Physiological responses of glycophytic and halophytic grasses *Poa pratensis*, *Poa juncifolia*, and *Puccinellia nuttalliana* to salt stress

By

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ABSTRACT

Responses of two halophytic grasses *Puccinellia nuttalliana* and *Poa juncifolia* to treatments with NaCl were compared with the glycophytic grass *Poa pratensis* to better understand salt tolerance mechanisms in these plants. The experiments were carried out under controlled-environment conditions with hydroponically-grown seedlings. The plants were subjected to 50, 150, and 300 mM NaCl treatments for up to 10 days and compared with their respective untreated controls (0 mM NaCl). At the lower NaCl concentrations, shoot and root dry weights were drastically reduced in *Poa pratensis*, but increased in *Puccinellia nuttalliana* and *Poa juncifolia*. The examined NaCl treatment concentrations had either no effect (*Puccinellia nuttalliana*) or little effect (*Poa juncifolia*) on the net photosynthesis and transpiration rates in the halophytic plants, but severely decreased the gas exchange parameters in *Poa pratensis*. Similarly, to growth and gas exchange, shoot water content in *Puccinellia nuttalliana* was not affected even by the highest, 300 mM NaCl concentration, while *Poa pratensis* showed decreased shoot water content in all examined NaCl treatments and *Poa juncifolia* in 150 and 300 mM NaCl. Cell hydraulic conductivity in *Poa pratensis* also showed high sensitivity to NaCl and drastically decreased in all examined treatments. Cell hydraulic conductivity in *Poa juncifolia* was less affected by NaCl compared with *Poa pratensis* and it increased in response to NaCl treatments in *Puccinellia nuttalliana*. Both *Puccinellia nuttalliana* and *Poa juncifolia* accumulated less Na in their shoot tissues compared with *Poa pratensis* and maintained relatively higher K concentrations in roots. *Puccinellia nuttalliana* also accumulated more P and Mg in the shoot and root tissues compared with the two other

examined grass species. The results demonstrate the importance of restricting root to shoot Na transport and maintenance of aquaporin-mediated water transport in salt tolerance of the two studied halophytes.

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List of symbols and abbreviations

ψ_w	Shoot water potential
ANOVA	Analysis of variance
AQP	Aquaporin
chl	Chlorophyll
DW	Dry weight
E	Transpiration rate
gs	Stomatal conductance
L_p	Cell hydraulic conductivity
Lpr	Root hydraulic conductivity
Pi	Inorganic phosphorus
Pn	Net photosynthesis rate
SE	Standard error
CPP	Cell pressure probe
$T_{1/2}$	Half time of water exchange
MIP	Major intrinsic Proteins
ANOVA	Analysis of variance
Na	Sodium chemical element
Ca	Calcium chemical element
Cl	Chloride chemical element
K	Potassium Chemical element
Mg	Magnesium chemical element
P	Phosphorus
Mg Kg $^{-1}$	Miligram per kilogram
mM	Millimolar
MIP	Major intrinsic proteins
SPAC	Soil – plant – air- continuum

1 Introduction

Elevated soil salinity is among the most challenging problems that plants must cope with in many agricultural and reclamation sites. Many areas of the world are affected by soilor water with high concentrations of salts including NaCl. Salt stress affects many physiological processes in plants and results in growth inhibition and plant mortality (Qados 2011). The ability of plants to tolerate salt is determined by different biochemical pathways that maintain the acquisition of water, protect chloroplast functions, and maintain ion homeostasis (Sun et al. 2009).

Most of the agricultural plant species and plants that are used for land reclamation are salt-sensitive glycophytes, which are unable to to grow in environments with high salt concentrations due to lack the adapation. Unlike glycophytes, halophytic plants can tolerate high salt concentrations. These plants can survive and reproduce in environments with salt concentrations that are higher than 200 mM. Halophytes form only about 1% of the world's flora (Flower et al. 2015).

The difference between halophytes and glycophytes in terms of salt tolerance is related to the presence of specific adaptive strategies which enable halophytes to survive in the environments with high concentrations of salt. Since glycophytes do not posses these adaptive mechanisms, they are unable to survive in the environments in which halophytes thrive (Flowers et al. 1986).

Most halophytes show optimal growth in the presence of salt and, for some halophytes, Na is considered to be an essential element which withot, the plants can not reach to the optimum growth and complete their life cycle. The tolerance of all halophytes to NaCl depends on controlling the uptake and compartmentalization of Na^+, K^+ and Cl^- and also the synthesis of organic compatible solutes. Some halophytes also form salt glands through which they remove salt. At the cellular level, H^+- ATPases present in the plasma membrane and tonoplast, as well as the tonoplast H^+- PPiase, provides the trans-membrane proton motive force used by various secondary transporters (Flowers and Colmer 2008).

Many landscape plants including some of the turf grass species can survive in the soil with high salt concentrations and can be used for recycling irrigation water or soils with high level of salt (Alshammary et al. 2004). Therefore, salt-tolerant C_3 and C_4 turf grasses have important practical uses (Qian et al, 2000).

Most species belong to the Poacae family are wildly distributed in all over the world due to their climatic adaptability (Carter and Spiering 2002). Poceae species vary in terms of salt tolerance and they range from being salt sensitive to highly tolerant (halophytic). Kentucky bluegrass (KBG; *Poa pratensis* L.) is considered to be a turfgrass species of the Poaceae family that is salt sensitive while *Poa juncifolia* (alkali bluegrass) and *Puccinellia nuttalianna* (alkali grass) are the species considered to be halophytic plants (Harivandi et al. 1992).

