Physiological Response of *Populus balsamifera* and *Salix eriocephala* to Salinity and Hydraulic Fracturing Wastewater for Potential in Phytoremediation

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submitted by Michael Bilek in partial fulfillment of the requirements for

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in Forestry

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Abstract

Agriculturally productive land is degrading at an alarming rate due to a rapidly increasing population affecting the extent of industrial pollution and soil salinization. It is estimated that more than 10% of global landmass is salt-affected, which results in lowered crop yield and disrupted local environments. Hydraulic fracturing (fracking), has recently seen increased frequency of use, but its environmental effects are poorly studied. This study examines the efficacy of *Populus balsamifera* L. and *Salix eriocephala* Michx. for their phytoremediation potential on saline and fracking wastewater polluted soils. Three growth trials were performed to screen for tolerance and quantify physiological responses to abiotic stress: a screening trial with thirty-one poplar and willow genotypes grown for eight weeks on 0, 30, and 80 mM NaCl, a second salinity trial with two poplar, five willow, and one hybrid willow genotypes grown for twelve weeks with 0, 20, 40, and 60 mM NaCl, and a fracking trial consisting of three willow and one hybrid willow genotypes treated for eight weeks with fracking wastewater dilutions. Poplar genotypes were susceptible to salinity, showing significant reductions in growth and failing to survive at 60 and 80 mM NaCl treatments. Poplar sensitivity is likely due to its inability to restrict sodium transport to aerial tissues. Native and hybrid willows did not experience mortality when grown at or below 60 mM NaCl treatments, and showed no reduction in height at 20 mM NaCl. Hybrid willow (Lev-13) accumulated the most biomass while native willow genotypes (Cam-2 and St-2) showed the smallest reductions in growth with increasing treatment. Water-use efficiency increased significantly with salinity treatment in native and hybrid willow genotypes. Stachyose and raffinose content tripled in leaf and root tissues respectively, suggesting use in oxidative defense. Tolerant willow genotypes excluded sodium

from leaf tissues and maintained higher K:Na ratios. In the fracking wastewater trial, the two willow genotypes Cam-2 and St-2 displayed limited necrosis, resistance to biomass loss, and survived eight weeks of treatment, while the hybrid did not survive the highest treatment. These results identify two candidate native willow genotypes for further study and use in phytoremediation field-trials.

Lay Summary

Soil degradation is a process that lowers the productivity of land, and can result from improper irrigation of farmlands and industrial pollution. Less productivity means that more land is needed to feed and clothe our growing global population – using our limited terrestrial resources increases the probability soil degredation. Therefore, it is necessary to find ways to revitalize marginal land using stress-resistant plants, a process called phytoremediation. The focus of this work is to examine poplar and willow trees for their efficacy at surviving, growing, and ultimately repairing marginal land. We found that native willows are capable of withstanding toxic amounts of salts, and described changes in their growth and physiology. Ultimately, this information will assist AAFCs poplar and willow breeding program to advance rapid selection and to deploy for phytoremediation, and should prove useful for maintaining the health of people and their environment for the benefit of future generations.

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Preface

My contributions to this work include the design and implementation of experiments, and the collection and analysis of data. The Mansfield lab assisted with greenhouse harvests, and Dr. Letitia Da Ros aided in the generation of R code for data analysis, as well as thesis edits. Xinyi Huang contributed to plant care and data collection during the second and third experiments. Shawn Mansfield and Raju Soolanayakanahally identified and designed the research program. Shawn Mansfield, Robert Guy, and Raju Soolanayakanahally contributed to editing of this thesis.

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For my parents, who were bored one evening and set this whole thing in motion.

Chapter 1: Introduction

One of mankind's next grand challenges will be clothing and feeding an ever-growing global population while managing finite land resources. While demand for agricultural products increases, soil quality and availability decrease annually. This in-turn results in significant economic loss, with estimated annual global losses of approximately $27.3 billion USD due to diminished crop yield (Qadir et al., 2014). Soil salinization and industrial pollution are key factors in the decline of the quality arable land, and methods of ameliorating these contaminants are traditionally costly and laborious (Pilon-Smits, 2005). Phytoremediation, the process of using plants to improve the quality of affected soils, has garnered recent attention as a cost effective alternative to manual or chemical methods of soil remediation (Mirck and Zalesny, 2015; Pulford and Watson, 2003; Volk et al., 2006). The objective of this work is to examine and quantify the impact of salinity and hydraulic fracturing wastewater on the growth, gas exchange, and nutrient balance of *Populus balsamifera* L. (balsam poplar) and *Salix eriocephala* Michx (heartleaf willow). Elucidating the saline stress responses of these species will contribute to our knowledge of how plants persist on marginal land, as well as help secure the health of native ecosystems for future generations.

1.1 Defining soil salinity

It is estimated that globally, up to 10% of biologically productive landmass is currently salt-affected to an inhibitory extent, resulting in diminished crop yield and substantial agro-economical losses (Wicke et al., 2011). While the majority of salts in soils exist in low

concentrations, vast areas such as in Australia and the Middle East can be seriously affected (Elhag, 2016; Lambers, 2003). In North America, primarily the western U.S. and Canadian prairies experience detrimental salt accumulation (Nachshon, et al., 2014). The classification of salinity varies by region, and three types of soil salinity have been defined: saline, alkali (sodic), and saline-alkali (saline-sodic) (Allison et al., 1954). Saline soils are characterized by salts being the dominant ions in the soil, whereas sodic soils have a high percentage of exchangeable sodium; saline soils are the most prevalent, globally (Wicke et al., 2011). Salts that accumulate in soils can be comprised of Na^+, Mg^{2+}, Ca^{2+}, or K^+ cations with Cl^-, SO_4^{2-}, CO_3^{2-}, or HCO_3^- anions (Volkmar et al., 1998). Electrical conductivity (EC) is commonly used to measure the degree of salinity in a soil solution, with <4 dS m^{-1}, 4-8 dS m^{-1}, and >8 dS m^{-1} representing low, moderate, and high levels, respectively.

1.1.1 Causes of soil salinity

Salinization occurs via mineral weathering and chemical reactions, either through aboveground erosion due to rainfall or belowground movement of water via the hydrologic cycle. As rain falls, water can react with carbon dioxide in the air to form naturally acidic carbonic acid, which can release ions from exposed minerals (Parihar et al., 2015). These salt ions subsequently accumulate in the topsoil layer or are transported through streams and rivers towards the ocean, where the salts are deposited in coastal regions. The flow of underground water via the hydrologic cycle also releases minerals which accumulate in subsurface soils (Heagle et al., 2013). Capillary action, driven by evaporation at the surface soil level, drives the movement of belowground water upwards, depositing salts in topsoil regions (Shokri-Kuehni et al., 2017).

Human activities can exacerbate these processes; deforestation increases the rate of salt deposition on the surface soil layer by removing deep root zones. Root systems act as a barrier for salt deposition by allowing water to travel to the surface via evapotranspiration while excluding salt uptake (Jayawickreme et al., 2011). Most commercial annual crop species produce shallow root systems compared to perennial tree species, leaving agricultural land susceptible to salinity. Farmlands may also experience increasing salinity due to the reuse of irrigation water: the tendency of irrigation water to salinize has plagued man since the dawn of agriculture (Jacobsen and Adams, 1958). As water evaporates or is used by crops, salts are excluded at the rhizosphere and accumulate in irrigation water over time. Irrigation water may be reused many times, especially in areas with limited access to sources of fresh water. While farming and forestry practices facilitate the accumulation of salts in the topsoil via removal by deep root systems, salt generation via mineral erosion may also have anthropogenic sources.

1.1.2 Hydraulic fracturing and wastewater

Oil and gas industries release large amounts of pollutants in pursuit of target materials (Castro-Larragoitia et al., 1997). Mine tailings – dump sites for excavation byproducts and wastes – often disperse with wind and rain. The constituents of mining runoff vary with target resources, and lead to disastrous health and environmental effects (Mukherjee and Bhattacharya, 2001). As global oil and gas reserves deplete, companies have invested in hydraulic fracturing, ‘fracking’, as a method to reach hard-to-access underground gas reservoirs. Fracking works by first drilling down, then horizontally to target porous rock with trapped gases. Water, containing lubricants, biocides, and ceramic beads called “proppants”, is pumped at high pressures to expand rock and

shale, whereby the proppants hold the fissures open and natural gas is collected (Gregory et al., 2011). After the gas is gathered, the 'flowback' water is then collected and disposed. Flowback water contains not only the industrial pre-treatments, but releases salts (and heavy metals) at a high concentration, often more concentrated than seawater (Blauch et al., 2009). Due to its potential harmful impact on the environment, fracking has received worldwide attention as more gas and oil companies seek its use. Thousands of spills have occurred causing environmental contamination; there is concern that fracking wastewaters may leach into freshwater sources and pose public health concerns (Gordalla et al., 2013; McLaughlin et al., 2016; Wright and Muma, 2018). Whether it be for public health or ecological restoration, non-destructive and cost-efficient methods of ameliorating both salts and heavy metals must be considered.

1.2 Phytoremediation

As salinity spreads, the economic detriment becomes exacerbated: crop yield reduces, fertilizer use increases, biodiversity declines, and available arable land diminishes, all of which translates to economic loss and limitation to the production of food, fuel, fibre and fodder. Traditional methods of remediation are cumbersome and expensive, such as excavation or chemical treatment soils (Pilon-Smits, 2005). In the last two decades, an inexpensive, environmentally friendly method of ameliorating polluted soils has garnered attention: phytoremediation.

1.2.1 Types of phytoremediation

Broadly, phytoremediation is the process of using plants to improve the condition of saline or polluted soils. The type of phytoremediation depends largely on the target pollutant and physiological action of the plant. Common pollutants can be metals, salts, and organic or inorganic compounds, and may come from natural or anthropogenic sources. For example, industrial runoff has been shown to be naturally filtered by wetland root systems, a process termed rhizofiltration (Mickle, 1993). Phytostabilization is a practice whereby mobile pollutants are immobilized and made biologically unavailable by root interactions (Wai Mun et al., 2008). The removal of pollutants from soil is often the primary goal in phytoremediation. Phytoextraction is a method of accumulating trace elements into aerial plant tissues for ease of disposal, which to date has yielded limited results (Robinson et al., 2015). Phytoremediation may also be as simple as the establishment of pioneer species; in cases of soil salinization, afforestation may act to restore deep root systems and thereby lower the local water table, restricting the upward movement of salts and restoring habitat diversity. A secondary goal for phytoremediation is generating biomass for bioenergy feedstocks or other classical industries, such as pulp and paper.

1.2.2 Biomass production

One issue with soil remediation is that it is a lengthy, economically unproductive process. One solution is to utilize trees for bioenergy production (Kalinoski et al., 2017; Rowe et al., 2013). Many tree species can produce biomass by coppicing, a method of harvesting aerial woody

biomass while allowing intact roots to produce new shoots. A short rotation coppice (SRC) system has been explored that allows landowners to simultaneously remediate land for future productivity and produce income on a harvest cycle of two to five years (Michels et al., 2018). Depending on the target pollutant, bioenergy may not be the only product of SRC. Some species may exhibit luxury uptake of fertilizer runoff, which may produce nutrient rich biochar as a secondary product of bioenergy processes (Da Ros et al., 2018). Furthermore, provided the pollutant is not toxic, secondary products can be manufactured through the harvest of wood and fibre (Volk et al., 2006). The productivity of phytoremediation activities is highly dependent on growth, and therefore, physiological response to abiotic stress and acclimatization.

A quality phytoremediator must fulfill a variety of roles: it must be well suited to a given climate, resist the targeted abiotic stress, and should also provide some form of agricultural product. Understanding the mechanisms of abiotic stress response is imperative to finding a viable phytoremediation candidate.

1.3 Abiotic stress response

Soil salinity can negatively impact the vast majority of plant species. When plants undergo salt stress, effects can include lower water and nutrient uptake, diminishing photosynthetic rates, and reductions in growth. Since soil salinity is extremely common, especially in low dosages, plants have evolved a variety of strategies to tolerate salinity or heavy metal stresses. Although a great deal of work has been conducted examining numerous abiotic stress responses in commercial crop and model species, such as rice, wheat, barely, and *Arabidopsis thaliana* (Britto et al., 2004;

Horie et al., 2009; Munns and James, 2003; Vighi et al., 2017), much work is needed on species targeted to directly combat soil salinization for phytoremediation strategies.

1.3.1 Water potential

Water availability and uptake are critical to plant health and growth. While water uptake is mostly a passive process, plants may employ several strategies to improve water uptake and prevent water loss in instances of drought and salinity. Plants take up water and attract nutrients via the transpirational process whereby water flows from a high water potential in soils to a lower water potential outside the leaf stomata. The total water potential of a solution (Ψ_w) reflects several component potentials:

$$\Psi_w = \Psi_s + \Psi_p + \Psi_g$$

Where Ψ_s, Ψ_p, and Ψ_g are solute, pressure, and gravity potential, respectively (Taiz and Zeiger, 2010). A low solute potential in root cells generated by accumulation of sugars generates a low water potential that initiates a flow of water towards the endodermal layer. Water enters the transpiration stream symplastically via aquaporins, and flows upward through the xylem due to the greatly negative hydrostatic pressure generated by the mesophyll tissue and the stomatal air boundary layer. The flow of water through the plant is critical for solute transport and gas exchange. As water exits the stomatal pore via evaporative processes, carbon dioxide enters through stomatal openings via diffusion and is fixed for use in the photosynthetic pathway. The ratio of carbon dioxide gain per unit water lost through stomata is water-use efficiency (WUE),

and is regulated to prevent excess water loss when the water potential in the soil drops in saline conditions. WUE varies by species photosynthetic strategy, photosynthetic capacity, water availability in the soil, and stomatal activity (Lawson and Blatt, 2014). Water availability and stomatal response can be negatively affected by soil salinity with consequent effects on WUE.

1.3.2 Plant response to osmotic stress

Salinity lowers the water potential of the soil upon deposition by lowering the osmotic potential, which greatly reduces the plant's ability to take up water and threatens cells with dehydration. A reduction in available water requires cellular osmotic adjustment throughout the whole plant (Lamsal et al., 1999). Plants can detect the change in osmotic potential within minutes, which triggers the release of Ca^{2+}, a messenger for many drought and salt stress responses (Chen and Polle, 2010; Fricke et al., 2004). Calcium induces the synthesis of abscisic acid (ABA), which stabilizes root growth, signals for the production of solutes used in osmotic adjustments, and initiates adjustments in stomatal aperture to reduce the loss of water (Leung and Giraudat, 1998). It is estimated that 98% of water lost by plants is through the stomata (Lawson and Blatt, 2014). While complete stomatal closure is useful in curtailing immediate water loss, it is not viable as a long-term strategy as indefinite closure will halt photosynthesis and metabolic activities.

If soil osmotic potential remains low, plants must adjust their own osmotic potential to facilitate water uptake and maintain photosynthetic rates. Accumulation of Na^+ from the soil may serve as a quick, energetically inexpensive method of restoring osmotic balance, however, utilizing Na^+ for osmotic balance will inevitably lead to ion toxicity if not sequestered in the tonoplast or

apoplastic space (Blumwald, 2000). Plants may produce organic solutes called osmolytes in response to soil salinity in order to lower their osmotic potential and protect cellular components against toxicity (Morgan, 1984; Yancey et al., 1982). Sugars, sugar alcohols, ions, or charged metabolites can all act as osmolytes, including sucrose, fructose, methylated inositols, proline, and glycine betaine (Hasegawa et al., 2000). The accumulation of cellular osmolytes has several benefits: osmolytes lower cellular osmotic potential to facilitate water uptake, act as enzyme chaperones in high Na^+ concentrations, function as antioxidants, and may be later metabolized to refund the energy and nutrient costs of production (Delauney et al., 1993; Lokhande et al., 2011).

1.4 Nutrient uptake

Plant growth and development is highly dependent on nutritional balance. Soil nutrients such as potassium, calcium, nitrogen, magnesium, and phosphorus function in a variety of metabolic processes. Salts are known to disrupt nutrient uptake and alter plant tissue concentrations. Nutrients are absorbed into root tissues through passive or active transport. Passive transport of solutes involves soil nutrients being carried by water bulk flow or through specific or non-specific ion channels. Active transport requires nutrients to come into contact with root hairs and bind to specialized transport proteins on root cell membranes. Saline soils alter the way in which nutrients are absorbed by roots: a reduction in water potential by salt species may severely hinder nutrient availability by limiting bulk flow of water into roots (Aroca et al., 2012). In addition, positively charged sodium ions can bind to negatively charged soil particles, which facilitates nutrient leaching from the soil such as K^+, Ca^{2+}, Mn^{2+}, and Mg^{2+} to the detriment of overall plant health.

1.4.1 Plant nutrient concentrations

A great deal of research has been conducted in crop and tree species on how salinity affects nutrient concentrations in different plant tissues (Ehlting et al., 2007; Lv et al., 2012; Suarez and Grieve, 1988). Nutrient uptake depends on the availability in the soil solution, ion mobility, and transport capabilities of the species. Sodium is a mobile ion that plants will preferentially store in root and developed leaf tissues, a strategy that both protects developing leaves and enhances water uptake capabilities (Imada et al., 2009; Quintero et al., 2008). Calcium, critical in its role in various stress responses, has been shown to decrease in plant tissues in the presence of saline conditions (Major et al., 2017). Arguably, the most important soil nutrient is nitrogen, which is used for amino acid and protein production, and comprises nitrogenous bases used in nucleotides. Soil salinity generally inhibits nitrogen uptake, as Cl^- competes with NO_3^- at membrane transporters (Chen et al., 2010). Cellular potassium helps to regulate osmotic homeostasis, and activates cytosolic and photosynthetic enzymes, however, significant amounts of K^+ can be lost from tissues in saline environments (Nassery, 1979).

1.4.2 Potassium transporters and the SOS pathway

Potassium is an abundant ion that has roles in osmotic balance, guard cell aperture control, enzymatic reactions, and ATP production (Boer, 1999; Nassery, 1979). During salinity stress, mechanisms regulating sodium and potassium balance are heavily intertwined. In the presence of salinity stress, K^+ can be depleted while Na^+ quickly overwhelms cells with toxic effects. Ion

balance is imperative to cell health, and therefore, plants have evolved highly specialized methods of regulating this balance. With access to newer and more sensitive technologies, a great deal of work has been done in the last twenty years to identify genes and proteins associated with sodium and potassium transport (Hosoo et al., 2014; Munns and Tester, 2008; Shabala et al., 2006).

Potassium uptake from the soil can be a passive or active process depending on cellular electrochemical gradients. Active uptake requires electrochemical energy: plant cells preferentially maintain a neutral cytosolic pH, utilizing H^+-ATPases to pump H^+ into the vacuole or the extracellular space. The proton gradient creates a useful electrochemical gradient that facilitates the import or export of solutes via symport and antiport activities (Shabala et al., 2006). Two main transporter families have been identified for K^+ transport: the KUP/HAK/KT and HKT families (Horie et al., 2009; Hosoo et al., 2014). Potassium enters cells through voltage-gated channels as well as H^+/K^+ symporters, then it is sequestered into the vacuole for osmotic adjustment and storage. Sodium may interfere with these potassium transporters: at high extracellular Na^+ concentrations, Na^+ can enter the cytosol through competition with K^+ for transport binding sites, as well as non-selective cation channels (Sauer et al., 2013). This competition may severely limit the potential uptake of potassium and lead to deficiencies such as loss of plant turgor or photoinhibition. Therefore, it is necessary for plants to control the movement of sodium within cells.

1.4.3 Compartmentalization

A common strategy that plants employ to tolerate extensive salinity is to sequester ions away from metabolically active sites. When excess sodium enters a cell, it is preferentially exuded into the apoplast or vacuole by the action of the Salt Overly-Sensitive (SOS) pathway transport proteins (Lv et al., 2012). At high Na^+ levels, Ca^{2+} enters the cell from the cell wall or vacuole. Calcium will then initiate the production and insertion of SOS1, a Na^+/H^+ antiporter inward into the vacuole, and outward on cell membranes (Shi et al., 2000). Calcium also induces changes in voltage-gated cation channels, closing inwardly rectifying Na^+ channels and outwardly rectifying K^+ channels (Cheng et al., 2004). Sodium may be translocated to older leaves in order protect developing leaf tissues due to its high mobility.

1.5 Photosynthesis and salinity

Photosynthesis is the process that allows plants to convert light energy and carbon dioxide into chemical energy available for growth and metabolic processes. Soil salinity inhibits photosynthetic processes via both a reduction in gas exchange and cellular toxicity. Gas exchange reduction is the primary influencer of photosynthetic rate, as stomatal closure directly reduces transpiration and CO_2 assimilation (Abbruzzese et al., 2009; Netondo et al., 2004). Accumulation of Na^+ and Cl^- in the cytosol can result in significant constraints: salt ions can alter the structure and function of chloroplast membranes, and reduce photosynthetic pigment content (Parihar et al., 2015). Many studies have shown a significant reduction in chlorophyll content, production, and quantum yield (as indicated by chlorophyll fluorescence) in a variety of crop

species subject to saline environments (Ali et al., 2004; Jiang et al., 2006; Rani et al., 2017). Additionally, leaf senescence caused by tissue-level damage reduces the whole plant photosynthetic levels, and ultimately limits growth.

1.5.1 Respiration and salinity

Abiotic stress response is an energetically demanding process. Glycolysis converts energy in the form of sugars into ATP, which is used to fuel osmolyte production and active transport proteins. Mitochondrial ATP production can easily be affected by salinity, as positively charged Na^+ ions are drawn toward the naturally negative charge of the mitochondrial membrane (Othman et al., 2017). Mitochondria naturally produce reactive oxygen species (ROS) as electrons travel through the electron transport chain and bind with oxygen, however, excess Na^+ can increase the abundance of ROS dramatically, impairing membrane function (Kurusu et al., 2015). Mitochondria may increase the abundance and/or activity of the enzyme alternative oxidase to lower ROS evolution, but this strategy reduces rates of ATP synthesis (Feng et al., 2013).

