluble Ca and P were also reduced at pH 8.5 and 9.0 (Table 2.1). Since KOH was used to adjust solution pH, the concentration of K increased with increasing pH (Table 2.1). Concentrations of other measured elements in the 25% Hoagland's solution were relatively little affected by pH treatments (Table 2.1).

2.3.2 Plant dry weights and chlorophyll concentrations

Total dry weights of aspen and tamarack were sharply reduced at pH 7 and higher (Fig. 2.1a,e). The pH treatments had little effect on root and shoot dry weights in white spruce (Fig. 2.1c). In aspen, shoot to root dry weight ratios were significantly reduced at pH 8.0 and higher compared with the low pH treatments (Fig. 2.1b).

Chlorophyll concentrations in old and young leaves of aspen were drastically reduced at pH 7 and higher, and the reductions were greater in young leaves (Fig. 2.2a). Moderate reductions in chlorophyll concentrations were observed with increasing pH, starting at pH 6 in young and old needles of white spruce (Fig. 2.2c). With tamarack, reductions in chlorophyll concentrations were also observed at pH 6 and higher in old and young needles and the effects were more pronounced at the pH range from 8 to 9 (Fig. 2.2e). Overall, in aspen and tamarack, the decrease in total chlorophyll concentrations with increasing pH appeared to be largely related to the reductions in chlorophyll-a (Fig. 2.2b,f), while the chlorophyll a:b ratios were similar across the studied pH in white

spruce (Fig. 2.2d).

2.3.3 Gas exchange and shoot water potential

In aspen, the highest net photosynthetic rates were measured at the lowest pH (Fig. 2.3a). Significant reductions in net photosynthesis occurred at pH 7.5 - 9 (Fig. 2.3a). In white spruce, there were no significant differences in net photosynthesis across the studied pH range of 5 to 9 (Fig. 2.3c). In tamarack, a significant decrease in net photosynthetic rate occurred from pH 6 to 9 (Fig. 2.3e).

In all of the three species, transpiration rates decreased starting at pH 6 (Fig. 2.3b,d,f). The reduction was greater in white spruce compared with aspen and tamarack (Fig. 2.3b,d,f).

The pH treatments differently affected shoot water potentials in the three plant species studied. In aspen, shoot water potentials were significantly decreased at pH 8.5 and 9.0 compared with the lower pH treatments while in tamarack a general increasing trend (values becoming less negative) was observed for shoot water potentials with increasing pH (Fig. 2.4). In white spruce, there was no statistically significant effect of pH on shoot water potentials (Fig. 2.4).

2.3.4 Elemental concentrations in old and young leaves

In both old and young leaves of aspen, the concentrations of P, Ca, Fe, Mn and Zn all decreased when the roots were exposed to high pH (Fig. 2.5a). Concentrations of P, Ca and Zn decreased from pH 8.0, while Mn decreased from pH 7.5 and Fe from pH 7.0 (Fig. 2.5a). For Fe, Ca and Mn, the reduction was more prominent in young leaves especially at pH 8.0 and higher (Fig. 2.5a). For white spruce, the concentrations of all of

these five elements decreased at high pH in young needles, while only Ca and Mn decreased in old needles (Fig. 2.5b). In tamarack, there were significant reductions with increasing pH observed for all of the five elements except for Fe which showed similar Fe concentrations at all pH levels examined (Fig. 2.5c). There was also a large decrease in the concentration of Mn in old and young needles of tamarack in pH treatments of 6.0 - 9.0 compared with pH 5.0 (Fig. 2.5c).

2.4 Discussion

Since the objective of the study was to examine plant responses to pH, I used a relatively simple solution culture system to control pH in the root zone. While this system offers the benefits of minimizing complex biological and chemical interactions with pH and makes it possible to precisely control pH in the root zone, it cannot fully represent plant responses to changes in the pH of the soil. Similarly to other controlled-environment studies, the simplicity of the system may not take into account the complex soil dynamics and the potential effects of factors such as rhizosphere microorganisms (Calvo-Polanco et al. 2009; Siemens and Zwiazek 2011), soil structure (Calvo-Polanco et al. 2008) and possible differences in root structure (Wan and Zwiazek 2001) in affecting plant responses to pH.

Relatively stable pH of treatment solutions which was maintained during the experiment allowed the experimental results to be related to treatment effects. As expected, at high pH treatments, the concentrations of divalent cations were reduced in the 25% Hoagland's solution (Table 2.1). There was also a severe reduction in soluble Fe at the highest pH of 9.0. The availability of the above elements has been reported frequently to be reduced in alkaline soils (Srivastava and Sethi 1981; Parker and Walker

1986; Russell 2008; Marschner 2012). However, since in this study, Fe was provided in the chelated form of FeEDTA, which is not normally present in this form in the soil, Fe availability in high pH soils may be actually lower (Marschner 2012).

The effects of pH on growth parameters varied with tree species. The total dry weight of aspen and tamarack seedlings was reduced at pH 7.0 and higher compared with the lower root zone pH. This growth reduction at high pH was accompanied by a decrease in net photosynthetic and transpiration rates. In white spruce, there was no statistically significant effect of pH on seedling dry weights and on net photosynthesis. However, there was a significant reduction in transpiration rates, indicating that the water use efficiency of white spruce increased in high pH treatments. The reductions in transpiration rates had no apparent effect on shoot water potentials in white spruce, but were accompanied by either a decrease or an increase in shoot water potentials of tamarack and aspen, respectively. The decrease in shoot water potentials in aspen at pH 8.5 and 9.0 could also represent a possible decrease in osmotic potentials due to greater K uptake from the solution compared with white spruce and tamarack. Also, interestingly, although the leaf chlorophyll reductions at high pH were more pronounced in tamarack and aspen compared with white spruce, needle chlorophyl concentrations in white spruce were also higher at pH 5.0 compared with higher pH. The effect on chlorophyll, combined with transpiration reductions at high pH in white spruce, suggest that longer-term effects of high pH on growth in white spruce can also be expected.

For nutrient uptake, plant roots need to maintain a proton gradient between the cytosol and apoplast with the pH of the cytosol commonly measuring about 7.2 and that of the apoplast between 5.0 and 6.5 (Grignon and Sentenac 1991; Rengel 2002). For the

plants which are more tolerant of high pH, including white spruce, the ability to maintain a pH gradient when subjected to high root zone pH, could offer an explanation for their higher pH tolerance. It was reported that high OH^- concentration can by itself limit root growth (Kopittke and Menzies 2004). In the present study, white spruce did not develop obvious nutrient deficiency symptoms, and its dry weight was only slightly reduced by high pH (Fig. 2.1c). It could be speculated that white spruce may have a mechanism to maintain the pH gradient, perhaps through the enhanced plasma membrane H^+-ATPase activity. Therefore pH in the apoplastic space in the roots could be actually lower than in the external solution. It is possible that maintenace of the proton gradient may be easier in real soil environments than in solution culture because plant roots interact with a localized rhizosphere in the soil rather than the bulk solution of solution culture. However, additional studies carried out in plants grown in the soil would be required to confirm this hypothesis.

There were similarities in the dry weight and leaf chlorophyll concentrations patterns in seedlings in responses to pH. Also, the effects on chlorophyll concentration were more pronounced in young compared with old leaves. These results suggest that leaf chlorosis observed in many plants at high pH was not likely due to deficiencies caused by mobile essential elements such as magnesium. The effect of pH on chlorophyll concentrations in young leaves and the resulting difference in chlorophyll concentrations between young leaves and old leaves were especially noticable in aspen. As deciduous trees, aspen and tamarack seasonally shed their leaves and, therefore, they may suffer more severe deficiency of the less mobile elements such as Fe under high pH conditions in the long run. In both aspen and tamarack, especially in young leaves, the ratio of

chlorophyll-a to chlorophyll-b decreased by several-fold at the pH higher than 7, which may indicate that possible differences in the effects of high pH on the synthesis of chlorophyll-a compared with chlorophyll-b.

Net photosynthetic rates in aspen were significantly reduced starting at pH 7.5 and the largest decline in photosynthesis was between pH 7.0 and 7.5. However, the leaf cholorophyll concentrations in aspen showed the greatest decline between pH 6.0 and 7.0 and there was no further significant decline from pH 7.0 to pH 7.5 in older leaves. Therefore, it appears that leaf chlorophyll concentrations were not a major determinant of the decline in net photosynthesis of aspen at this pH. In tamarack, the highest net photosynthesis was measured at pH 5.0 with significant decreases starting at pH 6.0. Similar to aspen, the decreases in net photosynthesis in tamarack could not be simply explained by the decreases in leaf chlorophyll concentration, but in contrast to aspen, they were better reflected by decreases in transpiration rates.

Decreases in transpiration rates at high pH were observed in all three of the studied tree species suggesting that high pH affected plant water balance. Shoot water potentials responded differently to high pH depending on the plant species. The decrease in water potentials in aspen at high pH that occurred in spite of the decrease in transpiration rates could be partly explained by a possible higher uptake of K from solution culture compared with white spruce and tamarack. However, differences in K uptake cannot explain the increase in shoot water potentials in tamarack at high pH. It appears that the effectiveness of stomatal control in plants subjected to high root zone pH varies between the tree species. It is also conceivable that pH may have differently affected root water uptake and root hydraulic conductivity in the different plants. Since

root water transport is largely controlled by the cell-to-cell pathway involving aquaporins (Aroca et al. 2006; Lee et al. 2010), and aquaporins are sensitive to changes in pH (Tournaire-Roux et al. 2003; Kamaluddin and Zwiazek 2004; Törnroth-Horsefield et al. 2006; Siemens and Zwiazek 2011), a reduction in the aquaporin-mediated water transport in pH-sensitive plants could have an immediate impact on plant water balance.

I focused tissue analysis on the essential elements that may be limiting in boreal forest soils and did not analyze N since this was the subject of another study. However, under the same experimental conditions as those used in the present experiment, I did not find pH-related differences in N uptake between tamarack, white spruce and aspen.

Due to high phloem mobility (Marschner 2012) and high requirement for growing tissues, P concentrations in young leaves of aspen and tamarack were higher than in old leaves. Aspen had the highest P concentration at pH 7.5 in young leaves, which may also reflect the biomass dilution effect at the lower pH, as seedling dry weight was higher below pH 7.5.

Since even low concentration of Ca can strongly enhance callose formation which can block the pholem transport (Kauss 1987), the mobility of Ca in phloem is quite low (Marschner 2012). Thus, in all three of the studied species, Ca concentrations were lower in young leaves compared with old leaves, especially at high pH conditions.

Plants growing in high pH soils often develop leaf chlorosis as a result of Fe deficiencies (Mengel and Geurtzen 1986). In this study, the effect of high pH on foliar Fe concentrations was more pronounced in aspen compared with white spruce and tamarack. Interestingly, the decrease in Fe concentrations in aspen was greater in young, compared

with old leaves. On the other hand, a decrease in Fe concentrations was measured in old needles of tamarack at pH 8.0 - 9.0 compared with lower pH treatments, but there was no significant effect of pH on Fe concentrations in young needles of tamarack. In white spruce, none of the pH treatments had a significant effect on Fe concentrations in old needles, but Fe reductions were measured in young needles at high pH. Fe is regarded as an intermediate mobile element in plants (Marschner 2012). Our study suggests that under high pH conditions, tamarack may be able to translocate higher amounts of Fe from old to young leaf tissues compared with aspen and white spruce. This may reflect differences in habitats and the resulting adaptations between the trees species. The differences in Fe foliar concentrations of the different plant species probably also reflect reduction processes during Fe uptake and assimilation in plants. This may also be caused by an inhibition of ferric chelate reductase, which is required for Fe uptake in plants and which has been reported to have a decreased activity at high soil pH (Chaney et al. 1972; Mengel 1994). It should be noted that the solubility of chelated Fe in Hoagland's nutrient solution was not significantly altered by pH as high as 8.5, which likely moderated the effects that could be expected in the real soil environment.

Manganese is also a largely immobile element (Marschner 2012) with reduced plant uptake at high pH. However, in tamarack, Mn concentration was higher in young leaves than in old leaves suggesting a retranslocation from older tissues.

Similarly to Fe, Zn is considered to have an intermediate mobility in plants (Marschner 2012) and its concentration in aspen and white spruce was similar in old and young leaves. However, in tamarack, the concentration of Zn was higher in young leaves compared with old leaves suggesting its mobility and possible translocation from older

tissues. The patterns of distribution of elements in leaves also depend on the intensity of transpiration stream and possible differences in transpiration rates between young and old leaves (Marschner 2012). On the other hand leaf transport of other elements should be similarly affected by the transpiration.

In conclusion, in the controlled-environment study, white spruce showed greater tolerance to eight weeks of high pH treatments compared with aspen and tamarack. However, the observed reductions in nutrient foliar concentrations are likely to affect growth in the longer term in all three tree species used in this study. High net photosynthesis rates and biomass production in white spruce under high pH conditions were likely possible due to an increase in photosynthetic water use efficiency, following a reduction in transpiration rates. Although differences were noted between the species in leaf chlorophyll concentrations and the uptake and distribution of P, Ca, Fe Zn, and Mn, there was no clear relationship between their concentrations and plant growth and photosynthesis. The high pH stress likely involves root water transport processes which may be responsible for large decreases in transpiration rates and, consequently, plant water balance. The results also confirm that high root zone pH can affect water balance in some plant species. Tamarack demonstrated greater capacity to translocate Fe, Mn, and Zn under high pH conditions than aspen and white spruce. Although the uptake and assimilation of essential elements such as Fe and Mn may contribute to plant tolerance of high soil pH, I did not find a direct relationship between growth and foliar nutrient concentrations that would explain the observed differences in growth. However, since the solution culture conditions may not be fully representative of soil conditions, more

research also needs to be carried out with plants growing in soils to learn about the potential effects of root environment on the responses of plants to pH.

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2.6 Tables

Table 2. 1 Concentrations of selected essential elements remaining soluble in 25% Hoagland's solution at different pH. The values are means (n=4) ± SE.

	B (μM)	Mg (mM)	P (mM)	K (mM)	Ca (mM)	Fe (μM)	Mn (μM)	Cu (μM)	Zn (μM)	Mo (μM)
pH 5.0	0.11± 0.0028	9.31± 0.1466	21.28± 1.7142	74.69± 0.0542	50.74± 0.6593	0.49± 0.0770	0.047± 0.00070	0.016± 0.0039	0.060± 0.0018	0.016± 0.00058
pH 6.0	0.11± 0.0029	9.59± 0.1704	21.16± 1.5961	79.96± 0.6019	51.16± 0.6440	0.48± 0.0818	0.047± 0.00086	0.015± 0.0041	0.058± 0.0016	0.016± 0.00070
pH 7.0	0.11± 0.0014	9.53± 0.2257	21.09± 1.7005	94.28± 1.1700	50.87± 0.4879	0.46± 0.0959	0.046± 0.00101	0.012± 0.0027	0.048± 0.0006	0.016± 0.00067
pH 7.5	0.11± 0.0025	9.30± 0.2385	20.86± 1.3944	98.15± 0.5862	51.00± 0.6164	0.47± 0.0831	0.043± 0.00151	0.007± 0.0014	0.012± 0.0009	0.016± 0.00073
pH 8.0	0.10± 0.0044	8.91± 0.2969	17.84± 0.4832	102.99± 2.7335	45.38± 1.0258	0.45± 0.0803	0.018± 0.00594	0.006± 0.0009	0.004± 0.0007	0.017± 0.00059
pH 8.5	0.11± 0.0037	8.55± 0.1446	10.99± 0.2179	115.74± 2.8332	34.22± 2.5366	0.41± 0.0585	0.003± 0.00056	0.006± 0.0010	0.005± 0.0007	0.017± 0.00066
pH 9.0	0.11± 0.0028	7.99± 0.1581	5.54± 0.1348	128.53± 3.7802	25.25± 2.3827	0.28± 0.0737	0.001± 0.00008	0.005± 0.0009	0.004± 0.0007	0.016± 0.00068

2.7 Figures

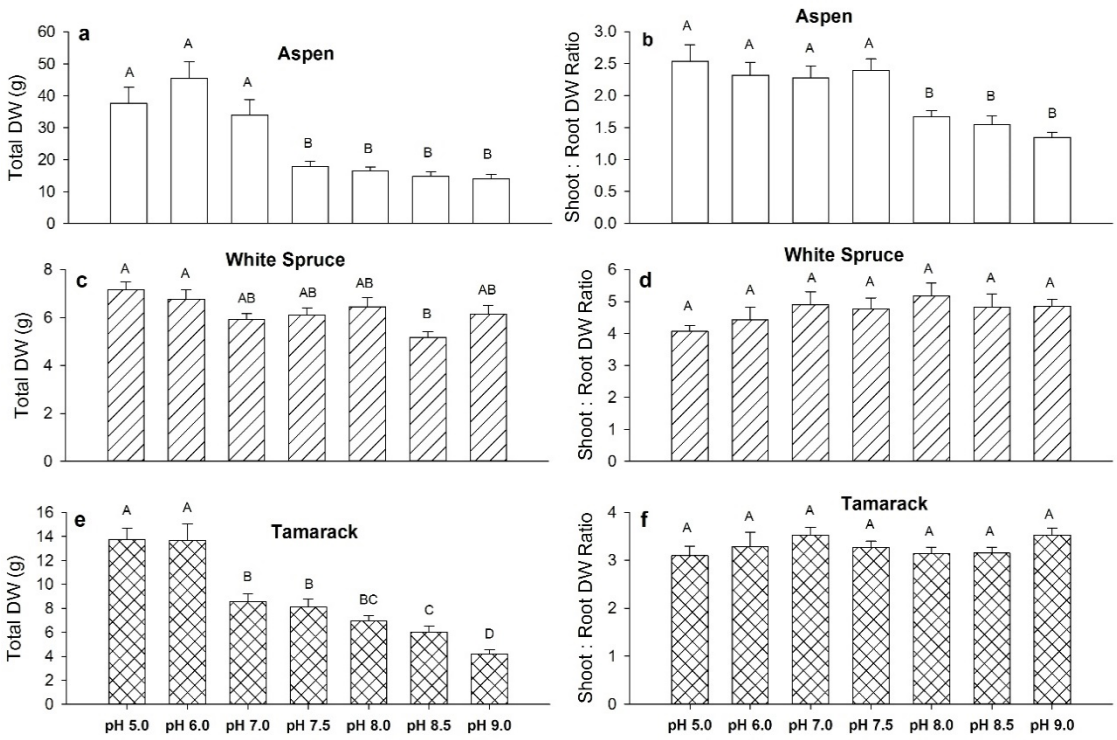

Figure 2. 1 Effects of pH on total dry weights and shoot to root ratios in aspen, white spruce, and tamarack. Different letters above the bars indicate significant differences (α=0.05) between treatments within each plant species. Means (n=18) ± SE are shown.

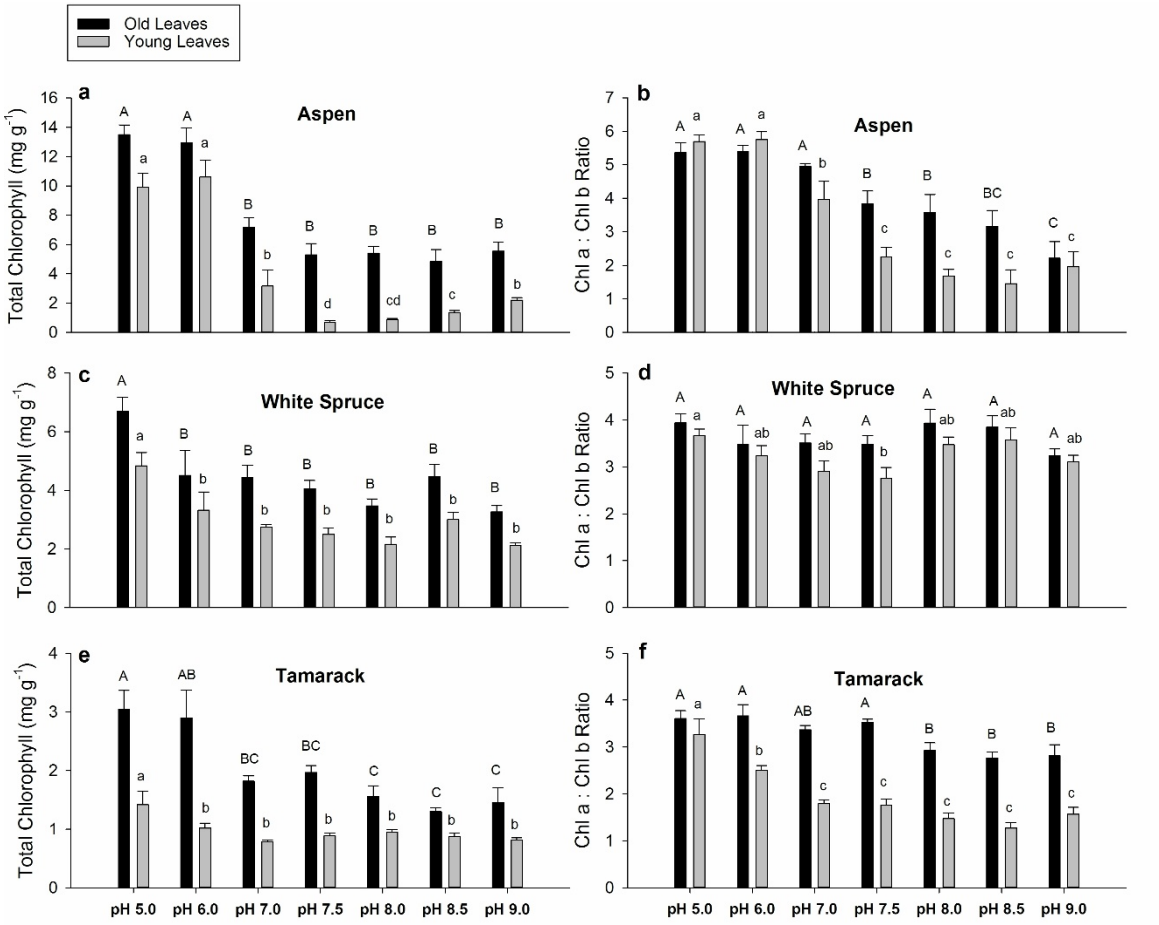

Figure 2. 2 Effects of pH on total chlorophyll concentrations (chlorophyll-a + chlorophyll-b) and ratios of chlorophyll-a to chlorophyll-b in old and young leaves of aspen, white spruce, and tamarack. Different letters above the bars (uppercase letters for old leaves and lowercase letters for young leaves) indicate significant differences (α=0.05) between treatments within each plant species. Means (n=6) ± SE are shown.

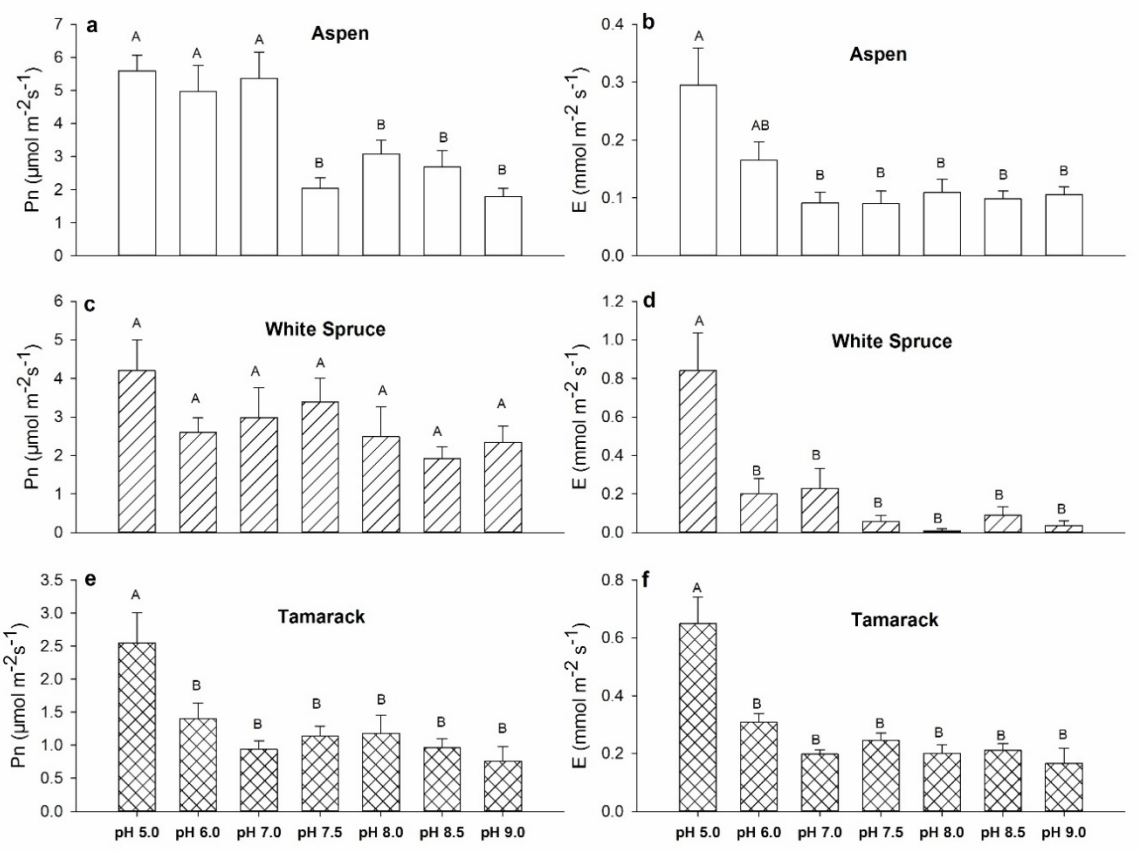

Figure 2. 3 Effects of pH on net photosynthesis (Pn) and transpiration (E) rates in aspen, white spruce, and tamarack. Different letters above the bars indicate significant differences (α=0.05) between treatments within each plant species. Means (n=6) ± SE are shown.

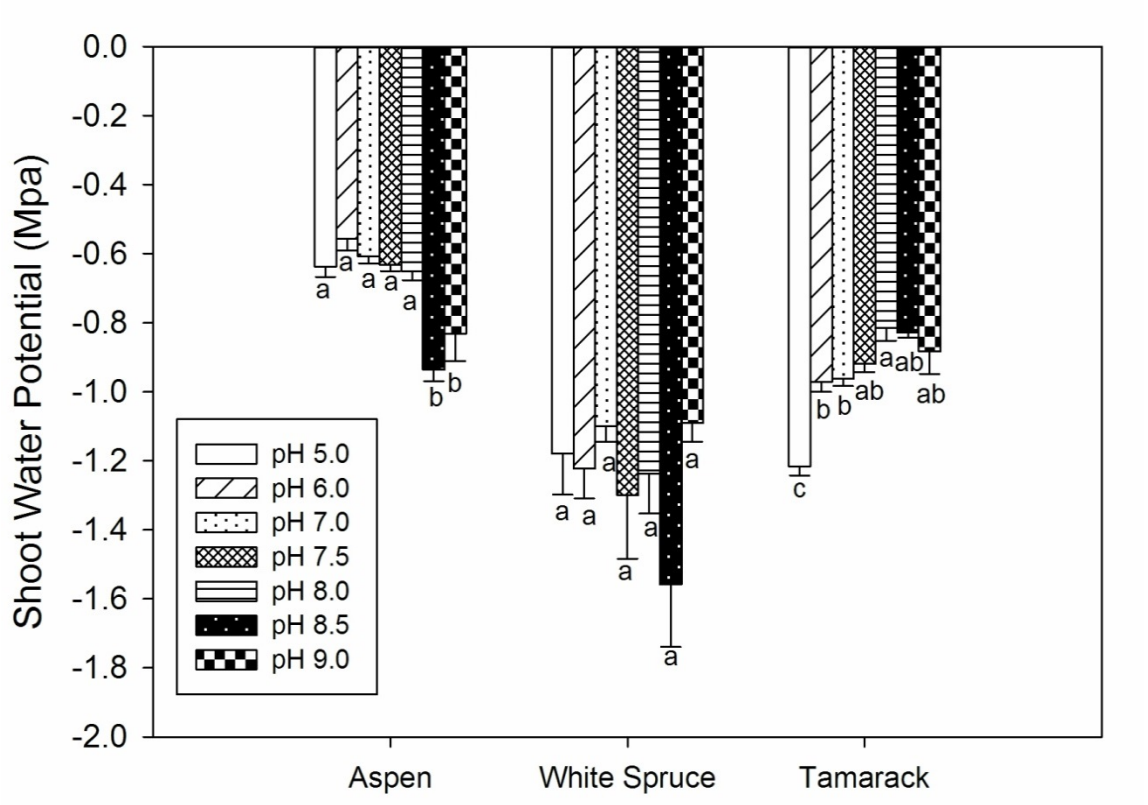

Figure 2. 4 Effects of pH on shoot water potentials in aspen, white spruce, and tamarack. Different letters above the bars indicate significant differences (α=0.05) between treatments within each plant species. Means (n=6) ± SE are shown.

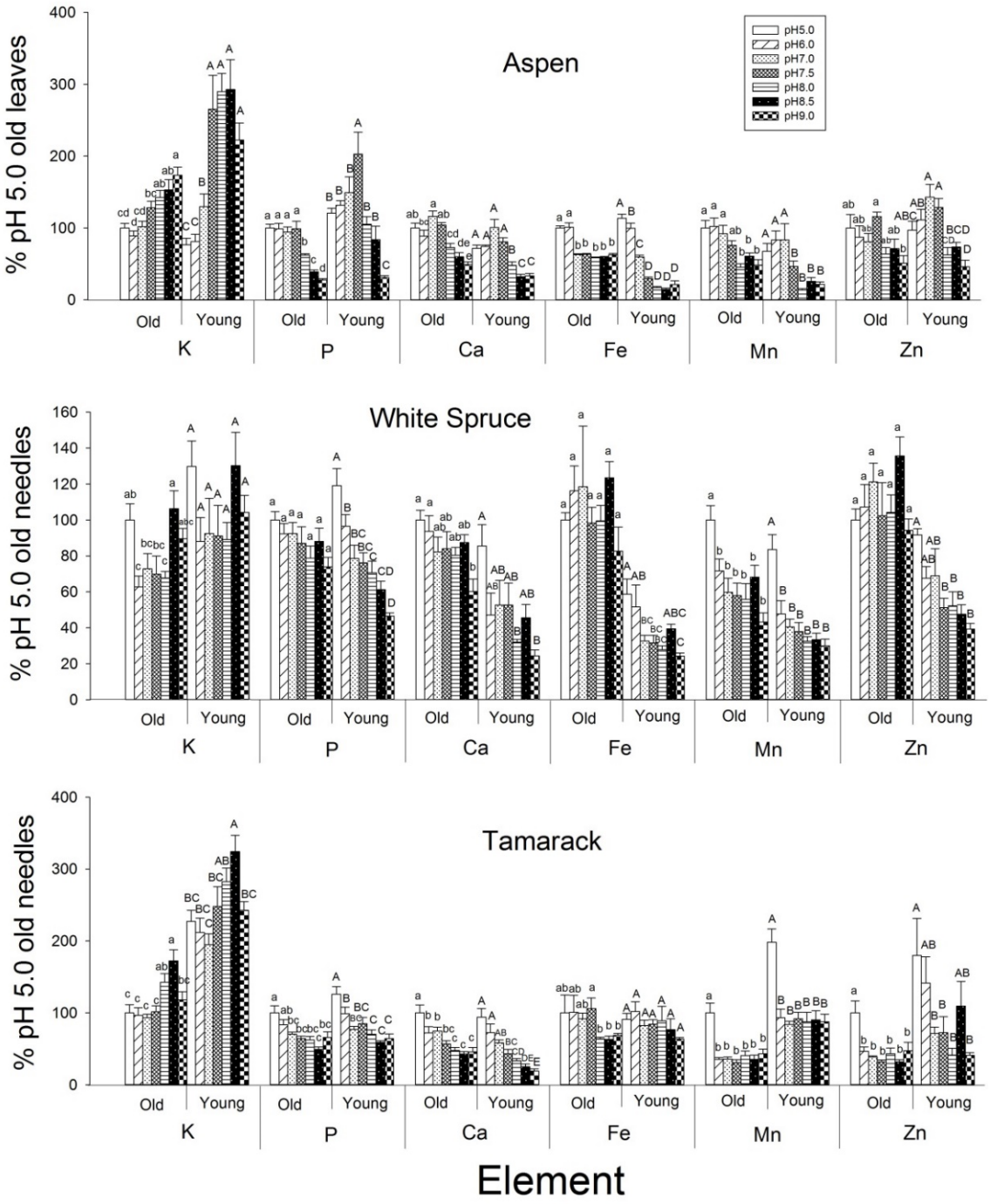

Figure 2. 5 Effects of pH on P, Ca, Fe, Mn and Zn concentrations in young and old leaves of aspen, white spruce, and tamarack seedlings, presented as the percentages of values measured at pH 5.0 in old leaves. Different letters above the bars (uppercase letters for young leaves and lowercase letters for old leaves) indicate significant differences (α=0.05) between treatments within each plant species. Means (n=6) ± SE are shown.

Chapter 3*♣

Responses of jack pine (*Pinus banksiana*) seedlings to root zone pH and calcium

3.1 Introduction

It is estimated that about 25-30% of the world land surface is calcareous (Wallace and Lunt 1960). Calcareous soils are characterized by ample Ca supply and high soil pH, usually in the range of 7.5-8.5 (Marschner 2012). Besides natural calcareous soils, human activities can also produce alkaline soils with high Ca concentrations (Renault et al. 2000). In the reclaimed areas following open-pit oil sands mining in northeastern Alberta, Canada, the pH of reclaimed soil is commonly higher than 8.0 (Howat, 2000). Since gypsum is also added to accelerate tailings consolidation process (Ramos-Padrón et al. 2010), the soil Ca content in some reclamation sites can exceed 400 mg kg^{-1} compared with less than 6 mg kg^{-1} typically found in the surrounding mixedwood forests (Visser 2005).

High soil pH affects many different processes in plants in a complex manner. A common problem associated with high soil pH is reduced availability of certain essential

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*A revised version of this chapter has been published. Zhang W, Xu F, Zwiazek JJ (2015) Responses of jack pine (*Pinus banksiana*) seedlings to root zone pH and calcium. Environmental and Experimental Botany 111: 32-41.

♣ I examined six species (aspen, white spruce, dogwood, blueberry, bearberry, jack pine) in this study. In the published article I only used the results of jack pine. The results of other species are in appendix 3.

elements including Fe, Mn, P, Zn, B, and Cu (Berger 1962; Yang et al. 1994; Valentine et al. 2006; Marschner 2012; Zhang et al. 2013). High soil pH can also reduce root water flux (Tang et al. 1993a; Kamaluddin and Zwiazek 2004; Siemens and Zwiazek 2011). Reductions in nutrient and water uptake under high pH may lead to stomatal closure (Tang et al. 1993a; Kamaluddin and Zwiazek 2004), decreased shoot water potential (Tang et al. 1993a), and reduced leaf concentrations of photosynthetic pigments (Zhang et al. 2013) leading to growth reductions (Bertoni et al. 1992; Tang et al. 1992).

The plant root intracellular pH is affected by pH of the root growth medium (Felle and Hanstein 2002). According to the acid growth theory, root cell elongation is induced by acidification of apoplastic pH (Rayle and Cleland 1992). The elongation of root cortex cells in *Lupinus angustifolius* L. was reported to be inhibited at pH 7.5 (Tang et al. 1993c). Calcium accumulation in the cell wall is thought to enhance cell wall rigidity by cross-linking pectin chains (Hepler 2005). Therefore, Ca and protons appear to have opposing roles in the processes leading to cell elongation and the consequence of high Ca concentrations may be the inhibition of cell elongation (Tang et al. 1993b; Hepler 2005).

In addition to affecting cell wall properties, Ca plays a major role in maintaining membrane integrity and selectivity (Grattan and Grieve 1998). Treatment of plants with Ca can significantly ameliorate the negative effects of abiotic stresses including salinity and aluminum toxicity (Kinraide and Parker 1987; Cabanero 2004), by reducing plasma membrane permeability and preventing influx of toxic ions into the cytosol (Cramer 2002). Ca^{2+} is a versatile signaling ion linking environmental and developmental stimuli and physiological responses of plants (Hepler 2005; Bickerton and Pittman 2012). The cytosolic [Ca^{2+}] is approximately 0.1 μM while in the apoplast and storage compartments,

such as vacuole and endoplasmic reticulum, [Ca^{2+}] ranges from 0.1 to 10 mM (Hepler 2005). Indeed, cytosolic [Ca^{2+}] elevation is a ubiquitous feature of the signaling network when plants are exposed to almost all biotic and abiotic stresses (Bose et al. 2011). Calcium also affects water uptake as Ca^{2+} is involved in the opening and closing of aquaporin (Cabanero et al. 2006) and regulates guard cell turgor and stomatal aperture (Webb et al. 1996). However, despite the importance of Ca in plant functioning, the effects of Ca on the responses of plants to high pH have not been thoroughly studied.

Jack pine (*Pinus banksiana* Lamb.) is a native tree species of Canadian boreal forest, and is an early successional species found in sandy and nutrient-poor sites (Cayford et al. 1967). It is among the conifer tree species showing stunted growth in calcareous soils in southeastern British Columbia and western Alberta, Canada (Kishchuk 2000) and one of the main tree species considered for the revegetation of the oil sands areas affected by high pH and Ca (Renault et al. 2000; Apostol and Zwiazek 2004; Calvo Polanco et al. 2008). In the present study, I carried out two controlled-environment experiments to examine the growth and physiological responses of jack pine seedlings to neutral and high root zone pH in the presence of different Ca concentrations. I hypothesized that high Ca concentration would aggravate the effects of high pH through inhibited root growth and the resulting impairment of root function.

3.2 Materials and methods

3.2.1 Plant material and growth conditions

One-year-old container-grown (415D styroblocks™, Beaver Plastics, Acheson, AB, Canada) jack pine (*P. banksiana* Lamb.) dormant seedlings were obtained from the

Boreal Horticultural Services Ltd., Bonnyville, AB, Canada. Seedling roots were gently washed to remove the potting medium and placed in 25% modified Hoagland's solution (Epstein 1972) for two weeks in a controlled-environment growth chamber before applying pH and Ca treatments. Environmental conditions in the growth chamber were 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) at the top of the seedlings provided by full spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada).

I investigated the effects of root zone pH and the effects of Ca on seedling responses to pH in two experiments. In Experiment 1, I examined the responses of jack pine seedlings to six root zone pH levels of 6, 7, 7.5, 8, 8.5, and 9. In Experiment 2, I selected three pH levels: 6.5, 7.5, and 8.5, and four Ca levels: 0.25, 1, 5, and 10 mM Ca, to study their combined effects on jack pine seedlings. These Ca treatment concentrations corresponded to 0.25, 1, 5, and 10x Ca concentration present in 25% modified Hoagland's solution. The quantity of $Ca(NO_3)_2 \cdot 4H_2O$ added in the nutrient solution was calculated from the formula of modified Hoagland's solution (Epstein 1972). In both experiments, plants were treated for 8 weeks. The hydroponic set-up for each treatment consisted of a 120 L opaque pail connected to three (Experiment 1) or two (Experiment 2) 30 L low-density polyethylene (LDPE) tubs through the PVC tubing. In each pail, a pump (Model 9.5 950GPH, Danner MFG Inc., New York, NY, USA) circulated 120 L of 25% Hoagland's solution between the pail and the tubs (Zhang et al. 2013). Each tub was covered with a Styrofoam lid containing 3.8 cm holes through which seedlings were placed in nutrient solution and secured with foam plugs. Six (Experiment 1) or nine

(Experiment 2) jack pine seedlings were placed in each tub. A pH controller (PHCN-70, Omega Engineering Inc., Laval, QC, Canada) connected with a pH electrode (Orion 9106 BNWP, Thermo Scientific, Rochester, NY) was used to continuously control the solution pH. An electronic valve (Model 8260G071 120/60 ASCO Valve, Inc., Florham Park, NJ, USA) was controlled by the pH controller and connected to a 5 % (w/v) KOH solution container. A plastic ball valve (Model R-01377-84, Cole-Parmer Canada Inc., Montreal, QC, Canada) was connected to the electronic valve to release 5% (w/v) KOH solution slowly to the nutrient solution to maintain the desired pH levels (Zhang et al. 2013). During the experiments, the variation of solution pH was maintained within ± 0.1 range. The nutrient solution was replaced every two weeks. Concentrations of dissolved O_2 were measured with the oxygen electrode (YSI 5000, YSI Inc., Yellow Springs, OH, USA) and were no less than 6 mg L^{-1} throughout the experiment.

