University of Alberta

Effects of NaCl on growth and physiology of *Pinus leiophylla* seedlings

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Renewable Resources

Fall 2009
Edmonton, Alberta

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Dedication

To my wife Virginia Jacob Cervantes

and

To the memory of my father

also

To the memory of my aunts Marce and Guadalupe

Abstract

Identification of salt- resistant tree species and genotypes is needed for rehabilitation of lands affected by salinity in Mexico. This dissertation consists of four studies for analyzing the responses of *Pinus leiophylla* seedlings to salt. In the first study, resistance to salt stress was studied in six-month-old seedlings from eight different sources of seed collected from the areas with contrasting precipitation levels. Plants from the xeric areas were shorter and had smaller stem diameters but were less sensitive to salt stress and recovered faster from salt injury compared with the plants from mesic sites, suggesting that morphological and physiological adaptations to drought were helpful with salt stress resistance. In the second study, fascicle needle production and tissue ion accumulation were examined in NaCl-treated three-month-old seedlings from two populations of the xeric origin and two populations from the mesic areas. Seedlings from the xeric population of San Felipe developed fewer fascicles and had shorter needles compared with seedlings from the remaining three populations. NaCl treatment delayed the emergence of fascicles and reduced the fascicle needle production and needle length. However, the extent of needle injury and ion accumulation in shoots were lower in the San Felipe seedlings compared with the other studied populations. In the third study, the effects of branch pruning and seedling size on total transpiration and accumulation of Na^+ and Cl^- in tissues were examined. Total plant transpiration, as affected by plant size and branch pruning, was correlated with Na^+ and Cl^- needle concentrations and needle necrosis. Branch pruning reduced ion accumulation in the shoots and needle necrosis levels in short seedlings but not in the tall seedlings. In the fourth study, sprouting and physiological responses of 16 month-old-seedling to salt were examined. NaCl treatment concentrations of 100, 150, and 200 mM reduced gas exchange and root hydraulic conductance, caused needle injury and triggered sprouting of adventitious shoots. Sprouting from the upper parts of the main stem and lateral branches was three times greater with 100 and 150 mM NaCl compared with 200 mM NaCl treatment but, at the base of the stem, sprout numbers were similar for all NaCl treatments.

Acknowledgments

I want to thanks to Consejo Nacional de Ciencia y Tecnologia (CONACYT), Colegio de Postgraduados, and the University of Alberta for all financial support given through scholarships and for permit me conclude this goal. Special thanks to the Department of Renewable Resources for all help and facilities. My appreciation to my supervisor Dr. Janusz Zwiazek for receiving me in his work team, giving me the freedom to develop this research, all unconditional help during my PhD program, and his friendship.

To my committee members: Dr. Francis Yeh and Dr. Nat Kav for the comments and suggestions to improve my thesis and the opportunity to defend my thesis.

The work in the laboratory and office always was pleasant and fun thanks to my workmates: Adriana Almeida, Adriana Arango, Aurea Siemens, Mihaela Voicu, Monica Calvo, and Hai Naguyen. I would like to thanks to Irma Diaz, Valentin Reyes, Dr. Maciej Grseiziak, Dra. Seonghee Lee, Dr. Tomasz Gradowski, Dra. Kim Metiever, Monica Molina, Pak Chow, and Xin Zhang for their support and friendship. The seed for this research was generously provided by Dr. Javier Lopez of the Colegio de Postgraduados which is geatly appreciated.

Spetial thanks to my wife Virginia Jacob-Cervantes for being with me during the good and bad times of this journey. This goal wouldn’t have been done without you.

Finally thanks to my family, the Jimenez, Casas, Nieto and Jacobs, for the support and the encouraging words during my doctoral program.

Never give up!

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Chapter 1. General introduction and literature review

1.1. Introduction

Salinity is one of the more lethal abiotic stresses since it induces a number of secondary stresses including water deficit stress, ionic stress, and nutritional imbalance. It has been estimated that more than one-third of irrigated land in the world is presently affected by salinity. This figure excludes the regions classified as arid and desert lands which comprise 25% of the total land of our planet (Abrol et al.1988). Salinization is a complex process since it may involve different sources and forms of salt, climatic factors, landscape features and human activities. In the last few decades, the clearance of native trees and the conversion of forests to agricultural lands have aggravated the salinization problems. The subsequent loss of these lands to agriculture due to excessive salinization elevates the risks associated with global warming and reduces potential to meet the needs of the world population which is projected to increase by 1.5 billion in the next 20 years (Blumwald et al. 2004).

In Mexico, the conversion of forest land to agriculture and livestock production has been the dominant force of deforestation during the last three decades in all regions and forest types. Between 1970 and 1990, agricultural lands increased by 39% and the area dedicated to pasture more than doubled, while forests declined by 13% (Segura 2000). This has had an important impact on soil compaction and erosion, leaching, decreased soil fertility, diminished natural plant regeneration, establishment of undesirable weedy plants, disrupted hydrological cycle, and salinization (Ezcurra 1991).

According to several sources, the salinization of agricultural soils (irrigated and nonirrigated) in Mexico is estimated to affect 1.5 million hectares, and in other types of disturbed land approximately 1 million hectares have elevated levels of salinity (Ortiz 1992). Often, low soil fertility and weed infestation result in land abandonment of areas with high salinity so that forest restoration is needed to avoid further soil erosion and loss of biodiversity. Forest restoration should aim at recreating, whenever possible, the full spectrum of forest ecosystem attributes, structure, and functions of the original forest. If such aims can not be achieved, a reasonable goal is to maximize the number of native species and self-sustainability of the forest, particularly when the level of degradation or lack of resources prevents full restoration.

Although there has been less research on the improvement of salt resistance in trees compared with agricultural crop plants, the exploitation of natural genetic variations of trees is a potentially effective approach to develop salt- resistant lines of some forest tree species. Several studies have been carried out to test salt resistance and the genetic variability of physiological responses of trees to salinity in order to select adapted trees for salt-affected sites. There is also evidence for genetic variation for salt resistance among different tree species including: *Pinus*, *Acer*, *Quercus*, *Eucalyptus*, *Casuarina*, and *Taxodium* (Allen et al. 1994; Saur et al. 1995; Loustau et al. 1995). Therefore, the first key issue that needs to be addressed to improve the restoration efforts of forested lands affected by salinity is to investigate the patterns of genetic variation among populations to match seedling genotypes with ecological conditions of the deteriorated sites.

In Central Mexico, *Pinus leiophylla* is one of the species most affected by the conversion of forests to agricultural use and, in the last few decades, the populations of this species have been reduced or totally eliminated in some sites (Eguiluz-Piedra 1978; Musálem and Martínez-García 2003). *P. leiophylla* is native to Mexico with a wide natural range, and is considered a valuable species for rehabilitation of disturbed sites, soil conservation and erosion control. As a pioneer species, it is able to tolerate stress conditions such as drought and frost, and has high ability to survive in sites with frequent fires (Perry 1991; Eguiluz-Piedra 1978). In several countries of Asia, Africa, an the Caribbean, as well as in Chile and Australia, *P. leiophylla* has been planted as a reclamation species (Rodríguez-Franco 2002). Therefore, it may also be a good candidate for the restoration of sites affected by salinity. However, little is known about salt resistance of this species.

The main objectives of the present study were to 1) assess the variability at the population level in the responses of *P. leiophylla* to NaCl, 2) examine the effects of NaCl on *P. leiophylla* growth and physiology, and to 3) determine potential salt- resistance traits and mechanisms in this tree species. This study provides the foundation required for the selection of seed sources that are likely to be superior in salt-affected areas and to establish restoration and management programs with *P. leiophylla* on these challenging sites.

Several similarities between salt stress and water-deficit stress effects on plants have been reported (Munns 2002; Liming et al. 2002; Wang et al. 2003; Lea et al. 2004) so that some drought resistance traits may help with salt resistance in plants. Differences in the responses to severe drought stress conditions were observed in *P. leiophylla* populations from mesic and xeric sites (Martínez-Trinidad 2002). Therefore, I hypothesized that the traits that are responsible for drought resistance in populations from xeric sites may also confer salt resistance to *P. leiophylla* seedlings. The results of testing this hypothesis are presented in Chapter 2 where the main objectives were: 1) determine the growth potential among populations, 2) estimate the differences among populations in response of eight populations of *P. leiophylla* from Central Mexico to NaCl, 3) determine the ability of plants to recover from NaCl injury, 4) identify salt resistance characteristics in plants from different populations and relate them to climatic conditions (precipitation) of the geographic origin.

To understand the mechanisms of NaCl resistance exhibited by some populations of *P. leiophylla*, the effects of NaCl-induced osmotic and ionic stresses were examined in *P. leiophylla* seedlings from the populations showing different degrees of resistance to salt stress. I tested the hypothesis that seedlings from xeric sites would be more resistant to NaCl by reducing ion accumulation in the tissues and this is reported in chapter 3. The main objectives of this study were to: 1) analyze the effects of NaCl on (i) production of fascicle needles and needle extension during the early growth, (ii) the accumulation of Cl^- and Na^+ in roots and shoots, (iii) the extent of needle injury in three month-old-seedlings from xeric and mesic sites, and 2) to evaluate the recovery of gas exchange and root hydraulic conductance after exposure to NaCl.

Results of the three chapters indicated that plant morphology was a factor involved in salt resistance. The size of a plant and its transpiration area may affect ion translocation from roots to shoots through the transpiration stream (Moya et al. 1999). Therefore, the hypothesis that low transpiration rates reduce accumulation of Na^+ and Cl^- in shoots of salt-treated *P. leiophylla* seedlings was tested (Chapter 4). The objectives of this study were: 1) to determine the effects of branch pruning on transpiration rates and on accumulation of Na^+ and Cl^- in roots and shoots of one-year-old *Pinus leiophylla* plants treated with NaCl and 2) to compare the effects of NaCl on seedlings of different size.

P. leiophylla has the ability to sprout and produce adventitious shoots in response to defoliation and injury caused by pests, fire, pruning or others stress factors (Lanner 2002; Rodríguez-Trejo and Fulé 2003). This little studied response may increase the survival of this species in salt-affected areas. Therefore, I tested the hypothesis that the salt-induced needle injury stimulates formation and flushing of adventitious buds and subsequent growth of adventitious shoots and that the level of needle injury determines the extent of adventitious shoot growth (Chapter 5). In this study, I examined the effects of different NaCl concentrations on gas exchange, root hydraulic conductance, needle injury, shoot growth, and sprouting of shoots in 16-month-old *P. leiophylla* seedlings.

The findings and implications of this research as well as its potential practical applications are discussed in Chapter 6.

1.2. Literature Review

1.2.1. Pinus leiophylla Schiede ex Schltdl. & Cham.

1.2.1.1. Taxonomy and common names

This species is classified in the section *Leiophyllae* of the subgenus Diploxilon or hard pines, where *P. chihuahuana* Engelm. and *P. lumholtzii* Rob. *et* Fern. are also included (Perry 1991). Some authors have considered *P. chihuahuana* to be the variety of *P. leiophylla* as there are many similarities between the two tree species and since *P. leiophylla* was described earlier than *P. chihuahuana* (Musálem and Martínez-García 2003). In Mexico, *P. leiophylla* is known under different common names depending on the place of origin. The common names include pino chino, pino prieto, ocote chino, palo otomite, and pino saguaco (Martínez 1948).

1.2.1.2. Distribution

P. leiophylla is widely distributed in Mexico (Figure 1.1) with a natural range from 17° 30' to 31° 20 LN and 96° 30 to 111° 30' LW. This range includes Sonora and Chihuahua in North Mexico through Sierra Madre Occidental into Oaxaca and along the Volcanic Axis in Central Mexico, for the total of 17 out of 32 states in Mexico (Echenique-Manrique and Díaz-Gómez 1969). Although the trees of this species grow on many marginal sites with thin sandy loam soils, low levels of organic matter and pH of 5.5-6.5, the best growth conditions are offered by well-drained soils of volcanic origin (Rodríguez-Franco 2002). The trees are found at elevations ranging from 1600 to 3000 m in temperate to warm-temperate climates where the annual temperature varies from -15 to

38°C and with a rainfall from 600 to 1500 mm from May to October (Eguiluz-Piedra 1978). The species is commonly associated with other pines including *P. engelmannii*, *P. arizonica*, *P. teocote*, *P. lumholtzii*, *P. montezumae*, *P. ayacahuite*, *P. pseudostrobus*, *P. devoniana*, and *P. patula*, as well as *Abies religiosa*, *A. guatemalensis*, *Juniperus* sp., *Arbutus* sp., *Quercus* sp., and other species of hardwoods (Santillán-Pérez 1991).

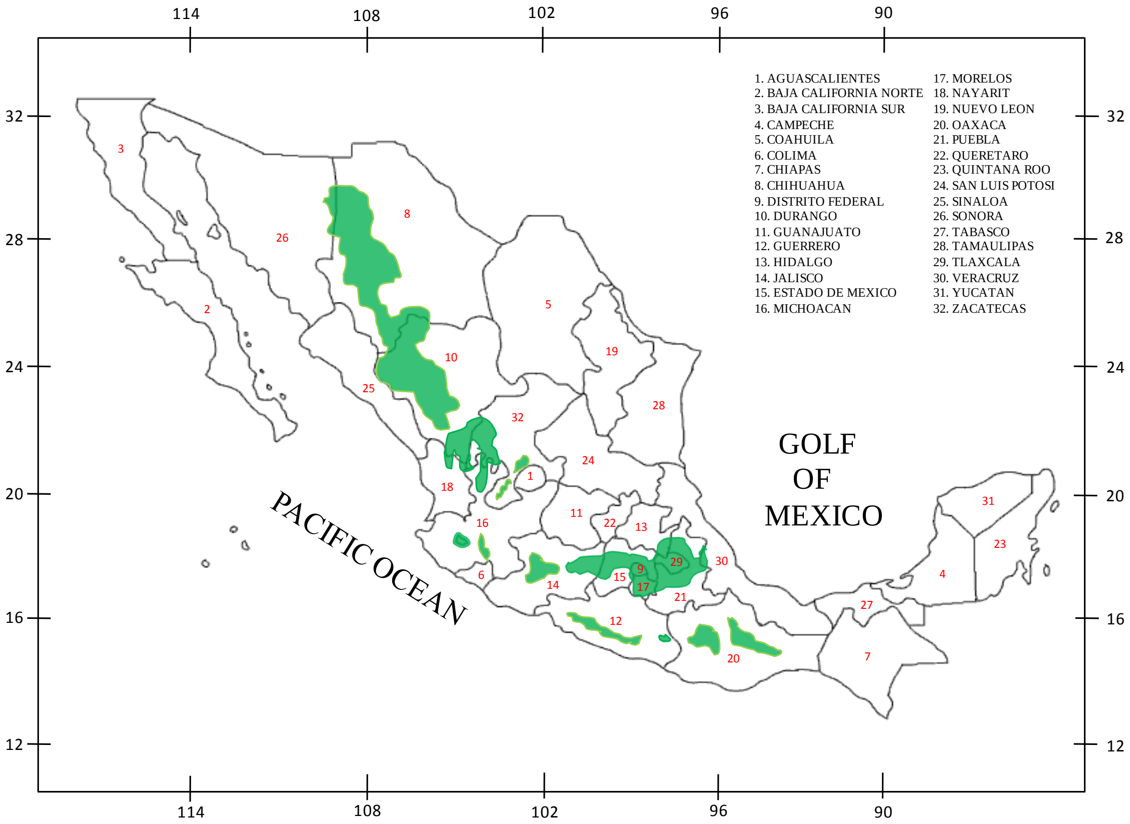

Figure 1.1. Natural distribution of *Pinus leiophylla* in Mexico (modified from Perry 1991; Crichfield and Little 1966).

1.2.1.3. Description of trees

Trees of this species reach moderate heights of 20-35 m and up to 80 cm in breast-height diameter with an irregular crown. The bark is thin and grey in young trees, but becomes black, thick, rough, and scaly with tree age (Perry 1991). Branches are long, slender, spreading or ascending, the lower ones are curved downwards; with clusters of short shoots on the trunk and branches (Figure 1.2). The needles are present in fascicles of five and are 12-20 mm long with 5-6 early-deciduous scales, so that mature fascicles have no sheath. They persist for 2 to 3 years and their margins are minutely serrulate. They are typically light green with 4-8 stomata lines on the convex abaxial face and 3-4 lines on the adaxial faces. Each needle has 1-4 medial resin canals, and the stele is round with 2 vascular bundles (Martínez 1948; Santillán-Pérez 1991).

Figure 1.2. Fifteen-year-old *Pinus leiophylla* trees in a seed orchard at Montecillo Edo. de Mexico.

The ovoid cones are sub-terminal or lateral and arranged in clusters of 1-7. They are 4-6-cm long on slender stalks that are 1.3-1.9-cm long. The cones ripen in the third year and can remain unopened on the branches for several years. When grown in their own range, *P. leiophylla* trees flower from February to April and seeds can be collected during October-February. Epicormic and adventitious shoots may sprout on the trunk and from the root collar after fire or cutting (Perry 1991). The wood has a specific gravity of 0.44 to 0.51, and is relatively dense, heavy, hard, and very resinous with pale brown heartwood (Echenique-Manrique and Díaz-Gómez 1969).

1.2.1.4. Uses

The wood of *P. leiophylla* is considered to be of medium quality and good for pulping. The main problem with wood is the presence of numerous knots that are present along the trunk of the tree. However, *P. leiophylla* is also known to be one of the best producers of resin of all trees in the Volcanic Axis (Villaseñor et al. 1962), and resin is the main commercial product from the trees. The trees are also used for fuel, round wood, building poles, sawn or hewn building timbers, beams, railway ties, containers, rustic furniture and boxes (Moreno-Gómez 1985). *P. leiophylla* is considered to be a valuable species for rehabilitation of disturbed sites, soil conservation, and erosion control. As a pioneer species, it is able to grow under unfavorable environmental conditions including drought and frost, and it has the ability to survive on sites with frequent fires (Perry 1991; Eguiluz-Piedra 1978). In several countries of Asia, Africa, and Caribbean as well as in Chile and Australia, *P. leiophylla* has been planted for reclamation and revegetation purposes (Rodríguez-Franco 2002).

1.2.2. **Deforestation and land salinization**

In deforested areas, the replacement of perennial vegetation with annual crops and the irrigation systems using salt-rich water, change the hydrological balance and create ideal conditions for soil salinization. Clearing the trees and other deep-rooted native vegetation, and replacing them with shallow-rooted agricultural pastures and crops that do not use all the rainfall, allows rainwater to escape below the roots rising water tables and, consequently, salts stored in the subsoil are taken up to the rhizosphere zone. When this happens and water comes close to the surface, it evaporates leaving behind salts which are deposited on the soil surface (Ruprecht and Schofield 1991; Hatton et al. 2003; Rengasamy 2006). On the other hand, irrigation water adds appreciable amounts of salt and contributes to salt accumulation in the root zone; mainly when there is poor water quality, low hydraulic conductivity of soil layers, and high evaporative conditions (Rengasamy 2006). The interaction between irrigation and rising saline groundwater in deforested areas may further increase the risk of soil salinization.

Almost 20% of the earth land mass and nearly half of all irrigated land are affected by salinity (Wang et al. 2003). Increased salinization of arable land is expected to have devastating global effects, with predictions of 30% arable land loss within the next 25 years, and up to 50% by the year 2050 (Yan et al. 2005). In Mexico, forest land conversion to agriculture and livestock production has been reported as the dominant force of deforestation during the last three decades in all regions and forest types. Between 1970 and 1990, area of agricultural land increased by 39% and the area dedicated to pastures more than doubled, while forest area declined by 13% (Masera 1996; Segura 2000). These patterns in land use change are bound to increase soil salinization and erosion problems and, therefore, reforestation programs must consider salt resistance as one of the main factors that will affect reforestation success in these areas.

1.2.3. Effects of soil salinity on plants

According to Munns and Tester (2008), soils are considered to be saline when the EC is 4 dS/m or higher, (equivalent to approximately 40 mM NaCl), at which concentration the productivity of most crops is significantly reduced. Elevated soil salinity reduces or completely inhibits plant growth and may kill cells and tissues because salt in the soil solution limits the ability of the plant to take up water, the condition known as osmotic stress or physiological drought. Under conditions of prolonged exposure to salinity, accumulation of high salt levels in the transpiring leaves may cause injury which usually results in the chlorosis and necrosis of the leaf tissues, brought about by the ionic stress or salt-specific effects (Hasegawa et al. 2000).

It is difficult to separate the specific effects of ion toxicity from those induced by osmotic stress in plants under salt stress; however, reductions in growth of leaves, shoots and roots have been proposed to be the main result of osmotic stress whereas visible injuries (e.g. Chlorosis and necrosis) and senescence of young and old leaves are thought to be the result of ion/salt-specific effects (Munns 2002). Osmotic stress effects can be detected in a few minutes or hours; while ionic stress effects occur after a prolonged period of exposure to salinity and, depending on the species, could take weeks or months before the symptoms can be detected (Munns and Tester 2008). A reduction of stomatal conductance induced by salt-induced osmotic stress is a common response in most woody plants. In studies with *Picea mariana*, *Pinus pinaster* and *P. banksiana* seedlings treated with NaCl, a decrease in stomatal conductance was attributed to the inhibition of root water conductance (Loustau et al. 1995; Redfield and Zwiazek 2002; Apostol and Zwiazek 2003) where water channels play an important role in the water uptake (López-Berenguer et al. 2006). An accumulation of abscisic acid (ABA) in leaves in response to salinity indicates that ABA may be involved in the regulation of stomatal opening as a signal of root water deficit caused by soil salinity (Fricke 2004). Since stomata play a central role in the loss of water and exchange of CO_2, they probably provide the main control of photosynthesis (Jarvis and Davis 1998; Jones 1998) so that the stomatal closure is one of the main factors responsible for the reduction of photosynthesis. In *P. ponderosa*, photosynthesis declined due to low hydraulic conductance (Hubbard et al. 1999). Stomatal closure may explain the reduction of photosynthesis observed in many species exposed to saline conditions.

In woody plants, chlorosis and necrosis in leaves are the main symptoms of ion toxicity caused by high concentration of Na^+ and Cl^- in the tissues (Azza Mazher et al. 2007). Usually, initial Cl^- effects are seen as marginal chlorosis of leaves, followed by browning; whereas Na^+ effects are evident by leaf mottling and necrotic patches. In the end, both Na^+ and Cl^- contribute to the formation of necrotic tissues followed by leaf shedding (Kozlowski 1997). Studies on effects of salinity on needle anatomy in white-cedar and white spruce, showed fragmented cuticles, disrupted stomata, collapsed cell walls, disorganized or disintegrated protoplasts, coarsely granular cytoplasm, disintegrated chloroplasts, disintegrated nuclei, and disorganized phloem. These changes were attributed to ion toxicity of the salt (Kutscha 1997).

Indices and symptoms of salt injury vary among species and depend on the concentration of salts in the soil solution. Necrosis was detected in old needles of *Larix laricina* seedlings exposed to 30 mM NaCl (Renault 2005) and in *P. banksiana* seedlings exposed to 60 mM NaCl (Apostol et al. 2002); whereas no foliar damage was reported in *P. pinaster* seedlings subjected to 50, 150 and 250 mM NaCl (Loustau et al. 1995). In general, woody plants such as avocado, grapevines, citrus as well as several species of genera *Eucalyptus*, *Casuarina*, *Quercus*, and conifers, tend to accumulate high concentrations of Cl^- and not Na^+ in leaves so that Cl^- toxicity is more important than Na^+ toxicity (Townsend 1980; Allen 1994 a; Moya et al. 1999). Redfield and Zwiazek (2002) observed a strong correlation between root electrolyte leakage and Cl^- but not Na^+ tissue concentration in *Picea mariana* seedlings treated with NaCl. In *Picea abies* and *P. glehnii* affected by de-icing salt, the concentration of Cl^- in leaves was higher than Na^+, and the damage and loss of half of the needles by trees was attributed to Cl^- (Kayama et al. 2003). It is believed that Na^+ is effectively stored in the roots and stems of woody plants. Thus Cl^-, which is more easily transported by cells and mobile, becomes the more significant toxic component of the salt solution (Munns and Tester 2008).

1.2.4. Regulation of Na^+ and Cl^- transport

1.2.4.1. Roots and absorption of ions

Studies of growing roots of *Pinus banksiana* and *Pinus taeda* have revealed three morphologically distinct zones known as the white zone, condensed tannin zone, and cork zone (Peterson et al. 1999). Each root zone is characterized by a different level of development and maturation which define the permeability to water and uptake of minerals (Enstone et al. 2001). The white zone is proximal to the root cap and is highly efficient for water and ion uptake due to the prescence of live cortical cells; whereas in the condensed tannin zones, dead cortex potentially could present a barrier for water and ion uptake. In the cork zone, the degree of maturation of cork cells reduces the sites for water and ion uptake so that no ion and little water absorption is thought to take place in this zone.

Absorption of water and ions occurs mainly in the white zone where the soil solution is in contact with epidermal cells and then water and ions flow via the radial transport into cortical parenchyma, endodermis, pericycle and stelar parenchyma using apoplastic (cell walls) and cell-to-cell (transmembrane and plasmodesmata) pathways. From the apoplast, water and ions can be transported into the cells of epidermis and cortex through the cell membranes and then move inward via symplast; or move all the way to the endodermis along the cell walls. However, soil solution cannot move through the apoplast all the way into the stele because of the presence of the Casparian strips in the radial walls of endodermal cells. The Casparian strip is a belt of waxy materials that are impermeable to water so that water and minerals have to cross the plasma membrane of an endodermal cell to detour the Casparian strip and pass into the stele.

1.2.4.2. Na^+ and Cl^- transport across membrane

The movement of cations and anions from soil solution to the cytoplasm is controlled by the permeability of cell membranes which contain transport proteins that facilitate the passage of ions (Figure 1.3).

The transport of Na^+ across the cell membrane is passive and requires membrane proteins to facilitate the passage down the electrochemical gradient. Studies with roots of monocot plants exposed to high Na^+ concentrations reveled that the Na^+ influx into roots may be mediated by voltage-independent protein channels or non-selective cation protein channels (Roberts and Tester 1997; and Davenport and Tester 2000). HKT-type transporters that were analyzed in several plants have been shown to mediate two types of Na^+ transport, K^+- Na^+ co-uptake and Na^+ influx at high Na^+ concentrations (Gassmann et al. 1996; Ren et al. 2005; Tholema et al. 2005; Platten et al. 2006).

Several studies have indicated that Cl^- influx across cell membrane may be mediated by both active and passive processes, depending on the external Cl^- concentration, and facilitated by non-selective channels and carriers of other ions (White and Broadley 2001). Active Cl^- transport across the plasma membrane dominates Cl^- influx to root cells at low Cl^- concentrations in the soil solution. The participation of a $Cl^-/2H$ symporter in the Cl^- active transport was confirmed by electrophysiological studies of root-hair cells, where these Cl^- channels mediated either influx or efflux of Cl^- across the plasma membrane (Munns and Tester 2008). Similarly, biochemical and electrophysiological studies demonstrated that Cl^- channels can mediate Cl^- fluxes in either direction across the tonoplast and that a Cl^-/nH antiport mediates Cl^- influx to the vacuole (White and Broadley 2001). On the other hand, passive Cl^- influx to root cells occurs under high salinity conditions, where excessive Na^+ levels in the extracellular fluid depolarize the membrane, facilitating the uptake of Cl^- through anion channels (Niu et al. 1995). There is evidence that both active and passive Cl^- transport occur also at the tonoplast (Kaestner and Sze 1987). Studies with citrus genotypes subjected to high salt concentrations indicated that Cl^- uptake is a passive process and that an increase of Cl^- root influx was linearly correlated with the increase of external NaCl concentration (Moya et al. 1999). Similar results were found in *Pinus banksiana*, where the increase in membrane electrolyte leakage due to excessive salt significantly contributed to the Na^+ uptake which was closely associated with the increase in Cl^- influx (Franklin and Zwiazek 2004).

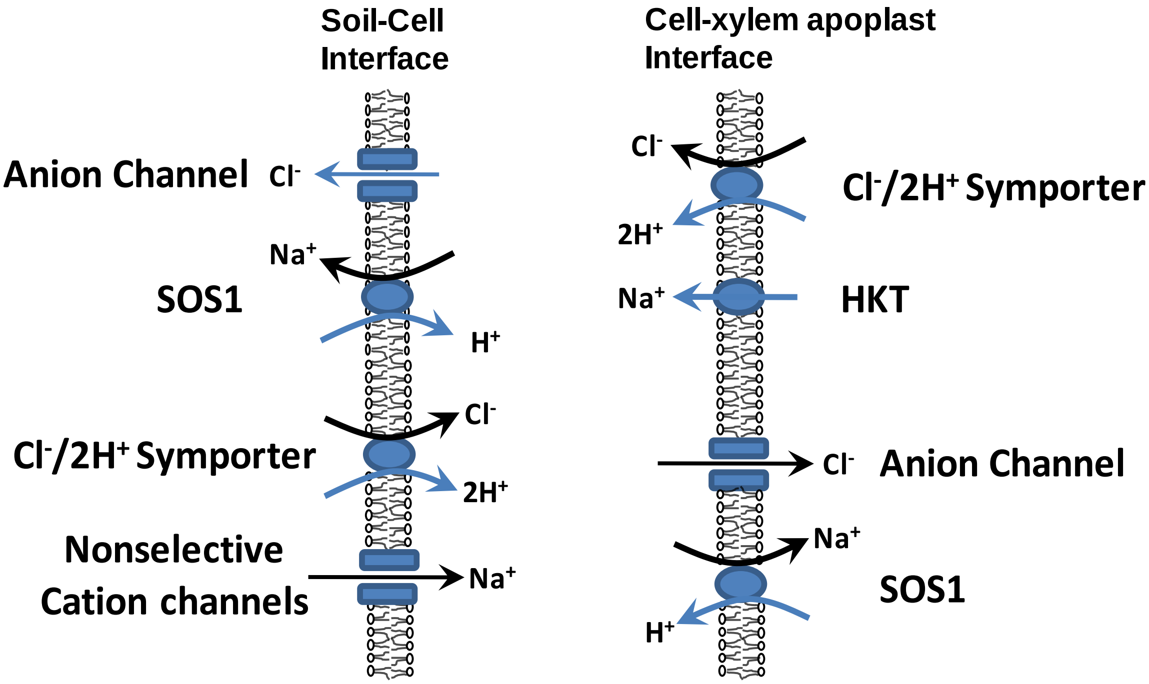

Figure 1.3. Movement of Na^+ and $\mathbf{Cl^-}$ across membrane by protein channels and carriers at the soil-root cell, and stelar cell-xylem vessel interfaces. Adapted from Munns and Tester (2008).