1.5.2 Reactive oxygen species

ROS are chemically reactive free radicals containing oxygen that are capable of participating in oxidation reactions. Many of these are products of respiration, such as hydrogen peroxide, superoxide, and hydroxyl radicals, and are used in cellular signaling and metabolism (Vighi et al., 2017). Excess ROS can cause accelerated rates of membrane degradation through peroxidation, affecting DNA and protein structure, as well as acting as signals for apoptosis. On

the macro level, ROS can cause leaf curl and necrosis. Both sodium salts and heavy metals increase the rate of ROS evolution in plants. In response to elevated ROS levels, plants employ a large variety of antioxidants such as superoxide dismutase, ascorbate peroxidase, glutathione, glycine betaine, and proline (Othman et al., 2017). Proline is one of the most common plant osmolytes and offers incredible utility in stress response: it can harvest free radicals, chaperone enzymes, restore osmotic balance, and can be later metabolized to recover its energy and nutrient production costs (Kishor and Sreenivasulu, 2014). Both sugars and polyols, such as sucrose, raffinose, and inositols, are common in abiotic stress responses and are employed as stress response indicators with relative ease (Wu et al., 2013). Osmolyte generation in response to salts and heavy metals is therefore an important quality to consider when choosing candidates for phytoremediation projects (Chen and Polle, 2010; Munns and Tester, 2008; Stobrawa and Lorenc-Plucińska, 2007)

1.6 Poplar and willow in phytoremediation

The genera *Populus* and *Salix* are woody dicots that together comprise about 430 species and inhabit temperate zones in the Northern Hemisphere. Many researchers are now examining their phytoremediation potential, as poplar and willow exhibit many desirable traits (Chen and Polle, 2010; Hangs et al., 2011; Sixto et al., 2005; Yoon et al., 2014). These species are fast-growing, develop deep root systems, can be coppiced, are widespread in Europe, North America, and Asia, and have a wealth of genetic information available (Tuskan et al., 2006). Poplar and willow plantations have been established for use in both wood product production and SRC for bioenergy applications. Furthermore, willow and poplar are known to be moderately tolerant to

abiotic stresses. Their ability to propagate by cuttings coupled with genotypic variance allows researchers to examine and choose highly stress tolerant individuals. Together, these factors combined highlight the members of these genera as quality candidates for phytoremediation applications.

1.7 Rationale

With a large wealth of species and genotypic variation, there is much work to be done in assessing the value of poplar and willow for phytoremediation applications. While abiotic stress tolerance work has been done on many poplar and willow varieties throughout Europe and Asia, a gap remains in knowledge regarding two species with a widespread Canadian range: *Populus balsamifera* and *Salix eriocephala* (Chen and Polle 2010). Work has been done on balsam poplar varieties examining clinal variation in physiology (Soolanayakanahally et al., 2009), however, recent evaluations have shifted focus towards abiotic stress tolerance. Metabolite profiling of poplar varieties indicated an increase of compatible osmolytes in response to drought stress and demonstrated the extent of the inherent variation (Barchet et al., 2014). While there is evidence that both species have potential for phytoremediation strategies, work remains to characterize and document the physiological responses to salinity.

1.8 Objectives and Scope

Numerous factors work in tandem to determine stress resistance, and one response alone does not generally confer "tolerance". Stress response, as discussed earlier, is the result of maintaining water potential, producing osmolytes, sustaining growth, and resisting cellular toxicity. Therefore, when determining the phytoremediation potential of a species, all of the aforementioned responses must be considered.

The objective of this research was to measure and quantify the impact of salinity and application of fracking wastewater on the tolerance and growth potential of *Populus balsamifera* L. (balsam poplar) and *Salix eriocephala* Michx. (heartleaf willow) genotypes. By examining the abiotic stress responses of these genotypes, insights can be gained into how members of Salicaceae resist stress and identify potential candidates for phytoremediation of saline and industrially-contaminated sites in Canada. We hypothesize that the members of Salicaceae (*Populus* and *Salix*) have cellular mechanisms to cope with salinity by maintaining high levels of osmolytes and restricting the toxic accumulation of salts. Throughout this work, the following objectives were pursued:

Objective 1: Screen for salinity tolerance in selected poplar and willow genotypes by measuring survival, growth, and photosynthetic response.

Objective 2: Observe changes in soluble sugar production and nutrient accumulation to quantify stress response at varying salinity levels.

Objective 3: Test the survival, growth, biomass, gas exchange capacity, sugar production, and nutrient uptake of candidate genotypes on diluted fracking wastewater.

In order to achieve these objectives, three greenhouse growth trials were performed. The first trial screened for tolerance in thirty-one poplar and willow genotypes by observing growth rates and survivability at three salinity levels to narrow the focus to a more manageable number of genotypes for in-depth analyses. A second growth trial examined eight genotypes at four salinities, and quantified their growth rates, gas exchange, tissue nutrient composition, and soluble sugar production. To conclude the study, four final genotypes were chosen based on previous physiological metrics for survival potential in fracking-wastewater supplemented irrigation.

Chapter 2: Materials and Methods

2.1 Greenhouse

Three greenhouse growth trials were conducted between February 2016 and June 2018 in the Horticulture Greenhouse of the University of British Columbia (49.26°N 123.25°W; elevation 82 m).

2.1.1 Cutting acquisition and storage

Dormant cuttings of seventeen native balsam poplar, fifteen native heartleaf willow, and one hybrid willow genotypes were harvested from the Agriculture and Agri-Foods Canada (AAFC), Agroforestry Development Centre in Indian Head, Saskatchewan (Table 2.1). Genotypes were selected based on biomass accrual ability and clinal variation in phenology (Figure 2.1) (Keller et al. 2011). Selected dormant cuttings were ordered one month before each trial, shipped frozen, and then stored at +4° C until planting.

Table 2.1: Poplar, willow, and hybrid willow genotypes used in salinity trials. Superscripts indicate genotypes that were grown in experiments 2 and 3, no superscript indicates growth in only the initial screening trial.

Native Poplar	Native Willow	Hybrid Willow
Boyle - 6	Camrose – $2^{(2,3)}$, 5, 7	LEV-D5
Fort McMurray - 1, 2, 6	Drumheller - 2, $4^{(2)}$, 6	(*S. discolor*) × India – $13^{(2,3)}$
Fort Nelson - 1, 5, 7	La Ronge - 1, $3^{(2,3)}$, $5^{(2)}$	
Grand Prairie - 2, $10^{(2)}$	Prince Albert – 1, 2, 4	
La Ronge – $3^{(2)}$, $5^{(2)}$	Stettler – $2^{(2,3)}$, 3, $4^{(2)}$	
Turtleford - 3, 4, 7		
Watson Lake - 1, 5, 7		

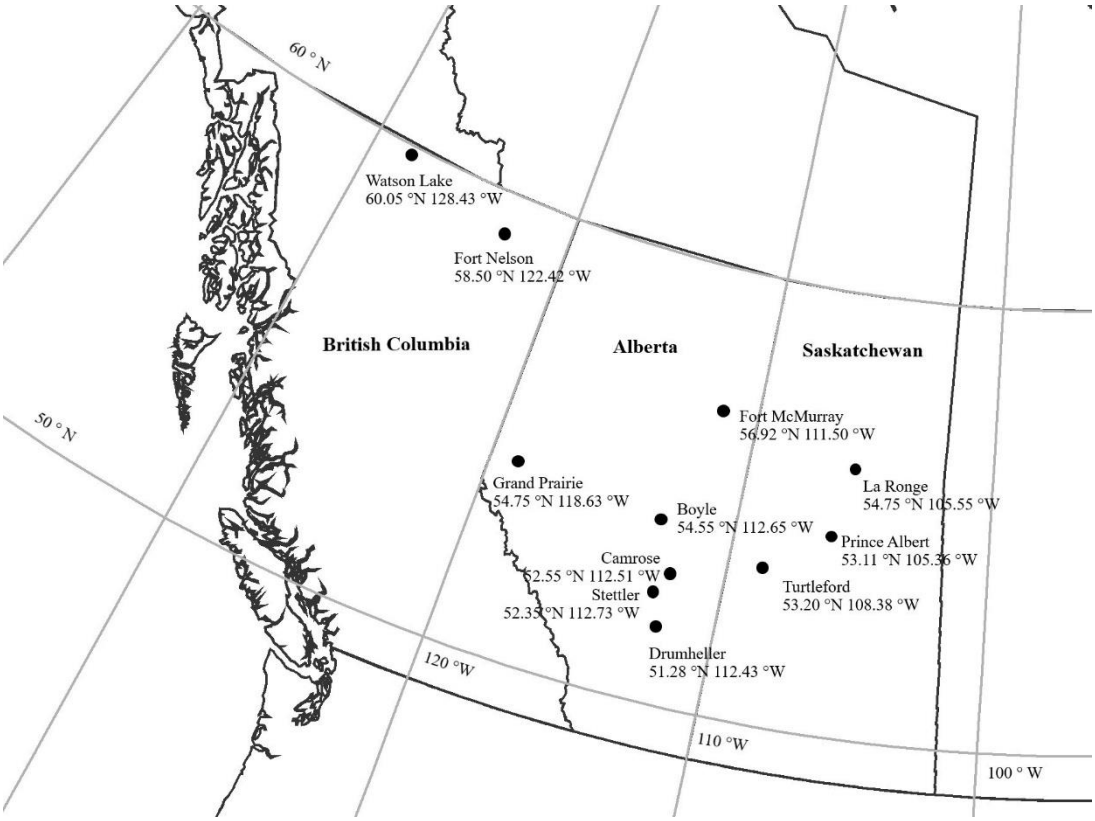


Figure 2.1. Native poplar and willow provenances from British Columbia, Alberta, and Saskatchewan employed in greenhouse growth trials at the University of British Columbia.

2.1.1 Planting

The planting protocol was consistent for all three growth trials. Cuttings were retrieved from +4° C storage and a fresh cut was given for each cuttings on the bottom using clippers. Cuttings were then dipped in 0.4% IBA Stim-Root No. 2 rooting powder (Brampton, Ontario, Canada), immediately placed in 2-gallon pots filled with perennial mix consisting of 50% peat, 25% crushed bark, and 25% pumice as a growth media, and watered with half-strength fertilizer-supplemented water for one month (Table 2.2). After bud break, new shoots were gradually pruned to allow growth of a single dominant stem.

Table 2.2: University of British Columbia horticulture greenhouse stock fertilizer mix

UBC Horticulture Fertilizer Mix

	NH_4	K	Na	Ca	Mg	NO_3	Cl	S	HCO_3	P	Si
mmol/L	0.1	5.9	0.4	6.4	3.2	16.3	2.3	3.3	0.1	1.42	0.05

	Fe	Mn	Zn	B	Cu	Mo
µmol/L	39	1	2.7	39	0.9	1

2.1.2 Treatment conditions

All experiments utilized a randomized block design. Average greenhouse temperature was 23° C during daytime and 18° C at night, with an 18h day : 6h night photoperiod. A red/white/blue LED Philips Green (Markham, Ontario, Canada) lighting system was employed for all trials with

an average light intensity of 147 µmol/sec/m^2. For the duration of all three experiments, the control treatments consisted of half-strength stock fertilizer solution growing under the same conditions as the treated trees (Table 2.3).

In the initial screening trial, four replicates per genotype (Tables 2.1 and 2.3) were randomly assigned to each of three treatments. Sodium chloride was weighed and subsequently dissolved into warm water and mixed with half-strength fertilizer solution to match targeted molarities (Table 2.3). Trees were treated for eight weeks, with height, diameter, and survival recorded weekly.

From the initial trial, eight genotypes were selected for further, more in-depth evaluation. In this trial, 10 replicates per genotype (eight genotypes; Table 2.1 and Table 2.3) were assigned randomly to each of the four treatments in Experiment 2. Sodium chloride was mixed into half-strength fertilizer as previously described (Table 2.3). Trees were treated for 12 weeks, with height, diameter, and survival recorded bi-weekly. Gas exchange was measured at weeks six and ten of treatment, and all biomass was harvested after 12 weeks of treatment.

For the final fracking wastewater trial, 10 replicates per genotype (four most promising genotypes selected from Experiment 2) were assigned randomly each of three treatments (Tables 2.1 and 2.3). The fracking wastewater (Table A.1) was diluted in half-strength fertilizer to match the electrical conductivities of 20 and 60 mM NaCl solution from Experiment 2 (Table 2.3). Trees were treated for 8 weeks with height, diameter, and survival recorded bi-weekly. Gas

exchange was measured at week 6, and all biomass was harvested at the conclusion of 8 weeks of treatment.

All trees were dripline fed with 2L/h emitters, with an average of 15 minutes per watering event. Cuttings were watered once a week for one month with fertilizer solution to allow uniform establishment. Water frequency increased to once every 4 days at the beginning of the treatment, once every 2 days from weeks 4-8, and once daily from weeks 8-12.

Table 2.3: Target molarity, NaCl/L, and average EC of treatments for all experiments.

	Treatment	NaCl (mM)	NaCl (g/L)	Average EC (mS cm^{-1})
Screening Trial	Control	0.2	0.005	0.7
	Low	30	1.75	4.2
	High	80	4.68	7.0
Experiment 2	Control	0.2	0.005	0.7
	Low	20	1.17	2.3
	Moderate	40	2.34	4.5
	High	60	3.51	6.4

	Treatment	Na (mM)	Cl (mM)	Na (g/L)	Cl (g/L)	Average EC (mS cm^{-1})
Fracking Trial	Control	0.2	1.2	0.005	0.041	0.7
	Low	16.6	11.3	0.382	0.400	2.6
	High	72.8	34.4	1.167	1.221	6.7

2.2 Measurements

2.2.1 Survival, Height, and Diameter

Mortality of an individual was visually determined when necrosis covered approximately 50% of the leaf tissue, and growth was no longer occurring. Height was measured from the soil surface to the distal leaf, and diameter was measured using an electronic caliper at the base of the primary shoot emerging from the cutting.

2.2.2 Gas exchange and chlorophyll fluorescence measurements

A LI-6400XT portable photosynthesis system (LI-COR Biosciences, Lincoln, Nebraska, USA) was used to measure leaf gas exchange at weeks six and ten of treatment. Five replicates per genotype, per treatment, were randomly selected for measurement. All trees were watered with their corresponding treatments prior to taking measurements. The first fully expanded, mature leaf was chosen for measurements, and the plastochron index was as follows: leaf 3-4 for poplar, leaf 7-9 for willow, and leaf 9-11 for hybrid willow. The LI-COR chamber settings were set to the following: LEDs were set to deliver 1000 μmol m^{-2} s^{-1} quantum, relative humidity was 50-60%, sample chamber CO_2 was 400 ppm, chamber temperature was set at 23° C, and the flow rate was set to 500 μmol/s. The sample chamber enclosed the majority of the leaf and was permitted to stabilize for 9 minutes before taking readings. All measurements were taken between the hours of 8:00 and 13:00. Net photosynthetic rate (A), stomatal conductance (g_s),

intercellular CO_2 (C_i), and transpiration rate (E) were quantified. Instantaneous water use efficiency (WUE) was calculated using the following formula:

$$WUE = A/E$$

The ratio of variable to maximum chlorophyll fluorescence (Fv/Fm) was measured with an OS-30p Chlorophyll Fluorometer (OPTI-Sciences, Hudson, NH, USA) concurrently with gas exchange using the same plastochron index. Leaves were allowed to adapt to darkness for a minimum of 15 minutes using light-blocking clips before measurements were taken.

2.2.3 Tissue sampling and biomass harvest

All trees were harvested over the course of 9 days between the hours of 10:00 and 14:00. The first three youngest leaves and three fully mature leaves were removed, weighed, wrapped in tin foil, and frozen in liquid nitrogen. Stems were separated from roots by making a cut between the original cutting and the new growth. The shoot was weighed in its entirety, then stripped of leaves and weighed again. Bark was removed from the first 4 centimeters from the base of the shoot, weighed, deposited in a 2 mL cryotube, and stored in liquid nitrogen. Xylem scrapings were subsequently taken from the bare stem section, weighed, placed in 2 mL cryotubes, and stored in liquid nitrogen. The remainder of the fresh leaves and bark was stored in paper bags.

The fresh weight of the stems were recorded and subsequently labelled and stored for future dry weight recording.

Roots were removed from the pots and vigorously shaken to remove the bulk of the growth media. The roots were then washed in warm, followed by cold water to remove as many growth media particles as possible. Approximately 3 g of the outermost root tissue was removed, dabbed dry with paper towel, weighed, wrapped in tin foil, and stored in liquid nitrogen. The remaining root tissue was left to drip dry for approximately 20 minutes, and fresh weight was recorded. Roots were subsequently stored in labelled paper bags for future dry weight recording.

The biomass samples that had been stored in liquid nitrogen were transferred into a -80°C freezer for long term storage. The remaining leaf and root biomass were dried at 60°C for two days, while the stems were dried at room temperature for 1 month. After drying, all tissue was weighed.

2.2.4 Elemental analysis

Three replicates from all genotypes and treatments were randomly selected for tissue elemental analysis of both leaves and roots. Dried leaves from an individual tree were pooled and ground into a fine powder using a Black and Decker© coffee grinder. Dried root tissue from the same

replicates was individually pooled, rough ground using a Wiley mill, then finely ground into a powder utilizing a 2010 SPEX Sample Prep Geno/Grinder (Metuchen, NJ, USA) at 1500 RPM for 90 seconds. About 1.5-2 grams of leaf and root powder were sealed in labelled plastic snap-cap vials and sent to AGVISE Laboratories (Northwood, ND, USA) for inductively coupled plasma mass spectrometry analyses (Havlin and Soltanpour, 1980). The following elements were quantified in percentages: N, P, K, S, Ca, Mg, Na, and Cl. The following elements were quantified in ppm: Zn, Fe, Mn, Cu, and B. Additionally, Pb and Ni were quantified for the fracking wastewater trial.

2.2.5 Non-structural carbohydrate determination

Non-structural carbohydrates were quantified employing methodology developed in the Mansfield Laboratory at the University of British Columbia (Da Ros, 2018). Soluble sugars were analyzed on three replicates for each genotype and treatment, chosen randomly. Frozen root and mature leaf tissues (see plastochron index in section 2.3.1) were ground in a Geno/Grinder using liquid nitrogen at 1500 RPM for 90 seconds. Ground tissues were wrapped in tin foil and freeze dried using a Labconco FreeZone 4.5 freeze drier for 24 hours. Fifty microliters of 10 mg/mL galactitol internal standard was added to 50 and 40 milligrams of freeze-dried root and leaf tissue, respectively. The tissues were then suspended in 4 mL of 12:5:3 methanol:chloroform:water (MCW) solution, and left to extract overnight at +4°C.

After overnight extraction, solutions were centrifuged at 6000 RPM for 10 minutes, the supernatant collected, and the pellet was resuspended in 4 mL of MCW solution. These steps were repeated twice more for a total of 12 mL supernatant, while the remaining tissue was dried at 55 °C and stored for starch determination. Five milliliters of NanoPure water was added to the supernatant, vortexed, and centrifuged for 6 minutes at 4000 RPM. Eight to ten milliliters of the uppermost aqueous layer was collected and the organic layer was disposed. Two milliliters of the solution was then vacuum centrifuged overnight. The resulting pellet was resuspended in 1 mL of NanoPure water and filtered using 4.5 µm filters into glass vials for high-performance liquid chromatography (HPLC) analyses.

Non-structural carbohydrates were quantified utilizing a Dionex CarboPac PA1 guard and column via HPLC with pulsed amperometric detection (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The HPLC program was set to the following conditions: temperature at 30 °C, flow rate at 0.7 mL/min, and the injection volume was 15 µL. Sugars were separated from 0 - 44 minutes using an eluent comprised of 8% 0.2M sodium hydroxide, 82% degassed NanoPure water, and 10% 20 mM sodium acetate. The column was washed in 100% 0.2 M sodium hydroxide from 44 – 64 minutes.

2.2.6 Starch analysis

Five milliliters of 4% sulfuric acid was added to 25-50 mg of dried residual tissue from the soluble sugar extraction and autoclaved for 210 seconds. The reaction was then centrifuged at 500 RPM after cooling, and the supernatant collected. The samples were prepared for HPLC by adding 50 µL of fucose stock (10 mg/mL) as internal standard into 950 µL of sample and passing them through a 4.5 µm filter. Samples were run against glucose standards.

Starch quantification was achieved using a Dionex CarboPac PA1 guard and column via HPLC with pulsed amperometric detection. The mobile phase was 100% NanoPure water from 0 – 35 minutes at a flow rate of 1 mL/minute. The column was washed with 1 M sodium hydroxide from 44 – 64 minutes.

2.3 Statistical analyses

All statistics were calculated using R, version 3.3.1. Linear mixed-effect models were fitted to test response variables, with ‘treatment’ as a fixed effect, and ‘replicate’ as a random effect. Comparisons at the species level were made only in Experiment 1 to compare height and growth among treatments, and in Experiment 2 to compare growth patterns in control treated height and biomass; all other comparisons among treatments were examined at the genotype level.

All p-values were adjusted using the Bonferroni method, with α set to 0.05. Type (III) ANOVA was used to test for interactions between means, and Wilks-Shapiro tests were used to test normality.

Chapter 3: Results

3.1 Screening trial

Seventeen native balsam poplar and fifteen native willow genotypes were grown for eight weeks while being subjected to either a control (0) treatment, or 30 or 80 mM NaCl solution supplemented with fertilizer solution (Tables 2.1 and 2.2).