3.2.2 Elemental analysis of nutrient solution

To quantify the solubility of selected essential elements at different pH and Ca levels in Experiment 2, 1 L of 25 % modified Hoagland's solution without Ca was prepared in triplicate (n=3), then $Ca(NO_3)_2 \cdot 4H_2O$ was added to adjust Ca concentrations to 0.25, 1, 5, and 10 mM. The solution pH was then adjusted to 6.5, 7.5 and 8.5 with 5 % KOH (w/v) or 1 % H_2SO_4 (v/v). For each treatment, three 20 mL nutrient solution samples were filtered with 0.45 μm Polyvinyl Difluoride (PVDF) syringe-driven filter unit (EMD Millipore Corporation, Billerica, MA, USA). The concentrations of B, P, K, Ca, Fe, Mn, Cu, and Zn in filtered solution were measured with the inductively coupled plasma mass spectrometry (ICP-MS) (Zarcinas et al.1987) in the Radiogenic Isotope Facility of the University of Alberta.

3.2.3 Dry weights and needle chlorophyll concentrations

Shoot and root dry weights were determined for all seedlings from each treatment (n=18). Roots and stems were dried in an oven at 70 ^{o}C for 72 h. Needles were divided into old and young needle classes. Needles that were fully elongated before the onset of treatments were regarded as old needles, while the needles which started elongating after the onset of treatments were regarded as young needles. Needles were separated from stems and immediately placed in an ultra-low temperature freezer at −80 ^{o}C, and freeze-dried for 72 h before storage. To determine shoot dry weights, the dry weights of all needles and stems from each plant were added.

Chlorophyll-a (chl-a) and chlorophyll-b (chl-b) concentrations of old and young needles were determined in six randomly selected seedlings per treatment (n=6). The freeze-dried needles were ground with a Thomas Wiley Mini-Mill (Thomas Scientific, NJ, USA). Chlorophyll was extracted from pulverized leaf samples (10 mg dry weight) with 8 mL dimethyl sulfoxide (DMSO) at 65 ^{o}C for 22 h. Chlorophyll concentrations were measured in DMSO extracts with a spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden), at 648 nm and 665 nm for chlorophyll-a and chlorophyll-b. Total chlorophyll concentration was calculated using Arnon's equation (Sestak et al. 1971).

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

After 8 weeks of treatment, six jack pine seedlings (n=6) were randomly taken from each treatment for the measurements of Pn and E. For the measurements, about 5-cm distal parts of needles were inserted in the leaf chamber of an infrared gas analyzer (LCA-4, Analytical Development Company Ltd., Hertfordshire, UK) with an auxiliary

LED bulb (400 μmol m^{-2} s^{-1} PPFD) as previously described (Nguyen et al. 2006). The reference CO_2 concentration was 400 μmoL and the flow rate was 250 μmoL s^{-1} in the leaf chamber. Measurements were conducted between 9:00 - 12:00 h. To determine needle areas, needles in the leaf chamber were cut with scissors and scanned. The needle areas were calculated following scanning using the Sigmascan Pro 5.0 computer software (Systat Software, San Jose, CA, USA).

3.2.5 Root cortex cell dimensions

Since high pH levels (pH 8, 8.5, and 9) in Experiment 1 resulted in a high degree of root tip necrosis, only plants subjected to pH levels of 6, 7, and 7.5 were taken to examine root cell dimensions. Similarly, due to high root mortality in Experiment 2, only plants from the following treatments were taken for root microscopic examinations at the end of treatments: pH 6.5+0.25 mM Ca, pH 6.5+1 mM Ca, pH 6.5+5 mM Ca, pH 7.5+0.25 mM Ca, pH 7.5+1 mM Ca, and pH 8.5+1 mM Ca. To determine the dimensions of root cortex cells in both experiments, free-hand longitudinal sections of roots were made in selected Ca and pH treatments with a razor blade approximately 10 mm away from the root tip. For each treatment, more than 15 cells were measured. Sections were stained with 0.05% Toluidine Blue O (TBO) for 30 s (Peterson et al. 2008). Photographs were taken to determine cell dimensions using a light microscope (Carl Zeiss, Göttingen, Germany) with a digital camera attachment.

3.2.6 Needle elemental analysis

Since the chlorophyll concentrations of young needles were either equal to or lower than those in old needles, only young needles were analyzed for the concentrations

of P, Fe, Mn, Cu, Zn, B, which have been frequently reported to be deficient in plants growing in calcareous soils (Berger 1962; Yang et al. 1994; Valentine et al. 2006; Marschner 2012; Zhang et al. 2013). Additionally, needle concentrations of K and Ca were analyzed since KOH and $Ca(NO_3)_2$ were added to treatment solutions. Pulverized young needle samples (0.2 g dry weight) were digested with 10 ml 70% HNO_3 and diluted with Milli-Q water to 40 ml. Extracts were then filtered and analyzed by ICP-MS (Zarcinas et al. 1987) in the Radiogenic Isotope Facility at the University of Alberta, Edmonton, AB, Canada.

3.2.7 Statistical analysis

All data were analyzed using SAS GLM model (Version 9.2, SAS Institute Inc., Cary, NC) to determine statistically significant ($p \leq 0.05$) differences between treatments. For Experiment 1, one-way ANOVA was used with pH treatment as the main factor. For Experiment 2, a two-way ANOVA was used with pH and Ca treatments as the main factors. The data that did not meet the ANOVA assumptions of normality of distribution and homogeneity of variance were transformed with a log10 function. Comparisons between different treatment means were conducted using Student-Newman-Keuls (SNK) test.

3.3 Results

3.3.1 Elemental analysis of nutrient solution

Increasing pH and Ca resulted in large reductions of soluble Mn, Cu, and Zn in 25% Hoagland's solution (Table 3.1). Soluble Ca concentrations increased linearly with increasing amounts of added Ca (Table 3.1). At pH 7.5 and 8.5 of 5 and 10 mM Ca

treatments, soluble P concentrations drastically decreased (Table 3.1). Since KOH was used to increase solution pH, at pH 7.5 and 8.5, K concentration slightly increased. The concentrations of B and Fe were relatively little affected by the pH and Ca treatments in the 25% Hoagland's solution (Table 3.1). Fe was provided in the chelated form of Fe-EDTA, hence, there was little effect of pH on its solubility (Table 3.1).

3.3.2 Total dry weights and shoot:root dry weight ratios

In Experiment 1, total dry weights (Fig. 3.1a) of jack pine seedlings were significantly higher at pH 6.0 compared with the higher (pH 7.0 - 9.0) pH treatments, while shoot:root dry weight ratios were significantly lower at pH 6.0 compared with the other pH levels (Fig. 3.1b).

In Experiment 2, the effects of Ca on seedling dry weights were pH-dependent (Fig. 3.1c) with significant pH and Ca interactions (Table 3.2). At pH 6.5 and 7.5, the highest total dry weights were recorded in plants supplied with 0.25 mM Ca compared with plants from the higher Ca treatment levels (Fig. 3.1c). However, at pH 8.5, plants supplied with the 1 mM Ca level had higher total dry weights compared with the plants supplied with 0.25, 5 and 10 mM Ca (Fig. 3.1c). There was relatively little effect of Ca on shoot:root ratios with the exception of 10 mM Ca treatment at pH 6.5 which had higher shoot:root ratios compared with 0.25 mM Ca treatment (Fig. 3.1d).

3.3.3 Leaf chlorophyll concentrations

In Experiment 1, total chlorophyll concentrations in both young and old needles of jack pine seedlings were over two-fold higher at pH 6.0 compared with the higher pH treatments (pH 7.0 - 9.0) (Fig. 3.2a). Similar effects of pH were observed for chlorophyll

a:b ratios which decreased in higher pH treatments (Fig. 3.2b).

In Experiment 2, there was a significant effect of pH, Ca, and their interactions on needle chlorophyll concentrations and chlorophyll a:b ratios (Table 3.2). Increasing Ca concentrations decreased total chlorophyll needle concentrations in all examined pH levels and the effect was similar in young and old needles (Fig. 3.2c). However, at pH 8.5, only the highest, 5 and 10 mM Ca concentrations significantly decreased needle chlorophyll concentrations (Fig. 3.2c). Chlorophyll a:b ratios showed similar trends as the total chlorophyll concentrations for the different pH and Ca treatments (Fig. 3.2d).

3.3.4 Net photosynthesis (Pn) and transpiration (E) rates

Both Pn (Fig. 3.3a) and E (Fig. 3.3b) sharply decreased above pH 6.0 and the magnitude of these decreases was similar at all pH levels examined (7.0 - 9.0). There were significant interactions between pH and Ca on Pn and E (Table 3.2). Of the Ca and pH treatments examined, the highest Pn (Fig. 3.3c) and E (Fig. 3.3d) were measured in plants subjected to 0.25 mM Ca treatment at pH 6.5. Both increased Ca concentrations and higher pH (7.5 and 8.5) treatments resulted in over four-fold reductions in both gas exchange parameters (Fig. 3.3c, d).

3.3.5 Root cortex cell dimensions

In Experiment 1, lengths of the root cortex cells sharply decreased at pH 7 and 7.5 compared with pH 6 (Fig. 3.4a). However, there was relatively little difference between the treatments in cell width resulting in low cell length:width ratios at pH 7.0 and 7.5 compared with pH 6.0 (Fig. 3.4b). In Experiment 2, the effects of pH, Ca and their interaction on root cell dimensions were significant ($p < 0.0001$) (Table 3.2). At the same

Ca levels (1 mM Ca), an increase in pH resulted in a decrease in cell lengths (Fig. 3.4c). At pH 6.5, increasing Ca concentrations from 0.25 to 1 and 5 mM Ca resulted in large decreases in cell length, but had less effect on cell width (Fig. 3.4c). An opposite trend was observed at pH 7.5 with greater root cortical cell lengths in the 1 mM Ca treatment compared with 0.25 mM Ca (Fig. 3.4c). Similar trends to cell length were observed for all cell length:width in all treatments (Fig. 3.4d).

3.3.6 Foliar elemental concentrations

In Experiment 1, of the eight elements examined, K showed a significant increase at higher pH as KOH was used to adjust pH of the solution (Fig. 3.5a). Both B and Ca showed reductions in needle concentrations with increasing pH (Fig. 3.5b,d). Phosphorus needle concentrations also showed relatively little response to pH treatments with the exception of pH 7.5 which significantly reduced needle P concentrations (Fig. 3.5c). However, both Zn and Cu concentrations showed a declining trend when solution culture pH was raised above pH 6.0, although the differences were not significant (Fig. 3.5g,h). The increase in pH from 6.0 to 7.5 resulted in a decrease in Mn needle concentrations, however, this trend was reversed at pH 8.0, 8.5, and 9.0 (Fig. 3.5f).

In Experiment 2, there was relatively little effect of Ca treatments on K needle concentrations (Fig. 3.6a). At all of the pH levels examined (6.5, 7.5, and 8.5), Ca needle concentrations were similar in both lower (0.25 and 1 mM Ca) and higher (5 and 10 mM Ca) concentration treatments. The needle Ca concentrations were two- to three-fold higher in 5 and 10 mM Ca treatments at all pH levels compared with 0.25 and 1 mM Ca treatments (Fig. 3.6b). Needle P concentrations increased with increasing Ca concentration treatments at pH 7.5 and 8.5, but not in the 6.5 pH treatment (Fig. 3.6c).

There was no consistent effect of pH and Ca treatments on needle B and Fe concentrations (Fig. 3.6d,e). There was little effect of Ca on Mn and Cu concentrations in jack pine needles (Fig. 3.6f,g). However, Zn needle concentrations at pH 6.5 were the highest in the lowest 0.25 mM Ca treatment (Fig. 3.6h).

3.4 Discussion

I used the semi-automated pH controlling system in hydroponics (Zhang et al. 2013; Calvo-Polanco et al. 2014) to study the responses of jack pine seedlings to Ca at different root zone pH levels. The growth and physiological responses observed clearly demonstrated high sensitivity of jack pine seedlings to high pH and significant interactions between pH levels and Ca treatment concentrations. Reductions in total seedling dry weights with increasing pH levels were largely due to the decreases in root dry weights resulting in the higher shoot to root dry weight ratios. High root zone pH can affect root growth by inhibiting root cell elongation and the formation of lateral roots and root hairs (Tang et al. 1993c; Canmore-Neumann et al. 1996). The decreases in dry weights at high pH were accompanied by decreases in total chlorophyll concentrations, chlorophyll a:b ratios and reductions in transpiration and net photosynthesis rates, which are commonly affected in plants by high pH (Zhang et al. 2013). Many of the observed effects of high pH on plant growth and physiological processes could be caused by the reductions in plant water transport triggered by the inhibitory effects of high pH on root aquaporin activity (Tournaire-Roux et al. 2003; Kamaluddin and Zwiazek 2004) and the reduced root system size (Tang et al. 1993a; Zhang et al. 2013).

At all three pH levels examined in this study, elevated Ca concentrations significantly reduced plant dry weights and needle chlorophyll concentrations. Since

excessive Ca in cells is deposited in the form of crystals in the vacuoles, high concentrations of Ca are usually not considered highly toxic to plants unless they are present in the cytosol. Calcium crystals serve as a localized Ca sink and may take up as much as 90% of total Ca in a plant (Nakata 2003). In some plant tissues, Ca can reach more than 10% of dry weight (Marschner 2012). Precise regulation of Ca^{2+} is essential in the cytosol since Ca^{2+} acts as a signalling molecule triggering a cascade of physiological events (Rengel 1992). Ca^{2+} concentrations are controlled by Ca^{2+} permeable channels, Ca^{2+}-pumps and proton-coupled Ca^{2+} exchangers on the plasma membrane (White 2000; Bickerton and Pittman 2012). If the cytosolic [Ca^{2+}] regulation processes are disrupted by stress factors, such as high pH, the cell processes regulated by [Ca^{2+}] could be altered triggering physiological responses in plants. Therefore, plant responses to high Ca soil concentrations may depend on the ability of plants to reduce cytosolic [Ca^{2+}] by reducing Ca uptake and sequestering Ca outside of the cytosol.

Interestingly, seedling dry weights were reduced by the higher Ca concentrations, especially at the lower pH. At pH 6.5 and 7.5, the highest seedling dry weights were measured in the 0.25 mM Ca treatment suggesting that the composition of Hoagland's solution (1 mM Ca) is likely higher than the optimum requirement for growth of jack pine seedlings under the study conditions. However, at pH 8.5, 1 mM Ca concentration was required for the maximum effect on dry weights. Since genetic variations of jack pine are high, differences in chlorophyll concentrations and transpiration rates of the seedlings from the different seed sources are common (Dancik and Yeh 1983; Xie and Knowles 1991), which could explain the differences in responses of seedlings in Experiments 1 and 2 to 1 mM Ca treatment at pH 8.5. The cytosolic pH of plants is about

7.2 while the apoplastic pH has been reported to measure usually between 5 and 6.5 (Grignon and Sentenac 1991; Rengel 2002). This pH gradient creates a negative membrane potential and makes it possible for plant cells to passively transport cations (Rengel 2002; Taylor et al. 2012). The root zone pH could increase apoplastic pH and disrupt the pH gradient (Felle and Hanstein 2002). For high pH-sensitive species, the consequences of higher apoplastic pH may include the inhibition of activity of membrane-bound enzymes and increased membrane leakiness (Zieslin and Snir 1989). It is possible that in pH 8.5+1 mM Ca treatment, the higher concentration of Ca^{2+} in treatment solution compared with pH 8.5+0.25 mM Ca, may help maintain membrane integrity and protect the membranes against high pH effects.

Effects of pH and Ca treatments on needle chlorophyll concentrations largely paralleled the effects on dry weights with the exception of pH 8.5+0.25 mM Ca treatment which had similar effect on chlorophyll concentrations as pH 8.5+1 mM Ca treatment. The chlorophyll a:b ratios significantly decreased starting at pH 7.0 and higher indicating that the chlorophyll reduction at higher pH was mainly due to chlorophyll-a decrease. Numerous studies have reported chlorophyll-b to be more stable under various environmental conditions than chlorophyll-a and its degradation rate is approximately 2.5 times slower compared with chlorophyll-a (Koca et al. 2007). Results of this study clearly demonstrate that higher Ca concentrations could aggravate the effects of pH on Pn. In Experiment 2, Pn in pH 6.5+0.25 mM Ca treatment was several-fold higher compared with other treatments. The decreases in chlorophyll concentrations could contribute to Pn reductions in the high pH and high Ca concentration treatments. However, the similarities in patterns and the magnitude of Pn and E reductions suggest that stomatal limitations

were likely the most significant contributor to the decrease in Pn by high pH and Ca. The reduced stomatal conductance could be the consequence of the inhibitory effect of high pH on the root aquaporin activity and its impact on root water flux (Tournaire-Roux et al. 2003; Kamaluddin and Zwiazek 2004).

In an experiment with *L. angustifolius* (Tang et al. 1993b), increasing Ca concentration in a buffered solution at either pH 5.2 or 6.6 only slightly decreased root elongation rates. Contrary to that, I have found that both pH 7 and higher and 5 mM Ca at pH 6.5 treatments significantly inhibited root cortex cell elongation in jack pine seedlings (Fig. 3.4a,c). Since cell length was more affected than cell width, the cell length to width ratio decreased in high pH and high concentration Ca treatments (Fig. 3.4b,d). The inhibition of cell elongation was likely the factor irresponsible for the observed growth decreases. Since the root cells that we examined were approximately 10 mm above the root tip where the cells were fully elongated, developmental stage was not a factor contributing to the differences in cell lengths between the treatments. The inhibitory effect of high pH on root cell elongation has been reported in *L. angustifolius* (Tang et al.1992; Tang et al. 1993c; Tang et al. 1996). It has been also long recognized that high concentrations of Ca can inhibit cell elongation (Cooil and Bonner 1957; Cleland and Rayle 1977). In the cell elongation process, acidification of the cell wall has been proposed to be required for wall loosening and extension (Taiz 1984). The inhibitory effect of high Ca concentrations on plant cell elongation was explained by the formation of Ca bridges between pectin chains and the resulting mechanical stiffening of the cell walls which, in turn, prevents wall extension (Tagawa and Bonner 1957; Burstrom 1968). Another hypothesis that has been proposed to explain the inhibition of cell elongation by

Ca is an inhibition of the biochemical cell wall-loosening processes (Cleland and Rayle 1977). However, there is still little experimental support for both theories and the detailed mechanisms of Ca effects on cell elongation remain to be uncovered (Hepler 2005).

Since pH affects the availability of mineral nutrients, plants may suffer from nutrient deficiencies when grown at very low or high pH. In Experiment 1, of the analyzed elements, only B, Zn and Ca needle concentrations were significantly reduced by increasing solution culture pH. Leaf chlorosis due to Fe deficiency is a common problem in high pH soils (Korcak 1987). In the present study, I used Fe-EDTA, which is little affected by pH of the nutrient solution (Zhang et al. 2013). In our earlier study (Zhang et al. 2013), high pH of the mineral nutrient solution which contained Fe-EDTA decreased foliar Fe concentrations in trembling aspen (*Populus tremuloides*) and white spruce (*Picea glauca*), but had little effect on Fe concentrations in young needles of tamarack (*Larix laricina*). Therefore, jack pine and tamarack may have similar mechanisms which enable them to absorb chelated Fe at high pH.

In the nutrient solution, the concentrations of soluble Mn, Cu, and Zn sharply decreased at high pH. Potential deficiencies of these nutrients could be of concern in the longer-term for plants growing in high pH and Ca-rich soils. However, calcium treatments had little effect on the needle concentrations of B, Fe, Mn, Cu, Zn, and K in jack pine seedlings regardless of the solution culture pH indicating that the concentrations of these micro nutrients remaining soluble in the solution still sufficiently high to support growth of seedlings over the treatment period. As expected, Ca treatments affected Ca and P tissue concentrations. It is interesting that in Experiment 1 at pH 6, needle Ca concentration was similar to that of 5 and 10 mM Ca treatments of Experiment 2.

However, at pH 6.5 - 8.5 in the treatments with 0.25 and 1 mM Ca, needle Ca concentrations were about 50% lower. This suggests that Ca uptake was disrupted at the pH levels above 6.0, likely exceeding the pH of root apoplast. Although soluble P concentrations were reduced from 0.5 mM at pH 6.5+0.25 mM Ca to 0.03 mM at pH 8.5+10 mM Ca, the needle P concentrations were not negatively affected. This was likely due to lower growth rates of seedlings subjected to this treatment. Dry weights of seedlings subjected to pH 7.5 and 8.5 with 10 mM Ca were lower compared with other treatments. However, at pH 6.5, dry weights also decreased with increasing Ca levels and the needle P concentrations were similar for all Ca treatments suggesting that the uptake of P was not significantly affected.

In conclusion, the results of the study demonstrated that Ca aggravated the effects of high pH on jack pine seedlings. The effects of Ca and high pH included the inhibition of root cell elongation and profound decreases in net photosynthesis, transpiration rates and needle chlorophyll concentrations. The effects of high pH and Ca on photosynthesis could be largely explained by the effects on transpiration rates suggesting that water transport processes were among the targets for both factors. Mapping of Ca distribution in plant cells and examining plasma membrane Ca permeability under high pH conditions may be helpful to shed more light on the processes involved in plant responses to Ca at different pH levels. Since high soil Ca levels may aggravate the effects of high pH, it should be considered as an important factor in revegetation areas.

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3.6 Tables

Table 3. 1 Concentrations (means ± SE, n=3) of selected essential elements remaining soluble in 25% Hoagland's solution with added 0.25, 1, 5, and 10 mM Ca at pH of 6.5, 7.5, and 8.5 in Experiment 2 (n=3).

Treatment	B (μM)	P (mM)	K (mM)	Ca (mM)	Fe (μM)	Mn (μM)	Cu (μM)	Zn (μM)
pH 6.5 + 0.25 mM Ca	5.50 ±0.01	0.50 ±0.003	1.74 ±0.007	0.22 ±0.0006	3.61 ±0.04	0.47 ±0.0043	0.20 ±0.005	0.53 ±0.007
pH 6.5 + 1 mM Ca	6.06 ±0.01	0.50 ±0.002	1.78 ±0.046	0.85 ±0.0017	3.69 ±0.02	0.51 ±0.0042	0.14 ±0.001	0.56 ±0.007
pH 6.5 + 5 mM Ca	6.03 ±0.11	0.51 ±0.003	1.65 ±0.018	4.52 ±0.0213	3.54 ±0.08	0.50 ±0.0060	0.14 ±0.007	0.51 ±0.005
pH 6.5 + 10 mM Ca	5.77 ±0.05	0.49 ±0.003	1.75 ±0.019	8.76 ±0.0036	3.59 ±0.14	0.50 ±0.0051	0.13 ±0.002	0.52 ±0.004
pH 7.5 + 0.25 mM Ca	5.30 ±0.13	0.50 ±0.004	2.04 ±0.013	0.22 ±0.0004	3.35 ±0.06	0.46 ±0.0020	0.17 ±0.005	0.48 ±0.005
pH 7.5 + 1 mM Ca	6.05 ±0.06	0.50 ±0.002	2.00 ±0.013	0.84 ±0.0038	3.35 ±0.01	0.50 ±0.0042	0.10 ±0.003	0.37 ±0.013
pH 7.5 + 5 mM Ca	5.84 ±0.03	0.31 ±0.026	1.95 ±0.005	4.28 ±0.0501	3.57 ±0.02	0.18 ±0.0276	0.06 ±0.008	0.03 ±0.004
pH 7.5 + 10 mM Ca	5.79 ±0.10	0.25 ±0.003	2.16 ±0.016	8.36 ±0.0245	3.49 ±0.08	0.21 ±0.0045	0.05 ±0.001	0.03 ±0.001
pH 8.5 + 0.25 mM Ca	5.32 ±0.06	0.50 ±0.001	2.30 ±0.003	0.22 ±0.0008	3.05 ±0.41	0.41 ±0.0096	0.11 ±0.004	0.11 ±0.013
pH 8.5 + 1 mM Ca	6.09 ±0.05	0.34 ±0.001	2.34 ±0.025	0.65 ±0.0050	3.17 ±0.03	0.03 ±0.0010	0.05 ±0.002	0.03 ±0.005
pH 8.5 + 5 mM Ca	5.82 ±0.02	0.05 ±0.001	2.46 ±0.023	4.00 ±0.0149	3.10 ±0.17	0.05 ±0.0001	0.04 ±0.001	0.03 ±0.002
pH 8.5 + 10 mM Ca	5.51 ±0.01	0.03 ±0.001	2.56 ±0.009	8.12 ±0.0307	2.69 ±0.05	0.10 ±0.0011	0.05 ±0.003	0.05 ±0.006

Table 3. 2 ANOVA table showing effects of pH and Ca treatments on the measured parameters in Experiment 2.

p-value	dw	st : rt	Pn	E	Y-Chl	Y-a:b	O-Chl	O-a:b	Length	Width	L/W
pH	0.3834	0.0006	<0.0001	<0.0001	0.0003	<0.0001	0.0026	<0.0001	<0.0001	<0.0001	<0.0001
Ca	<0.0001	0.0570	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
pH*Ca	<0.0001	0.0005	<0.0001	<0.0001	<0.0001	0.0020	0.0002	0.0002	<0.0001	<0.0001	<0.0001

p-value	B	Fe	Mn	Cu	Zn	P	K	Ca
pH	0.9606	0.0013	0.0084	0.0009	0.9339	0.2684	0.0179	0.4058
Ca	0.4190	0.8185	0.0301	0.2807	0.0006	<0.0001	0.0090	<0.0001
pH*Ca	0.0304	0.3339	0.4196	0.0373	0.0091	<0.0001	0.3396	0.6050

Abbreviations are: dw − dry weight; st:rt − shoot : root dry weight ratio; Pn − net photosynthetic rate; E − transpiration rate; Y-chl − chlorophyll concentrations in young needles; Y-a:b − chlorophyll a:b ratios in young needles; O-Chl − chlorophyll concentrations in old needles; O-a:b − chlorophyll a:b ratios in old needles; length − root cortical cell length; width − root cortical cell width; L/W − root cell length/width ratios.

3.7 Figures

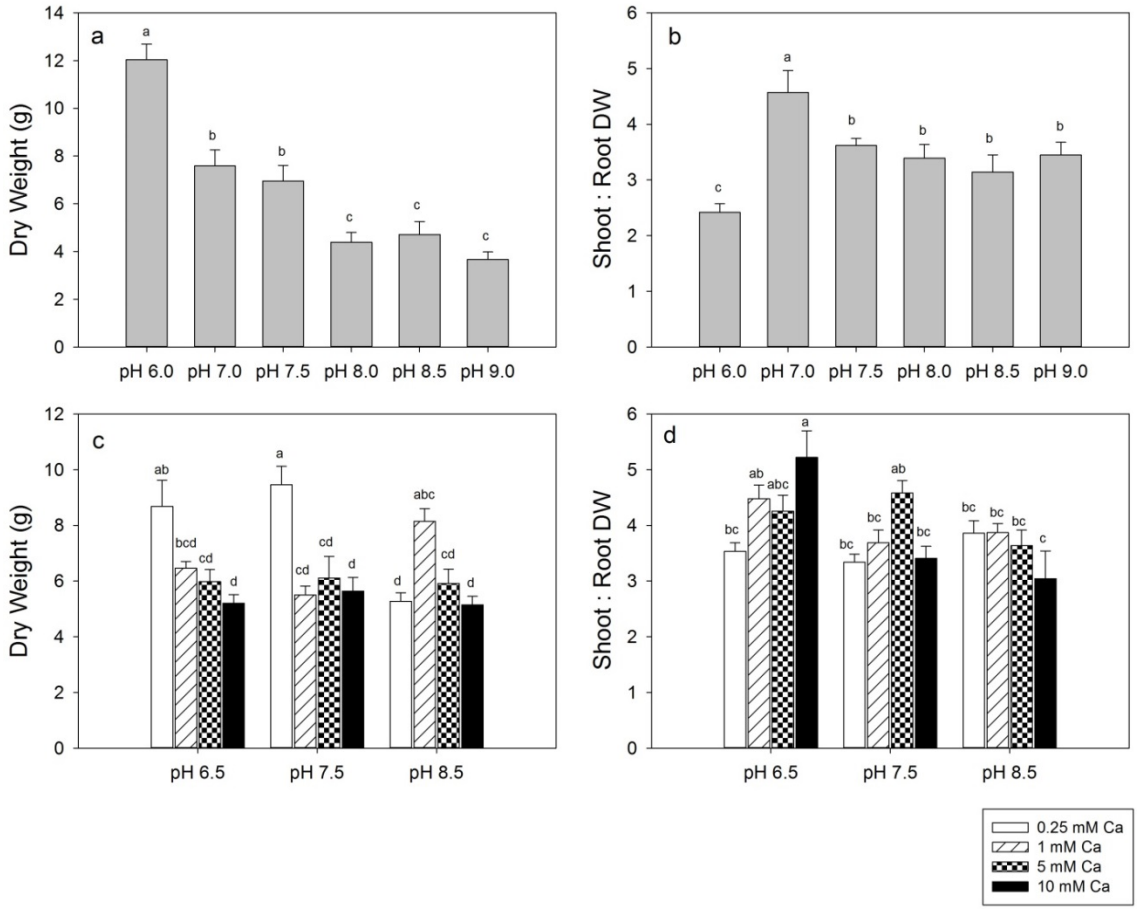

Figure 3. 1 Effects of pH and Ca treatments on total dry weights and shoot:root dry weight ratios in jack pine seedlings. Different letters above the bars indicate significant differences (α=0.05) between treatments determined by the Student-Newman-Keuls test. Means (n = 18) ± SE are shown.

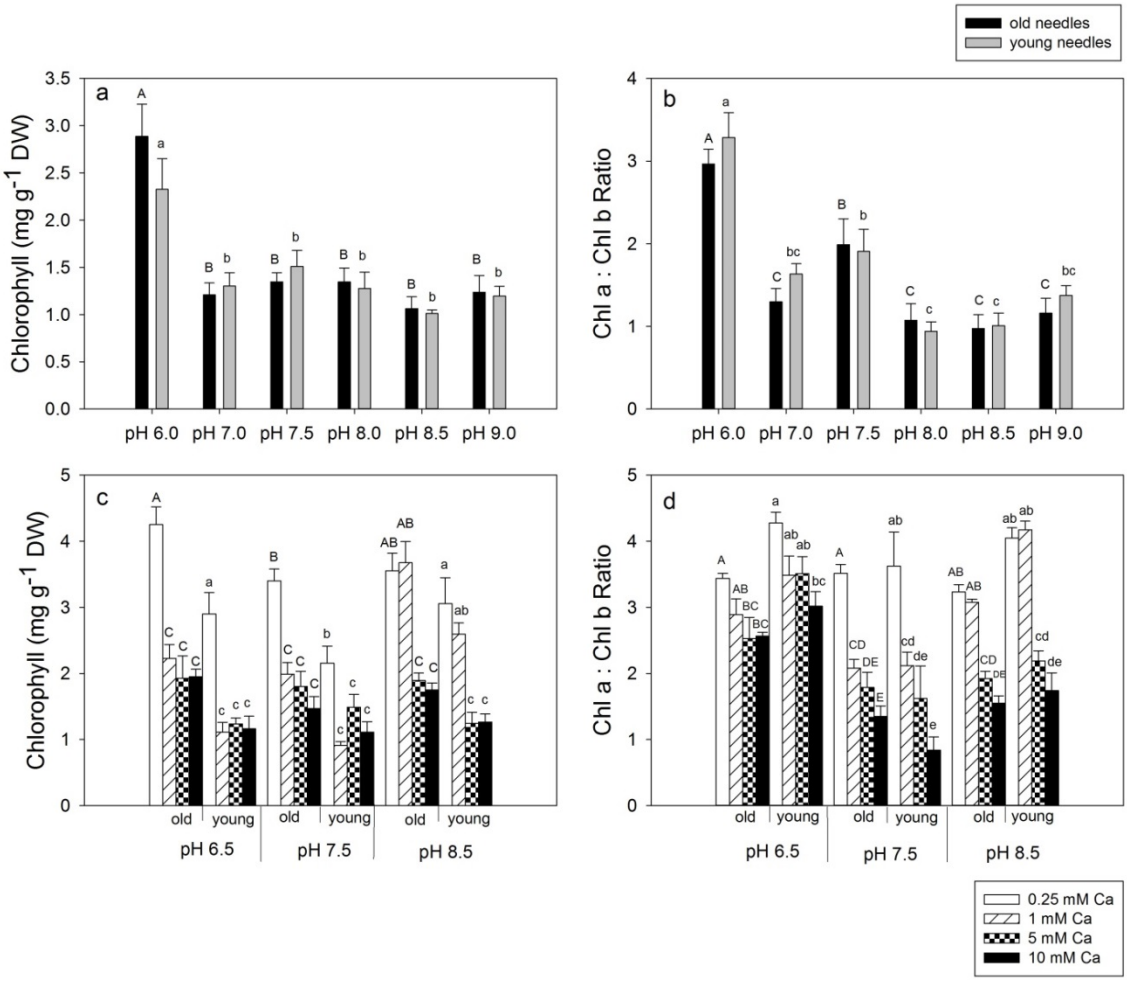

Figure 3. 2 Effects of pH and Ca treatments on total chlorophyll concentrations (chlorophyll-a + chlorophyll-b) and chlorophyll a:b ratios in old and young needles of jack pine seedlings. Different letters above the bars (uppercase letters for old leaves and lowercase letters for young leaves) indicate significant differences (α=0.05) between treatments determined by the Student-Newman-Keuls test. Means (n = 6) ± SE are shown.

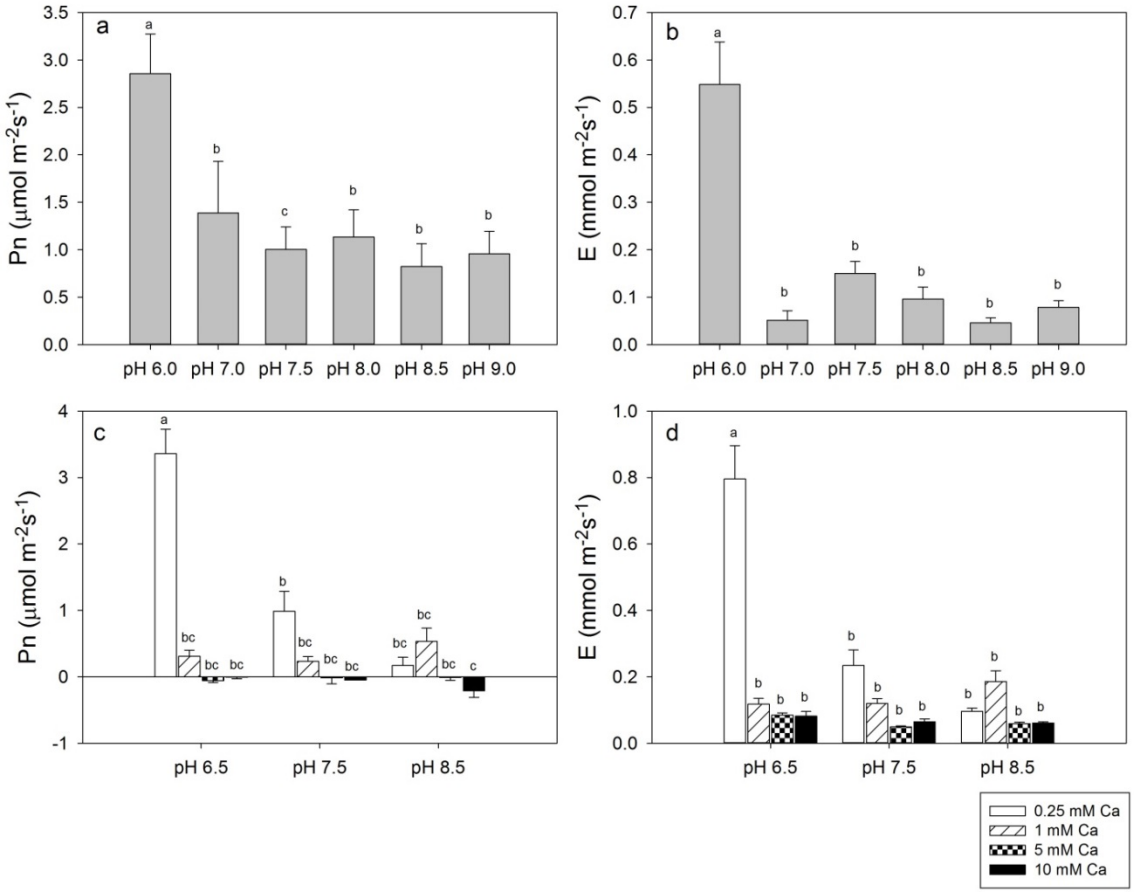

Figure 3. 3 Effects of pH and Ca treatments on net photosynthesis (Pn) and transpiration rates (E) in jack pine seedlings. Different letters above the bars indicate significant differences (α=0.05) between treatments determined by the Student-Newman-Keuls test. Means (n = 6) ± SE are shown.