The main secondary stresses that plants experience as a result of exposure to salt are water deficit, ion toxicity, and inhibition of nutrient uptake (Greenway and Munns 1980, Tester and Davenport 2003). In transpiring plants, water gets from the soil to the root xylem mostly through apoplastic path and its flow is driven by hydrostatic pressure gradient. However, water movement can change when transpiration is restricted during stress conditions such as salinity. Under these circumstances, more of the water follows the cell-to-cell path, flowing across membranes of the living cells (Steudle 2000). Salinity can also upset plant water relations when water availability from soil solution is restricted as a result of lowered osmotic potential (Munns 2005).Stomatal closure is often observed in salt-affected plants in order to limit water losses (Fricke et al. 2004).

Root water uptake is among the most sensitive processes in plants to salinity. Even very low soil salt concentrations can drastically reduce root water uptake due to the effect on root aquaporins. Concentrations of NaCl as low as 10 mM have been shown to inhibit aquaporin-mediated water transport within several minutes in some plants (Lee et al. 2015).

Aquaporins (AQP) are channel proteins present in the intracellular and plasma membranes. They belong to the group of major intrinsic proteins (MIP) with molecular weights of 26–34 kDa and play an important role in controlling and transporting water

and other small neutral solutes including urea, boric acid, silicic acid, ammonia, and carbon dioxide in plant cells. The main role of aquaporins in plants is water transport. They have remarkable features to provide an efficient and specific water flow and transport water into and out of the cells (Tyerman et al. 2002). The initial and almost immediate effect of NaCl is a decrease in root hydraulic conductivity due to the inhibition of aquaporin-mediated water transport resulting in a reduction of stomatal conductance and photosynthesis (Lee et al. 2010). However, in halophytes, the aquaporin-mediated water transport appears to be relatively less sensitive to salt since halophytic plants do not suffer from salt-induced water stress. The processes leading to this salt-resistance of aquaporins in halophytes are presently unknown.

Salt stress is highly complex and involves different physiological processes in plants. In the presence of salt, all major processes such as photosynthesis and respiration can be affected in both halophytes and glycophytes (Greenway and Osmond 1972). In addition, growth rate can be reduced even by low salt concentrations in all plants including halophytes (Greenway and Munns 1980). This growth reduction can be the result of changes in ionic balance, water status, and mineral nutrition (Munns and Termaat 1986) which affect growth by inhibiting net photosynthesis (PN), stomatal conductance (gs), transpiration (E) and other physiological processes (Sharma et al. 2005). The rate of photosynthetic CO_2 assimilation is reduced during salt stress due to reduced stomatal conductance (Farquhar et al. 1982). Non-stomatal photosynthetic reduction can be also caused by a direct effect of NaCl on photosynthetic apparatus in both halophytic and glycophytic plants (Ball and Farquhar 1982).

The principal objective of the present study is to examine the effects of NaCl on physiological pocesses in three related grass species *Poa pratensis, Poa juncifolia and Puccinellia nuttalianna* which had been previously demonstrated to widely vary in their sensitivity to salt. The study was carried out to lay foundations for future research aimed at understanding the mechanisms that lead to salt tolerance of aquaporin-mediated water transport in halophytic plants. In the present study, growth, water relations, gas exchange, and tissue Na accumulation was examined in the three grass species that were exposed to different concentrations of NaCl in hydroponics under controlled-environment conditions.

I examined the hypothesis that in the halophytic grass species of *P. juncifolia* and *P.nuttalliana*, hydraulic conductivity of root cells would be less affected by NaCl compared with the glycophytic grass *P. pratensis*, leading to higher rates of transpiration, net photosynthesis and growth. Halophytes play increasingly important roles as model plants for understanding plant salt tolerance, as genetic resources contributing towards the goal of improvement of salt tolerance in crops, and reclamation plants as well as 'niche crops' in their own landscapes with saline soils. It is envisioned that the knowledge generated through this study will help improve understanding of salt tolerance in plants and may be applied in the future to assist with the revegetation efforts of areas affected by salinity. This knowledge can be also used to assist with the management of native species biodiversity in saline environments and rehabilitation of plant-depleted sites.

2 Literature Review

2.1. Salinity

2.1.1. Soil salinity

Salinity is a major environmental problem that can affect growth, development and productivity of plants. Saline soils are mainly dominated by Na^+ and Cl^- ions, in which NaCl constitutes from 50 to 80% of the total soluble salts (Rengasamy 2010). Soil salinity is a common environmental problem that can lead to salt accumulation and reduced crop production in irrigated lands in all climatic regions (Singh and Chatrath 2001). Most of the soils and irrigation waters, even those considered to be of good quality soil and water, still contain significant amounts of dissolved salts. The irrigation water often contains various ions contributing to overall salinity including calcium (Ca^{2+}), magnesium (Mg^{2+}), and sodium (Na^+). When water evaporates, Ca^{2+} and Mg^{2+} often precipitate into carbonates, and Na^+ becomes dominant in the soil (Serrano et al. 1999). High concentration of Na^+ in the soil can affect nutrient and ion balance in the soil (Grattana and Grieveb 1999). As a result, increasing the cations and their salts, especially NaCl, in the soil can create low soil osmotic potential that can reduce or even prevent water flux to the root and cause water deficit. Soil salinity can also lead to soil sodicity and damage to soil structure. Therefore, plants growing in saline soils not only suffer from high salt concentrations, but are also affected by some level of hypoxia (Singh and Chatrath 2001). Soil structure and different environmental factors such as temperature and vapor pressure deficit may affect plant salt responses (Chinnusamy et al. 2005). Since different salts can be harmful to plants, in this thesis, the term salinity stress is reffered to stress induced specifically by the most common injurious salt, sodium chloride (Rengasamy 2010).