3.1.1 Mortality

After eight weeks of treatment, poplar genotypes generally displayed approximately 66% mortality at 30 mM NaCl (Figure 3.1) and 100% mortality at 80 mM NaCl, whereas willow genotypes showed no mortality at 30 mM NaCl and only ~7% mortality at 80 mM NaCl. Poplar genotype LR-5 exhibited the greatest survivorship after 8 weeks of 30 mM NaCl treatment, while genotypes FN-1, FN-5, and WL-1 suffered complete mortality after six weeks of treatment. Only two willow genotypes, LR-1 and PAL-4, experienced mortality at the conclusion of the highest salinity treatment.

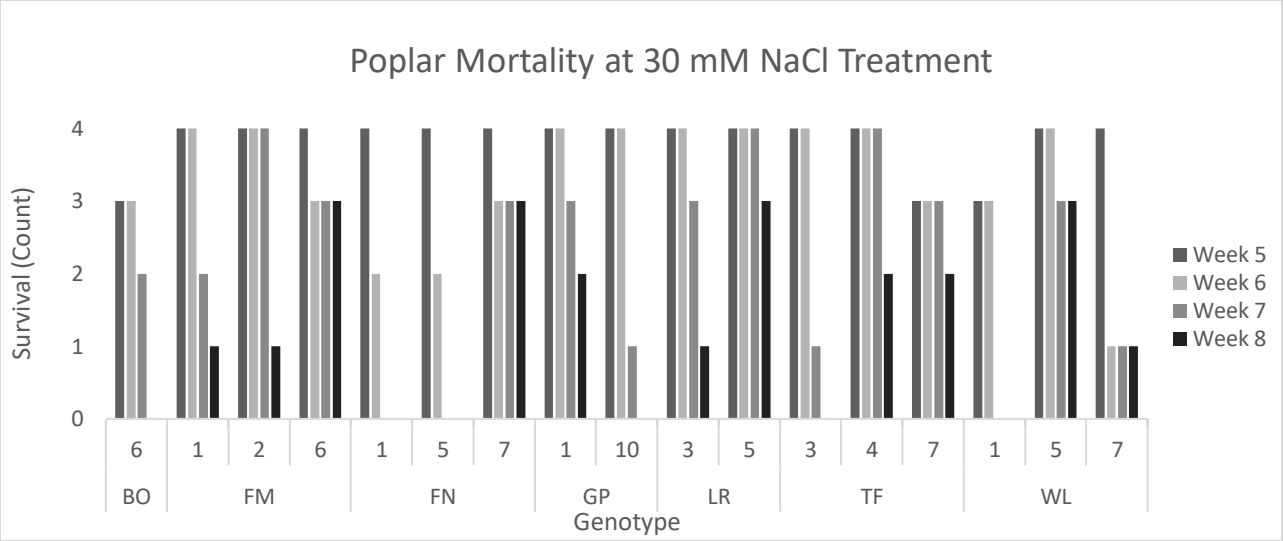
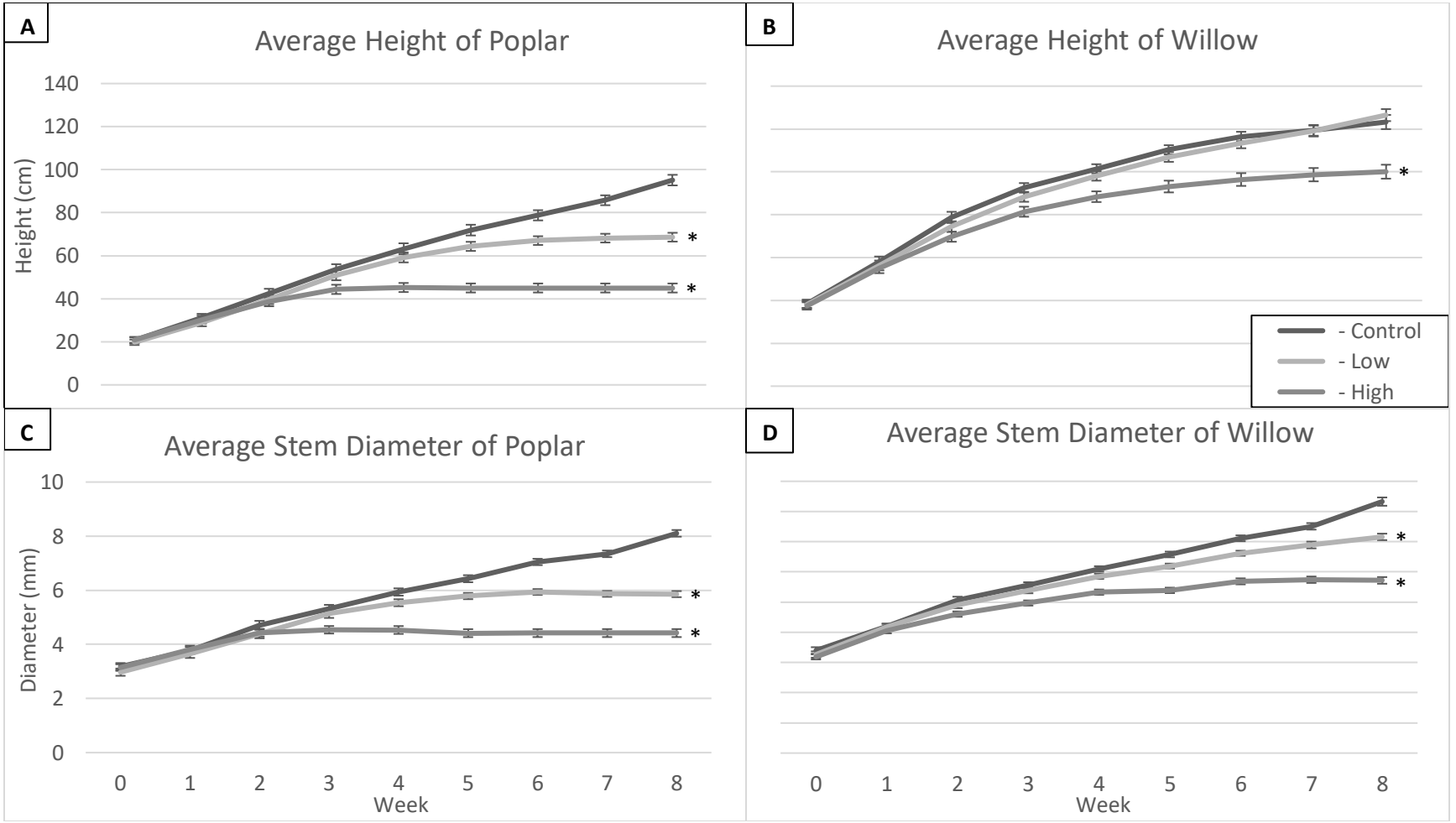


Figure 3.1: Poplar mortality at 30 mM NaCl treatment after eight weeks of treatment (n=4 for each genotype and treatment).

3.1.2 Height and diameter

Willow genotypes grew, on average, taller with greater stem diameters in all treatments over the 8-week growth trial compared to poplar genotypes grown under similar conditions (Figure 3.2A and B). Although willow genotypes generally exhibited a significant reduction in height growth at 80 mM NaCl compared to control treatments, they did not appear to experience significant growth retardation at 30 mM NaCl ($p < 0.05$). Poplar clearly showed a significant reduction in height (27.9% and 52.7%) at low and high salinity compared to control treatment, respectively (Figure 3.2A). Willows, on average, displayed a 2.6% increase and 18.8% decrease at low and high salinity treatments, respectively (Figure 3.2B). Stem diameter was affected by salinity to a lesser extent than height in poplar, with decreases of 27.7% and 45.5% at low and high salinity, respectively (Figure 3.2C). Willow stem diameter was more susceptible to salinity stress than

height, displaying decreases of 14.0% and 31.3% at 30 and 80 mM NaCl treatments, respectively (Figure 3.2D). At the highest salinity treatment, both height and diameter diverged from the control and low treatments between weeks two and three in both poplar and willows. However, with low salinity treatment, poplar height and diameter decreased earlier than willows compared to control treatment.



Figures 3.2: Weekly height (A, B) and stem diameter growth (C, D) for poplar and willow genotypes, averaged by species (+/- SEM). Control, low, and high treatments are 0, 30, and 80 mM NaCl supplemented with fertilizer solution, respectively (n=4 for each genotype and treatment). Asterisks represent statistical significance from control treatments at the termination of the growth trial ($p < 0.05$).

3.2 Experiment 2

Two native balsam poplar, five native willow, and one hybrid willow genotypes were selected and grown for 12 weeks while being treated with either a control (0) treatment, or 20, 40, or 60 mM NaCl solution supplemented with fertilizer solution (Tables 2.1, 2.2 and 2.3).

3.2.1 Mortality

Willow and hybrid willow genotypes showed no mortality over 12 weeks of treatment at 0, 20, 40, and 60 mM NaCl. Of the two poplar genotypes tested, GP-10 had 11.1% mortality at 20 and 40 mM NaCl at the conclusion of the trial, while both GP-10 and LR-5 experienced complete mortality at 60 mM NaCl. Poplar mortality was first recorded between weeks four and six of treatment.

3.2.2 Height and diameter

Control treated poplar, willow, and hybrid willow trees varied significantly from one another in both height and diameter at the conclusion of the trial ($p < 0.05$). Willow trees had the highest average height growth at all treatments, followed closely by the hybrid willows (Figure 3.3). Additionally, hybrid willow trees had thicker stem diameters than both poplar and willow genotypes in all treatments (Figure 3.4). Height growth rate declined over time in all genotypes, beginning to plateau after eight weeks of growth.

Poplar experienced significant reductions in stem diameter at all treatments and showed diminished height at 40 and 60 mM NaCl; GP-10 was more susceptible to growth reduction that LR-5. The hybrid willow displayed significant reduction in height with the highest salinity treatment at the conclusion of the trial, but diameter was reduced at both the 40 and 60 mM NaCl treatments ($p < 0.05$). Willow suffered significant reductions in both average height and stem diameter at all treatment levels, but genotypic response varied.

Both native and hybrid willow trees had between 12 - 17% reductions in height at 60 mM NaCl following 12 weeks of treatment; however, willow genotype LR-3 experienced an increase in average height at both 40 and 60 mM NaCl compared to the control treatment. The average height of poplar at the conclusion of the highest salt treatment suffered significantly, with a reduction of 52.7% and 60.0% for GP-10 and LR-5, respectively (Figure 3.3). Average stem diameter varied more than height, with willow and the hybrid willow genotypes experienced reductions of 17 – 38% at 60 mM NaCl at week 12 of treatment, while poplars GP-10 and LR-5 had reductions of 59.8 and 55.9%, respectively (Figure 3.4).

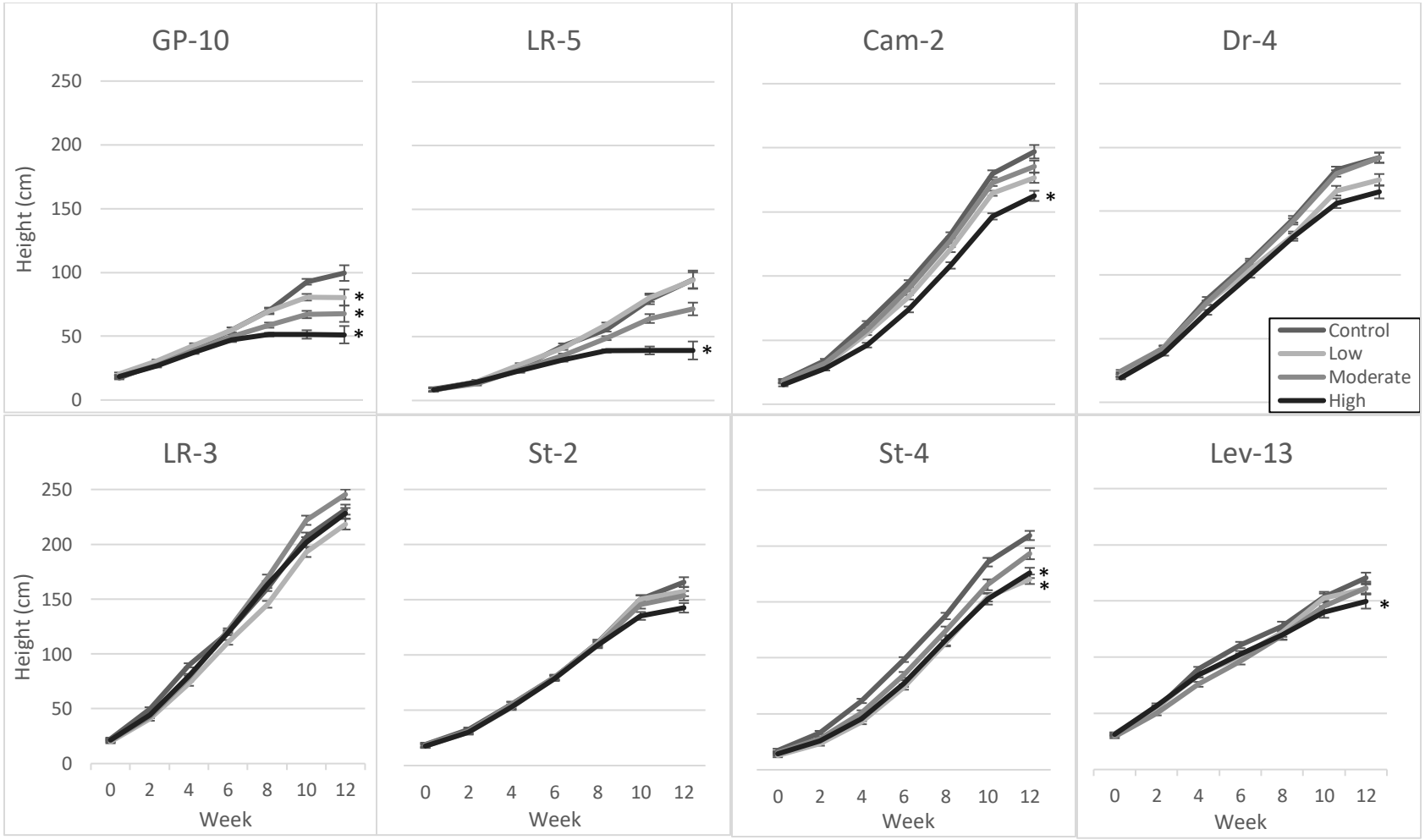


Figure 3.3: Biweekly average height (+/- SEM) of the eight poplar, willow, and hybrid willow genotypes. Control, low, moderate, and high treatments represent 0, 20, 40, and 60 mM NaCl, respectively (n=9 for each genotype and treatment). Asterisks denote significance from control treatment at the conclusion of the trial ($p < 0.05$).

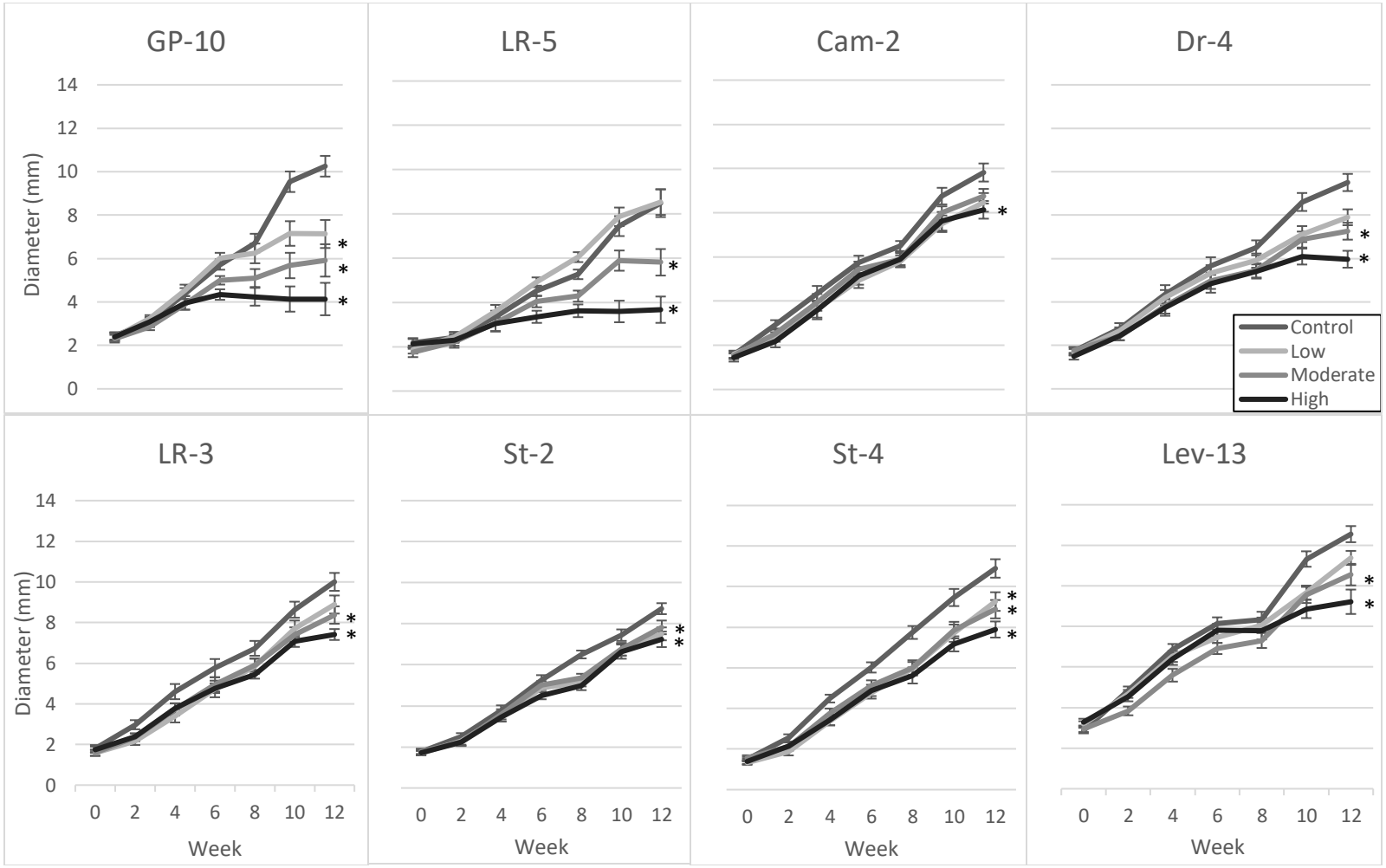


Figure 3.4: Biweekly average stem diameter (+/- SEM) of the eight poplar, willow, and hybrid willow genotypes. Control, low, moderate, and high treatments represent 0, 20, 40, and 60 mM NaCl, respectively (n=9 for each genotype and treatment). Asterisks denote significance from control treatment at the conclusion of the trial ($p < 0.05$).

3.2.3 Dry biomass

The hybrid willow trees had larger average dry biomass across all treatments compared to native poplar and willow species; despite similar heights, control hybrid willow trees had more than double the total average biomass compared to control poplar trees (Table 3.1A). Dry leaf mass differed significantly ($p < 0.05$) at all treatments in poplar genotypes compared to control treatment. A high degree of genotypic variation was observed in leaf biomass of willows with increasing salinity treatments (Table 3.1B). Willow genotypes Cam-2, LR-3, and St-2 did not have a statistically significant reduction in stem mass up to 40 mM NaCl, nor did poplar genotype LR-5 ($p < 0.05$). Poplar genotypes experienced immediate loss of root biomass with increasing salinity, while willow and hybrid willow genotypes Cam-2 and Lev-13 did not experience significant root biomass loss until the moderate (40 mM NaCl) treatment.

Poplar trees suffered the greatest reduction in total dry stem biomass at the highest treatment application, with up to 95% less biomass compared to control (Fig 3.1C). Even at the lowest salinity treatment, poplar genotypes experienced a 55% reduction in dry biomass. The total average dry stem and leaf biomass of poplar genotypes LR-5 and GP-10 differed significantly ($p < 0.05$) from one another under the control treatments. LR-5 was also more resistant to biomass reduction than GP-10, having a decrease of 33% compared to that of GP-10, which displayed a 65% reduction. Root biomass of the poplar genotypes treated with salt were all significantly reduced from control, but were not significantly different from one another (Fig 3.1D).

Total biomass, leaf, stem, and root masses of willow genotypes all decreased significantly with the highest salinity treatment. More specifically, average stem biomass had the greatest variability among control treated willow genotypes. Willow genotypes Cam-2 and St-2 had the smallest reduction (39.9% and 32.7%, respectively) in dry biomass at the highest salinity treatment compared to their corresponding controls, while Dr-4 had the greatest reduction (74.5%).

Table 3.1: Average total dry biomass (A), leaf (B), stem (C), and root biomass (D) of poplar, willow, and hybrid willow genotypes. Control, low, moderate, and high treatments represent 0, 20, 40, and 60 mM NaCl, respectively (n=9 for each genotype and treatment). Superscripts indicate significant differences between treatments ($p < 0.05$).