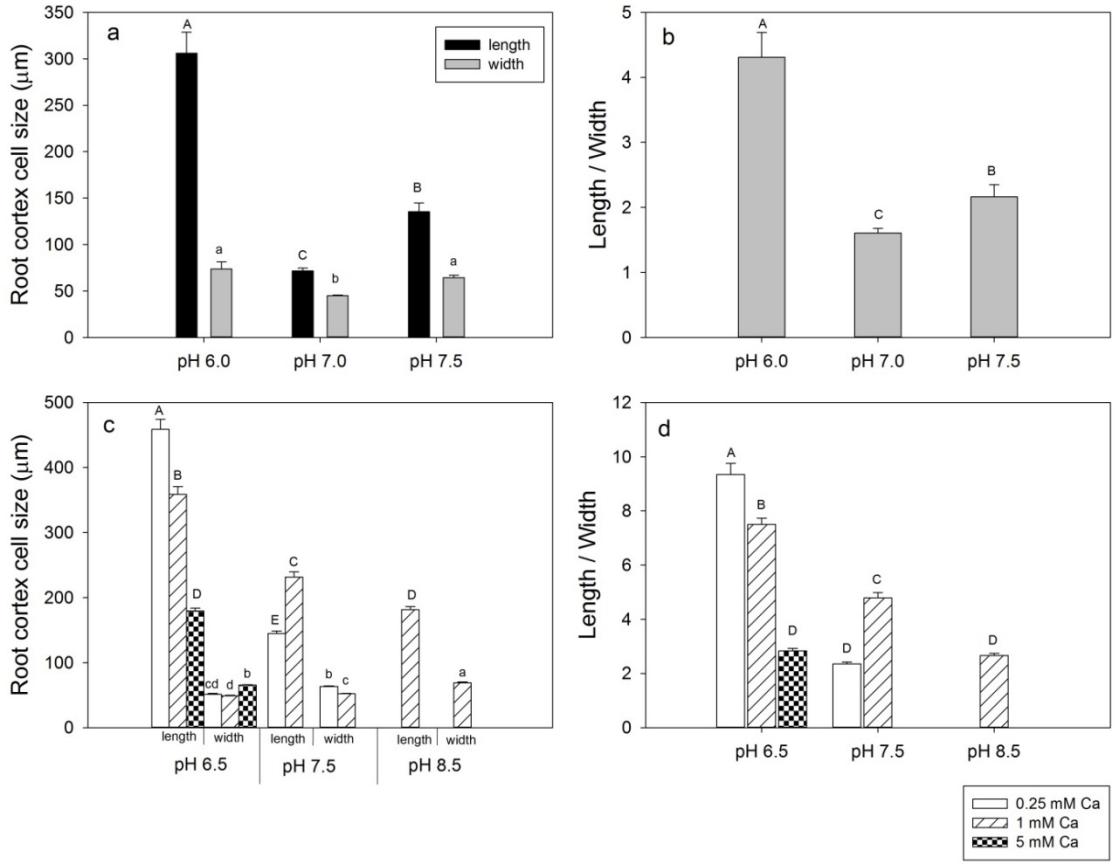

Figure 3. 4 Effects of pH and Ca treatments on length, width and length:width ratios in root cortex cell of jack pine seedlings in Experiment 1 (a, b) and Experiment 2 (c, d). Different letters above the bars indicate significant differences (α=0.05) between treatments determined by Student-Newman-Keuls test. Means (n>15) ± SE are shown.

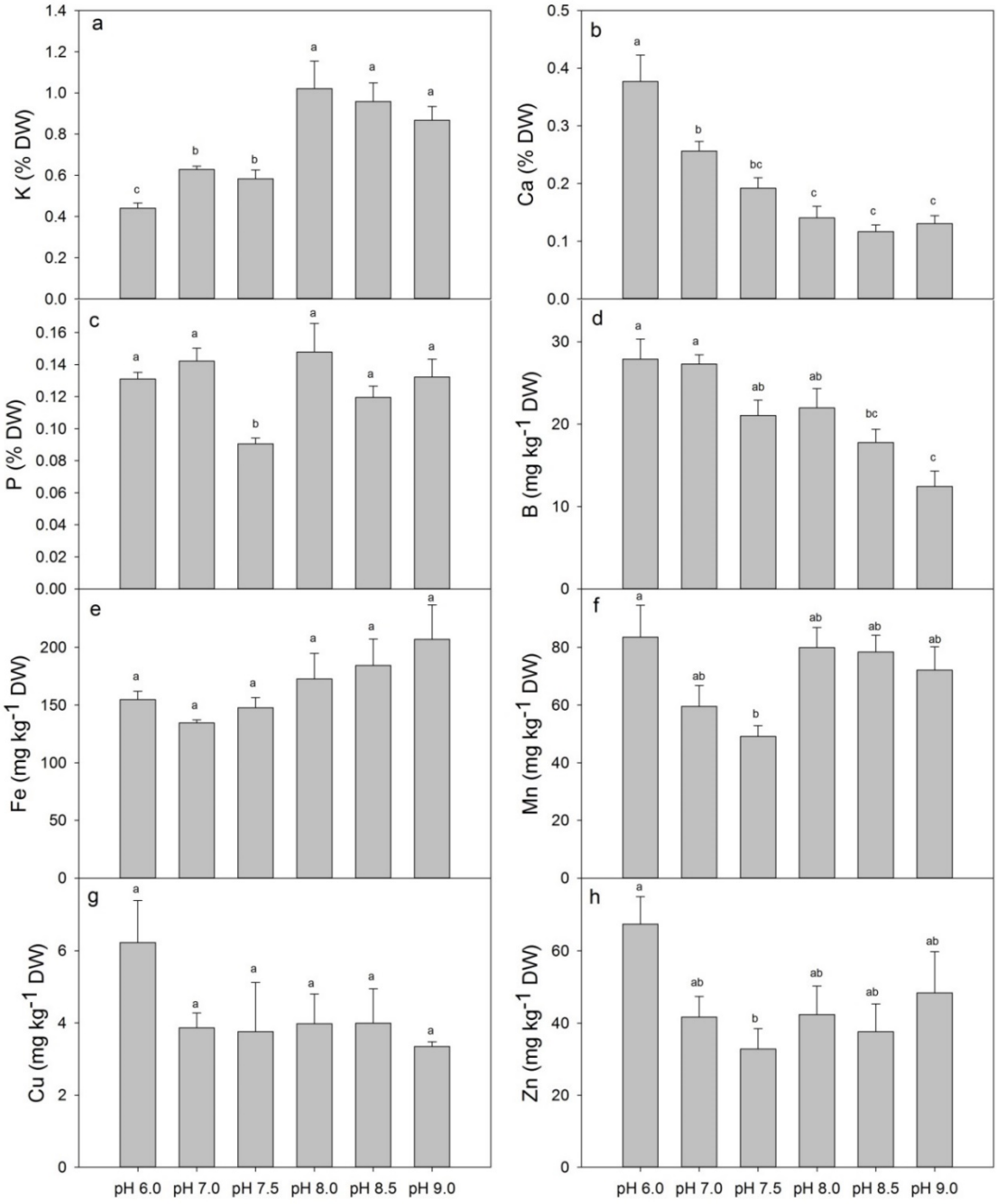

Figure 3. 5 Effects of root zone pH on K, Ca, P, B, Fe, Mn, Cu and Zn concentrations in young needles of jack pine seedlings in Experiment 1. Different letters above the bars indicate significant differences (α=0.05) between treatments determined by Student-Newman-Keuls test. Means (n=6) ± SE are shown.

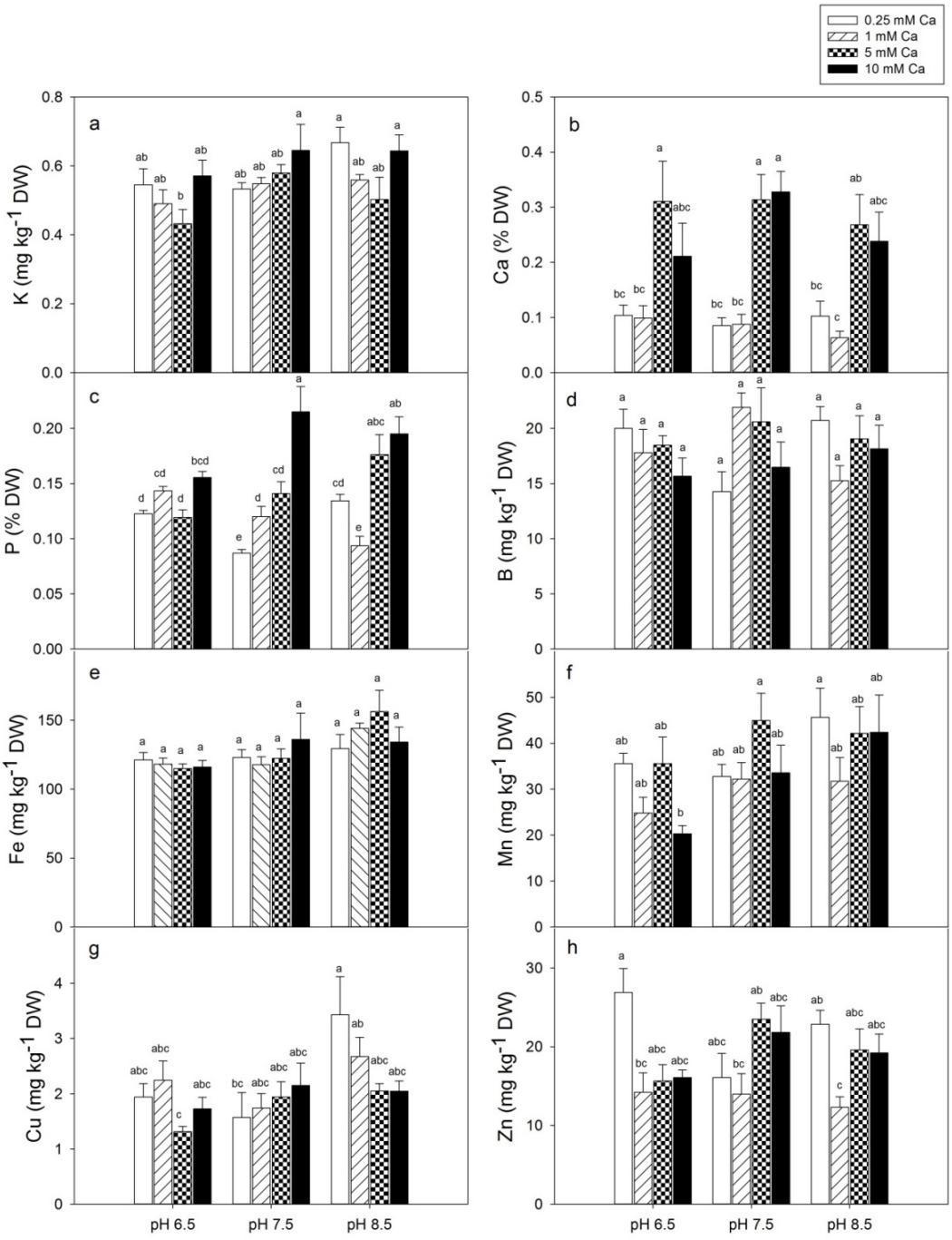

Figure 3. 6 Effects pH and Ca treatments on K, Ca, P, B, Fe, Mn, Cu and Zn concentrations in young needles of jack pine seedlings in Experiment 2. Different letters above the bars indicate significant differences (α=0.05) between treatments. Means (n=6) ± SE are shown.

Chapter 4

Effects of phosphorus and calcium availability on plant responses to high pH in split-root hydroponic system

4.1 Introduction

Phosphorus is one of the most frequently limiting nutrients to plant growth (Schachtman et al. 1998; Shen et al. 2011). Calcareous soils, which cover 25-30% of the world's land surface (Wallace and Lunt 1960), are characterized by high Ca levels and high soil pH ranging from 7.5 to 8.5 (Marschner 2012). Phosphorus availability is extremely low in calcareous soils, due to its adsorption to soil particles and clay minerals as well as surfaces of calcium carbonates (Rausch and Bucher 2002; Jing et al. 2010). The concentration of available soil P in calcareous soils is usually lower than 10 μM (Bieleski 1973), whereas in plant tissues, the concentration of P is approximately 5 to 20 mM (Raghothama 1999). In addition, the diffusion rate of P in soil is extremely low, with the diffusion coefficient of 10^{-12} to 10^{-15} m s^{-1} (Schachtman et al. 1998).

In the oils sands reclamation areas in Northeast Alberta, Canada, the soil Ca concentration in some reclamation sites can exceed 400 mg kg^{-1}, compared with less than 6 mg kg^{-1} typically found in natural soils (Visser 2005). This is partly due to the use of gypsum in tailings management processes (Ramos-Padrón et al. 2010). Therefore, P content in reclaimed soils has also been reported to be lower than in undisturbed soils (Visser 2005). Moreover, salinity is another severe problem that adversely affects plant growth in oil sands reclamation areas. The soil electrical conductivity (EC) at some reclamation sites can be as high as 10 mS cm^{-1} (Howat 2000; Kessler et al. 2010).

High root zone pH can inhibit root cell elongation and the formation of lateral roots and root hairs (Tang et al. 1993; Canmore-Neumann et al. 1996). It is thought that plants absorb soil P mainly by root hairs and root tips (Gahoonia et al. 1997; Raghothama 1999), therefore, high soil pH might also inhibit P absorption at the root surface. The major functions of P in plants are related to their presence as part of nucleic acids and phospholipids as well as the phosphorylated intermediates of energy metabolism (Shen et al. 2011; Marschner 2012). In P-deficient plants, the most obvious effects are reductions in leaf expansion (Fredeen et al. 1989), number of leaves (Lynch et al. 1991) and shoot-to-root ratios, since shoot growth is usually more inhibited than root growth (Marschner 2012). The strategies of plants to cope with low soil P availability include associations with mycorrhizae (Bolan 1991), formation of cluster roots (Lambers et al. 2006), and exudation of organic acids (Marschner 1995).

In addition to P, high pH of calcareous soil can also induce low availability of micronutrients such as Fe and Mn (Mengel 1994; Dordas 2009; Zhang et al. 2013). Both Fe and Mn can profoundly affect photosynthetic rate, as Fe plays a key role in chlorophyll synthesis and Mn is required for water photolysis in photosystem II (Marschner 2012). As plants only need very low amounts of micronutrients for growth, part of the root system in contact with suitable soil pH might supply enough micronutrients for the whole plant. Plant roots can change the soil pH in the rhizosphere by 1-2 units due to H^+/OH^- release, respiration, organic acid extrusion and redox reactions (Nye 1981; Hinsinger et al. 2003). Split-root system is often used to study plant responses to different root environments. In such studies, roots of individual plants are divided between two compartments in which soil conditions such as osmotic potentials, salt concentration, or different nutrients can be controlled (Zekri and Parsons 1990).

In the present study, I investigated the growth and physiological responses of six boreal forest tree species to root medium pH, osmotic stress, and different P and Ca levels using a split-root hydroponic system. The main purpose of this study was to investigate the effects of increased P supply and heterogeneous root zone pH on plant responses to high pH and Ca treatments. I hypothesized that high levels of P and low root zone pH applied to one part of the root system while the other part is subjected to high pH, would help plants cope with the high pH conditions and high Ca concentrations.

4.2 Materials and methods

4.2.1 Plant material and growth conditions

One-year-old, container-grown paper birch (*Betula papyrifera* Marsh.), jack pine (*Pinus banksiana* Lamb.), green alder [*Alnus viridis* (Chaix.) D.C.], trembling aspen (*Populus tremuloides* Michx.), black spruce [*Picea mariana* (Mill.) B.S.P.], and white spruce [*Picea glauca* (Moench) Voss] dormant seedlings were obtained from the Boreal Horticultural Services Ltd., Bonnyville, Alberta, Canada. These seedlings were grown from seeds collected in different locations close to Fort McMurray, Alberta, Canada. Seedling roots were gently washed to remove the potting medium and placed in 50% modified Hoagland's solution (Epstein 1972) for two weeks in a controlled-environment growth room before the commencement of treatments. Environmental conditions in the growth room were: 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) at the top of the seedlings provided by the full spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada).

Two 11 L plastic tubs (18 x 31 cm and 40 cm deep) were glued side-by-side to form a split-root growth container. Four split-root growth containers were connected to two 40 L plastic pails (dimensions 31 x 40.5 cm and 62 cm deep) containing a circulating pump (Model 9.5 950GPH, Danner MFG Inc., New York, USA) and a pH electrode immersed in treatment solution. The pump continuously circulated the solution between the tubs and the pail. The pH electrode was connected to a pH controller (PHCN-70, Omega Engineering Inc., Laval, QC, Canada) for a continuous control of solution pH. The pump in one of the two pails (left) was connected with all of the left tubs of split-root growth containers by the PVC tubing, and the same for the other (right side) pail and the right side tubs. All tubs had spouts installed into their sides to facilitate drainage and circulation of nutrient solution. Styrofoam lids were used to cover the split-root containers. Along the center of each lid, 8 holes (diameter 3 cm) were cut, so that seedling roots could be slipped into the nutrient solution through the lid. Foam plugs were fitted around the stems, and inserted into the holes to hold the stems in place while the roots were immersed in solution, with the stems protruding through the lid. In the first two weeks, all roots were in one side of the split-root container. Two weeks later, when the roots grew longer, they were divided evenly and placed in each of the two parts of the split-root container. The plants of six species were divided into two groups: paper birch, jack pine and black spruce formed one group, and green alder, aspen and white spruce formed another group. These two groups were studied separately in two independent experiments. In each split-root container, eight seedlings from the three species were sequentially placed so that the seedlings of the different species were always separated. For each treatment, there were ten to eleven seedlings per species.

4.2.2 Treatments

The seedlings were treated with two pH levels (5 and 9), two Ca levels (2 mM and 50 mM) and two P levels (0.5 mM and 15 mM). The combinations of treatment factors are illustrated in Figure 4.1. In each set-up, the pump circulated 70 L 50% modified Hoagland's solutions between one pail and four connected tubs. The amounts of $Ca(NO_3)_2$ and $NH_4H_2PO_4$ were calculated from the formula of modified Hoagland's solutions (Epstein 1972) to achieve the required P and Ca solution concentrations. The solution pH was adjusted by adding 5% (w/v) KOH or 1% (v/v) H_2SO_4 using the automatic pH controller. During the experiments, the variation of solution pH was maintained within ± 0.1 range. The treatment lasted for 6 weeks. The nutrient solutions were replaced every 2 weeks.

4.2.3 Elemental analysis of nutrient solution

To quantify the solubility of essential elements in the solution, 1 L of each of the six treatment solutions were prepared. The solution pH was then altered to 5 and 9 with 5 % KOH (w/v) or 1 % H_2SO_4 (v/v). For each treatment solution, three 20 ml nutrient solution samples were filtered with 0.45 μm Polyvinyl Difluoride (PVDF) syringe-driven filter unit (EMD Millipore Corporation, Billerica, MA, USA). The analyses of soluble elements in filtered solutions were carried out with the inductively coupled plasma mass spectrometry (ICP-MS) (Zarcinas et al. 1987) in the Radiogenic Isotope Facility of the University of Alberta. The electrical conductivity of solutions was assayed with the conductivity meter (Model Accumet Research AR20, Fisher Scientific, Ottawa, ON, Canada).

4.2.4 Shoot height and dry weight

Immediately before and after six weeks of treatments, seedling heights were measured from the root collar to the shoot tip. The relative shoot height growth was calculated by dividing

the difference in the initial and final values by the initial value. Shoot and root dry weights were determined for all seedlings from each treatment (n =10). For roots grown in pH 9-50 mM Ca, at the end of the experiment, Ca precipitation was observed on the root surface. Therefore, the roots were soaked in 1% HCl for 8 h before drying. Roots and stems were dried in an oven at 70°C for 72 h. The leaves (or needles) were divided into old and young classes. The leaves that were fully elongated before the onset of treatments were regarded as old leaves while the young leaves were those that started elongating after the onset of treatments and were close to the shoot tips. The leaves were separated from stems and immediately placed in an ultra-low temperature freezer at -80^{o}C for storage before freeze-drying for 72 h. When dry, all leaves and stems from each plant were combined to determine shoot dry weights.

4.2.5 Net photosynthesis (Pn) and transpiration (E) rates

After six weeks of treatments, for each species, eight seedlings (n = 8) were randomly taken from each treatment group for the measurements of Pn and E. The measurements were conducted with the infrared gas analyzer (LI-6400, LI-COR, Lincoln, Nebraska USA) at 400 μmol m^{-2} s^{-1} PPFD. For conifer measurements, about 3-cm distal parts of needles were inserted in the leaf chamber. To determine needle areas in black spruce, white spruce and jack pine, the needles in the leaf chamber were cut with scissors and scanned. The needle areas were calculated using the Sigmascan Pro 5.0 computer software (Systat Software, San Jose, CA).

4.2.6 Leaf chlorophyll concentrations

Chlorophyll-a (chl-a) and chlorophyll-b (chl-b) concentrations of old and young leaves were determined in eight randomly selected seedlings per treatment (n = 8). The freeze-dried leaves were ground with a Thomas Wiley Mini-Mill (Thomas Scientific, NJ, USA). For paper

birch, jack pine, aspen, black spruce and white spruce, chlorophyll was extracted from pulverized leaf samples (10 mg dry weight) with 8 ml dimethyl sulfoxide (DMSO) at 65℃ for 22 h. Chlorophyll concentrations were measured in DMSO extracts with a spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden), at 648 nm and 665 nm for chlorophyll-a and chlorophyll-b. Total chlorophyll concentration was calculated using Arnon's equation (Sestak et al. 1971). Since the DMSO extracts of green alder leaves were black, which interfered with chlorophyll analysis, chlorophyll was extracted from green alder leaves with 8 ml methanol at 55℃ for 24 h, and measured at 652 nm and 665 nm for chlorophyll-a and chlorophyll-b with a spectrophotometer (Wellburn 1994).

4.2.7 Elemental concentrations in old and young leaves

Since leaf chlorosis mainly occurred in young leaves, the elemental analyses were carried out in young leaves. Six seedlings were randomly selected per species from each treatment (n = 6). For all 6 species P, K, Ca, Fe and Mn were analyzed in leaves. For green alder, jack pine and black spruce, nitrogen was also analyzed. For P, K, Ca, Fe and Mn analysis, pulverized leaf samples (0.2 g dry weight) were digested with 10 ml 70% HNO_3 and heated for 10 minutes at 185℃ in a microwave oven (Mars 5 Microwave Accelerated Reaction System, CEM, Matthews, NC, USA) and diluted with Milli-Q water to 40 ml. The extracts were then filtered and analyzed by ICP-MS in the Radiogenic Isotope Facility of the University of Alberta (Zarcinas et al. 1987). For nitrogen concentration analysis, pulverized leaf samples (3 mg dry weight) were wrapped in aluminum foil, and analyzed in the Biogeochemical Analytical Service Laboratory (BASL) in University of Alberta by CHN analyzer (CE440 Elemental Analyzer).

4.2.8 Statistical analysis

The data for the root dry weights in the left and right sides of the containers were analyzed using paired t test by SAS (Version 9.2, SAS Institute Inc., Cary, NC). All other data were analyzed using SAS GLM model to determine statistically significant ($p \leq 0.05$) differences between treatments. The data that did not meet the assumptions of normality of distribution and homogeneity of variance were transformed with a log10 function. Comparisons between different treatment means were conducted using Student-Newman-Keuls (SNK) test.

4.3 Results

4.3.1 Elemental analysis and electric conductivity of nutrient solution

The actual P concentration in the 15 mM treatment solution was about 14 mM, due to the reaction of P with other elements; while for the 0.5 mM P treatment solution, the measured concentration was also 0.5 mM (Table 4.1). The measured Ca concentration of 2 mM treatment solution was about 1.9 mM, while for the 50 mM treatment solution it was about 40 mM (Table 4.1). In pH 9-15 mM P treatment solution K concentration was 24.6 mM, which was higher than in the other solutions (3-4 mM). Iron concentration was reduced only in pH 9-50 mM Ca treatment solution, with a reduction of about two-fold compared with the other treatment solutions. In 15 mM P treatment solutions, Mn concentration decreased from 1 μM at pH 5 to 0.26 μM at pH 9, while Mg concentration decreased from 0.53 μM at pH 5 to 0.03 μM at pH 9 (Table 4.1). For Zn, the concentration decreased by about two-fold at pH 9 compared with pH 5 in all treatment solutions (Table 4.1).

The electrical conductivity of 50 mM Ca solution was about 10 mS cm^{-1}, and in pH 9-15 mM P solution it measured 3.65 mS cm^{-1}, due to the relatively higher amount of KOH that was used to increase the solution pH (Table 4.1).

4.3.2 Relative shoot height growth (RSHG)

No significant differences were found for RSHG between the treatments in white spruce and black spruce (Fig. 4.2e,f). For other species of pH 9:9 treatments, the RSHG was lower compared with pH 5:9 (or 9:5) and pH 5:5 treatments (Fig. 4.2a,b,c,d). In pH 9:9 treatments, for all species, the Ca and P concentrations had no significant effect on RSHG (Fig. 4.2). For paper birch in pH 9:5 treatments, RSHG was similar to pH 5:5 treatments, while for the pH 5:9 treatments, RSHG was slightly lower than pH 5:5 (Fig. 4.2a). For jack pine in 50 Ca:0.5 P-pH 5:9 treatment, RSHG was markedly lower compared with the other pH 5:9 (or 9:5) and pH 5:5 treatments (Fig. 4.2b). For green alder and aspen, all pH 5:9 (or 9:5) treatments had similar RSHG that were higher than pH 9:9 treatments and lower than pH 5:5 treatment (Fig. 4.2c,d).

4.3.3 Total dry weights

In jack pine, black spruce and white spruce, there were no clear differences in total dry weights between treatments. The only exceptions were slightly higher dry weights in jack pine exposed to 2 Ca:0.5 P and 2 Ca:15 P of pH 5:9 treatments compared with the remaining treatments. Also, total dry weights were slightly higher in black spruce exposed to 2 Ca:0.5 P-pH 5:9 treatment than in the plants subjected to other treatments (Fig. 4.3b,e,f). The most prominent difference in paper birch was higher total dry weight in 2 Ca:0.5 P-pH 9:5 and pH 5:5 treatments compared with the other treatments (Fig. 4.3a). In green alder, total dry weights were slightly higher at pH 5:5 compared with the remaining treatments (Fig. 4.3c). In aspen, total dry weights were reduced by the 50 Ca:15 P and 2 Ca:15 P treatments of pH 5:9 and by all pH 9:9 treatments compared with other experimental groups (Fig. 4.3d).

4.3.4 Root dry weights

In paper birch, only in 50 Ca:15 P-pH 9:9 treatment, the root dry weights in the Ca side of the container were significantly higher compared with the P side of the container, and in 2 Ca treatment they were also higher than in 15 P treatment at pH 9:9. For pH 5:9 (or 9:5) treatments, generally, the root dry weights of the pH 5 side was higher compared with the pH 9 side, except in the 50 Ca pH 5 treatment where it was lower than in 0.5 P pH 9 (Fig. 4.4a).

For jack pine, root dry weights in the Ca side of the containers were significantly higher compared with the P side in the 50 Ca:0.5 P-pH 5:9 treatment (Fig. 4.4b).

For green alder, root dry weights in the Ca side of the container were significantly higher than in the P side in the 2 Ca:15 P-pH 5:9, 2 Ca:0.5 P, and 2 Ca:15 P of pH 9:9 treatments. In 2 Ca: 0.5 P-pH 9:5 treatment, root dry weight was significantly higher in P side than Ca side. In pH 5:9 (or 9:5) treatments, root dry weights in the Ca side were generally higher when at pH 5, except in the 50 Ca and 15 P of pH 5 treatment where there was no difference between the Ca and P sides (Fig. 4.4c).

Root dry weights in aspen were significantly higher in the 2 Ca pH 5 than 0.5 P and 15 P of pH 9, 2 Ca pH 9 than 15 P pH 5, and 0.5 P pH 9 than 50 Ca pH 5 treatment (Fig. 4.4d). In the pH 9:9 treatments, P and Ca concentration had little effects on root dry weights (Fig. 4.4d).

In black spruce, root dry weight was only significantly higher at 15 P pH 5 than 2 Ca pH 9, and generally, the root dry weights were similar in all treatments (Fig. 4.4e).

For white spruce, root dry weights were significantly higher when exposed to 0.5 P of pH 5 compared with 2 Ca of pH 9, and 2 Ca compared with 15 P of pH 9:9 treatments (Fig. 4.4f). For the remaining treatments Ca and P sides had similar root dry weights (Fig. 4.4f).

4.3.5 Net photosynthesis (Pn) and transpiration (E) rates

In paper birch and aspen, Pn was significantly reduced for all pH 9:9 treatments, and it was reduced more by the 50 Ca:15 P-pH 9:9 treatment than other pH 9:9 treatments in paper birch. For pH 9:5 treatments, Pn was similar to pH 5:5 treatment, while in pH 5:9 treatments, Pn was moderately reduced compared with pH 5:5 treatment (Fig. 4.5a,d).

For jack pine and black spruce, there were no significant differences found across all treatments. In jack pine subjected to pH 9:9 treatments and 50 Ca:0.5 P-pH 5:9 treatment, Pn was reduced to zero and negative values (Fig. 4.5b,e).

In green alder and white spruce, Pn was at the highest level when the seedlings were subjected to pH 5:5 treatments and was moderately reduced by the 2 Ca:0.5 P-pH 9:5 treatment in green alder and by pH 9:5 treatments in white spruce (Fig. 4.5c,f).

For all species, the patterns of E were similar to those of Pn in all treatments (Fig. 4.6). Minor differences included those in aspen seedlings subjected to 50 Ca:15 P-pH 9:9 treatments in which E was more reduced compared with the other pH 9:9 treatments, while for Pn the values were similar for all pH 9:9 treatments (Fig. 4.6d).

4.3.6 Chlorophyll concentrations

In paper birch, green alder, aspen and white spruce, chlorophyll concentrations were lower in young leaves compared with old leaves (Fig. 4.7a,c,d,f). In paper birch, chlorophyll concentrations were severely reduced by all pH 9:9 treatments, and also moderately reduced by 50 Ca:15 P, 2 Ca:0.5 P and 50 Ca:0.5 P treatments of pH 5:9 (Fig. 4.7a). In jack pine, chlorophyll concentrations were mainly reduced by the 50 Ca:0.5 P-pH 5:9 treatment and by all pH 9:9 treatments (Fig. 4.7b). In green alder, aspen, and black spruce, chlorophyll concentrations were mainly reduced by the pH 9:9 treatments (Fig. 4.7c,d,e). In white spruce subjected to pH

9:9 treatments, chlorophyll concentrations in young needles were reduced by about two-fold compared with pH 5:5 treatments (Fig. 4.7f), while in pH 5:9 treatments, the reductions were about 30% (Fig. 4.7f).

4.3.7 Elemental concentrations

Leaf N concentrations were little affected by the treatments (Fig. 4.8). In jack pine, needle N concentrations were slightly higher in seedlings subjected to 2 Ca:0.5 P-pH 5:9 and 2 Ca:15 P-pH 9:5 treatments and to pH 5:5 treatments (Fig. 4.8a). In green alder, leaf N concentrations were slightly elevated in seedlings that were subjected to 2 Ca:15 P-pH 9:5 and 2 Ca:15 P-pH 9:9 treatments, and reduced by the 50 Ca:15 P-pH 9:9 treatment compared with other treatments (Fig. 4.8b). However, the difference between highest and lowest concentrations was only about 20%. For black spruce, the differences in N concentrations were also minor, with slightly higher N concentrations measured in pH5:5 treatments (Fig. 4.8c).

In paper birch, leaf K concentrations were similar in all pH 9:9 treatments and were about two-fold higher compared with pH 5:5 (Fig. 4.9a). For the pH 5:9 (or 9:5) treatments, leaf K concentrations were intermediate between pH 5:5 and pH 9:9 with the exception of 50 Ca:15 P and 2 Ca:15 P of pH 5:9 treatments, which were similar to pH 5:5 treatments (Fig. 4.9a). In jack pine, there were no significant differences in needle K concentrations across all treatments (Fig. 4.9b). In green alder, black spruce, and white spruce, K concentrations were generally moderately lower in pH 5:9 treatments, while there were no significant differences for the remaining treatments (Fig. 4.9c,e,f). In aspen, leaf K concentrations were moderately lower in 50 Ca:15 P, 2 Ca:0.5 P and 2 Ca:15 P treatments of pH 5:9. There was also a small reduction in pH 5:5 treatment and in the 50 Ca:15 P-pH 9:9 treatment compared with 50 Ca:0.5 P-pH 5:9 treatment (Fig. 4.9d).

Paper birch, green alder and aspen showed similar patterns in leaf Ca concentrations in response to the applied treatments. Leaf Ca concentrations were the highest in 50 Ca:15 P-pH 5:9, 50 Ca:15 P and 50 Ca:0.5 P of pH 9:9 treatments and the lowest in the 2 Ca:0.5P-pH 9:5 treatment (Fig. 4.10a,c,d). In jack pine, needle Ca concentrations were the highest in the 50 Ca:15 P-pH 5:9 treatment. Other treatments with slightly elevated Ca concentrations compared to pH 5:5 treatment included 50 Ca:0.5 P-pH 5:9 and 50 Ca:15 P-pH 9:9 (Fig. 4.10b). In black spruce and white spruce, needle Ca concentrations were higher in 50 Ca:15 P and 50 Ca:0.5 P of pH 5:9, and 50 Ca:0.5 P-pH 9:9 treatments, while for the remaining treatments, Ca concentrations were not significantly different (Fig. 4.10e,f).

In paper birch, leaf P concentrations were about four-fold higher in 2 Ca:15 P-pH 5:9, pH 9:5 and pH 5:5 treatments compared with the remaining treatment groups (Fig. 4.11a). In jack pine, needle P concentrations were about 20 to 30% higher in 2 Ca:15 P-pH 9:5 treatment compared with other treatments (Fig. 4.11b). In green alder, leaf P concentrations were higher in pH 9:5, pH 5:5 and 50 Ca:15 P-pH 9:9 treatments compared with the remaining treatments (Fig. 4.11c). In aspen, P concentrations were the highest in pH 5:5 treatment and were reduced by about two-fold in the 50 Ca:15 P-pH 5:9 treatment than pH 5:5 (Fig. 4.11d). In black spruce, needle P concentrations were slightly higher in the 2 Ca:15 P-pH 9:5 treatment than the other treatments (Fig. 4.11e). In white spruce, needle P concentrations was significantly higher in 2 Ca:15 P-pH 9:5 compared with other treatments(Fig. 4.11f).

In paper birch, leaf Fe concentrations were lower in all pH 9:9 treatments, and in 50 Ca:0.5 P-pH 5:9 and 2 Ca:15 P-pH 9:5 treatments compared with other treatments (Fig. 4.12a). In jack pine, Fe concentrations were about 20% lower in 50 Ca:0.5 P-pH 5:9 and 2 Ca:0.5 P-pH 9:9 treatments compared with other treatments (Fig. 4.12b). Green alder leaf Fe concentrations

were similar in all treatments, and in aspen, Fe concentrations were slightly, but significantly, higher in the 50 Ca:15 P-pH 5:9 treatment compared with other treatments (Fig. 4.12c,d). In black spruce, Fe concentrations were about 20% higher in pH 5:5 treatment compared with other treatments, while in white spruce, Fe concentrations were about 10% higher in 2 Ca:0.5 P-pH 9:5 and pH 5:5 treatments compared with other treatments (Fig. 4.12e,f).

Leaf Mn concentrations of paper birch were higher in 2 Ca:0.5 P and 2 Ca:15 P treatments of pH 5:9, and pH 5:5 treatments compared with other treatments (Fig. 4.13a). In jack pine, there were no significant differences present in Mn concentrations between the treatments (Fig. 4.13b). In green alder, at 2 Ca:0.5 P, 2 Ca:15 P of pH 5:9 and pH 9:5, and pH 5:5 treatments, Mn concentrations were higher compared with the remaining treatments (Fig. 4.13c). In aspen, Mn concentrations was significantly higher at 2 Ca:15 P-pH 9:5 than other pH 5:9 (or 9:5) treatments (Fig. 4.13d). In both black spruce and white spruce, there was generally only minor differences in Mn concentrations between treatments (Fig. 4.13e,f).

4.4 Discussion

In the present study, I investigated the effects of Ca and P application at different root zone pH on growth and physiological responses in seedlings of six boreal forest tree species. The environmental concerns in oil sands reclamation sites include high soil pH, excessive Ca levels, high soil electrical conductivity, and salinity (Howat 2000; Visser 2005). Since salinity effects on oil sands reclamation plants have been extensively studied (Renault et al. 1998; Renault et al. 2001; Calvo-Polanco et al. 2009), the present research focused on the effects of pH in association with elevated Ca and P levels.

The 50 mM Ca treatment solutions had a rather high EC (about 10 mS cm^{-1}). As a large

amount of KOH was used to adjust 15 mM P solution to pH 9, the EC of 15 P-pH 9 treatment solutions were also relatively high (3.65 mS cm^{-1}). In these two types of treatment solutions the osmotic potential was likely sufficiently low to create a significant osmotic effect and alter root water uptake. In the six studied species, the shoot height growth and dry weights of black spruce and white spruce seedlings were little affected by the applied treatments. These results demonstrate that both spruce species are relatively resistant to high pH and osmotic stress compared with the other studied species, which is in accordance with the results of the study presented in Chapter 2 and 3. Of the applied treatment factors, it appears that high pH was the main factor responsible for the effects on plant growth and physiological parameters including chlorophyll concentrations, net photosynthesis and transpiration rates, while the effects of P and Ca were relatively modest, especially in pH 9:9 treatments.

In hydroponic culture, high pH severely reduced root growth in aspen and tamarack (Chapter 2). However, in the present study, root zone pH 5 and 9 had little effect on root dry weights in the Ca and P side of the container in the same seedling. Only in jack pine seedlings subjected to 50 Ca:0.5 P-pH 5:9 treatment, in green alder subjected to 2 Ca:15 P-pH 5:9 and 2 Ca:0.5 P-pH 9:5, in aspen subjected to 2 Ca:0.5 P and 2 Ca:15 P of pH 5:9, in black spruce subjected to 2 Ca:15 P-pH 9:5, and in white spruce subjected to 2 Ca:0.5 P-pH 9:5 treatment, the root dry weights were significantly higher at pH 5 compared with pH 9. In aspen seedlings subjected to 50 Ca:0.5 P-pH 5:9 and 2 Ca:15 P of pH 9:5 treatments, root dry weights in pH 9 were higher than in pH 5. In the 50 Ca-pH 5 treatment, roots were exposed to low osmotic potential (EC 10 mS cm^{-1}) and the resulting osmotic stress could have affected root growth. In the present study, when Ca was supplied only in the pH 9 medium, the roots morphology and function was likely altered by high pH and it is plausible that these changes could facilitate root

Ca uptake and support growth processes. Ca treatments of plants can ameliorate salinity (Cabañero et al. 2004) and aluminum (Kinraide and Parker 1987) stresses in plants, probably due to the role of Ca in maintaining membrane integrity and selectivity (Grattan and Grieve, 1998). As high pH could increase membrane permeability (Zieslin and Snir 1989), in this study, Ca might also help aspen seedlings resist high pH stress.

A value of 4 mS cm^{-1} of soil EC is widely regarded as a threshold of salinity stress for plants, partly because EC higher than this value imposes osmotic stress on plants and affects water uptake (Richards 1954; Sairam and Tyagi 2004). However, in hydroponic culture, due to lack of buffering capacity of soils, the threshold of EC values to induce stress on plants is probably lower than 4 mS cm^{-1}. Therefore, roots exposed to 50 mM Ca (EC 10 mS cm^{-1}) were exposed to osmotic stress, and in 15 mM P solution at pH 9 (EC 3.65 mS cm^{-1}) osmotic stress was probably also a significant factor. In all species examined, the transpiration rates of plants subjected to 50 Ca:15 P treatments in both pH 5:9 (or 9:5) and pH 9:9 were reduced compared with 2 Ca:0.5 P-pH 5:5 group, indicating that the treatment, at least initially, affected plant water balance and suggesting osmotic effects. In aspen exposed to 50 Ca:15 P treatment at pH 5:9, transpiration was higher than that in the same treatment at pH 9:9, which demonstrates that high pH aggravated the negative effects of osmotic stress on transpiration. High soil pH was reported to reduce root water uptake (Kamaluddin and Zwiazek 2004), likely due to the effect of pH on the function of aquaporins (Tournaire-Roux et al. 2003; Siemens and Zwiazek 2011).

The critical needle concentration values for P and Ca deficiencies were reported to be 0.14% and 0.1%, for white spruce trees (Ballard 1986). Therefore, in this study, white spruce did not likely suffer from Ca deficiency in all treatments. For treatments of 2 Ca:0.5 P, 50 Ca:0.5 P, 2 Ca:15 P of pH 5:9, and 2 Ca:0.5 P, 2 Ca:15 P of pH 9:9, white spruce appeared to suffer from P

deficiency. There is no readily available information for the tissue requirements for these elements in the other tree species examined in the present study.

