The effects of NaCl on a native boreal nitrogen-fixing shrub: *Elaeagnus commutata* (wolf willow)

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Abstract

Salinity is a major abiotic stress that reduces the growth and survival of most plants. In Canada, elevated levels of NaCl pose reclamation challenges for habitats disturbed by the oil sand industry. I examined the salt tolerance of *Elaeagnus commutata* (wolf willow), a native nitrogen fixing boreal shrub that has potential for land reclamation, by conducting greenhouse hydroponics experiments with seedlings (uninoculated or inoculated with *Frankia*), exposed to 0, 50 and 100 mM NaCl for 12 weeks. All plants survived 100 mM NaCl, suggesting that *E. commutata* is a moderately salt tolerant plant. Despite reductions in performance of salt stressed seedlings, they were still able to maintain relatively high levels of physiological activities and shoot/root growth. Plant mostly maintained nutritional balance even with elevated levels of Na^+ and Cl^- in their tissues. However, salinity inhibited root nodule formation and severely reduced root nitrogen fixation. To confirm the suitability of *E. commutata* as a potential species for saline land reclamation, field studies should be conducted.

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CHAPTER 1. INTRODUCTION

As the global consumption and demand for fossil fuel continuously increases, the Canadian province of Alberta, which has one of the largest energy reserves in the world, plays an important part globally in the mining of energy resources (Government of Alberta 2008). The majority of the energy reserves in Alberta is found within the oil sands. The largest reserve is the Athabasca oil sands, which is located in the northeastern region of Alberta. Together with the Peace River oil sands, and the Cold lake oil sands, the three deposits cover a boreal forest area of 140,200 square kilometers, and are estimated to contain 1.7 trillion barrels of bitumen (Alberta department of energy 2007).

The mining of oil sand is certainly not without environmental concerns. Oil sand mining involves the extraction of naturally occurring crude bitumen. Eighty percent of bitumen production occurs through deep ground in-situ extraction methods, while 20% of the bitumen is produced through surface open-pit mining (Government of Alberta 2008). In-situ extraction uses steam heating methods to separate sand from bitumen deep underground; the bitumen is then pumped above-ground through wells. In-situ extraction creates smaller landscape disturbances compared to that of surface mining (Government of Alberta 2008). One of the most common surface mining extraction methods involves an extraction process named "Clark hot water extraction" which results in large amounts of aqueous fine tailings with suspended silt and clay (FTFC 1995; Renault et al. 2004). The consolidation of these fine tailings requires the addition of coagulants such as gypsum ($CaSO_4$), which helps to separate consolidated tailings (CT) from tailing waters. This process leaves CT tailing waters with relatively high ion content, particularly Na^+ which is added during the hot water extraction process and from ion exchange with Ca^{2+}, Cl^- from leaching of the oil sands, and SO_4^{2-} from the addition of gypsum (Renault et al. 1998, 2004; MacKinnon et al. 2000). Additionally, the exposed overburden layer (removed in order to access oil sands) is also

known to contain relatively high levels of salt (Kessler et al. 2010). Salinity is therefore one of the defining characteristics of oil sand tailing materials that could pose challenges for the reclamation of these areas back to self-sustainable ecosystems. The Government of Alberta (2000) issued an "Environmental protection and enhancement act" which specified that land operators (referring to registration holders of lands who carry out activities on the land) have the duty to conserve and reclaim the lands that they operate on. This means that by law, the oil sand companies are required to reclaim lands that are disturbed by oil sand industry.

In general, salt stress is one of the main abiotic stresses that negatively impacts the survival and growth of plants. It is known to affect plants through osmotic stress, ionic toxicity and oxidative stress (Fadzilla et al. 1997; Munns and Tester 2008). High levels of salts such as NaCl in soil not only prove problematic for crop production worldwide (Vicente et al 2004), but also pose significant threat for natural habitats (Purdy et al. 2005). The reclamation of salt disturbed habitats is crucial for sustaining natural ecosystems.

The reclamation of saline habitats requires plant species that are salt tolerant. In addition, nitrogen fixing plants are often considered for reclamation due to their ability to fix nitrogen which gives them advantage in their establishment on degraded nutrient poor lands (Khamzina 2009). In order to determine if certain nitrogen fixing plants can be used for reclamation, their salt tolerance and nitrogen fixation properties should be thoroughly examined. Most of the studies done on the effects of salinity on nitrogen fixing plants have focused on legume crop species, few studies focused on utilizing nitrogen fixing plants of northern climates for saline land reclamation.

Actinorhizal plants are a group of mostly woody nitrogen fixing plants that are found across eight plant families and are widely distributed across of world. They are characterized by their natural associations with *Frankia* bacteria (there are generally no recognized species in this genus)

forming root nodules for nitrogen fixation (Wall 2000). Actinorhizal plants contribute significantly to the biologically fixed nitrogen input in ecosystems (Torrey 1978; Santi et al. 2013). Some of these plants have been used for reclamation of saline habitats in mostly in eastern Asia (Qin et al. 2010 a, b; Maimaiti et al. 2014). A number of studies have been conducted worldwide on actinorhizal plants such as *Shepherdia argentea*, as well as several species from the genus *Casuarina* and *Elaeagnus*, which show relatively high levels of salt tolerance and reclamation potential (Tani and Sasakawa 2003; Qin et al. 2010a, b; Sayed 2011; Maimaiti et al. 2014). However, these studies have mostly neglected to investigate salinity interaction with nitrogen fixation in actinorhizal plants with the exception of *Casuarina* species (Ng 1987). In addition, no study has been conducted on salt tolerance of boreal actinorhizal plants that are native to North America - a group of plants that are critical for reclamation use in the boreal forest.

Elaeagnus commutata (wolf willow) is a boreal actinorhizal shrub that is native to Canada. Like all actinorhizal plants, *E. commutata* can form symbiotic relationships with *Frankia* for nitrogen fixation (Mirza et al. 2009). The shrub is also known for its fast growth and easy propagation (Corns and Schraa 1962). *E. commutata* is often found to be adaptive to relatively infertile sandy land and are resilient to drought stress. Due to these characteristics, the potential for *E. commutata* to be utilized as a reclamation species should not be overlooked. Can *E. commutata* shrubs sustain themselves in saline habitats as it is the case with some other actinorhizal shrubs? Unfortunately, no study has been conducted to investigate the salt tolerance, nodulation formation, and nitrogen fixation in *E. commutata*. Therefore, the objective of my thesis research was to examine the degree to which various levels of a selected salt "NaCl" affects the survival, growth and physiology of *E. commutata*. In addition, I also looked into the effects of NaCl on nitrogen fixation rates and nodule formation in *E. commutata*.

CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

In this review, I will first discuss the topic of soil salinity, focusing on NaCl and how components of NaCl stress (ionic and osmotic stress) affects the growth and physiology of plants. I will discuss different morphological and physiological responses as well as common salt tolerance mechanisms that plants utilize in response to salinity. I will focus on actinorhizal plants – a group of mostly nitrogen fixing shrubs that may have significant potential to be used as reclamation species on saline lands. The nature of the actinorhizal plant – *Frankia* bacteria symbiosis, the roles of actinorhizal plants in developmental stages of ecosystems, the salt tolerance of certain actinorhizal plant species and some current findings on how nitrogen fixation in these plants may be affected by salinity, will be discussed. Finally, I will introduce *Elaeagnus commutata* (wolf willow), a native boreal actinorhizal shrub as my subject of research and explain the objectives of my thesis research.

2.2 Soil salinity

Soil salinity is one of the main abiotic stresses that negatively impacts the physiological functions and limits the growth and survival of plants. The rise in soil salinity globally can be attributed to many factors, some of which are of anthropogenic causes. Global climate change, for example, causes frequent drought in many regions, which is often associated with higher soil salinity (Araújo et al. 2006; Koyro et al. 2008). Some of the other causes of soil salinity include mining, where weathering of minerals can result in elevated salinity in mine tailings (Mendez and Maier 2008; Young et al 2015), deforestation, which can mobilize ground salts, the use of de-icing salt in winter, which can lead to damage of the roadside ornamental plants (Devecchi and Remotti 2004), and excessive irrigation practices, which can result in waterlogging with poor drainage,

bringing up significant amount of ground salt to the plant root zone (Lambers 2003; Ashraf and Athar 2009). Irrigation with saline water is also problematic, since salt will accumulate in soil long after water evaporates (Koyro 2006). Soil salinity is becoming an escalating worldwide problem. It has been reported that close to 400 million hectares of lands, and around 45 million hectares of irrigated land (accounting for 20% of all irrigated land) are affected by various levels of soil salinity (Lambers 2003; Hasanuzzaman et al. 2014).

Soil salinity is defined by the presence of high amounts of soluble salts (NaCl, Na_2SO_4, $CaCl_2$, $MgCl_2$, $CaSO_4$ etc.) in soil. Although many of these salts, such as Na_2SO_4, Na_2CO_3, and NaCl are confirmed to negatively affect the survival, growth and physiological functions of plants (Abd El-Samad and Shaddad 1996; Renault 2005), NaCl salinity is the most commonly spread salt around the world (Munns and Tester 2008). As a result, most of the studies on plant salinity tolerance have focused on NaCl. Saline soil typically refers to soil with EC (electrical conductivity) >4 dS/m, which is estimated to be comparable to 40 mM of NaCl (Munns and Tester 2008). One of the primary concerns is that soil salinity leads to loss of crop production. The majority of plants (including most commonly farmed crop species) are very sensitive to saline soil conditions (>40 mM NaCl) (Shannon and Grieve 1999). The reduction in crop biomass production becomes a devastating problem, especially in a world with a booming population (Vicente et al. 2004). Irrigated lands (which accounts for 1/3 of world's crop production) also happen to be most prone to soil salinization (Munns 2002). Aside from the reduction in crop yield, soil salinization can also lead to disturbances in natural habitats. Since plant species are affected by salinity to varying degrees, soil salinity can change the dynamics of community species interactions, altering species composition and decreasing biodiversity within natural ecosystems (Gough et al. 1994; Purdy et al. 2005).

Soil salinization threatens a majority of the world's most populous nations. It is becoming a widely recognized problem in Canada as well. Some areas of the Canadian prairies are affected by various levels of soil salinity, with an estimated area of ~3.5 Million ha of subsoil being affected by moderate to severe levels of salinity (Wiebe et al. 2007; Renault 2012).

In Alberta, the oil sand industry is an important sector of the Canadian economy. Oil sand mining involves the extraction, processing and refinement of naturally occurring bitumen. The bitumen extraction process often utilizes a "Clarks hot water extraction" method that results in the production of a crude oil product, along with a large amount of relatively saline tailings and tailing water with high levels of ions, such as Na^+, Cl^- and SO_4^{2-} (Renault et al. 1998, 2004). The relatively high concentrations of ions result in high EC values ranging from 2.2 to 8.8 dS/m in tailings and tailing waters that could potentially pose challenges for the reclamation of oil sand disturbed habitats in Alberta (Renault et al. 1998). In order to reclaim and restore these disturbed habitats back to sustainable ecosystems, native boreal plant species that are relatively salt tolerant should be planted to stabilize and improve soil conditions to the point that other species can survive. Obtaining knowledge on the degree of salt tolerance in potential plant species that can be used for such reclamation purposes is crucial. There have been a few studies which examined the salt tolerance of boreal woody species such as white spruce (*Picea glauca*), aspen (*Populus tremuloides*), jack pine (*Pinus banksiana*) and black spruce (*Picea mariana*) showing very wide ranges of salinity tolerance for these species (Howat 2000). However, many of these studies were observational, and may not be comprehensive or precise enough to confirm the sustainability of these species over time in saline environments. In addition, there has been a lack of study focusing on nitrogen fixing boreal plant species which may prove to be suitable for the reclamation of saline disturbed lands into sustainable boreal ecosystems.

As soil salinity becomes a major concern, researchers around the world have been interested in investigating how salt would affect different plant species. Plant species vary greatly in their ability to cope with salinity, as well as their mechanisms for salt tolerance. It has been the goal of many scientists to study various responses different plants utilize in dealing with salinity (Munns and Tester 2008). Acquiring this knowledge can help tremendously in dealing with loss in global crop production (due to soil salinity), as well as in finding suitable species that are relatively salt tolerant for the conservation and reclamation of saline disturbed habitats (Colmer et al. 2006; Maimaiti et al. 2014). Most of the studies conducted on the effects of salinity on various plants focus on NaCl. Thus, throughout my thesis, the term "salinity" will refer to NaCl specifically.

2.3 Effects of salinity on plants

2.3.1 Ionic, osmotic and oxidative stresses

Salinity stress tends to adversely impact the survival, growth and general physiology of plants. Plants are usually affected through two main ways: ionic and osmotic stress. Osmotic stress refers to the limitation in water uptake by plants as a result of a lower soil water potential caused by the presence of salts. Lower water uptake tends to cause reduced plant shoot growth, with fewer, smaller leaves and stunted heights, as well as reduced leaf photosynthesis and transpiration (Yeo et al. 1991; Vicente et al. 2004; Koyro 2006). On the other hand, accumulation of ions (Na^+ and Cl^-) in plant shoot tissues can be highly toxic. This ionic toxicity represents the other main component of salinity stress. The presence of higher concentrations of Na^+ can lead to the replacement of K^+ by Na^+ for enzyme activities, thereby disrupting many plant biochemical processes (Gupta and Huang 2014). The high concentration of Na^+ and Cl^- can also lead to alterations in cellular protein structures by interfering with amino acid non-covalent interactions, resulting in inefficient cellular function (Chinnusamy et al. 2005). Plants suffering from ionic

toxicity often display signs of leaf and meristem damage, such as leaf scorching, senescence and necrosis of older leaves (Shannon and Grieve 1999; Munns and Tester 2008). In addition, osmotic stress results in the reduction in leaf photosynthetic rates, which can lead to production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and singlet oxygen. These ROSs are known to cause oxidative damage to plant tissues (Fadzilla et al. 1997; Gupta and Huang 2014).

Higher salinity also induces a nutritional imbalance in plants. The presence of high concentrations of Na^+ ions can lead to reduced uptake of K^+ and Ca^{2+} (due to direct competition for common cation transporters), both of which are essential nutrients playing important roles in plant growth, development, and major metabolic functions (Rengel 1992; Adams and Ho 1993; Hasegawa et al. 2000; Gupta and Huang 2014). Studies have also demonstrated that in some plants, the presence of higher concentrations of Cl^- under saline conditions can reduce the uptake of NO_3^-, thus affecting overall plant nitrogen uptake (Liu and Shelp 1995; Bar et al. 1997; Frechilla et al. 2001).

2.3.2 Sensitivity to salinity – glycophytes and halophytes

Most plant species in general, including commonly consumed domesticated crop are very sensitive to the osmotic and ionic stress caused by soil salinity. The plants that can only grow and survive in conditions of relatively low soil salinity are classified as “glycophytes”. Most glycophytes become susceptible when soil salinity reaches 40 mM NaCl -- the threshold level for “saline soil” (Chinnusamy et al. 2005). Although all glycophytes are sensitive to high salt levels, among these plants, levels of salt tolerance still do vary greatly (Munns and Tester 2008). Onion (*Allium cepa*), celery (*Apium graveolens*) and lettuce (*Latuca sativa*) plants start to suffer decline in their growth rates at salinity levels ranging between 10 to 20 mM NaCl (Shannon and Grieve

1999), while in certain cereal crops, such as *Triticum aestivum* (common wheat) and *Hordeum vulgare* (barley), the threshold salinity levels (at which point there would be decline in growth) can be as high as 60 to 80 mM NaCl (Chinnusamy et al. 2005). In addition, the degree of plant salt tolerance can vary on an intraspecific level. Factors affecting plant performance under salinity can include differences in plant provenances, cultivars, age and developmental stages (Lutts et al. 1996; Niknam and McComb 2000; Renault 2012).

In contrast to glycophytes, some plants species are capable of surviving and sustaining growth in soil with relatively high levels of salinity. These plants are generally referred to as "halophytes". Not only are halophytes capable of surviving under such conditions, some species can have stimulated growth (faster shoot growth rates) under high salinity -up to 200 mM NaCl (Yeo and Flowers 1980). Although halophytes make up of only 1% to 2% of world's flora, they are found in a number of plant families (Flowers and Colmer 2008), which include wild plant relatives to known crops such as wheat (Colmer et al. 2006).

Considering that the majority of domesticated plants are highly sensitive to salinity, this makes it important to understand the salt tolerance properties of plant species. There is a large number of studies conducted on different halophytes, and various relatively salt tolerant glycophyte plants in general that have investigated the specific tolerance mechanisms that plants employ to deal with high salinity (Flowers 1985; Koyro 2006; Agarie et al. 2007; Redondo-Gomez et al. 2007). Although numerous studies have demonstrated that higher levels of salinity tend to negatively affect plant growth and survival, they do trigger a large variety of coping mechanisms among different plant groups, which includes both morphological and physiological adaptations.

2.4 Plant responses to salinity

2.4.1 Plant morphological responses to salinity

Plants utilize a variety morphological adaptations to deal with soil salinity. One common adaptation to higher salinity is an increase in the plant root: shoot ratio, this involves plants directing energy more toward root growth instead of shoot growth to maintain the uptake of water and nutrients (Cheeseman 1988; Albacete et al. 2008). This can be crucial for plants exposed to salinity, as the maintenance of water uptake is required for plant growth/photosynthesis, nutritional transport, and maintaining plant turgor. In desert regions (associated often with drought and high salinity), for example, root development is often highly promoted for some halophyte species, to the extent that root mass can represent over 75% of the plant biomass (Yi et al. 2007; Weber 2009).

Plants growing in dry regions that are constantly exposed to drought and high levels of salinity often develop thick, fleshy leaves with relatively low surface to volume ratio as a way to minimize water loss through transpiration. These thick leaves also store large quantities of water to ensure normal plant metabolism (Wang et al. 2004). This feature is referred to as leaf succulence (Weber 2009). Some plants subjected to drought stress (especially in arid environments) develop a layer of surface cuticular wax (leaf wax) that is composed mostly of hydrocarbons and fatty acids, which acts as a barrier to prevent excess loss of water through transpiration from leaf surface. The development of cuticular wax is often found directly related to levels of drought resistance in plants (Islam et al. 2009; Gonzales et al. 2012). Salinity stress is often found to induce higher plant leaf cuticular wax deposition as well, this is likely because salinity stress is often associated with drought stress due to the reduction in water availability to plants. Increased deposition of leaf wax in response to salinity was found in *Arabidopsis thaliana*, tobacco plants (*Nicotiana tabacum*) as well as in Rangpur (*Citrus* x *limonia*) (Hadli and Raijadhav 2004; Cameron et al. 2006; Kosma et

al. 2009).

2.4.2 Plant physiological responses: ion exclusion

Some plants have evolved ion exclusion mechanisms to prevent the excessive buildup of Na^+ in shoot tissues, since their accumulation can be highly toxic. It has been found that the majority of mangrove species have the ability to exclude ~99% of salt from their environment (Aslam et al. 2011). Although the specific tissue/cell types involved in the process of excluding salt from leaves is unclear, the exclusion of salts likely occurs at root level and by the selective uptake and transport of other essential nutrients, such as K^+ (Munns 2002). In some plants, such as *Cornus sericea* (red-osier dogwood), relatively high levels of salt can be stored in the roots to limit ionic damage to plant shoot tissues (Renault 2004; Renault 2012). Ion exclusion as a salt avoidance mechanism is widely documented in other species such as *Chenopodium album*, and *Chenopodium schraderianum* (Bradley and Morris 1991; Reimann 1992).

When Na^+ does build up in leaves to a toxic level, another ion exclusion mechanism that some plants utilize is “leaf shedding”. Leaf shedding refers to the dropping of plant older leaves which have accumulated high salt content to get rid of excess salt. This reduced lifespan of leaves ensures the production of newer leaves that function more efficiently with reduced ionic toxicity (Suarez and Medina 2005; Aslam et al. 2011). Another mechanism utilized by some plants (such as *Chloris gayana*, as known as Rhodes grass) to get rid of excess salt is through salt excretion via salt glands (Liphschi et al. 1974). Salt glands secrete fluid of high salt concentrations to the leaf surface, after the water evaporates, salts are left on the leaf surface until they drop to the ground or are taken away by wind. In addition to salt glands, halophytes such as *Mesembryanthemum crystallinum* (ice plant) and *Atriplex portulacoides* (sea purslane) can modify their epidermal hair cells into “salt bladders”, which can sequester large amount of salt for elimination (Agarie et al.

2007; Redondo-Gomez 2007).

Although ion exclusion mechanisms are commonly documented, many plants do not actually utilize ion exclusion as salt tolerance mechanisms. Touchette et al. (2009) conducted a study on two salt marsh species (*Spartina alterniflora* and *Juncus roemerianus*) and found that while the *Juncus* plants utilized salt avoidance/exclusion mechanisms, *S. alterniflora* tends to accumulate salt in shoot tissues. *Atriplex nummularia* accumulates Na^+/Cl^- at higher concentrations in shoot tissues in comparison to the roots (Araújo et al. 2006). This character is distinct from plants that utilize ion exclusion where we would expect higher concentrations of salt in roots as opposed to shoot tissues.

2.4.3 Plant physiological responses: ion inclusion

Under high soil salinity, some plants can utilize an ion inclusion mechanism as a physiological adaptation that improves salt tolerance. The uptake of Na^+ and Cl^- at the root level can occur through non-specific cation/anion channels, as well as K^+ transporters (Blumwald et al. 2000; Hasegawa et al. 2000). However, since the accumulation of Na^+ and Cl^- can be highly toxic in leaf tissues, the compartmentalization of these ions into cell vacuoles must occur to prevent their accumulation in the cytoplasm. The active transportation of Na^+ into vacuoles is an energy costly process, which involves the coordination of V-ATPase, and H^+/Na^+ exchangers at the level of vacuolar tonoplast (Rea et al. 1992).

The compartmentalization of Na^+/Cl^- in cell vacuoles creates osmotic pressure that must be balanced out in the cell cytoplasm. Many plants actively produce energy costly compatible solutes to achieve this (Flowers and Colmer 2008). Although there is a variety of compatible solutes that different plants tend to produce, sugars (such as sucrose), amino acids (such as proline), sorbitol, and glycine betaines are some of the most common ones (Munns and Tester 2008). Apart from

osmobalancing, the synthesis of compatible solutes serves many other functions. They can act as osmoprotectant as a response to general osmotic stress, as well as stabilizing subcellular protein structures and membrane proteins (Hare et al. 1998; Roychoudhury et al. 2013).