2.1.2. Effects of salinity on plants

Salinity can affect plants in different ways and induce ion toxicity, osmotic balance and nutrient deficiency. Therefore, under salinity stress, plants need to reduce the entry of Na and Cl ions and minimize their concentration in the cytoplasm (Hasegawa et al. 2000). Water deficit happens immediately once the plant is exposed to salts. Water deficit can be caused by a decrease of the osmotic potential in the soil solution and it can easily disturb the ability of roots to absorb water. Therefore, major processes such as photosynthesis, cell expansion, cell division, and stomatal movements can be disrupted. Plant responses to water deficit depend on different factors such as the amount of water lost, the rate of loss, and the duration of stress condition (Bray 1997)

During the long-term exposure to salt, plants experience more severe problems such as ionic stress due to a high concentration of Na^+ and ion accumulation reaches to toxic level. Therefore, all major metabolic processes are disrupted (Cramer and Nowak 1992). In addition, the accumulation of sodium in photosynthetic tissues can have a negative impact on photosynthetic pigments such as chlorophylls (Davenport et al. 2005).

Marschner (1995) introduced nutrient imbalance as the third factor affecting plant growth under salinity condition. This can happen as a result of the disruption of mineral nutrients uptake especially calcium (Marschner 1995).

Two phase model of salinity is another theory suggested by Munns (1995). Base on this model, salt sensitivity or salt tolerance of plants is determined by the rate of toxicity level in the leaves.

During the phase one, both halophyte and glycophyte plants show growth reduction due to the osmotic effect of salt solution around the roots. Salinity decrease the soil osmotic potential and this effect increases immediately after exposure to salt, therefore, makes it difficult for the roots to absorb water from the soil. Ionic stress occurs due to high Na^+ concentrations which affect plants by disrupting protein synthesis and interfering with enzyme activity causing serioius injuries in leaves such as chlorosis and necrosis of older leaves (Munns and Termaat 1986). During this phase, mature leaves of plants die and the photosynthetic rate is severely decreased (Munns and Termaat 1986).

Long term salinity affects shoot growth more than root growth due to preventing leaf and stem development. Shoot growth reduction decrease the water use by plant and provides an opportunity to preserve soil moisture and minimizes salt uptake (Munns and Tester 2008). Na^+ moves in the transpiration stream and accumulates mainly in the leaves rather than in the roots of the plant (Munns 2002). Na^+ accumulation is especially toxic to older leaves since, unlike younger leaves, older leaves are no longer able to expand and dissolve the arriving salt. Therefore, the rate of leaf loss will be greater than the rate of leaf production and plant photosynthetic production will be decreased (Munns and Tester 2008). The reduction of photosynthetic rates in glycophytic plants can also increase the production of reactive oxygen species (ROS). The process of ROS removal by the antioxidant compounds can be fairly impaired by salt stress and lead to ROS accumulation (Foyer and Noctor 2003).

2.1.3. Avoidance and tolerance of salinity stress

Many plants have evolved several mechanisms either to exclude salt from their cells or to tolerate salt presence within the cells. Salt tolerance is more common than salt avoidance in plants and it can differ greatly between plant species or even at the different growth stages (Shannon et al. 1994). Plants have developed different biochemical and physiological mechanisms in order to cope with salt stress. All plants need to regulate cellular Na^+, Cl^- and K^+ concentrations in order to adjust to the external water potential (Amor et al. 2005). General mechanisms involved in salt tolerance include osmotic adjustment, removing toxic ions (Ashraf et al. 2010), accumulation of metabolites, ion homeostasis, and removing activated oxygen species (AOS). In addition, halophytes have developed specific salt tolerance mechanisms that allow them to grow and survive longer in saline environment (Ben Ahemd et al. 2010).

Salt tolerance mechanisms can be also related to genetic traits. A plant species tolerance for salinity will be changed by a sudden exposure to salinity, even if the species is salt tolerant (Gupta and Haung 2014). The mechanisms of genetic control of salt tolerance in plants appear to be highly complex and are not well understood. There are many different genes controlling stress tolerance in the different plant species that can be

affected by different environmental conditions such as salinity. Genetic variation can be determined by measuring the responses of different genotypes to abiotic stresses. For instance, salt tolerance, can be examined by comparing percent biomass production in saline versus non-saline environment at certain period of time (Munns 2002).

Salinity tolerance level can increase or decrease depending on other environmental factors. It can also change at different growth stages. Some plant species have maximum sessitivity to salinity during seed germination (Croser et al. 2001) while other species are more sensitive during the reproductive growth (Marschner et al. 1986).

Osmotic tolerance is among the most common salinity tolerance mechanisms in plants (Munns and Tester 2008). The growth of plants growing under salinity conditions is restricted by the osmotic effect regardless of the capacity to exclude salt. Therefore, similarly to drought conditions, the processes of cell expansion and stomatal conductance can be severely reduced in salt-stressed plants (James et al. 2008).

It is interesting that, although mineral nutrient imbalance has been postulated to be one of the three main factors affecting plant growth under salinity stress conditions (Marschner 1995), controlled-environment studies demonstrated that the availability of plant nutrients does not affect plant response to osmotic stress (Hu et al. 2007).

2.1.4. Ion homeostasis and salt tolerance

Ion fluxes can regulate the concentration of different ions whithin the tissues. During salt stress, ion fluxes can be affected and the ion balance is disrupted (Volkov 2015). Salt Overly Sensitive pathway (SOS) is considered as an important signaling pathway for maintaining ion homeostasis and sodium exclusion at tcellular levl (Ji et al. 2013; Akhter et al. 2003). There are three major protein structure involved in this pathway; SOS1, SOS2, and SOS3. SOS1 responsible for encoding a plasma membrane Na^+/H^+ antiporters, is considered a the most important SOS and plays an essential role in sodium exclusion. Studies demonstrated that overexpression of SOS1 can enhanced salinity tolerance in Arabidopsis (Yang et al. 2009). SOS2 genes encode serine/threonine kinase which are activated by salt stress. SOS 2 seems to be important in regulating ph and salt tolerance in plants (Liu and Zhu 1998). SOS3 is considered as Ca sensing protein

which has no enzymatic activity by itself and can only interact with SOS2 and cause salt tolerance. All together, these protein structures play an importan role in salt tolerance in some plant species such as Arabidopsis (Shi et al. 2003).