In soil solution, P availability seldom exceeds 10 μM (Bieleski 1973). The optimum pH for phosphorus uptake in plants is between pH 5 and 6 (Furihata et al. 1992). Phosphate anions are co-transported with H^+ into cytosol, due to the negative plasma membrane potential created by the cytosolic and apoplastic pH gradient (Ullrich-Eberius et al. 1984). Therefore, high root zone pH can reduce root phosphorus uptake and lead to P deficiency. To counteract this effect, I used 0.5 and 15 mM P which are much higher than the P concentrations in soil solution, with the expectation that the oversupply of P could offset the inhibitory effect of high root zone pH on P uptake. Since P is a component of nucleotides and phospholipids, P deficiency profoundly affects plant growth. In the present study, RSHG in paper birch was much higher when P was supplied at pH 5 compared with pH 9. However, in paper birch subjected to 2 Ca:15 P treatments, pH 5:9 and pH 9:5 had similar leaf P concentrations, yet RSHG in pH 9:5 treatment was much higher compared with pH 5:9 treatment. This demonstrates that P can be absorbed in paper birch equally well at pH 5 and 9, but probably in different forms since the pH gradient across the plasma membrane was likely disrupted by the high root zone pH (Felle and Hanstein 2002). It was suggested that $H_2PO_4^-$ may be the dominant form for plant P uptake compared with HPO_4^{2-} or PO_4^{3-} (Schachtman et al. 1998). Therefore, at high pH, P utilization rate for new cell synthesis might be reduced and affect plant growth. However, only in paper birch, RSHG was higher when P was supplied at pH 5 compared with pH 9, and in the other species it was the opposite. This means that utilization of P at different pH may vary between different plant species.

Although the plants were treated with high concentrations of both Ca and P, in plants provided with additional Ca, the leaf Ca concentration could be much higher than 2 Ca:0.5 P-pH

5:5 group; while in plants provided with additional P, the leaf P concentrations were the same as in 2 Ca:0.5 P-pH 5:5 group. Phosphorus toxicity seldom exists in plants, as plant can down regulate expression of phosphate transporters (Dong et al. 1999). Two phosphate transport systems have been reported in plants, and when P is not deficient in the growth medium, the low affinity system may be activated (Schachtman et al. 1998). Calcium toxicity is also rare in plants since excessive Ca can be stored in vacuoles as oxalate crystals (Nakata 2003).

Leaf chlorisis is a frequent symptom in plants growing in alkaline soils, and is mostly caused by Fe or Mn deficiencies (Mengel and Geurtzen 1986; Mengel 1994; Dordas 2009). In the previous study (Chapter 2), leaf chlorophyll concentrations decreased significantly at high root zone pH in high pH sensitive species including aspen and tamarack. In the present study, leaf chlorophyll concentrations at pH 5:9 (or 9:5) treatments were higher compared with pH 9:9 treatments, especially for young leaves. This confirms my hypothesis that partial low root zone pH could ameliorate the high pH stress in plants since it is more likely caused by the nutrient deficiencies than by direct or indirect toxicities. In the previous study (Chapter 2), leaf Fe concentrations in aspen and white spruce were reduced by more than 60% at pH 9 compared with pH 5 and the reductions of Mn were also substantial at high pH. However, in this study, compared with the pH 5:5 treatments, Fe concentrations in pH 9:9 treatments were reduced by about 40% in paper birch, and about 20% in black spruce and white spruce. The reductions in Mn leaf concentrations were only found in green alder in 2 Ca:0.5 P and 50 Ca:0.5 P of pH 9:9 treatments. None of these reductions were associated with the reductions in leaf chlorophyll concentrations, suggesting that the leaf chlorosis induced by pH 9:9 treatments in this study was not due to Fe or Mn deficiency. However, it could be argued that the foliar Fe distribution rather than total leaf Fe concentration is more important to Fe-induced chlorosis (Mengel 1994). Since

apoplastic pH of leaf cells was high, the Fe uptake from xylem sap might be inhibited, which could be due to reduced activity of ferric chelate reductase (Chaney et al. 1972).

In conclusion, there were no substantial beneficial effects of increasing P supply to the studied plants that were subjected to high root zone pH environment. However, exposure of a part of the root system to low pH could partly alleviate the effects of high pH including the effects on leaf chlorophyll concentrations possibly through micronutrients supply. These results demonstrate that the effects of high pH are more likely caused by nutrient deficiencies than direct or indirect toxicity factors. Therefore, plants growing in heterogeneous alkaline soils in oil sands reclamation areas, with the roots partly exposed to lower pH, should survive and grow better compared with plants growing in the soil with uniformly high root zone pH. High root zone pH profoundly affected P uptake in some of the studied tree species including paper birch and green alder. Plant roots may also develop some features to maintain Ca uptake under high pH conditions. I found white spruce and black spruce to be more resistant to high pH and high Ca concentrations compared with the other tree species examined in this study. Therefore, white spruce and black spruce should be expected to outperform other trees in oil sands reclamation sites where high soil pH and elevated Ca levels are of concern.

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4.6 Tables

Table 4. 1 Mean value of elemental concentrations and electrical conductivity (EC) of treatment solutions (n=3)

Treatments	B (μM)	Mg (mM)	P (mM)	K (mM)	Ca (mM)	Fe (μM)	Mn (μM)	Cu (μM)	Zn (μM)	EC (mS cm^{-1})
pH5-0.5mM P	12.457	0.509	0.505	3.045	0.007	7.499	1.010	0.238	0.973	0.65
pH9-0.5mM P	12.305	0.498	0.508	3.947	0.000	6.530	0.819	0.132	0.400	0.67
pH5-15mM P	18.163	0.530	13.78	3.346	0.003	7.569	1.016	0.286	0.969	2.01
pH9-15mM P	17.114	0.029	13.76	24.622	0.004	6.163	0.259	0.320	0.583	3.65
pH5-2mM Ca	11.948	0.543	0.002	3.216	1.907	6.882	1.055	0.268	1.052	1.02
pH9-2mM Ca	12.029	0.547	0.000	3.358	1.936	5.067	0.948	0.103	0.535	1.06
pH5-50mM Ca	9.746	0.854	0.000	2.942	41.222	7.686	2.258	0.238	0.870	10.00
pH9-50mM Ca	7.974	0.805	0.056	3.142	40.649	3.019	2.036	0.118	0.366	10.40

4.7 Figures

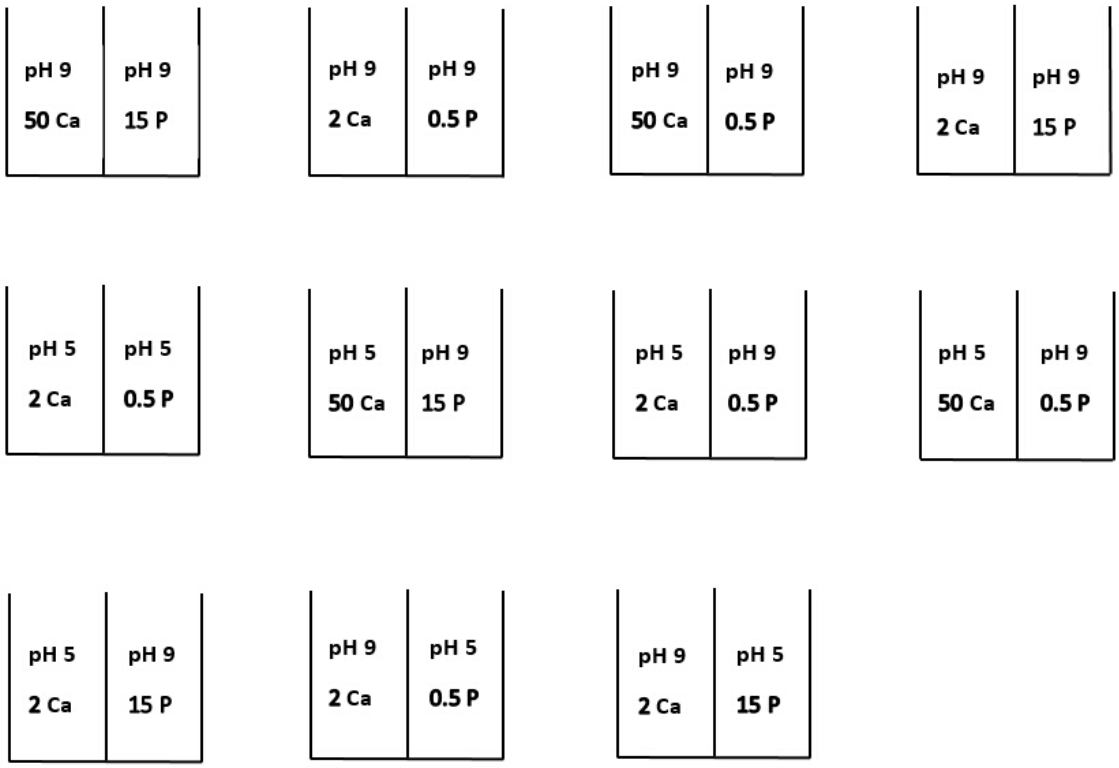

Figure 4. 1 Illustration of treatments in split-root containers. The unit for Ca and P concentrations is mM.

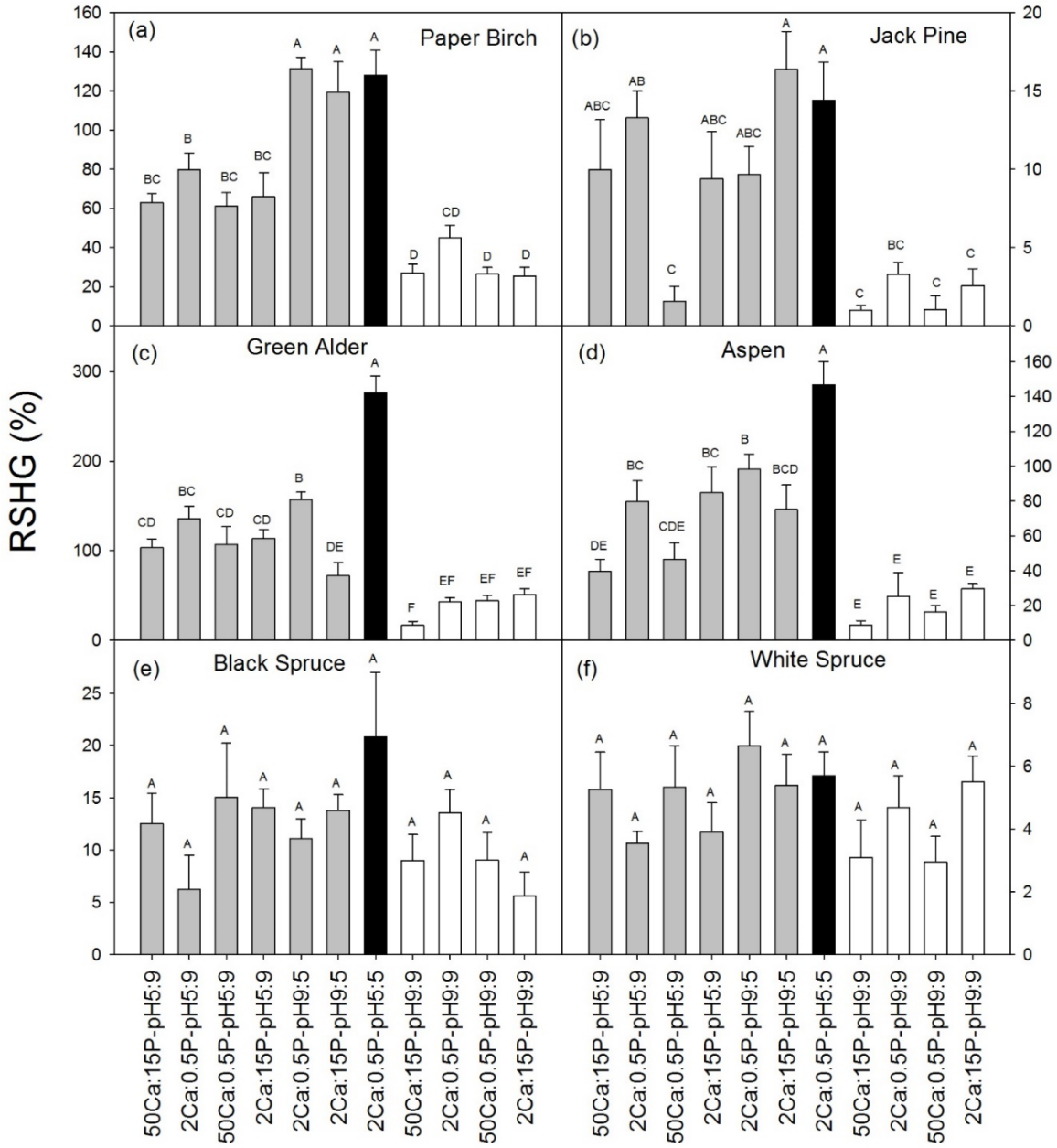

Figure 4. 2 Effects of pH, P and Ca treatments in split root design on relative shoot height growth (RSHG) of paper birch, jack pine, green alder, aspen, black spruce and white spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 10$) ± SE are shown.

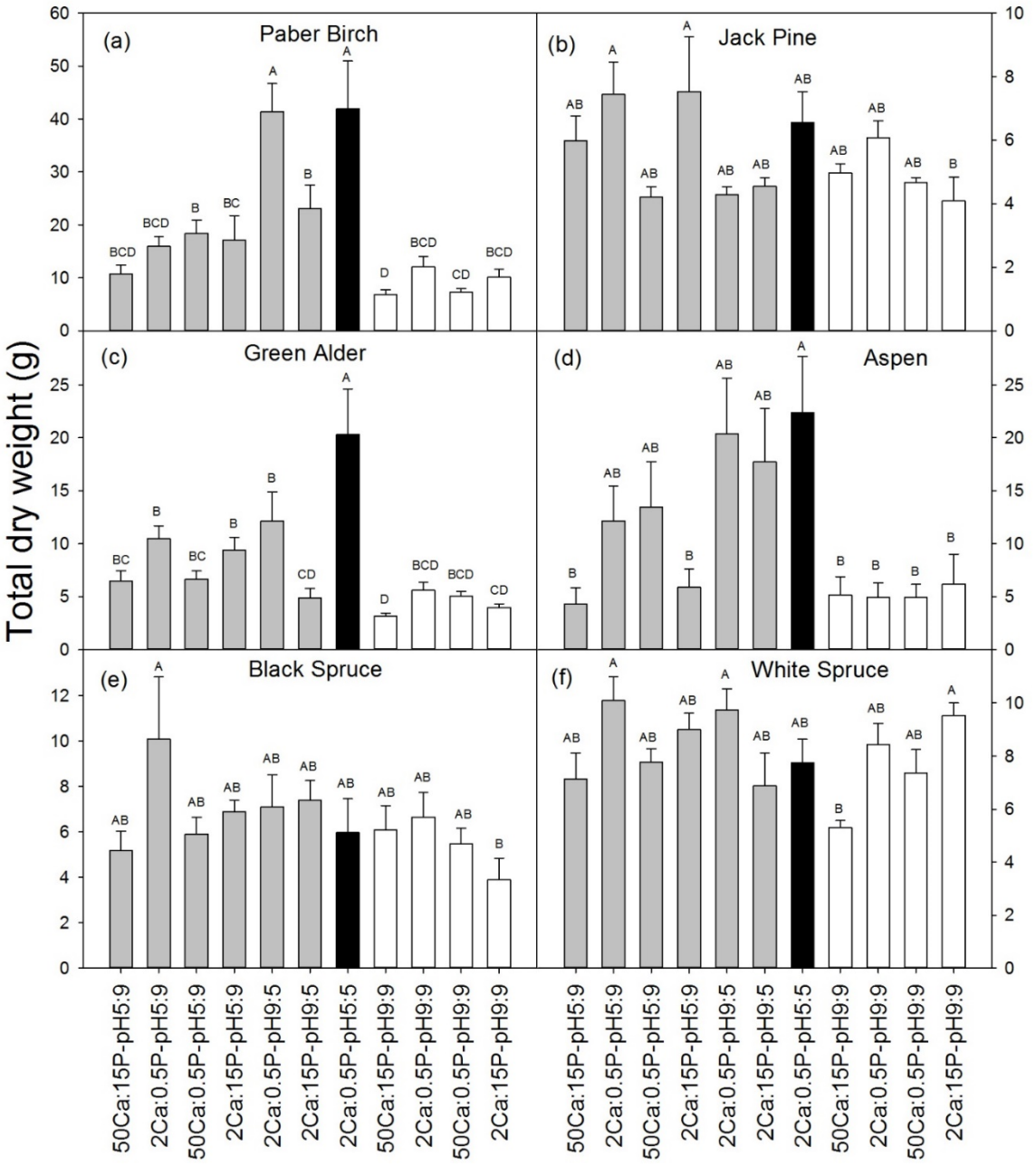

Figure 4. 3 Effects of pH, P and Ca treatments in split root design on total dry weights of paper birch, jack pine, green alder, aspen, black spruce and white spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 10$) ± SE are shown.

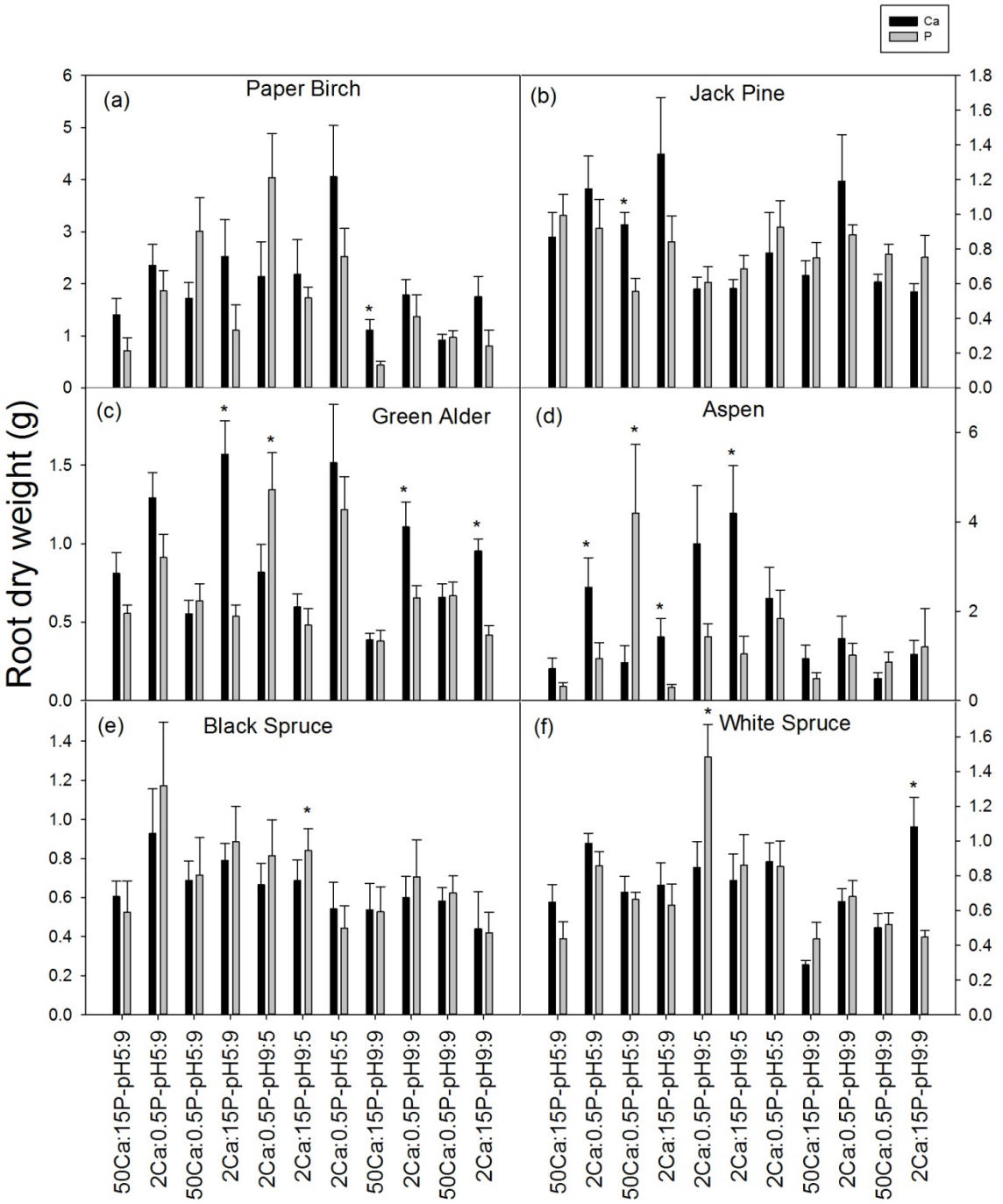

Figure 4. 4 Effects of pH, P and Ca treatments in split root design on root dry weights in Ca and P side of paper birch, jack pine, green alder, aspen, black spruce and white spruce seedlings. The asterisk above the bars indicate significant differences (α = 0.05) between leaf and side root dry weight determined by the unpaired t test. Means (n = 10) ± SE are shown.

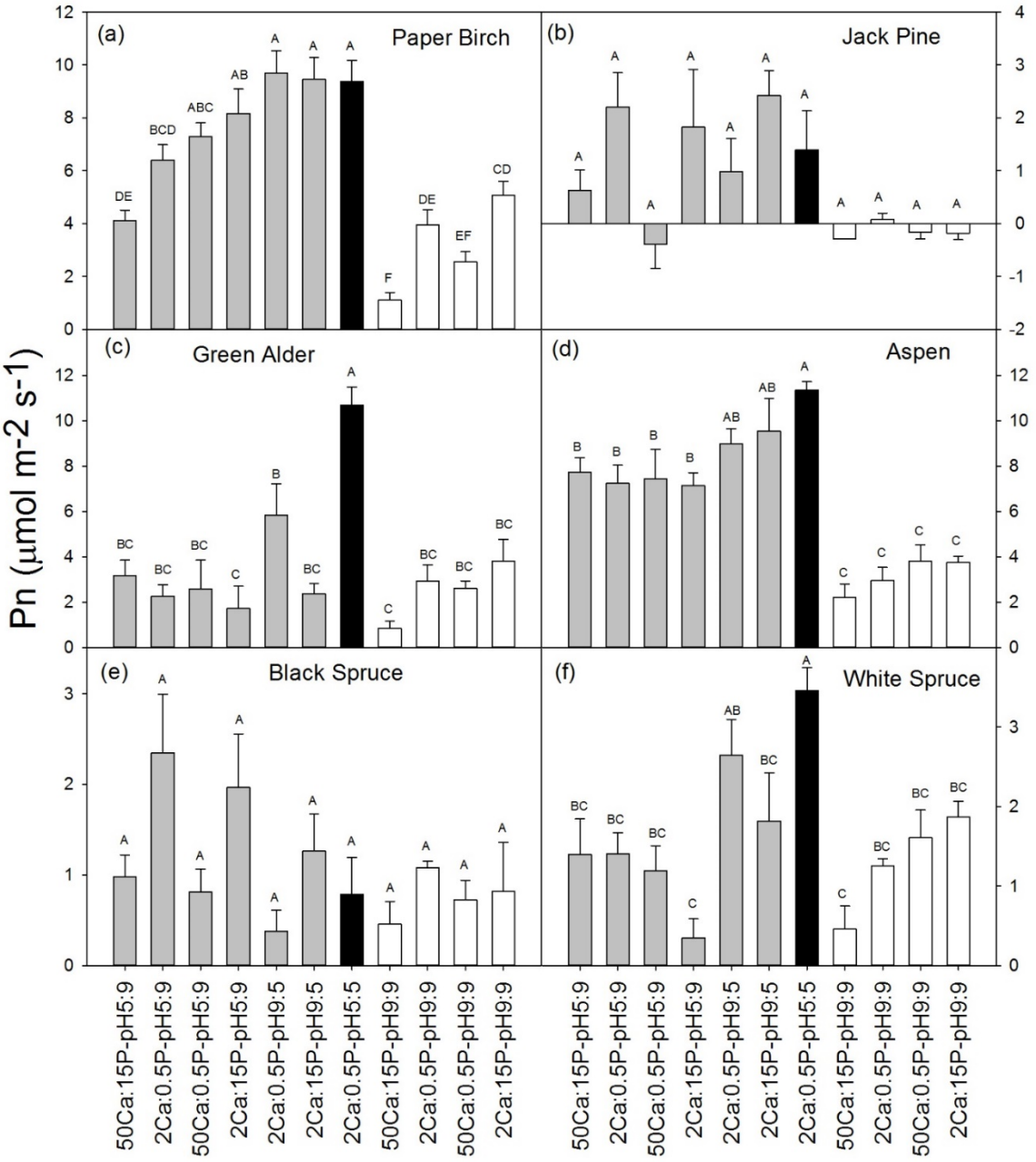

Figure 4. 5 Effects of pH, P and Ca treatments in split root design on net photosynthetic rate (Pn) of paper birch, jack pine, green alder, aspen, black spruce and white spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 8$) ± SE are shown.

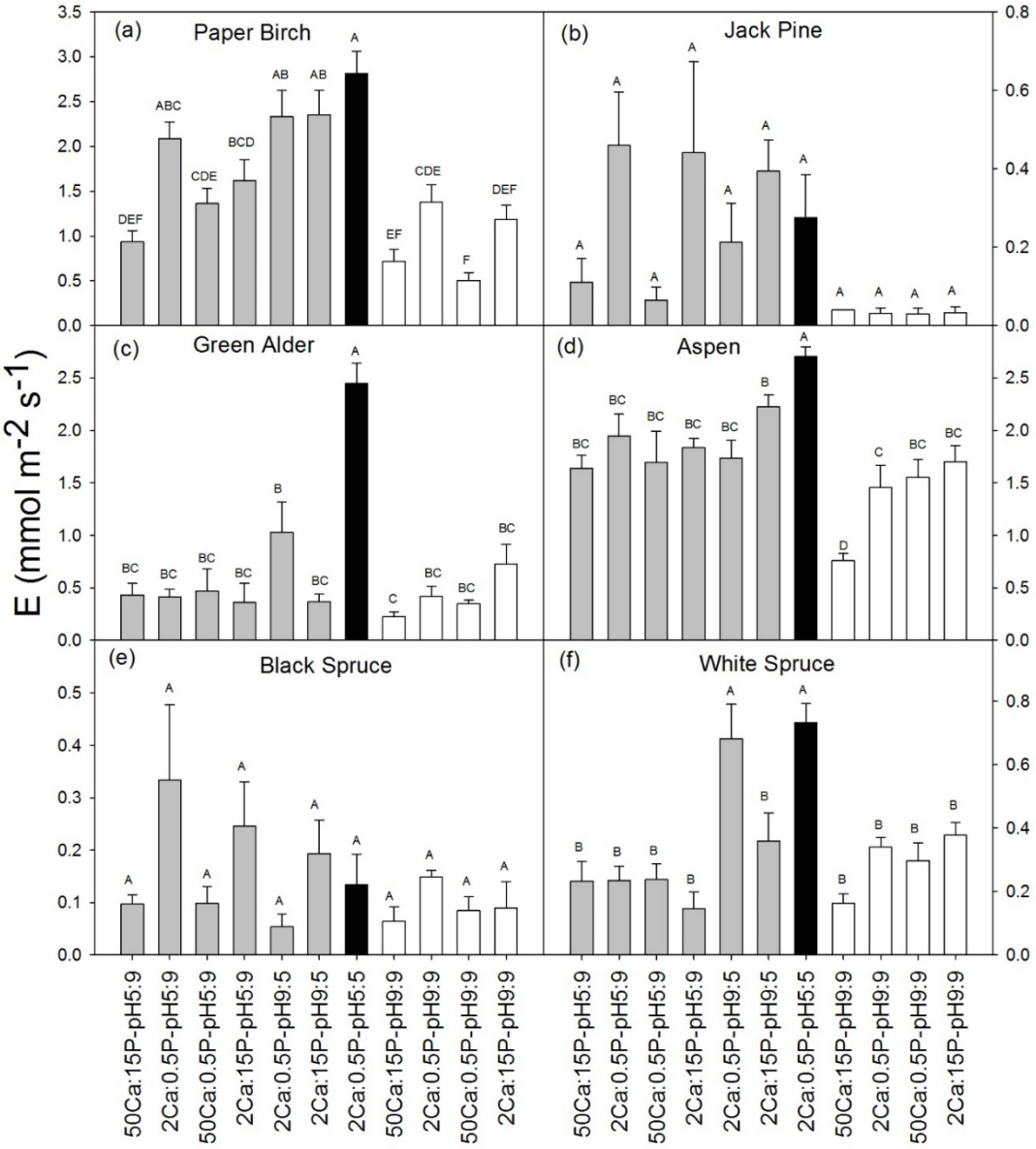

Figure 4. 6 Effects of pH, P and Ca treatments in split root design on transpiration rate (E) of paper birch, jack pine, green alder, aspen, black spruce and white spruce seedlings. Different letters above the bars indicate significant differences (α = 0.05) between treatments determined by the Student-Newman-Keuls test. Means (n = 8) ± SE are shown.

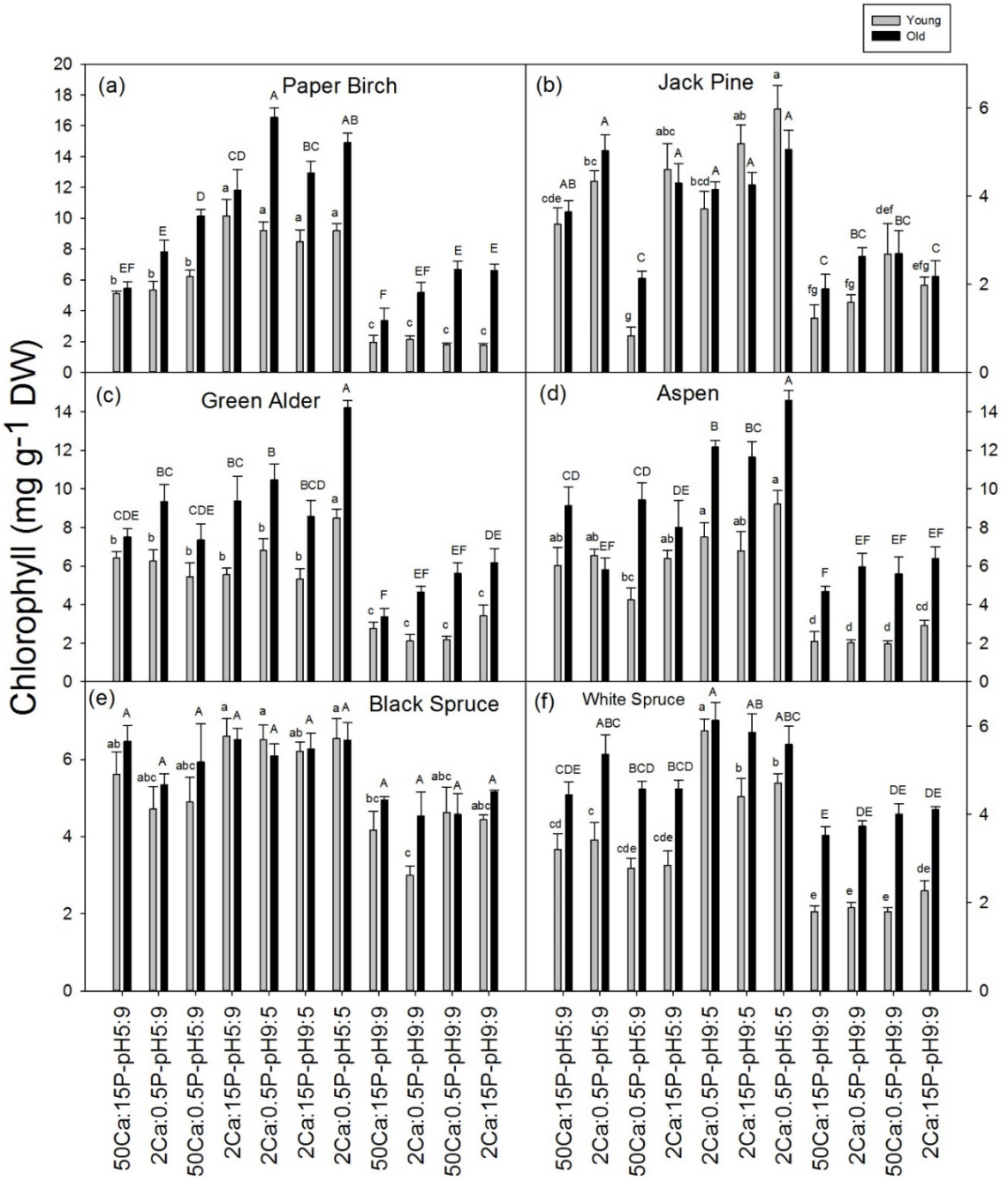

Figure 4. 7 Effects of pH, P and Ca treatments in split root design on leaf chlorophyll concentrations of paper birch, jack pine, green alder, aspen, black spruce and white spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 8$) ± SE are shown.

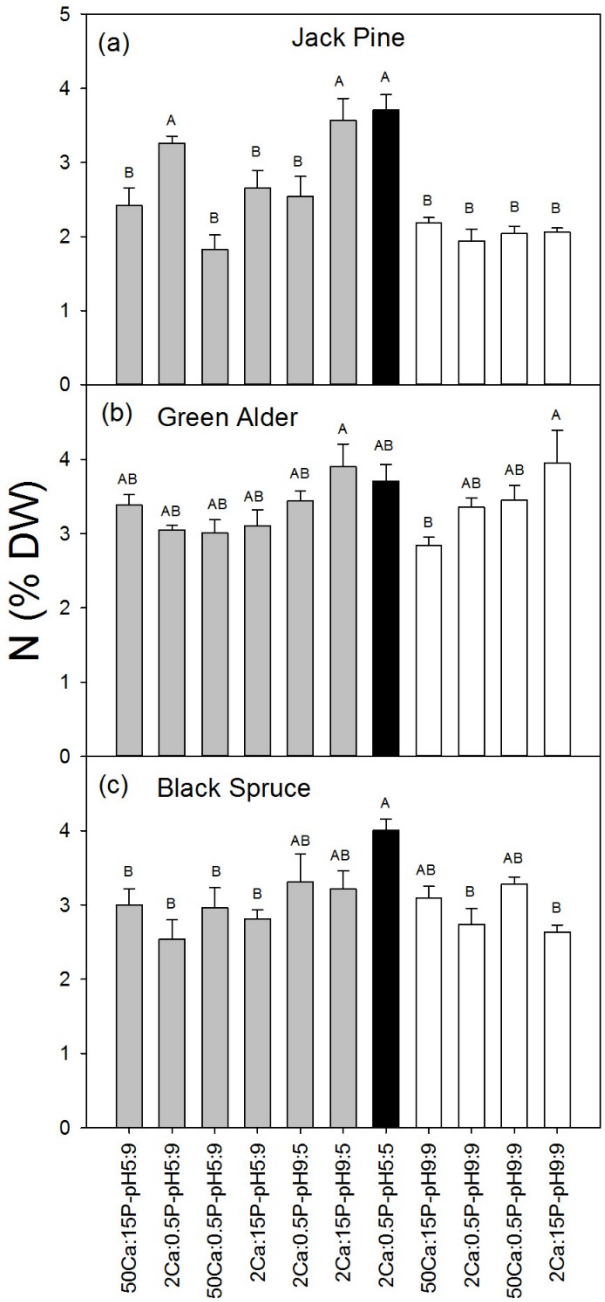

Figure 4. 8 Effects of pH, P and Ca treatments in split root design on leaf nitrogen (N) concentrations of jack pine, green alder and black spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 6$) ± SE are shown.

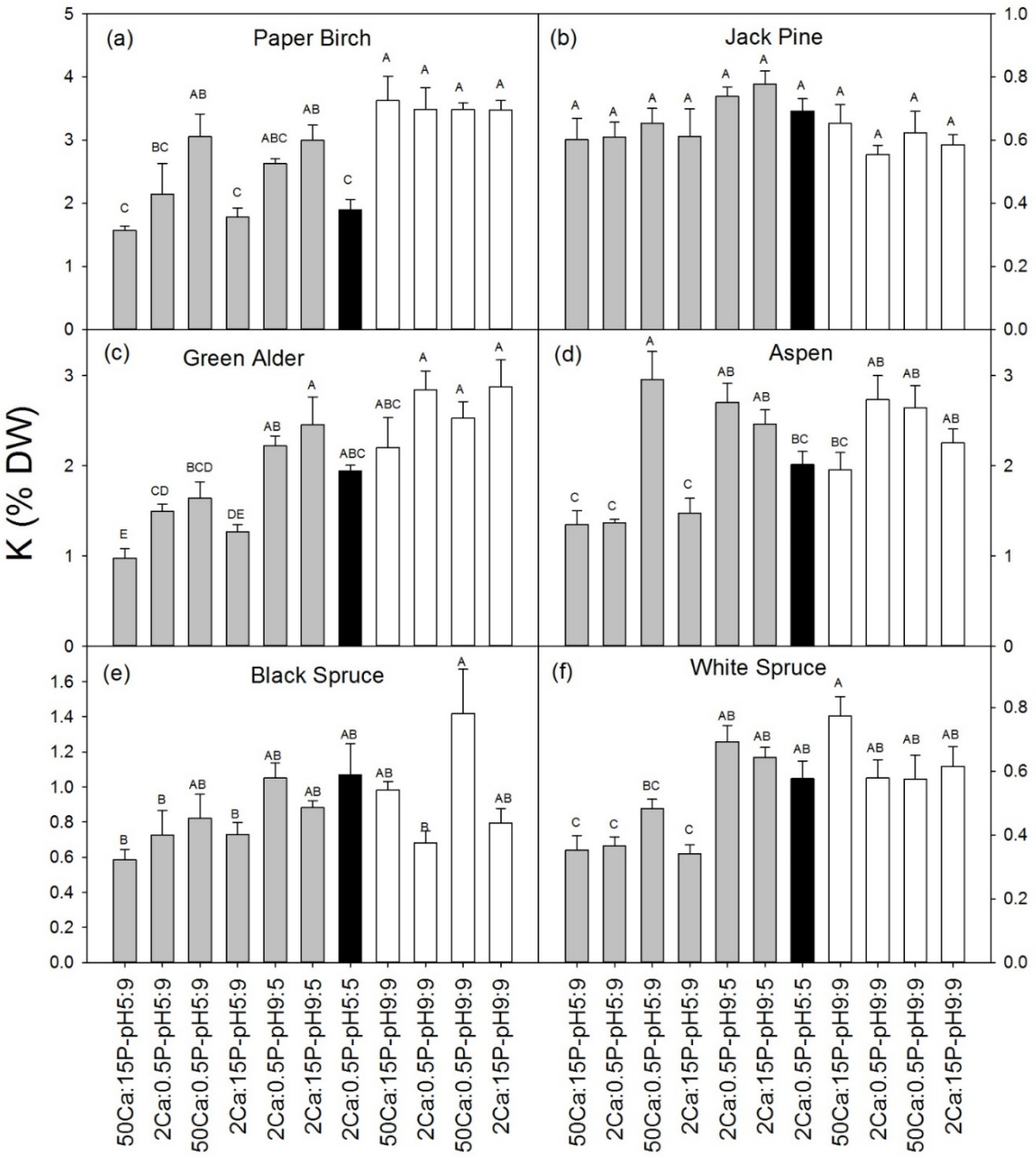

Figure 4. 9 Effects of pH, P and Ca treatments in split root design on leaf potassium (K) concentrations of paper birch, jack pine, green alder, aspen, black spruce and white spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 6$) ± SE are shown.