A — Total Dry Biomass (g)

Species	Genotype	Control	Low	Moderate	High
Poplar	GP-10	47.1^{a}	16.4^{b}	9.9^{bc}	2.3^{c}
	LR-5	23.8^{a}	15.9^{b}	8.3^{bc}	0.9^{c}
Willow	Cam-2	64.1^{a}	45.9^{ab}	49.5^{b}	38.5^{b}
	Dr-4	61.9^{a}	49.8^{ab}	36.9^{b}	15.8^{c}
	LR-3	71.2^{a}	54.7^{a}	48.5^{ab}	31.8^{b}
	St-2	46.5^{a}	40.1^{ab}	39.4^{ab}	31.3^{b}
	St-4	72.7^{a}	44.5^{b}	43.2^{bc}	28.7^{c}
Hybrid	Lev-13	90.6^{a}	67.2^{b}	53.7^{bc}	42.6^{c}

B — Dry Leaf Biomass (g)

Species	Genotype	Control	Low	Moderate	High
Poplar	GP-10	25.8^{a}	9.0^{b}	5.6^{bc}	1.1^{c}
	LR-5	12.8^{a}	8.6^{b}	3.3^{c}	0.3^{c}
Willow	Cam-2	21.8^{a}	14.8^{ab}	17.2^{b}	14.2^{b}
	Dr-4	20.3^{a}	17.9^{a}	13.2^{b}	5.4^{c}
	LR-3	18.2^{a}	14.1^{a}	12.4^{ab}	7.3^{b}
	St-2	15.7^{a}	14.8^{a}	14.7^{a}	12.8^{a}
	St-4	24.1^{a}	15.5^{b}	13.4^{b}	9.2^{c}
Hybrid	Lev-13	35.7^{a}	23.9^{b}	20.3^{b}	18.7^{b}

C — Dry Stem Biomass (g)

Species	Genotype	Control	Low	Moderate	High
Poplar	GP-10	10.6^{a}	3.6^{b}	2.5^{b}	1.1^{b}
	LR-5	6.7^{a}	5.5^{ab}	3.0^{ab}	0.4^{b}
Willow	Cam-2	26.3^{a}	20.0^{a}	21.4^{ab}	14.7^{b}
	Dr-4	21.2^{a}	17.4^{ab}	15.5^{b}	6.2^{c}
	LR-3	28.6^{a}	24.6^{a}	29.1^{ab}	18.2^{b}
	St-2	19.0^{a}	15.0^{ab}	14.4^{ab}	11.6^{b}
	St-4	27.8^{a}	16.2^{b}	19.6^{b}	13.7^{b}
Hybrid	Lev-13	29.7^{a}	23.7^{b}	20.7^{b}	12.8^{c}

D — Dry Root Biomass (g)

Species	Genotype	Control	Low	Moderate	High
Poplar	GP-10	4.6^{a}	0.7^{b}	0.5^{b}	
	LR-5	2.1^{a}	0.7^{b}	0.3^{b}	
Willow	Cam-2	8.4^{a}	6.6^{ab}	6.5^{ab}	4.7^{b}
	Dr-4	8.7^{a}	5.1^{b}	3.3^{bc}	1.3^{c}
	LR-3	12.4^{a}	8.1^{ab}	6.2^{b}	3.7^{b}
	St-2	7.6^{a}	4.9^{b}	4.3^{b}	2.6^{b}
	St-4	10.8^{a}	5.5^{b}	4.6^{bc}	2.4^{c}
Hybrid	Lev-13	11.3^{a}	11.1^{a}	8.6^{a}	4.1^{b}

3.2.4 Gas exchange

At week six of the salinity treatments, control and treated poplar, willow, and hybrid willow genotypes had significantly different ($p < 0.05$) rates of net photosynthesis, transpiration, and water-use efficiency (WUE). Average net photosynthetic and transpiration rates decreased with treatment, while WUE increased (Figures 3.5 and 3.6). Net photosynthetic rate decreased significantly with increasing salinity in willow genotypes Dr-4 and St-4, but not in the poplar or hybrid willow genotypes (Figure 3.5A). Transpiration decreased more than photosynthesis, with average transpiration decreasing dramatically in Cam-2, Dr-4, and St-2 compared to the control treatment (3.5C). Consequently, average WUE increased with all treatments at week six compared to control, significantly in willow genotypes Dr-4 and St-2 ($p < 0.05$). Intercellular CO_2 decreased with salinity treatment; significantly at low, moderate, and high salinities in GP-10, while willow genotypes St-2 and St-4 were only affected at high treatments, and at low and high treatments in the hybrid willow genotypes. Intercellular CO_2 in the hybrid genotype varied greatly at the moderate treatment level, resulting in no significant difference from control.

Comparing weeks six and ten of the treatment, net photosynthesis and transpiration were significantly higher at week ten regardless of treatment (including control and salinity treatment), whereas no significant differences were observed in WUE (Figure 3.5 and 3.6). The hybrid willow genotype Lev-13 had significant differences in net photosynthesis, transpiration, intercellular CO_2 and WUE at week 10 of the salinity treatments ($p < 0.05$). Photosynthetic rate did not differ among treatments at week 10 of the treatment compared to week six, and only Cam-2 and Lev-13 displayed significant reductions with treatment (Figure 3.5B). Average

intercellular CO_2 was lower at week 10 of treatment compared to week six, and was significantly lower in St-2 and St-4 at the highest salinity treatment (Figures 3.6A and 3.6B).

At week six of the treatment, willow genotypes Cam-2 and St-2 had the greatest change in transpiration rate at 20 mM NaCl compared to control treatments, displaying a 29.5% and 49.1% reduction, respectively (Figure 3.5C). Cam-2 and St-2 also had the greatest percent increase in WUE with increasing treatment (Figure 3.6A). At week ten of the treatment, the hybrid genotype Lev-13 had the greatest reduction in photosynthesis and transpiration at 60 mM NaCl, and the greatest WUE.

Dark adapted chlorophyll fluorescence (Fv/Fm) was measured utilizing the first mature leaf for each genotype and treatment, and no differences were observed: all genotypes at all treatments measured between 0.78 and 0.83.

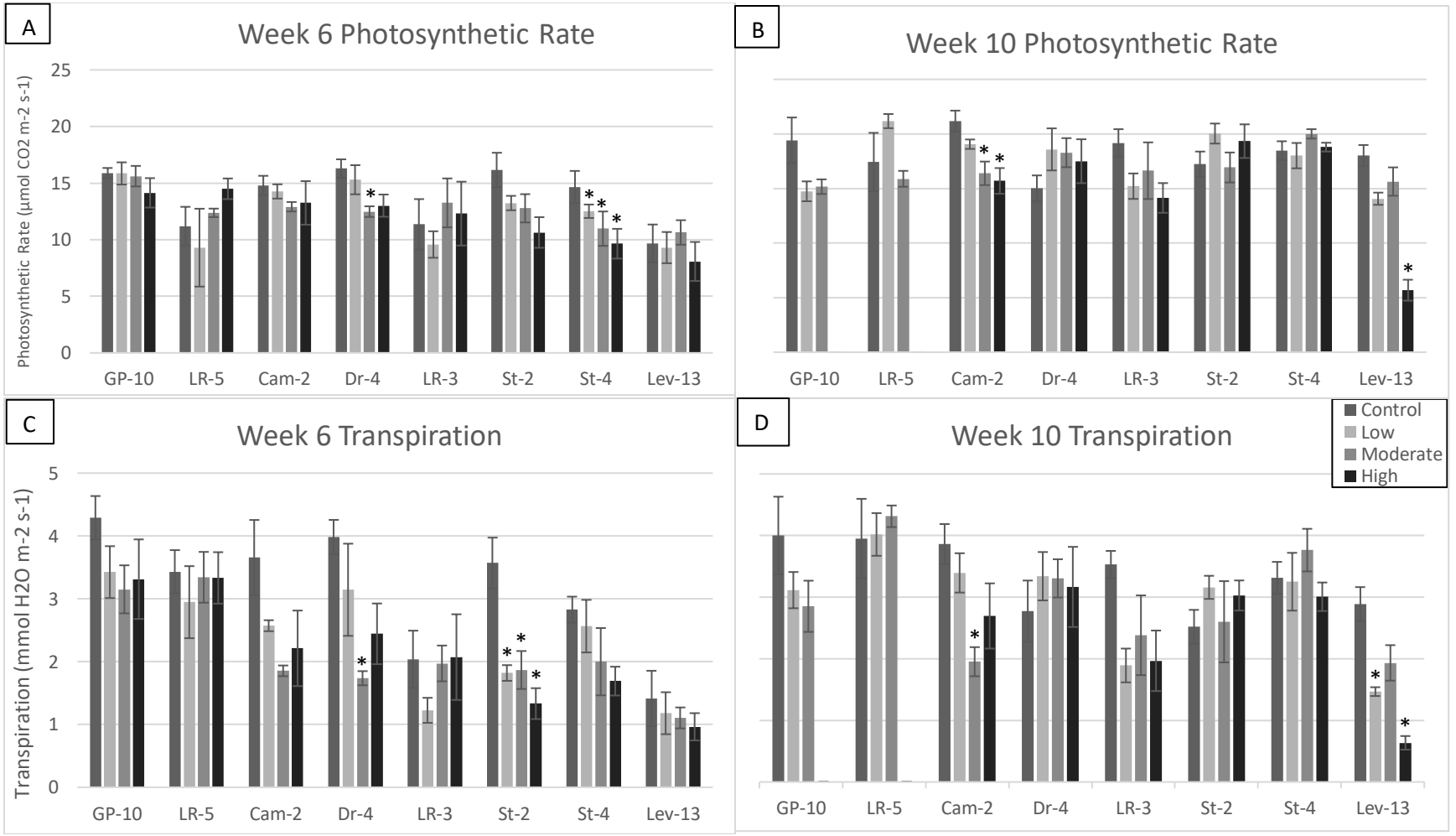


Figure 3.5: Photosynthetic (A, B) and transpiration rates (C, D) (+/- SEM) of poplar, willow, and hybrid willow genotypes at weeks 6 and 10 of treatment. Control, low, moderate, and high treatments represent 0, 20, 40, and 60 mM NaCl, respectively (n=5 for each genotype and treatment). Asterisks represent significant difference from control treatments ($p < 0.05$).

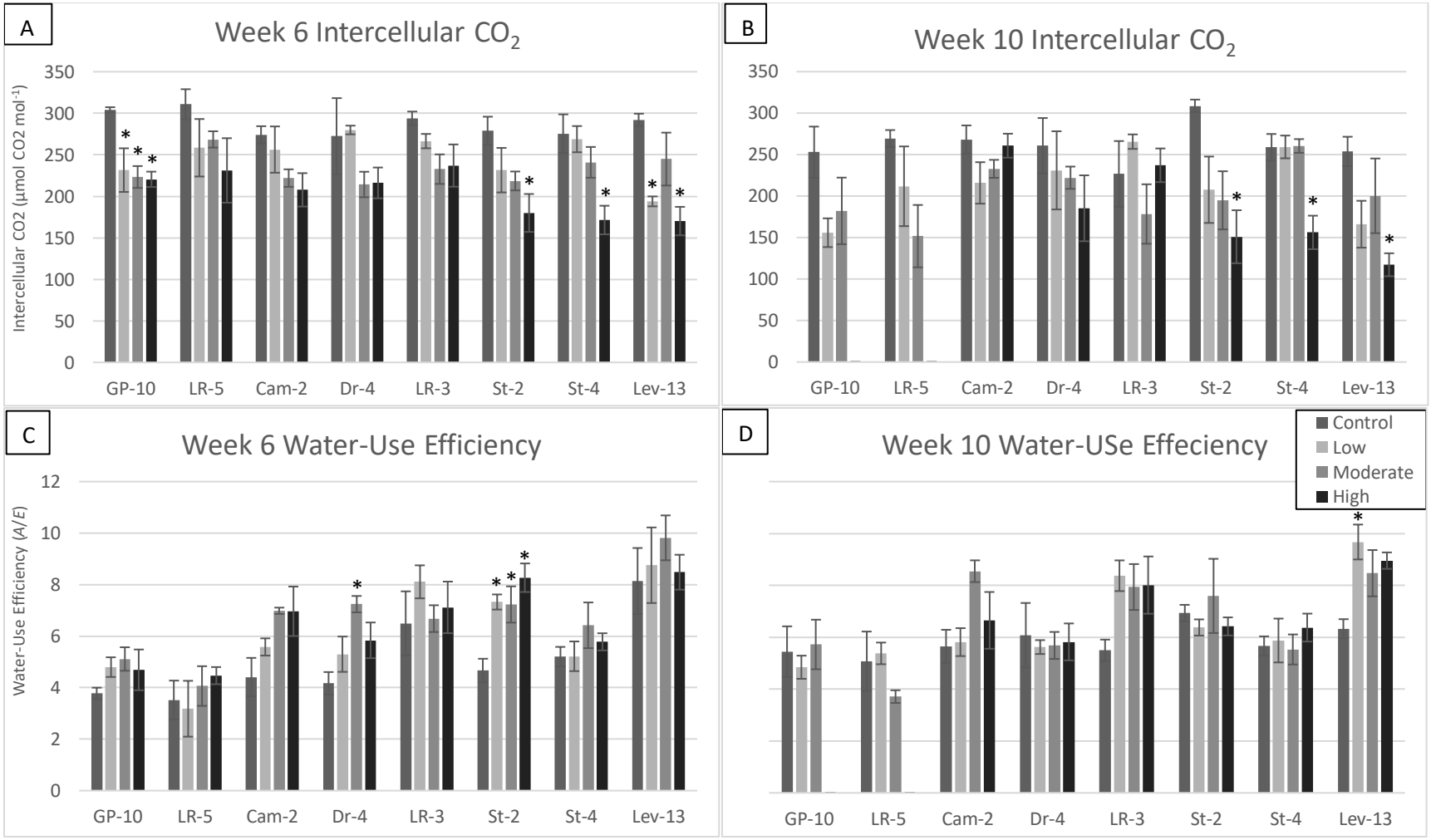


Figure 3.6: Assimilation rate (A, B) and instantaneous water-use efficiency (C, D) (+/- SEM) of poplar, willow, and hybrid willow genotypes at weeks 6 and 10 of treatment. Control, low, moderate, and high treatments represent 0, 20, 40, and 60 mM NaCl, respectively (n=5 for each genotype and treatment). Asterisks represent significant difference from control treatments ($p < 0.05$).

3.2.5 Compatible solutes

There was little statistical significance found in the sugar content of poplar, willow, and hybrid willow leaves with increasing salinity treatment: stachyose increased significantly in poplar genotype GP-10, and fructose decreased significantly in willow genotype St-4 ($p < 0.05$; Tables 3.2 and 3.3). An average increase in leaf sucrose was observed in poplar leaves, 35% and 28% in GP-10 and LR-5, respectively. Genotypic variation was prevalent between sugar content of willow leaves, but stachyose rose consistently amongst all willow genotypes with increasing salinity.

In poplar roots, significant increases were seen in myo-inositol and glucose concentration in GP-10, and raffinose in LR-5 in response to salinity treatments ($p < 0.05$; Table 3.2). Genotypic variation was less pronounced in willow roots than in leaves, as myo-inositol, sucrose, and raffinose content increased with salinity amongst all genotypes (Table 3.4). Hybrid willows displayed no significant differences in sugar content in leaf or root tissues, and starch did not differ significantly in any genotype with salinity treatment.

Table 3.2: Soluble sugar and starch contents in poplar leaf and root tissues. Control and moderate treatments are 0 and 40 mM NaCl, respectively (n=3 for each genotype and treatment). Asterisks denote significant difference from control treatments ($p < 0.05$).

Poplar Leaf Tissue

Control

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Poplar	GP-10	7.8	13.4	49.9	6.6	1.4	0.7	1.6
	LR-5	11.6	30.6	83.6	9.5	0.6	1.3	1.2

Moderate

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Poplar	GP-10	12.7	11.4	67.6	7.9	1.1	3.2*	2.2
	LR-5	15.5	30.9	107.3	14.2	1.3	6.6	1.0

Poplar Root Tissue

Control

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Poplar	GP-10	1.2	4.3	23.1	2.9	0.8	0.1	1.3
	LR-5	0.8	6.1	21.1	6.9	0.4	0.1	1.4

Moderate

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Poplar	GP-10	3.1*	8.0*	22.4	3.7	0.5	0.2	1.4
	LR-5	1.7	8.2	33.2	2.9	1.4*	0.6	2.1

Table 3.3: Soluble sugar and starch contents in willow and hybrid willow leaf tissues. Control and high treatments are 0 and 60 mM NaCl, respectively (n=3 for each genotype and treatment). Asterisks denote significant difference from control treatment ($p < 0.05$). One sample outlier was removed for Dr-4 glucose in the high fracking wastewater treatment.

Willow Leaf Tissue

Control

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Willow	Cam-2	13	8.1	78.1	10.1	2.2	1.2	6.3
	Dr-4	7.0	3.6	50.3	2.7	1.7	0.9	4.5
	LR-3	8.2	9.4	43.1	12.2	0.9	0.5	6.0
	St-2	7.2	6.9	43.8	6.9	1.3	0.4	4.6
	St-4	7.3	6.7	59.6	7.7	3.4	0.6	7.6
Hybrid	Lev-13	10.9	11.7	48.8	10.7	0.8	1.8	9.0

High

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Willow	Cam-2	8.7	7.0	61.5	8.1	1.2	3.6	7.4
	Dr-4	12.3	9.9	65.7	10.1	1.0	3.0	6.7
	LR-3	11.7	14.7	61.2	17.6	0.9	2.5	6.7
	St-2	7.9	4.4	43.7	3.9	0.9	1.5	5.1
	St-4	6.8	4.1	39.9	3.3*	1.0	1.5	10.3
Hybrid	Lev-13	9.0	10.4	53.7	9.0	0.8	1.1	8.6

Table 3.4: Soluble sugar and starch content in willow and hybrid willow root tissue. Control and high treatments are 0 and 60 mM NaCl, respectively (n=3 for each genotype and treatment). Asterisks denote significant difference from control treatment ($p < 0.05$).

Willow Root Tissue

Control

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Willow	Cam-2	0.7	2.9	15.0	2.5	0.4	<0.1	3.6
	Dr-4	0.6	2.4	22.8	2.4	0.6	0.4	3.5
	LR-3	0.3	2.9	16.4	3.2	0.3	<0.05	2.2
	St-2	0.8	2.1	19.6	1.9	0.7	0.1	4.2
	St-4	1.3	3.2	19.8	4.0	0.7	0.1	1.7
Hybrid	Lev-13	2.0	3.7	27.6	4.2	0.8	0.1	3.7

High

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Willow	Cam-2	3.2*	3.1	31.7*	3.1	1.2*	0.1	6.1
	Dr-4	1.5	3.1	25.1	2.0	1.6	0.1	1.1
	LR-3	2.5	3.4	35.3	3.9	2.4*	0.3	2.1
	St-2	2.9	3.1	34.6*	3.5	1.7	0.2	2.1
	St-4	1.9	2.4	24.1	2.5	1.0	<0.05	1.6
Hybrid	Lev-13	2.5	2.6	23.5	2.5	0.7	<0.05	1.5

3.2.6 Elemental analysis

Poplar genotype GP-10 accumulated ten times as much sodium in its leaves than LR-5 (Figure 3.7A). Hybrid willow genotype Lev-13 did not accumulate significant quantities of sodium in leaf tissues at any treatment, whereas poplar and willow genotypes did at the highest salinity treatment. Generally, sodium is stored in significantly greater quantities in root tissues compared to leaves; hybrid genotype Lev-13 stored the greatest amount of sodium in its roots, with a 150% increase at the highest salinity treatment compared to the corresponding control treatment (Figure 3.7B). Chloride levels in both leaf and root tissues were significantly higher under all salinity treatments compared to control-treated trees ($p < 0.05$), and reached as much as 5% of the dry leaf content in Dr-4, while LR-3 accumulated (3%) the least (Figure 3.7C and 3.7D).

Average potassium levels increased in the leaves and decreased in root tissues, except in poplar leaves, where potassium content remained constant (Figure 3.8A and 3.8B). Willow leaf potassium content increased significantly under the highest salinity treatments compared to control, in LR-3 (Figure 3.8C). Leaf potassium in Lev-13 and Dr-4 increased significantly at both the moderate and severe treatments ($p < 0.05$). The hybrid willow genotype displayed an immediate, significant drop in root potassium concentration at low salinity, decreasing by 45% compared to the corresponding control trees. Increases in leaf calcium concentration were significant in only Cam-2, but root calcium content was consistent among all genotypes, except St-4, which experienced significant reduction at the highest salinity treatment (Figure 3.8D).

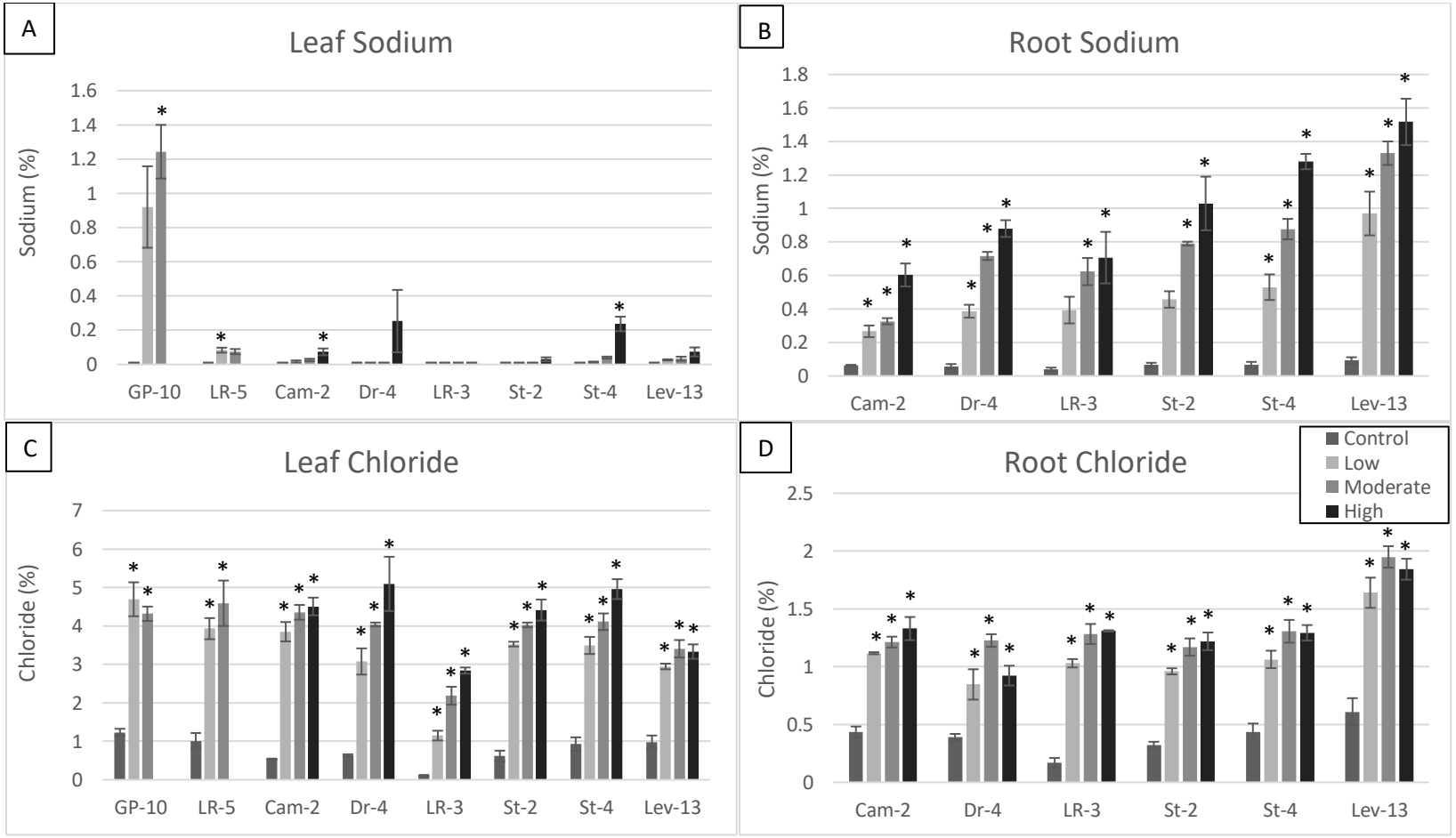


Figure 3.7 Sodium (A, B) and chloride (C, D) content of leaf (A, C) and root (B, D) tissues (+/- SEM) in poplar, willow, and hybrid willow genotypes. Poplar root tissue is omitted due to lack of tissue resulting from higher mortality rates. Control, low, moderate, and severe treatments represent 0, 20, 40, and 60 mM NaCl, respectively (n=3 for each genotype and treatment). Asterisks denote significant difference from control treatment ($p < 0.05$).