All these structure inteact with eachother through phosphorylation processes and activated kinases int certain areas of protein structures. During salinity, High concentration of sodium cause binding between SOS1 and SOS3. SOS2 is activated by SOS3 and this complex is responsible for phosphorylating SOS1 and reducing Na toxicity inside the cells (Matysilk et al, 2002). Recently, SOS4 and SOS5 have also been identified. These structures appear to be important in maintaining cell eaxpansion and cell wall structures during salt stress (Mahajan et al. 2008).

2.2. Water relations

2.2.1. Root water flow

One of the most imortant functions of roots in plants is to provide water from the soil to the whole plant. The processes of water absorption and water movement are completely different from the ion movement, because unlike water transport, ion transport requires active pumping across the plasma membrane into the cytoplasm. In transpiring leaves, water moves passively through the root due to water potential gradient created by transpiration and the rate of water movement is mainly affected by the root anatomy (Steudle et al. 1993). Composite transport model (CTM) is an excellent model to describe the differences in water movement through individual cells as well as through different tissues. This model also describes the roles of apoplastic and non-apoplastic water pathways in the roots (Steudle and Frensch 1996). According to this model, water moves through tissues with different resistance. Therfore, the magnitude of the osmotic and hydrostatic forces predicts whether appoplastic (with low resistance) or cell-to-cell pathway (symplastic or transcellular, with high resistance) is going to be the main water flow pathway. In addition to understanding the limiting factors and resistance involved in radial water flow, it is also important to understand how these pathways should be

changed in order to regulate the the root hydraulic conductivity (Steudle and Peterson 1998). The discovery of aquaporins helped shed more light on the of the cell-to-cell water transport in plants (Maurel 1997).

2.2.2. Water movement in plants

Water moves throughout plants via the soil–plant-air continuum (SPAC), however, this process can be restricted by different driving forces (Tyree and Ewers 1991). Thus, the regulation of root hydraulic conductivity (L_p) (conductance per unit root surface area) has been subject to numerous studies. Environmental conditions such as salt stress or low soil temperature can easily affect the rate of water flow (Sparks and Black 1999). Water movement from the soil to the roots is generally driven by a water potential gradient created by transpiration and the most resistant part of water flow in the plant is thought to be the radial movement of water across roots (Steudle and Peterson 1998). The efficiency of root water uptake can be affected by different factors such as total root surfaces area and number of roots (North and Nobel 1997; Suku et al. 2014).
The properties of water that affect water movement processes in plants are: hydrogen band formation, polarity, and viscosity (Passioura 2010).

Hydrogen bound formation and polarity of water molecules are responsible for the cohesion and adhesion processes that facilitate water movement through the xylem elements. The cohesion – tension theory states that high tensile strength makes it possible for water to move as a column in the xylem and the driving force for water ascent in the plant is generated by surface tension at the evaporating surfaces, mostly in the leaves (Tyree 1997). Leaf transpiration is the main driving force that causes water to move up in the tracheary elemets like a long hydraulic rope (Sack and Holbrook 2006). Water also moves in the phloem according to the pressure - flow theory. Water moves from the photosynthetic sources (mainly leaves) to sinks (mostly non-photosynthetic plant parts) due to an osmotic pressure gradient caused by loading of sugars into the sieve elements (Kramer and Boyer 1995).

2.2.3. Water movement in roots

Water is absorbed from the soil mainly by the youngest parts of the root including root hairs. The absorbed water moves across the root through the cortex and passes through the endodermis, pericycle and finally into the xylem elements. Then, it moves up through the plant (Rieger and Motisi 1990, Steudle and Peterson 1998).

There are three major pathways involved in water movement across the root; apoplastic, symplastic and transmembrane pathways. Since the symplastic and transmembrane pathways are experimentally difficult to separate, they are referred to as the cell-to-cell pathway (Steudle and Peterson 1998). These pathways can function either separately or in combination and cause different water transport rates. Identifying relative contributions of these water movement pathways can be important for understanding environmental constraints to water transport. It also provides an opportunity to manipulate water movement by altering the pathways without any anatomical changes, However, identifying the dominant water pathway does not seem to be easy as water usually follows different radial pathways when influenced by different driving forces (Bramley et al. 2007).

2.2.3.1. Symplastic water pathway

This pathway consists of the network of cytoplasm of all plant cells that are interconnected by specific structures called plasmodesmata. Plasmodesmatas are narrow strands of cytoplasm that interconnect the neighboring protoplasts of the plant cells. In this pathway, water first enters to the plasma membrane and then passes through one cell to another (Stedule and Peterson 1998). Therefore, the ability of plasma membrane water chnnels to change seems crucial in regulating water movement in this pathway (Raven et al. 2005). Osmotic pressure induces water flow through this pathway, thus, the reflection coefficient of the root would have a value of about 1 (Stedule and Peterson 1998).

2.2.3.2. Apoplastic water pathway

In this pathway, water moves through the intercellular spaces and cell walls and water movement does not involve passing through the cell membranes and the protoplasts. This pathway helps with water and ion transport from the soil through the roots and xylem. In plants with secondary growth, apoplast is considered to be the main water transport pathway in the root cortex. Apoplastic water flow can continue through the root cortex until it reaches tthe Casparian bands of the endodermal (or exodermal, if present) cells. These structures consist of deposited hydrophobic suberin and lignin in the cell walls. They are located in the radial and transverse (end) walls of the cells to prevent uncontrolled movement of ions and water through the apoplast (Schreiber et al. 1996). Therefore, the cell-to-cell pathway becomes the dominant pathway for water and ion transport through the cortex. These findings were based on the experiments using dyes which demonstrated apoplastic barriers of water movement in the roots (Peterson et al. 1981). Dyes were deposited in the intercellular spaces and cell walls of the cortex and showed that water movement through the apoplastic pathway was blocked. However, a purely apoplastic path to the xylem was demonstrated along the margins and lateral roots (Peterson et al. 1981). Some evidence also suggests that there is a positive correlation between the number of passage cells in the endodermis and root hydraulic conductivity (Peterson and Enstone 1996).