In response to salinity induced oxidative stress, plants tend to produce antioxidant compounds (glutathione, ascorbate etc.) and antioxidant enzymes (such as superoxide dismutase) to alleviate oxidative damage (Chinnusamy et al. 2005). In addition, it has also been found that the increased production of some compatible solutes, namely proline and glycine betaine, can also help lowering the amount of free radicals accumulated in plants (Hong et al. 2000; Chinnusamy et al. 2005).

2.5 Actinorhizal plants

2.5.1 Actinorhizal plants and *Frankia* symbiosis

Since salinity negatively impacts the survival and growth for most plants, and salinity stress currently poses challenges to the reclamation of Alberta oil sand tailings back to sustainable boreal forest ecosystems. Native salt tolerant boreal forest species are needed for the reclamation effort of these disturbed areas. However, the salt tolerance properties of native boreal plants are generally not very thoroughly studied, especially when it comes to boreal nitrogen fixation species.

Actinorhizal plants are a group of mostly woody, nitrogen fixing perennial plants that are defined by their ability to form symbiotic relationships with actinobacteria of the Genus-*Frankia* (Dawson 2007). Root nodules form as a result of the mutualistic symbiosis between *Frankia* where nitrogen fixation by the bacteria takes place. Actinorhizal plants is a fairly diverse group that consists of more than 200 species, found across eight plant families (*Betulaceae*, *Casuarinaceae*, *Coriariaceae*, *Datiscaceae*, *Elaeagnaceae*, *Myricaceae*, *Rhamnaceae*, and *Rosaceae*) and are widely distributed across the world (Wall 2000; Diagne et al. 2013). Likely due to their nitrogen

fixing capabilities, these plants are often found to be resilient to low nitrogen habitats, and are often found in marginally fertile lands (Pawlowski and Sirrenberg 2003). Actinorhizal plants are important for the productivity of ecosystems, as they contribute to a large proportion of total biologically fixed nitrogen input (Torrey 1978). With global climate change and other anthropogenic factors leading to increasing amount of land degradation and loss of biodiversity, actinorhizal plants are becoming important species to be studied and potentially utilized for conservation and restoration of disturbed and degraded lands (Visser et al. 1991).

Frankia is a nitrogen fixing filamentous bacteria (actinomycete) found free-living in soil that can also infect the root systems of a variety of plants (actinorhizal plants) from different groups. Aside from rhizobium-legume associations, *Frankia*-actinorhizal plants association is the other major nodule forming symbiotic relationship found in nature (Pawlowski and Demchenko 2012).

Root nodules are sites of nitrogen fixation in actinorhizal plants, they form after *Frankia* infection occurs at plant roots. The anatomy of theses nodules closely resembles the general anatomy of roots. Root nodules retain a central vascular system, as well as an apical meristem which contribute to the development and growth of the nodules (Wall 2000). Within the nitrogen fixing part of nodules, cells are divided into infected and uninfected cells. Infected cells contain differentiated bacterial vesicles where N-fixation actively takes place. Uninfected cells are likely locations where the assimilation of fixed nitrogen occurs (Laplaze et al. 1999; Wall 2000).

2.5.2 Actinorhizal plants and ecosystem development

Actinorhizal plants, due to their ability to grow on soil with lower nitrogen, as well as their abilities to withstand various environmental stresses, such as drought and salinity, play important roles in the post disturbance development of natural habitats (Pawlowski and Sirrenberg 2003, Diagne et al. 2013). Typically, disturbed lands usually suffer from lack of nitrogen, thus the ability

for actinorhizal plants to fix nitrogen gives them advantage in these environments. These plants are often described as "pioneer species" that colonize disturbed areas following a natural or man-made disturbance. They are the early settlers that contribute to the successional development of natural communities (Benson and Silvester 1993). The growth of actively N-fixing actinorhizal plants progressively improves soil conditions so that other species can eventually establish themselves (Bargali 2011; Diagne et al. 2013). These characters make actinorhizal plants uniquely suitable to be used as revegetation/ reclamation species.

The amount of nitrogen fixed by actinorhizal plants is ecologically significant. The nitrogen fixation rates for high performing actinorhizal plants can be between 240 to 350 kg/ha/year, which makes them comparable to some of the highest performing nitrogen fixing legumes (Wall 2000; Santi et al. 2013). The role of actinorhizal plants as nitrogen fixers are even more important in colder regions where legumes are not as prominent. One group of actinorhizal plants that are known for having high nitrogen fixation is alders (*Alnus*), which are very widely distributed shrubs and trees in both North America and Europe (Benson and Silvester 1993). Nitrogen fixation rates vary between *Alnus* species, with reported amounts of 279 kg/ha/year for *Alnus acuminata* (Diagne et al. 2013), between 140 to 300 kg/ha/year for *Alnus rubra*, and as high as ~ 300 kg/ha/year for some of the other species (Bargali 2011). *Alnus* species are more thoroughly studied compared to many other actinorhizal plants because they're wide spread and commonly found.

2.5.3 Salinity and nitrogen fixation in legumes and actinorhizal plants

In order to examine whether certain actinorhizal plants are suitable to be used for reclamation of saline habitats, it is important to look at how nodule formation and nitrogen fixation rates in these plants are affected by high levels of salinity. However, most salt tolerance studies on

actinorhizal plants often neglect to investigate how their capability to fix nitrogen are affected by higher levels of salinity.

There has been plenty of work on the effects of salinity on nitrogen fixation in legume crop plants. Although higher salinity tends to reduce nitrogen fixation in a variety of legume-*Rhizobium* associations, the effects of salinity vary depending on different host legume plant species, as well as different rhizobia species/strains. For example, studies have demonstrated that pea (*Pisum sativum*) suffered more severely reduced growth, nodulation, nodule mass and nitrogenase activity at 50 mM and 100 mM NaCl compared to soybeans (*Glycine max*) and faba-beans (*Vicia faba*). (Delgado et al. 1994; Serraq et al. 1998, Bolanos et al. 2006). Interestingly, host legume plants often tend to be less salt tolerant than their associated Rhizobium bacteria (Singleton and Bohlool 1983; El-Shinnawi et al. 1989). However, the salt tolerance of the host plants is important to successful initiation of root nodulation under salinity (Singleton and Bohlool 1984; Zahran 1999). One study found that cultivars of chick peas (*Cicer arietinum*) that are more salt tolerant have also shown higher rates of nodulation and nitrogenase activity under the same levels of salinity compared to more susceptible cultivars (Garg and Singla 2004). In addition, different Rhizobium species and strains also vary significantly in their ability to tolerate salinity (Zahran 1999).

These findings for Legume-*Rhizobium* associations provide insights to *Frankia*-actinorhizal associations as well. The extent to which nitrogen fixation rates are affected by salinity in actinorhizal plants could very well be species specific. Although little has been done on salinity interaction with nodulation and nitrogen fixation in actinorhizal plants, Dawson and Gibson (1987) have shown that for *Frankia* isolates from salt tolerant *Casuarina* species, bacterial growth rates (grown on medium) and nitrogenase activity remained unaffected at up to 200 mM NaCl, while

Frankia isolates from other actinorhizal plants (*Alnus rubra* and *Elaeagnus umbellata*) were heavily affected at 200 mM NaCl. Furthermore, different *Frankia* strains isolated from the same species could also vary greatly in their sensitivity to various salt levels (Tani and Sasakawa 2003). Due to variations between plant species as well as *Frankia* strains, more studies are needed to thoroughly investigate and document the effects of salinity on nitrogen fixation among different actinorhizal plants.

2.5.4 Salt tolerance of actinorhizal plants

Casuarina is a group of actinorhizal plants that are native to Southeast Asia and Australia and are widely planted in tropical regions all over the world. They have been used for improvement of habitats with harsh conditions due to their nitrogen fixation ability, fast growth and the improve soil fertility (Sayed 2011).

In terms of salt tolerance, Tani and Sasakawa (2003) have found that in *C. equisetifolia*, although high salinity (300 mM NaCl) seemed to inhibit seed germination rates, the survival rates of seedlings remained high (>80%) and root nodulation can still occur at 300 mM NaCl. In fact, it was shown in an earlier study that the growth rates of *C. equisetifolia* can be stimulated at moderate salt levels (50 to 100 mM NaCl; Ng 1987). Similarly, a high degree of salt tolerance (150 mM NaCl to 600 mM NaCl) was found in several other *Casuarina* species (Hyland 1983; Niknam and McComb 2000). Studies have also shown that the *Casuarina* species *C. obesa* and *C. cristata* exhibit high levels of drought tolerance and tend to inhabit arid and semi-arid regions with low precipitation (El-Lakany 1983; Sayed 2011).

Aside from *Casuarina* spp., actinorhizal plants from the *Elaeagnaceae* (*Shepherdia*, *Elaeagnus*) family have also been studied to determine their salt tolerance. Several plant species from this family (*Shepherdia argentea*, *Elaeagnus angustifolia*) have demonstrated high levels of

salt and drought tolerance, which indicates great potential in their suitability to be used for the reclamation of saline land.

Silver buffaloberry (*Shepherdia argentea*) is an actinorhizal shrub that is native to many boreal communities in North America. It was introduced to China in 2002 as a reclamation species to improve the conditions in the China western plateau area, which has high degrees of salinization and drought. *Shepherdia argentea* was widely recognized as a cold, drought and salt tolerant plant, and it has since been planted in large scale in coastal area as well as inland plateaus in China (Qin et al. 2009). Qin et al. (2010 a, b) thoroughly examined the salt tolerance properties of *S. argentea*. It was found that despite severe reductions in physiological properties (photosynthesis, transpiration, leaf water potential etc.), seedlings were able to survive up to 600 mM NaCl for a period of 30 days. Although *Shepherdia argentea* is a plant native to North America, its utilization for land reclamation or related purposes has not been widely documented.

Several species within the genus *Elaeagnus,* such as *Elaeagnus angustifolia*, have some economical values in small scale fruit productions, and for ornamental uses (Ayaz et al. 1999). In recent years, a few studies have demonstrated potentials for these plants to be used for reclamation of saline disturbed habitats.

Elaeagnus angustifolia is a widely spread shrub native to northern and central Asia. It is generally considered to be salt and drought tolerant, and it has been used in China for reclamation of saline lands. *E. angustifolia* is commonly found naturalized in arid areas, which includes heavily irrigated lands that suffered from soil salinity (Maimaiti et al. 2014; Zhao et al. 2014). A study has shown that *E. angustifolia* can survive up to 300 mM NaCl, and does not exhibit ionic toxicity symptoms at up to 200 mM NaCl. The plant was recommended to be used for afforestation (for disturbed areas) as well as for phytoremediation of degraded saline land based on these

characteristics (Maimaiti et al. 2014). To thoroughly examine its potential to be used for afforestation, Khamzina et al (2009) conducted a field experiment which compared plots with *E. angustifolia* and mixed plantations, to similarly set up plots with non-nitrogen fixers poplar (*Populus euphratica*) and Siberian elm (*Ulmus pumila*). It was shown that *E. angustifolia* plants are highly efficient nitrogen fixers. The nitrogen fixation rates increased from 20% to ~100% (% Ndfa: Nitrogen derived from atmospheric N) in a duration of five years, despite the saline and low phosphorus conditions which characterizes the degraded agriculture land where the study took place. Compared to *P. euphratica* and *U. pumila, E. angustifolia* had an average of two to three times higher N content across different plant tissues. Over a period of five years, *E. angustifolia* plots also accumulated higher soil nitrogen content compared to the non-nitrogen fixer plots.

As is the case for *Elaeagnus angustifolia*, the ornamental ground cover shrub *Elaeagnus pungens* was shown to be fairly salt tolerant as well, they were able to survive under 250 mM NaCl with little leaf tissue damage (Devecchi and Remotti 2004).

2.6 Wolf Willow (*Elaeagnus commutata*)

Wolf willow (*Elaeagnus commutata*) is a boreal actinorhizal perennial shrub that is native to Canada and is found in all provinces except for Atlantic regions, as well as some parts of the US (USDA 2016). The common name “wolf willow” likely came from the flexible “willow like” branches that characterizes the plant. *E. commutata* has been used for conservation purposes such as for the stabilization of soil along river banks (pfaf 2016). A manual for the usage of plant species reclamation in Alberta rated *E. commutata* with “medium” suitability for salt tolerance, and suggested that the plant is well adapted to the tailing sand in the Fort McMurray area in Alberta (Hardy BBT Limited 1989).

Like all other actinorhizal plants, *E. commutata* can form symbiotic association with

nitrogen fixing *Frankia* forming root nodules for nitrogen fixation (Mirza et al. 2009). Although relatively wide spread, *E. commutata* is not known for significant economic values. However, people of the Blackfoot (Niitsitapi) tribe included the edible fruits as a part of their diet (Nature Alberta, 2016). In addition, the bark of *E. commutata* has been suggested to have medicinal applications for treatment of frostbite and syphilis (Pfaf 2016). Since *E. commutata* is a native nitrogen fixing shrub, the potential for it to be used as a reclamation species for saline disturbed habitats should not be overlooked.

E. commutata has been described to grow and spread vigorously through seeds and suckers. It can also easily be propagated vegetatively (Corns and Schraa 1962). The shrub is highly adaptive to different soil conditions. It grows well both in soil with high moisture, as well as in drier, sandy and relatively nutrient poor habitats (Pfaf 2016). It is able to stay competitive in sandy habitats likely due to its nitrogen fixing capability, as well as a certain degree of drought tolerance (Natural Resources Conservation Service 1999). It has also been suggested that *E. commutata* is relatively salt tolerant (which would be consistent with many other *Elaeagnaceae* species) (Natural Resources Conservation Service 1999).

A study has shown an increase in the nitrogen content and overall vegetative growth in forage plants, including grasses, sedges, and forbs growing within a *E. commutata* dominated community, in comparison to adjacent communities dominated by aspen poplar (*Populus tremuloides*) and balsam poplar (*Populus balsamifera*) (Whysong and Bailey 1975). This suggests that *E. commutata* is capable of providing large quantity of nitrogen input within their communities. Visser et al. (1991) planted AMF (arbuscular mycorrhiza fungus) and *Frankia* inoculated and uninoculated *E. commutata* seedlings on oil sand tailings in order to examine field performance of *E. commutata* in disturbed habitats. It was found that inoculation (with *Frankia* and AMF) led to

increase in growth rates ranging from 1.5 to 6 times greater as indicated by parameters such as shoot and root biomass, plant height and root nodule mass compared to uninoculated seedlings over two growing seasons.

2.7 Conclusions

Although there have been studies on the salt tolerance properties of a few actinorhizal plants in certain parts of the world, little research has been done on boreal nitrogen fixing shrubs native to Canada such as *E. commutata*, despite studies demonstrating the potential of *E. commutata* being used to improve soil conditions. How salinity interacts with nitrogen fixation and nodulation in actinorhizal plants is generally poorly understood. In order to determine if *E. commutata* can be used as a potential reclamation species for the oil sand disturbed habitat in Alberta, we need to obtain knowledge on the salt tolerance and nitrogen fixation properties of *E. commutata*, as well as the physiological adaptations that *E. commutata* may utilize to deal with salinity stress. The effort to study *E. commutata* not only can potentially establish it as a suitable reclamation species, it will also further our knowledge in the stress tolerance properties of actinorhizal plants, as well as in the area of *Frankia*-actinorhizal plant symbiosis.

CHAPTER 3. MATERIALS AND METHODS

3.1. Effects of NaCl on the survival, growth and physiology of *E. commutata* seedlings (experiment 1)

3.1.1. Plant materials and growth conditions

Wolf willow (*Elaeagnus commutata* Bernh. ex Rydb.) moistened seeds (collected near Fort McMurray, Alberta, Canada by Farrah Terpstra from Halcyon Tech) were stratified at 5 ^{o}C for three months. The seeds were then planted in a soil mixture that consisted of a 2:1:1 ratio of peat moss, perlite and sand. After germination, seedlings were maintained in a greenhouse with 16 hours of supplemental sodium halide light. Greenhouse temperature was set to vary from 18 to 22 ^{o}C. Seedlings were watered two to three times per week. Modified Rorison's nutrient solution (Hendry and Grime 1993; Appendix 3) with half nitrogen strength (2 mM NO_3^-) was used to fertilize the seedlings once every two weeks. Seedlings were grown in the pots with soil mix for five months prior to inoculation with *Frankia*.

3.1.2. Experimental design and set up

The five month old seedlings were transplanted into hydroponic systems. Each hydroponic system consisted of two stacked 10 liter containers with the upper one containing a calcined clay planting medium "Turface" (Turface Athletics). The lower container was filled with half strength Rorison's nutrient solution (Appendix 3), and a submersible pump to cycle the solution constantly throughout the system (Figure 3.1).

Figure 3.1: Hydroponic systems set up for experiment 1. Each system consisted of two stacked buckets, the upper one contained transplanted seedlings and planting medium, while the lower one contained nutrient/salt solutions. Submersible pumps were used to cycle solution throughout each system. Each treatment consisted of four replicate hydroponic systems. Each hydroponics system contained five transplanted seedlings.

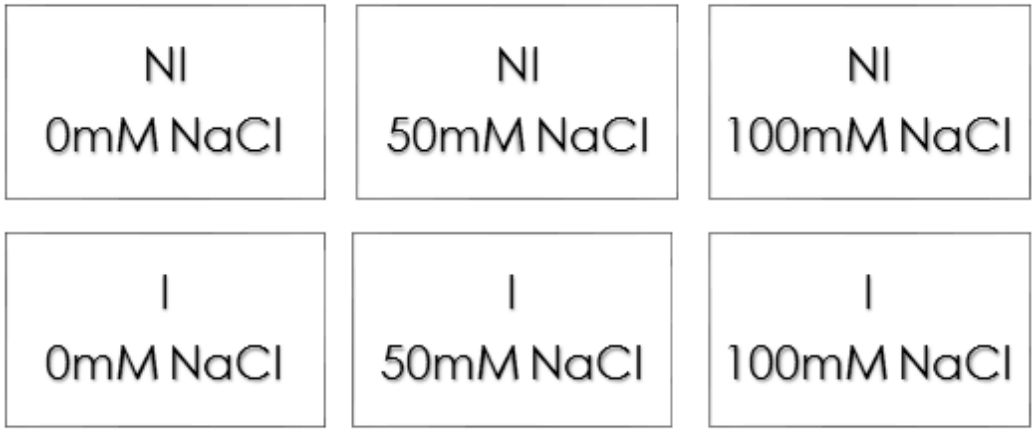


Figure 3.2: Treatment combinations for experiment 1. Each hydroponic system had plants inoculated with *Frankia* or not (NI-Non-inoculation, or I-Inoculation with *Frankia*) and one of three salt levels (0 mM, 50 mM, or 100 mM of NaCl).

A total of 24 hydroponic systems were made, providing four replicates for six experimental treatments. The treatments involved a combination of *Frankia* inoculation (inoculated or non-inoculated, from local field collection of *E. commutata* root nodules around Winnipeg, Manitoba, Canada) and salt treatments of 0 mM, 50 mM, or 100 mM NaCl in the nutrient solution. These concentrations were chosen because they're comparable to the NaCl concentrations found in oil sand tailings (Davis et al. 2014; Figure 3.2).

Five seedlings were planted into each hydroponic system for a total of 120 seedlings. Seedlings were at first exposed to only nutrient solution for four weeks to ensure that they acclimate to the new environment. The *Frankia* inoculation was done directly in the hydroponic systems two weeks before salt treatments were applied. Nodules were collected from *E. commutata* plants in a local field around Winnipeg, Manitoba, these nodules were washed with water, disinfected with a 30% bleach solution, and they were then crushed with pestle and mortar and diluted with 150 ml of distilled water. 2 ml of diluted crushed nodule was injected with a syringe around each seedling in the hydroponic systems. One month after inoculation, seedlings were exposed to salt treatments (0 mM, 50 mM or 100 mM of NaCl directly added into nutrient solution. For the 100 mM NaCl treatment, NaCl was added over two days in 50 mM increments). Plants grew in their salt treatments for a period of 12 weeks. Each hydroponic system was flushed with 5 L of distilled water thoroughly every week to prevent the accumulation of salt in the Turface. At the same time, built up of algae on the surface of the turface was removed, the nutrient solution within each hydroponic system (with or without salt) was also replaced.

3.1.3. Plant physiological parameter measurements

To assess the general effects of different levels of salinity on the physiological function of *E. commutata*, I made measurements on the plant physiological parameters listed below at 1, 4,

8, and 12 weeks into the salt treatment. For these measurements, two plants (out of five) were randomly chosen from each system, and mature, relatively new leaves were sampled to maintain consistency in the measurements.

1. Gas exchange parameters (leaf photosynthesis, leaf transpiration and leaf stomatal conductance) were measured to examine the extent to which plant key physiological functions were affected by salinity stress. These were measured using a portable Infrared gas analyzer (LI-COR LI-6400), with PAR set at 700 µmol $m^{-2}s^{-1}$, reference CO_2 concentration at 400 µmol mol^{-1}, and a flow rate at 400 µmol s^{-1}. These measurements took place in the morning starting between 9-10 AM until around noon time.
2. Leaf chlorophyll fluorescence was measured to determine if salinity stress induced any damage to plant photosynthetic machinery in photosystems II. The measurements were done using a portable chlorophyll fluorometer to assess the efficiency of plant photochemistry (Opti-Sciences OS-30P). These measurements usually took place at around the same time (between 9 to 10AM to noon) as for the gas exchange parameter measurements. Before taking measurements, leaf parts being measured were covered by clips for 30 minutes, to ensure leaves were dark adapted for the minimum fluorescence measurement. The fluorometer was set with a saturation pulse of 1800 µmol m^{-2} s^{-1}. During fluorescence measurements, minimum fluorescence (Fo) and maximum fluorescence (Fm) were recorded, and maximum quantum efficiency (Fv/Fm) for each measured leaf was calculated as: (Fm-Fo)/Fm.
3. Leaf water potential was measured to examine plant water status and to determine how salinity changes water availability in the leaves of these plants. This was done by placing leaves into a pressure chamber (PMS instrument) and the pressure required to force leaf

sap out of the petioles was measured (Boyer 1967). Relative water content (RWC) was calculated on collected leaves using the fresh weight (Fw) and the turgid weight (Tw) after submerging the leaves in distilled water for one day (in the dark at 4 ^{o}C) and the dry weight (Dw) of after freeze drying (Labconco freeze drier, USA). The calculation for RWC follows the formula: 100% × (Fw − Dw)/ (Tw− Dw) (Qin et al. 2010a).