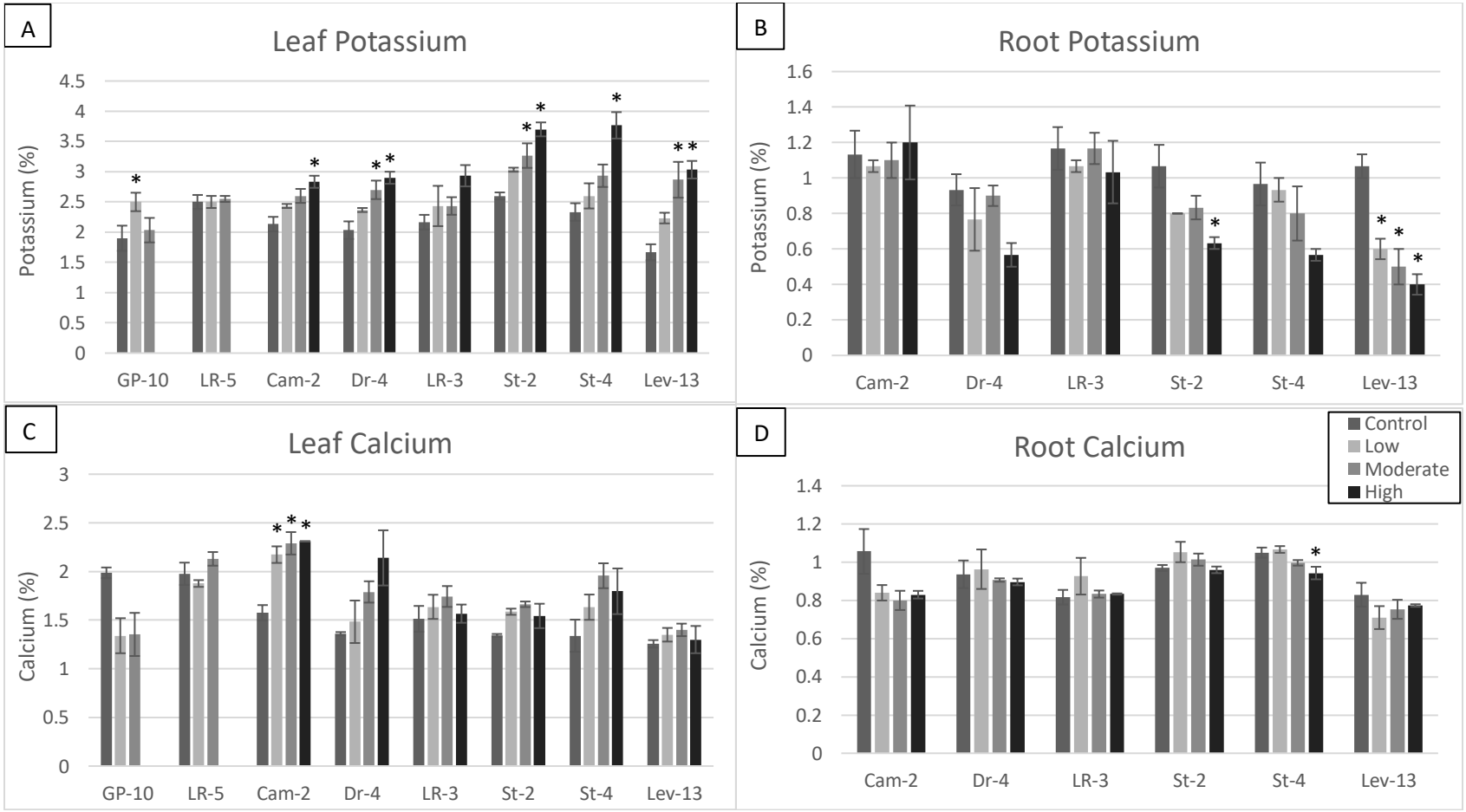


Figure 3.8 Potassium (A, B) and calcium (C, D) content of leaf (A, C) and root (B, D) tissues (+/- SEM) in poplar, willow, and hybrid willow genotypes. Poplar root tissue is omitted due to lack of tissue resulting from higher mortality rates. Control, low, moderate, and severe treatments represent 0, 20, 40, and 60 mM NaCl, respectively (n=3 for each genotype and treatment). Asterisks denote significant difference from control treatment ($p < 0.05$).

3.3 Fracking trial

Three native willow and one hybrid willow genotypes were grown for eight weeks while being treated with either a control (no), low, or high concentrations of fracking solution supplemented with fertilizer solution (Tables 2.1 and 2.3).

3.3.1 Mortality

The three willow genotypes showed complete survival after eight weeks treatment with fracking wastewater, regardless of concentration. The hybrid willow genotype, however, experienced complete mortality with the high fracking treatment at the termination of the growth trial.

3.3.2 Height and diameter

Both the native and hybrid willow genotypes had significantly reduced height and diameter growth when subjected to the high fracking treatments compared to the corresponding control treatment, but no effects were apparent at low treatments except in St-2 (Figure 3.9). Cam-2 and Lev-13 both had the largest reduction in height at the high fracking treatment, displaying 34.7% and 34.5% reductions respectively, while LR-5 and St-2 were reduced by 29.0% and 29.8%, respectively. Stem diameter was reduced by 33% at the highest treatment in all genotypes except St-2, which experienced a 23.4% reduction.

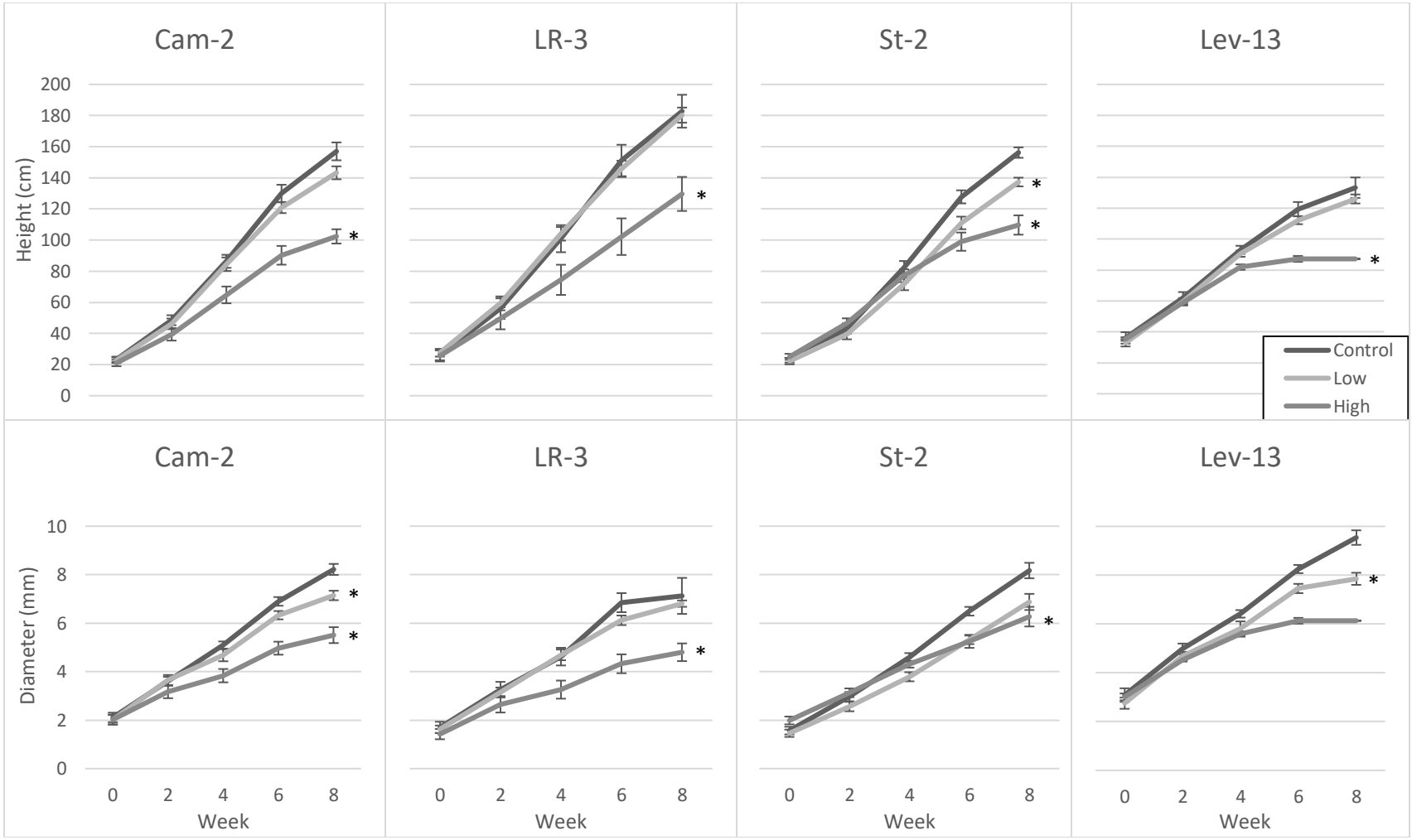


Figure 3.9: Height and stem diameter of native and hybrid willow genotypes (+/- SEM) at control, low, and high fracking treatments over an eight week period (n=8 for each genotype and treatment). Asterisks represent singificance from control treatments ($p < 0.05$).

3.3.3 Dry biomass

All four genotypes experienced significantly diminished leaf, stem, root, and average total biomass (Table 3.5) at the highest fracking wastewater treatment compared to their control treatments ($p < 0.05$). The hybrid willow Lev-13 gained the greatest biomass over eight weeks in the control treatment, but was surpassed by native willow genotypes with fracking treatment. LR–5 had the highest reduction in total biomass at the high fracking treatment, with an 80% loss compared to control trees, while St–2 had the smallest biomass reduction (58.3%). Leaf sensitivity varied greatly between genotypes; St-2 displayed a 50% reduction in leaf biomass while LR-5 had a 83% reduction.

Table 3.5: Average total dry biomass (A), leaf (B), stem (C), and root (D) biomass of willow and hybrid willow genotypes (n=5 for genotypes Cam-2 and St-2, and n=8 for genotypes LR-3 and Lev-13). Asterisks denote significance from corresponding control treatment ($p < 0.05$).

A Total Dry Biomass (g)

Species	Genotype	Control	Low	High
Willow	Cam-2	36.7^{a}	26.5^{b}	11.3^{c}
	LR-3	40.2^{a}	28.2^{b}	7.9^{c}
	St-2	39.6^{a}	25.5^{b}	16.5^{c}
Hybrid	Lev-13	47.3^{a}	26.4^{b}	

B Dry Leaf Biomass (g)

Species	Genotype	Control	Low	High
Willow	Cam-2	18.0^{a}	12.4^{b}	5.5^{c}
	LR-3	15.7^{a}	10.3^{b}	2.6^{c}
	St-2	17.6^{a}	11.9^{b}	8.8^{b}
Hybrid	Lev-13	24.9^{a}	11.4^{b}	

C Dry Stem Biomass (g)

Species	Genotype	Control	Low	High
Willow	Cam-2	16.0^{a}	11.6^{b}	4.8^{c}
	LR-3	20.2^{a}	14.9^{b}	5.5^{c}
	St-2	18.2^{a}	10.8^{b}	6.3^{c}
Hybrid	Lev-13	18.4^{a}	12.0^{b}	

D Dry Root Biomass (g)

Species	Genotype	Control	Low	High
Willow	Cam-2	2.7^{a}	2.5^{a}	1.3^{b}
	LR-3	4.3^{a}	3.1^{b}	0.9^{c}
	St-2	3.8^{a}	2.8^{ab}	1.8^{b}
Hybrid	Lev-13	4.0^{a}	3.0^{b}	

3.3.4 Gas exchange

Photosynthetic rates decreased significantly with fracking wastewater treatment in two willow genotypes (Cam-2 and St-2; Figure 3.10A). St-2 exhibited the greatest reduction in photosynthetic rates at the high fracking treatment (88% reduction at high treatment compared to control; $p < 0.05$). Transpiration rates mirrored photosynthesis, but only St-2 was significantly different at the highest fracking wastewater treatment (Figure 3.10B). No significant differences were observed in intercellular CO_2 concentrations. The water-use efficiency of LR-3 and Lev-13 both decreased with treatment, while Cam-2 and LR-3 maintained levels similar to their control treatments (Figure 3.11B).

Dark adapted chlorophyll fluorescence (Fv/Fm) was measured utilizing the first mature leaf for each genotype and treatment, and no differences were observed: all genotypes at all treatments measured between 0.78 and 0.83.

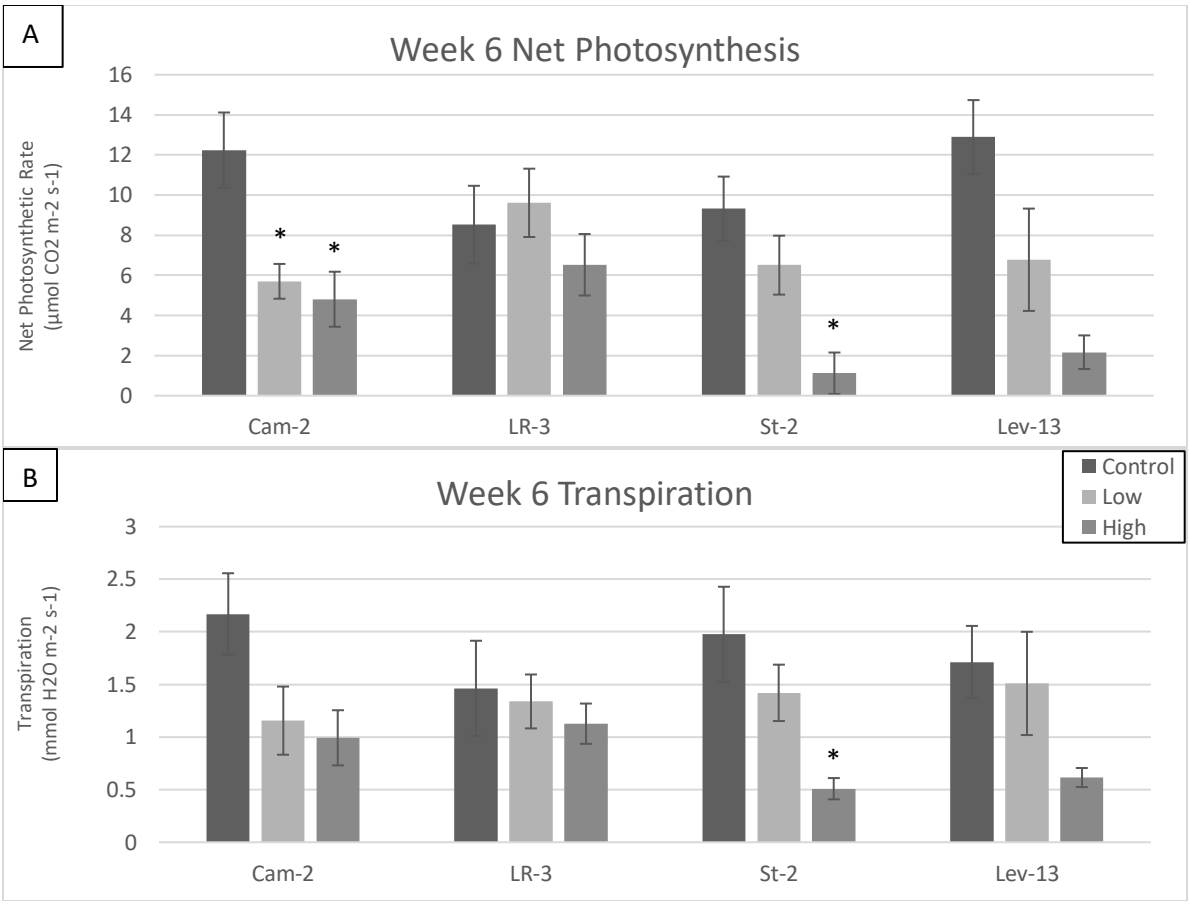


Figure 3.10: Net photosynthetic rate (A) and transpiration rate (B) (+/- SEM) of willow and hybrid willow genotypes at control, low, and high fracking wastewater treatments following six weeks growth (n=5 for genotypes and treatments). Asterisks denote significant difference from control treatment ($p < 0.05$).

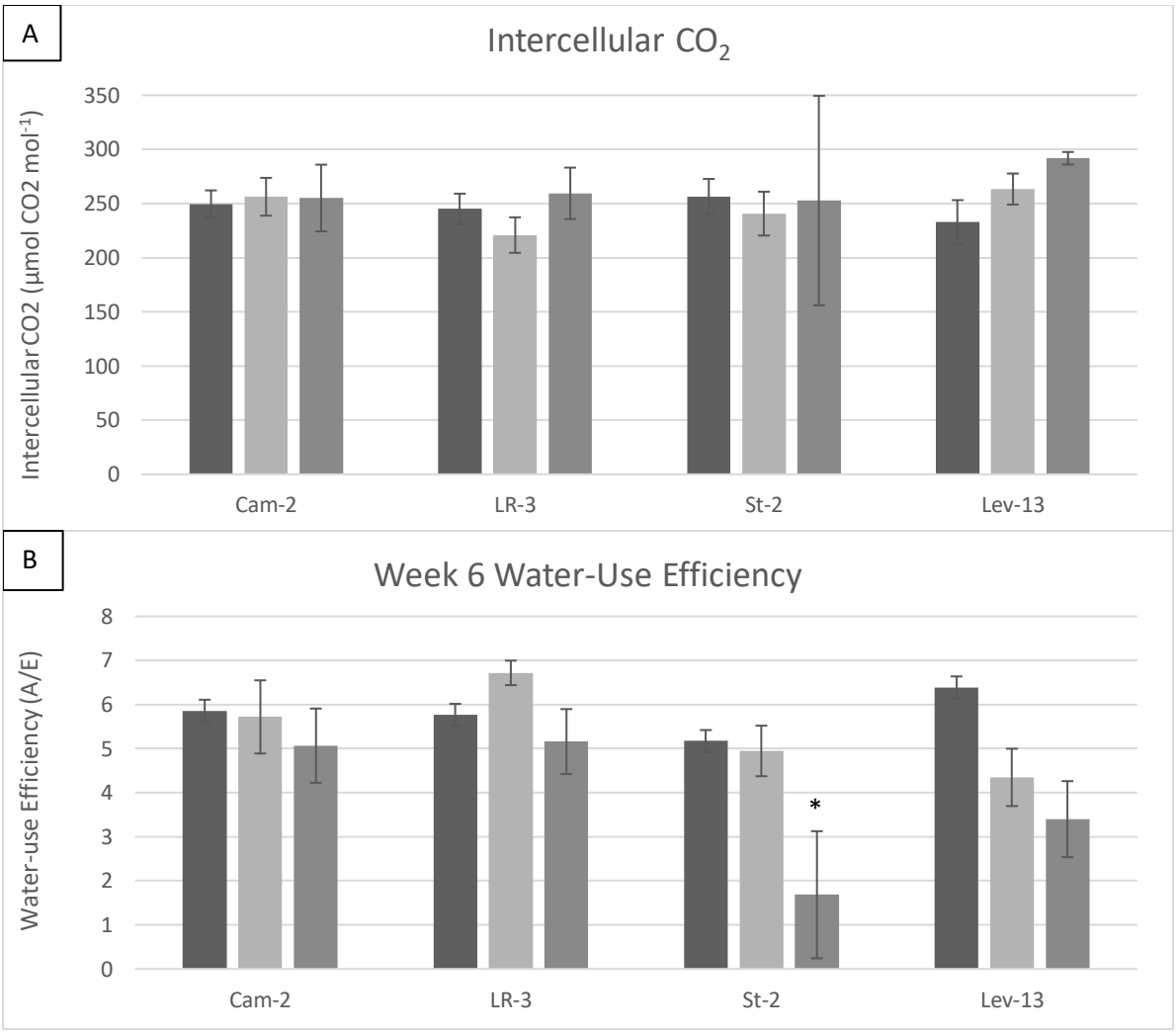


Figure 3.11: Intercellular CO_2 (A) and instanteous water-use efficiency (B) (+/- SEM) of willow and hybrid willow genotypes at control, low, and high fracking wastewater treatments following six weeks growth (n=5 for genotypes and treatments). Asterisks denote significant difference from control treatment ($p < 0.05$).