1.2.4.3. Salt resistance mechanisms in plants

Research performed in the last few decades has demonstarted that plants resist salt by avoidance and tissue tolerance mechanisms. Avoidance mechanisms keep Na^+ and Cl^- levels low in root tissues and in the transpiration stream, avoiding accumulation and toxicity of ions in shoot tissues. Dilution of salt inside the plant through the development of succulent tissues and salt exclusion by glands are also regarded as avoidance mechanisms, while tissue tolerance is of importance when Na^+ and Cl^- are sequestered or compartmentalized inside or outside of cells to avoid toxicity and injury to the plant (Levitt 1972; Allen et al. 1994 a; Munns 2002).

1.2.4.4. Salt Overly Sensitive (SOS) signal transduction pathway

SOS signal pathway consists of three components (SOS1, SOS2 and SOS3) that function in a hierarchical sequence to control ion homeostasis and salt tolerance, and its activation is calcium (Ca^{2+})-dependent (Zhu 2002). In high concentrations, NaCl induces influx of Ca^{2+} which quickly increases the cytosolic Ca^{2+} concentration. This process promotes the link between Ca^{2+} and SOS3 (a myristoylated calcium-binding protein) and permit the interaction with SOS2 (a serine/threonine protein kinase) and activation of this kinase complex (Ishitani et al. 2000) as shown in Figure 1.4. The SOS3/SOS2 kinase complex phosphorylates and activates the transport activity of the plasma membrane Na^+/ H^+ exchanger and increases the SOS1 gene transcript levels. Furthermore, kinase complex may also inhibit Na^+ influx by downregulating the low affinity Na^+ transporter HKT1 genes (Halfter et al. 2000; Zhu 2002).

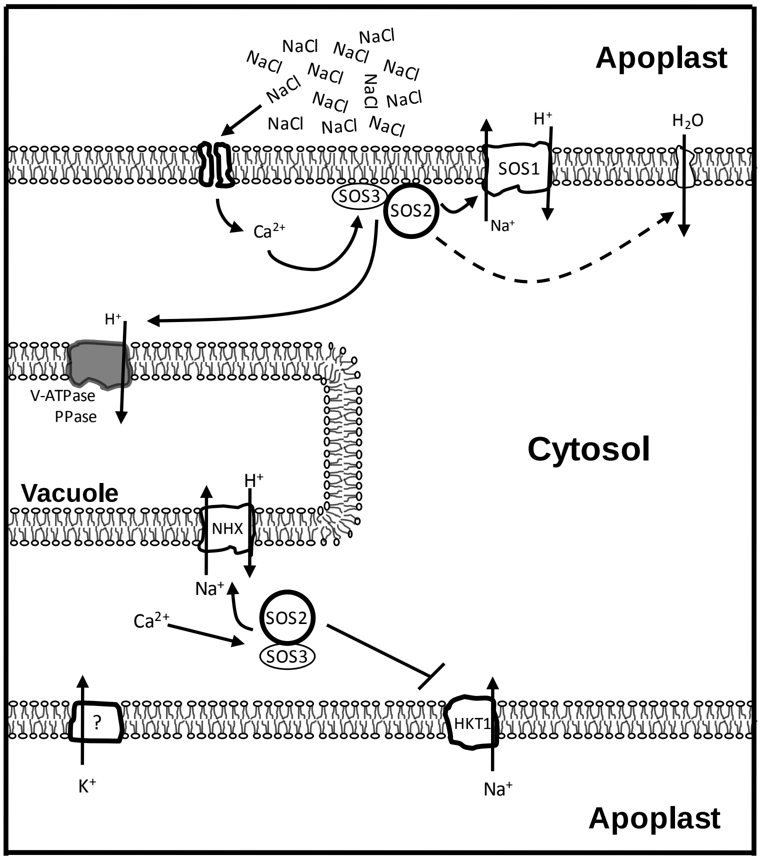

Figure 1.4. Control of ion homeostasis, to increase salt tolerance, by SOS signal pathway. Activation of Ca^{2+} channels by salt causes an increase of cytosolic Ca^{2+} which promotes the establishment of the SOS2 and SOS3 complex. This complex is involved in the activities of several transporters that may include water channels between other. Adaptated from Zhu (2002).

Na^+/ H^+ antiporter exchanges Na^+ for H^+ across membranes, pumping Na^+ out of the cytoplasm to apoplast and permitting the influx of H^+ to cytoplasm (Tomoaki and Schroeder 2004). This implies that the antiporter is involved in the regulation of cytosolic pH and cell turgor (Wang et al. 2003). Under NaCl stress, an immediate effect is the vacuolar alkalization due to the influx of Na^+ and efflux of H^+ (Fukada-Tanaka et al.

2000). In salt tolerance, the participation of Na^+/ H^+ antiporters has been confirmed (Wu et al. 2004). SOS1 transcripts have been localized in the epidermal and stelar cells of roots under saline conditions (Shi et al. 2002).

1.2.4.5. Vacuolar sequestration of Na^+ and Cl^-

Na^+ and Cl^- sequestration or compartmentation is a means of preventing toxicity in the cytosol and helping achieve osmotic homeostasis because Na^+ can be used as an osmolyte. It is estimated that sequestration may maintain the cytosolic concentrations of ions as low as 10-30 mM. This mechanism is essential for salt tolerance of not only halophytes but also glycophytic plant species (Flowers et al. 1977). The pumping of Na^+ into vacuoles is performed by a vacuolar Na^+/ H^+ antiporter while Cl^- may be transported by a channel or carrier that exchange protons (H^+) which could be a kind of H^+/anion antiporter (Munns 2002). Na^+/H^+ antiport activities were detected years ago. The molecular nature of the antiporters was revealed when several genomic sequences with similarities to microbial and animal Na^+/H^+ antiporters appeared in the GenBank of *Arabidopsis*. Several groups then characterized these Na^+/H^+ antiporter sequences (Apse et al. 1999), categorized in groups of the AtNHX antiporter family of tonoplast. The vacuolar Na^+/H^+ antiporters appear to form a multigene family that might show different temporal or spatial expression of the various isoforms. Overexpression of vacuolar transporters plays an important role substantially improving plant salt tolerance (Zhu 2001).

1.2.4.6. Control of Na^+ in the xylem

Tester and Davenport (2003) described two other strategies to prevent Na^+ accumulation in shoots. The first strategy refers to the control of influx and efflux of Na^+ from cells in the stele into the apoplast and xylem, where a type of Na^+/ H^+ antiporter may be involved, since SOS1 is expressed in stelar cells. According to Tester and Davenport (2003), at moderate Na^+ concentrations, the Na^+/ H^+ antiporter might function in active efflux of Na^+ to the xylem; while in high concentrations, the efflux may be reduced and controlled actively. The participation of ABA should be considered in the stimulation of the antiporter, since ABA can stimulate H^+ extrusion into the xylem (Clarkson and Hanson 1986). In the second mechanism, protein porters work in reverse to remove Na^+ from xylem sap. In the removal mechanisms, Na^+/H^+ symporter and Na^+ uniporter have been proposed (Munns 2002; Shi et al. 2002). In addition, transfer cells could play a role during the removal process; that kind of cells in the roots may increase in number to remove more Na^+ back out of the xylem. In roots, the removed Na^+ could go to vacuolated cells in mature roots and stems (Yeo et al. 1977). However, there is little evidence to support these strategies of Na^+ exclusion and many mechanisms involved in such a process are unknown.

1.2.4.7. Hydrogen pumps

The activity of proton pumps (H^+-pumps) also plays an essential role in ion transport. The H^+ -pump (H^+ ATPase and H^+ pyrophosphatase) membrane proteins are important in the movement of ions because they provide the needed energy. The H^+-pumps establish the electrochemical potential gradient (proton motive force) with an inside negative plasma membrane potential of -120 to -200 mV that facilitates electrophoretic ion flux across cellular membranes (Poole 1988). Under moderately saline conditions, the H^+ electrochemical potential gradients could facilitate ion flux and reduce the Na^+ and Cl^- levels in the cytosol from 10^2- to 10^3-fold, relative to the apoplast or the vacuole (Hasegawa et al. 2000). The H^+-pump localized in the plasma membrane is a P-type ATPase and is responsible for the pH and membrane potential gradient across this membrane; whereas in the tonoplast, there is a P-type H^+-ATPase and a pyrophosphatase that establish the membrane potential in the tonoplast and regulate the vacuolar pH (Yokoi et al. 2002).

1.2.4.8. Cellular adjustment and protection against salinity

An increase of organic solutes and proteins in the cytoplasm has been reported as a response of plants aimed at avoiding cellular damage and improving water balance.

Accumulation of compatible organic solutes that do not inhibit metabolic reactions, in response to salinity has been observed in several species. The tolerance of salinity and drought exhibited by *Populus euphratica* Oliv. coincided with an accumulation of organic solutes that promoted osmotic adjustment and salt tolerance (Watanabe et al. 2001). Compounds involved in the protection of structures and osmotic balance are considered to act as osmolytes. The solutes with osmolyte functions that accumulate in the cytosol are most commonly mono- and disaccharides including fructose and sucrose, sugar alcohols such as glycerol and methylated inositols, and complex sugars such as trehalose, rafinose and fructans, as well as ions such as K^+ and charged metabolites such as glycine betaine, proline and ectoine (Hasegawa et al. 2000).

The accumulation of such solutes varies within and between species (Yokoi et al. 2002). Although the synthesis of these organic solutes is costly to the plant and may affect plant growth, this strategy increases chances of survival and recovery of plants exposed to salt stress.

Proteins synthesized in response to salinity have been identified in several plants. These proteins include osmotins, dehydrins or late embryogenesis abundant (LEA) proteins, chaperones, and reactive oxygen species (ROS) proteins (Tester and Davenport 2003; Mahajan and Tuteja 2005). The accumulation of these proteins in response to salt stress suggests their involvement in salt stress adaptation. They may be involved in stabilization of macromolecules and membranes, protection against osmotic effects, reduction of lipid peroxidation, protection of the photosynthetic electron transport, and in detoxifying or preventing the generation of ROS (Moons et al. 1995; Zhu 2002; Anzlovar and Dermastia 2003; Ndimba et al. 2005).

ROS scavenging plays an important role in the repair systems. Environmental stresses affect the efficiency of photosynthetic electron transport and result in changes in the redox state of chloroplasts and the production in intracellular levels of ROS: singlet oxygen (${}^1O_2$), superoxide ($O^{\cdot}/2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH) (Foyer and Noctor 2003). Sensing changes of ROS concentrations as a result of metabolic disturbances are used by plants to activate stress defensive responses, enzymic and non-enzymatic processes, for scavenging of ROS (Pauly et al. 2006). The enzymes overproduced to minimize the concentration of ${}^1O_2$ and H_2O_2 include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione-synthesizing enzymes. Scavenging of $O^{\cdot}/2$ and by SOD results in the production of H_2O_2, which is removed by ascorbate peroxidase or catalase. ROS removal is a very important process allowing avoids the synthesis of a very toxic hydroxyl radical. Radical OH is synthesized by the combination of $O^{\cdot}/2$ and H_2O_2 in the presence of trace amounts of Fe^{2+} and Fe^{3+} and it can damage chlorophyll, proteins, DNA, lipids and other important macromolecules, and negatively affect plant metabolism, growth and yield (Sairam and Tyagi 2004; Christophe et al. 2004).

1.2.5. **Genetic variations in salt stress resistance of woody plants**

Some woody plants may adapt to salinity conditions through both avoidance and tolerance mechanisms. For example, mangrove species and *Tamarix* can exclude up to 90% of the salt due to their efficient system of restricting salt uptake through the development of a passive root membrane filtration system and glands (Shannon 1997; Kozlowski 1997). *Populus euphratica* has the ability to restore ion homeostasis by compartmentalizing excessive sodium in the vacuole and apoplast, protecting the cytoplasm and the trees can produce succulent leaves by increasing the number and size of cells (Ottow et al. 2005).

Exclusion is probably the most important mechanism of resistance operating in woody plants under saline conditions (Allen et al. 1994 a; Munns 2002). For example, avocado from Mexican rootstocks were less salt- resistant than Guatemalan stocks because the less resistant trees contained high concentrations of chloride in their leaves (Downton 1978). In citrus and *Vitis* spp., salt exclusion has been tested by the ability for reducing the chloride concentration in tissues (Downton 1977).

The ability to exclude ions in *Citrus*, *Poplar* sp. *Eucalyptus* sp., *Pinus radiata*, and several annual species has been attributed to early formation of Casparian bands, lipid concentration, and sequestration of chloride ions in vacuoles of ray cells and in the lumen and cell walls of tracheids (Allen et al. 1994 a; Chen et al. 2002). In *Citrus*, the capacity to restrict foliar accumulation of Na^+ and Cl^- has been improved through crosses between selected hybrids which also permits studies about the inherence and mechanisms of salt exclusion in this species (Sykes 1992).

Salt exclusion reduces the rate at which salt accumulates in transpiring organs, regulating ion movement to the tissue and protecting the cellular metabolism. In many plants, salt resistance depends on the ability of the root system to restrict Na^+ and Cl^- transport to the shoots; some woody plants such as *Pinus radiata and Quercus robur* have shown the ability of the root system to restrict Na^+ transport to the shoots, limiting the extent of shoot injury (Foster et al. 1977; Myers et al. 1998). In contrast, Cl^- uptake and transport appeared to be less controlled than that of Na^+ (Foster and Sands 1977; Myers et al. 1998). *Pinus banksiana* is able to retain Na^+ and Cl^- in roots for a short time, but when the exposure to salt is prolonged, both Na^+ and Cl^- are translocated to shoots (Franklin and Zwiazek 2004). In high concentrations, Na^+ and Cl^- may affect negatively the plasma membrane and proteins. In high concentrations, Cl^- produces membrane injury which affects its permeability and structure (Renault et al. 2001; Redfield and Zwiazek 2002). Studies with *Pinus banksiana* have shown that the loss of Cl^- exclusion mechanism and the presence of Cl^- results in an increase in the translocation of Na^+ and other cations to the shoot due to the increase in membrane leakiness (Franklin et al. 2002; Apostol and Zwiazek 2003).

Genetic variation in salt resistance has not been as extensively studied in woody plant species as in herbaceous species. However, there are interesting studies in woody plants that have demonstrated the genetic potential of several species for the reclamation of abandoned agricultural areas and deforested lands. Inter- and intra-specific variation to salt- resistance was studied in Na^+-treated seedlings (0, 25, 50, 75 mM) of *Pinus contorta, Pinus banksiana, Picea glauca*, *Populus tremuloides,* and *Alnus rubra* which exhibited a significant interaction between salt treatments and seed lots within plant species as well as between salt treatments and plant species was observed for weight and necrosis, indicating that the plant genotype responded differently to salt treatments (Khasa et al. 2002). Similar variation was observed in leaf number and height of sixteen *Eucalyptus* species subjected to salt treatments. Species with an ability to maintain a relatively large number of leaves and showing a relatively large height growth when subjected to salinity appeared to be more resistant to salt (Sun and Dickinson 1993). Resistant and sensitive genotypes were detected in five species of *Casuarina* grown under non-saline drained, saline drained, non-saline waterlogged and saline waterlogged conditions and their resistance assessed by survival and relative growth after 12 weeks. The salinity resistance exhibited by some genotypes was associated with the exclusion of Na^+ and Cl^- while relatively sensitive genotypes accumulated salt in the shoots (Van Der Moezel et al. 1989). Poplar clones have shown variation in sensitivity to salinity. Less sensitive clones were more vigorous and had significantly lower leaf and root Na^+

accumulation than more sensitive genotypes (Fung et al. 1998). Genetic variation was demonstrated in seedlings of 15 open-pollinated families of baldcypress (*Taxodium distichum)* subjected to salinity and flooding stress. Significant variation was found among salinity levels, families, and salinity x family interactions for leaf area and total biomass (Allen et al. 1994 b).

Despite considerable inter and intra specific genetic variations in salt resistance of tree species, there has been little progress in the identification of salt- resistant species, genes controlling salt resistance and level of heritability. These studies are important to establish selection criteria based on inter-specific variation and intra-specific variation for the development of salt- resistant lines to be used for reclamation of saline habitats.

1.2.6. **References**

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Chapter 2. Salt resistance of eight *Pinus leiophylla* seedling populations from sites in Central Mexico differing in precipitation conditions

2.1. Introduction

Deforestation followed by intensive agriculture has created soil salinization problems in Central Mexico. The deforested sites are characterized by hot and dry conditions with low levels of precipitation and, therefore, require intensive irrigation (Alcocer et al. 2000). It has been well documented that excessive irrigation greatly contributes to soil salinization (Rengasamy 2006). High soil salt levels affect water and ionic balance in plants and result in growth reductions, injury and death. Therefore, salt and drought resistance are among the most desirable characteristics of plants designated for the restoration of these degraded areas. Salt resistance in plants varies by species and genotypes within the species (Allen et al. 1994; Khasa et al. 2002; Niknam and McComb 2000; Marcar et al. 2002). Selection of salt-resistant genotypes for land restoration should be preferably carried out among native vegetation which is adapted to local environmental conditions. In addition, land restoration with native vegetation guarantees the conservation of forest ecosystems and reduces problems associated with pests, diseases, as well as competition from invasive and exotic species.

Pinus leiophylla Schl. *et* Cham. is one of the main tree species which have been affected by deforestation in many parts of Mexico. Trees of this species are also recommended for revegetation, soil conservation and erosion control (Rodríguez-Franco 2002). *P. leiophylla* is considered a pioneer species with wide distribution in Mexico ranging from subtropical to hot-temperate and temperate climates (Santillán-Pérez 1991). The trees grow on marginal sites, in areas covered by volcanic rocks and in thin sandy loam soils with low levels of organic matter and pH of 5.5 to 6.5 (López-Upton 2002). In addition, *P. leiophylla* can tolerate drought, low-intensity fire and frost (Perry 1991; Musálem and Martínez-García 2003; Rodríguez-Trejo and Fulé 2003).

The wide distribution of this species in Mexico is evidence of its adaptability to different environments. Studies with other conifer species have shown that the resistance level to frost, drought and salinity conditions varies with the geographic origin (Loustau et al. 1995; Benowicz et al. 2001; Calamassi et al. 2001; Cregg and Zhang 2001; Mitton and Jeffry 2004). Geographic variation in drought resistance has been studied in *P. leiophylla* populations which showed high water use efficiency during and after drought treatments (Martínez-Trinidad et al. 2002).

Since salt induces water deficit stress in plants, genotypes with adaptations to drought conditions may also be more resistant of salinity conditions. In the present study, I hypothesized that these adaptive features to drought would also be beneficial for salt resistance. The present study was conducted to generate knowledge required to develop early selection criteria for reforestation of sites with elevated salt levels. The main objective of the study was to identify salt resistance characteristics in *P. leiophylla* seedlings from different populations and relate these characteristics to the climatic conditions and geographic origins of the populations.

2.2. Materials and Methods

2.2.1. Plant material and growth conditions

Seeds of *Pinus leiophylla* Schl. *et* Cham., provided by the Colegio de Postgraduados, Programa Forestal (Montecillo, Edo. de Mexico), were collected from open-pollinated trees growing of eight populations from three states that are characterized by different precipitation levels in the central region of Mexico (Table 2.1). In February 2005, seeds were soaked in tap water for 24 h and placed at 4ºC for another 24 h to enhance and accelerate germination. The seeds were then transferred to Petri dishes containing moist sand and germinated in the dark at 26 °C. After radicle emergence from the seed coat, seedlings were transferred to Spencer-Lemaire root trainers (170 mL volume, Spencer-Lemaire Industries Ltd. Edmonton, AB, Canada) filled with a mixture of peat moss and sand (2:1 by volume). The seedlings were grown in a greenhouse with day/night temperatures of 22/10°C, 60-70% relative humidity, and a 16-h photoperiod. Plants were irrigated with distilled water every three days and fertilized every 8 days with a solution containing 6 g L^{-1} of 30-10-10 (N-P-K) commercial fertilizer (Plant Prod® Water-Soluble Fertilizer, Spectrum Brands Inc.).

2.2.2. NaCl treatment

In July 2005, six-month-old plants were rearranged within the cells of root trainers in a randomized complete block design with six replicates per population per block, giving 8 blocks and 384 plants in total (Figure 2.1 a). For NaCl treatments, plants were divided into a treatment group which was provided with mineral nutrient solution supplemented with NaCl and a control group which was provided with mineral solution without NaCl.

The plants were treated by immersing the trays with seedlings in the respective treatment solution for 24 h every 4 days. To prevent osmotic shock, the plants were treated with increasing NaCl concentrations. The starting concentration of 30 mM NaCl was increased by 15 mM at each subsequent immersion in NaCl solution (every 4 days) until the final concentration of 90 mM NaCl was reached. The 90 mM NaCl treatment solution had an electrical conductivity of 8.42 dS m^{-1} compared with 0.82 dS m^{-1} for the control solution. Electrical conductivity of the soil leachate was checked every 8 days for randomly selected plants and distilled water was added every 10 days to maintain similar electrical conductivity levels throughout the treatments. The NaCl treatments were applied for 60 days and the measurements were taken every 20 days, during this period. After 60 days of salinity treatment, the seedlings were subjected to a recovery period of 60 days. During the recovery period, plants were provided every four days with nutrient solution and every 15 days with distilled water to avoid the accumulation of salts (soil electrical conductivity of 0.80-1.03 dS m^{-1}).

2.2.3. Growth measurements

At the time of harvest (20, 40, 60 days), root collar diameter (D), height (H), shoot dry mass (SDM), root dry mass (RDM), and the number of new roots (NR) were determined. Shoots were separated from roots and the roots were gently washed with cold tap water to remove the soil. New roots (NR) $\geq$ 1 cm in length were counted and root collar diameters were measured with a digital caliper ($\pm$ 0.01 mm). Shoots and roots were dried at 70 °C for 48 h and weighed.

2.2.4. Gas exchange and root hydraulic conductance

Net photosynthesis (P_n) and stomatal conductance (g_s) were measured after 40 days of NaCl treatment. Measurements were taken in the greenhouse between 9:00 and 11:00 with a portable infra-red gas analyzer (LCA4, Analytical Development Company Limited, Hoddesdon, UK) on the distal 3 cm of terminal shoots. Temperature in the cuvette varied between 26.8 to 28.3 °C and the irradiance of 586 to 589 µmol m^{-2} s^{-1} photosynthetic photon flux density was provided by supplemental light (Sylvania flood USA 150 W). Following the measurements, fascicles were removed and needle surface areas obtained by computer scanning (Sigma Scan Pro 5.0, SPSS Inc.).

Root hydraulic conductance (K_r) was measured using the hydrostatic pressure method (Siemens and Zwiazek 2003). The root system was excised 3 cm above the root collar, immersed in distilled water and placed in a Scholander pressure chamber (PMS Instruments, Corvallis, OR). The stem protruding through the lid of the chamber was connected to a graduated pipette to collect xylem sap. The chamber was pressurized with compressed air to 0.3 MPa and after 5 min of equilibration time, the steady-state water flow (Q_v) was measured for 15 min. Pressure was then increased to 0.6 MPa and 0.9 MPa and Q_v measured at both pressures. K_r was calculated from the regression line of Q_v plotted against hydrostatic pressure and expressed as m^3 H_2O s^{-1} MPa^{-1}x 10^3.

2.2.5. Needle injury and production of needles

After 60 days of NaCl treatment and recovery period, Needles were removed from each seedling and necrotic parts of the needles separated from green, healthy areas. The green needles were weighed to determine fresh mass and dried at 70 °C for 24 h. Both green and necrotic needles were weighed to determine needle necrosis which was expressed as a dry weight ratio of necrotic to green needle tissues.

2.2.6. Statistical analysis

The residuals of trait data were analyzed by the Shapiro-Wilkins test for fit to the normal distribution using the UNIVARIATE-SAS procedure. Data were subjected to analysis of variance with GLM-SAS procedure, mean separation of main effects was carried out with Duncan's multiple range test, and correlation analysis was conducted with CORR procedure and variance components were estimated with VARCOMP method=type1-SAS/PC for Windows 8.2 (SAS institute Inc., 2001).

Total height and diameter of untreated plants and needle necrosis were analyzed using the statistical model:

$$Y_{ijkl} = \mu + B_i + St_j + P_{k(j)} + e_{ijk}$$

Where Y_{ijkl} is the plant l from state j of the population k in the block i; μ is the overall mean, B_i is the block effect, St_j is the state effect, $P_{k(j)}$ is the population within state effect, and e_{ijk} is the error.

Growth and physiological traits of plants under saline treatment were subject to the statistical model:

$$Y_{ijklm} = \mu + B_i + T_j + T*B_{ji} + St_k + T*St_{jk} + P_{l(k)} + T*P_{jl(k)} + e_{ijk}$$

Where Y_{ijklm} is the plant m of the population l from the state k in the treatment j of the block I, B_i is the random block effect, T_j the fixed treatment effect, $T*B_{ji}$ the treatment X block interaction, St_k the fixed state effect, $T*St_{jk}$ the treatment X state interaction effect, $P_{l(k)}$ is the population within state effect, $T*P_{jl(k)}$ the treatment X population within state, and e_{ijk} the error.

2.3. Results

2.3.1. Potential growth among populations

Stem diameter and height values were significantly different among states and populations (Figure 2.2 a and b).The variation in diameter and height among populations wthin states was 53.9 % and 81.3 % of total variance, respectively (Table 2.2).

Plants from Estado de Mexico showed the highest stem diameter and heights (5.2 mm and 9.4 cm, respectively) and those from Tlaxcala had the lowest values (4.3 mm and 6.9 cm, respectively). Puebla populations had intermediate values (Table 2.3).

Seedlings of the populations from the areas with high annual precipitation were taller on average than those from the low precipitation sites (R^2=0.62), and the same tendency was present for stem diameter (R^2=0.59). Two populations from Estado de Mexico (3 and 4) and one from Puebla (8) from sites with high annual precipitation had 52.4 % and 29.3% greater heights and stem diameters, respectively than the Tlaxcala (6) and Puebla (7) populations from drier sites. Intermediate values were observed for the two populations from Estado de Mexico (1 and 2) and one from Tlaxcala (5) (Figure 2.4).

2.3.2. Growth

The morphological (H, D, NR, SDM, and RDM) parameters were significantly reduced by the NaCl treatment, as compared with the control (Table 2.4, Figure 2.1 e and f). The NR decreased over time until almost zero after 60 days of NaCl treatment. However, new root production resumed during the recovery period, and after 60 days of stress relief, the treated seedlings had 49% of the number of new roots found in control plants (Figure 2.5 a).

The same tendency as for roots was found for height and stem diameter, although NaCl never completely stopped seedling height or dimaeter growth. After 20, 40 and 60 days of NaCl treatment, height was reduced by 13.1, 40.6, and 41.9%, respectively; whereas stem diameter showed little reduction after 20 days but became significant on days 40 and 60 by 39.9 and 47.9 %, respectively (Figure 2.5 b and c). In contrast to height, stem diameters continued to increase under NaCl treatment and the stems increased their diameter by 64% from the end of treatment to the end of the recovery period (Figure 2.5 b).

The effect of NaCl on height and diameter varied significantly between populations during the NaCl treatment and recovery (Table 2.2). Populations from the state of Tlaxcala had the lowest percentage reduction of D, H and NR (Figure 2.3). This is in sharp contrast with the states of Mexico and Puebla that had populations with the greatest percentage reductions (Figure 2.3).

After 60 days of NaCl treatment, the greatest reductions in H and D were present in populations from Estado de Mexico (2, 3 and 4) and Puebla (8). These reductions ranged from 42% to 54% for H and from 52% to 60% for D. The lowest reductions in H ranged from 26 to 29% and those in D ranged from 27 to 36% in populations from Tlaxcala (6) and Puebla (7) (Figure 2.6). The reduction in NR measured between 81 and 91% and was similar for all populations at the end of the 60-day 90 mM NaCl treatment. However on day 40, Tlaxcala populations had the smallest reductions in NR varying between 50 and 55%, whereas the rest of the populations had reductions ranging from 60 to 70 % (Figure 2.6). During the recovery period, reductions were smaller as treated plants resumed stem diameter growth and produced new roots so that the differences with control plants were reduced. Population 6 (Tlaxcala) showed the greatest recovery in NR illustrated in Figure 2.6 as the smallest reduction value (18%) in contrast with the rest of the populations that had large reductions (between 42 and 72%) (Figure 2.6). The populations 6 and 7 had smaller reductions in D (23 and 30%, respectively) than the rest of the populations (> 42%) (Figure 2.6).

After 60 days, NaCl treatment reduced root and dry mass in all populations. The range of dry mass reduction among populations relative to the control was between 34% and 55% DW for roots and between 24% and 35% DW for shoots. Compared with the control, population 6 had the smallest reductions with 34% for roots and 24% for shoots. In contrast, population 3 had the greatest reduction of root and shoot dry mass at 55% and 35%, respectively. Among the states, plants from Estado de Mexico exhibited 10% and 5% greater reduction of root and shoot dry mass, respectively, compared with plants from the Tlaxcala and Puebla States (Table 2.5).

2.3.3. Root hydraulic conductance and gas exchange

Root hydraulic conductance (K_r) was significantly reduced by the NaCl treatment (Figure 2.7). Populations 1, 6 and 7 showed low K_r values in comparison with the remaining populations. Maximum reduction in K_r was reached after 60 days of NaCl treatment in all populations (Figure 2.7). In populations 1, 6 and 7, K_r was reduced by less than 55%, whereas in the remaining populations, the reduction was greater than 80% (Figure 2.7).

Effects of NaCl on stomatal conductance (*gs*) and net photosynthesis (P_n) are shown in Table 2.6. After 40 days of treatment, there were significant reductions in *gs* and P_n compared with the control. However, seedlings from different states and populations did not show significant differences in *gs* and P_n in response to NaCl treatment (Table 2.4). Seedlings of the population 4 (from Estado de Mexico) and population 5 (from Tlaxcala) showed reduction trends in *gs* (Table 2.6).