3.1.4. Plant Growth and nitrogen fixation

All seedlings were harvested after 12 weeks of salt treatments. Just before harvesting, plant heights and stem diameters were measured, the proportions of injured leaves (visually estimated as a percentage of all leaves showing leaf tissue necrosis) were also noted for all plants in all hydroponic systems. Each plant was washed with distilled water, and then divided into main stem, main stem leaves, lateral stems, lateral leaves, roots and nodules. Fresh plant tissues were freeze dried and weighed. Plant dry tissues were grounded into powder (with Black & Decker standard coffee grinder) for analyses of cations, chloride and chlorophyll pigment content.

To estimate whole plant nitrogen fixation by acetylene reduction, fresh roots for each plant were incubated with 10% acetylene gas in 250 ml glass jars for one hour (Markham and Zekveld 2007). At the end of the root incubation period, 5 ml of gas was extracted from each jar and injected into a 450-GC Gas Chromatograph (Bruker Corporation) with a 0.25 ml sampling value and Haysep T Column to estimate ethylene and acetylene concentrations. This acetylene reduction assay is based on the finding that nitrogenase, the enzyme utilized by bacteria to fix nitrogen, reduces acetylene to ethylene. Therefore, the amount of acetylene reduced to ethylene during a plant root incubation period provides an estimation of enzymatic activity, and thereby

root nitrogen fixation rates (Dilworth 1966). Control incubators with only acetylene were set up to estimate the amount of ethylene present in the acetylene gas.

3.1.5. Chlorophyll pigment analysis

Freeze-dried powdered leaf tissues were used for chlorophyll pigment analysis. 6 ml of 80% methanol was added to 20 mg leaf samples to extract pigments. The samples were left in the dark for a full day to complete the extraction process. The absorption of the supernatant was then determined with a spectrophotometer (Ultrospec 2100pro) at 650 nm and 665 nm. Chl a and b pigment contents were calculated as follows (MacKinney 1941; Sivasankaramoorthy 2014):

Chl a = 16.5 * A(665nm) – 8.3 * A(650nm)

Chl b = 33.8 * A(650nm) - 25.5 * A(650nm)

3.1.6. Proline analysis

Mature leaves (leaf # 8-10 from the apex of main shoot) were collected from each plant in all hydroponic systems right before the plant harvest took place. Leaves were frozen in liquid nitrogen immediately after collection and stored at -80 ^{o}C. The proline analysis was slightly modified from the process described by Sofo et al. (2004). Leaf tissues (from frozen) were ground into powder with liquid nitrogen. The tissues were then homogenized with 10 ml of 3% sulphosalicylic acid. The samples were centrifuged at 4900X g for 5 min for separation of different phases, and 1 ml of the supernatant was and added to 2 ml of extraction reagent (20 ml distilled water, 30 ml acetic acid, and 1% ninhydrin) and incubated in a 100 ^{o}C hot water bath for 1 hour. The samples were then cooled on ice and 3 ml of toluene was mixed into each sample, and vortexed for 10 seconds. After phase separation was complete, the upper organic layer, which contains toluene and chromophores, was retained. The absorbance of the upper phase

from each sample was determined at 520 nm with a spectrophotometer (Ultrospec 2100pro) with toluene acting as a blank. Proline concentrations were calculated with prepared proline standard curves.

3.1.7. Ion analyses

Powdered dry leaf, stem and root tissue samples (0.5g) from plants harvested were sent out to Stratford Agri Analysis (Stratford, ON) to be analyzed for total N, Na, K, Mg, and Ca. Total Nitrogen content was analyzed with Leco TruSpec N nitrogen analyzer while the other elements were analyzed with DCP (Direct Current Plasma) analysis.

For Cl^- analysis, 50 mg of powdered leaf, stem and root tissues samples were mixed with 10 ml of 0.5 M HNO_3. Samples were then shaken for 30 minutes. 200 µl of Ionic Strength Adjuster (ISA = 5 M $NaNO_3$) was added to each sample before chloride measurements were determined with a chloride ion sensitive probe (Accumet, USA). Measurements were converted into % of plant tissue for data presentation.

3.1.8. Total protein analyses

For total protein content analysis, as in the case with proline, mature leaves were collected just before plant harvest took place. Leaves were frozen in liquid nitrogen immediately after collection and were stored at -80 ^oC. Leaf tissues were ground in liquid nitrogen using mortars and pestles. The ground leaf samples were then incubated with 25 ml of 0.05 M cold potassium phosphate buffer (pH = 7) containing 1% polyvinylpyrrolidone (PVP), 1 mM L-ascorbic acid, and 1 mM EDTA. Samples were centrifuged at 15000 g for 20 minutes (at 4 ^oC). The supernatant (200 µl) was pipetted into new test tubes, and 5 ml of Comassie Brilliant Blue reagent was poured into the tubes. Absorbance readings were recorded at 595 nm with

spectrometer (Ultrospec 2100pro) (Bradford 1976). Bovine serum albumin (BSA) was used for creating standard curves for total protein determination.

3.1.9. Statistical analyses

Least squared models were used to examine both the individual and interactive effects of salinity levels and inoculation (presence or absence of *Frankia* symbiosis) on plant performance. Inoculation was considered a categorical variable and salinity levels a continuous variable. The alpha value was set at 0.05. For these tests we assumed normal distribution of data. The assumption of homogeneous variances was visually tested by examining residual plots. After determining that inoculation had no effect on any of the parameters measured, the inoculation effect was dropped from the model and linear regression analysis was then used to analyze solely the effects of salinity on plant performances. For physiological parameters which were measured over a period of 12 weeks, I have also conducted repeated measure analysis where each hydroponic system is treated as random variables to look at the effects of time, and whether there were any interactive effects between time and salinity on plant physiological parameters. All statistical analyses were conducted with JMP 10 software (SAS Institute, USA).

3.2. Effects of NaCl (100 mM) on plant performance and root nitrogen fixation of *E. commutata* seedlings (experiment 2)

3.2.1. Plant material

E. commutata seedlings used in this experiment were planted at the same time, and grown under the same conditions as those in hydroponic experiment 1. When this experiment took place, these seedlings were around one-year-old. Seedlings were inoculated with *Frankia* one month before being transplanted into hydroponic systems (unlike in experiment 1). Seedlings were cut back at the time of transplant due to overgrowth as a result of their age. The main

branches were trimmed back to a point where there were 7-8 buds remaining, while lateral branch leaves were all removed.

3.2.2. Experimental design and set up

Data from the first hydroponics experiment testing the effects of NaCl on plant growth and physiology also showed low nodulation rates on all seedlings. It is likely that the low nodulation may be due to easy access of nutrients for *E. commutata* seedlings in the hydroponic systems compared to their natural settings. This experiment was then designed to further investigate how salinity affects nitrogen fixation in *E. commutata*.

Only seedlings that were nodulated were selected and the hydroponic systems contained very low nitrogen (0.2 mM) Rorison's nutrient solution. Due to a limitation in plant numbers, salt levels were adjusted to only 0 mM and 100 mM NaCl and only one seedling was transplanted into each system. A total of eight hydroponic systems were made providing for four replicates at each salt level. The treatments (control and salt) lasted for a duration of 12 weeks.

3.2.3. Leaf photosynthesis – light response curves

Instead of measuring leaf photosynthesis at a fixed PAR as in experiment 1, leaf photosynthesis was measured across a range of light intensities to produce light response curves. This could provide further insight on how plant photosynthesis performs under different light conditions. Measurements took place in week 8 and week 12 of the salt treatments. The measurements took place between 9-10AM to noon. A portable photosynthesis system (LI-COR LI-6400) was used for measurements, with light intensities of 0, 50, 100, 200, 500, 1000 and 1500 µmol photon m^{-2} s^{-1}, a reference CO_2 concentration at 400 µmol mol^{-1}, with a flow rate of 400 µmol s^{-1}. For smaller leaves that could not cover the entire area of the leaf chamber, leaf

areas were determined with a leaf area meter (Li-Cor LI-3000), and were taken into account when calculating for photosynthesis rates. For each run of measurements light photosynthesis parameters were estimated by fitting the data to the simple exponential model (equation two) in Chalker (1981). Average values of these parameters were used to construct light response curves for each treatment.

3.2.4. Plant growth and nitrogen fixation

Seedlings were harvested after 12 weeks of salt treatments. Plant heights and stem diameters were measured prior to harvesting and the proportion of injured leaves were noted for all plants. Each seedling was washed with distilled water, and then divided into leaves, stems, root and nodules. The root system for each plant was incubated within 10% acetylene gas in 250 ml glass jars for one hour to estimate the rate of nitrogen fixation (as in the previous experiment). Selected nodules were preserved in 70% alcohol for the purposes of nodule sectioning and microscopy (see below). Harvested fresh tissues were freeze dried, and weighed.

3.2.5. Root nodule sections and microscopy

Nodules were sectioned longitudinally by hand to examine their infection by *Frankia*. Nodule slices were stained with Fabils stain (0.5% aniline blue, 0.5% basic fuchsin, iodine and potassium iodide in lactophenol) for around 1 min, then mounted on slides with lactophenol and observed under light microscope. The aniline blue portion of the Fabils stains *Frankia* bacterial vesicle walls found in infected root cells. The other portions of the stains, iodine and basic fuchsin, are respectively known to be used to stain starch and cell nuclei.

3.3. Effects of high levels of NaCl on the survival of *E. commutata* cuttings (experiment 3)

3.3.1. Plant material

The goal of this experiment was to examine the ability of *E. commutata* to survive higher salinity levels beyond of what is present in the oil sand tailings. *E. commutata* cuttings were collected from near Fort McMurray, Alberta (Canada). These cuttings were stored at 4°C for several months were divided into 5-7 cm segments (each included two or three buds), dipped with STIM-ROOT 0.8% IBA rooting powder (Master Plant-Prod Inc.), and planted into trays containing perlite: vermiculite (1:1, V/V) as rooting medium. The cuttings were intermittently misted with water for around 10 seconds every 10 minutes with automatic misting systems until the majority of them flushed and developed roots. Healthy cuttings were transplanted from rooting medium into plugs that contained soil mixture that consisted of 2:1:1 volumes ratio of peat moss: sand: perlite. From this point on, cuttings were routinely watered two to three times a week, and fertilized with half N strength Rorison's nutrient solution (Appendix 3) every two to three weeks. These cuttings grew in the same greenhouse where the *E. commutata* seedlings used in experiment 1 and 2 were grown in. Growth conditions were identical as described in section 3.1.1.

3.3.2. Experimental design and set up

E. commutata cuttings were more than one-year-old when this experiment took place. Non nodulated cuttings were selected for this experiment. The cuttings were grouped into nine replicates with cuttings within each replicate randomly assigned to receive a salinity treatment of different NaCl levels. Cuttings were exposed to treatments for a total of 16 weeks.

A step-wise method was used to progressively apply NaCl. This was to provide cuttings an adjustment period and to prevent them from being killed off by high levels of osmotic stress as a result of high salinity. The NaCl was applied at 100 mM increments, each application involved adding 100 mM NaCl into 100 ml of six times diluted Rorison's nutrient solution. A total of 600 ml diluted Rorison's nutrient solution was applied to all plants (with 0.33 mM nitrate). Applications were spaced two to three days apart. This meant that the control cuttings (0 mM NaCl) would have received a total of 6 applications of only diluted nutrient solution (without NaCl), while cuttings assigned with higher NaCl treatments would have received, 2, 4 or 6 applications of 100 mM NaCl in 100 ml diluted nutrient solution. Collection cups were placed underneath the plugs to collect leachate from the plugs. This was routinely poured back into the plugs to maintain overall consistency in salinity and nutrient levels. Cuttings were watered every two to three days with 100 ml of distilled water for 16 weeks.

3.3.3. Stem height and leaf chlorophyll fluorescence measurements

In this experiment, week 1 represents the time which all 6 incremental salt applications were complete for cuttings receiving the highest levels of NaCl (6 applications of 100 mM NaCl in 100 ml nutrient solution). For cuttings receiving more intermediate levels of NaCl (with 2 applications and 4 applications of 100 mM NaCl, the assigned salinity levels would have been reached 1 to 2 weeks prior to "week 1".

Plant height measurements were taken in week 1 of the treatments, and then again after week 16 of the treatment applications. Heights were taken from where shoots branched off from the cuttings to the tips of the shoots. Leaf chlorophyll fluorescence measurements were also taken after 16 weeks with a fluorometer (Opti-Sciences OS-30P), as described previously.

3.3.4. Survival and leaf injury

The condition of all 36 cuttings was monitored daily and the date at which each cutting died was recorded. A final tally was taken at week 16. Leaf injury (%) was estimated visually by me as a proportion of all leaves displaying leaf tissue necrosis, with increments at around 5% (ex. 15%, 20% or 25% leaf injury), these data were recorded for all surviving cuttings at week 16.

CHAPTER 4. RESULTS

4.1. Effects of NaCl on the survival, growth and physiology of *E. commutata* seedlings (experiment 1)

4.1.1. Plant root nodulation

Seedlings harvested from Experiment 1 showed poor nodulation. Out of 60 inoculated plants, only five were nodulated. Salinity seemed to inhibit nodulation, as all of the nodulated plants were from 0 mM NaCl treatments (Table 4.1). Among the nodulated plants, there was only one nodule active in fixing nitrogen at the time of the harvest. Likely, due to the low nodulation rates, inoculation did not have significant effects in any of the plant parameters which were measured. Therefore, for data analyses, I grouped inoculated plants and non-inoculated plants together, focusing on how *E. commutata* seedlings were affected by intermediate levels of NaCl.

4.1.2. Plant survival and physiological parameters

At the end of the 12-week duration of NaCl treatments, all 120 *E. commutata* seedlings survived, including those subjected to the highest level of NaCl. Plant physiological parameters were measured on weeks 1, 4, 8 and 12 throughout the duration of the salt treatments. For leaf photosynthesis, salinity led to significant reductions in week 1 ($p = 0.0002$, $r^2 = 0.495$) and in week

12 ($p = 0.0041$, $r^2 = 0.330$); with 100 mM NaCl, the reduction rates in leaf photosynthesis were 26% and 27.3%, respectively, compared to control plants. NaCl treatments did not lead to reductions in photosynthesis in week 4 or week 8 (Figure 4.1). There was a significant effect of time (weeks of treatment; $p = 0.0001$), as well as an interactive effect between time and NaCl ($p = 0.0068$). Leaf photosynthesis declined as time progressed, control plants had an average PS rate of 13.23±0.63, 12.23±0.72, 7.96±0.54 and 7.40±0.50 µmol CO_2 m^{-2} s^{-1} for weeks 1, 4, 8 and 12, respectively.

NaCl treatments led to significant reductions in leaf transpiration rates in all weeks of treatments ($p = 0.0011$, $r^2 = 0.405$ in week 1, $p = 0.0015$, $r^2 = 0.387$ in week 4, $p = 0.0178$, $r^2 = 0.239$ in week 8, $p = 0.0001$, $r^2 = 0.530$ in Week 12). Plants treated with 100 mM NaCl had transpiration rates reduced by 36.2%, 22.6%, 25.7%, and 47.5%, in weeks 1, 4, 8 and 12 respectively, compared to control plants (Figure 4.2). Time led to significant declines in leaf transpiration ($p = 0.0001$), transpiration rates dropped from an average of 3.27±0.25 mmol H_2O $m^{-2}s^{-1}$ in week 1, to 2.61±0.18 mmol H_2O $m^{-2}s^{-1}$ in week 12 for control plants.

NaCl treatments also led to significant reductions in stomatal conductance throughout the 12 weeks of treatments ($p = 0.0009$, $r^2 = 0.418$ in week 1, $p = 0.0014$, $r^2 = 0.391$ in week 4, $p = 0.0168$, $r^2 = 0.243$ in week 8, and $p = 0.0001$, $r^2 = 0.544$ in week 12). At 100 mM NaCl, plant stomatal conductance was reduced by 45.6%, 30.8%, 29.3% and 51.7% for weeks 1, 4, 8 and 12, respectively, compared to control plants (Figure 4.3). There was a significant effect of time ($p = 0.0001$), and an interactive effect with time and NaCl ($p = 0.0398$) on stomatal conductance. Stomatal conductance declined progressively with time, from an average of 0.159±0.017 mmol H_2O $m^{-2}s^{-1}$ in week 1 to 0.083±0.006 mmol H_2O $m^{-2}s^{-1}$ in week 12 for control plants.

Although NaCl did not affect leaf chlorophyll fluorescence maximum quantum yield ratios (Fv/Fm) after weeks 1, 4, and week 8, in week 12, leaf Fv/Fm ratio increased by 3.5% for 100 mM NaCl treated plants compared to control plants ($p = 0.0085$, $r^2 = 0.286$; Figure 4.4). None of these Fv/Fm ratios were low enough (<0.7) to indicate plant stress (Ritchie 2006).

NaCl led to significant reductions in leaf relative water content in week 1 ($p = 0.0003$, $r^2 = 0.478$), week 4 ($p = 0.0131$, $r^2 = 0.259$), and week 8 ($p = 0.0008$, $r^2 = 0.421$) of the experiment, the percent of reduction at 100 mM NaCl being 4.4%, 3.9% and 4.2%, for weeks 1, 4, and 8 respectively, compared to control plants. However, NaCl exposure did not lead to a reduction in leaf relative water content in week 12(Figure 4.5). Salinity reduced leaf water potential in week 4 ($p = 0.0149$, $r^2 = 0.251$), week 8($p = 0.0024$, $r^2 = 0.361$) and week 12 ($p = 0.0256$, $r^2 = 0.216$). Reduction rates at 100 mM NaCl were 25.5%, 24.6% and 10.6% at week 4, 8 and 12, respectively (Figure 4.6).

Table 4.1: Number of nodulated plants, and numbers of actively functioning nodules for *E. commutata* seedlings harvested from experiment 1 (total = 120 plants). The *E. commutata* seedlings were 5 months old at the beginning of the treatments, they were subjected to control or salt treatment for 12 weeks. The treatments incorporated inoculations (I) or non-inoculation (NI) with *Frankia*, combining with 3 salt levels (0, 50 and 100 mM NaCl).

Treatment	Nodulated Plants/Total Plants	Active nodules
I-0 mM NaCl	5/20	1
NI-0 mM NaCl	1/20	0
I-50 mM NaCl	0/20	0
NI-50 mM NaCl	0/20	0
I-100 mM NaCl	0/20	0
NI-100 mM NaCl	0/20	0

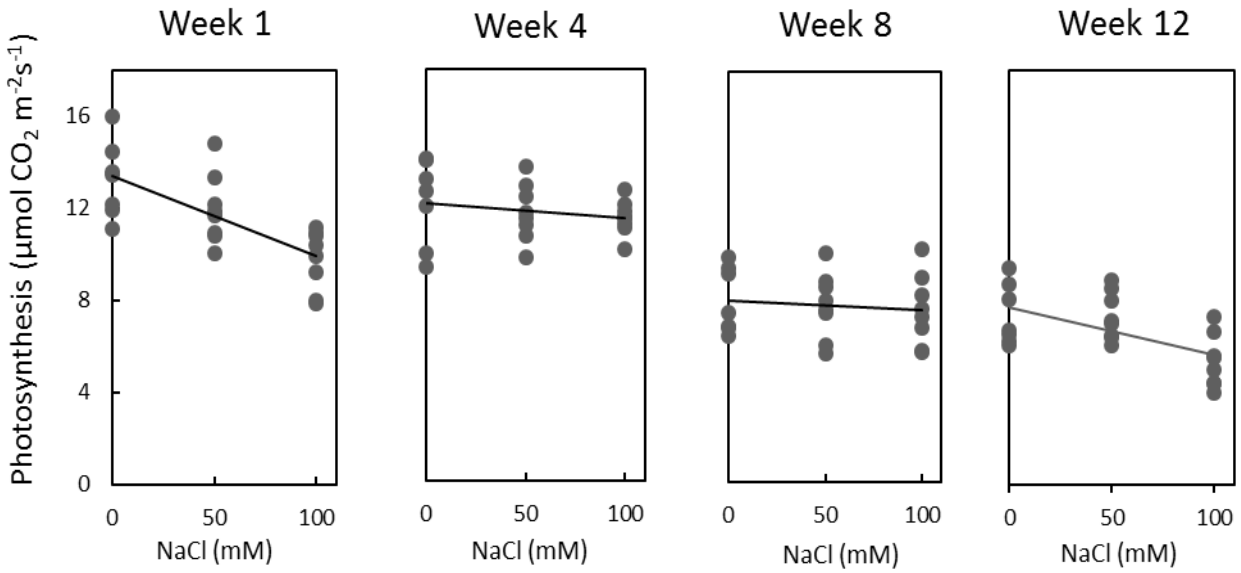


Figure 4.1: Leaf photosynthesis rates measured at week 1, week 4, week 8, and week 12 for *E. commutata* seedlings subjected to 0, 50 and 100 mM NaCl in hydroponic systems. Linear regression analysis was used for statistical analysis for all weeks of measurement. Least squares fit lines are shown for each time period (n=7 for 0 mM NaCl, n=8 for 50 and 100 mM NaCl). Significant reduction effect with salinity was found in week 1 and week 12, slopes of least squares fit lines are -0.0347, and -0.0207 for week 1 and week 12, respectively. For week 4 and week 8, salinity led to no significant change in photosynthesis.

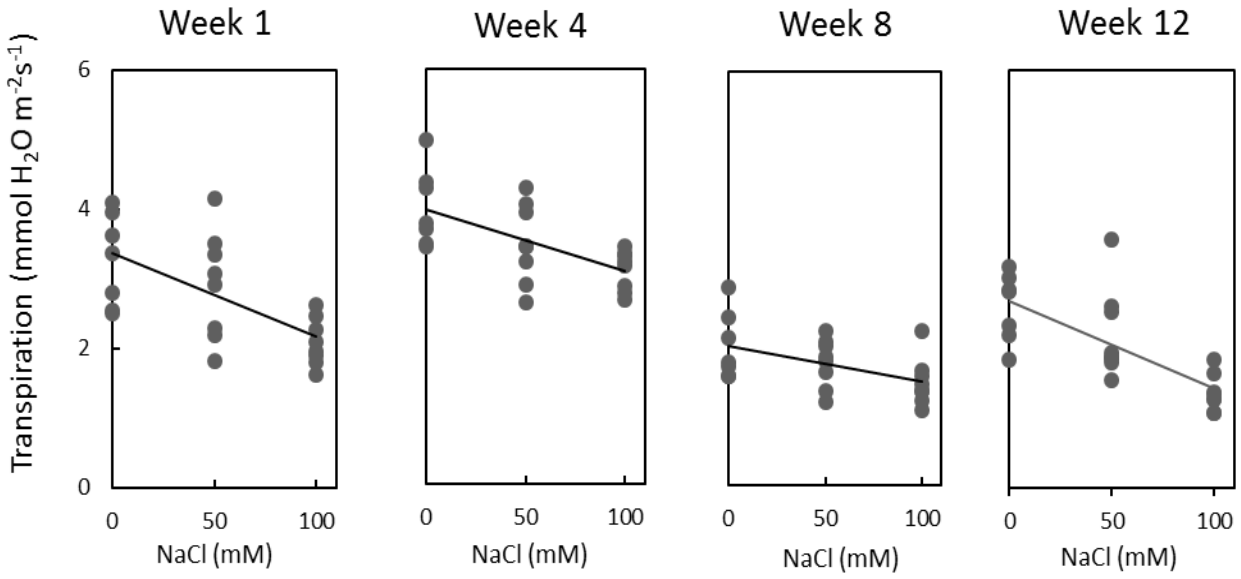


Figure 4.2: Leaf transpiration rates measured at week 1, week 4, week 8, and week 12 for *E. commutata* seedlings subjected to 0, 50 and 100 mM NaCl in hydroponic systems. Linear regression analysis was used for statistical analysis for all weeks of measurement. Least squares fit lines are shown for each time period (n=7 for 0 mM NaCl, n=8 for 50 and 100 mM NaCl). Significant reduction effect with salinity was found in all weeks of measurement, slopes of least squares fit lines are -0.012, -0.009, -0.0052, and -0.0125 for weeks 1, 4, 8 and 12, respectively.