3.3.5 Compatible solutes

Glucose, fructose and raffinose content of leaf tissues declined in both willow and hybrid willow trees at both low and high fracking wastewater treatments (Table 3.6). In addition, the hybrid willow genotype (Lev-13) had significantly diminished myo-inositol content in leaf tissue ($p <$

0.05). In root tissues, treatment effects were observed in myo-inositol and sucrose contents, both increasing when the trees were grown at high fracking wastewater (Table 3.7). No other significant differences were observed in root sugar contents, nor in the starch content of roots or leaves.

Table 3.6: Soluble sugar content of willow and hybrid willow leaf tissues grown for eight weeks at control, low, and high fracking wastewater treatments (n=3 for genotypes and treatments). Asterisks indicate significant treatment effect compared to control ($p < 0.05$). Lev–13 is omitted at the high fracking wastewater treatment due to limitations in available tissue.

Leaf Tissue

Control

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Willow	Cam-2	10.5	23.2	64.3	19.0	4.2	0.6	2.4
	LR-3	14.1	25.1	91.8	21.5	5.3	0.6	2.0
	St-2	13.8	18.1	100.3	16.2	4.8	1.5	2.6
Hybrid	Lev-13	15.1	17.8	92.5	14.5	4.8	1.3	4.9

Low

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Willow	Cam-2	9.5	10.6	84.0	11.7	1.8	0.8	2.3
	LR-3	15.2	23.1	96.7	18.9	3.8	1.5	4.3
	St-2	11.0	16.6	71.0	14.6	1.9	1.0	2.2
Hybrid	Lev-13	7.3*	10.8*	97.3	8.5*	0.9	1.0	2.7

High

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Willow	Cam-2	11.1	12.8	95.6	11.9	1.1	0.9	3.6
	LR-3	11.4	9.9	80.3	8.2*	0.6	0.7	3.1
	St-2	15.6	14.7	111.0	11.1	1.8	1.2	3.6

Table 3.7: Soluble sugar content of willow and hybrid willow root tissues grown for eight weeks at control, low, and high fracking wastewater treatment (n=3 for genotypes and treatments). Asterisks indicate significant treatment effect compared to control ($p < 0.05$). Lev–13 is omitted at the high fracking concentration due to limitations in available tissue.

Root Tissue

Control

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Willow	Cam-2	2.3	6.4	9.8	3.4	1.0	0.3	2.1
	LR-3	1.6	4.6	14.0	3.9	1.6	0.4	1.9
	St-2	2.9	5.7	18.0	2.5	2.9	1.0	1.9
Hybrid	Lev-13	2.6	5.8	17.1	4.7	1.1	0.5	2.1

Low

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Willow	Cam-2	3.5	5.3	40.1	2.6	1.6	0.4	2.6
	LR-3	2.6	5.3	25.1	3.7	1.9	0.4	2.5
	St-2	2.6	6.7	20.0	4.5	1.6	0.5	1.6
Hybrid	Lev-13	4.2*	7.4	20.5	4.2	0.6	1.3	2.7

High

Species	Genotype	Myo-Inositol (mg/g)	Glucose (mg/g)	Sucrose (mg/g)	Fructose (mg/g)	Raffinose (mg/g)	Stachyose (mg/g)	Starch (%)
Willow	Cam-2	5.0	5.7	46.3*	4.8	1.3	0.2	2.0
	LR-3	2.7	5.2	17.6	2.5	1.6	0.1	2.5
	St-2	3.8	9.3	34.0	5.0	0.7	0.1	1.6

3.3.6 Elemental Analysis

In leaf tissues, sodium concentration increased significantly in only the hybrid willow genotype with increasing fracking wastewater treatment ($p < 0.05$) (Figure 3.12A). Sodium was significantly higher in the root tissues of all genotypes with increasing treatment, and the hybrid willow Lev-13 accumulated about five times as much sodium at the lowest treatment compared with pure willow genotypes (Figure 3.12B). Chloride content of both roots and leaves were significantly higher with fracking wastewater treatments compared with control treated trees in all genotypes, with the hybrid willow accumulating more than pure willows at the lowest fracking wastewater treatment (Figures 3.12C and 3.12D).

Potassium content was unchanged at all fracking wastewater dilutions compared to control treatment in roots and leaf tissue, except in the hybrid willow genotype (Figures3.13A and 3.13B). Calcium concentration increased significantly in leaf tissues of pure willows, but not the hybrid, and was unchanged in roots compared with control treatments ($p < 0.05$). Three metals were found in the stock fracking wastewater, Cu^{2+}, Pb^{2+}, and Ni^{2+}, but were not accumulated in significant quantities compared with control treatments (Tables A.4 and A.5).

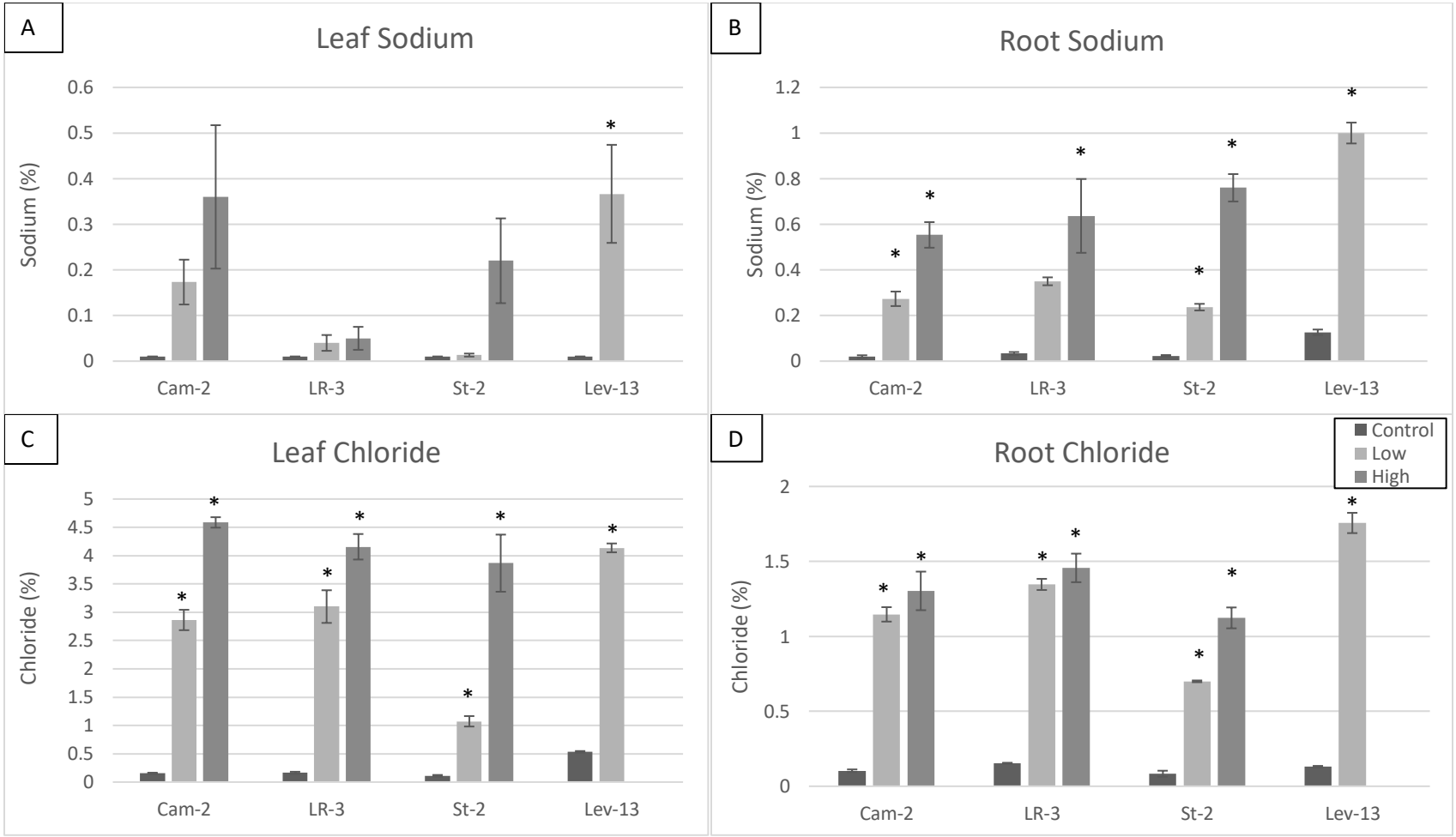


Figure 3.12 Sodium (A, B) and chloride (C, D) content of leaf (A, C) and root (B, D) tissues (+/- SEM) in willow and hybrid willow genotypes grown for eight weeks on control, low, and high fracking wastewater dilutions (n=3 for each genotype and treatment). Hybrid leaf and root tissue is omitted due to lack of tissue resulting from higher mortality rates. Asterisks denote significant difference from control treatment ($p < 0.05$).

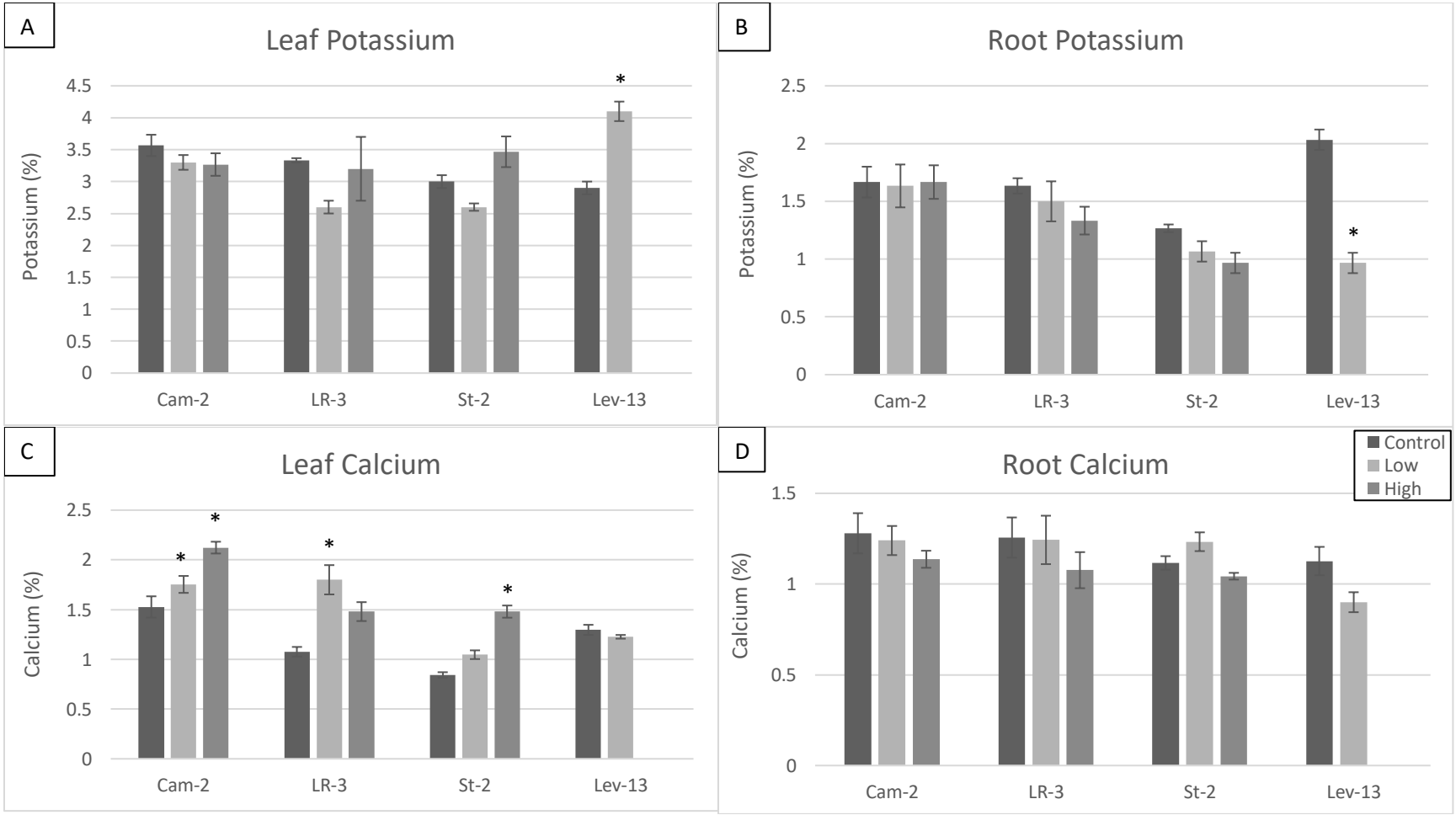


Figure 3.13 Potassium (A, B) and calcium (C, D) content of leaf (A, C) and root (B, D) tissues (+/- SEM) in willow and hybrid willow genotypes grown for eight weeks on control, low, and high fracking wastewater dilutions (n=3 for each genotype and treatment). Hybrid leaf and root tissue is omitted due to lack of tissue resulting from higher mortality rates. Asterisks denote significant difference from control treatment ($p < 0.05$).

Chapter 4: Discussion

Members of *Populus* and *Salix* have garnered recent attention for their remarkable confluence of useful traits for phytoremediation applications, such as abiotic stress tolerance, ease of genotypic propagation, widespread natural ranges, and expansive growth. Although there has been admirable progress in examining poplar and willow for phytoremediation and bioenergy applications, work remains to characterize the physiological stress responses of these same trees when grown under adverse growing conditions (Chen and Polle, 2010).

The principal objective of this work was to screen for salinity stress tolerance from a large selection of *P. balsamifera* and *S. eriocephala* genotypes for potential use in phytoremediation applications. A growing body of work has just begun to elucidate the salinity stress response of poplar and willow species (Chen et al., 2002; Dimitriou and Aronsson, 2010; Fung et al., 1998; Imada et al., 2009; Michels et al., 2018). Despite this, there have been no reports detailing the stress response of balsam poplar to salinity. Therefore, an investigation into the growth and development of balsam poplar under varying salinities should be a welcome contribution to this growing field of study. The salinity tolerance of *Salix eriocephala* has been recently studied in limited capacity, as well as its phytoextraction potential in polluted sites in eastern Canada (Major et al., 2017; Mosseler and Major, 2017). Additionally, the harmful potential of fracking wastewater has undergone only cursory study (Blauch et al., 2009; Folkerts et al., 2017; Gordalla et al., 2013; Wright and Muma, 2018). Quantifying the physiological stress responses of *S. eriocephala* in response to fracking wastewater should contribute to our understanding how plants may react to fracking spills.

The primary goals of this research were: i) to screen for salinity tolerance in *P. balsamifera* and *S. eriocephala* by subjecting genotypes to low and high salinity treatments for extended periods in greenhouse growth trials, examining growth and survival, ii) to examine and quantify the physiological stress responses of poplar and willow, such as gas exchange, biomass, non-structural carbohydrate production, and mineral uptake under salinity stress, iii) to test the survival of genotypes when grown with supplemented fracking wastewater soils while characterizing physiological stress responses, and iv) to ultimately identify potential candidate genotypes for phytoremediation applications in the field.

4.1 Salinity trials

4.1.1 Survival, growth, and biomass

Survival in saline conditions is a key determinant for the applicability of a species or genotype for phytoremediation strategies. To test the limits of salinity tolerance of balsam poplar and heartleaf willow, salinity treatments at 30 and 80 mM NaCl were initially chosen based on the salt tolerance of closely related species (Allison et al., 1954; Ogle and St. John, 2010; Tang et al., 2010). In the screening trial, differences in survival were apparent between the two species: balsam poplar exhibited rampant mortality at both low and high salinity treatments after eight weeks of treatment, indicating a low threshold for tolerance, whereas willows experienced no mortality at low treatments and comparatively low mortality at high treatments (Figure 3.1). A salinity trial in 2017 using an eastern Canadian population of *Salix eriocephala* reported only 40% survival after 25 days of being treated with 3.0 mS^{-1} saline solution (Major et al., 2017),

which is a lower salinity treatment and faster mortality rate than in both of our salinity trials. One potential reasoning could be the differences in annual precipitation amounts between eastern (>1000mm) and western (<450mm) Canada; salts rarely come in contact with root zones in eastern Canada. At the same time, on Canadian prairies with frequent episodes of dry spells, salts are pulled to surface due to evaporation, thus implying to some extent the differences in selection pressure between eastern and western *S. eriocephala* populations to deal with salts in their root zones. In addition, it has been recently suggested that *S. eriocephala* should be recognized as two separate sub-species, and molecular data seems to support the notion of distinct populations across North America (Murphy, E.K., Unpublished manuscript). Genotypic variation was evident in both survival and growth, allowing us to select individuals for further testing. Tolerant and sensitive genotypes were chosen for future comparison based on mortality and relative growth retardation in the presence of salt, the severity of which can indicate salinity sensitivity (Noble and Rogers, 1992).

In Experiment 2, all three species varied significantly from one another ($p < 0.05$) in both growth and total biomass under control treatments due to differences in inherent developmental patterns; poplar genotypes were shorter with few broad leaves, while willows produced long, spindly stems, and the willow hybrid grew thick stems with abundant leaves (Figure 4.1). Marginal leaf necrosis was observed in varying severities (Figure 4.2), consistent with previous observations when similar species were subject to salinity trials (Sixto et al., 2005; Tanou et al., 2009). Interestingly, most willow genotypes produced new foliage after salinity-induced leaf senescence, an observation which has previously been reported (Renault et al., 1998), whereas

poplar genotypes did not. This may be an important adaptive mechanism for recovering from salinity damage and long-term survival in saline environments.

Average height and diameter varied significantly between treatments for poplar, willow, and willow hybrids, indicating the toxic effects of salinity. Comparing control treatments to the lowest salinity level in Experiment 2, poplar genotype GP-10 had the greatest reduction in stem height, stem diameter, and dry biomass, demonstrating significant losses at even the lowest salinity treatment. One poplar and two willow genotypes exhibited an equal or greater average height at 20 mM NaCl compared to control (Figure 3.3): despite these genotypes having a taller average height, biomass still decreased due to reduced stem diameter, as well as displaying significant signs of leaf necrosis and senescence (Table 3.1).

The single hybrid genotype employed in these studies accrued the most biomass at all treatments compared to both the poplar and willow genotypes, which is an important trait in salt remediation for the uptake and dilution of salts, as well as for a potential sources of fibre or biomass for industrial applications (Imada et al., 2009; Yeo et al., 1985). All species and genotypes experienced statistically significant declines in biomass accumulation with increasing treatments except in two instances: willow genotypes LR-3 and St-2 did not experience significant reductions in leaf, stem, or total average biomass up to 40 mM NaCl ($p < 0.05$; Table 3.1). The maintenance of aerial tissues indicates that these willows would likely be good candidates for biomass production on a wide range of saline soils. Hybrid willow Lev-13, in addition to producing the greatest overall biomass, produced root biomass at low and moderate (20 and 40 mM NaCl) salinity equal to its corresponding control treatment, which could prove to

be productive on slightly saline sites. It should be noted that due to the density of the soil substrate, root biomass was lost in the process of harvesting and the washing protocol. Therefore, root biomass totals may not represent the total absolute amount of tissue; however, due to the consistency of the protocol, percent reductions in biomass may be calculated.

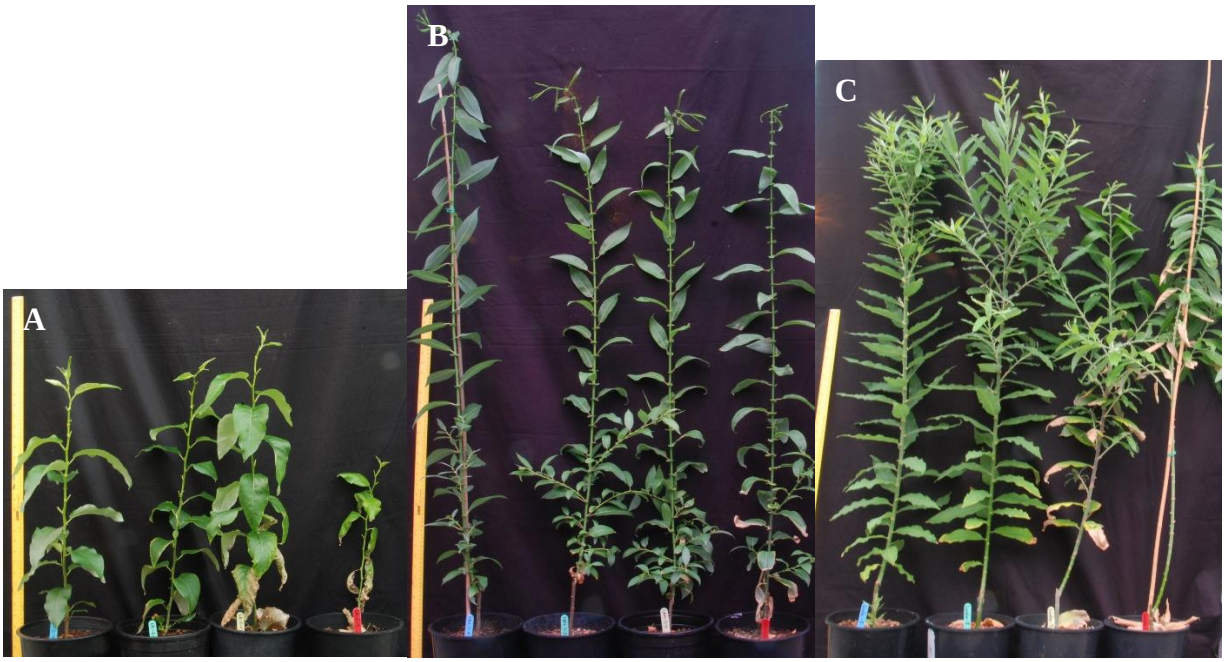


Figure 4.1: Representative poplar (A), willow (B), and hybrid willow (C) trees following ten weeks of growth in a saline environment. Treatments from left to right are 0, 20, 40, and 60 mM NaCl, respectively.