The apoplastic pathway is considered to be much faster than the cell-to-cell pathway as the apoplast consist of non-living parts which are not influenced by the metabolic state of the root. In the purely apoplastic pathway, cell membranes are not involved and osmotic gradients have little effect on water transport, therefore, the reflection coefficient of the root has a value close to 0 (Bramley et al. 2007).

2.2.3.3. Intercellular (transmembrane) water pathway

Similarly, to symplastic pathway, transmembrane pathway involves water movement between cells. However, unlike the symplastic pathway, in the transmembrane pathway water has to cross the cell membrane either through the lipid bilayer or through

special protein structures referred to as water channels or aquaporins (AQPs) (Steudle and Peterson 1998).

Most mature plant cells contain a large single vacuole and there is a thin layer of cytoplasm between the plasma membrane and vacuolar membrane (tonoplast). Therefore, in some cases the transmembrane pathway as also involves water transport across the tonoplast between the cytoplasm and the vacuole and across the vacuole (Johansson et al. 2000). AQPs in the plasma membrane and the tonoplast, together with ion transporters and osmolytes play an important role in maintaining proper cytosolic osmolarity. Similar to symplastic pathway, osmotic and turgor pressures are primary driving forces that cause water to move through membranes, thus, the reflection coefficient of the root has a value of approximately1(Bramley et al. 2007).

2.2.4. Root anatomy and transport processes

Roots are formed as a series of tissues from the epidermis to the xylem elements with different amounts of resistance (Steudle and Peterson 1998). Therefore, the anatomy of roots is important in determining their physical properties including hydraulic conductivity. The first complication is that root anatomy is highly variable between different plant species or even the same species in different habitats. Root properties can be affected by different amounts of secondary structures and and in the development of various structures such as formation of aerenchyma and endo or exodermis (with Casparian bands, suberin lamellae, and thickened, modified walls), as well as the development of lateral roots. This complexity and variability of root structure makes it difficult to study root systems. Therefore, the results obtained from one experiment cannot always be applied to all roots and in order to understand the process of water transport at the cellular level, the anatomy of studied plants species must be known (Fsicus and Markhart 1979).

2.2.5. Root hydraulic conductivity

Root hydraulic conductivity (L_p) is an important parameter characterizing water transport capacity of the roots. L_p measures water transport per unit surface area (sometimes also volume or weight) and per driving force (Steudle and Peterson 1998). Other parameters of water transport include root water flow rate (*Q*v, volume of water transported over time), Water flux (*J*v, volume of water transported per surface area or volume over time), and root hydraulic conductance (*K*r), volume of water transported over time per unit driving force) (Wan and Zwiazek 1999). L_p has been often correlated with anatomical structures of the root radial path in plants with contrasting root anatomy. L_p was found to be lower in roots that are greater in diameter or they have developed secondary structures such as the suberized exodermis (Gambetta et al. 2013). Therefore, it can be concluded that larger roots and the presence of secondary structures in the cell walls, such as suberin, can decrease L_p. There are also osmotic flows driven by gradients in osmotic pressure, however, these can only happen in the presence of membranes. Therefore, hydraulic conductance can be important when water moves across the cell membranes and osmotic water flow is not as important in the appoplast pathway because of the absence of selectivity between water and solutes which is important in the osmotic flow process. Therefore, the root reflection coefficient is almost zero (Wallach et al. 2010).

2.2.6. Effect of salinity on water relations

The effect of radial water flow on hydraulic circuit can be demonstrated by examining plant responses when the hydraulic driving forces are limited due to environmental stress conditions. For example, during salt stress, high Na^+ concentration, in addition to water deficit, can severely reduce growth rate. High salinity causes ion toxicity in plant tissues and reduces soil water potential which makes water absorption more difficult for the plant (Munns and Tester 2008). Therefore, regulation between ion redistribution and the water flow pathway is important for salt tolerance. Some studies also suggest that there is a correlation between ion distribution specifically Na^+ and K^+

and membrane pathways, however, it is important to know how water pathways resistances interact to improve plant salt tolerance (Peng et al. 2004). For instance, *Beta vulgaris* of the Chenopodiaceaea family is considered to be a halophytic or moderately salt tolerant glycophyte, (Clarke et al. 1993). Under salt stress, this plant is able to control ion and water uptake and shows osmotic adjustment, therefore, water potential decrease caused by the salinity can be overcome by the osmotic regulation processes and the plant will be able to absorb enough water from the saline soil and maintain turgor pressure (Rohatgi et al. 2008).

During the drought stress or any other environmental condition that upsets water balance, the root water pathways can be quickly altered through changes in the aquaporin-mediated water transport and, consequently, L_p (Maurel et al. 2010). In response to salt stress, L_p is reduced through post-translational and transcriptional changes of aquaporins and changes in the root anatomy and suberin deposition (Muries et al. 2011). Abiotic stresses may profoundly affect water permeability of cell membranes in response to changing conditions (Alleva et al. 2006). Also plants grown in different environments show profound differences in membrane water permeability which is often demonstrated by the differences in aquaporin expression and composition such as those found in related plant species that vary in salt tolerance (Skorupa-Kłaput et al. 2015).