Survival of plants was not affected in any of the studied populations by the 60-day 90 mM NaCl treatment, and shoot tips showed no signs of injury. However, needle tip necrosis appeared in older needles after 40 days of NaCl treatment and increased over time (Figure 2.1. c and d). Needle necrosis was about 42% higher in populations from mesic sites (2, 3, 4 and 8) compared with those from the drier sites (6 and 7) (Figure 2.8).

After three months of recovery, new roots and needles were produced in plants of all populations (Figure 2.3 and 2.9). However, there were significant differences among populations in numbers of new roots produced at the end of the recovery period. There was a strong correlation between needle necrosis and the production of new roots (Figure 2.9 a) and new needles (Figure 2.9 b). Populations 6 and 7, which had less extensive needle necrosis, showed greater production of new roots and needles compared with the populations with more extensive needle necrosis (Figure 2.9).

The K_r increased proportionally to the increase in the number of new roots (Figure 2.10). Populations from the drier sites (1, 2, 3 and 4) showed a greater number of new roots and higher K_r values compared with the populations from mesic sites (5,6,7 and 8) (Figure 2.10).

2.4. Discussion

There were differences in growth and sensitivity to NaCl between different populations of *P. leiophylla*. The variations in shoot growth have been studied extensively in seedlings and mature trees of numerous species of conifers (Jayawickrama et al. 1998; Kaya et al. 1994; Ettl and Petterson 1995; Benowicz et al. 2001). In some conifers, such variations are associated with temperature and moisture regimes (Aitken 2000). Previous studies have demonstrated a relationship between productivity and stress resistance in various tree species (Loustau et al. 1995, Niu et al. 1995). Trees from xeric areas exhibited slower growth compared with trees from mesic sites (Oleksyn et al. 2000; Tognetti et al. 2000; Cregg and Zhang 2001). This relationship was also present in *P. leiophylla* (Figure 2.4) with seedlings from the three mesic Estado de Mexico sites being taller and having greater stem diameter compared with seedlings from the drier region of Tlaxcala and Puebla (Table 2.3). These differences in growth are likely the result of adaptations to drought.

Since water deficit is among the main effects of salt stress (Munns 2002), I hypothesized that the adaptive traits to drought in *P. leiophylla* populations would also help trees tolerate salt stress. The ability of *Picea mariana* seedlings to maintain cell turgor when treated with Na_2SO_4 helped the seedlings avoid needle injury (Redfield and Zwiazek 2002). Similar responses were reported for *P. pinaster* genotypes from drier seed sources, which showed a greater ability to maintain low osmotic potential and greater shoot elongation under salt stress compared with the genotypes from mesic sites (Lostau et al. 1995). Authors of a study of geographic variation in response to soil salinity in European populations of *Picea abies* recommended that genotypes exhibiting low transpiration rates and high water-use-efficiency should be considered for early selection of salt- resistant families (Bozhko et al. 2003). In our study, the extent of needle necrosis and the reductions in hydraulic conductance and gas exchange by the NaCl treatment varied between different populations. The growth reduction and needle necrosis caused by NaCl were less evident in the slower-growing seedlings from drier sites compared with faster-growing seedlings from the mesic sites (Figure 2.6, Figure 2.8). A similar relationship was found in *Pseudostuga menziesii* and *Picea glauca* where slower-growing seedlings were more resistant to heat and drought than faster growing seedlings (Bigras 2000; 2006).

Production of new roots in *P. leiophylla* seedlings was one of the growth parameters sensitive to NaCl with all populations significantly decreased after 20 days of NaCl treatment (Figure 2.5, 2.6). Depending on the conifer species and age, root growth inhibition may occur when electrical conductivity of the growth medium is above 2.5 dS m^{-1} (Jacobs and Timmer 2005). In the present study, *P. leiophylla* was subjected to 8.42 dS m^{-1} (90 mM NaCl). It is likely that the reduction of soil osmotic potential combined with ionic toxicity of NaCl affected water uptake (Bernstein 2002) and the permeability of cell membranes (Apostol and Zwiazek 2003). This, in turn, led to water deficit stress and nutritional imbalance (Saur et al. 1995; Franklin and Zwiazek 2004) affecting root growth.

Although the reduction of root growth appears to be the principal factor that is responsible for the decrease in root hydraulic conductance (Figure 2.7); the inhibition of water channels (aquaporins), suberization of cortical cell, membrane injury, and low soil water potential should be also considered as possible important factors responsible for this decrease (Kozlowski 1997; Steudle 2001; Apostol et al. 2002; Boursiac et al. 2005).

Root hydraulic conductance responded differently in different populations to the NaCl treatment. After 20 days of treatment, seedlings from mesic sites (populations 3, 4 and 8) had the highest values of root hydraulic conductance that corresponded to high stomatal conductance (Figure 2.7). However, after 40 days, there was a greater reduction in hydraulic conductance in these populations when compared with seedlings from the drier sites (Figure 2.7). These responses among populations may be related with the ability of seedlings of xeric to control the deficit-water stress more efficiently than those from mesic sites; however, further studies are needed to know the mechanisms involved in the root hydraulic conductace response by the seedlings from xeric sites.

The decrease in stomatal conductance by NaCl resulted in reduced CO_2 needle uptake and photosynthetic rates (Table 2.6), as well as reduced dry mass (Table 2.5). Stomatal closure is the most common cause of photosynthetic reduction in conifers exposed to various environmental stresses including hypoxia, NaCl (Apostol and

Zwiazek 2003), drought (Bigras 2005) and temperature (Teskey and Will 1999). Although reductions of *gs* and P_n were not statistically different between the studied populations of *P. leiophylla* (Table 2.4), there was a trend for greater reductions of *gs* in populations from the Estado de Mexico compared with the populations from Puebla and Tlaxcala (Table 2.6). However, the absence of differences in P_n between the populations would suggest that other NaCl-sensitive processes, such as cell elongation, could play a greater role in dry mass reductions observed in treated plants (Table 2.5).

Needle tip necrosis is one of the indicators of salt injury. This symptom appears first on the tips of old needles with the extent depending on the concentration and time of exposure to salt. In later stages, needle injury increases toward the needle base and, eventually, appears in new needles (Renault 2005). The exact mechanisms by which salt causes needle necrosis are still unknown. However, there is a strong correlation between needle necrosis and Cl^- needle concentrations (Franklin and Zwiazek 2004). It would appear that the accumulation of Cl^- in tissues plays a central role in making the tissues more susceptible to Na^+ accumulation and injury (Apostol et al. 2002; Franklin and Zwiazek 2002). In our experiment, older needles of *P. leiophylla* showed needle necrosis after 50 days of 90 mM NaCl treatment and needle necrosis increased until the harvest time (60 days). However, younger needles and lateral and apical shoot tips did not show signs of visible injury and the plants continued producing new needles during recovery from stress.

The present results indicate that the extent of needle necrosis was a fundamental factor affecting the recovery of plants. The negative correlation between percentage of needle necrosis and production of new roots and needles demonstrated that genotypes from populations 6 and 7 had the ability to avoid damage to old needles which was reflected by the production of new roots and needles (Figure 2.9). This could also indicate an importance of the loss of photosynthetic area for growth recovery. New root production was a key factor in the re-establishment of hydraulic conductance and, therefore, in the recovery of plants. There was also a strong correlation between new root production and root hydraulic conductivity in plants recovering from stress (Figure 2.10), pointing to the importance of new root production for water uptake following stress. The relationship between new root production and survival of planted seedlings was studied in commercial plantations of conifers and the results indicated that greater root production increased the rate of seedling survival following planting (Simpson 1990; Grossnickle 2005).

In conclusion, *P. leiophylla* seedlings of the populations growing in dry sites were smaller, more resistant to NaCl, and had greater recovery capacity than plants from the wetter sites, suggesting that the traits responsible for drought adaptation were likely contributing to salt resistance in *P. leiophylla* seedlings.

2.5. References

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Table 2.1. Geographic location, altitude and mean annual precipitation (PP) of seed collection sites

No.	Population	State	Location		Altitude	PP
			Lat. N.	Lat. W.	(m)	(mm)
1	Tequesquinahuac	Estado de México	19°27’	98°47’	2700	750
2	Amecameca	Estado de México	19°10’	98°46’	2550	936
3	Tlalmanalco	Estado de México	19°11’	98°47’	2550	1072
4	Tlamacas	Estado de México	19°03’	98°40’	2700	1186
5	La Malinche	Tlaxcala	19°19’	98°00’	2780	800
6	San Felipe	Tlaxcala	19°28’	98°32’	2630	822
7	Calpan	Puebla	19°06’	98°26’	2330	884
8	S M Atepetzingo	Puebla	19°21’	98°35’	2500	950

Table 2.2. Variance components for diameter (D) and height (H) of untreated *P. leiophylla* plants

Source of Variation	d.f.	Variance components (%)		EMS
		D	H	
B	2	0.2	0	$\sigma_e^2 + 16\sigma_B^2$
St	2	3.2 *	6.1*	$\sigma_e^2 + 6\sigma_{P(St)}^2 + 15\sigma_{(St)}^2$
P(St)	5	53.9 *	81.3 *	$\sigma_e^2 + 6\sigma_{P(St)}^2$
Error	38	42.7	12.6	σ_e^2

B=Block, St=State, and P(St)=Population within State; Degree of freedom (d.f.); Expected means square (EMS); *=significant at α=0.05%.

Table 2.3. Means of stem diameter and height for seedlings from three different states in Mexico used in this experiment. Different letters indicate significant differences at α=0.05

State	Stem diameter (mm)	Height (cm)
Estado de México (n=96)	5.20 a	9.44 a
Puebla (n=48)	4.63 b	7.91 b
Tlaxcala (n=48)	4.32 b	6.89 c

Table 2.4. Analysis of variance (F values) for height (H), stem diameter (D), number of new roots (NR), shoot dry mass (SDM), root dry mass (RDM), root hydraulic conductance (K_r), net photosynthesis (P_n) and stomatal conductance (gs) in 8 populations of *P. leiophylla* after 20, 40, 60 of treatment with 90 mM NaCl and 120 days (or recovery period of 60 days)

Trait	Source of Variation				
	S (1)	St (2)	S*St (2)	Pop(St) (5)	Sal*Pop(St) (5)
20 days					
H	15.0 *	31.5 *	1.7	38.8 *	1.6
D	3.1	10.2 *	0.1	10.9 *	0.1
NR	12.3 *	7.0 *	0.1	4.8 *	0.4
K_r	36.9	40.9	4.0	116.0	0.9
40 days					
H	250.4 *	18.1 *	9.0 *	24.8 *	6.8 *
D	168.8 *	6.3 *	0.8	8.2 *	4.4 *
NR	190.8 *	3.8 *	4.6 *	1.8	0.1
K_r	339.5 *	51.8 *	4.7	78.0 *	16.4 *
P_n	206.3 *	2.7	0.8	5.2 *	0.5
gs	26.9 *	0.7	2.2	2.8	1.3
60 days					
H	609.7 *	43.0 *	27.5 *	42.7 *	20.5 *
D	458.6 *	8.4 *	7.4 *	8.1 *	8.8 *
NR	678.0 *	4.6 *	4.6 *	1.4	1.3
K_r	3854.0 *	19.7 *	27.1 *	60.0 *	87.4 *
RDM	312.1 *	9.4 *	4.1 *	3.4 *	1.7
SDM	346.7 *	62.8 *	33.1 *	18.3 *	4.0 *
120 days					
H	653.8 *	45.4 *	30.6 *	42.2 *	21.4 *
D	365.7 *	20.0 *	8.2 *	9.6 *	8.7 *
NR	552.2 *	6.5 *	19.0 *	1.1	11.1 *
K_r	932.6 *	5.1 *	13.4 *	21.6 *	35.6 *

( ) = degree of freedom; * = significant difference at $p \leq 0.05$

Table 2.5. Root and shoot dry mass of seedlings from eight *P. leiophylla* populations treated with 90 mM NaCl for 60 days. Means ± SD are shown (n = 6). State/Population (S/P), 0 mM NaCl (control, C), 90 mM NaCl (T)

	Root (g)			Shoot (g)		
S/P	C	T	Reduction (%)	C	T	Reduction (%)
E. Mexico	**8.8 ± 2.1**	**4.2 ± 0.5**	**52.3**	**36.4 ± 4.1**	**25.3 ± 1.9**	**30.5**
1	7.4 ± 1.6	3.8 ± 0.5	48.6	30.7 ± 3.5	24.5 ± 1.5	20.2
2	9.5 ± 1.0	4.2 ± 0.4	55.8	38.4 ± 1.5	27.0 ± 1.6	29.7
3	10.0 ± 3.2	4.7 ± 0.5	53.0	37.9 ± 2.0	24.9 ± 1.8	34.3
4	8.7 ± 1.6	4.2 ± 0.4	51.7	38.7 ± 2.4	25.0 ± 1.7	35.4
Tlaxcala	**7.6 ± 1.9**	**4.2 ± 0.7**	**44.7**	**29.4 ± 3.1**	**22.0 ± 3.1**	**25.2**
5	8.9 ± 1.5	4.2 ± 0.8	52.8	31.8 ± 2.5	23.6 ± 3.2	25.8
6	6.4 ± 1.4	4.2 ± 0.8	34.4	27.1 ± 1.5	20.6 ± 2.2	24.0
Puebla	**6.5 ± 1.7**	**3.6 ± 0.5**	**44.6**	**29.5 ± 4.0**	**21.3 ± 3.5**	**27.8**
7	6.2 ± 1.8	3.4 ± 0.5	45.2	26.5 ± 2.9	18.5 ± 1.2	30.2
8	6.8 ± 1.6	3.9 ± 0.5	42.6	32.7 ± 1.9	24.2 ± 3.5	26.0

Table 2.6. Stomatal conductance (*gs*) and net photosynthesis (P_n) in *P. leiophylla* seedlings treated for 40 days with 90 mM NaCl Means±SE are shown (n = 6)

Population	***gs* (mmol m^{-2} s^{-1})**			**P_n (µmol m^{-2} s^{-1})**		
	[NaCl]			**[NaCl]**		
	0 mM	90 mM	Reduction (%)	0 mM	90 mM	Reduction (%)
E. Mexico	**199.8 ± 22.3**	**137.3 ± 29.0**	**31**	**21.5 ± 5.6**	**8.0 ± 2.7**	**63**
1	214.1 ± 73.9	178.9 ± 30.3	16	28.6 ± 4.1	11.6 ± 3.3	60
2	170.5 ± 23.5	134.9 ± 20.3	21	21.7 ± 7.8	8.8 ± 2.9	60
3	219.8 ± 45.7	120.6 ± 29.8	45	20.5 ± 3.9	6.1 ± 2.1	70
4	194.7 ± 18.0	114.5 ± 28.0	41	15.0 ± 8.4	5.7 ± 3.2	62
Tlaxcala	**186.1 ± 5.2**	**160.5 ± 0.3**	**14**	**22.4 ± 6.6**	**9.7 ± 1.5**	**56**
5	182.4 ± 23.7	160.4 ± 48.7	12	17.6 ± 5.9	8.6 ± 2.4	52
6	189.8 ± 33.6	160.7 ± 12.9	15	27.0 ± 8.0	10.7 ± 2.9	60
Puebla	**176.6 ± 28.1**	**143.0 ± 16.7**	**19**	**19.6 ± 0.3**	**7.8 ± 0.2**	**60**
7	196.5 ± 49.6	154.8 ± 45.1	21	19.4 ± 7.5	7 .9 ± 5.5	59
8	156.7 ± 38.1	131.2 ± 33.4	16	19.8 ± 8.1	7.6 ± 2.6	61

Figure 2.1. *P. leiophylla* seedlings at the begining of NaCl-treatment (a), treated and control seedlings after 40 days of treatment (b), Needle necrosis in NaCl-treated seedlings after 60 days under treatment of NaCl (c) y (d), Effects of NaCl treatment on reduction of shoot and root biomass after 60 days of treatment (e) and (f).

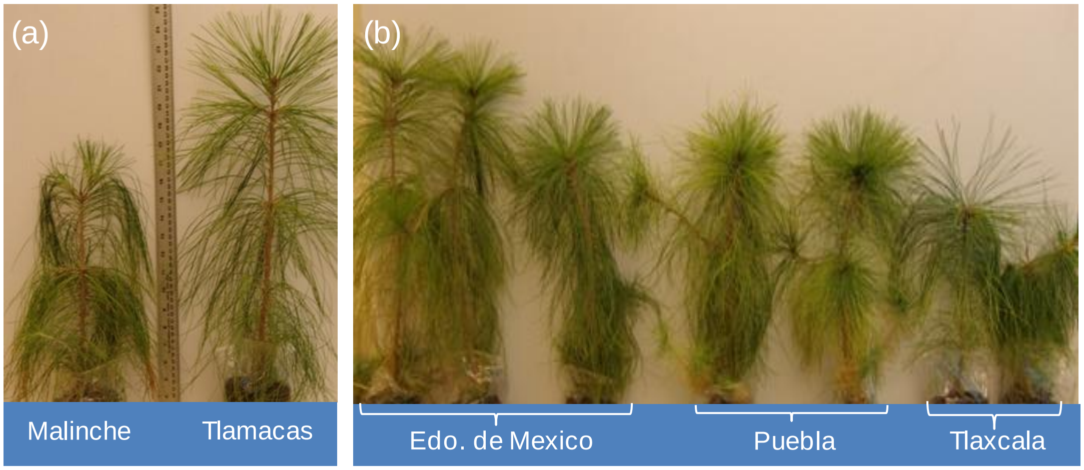

Figure 2.2. Variation in seedling height among the populations tested in this study. Between two populations from xeric site (Malinche) and mesic site (Tlamacas) (a); and among populations from different states (b).

Figure 2.3. NaCl-treated plant from the Malinche population at the end of recovery period showing injury in old fascicle needles and new needles production at the top of the stem.

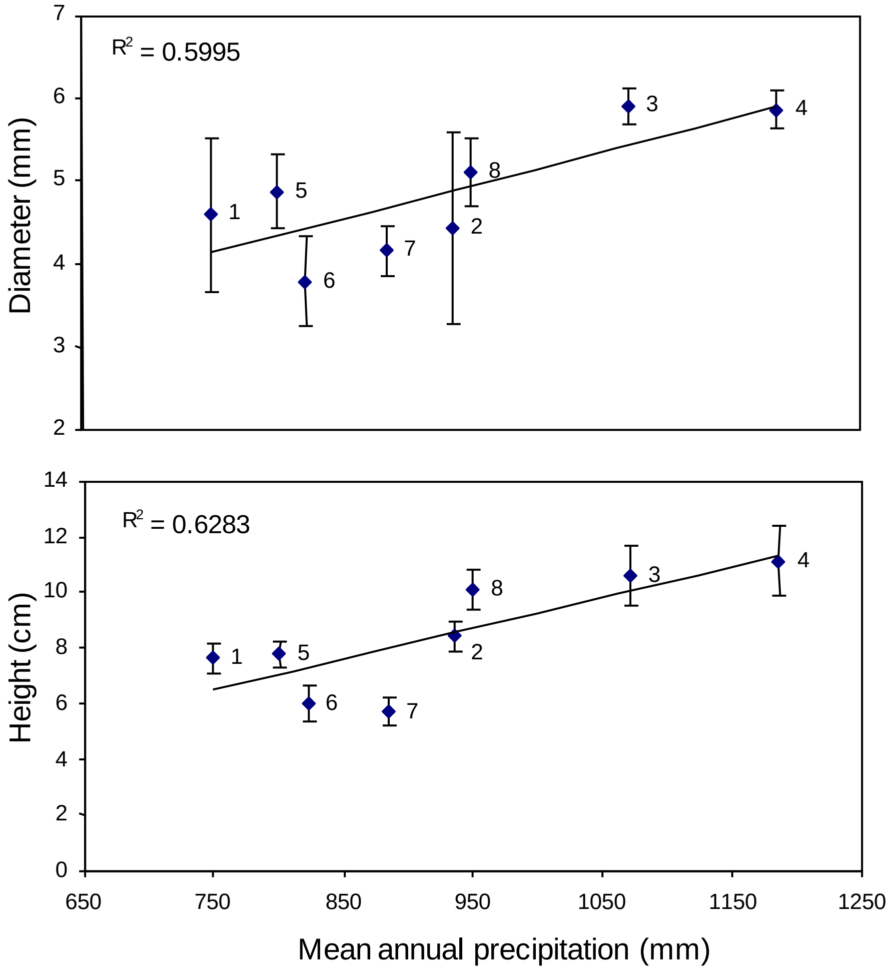

Figure 2.4. Relationship between the precipitation level and mean stem diameter and height in seedlings from 8 populations of *P. leiophylla*. Vertical bars are standard error of means (n = 6).

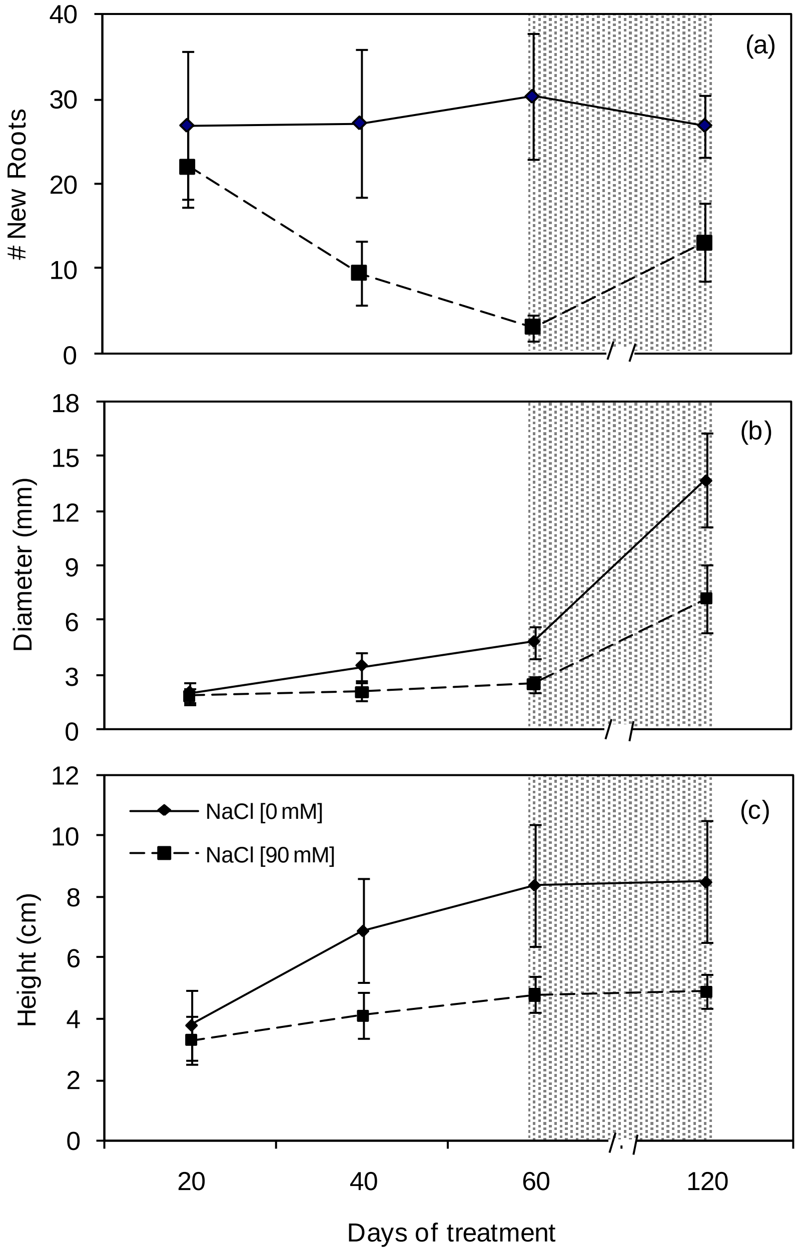

Figure 2.5. Effect of NaCl on number of roots (a), stem diameter (b) and height (c) in six-month-old *P. leiophylla* seedlings from all states. Shaded areas represent recovery time. Vertical bars are SE (n = 48).

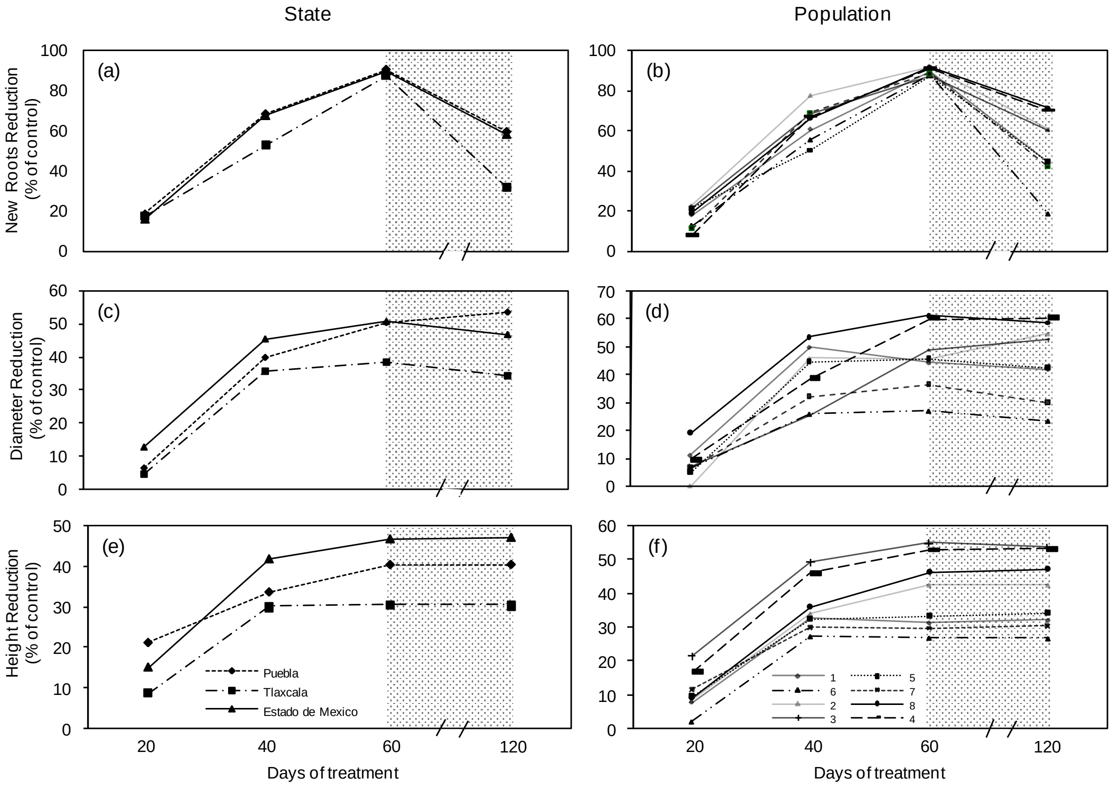

Figure 2.6. Percent reduction in new roots, stem diameter, and height in 6 month-old *P. leiophylla* plants from 8 populations treated for 20, 40 and 60 days with 90 mM NaCl and after 60 days of recovery. Shaded areas represent recovery time.

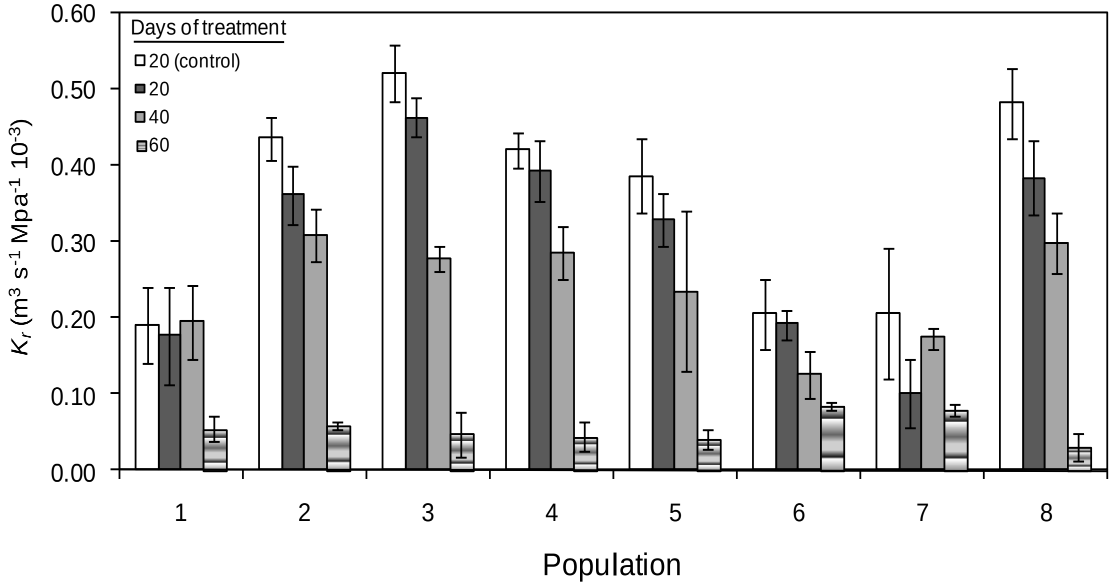

Figure 2.7. Effects 20, 40, and 60 days of 90 mM NaCl treatment on root hydraulic conductance (K_r) in different populations of *P. leiophylla* seedlings. Vertical bars are SE (n=6).