Two native willow genotypes, Cam-2 and St-2, stood out from the rest as they exhibited only marginal decreases in average dry biomass with increasing salinity, displaying 39.9% and 32.6% reductions at high salinity, respectively (Table 3.1). In contrast, susceptible willow genotypes, such as Dr-4 and LR-3, experienced up to 80% losses in dry biomass at the 60 mM NaCl treatment. Maintenance of growth under stress suggests an innate ability to limit Na^+ toxicity from interfering with cellular metabolism and photosynthetic activities (Krasensky and Jonak, 2012; Neumann, 1997).

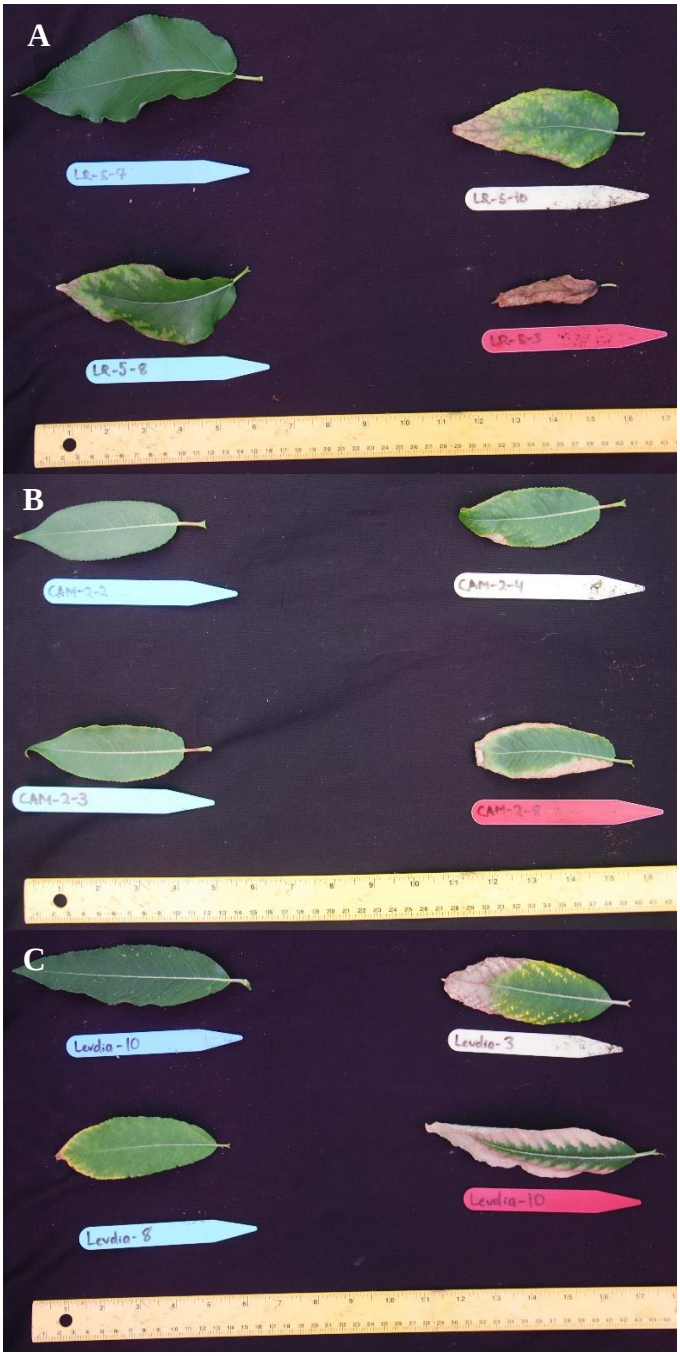


Figure 4.2: Representative poplar (A), willow (B), and hybrid willow (C) leaves after 12 weeks of growth in saline environments. Treatments are 0 (top left), 20 (bottom left), 40 (top right) and 60 (bottom right) mM NaCl, respectively.

4.2 Gas exchange

An inspection of gas exchange at varying salinities can provide valuable insight into the stress response of poplar and willows. Following six weeks of treatment, three general trends were apparent in most genotypes in response to increasing salinity: a slight decrease in net photosynthesis, reduced intercellular CO_2 concentrations, and a substantial decrease in transpiration rates (Figure 3.5). Examining individual genotypes, photosynthesis and transpirational rate dropped significantly in only two genotypes, Dr-4 and St-4 ($p < 0.05$). This may contribute to the stress resistance in saline soils of these willow genotypes, as tight regulation of transpiration is an advantageous trait to conserve water in saline soils where water potential is low and therefore may be taken up at a slower rate (Joshi-Saha et al., 2011). A similar salinity trial using *S. eriocephala* reported an increase in net photosynthesis with mild salt treatment, while our willows experienced a decrease with treatment (Major et al., 2017). Differences in photosynthetic response may be due to the divergence of eastern and western *Salix* populations over time, and their inherent strategies to combat adverse environmental conditions.

Dark adapted Fv/Fm measurements were taken at week six in both salinity and fracking wastewater experiments, and no differences were observed between control, salinity, and fracking wastewater treatments in or between any genotype; all measurements were between 0.78 and 0.83, indicating an "unstressed" plant despite necrotic symptoms clearly visible in lower tissues of treated individuals. Unobserved differences in Fv/Fm may be due to our choice of leaf for measurement: the first fully mature leaf may not accumulate sodium in toxic

quantities because sodium is a mobile nutrient, which could be restricted to older leaves. While Fv/Fm measurements have been suggested to represent a measure of plant health in cases of soil salinity, lack of observable difference between salinity treatments seems to suggest the contrary (Amirjani, 2011; Jamil et al., 2014; Murchie and Lawson, 2013), and therefore may be an over-representation of the measure. Intercellular CO_2 decreased significantly with treatment at both weeks 6 and 10, which indicates that net photosynthetic rate is primarily being limited by stomatal closure rather than direct interference with photosynthetic systems (Flexas et al., 2004).

Average transpiration and net photosynthetic activity increased at week 10 compared to week 6, which appears to occur as plants accumulate greater whole-plant leaf area (Ben-Gal et al., 2003). The hybrid willow genotype had the lowest photosynthetic and transpiration rates at weeks six and ten, as well as the highest WUE (Figures 3.5 and 3.6). Despite bearing the lowest photosynthetic rate, the hybrid willow produced a far greater number of leaves than the poplars and willows employed in this study, which likely resulted in greater total photosynthesis (Williams, 1946). This strategy, however, did not seem to hold up during high salinity treatments, as its average biomass was reduced by more than half at the highest salt treatment. In fact, rampant and early leaf senescence observed in the hybrid willow, the poplar genotypes, and willow genotypes Dr-4 and LR-3 likely resulted in stunted biomass accrual due to a loss in whole-plant photosynthetic capacity (Neumann, 1997). In contrast, native willow genotypes Cam-2 and St-2 both lost the smallest percentage of biomass in response to the increasing salinity, and also displayed limited leaf necrosis as well as the greatest capacity to limit transpiration compared to control treatments.

4.2.1 Compatible solutes

In order to overcome the lowered water potential induced by saline soils in long-term conditions, internal osmotic adjustments can be made through the synthesis of compatible solutes (Bernstein, 1975; Bohnert and Shen, 1999; Chen and Jiang, 2010). In leaf tissues, increases in stachyose content were observed in poplar and willow genotypes in response to increasing salinity (Tables 3.2 and 3.3). Stachyose, in addition to restoring the osmotic potential of leaves, has been shown to have antioxidative properties (Nishizawa-Yokoi et al., 2007). The ability to scavenge free-radicals is integral to manage the reactive oxygen species generated by salinity, and may protect photosynthetic systems as well as the function of membrane-bound transporters (Tang et al., 2010; Wang et al., 2007).

In root tissues, average raffinose content was higher in native hybrids with increased salinity, and sucrose content increased only in native willow genotypes (Tables 3.2 and 3.3). Both sucrose and raffinose are mobile, non-reducing sugars that may be transported from leaves to root to assist in salinity tolerance. Sucrose can be used as an osmotic regulator, be metabolized to yield energy, or used in the production of osmolytes such as raffinose and stachyose (Gilbert et al., 1997; Yancey et al., 1982). Raffinose, a trisaccharide precursor to stachyose, has been previously shown to act as an osmoprotectant with environmental stresses (Barchet et al., 2014; Tattini et al., 1996). Maintaining relatively high sucrose and raffinose concentrations in root tissues could be particularly advantageous in salinity tolerance, as roots are the first point of contact with soil salts, and consequently need to maintain high osmotic potential, ultimately limiting the movement of salts into aerial tissues (Chen et al., 2002; De Boer and Volkov, 2003).

4.2.2 Nutrient and mineral balance

Sodium, chloride, potassium, and calcium are intertwined in instances of soil salinity and tolerance responses (Lessani and Marshner, 1978; Liu and Zhu, 1998). Sodium ions are thought to be the primary antagonist in cases of toxic levels of salinity due to their capacity to degrade membranes and compete with potassium for enzyme binding sites (Blumwald, 2000). In this study, sodium is present in far greater quantities in root tissues than in leaves (Figure 3.7). This is likely because sodium is a readily available ion for osmotic adjustment to facilitate water uptake in roots, and because a common glycophyte tolerance strategy is to limit the transport of sodium to aerial tissues (Apse and Blumwald, 2007). Poplar genotype GP-10 and willow genotypes Dr-4 and St-4 experienced major biomass reduction and leaf senescence when subjected to salinity treatments, and also displayed the greatest leaf sodium contents (Figure 3.7). This suggests an inability to restrict sodium transport to leaf tissues where sodium can reach toxic levels, and likely contributed to the observed necrosis and induced senescence. Variation in leaf sodium restriction ability was evident: GP-10 accumulated sodium at the lowest salinity treatment, whereas Dr-4 and St-4 accumulated sodium in leaves at only the highest salt treatment.

Maintenance of a high K:Na ratio is indicative of salinity tolerance in glycophytes, as it can prevent ion competition and ensure proper enzyme function (Hasegawa et al., 2000; Parida and Das, 2005). Leaf potassium concentration increases steadily with saline treatment in willow and hybrid willow genotypes, but did not in the salt-sensitive poplar (Figure 3.8). Interestingly, potassium decreased in root tissues with increasing salinity, suggesting translocation of potassium from roots to leaf tissues (Boer, 1999; Hafsi et al., 2007). Calcium is a secondary

messenger that plays an integral role in the Salt Overly Sensitive (SOS) pathway, which mediates K:Na balance in plant cells (Rengel, 1992). In poplar and hybrid willow leaves, calcium was the same at all treatment levels. However, calcium significantly increased with salt treatment in the willow genotype Cam-2, which may have a role in the activation of membrane-bound transporters.

Chloride is most commonly associated with sodium in saline soils and, while an essential micronutrient, can be toxic in high quantities (White and Broadley, 2001). Chloride accumulated in large concentrations in poplar, willow, and hybrid willow leaf tissues, for example, up to 5% in Cam-2 (Figure 3.7). Interestingly, chloride content was much lower in all root tissues when compare to corresponding tree leaf tissue. Chloride is useful in both maintaining cell turgor and can be used as an abundant, energetically 'cheap' osmotic adjustment, which could explain the massive chloride contents observed in the leaf tissues, but the concentration observed is well above glycophyte toxicity levels (White and Broadley, 2001).

4.3 Fracking wastewater trial

Studying plant response to fracking wastewater presents a unique challenge for researchers: the constituents of fracking wastewater vary by drill site due to the geological makeup of the gas reserve and the site-specific combination of industrial additives (Wright and Muma, 2018). However, two stresses ubiquitously present in fracking wastewater are salts and metals. Without a large catalogue of phytoremediators tuned to resist a precise combination of stresses, plants that can withstand a variety of stresses will be desired. Willows have been previously shown to

be moderately tolerant of both salt and metal stressors, and make an attractive subject for phytoremediation research (Pulford et al., 2002; Pulford and Watson, 2003). This study is the first to directly test the effect of fracking wastewater on willow health and physiology.

4.3.1 Survival, growth, and biomass

The fracking wastewater provided for our study is a complex mixture of salts, metals, industrial lubricants and biocides, the elemental composition of which is listed in Table A1. The fracking wastewater treatment levels were initially chosen to match the EC of the low and high treatments from Experiment 2 (Table 2.3). However, the highest fracking wastewater treatment actually contained a sodium concentration of 72.8 mM, a concentration between the highest treatments of the screening trial and Experiment 2. Hybrid willows suffered complete mortality after eight weeks of growth in the presence of the highest fracking wastewater treatment, a full month before the conclusion of the previous salinity trial. This could indicate that the sodium content may have been overwhelming at this concentration, as biomass declined significantly at 60 mM NaCl compared to the control in the previous trial, or that additional stressors may have influenced its early demise (Zurayk et al., 2001). The other three willow genotypes, Cam-2, LR-3, and St-2, survived eight weeks of both high salinity and fracking wastewater treatments.

The tree heights of control treated willow and hybrid willow genotypes closely mirror the growth from the previous salinity trial (Figure 4.3). Significant reductions in height were apparent in all genotypes at the high fracking wastewater treatment (Figure 3.9). In fact, the average height at week eight of the highest fracking wastewater treatment for willow genotype Cam-2 is between

the average heights of the same genotype grown at 60 and 80 mM NaCl. This indirectly suggests that sodium content is a primary stressor in fracking wastewaters, and that its other constituents may only contribute as mild stressors at these dilutions, for these specific trees.

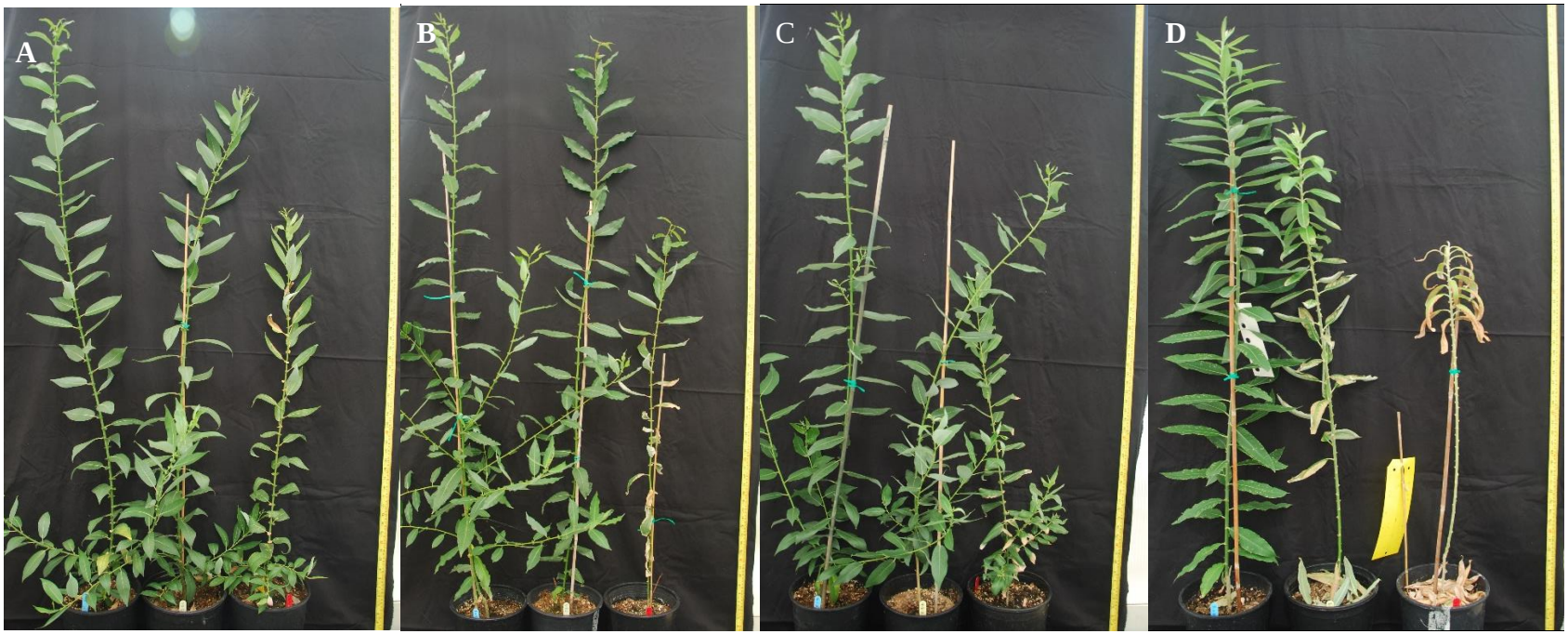


Figure 4.3: Willow genotypes Cam-2 (A), LR-3 (B), St-2 (C), and hybrid genotype Lev-13 (D) grown in soil supplemented with diluted fracking wastewater treatment. From left to right, treatments in each photo represent control, low, and high treatments.

While the height growth patterns of willow and hybrid willow genotypes were similar throughout the fracking wastewater trial, variations were more readily apparent in biomass production. Willow genotype LR-3 suffered the greatest reductions in mass at the highest fracking wastewater treatment in every tissue measured, ~80% loss in leaf, stem, and root tissues (Table 3.5). Cam-2 and St-2 genotypes consistently had a lower percentage of biomass reduction in all measured tissues compared to LR-3 and Lev-13. Interestingly, rates of necrosis and senescence were diminished in Cam-2 and St-2 in the fracking trial compared to both other salinity trials (Figure 4.4). Reduced biomass production despite a 'healthy' appearance is likely due to the increased energy demand of resisting salt and industrial byproduct stresses, such as active transport of ions and production of ROS scavengers and phytochelators (Prasad and Hagemeyer, 1999). Leaf litter from phytoremediators can contribute to the salinization or metal deposition to top soils. Therefore, an advantageous trait for phytoremediators is diminished rates of leaf necrosis and senescence (Imada et al., 2009; Michels et al., 2018). With their high survival rates, relatively strong maintenance of height and biomass, and lack of damaged tissues, Cam-2 and St-2 could potentially be an excellent pioneer species for growth on marginal lands and fracking wastewater spill sites.

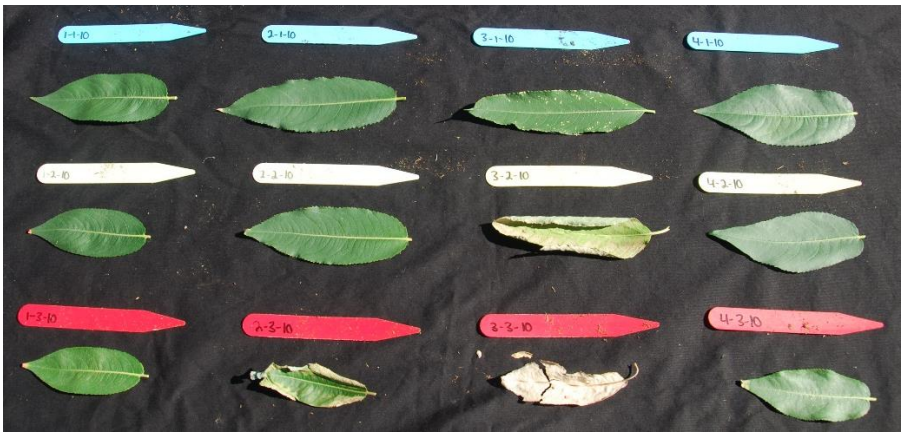


Figure 4.4: Leaves of willow and hybrid willow trees grown in soil supplemented with diluted fracking wastewater treatments. Genotypes from left to right are Cam-2, LR-3, Lev-13, and St-2. Treatments from top to bottom represent control, low, and high fracking wastewater treatments.

4.3.2 Gas exchange

Gas exchange measurements can be used to examine the health and physiological status of plants under abiotic stress, and employing it to report on the tree response to fracking wastewater is a novel contribution to the field. Gas exchange measurements were taken at the sixth week post initial treatment in both salinity and fracking wastewater trials for comparison. Net photosynthetic rates were lower when the trees were subject to fracking fluid than when subject to salinity; negative photosynthetic rates were recorded in individual St-2 trees at high fracking concentrations, indicating that respiration was greater than photosynthetic activity. It has been suggested that under mild heavy metal stress, respiration will increase as metals interfere with metabolic processes (Prasad and Hagemeyer, 1999), which may contribute to the significantly lower, but not fatal, biomass and photosynthesis rates seen in Cam-2 and St-2.

Transpiration mirrored the observed decrease in photosynthesis, however, intercellular CO_2 remained constant throughout the fracking wastewater treatments in all genotypes, which indicated that heavy metals or industrial additives present in the fracking wastewater treatments may have interfered with enzymes involved in photosynthesis and respiration (Gordalla et al., 2013; Prasad and Hagemeyer, 1999; Seregin and Kozhevnikova, 2006). Hybrid genotype Lev-13 and native willow genotype St-2 failed to maintain high water-use efficiency as seen in the previous salinity trials which could be a symptom of the additional metal stresses (Figure 3.11B). LR-3 once again remained unchanged in photosynthetic rate, transpiration, and water-use efficiency, but experienced biomass reduction with salinity compared to controls, which suggests that this genotype lacks robust defensive systems, unlike Cam-2 and St-2.