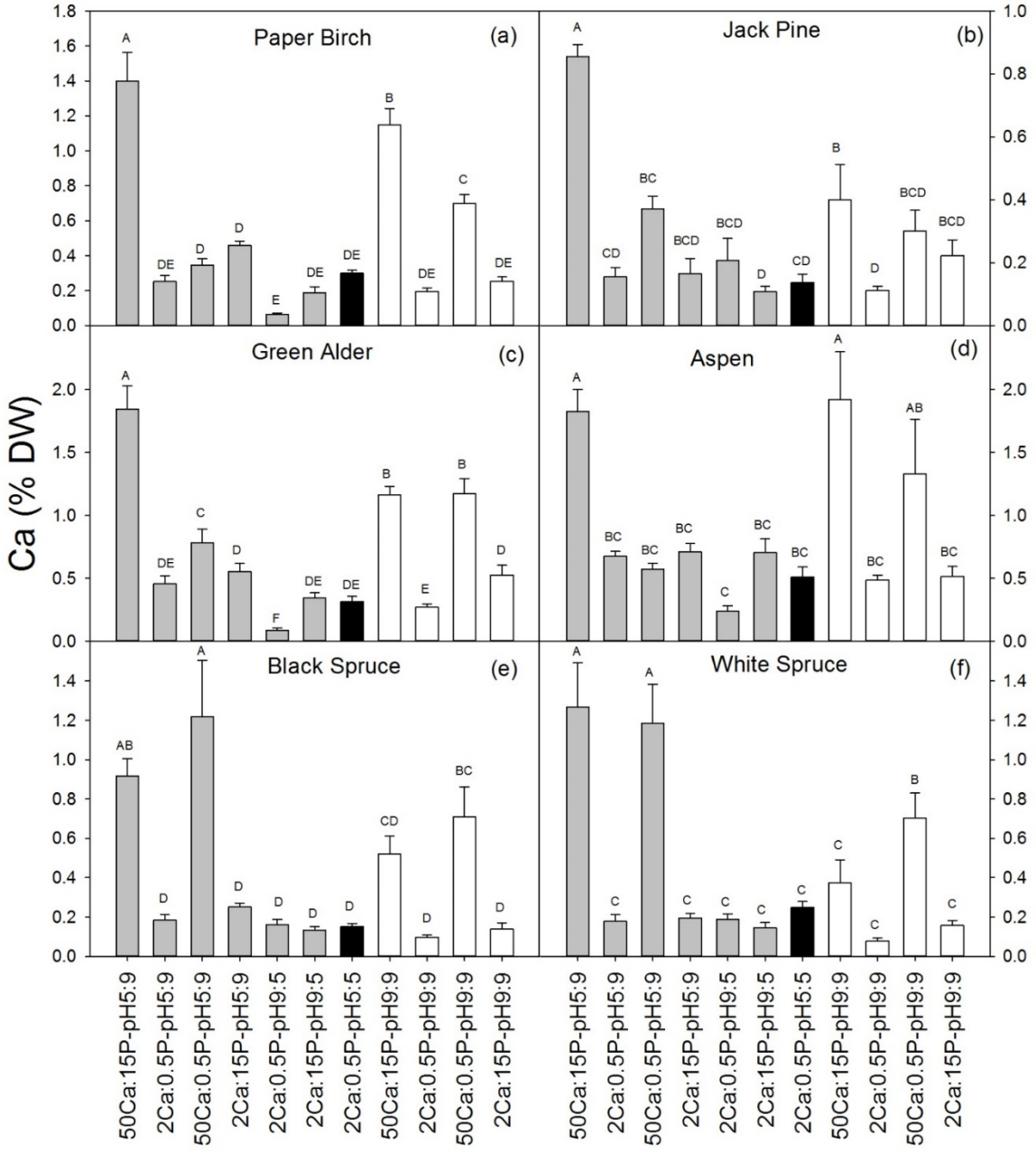

Figure 4. 10 Effects of pH, P and Ca treatments in split root design on leaf calcium (Ca) concentrations of paper birch, jack pine, green alder, aspen, black spruce and white spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 6$) ± SE are shown.

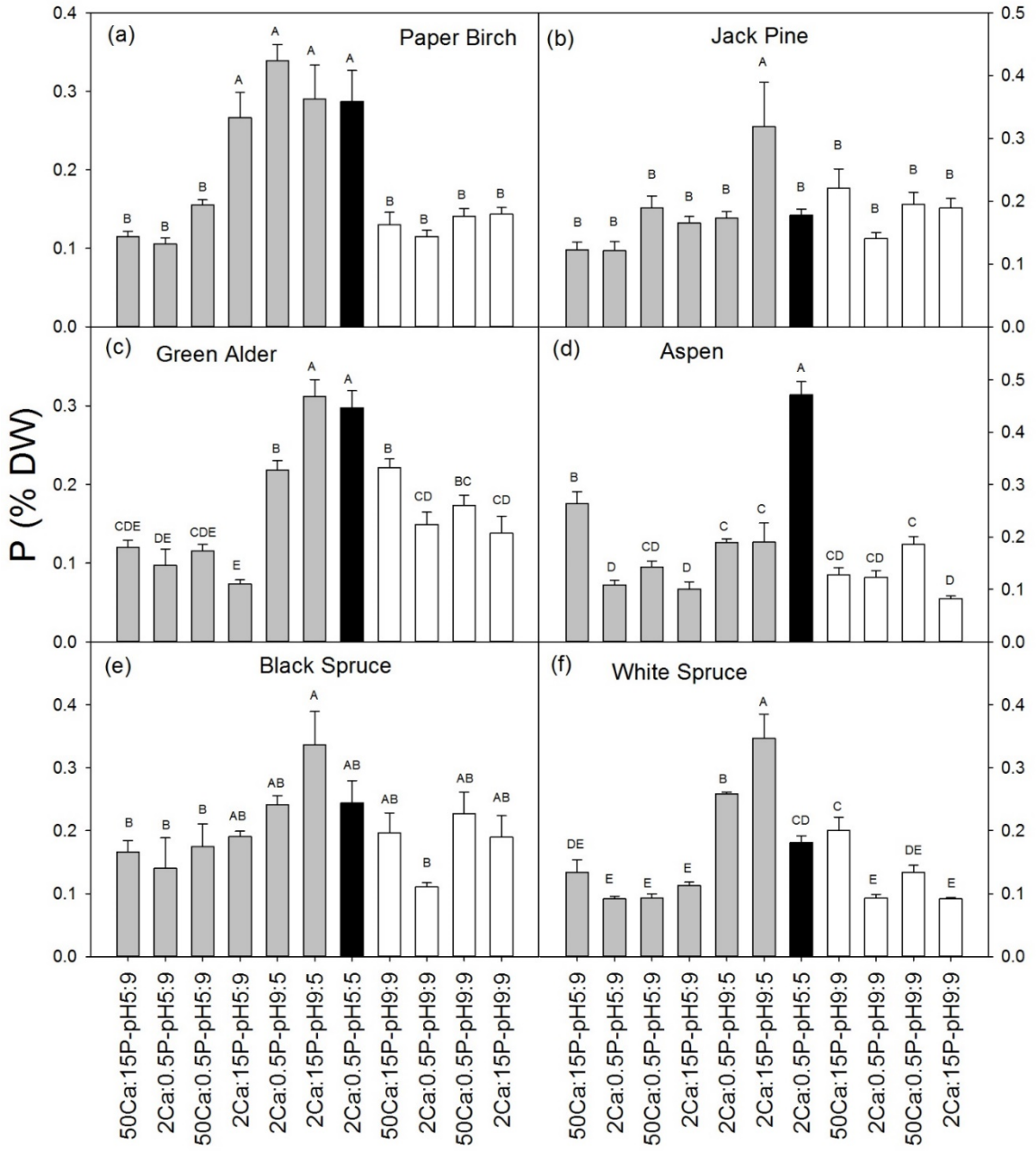

Figure 4. 11 Effects of pH, P and Ca treatments in split root design on leaf phosphorus (P) concentrations of paper birch, jack pine, green alder, aspen, black spruce and white spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 6$) ± SE are shown.

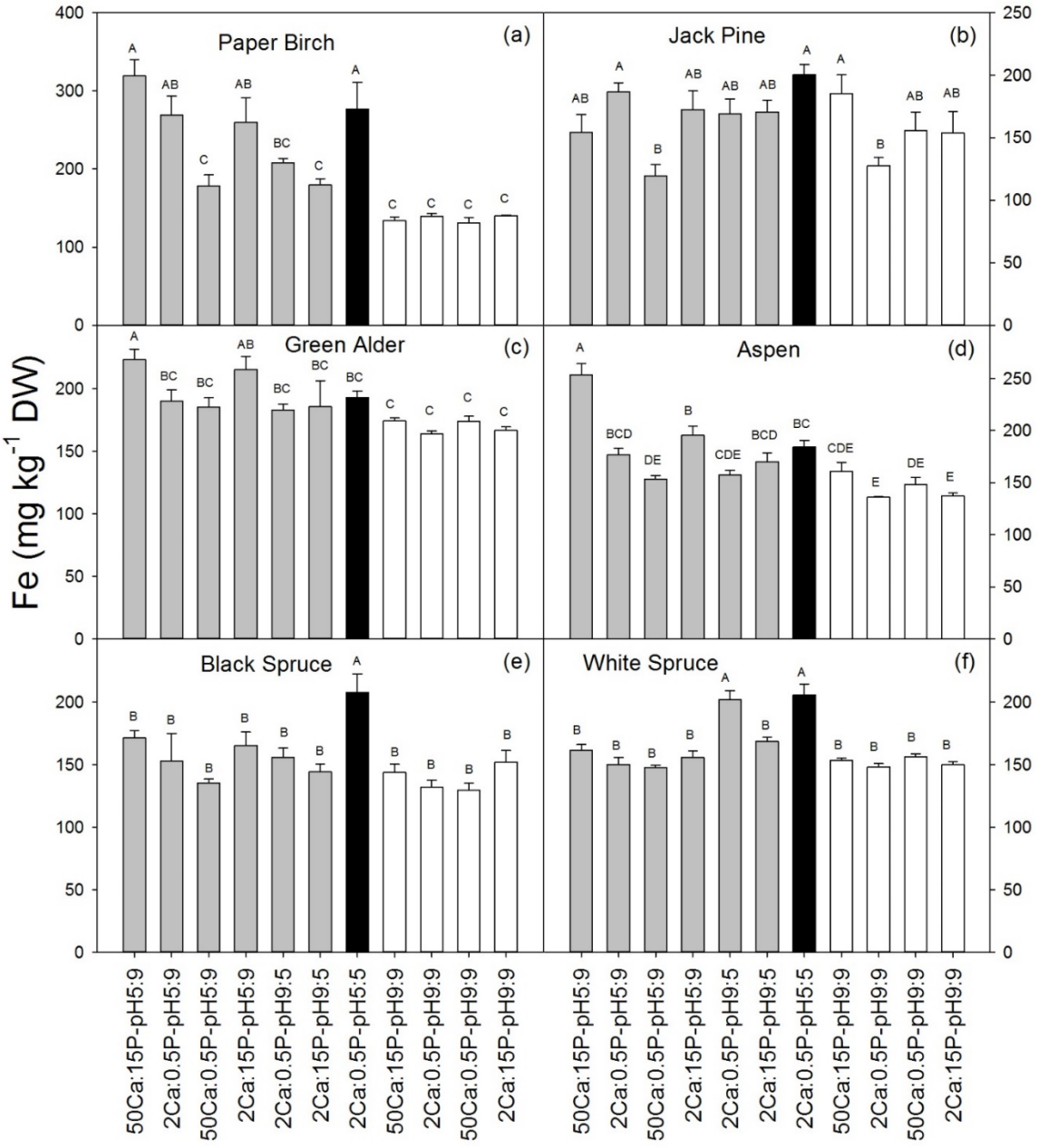

Figure 4. 12 Effects of pH, P and Ca treatments in split root design on leaf iron (Fe) concentrations of paper birch, jack pine, green alder, aspen, black spruce and white spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 6$) ± SE are shown.

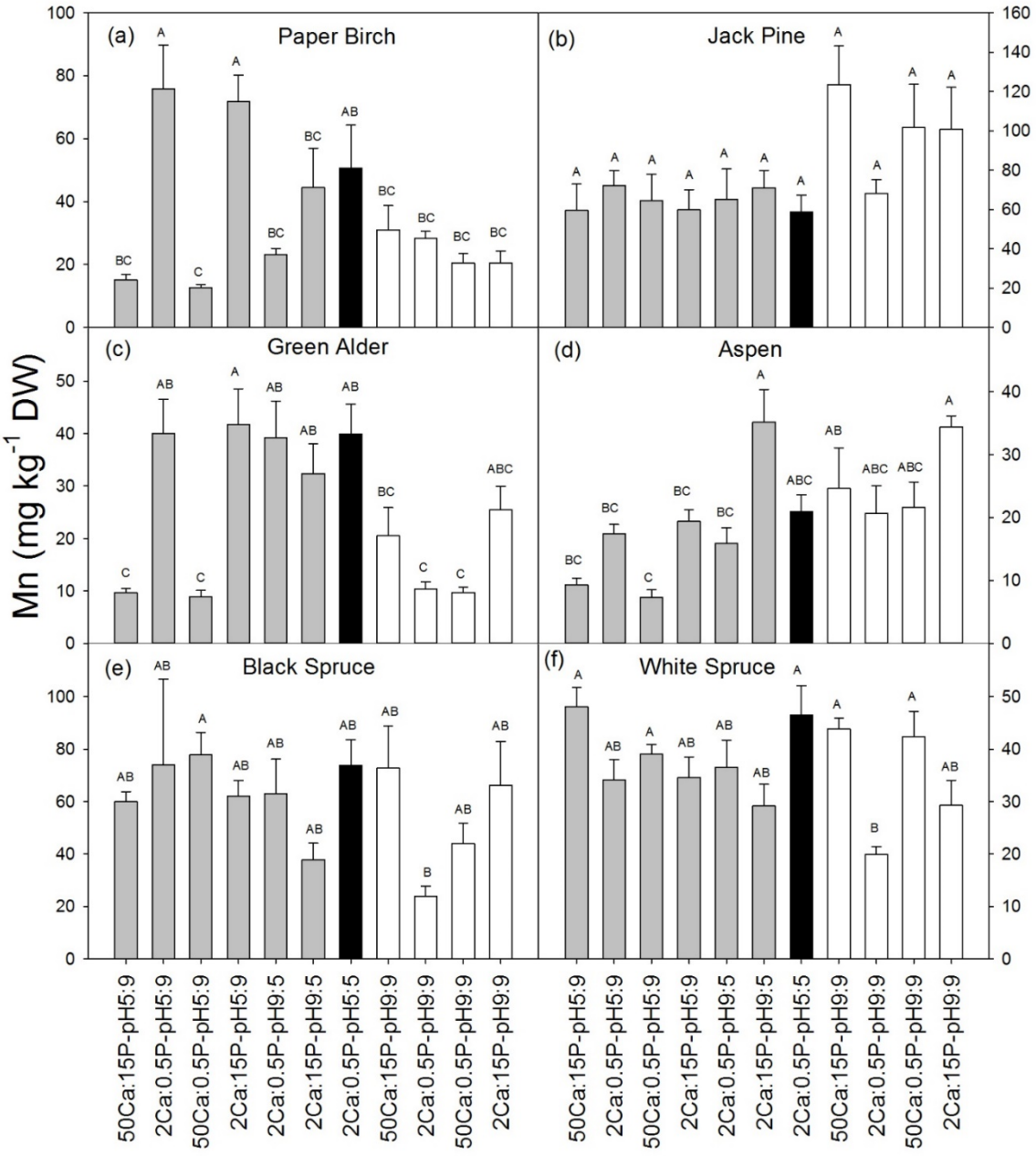

Figure 4. 13 Effects of pH, P and Ca treatments in split root design on leaf manganese (Mn) concentrations of paper birch, jack pine, green alder, aspen, black spruce and white spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 6$) ± SE are shown.

Chapter 5

Effects of root medium pH on root water transport and apoplastic pH in dogwood (*Cornus stolonifera*) and paper birch (*Betula papyrifera*) seedlings

5.1 Introduction

Although numerous studies have reported deleterious effects of high pH on plant growth, nutrient uptake and water relations (Tang et al. 1993b; Kopittke and Menzies 2005; Zhang et al. 2013; Calvo-Polanco et al. 2014), the exact mechanisms of high pH tolerance that have been reported for some species of woody plants remain largely unknown (Kamaluddin and Zwiazek 2004; Kopittke and Menzies 2004). High root zone pH can directly affect plants due to elevated concentrations of [OH^-] (Zieslin and Snir 1989; Kopittke and Menzies 2004), or indirectly, by affecting nutrient availability in soil solution (Brady and Weil 1996). In previous chapters of this thesis, both the direct and indirect effects of high pH on growth and physiological processes of plants were investigated. In this study, I examined some of the more detailed mechanisms which could explain why high root zone pH may differently affect various plants.

The inhibitory effects of high pH on plant growth is highly pronounced in roots, however, the effect varies between plant species (Bertoni et al. 1992; Tang et al. 1992; Zhang et al. 2013). In an alkaline-sensitive species of lupin (*Lupinus angustifolus* L), root growth was significantly decreased by high root root zone pH, while the alkaline-tolerant species of pea (*Pisum sativum* L) was less affected (Tang et al. 1992; Tang et al. 1993b).

Iron-induced chlorosis is a typical symptom in plants growing in high pH soils (Schinas and Rowell 1977; Mengel 1994). Decreased Mn availability with increasing soil pH is also an established concern to plants (Parker and Walker 1986). Both Fe and Mn are involved in photosynthetic activities (Marschner 2012); therefore, their deficiencies could significantly reduce photosynthetic rates (Zhang et al. 2013).

Plant apoplast is the space outside of the symplast. It includes cell walls as well as intercellular and xylem lumen spaces (Canny 1995). The apoplastic pH conditions can significantly affect important plant cellular processes such as nutrient uptake and water relations (Steudle 2000; Sattelmacher 2001). The cytosolic pH of plants has been reported to be 7.2 for most terrestrial plant species, while the apoplastic pH is usually between 5 and 6.5 (Grignon and Sentenac 1991; Rengel 2002). The apoplastic pH in roots can be altered by root medium pH by 1.5 units, which may disrupt the pH gradient between cytosol and apoplast (Felle and Hanstein 2002). Iron-related leaf chlorosis in alkaline soils may be caused by high apoplastic pH as it can affect Fe mobilization in leaf apoplast (Mengel et al. 1994). Iron uptake by plants is strongly linked to ferric chelate reductase (FCR) activity, which may be reduced by high apoplastic pH (Chaney et al. 1972; Mengel 1994). Xylem sap pH was found to be increased in plants growing under drought conditions, which was proposed to serve as a stress signal through which the plant communicates to the shoot the fact that the roots are in contact with dry soil (Davies and Zhang 1991; Wilkinson and Davies 1997).

Reductions of stomatal conductance, shoot water potential and root water flux have been widely reported for plants growing under high root zone pH conditions (Tang et al. 1993a; Tang and Turner 1999; Kamaluddin and Zwiazek 2004). These effects of

alkaline conditions on plant water relations may be caused by changes in aquaporin (AQP) activity, as AQPs extensively mediate the cell-to-cell pathway for root water transport (Steudle and Peterson 1998; Voicu and Zwiazek 2004; Aroca et al. 2006). AQP activity is thought to be affected by pH since cytosolic pH can alter the structure of AQPs (Tournaire-Roux et al. 2003; Kamaluddin and Zwiazek 2004; Törnroth-Horsefield et al. 2006).

In this study, I investigated the effects of root zone pH on several important growth and physiological parameters in paper birch (*Betula papyrifera*) and dogwood (*Cornus sericea*). In earlier studies (Chapter 3, 4), I found paper birch to be relatively sensitive and dogwood to be relatively tolerant to high pH. I tested the hypothesis that the relatively higher pH resistance in dogwood compared with paper birch involves several key mechanisms including: i) less resistance of root water transport processes, ii) maintenance of ferric chelate reductase activity, and iii) maintenance of relatively stable xylem sap pH.

5.2 Materials and Methods

5.2.1 Plant material and experimental conditions

One-year-old container-grown (415D styroblocks™, Beaver Plastics, Acheson, AB, Canada) dogwood (*Cornus stolonifera*) and paper birch (*Betula papyrifera*) dormant seedlings were obtained from Boreal Horticultural Services Ltd., Bonnyville, Alberta, Canada. The seedlings were grown hydroponically in a controlled-environment growth chamber. 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 μmol

m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at the top of the seedlings provided by the full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada). The set-up consisted of three 120 L pails containing each of the three treatment solutions [25% Hoagland's solution (Epstein 1972) at pH 5, 7, or 9] and each pail was connected to three replicated 30 L tubs by PVC tubing. A water pump was used to circulate 25% modified Hoagland's solution between the pail and tubs. The tubs were covered with Styrofoam boards, each containing 20 holes, 3-cm in diameter. In each tub, 6 seedlings per species were placed for a total of 12 seedlings. A pH controller (Model 9.5 950GPH, Danner MFG Inc., New York, USA) was used to automatically adjust solution pH by releasing the required amount of 5% (w/v) KOH or 1% (v/v) H_2SO_4. A randomized complete block design (RCBD) was used in this study. Three pails with 25% Hoagland's solution of pH 5, 7 and 9, respectively, were placed at the left and right sides of growth chamber, with each side serving as a block. The plants were subjected to the different solution pH treatments for 6 weeks and every 2 weeks the nutrient solutions were replaced.

5.2.2 Dry weights and gas exchange determinations

At the end of the treatments, twelve seedlings per species were harvested from each block and roots and shoots dried in an oven at 70 °C for 72 h (n = 24). Four seedlings from each treatment in each block (n = 8), were randomly selected for the measurement of net photosynthesis rate (Pn), transpiration rate (E), and stomatal conductance (gs), with an infrared gas analyzer (LI-6400, LI-COR, Lincoln, Nebraska USA). The reference CO_2 concentration was 400 μmol and the flow rate was 200 μmol s^{-1} in the leaf chamber. The leaf chamber temperature was kept at 20 °C and the

photosynthetic photon flux density (PPFD) was set to 400 μmol $m^{-2}s^{-1}$. Measurements were conducted between 9:00 to 12:00 h. Water use efficiency (WUE) was calculated by dividing Pn by E.

5.2.3 Measurements of ferric chelate reductase (FCR) activity

Enzyme assay solution of 0.2 mM $CaSO_4$, 0.1 mM Fe-EDTA and 0.2 mM BPDS (bathophenanthrolinedisulfonic acid) (Cohen et al. 1997) was prepared and adjusted to pH 5, 7 and 9 by adding 1% (v/v) H_2SO_4 or 5% (w/v) KOH. Root tips of 3 cm length (about 0.1 g) were excised from three seedlings per species per block from each pH treatment, briefly rinsed with distilled water, and then immersed in 10 ml assay solution of different pH (n = 6). Samples were shaken in the dark at room temperature for 1 h. After that, the absorbance in each solution was measured spectrophotometrically (Ultrospec, Pharmacia LKB, Uppsala, Sweden) at 535 nm. The molar concentration of the Fe(II)-BPDS complex was calculated using the extinction coefficient of 22.14 mM^{-1} cm^{-1} (Cohen et al. 1997).

5.2.4 Root hydraulic conductivity (Lpr) measurements

Three seedlings from each pH treatment were randomly selected from each of the two blocks to measure root hydraulic conductivity (n = 6). The shoots of seedlings were excised about 2 cm above root the collar and bark was peeled off. The roots were kept in an aerated nutrient solution at the treatment pH and attached to a high pressure flow meter (HPFM, Dynamax Inc., Houston, TX) for the measurement of root hydraulic conductance (Kr) (Kamaluddin and Zwiazek 2002). The root system was gradually pressurized to 0.5 MPa. After the measurements, root systems were immersed in water in

a graduated cylinder to measure the displaced water volume as a measure of root volume (Kamaluddin and Zwiazek 2004). Lpr was calculated by dividing Kr by root volume (Calvo-Polanco et al. 2012).

5.2.5 Determinations of xylem sap pH in stems and roots

After 6 weeks of pH treatments, three seedlings per species of each pH level randomly selected in each block were taken to measure xylem sap pH (n = 6). Before the measurements, seedlings were kept in aerated pH treatment solutions. Shoots were excised at root collar with a sharp razor blade. The bark was peeled off from around the excision area in stems and roots which were then placed in the Scholander-type pressure chamber (PMS instruments, Corvallis, OR, USA) with the ends protruding through the lid. A 1 ml pipette tip was cut to fit the diameter of the stem and the stem was inserted into the opening of the pipette tip. A 10 ml syringe was cut into about 3 cm segments. A PVC tubing of about 2 cm in length and 3 mm diameter connected the pipette tip with the syringe which was used to collect xylem sap.

To collect xylem sap, a pressure of 0.2 MPa above the balancing pressure (the pressure at that xylem sap come out from cut surface) was applied. The first droplet of xylem sap was discarded to avoid contamination from injured cells. The xylem sap was transferred with a pipette into 1.5 ml Eppendorf tubes for storage. For stems, the sap was collected until it stopped coming out of the surface. For roots, about 1.5 ml of xylem sap was collected from each sample. The tubes with sap were immediately frozen in liquid nitrogen and stored in the freezer at -80°C (Liang and Zhang 1997). Since for some seedling stems less than 0.3 ml xylem sap could be collected, a small volume pH probe

(B-713 LAQUAtwin Compact pH Meter, HORIBA Instruments Incorporated, Irvine, California) was used for measuring the pH of xylem sap.

5.2.6 Determinations of relative abundance of K, Ca, Fe, Mn in cell walls and protoplasts of paper birch

Three paper birch leaves from three seedlings were collected in each block (n = 6) at pH 5 and 9 and immediately frozen in liquid nitrogen. The leaves were excised into round discs and mounted on SEM stubs with a conductive carbon adhesive. The leaf discs per treatment were frozen in liquid nitrogen and prepared for the measurements of relative abundance of K, Ca, Fe, and Mn in the cell walls and protoplasts using the scanning electron microscope (SEM) (Zeiss EVO MA 15) equipped with a Bruker Silicon Drift Detector for Energy Dispersive X-Ray analysis/mapping with a peak resolution of 125 eV. The relative abundance of K, Ca, Fe and Mn in the cell walls and protoplasts was expressed as % total. For each leaf, two cells were randomly selected for the measurements and their means were used for each leaf sample.

5.2.7 Statistical analysis

Data of stem and root xylem sap pH at each treatment were analyzed using Student's t test by SAS (Version 9.3, SAS Institute Inc., Cary, NC). All other data were analyzed with SAS mixed model to determine statistically significant ($p \leq 0.05$) differences between treatments, and block was regarded as a random factor. Data that did not meet the assumptions of normality of distribution and homogeneity of variance were transformed with a log10 function. Comparisons between different treatment means were conducted using Lsmeans statement and Pdiff option of SAS.

5.3 Results

5.3.1 Dry weights and net photosynthesis rate

Total seedling dry weights in paper birch were the highest at pH 7 followed by pH 5 and 9 (Fig. 5.1a). At pH 9, dry weights were reduced by about two fold compared with pH 7 (Fig. 5.1a). In dogwood, the seedling dry weights were similar at pH 5 and 7, while at pH 9 the dry weights were reduced by about two fold compared with the lower pH (Fig. 5.1b).

Unlike dry weights, Pn in paper birch progressively decreased with increasing pH. With each pH increased from pH 5 to 7 and 9, Pn values decreased by about one-half (Fig. 5.1c). In dogwood, Pn in seedlings subjected to pH 7 and 9 treatments was lower compared with pH 5 (Fig. 5.1d). There was no statistically significant difference in Pn between the seedlings subjected to pH 7 and 9 (Fig. 5.1d).

5.3.2 Transpiration rate and stomatal conductance

In both paper birch and dogwood, E was similar at pH 5 and 7 (Fig. 5.2a,b). In paper birch, a 60% reduction in E was measured in seedlings subjected to pH 9 (Fig. 5.2a), while in dogwood, the reduction was less than 30%(Fig. 5.2b). Similar patterns of response, but with more pronounced reductions than for E were measured for gs in both tree species (Fig. 5.2c,d).

5.3.3 Ferric chelate reductase (FCR) activity and root hydraulic conductivity (Lpr)

For both paper birch and dogwood, FCR activity at pH 5 was drastically lower compared with pH 7 (Fig. 5.3a). However, in paper birch, FCR activity was similar at pH

7 and 9, while in dogwood the activity was lower by about three-fold at pH 9 compared with pH 7 (Fig. 5.3a, b). A slight difference in FCR activities in dogwood at pH 5 and 9 was not statistically significant (Fig. 5.3b).

At all studied pH levels, Lpr was several-fold higher in dogwood compared with paper birch (Fig. 5.3c, d). In paper birch, Lpr decreased with increasing pH (Fig. 5.3c). However, the differences between pH 7 and pH 5 as well as between pH 7 and pH 9 were not statistically significant (Fig. 5.3c). In dogwood, Lpr was several-fold higher at pH 7 compared with pH 5 and 9 (Fig. 5.3d). No significant difference was found between pH 5 and 9 (Fig. 5.3d).

5.3.4 Stem xylem sap pH and root and water use efficiency (WUE)

No significant differences were found for either stem or root xylem sap pH between the different pH treatments in both paper birch and dogwood (Fig. 5.4a,b). Only for paper birch at pH 7, stem xylem sap pH was significantly higher compared with root xylem sap pH (Fig. 5.4a). In both paper birch and dogwood, WUE was the highest at pH 5 and the lowest at pH 7 (Fig. 5.4c,d). However, in paper birch, WUE was significantly different between all pH treatments, and in dogwood significant difference occurred only between pH 5 and 7 (Fig. 5.4c,d).

5.3.5 Relative abundance of K, Ca, Fe, Mn in cell walls and protoplasts of paper birch

The relative abundance of K was significantly higher at pH 9 than 5 in cell walls and protoplasts, while at both pH levels, there was no significant difference between the cell walls and protoplast (Fig. 5.5a). There was no significant difference for the relative abundance of Ca at pH 9 and 5 and between the cell walls and protoplasts (Fig. 5.5b).

The cell wall relative abundance of Fe at pH 5 was significantly higher than in the protoplast, and at pH 9, there were no significant differences between the cell wall and protoplasts (Fig. 5.5c). In the protoplasts, relative abundance of Fe showed an increasing trend with increasing pH, while in the cell walls, it had a decreasing trend with increasing pH (Fig. 5.5c). No significant differences were found in Mn relative abundance between pH 5 and pH 9 and between the cell walls and protoplasts (Fig. 5.5d).

5.4 Discussion

In the study presented in Chapter 3, dogwood appeared to be more resistant to high root zone pH under moderate Ca supply compared with aspen. The study in Chapter 4 also showed that aspen and paper birch had similar levels of high pH tolerance. Therefore, I expected dogwood to exhibit greater tolerance of high pH compared with paper birch. In the present study, although both paper birch and dogwood showed dry weight, transpiration, and stomatal conductance reductions at pH 9, and photosynthesis declined at pH 7 and 9, the decreases in these parameters in paper birch were indeed greater compared with dogwood.

In both species, Pn reductions occurred at pH 7 and 9, while significant dry weight decreases were recorded only at pH 9. This might be due to the relatively short treatment duration and because differences in Pn may not always be captured in a single measurement. Pn is affected by stomatal opening and efficiency of the photosynthetic system. Since there were no reductions in gs at pH 7, the Pn reduction at pH 7 likely reflects the reductions in the photosynthetic processes not related to CO_2 availability. In Chapter 2, aspen (*Populus tremuloides*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) showed reductions in leaf Fe or Mn, or both at pH 7. The Pn reduction at

pH 7 in the present study in paper birch and dogwood may be also due to Fe or Mn deficiency and the resulting leaf chlorosis and (or) reduced efficiency of electron transport processes involving Fe. Other studies presented in this thesis have found that pH-sensitive plants negatively responded to pH 6.5 and higher. This may be related to the apoplastic pH which usually measures pH 6.5 or lower in plants (Grignon and Sentenac 1991; Rengel 2002). Therefore, plants may be negatively affected by the root medium pH which is higher than apoplastic pH of the root cells.

In paper birch, Lpr decreased with increasing root zone pH, while E was significantly reduced only at pH 9. This demonstrates that contrary to pH 9, the reduction of Lpr at pH 7 was not sufficient to affect E. In dogwood, both Lpr and E were about thirty percent lower at pH 9 compared with pH 5, although the differences in Lpr were not statistically significant. In dogwood, the increase of Lpr at pH 7 could be due to the effect on the activity of root aquaporins. The water transport pathways of roots can be divided into apoplastic and cell-to-cell pathways which merge at the exodermis and endodermis due to the existence of water impermeable Casparian strips (Steudle and Peterson 1998; Steudle 2000). The aquaporins in the exodermis and endodermis could potentially act as valves for root water transport. It was reported that at root medium pH 4 and 8, Lpr was significantly lower in paper birch compared with pH 6 (Kamaluddin and Zwiazek 2004). The optimum pH for aquaporin activity has not been established, but a decrease of cytosolic pH from 7.6 to 6.6 was reported to induce aquaporin closure and result in decreased water transport of plant cells (Tournaire-Roux et al. 2003). It is possible that under moderate stress of pH 7, dogwood may increase the abundance and (or) the activity of aquaporins and increase Lpr. However at pH 9, the activity of

aquaporins is likely to be reduced due to effects on protein structure (Dill 1990) and result in Lpr reduction. In both paper birch and dogwood, WUE decreased at pH 7 and then, moderately increased at pH 9. This suggests that high pH triggered plant responses to more efficiently use water resources compared with neutral pH.

In the Fe uptake processes of plants, Fe(III)-chelate is reduced to Fe(II) by FCR and then, the Fe(II) transporter moves Fe across the plasma membrane into cells (Chaney et al. 1972; Walker and Connolly 2008). Fe(III) reduction is considered to be the rate-limiting step for Fe uptake in nongraminaceous plants (Grotz and Guerinot 2002). In tomato (*Lycopersicon esculentum* Mill.) roots, the optimum pH for FCR activity was reported to be 6.5 (Holden et al. 1991). In the present study, the FCR activity increased at pH 7 and 9 compared with pH 5 in both paper birch and dogwood, which is inconsistent with previous studies in sugar beet and tomato (Susin et al. 1996; Holden et al. 1991). Since in these studies, FCR activity decreased at pH>6.5. It is possible that the effect of pH on FCR may vary among plant species. Iron deficiency and accompanied leaf chlorosis are typical symptoms of plants growing in high pH soils (Mengel 1994; Zhang et al. 2013). In the modified Hoagland's solution, Fe is in the chelated form, and at pH 5 and 7, soluble Fe concentrations in Hoagland's solution were similar, while at pH 9, soluble Fe concentration was reduced by about 40% compared with pH 5 (Chapter 2). Thus, at pH 7, leaf Fe deficiency was not likely caused by the inadequate concentration of Fe in nutrient solution. As FCR activity was not reduced, other Fe transport processes from roots to leaves are likely to be affected by pH. The increase of FCR activity in both species at pH 7 and 9 could be a general stress response in the two studied species,

possibly, due to enhanced FCR abundance in the plasma membranes of epidermis cells. However, further studies are needed to test this hypothesis.

The methods to determine apoplastic pH directly in apoplastic fluid have some drawbacks including low volumes of fluid, contamination by cytoplasmic fluid, and effects of dehydration on apoplastic pH (Hartung et al. 1988; Hartung et al. 1992). I used a pH probe specialized for measuring a small volume of sample to measure the xylem sap. Changes of xylem sap pH caused by environmental stimuli should be relatively small since plants need to maintain regular metabolic activities, thus a large number of samples are needed to detect a statistically significant change. Probably due to the relatively small number of samples, with the exception of the differences between stem and root xylem sap pH at pH 7 in paper birch, there were no other significant differences in xylem sap pH of stems and roots between the different pH treatments. In paper birch at pH 5 and 7, and in dogwood at all pH levels, mean values of stem xylem pH were higher than in the roots. The higher root xylem sap pH compared with stems in paper birch at pH 9 was likely due to the effect of treatment solution pH. This implies that dogwood may have a better ability to maintain optimum apoplastic pH compared with paper birch, which could partially explain greater tolerance of dogwood to high root zone pH.

I measured the relative abundance of several elements using the x-ray microanalysis method in the leaf cell protoplasts and cell walls in paper birch. The results of x-ray microanalysis in this study showed that of the analyzed elements (Ca, Fe, Mn, and K), only K mass percentage significantly increased at both the cell wall and protoplast at pH 9 compared with pH 5. Thus, the results of relative abundances measured by x-ray microanalysis for K were consistent with the concentration analysis by

dry weight (Chapter 4). On the other hand, when K, Ca, Fe and Mn leaf concentrations were measured in the earlier study (Chapter 4) in aspen and paper birch subjected to pH treatments, the concentrations of Ca, Fe, and Mn decreased at pH 9 and K concentrations increased at pH 9. The X-ray microanalysis method may be affected by the dehydration of cells due to applied vacuum and high-energy electronic beam. However, the assumption is that all samples would be subjected to a similar error.

The plant apoplast and vacuole have similar Ca concentrations ranging from 0.1 to 10 mM, which is much higher than the 0.1 μM Ca of cytosol (Hepler 2005). In the present study, the relative abundance of Ca in paper birch was similar in the protoplasts and cell walls at pH 5. At pH 9, the relative abundance of Ca was slightly higher in the cell walls than in the protoplast, which might be because less Ca^{2+} was replaced by H^{+} due to the likely increase in leaf apoplastic pH (Rayle and Cleland 1992; Hepler 2005). At pH 9, Fe and Mn relative abundance was lower in the cell walls than in the protoplasts. This is consistent with the assumption of high apoplastic pH reductions in Fe and Mn availabilities. The relatively large difference of relative abundance of Fe in the cell walls at pH 5 and 9 are likely caused by different leaf apoplastic pH. However, leaf apoplastic pH needs to be measured to further explain the distribution of these elements in apoplast and inside the cell.

In conclusion, the present study compared physiological responses to pH in dogwood, a relatively tolerant plant species to high pH, and paper birch, a relatively sensitive plant species to high pH. The results demonstrate that the reasons for greater high pH tolerance of dogwood compared with paper birch likely include enhanced ability to maintain high root hydraulic conductivity and maintain high ferric chelate reductase

activity under high pH conditions. The ability of dogwood to maintain optimum apoplastic pH might also be higher than in paper birch and affect the uptake of Fe and Mn by leaf cells. Future studies should focus on the function of root aquaporins under different root zone pH conditions. The results of this study shed more light on the mechanisms of high pH tolerance in plants.