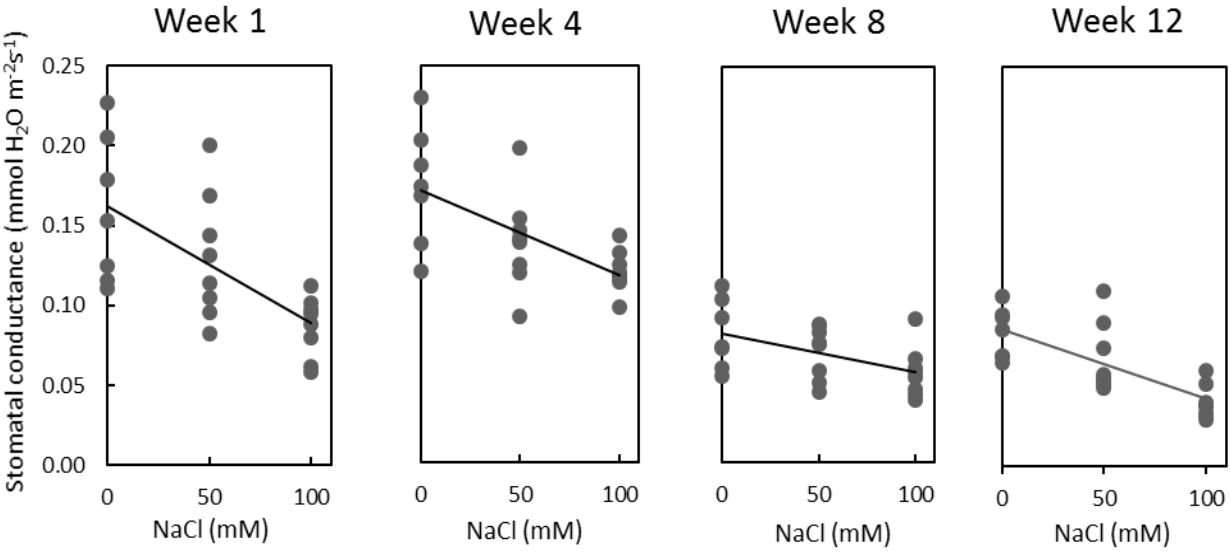


Figure 4.3: Stomatal conductance rates measured at week 1, week 4, week 8, and week 12 for *E. commutata* seedlings subjected to 0, 50 and 100 mM NaCl in hydroponic systems. Linear regression analysis was used for statistical analysis for all weeks of measurement. Least squares fit lines are shown for each time period (n=7 for 0 mM NaCl, n=8 for 50 and 100 mM NaCl). Significant reduction effect with salinity was found in all weeks of measurement, slopes of least squares lines are -0.0007, -0.0005, -0.0002, and -0.0004 for weeks 1, 4, 8 and 12, respectively.

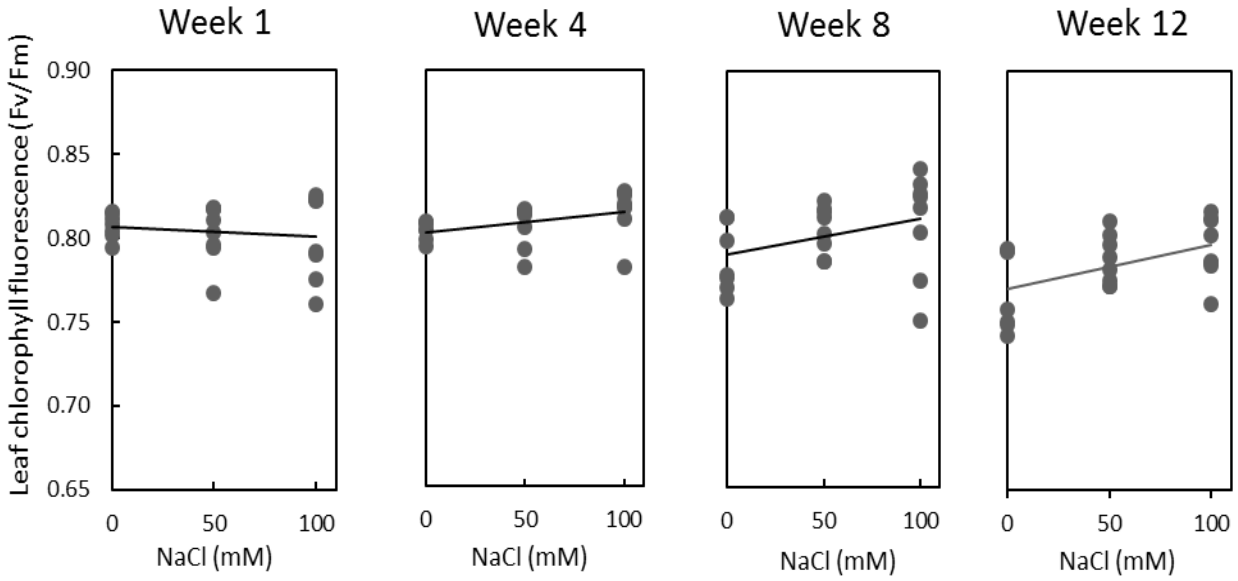


Figure 4.4: Leaf chlorophyll fluorescence (maximum quantum yield: Fv/Fm) measured at week 1, week 4, week 8, and week 12 for *E. commutata* seedlings subjected to 0, 50 and 100 mM NaCl in hydroponic systems. Linear regression analysis was used for statistical analysis for all weeks of measurement. Least squares fit lines are shown for each time periods (n=7 for 0 mM NaCl, n=8 for 50 and 100 mM NaCl). The slope of the least squares fit line for week 12 is 0.0003, in week 1, 4 and week 8, salinity did not lead to any changes in chlorophyll fluorescence.

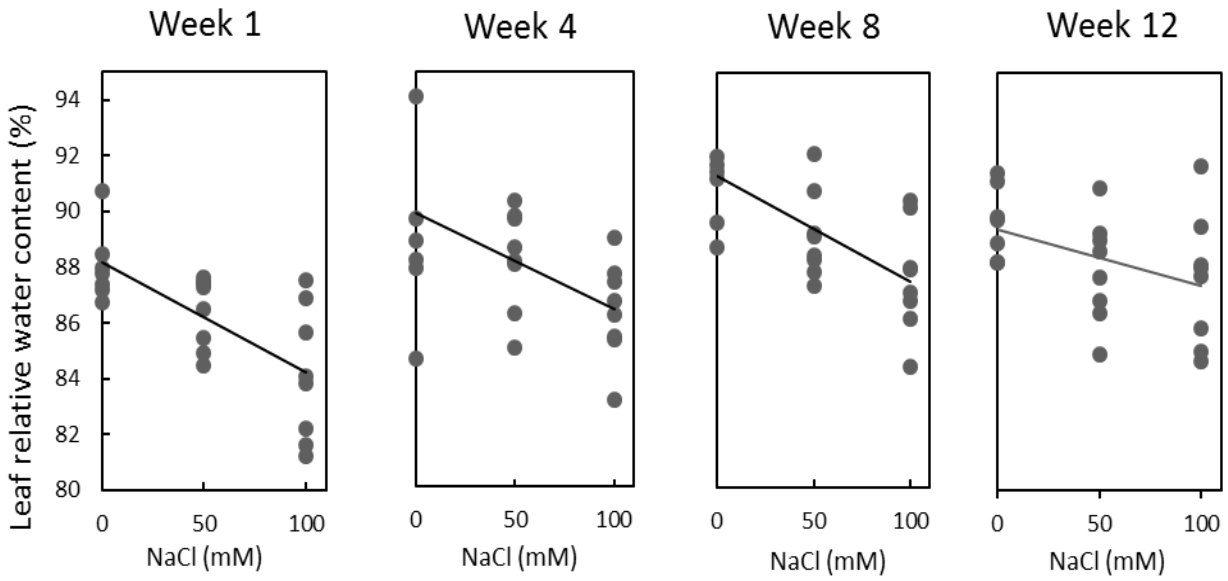


Figure 4.5: Leaf relative water content measured at week 1, week 4, week 8, and week 12 for *E. commutata* seedlings subjected to 0, 50 and 100 mM NaCl in hydroponic systems. Linear regression analysis was used for statistical analysis for all weeks of measurement. Least squares fit lines are shown for each time period (n=7 for 0 mM NaCl, n=8 for 50 and 100 mM NaCl). Significant reduction effect with salinity was found in week 1, 4 and 8, slopes of least squares fit lines are -0.0391, -0.0349, and -0.0385 for weeks 1, 4 and 8, respectively. Salinity led to no change in leaf relative water content in week 12.

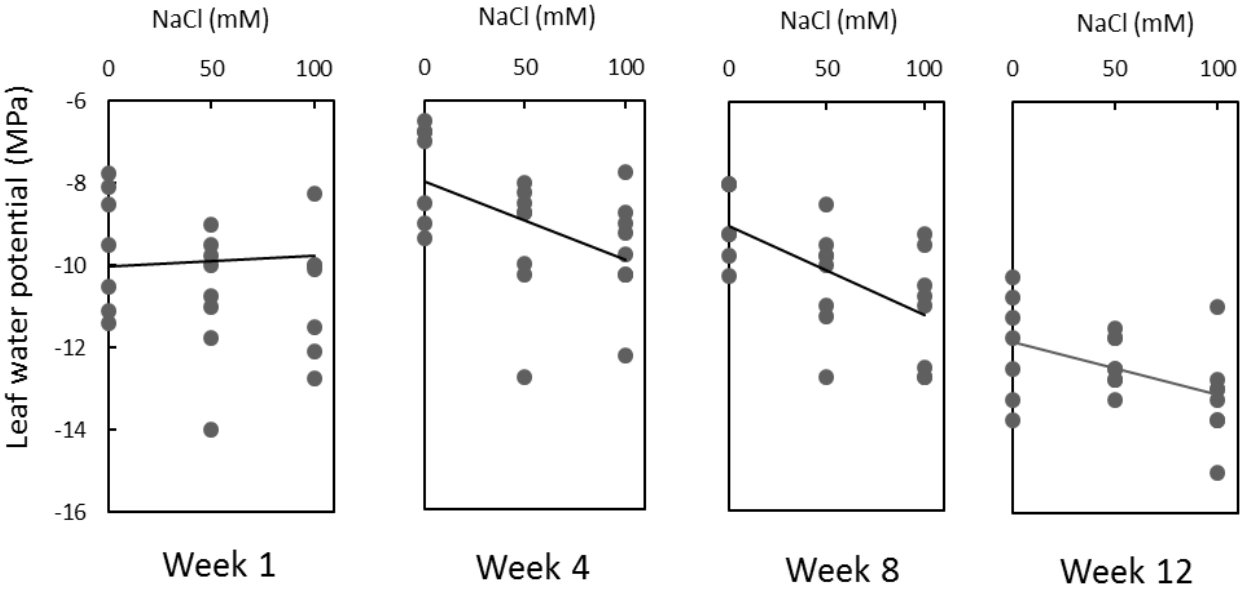


Figure 4.6: Leaf water potential measured at week 1, week 4, week 8, and week 12 for *E. commutata* seedlings exposed to 0, 50 and 100 mM in hydroponic systems. Linear regression analysis was used for statistical analysis for all weeks of measurement. Least squares fit lines are shown for each time period (n=7 for 0 mM NaCl, n=8 for 50 and 100 mM NaCl). Significant reduction effect with salinity was found in week 4, 8 and 12, slopes of least squares fit lines are -0.0193, -0.0218, and -0.0127 for weeks 4, 8 and 12, respectively. In week 1, salinity did not lead to a significant difference in leaf water potential.

4.1.3. Plant growth parameters and injury

NaCl did not have any effect on leaf injury for leaves that were still intact on the seedlings ($p = 0.5363$; Table 4.2). However, the proportion of plant shed leaf (litter found in hydroponic systems as a percentage of plant shoot biomass) increased with increasing NaCl concentration ($p = 0.0143$) to reach 66.7% with the 100 mM treatment (Figure 4.7).

Salinity led to significant decline in plant total biomass ($p = 0.0001$), plant height ($p = 0.0001$), and plant stem width($p = 0.0017$; Table 4.2). The decrease in biomass with higher concentration of NaCl was found in both shoot (stems + leaves; $p = 0.0001$) and root tissues ($p = 0.0001$), reaching 33.7% and 36%, respectively at 100 mM NaCl compared ot controls (Figure 4.8). There was no difference in plant shoot : root ratio between different NaCl levels. Interestingly, the decline in plant shoot biomass was mainly represented as a reduction in the plant main shoot as opposed to lateral shoots. The proportion of plant lateral shoot biomass (as a % of shoot biomass) increased from 32% at 0 mM NaCl to 43.5% at 100 mM NaCl ($p = 0.0043$; Figure 4.9). Plant main shoot biomass and plant main branch leaf biomass declined by 49.3% ($p = 0.0001$) and 46.9% ($p = 0.0001$), respectively at 100 mM NaCl, while plant lateral branch biomass (stems and leaves) was not affected by NaCl (Table 4.2).

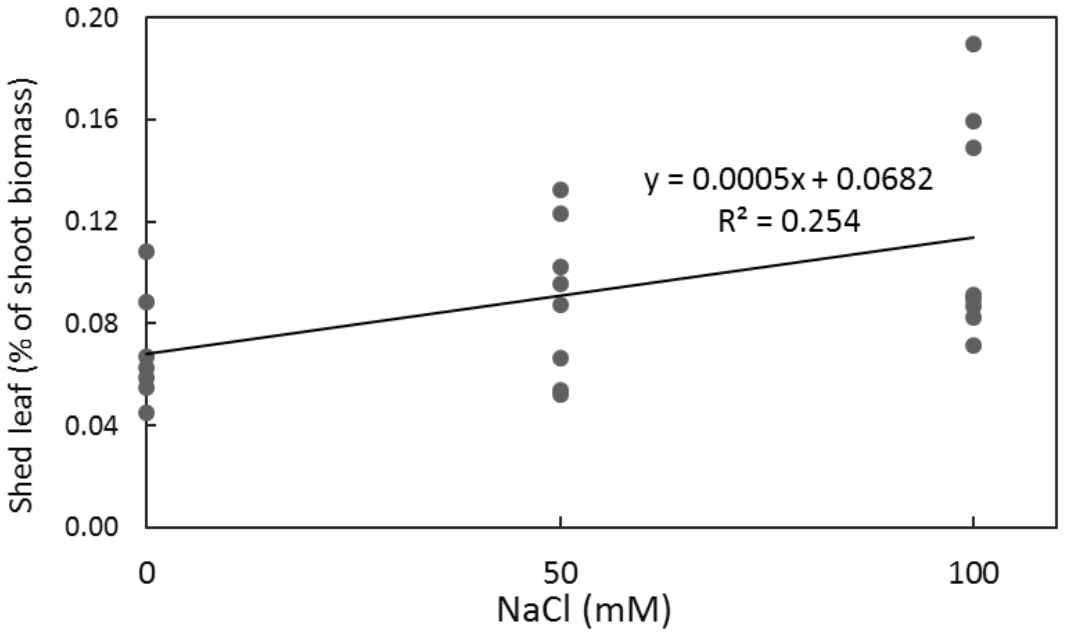


Figure 4.7: Plant shed leaf as a percentage of plant total shoot biomass (stem + leaves) for *E. commutata* seedlings subjected to 0, 50 and 100 mM NaCl in hydroponic systems (n=7 for 0 mM NaCl, n=8 for 50 and 100 mM NaCl).

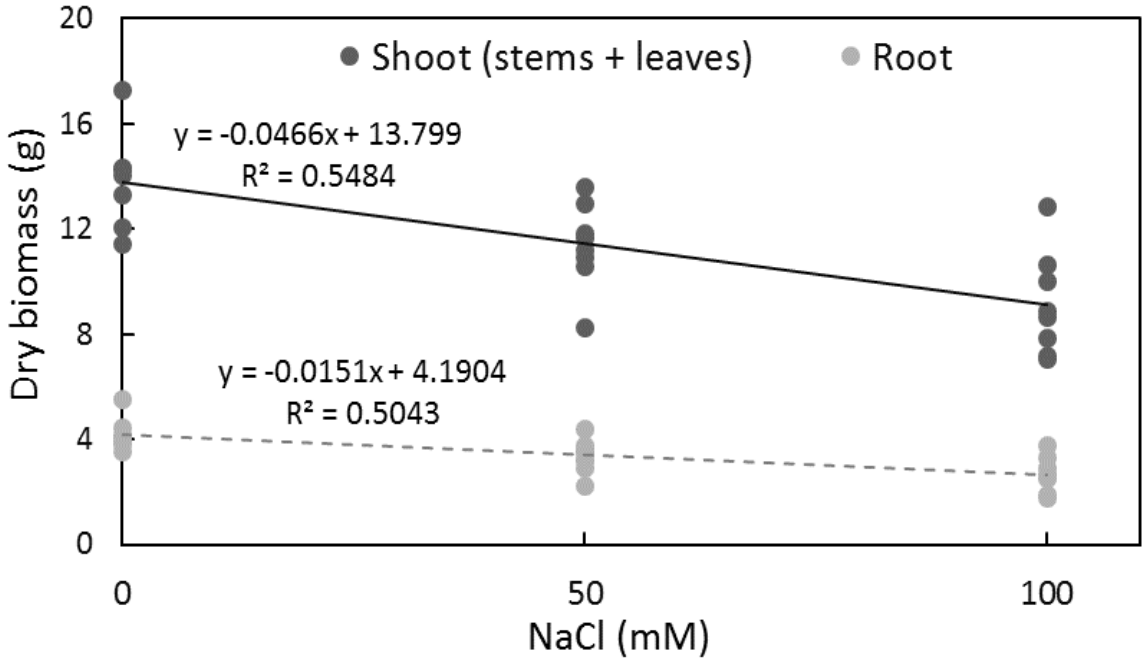


Figure 4.8：Plant shoot (stem + leaves) and root dry biomass of *E. commutata* seedlings subjected to 0, 50, 100 mM NaCl in hydroponic systems (n=7 for 0 mM NaCl, n=8 for 50 and 100 mM NaCl).

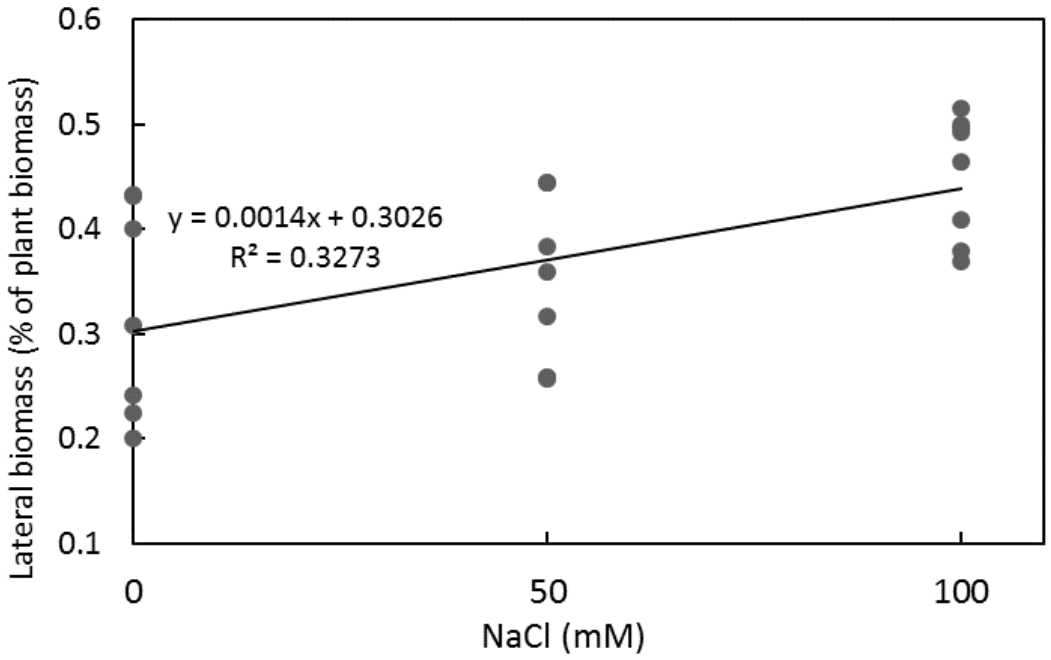


Figure 4.9: Plant lateral shoot biomass (lateral branch stem + leaves) as a percentage of plant total shoot biomass (stems + leaves) for *E. commutata* seedlings subjected to 0, 50 and 100 mM NaCl in hydroponic systems (n=7 for 0 mM NaCl, n=8 for 50 and 100 mM NaCl).

Table 4.2 : Plant growth parameters (mean ± SE) for harvested wolf willow (*Elaeagnus commutata*) seedlings. These seedlings were subjected to salinity (0,50 and 100 mM NaCl) treatments in hydroponic systems. Data analysis was performed using linear regression analysis, p and r^2 values are displayed. (n=7 for 0 mM NaCl; n=8 for 50, 100 mM NaCl). Bolded values represent significant changes with NaCl.