2.3. Structure and functions of plant aquaporins

Aquaporins (AQPs) are channel proteins of intercellular and plasma membranes. They belong to the major intrinsic protein (MIP) family with molecular weights of 26–34 kDa (Johansson et al. 2000). AQPs play an important role in controlling of the transport of water and other small solutes and gases in cells. Over 150 MIPs have been identified in different organisms such as bacteria, animals and plants. Therefore, an abundance and existence of aquaporins can show their physiological importance among different organisms (Johansson et al. 2000).

The main role of AQPs in plants is water transport. They have remarkable features to provide an efficient and specific water flow and they transport water into and

out of the cell with different physiological functions. Generally, aquaporins have a highly protected structure among the animals, plants, yeast, and bacteria. In plants, in addition to having different physiological function, AQPs also play an important role in response to abiotic stresses (Tyerman et al. 2002).

All MIPs have at least six nonpolar regions with the N and C-termini facing the cytosol. These structures are considered to be helical regions that are packed together and, as a part of their structure, they also have five loops (A–E) joining the transmembrane helices (Fotiadis et al. 2001). Two conserved loops (loops B and E) are extremely hydrophobic and contain an internal repeat of Asn-Pro-Ala residues that form (NPA) sequence, which is extended into the pore from both sides of the membrane. This motif is the most important feature in all aquaporins (Postaire et al. 2007). Loop C also connects to the loop B and E. This connection is functionally necessary for water permeability. Symmetry has also been observed between the two halves of the protein.

Aquaporin polypeptides usually form homotetramers in the membrane and each monomer forms a single water pore (Chaumont et al. 2005). Through the electrostatic forces, water molecules move toward the center of the channel. Water is also considered to flow across the water channel pore in both directions down its potential gradient (Chrispeels et al. 1994).

2.3.1. Plant aquaporins

AQPs in higher plants are divided into five main homologous subfamilies including Plasma Membrane Intrinsic Proteins (PIPs), Tonoplast Intrinsic Proteins (TIPs), Nodulin 26-like Intrinsic Proteins (NIP), Small basic Intrinsic Proteins (SIPs) and X Intrinsic Proteins (XIPs). Different members of the AQP family have been identified in archaea, eubacteria and eukaryotes, including fungi, animals and plants. AQPs have a very diverse structure in different plants (Weig et al. 1997). The TIP, PIP, NIP and SIP subfamilies have been identified in almost all plants including the moss (bryophyte) *Physcomitrella patens* (Kaldenhoff et al. 2006).

2.3.1.1. Tonoplast Intrinsic Proteins (TIPs)

Although plant vacuoles are generally considered to be a cellular storage compartment, they can have multiple functions. Vacuoles may contain hydrolytic enzymes or store proteins and different secondary metabolic products (Javot et al. 2002). TIPs are considered as the most abundant aquaporins in the tonoplasts. They were the first proteins with aquaporin function that have been identified in the vacuolar membranes of *Arabidopsis thaliana* (Johanson et al. 2001, Gattolin et al. 2010). It is clear that TIPs are responsible for osmoregulation and non-limiting water flow through the membranes. In addition, they can be involved in transporting small solutes and gases and they may link TIPs to important metabolic cycles such as the urea cycle or amino acid synthesis (Kaldenhoff et al. 2006).

2.3.1.2. Nodulin 26-Like Intrinsic Proteins (NIPs)

Plants of the Leguminosae family can be infected by nitrogen-fixing bacteria, especially when there is not enough nitrogen in the soil. This infection can lead them to the formation of nitrogen fixing root structures called nodules. The formation of nodules is the result of symbiotic relationship between the plant and bacteria. In this case, the plant provides the bacteria with reduced carbon to support the energy it requires for the reduction of atmospheric nitrogen to ammonia (Udvardi and Day 1997). During the formation of nodules, different proteins called nodulins are expressed by the plant and are transferred to the symbiosome membrane. Soybean Nodulin 26 (Nod26) is considered to be the major integral protein of symbiosome membrane. These proteins belong to the MIP cluster and form approximately 10% of the total membrane protein Although, Nod26 and other NIP proteins have the same role in the transport of water and other small solutes, compared with other aquaporins family, NIPs have a lower rate of water transport. In addition, NIPs in nonlegume plants are considered to have different physiological functions from Nod 26, which is only expressed in nodules (Fortin et al. 1985).

2.3.1.3. Small basic Intrinsic Protein (SIPs)

SIPs are proteins of the small basic intrinsic subfamily and the smallest in the MIP group in plants. Proteins of this subfamily are very basic. The main reason for their small size is a very short cytosolic N-terminal region compared to the other plant AQPs (Johanson and Gustavsson 2002). The fusions of Arabidopsis SIPs were expressed in suspension cultured cells to localize SIPs and it was shown than most of them are located in the ER, however, a few of them were also identified in the plasma membranes and tonoplasts (Gerbeau et al. 2002). Heterologous expression system in yeast also led to identifying two subgroups of SIPs, including SIP1; 1 and SIP1; 2, as AQPs, however, SIP2; 1 showed only a slow water transport into membrane vesicles (Abascal et al. 2014).

2.3.1.4. Plasma membrane Intrinsic Proteins (PIP)

PIPs are the largest plant AQP subfamily with 13 known members in Arabidopsis and 14 in maize (Chaumont et al. 2000). The majority of PIPs have been identified in the plasma membrane. Phylogenetic analysis also classified PIPs into two subgroups, PIP1 and PIP2. The difference between these two groups is the length of the N- and C-termini as well as their water permeability. In some plants, another PIP subfamily called PIP3 has been reported (Schuurmans et al. 2003).

2.3.2. Responses of AQPs to environmental stresses

Numerous studies have confirmed that the membrane abundance of AQPs can be regulated by different environmental factors including abiotic stresses. Plant water relations can be disrupted by drought or high salinity. Therefore, plants must develop various adaptive responses to handle the environmental stresses. Preserving water balance under harsh environmental conditions can be a difficult and crucial challenge for plants (Mahdieh et al. 2008).