4.3.3 Compatible solutes

While a wealth of literature exists on antioxidant and phytochelator production in response to specific heavy metal stresses, variation in non-structural carbohydrate content is poorly studied. The three metals that were quantified in the greatest concentration in the fracking wastewater were copper, lead, and nickel, but were well below toxic levels (Table A.1). However, complex industrial additives and byproducts are present in fracking wastewater which may influence the highly sensitive and specific physiological response of plants to stress (Richter et al., 2018). In the root tissue isolated from the willow and hybrid genotypes, an increase occurred in both myo-inositol and sucrose at the low and high fracking treatments (Table 3.7). In a study that examined the effect of copper toxicity on cucumber plants, sucrose was shown to increase significantly in leaf tissues but not roots, suggesting an inhibition of transport mechanisms (Alaoui-Sossé et al.,

2004). With the observed increase of sucrose in the willow roots, it can be inferred that sugar transport mechanisms remain functional and that root tissues are likely utilizing sucrose as an osmolyte in the presence of both salts.

Fructose is utilized in the production of raffinose, and a decrease of both were apparent in the leaf tissues of willow and hybrid willow genotypes compared to their control treatments (Table 3.6). Fructose and raffinose concentrations were unaffected by salt treatment in the leaves of our previous salinity trial, which suggests that the addition of industrial contaminants present in the fracking treatment may inhibit raffinose production, in accordance with a previous study of poplar grown near an industrial copper production site (Stobrawa and Lorenc-Plucińska, 2007). Average sucrose content increased with the fracking wastewater treatments in leaves and roots with increasing fracking wastewater supplementation (Tables 3.6 and 3.7). Carbon allocation is likely being diverted away from biomass production, as sucrose can be used in respiration that fuels ion transport systems and antioxidant defense (Jacoby et al., 2011).

The hybrid genotype Lev-13 experienced dramatic changes in leaf sugar content at the low fracking treatment compared to the willows, displaying a 50% drop in myo-inositol and glucose, and 80% drop in raffinose. Interestingly, while willow genotype LR-3 experienced the greatest biomass loss and necrosis, its sugar content was slower to change at the low fracking treatment. However, both Cam-2 and St-2 maintained higher carbohydrate contents at the highest fracking treatment, displaying greater resistance to a more severe treatment.

4.3.4 Elemental analysis

Sodium and chloride accumulation in leaf and root tissues following fracking wastewater treatment showed similar patterns to those observed with the NaCl treatments: trees tended to exclude sodium from leaf tissues while storing greater quantities in roots (Figure 3.11). Although Cam-2 had a higher average sodium concentration in leaves with diluted fracking wastewater treatments compared with the previous salinity trial, statistical significance was not observed due to high variation in sodium concentrations at the high fracking wastewater treatment. Additionally, the hybrid willow genotype accumulated more sodium in leaf tissues at the lowest fracking wastewater treatment compared with the hybrid willow treated with 60 mM NaCl in the previous trial. This suggests that something else present in the fracking wastewater was interfering with the capacity of the hybrid willow to exclude sodium from leaf tissues. However, no toxic accumulation of the detected metals in the fracking wastewater (Cu^{2+}, Pb^{2+}, and Ni^{2+}) was observed in plant tissues (Figures A.4 and A.5). A possible explanation may be that industrial additives such as lubricants, biocides, or polycyclic aromatic hydrocarbons (not quantified in the fracking wastewater stock in this study), may have had a detrimental effect on the salt tolerance mechanisms of willows (Folkerts et al., 2017; Gordalla et al., 2013; Robinson et al., 2015).

Although it would appear that salinity is the primary stressor in fracking wastewater (Table A.1), potassium and calcium concentrations did not follow analogous patterns compared with the salinity trial. In the second experiment, potassium clearly increases with salinity treatment in leaves and decreases in roots (Figures 3.8A and 3.8B). Potassium concentration was observed to

be unresponsive to fracking wastewater treatments, but was present in higher concentrations than in the second salinity trial. The higher potassium concentration is likely due to potassium present in the fracking wastewater (Table A.1) in addition to the fertilizer supplement, which may have contributed to the tolerance observed in the willow genotypes (Shabala et al., 2006). Calcium accumulation in both leaves and roots is similar in the fracking wastewater trials as in the salinity trials despite its greater abundance in fracking wastewater, suggesting that it may be in a biologically unavailable form or that willows do not accumulate more in response to stress.

Both Cam-2 and St-2 survived and displayed minimal stress symptoms with fracking wastewater treatments despite a relatively severe amount of salinity, while the hybrid genotype accumulated more sodium and chloride at a less severe saline concentration than the previous salinity trial. These results demonstrate that Cam-2 and St-2 possess a capacity for tolerance to the multiple stressors present with fracking wastewater.

Chapter 5: Conclusion

5.1 Candidate genotypes for phytoremediation of saline and fracking-polluted sites

This work presents the growth and physiological stress responses of two ecologically and economically important tree species found on the Canadian landscape, *Populus balsamifera* and *Salix eriocephala*, to salinity and fracking wastewater. By screening for tolerance among thirty-one genotypes, two candidates willow genotypes displayed traits that make them strong candidates for phytoremediation strategies aimed at combatting saline environments: Cam-2 and St-2.

The first salinity trial performed tested poplar and willow genotypes at salinity levels near the reported tolerance limits of related species. It became quickly apparent that *P. balsamifera*, which to our knowledge has not been reported on previously, was largely salt-sensitive. Among the *P. balsamifera* genotypes screened, within the first 30 days even the lowest salinity treatment display high rates of necrosis and senescence, as well as a severe stunting of growth. Few poplar remained following two months of treatment, clearly demonstrating that these genotypes would not represent suitable prospects for phytoremediation applications. Native willow genotypes, however, not only survived high salinity growth environments, but displayed limited suppression of growth in response to low treatment levels. Genotypic variation was evident in both species, which would allow us to further compare sensitive to tolerant genotypes within both species. The second salinity trial permitted an examination and quantification of the physiological responses of poplar and willows to a range of salinities. Three months of salinity treatment

clearly showed that long-term survival of willow genotypes on moderately saline soils would be feasible. Furthermore, a full harvest highlighted the abiotic stress tolerance of genotypes Cam-2 and St-2 to these adverse conditions, as shown by their ability to continue to accumulate biomass, which would be an added asset for fibre production. While the hybrid genotype introduced in this experiment could also be considered for phytoremediation on slightly saline soils due to its comparatively large root and shoot biomass production, it proved to experience considerable losses at moderate to high salinity levels and might suffer more over a longer period of salinity stress.

Gas exchange, non-structural carbohydrates, and elemental uptake were examined in an effort to explain, in part, the differences in tolerance between species and genotypes. One feature common to the more tolerant genotypes is the ability to restrict water loss by regulating stomatal closure, which is a trait shared by known salt-tolerant species. Furthermore, water-use efficiency was much higher in willow genotypes in response to salinity compared to the corresponding poplar genotypes tested, pointing to more efficient water regulation in willows. Next, we examined the leaf and root tissues, and found that soluble sugars may aid in water uptake and regulation: raffinose and stachyose, known to be employed in osmotic adjustment and free radical scavenging, were in present in greater quantities in high salinity-treated roots and leaves, respectively, and could potentially aid in salinity tolerance in these genotypes. Differences were also found in the concentration and distribution of elements involved in salt toxicity: poplar genotype GP-10, the most sensitive of those examined (Experiment 2) was unable to restrict sodium from entering its leaves in toxic concentrations. Furthermore, the willows were able to maintain higher potassium content in their leaves, suggesting a tighter regulation of their

transport systems than the corresponding poplar species. From this experiment, balsam poplar was entirely ruled out of further phytoremediation applications specific to salt remediation, while the three most promising willow genotypes and a hybrid willow genotype were chosen: Cam-2, LR-3, St-2, and Lev-13.

The final growth trial examined the growth and stress response of willows to a previously unstudied toxicological threat: fracking wastewater. The same assessments were made as those in the previous salinity trials in order to compare stress responses. Growth was similar between the low fracking treatment and 20 mM treatment in the four genotypes, however, the high fracking wastewater treatment proved fatal to the hybrid genotype after eight weeks growth, which had previously survived twelve weeks growth at high salinity. Biomass was severely reduced in genotypes LR-5 and Lev-13, but Cam-2 and St-2 continued to accrue biomass, although at a reduced rate, and displayed limited leaf necrosis. The ability to retain their foliage is a valuable trait as it provides a means for carbon assimilation and reduces topsoil salt deposition (recycling of the salt initially removed from the soil). Photosynthesis was inhibited more than with the salt only stress trials, and respiration was increased in the two more tolerant willow genotypes. Moreover, fracking wastewater inhibited the production of raffinose and stachyose that was observed with salinity treatments, however, sucrose content increased, likely fueling respiration and abiotic stress defenses. Together, thirty-one poplar and willow genotypes were narrowed to two native willow genotypes that demonstrated the ability to resist and survive long term exposure to abiotic stresses that are common to marginal land on the Canadian landscape.

5.2 Relevance

Poplar and willow have garnered interest as potential phytoremediators, particularly throughout Europe and China. This study expands our knowledge of phytoremediation in North America, and also is the first, to our knowledge, to describe the effect of salinity on *P. balsamifera*, a pan-Canadian species. While the specific mechanisms of salt toxicity require further research, this study has identified tolerant genotypes that could prove useful in both practical phytoremediation operations as well as interesting subjects for future study.

This is also the first study to examine the effects of fracking wastewater on plant species. Globally, as the oil and gas reserves are depleted and fracking becomes more widely used, research will be necessary to understand the effects of its byproducts on the environment, and maybe more importantly, to find tools to ensure that local environments around drill sites are returned to their pre-drill conditions. This study highlights the dual effects of salinity and heavy metal stress on plant health while also quantifying phytoextraction potential. Being able to ameliorate pollution while also being productive on marginal land would benefit local economies and preserve environmental health for future generations.

5.3 Future research

This study serves as a foundation by which many avenues of continuing research should be directed. First and foremost, a field trial on a mining or fracking spill site could be performed as a proof-of-concept over a several year span. In so doing, yearly coppicing could test the recovery

of genotypes while providing tissues for assessment and use/testing in other secondary industries, such as biofuel applications. Soil samples could also be tested yearly to determine the efficacy of phytoremediation on salts or heavy metals. Additionally, further testing on aerial tissues could provide insight into antioxidants produced in response to abiotic stress, as well as phytoextraction capacity outside of a greenhouse environment.

Quantitative trials on specific heavy metals found in fracking solutions may also yield insight into the stress response in the absence of salinity; while work has been done on a variety of heavy metals, work remains to characterize stress response in poplar and willow, especially in the context of phytoremediation. Root tissues play an extremely important role in salt or metal tolerance and require new methods of study due to the difficulty of direct observation. In that regard, a new technology, synchrotron x-ray, could be employed to examine root systems in potted soil to observe and quantify the localization of each element, and elemental species. This could provide valuable insight into the abiotic stress response of poplar and willow and elucidate some of the more clandestine function of roots.

In a collaboration with Xinyi Huang (PhD candidate in the Mansfield Lab), who is working with willows treated with sulphate salts, a dominant salt on Canadian prairies (unpublished data), RNAseq will be utilized to compare the expression patterns of genes in control and salt treatments, between saline and sulfate salts, as well as among genotypes. Both root and leaf tissue transcripts will be analyzed to compare areas of high or low expression in order to identify genes of interest related to growth, abiotic defense, and ion transport. Though this collaboration, a wealth of new genetic information may be revealed for salt tolerance in poplar and willows,

and provide candidate genes for the Agriculture and Agri-Food Canada poplar and willow breeding program to rapidly select trees for most unproductive landscapes.

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Appendices

Appendix A

Table A.1: Fracking wastewater composition

Element	Concentration (mg/L)
Calcium	1960
Magnesium	239
Sodium	21000
Potassium	709
Iron	53.8
Zinc	< 1
Arsenic	< 0.04
Cadmium	< 0.01
Chromium	< 0.04
Copper	0.084
Lead	0.0619
Nickel	0.1001

Table A.2 Element composition of poplar, willow, and hybrid leaves at control and 60 mM NaCl treatments.

Leaf Tissue

Control

Species	Genotype	Na (%)	Cl (%)	K (%)	Ca (%)	N (%)	P (%)	S (%)	Mg (%)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)
Poplar	GP-10	0.0	1.2	1.9	2.0	2.2	0.3	0.3	0.4	63.7	54.3	44.0	1.7	29.3
	LR-5	0.0	0.1	2.2	1.5	2.8	0.4	0.9	0.4	174.7	50.3	55.3	2.7	43.7
Willow	Cam-2	0.0	0.5	2.1	1.6	2.3	0.3	0.8	0.4	73.0	52.0	36.0	3.0	40.3
	Dr-4	0.0	0.6	2.0	1.4	2.5	0.3	0.7	0.5	93.7	47.0	41.3	1.3	44.7
	LR-3	0.0	1.0	2.5	2.0	3.2	0.3	0.3	0.4	66.7	62.3	52.3	2.3	32.0
	St-2	0.0	0.6	2.6	1.3	2.6	0.3	1.0	0.4	79.7	53.3	45.0	2.0	40.3
	St-4	0.0	0.9	2.3	1.3	2.6	0.2	0.8	0.4	85.3	40.7	40.0	2.0	39.0
Hybrid	Lev-13	0.0	1.0	1.7	1.3	2.3	0.3	0.3	0.5	84.3	53.0	49.3	2.0	35.3

60 mM NaCl

Species	Genotype	Na (%)	Cl (%)	K (%)	Ca (%)	N (%)	P (%)	S (%)	Mg (%)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)
Poplar	GP-10	1.2	4.3	2.0	1.4	2.5	0.3	0.2	0.3	90.3	50.3	63.7	3.3	24.0
	LR-5	0.1	4.6	2.6	2.1	3.1	0.4	0.3	0.4	144.5	60.5	73.5	3.5	34.5
Willow	Cam-2	0.1	4.5	2.8	2.3	2.5	0.3	0.6	0.4	140.0	71.3	83.7	3.7	53.3
	Dr-4	0.3	5.1	2.9	2.1	2.6	0.5	0.5	0.5	162.3	59.0	88.3	5.7	58.0
	LR-3	0.0	2.8	2.9	1.6	3.3	0.4	0.7	0.4	169.0	53.7	77.7	5.3	39.3
	St-2	0.0	4.4	3.7	1.5	2.7	0.3	0.4	0.4	102.7	50.3	79.3	3.0	45.7
	St-4	0.2	5.0	3.8	1.8	2.6	0.3	0.5	0.5	116.3	49.0	93.7	5.0	52.7
Hybrid	Lev-13	0.1	3.3	3.0	1.3	3.0	0.3	0.2	0.3	158.0	49.7	75.7	4.0	30.3

Table A.3: Element composition of poplar, willow, and hybrid roots at control and 60 mM NaCl treatment. Poplar are excluded at 60 mM NaCl due to lack of available tissue.

Root Tissue

Control

Species	Genotype	Na (%)	Cl (%)	K (%)	Ca (%)	N (%)	P (%)	S (%)	Mg (%)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)
Poplar	GP-10	0.0	0.7	1.4	0.9	1.3	0.3	0.1	0.2	46.3	245.0	20.3	15.0	11.3
	LR-5	0.0	0.3	1.4	1.0	1.3	0.3	0.1	0.2	50.7	157.3	22.3	10.0	10.7
Willow	Cam-2	0.1	0.4	1.1	1.1	1.3	0.3	0.3	0.2	83.0	402.7	32.3	9.3	12.3
	Dr-4	0.1	0.4	0.9	0.9	1.2	0.3	0.2	0.2	88.3	219.7	21.3	8.7	10.7
	LR-3	0.0	0.2	1.2	0.8	1.0	0.2	0.2	0.2	73.0	154.7	13.7	7.3	9.7
	St-2	0.1	0.3	1.1	1.0	1.1	0.3	0.2	0.2	58.3	158.3	18.3	6.3	10.0
	St-4	0.1	0.4	1.0	1.1	1.2	0.3	0.2	0.2	73.0	211.3	16.0	7.0	11.3
Hybrid	Lev-13	0.1	0.6	1.1	0.8	1.0	0.2	0.2	0.2	65.7	157.3	17.7	8.3	11.7

60 mM NaCl

Species	Genotype	Na (%)	Cl (%)	K (%)	Ca (%)	N (%)	P (%)	S (%)	Mg (%)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)
Willow	Cam-2	0.6	1.3	1.2	0.8	1.6	0.4	0.3	0.2	132.3	222.3	45.3	10.3	14.0
	Dr-4	0.9	0.9	0.6	0.9	1.6	0.5	0.3	0.1	210.3	230.3	42.3	16.0	14.7
	LR-3	0.7	1.3	1.0	0.8	1.6	0.3	0.3	0.1	87.0	239.3	42.3	9.3	13.3
	St-2	1.0	1.2	0.6	1.0	1.6	0.4	0.4	0.1	100.7	309.3	70.7	11.7	13.7
	St-4	1.3	1.3	0.6	0.9	1.6	0.5	0.4	0.1	120.0	177.0	44.3	15.3	13.3
Hybrid	Lev-13	1.5	1.8	0.4	0.8	1.2	0.3	0.3	0.1	91.0	247.3	27.0	11.0	15.3

Table A.4 Element composition of willow and hybrid leaves at control, low, and high fracking wastewater treatment. Hybrid willows are excluded at the high treatment due mortality at the highest treatment.

Leaf Tissue

Control

Species	Genotype	Na (%)	Cl (%)	K (%)	Ca (%)	N (%)	P (%)	S (%)	Mg (%)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)	Pb (ppm)	Ni (ppm)
Willow	Cam-2	0.0	0.2	3.6	1.5	3.1	0.4	1.2	0.8	202.0	590.3	174.7	4.3	63.0	< 1	0.6
	LR-3	0.0	0.2	3.3	1.1	3.6	0.5	1.0	0.7	181.3	255.3	138.3	4.7	59.0	< 1	0.9
	St-2	0.0	0.1	3.0	0.8	3.7	0.4	1.0	0.6	109.0	218.3	109.0	3.0	41.0	< 1	0.6
Hybrid	Lev-13	0.0	0.5	2.9	1.3	3.4	0.4	0.4	0.6	144.3	251.0	143.3	4.0	58.0	< 1	0.9

Low

Species	Genotype	Na (%)	Cl (%)	K (%)	Ca (%)	N (%)	P (%)	S (%)	Mg (%)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)	Pb (ppm)	Ni (ppm)
Willow	Cam-2	0.2	2.9	3.3	1.8	2.8	0.4	1.0	0.9	240.3	494.7	265.7	4.3	69.3	< 1	0.8
	LR-3	0.0	3.1	2.6	1.8	3.3	0.5	0.9	1.1	242.0	288.0	192.7	3.7	78.0	< 1	1.4
	St-2	0.0	1.1	2.6	1.0	3.6	0.4	0.9	0.7	158.3	215.0	186.3	3.0	44.7	< 1	0.8
Hybrid	Lev-13	0.4	4.1	4.1	1.2	2.9	0.3	0.3	0.7	155.3	223.3	234.3	4.7	58.3	< 1	1.0

High

Species	Genotype	Na (%)	Cl (%)	K (%)	Ca (%)	N (%)	P (%)	S (%)	Mg (%)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)	Pb (ppm)	Ni (ppm)
Willow	Cam-2	0.4	4.6	3.3	2.1	2.8	0.4	0.8	1.0	302.0	653.0	353.3	5.0	105.7	< 1	0.8
	LR-3	0.1	4.2	3.2	1.5	3.3	0.5	0.5	0.8	316.3	284.0	316.7	8.0	85.3	< 1	0.9
	St-2	0.2	3.9	3.5	1.5	3.0	0.3	0.7	0.8	247.7	170.7	217.3	3.0	60.7	< 1	0.7

Table A.5 Element composition of willow and hybrid roots at control, low, and high fracking wastewater treatment. Hybrid willows are excluded at the high treatment due mortality at the highest treatment.

Root Tissue

Control

Species	Genotype	Na (%)	Cl (%)	K (%)	Ca (%)	N (%)	P (%)	S (%)	Mg (%)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)	Pb (ppm)	Ni (ppm)
Willow	Cam-2	0.0	0.1	1.7	1.3	2.7	0.7	0.3	0.3	159.7	430.7	76.7	8.0	17.7	< 1	1.0
	LR-3	0.0	0.2	1.6	1.3	3.2	0.6	0.3	0.3	137.3	280.7	57.3	7.3	17.0	< 1	0.6
	St-2	0.0	0.1	1.3	1.1	2.6	0.5	0.2	0.2	108.7	137.0	43.3	6.3	12.7	< 1	0.4
Hybrid	Lev-13	0.1	0.1	2.0	1.1	2.5	0.9	0.3	0.3	93.3	247.0	22.0	7.3	15.7	< 1	0.8

Low

Species	Genotype	Na (%)	Cl (%)	K (%)	Ca (%)	N (%)	P (%)	S (%)	Mg (%)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)	Pb (ppm)	Ni (ppm)
Willow	Cam-2	0.3	1.1	1.6	1.2	2.4	0.7	0.3	0.4	214.7	504.0	235.7	9.0	16.7	< 1	1.3
	LR-3	0.4	1.3	1.5	1.2	2.8	0.6	0.4	0.3	175.7	338.0	113.7	7.0	16.0	< 1	0.9
	St-2	0.2	0.7	1.1	1.2	2.3	0.6	0.2	0.2	143.3	241.7	155.3	8.7	14.3	< 1	0.6
Hybrid	Lev-13	1.0	1.8	1.0	0.9	2.3	0.8	0.3	0.3	98.3	499.7	102.0	6.7	16.0	< 1	0.8

High

Species	Genotype	Na (%)	Cl (%)	K (%)	Ca (%)	N (%)	P (%)	S (%)	Mg (%)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)	Pb (ppm)	Ni (ppm)
Willow	Cam-2	0.6	1.3	1.7	1.1	2.3	0.7	0.5	0.4	383.0	671.7	727.7	12.0	20.0	< 1	1.9
	LR-3	0.6	1.5	1.3	1.1	2.2	0.6	0.4	0.3	322.3	535.7	540.7	8.7	17.0	< 1	1.3
	St-2	0.8	1.1	1.0	1.0	2.6	0.7	0.3	0.3	224.7	378.3	420.7	11.7	16.3	< 1	1.0