	NaCl (mM)				
	0	**50**	**100**	**P-value**	**r^2**
Total biomass (g)	**18.06±0.95**	**14.78±0.78**	**11.87±0.91**	**0.0001**	0.546
Plant height (cm)	**70.23±1.32**	**63.24±2.81**	**48.73±2.06**	**0.0001**	0.680
Plant stem width (mm)	**7.60±0.32**	**7.08±0.20**	**6.25±0.28**	**0.0017**	0.381
Shoot : root ratio	4.09±0.11	4.05±0.09	3.89±0.18	0.2910	0.053
Main shoot biomass (g)	**8.42±0.37**	**6.83±0.48**	**4.27±0.31**	**0.0001**	0.718
Lateral shoot biomass (g)	5.39±0.67	4.57±0.53	4.89±0.46	0.5528	0.017
Leaf biomass (Main shoot; g)	**3.52±0.19**	**2.93±0.21**	**1.87±0.14**	**0.0001**	0.658
Leaf biomass (Lateral shoot; g)	3.70±0.48	3.18±0.33	3.37±0.31	0.5691	0.016
Leaf Injury (%)	6.57±3.98	3.79±1.95	4.29±1.16	0.5363	0.018

4.1.4. Leaf proline and protein content

The leaves of the *E. commutata* seedlings contained low amounts of proline. At 0 mM NaCl, the average proline content was 6.13±3.21µg/g leaf tissue, which corresponds to only about 0.0006% of leaf tissue content. There were no differences in leaf proline content between different NaCl treatments ($p = 0.9848$; Figure 4.10a).

It was determined that at 0, 50 and 100 mM NaCl, leaf protein content was 14.01±0.65 14.42±1.09, and 13.69±0.83 mg/g, respectively. These numbers correspond to around 1.3% to 1.4% of the plant leaf tissue content. Leaf protein content also did not change significantly with NaCl treatments ($p = 0.7458$; Figure 4.10b).

4.1.5. Leaf chlorophyll pigment content

Both chlorophyll a, and chlorophyll b content increased with increasing NaCl concentration ($p = 0.0001$, $r^2 = 0.499$ for Chl a; $p = 0.0001$, $r^2 = 0.559$ for Chl b). At 100 mM NaCl, chlorophyll a content increased by 50% compared to control plants, while chlorophyll b content more than doubled (Figure 4.11).

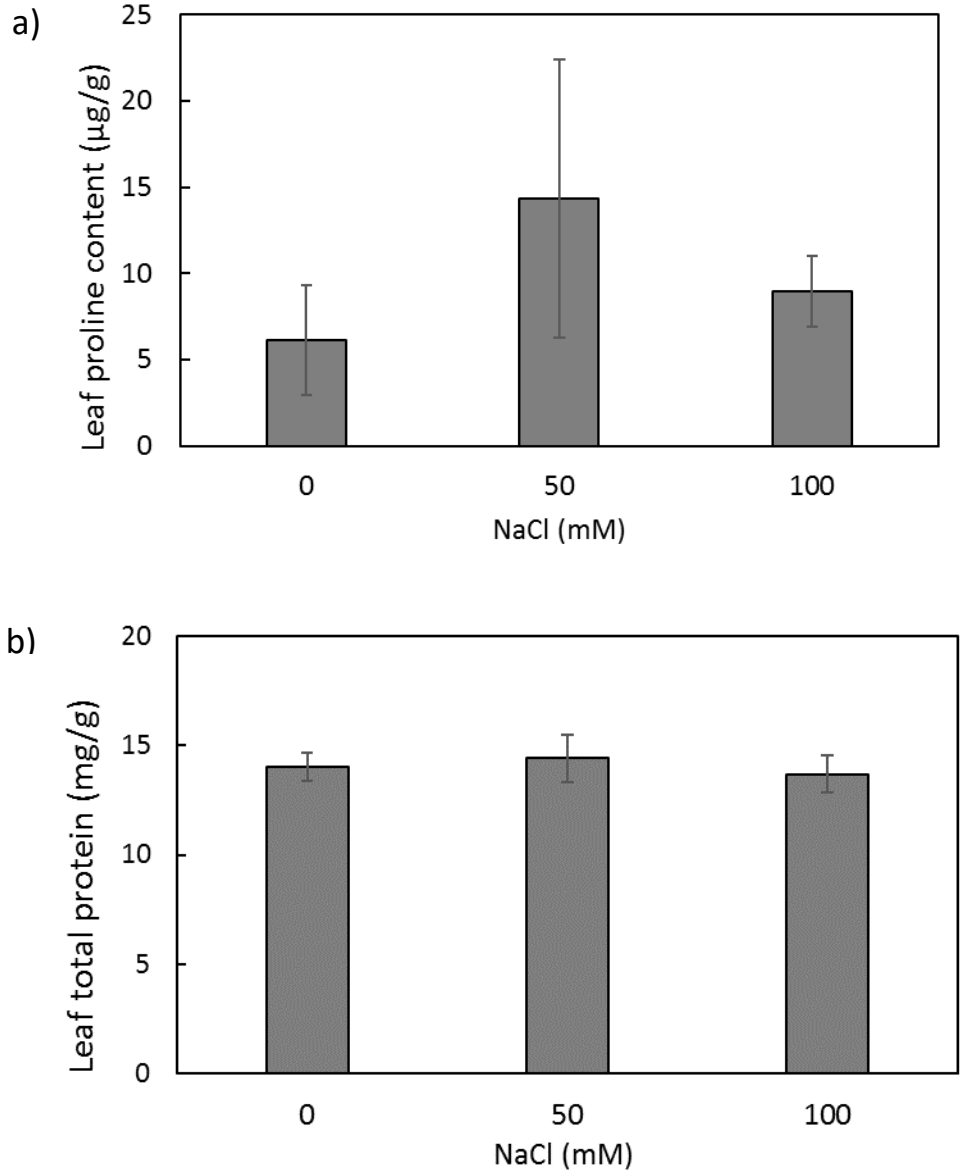


Figure 4.10: a). Plant leaf proline content (µg proline/ g fresh leaf tissue); b) Plant leaf total protein content (mg protein/g fresh leaf tissue) analysed from *E. commutata* (wolf willow) leaves exposed to 0, 50 and 100 mM in hydroponic systems. Linear regression analysis was used for statistical analysis. Bars represent mean ± SE (n=7 for 0 mM NaCl, n=8 for 50 and 100 mM NaCl).

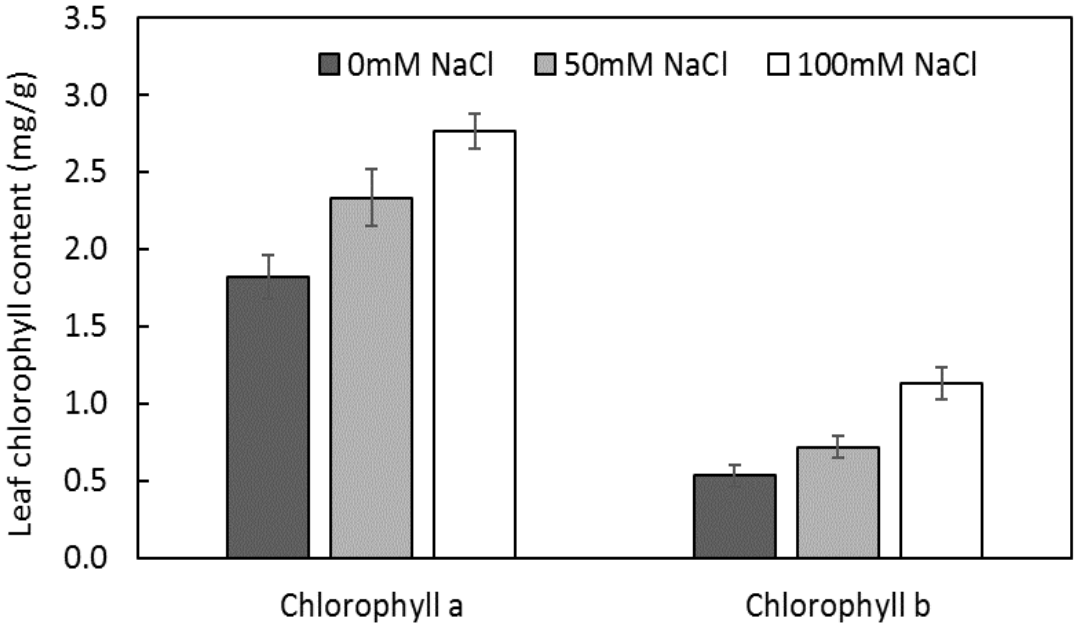


Figure 4.11: Plant leaf chlorophyll a and chlorophyll b content (mg pigment / g leaf dry tissue) of *Elaeagnus commutata* (wolf willow) leaves subjected to 0, 50 and 100 mM NaCl in hydroponic systems. Linear regression analysis was used for statistical analysis. Bars represent mean ± SE (n=7 for 0 mM NaCl, n=8 for 50 and 100 mM NaCl).

4.1.6. Plant elemental analyses

An increase in plant nitrogen content was detected with higher levels of NaCl in leaf and root tissues. At 100 mM NaCl, plant tissue nitrogen content increased by 69.9% in leaf tissue ($p = 0.0003$, $r^2 = 0.749$), and 24.8% in root tissue ($p = 0.0201$, $r^2 = 0.433$) compared to control plants (0 mM NaCl; Figure 4.12).

As expected, plant concentrations of Na and Cl both increased with salinity exposure for all plant tissues. At 100 mM NaCl, Na concentrations were 4.9 times higher in plant leaf tissues ($p = 0.0029$, $r^2 = 0.604$), 10.5 times higher in plant stem tissues ($p = 0.0001$, $r^2 = 0.909$), and almost 19.8 times higher in plant root tissues ($p = 0.0001$, $r^2 = 0.967$) compared to that of control plants (Figure 4.13). For Cl, the 100 mM NaCl treatment increased Cl concentrations in leaves by 4.2 times ($p = 0.0046$, $r^2 = 0.568$), in stems by 9.9 times ($p=0.0001$, $r^2=0.777$), and in roots by 3.2 times ($p = 0.0018$, $r^2 = 0.639$) compared to control plants (Figure 4.14). In addition, at each salinity level, plant root tissues accumulated much higher amounts of both Na and Cl compared to leaf or stem tissues. At 100 mM NaCl, Na content in root tissues are six to seven times higher than that of leaf and stem tissues. Root tissues also contained more than doubled the amount of Cl content compared to leaf tissues, and almost nine times the amount of Cl content compared to stem tissues (Figure 4.13, 4.14).

In plant leaf tissues, Ca content declined under NaCl treatments ($p = 0.0018$). At 100 mM NaCl, leaf tissue Ca content declined by 37.3% compared to control plants. However, Ca content in stem and root tissues were not affected by the NaCl treatments. For K content, which is an essential plant nutrient like Ca, higher NaCl treatments did not result in any changes in leaf, stem

or root tissues (Table 4.3). Higher level of NaCl also resulted in the decline in both Phosphorus (P) and Boron (B) contents in leaf tissues ($p = 0.0001$ for both) and root tissues ($p = 0.0001$ for Phosphorus, $p = 0.0066$ for Boron; Table 4.3).

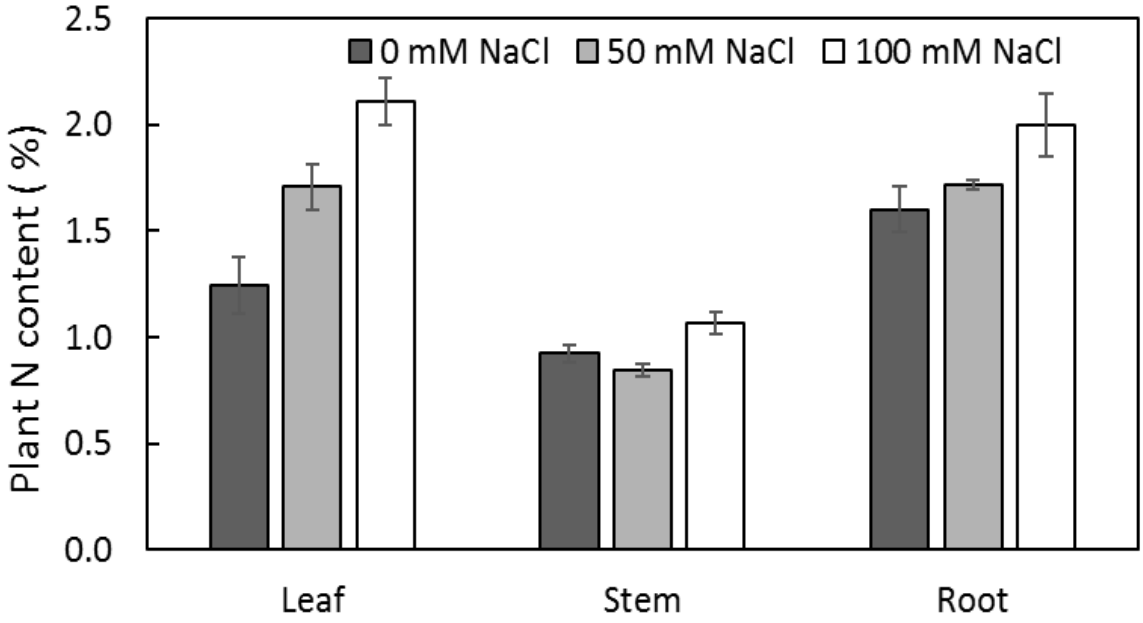


Figure 4.12: Plant nitrogen content (% of plant tissue) of *Elaeagnus commutata* (wolf willow) leaf, stem and root tissues exposed to 0, 50 and 100 mM NaCl in hydroponic systems (mean ± SE, n=4).

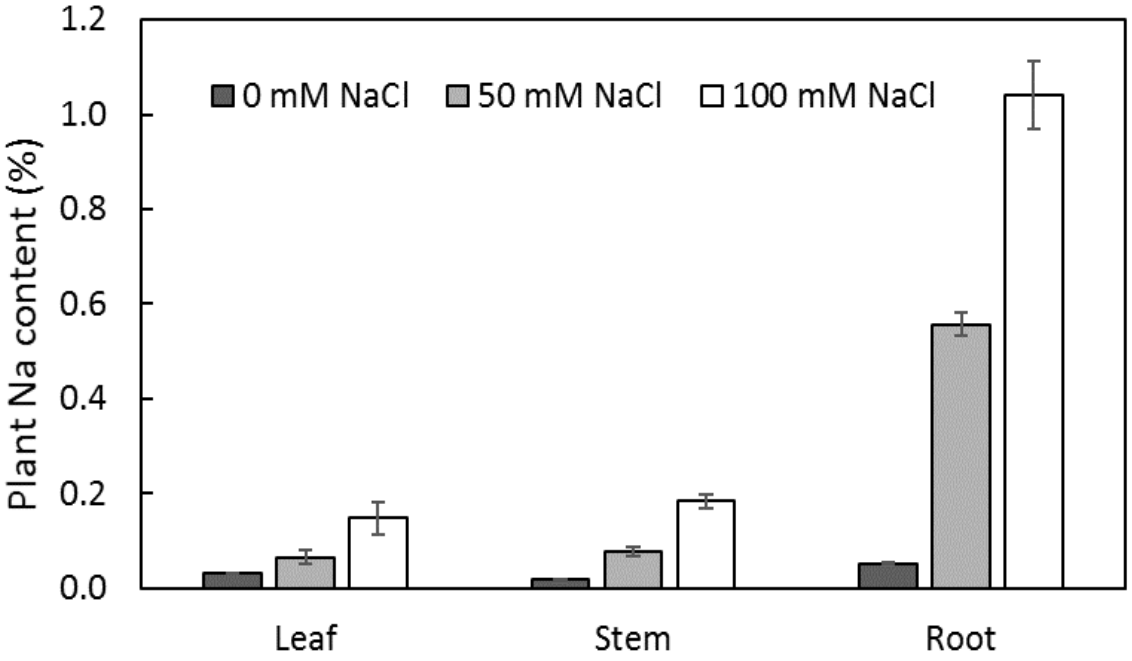


Figure 4.13: Plant Na content (% of plant tissue) of *Elaeagnus commutata* (wolf willow) leaf, stem and root tissues treated with 0, 50 and 100 mM NaCl in hydroponic systems (mean ± SE, n=4).

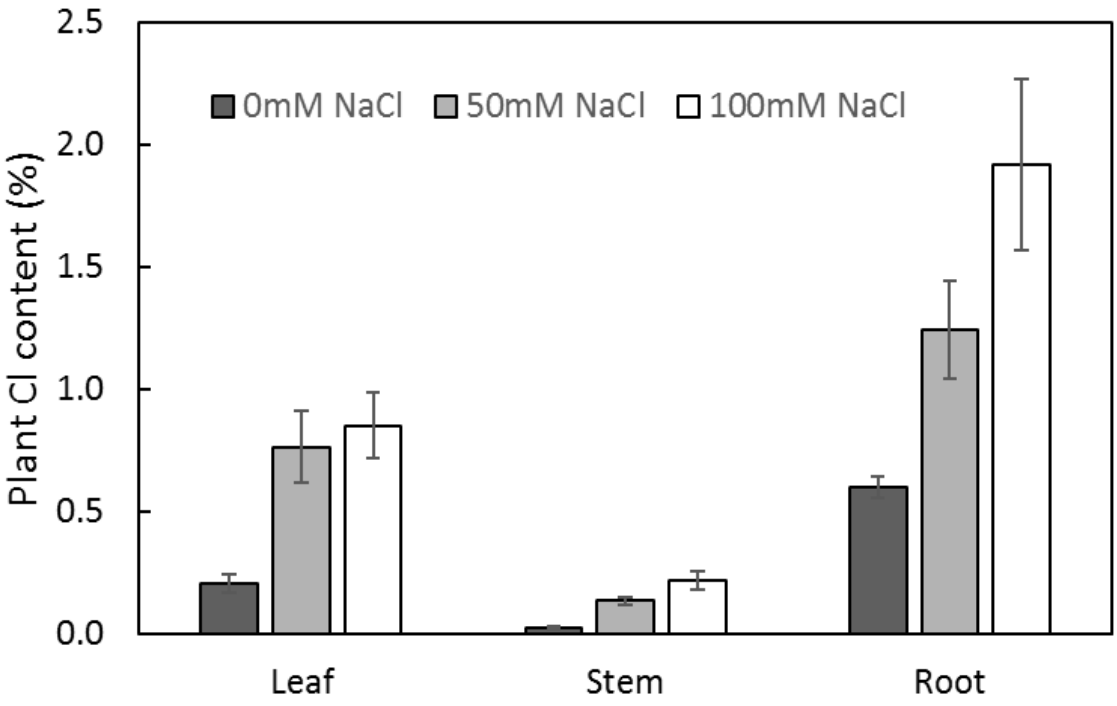


Figure 4.14: Plant chloride (Cl^-) content (% of plant tissue) of *Elaeagnus commutata* (wolf willow) leaf, stem and root tissues subjected to 0, 50 and 100 mM NaCl in hydroponic systems (mean ± SE, n=4).

Table 4.3 : Ionic content of leaf, stem and root tissues from wolf willow (*Elaeagnus commutata*) seedlings. These seedlings were subjected to salinity (0,50 and 100 mM NaCl) treatments in hydroponic systems for 12 weeks. Data represented Mean ± Standard error. Data analysis was performed using linear regression analysis, p and r^2 values are displayed. (n=4). Significant differences with salinity are in bold.

		0 mM NaCl	50 mM NaCl	100 mM NaCl	P-value	r^2
Leaf	K (%)	1.63±0.10	2.08±0.16	2.07±0.14	0.0561	0.318
	Ca (%)	**1.10±0.06**	**0.93±0.03**	**0.69±0.10**	**0.0018**	**0.641**
	Mg (%)	0.44±0.02	0.40±0.02	0.36±0.05	0.1004	0.247
	P (%)	**0.68±0.04**	**0.53±0.01**	**0.39±0.02**	**0.0001**	**0.858**
	Fe (mg kg^{-1})	176±24	662±143	608±204	0.0750	0.283
	Mn (mg kg^{-1})	1289±83	1140±52	960±198	0.0840	0.269
	Cu (mg kg^{-1})	8.65±0.80	12.33±1.48	12.71±1.85	0.0731	0.286
	Zn (mg kg^{-1})	46.3±1.9	61.3±5.7	58.6±9.8	0.2260	0.143
	B (mg kg^{-1})	**87.5±2.9**	**75.6±1.6**	**61.7±3.5**	**0.0001**	**0.823**
Stem	K (%)	**0.91±0.03**	**0.95±0.03**	**1.11±0.06**	**0.0100**	**0.501**
	Ca (%)	0.13±0.00	0.13±0.00	0.16±0.02	0.1034	0.243
	Mg (%)	**0.07±0.00**	**0.07±0.00**	**0.08±0.01**	**0.0297**	**0.391**
	P (%)	0.28±0.01	0.26±0.01	0.31±0.01	0.1910	0.164
	Fe (mg kg^{-1})	**70.4±3.5**	**106.7±12.9**	**164.7±28.9**	**0.0036**	**0.589**
	Mn (mg kg^{-1})	**84.1±4.76**	**103.1±4.3**	**116.1±12.6**	**0.0170**	**0.450**
	Cu (mg kg^{-1})	9.78±1.61	8.65±0.36	13.55±1.71	0.1119	0.233
	Zn (mg kg^{-1})	**14.0±0.6**	**16.6±0.4**	**30.0±4.4**	**0.0023**	**0.621**
	B (mg kg^{-1})	13.7±0.6	13.4±1.2	12.0±0.52	0.1199	0.224
Root	K (%)	1.62±0.06	1.45±0.08	1.60±0.10	0.9075	0.001
	Ca (%)	0.35±0.05	0.27±0.03	0.31±0.03	0.5790	0.032
	Mg (%)	**0.56±0.05**	**0.29±0.03**	**0.28±0.02**	**0.0025**	**0.616**
	P (%)	**0.98±0.07**	**0.66±0.06**	**0.67±0.04**	**0.0001**	**0.898**
	Fe (mg kg^{-1})	1118±171	965±33	1319±231	0.4261	0.064
	Mn (mg kg^{-1})	**1530±269**	**857±136**	**883±113**	**0.0434**	**0.348**
	Cu (mg kg^{-1})	27.2±1.9	23.8±2.9	32.8±5.7	0.3780	0.078
	Zn (mg kg^{-1})	61.0±9.0	57.2±14.0	79.1±13.6	0.3192	0.099
	B (mg kg^{-1})	**22.8±0.7**	**18.7±1.0**	**17.6±1.1**	**0.0066**	**0.538**

4.2. Effects of NaCl (100 mM) on plant performance and root nitrogen fixation of *E. commutata* seedlings (experiment 2)

4.2.1. Plant biomass and injury

The total biomass of *E. commutata* seedlings in experiment 2 was significantly reduced by NaCl ($p = 0.0075$). Plants subjected to 100 mM NaCl had 72.1% decline in total biomass compared to control plants. The decline in total plant biomass was mainly attributed to the decline in plant shoot biomass ($p = 0.0068$). Plant shoot: root ratio also declined significantly with NaCl treatments ($p = 0.0024$). The proportion of litter to total plant shoot biomass increased drastically in presence of NaCl ($p = 0.0468$), indicating significant increase in plant leaf injury. However, there were large variations in litter proportion values among plants treated with 100 mM NaCl (Table 4.4).