In some glycophytes, very low concentrations of NaCl inhibit AQP-mediated water transport within several minutes following salt application (Lee et al. 2015). The effect of salt on transport through AQPs leads to the reduction of root hydraulic

conductivity (L_p) and, subsequently, stomatal conductance, photosynthesis, and growth (Lee et al. 2010).

During abiotic stress, certain AQP isoforms are expressed only in specific tissues. Over-expression of AQP genes has been a great help to understand plant water relations under stress conditions. Molecular analysis of regulation of the whole AQP family have often revealed complex transcriptional and posttranslational responses, with sometimes opposite patterns between isoforms. However, some studies indicate that the abundance of AQP transcripts and the encoded proteins are not necessarily correlated. For instance, the number of mRNAs encoding some PIP2 isoforms was temporarily increased in response to an osmotic stress while the transcripts for several TIP isoforms were continually expressed, but the protein levels of all these isoforms remained constant, suggesting the occurrence of post-transcriptional regulations for PIPs (Maurel 2007).

Numerous studies have confirmed that increasing AQP expression in transgenic plants can induce higher resistance to stresses, however, in some cases, negative effects on stress resistance have been observed when an AQP was over-expressed in a heterologous plant species (Sade et al. 2009).

2.4. Halophytes

Halophytes are plants that can tolerate high salt concentrations. These plants, which form about 1% of the world flora, can survive and reproduce in the environments where salt concentration is higher than 200 mM (Flowers et al. 2015). Unlike halophytes, glycophyte plants are sensitive to salt and cannot tolerate salinity (Munns and Termaat 1986). All glycophytes, as well as many halophytic plants, grow normally in the absence of salt and only extreme halophytes are able to grow at higher concentration of salt, however, their growth rate can also be affected by high salinity (Flowers et al. 1977).

Halophytes are defined differently by different authors. Scholander (1962) defines halophytes as plants that can grow normally either in salt habitats or in ordinary soil. Stocker (Stocker 1928) describes halophytes as plants that can tolerate salt concentrations of over 0.5% at any period of their life. More simply, Dansereau (1957) believed that

halophytes are plants that can grow solely in saline soils. Without doubt, these definitions can not be very complete since the levels of salt tolerance in different species are not the same and usually, there is a continuous range from the least to the most salt-tolerant species. Studies show that some non-halophyte plants such as sugar beet can also survive and complete their life cycle in saline habitats. Halophytes are also sometimes called euhalophytes because some of them can increase their productivity by increasing the concentration of salt and even grow better under salinity conditions than under fresh water conditions (Khan et al. 2000).

Clearly, halophytes should have special morphological and anatomical adaptations as well as different physiological processes that enable them to cope with saline environments. Halophytes can also improve the saline soils and can be used for phytoremediation of the saline areas, but also to provide food, fuel, wood, and industrial raw materials (Melcher et al. 2001).

2.4.1. Classification of halophytes

Based on various factors, there are different classifications for halophytes. Chapman (1942) classified halophytes into two different groups (i) Miohalophytes (plants that grow in the habitats of low salinity (below 0.5% NaCl) and (ii) Euhalophytes (plants that can grow in habitat with high concentration of salt. However, one of the most widely accepted classifications of halophytes is based on the salt demand level and ecological aspects. In this classification, halophytes are divided into (i) obligate, (ii) facultative, and (iii) habitat-indifferent categories (Hasegawa et al. 2000).

Obligate halophytes can grow only in salty habitats and they need salt in order to survive. They usually have the optimum growth and development in high concentrations of salt (more than 200 mM). Most plants belong to Chenopodiaceae family are considered as obligate halophytes. A facultative halophyte is a plant which can grow in saline areas, but prefer to avoid salt (Wang et al. 2004). In other words, facultative halophytes have optimum growth and development at moderate salinity and their growth will be reduced at both low and high concentrations of salt (Rao et al.2004). Most of the Poaceae, Cyperaceae, and Brassicaceae species belong to this group. Intolerant

halophytes (habitat indifferent halophytes) are plants that normally grow better in salt-free soils, but they are still able to cope with low to moderate concentrations of salt and compete with the species that are sensitive to salt. *Chenopodium glaucum, Myosurus minimus,* and *Potentilla anserina* are examples of this group of halophytes (Hasegawa et al. 2000)

2.4.2. Effects of salinity on halophytic plants

Although halophytes are considered to be salt-tolerant plants, salinity can still negetively impact their growth and survival. Many functional proteins such as those involved in signaling and signal transduction, ion transport, as well as energy metabolism can be easily affected by salt stress. In addition, salinity can have a negative effect on all major processes such as photosynthesis, protein synthesis, energy and lipid metabolism, and water relations. Seed germination and young seedling growth, which is sensitive to salt in glycophytes (Croser et al. 2001) are also sensitive to salt in halophytes (Flower et al. 1977). Therefore, it seems vital for both halophytes and glycophytes to evolve different salt tolerance mechanisms at different stages of development (Medina et al. 1997).

2.4. 3. Salt tolerance mechanisms in halophytes

In addition to general salt tolerance mechanisms that are found in most plants, halophytes have developed some specific salt tolerance mechanisms that allow them to survive in the high salinity environments. The important factor in salt tolerance of halophytes is the ability to control the uptake of Na^+ and Cl^- and maintain cytoplasmic K^+ and Mg^{2+} concentrations at a certain level required for the activation of essential enzyme activities (Melcher et al. 2001). The distribution, exploitation and physiology of salt tolerance of halophytes have been thoroughly studied.

Halophytes are able to control Na^+ uptake into cell vacuoles in order to drive water into the plant against a low external water potential. The process of Na^+ and Cl^- entry into the halophyte cells are not very well understood. However, it is still widely

accepted that salt tolerance in halophytes is controlled by other processes such as osmotic adjustment, succulence, selective transport and uptake of ions, enzyme responses, and salt excretion (Lee et al. 2008). According to the classification proposed by Walter (1961), halophytes have three specific salt tolerance mechanisms including (i) salt exclusion, (ii) salt excretion, and (iii) salt accumulation.