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5.6 Figures

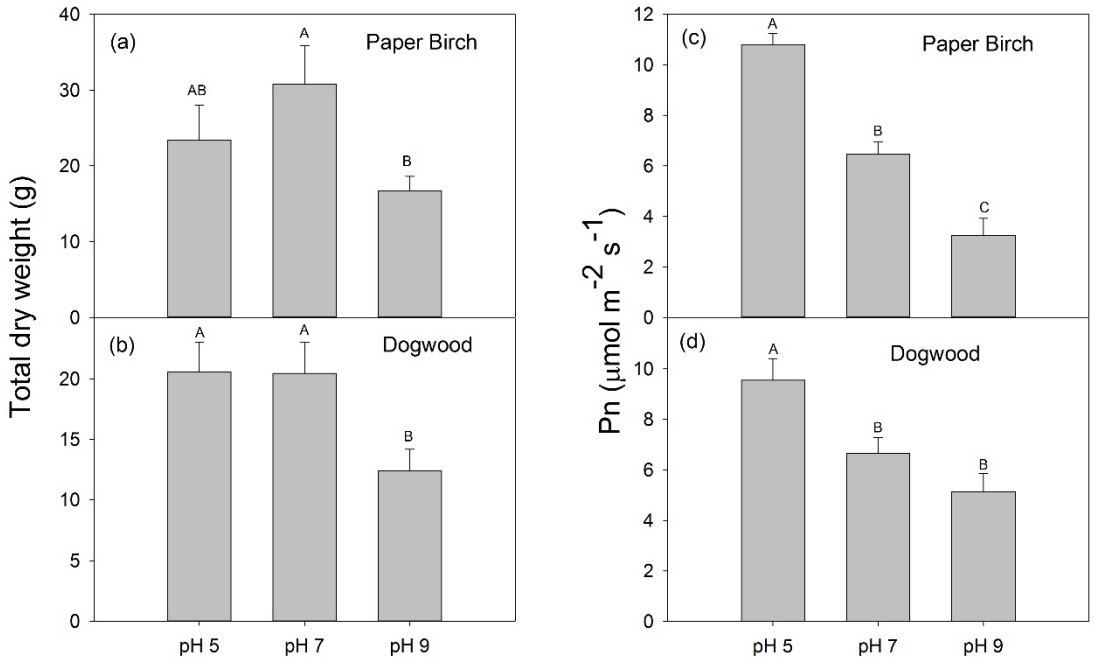

Figure 5. 1 Effects of root zone pH on total dry weight (n = 24) and net photosynthesis rate (Pn) (n = 8) of paper birch and dogwood. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments within each plant species. Means ± SE are shown.

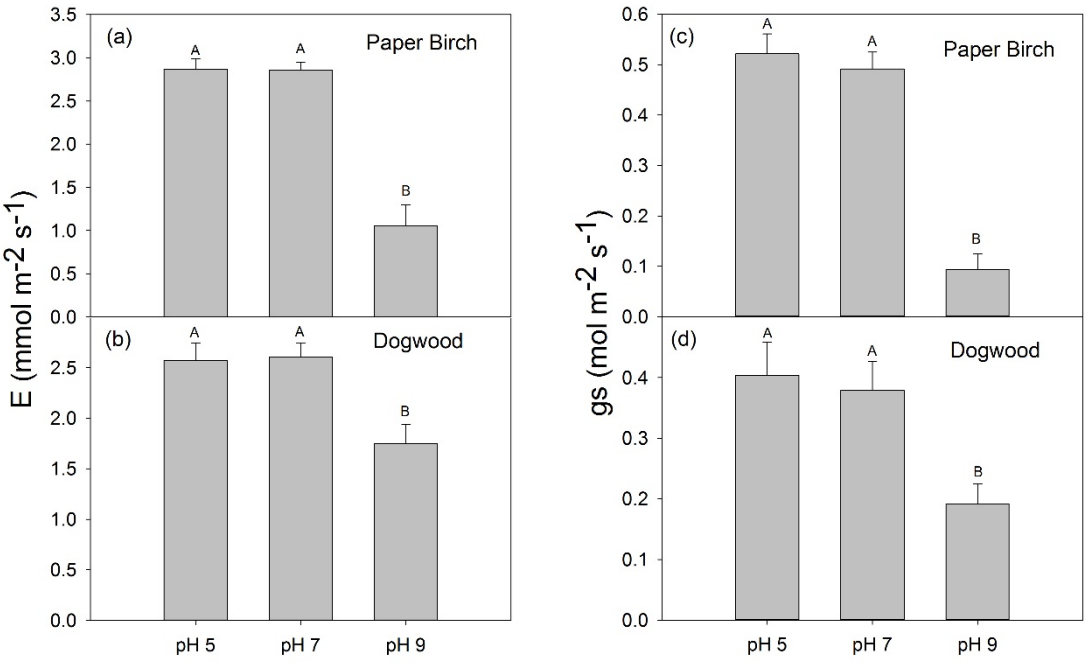

Figure 5. 2 Effects of root zone pH on transpiration rate (E) (n = 8) and stomata conductance (gs) (n = 8) of paper birch and dogwood. Different letters above the bars indicate significant differences (α = 0.05) between treatments within each plant species. Means ± SE are shown.

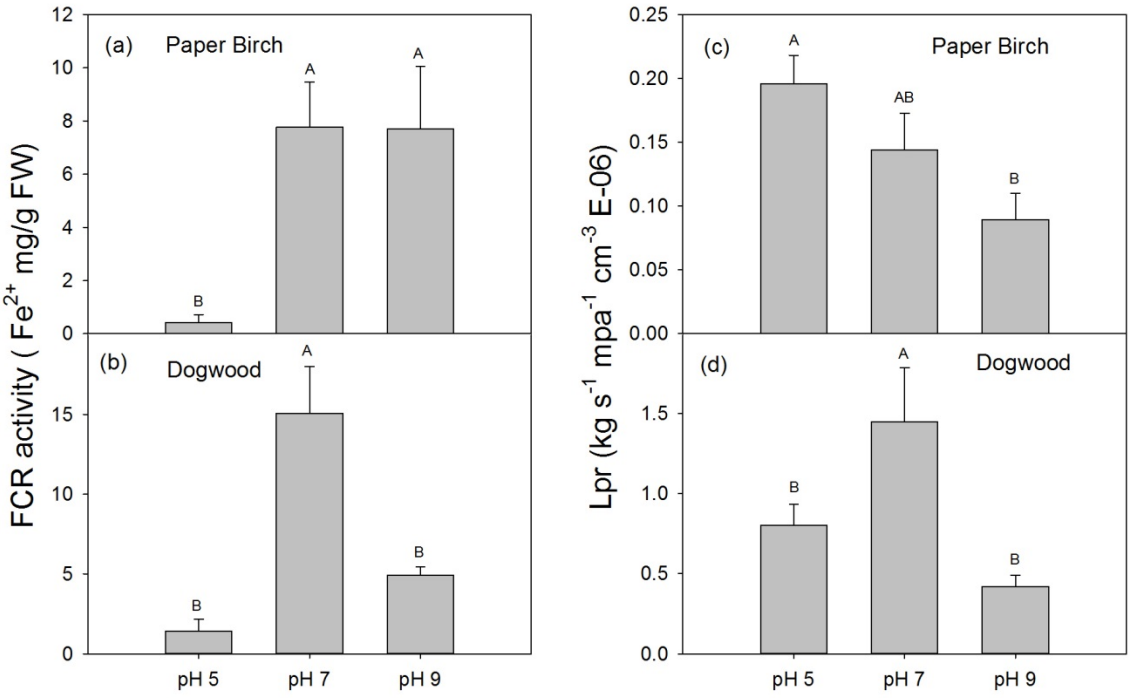

Figure 5. 3 Effects of root zone pH on ferric chelate reductase (FCR) activity (n = 6) and root hydraulic conductivity (Lpr) (n = 6) of paper birch and dogwood. Different letters above the bars indicate significant differences (α = 0.05) between treatments within each plant species. Means ± SE are shown.

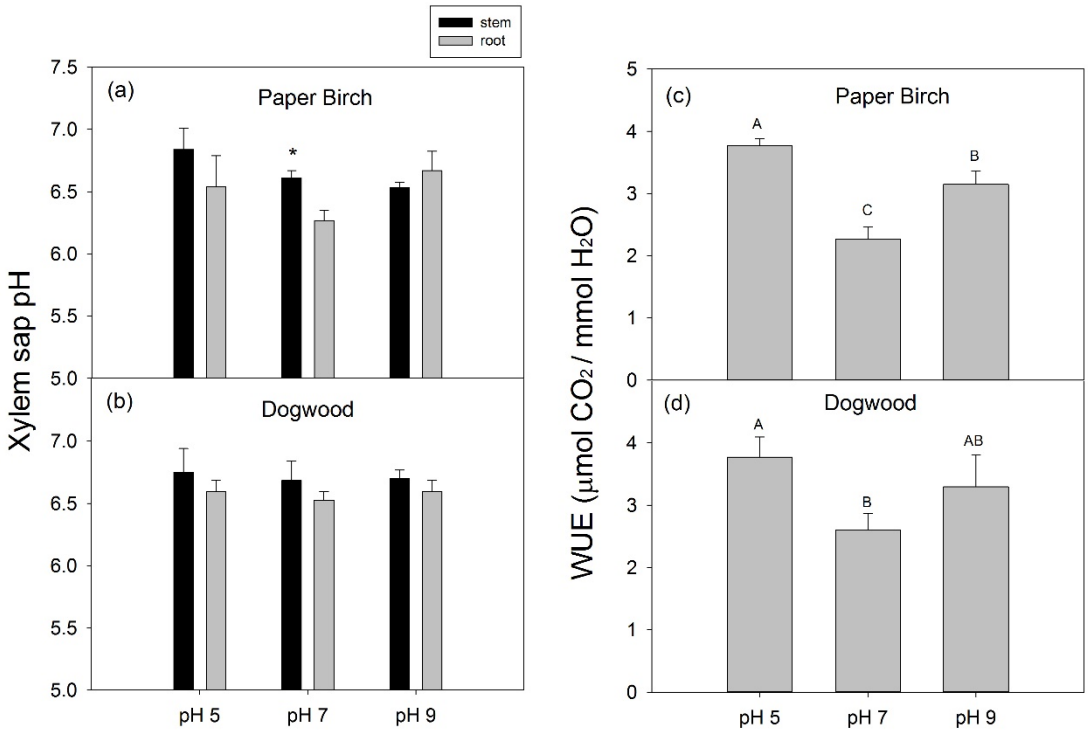

Figure 5. 4 Effects of root zone pH on stem and root xylem sap pH (n = 6) and water use efficiency (WUE) (n = 8) of paper birch and dogwood. Different letters and asterisk above the bars indicate significant differences (α = 0.05) between treatments within each plant species. Means ± SE are shown.

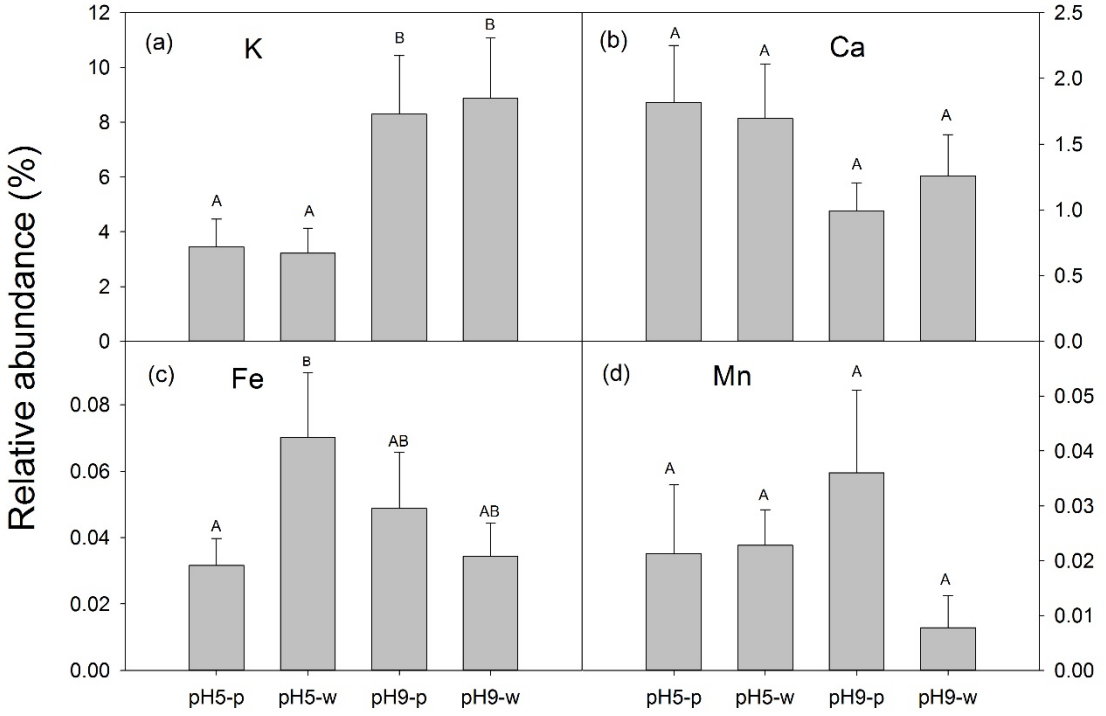

Figure 5. 5 Effects of root zone pH on relative abundance of K, Ca, Fe and Mn at leaf cell protoplast and cell wall of paper birch (n = 6). Different letters above the bars indicate significant differences (α = 0.05). Means ± SE are shown. (p - protoplast; w - cell wall)

Chapter 6

General discussion and conclusion

6.1 Summary of findings

The soil factors that are commonly present in oil sands reclamation areas and which can hamper their revegetation include high soil pH, high soil calcium levels, and the associated problems related to plant nutritional and water imbalance (Howat 2000; Visser 2005; Ramos-Padrón et al. 2010). In this research I investigated the effects of high root zone pH as well as calcium and phosphorus on growth and physiological responses of ten major boreal forest tree species. The studies aimed at developing knowledge required to improve oil sands reclamation following open-pit mining activities. To examine the processes which affect high pH tolerance of plants, I carried out several controlled-environment studies to investigate the mechanisms of high pH tolerance in the absence of other confounding environmental factors. I used a hydroponic system consistently through this project to remove the physical, chemical and biological soil factors which could interfere with the interpretation of the observed responses in plants. However, the results and conclusions obtained from these studies should be further tested in more complex soil systems and in field experiments before unequivocal recommendations can be provided to oil sands reclamation practitioners.

In Chapter 2, I studied the effect of root zone pH, as the sole factor, on growth and physiological responses of trembling aspen (*Populus tremuloides*), white spruce (*Picea glauca*) and tamarack (*Larix laricina*) seedlings. I tested the hypothesis that the differences in high pH tolerance between white spruce, tamarack and trembling aspen are

largely due to the differences in nutrient uptake and nutrient allocation mechanisms. I found the leaf chlorophyll reductions in aspen and tamarack at high pH to be associated with deficiencies of Fe, Mn and Zn as well as P and Ca. However, the deficiencies of these nutrients were also observed in white spruce, but were not severe enough to induce needle chlorosis. Plant water relations were also found to be disrupted by high root zone pH and the transpiration rates of the examined plants decreased sharply as a result of high root zone pH. White spruce was generally more tolerant to high root zone pH compared with the two deciduous species.

Due to the abundance of calcium in calcareous soils and in some of the oil sands reclamation sites, in Chapter 3, I investigated the confounding effects of high pH and high calcium levels in the root zone of six tree and shrub species of the boreal forest including blueberry (*Vaccinium myrtilloides*), bearberry (*Arctostaphyllos uva-ursi*), dogwood (*Cornus stolonifera*), white spruce (*Picea glauca*), trembling aspen (*Populus tremuloides*), and jack pine (*Pinus banksiana*). Due to the role of calcium in the function of cell walls, as proposed by the acid growth theory, I formulated the hypothesis that calcium would aggravate the effects of high pH through the inhibition of cell elongation in roots and the resulting impairment of root function. The results demonstrate that calcium aggravated the effects of high pH in dogwood, aspen and jack pine seedlings, while in blueberry, bearberry and white spruce, the confounding effects of calcium were relatively minor. The confounding effects of calcium on high pH responses in plants are likely due to the inhibition of root cortex cell elongation by both factors, which was especially prominent in jack pine, as well as the inhibitory effects on gas exchange and nutrient uptake. Thus, in reclamation areas with high calcium levels, these factors must

be considered to alleviate the combined effect of high pH and excessive calcium on plants when preparing reclamation strategies. Among the studied species, dogwood showed relatively high growth rates and overall resistance to high pH and high calcium levels. Therefore, dogwood should be considered as a potential candidate species for reclamation in areas where high soil pH and excessive calcium levels are present.

Since phosphorus deficiency is widely reported in plants growing in calcareous soils, in Chapters 2 and 3, I examined the effects of root zone pH on phosphorus nutrition in trembling aspen, white spruce, jack pine, tamarack, red osier dogwood, blueberry, and bearberry seedlings. The results showed reductions of leaf phosphorus concentration in aspen, white spruce, tamarack and dogwood seedlings under high root zone pH conditions. In the rhizosphere, soil pH distribution is usually not homogeneous and can change largely due to metabolic root activities, which may affect solubility and uptake of micronutrients by plants. In Chapter 4, I studied the effects of supplemental phosphorus nutrition and heterogeneous root zone pH conditions on the responses of six plant species including paper birch (*Betula papyrifera*), jack pine (*Pinus banksiana*), green alder (*Alnus viridis*), trembling aspen (*Populus tremuloides*), black spruce (*Picea mariana*), and white spruce (*Picea glauca*) to different calcium levels. Since high concentrations of soluble calcium and phosphorus cannot be achieved in the same solution due to precipitation of calcium phosphates, I used a split-root experiment setup in this study. I formulated a hypothesis that phosphorus oversupply and partial low root zone pH would benefit plants exposed to high root zone pH and elevated calcium levels. After 6 weeks of treatments, I found that the beneficial effects of increased phosphorus supply for plants exposed to high root zone pH were generally limited, and that the exposure of part of the

root system to low pH while keeping the remaining part exposed to high pH can increase leaf chlorophyll concentrations, possibly due to the effects on micronutrient uptake. Consistent with the results of Chapters 2 and 3, the results of the study in Chapter 4 showed that white spruce and black spruce were more resistant to high pH and high calcium levels compared with jack pine, paper birch, aspen and green alder.

In the first three research chapters (Chapters 2, 3, and 4), I studied various growth parameters, gas exchange, leaf chlorophyll concentrations, and elemental concentrations in several boreal forest plant species in response to different root zone pH, calcium and phosphorus levels. In Chapter 5, I investigated possible underlying mechanisms of plant tolerance to high root zone pH by comparing a species considered to be relatively sensitive to high pH (paper birch) with the species considered to be relatively tolerant to high pH (dogwood). I hypothesized the differences in responses of paper birch and dogwood to high root zone pH are partly due to the different responses in root water transport and apoplastic pH as well as the activity of ferric chelate reductase. I found that differences in the function of aquaporin and ferric chelate reductase are among the possible factors which may be responsible for greater high pH tolerance in dogwood compared with paper birch. Another reason that likely accounts for the high pH tolerance in dogwood is possibly related to the maintenance of lower apoplastic pH, which can help with the uptake and transport of Fe and Mn to leaf cells and maintain photosynthesis and growth.

6.2 General discussion

The hydroponic systems used for the different controlled-environment experiments in this thesis provided the possibility of precise control of treatment factors.

However, soil conditions are much more complex compared with solution culture. The pH buffering capacity of soil is much higher than in nutrient solutions. For example, plants of some of the species examined in this thesis project had been reported to grow well at pH 6.5 in the soil, while in the present hydroponic studies, they showed reduced growth and stress symptoms at the same pH. Therefore, it is possible that plant responses to pH may be modulated in the soil and the plants may perform better compared with solution culture. The microbial activities in soil, especially mycorrhizal associations, can significantly affect plant responses to various soil physical and chemical factors. It has been well established that mycorrhizas can enhance water and nutrient uptake in colonized plants. However, the effects of mycorrhizas on plants could not be examined in hydroponic culture. Other soil physical factors including compaction and particle size can also have major effects on plant growth. Effects of high soil pH and these factors on plants should also be tested in field experiments where various combinations of these factors may be present. In the oil sands reclamation areas, plants must cope with numerous other environmental stresses including salinity, temperature extremes, and periods of drought. All of these environmental factors could affect plant survival and growth and affect oil sands revegetation efforts.

The results of the study in which I compared responses of ten boreal forest plant species to high root zone pH showed that white spruce, black spruce and dogwood were relatively more resistant to high pH compared with the remaining seven species. However, the mechanisms of high pH tolerance in white and black spruce and dogwood may also be different. As the growth rate of white spruce is probably the lowest among the studied species and, in Chapter 2, white spruce showed reductions of foliar elemental

concentrations in response to high pH, and these reductions did not impact growth over the study period of 8 weeks. Since the successful reforestation process may take several decades (Grant et al. 2008), the long-term effects of nutrient deficiencies on slow-growing species need to be considered when preparing reclamation strategies. However, another major consequence of high pH stress in plants includes high concentration of [OH^-] in apoplastic space, which can significantly reduce root growth by inhibiting cell elongation in the root cortex (Rayle and Cleland 1992; Hager 2003), and which was also confirmed in Chapter 3 for jack pine. The root growth of white spruce seedlings was less affected compared with aspen and jack pine, and this implies that white spruce may have some mechanisms to maintain low apoplastic pH in the root cortex. I propose the hypothesis to be addressed by future studies that the epidermal cells in roots of white spruce are modified to prevent high medium pH from affecting the root cortex.

The reasons for tolerance of high root zone pH in dogwood may be related to the architecture of the root system. Unlike many other species, dogwood does not form taproots, and its fibrous root system has more short roots and root hairs which form a ball-like structure in hydroponic culture. This structure might enhance the pH buffering capacity of the dogwood root system, as the densely tangled roots reduce the flow of the nutrient solution and ameliorate the effects of high medium pH on the inner roots. Modifying root system architecture could offer an interesting option to alter plant responses to abiotic stresses originating in the soil, however, these ideas need to be further explored in future studies. Differences in root architecture between the studied species, which were observed in the hydroponic set-up, however, may not be present in the soil. Therefore, future studies should include a solid root medium to confirm the

results of hydroponics experiments.

In Chapter 3, I reported that high calcium concentrations could also inhibit root cell elongation in a similar way as high pH. Although this had been previously reported for other plants (Cleland and Rayle 1977), in the current model of "acid growth theory", which is most widely accepted to explain the plant cell elongation processes, the role of calcium in the cell walls was not clearly interpreted (Rayle and Cleland 1992; Hager 2003). Due to the relatively large amounts of calcium in the cell walls (Hepler and Winship 2010), it is plausible that calcium also plays an important role in plant cell elongation, which should receive more attention in future studies on plant cell elongation and differentiation.

Contrary to my original hypothesis, increasing phosphorus supply to the root zone had only a minor effect on plants exposed to high pH. It is possible that other nutrient limitations prevented supplemental phosphorus nutrition from having an effect, according to the law of the minimum. Deficiencies of other nutrients might also significantly affect phosphorus uptake (Adriano et al. 1971). The interactions of different nutrients on plant growth under stress conditions needs to be further studied and supplementing plants that grow in high pH soils with a combination of nutrients may be an effective strategy to help plants cope with high pH conditions.

6.3 Suggestions for oil sands reclamation

I carried out studies on the effects of root zone pH, calcium, and phosphorus nutrition in plant species that are commonly used for the reclamation of oil sands areas. Among the studied species, I found white spruce, black spruce and dogwood to be

generally more resistant to high root zone pH than the remaining studied species. The current policy requires oil sands operators to restore the disturbed ecosystem to its original state, including a degree of biodiversity (Alberta-Environment 2010). This means that as many native plant species as possible should be present in the reclaimed areas and the reclaimed areas need to become self-sustainable over time. Plants have some potential to lower rhizosphere pH and optimize their growth through the activities involving proton pumping and extruding organic acids from the roots (Hinsinger et al. 2003). Due to its fast growing rate and stress resistance, dogwood is a desirable candidate reclamation species for oil sands reclamation areas and could likely be successfully planted in high pH reclamation sites before the soil pH is lowered and made more suitable to other plants.

In this thesis, I found that the deficiencies of micronutrients, including Fe, Mn and Zn, are correlated with reduced chlorophyll concentrations and photosynthesis leading to decreased growth rates. Due to high soil pH conditions, the utilization rate of these nutrients for plants is low in the rhizosphere (Marschner 2012). Since in agricultural and horticultural practices, the foliar applications of micronutrients have been proven effective in reducing leaf chlorosis and increasing yield (Modaihsh 1997; Dordas 2009), this method should also be tested in oil sands reclamation areas. In some reclamation areas, fertilizers with N, P, and K are already applied by aerial spraying and, therefore, the micronutrients could be added to these applications with relatively low cost. Both Fe and Mn are strongly linked to photosynthetic activities (Marschner 2012), and even very small amounts of these micronutrients might prominently enhance photosynthetic rates (Horesh and Levy 1981). If plants under high pH conditions could maintain high

photosynthetic rates, they may also be able to utilize the captured energy for stress resistance, and the root activity will over time help reduce soil pH.

6.4 Suggestions for future research

Although I have already mentioned above some of the additional studies that could help solve the questions raised through this research, I listed below the highest priority areas that require more research to understand plant resistance to high soil pH conditions:

1) Examine the combined effects of high root zone pH and salinity on the growth and physiological responses of major boreal forest plant species.

2) Examine the H^+-ATPase activity and expression of high pH tolerant and sensitive plant species under high root zone pH conditions.

3) Examine changes in aquaporin function and expression patterns in relation to cell and root hydraulic conductivities in plants subjected to high root zone pH conditions.

4) Study changes in leaf and root apoplastic pH using refined microtechniques in high pH tolerant and sensitive species exposed to high soil pH.

6.5 References

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Appendix 1

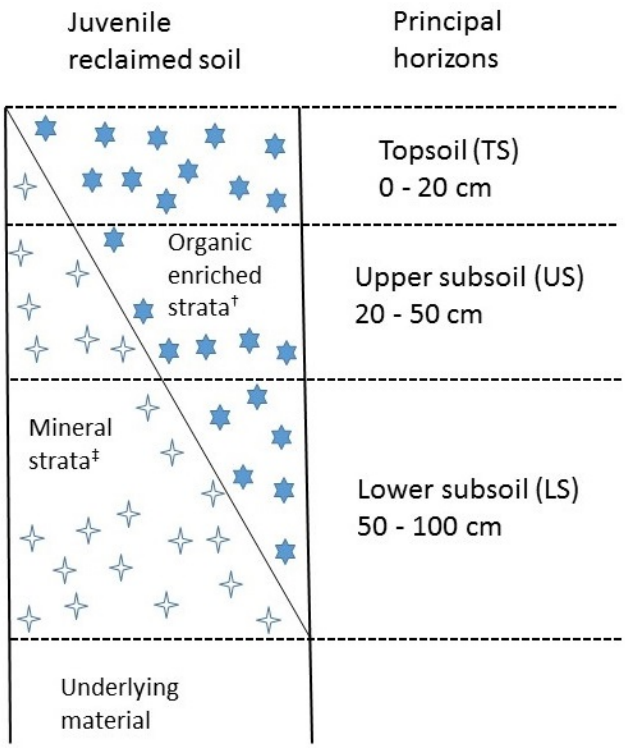

Figure a1. 1 *Schematic diagram of the principal horizons applied to idealized reclaimed soil[§] profiles.

[†]Mineral horizons are defined as those having less than 17% total organic carbon (TOC).

[‡]Organic-enriched strata are mineral horizons containing organic matter (i.e., peat/mineral mixes and shallow soil salvage). In the cases where the surface strata of a reclaimed soil or natural mineral soil with an O layer contain 17% or more TOC it is not considered to contribute to the moisture regime of the soil.

[§]These profiles are generalizations. Each soil type presented is characterized by wide ranges of variability in horizon thickness and development.

*Referred from: CEMA (2006) Land Capability Classification System for Forest Ecosystems in the Oil Sand. 3rd ed. Volume 1: Field Manual for Land Capability Determination. Alberta Environment, Edmonton, AB.

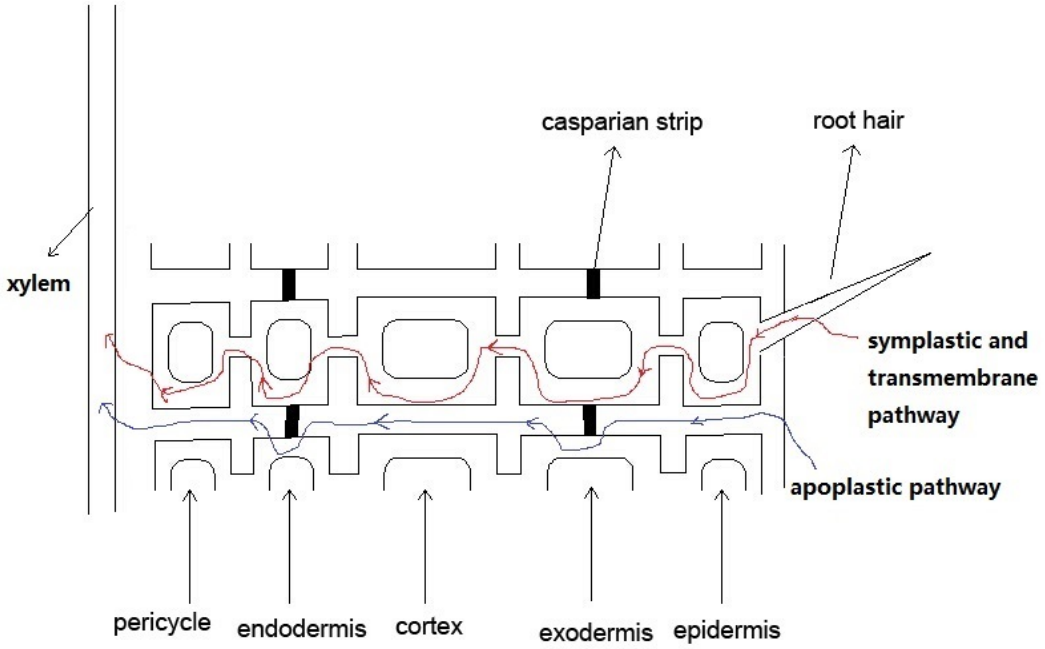

Figure a1. 2 Water uptake pathways in the root. Through the cortex, water may travel via the apoplast, the transmembrane, and the symplast pathway. In the symplast pathway, water flows between cells through the plasmodesmata without crossing the plasma membrane. In the transmembrane pathway, water moves through the plasma membranes, with a short visit to the cell wall space. At the endodermis and exodermis, the apoplast pathway is blocked by the Casparian strip.

Appendix 2

(Supplementary results of Chapter 2)

Table a2. 1 Pearson correlation coefficients of foliar elements concentration and growth and physiology parameter in aspen. Asterisk labeled means significantly correlated (P-value<0.05). Absolute value of correlation coefficient higher than 0.6 is regarded as highly correlated, marked as bold and underlined.

	pH	RDW	SDW	Pn	E	oCha	oChb	yCha	yChb
oB	0.092	-0.119	-0.127	0.028	-0.132	-0.139	-0.201	-0.211	-0.254
oMg	-0.232	0.012	0.070	0.079	-0.048	0.022	-0.277	-0.092	-0.307
oP	***-0.762**	*0.336	*0.546	*0.515	*0.31	***0.607**	0.015	*0.456	0.198
oK	***0.728**	*-0.318	*-0.495	*-0.569	-0.175	***-0.648**	0.142	*-0.566	*-0.358
oCa	*-0.595	*0.393	*0.407	0.176	*0.418	-0.014	0.305	0.061	0.061
oFe	***-0.823**	*0.186	*0.463	*0.542	***0.624**	***0.923**	*0.465	***0.89**	***0.785**
oMn	***-0.673**	0.243	*0.369	*0.474	*0.29	***0.673**	0.196	***0.702**	*0.543
oCu	0.126	-0.089	-0.106	-0.151	-0.189	-0.172	-0.210	-0.137	-0.268
oZn	*-0.385	-0.062	0.088	0.094	0.257	0.308	0.085	*0.33	0.173
oMo	*-0.474	0.011	0.120	0.253	0.175	*0.403	0.051	*0.335	0.162
yB	0.183	-0.234	-0.241	-0.056	-0.248	-0.318	-0.241	*-0.366	*-0.45
yMg	-0.115	-0.016	0.063	-0.026	-0.169	-0.172	*-0.448	-0.248	*-0.455
yP	*-0.373	0.099	*0.324	0.175	0.016	0.120	*-0.271	-0.002	-0.250
yK	***0.685**	*-0.411	*-0.485	*-0.537	*-0.375	***-0.786**	*-0.319	***-0.714**	***-0.625**
yCa	*-0.554	0.277	*0.372	*0.358	0.095	*0.377	-0.081	*0.358	0.158
yFe	***-0.923**	0.237	*0.496	***0.667**	***0.607**	***0.907**	*0.34	***0.903**	***0.771**
yMn	*-0.578	0.175	0.248	*0.431	0.191	*0.547	0.120	***0.669**	*0.566
yCu	0.088	-0.087	-0.046	-0.087	-0.264	-0.158	-0.207	-0.178	*-0.316
yZn	*-0.418	0.148	0.279	0.249	-0.015	0.263	-0.218	0.277	0.064
yMo	*-0.451	0.012	0.108	0.303	0.111	*0.369	0.020	*0.397	0.249

Abbreviations: RDW-root dry weight; SDW-shoot dry weight; Pn-net photosynthesis rate; E-transpiration rate; oCha-chlorophyll a concentration in old leaves; oChb-chlorophyll b concentration in old leaves; yCha-chlorophyll a concentration in young leaves; yChb-chlorophyll b concentration in young leaves; elements symbol with o means in old leaves, with y means in young leaves

Tabel a2. 2 Pearson correlation coefficients of foliar elements concentration and growth and physiology parameter in tamarack. Symbol meaning and abbreviation is the same as in table a2.1.

	pH	RDW	SDW	Pn	E	oCha	oChb	yCha	yChb
oB	-0.005	-0.117	-0.263	0.251	0.105	0.036	0.035	0.057	0.089
oMg	*-0.418	*0.429	*0.404	0.193	0.209	*0.411	*0.385	*0.281	-0.039
oP	***-0.664**	*0.441	*0.476	***0.61**	***0.676**	***0.749**	***0.757**	***0.652**	-0.145
oK	*0.466	-0.123	-0.205	-0.181	-0.212	*-0.417	*-0.345	-0.157	*0.592
oCa	*-0.75	*0.51	*0.523	***0.631**	***0.609**	***0.672**	***0.652**	*0.555	*-0.257
oFe	-0.199	0.172	*0.296	0.028	0.043	0.222	0.193	0.146	0.044
oMn	*-0.526	*0.314	*0.342	***0.636**	***0.639**	*0.439	*0.512	***0.787**	0.249
oCu	0.297	-0.211	-0.146	-0.064	-0.198	-0.193	-0.131	-0.039	0.282
oZn	*-0.561	*0.417	*0.452	***0.733**	***0.735**	*0.552	*0.569	***0.777**	0.063
oMo	-0.061	0.107	0.145	0.067	0.082	-0.027	0.036	0.205	*0.356
yB	*-0.491	*0.343	0.221	*0.426	*0.423	*0.389	*0.39	0.211	-0.064
yMg	*-0.529	*0.467	*0.479	*0.363	*0.324	*0.464	*0.45	0.225	-0.038
yP	***-0.761**	*0.453	*0.497	***0.718**	***0.668**	***0.758**	***0.78**	***0.614**	-0.071
yK	*0.401	-0.135	-0.151	-0.061	-0.173	-0.171	-0.108	-0.162	***0.617**
yCa	***-0.837**	*0.585	***0.608**	***0.705**	***0.655**	***0.708**	***0.697**	*0.53	-0.265
yFe	*-0.395	*0.566	*0.553	0.229	0.268	0.245	0.230	0.312	0.087
yMn	*-0.594	*0.35	*0.373	***0.74**	***0.67**	*0.496	*0.562	***0.611**	0.110
yCu	*-0.513	***0.651**	*0.598	*0.452	*0.48	*0.402	*0.436	*0.416	0.124
yZn	*-0.573	0.247	*0.339	***0.667**	***0.638**	*0.552	*0.527	*0.564	-0.067
yMo	*-0.494	*0.377	*0.511	*0.47	*0.452	***0.643**	***0.668**	*0.486	0.162

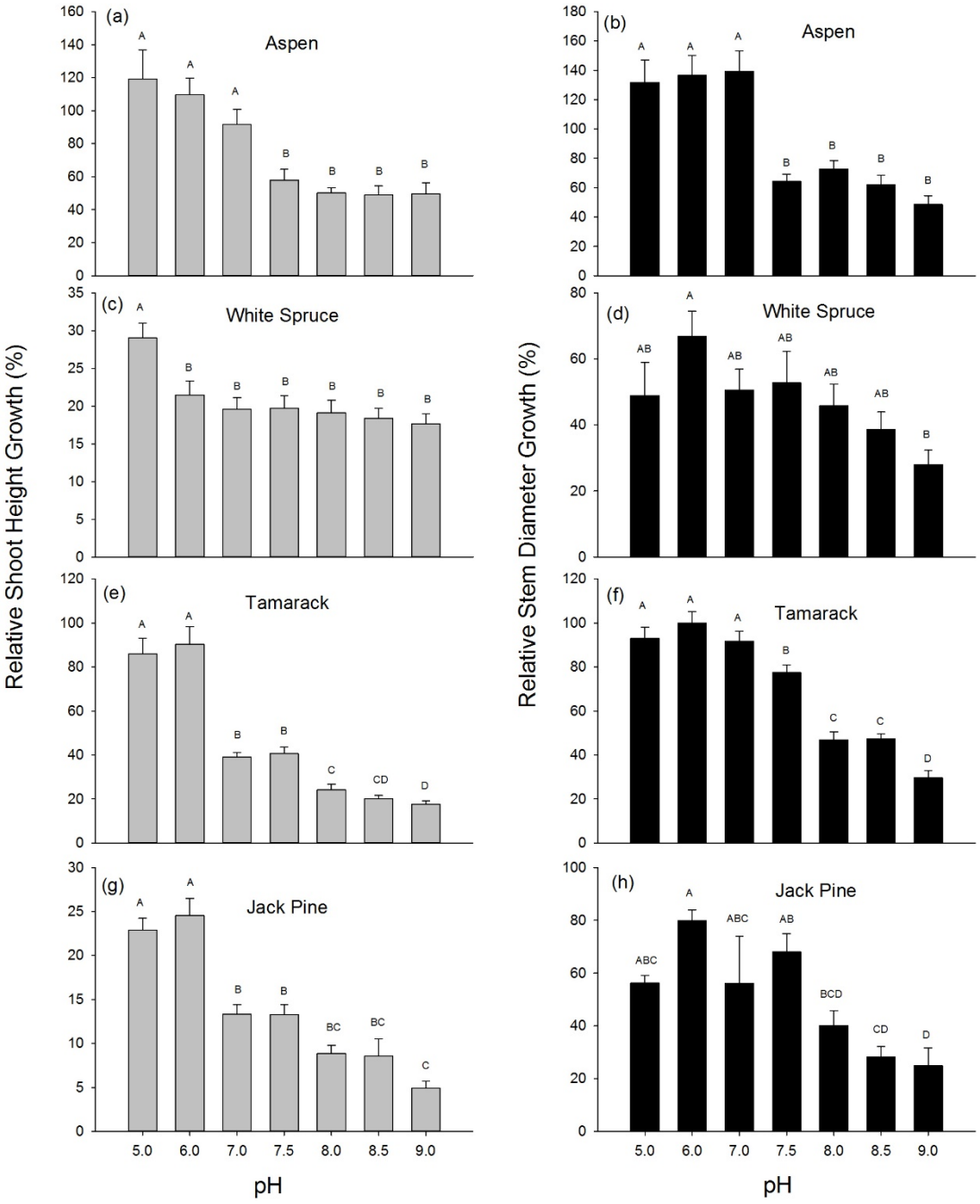

Figure a2. 1 Effects of pH on relative shoot height growth and relative stem diameter growth* in aspen, white spruce, tamarack and jack pine seedlings. Different letters above the bars indicate significant differences (α=0.05) between treatments within each plant species. Means (n=18) ± SE are shown.