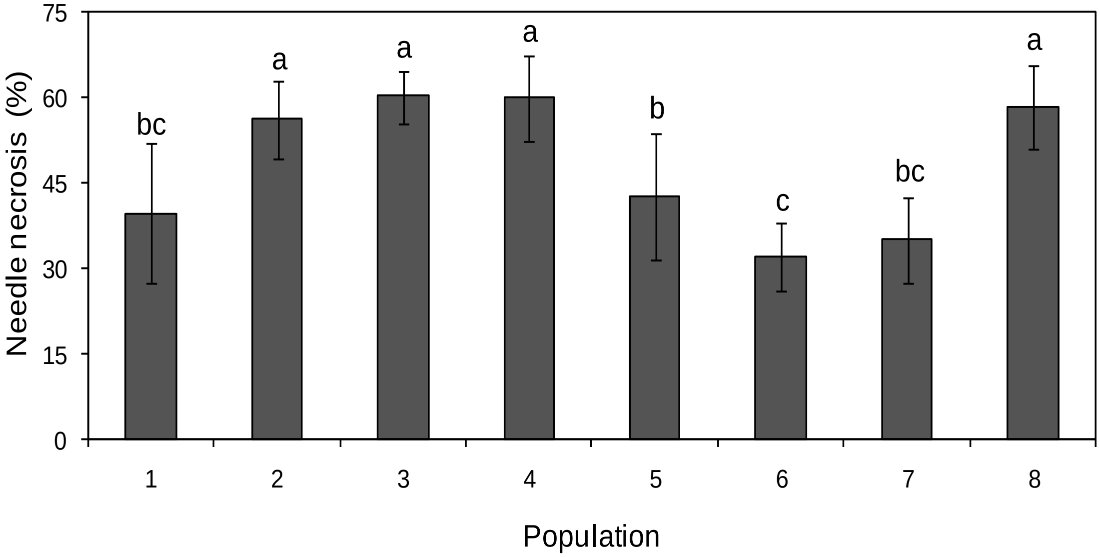

Figure 2.8. Needle necrosis (% needle dry mass) in seedlings of different populations treated with 90 mM NaCl for 60 days. Means (n=6) ± SE are shown.

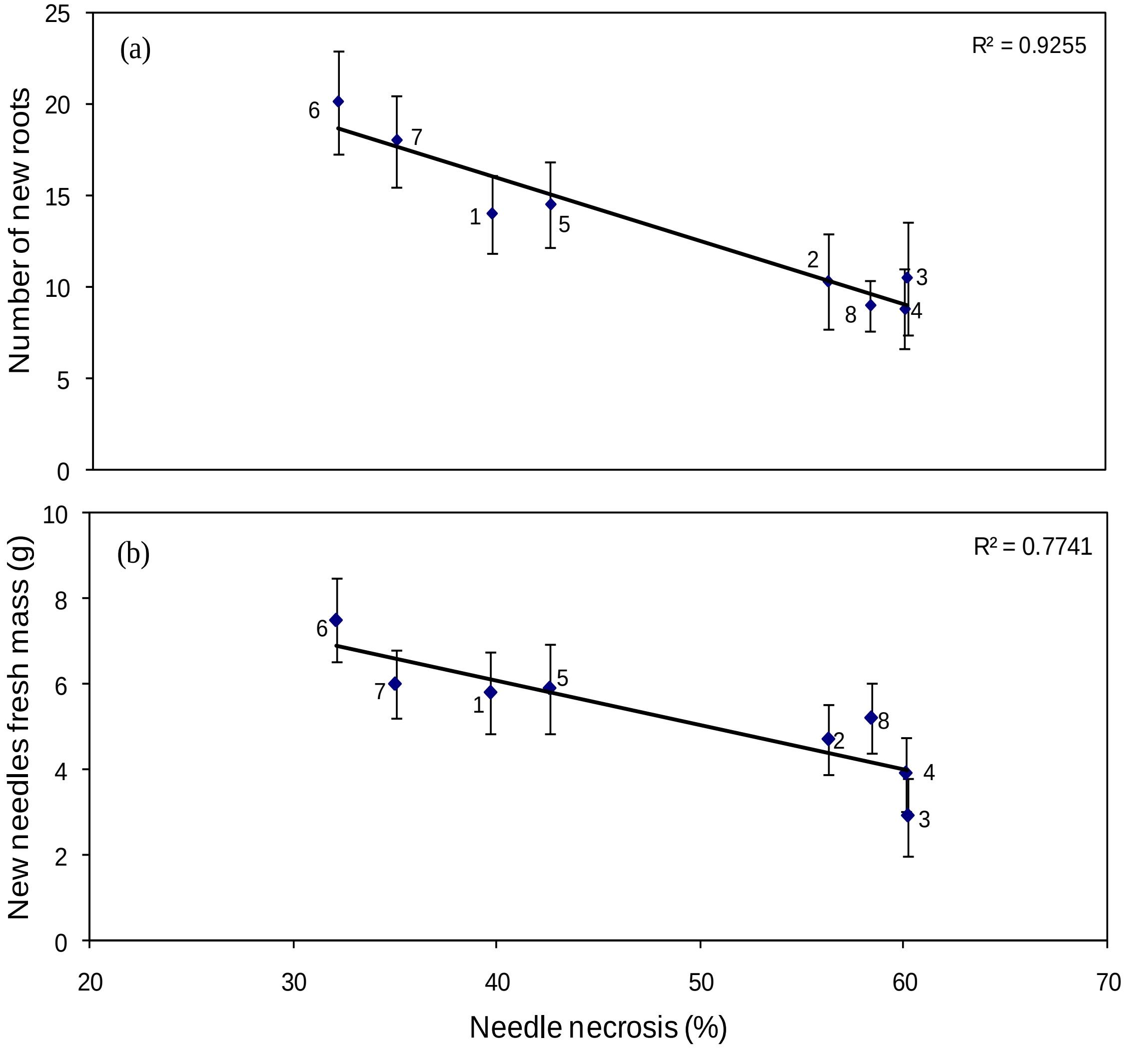

Figure 2.9. Relationship between needle necrosis and new roots (a) and new needles (b) in different populations of *P. leiophylla* after recovery period. Means (n=6) ± SE are shown.

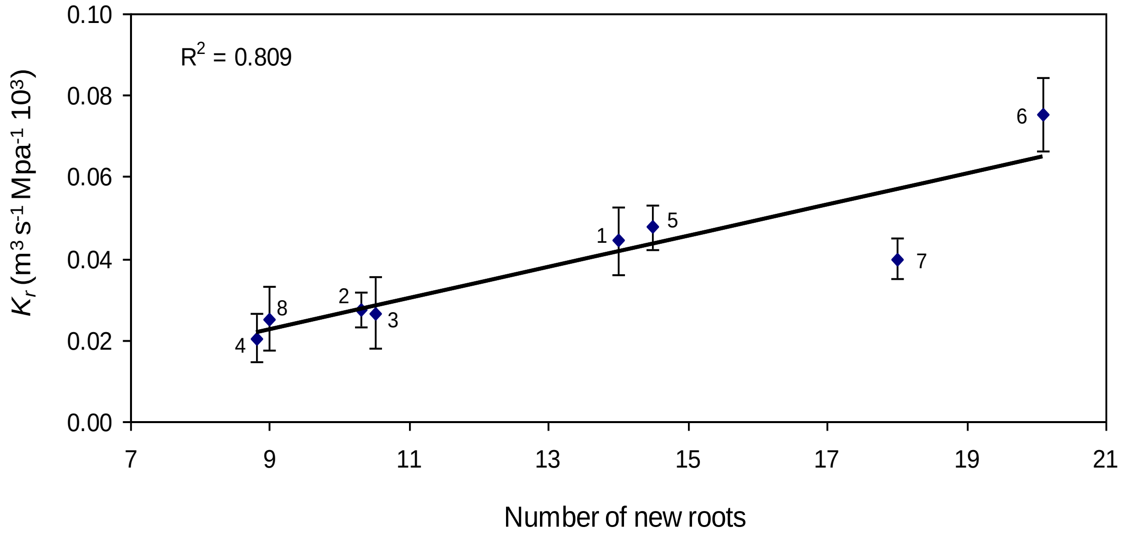

Figure 2.10. Relationship between the number of new roots and root hydraulic conductance (K_r) in different populations of *P. leiophylla* after recovery period. Means (n=6) ± SE are shown.

Chapter 3. Effect of NaCl on growth of fascicle needles, salt uptake and gas exchange in seedlings from four populations of *Pinus leiophylla* in Central Mexico

3.1. Introduction

Soil salinization in Mexico has increased as a consequence of forest land conversion for agricultural activities. Salt build up in the root zone is largely the consequence of replacement of native deep-rooted vegetation with annual crop plants which require frequent irrigation (Feikema et al. 1999). Excessive soil salinity reduces root water uptake and causes ion toxicity when salt accumulates in the tissues (Allen et al. 1994).

Leaf emergence and expansion processes are among the most sensitive processes affected in plants in response to salt stress (Neves-Piestun et al. 2001; Munns 2002; Suárez and Medina 2005). In trees which produce dormant buds, leaf primordia expand rapidly during bud opening as leaf cells absorb water into the vacuoles. The process of leaf expansion is followed by the leaf maturation events which include the synthesis of chlorophyll, cutin and wax (Mauseth 1998). Salt may interfere with water uptake that is needed for the turgor-driven cell expansion (Neves-Piestun et al. 2001; Meinzer et al. 2008). In addition to water deficit stress, salt produces direct ion toxicity effects when the salt build up in the tissues exceeds a threshold level. Ion toxicity symptoms often include chlorotic and necrotic lesions at the tips and margins of older leaves (Munns and Tester 2008). Glycophytes tolerate limited amounts of salt by sequestering it in the areas that are less sensitive to salt, such as the vacuoles (Sands and Clarke 1977; Apostol et al. 2002;

Navarro et al. 2007). However, the main mechanism of salt resistance in glycophytes, including pines, appears to be the restriction of salt uptake (Allen et al. 1994).

In some areas of Central Mexico, reforestation and land restoration programs require salt- resistant genotypes to ameliorate the increasing soil salinization caused by anthropogenic factors such as deforestation and agricultural activities (Castillo and Toledo 2000; Velázquez et al. 2002). *Pinus leiophylla* Schltdl. & Cham. is among the most affected tree species by deforestation in Central Mexico and in some areas *P. leiophylla* populations have been totally eliminated (Eguiluz-Piedra 1978). This species is widely distributed in Mexico and has the ability to tolerate drought, low intensity fire and frost. In addition, *P. leiophylla* trees can grow in the areas covered by volcanic rocks and on marginal sites with low levels of organic mater (Perry 1991; Musálem and Martínez-García 2003; Rodríguez-Franco 2002).

In my previous study (Chapter 2), I observed that the populations of *P. leiophylla* from xeric sites were more salt–resistant compared with those from mesic sites indicating that the adaptive traits to drought are also likely involved in salt-resistance in *P. leiophylla* seedlings. The main objective of the present study was to examine the effect of NaCl on the production of fascicle needles and needle extension, accumulation of Cl^- and Na^+ in roots and shoots, extent of needle injury, gas exchange, and root hydraulic conductivity in three months-old-seedlings from *P. leiophylla* populations growing in xeric and mesic sites in Central Mexico. I tested the hypothesis that NaCl would have a greater effect on needle growth in seedlings from the mesic site populations compared with those from the xeric site, due to reduced ion uptake and greater water use efficiency.

3.2. Materials and Methods

3.2.1. Plant material and growth conditions

Pinus leiophylla Schltdl. & Cham. seeds of four populations, provided by the Forestry Program of Colegio de Postgraduados in Mexico, were collected from open-pollinated trees growing in Central Mexico (Table 3.1). In April 2006, the seeds were washed in cold running tap water for 60 minutes; then rinsed with a Tween solution (2 drops of Tween/L water) for 30 minutes; and hydrated overnight in cold tap water. They were then transferred to Petri dishes containing moist sterilized sand. The Petri dishes were placed in a growth chamber at 20°C for seed germination. Immediately after seed germination, the seedlings were moved to Spencer-Lemaire root trainers (170 mL volume, Spencer-Lemaire Industries Ltd. Edmonton, AB, Canada**)**, containing a mixture of peat moss and sand (2:1, by volume). The seedlings were grown under controlled-environment conditions in a growth chamber that was set up to 75% relative humidity, 18/24 °C day/night temperature, 18-h photoperiod and photosynthetic photon flux density of approximately 300 µmol m^{-2} s^{-1} at the seedling level. Water was supplied to seedlings twice to three times per week and modified Hoagland's nutrient solution (Epstein 1972) once every two weeks by placing the containers with seedlings in 25 L tubs and soaking the soil for 4 h.

3.2.2. Treatments

Three months after germination, seedlings without fascicle needles were selected (Figure 3.1) and subjected to 50 mM NaCl (S_l) or 75 mM NaCl (S_2) treatments by increasing the concentration of NaCl by 25 mM every 72 h until the concentration indicated was achieved. Treatment solutions were applied for 72 days by placing the containers with seedlings in NaCl solutions (distilled water, S_0, as control) twice a week for 4 h. All seedlings were fertilized with nutrient solution as above every 16 days. Salt accumulation in the soil was prevented by flushing the soil with distilled water every 15 days.

Soil salinity was monitored every week using “the pour-through technique” described by Landis and Dumroese (2006). Briefly, immediately after treatments were supplied and soil totally saturated (field capacity), 150 mL of distilled water was poured onto the soil surface to obtain 100 mL of soil leachate that was collected in a plastic container and its electrical conductivity (EC) was determined with a conductivity meter (AR20, Fisher Scientific Accument, Canton, MA). Four randomized samples per treatment were used for the measurements.

After the salinity treatments, seedlings were subjected to a recovery treatment for 15 days. For the recovery treatment, the soil was flushed with distilled water, and all seedlings were provided with nutrient solution twice per week. The soil electrical conductivity was between 0.80 and 1.1 dS m^{-1} during the recovery period.

The experiment had a randomized complete block design with four blocks per treatment solution and each block contained 4 plants per population.

3.2.3. Growth of fascicle needles and needle injury

Number of needle fascicles with needles > 1 cm long were counted daily. Each fascicle contained consistently 5 needles. The fascicle needle lengths were measured at the end of the treatments. Injury to the primary and fascicle (secondary) needles was recorded as no injury (< 15% of the total needle area with necrosis) or injury (> 15%). Lengths of the fascicle needles were measured in 5 randomly-sampled needles from the lower part of the seedling (approximately 5 cm above the root collar). The measurements were taken from the tip of the longest needle to the edge of the fascicle sheath.

3.2.4. Analysis of total chlorophyll and tissue Na^+ and Cl^- concentrations

The analysis of total chlorophyll and tissue Na^+ and Cl^- concentrations were carried out in seedlings treated for 72 days (six randomly-selected plants per treatment, n = 6). Total chlorophyll in the secondary needles was determined according to Hiscox and Israelstam (1979). Needle samples containing 0.1 g fresh wight (FW) were extracted in screw-capped vials containing 7 mL dimethyl sulfoxide (DMSO). The vials were placed in an oven at 65 °C for 25 min and the extract was diluted with 7 mL of DMSO and filtered. Total chlorophyll was determined spectrophotometrically and calculated using Arnon's equations (Arnon 1949).

For the Na^+ and Cl^- analysis, roots were separated from shoots and were gently washed with running water followed by rinsing with deionized water. The roots and shoots were dried in an oven at 70 °C for 24 and 48 h, respectively. Chloride was extracted with hot water by placing 50 mg dry weigth (DW) of ground tissue in tubes containing 10 mL of deionized water and incubating at 80 °C for 5 min. The samples were then shaken for 20 min and centrifuged at 1,650 *g* for 10 min. The extraction was repeated and the extracts were combined and filtered through a 45-µm Millipore filter and analyzed by ion chromatography (Dionex, DX-600, CA, USA). For Na^+ analysis, 50 mg DW ground tissue was extracted using the sulfuric acid-hydrogen peroxide wet digestion method (Richards 1993). Briefly, ground tissue samples of 50 mg DW were placed into glass digestion tubes to which 5 mL of concentrated H_2SO_4 and 1 mL H_2O_2 were added. The digestion tubes were placed in a heater at 360 °C for 30 min, then tubes were removed and cooled. The process was repeated before the extract was diluted with deionized water to a volume of 50 mL and the Na^+ content was determined with an atomic absorption spectrometer (Spectra AA880, Varian Inc., Mississuaga, ON, Canada).

3.2.5. Gas exchange and root hydraulic conductance

Net photosynthesis (P_n) and stomatal conductance (*gs*) were measured in individual fascicles, each containing 5 fully-developed needles. The fascicles were randomly selected about 3 cm below the shoot apex. The measurements were carried out in 6 treated and 6 control seedlings after 72 days of NaCl treatments and at the end of the 15-day recovery period. Measurements were taken in the growth chamber with a portable infra-red gas analyzer (LCA4 Analytical Development Company Limited, Hoddesdon, UK) two hours after the lights turn on in the chamber. During measurements, the leaf temperature in the sample chamber varied between 22 and 25 °C and the irradiance was 300 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density at the seedling level. Needle surface areas were obtained after computer scanning (Sigma Scan Pro 5.0, SPSS Inc.) and the P_n and *gs* values were expressed on a needle area basis.

For root hydraulic conductance, the seedlings were moved to the laboratory where the stems were cut 3 cm above the root collar and attached by sealed connectors to a high-pressure flow meter (HPFM, Dynamex, Houston, Texas). The system was pressurized to 0.5 MPa for the determination of hydraulic conductance as described by Tyree *et al.* (1995). Flow (F), and applied pressure (Pi) were registered and the slope of the relationship between F and Pi was taken as the root hydraulic conductance (K_r) expressed in kg s^{-1} MPa^{-1}.

3.2.6. Statistical analysis

Percentage of seedlings with fascicle needles, necrosis in primary needles and fascicle needles were analyzed by Chi-Square using the CATMOD-SAS/PC program for Windows version 8.2 (SAS Institue, Inc., 2001) based on the following model:

$$Y_{ijk}=\mu+P_i+T_j+P_i*T_j+\varepsilon_{ijk} ,$$

where Y_{ijk} was the individual observation, μ was the overall mean, P_i was the fixed effect of population, T_j was the fixed effect of salinity treatment, P_i*T_j was the fixed effect of the interaction between population and salinity treatment, and ε_{ijkl} was the experimental error.

Seed and seedlings size data were analyzed by GLM-SAS/ PC for Windows version 8.2 (SAS institute, Inc., 2001) following the general linear model procedure:

$$Y_{ij}=\mu+P_i+\varepsilon_{ij} ,$$

where Y_{ij} was the individual observation, μ was the overall mean, P_i was the fixed effect of population, and ε_{ij} was the experimental error.

For number of fascicles, needle length, chlorophyll, ion concentrations, gas exchange and root hydraulic conductance:

$$Y_{ijkl}=\mu+T_i+B_j+T_i*B_j+P_k+T_i*P_k+T_i*P_k*B_j+\varepsilon ijkl ,$$

where Y_{ijkl} was the individual observation, μ was the overall mean, T_i was the fixed effect of salinity treatment, B_j was the random effect of the block, T_i*B_j was the interaction effect between salinity treatment and block, P_k was the fixed effect of population, T_i*P_k

was the interaction effect between salinity treatment and population, $T_i*P_k*B_j$ was the interaction effect between NaCl treatment, population and block, and ε_{ijkl} was the experimental error.

3.3. Results

3.3.1. Size of seeds and seedlings

Seeds and seedlings of different *P. leiophylla* populations varied significantly in size (Figure 3.1). The seeds from San Felipe population were smaller in length and width and had lower weight than the other populations (Table 3.2). Seedling heights and stem diameters of the three-month-old seedlings showed a similar tendency with the San Felipe seedlings being about 42 % and 17.2 % shorter and of smaller stem diameter compared with the other populations (Table 3.2).

3.3.2. Length and production of needle fascicles

The emergence and expansion of fascicle needles in seedlings of S_0, S_1 and S_2 treatments from the four populations started after 8 days of treatment (Figure 3.2). The only exception was in San Felipe seedlings treated with S_2, where the initiation of fascicle needles showed a delay of about 16 days (Figure 3.3).

During the experiment, control seedlings from Tlamacas and Malinche populations showed a significantly higher rate of fascicle production when compared with the other populations (Table 3.3). After 24 days of treatments, about 90% of the seedlings in these two populations had fascicle needles, in contrast with 38% in the San Felipe population. On day 48 100% was reached in the populations of Tlamacas, Malinche, and Tlalmanalco. In contrast, the population of San Felipe reached 100% on day 56. Although both S_1 and S_2 did not have a significant effect on fascicle needle production during the experiment (Table 3.3), a two-week delay in reaching 100% of the seedlings with fascicles was observed in S_1 and S_2 seedlings when compared with S_0 (control) (Figure 3.3).

The effects of NaCl treatments and populations were significant for the number of fascicle needles produced at the end of treatments. San Felipe seedlings showed consistently the lowest production of fascicle needles throughout the 72 days of treatments (Figure 3.3). At the end of the treatments, San Felipe seedlings had about 50% fewer fascicle needles than Tlamacas and Tlalmanalco seedlings of S_0, S_1 and S_2 treatments (Table 3.5). On the other hand, Malinche seedlings showed higher sensitivity to NaCl in terms of fascicle needles production compared with the other populations and showed a reduction of about 30 % in S_1 and S_2 treatments compared with S_0 control whereas the reduction in the remaining populations was about 17 % (Table 3.5).

Seed source and NaCl treatments had significant effects on the length of fascicle needles (Table 3.4). After 72 days of treatments, needles on control seedlings (S_0) from San Felipe population were 35% shorter than those in the other populations (Table 3.5). NaCl treatments triggered significant reductions in length of the fascicle needles in all of the four studied populations. A slight reduction was caused by S_1, whereas a major reduction occurred in response to S_2 treatment. The S_2 treatment reduced needle length in the Tlamacas population by 20% in comparison with the control plants and by approximately 12% in the remaining populations (Table 3.5).

3.3.3. Needle necrosis

Both NaCl treatments caused significant needle tip necrosis of the primary and fascicle needles (Figure 3.4), however, the extent of necrosis varied between the populations. The percentage of seedlings with necrosis of the primary needle increased significantly faster in Tlalmanalco, Tlamacas and Malinche seedlings compared with San Felipe seedlings (Table 3.3 and Figure 3.6). After 40 days, 100% of Tlalmanalco seedlings subjected to S_1 and S_2 treatments had needle tip necrosis while 20 and 38% of the San Felipe seedlings showed needle necrosis when exposed to S_1 and S_2 treatments, respectively. After 72 days of NaCl treatments, fewer than 50% of San Felipe seedlings exhibited tip necrosis of the primary needles (3.6 A, B).

After 56 days of S_1 and S_2 treatments, there was no fascicle needle tip necrosis in San Felipe seedlings while in other populations, the percentage of seedlings with fascicle needle necrosis varied from 10%t o over 50% in S_1, and from 20% to 70% under S_2 (Figure 3.5 and 3.6 C, D). For the S_1 treatment, tip necrosis of the fascicle needles was observed in San Felipe seedlings only after 72 days of treatment (Figure 3.6 C, D). At that time, 100% of the Tlalmanalco seedlings under S_1 and S_2 treatments had necrotic fascicle needles (Figure 3.6 C, D).

3.3.4. Total needle chlorophyll and Na^+ and Cl^- tissue concentrations

The reductions in needle chlorophyll a and b contents by NaCl treatments varied significantly among populations and treatments (Table 3.6 and 3.7). In seedlings from the San Felipe and Malinche populations, chlorophyll a concentrations decreased more under the S_2 treatment, while in Tlalmanalco and Tlamacas the reductions were similar for S_1

and S_2 treatments (Tables 3.6 and 3.7). Chlorophyll b concentration was reduced more by S_2 compared with S_1 in all populations (Tables 3.6 and 3.7). Among the studied populations, San Felipe seedlings had consistently the highest chlorophyll a and b levels, and Tlalmanalco seedlings showed the lowest chlorophyll concentrations in both NaCl treatments (Table 3.7).

Na^+ and Cl^- levels increased significantly in the root and shoot tissues of S_1 and S_2 seedlings compared with S_0 (Figure 3.7, Table 3.6). In the roots, concentrations of Na^+ and Cl^- were not significantly different between the four studied populations and were similar in seedlings exposed to S_1 and S_2 treatments. Shoots accumulated higher concentrations of Na^+ and Cl^- compared with roots and the seedlings subjected to S_2 treatment showed higher levels of shoot Na^+ and Cl^- compared with S_1 (Figure 3.7). The levels of Cl^- were almost twice as high as those of Na^+ in the shoot tissues (Figure 3.7). Of the four studied populations, San Felipe had the lowest while Tlalmanalco had the highest shoot Na^+ and Cl^- concentrations (Figure 3.7).

3.3.5. Gas exchange and root hydraulic conductance

NaCl treatments significantly affected water relations and reduced the needle stomatal conductance (*gs*), net photosynthesis (P_n) and root hydraulic conductance (K_r) (Figures 3.8 and 3.9). For all populations, the greatest reduction in P_n and *gs* occurred under S_2, but the extent of reduction varied significantly among the populations (Table 3.6).Under S_2 treatment, the Tlalmanalco seedlings exhibited reductions of 69 and 94% in P_n and *gs,* respectively, compared with S_0; and these reductions were significantly greater than the reductions in the San Felipe seedlings (Figure 3.8). After 15 days of recovery from NaCl treatments, the levels in P_n and gs were still lower in comparison with the S_0 treatment (Figure 3.8).

At the end of the recovery period, K_r was significantly reduced by the NaCl treatments in all populations. Compared with the control, Tlalmanalco and Malinche seedlings showed a reduction in K_r of 60% and 71 % under S_1 and S_2 treatments, respectively, whereas in the San Felipe seedlings the reductions were 30 % in S_1 and 51 % under S_2 treatments (Figure 3.9).

3.4. Discussion

Differences in size of seeds and seedlings as observed among the four *P. leiophylla* populations (Table 3.2) have also been observed in natural populations of other conifer species (Castro 1999; Grotkopp et al. 2004). Adverse environmental conditions have an impact on seed size (Sutton et al. 2002; Wahid et al. 2006). In the present study, the seeds from the San Felipe population of the xeric origin (Table 3.1) were the smallest of the four populations studied, however, the seed size of the Malinche population, also of the xeric origin, was similar to the populations from mesic sites (Table 3.2). Therefore, factors in addition to precipitation appear to be involved in determining the seed size in *P. leiophylla* populations. In *Austrocedrus chilensis,* the level of precipitation did not affect the weight of seed, but latitude had a strong correlation with seed size (Pastorino and Gallo 2000). The difference in seed size observed among the four *P. leiophylla* populations may explain the differences in seedling shoot lengths and stem diameters between the San Felipe seedlings and the seedlings from the other three populations (Table 3.2). Several studies with tree species have indicated that size of seed has an important effect on seedling germination and early growth (Salazar 1986; Pastorino and Gallo 2000). Kandya (1978) and Castro (1999) found in *P. oocarpa* and *P. silvestris*, respectively, a strong correlation between seed size and seedling growth over one or several growing seasons, with the larger seeds giving rise to larger seedlings and maintained over several years in *P. silvestris*.

The variation in fascicle needle production among the four *P. leiophylla* populations, where the San Felipe seedlings had fewer and shorter fascicle needles than the other provenances (Table 3.5), may be one of the climatic adaptations. High variation in needle production has been found as a result of environmental factors. In *P. palustris*, the start of needle elongation and needle growth rate was earlier at the mesic site than xeric site (Sheffield 2003); and in *P. resinosa*, the initiation and number of fascicle needles was reduced during dry seasons (Garret and Zahner 1973). It has been suggested that fewer needles might be an important drought adaptation that helps reduce transpirational water loss (Haller 1965; Cole et al. 2008).

In this study, the delay of new fascicle emergence and reduction in the number and length of fascicles in *P. leiophylla* seedlings treated with NaCl could be attributed to the osmotic stress created by NaCl. Slow needle emergence has been observed in drought-stressed trees (Kirongo et al. 2002) and in salt-stressed plants where it was thought to be caused by osmotic stress (Munns and Tester 2008). In pine species, the formation of fascicle needles is determined during the formation of apical buds, where cataphyll primordia develop into dwarf shoot primordia giving rise to fascicle needles (Cannell and Willett 1975). In the present study, although the number of cataphyll primordia is genetically determined in the bud, before NaCl treatment, apparently, not all of the primordial developed into fascicles in treated seedlings. Therefore, the number of fascicles was reduced by less of 15% in seedlings from San Felipe, Tlalamanalco, and Tlamacas and by 30% in Malinche seedlings (Figure 3.3 and Table 3.5). This effect has also been observed in other plant species including *Solanum quitoense* and *Avicennia germinans,* where the leaf number was reduced by more than 50% in high salinity (Suárez and Medina 2005; Flórez et al. 2008). It has been proposed that under salinity conditions, foliar primordia or buds fail to develop and remain quiescent due to the osmotic effect of salt, and fewer leaves are formed (Munns and Tester 2002).

The reduction of needle extension observed in seedlings of the four populations of *P. leiophylla* treated with NaCl (Table 3.5) has been considered to be an immediate response to salinity in several plant species (Neves-Piestun and Bernstein 2001; Suárez and Medina 2005; Flórez et al. 2008). In San Felipe and Tlalmanalco seedlings, the length of needles was reduced by 14 and 12%, respectively and in Tlamacas and Malinche seedlings by 19%. Some authors have suggested that the reduction in leaf length of salt-stressed plants is due to an immediate loss of turgor which can inhibit cell elongation (Yokoi et al. 2002; Meinzer et al. 2008). However, plant cells can also restore the original turgor and cell volume by an accumulation of osmotically-active solutes before tissue salt concentrations reach toxic levels (Munns 2002; Mahajan and Tuteja 2005). Therefore, osmotic adjustment combined with restriction of salt uptake and salt sequestration may be the factors involved in this response. Other factors, including hormonal status may be also involved in controlling the growth responses of salt-stressed plants (Munns and Tester 2008). In the present study, NaCl treatments did not have a major effect on the number of fascicles and length of secondary needles which could suggest that *P. leiophylla* seedlings could minimize the impact of osmotic stress. There is evidence that *P. leiophylla* is relatively resistant to drought stress (Vargas-Hernández et al. 1986) and that drought stress resistance of drought resistant populations is due to reduced water consumption (Martínez-Trinidad et al. 2002).

In the present study, the survival of *P. leiophylla* seedlings was not affected by NaCl treatments. However, needle injury was detected in both primary and secondary needles. Needle chlorosis was followed by tip necrosis that appeared in some seedlings after 16 days of NaCl treatments in the primary needles and 56 days of treatments in the secondary needles (Figure 3.6). Accumulation of Na^+ and Cl^- in toxic levels could lead to leaf injury (Kozlowski 1997). Salt is transported from the roots to the shoots through the transpiration stream. Over time, salts would gradually accumulate in the leaves. Therefore, tissue salt accumulation will depend on the transpiration intensity and duration of exposure to salt. Since the older needles were exposed to NaCl for the whole duration of treatments, they could be expected to contain higher Na^+ and Cl^- levels. Also, the old leaves are no longer expanding and, therefore, the salt arriving in them is not diluted during growth as in young growing leaves (Munns and Tester 2008). On the other hand, studies with tamarack and jack pine seedlings showed that both old and new needles contained similar concentrations of Na^+ and Cl^- ions but old needles showed first the effects of toxicity (Franklin and Zwiazek 2004; Renault 2005) suggesting that they are more sensitive to NaCl.