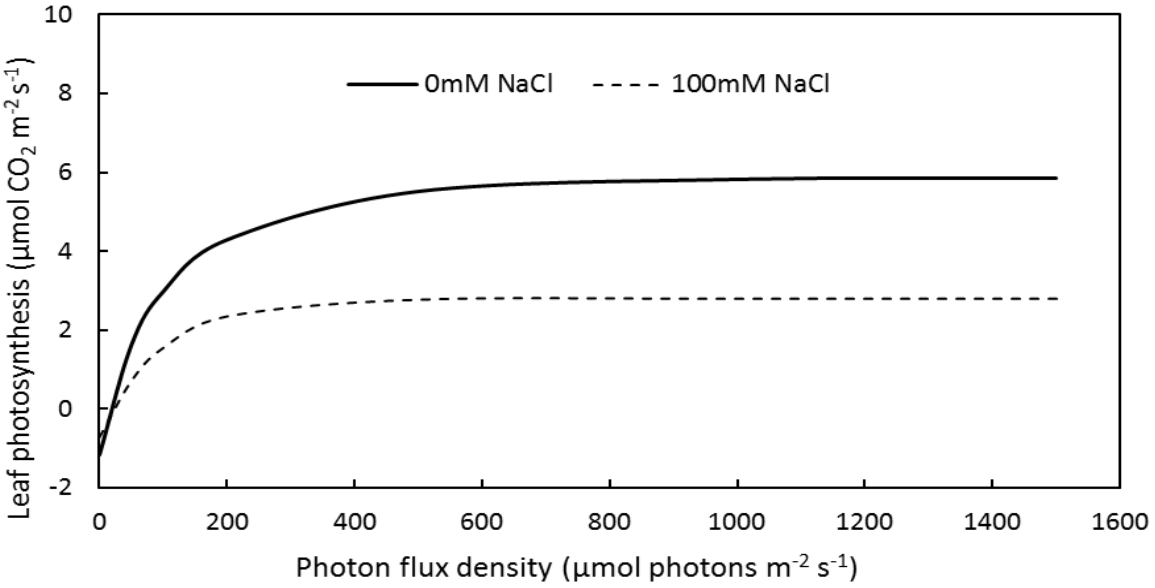
4.2.2. Leaf gas exchange parameters

Simulated light response curves for seedlings exposed to 100 mM NaCl compared to control plants showed marked decline overall in leaf photosynthesis rates across different light photon flux densities (0 to 1500 µmol Photon m^{-2} s^{-1}) in week 8 and week 12 of treatments (Figure 4.15a, b). It was found that 100 mM NaCl led to a decline in plant Pmax (maximum photosynthesis rate; $p = 0.0213$) in week 12, but not in week 8 (Table 4.5). However, irradiance of saturation (IK), and respiration rate (R) were not affected by NaCl in either week 8 or week 12 (Table 4.5). Exposure to 100 mM NaCl also led to declines in leaf transpiration ($p = 0.0308$), and stomatal conductance ($p = 0.0429$) in week 8, 100 mM NaCl treated plants had a 46.9% and 55.2% reduction in leaf transpiration, and stomatal conductance, respectively, compared to control plants (Figure 4.16a, Figure 4.17a). However, in week 12, NaCl did not significantly reduce leaf transpiration or stomatal conductance (Figure 4.16b, Figure 4.17b).

Table 4.4 : Plant biomass parameters (total biomass, shoot biomass, root biomass, shoot:root, litter proportion, and nodule allocation) of wolf willow (*Elaeagnus commutata*) seedlings subjected to control (0 mM NaCl) and salt (100 mM NaCl) treatments in hydroponic systems. Data represented Mean ± Standard error. Data analysis was performed using linear regression analysis, p values are displayed (n=4).

	0 mM NaCl	**100 mM NaCl**	**p-value**
Total biomass	**14.97±2.74**	**4.17±0.89**	**0.0075**
Shoot biomass	**12.15±2.30**	**2.64±0.46**	**0.0068**
Root biomass	2.82±0.46	1.53±0.43	0.0873
Shoot: Root	**4.27±0.44**	**1.87±0.20**	**0.0024**
Litter proportion	**0.03±0.01**	**0.34±0.24**	**0.0468**
Nodule allocation	0.05±0.01	0.04±0.01	0.2589

(a)



(b)

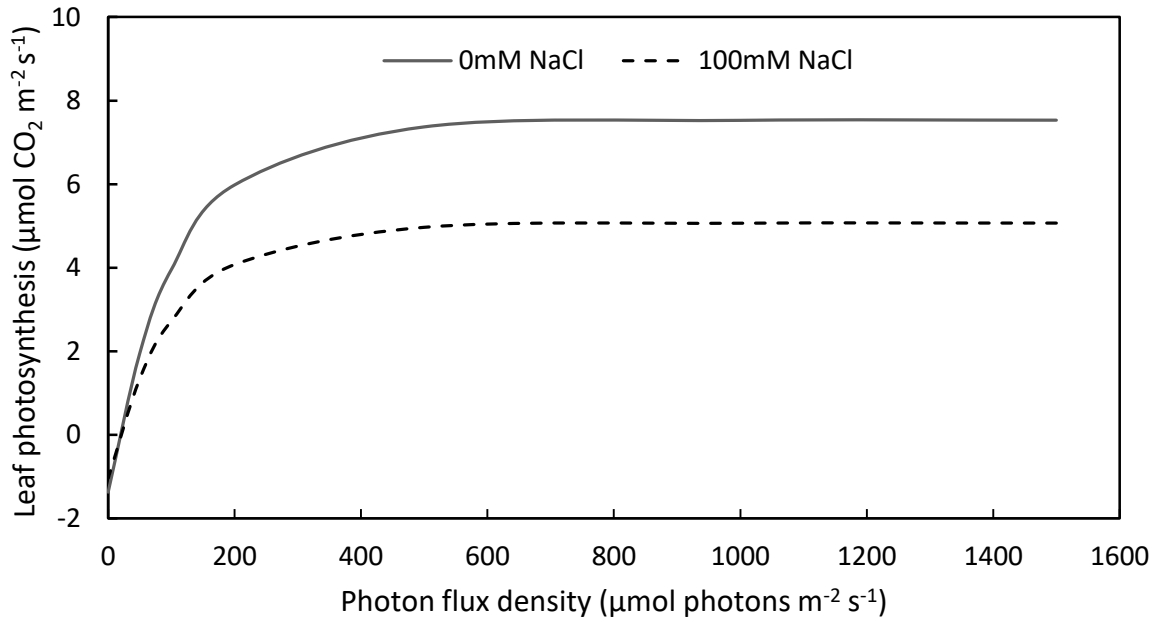
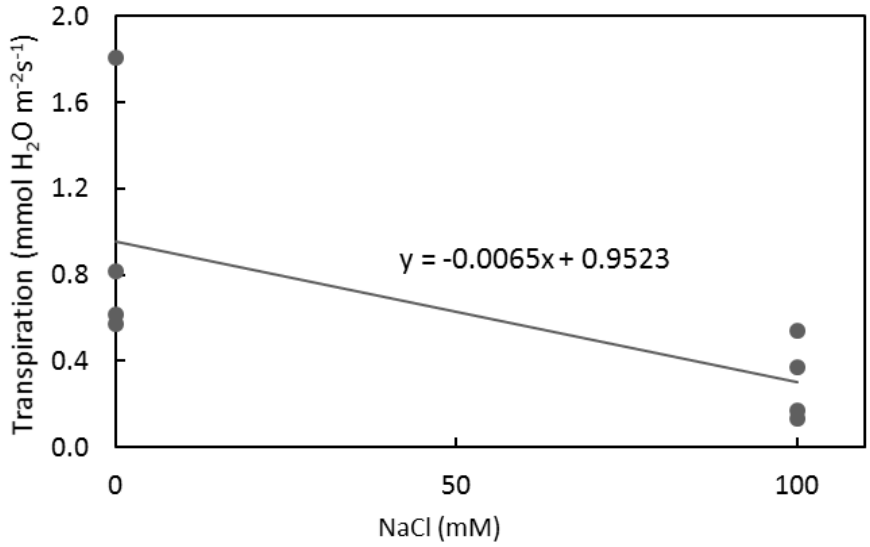


Figure 4.15: Light response curves for *E. commutata* seedlings treated with 0 mM NaCl and 100 mM NaCl. Measurements took place after 8 weeks (a) and 12 weeks (b) of treatments. Simulated light response curves were constructed with a method outlined in equation 2 from Chalker (1981) based on calculated Pmax, IK and R values. Curves displayed represent averaged simulated curves from all replicates (n=4).

Table 4.5 : Photosynthesis light response curve variables (Pmax, IK and R) that were used to construct simulated light response curves calculated for seedlings using week 8 and week 12 photosynthesis measurements. Seedlings were subjected to control (0 mM NaCl) and salt (100 mM NaCl) treatments in hydroponic systems. Data represented Mean ± Standard error. Data analysis was performed using linear regression, p values are displayed (n=4, (*)n=2 for 100 mM NaCl in week 8).

	0 mM NaCl	100 mM NaCl	P-values
		Week 8*	
Pmax ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	7.01±2.13	3.56±1.28	0.3520
IK ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$/ μmol photons $m^{-2}\ s^{-1}$)	106.38±39.74	100.51±10.24	0.9266
R ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	1.15±0.22	0.75±0.30	0.3509
		Week 12	
Pmax ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	**8.91±0.80**	**6.10±0.43**	**0.0213**
IK ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$/ μmol photons $m^{-2}\ s^{-1}$)	109.97±19.08	112.50±20.72	0.9312
R ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	1.37±0.15	1.04±0.14	0.1567

(a).



(b)*.

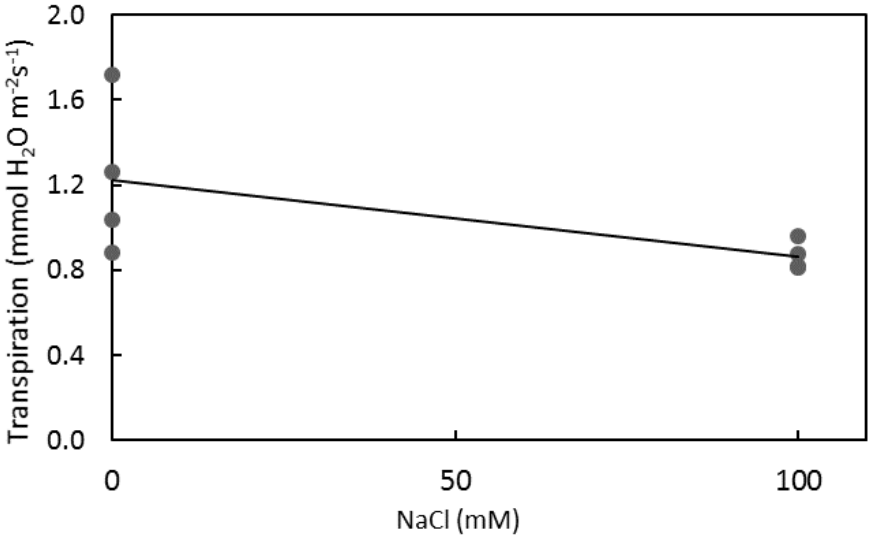
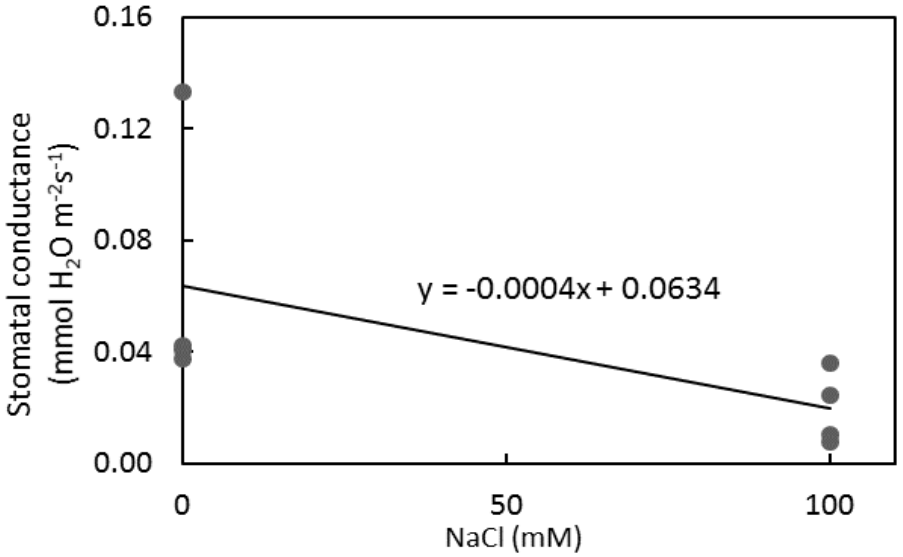


Figure 4.16: Leaf transpiration rates for *E. commutata* seedlings treated with control (0 mM NaCl) and salt treatment (100 mM NaCl) measured at light intensity of 500 µmol photons m^{-2} s^{-1}. Measurements took place after 8 weeks (a) and 12 weeks (b) of treatments. Data was log transformed (n=4). Least square fit lines were displayed. (*) In week 12, NaCl treatment led to no significant change in leaf transpiration rates (p=0.0753).

(a).



(b)*.

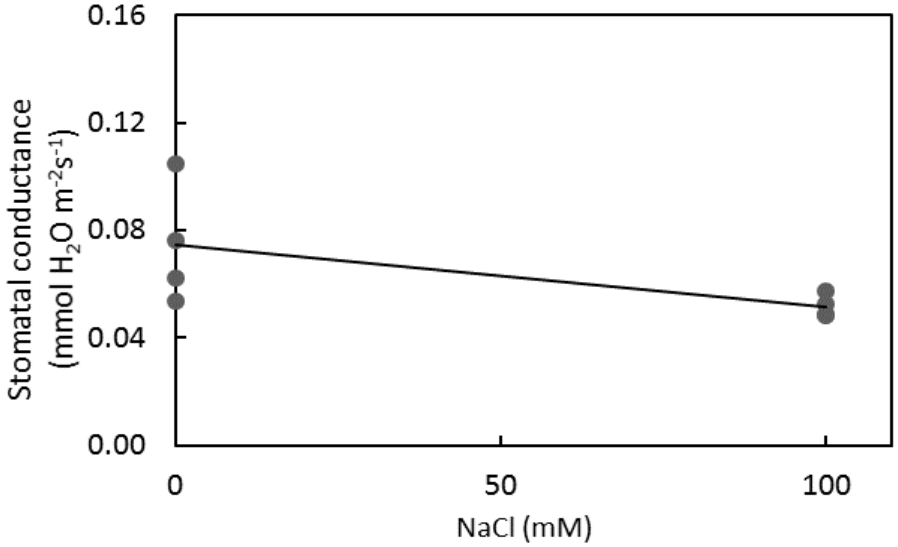


Figure 4.17: Leaf stomatal conductance for *E. commutata* seedlings treated with control (0 mM NaCl) and salt treatment (100 mM NaCl) measured at light intensity of 500 µmol photons m^{-2} s^{-1}. Measurements took place after 8 weeks (a) and 12 weeks (b) of NaCl treatments. Linear regression was used for statistical analysis (data was log transformed, n=4). Least square fit lines were displayed. (*) In week 12, salt treatment led to no significant difference in stomatal conductance (p=0.069).

4.2.3. Root nitrogen fixation

For whole plant nitrogen fixation rates, it was found that although plants subjected to 100 mM NaCl were still actively fixing nitrogen, their fixation rates were drastically reduced in comparison to control plants ($p = 0.0056$). 100 mM NaCl exposed plants had root nitrogen fixation reduced by 98% compared to control plants. In addition, nitrogen fixation per root nodule mass, and nitrogen fixation per plant biomass were reduced by 92% and 95%, respectively, for 100 mM NaCl treated plants in comparison to control plants (Table 4.6). These indicated that exposure to 100 mM NaCl significantly reduces nitrogen fixation in *E. commutata* seedlings.

4.2.4. Root nodule infection

Harvested plant nodules were sectioned, stained and observed under light microscope. Bacterial infected cells (filled with nitrogen fixing bacterial vesicles) were stained blue with Fabils stain. Root nodules of control plants (Figure 4.18a, b) had higher numbers of actively nitrogen fixing cells (stained blue, as evident in the microscopy images). In comparison, nodules from salt treated plants (Figure 4.18c, d) had much lower numbers of nitrogen fixing cells. These observations indicated that 100 mM NaCl seemed to reduce the functionality of *E. commutata* nodules.

Table 4.6: Whole plant root nitrogen fixations (measured by ethylene production), nitrogen fixation per nodule mass, and nitrogen fixation per plant biomass for *E. commutata* seedlings treated with 0 mM NaCl, or 100 mM NaCl in hydroponic systems. Data represent average ethylene production per hour (Mean ± SE; n=4).

	0 mM NaCl	100 mM NaCl
Ethylene production (µmol/hr）	26.02 ± 6.06	0.47 ± 0.32
Ethylene production per nodule mass (µmol/hr/g nodule)	33.75±2.65	2.78±1.25
Ethylene production per plant mass (µmol/hr/g plant)	1.72±0.29	0.09±0.04

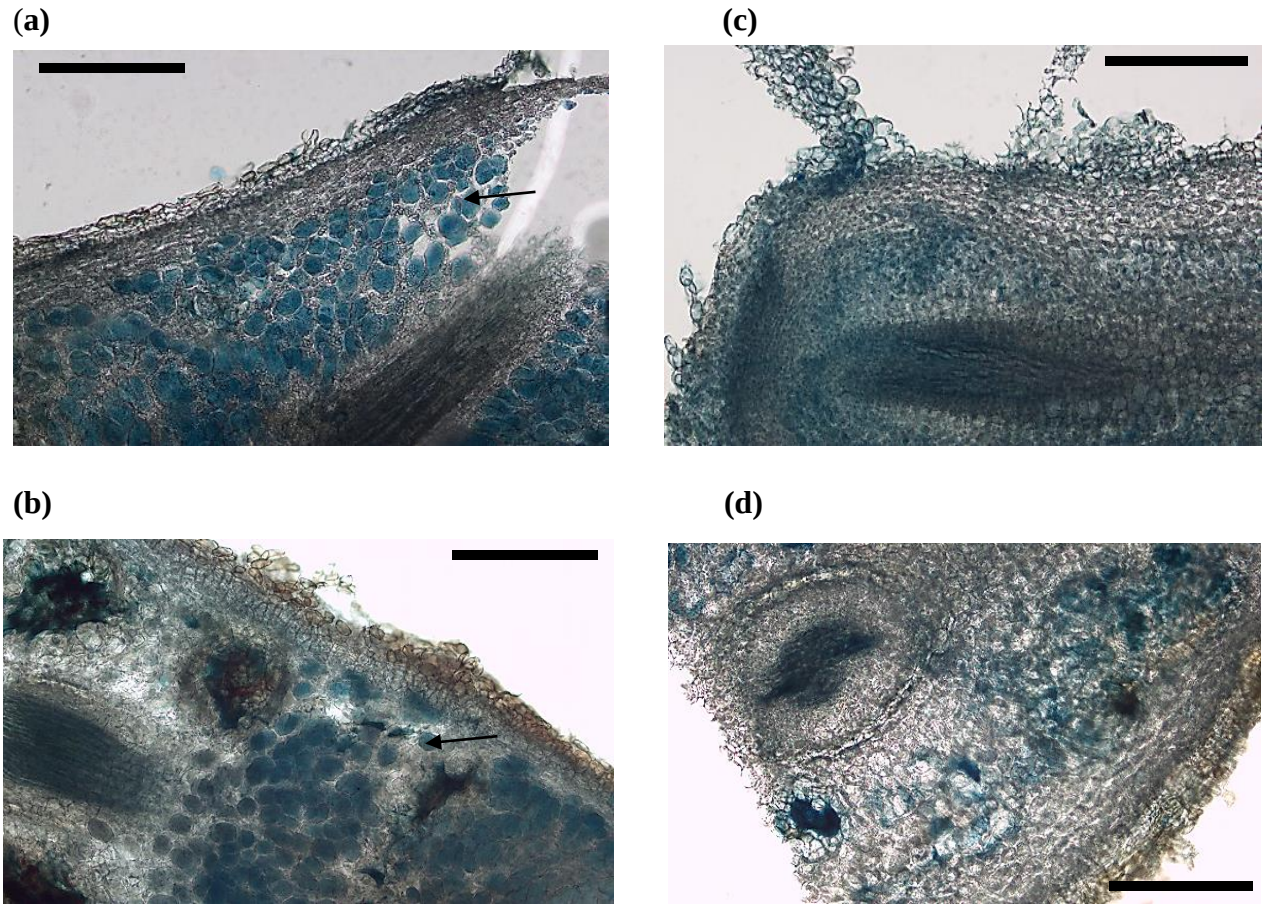


Figure 4.18: Root nodule hand sections stained with Fabil stain (aniline blue 0.5%, basic fuchsin 0.5% and 3g of iodine and potassium iodide in lactophenol). The photos were taken under viewing with light microscope under 10X objective lens (total magnification 100X). Root nodules were from plants that were subjected to either 0 mM NaCl (a and b) or 100 mM NaCl (c and d) for 12 weeks. Arrows are pointed to blue stained cells, which contain bacterial vesicles where nitrogen fixation takes place. Scale bars (a – d) = 260 µm.

4.3. Effects of high levels of NaCl on the survival of *E. commutata* cuttings (experiment 3)

At end of the 16 weeks of NaCl exposure, the proportions of cuttings still alive were 100%, 66.7%, 33.3% and 44.4% for cuttings receiving 0, 2, 4, and 6 applications of 100 mM NaCl (each applied with NaCl mixed in 100 ml diluted nutrient solution), respectively. Although the survival rates declined with increased levels of NaCl, even at the highest level of NaCl, several *E. commutata* cuttings remained alive after being exposed to the salt treatment for 16 weeks (Figure 4.19).

It was found that the estimated proportion of injured leaves (as a % of total leaf area) significantly increased with higher NaCl ($p = 0.0001$) at the end of the 16 weeks of salt exposure. The average leaf injury for cuttings subjected to 6 applications of 100 mM NaCl was more than 6 times higher compared to that of control plants (Figure 4.20).