* The relative shoot height and stem diameter growth were calculated by dividing the difference in the initial and final values by the initial value. Same in appendix 3.

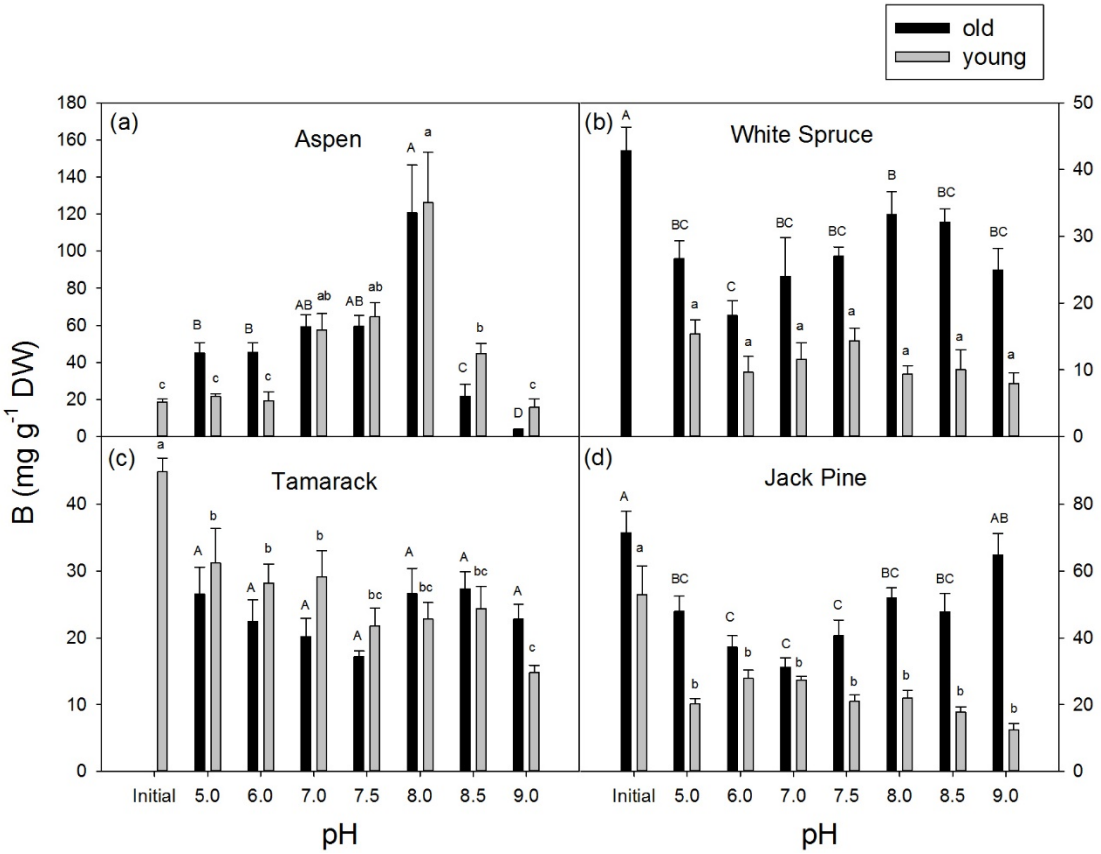

Figure a2. 2 Effects of pH on B concentrations in young and old leaves of aspen, white spruce, tamarack and jack pine seedlings. Initial values were measured in the beginning of treatment, same in the following figures. Different letters above the bars indicate significant differences (α=0.05) between treatments within each plant species. Means (n=6) ± SE are shown.

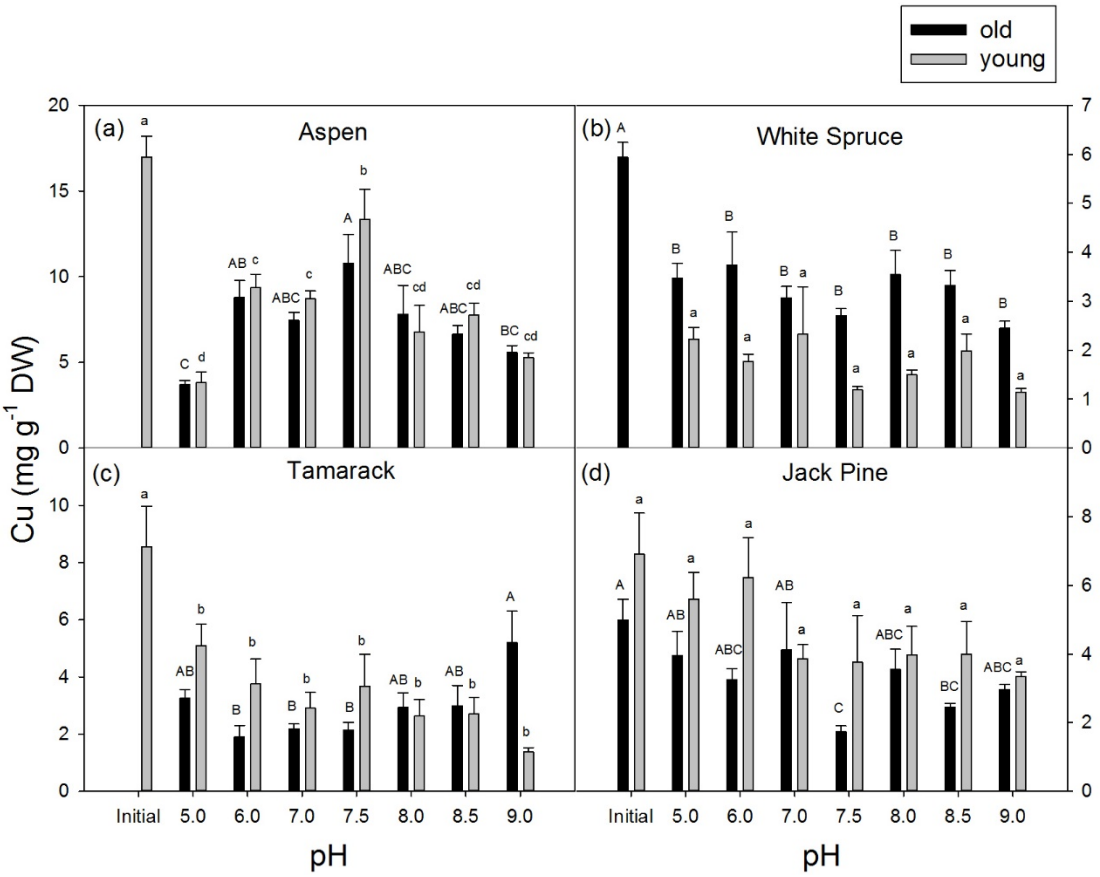

Figure a2. 3 Effects of pH on Cu concentrations in young and old leaves of aspen, white spruce, tamarack and jack pine seedlings. Different letters above the bars indicate significant differences (α=0.05) between treatments within each plant species. Means (n=6) ± SE are shown.

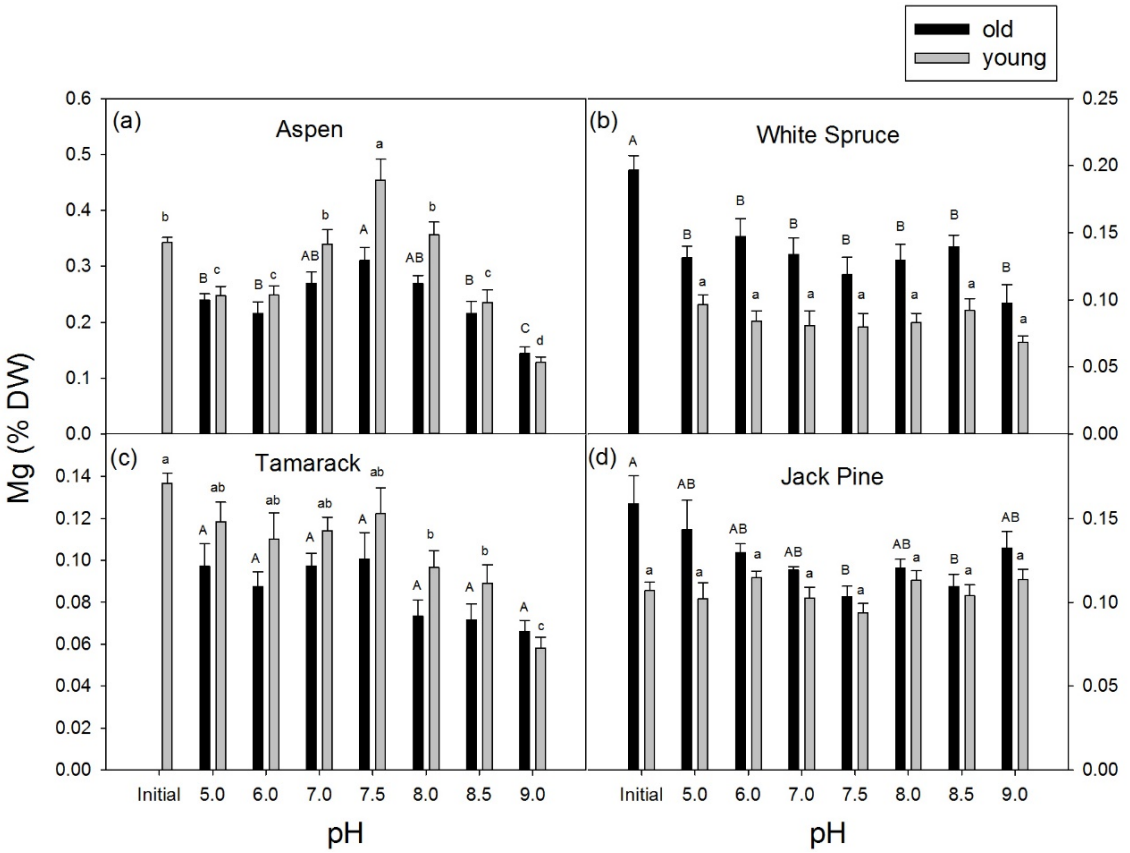

Figure a2. 4 Effects of pH on Mg concentrations in young and old leaves of aspen, white spruce, tamarack and jack pine seedlings. Different letters above the bars indicate significant differences (α=0.05) between treatments within each plant species. Means (n=6) ± SE are shown.

Appendix 3

(Supplementary results of Chapter 3)

Table a3. 1 ANOVA table showing effects of pH and Ca treatments on measured parameters for blueberry, bearberry, dogwood, white spruce and aspen seedlings.

Blueberry

p-value	DW	Y-Chl	Fe	Mn	P	K	Ca
pH	0.8336	0.4899	<0.0001	0.1699	0.2055	<0.0001	0.0026
Ca	0.1653	0.0577	0.3549	0.0942	0.4747	0.0356	<0.0001
pH*Ca	0.1979	0.1868	0.0189	0.1567	0.0611	0.0193	0.0074

Bearberry

p-value	DW	Y-Chl	Fe	Mn	P	K	Ca
pH	0.1762	0.0003	<0.0001	0.3454	0.0135	0.4245	<0.0001
Ca	0.2667	0.0235	0.0153	0.0485	0.5920	0.6583	<0.0001
pH*Ca	<0.0001	0.0047	0.0002	0.7983	0.0589	0.1417	0.0001

Dogwood

p-value	DW	Pn	E	Y-Chl	Fe	Mn	P	K	Ca	N
pH	0.0014	<0.0001	<0.0001	<0.0001	<0.0001	0.0060	<0.0001	<0.0001	<0.0001	<0.0001
Ca	0.9279	0.2830	<0.0001	<0.0001	<0.0001	<0.0001	0.0701	<0.0001	<0.0001	0.3338
pH*Ca	0.0179	<0.0001	<0.0001	0.3597	0.0913	<0.0001	0.0012	<0.0001	<0.0001	0.1547

White Spruce

p-value	DW	Pn	E	Y-Chl	Fe	Mn	P	K	Ca	N
pH	0.0026	0.0009	0.0069	0.0049	0.3775	0.1797	0.0063	0.0008	<0.0001	0.0748
Ca	0.0002	0.5847	0.1508	0.0017	0.0597	0.2591	0.1046	0.0476	<0.0001	0.1125
pH*Ca	0.1404	0.4083	0.5539	0.0025	0.5709	0.2483	0.1681	0.2600	0.0640	0.4509

Aspen

p-value	DW	Pn	E	Y-Chl	Fe	Mn	P	K	Ca	N
pH	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0004
Ca	0.6366	0.0115	0.0040	0.0193	0.1193	0.0012	0.0056	<0.0001	<0.0001	0.7108
pH*Ca	0.0278	0.0077	0.7946	0.0029	0.0114	0.0001	0.0002	0.0002	0.0042	0.7033

Abbreviations are: dw − dry weight; Pn − net photosynthetic rate; E − transpiration rate; Y-chl − chlorophyll concentrations in young leaves.

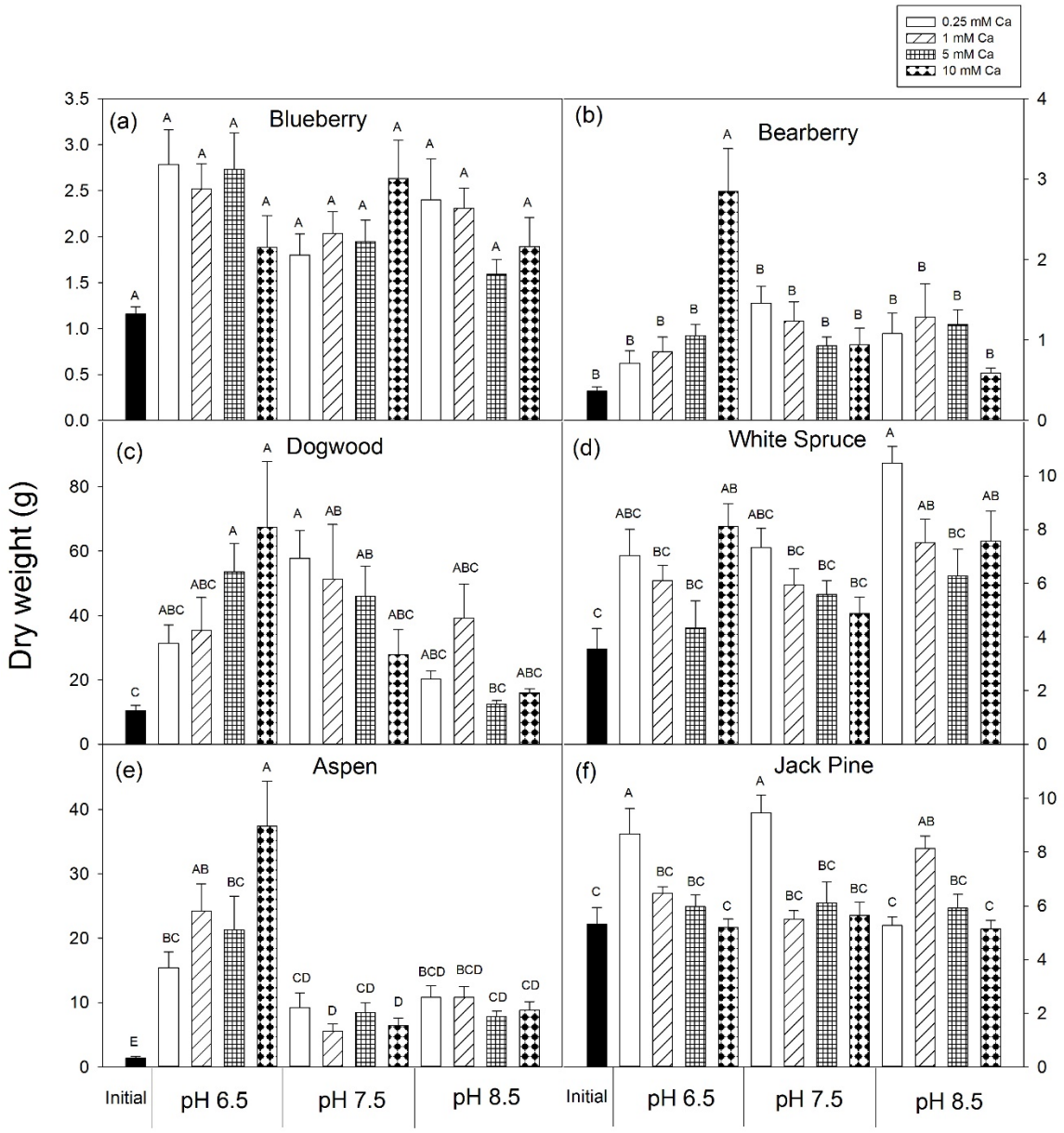

Figure a3. 1 Effects of pH and Ca treatments on total dry weights of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Initial values were measured in the beginning of treatment, same in the following figures. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 18$) ± SE are shown.

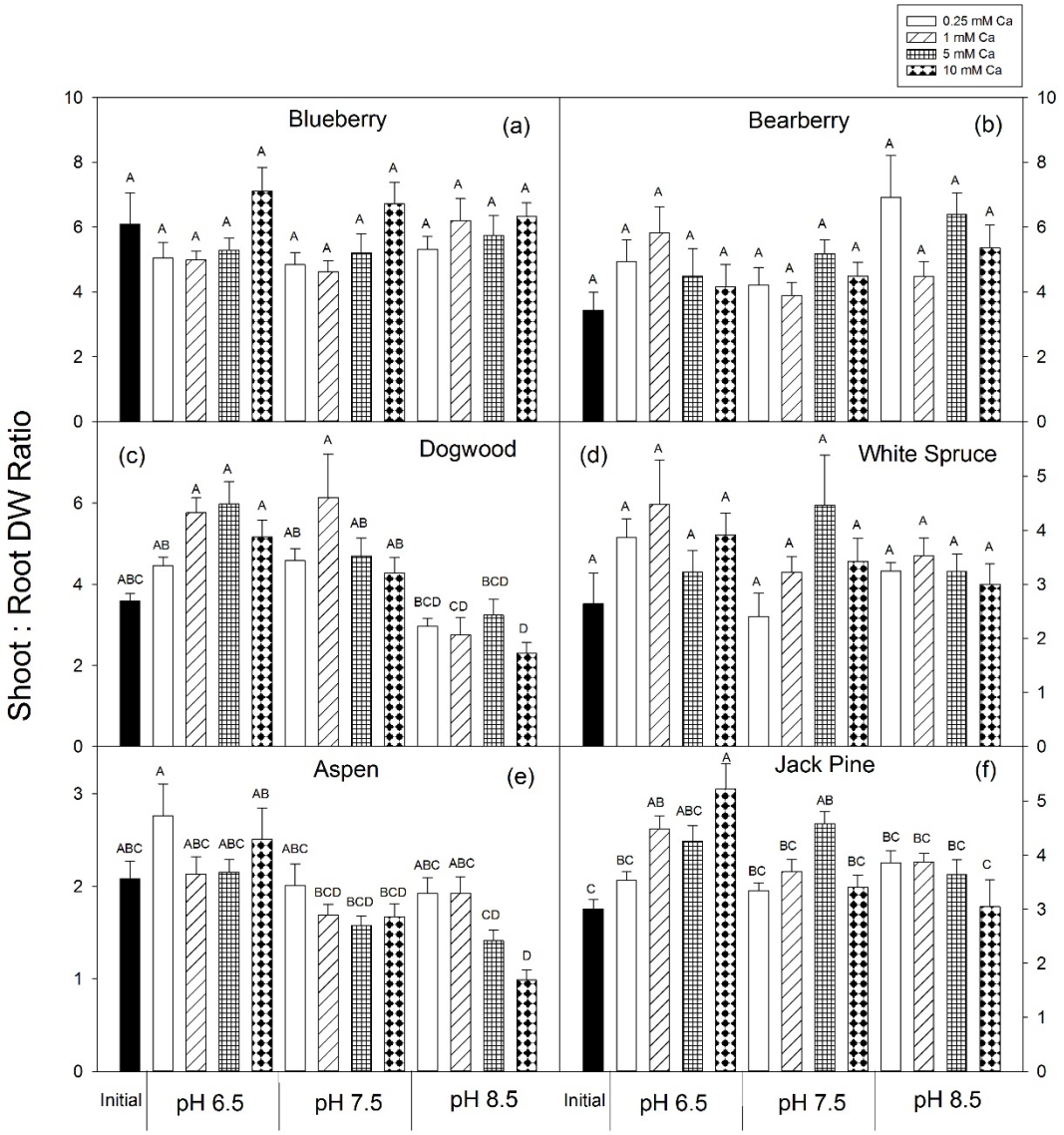

Figure a3. 2 Effects of pH and Ca treatments on shoot:root dry weight ratios of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 18$) ± SE are shown.

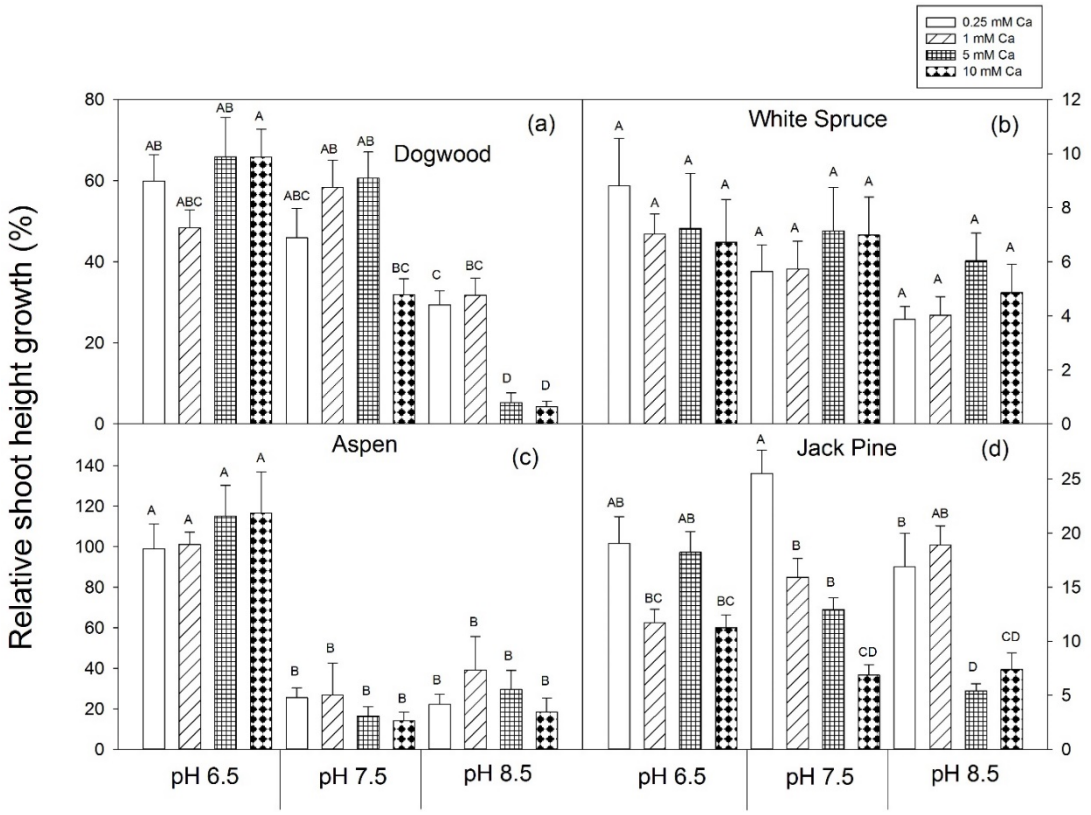

Figure a3. 3 Effects of pH and Ca treatments on relative shoot height growth of dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 18$) ± SE are shown.

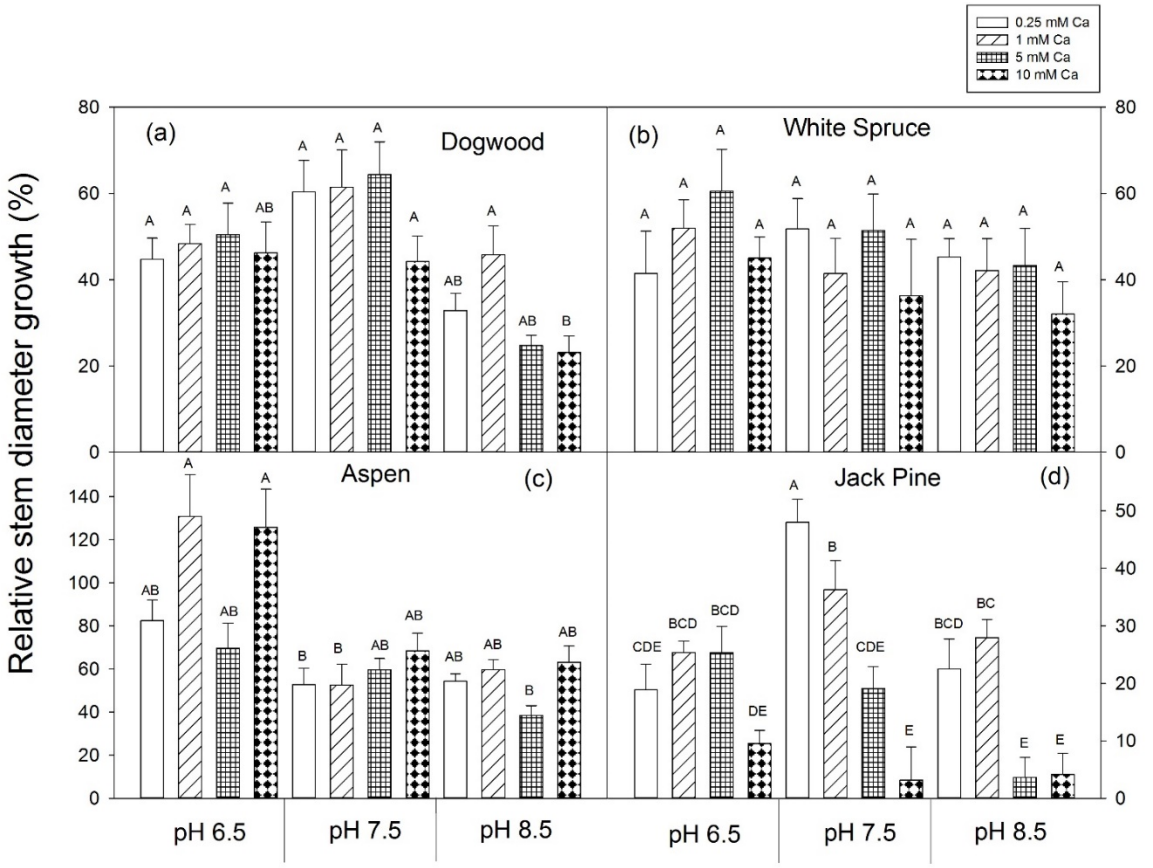

Figure a3. 4 Effects of pH and Ca treatments on relative stem diameter growth of dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 18$) ± SE are shown.

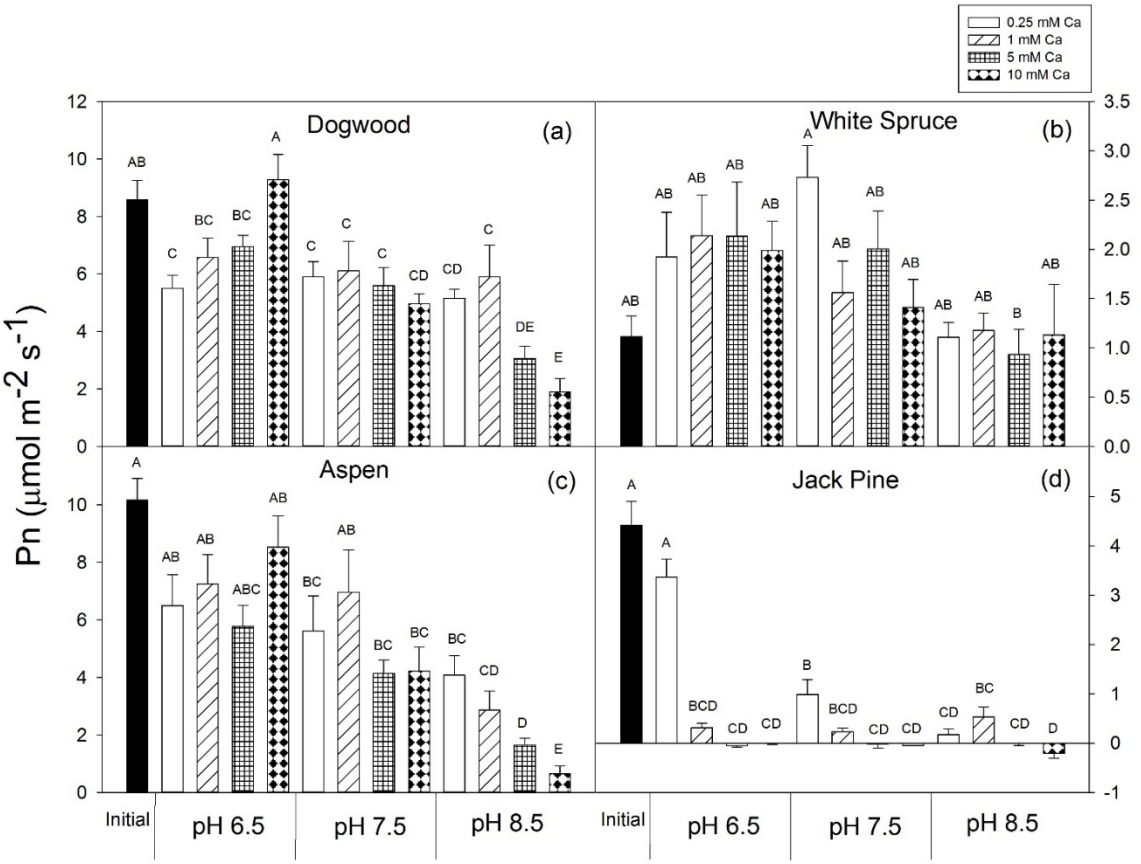

Figure a3. 5 Effects of pH and Ca treatments on net photosynthesis rates (Pn) in dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 6$) ± SE are shown.

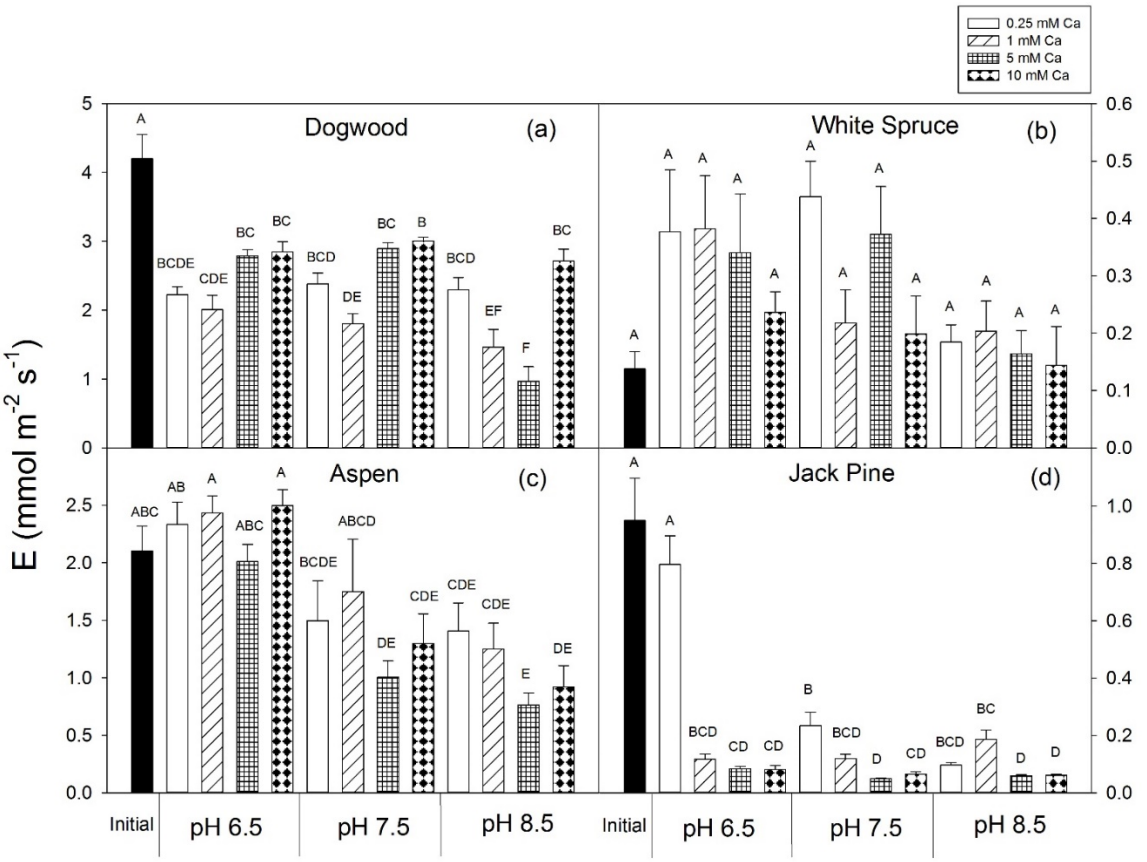

Figure a3. 6 Effects of pH and Ca treatments on transpiration rates (E) in dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 6$) ± SE are shown.

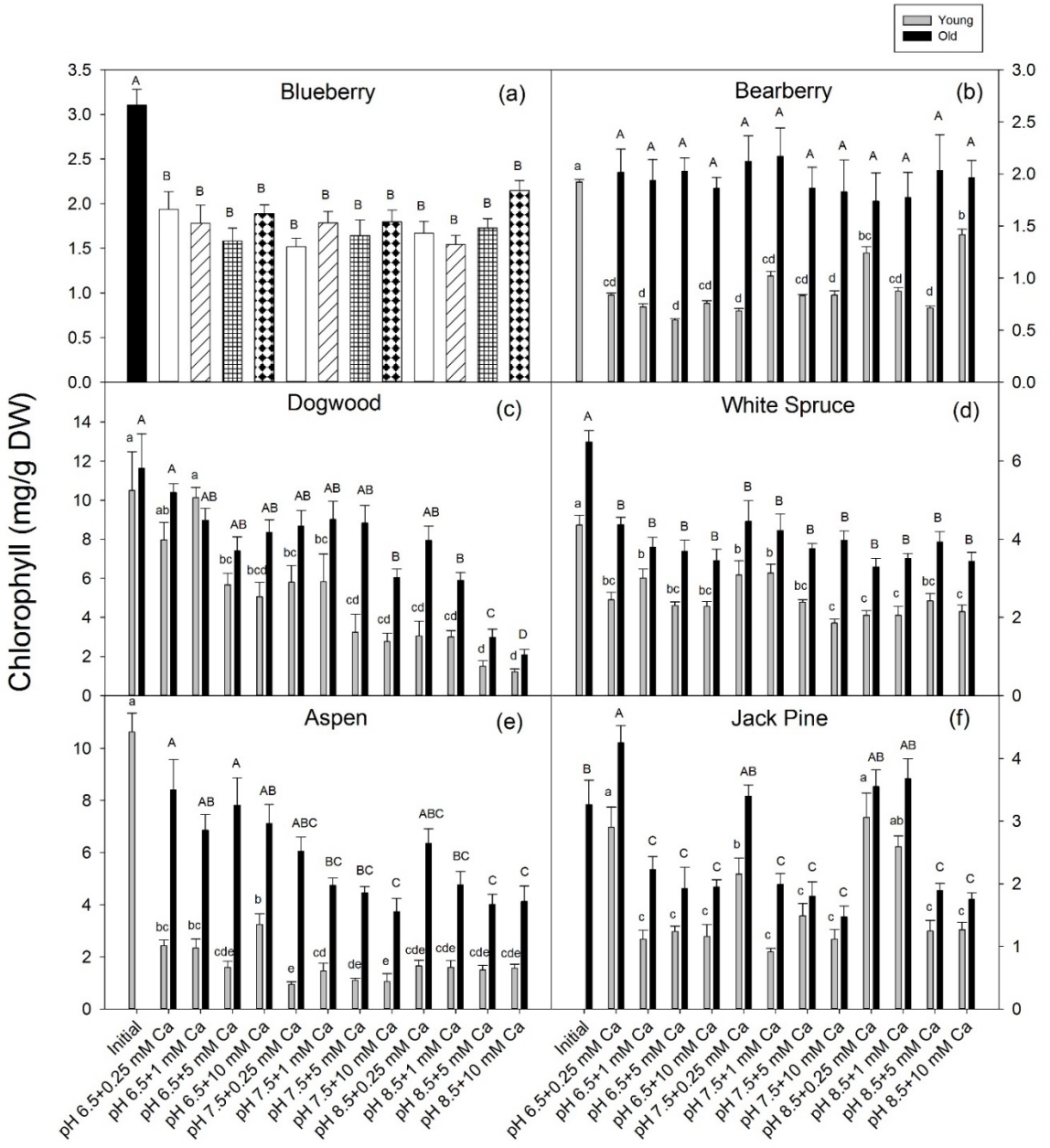

Figure a3. 7 Effects of pH and Ca treatments on total chlorophyll concentrations (chlorophyll-a + chlorophyll-b) in old and young leaves of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars (uppercase letters for old leaves and lowercase letters for young leaves) indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 6$) ± SE are shown.