The percentage of plants with needle necrosis varied significantly between the populations. Despite the highest rates of stomatal conductance, NaCl-treated San Felipe seedlings had the lowest Na^+ and Cl^- shoot concentrations, the lowest percentage of seedlings with needle necrosis and the highest needle chlorophyll concentrations among the studied populations. These results suggest that there is no simple relationship between the water use and shoot accumulation of Na^+ and Cl^- in *P. leiophylla* seedlings. Although, correlation between the transpiration rates and salt uptake may not always be present (Franklin and Zwiazek 2004; Chen et al. 2002), the root-to-shoot ion transport is often affected by the rate of transpiration such that high rates of transpiration may increase the levels of ions in the leaves should high concentrations of ions be present in the xylem (Kramer 1983). In present study, the San Felipe seedlings showed slower development of fascicle needles, shorter needles, fewer needles, and lower root hydraulic conductance in comparison to the other populations. This might suggest that lower transpirational area and root water flow were likely contributing to the lower rates of Na^+ and Cl^- uptake in the San Felipe seedlings when compared with the other populations. Since Na^+ and Cl^- exclusion has been considered the most important mechanism of salinity resistance in glycophytes (Chen et al. 2002), it is possible that the San Felipe seedlings could have limited Na^+ and Cl^- loading into the xylem during root radial transport and sequestered ions in the cortical cells more efficiently than the other populations. Thus, further research is needed to know the extent of restriction of ions in the radial transport and to determine its contribution in the restriction of ion transportation and in the toxicity of seedling tissues.

Both NaCl treatments reduced the stomatal conductance and this reduction was accompanied by a decrease in the root hydraulic conductance. Water channels (aquaporins) have been shown to play a key role in the regulation of water transport in response to stress (Maurel and Chrispeels 2001; Vera et al. 2004). Although, the reduction of activity and abundance of aquaporins by NaCl have decreased the water flux (Carvajal et al. 1999; Lopez et al. 2006), mortality of functional roots played a major role in the reduction of root hydraulic conductance in NaCl-treated plants (Apostol et al. 2002). In an earlier study (Chapter 3.2), NaCl-treated *P. leiophylla* seedlings showed a strong correlation between the root hydraulic conductance and number of new roots. Although in the present study, NaCl treatments reduced the root hydraulic conductance in all examined populations, the effects on stomatal conductance varied significantly between the populations. In the San Felipe seedlings, stomatal conductance was less affected by NaCl than in the other genotypes, likely due to the lower Na^+ and Cl^- shoot concentrations and lower extent of needle injury.

In conclusion, the results of this study confirmed NaCl-resistance observed in plants from San Felipe, one of the driest sites (Chapter 2). These seedlings were slower-growing, but more salt-resistant compared with seedlings from the fast-growing populations from the mesic sites. The San Felipe seedlings developed fewer fascicle needles and had shorter needles than the seedlings from the other examined populations. These traits may be adaptations to drought and might explain the slow-early growth of these seedlings. The emergence of fascicle needles, and needle elongation showed little response to NaCl treatments and little difference between the examined populations.

However, the extent of needle injury and ion accumulation in shoots were lower in San Felipe seedlings compared with the other three populations, including the other xeric population (La Malinche). It indicates that the greater ability of San Felipe seedlings to avoid Na^+ and Cl^- shoot accumulation in shoots may be not only associated with adaptive drought traits conferring drought resistance but also with other traits of this population and therefore further studies are needed to understand the relationship between adaptive traits and salt- resistance in seedlings of this population.

3.5. References

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Table 3.1. Geographic location and mean annual precipitation of the seed collection areas with the four populations of *P. leiophylla* used in the study

Population	State	Location		Altitude (m)	Precipitation (mm)
		Lat. N.	Lat. W.		
San Felipe	Tlaxcala	19°28’	98°32’	2630	822
La Malinche	Tlaxcala	19°19’	98°00’	2780	800
Tlamacas	Estado de México	19°03’	98°40’	2700	1186
Tlalmanalco	Estado de México	19°11’	98°47’	2550	1072

Table 3.2. Size and weight of seeds (n = 90), and height and stem diameter of three-month-old seedlings (n = 48) from the four *Pinus leiophylla* populations used in the study. Means followed by different letters are significantly different at $\alpha = 0.05$ level

Population	Seeds			Seedlings	
	Length (mm)	Width (mm)	Weight (mg)	Height (cm)	Diameter (mm)
San Felipe	4.69 c	2.66 b	0.0078 c	2.5 b	1.78 b
Malinche	4.81 b	2.85 a	0.0100 a	4.3 a	2.15 a
Tlamacas	5.01 a	2.88 a	0.0100 a	4.3 a	2.00 a
Tlalmanalco	5.01 a	2.71 b	0.0096 b	4.8 a	2.15 a

Table 3.3. Sources of variation, degrees of freedom and Chi-Square values for plants with needle fascicles production (A), and number of plants with necrosis in primary and fascicle needles (B) after 24, 40 and 56 days of S_0 (control) S_1 (50 mM NaCl) and S_2 (75 mM NaCl) treatments. Pop, population; T, treatment; df, degree of freedom; * significant differences at $\alpha = 0.05$ level

Source	df	Chi-Square		
		24 days	40 days	56 days
A				
Pop	3	34.5 *	11.1 *	2.56
T	2	2.5	0.5	0.97
Pop*T	6	13.7 *	0.6	1.77
B	Primary needles			
Pop	3	1.96	8.6 *	10.2 *
T	2	4.1	28.8 *	43.6 *
Pop*T	6	1.7	6.6	4.0
	Fascicle needles			
		56 days	64 days	72 days
Pop	3	7.9 *	9.9 *	14.2 *
T	2	7.9 *	14.2 *	28.0 *
Pop*T	6	3.9	5.3	7.9

Table 3.4. F values for number of needle fascicle (NNF) and length of fascicle needles (LFN) in *P. leiophylla* seedlings subjected to NaCl treatments. * indicates significant differences at α = 0.05 level

Source	df	NNF	LFN
T	2	3.7*	3.5*
P	3	28.3*	37.1*
P x T	6	1.3	2.3*

Table 3.5. Number of needle fascicles (NNF) and length of fascicle needles (LFN) in four populations of *P. leiophylla* seedlings subject to S_0 (control), S_1 (50 mM NaCl) and S_2 (75 mM NaCl) treatments for 72 days (n=16). Means with different letters within the columns are significantly different as determined by the Duncan's standardized range test (α= 0.05)

Population	NNF			LFN (cm)		
	S_0	S_1	S_2	S_0	S_1	S_2
San Felipe	18.1 b	15.4 b	15.7 c	4.1b	3.6 b	3.5 b
Tlamacas	31.4 a	30.2 a	28.8 a	6.8a	5.5 a	5.4a
Malinche	32.1 a	22.4 b	22.1 a	6.7a	6.7 a	5.9a
Tlalmanalco	30.3 a	30.4 a	26.0 ab	6.0a	6.2 a	5.3a

Table 3.6. F values for chlorophyll a, b and total, sodium (Na$^+$), chloride (Cl$^-$), net photosynthesis (P_n), stomatal conductance (gs), and root hydraulic conductance (K_r) in three-month-old *P. leiophylla* seedlings subjected to NaCl treatments for 72 days and followed by a recovery period for 15 days. **, P≤ 0.01; *, P ≤ 0.05; T: Salinity treatment; P: Population; S: shoots; R: roots. P_n^1 *and* g_s^1 : measurements taken at the end of treatment period, and P_n^2 *and* g_s^2: at the end of recovery period

Source	df	Chlorophyll			Ions				Gas exchange				K_r
					Na$^+$		Cl$^-$						
		a	b	Total	S	R	S	R	P_n^1	g_s^1	P_n^2	g_s^2	
T	2	49.5**	8.5**	48.4**	263.0**	89.1**	218.0**	41.6**	51.4**	140.0**	19.6*	181.2**	139.4**
Bl	2	1.9	0.1	1.4	0.3	0.3	0.6	0.3	0.2	4.3*	0.3	1.1	2.0
T x Bl	4	0.5	0.3	0.4	1.3	1.3	0.9	0.6	1.2	1.4	0.3	0.3	0.6
P	3	10.6**	2.2	9.7**	19.5**	3.0*	14.6**	1.4	5.8**	7.5*	2.0	4.6**	0.9
T x P	6	1.7	3.2*	1.4	396.2**	423.4**	335.9**	148.6**	3.9**	2.4*	1.0	2.7*	5.3**
T x P x Bl	18	0.5	1.7	0.9	1.0	1.3	1.2	0.9	0.7	0.5	0.3	0.9	1.1

Table 3.7. Concentrations of chlorophyll a, b and total chlorophyll in needles from four populations of *P. leiophylla* seedlings subjected to 0 mM (S_0), 50 mM (S_1) and 75 mM (S_2) NaCl treatments for 72 days. Standard errors are shown in parenthesis. Means with different letters within the columns are significantly different as determined by the Duncan's standardized range test (α= 0.05)

Population	Chlorophyll (mg g^{-1}FW)								
	a			b			Total		
	S_0	S_1	S_2	S_0	S_1	S_2	S_0	S_1	S_2
San Felipe	0.53(0.06)	0.45(0.06)	0.37(0.06)	0.15(0.06)	0.15(0.01)	0.13(0.04)	0.69(0.09)	0.60(0.08)	0.50(0.09)
Malinche	0.47(0.05)	0.39(0.08)	0.32(0.02)	0.20(0.03)	0.11(0.04)	0.09(0.03)	0.67(0.03)	0.49(0.05)	0.41(0.03)
Tlamacas	0.54(0.08)	0.38(0.04)	0.37(0.03)	0.11(0.10)	0.13(0.04)	0.11(0.03)	0.65(0.16)	0.51(0.05)	0.48(0.03)
Tlalmanalco	0.47(0.04)	0.28(0.05)	0.28(0.04)	0.15(0.02)	0.10(0.02)	0.09(0.01)	0.62(0.07)	0.37(0.06)	0.37(0.05)

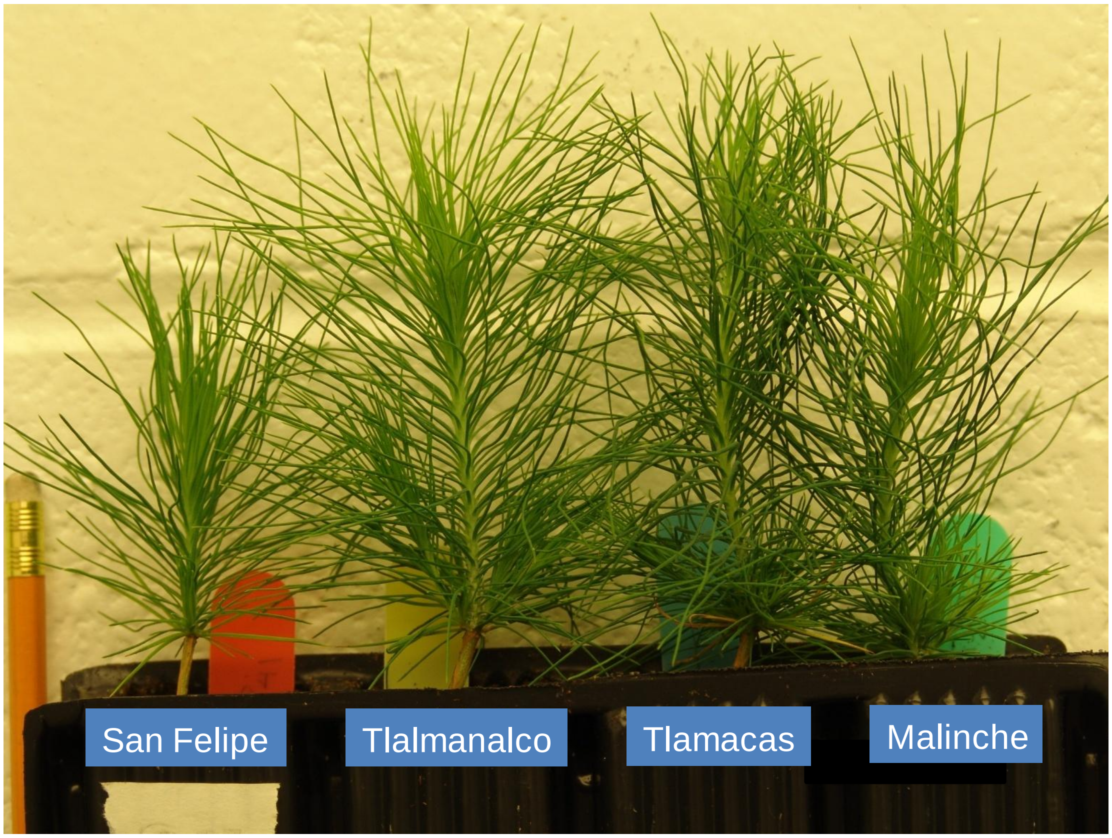

Figure 3.1. Three-month-old seedlings without fascicle needles from the four *Pinus leiophylla* populations tested in this study.

Figure 3.2. Emision and expansion of fascicle needles in *P. leiophylla* seedlings during the treatment of NaCl.

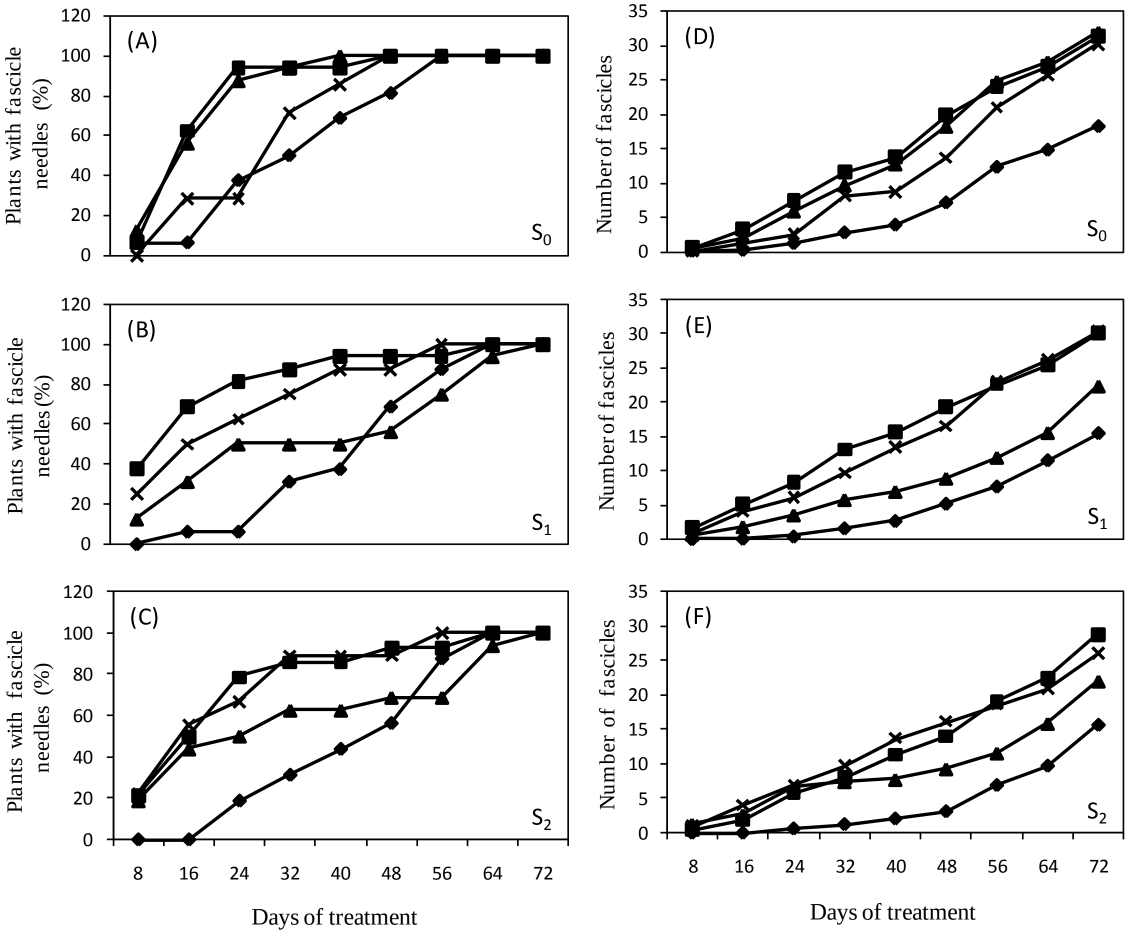

Figure 3.3. Percentage of seedlings with fascicle needles and mean numbers of fascicles in three-month-old *P. leiophylla* seedlings from San Felipe (♦), Tlamacas (■), Malinche (▲), and Tlalmanalco (×) exposed to S_0 (0 mM NaCl), S_1 (50 mM NaCl), and S_2 (75 mM NaCl) during 72 days (n= 16).

Figure 3.4. Necrosis in primary needles of seedlings of *P. leiophylla* caused by NaCl after 30 days of treatment.

Figure 3.5. Differences in the exent of needle damage caused by NaCl between the Tlamacas and Malinche seedlings that showed severe damage and the San Felipe seedlings with little necrotic foliar tissue, at the end of treatment.

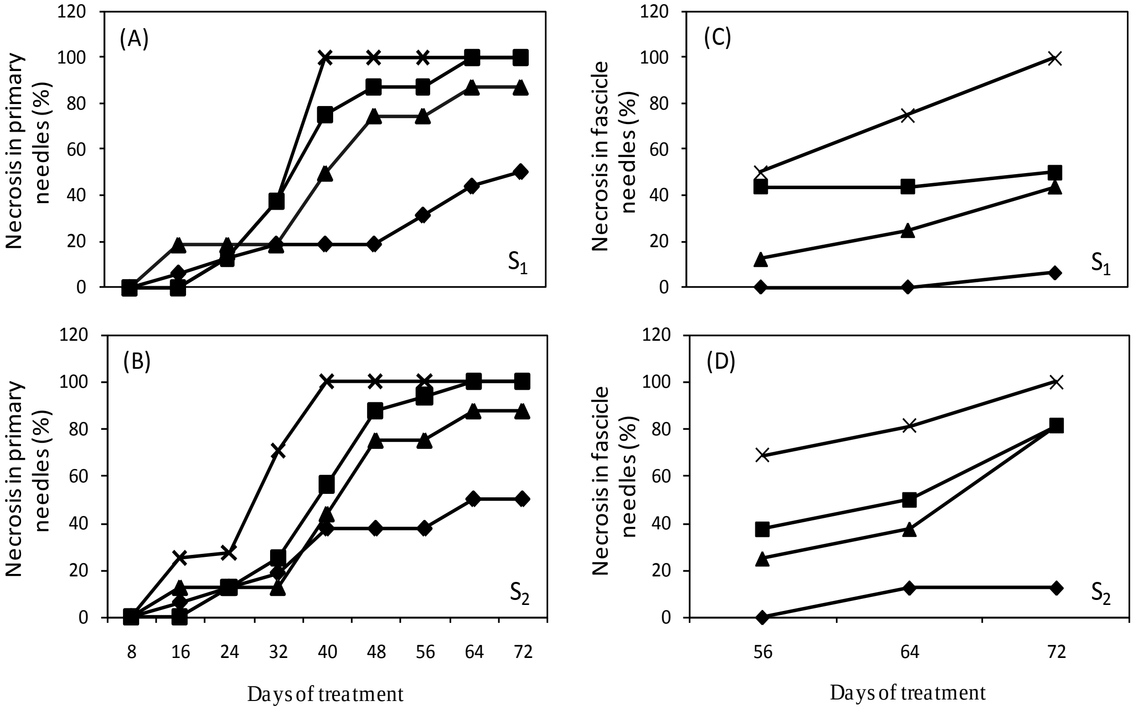

Figure 3.6. Percentage of plants with tip necrosis in the primary (A, B) and secondary (C, D) needles in three-month-old *P. leiophylla* seedlings from San Felipe (♦), Tlamacas (■), Malinche (▲), and Tlalmanalco (×) subjected to 50 mM (S_1) (A, C) and 75 mM (S_2) (B, D) NaCl (n=16).

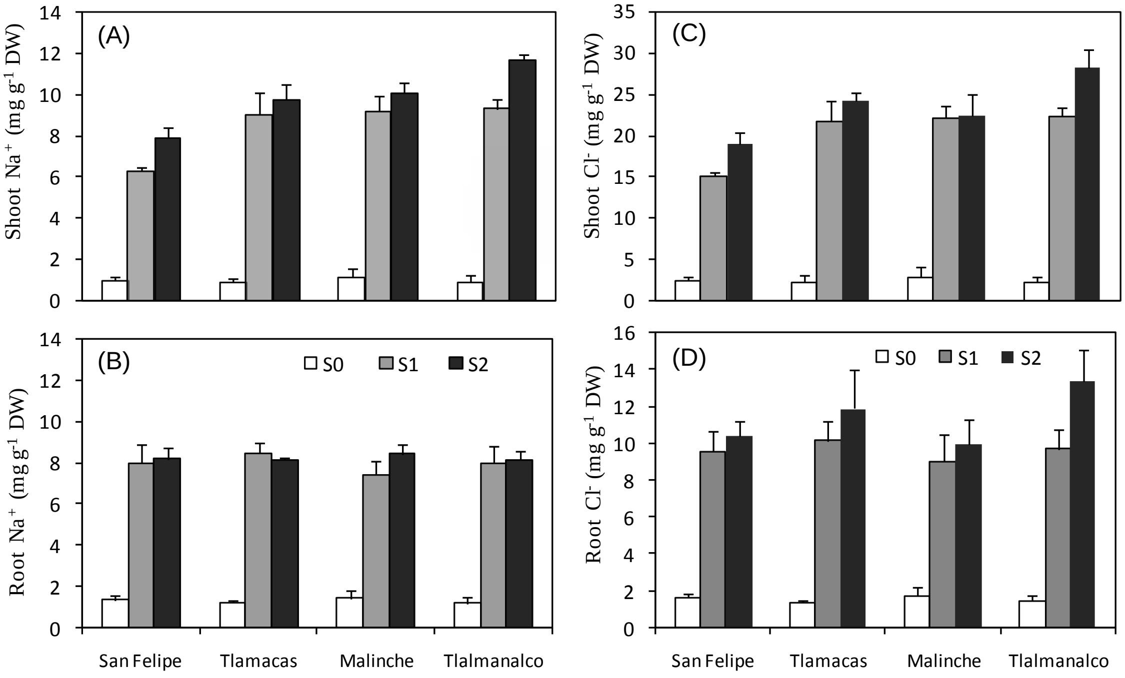

Figure 3.7. Na^+ and Cl^- concentrations in roots and shoots of three-month-old *P. leiophylla* seedlings from four populations subjected to 0 mM (S_0), 50 mM (S_1) and 75 mM (S_2) NaCl treatments for 72 days. Each data point represents mean (n=6) + SE.

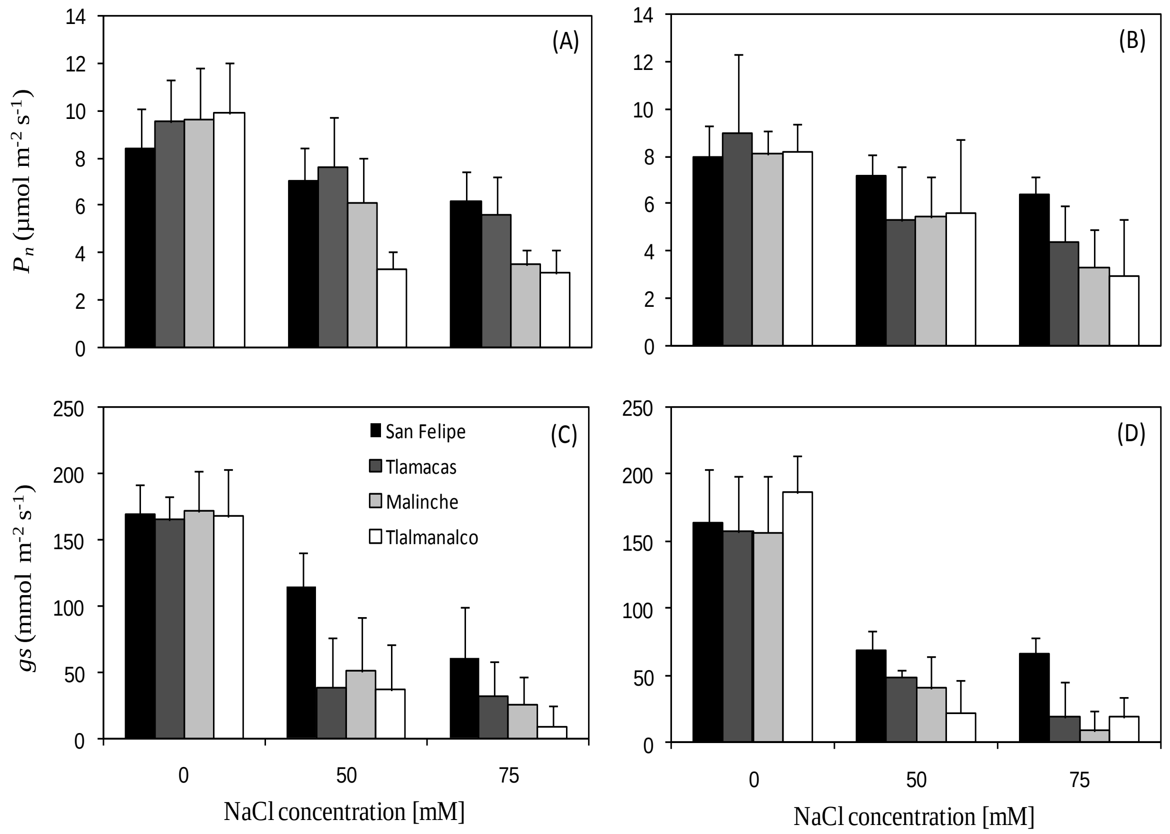

Figure 3.8. Net photosynthesis (P_n) and stomatal conductance (*gs*) of three-month-old *P. leiophylla* seedlings from four populations subjected to 0 mM, 50 mM and 75 mM NaCl treatments for 75 days (A, C) and after recovery period of 15 days (B, D). Each data point represents mean (n=6) +SE.

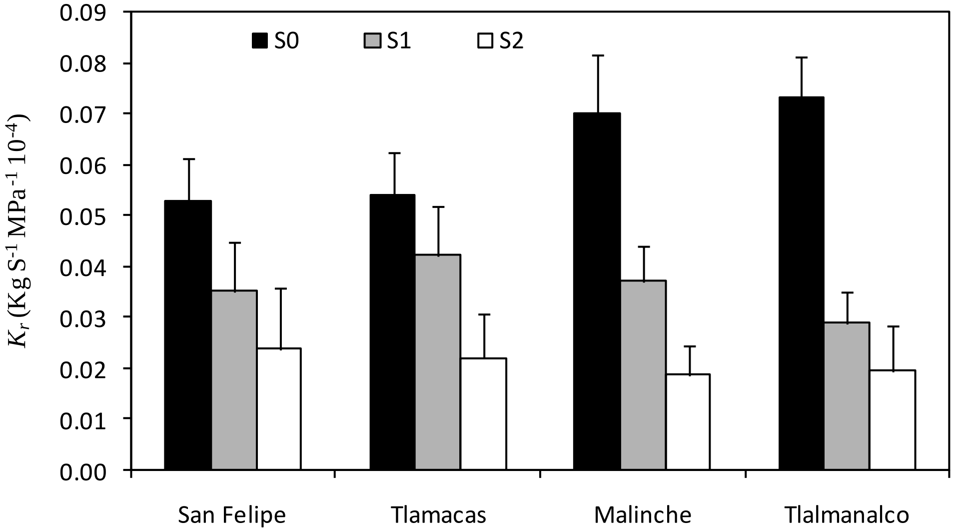

Figure 3.9. Root hydraulic conductance (K_r) in seedlings from four populations of *P. leiophylla* treated with NaCl for 72 days and following 15 days of recovery. Each data point represents mean (n=6) + SE.

Chapter 4. Effect of NaCl stress and branch pruning on transpiration and ion accumulation in shoots of *Pinus leiophylla* seedlings

4.1. Introduction

The increase of salt levels in soils due to irrigation is a serious challenge for agriculture (Ruprecht and Schofield 1991; Niknam and McComb 2000). In the subsoil layers, the rising groundwater can interact with the salts introduced by irrigation and take salts to soil surface which affect plant survival and productivity (Rengasamy 2006).

In Central Mexico, many agricultural areas have been abandoned due to salinity and soil degradation problems. Since these are largely deforested areas which originally supported forest ecosystems, attempts are being made for their reclamation and reforestation. Species with greater ability to survive salinity conditions must be identified for the reforestation of these lands. *Pinus leiophylla* is among the main forest tree species found in this region and, in some sites, natural populations of this species have been totally eliminated by deforestation (Musálem and Martínez-García 2003). This tree species has a wide natural distribution and high ability to adapt to diverse growth conditions including infertile soils, frost and drought. *P. leiophylla* has been introduced to Asia, Africa, Caribbean, North and South America, and Oceania and used for revegetation, soil conservation and erosion control (Rodríguez-Franco 2002; and López-Upton 2002). However, little is known about the ability of *P. leiophylla* to resist salt stress.