Interestingly, there were no significant differences in plant relative height growth (as a percentage of their initial height) for *E. commutata* cuttings subjected to different NaCl treatments between week 1 and week 16 of the experiment ($p = 0.1374$; Figure 4.21). Log transformation of the data was attempted for statistical analysis, however, this did not change the outcomes of the analysis.

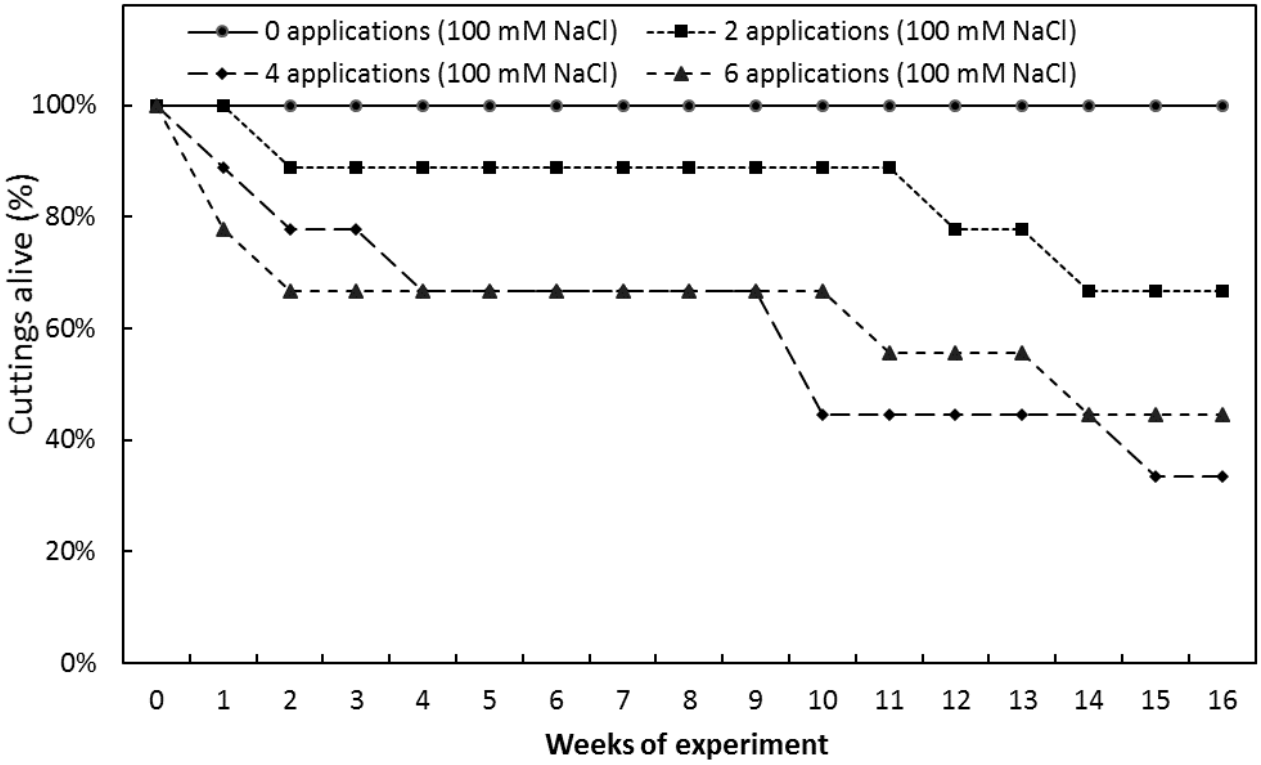


Figure 4.19: Proportion (%) of *E. commutata* cuttings that remained alive when subjected to 0, 2, 4 and 6 applications of 100 mM NaCl for a duration of 16 weeks (n=9). Week 1 represent the time which the highest salinity level (6 applications of 100 mM NaCl) was reached. 2 X (100 mM NaCl) and 4 X (100 mM NaCl) treated plants would have already been under assigned NaCl levels for 1 and 2 weeks, respectively, at "week 1".

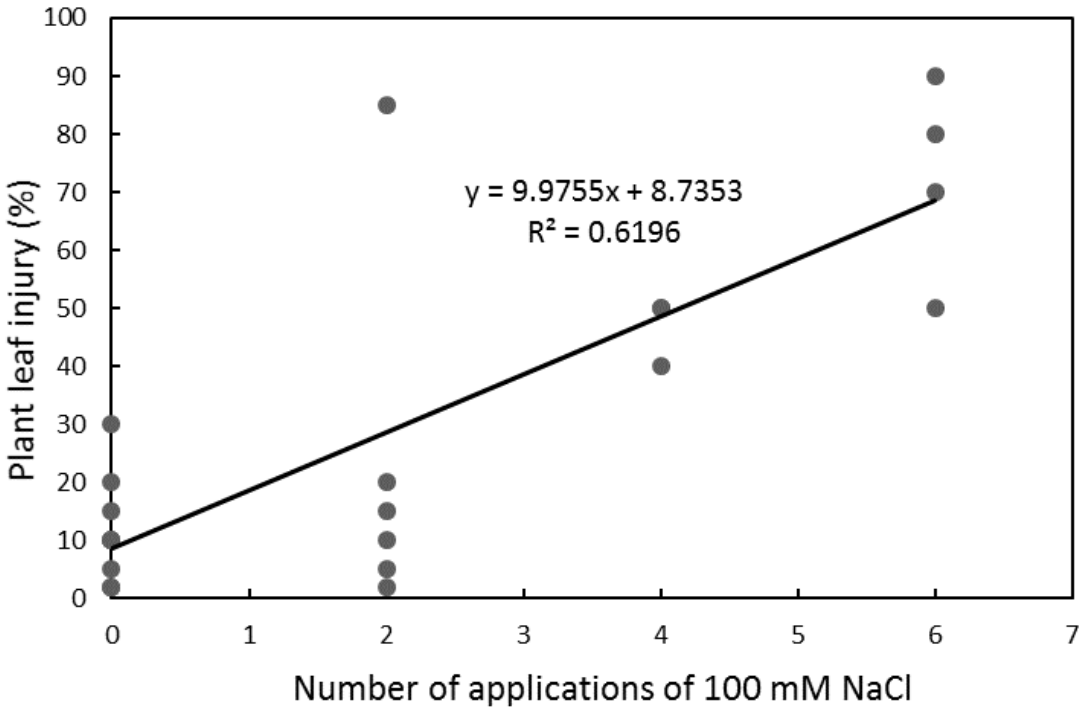


Figure 4.20: Estimated plant leaf injury (% of all leaves) for *E. commutata* cuttings at the end of treatments, dead plants were excluded from the analysis (n=9 for control plants, n=6 for 2 applications of 100 mM NaCl, n=3 for 4 applications of 100 mM NaCl, and n=4 for 6 applications of 100 mM NaCl).

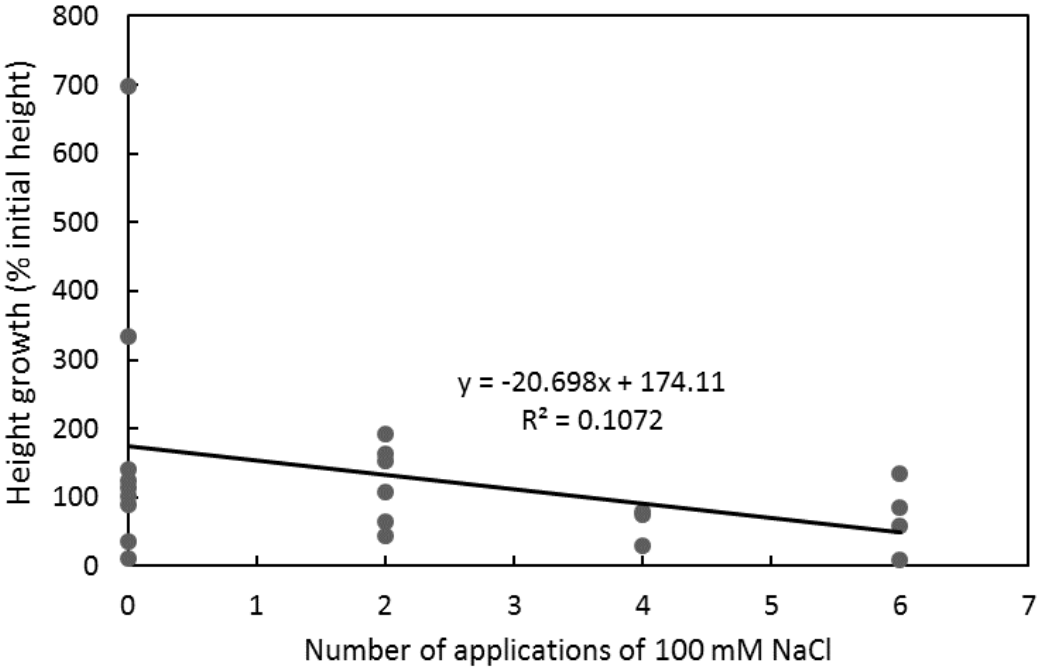


Figure 4.21: Plant height growth (calculated as increase in height as a % of initial height) for *E. commutata* cuttings exposed to different levels of salinity (0, 2, 4, and 6 applications of 100 mM NaCl) for 16 weeks. Dead cuttings at the end of treatments were excluded (n=9 for control, n=6 for 2 applications of 100 mM NaCl, n=3 for 4 applications of 100 mM NaCl, and n=4 for 6 applications of 100 mM NaCl).

CHAPTER 5. DISCUSSION

5.1. Effects of NaCl on the survival, growth and physiology of *E. commutata* seedlings (experiment 1)

In my first hydroponics experiment, *E. commutata* seedlings were subjected to salinity treatments (0, 50 and 100 mM NaCl) and all seedlings survived in all treatments for 12 weeks. This result indicated that *E. commutata* seedlings can sustain growth even under the exposure to 100 mM NaCl. This is in line with studies which have shown that other *Elaeagnus* species can survive under 200 to 300 mM NaCl (Devecchi and Remotti 2004; Maimaiti et al. 2014). This is also consistent with several studies conducted on the salt tolerance of other actinorhizal plants around the world, which show promising land reclamation potential for some species due to their ability to survive in saline environments. These species include *Shepherdia argentea*, a native actinorhizal shrub found within the *Elaeagnaceae* family (the same family that *E. commutata* belongs to), as well as several species within genus *Casuarina* (Ng 1987; Tani and Sasakawa 2003; Qin et al 2010a, b).

In order to examine the suitability for *E. commutata* to be used as a reclamation species for saline disturbed habitats, it is important to look at how root nodulation and nitrogen fixation rates are affected by salinity. In experiment 1, the nodulation rates were extremely low (Table 4.1). This low nodulation success was likely the reason for which the inoculation with *Frankia* did not significantly affect any of the plant growth and physiological parameters that were measured. Considering the fact that the other seedlings, which I inoculated and maintained in pots (to be transplanted and used for experiment 2) nodulated fairly well, and that those seedlings did not receive as much nutrient as the seedlings used in experiment 1, there is reason to believe that the low nodulation rates may be due to the relatively high availability of nitrogen (2 mM NO_3^-) to the seedlings in the nutrient solution that may have inhibited root nodulation. Kohls and

Baker (1989) found that at NO_3^- levels higher than 2.5 mM, nodulation of actinorhizal plants such as *Alnus glutinosa* and *Casuarina cunninghamiana* were completely inhibited, though inhibition of nodulation did not occur for *Elaeagnus angustifolia* with 2.5 to 3 mM NO_3^-. For my experiment, it is possible that direct access to nutrient solution further increased nitrogen availability to the plants, thus inhibiting root nodulation. The inhibition of nodulation could potentially occur through the reduction of root flavonoid production. In the legume-Rhizobium symbiosis, flavonoids release by roots are known to act as chemo-attractants for suitable rhizobia to trigger bacterial movement to plant root. In addition, flavonoids also play important roles in activation and regulation of nod (nodulation) genes expression in legumes (Abdel-Lateif et al. 2012). However, currently, the function of flavonoids in actinorhizal-*Frankia* associations is not well characterized.

Although result from experiment 1 suggested that *E. commutata* can survive up to 100 mM NaCl even without nitrogen fixation, this does not address the question of how nitrogen fixation in *E. commutata* is impacted by salinity. As a result, a second hydroponic experiment (experiment 2) was conducted to further investigate nitrogen fixation of *E. commutata* under salinity (this will be elaborated in section 5.2).

The physiological parameters of *E. commutata* seedlings (photosynthesis, transpiration, stomatal conductance, leaf water potential and relative water content) were monitored throughout the 12 weeks of salinity treatments, as these parameters are important indicators of plant growth performance and levels of stress. The seedlings went through high levels of growth in initial weeks and then the growth rates slowed down toward the end of the treatment, likely due to limitation of space in the hydroponic systems. This is reflected by the decline in physiological parameters even for control plants as time progressed. Repeated measure analyses also

confirmed this suggesting that time course (weeks of treatment) had significant effects on all measured physiological parameters.

For plant leaf photosynthesis, a 26% decline with salinity was found toward the beginning of the salt treatments (week 1) and then a 27% reduction toward the end of the salinity treatments (week 12). However, during the mid-treatment weeks (weeks 4 and 8), leaf photosynthesis was not significantly reduced with salinity (Figure 4.1). Salinity stress is known to lead to osmotic and ionic stress, both of which can result in reduction in plant photosynthesis. For osmotic stress, the reduction of water uptake as a result of high soil salinity (low soil water potential) can lead to closure of stomata as an adaptation for water conservation, which is important for plant maintenance of turgor pressure and plant nutrient transport (Yeo et al. 1991; Vicente et al. 2004). As a result of stomatal closure, gas exchange on leaf surface can be severely reduced which in turn leads to reduction in plant leaf photosynthesis (Lawlor and Cornic 2002). In addition, salt induced ionic stress can also potentially reduce plant leaf photosynthesis. Ionic stress is caused by the accumulation of high levels of Na^+ and Cl^- ions in plant leaf tissues. The presence of high levels of Na^+ can disrupt numerous biochemical processes by replacing K^+ for enzyme activation (Gupta and Huang 2014). High levels of Na^- and Cl^- may also lead to changes in subcellular protein structures by interfering with amino acid interactions (Chinnusamy et al. 2005). In addition, Na^+ interaction with Ca^{2+} can potentially lead to changes in membrane integrity and stability (Tavakkoli et al. 2010).

Osmotic stress tends to have more immediate impacts on plants in comparison to ionic stress, which is more pronounced overtime with the accumulation of Na^+, Cl^-. Munns (2002) described a “two phases growth responses” pattern commonly observed in certain wheat (*Triticum aestivum*) and maize (*Zea mays*) cultivars, which included an immediate phase of

growth reduction as a result of osmotic stress, and then a second phase of growth reduction occurring much later as a result of excessive accumulation of salt. This could apply to my seedlings as well, the decline in leaf photosynthesis at the beginning of the treatment could be mainly due to osmotic stress. After four weeks of treatments these plants may have started to recover by utilizing tolerance mechanisms such as osmoregulation, resulting in higher water uptake and the re-opening of stomata, this could lead to recovery in photosynthesis as well. The decline in photosynthesis toward the end of the treatments (week 12) could be explained, at least in part, by the accumulation of Na^+ and Cl^- in shoot tissues leading to ionic damage, my results from tissue elemental analyses also indicated increased leaf and stem Na^+ and Cl^- content. In order to separate the effects of osmotic stress from ionic stress, experiments would need to be designed so that plants are subjected to NaCl solutions and water solutions with the same water potentials (with non toxic compounds such as Polyethylene Glycol) (Demir and Mavi 2008).

My results have shown that at the beginning of the treatments (week 1), both leaf stomatal conductance and leaf transpiration were reduced with salinity. This is likely caused by osmotic stress with higher salinity reducing the amount of water uptake, which then causes the reduction in leaf transpiration and stomatal conductance due to stomatal closure, as discussed previously. Interestingly, as time progressed (week 4 and week 8), leaf stomatal conductance and transpiration were still reduced by salinity, unlike the case for photosynthesis, where there were strong signs of recovery from osmotic stress. However, a recovery trend was observed as the reduction in leaf transpiration and stomatal conductance was less in week 4 and week 8 compared to week 1 (Figure 4.2, 4.3). This somewhat supports the idea that plants recovered to a certain extent from osmotic stress (it varies depending on the physiological mechanisms) as time progressed to be able to sustain a relatively high level of stomatal gas exchange and leaf

transpiration under salinity (100 mM NaCl). However, at week 12, leaf transpiration and stomatal conductance for salt treated seedlings went through large declines again compared to control plants.

The results for plant leaf relative water content and leaf water potential have shown that plant water status changes with exposure to higher levels of NaCl (Figure 4.5, 4.6). As suggested by Maimaiti et al. (2014), the significant reduction in leaf relative water content showed that the lower soil water potential and the increase in plant salt uptake collectively led to higher levels of dehydration in plant leaf tissues. This also explains the reduction in leaf transpiration and stomatal conductance since closure of stomata was required to prevent further evaporative water loss. The reduction of leaf water potential with salinity in week 4 through week 12 suggested that *E. commutata* seedlings goes through osmotic adjustment when exposed to NaCl. This adjustment, which lowers plant osmotic potential helps to increase water uptake and reduce water loss. This is a commonly described salt tolerance mechanism found in a variety of plant species (Hasegawa et al. 2000; Khan et al. 2000; Qin et al 2010a). In addition, the delayed reduction in leaf water potential (week 4 to week 12) also suggests that the solute accumulation in the seedlings increased over time, which could explain the delayed effects of ionic toxicity on the physiological parameters in *E. commutata*.

Like in many other plants, the osmotic adjustment in *E. commutata* seedlings was at least partly accomplished through the accumulation of Na^+ and Cl^- in plant root and shoot tissues (Koyro 2006; Qin et al.2010a). This helps to lower plant water potential to achieve osmotic balance with the external environment, alleviating salinity induced osmotic stress. The uptake of Na^+ and Cl^- starts at the level of plant roots, through non-specific ion channels, and K^+ transporters (Hasegawa et al. 2000; Flowers and Colmer 2008). In addition, some plants also

synthesize compatible solutes, such as glycine betaine, proline, sorbitol, mannitol and sucrose in plant cell cytoplasm to achieve osmotic adjustment, although these organic solutes do not lead to toxicity damage in cell cytoplasm like Na^+ and Cl^-, the production of compatible solutes for osmoregulation can be an energy costly process (Munns 2002; Munns and Tester 2008; Flowers and Colmer 2008). Proline is important because it can also act as free radical scavengers to alleviate salinity induced oxidative stress, as well as acting as general osmoprotectant which helps to stabilize cellular structures such as membranes and proteins (Chinnusamy et al. 2005). My results showed that *E. commutata* seedlings subjected to NaCl did not show any increase in leaf proline content. This result is in contrast to many studies which indicated that plant production of proline increase with exposure to salinity (Tani and Sasakawa 2006; Nazarbeygi et al. 2011; Maimaiti et al. 2014). It is possible that *E. commutata* synthesizes other compatible solutes instead of proline. The high energy cost associated with synthesizing organic solutes may also be a factor preventing *E. commutata* from utilizing proline for osmotic adjustment (Chinnusamy et al. 2005).

Leaf chlorophyll fluorescence, a measurement of the maximum quantum efficiency (Fv/Fm) of photosynthesis is a widely used parameter that is indicative of stress induced photo inhibition and damages to the photosystem II (PSII) (Maimaiti et al. 2014). Leaf chlorophyll fluorescence measurements did not show any differences between salinity treatments with the exception of week 12, where salinity led to a slight increase in maximum quantum yield (Fv/Fm) (Figure 4.4). However, the Fv/Fm ratios measured across plants subjected to different NaCl treatments through the 12 weeks were not low enough to indicate stress, as it was defined by Ritchie (2006). Although studies have shown that some plant species have exhibited declined Fv/Fm ratios when exposed salinity at 70 to 100 mM NaCl (Ranjbarfordoei et al. 2006). For salt

tolerant halophytes such as *Cakile maritima*, maximum quantum efficiency actually increased slightly at 100 and 200 mM NaCl, these levels of salinity coincide with the optimal growth salinity range for this plant (Debez et al. 2008). Maimaiti et al. (2014) has also found that for *Elaeagnus angustifolia*, an actinorhizal shrub found within the same genus as *E. commutata*, the maximum quantum yield remained constant for plants exposed to 200 mM NaCl. This is a clear indication that the photosynthetic machinery of *E. commutata* is fairly tolerant of salinity-induced ionic stress. This finding suggests that the decline in leaf photosynthesis under salinity for *E. commutata* seedlings was not due to ionic damage to photosystem II. However, Na^+ in plant tissues can still displace K^+, thereby disrupting enzyme reactions, as well as regulation of stomatal opening (Gupta and Huang 2014). Na^+ could also displace Ca^{2+} on membranes, thereby altering membrane stability (Tavakkoli et al. 2010). These could also result in the decline in photosynthesis.

In my experiment salinity led to an increase in both Chlorophyll a and b pigments in *E. commutata* seedlings. The fact that chlorophyll pigment reduction did not occur at 100 mM NaCl is an indication of the relative salt tolerance of *E. commutata* physiological processes. Other studies on salt tolerant crop species like Pearl millet (*Pennisetum glaucum*) and wheat (*Triticum aestivum*), have also reported increases in chlorophyll a and b pigment with salinity at concentrations up to 160 mM NaCl (Reddy and Vora 1986; Hamada 1996). Studies on plants with low salt tolerance typically reveal a decline in chlorophyll pigment with salinity crop plants; examples include crops such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*) and pea (*Pisum sativum*) (Sudhur and Murthy 2004), as well as actinorhizal plants such as *Shepherdia argentea* (Qin et al. 2010a).