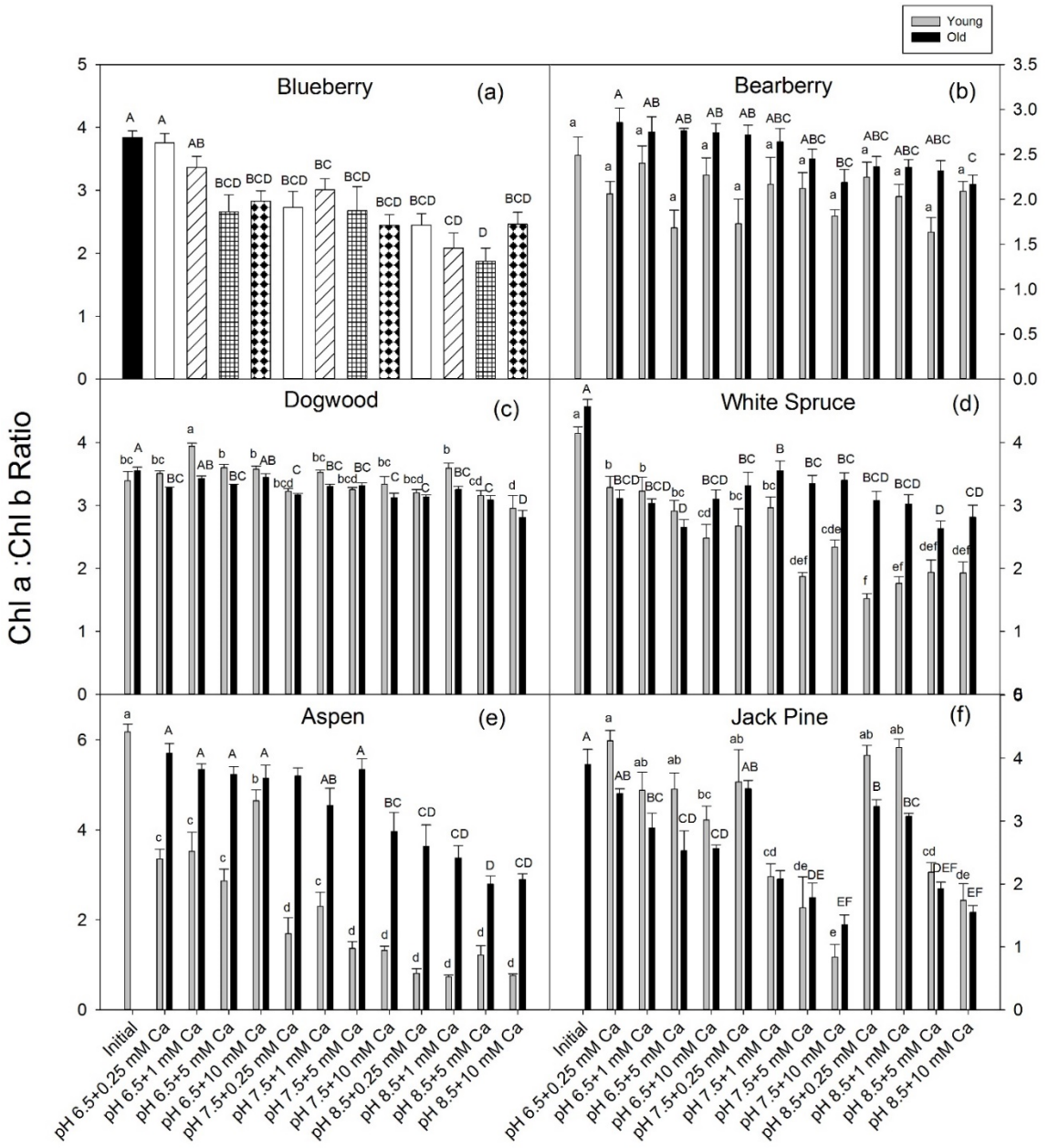

Figure a3. 8 Effects of pH and Ca treatments chlorophyll-a:b ratios in old and young leaves of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars (uppercase letters for old leaves and lowercase letters for young leaves) indicate significant differences ($\alpha = 0.05$) between treatments determined by the Student-Newman-Keuls test. Means ($n = 6$) ± SE are shown.

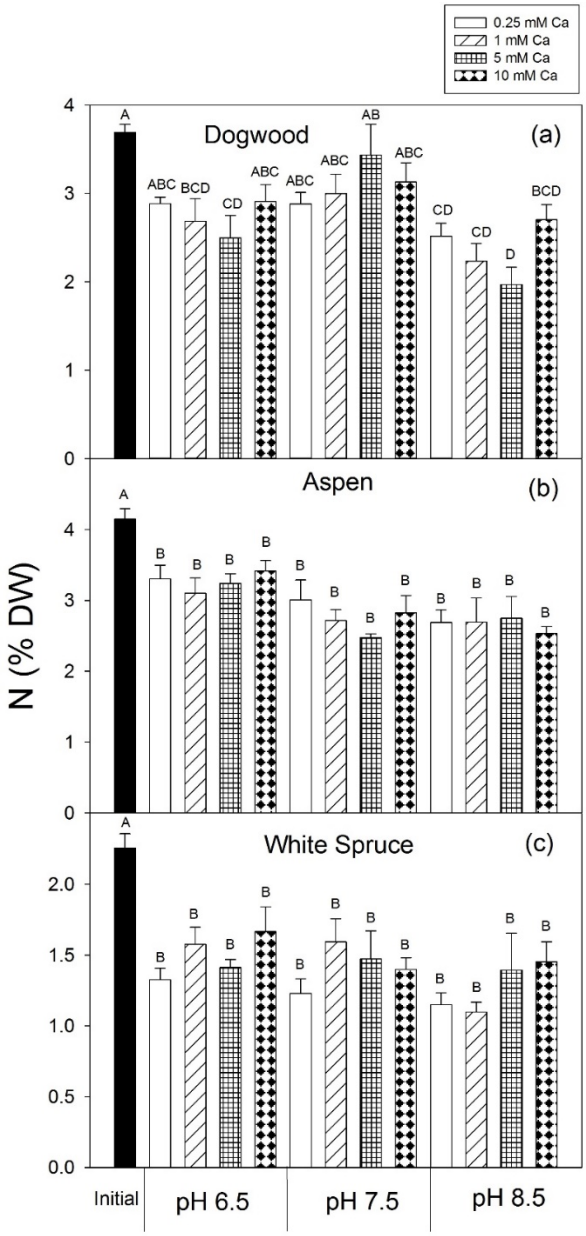

Figure a3. 9 Effects pH and Ca treatments on N concentrations in young leaves of dogwood, aspen and white spruce seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means (n=6) ± SE are shown.

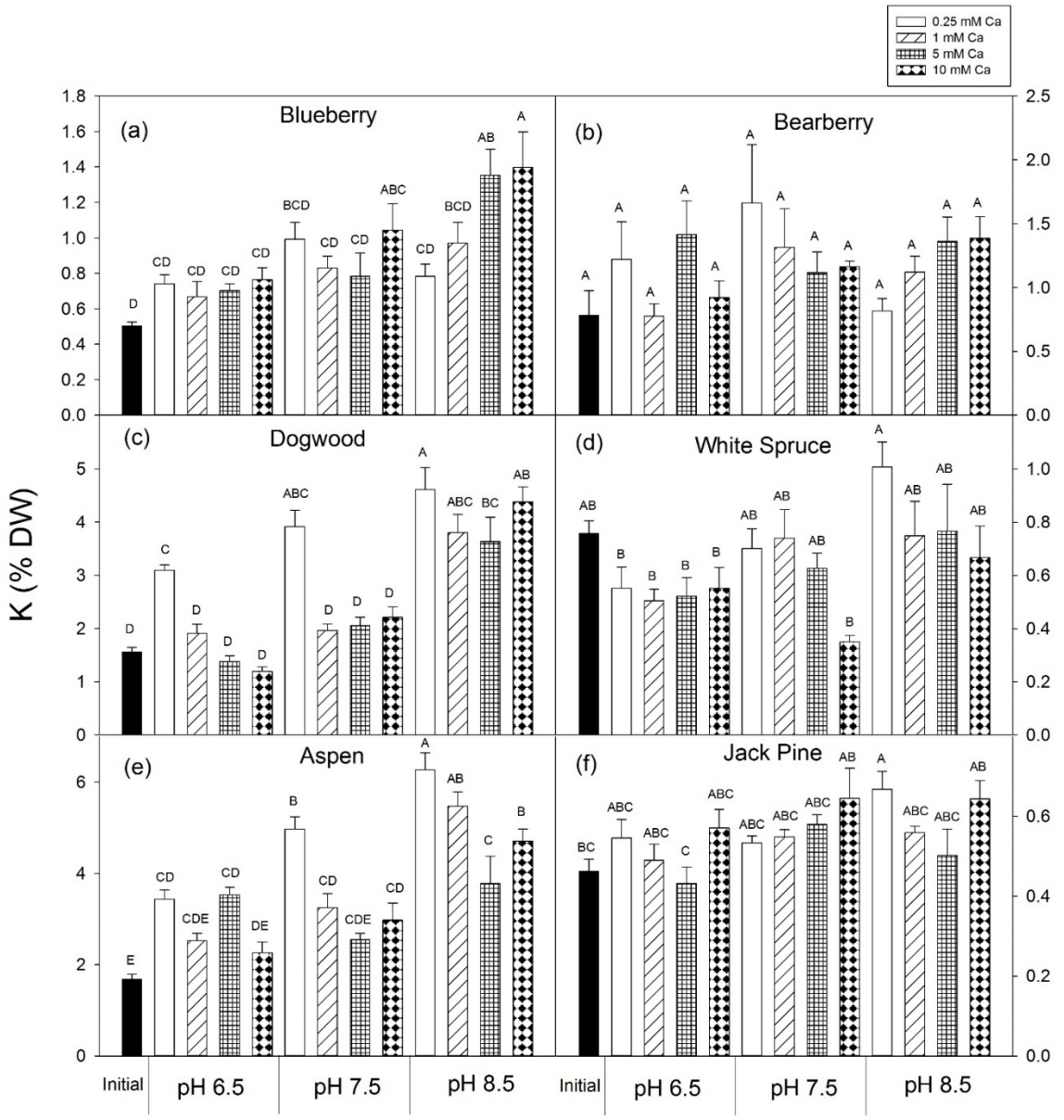

Figure a3. 10 Effects pH and Ca treatments on K concentrations in young leaves of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means (n=6) ± SE are shown.

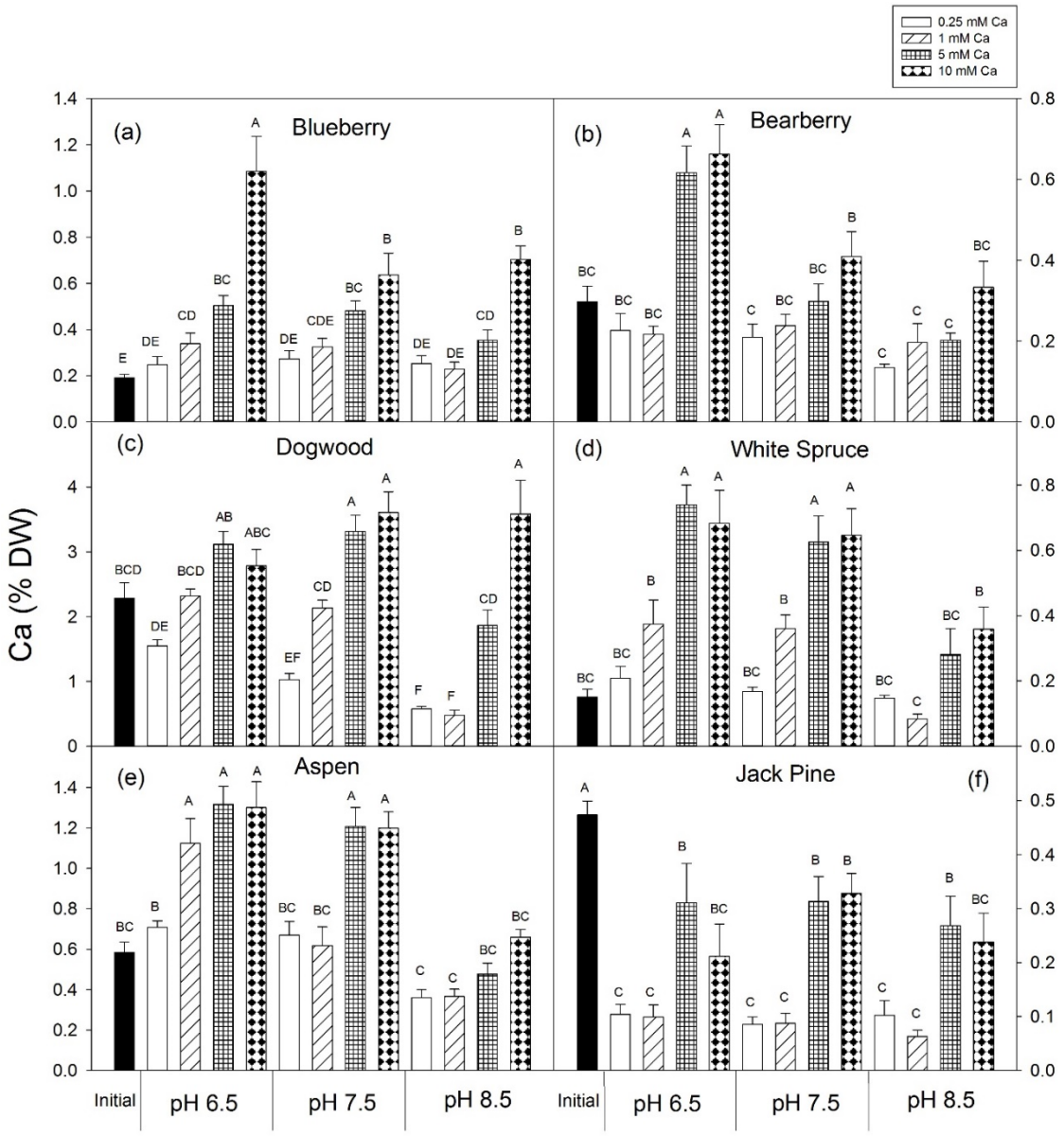

Figure a3. 11 Effects pH and Ca treatments on Ca concentrations in young leaves of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means (n=6) ± SE are shown.

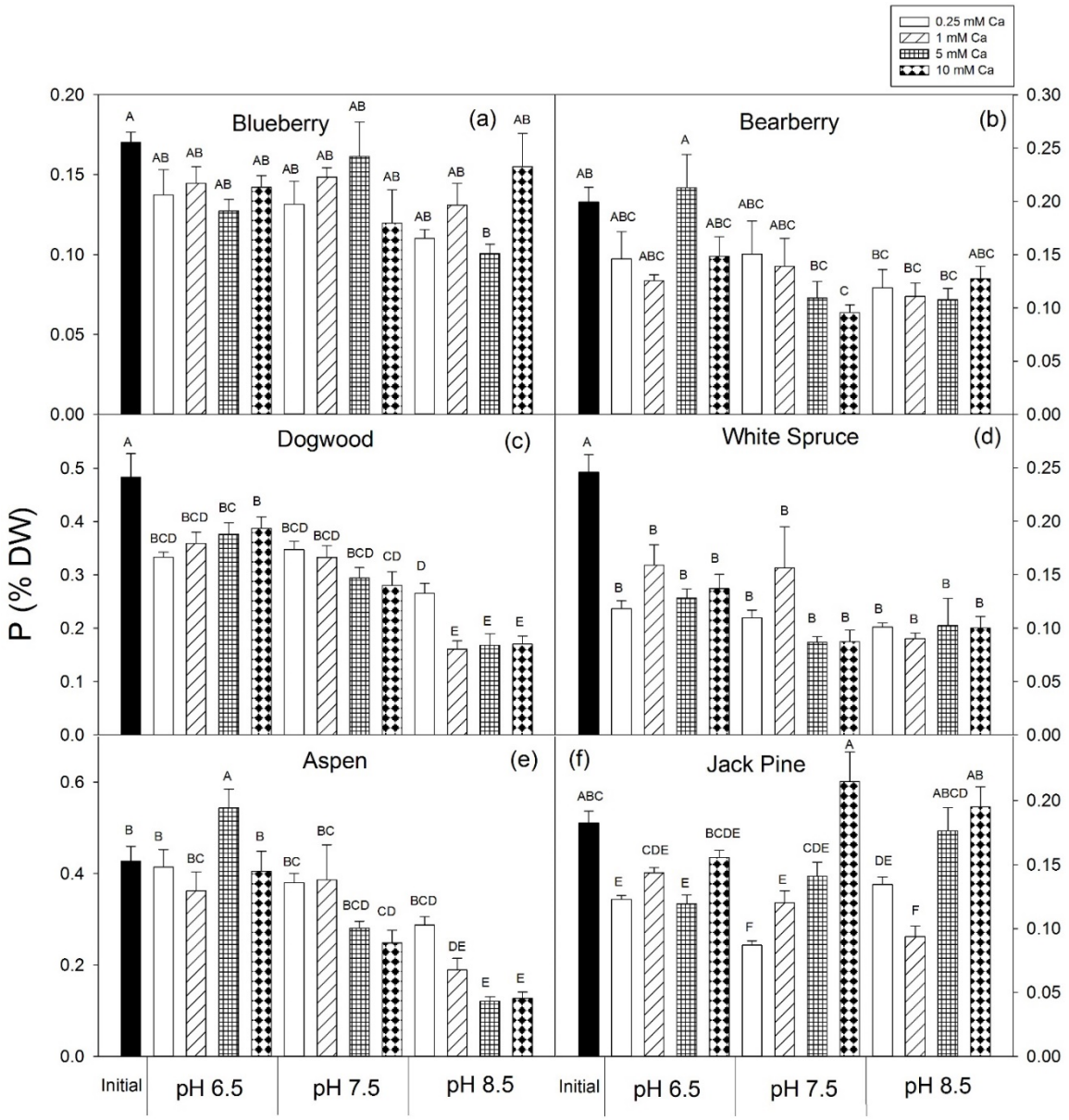

Figure a3. 12 Effects pH and Ca treatments on P concentrations in young leaves of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means (n=6) ± SE are shown.

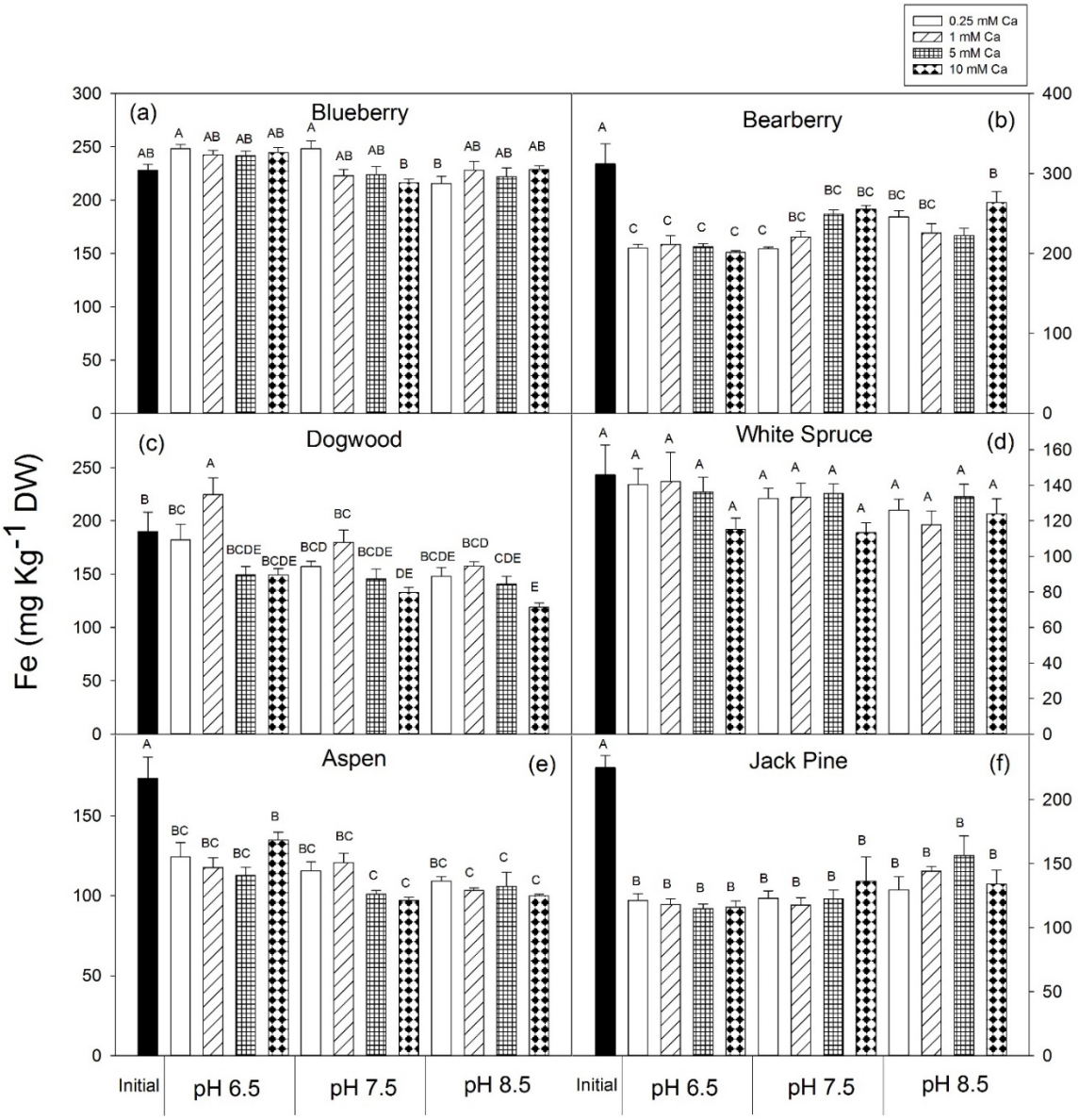

Figure a3. 13 Effects pH and Ca treatments on Fe concentrations in young leaves of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means (n=6) ± SE are shown.

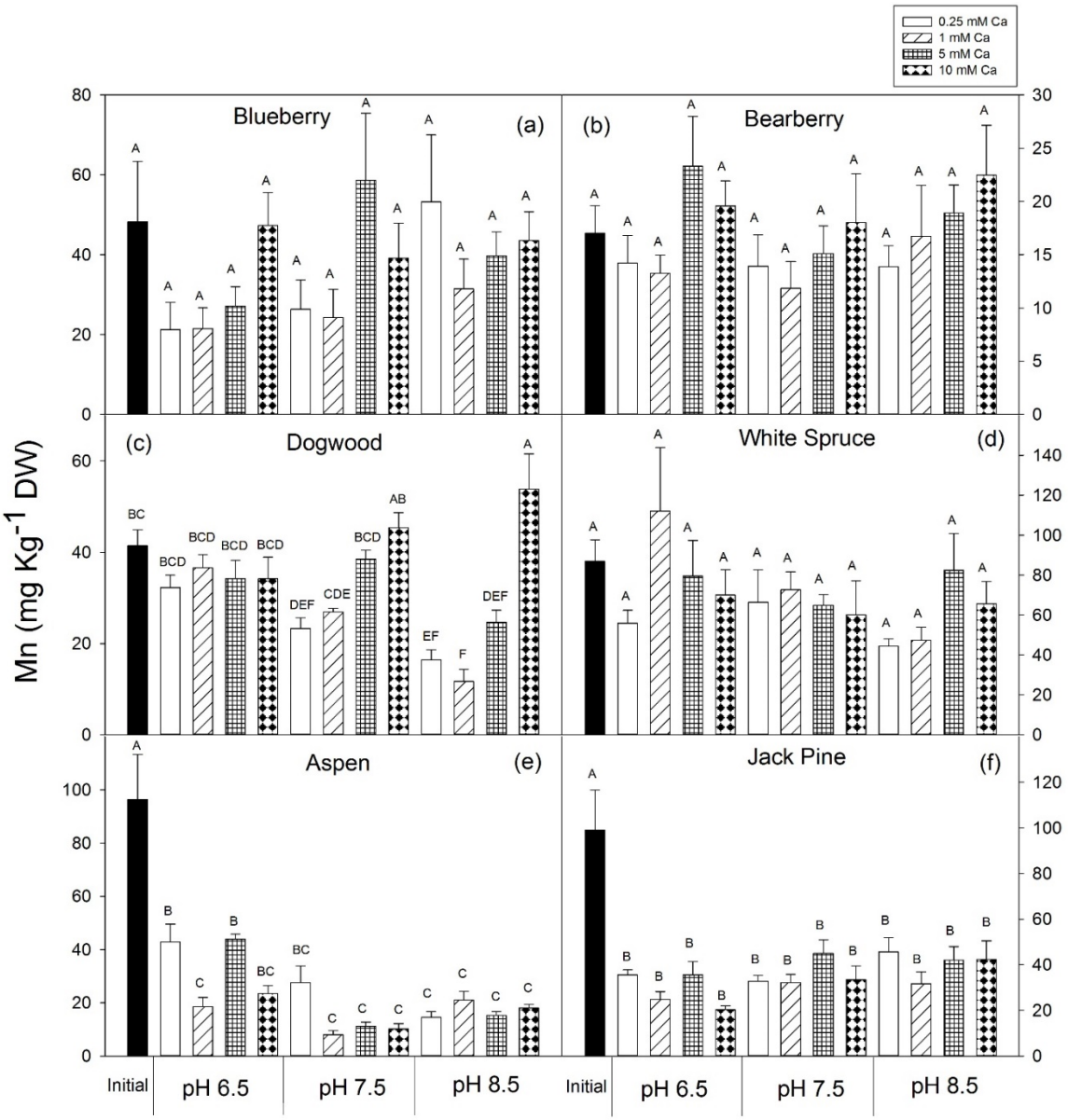

Figure a3. 14 Effects pH and Ca treatments on Mn concentrations in young leaves of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means (n=6) ± SE are shown.

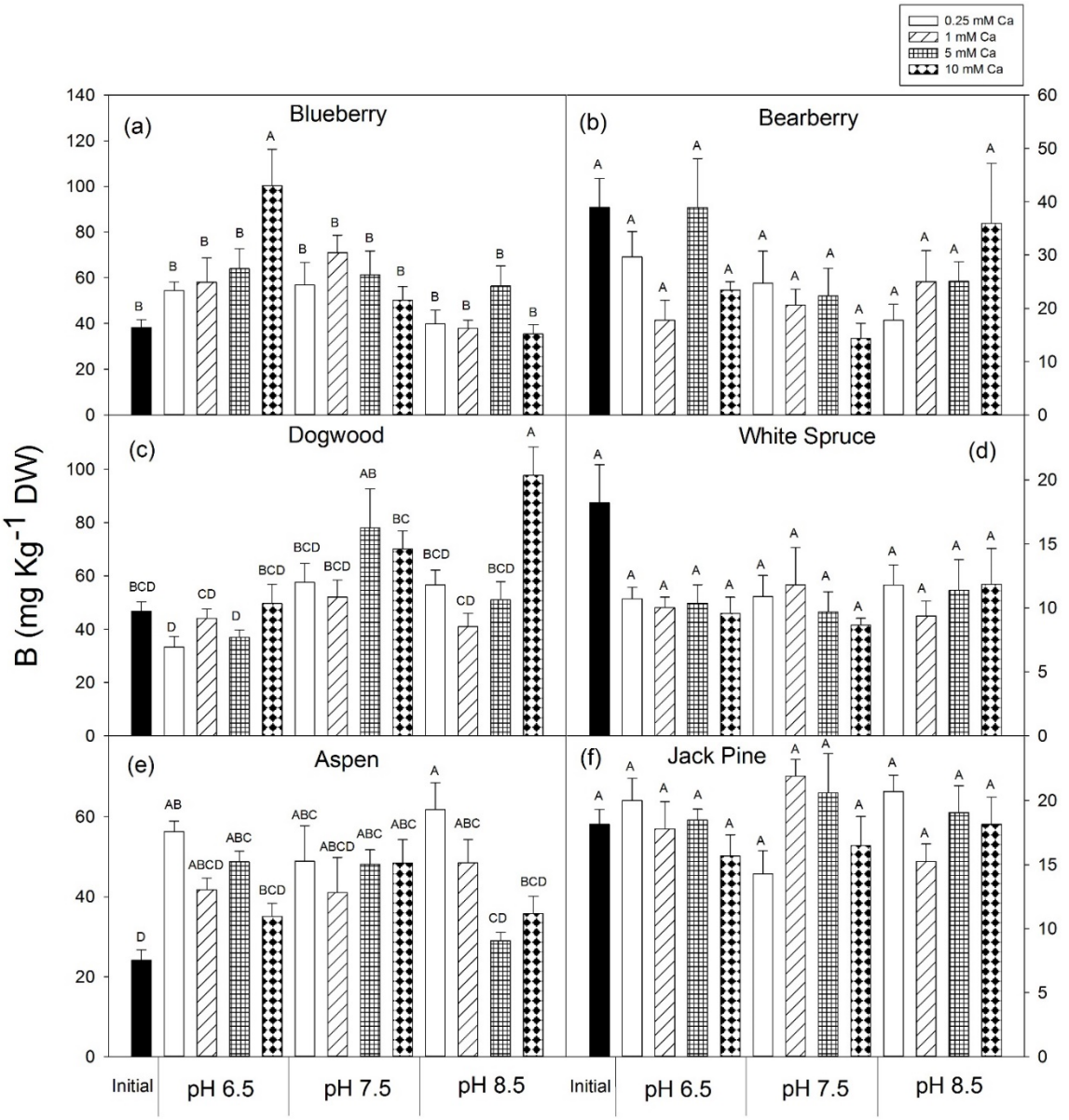

Figure a3. 15 Effects pH and Ca treatments on B concentrations in young leaves of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means (n=6) ± SE are shown.

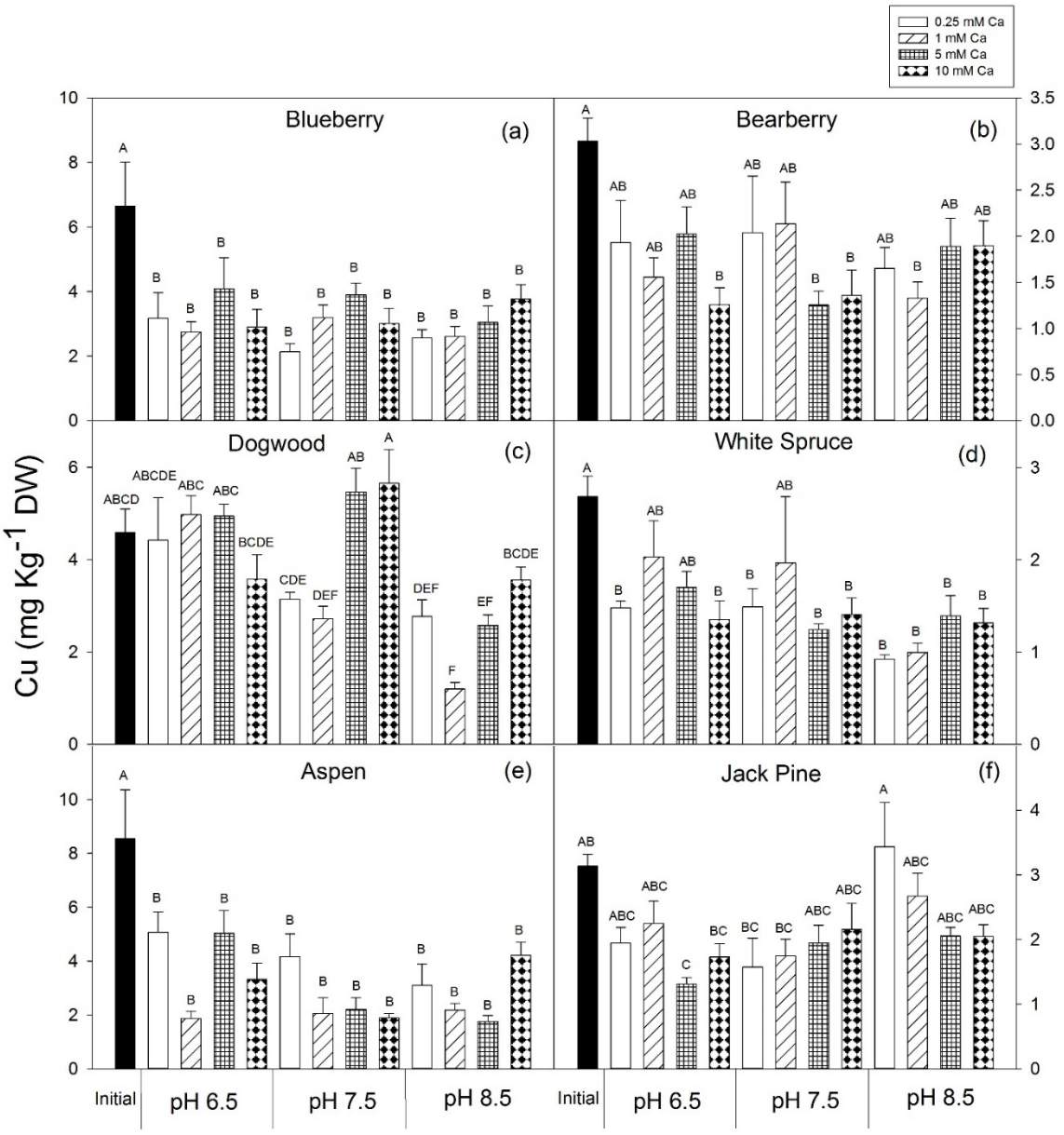

Figure a3. 16 Effects pH and Ca treatments on Cu concentrations in young leaves of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means (n=6) ± SE are shown.

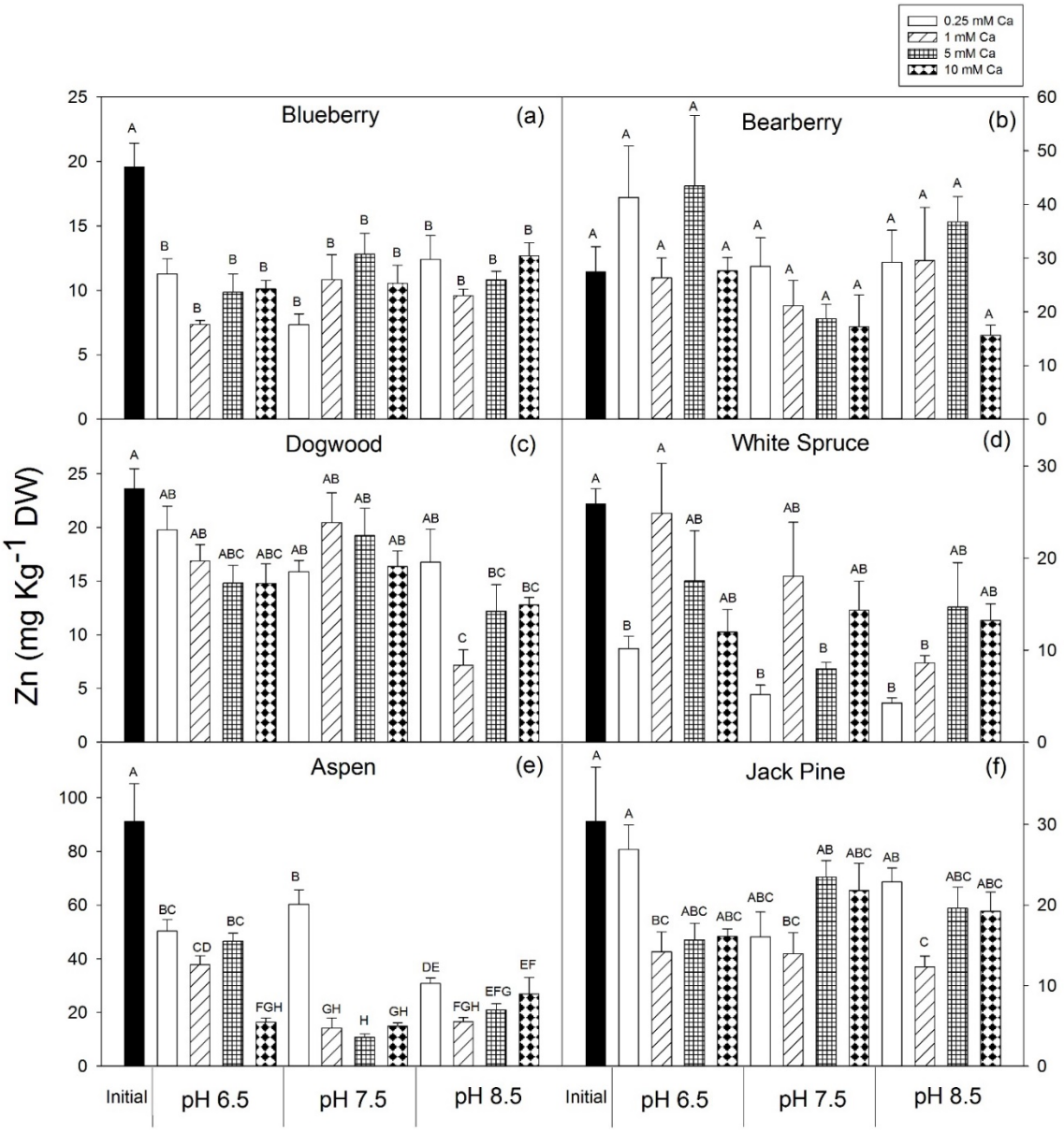

Figure a3. 17 Effects pH and Ca treatments on Zn concentrations in young leaves of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means (n=6) ± SE are shown.

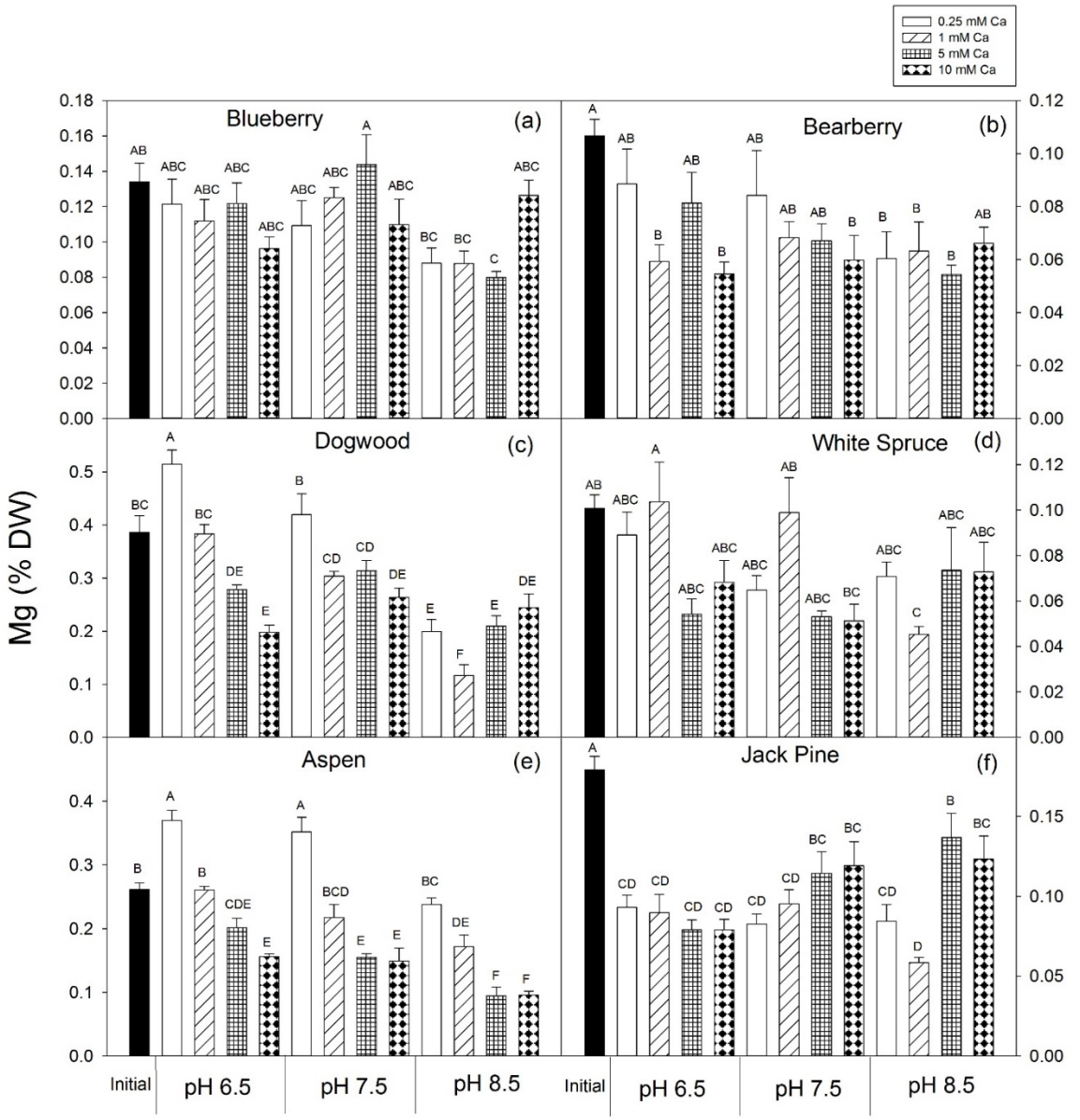

Figure a3. 18 Effects pH and Ca treatments on Mg concentrations in young leaves of blueberry, bearberry, dogwood, white spruce, aspen and jack pine seedlings. Different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means (n=6) ± SE are shown.