Salinity is detrimental to plants due to direct ion toxicity and osmotic effects which alter physiological processes and structures in plants and result in growth reductions, injury and death (Hasegawa et al. 2000). Several studies have pointed to the importance of ion toxicity in salt injury of conifer seedlings (Franklin and Zwiazek 2004; Apostol et al. 2002; Renault 2005). However, the link between plant morphological characteristics and salt resistance is not clear. The ability to restrict Na^+ and Cl^- uptake by shoots appears to play an important role in salt resistance of many studied woody plants (Allen et al. 1994). Since ion uptake and transport may be linked to transpiration (Davenport 2007) the ability of plants to reduce Na^+ and Cl^- in the transpiration stream may be part of the salt stress resistance mechanism (Jeschke 1982). Although a study with jack pine (*Pinus banksiana*) seedlings (Franklin and Zwiazek 2004) found no relationship between transpiration and salt uptake, other studies (O'leary 1965; Kramer and Boyer 1995) demonstrated that an increase in transpiration rates is accompanied by an increase in salt uptake and translocation to shoots. Therefore, reduced transpiration area could be beneficial for salt resistance of plants. Low transpiration rates and the ability to restrict radial salt transport in roots by blocking apoplastic pathway and sequestering Cl^- in vacuoles of the root cortex have been reported to be the main salt exclusion mechanisms in salt-tolerant tree species (Moya et al. 1999; Chen et al. 2003). Since root-to-shoot ion transport is the product of xylem ion concentration and the rate of transpiration (Chen et al. 2002 b), it is expected that low transpiration rates reduce the translocation of ions to shoots. In my previous studies Na^+ and Cl^- concentrations in shoots of *P. leiophylla* seedlings were low in small seedlings with few fascicles and short needles characteristic of the populations that were adapted to xeric sites. Therefore, in addition to being an important drought avoidance feature, the lower transpirational surface could also be a factor in decreasing root-shoot flux of salt.

A reduction of leaf area by pruning is an effective technique used by tree nurseries to reduce transpiration and improve seedling water status after planting in dry areas (Landis 2005). Branch pruning has been also successfully used to ameliorate drought stress in peach (*Prunus persica*) (Marsal et al. 2006) and Aleppo pine (*Pinus halepensis*) (Schiller and Cohen 1998). Therefore, if transpiration is one of the key factors affecting salt accumulation in shoots, the reduction of transpiration area by branch pruning could be helpful for plants growing in arid and salt-affected areas.

The objective of this study was to examine the effects of branch pruning and seedling size on total transpiration and on accumulation of Na^+ and Cl^- in roots and shoots of *Pinus leiophylla* seedlings treated with NaCl. We tested the hypothesis that the decrease in demand for water caused by branch pruning reduces Na^+ and Cl^- shoot uptake and prevents salt injury in *P. leiophylla* seedlings treated with NaCl.

4.2. Materials and Methods

4.2.1. Plant material and growth conditions

Seeds of *Pinus leiophylla*, collected in Tlaxcala State, Mexico (19°19’ N and 98°00’ W), were germinated and grown in Spencer-Lemaire root trainers (170 mL volume, Spencer-Lemaire Industries Ltd. Edmonton, AB, Canada) containing a mixture of peat moss and sand (2:1). The seedlings were placed in a controlled-environment growth room with 75% relative humidity, 24/18 °C temperature, 18-h photoperiod and photosynthetic photon flux density of approximately 300 µmol m^{-2} s^{-1}. Seedlings were watered three times per week and provided with modified Hoagland's mineral solution (Epstein 1972) once every three weeks by placing the containers in 25 L tubs and soaking the soil for 4 h to assure uniform soil moisture and nutrient distribution in all containers. The modified Hoagland's solution contained 224 mg L^{-1} N, 235 mg L^{-1} K, 160 mg L^{-1} Ca, 62 mg L^{-1} P, 32 mg L^{-1} S, 24 mg L^{-1} Mg, 50 mg L^{-1} Cl, 25 mg L^{-1} B, 2 mg L^{-1} Mn, 2 mg L^{-1} Zn, 0.5 mg L^{-1} Cu, 0.5 mg L^{-1} Mo, 20 mg L^{-1} Fe.

4.2.2. Branch pruning and NaCl treatments

When 11-months old, 32 short *P. leiophylla* seedlings (10-13-cm tall) and 32 tall seedlings (17-20-cm tall), before the beginning of shoot elongation, were selected. From each height group, 16 seedlings were randomly chosen and all basal branches were removed with a razor. The remaining 16 plants were left intact and served as control for the pruned group. Approximately 60 days after germination, *P. leiophylla* seedlings develop 4 basal branches, a few millimetres above the root collar. In 11-month-old plants, these branches contain about 25% of total shoot mass.

After branch pruning, the plants were grown in the growth room under aforementioned conditions for 30 days. Then they were transplanted to polyethylene bags (25 x 30 cm) filled with the same soil mixture as described above. Each bag was tied with an elastic band around the stem base to prevent evaporation from the soil, and two drainage holes (0.5 cm diameter) were made in the bottom of each bag. Every day, the elastic band was released for 15 min to improve soil aeration. Eight days before NaCl treatments, the plants were randomly placed into styroblocks^{TM} (PSB 24, 700 mL, Beaver

Plastics Ltd., Edmonton, Alberta, Canada) and arranged in a split plot design, with each styroblock containing two replicates of pruned and intact plants of each height group (sub plot) (Figure 4.1). Half of the plants (four styroblocks) was treated for 30 days with 150 mM NaCl (13.8 dS m^{-1} EC) and the remaining half (NaCl control) with 0 mM NaCl (0.38 dS m^{-1} EC). Both treatments were applied in a modified Hoagland's solution. Treatment solutions were applied twice per week by placing the styroblocks in plastic tubs and immersing them for 4 h in treatment solutions. Every 12 days, the soil in NaCl-treated and control plants was flushed with distilled water to avoid salt accumulation. After 30 days of NaCl treatment, soil was flushed again with distilled water to reduce the EC to about 0.38 dS m^{-1} and three days later, the styroblocks were immersed in nutrient solution for 4 h. For the following 15 days (recovery period), all plants were provided every four days with nutrient solution as above.

Soil electrical conductivity (EC) was measured once a week during NaCl treatments in randomly selected pots using "the pour-through technique" (Landis and Dumroese 2006). Briefly, two hours after immersing the styroblocks in treatment solution, distilled water was added to the soil surface to obtain 100 mL of leachate which was collected and its EC measured with a conductivity meter HI 8033 (Hanna Instruments Inc., Woonsocket, RI, USA).

4.2.3. Growth

Lengths of main new shoots were measured 30 days after the branches were pruned (before NaCl treatments were applied). All plants were harvested 45 days after the start of NaCl treatment (30 days of treatment plus 15 days of recovery). Roots were separated from shoots and rinsed with running tap water followed by distilled water to gently remove the soil and salt residues. After rinsing, new roots > 1 cm were counted and the roots, old and new needles, and stems were separated and oven-dried at 70°C to determine dry weights.

4.2.4. Visible injury

After 48 days, the extent of needle necrosis was determined for all plants. Needles were considered necrotic when the length of tip necrosis was equal to or greater than 25 mm. All necrotic areas were separated from green areas with scissors and their weights determined after drying. The extent of needle necrosis was calculated as percentage of weight of necrotic areas over the total needle weight.

4.2.5. Ion analysis

Chloride concentrations were measured in roots and shoots following hot water extraction as described by Apostol et al. (2002). Briefly, samples containing 50 mg of ground tissue were placed in tubes containing 10 mL of deionized water and incubated at 80°C for 5 min. The tubes were placed on an orbital shaker for 20 min and then centrifuged at 1,650 *g* for 10 min. This procedure was repeated and the extracts were combined, passed through a 45-µm Millipore filter, and analyzed using ion-exchange chromatography (Dionex, DX-600, CA, USA). Sodium concentrations were determined in root and shoot samples containing 50 mg of ground tissue as described by Richards (1993). The samples were extracted using sulfuric acid-hydrogen peroxide wet digestion and Na^+ content was determined with an atomic absorption spectrophotometer (Spectra AA880, Varian Inc., Mississuaga, ON, Canada).

4.2.6. Transpiration

Total transpiration was measured daily during the treatments and stress recovery using the gravimetric method (Atwell and Stout 1962). The sealed pots with seedlings were weighed every 24 h between 8:00 and 9:00 am using a digital balance and transpiration was calculated as the change in weight.

4.2.7. Gas exchange measurements and root hydraulic conductivity

Net photosynthesis (P_n) and stomatal conductance (g_s) were measured on days 7, 15, 30 (NaCl-treatment), and 45 (recovery period) in selected fascicles approximately 3 cm below the shoot apex in each plant of three randomly-selected blocks. At the time of measurements, the soil was at 100% field capacity. The measurements were taken in the growth room with a portable infra-red gas analyzer (LCA4 Analytical Development Company Limited, Hoddesdon, UK) two hours after the lights turn on in the chamber. During the measurements, leaf temperature in the cuvette was 23°C and photosynthetic photon flux density was 315 $\mu mol\ m^{-2}\ s^{-1}$. Needle surface areas were obtained by computer scanning (Sigma Scan Pro 5.0, SPSS Inc.) and net photosynthesis values expressed on a needle area basis.

After the recovery period, the plants that had been used for the gas exchange measurements were placed for 24 h in nutrient solution, drained and moved to the laboratory where the shoot of each seedling was excised about 3 cm above the root collar.

The stem was attached to a high-pressure flow meter (HPFM, Dynamex, Houston, Texas) and the hydraulic conductance measurement was taken in the transient mode (Tyree *et al.* 1995) over the range of 0 – 0.5 MPa as previously described (Calvo-Polanco et al. 2009). Flow (F), and applied pressure (Pi) were registered every few seconds, and the slope of the relationship between F and Pi was taken as root hydraulic conductance (K_r) expressed in kg s^{-1} MPa^{-1}.

4.2.8. Statistical analysis

The analysis of variance for elongation of new shoots, new roots and needle numbers, and for needle necrosis was conduced using GLM-SAS/ PC for Windows version 8.2 (SAS institute, Inc., 2001) according to the following general linear model:

$$Y_{ijkl}=\mu+P_i+B_j+PB_{ij}+T_k+TP_{ki}+PBT_{ijk}+\varepsilon_{ijkl}$$

Where Y_{ijkl}=individual plant value; μ= is the overall mean; P_i= is the fixed effect of *i*th branch pruning treatment; B_j= is the random effect of the *j*th block; PB_{ij}= is the random interaction of branch pruning with block; T_k= is the fixed effect of *k*th size of plant treatment; TP_{ki}= is the fixed interaction effect of size plant with branch pruning; PBT_{ijk}= is the random interaction effect of branch pruning with block and size of plant; and ε_{ijkl}= error.

Dry weight of shoots and roots, hydraulic conductance and accumulation of ions were analyzed by the following linear model:

$$Y_{ijklm}=\mu+S_i+B_j+SB_{ij}+T_k+TS_{ki}+TSB_{ijk}+P_l+PS_{il}+PT_{kl}+PST_{ikl}+PSTB_{ijkl}+\varepsilon_{ijklm}$$

Where S_i= is the fixed effect of *i*th salinity treatment; B_j= is the random effect of *jth* block; SB_{ij}= is the random interaction effect of salinity with block; T_k= is the fixed effect of size of plant; TS_{ki}= is the fixed interaction effect of size of plant with salinity; TSB_{ijk}= is the random interaction effect of size of plant with salinity and block; P_l= is the fixed effect of *l*th pruning treatment; PS_{il}= is the fixed interaction effect of pruning with salinity; PT_{kl}= is the fixed interaction effect of pruning with size of plant; PST_{ikl}= is the fixed interaction effect of pruning with salinity and size of plant; $PSTB_{ijkl}$= is the random interaction effect of pruning with salinity, size of plant, and block; and ε_{ijklm}= error.

Total transpiration and gas exchange, P_n and *gs*, were subject to repeated measures ANOVA using the split-plot design with time (weeks) as a subplot effect. The analysis was conducted using PROC MIXED-SAS/ PC for Windows version 8.2 (SAS institute, Inc., 2001), with the following linear model:

$$Y_{ijkl}=\mu+S_i+P_j+SP_{ij}+H_k+HS_{ik}+HP_{jk}+SHP_{ijk}+\mathrm{T}_l+\mathrm{TS}_{il}+\mathrm{TP}_{jl}+\mathrm{TH}_{kl}+\mathrm{TPH}_{jkl}$$

$$+\mathrm{TSH}_{ikl}+\mathrm{TSP}_{ijl}+\mathrm{TSPH}_{ijkl}+\varepsilon_{ijkl}$$

Where S_i= is the effect of the *i*th salinity treatment; P_j= is the effect of *j*th pruning treatment; SP_{ij}= is the interaction effect of salinity with pruning; H_k= is the effect of *k*th size of plant; HS_{ik}= is the interaction effect of size of plant with salinity; HP_{jk}= is the interaction effect of size of plant with pruning; SHP_{ijk}= is the interaction effect of salinity with size of plant and pruning; T_l= is the effect of *l*th time; TS_{il}= is the interaction effect of time with salinity; TP_{jl}= is the interaction effect of time with pruning; TH_{kl}= is the interaction effect of time with size of plant; TPH_{jkl}= is the interaction effect of time with pruning and size of plant; TSH_{ikl}= is the interaction effect of time with salinity and size of plant;TSP_{ijl}= is the interaction effect of time with salinity and pruning; $TSPH_{ijkl}$= is the interaction effect of time with salinity, pruning, and size of plant; and ε_{ijkl}= error.

Treatment means were compared using the Duncan's multiple range test.

4.3. Results

4.3.1. Effects of pruning and NaCl on growth

Branch pruning treatment (P_r) induced a significant increase in the new terminal shoot elongation (Figure 4.3 and 4.4). In S (Short)+Pr and T (Tall)+Pr plants, new shoots were 51% longer than those without pruning, respectively (Figure 4.4). Interaction between size of plant and branch pruning was significant (Table 4.1) so that the magnitude of the growth increase caused by branch pruning varied according to the height of the plants. The shoot length of T+Pr plants was 53% greater than that of S+Pr plants (Figure 4.4).

Root and shoot dry weights of plants treated with NaCl were significantly reduced compared with control plants (Table 4.2, 4. 3). The dry weights of roots and shoots in T plants were about 10% more reduced than in S+Pr plants (Table 4.3).

All NaCl-treated plants produced new needles; however needle production varied significantly among treatments (Table 4.3). The percentage of plants with new needles and the dry weights of new needles were higher in S+Pr plants compared with S, T and T+Pr plants (Table 4.4). For all treatments, S+Pr plants showed the greatest new needle production with 87% of plants producing new needles (0.15±0.07 g DW new needles per plant). In the remaining treatments, there were 50% or fewer seedlings with new needles and 0.035±0.05 g DW needles produced per plant (Table 4.4). A similar tendency was present for new root production with S+Pr plants having higher numbers of roots than plants that recived the remaining treatments (Table 4.4).

4.3.2. Effects of pruning and NaCl on total transpiration, P_n, gs, and K_r

The height of plants, branch pruning and NaCl treatments had a significant effect on total transpiration of plants throughout the NaCl treatment duration and recovery period, and significant interactions were detected between treatments (Table 4.5). Over the treatment period, T plants had the highest values of total transpiration, and S+Pr plants had the lowest values (Figure 4.5 a). The reduction in total transpiration during the NaCl treatment ranged from 24 to 36% in T+Pr plants and from 30 to 50% in S+Pr plants. Relative to the control, the NaCl treatment reduced total transpiration in all plants with similar trends for all plant groups except for T+Pr plants which exhibited the highest reductions in transpiration compared with other treatments (Figure 4.5 b). At the end of the recovery period, S+Pr plants increased the total transpiration values by more than 45% over their value observed at 32 days (end of treatment) in contrast with the other plants which showed increases of about 24%.

NaCl significantly reduced the levels of P_n and g_s by about 40% during the first 7 days of treatment and the reduction further increased over time (Figure 4.6, Table 4.5). The magnitude of this reduction varied with the height of plants and branch pruning treatment (Figure 4.6). In S+Pr plants the levels of P_n and *gs* were reduced less during NaCl-treatment compared with other plants and were the only ones that showed a slight increase in the levels of *gs* during recovery period. At the end of this period, the levels of

g_s and P_n in S+Pr plants were 45 and 50%, respectively, higher than in S, T, and T+Pr plants (Figure 4.6 b and d).

The levels of root hydraulic conductance (K_r) were significantly reduced by NaCl with the magnitude of reduction varying between plants of different treatments (Table 4.2). In short plants, branch pruning treatment affected the K_r values. In untreated plants, the K_r of S+Pr was lower than that measured in S plants while in treated plants, the K_r of S+Pr was less reduced compared with S plants. There was no effect of pruning on K_r of T and T+Pr plants (Figure 4.7).

4.3.3. Tissue concentrations of Na^+ and Cl^-, and visible injury

After 30 days of treatment, concentrations of Na^+ and Cl^- increased significantly in root and shoot tissues of all NaCl-treated plants (Figure 4.8, Table 4.6). In shoot tissues, the concentrations of Na^+ were almost two-fold higher and Cl^- about four-fold higher than in roots (Figure 4.8). In NaCl-treated plants, the ratios of Cl^-: Na^+ were approximately 3:1 in roots and of 7:1 in shoots (Figure 4.8). Plant height and branch pruning treatment had no significant effects on the concentrations of Na^+ and Cl^- in roots. However, in shoots, the concentrations of Na^+ and Cl^- in S+Pr plants were significantly lower compared with non-pruned plants (Figure 4.8). After 30 days of NaCl treatment, the concentrations of Na^+ and Cl^- in shoots of S+Pr plants were about 6 and 48 mg while those in the shoots of T+Pr, S, and T plants were over 10 and 65 mg, respectively (Figure 4.8).

Both seedling height and branch pruning had an effect on the extent of needle necrosis caused by NaCl (Table 4.4). Needle necrosis appeared in T+Pr, T, and S plants after 15 days of NaCl treatment whereas in S+Pr plants needle necrosis started appearing after 20 days of treatment (Table 4.4). At the end of the recovery period, S+Pr plants had less needle necrosis than the non-pruned seedlings and only 50% of S+Pr plants (18% total needle dry weight) compared with 100% of T+Pr, T and S (over 50% total needle dry weight) seedlings exhibited needle necrosis (Table 4.4 and Figure 4.3).

4.4. Discussion

In this study, branch pruning was used as a method to reduce seedling transpiration area in seedlings of two different height groups (short and tall) for testing the hypothesis that reduced transpiration in *Pinus leiophylla* seedlings will result in decreased salt injury due to reduced shoot uptake of Na^+ and Cl^-.

The increase in terminal shoot elongation that was triggered by branch pruning (Figure 4.4) could be due to changes in levels of endogenous growth regulators after the branch removal. In the conifer species investigated, changes in hormonal status were implicated in the increased allocation of carbohydrates to neighboring terminal buds following removal of lateral buds and branches which caused a significant growth of the terminal shoot (Little 1970; Rasmussen et al. 2003 a,b; Piene 2003). The role of endogenous growth regulators in the control of shoot elongation of conifers has been long established. It has been proposed that indole-3-acetic acid (IAA) determines the shoot elongation in *Pinus ponderosa* since the amount of stored carbohydrates translocated to terminal shoots depends on IAA concentration in tissues (Lanner and Connor 1988). Therefore, the reallocation of carbohydrates could have caused the increase in the growth of terminal shoots in pruned *P. leiophylla* seedlings. This response may have important implications during the establishment of seedlings in forest plantations.

Although the loss of water vapor from plants may involve any above-ground part of the plant, leaves are by far the principal site of transpiration (Raven et al. 2003). In the present study, reduced transpiration area caused by branch pruning significantly reduced the total water loss from both control and NaCl-treated plants (Figure 4.5). A similar effect was observed in a study with olive trees, where branch pruning reduced the transpiration levels and maintained relatively high soil water content through summer; in contrast with intact trees which depleted soil water and developed low shoot water potentials (Shelden and Sinclair 2000). In *Pinus halepensis* plantations and fruit orchards of peaches established on dry sites, branch pruning was an effective strategy to reduce plant transpiration and to improve water status of the plants during temporary water stress (Schiller and Cohen 1998; Marsal et al. 2006). This strategy could also offer some control over salt uptake by the leaves since transpiration may be a factor contributing to an accumulation of salt in shoots (Robinson 1997).

The reduction of total transpiration and g_s by NaCl in all treatment groups (Figure 4.5 and 4.6 c, d) may be explained by a direct ion toxicity and secondary water stress which are often induced in plants by NaCl (Allen et al. 1994; Hasegawa et al. 2000). Several studies with woody plants have reported that low osmotic potential of the soil solution generated by salinity inhibits root growth, induces suberization of cortical cells and inhibits root aquaporin activity which, in turn, reduces water uptake and results in plant water deficit (Loustau et al. 1995; Apostol et al. 2002; Boursiac 2005; Navarro et al. 2007). A common response of plants to water deficit or to salt conditions is the accumulation of endogenous abscisic acid (ABA) in the leaf tissues where it triggers stomatal closure (Liu et al. 2001; Johnson et al. 2001; Chen et al. 2002 a; Sairam and Tyagi 2004; Tallman 2004). However, when the ability of roots to sequester salt is exceeded and the transport of Na^+ and Cl^- ions from roots to shoots increases, then ion toxicity in leaves may play the principal role in the reduction of *gs* because salt accumulation in the mesophyll might increase the levels of intracellular CO_2, as a consequence of photosynthesis inhibition, which induces stomatal closing (Robinson et al. 1997). This mechanism could explain the gradual decrease of g_s observed in all plants treated with NaCl, and the difference in the reduction levels of g_s observed between S+Pr plants and S, T, and T+Pr plants at the end of treatment, where S+Pr plants with lower Na^+ and Cl^- concentrations in shoots (Figure 4.8) had higher g_s values compared with other NaCl-treated plants (Figure 4.6 d), indicating that Na^+ and Cl^- accumulation in leaf tissues contributed to stomatal closing.

It is possible that the lower Na^+ and Cl^- shoot concentrations in S+Pr plants compared with S, T, and T+Pr plants were due to lower transpiration caused by branch pruning (Figure 4.8). In jack pine seedlings, transpiration was not correlated with Na^+ uptake and transport and did not affect transport of mineral elements in plants (Tanner and Beevers 1996; Franklin and Zwiazek 2004). However, several other studies demonstrated that leaf salt accumulation is linked to transpiration rates. In citrus, Cl^- uptake and xylem concentrations were similar in Cl^--sensitive and Cl^--tolerant genotypes. However, leaf Cl^- concentrations differed and depended on both leaf biomass and transpiration rates suggesting that they may be determined by the intensity of xylem flow (Moya et al. 1999). Similar results were reported in salt-tolerant *Populus* genotypes, where the lower transpiration rates contributed to the control of salt transport to shoots (Chen et al. 2003). In the present study, all groups of NaCl-treated plants showed similar Na^+ and Cl^- concentrations in roots. However, S+Pr plants, which had the lowest shoot mass and the lowest total transpiration of all treatment groups, had also the lowest shoot Na^+ and Cl^- concentrations. It is interesting that branch pruning in tall plants was not effective in reducing Na^+ and Cl^- shoot concentrations as in the short plants (Figure 4.8). A likely explanation is that branch pruning treatment in tall plants reduced the shoot-to-root ratio less than in the short plants. It has been demonstrated that larger root systems tend to accumulate higher Na^+ and Cl^- amounts than smaller roots (Moya et al. 1999).

The differences in concentrations of Na^+ and Cl^- in shoot tissues are likely responsible for the variations in the extent of needle necrosis between different treatment groups. Shoots of S+Pr plants with lower shoot concentrations of Cl^- and Na^+ exhibited lower needle necrosis, in contrast to the shoots in other experimental groups that accumulated higher concentrations of both ions (Figure 4.8). Needle necrosis in NaCl-treated jack pine (Apostol et al. 2002) and tamarack (Renault 2005) was correlated with tissue Na^+ concentrations. Although both Cl^- and Na^+ in high concentrations may cause leaf injury, Cl^- appears to have an earlier impact than Na^+ in the initial development of chlorosis and necrosis of leaf tissues (Kozlowski 1997). In addition, tissue Cl^- concentrations are usually higher than those of Na^+ in salt-treated plants (Apostol and Zwiazek 2003; Franklin and Zwiazek 2004; Renault 2005; Navarro et al. 2007). Needle necrosis and resulting loss of photosynthetic tissue is usually translated into a decline of plant productivity. In our study, the dry weights of plants were significantly reduced by

NaCl treatment (Table 4.4) and were proportional to the level of needle necrosis. The least affected root and shoot dry weights were in S+Pr plants which also had the lowest levels of needle necrosis.

The level of needle injury in plants treated with NaCl may also affect the production of new roots and needles following salt stress (Table 4.4). The S+Pr plants with the lowest extent of needle necrosis also had the highest production of new roots and needles (Table 4.4). These results are consistent with our previous finding, where the level of needle injury caused by NaCl and the production of new roots and needles were highly correlated. It could be logically expected that greater loss of photosynthetic tissue results in reduced availability of photosynthates needed for the production of new needles and roots. Seedlings with more new roots have a greater ability to take up water needed for growth during the recovery period (Grossnickle 2005). In our study, the number of new roots in plants treated with NaCl was also correlated with root hydraulic conductance. The S+Pr plants had the highest production of new roots and showed the highest values in K_r (Figure 4.7). The recovery of K_r following salt stress ensures efficient water supply to transpiring new and old needles which increases the probability of survival after a long period of salt stress.

In conclusion, in the present study, total plant transpiration, as affected by plant size and branch pruning, was correlated with Na^+ and Cl^- needle concentrations and needle necrosis in NaCl-treated *P. leiophylla* seedlings. Branch pruning, in short seedlings, might be an effective treatment increasing the chances of survival of pine seedlings planted in salt-affected areas.

4.5. References

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Table 4.1. F values in the analysis of variance of the effects of plant height (H), and branch pruning (Pr) on shoot elongation (SEL); and after NaCl treatment on new roots (NR), new needles (NN), and needle necrosis (NEC). Significant levels are given as probability: * P<0.05; ** P<0.01

Source of Variation	**df**	**SEL**	**NR**	**NN**	**NEC**
H	1	87.6 **	10.4 **	11.4 **	51.4 **
Pr	1	66.8 **	26.1 **	6.2 *	74.7 **
H*Pr	1	10.5 **	16.6 **	7.8 *	7.9 *

Table 4.2. F values in the analysis of variance of the effects of NaCl (S), plant height (H), and branch pruning (Pr) on dry weight shoot (DWS), dry weight root (DWR), and root hydraulic conductance (K_r). Significant levels are given as probability: * $P<0.05$; **$P<0.01$

Source of Variation	df	DWR	DWS	K_r
S	1	212.2 **	167.0 **	2063.4 **
H	1	99.8 **	46.3 **	4.9 *
S*H	1	5.2 *	3.8 ns	109.4 **
Pr	1	95.2 **	122.9 **	2.7 ns
Pr*S	1	18.1 **	21.5 **	110.6 **
Pr*H	1	2.3 ns	1.6 ns	0.1 ns
S*Pr*H	1	1.1 ns	0.1 ns	66.5 **

Table 4.3. Effect of NaCl, plant height, and branch pruning on root and shoot dry weights. Means within columns followed by different letters are significantly different at α=0.05 based as determined by Duncan test

Parameter	**NaCl [mM]**	**Short plants**		**Tall plants**	
		No-Pr	**Pr**	**No-Pr**	**Pr**
Root dry weight (g)	0	1.55 a	1.05 a	1.96 a	1.49 a
	150	1.01 b	0.74 b	1.19 b	0.88 b
Reduction (% of control)		34.8	29.5	39.3	40.9
Shoot dry weight (g)	0	4.64 a	2.56 a	5.64 a	3.76 a
	150	2.74 b	1.71 b	3.13 b	2.22 b
Reduction (% of control)		40.9	33.2	44.5	40.1

Table 4.4. Effect of NaCl on needle injury and production of new needles and roots in relation to height of the plants (S and T) and branch pruning (Pr). Means within columns followed by different letters are significantly different at α=0.05 based as determined by Duncan test

Treatment	**Needle Necrosis (%)**	**Plants with needle necrosis (%)**	**New needles (g)**	**Plants with new needles (%)**	**Number of new roots**
S+Pr	18.4 c	50	0.15 a	87.5	24.7 a
S	55.2 b	100	0.05 b	50.0	12.6 b
T+Pr	50.4 b	100	0.03 b	25.0	15.1 b
T	69.1 a	100	0.03 b	37.5	13.7 b

Table 4.5. F values in the analysis of variance of the effects of NaCl (Sal), height of plant (H), branch pruning (Pr) and time (T) on total transpiration and gas exchange: net photosynthesis (*P_n*) and stomatal conductance (*g_s*) in *P. leiophylla* plants. Significant levels are given as probability: ns; *, P<0.05; **P<0.01

Source of Variation	**Total Transpiration**		**Gas exchange**		
				P_n	***gs***
	df	**F value**	**df**	**F value**	**F value**
Sal	1	5001.7 **	1	2384.4 **	1460.9 **
Pr	1	222.8 **	1	11.1 **	4.5 *
Pr*Sal	1	907.9 **	1	47.9 **	1.9 ns
H	1	116.2 **	1	58.2 **	0.1 ns
H*Pr	1	0.6 ns	1	3.0 ns	1.5 ns
H*Sal	1	857.5 **	1	62.9 **	29.4 **
H*Pr*Sal	1	7.3 **	1	0.2 ns	2.1 ns
T	5	5455.7 **	3	75.9 **	6.9 **
T*Size	5	40.8 **	3	4.8 **	0.3 ns
T*Pr	5	61.5 **	3	3.3 *	0.2 ns
T*Sal	5	2160.7 **	3	159.2 **	40.5 **
T*Size*Pr	5	3.1 **	3	7.5 **	1.1 ns
T*Size*Sal	5	97.7 **	3	8.1 **	3.8 *
T*Pr*Sal	5	113.1 **	3	2.6 ns	3.3 *
T*H*Pr*Sal	5	3.5 **	3	7.2 **	2.8 *

Table 4.6. F values in the analysis of variance of the effects of NaCl (Sal), height of plant (H), and branch pruning (Pr) on accumulation of Na^+ and Cl^- ions in roots and shoot tissues of *P. leiophylla*. Significant levels are given as probability: ns; *, P<0.05; **P<0.01

Source of Variation	**df**	**Na^+**		**Cl^-**	
		Root	**Shoot**	**Root**	**Shoot**
Sal	1	270.9 **	763.5 **	610.2 **	3329.1 **
H	1	0.04 ns	25.2 **	0.03 ns	39.6 **
H*Sal	1	0.01 ns	23.4 **	0.6 ns	40.3 **
Pr	1	1.7 ns	10.4 **	0.3 ns	9.3 **
Pr*Sal	1	0.8 ns	10.4 **	0.2 ns	11.1 **
Pr*H	1	0.4 ns	11.6 **	0.3 ns	27.2 **
Pr*H*Sal	1	0.4 ns	11.2 **	0.9 ns	26.5 **

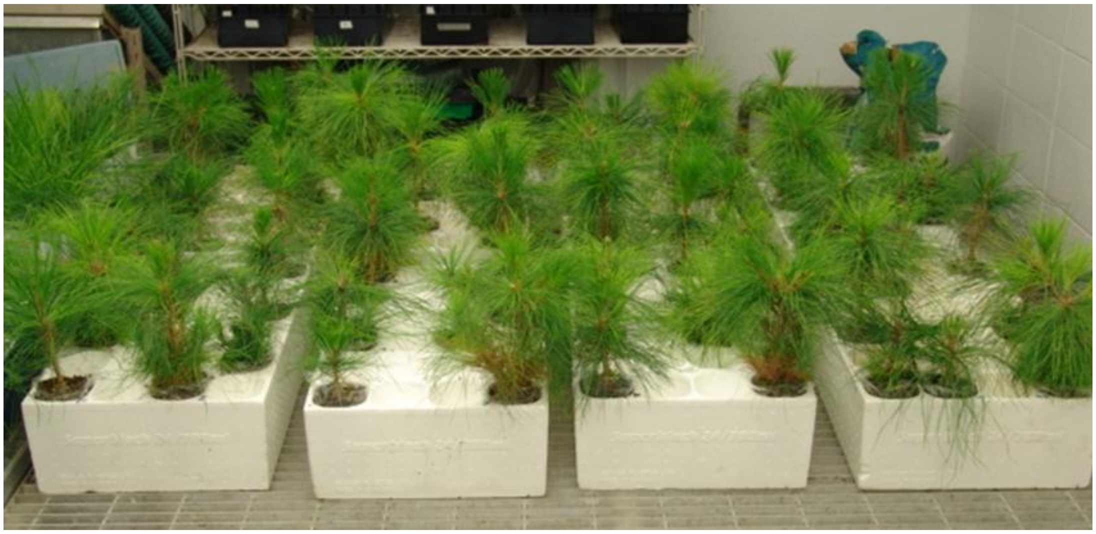

Figure 4.1. Pruning and no-pruning plants at the beginning of the NaCl treatment in growth chamber conditions.