In spite of the relative salt tolerance of the physiological processes, exposure to 100 mM NaCl led to moderate declines in overall plant biomass (34%), plant height (31%) and stem diameter (18%). The reduction in plant shoot and root biomass under salinity was at comparable levels, resulting in no change in the plant shoot: root ratio. Salinity induced growth reduction is one of the defining characters of salinity stress, with the exception of some halophytes, which can sustain high levels of growth at up to 200 mM of NaCl (Yeo and Flowers 1980). The decline in plant growth with salinity is very widely documented across different plants, as demonstrated by various studies, including several on actinorhizal plants (Tani and Sasakawa 2003; Gama et al. 2007; Koyro 2006; Renault 2012; Maimaiti et al. 2014). The decline in plant biomass, stunted height and reduced stem width is mainly attributed to a decline in plant photosynthetic rate, as a result of both osmotic stress and ionic toxicity (as previously discussed), which results in water deficiency, decreased gas exchange (stomatal closure), and damage to components of the photosynthetic pathway. In addition, salinity induced production of reactive oxygen species (ROS) can lead to oxidative damage to membrane protein and lipids, contributing to the decline in photosynthesis (Chinnusamy et al. 2005). In some plants, salinity can result in the reduction of K^+ and Ca^{2+} uptake, resulting in disruption of Ca^{2+} binding to plasma membranes, cell signal transduction, and also stomatal activity (due to Na^+ replacement of K^+), all these changes can contribute to further reduction in plant growth (Rengel 1992; Hasegawa et al. 2000; Debez et al. 2008). However, my results have shown that in *E. commutata*, salinity did not seem to lead to any major changes in nutritional balance (to be further discussed). Despite the reduction in plant growth, it should be noted that even under 100 mM NaCl, *E. commutata* seedlings still sustained relatively high levels of growth (retaining 66% of the biomass of the control plants) in the span of the 12 weeks of salt treatments. Furthermore, as pointed out by Koyro (2006), the decline in

plant growth can be interpreted as a coping mechanism to prevent the excessive loading and transport of NaCl to plant shoot tissues to avoid tissue damage. Therefore, the growth reduction may not be entirely due to the negative outcomes of salinity stress.

One of the more intriguing findings from my experiment is how plant biomass distribution in *E. commutata* seedlings changed with increasing salinity levels. The reduction in plant biomass was mainly attributed to reduction in leaf and stem biomass in the main stem of the seedlings, resulting in an increasing proportion of lateral branch biomass (Table 4.2). The increase in plant branching and lateral development with salinity is not documented in other plants. This finding provides some interesting topics for future research.

Salinity did not lead to any increase in leaf injury when I observed the leaves remaining on the plants after 12 weeks of treatment. However, when plant litter (shed leaves) was taken into account, seedlings exposed to salinity did have higher leaf injury compared to control plants (Figure 4.7). Salinity induced leaf injury is widely documented. Leaf scorching, leaf senescence, meristematic damage, and leaf necrosis are some of the common leaf injury symptoms as a result of salt-induced ionic stress (Shannon and Grieve 1999; Munns and Tester 2008). Compared to the closely related *Elaeagnus angustifolia*, it seems that *E. commutata* is more prone to leaf injury, and possibly more sensitive to ionic damage to leaf tissues, as *E. angustifolia* seedlings could sustain in 200 mM NaCl conditions for 30 days without showing leaf injury symptoms (Maimaiti et al. 2014). However, since a large portion of leaf injury in *E. commutata* seedlings were counted as shed leaves, this showed that perhaps *E. commutata* seedlings utilizes the shedding of old leaves (which may have accumulated more NaCl in comparison to younger leaves) as an ion exclusion mechanism to ensure the functionality of newer produced leaves with

reduced ionic toxicity as observed in some plant species such as *Avicennia germinans* (black mangrove) (Suarez and Medina 2005; Aslam et al. 2011).

My results show that in all parts of *E. commutata* tissues (root, stem and leaf), the levels of Na^+ and Cl^- increased significantly with increasing levels of NaCl. Interestingly, plant root tissues accumulated much higher concentrations of Na^+ and Cl^- at both 50 and 100 mM NaCl, in comparison to leaf and stem tissues. This shows that ionic exclusion from plant shoot is occurring in *E. commutata* as the storage of Na^+ and Cl^- ions mostly occurs in the roots. Studies have shown that in some plants, salt can be stored mostly in the root to limit damage to plant shoot tissues where photosynthesis takes place. This is thought to occur through selective transport to shoot tissues at the root level favouring essential nutrients, such as K^+, instead of Na^+ (Munns 2002). Ion exclusion from shoot tissues is widely documented in plant species such as *Chenopodium album*, *Chenopodium schraderianum* and *Cornus Sericea* (Renault 2004, 2012; Reimann 1992).

In comparison to synthesizing compatible solutes to achieve such purposes, utilizing Na^+ and Cl^- ions for osmotic adjustment is a more energy efficient process (Munns 2002). However, accumulation of high concentrations of Na^+ and Cl^- ions in plant shoot tissues can also lead to ionic toxicity. The presence of high levels of Na^+ in plant leaf tissues is problematic in many different ways, including replacing K^+ ions for the activation of many enzymes, leading to inefficiency in plant biochemical processes, as well as potentially altering subcellular protein structures, disturbing cellular functions (Chinnusamy et al. 2005). In addition, the presence of high levels of Na^+ and Cl^- are known to lead to nutritional imbalance, competing with the uptake of plant essential nutrients, such as K^+, Ca^{2+} and NO_3^-, which play important roles in plant growth and metabolism (Liu and Shelp 1995; Hasegawa et al. 2000; Gupta and Huang 2014).

My results have shown that the K content of *E. commutata* seedlings did not change with NaCl (at 50 and 100 mM NaCl) in plant leaf, stem or root tissues (Table 4.3). This is an important finding because maintaining a relatively stable cellular K^+/Na^+ ratio is crucial for the plant growth and survival under salinity stress, since K^+ plays a role in activation of enzymes in many plant metabolic activities, and as previously mentioned, Na^+ could replace K^+ causing disruptions of plant metabolism (Gupta and Huang 2014). Reduction in plant tissue K content under salinity is commonly found in crop plants such as faba bean (*Vicia faba*) and barley (*Hordeum vulgare*) (Tavakkoli et al. 2010, 2011), and even in the salt tolerant actinorhizal shrub *Shepherdia argentea*. On the other hand, my results for *E. commutata* are in agreement with findings from the closely related shrub *E. angustifolia*, and the highly salt tolerance halophyte *Plantago coronopus*, in which cases salinity did not lead to changes in plant tissue K content (Koyro 2006; Maimaiti et al. 2014). Although plant tissue Ca content declined in the leaf tissues, it remained consistent in stem and root tissues across different NaCl levels compared to control plants. A significant reduction in plant tissue Ca content could lead to change in plasma membrane stability due to Na^+ and Ca^{2+} interaction (Tavakkoli 2010), as well as disruptions in plant metabolic processes due to the roles Ca^{2+} plays in signal transduction pathways (Rengel 1992).

The presence of high levels of Cl^- ions could competitively limit the uptake and the transport of NO_3^-, thereby limiting plant tissue nitrogen content. Studies have found that under relatively high levels of NaCl, the accumulation of Cl^- ions could displace NO_3^- in plants like pea (*Pisum sativum*) and avocado (*Persea americana*) (Bar et al. 1997; Frechilla et al. 2001). Interestingly, my results contrasted these finding, and showed that in *E. commutata* seedlings, nitrogen content in plant leaf and root tissues increased significantly at 100 mM NaCl in

comparison to control plants. This result is encouraging because it can be concluded that under 100 mM NaCl, plant nitrogen content does not decrease as a result of the competitive uptake of NaCl. This is essential because nitrogen is a crucial part of plant growth and development, as nitrogen is a key component in plant chlorophyll pigments and amino acids. Nitrogen fixing plants are often preferred for land reclamation specifically due their ability to supply nitrogen in relatively nitrogen poor environments. As previously mentioned, my *E. commutata* seedlings were poorly nodulated in this experiment likely due to the relative high availability of NO_3^- in the nutrient solution. However, this does show that even in the absence of nitrogen fixation, *E. commutata* seedlings can survive under at least 100 mM NaCl, partly due to their ability to uptake nitrogen that is available to them. The increase in tissue nitrogen content with exposure to salinity is a trend that is not well documented in other species. My results have shown that the increase in plant tissue nitrogen content is not due to increase in proline, or total protein in plant tissues, as concentration for proline and total protein in plant tissues remained unchanged with salinity. It is possible, however, that *E. commutata* seedlings synthesize a large amount of nitrogenous compounds such as glycine betaine, and other quaternary ammonium compounds to be utilized as compatible solutes for osmotic adjustment as they are exposed to higher levels of NaCl, this, of course, remains to be tested (Flowers and Colmer 2008).

These results on plant tissues K, Ca and N content suggest that salinity induced nutritional imbalance does not seem to be a major problem for *E. commutata* seedlings. Despite the higher concentration of Na^+ and Cl^- found in all tissues with higher salinity, it seems that these seedlings are able to selectively uptake and transport essential nutrients to ensure high levels of plant metabolic functions. This characteristic reflects positively on the salt tolerance of *E. commutata*.

5.2. Effects of NaCl (100 mM) on plant performance and root nitrogen fixation of *E. commutata* seedlings (experiment 2)

This second experiment was conducted as a supplementary experiment to experiment 1 to investigate how salinity affects nitrogen fixation in *E. commutata*. From experiment 1, we determined that *E. commutata* can survive under 100 mM NaCl even without nitrogen fixation. However, for the purpose of examining the suitability of *E. commutata* being used for saline land reclamation, it is important to understand how its ability to fix nitrogen changes with salinity, as well as how that may affect the growth and physiology of *E. commutata* seedlings under salinity.

Seedlings exposed to 100 mM NaCl had significant reductions in plant shoot biomass. In this experiment, salinity did not actually lead to reductions in root biomass, resulting in a decrease in the shoot: root ratio (Table 4.4). Typically, a decrease in shoot: root ratio is seen as one of the common morphological adaptations to salinity, as plants may direct more energy toward root expansion for increased uptake of water and essential nutrients (Cheeseman 1988). However, in the case of this experiment, there could be another explanation. These seedlings were older than the ones used in the first experiment and thus had a more established root system when transplanted into hydroponic systems compared to seedlings used in experiment 1. The shoots were cut back due to overgrowth at the time of transplanting, creating an imbalance in shoot: root ratio. Throughout the experiment,100 mM NaCl treated plants had very limited shoot growth, resulting in the seedlings not being able to restore the shoot: root ratio equilibrium. This is evident when looking at results from experiment 1, the shoot: root ratios were at around 4 for seedlings under all treatments. In experiment 2, the shoot: root ratio for control plants was around 4 as well, but for salt treated seedlings, the shoot: root ratio was only 1.87. As a result,

there is no concrete evidence to suggest that the change in shoot: root ratio reflects an adaptation to salinity.

Seedlings exposed to 100 mM NaCl suffered from greater reductions in plant biomass compared to the seedlings in experiment 1. For overall plant biomass, 100 mM NaCl had led to an over 70% reduction in plant biomass, compared to only around 35% for seedlings in experiment 1. This result suggested that in terms plant growth, seedlings in experiment 2 were more vulnerable toward exposure to 100 mM NaCl. The most likely reason for this is that the nutrient solution in experiment 2 consisted of only 0.2 mM NO_3^- compared to the 2 mM in the nutrient solution from the first experiment. This was done to prevent the over abundance of N from inhibiting nitrogen fixation in the seedlings. Therefore, if salinity at 100 mM NaCl significantly reduces the capability of *E. commutata* seedlings to fix nitrogen, then as a result, seedlings would suffer from severely reduced growth due to nitrogen deficiency. This was clearly what occurred in this experiment.

Exposure to 100 mM NaCl led to significant reductions in plant photosynthesis across a range of applied photo light flux density (0 to 1500 µmol photons m^{-2} s^{-1}, Figure 4.15). This was expected and is in agreement with results from experiment 1. When looking at Pmax (maximum phosynthesis), exposure to NaCl led to declined Pmax only at week 12 but not in week 8 of experiment. This result is somewhat in line with the results of experiment 1 and showed that perhaps the decline in photosynthesis in the last week of experiment is the culmination of both the build up of ionic toxicity (due to accumulation of Na^+, Cl^- over time), combined with effects of salt induced osmotic stress.

Seedlings exposed to 100 mM NaCl suffered a 98% decline in whole plant nitrogen fixation compared to control plants. This can lead to significant reduction in the growth of these

plants and this could explain the disparity in growth and biomass reduction between these two experiments. Root nodules exposed to 100 mM NaCl had a lack of *Frankia* infected cells actively fixing nitrogen. Salinity at 100 mM NaCl may have led to a collapse of bacterial vesicles with infected nodule cells where nitrogen fixation typically takes place. Currently, there is a lack of study focusing on how salinity affects nitrogen fixation in actinorhizal plants in general with the exception of a few *Casuarina* species. Ng (1989) found that *Casuarina equisetifolia* can sustain consistent nitrogen fixation rates under 200 mM NaCl, and can nodulate successfully with NaCl below 500 mM NaCl. A similar degree of salt tolerance was described for isolated *Frankia* sp. strains Ceq1 from *C. equisetifolia* and CcI6 from C. *cunninghamiana* growing on culture (Tani and Sasakawa 2003; Oshone et al. 2013). These results are not in agreement with my results, and it suggests that the effects of salinity on nitrogen fixation in actinorhizal plants likely vary significantly between different actinorhizal-*Frankia* associations. Dawson and Gibson (1987) have demonstrated that at 200 mM NaCl, *Frankia* isolates from *Casuarina* did not suffer any reduction in bacterial growth rates, while *Frankia* isolated from *Alnus rubra*, and *Elaeagnus umbellata* (closely related to *E. commutata*), showed significant reduction in growth rates under the same salt level. The differences in salt tolerance of these *Frankia* isolates may be explained by the habitat differences between the actinorhizal plants that they were isolated from. *Casuarina* plants are known to grow in coastal areas close to sea water, as well as near salt swamps, these plants are highly adaptive to saline habitats (Ng 1987). On the other hand, while the native habitat of red alder (*Alnus rubra*) is the pacific northwest coastal region (Markham and Chanway 1998), they tend of grow in coastal inland areas instead of highly saline areas (Dang et al. 1994). The finding of Dawson and Gibson (1987), along with my result

suggests that *Frankia* sp. from non *Casuarina* actinorhizal plants are less salt tolerant compared to their *Casuarina* counterparts.

Although *E. commutata* can survive under 100 mM NaCl in the absence of nitrogen fixation their ability to fix and balance nitrogen may be compromised depending on salinity levels. Their growth rates under 100 mM NaCl may be dependent on nitrogen supply. These factors should be taken into consideration if this species is utilized for saline land reclamation. Due to variations in salt tolerance between different actinorhizal plants, as well as between different *Frankia* strains, more comprehensive studies on how salinity affects nodulation and nitrogen fixation in various actinorhizal plants (such as *Elaeagnus angustifolia*, *Elaeagnus umbellata*, *Alnus rubra*) including *E. commutata*, are very much needed.

5.3. Effects of high levels of NaCl on the survival of *E. commutata* cuttings (experiment 3)

The goal of this supplementary experiment was to test the ability of *E. commutata* to survive in NaCl levels beyond of that typically found in oil sand tailings (usually at 50 to 100 mM NaCl). The results of my experiment indicated that, as expected, the survival rates of *E. commutata* cuttings declined progressively with increasing salinity (Figure 4.19). The majority of the cuttings survived exposure to 2 applications of 100 mM NaCl for 16 weeks, while only a smaller portion of the cuttings survived when exposed to 6 applications of 100 mM NaCl. This indicated certain levels of resilience toward higher levels of salinity. This also shows that the salt tolerance levels in *E. commutata* cuttings are in line with those of closely related species within the *Elaeagnus* genus, such as *E. angustifolia*, and *E. pungens*. In these species, studies had confirmed that the seedlings could survive up to 200 to 300 mM NaCl with relatively low toxicity symptoms (Devecchi and Remotti 2004; Maimaiti et al. 2014). The degree of salt tolerance for *E. commutata* cuttings pales in comparison to that of *Shepherdia argentea* seedlings

(a boreal actinorhizal shrub within *Elaeagnaceae* family, like *E. commutata*). A similarly designed experiment with a step-wise salt application (similar to my experiment) conducted on *Shepherdia argentea* showed that all seedlings were capable of surviving up to 600 mM NaCl for 30 days (Qin et al. 2010a, b).

Results from this experiment suggest that *E. commutata* cuttings should be able to survive and grow at relatively moderate NaCl levels (50 mM to 100 mM) quite well, as it was the case for *E. commutata* seedlings in Experiment 1. This reflects positively on the potential for the cuttings being utilized for saline land reclamation. It seems that results for *E. commutata* cuttings and seedlings consistently show that while *E. commutata* plants are fairly tolerant of moderate levels of salinity, it is not a plant species that shows strong resilience towards very high levels of salinity. Studies have shown that there can be intraspecific variations in salinity tolerance, the degree of salt tolerance can differ depending on plant developmental stages, plant provenances and cultivars (Lutts et al. 1996; Niknam and McComb 2000; Renault 2012). Salt tolerance properties of the same plant species may also differ between cuttings and seedlings. Nearly all of the studies conducted on the effects of salinity on actinorhizal plants examined seedlings instead of cuttings. This could be due to concerns on low rooting success for cuttings. Studies comparing effects of salinity stress on cuttings and seedlings remained very limited. Sasse and Sands (1995) found that in *Eucalyptus globulus* (Tasmanian bluegum), cuttings seemed to be less resistant toward osmotic stress, this was likely due to differences in root system development compared to seedlings. On a different note, Lissner and Schierup (1996) found that with *Phragmites australis*, vegetatively propagated plants had higher survival rates under salinity compared to seedlings.

Due to differences in plant age, salinity levels, method of NaCl applications and experimental set up, my experiments on seedlings and cuttings cannot be compared to form a conclusion as to whether cuttings or seedlings are more tolerant to saline conditions. However, future studies can be designed to directly compare the salt tolerance properties of *E. commutata* cuttings and seedlings with the same planting medium and same salinity levels. This can provide more comprehensive knowledge on the reclamation potential of *E. commutata* as a species, as well as providing more information on how salinity affects seedlings and cuttings in actinorhizal plants in general.

CHAPTER 6. CONSLUSIONS AND RECOMMENDATIONS

The results from my three experiments on wolf willow (*Elaeagnus commutata*) seedlings and cuttings have provided a comprehensive assessment of the degree of salt tolerance in *E. commutata*. *Elaeagnus commutata* seedlings can survive and grow in at least 100 mM NaCl in a greenhouse; a level of salinity that would be equivalent to the highest levels found within the oil sand tailings in Alberta. Although this salt level (100 mM NaCl) was not directly tested, results suggest a comparable level of salt tolerance for *E. commutata* cuttings as well.

NaCl exposure led to decline in the physiological activities and biomass in *E. commutata*. However, at 100 mM NaCl, *E. commutata* were still able to maintain relatively high levels of physiological functions and growth, which indicated moderate levels of salt tolerance. Leaf injury was also generally contained to a small proportion (<20%) of all leaves.

Results from elemental analyses and changes in plant water status with salinity indicated an increased plant uptake of Na^+ and Cl^- under salinity. This did not lead to significant declines in plant K^+ and Ca^{2+}, which means that plant nutritional balance was not disturbed by higher

levels of NaCl. These plants were able to accumulate significantly more Na^+ and Cl^- ions in root tissue to avoid ionic damage to plant shoot tissues. Furthermore, salinity did not lead to reduction in plant nitrogen content, instead, plant nitrogen increased under salinity, suggesting that *E. commutata* can sustain nitrogen uptake under 100 mM NaCl even without nitrogen fixation. Finally, there was no evidence of ionic damage to photosystem II, neither did salinity lead to a decrease in chlorophyll pigments. All of these results confirm the capability of *E. commutata* to utilize a range of salt tolerance mechanisms to survive and grow under up to 100 mM NaCl.

Root nodulation was inhibited by salinity at both 50 and 100 mM NaCl. For nodulated plants, nitrogen fixation was severely reduced at 100 mM NaCl. Although as demonstrated by both experiment 1 and experiment 2, the plants can still survive under 100 mM NaCl without nitrogen fixation, the growth of *E. commutata* may be compromised with low nitrogen availability under salinity. If utilized for land reclamation, this would need to be taken into consideration.

Overall, *E. commutata* is a moderately salt tolerant plant that has good potential to be used as a reclamation species for saline habitats, such as the oil sand mine tailings in Alberta. My experiments did raise a few questions that remain to be addressed. For example, although I was able to rule out any increase in N compounds like proline and total protein as possible explanations for the observed increase in nitrogen content under salinity, we still need to determine what caused the increase in nitrogen under salinity. In addition, *E. commutata* may be more efficiently propagated with cuttings compared to seedlings. Viable seeds are often hard to obtain, and from my observations, seed germination rates are relatively low for *E. commutata*. My experiment did not directly compare the salt tolerance of *E. commutata* seedlings vs. cuttings, thus it would be interesting to investigate whether there is a difference in their levels of

salt tolerance in order to determine the most appropriate choice of reclamation strategy. Most importantly, my experiments were conducted in the greenhouse with controlled temperature, lighting, moisture, nutrient supply and planting medium. For real world applications, field experiments with oil sand mine tailings will be required to validate the results of my experiments and confirm the suitability of *E. commutata* for reclamation purposes.

In addition, other actinorhizal shrubs native from Canada, like Silver buffaloberry (*Shepherdia argentea*), and the closely related Canada buffaloberry (*Shepherdia canadensis*) should be taken into consideration as potential saline land reclamation species as well. Studies from Qin et al. (2010 a, b) have confirmed the high salt tolerance of *Shepherdia argentea* seedlings, however, field experiments are still needed to further examine whether the species can be used for reclamation applications. On the other hand, while a study has tested *Shepherdia canadensis* inoculated and uninoculated with *Frankia* in oil sand tailings (Visser et al. 1991), a comprehensive study on the salt tolerance and nitrogen fixation of *S. canadensis* has yet to be conducted.

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Appendices

Appendix 1. *E. commutata* seedlings in experiment 1 (week 12) subjected to 0 mM NaCl (left), 50 mM NaCl (center), and 100 mM NaCl (right).

Appendix 2. *E. commutata* cuttings (shoot and root) after harvest in experiment 3, these cuttings were subjected to 0 (control), 2, 4 and 6 applications of 100 mM NaCl (from left to right, respectively).

Appendix 3. Composition (chemicals and concentrations) of the modified Rorison's nutrient solution used for my experiments.

		Nutrient solution (concentrations)	
Elements	**Chemical**	**Full Nitrogen (mM)**	**Half Nitrogen (mM)**
Ca/N	$Ca(NO_3)_2 4H_2O$	2/4	1/2
Mg/S	$MgSO_4 7H_2O$	1/1	1/1
K/P	K_2HPO_4	2/1	2/1
Fe	Fe EDTA	0.053	0.053
Traces			
Mn	$MnSO_4\ 4H_20$	0.009	0.009
B	H_3BO_3	0.0045	0.0045
Mo	$Na_2MoO_4\ 2H_2O$	0.001	0.001
Zn	$ZnSO_4\ 7H_2O$	0.0015	0.0015
Cu	$CuSO_4\ 5H_2O$	0.0015	0.0015
Variation			
Ca (for half N)	$CaCl_2\ 2H_2O$	0	1