Figure 4.2. Difference in needle damage caused by the treatment of NaCl between tall pruned plants (T+Pr) and small pruned plants (S+Pr) after 32 days of treatment.

Figure 4.3. Effect of pruning treatment on the new terminal shoot length in tall pruned plants (T+Pr) compared with tall intact plant (T).

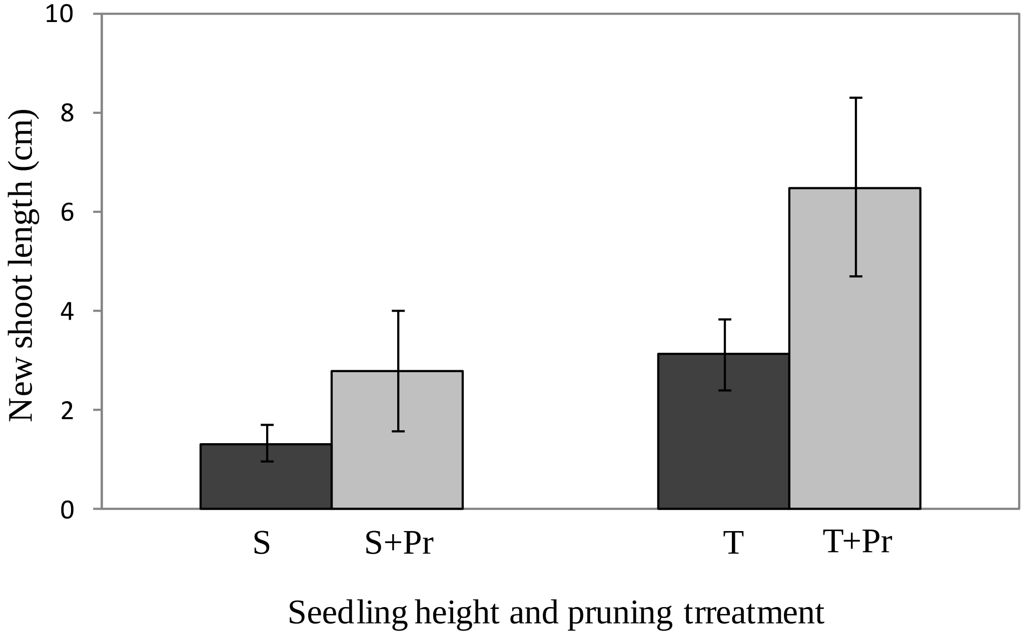

Figure 4.4. Effects of seedling height (S and T) and branch pruning (Pr) treatment on the new terminal shoot length in 11-month-old *Pinus leiophylla* plants. Means ± SE (n=16).

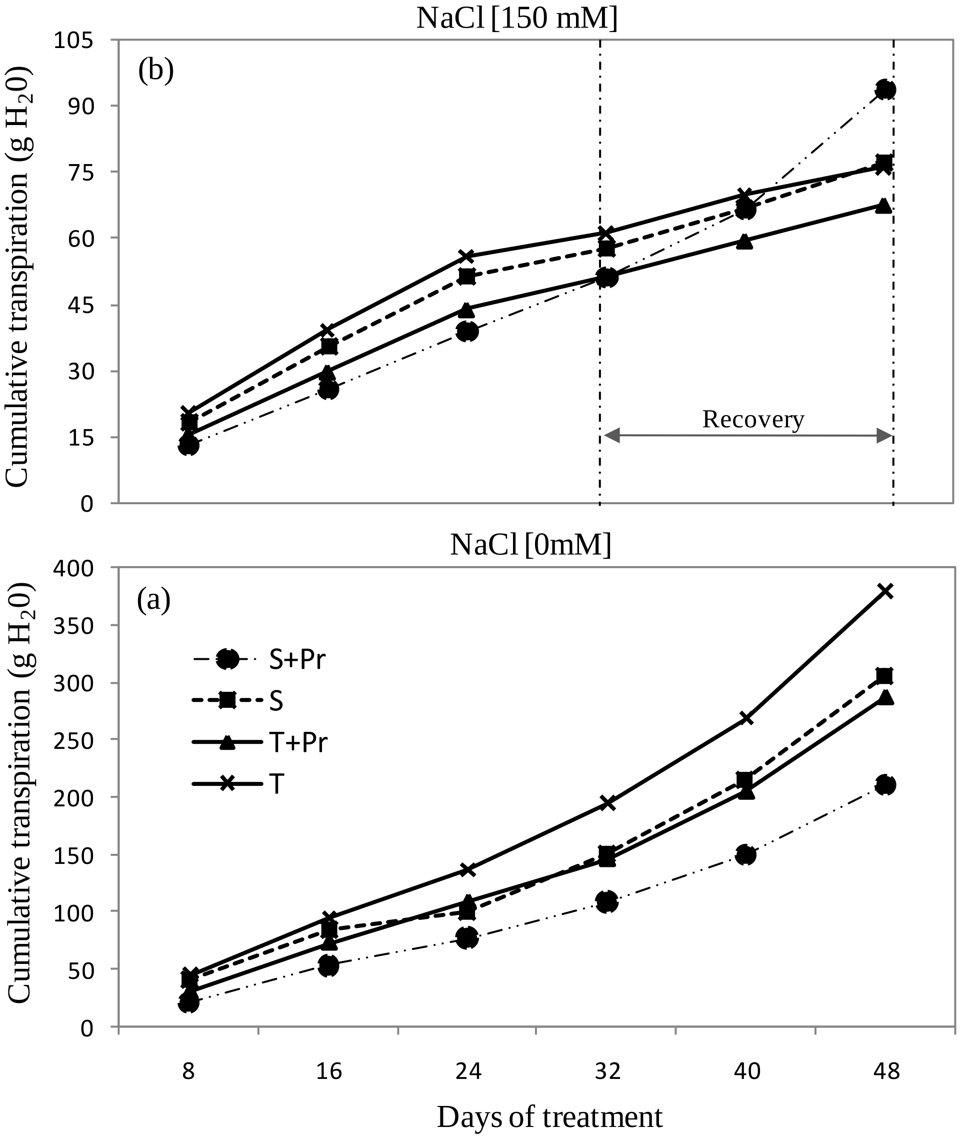

Figure 4.5. Average cumulative transpiration per plant in a) NaCl-untreated plants and b) NaCl-treated plants that were either pruned (Pr) on non-pruned (non-Pr) in short (S) or tall (T) seedlings. The plants were treated with 150 mM NaCl for 30 days. The recovery period is shown by the dashed lines (b), (n=8).

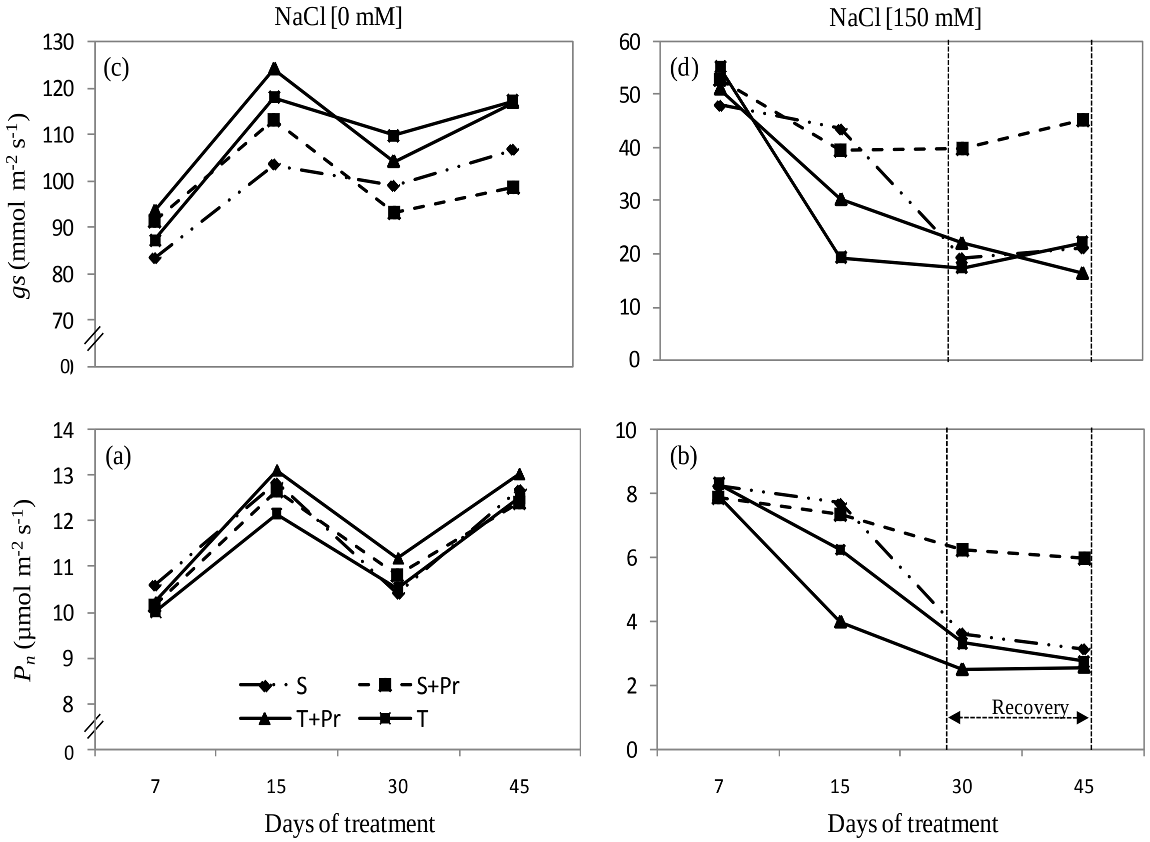

Figure 4.6. Effects of NaCl, seedling height, and branch pruning on net photosynthesis (P_n) (a,b), and stomatal conductance (g_s) (c,d) of *P. leiophylla* plants over 30 days of treatment with NaCl. Recovery period is shown between dashed lines (b-d) (n= 6).

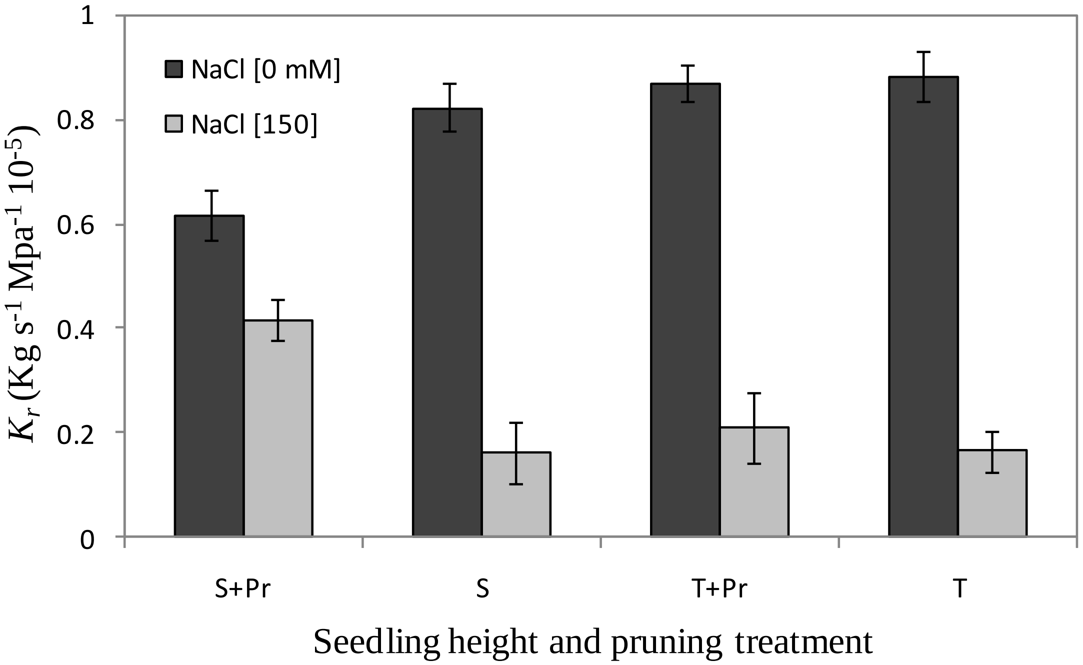

Figure 4.7. Effect of NaCl, seedling height, and branch pruning treatment on root hydraulic conductance (K_r) of *P. leiophylla* plants. Means (n=8) ± SE are shown.

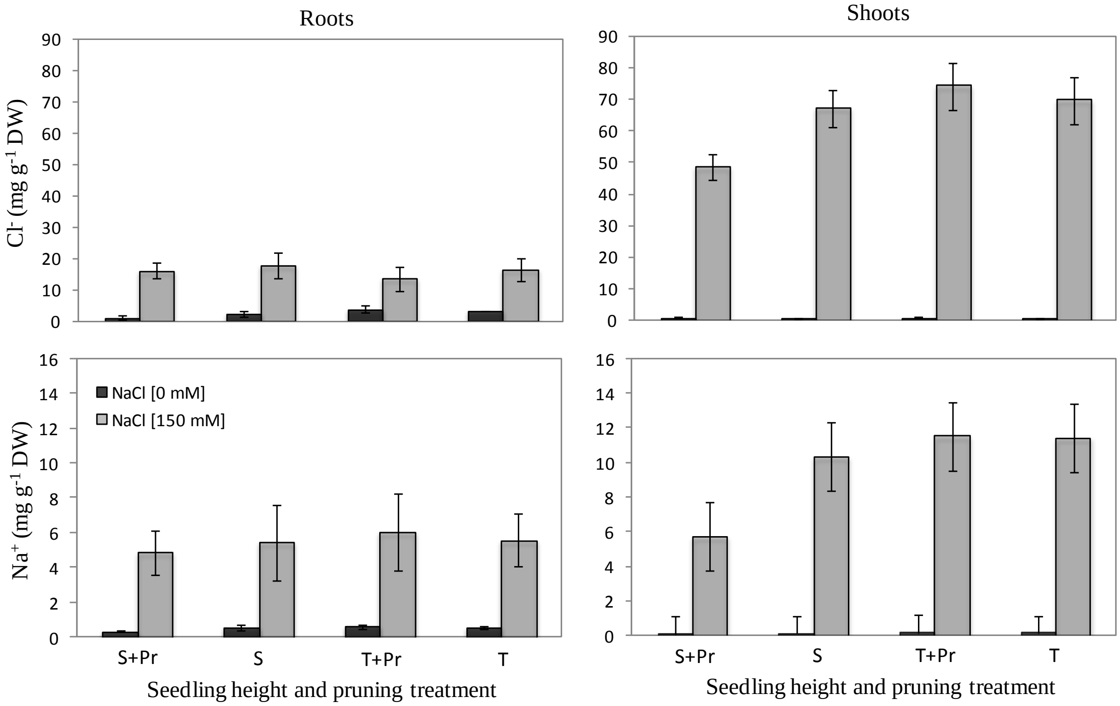

Figure 4.8. Accumulation of Na$^+$ and Cl$^-$ in roots and shoots of *P. leiophylla* short (S) and tall (T) plants and subjected to branch pruning (Pr) after being treated with NaCl for 30 days. Each point represents mean (n=8) ± SE.

Chapter 5. Adventitious sprouting of *Pinus leiophylla* in response to injury caused by salt stress

5.1. Introduction

Conversion of forests to agricultural lands that are used for annual crop production has intensified in recent years in Central Mexico (Messera 1996; Alix-García et al. 2005). Deforestation and subsequent agricultural land use increase soil salinity and accelerate land degradation (Bettenay 1986) which necessitates intensive reclamation efforts prior to reforestation. Selection of native tree species with salinity resistant may be necessary for the revegetation of these areas. *Pinus leiophylla* Schl. *et* Cham. is a widely-distributed tree species in Mexico with high potential for the reclamation of degraded land due to its adaptability to different environments and its resistance of stress conditions (Eguiluz-Piedra 1978; Musálem and Martínez-García 2003). *P. leiophylla* has been used in experimental plantations for erosion control and soil conservation purposes in Asia, Africa, the Caribbean, South America and Oceania (Rodríguez-Franco 2002).

Unlike many other pine species, *P. leiophylla* develops sprouts from the root collar and along the trunk and branches in response to defoliation, fire, pruning and other stress factors (Lanner 2002; Rodríguez-Trejo and Fulé 2003). This attribute is considered an important survival strategy under conditions that cause injury to aerial parts of trees and it may increase tree survival in disturbed areas (Del Tredici 2001).

Sprouts can be derived from epicormic buds that fail to flush during the growth season and remain dormant in the bark or from adventitious buds that may be formed from different living tissues as a result of injury. In *P. leiophylla* trees, the origin of sprouts is unclear. However, the number of buds has been reported to be the greatest at the root collar and to decrease with tree height (Del Tredici 2001) suggesting that these are adventitious buds produced as a result of environmental stimuli. The formation of adventitious buds may depend on factors such as the extent of injury, hormonal status, and accumulation of carbohydrates in the parenchyma cells of sapwood (Lanner 2002; Del Tredici 2001; Climent et al. 2004). Mature parenchyma cells may resume division in response to these external factors and revert to an undifferentiated state, in a process called dedifferentiation, which results in the formation of adventitious buds giving rise to adventitious shoots on various parts of the plant (Esau 1977; Bowes 1996).

In my earlier studies, direct NaCl toxicity and needle injury in *P. leiophylla* were among the most injurious effects of salt that appeard to be largely responsible for the reduction of photosynthetic rates (chapter 2, 3, and 4). However, the shoot growth in *P. leiophylla* was not inhibited even by the highest NaCl concentrations studied (Chapter 4). In the present study, I examined the effects of different NaCl concentrations on gas exchange, root hydraulic conductance, needle injury, shoot growth, and sprouting in shoots of 16 month-old *P. leiophylla* seedlings to test the hypotheses that 1) salt-induced needle injury stimulates the formation and flushing of adventitious buds and subsequent growth of adventitious shoots, and 2) the level of needle injury determines the extent of adventitious shoot growth.

5.2. Materials and Methods

5.2.1. Plant material and growth conditions

Seeds of *Pinus leiophylla* Schl. *et* Cham. were collected from open-pollinated trees growing in Tlaxcala State- Mexico (19°19’ N and 98°00’ W). The seeds were soaked in running water for 24 h and placed at 4ºC for another 24 h to enhance germination. The seeds were germinated in the dark at 26°C in Petri dishes containing moist sand. After radicle emergence from the seed coat, seedlings were transferred to Spencer-Lemaire root trainers (170 cm^3 vol, Spencer-Lemaire Industries Ltd., Edmonton, AB, Canada) filled with a mixture of peat moss and sand (2:1 by volume). The seedlings were grown in a controlled-environment growth room set to 75% relative humidity, 24/18 °C (day/night) temperature, 18-h photoperiod, and photosynthetic photon flux (PPF) of approximately 300 µmol m^{-2} s^{-1} at the seedling level. Seedlings were watered twice per week and fertilized every two weeks with modified Hoagland’s solution (Epstein 1972), containing 224 mg L^{-1} N, 235 mg L^{-1} K, 160 mg L^{-1} Ca, 62 mg L^{-1} P, 32 mg L^{-1} S, 24 mg L^{-1} Mg, 50 mg L^{-1} Cl, 25 mg L^{-1} B, 2 mg L^{-1} Mn, 2 mg L^{-1} Zn, 0.5 mg L^{-1} Cu, 0.5 mg L^{-1} Mo, 20 mg L^{-1} Fe. The nutrients were supplied by placing the containers with seedlings in 25-L tubs and soaking the soil for 4 h. After 14 months, the plants were transferred to 4-L pots using the same substrate and growth conditions as described above.

5.2.2. NaCl treatments

Sixteen-month-old *P. leiophylla* plants, before the beginning of shoot elongation, were subjected to 0 (control), 100, 150, and 200 mM NaCl treatments (0.71, 10.3, 15.1, 19.6 dS m^{-1} electrical conductivity, respectively). All treatments were prepared in distilled water. The treatments were applied by soaking the pots for 24 h every 3 days. All plants were fertilized every 15 days with the modified Hoagland's solution as above. Leachate electrical conductivity of randomly-selected pots was checked every 8 days and the soil was flushed with distilled water every 10 days to prevent salt accumulation. The treatments were applied randomly among blocks of four plants and were replicated three times resulting in 12 plants per treatment for a total of 48 plants. The treatments were carried out for 64 days followed by a recovery period of 30 days. At the start of the recovery period, the soil was flushed with distilled water and all plants were irrigated with destilled water and supplied with modified Hoagland's solution without NaCl. During the recovery period, soil electrical conductivity varied between 0.80 and 1.03 dS m^{-1}.

5.2.3. Survival, needle injury, and chlorophyll content

Needle injury and seedling survival were monitored daily. At the end of the recovery period, necrotic parts of the needles were separated from the green, healthy, tissues. The necrotic and green needle parts were then dried at 70°C for 24 h and weighed. The proportion of needle necrosis was calculated from the dry weights of green and necrotic needle tissues. Plants were considered dead when needle necrosis was above 70%, shoot apical meristems were necrotic and there was no new needle growth.

Needle chlorophyll contents in all plants were determined at the end of NaCl treatments according to Hiscox and Israelstam (1979). Six needles were randomly sampled from each part of the shoot (top, middle and bottom), cut into small pieces and mixed. Tissue samples, each containing 100 mg of needles, were extracted with 7 mL

DMSO at 65°C for 25 min. The extracts were diluted with 7 mL DMSO and the total chlorophyll concentrations were determined spectrophotometrically. Chlorophyll a and b were calculated using the Arnon's equation (Arnon 1949).

5.2.4. Gas exchange and root hydraulic conductance

Net photosynthesis (P_n) and stomatal conductance (g_s) were measured after 64 days of NaCl treatments and after the recovery period. For the measurements, six seedlings per treatment were chosen at random and four fascicles per seedling were measured. Each fascicle with five fully-grown needles was selected approximately 3 cm below the terminal shoot apex. Prior to the measurements, the pots with plants were soaked overnight in water to the field capacity. The measurements started two hours after the light turn on in the chamber and were taken with a portable infra-red gas analyzer equipped with the conifer sample chamber (LCA4, Analytical Development Company Limited, Hoddesdon, UK). During the measurements, leaf temperature in the sample chamber was 24 ± 1°C and PPF was 300 µmol m^{-2} s^{-1}. Needle surface areas were obtained after computer scanning and calculated using Sigma Scan Pro 5.0 software (SPSS, Jandel Scientific, San Rafael, CA, USA).

After the recovery period, the pots with plants were soaked overnight in water and the next morning, they were moved to the laboratory for the measurements of root hydraulic conductance (K_r) (Tyree et al. 1995). For K_r measurements, the shoots were excised about 3 cm above the root collar and the roots were connected through the cut stem to the high pressure flow meter (HPFM) via a piece of flexible high-pressure tubing.

The measurements were carried out in the transient mode with the roots pressurized up to 0.5 MPa (Calvo-Polanco et al. 2009).

5.2.5. Biomass, growth and sprouting of adventitious shoot

Stem diameter at the root collar and terminal shoot lengths were measured at the end of NaCl treatments and after the recovery period. Adventitious shoots greater than 2-cm in length were counted at the end of the recovery period. The plants were harvested at the end of the recovery period, their roots and shoots were separated, and new needles (grown after the onset of NaCl treatment) were removed from the stems. The tissues were then dried at 70°C (24 h for roots and 48 h for stems and needles) for dry mass determinations.

5.2.6. Statistical analysis

Gas exchange (P_n and gs) data were subjected to repeated measured ANOVA using the split-plot design with time (weeks) as a subplot effect. The analysis was conducted using the PROC MIXED-SAS/ PC for Windows version 8.2 (SAS institute, Inc., 2001), using the following general linear model model:

$$Y_{ijkl}=\mu+S_i+B_j+SB_{ij}+T_k+TS_{ik}+ TB_{jk}+TSB_{ijk}+\varepsilon_{ijkl}$$

where: μ is the overall mean, S_i is the fixed effect of the salinity treatment; B_j is the random effect of the block; SB_{ij} is the interaction effect between salinity treatment and block; T_k is the fixed effect of the time measured in weeks; TS_{ik} is the interaction between time and salinity treatment; TB_{jk} is the interaction between time and block; TSB_{ijk} is the interaction between time, salinity treatment, and block, and ε_{ijkl} is the experimental error.

Other variables studied were analyzed according to the following general linear model:

$$Y_{ijkl}=\mu+S_i+B_j+SB_{ij}+\varepsilon_{ijkl}$$

where: μ is the overall mean; S_i is the fixed effect of the salinity treatment; B_j is the random effect of the block; SB_{ij} is the interaction between salinity treatment and block; and ε_{ijkl} is the experimental error. Means were compared using Duncan's multiple range test.

5.3. Results

5.3.1. Survival, needle injury, and chlorophyll content

Seedlings that were subjected to 0, 100 and 150 mM NaCl for 64 days showed 100% survival compared with 67% survival for plants treated with 200 mM NaCl (Table 5.1). Injury symptoms started appearing on older needles after 24 days of treatment, first as needle tip chlorosis followed by the appearance of necrotic lesions about 10 days later. Although needle injury was observed in all NaCl-treated plants, the extent of injury was the greatest in plants exposed to 200 mM NaCl, with 80% of needle weight affected by necrosis (Table 5.1). The seedlings treated with 200 mM NaCl also had the lowest chlorophyll concentrations with chlorophyll a and b reduced to 58% and 87%, respectively, of the control level (Table 5.1). Chlorophyll a and b concentrations were reduced to 29% and 19% of the control level in plants treated with 100 mM NaCl and showed 40% of needle necrosis (Table 5.1). Control plants had no needle necrosis (Table 5.1).

5.3.2. Gas exchange and root hydraulic conductance

NaCl concentration, time and interaction had significant effects on P_n and *gs* of plants (Table 5.2). Both P_n and *gs* decreased with increasing salt concentration treatments, and the more rapid decrease occurred in 150 and 200 mM NaCl compared with 100 mM NaCl (Figure 5.1 a, b). At the end of the treatments, the *gs* values of plants treated with 100 and 150 mM were approximately 70 and 90%, respectively, lower than those of control plants (0mM NaCl) (Figure 5.1a). The *gs* of plants treated with 200 mM NaCl treatment was totally inhibited at the end of treatment (Figure 5.1 a). At the end of the recovery period, the *gs* values slightly increased in NaCl-treated plants, but were still several-fold lower compared with untreated controls (Figure 5.2 a). The P_n values of treated plants followed similar patterns as those shown by *gs* (Figure 5.1 b). At the end of the NaCl treatments, the P_n values of plants treated with 100, 150 and 200 mM were 42, 64 and 84% lower, respectively, than those of control plants. Similarly to *gs*, P_n and *Kr* values did not return to the control levels after 30 days of stress relief (Figure 5.2 b,c). The recovery of P_n, *gs* and K_r in plants treated with 200 mM were not measured due to low seedling survival and high extent of needle injury.

5.3.3. Biomass, growth and sprouting of adventitious shoot

After 64 days of treatments, shoot and root dry mass of plants treated with 100 mM, 150 mM, and 200 mM NaCl were significantly lower compared with the control, untreated, plants (Table 5.3). Shoot and root dry mass of treated plants decreased with increasing NaCl treatment concentrations (Table 5.3).

Terminal shoot length and stem diameter growth of treated plants significantly decreased with increasing NaCl concentration compared with control plants (Figure 5.3). Terminal shoot lengths of plants treated with 200 mM were 5.6 ± 1.4 cm and stem diameter 0.6 ± 0.1 mm compared with 7.1 ± 2.1 cm for shoot length and 2.9 ± 0.3 mm for stem diameter in control plants (means ± SE, n = 12) (Figure 5.3).

Plants treated with NaCl showed a significant increase in the adventitious shoot production (Table 5.4). Sprouting of adventitious shoots in plants was first observed 40 days after the start of NaCl treatments. The first adventitious shoots were produced at the base of the stem near the root collar (Figure 5.4), while sprouting of adventitious shoots from branches occurred towards the end of NaCl treatments (Figure 5.4). About 33% of plants treated with 200 mM NaCl produced adventitious shoots compared with 100% of seedlings that were treated with 100 and 150 mM NaCl (Table 5.4). Sprouting density was higher in the upper parts of the terminal shoot compared with the base of the terminal shoot or branches (Table 5.4). The production of needles was significantly reduced by increasing NaCl concentrations. The total dry mass of new needles was reduced by as much as 94% in the 200 mM NaCl treatment (Table 5.4).

5.4. Discussion

Compared with other plants that have been investigated (Fostad and Pedersen 2000; Akbar et al. 2000; Apostol et al. 2002; Renault 2004), *P. leiophylla* seedlings showed relatively high salt resistance. In the present study, there was no seedling mortality in NaCl concentrations as high as 150 mM (electrical conductivity of 15.1 dS m^{-1}), while in similar studies conducted with *Pinus banksiana* and *Larix laricina*, 60 mM NaCl was sufficient to produce severe injury and 30% seedling mortality (Fostad and Pedersen 2000; Apostol et al. 2002). Also, in citrus, the mortality of seedlings subjected to 8 dS m^{-1} EC NaCl treatment was 65% (Akbar et al. 2000). The needle chlorosis and necrosis in *P. leiophylla* are likely related to Na^+ and Cl^- tissue concentrations. In tamarack and jack pine treated with NaCl, the increase in needle concentrations of Na^+ and Cl^- corresponded to the applied NaCl concentrations and were correlated with the extent of needle injury (Apostol et al. 2002; Renault 2004; Calvo-Polanco et al. 2008).

Numerous studies have demonstrated that excessive salinity in the root zone of woody plants can adversely affect their water relations and cause injury. The effects of salinity on plants are associated with ion toxicity and low osmotic potential which triggers stomatal closure in an attempt to reduce water loss, which results in a decrease of photosynthetic rates (Allen et al. 1994; Hasegawa et al. 2000; Ashraf and Harris 2004). The reduction of P_n and gs in NaCl-treated *P. leiophylla* plants increased with increasing NaCl concentrations and progressed over time (Figure 5.1). Similar responses were observed in plants of *P. pinaster, Arbutus unedo, and Aster tripolium* subjected to a NaCl gradient where P_n and gs also was reduced progressively by NaCl in a concentration-dependent manner (Loustau et al. 1995; Robinson et al. 1997; and Navarro et al. 2007).

The growth reduction in NaCl-treated *P. leiophylla* seedlings is a frequently observed effect of salt on plants. The decrease of water uptake, followed by photosynthesis reduction, hormonal imbalance and tissue injury all contribute to growth inhibition in salt-treated plants (Hasegawa et al. 2000; Loustau et al. 1995; Apostol and Zwiazek 2003). Munns and Tester (2008) proposed that salt-induced osmotic stress has an immediate impact on a reduction of shoot growth and it occurs when salt concentration increases above approximately 40 mM, the threshold level for most plants; while ionic stress occurs later as a consequence of Na^+ accumulation in shoots.

Although the stem diameter growth and shoot elongation were reduced in NaCl-treated *P. leiophylla* seedlings, they were not completely inhibited by salinity. Loustau et al. (1995) reported that the ability to maintain stem elongation under salt stress by *Pinus pinaster* was associated with large decreases in osmotic potentials of cell and xylem saps, which maintained high cell turgor. This growth was likely supported by the mobilization of storage carbohydrates rather than by current photosynthates which were likely less available as a consequence of needle injury and reduced photosynthetic rates. Although some studies with young conifer seedlings have shown that stored carbohydrates are used for shoot elongation (Puttonen 1986), the relative contributions of stored carbohydrates and current photosynthates for the processes of shoot extension growth remain unclear. The removal of needles from *Pinus nigra* and *P. resinosa* prior to shoot extension reduced shoot elongation over the whole growth season but the reduction was much greater during the later part of the shoot elongation period. (Ford 1980). These results point to the importance of carbohydrates stored in stems and roots for shoot growth. Thus, the content of carbohydrate reserves and the mobilization of carbohydrates may determine the re-growth of shoots in plants previously affected by environmental stresses.

In our study, adventitious sprouting was observed at the stem-base, main stem and branches of all NaCl-treated *P. leiophylla* seedlings. This points to the ability of this species to sprout in response to salt injury similarly to trees damaged by fire (Perry 1991) and herbivory (Jasso-Mata and Jiménez-Casas 2001). Other pine species with a similar ability include *P. canariensis*, *P. oocarpa*, *P. merkussi*, *P. clausa*, *P. serotina*, *P. brutia*, *P. halepensis*, *P. pungens*, *P. hartwegii*, and *P. caribaea* var. *hondurensis* (Climent et al. 2004; Rodríguez-Trejo and Fulé 2003).

Needle damage or loss seems to be the main trigger the mechanisms leading to a formation of adventitious buds or release of epicormic dormant buds. It has been suggested that this damage modifies the levels of endogenous growth regulators and induces translocation of carbohydrate reserves needed to stimulate the formation and growth of buds (Del Tredici 2001; Lanner 2002). In *Pseudotsuga menziesii*, cytokinin sprayed on seedlings induced adventitious bud formation (Ross et al. 1983). Changes in endogenous cytokinin concentrations in *Pseudotsuga menziesii* were also correlated with the release of bud quiescence (Pilate et al. 1989). On the other hand, the sprouting ability of *Acer saccharum*, *Alnus rubra*, *Sequoiadendron giganteum*, *Pinus canariensis*, and other tree species has been explained by the carbohydrate reserves present in sapwood parenchyma (Moony 1973; Harrington 1984; Climent et al. 2004; O'Hara et al. 2008). Therefore, the resprouting capacity is likely affected by the quality and quantity of carbohydrates stored in the sapwood parenchyma cells (Climent et al. 2004; Puijalon et al. 2008).

Both 100 and 150 mM NaCl were more effective than 200 mM NaCl in stimulating sprouting of adventitious shoots from the lateral branches and from the upper parts of the main stem, but the number of adventitious branches that were produced from the stem base was similar for all NaCl treatments (Table 5.4). The sprouting from the stem base also took place in seedlings which suffered extensive damage to the shoots (Figure 5.4 d). Similar findings were reported for *Arbutus arizonica* where sprouting capacity decreased as a result of shoot injury severity that was caused by fire (Barton 2005). However, in *Sequoia gigantea*, sprouts were more frequent and larger on more severely pruned trees (O'Hara et al. 2008). These results indicate that the frequency and number of sprouts vary according to the severity and type of stress. In our study, accumulation of Na^+ and Cl^- in tissues and the injury level exhibited by plants treated with 200 mM likely affected the ability of plants to mobilize carbohydrates which, in turn, reduced the number of sprouts in stems and branches but not in the root collar section. Starch reserves are stored at the root collar in many conifers (White 1981; Chomba et al. 1995). Therefore, the buds close to the root collar likely had higher concentrations of carbohydrate reserves available. It is also interesting that these and other buds were maintained alive in seedlings exposed to high NaCl concentrations. A possible explanation may include the reduced degree of vascular connections to meristematic cells which could help these tissues avoid accumulation of toxic levels of Na^+ and Cl^- (Hasegawa et al. 2000).

The reduced levels of P_n, gs and K_r in NaCl-treated plants at the end of the recovery period indicate that seedlings may require a relatively long time to fully recover form the effects of salt (Figure 5.2), which may affect the ability of plants to produce new roots and needles to substitute the biomass loss caused by salinity. The adventitious branch sprouting in response to salt stress may contribute to the production of new photosynthetic tissues and consequently improve the P_n and *gs* levels to increase the photosynthate production needed for the re-growth of plants injured by salinity. Sprouting is considered as part of the plant regeneration strategy after damage caused by biotic and abiotic factors (Vesk and Westoby 2004; Wang et al. 2007).

In conclusion, NaCl treatments reduced growth, P_n, *gs*, and K_r, and caused needle injury, but stimulated adventitious sprouting in *P. leiophylla* seedlings. Sprouting from the upper parts of the main stem and lateral branches was greater in 100 and 150 mM NaCl compared with 200 mM NaCl treatment. However, sprouting from the base of the main stem was similar in all NaCl concentrations. These results suggest that *P. leiophylla* has regeneration potential even after exposure to high salt concentrations. Further research is needed to understand the factors that are involved in the induction and growth of adventitious branches and to determine the frequency, extent, and persistence of sprouting in plants exposed to longer-term soil salinity under field conditions.

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Table 5.1. Needle chlorophyll concentration (n = 6), needle necrosis and seedling survival (n=12) of seedlings subjected to NaCl-treatments for 64 days and in control plants (0 mM NaCl). Means in each column followed by the same letter are not significantly different at α = 0.05 as determined by Duncan test

[NaCl]	Chlorophyll (mg g^{-1})			Needle necrosis (% needle weight)	Survival (%)
mM	a	b	Total		
0	0.72 a	0.47 a	1.19 a	0	100
100	0.51 a	0.38 b	0.86 b	39.9 c	100
150	0.37 c	0.18 c	0.55 c	63.2 b	100
200	0.30 d	0.06 d	0.36 d	80.4 a	33.3

Table 5.2. F values in the analysis of variance of the effects of NaCl (Sal), block (B) and treatment duration (Time) on net photosynthesis (P_n) and stomatal conductance (*gs*) in plants treated for 64 days

Source	DF	Gas exchange	
		P_n	*gs*
Sal	3	745.8**	901.7**
B	2	1.1	4.0
Sal X B	6	0.9	3.3
Time	7	209.9**	497.5**
Time X Sal	21	42.3**	43.6**
Time X B	14	1.0	0.8
Time X B X Sal	42	1.1	0.7

**= significance level, $P \leq 0.01$

Table 5.3. Root, shoot, needle, and total dry mass in *P. leiophylla* seedlings subjected to NaCl treatments for 64 days and in control plants (0 mM NaCl) (n = 12). Means with different letters in the same column indicate significant differences at α = 0.05 level as determined by the Duncan's standardized range

[NaCl] mM	Roots (g)	Stem (g)	Needles (g)	Total (g)
0	39.8 a	14.8 a	46.8 a	101.4 a
100	21.9 b	9.6 b	37.9 b	69.6 b
150	15.6 c	6.2 c	30.9 c	53.0 c
200	10.7 c	5.9 c	24.2 d	40.9 d

Table 5.4. Number and percent of plants with adventitious shoots (in brackets) present at collar root, stem , and branches, and dry weight of new needles in seedlings subjected to NaCl treatments and in control plants (0 mM NaCl) (*n*=12). Values in each column followed by different letters are significantly different at α= 0.05 level as determined by the Duncan's standardized range

[NaCl]	Number of adventitious shoots							N.Needles
mM	Rooot Collar		Stem		Branches		Total	(g)
0	1.6 b	(75)	2.6 b	(67)	0.3 b	(25)	4.6 c	15.6 a
100	7.3 a	(100)	11.6 a	(100)	4.6 a	(100)	23.5 a	9.6 b
150	7.8 a	(100)	12.7 a	(100)	4.3 a	(100)	24.9 a	4.9 c
200	7.2 a	(100)	3.6 b	(33)	1.5 b	(33)	12.2 b	0.9 d

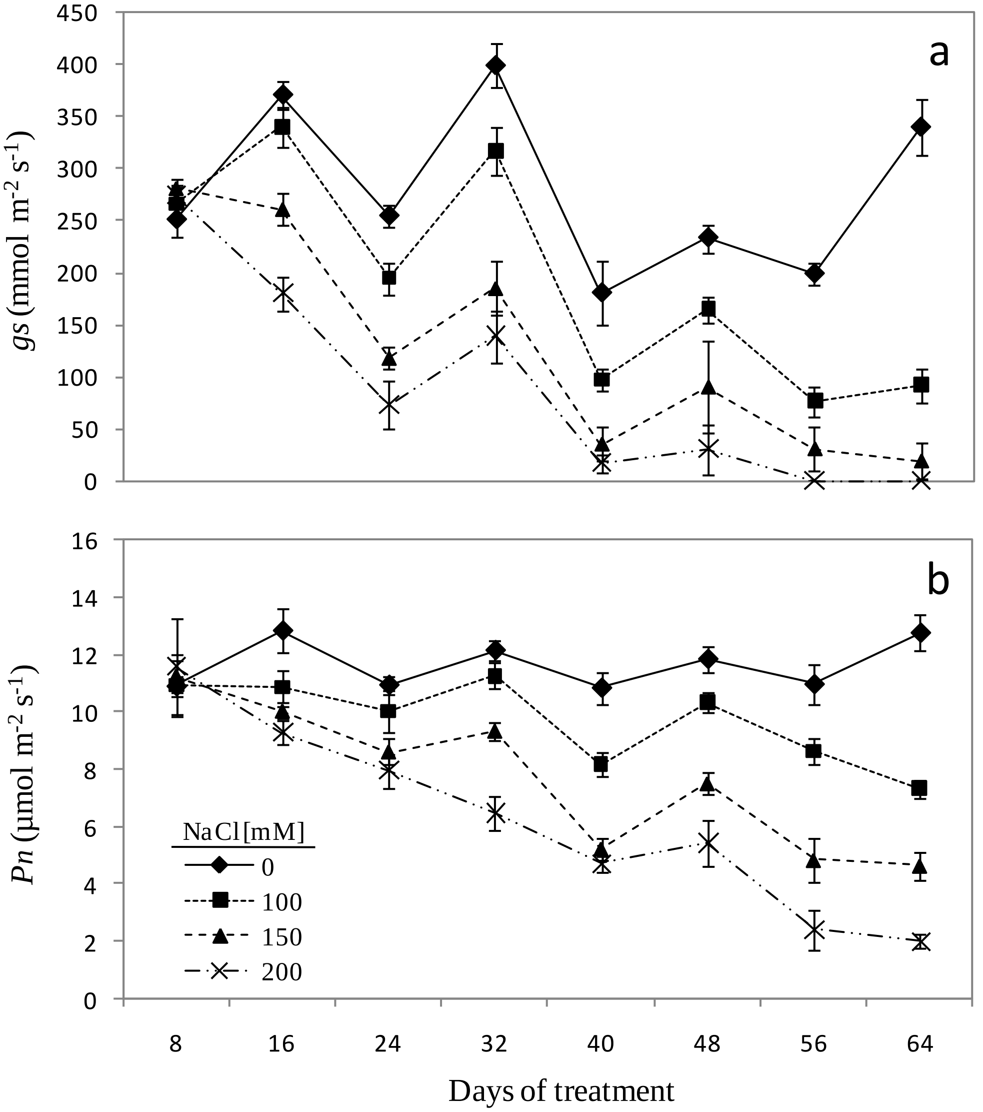

Figure 5.1. Net photosynthesis (P_n) and b stomatal conductance (*gs*) in seedlings over 64 days of 0 (control), 100, 150, and 200 mM NaCl treatments. Each point represents mean (n=6) ± SE.

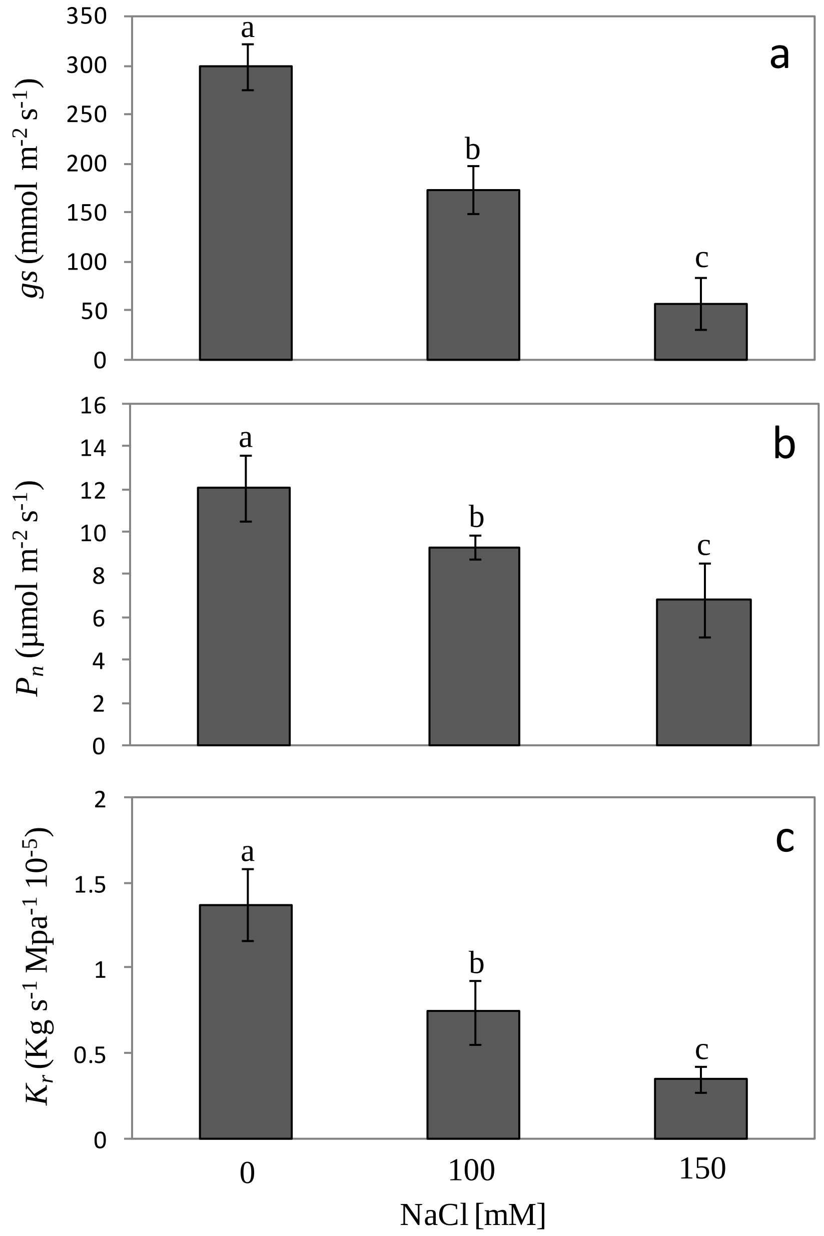

Figure 5.2. Stomatal conductance (*gs*) (a), net photosynthesis (P_n) (b), and hydraulic conductance (K_r) (c) in seedlings subjected for 64 days to 0 (control), 100, and 150 mM NaCl treatments following by 30 days of recovery. Bars are means (n=6) ± SE.

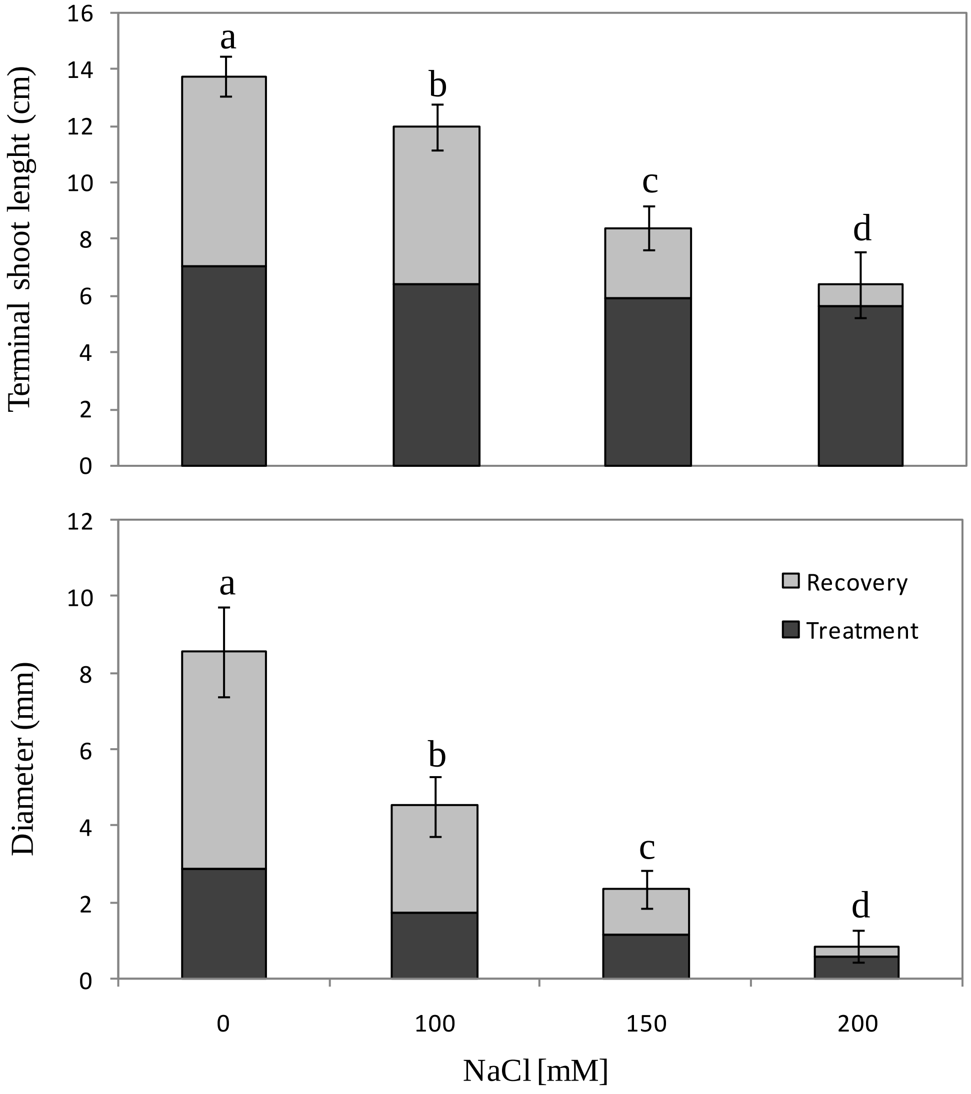

Figure 5.3. Effects of 0 (control), 100, 150, and 200 mM NaCl on stem diameter and terminal shoot length of seedlings after 64 days of treatments and after the recovery period. Bars are means (n=12) ± SE.

Figure 5.4. Adventitious shoots in NaCl-treated seedlings. Red arrows point to adventitious shoots in terminal shoots (a) and in branches (b) after 64 days of treatments, adventitious shoots at the stem base and along the stem (c); and adventitious shoots at the stem base in the plant with the shoot severely injured by the 200 mM NaCl-treatment (d).

Chapter 6. General Discussion and Conclusions

The research study focused on salt resistance of *Pinus leiophylla*. The studies of geographic variations among populations of contrasting localities have identified some of the morphological traits associated with NaCl resistance (Chapter 2 and 3). The branch pruning studies provided information about the mechanisms involved in the transport of Na^+ and Cl^- into shoots and about potential benefits of branch pruning for the improvement of planting performance in salt–affected sites (Chapter 4). The branch sprouting analysis provided insight into the adaptive ability of *P. leiophylla* to regenerate new branches after foliar damage caused by salinity and to promote the survival of trees affected by saline conditions (Chapter 5). This information will help to establish the management plans and criteria for early genetic selection of *P. leiophylla* for restoration of areas affected by salinity.

In the first study (Chapter 2), *P. leiophylla* seedlings from eight populations of different geographic locations in Mexico showed distinct differences in height and shoot diameter which suggest adaptation of the species to the local environmental conditions. This adaptation played an important role in salt resistance exhibited by seedlings of these populations. Seedlings from xeric sites were smaller but less sensitive to NaCl and had greater capacity to recover from injury compared with tall plants from the mesic sites, suggesting that morphological and physiological adaptive traits to drought likely contributed to NaCl resistance. Geographic variations in conifers are associated mainly with temperature and moisture regimes (Aitken 2000). Some conifers from xeric areas have exhibited slower growth, lower shoot/root dry mass ratio, earlier bud set and more resistance to drought stress compared with trees from the mesic sites (Oleksyn et al. 2000; Tognetti et al. 2000; Cregg and Zhang 2001). Studies of geographic variations in salt resistance of *Picea abies* and *Pinus pinaster* have demonstrated that adaptive traits to drought conditions can favor the resistance to salinity and it was recommend that genotypes from drier sites exhibiting low transpiration rates and with high water-use-efficiency should be considered for early selection of salt-resistant families (Loustau et al. 1995; Bozhko et al. 2003). Although it is unclear if the small size and slow growth of plants were the costs associated with stress resistance, the slow growth of *P. leiophylla* plants from dry sites might be a potential trait for early selection of salt-resistant genotypes for restoration and reforestation of saline soils.

The results of the second study (Chapter 3) confirmed NaCl resistance exhibited by the seedlings of San Felipe population and suggested that slow-growth of seedlings, production of fascicle needles and length of needles were important traits for salt stress resistance. Seedlings from San Felipe showed slower development of fascicle needles, shorter and fewer needles, and lower root hydraulic conductance than seedlings from the other populations. These might be considered as adaptive traits to drought, explain the slow-early growth of these seedlings. The delayed emergence of fascicles and the reduction of fascicle needle production as well as the elongation of needles in response to NaCl treatments were slight and similar among seedlings of the four populations but, the extent of needle injury and accumulation of Na^+ and Cl^- in shoots were lower in the San Felipe seedlings than in the other three populations. Lower transpirational area of seedlings from San Felipe likely resulted in lower transpiration rates which were reflected in the lower root water flow and lower rates of Na^+ and Cl^- uptake and transport by the seedlings. The root-to-shoot ion transport is often affected by the rate of transpiration so that high rates of transpiration might increase the levels of ions in leaves if high concentrations of ions were present in the xylem (Kramer 1983). However, since exclusion of Na^+ and Cl^- was considered the most important mechanism of salinity resistance (Chen et al. 2002), there is also the possibility that the San Felipe seedlings could have limited Na^+ and Cl^- loading into the xylem during root radial transport and sequestered ions into the root cortical cells more efficiently than the other populations. Therefore, further research is needed to examine the extent of restriction of radial transport of ions and determine the contribution of this mechanism to the restriction of ion transport and ameliorating salt toxicity in seedlings from San Felipe.

In the branch pruning study (Chapter 4), total plant transpiration as affected by plant size and branch pruning was correlated with the Na^+ and Cl^- needle concentrations and needle necrosis in the NaCl-treated *P. leiophylla* seedlings. The shoots of pruned small plants had the lowest shoot mass, total transpiration, and Na^+ and Cl^- shoot concentrations of all the treatment groups which was reflected in the low needle necrosis levels in these plants. These results suggest that transpiration was a mechanism involved in the translocation of ions into the shoots. In citrus, the Cl^- uptake and concentrations in the xylem were similar in the Cl^--sensitive and Cl^-- resistant genotypes. However, leaf Cl^- concentrations differed and depended on both leaf biomass and transpiration rates, suggesting that they might be determined by the intensity of xylem flow (Moya et al. 1999). Similar results were reported in salt- resistant *Populus* genotypes where the lower transpiration rates contributed to the control of salt transport to shoots (Chen et al. 2003). Branch pruning might be an effective treatment increasing the chances of survival of pine seedlings planted in salt-affected areas. These results indicate that branch pruning could be very useful during and after the establishment of *P. leiophylla* seedlings in situations where the salinity level in soil solution fluctuates during the year. Therefore, pruning effects in this species should be studied under field conditions to confirm the observed results and to include this practice in afforestation and reforestations programs for areas affected by salinity.

Finally, in Chapter 5, my results showed that sprouting ability of *P. leiophylla* in response to NaCl stress was an important attribute that might increase the trees probability of survival in salt-affected sites. NaCl treatments reduced growth, net photosynthesis, stomatal conductance, and root hydraulic conductance in *P. leiophylla* seedlings. It also caused needle injury but stimulated adventitious sprouting at the stem-base, stem and branches. Needle damage is likely the main cause that triggers the mechanisms leading to formation, development and release of adventitious buds in young *P. leiophylla* plants. In some tree species, the damage in shoots caused by stress factors modified the levels of endogenous growth regulators and induced the translocation of carbohydrate reserves needed to stimulate the formation and growth of buds (Del Tredici 2001; Lanner 2002). The quality and quantity of carbohydrates stored in the sapwood parenchyma cells were the determinant factors for sprouting (Climent et al. 2004; Puijalon et al. 2008). In my study, the number of adventitious shoots varied with the severity of needle injury. Sprouting from the upper parts of the stem and lateral branches was greater in the 100 and 150 mM NaCl compared with the 200 mM NaCl treatment. The injury level exhibited by plants treated with the 200 mM NaCl likely affected the ability of plants to mobilize carbohydrates which in turn, reduced the number of sprouts in stems and branches. These sprouting results are very interesting in terms of survival of *P. leiophylla* seedlings after salt stress exposition because it would allow the seedlings to recover the foliar mass loss caused by salts. Sprouting is an important attribute that allows of the establishment and persistence of many species on marginal and disturbed sites (Bond and Midgley 2003). Therefore, branch sprouting of *P. leiophylla* in response to salinity may be considered as a sign of recovery since this response was part of the plant regeneration strategy to survive after damage caused by biotic and abiotic stresses (Vesk and Westoby 2004; Wang et al. 2007). These results indicate that *P. leiophylla* has the potential to regenerate even when exposed to high salt concentrations. However, further research is needed to understand the factors that are involved in the induction and growth of adventitious branches and to determine the frequency, extent, and persistence of sprouting in plants exposed to longer-term soil salinity under field conditions.

To the best of my knowledge, the responses of *P. leiophylla* to salt stress had not been addressed in earlier studies elsewhere so that the results of my research provide important information about the potential performance of trees of this species under salt stress conditions.

The drought resistance traits such as slow height growth, slower development of fascicle needles, shorter and fewer needles, and lower root hydraulic conductance; and the sprouting ability were involved in the NaCl-resistance of *P. leiophylla* treated plants so that these traits may lay foundations for future early selection of salt resistant genotypes of this species. However, my studies were performed under controlled-environment conditions and assessed the relatively short-duration of salt treatments. It is plausible that the contribution of seasonal environmental fluctuations may affect plant responses so that field tests and longer-term salt treatments will be essential for validating the results reported here. Such studies will also allow the determining in the effectiveness of early selection for this pine species.

The field tests should include families and populations to understand the relative magnitude of family and provenance variations and establish the selection strategies to improve in salt-resistance. In these field studies, special emphasis should be placed on populations and families of Tlaxcala state, particularly San Felipe, which showed resistance to NaCl stress. Since some drought adaptive traits were correlated with NaCl resistance, more populations of *P. leiophylla* from the drier sites in Mexico should be included. I addition, It will be necessary to collect environmental data, analyze soil properties and determine the types of mycorrhizal associations for each seed source to understand the patterns of geographic variations and NaCl resistance among the populations. Finally, alternative management practices such as branch pruning and the application of mycorrhizal technology should be considered in *P. leiophylla* improvement programs aimed at increaseing saline resistance.

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