2.4.3.1. Salt exclusion

In most of plants growing under salinity conditions, Na^+ seems to reach toxic level before Cl^- does. For this reason, most studies mainly focused on Na^+ exclusion and Na^+ transport regulation within plants (Munns and Tester 2008). Both glycophyte and halophyte plants are not able to tolerate high concentration of salt in their cytoplasm. Therefore, the extra salt must be excluded from the cytoplasm to vacuole or older tissues in order to protect the plant from salt stress. This mechanism is considered to be essential for reducing the ionic stress in salt-stressed plants, especially in transpiring leaves. This process is also involved in down-regulation of the expression of certain ion channel and transporter genes in cells or tissues which controls the transport of Na^+ throughout the plant (Davenport et al. 2005).

Na^+ exclusion is an essential salt tolerance mechanism also in glycophytes including cereal crops such as rice, durum wheat, bread wheat, and barley.Exclusion of Na^+ from the leaves is due to low net Na^+ uptake by cells in the root cortex and the tight control of net loading of the xylem by parenchyma cells in the stele (Davenport et al. 2005, Munns and Tester 2008).

Vacuolar Na^+/H^+ antiporters play an important role in removing Na^+ and from cytosol and transporting it to the large vacuoles. Inside the vacuoles, these ions act as an osmoticum that can sustain water flow into the cell. There are two different types of H^+ pumps in the vacuolar membrane: Vacuolar type H^+-ATPase (V-ATPase) and the vacuolar pyrophosphatase. V-PPase is considered to be the most dominant H^+ pump in plant cells and plays an important role during non-stress conditions, however, during stress conditions, H^+- ATPase (V-ATPase) seems to be more more important for plant survival (Woodruff et al. 2007).

Salt exclusion in plants under salt stress mostly occurs in the root cortex with a very tight control of xylem loading by parenchyma cells in the stele (Saqib et al. 2005). The root system of some halophyte plants that use salt exclusion as a salt tolerance mechanism uses cell membranes to filter out salt. Root cell membranes prevent salt from entering root cells, but allow the water to pass through. This is the main salt tolerance mechanism in species such as *Rhizophora mucronata*, *Ceriops candolleana*, *Bruguiera gymnorrhiza*, and *Kandelia candel* (Sivritepe et al. 2003).

Salt exclusion is regarded as a very effective salt tolerance mechanism. Although the process of reducing the ion uptake appears to be very complex, some halophyte plants are equipped with well-developed transport system that can help them reduce the uptake and accumulation of salt in the upper part of the plant, especially in the transpiring leaves (Reef and Lovelock 2014).

Here are many studies suggesting that also in some glycophytes including rice, wheat, and barley, Na^+ exclusion is used as a mechanism of salt tolerance. However, the ability of salt exclusion is determined by several factors such as selectivity of uptake in root cells, or absorbing more K^+ rather than Na^+ by the stele cells into the xylem, therefore, removing the salt from the xylem (Munns and Tester 2008). The capacity of plants to sense Na^+ can be determined either by exteracellular factors such as membrane receptor or by intercellular factors such as membrane proteins or Na^+-sensitive enzymes in the cytoplasm (Hasegawa 2013).

2.4.3.2 Salt excretion

Salt excretion is one of the most important and efficient physiological adaptations to salt in halophytes. This mechanism enables halophytes to prevent excessive concentration of salt and adjust internal salt concentration through foliar glands (Yuan et al. 2016). Salt secretion in many halophyte plants is regarded as an important process for maintaining water balance. Studies show that salt crystals that are present on the surface of the leaves may help with the absorption of water from the air, which causes attracting water to the leaf surface by lowering the dew point, therefore facilitate liquid penetration into stomata by making the leaf surface less hydrophobic and reducing the water surface

tension (Carillo et al. 2011). However, not all plants have developed salt secretion mechanism and in non-salt secreting species, salt can accumulate in leaves.

Many halophytic plants such as *Aeluropus* (Poaceae), *Atriplex* (Chenopodiaceae), *Armeria* (Plumbaginaceae), form special salt glands and salt hairs. These structures are formed from a group of epidermal cells which are responsible for removing salt from the mesophyl cells below them. Mesophyll cells are connected by the plasmodesmata, so salt will be transferred to the leaf surface where the salt crystal layer is formed (Yuan et al. 2016).

2.4.3.3. Salt accumulation

Accumulation of salt and compatible solutes is another basic process for the protection and survival of halophytes under salinity conditions (Ashraf et al. 2010). Generally, soluble compounds such as carbohydrates play an important role in protecting plants under salinity stress through osmotic adjustment, detoxification of ROS, protection of membrane integrity, as well as protection of enzyme and other functional proteins. Many studies also proved that in halophytes, the leaf tissues are able to accumulate large amounts of salt ions in the vacuoles. This is the most common salt tolerance mechanisms in halophytes and it seems to be very important for creating a water potential gradient along root-shoot, maintaining low Na^+ concentration in cytoplasm and maintaining water movement throughout the plant (Saxena et al. 2013).

2.4.4 Salinity stress and water relations in halophyte plants

Plants growing in saline habitats are faced with big physiological challenges due to extremely negative water potentials in the pore water of the saline soil, causing water uptake more difficult than in non-saline soils. The ability to maintain water uptake is an important element of salt tolerance. Ion toxicity is one of the major physiological problems in plants under salinity stress. The high water uptake regulation in some plants under salinity stress suggests that the regulation of water uptake along with managing ion

transport is considered as an important factor in their salinity tolerance (Melcher et al. 2001).

Under salinity stress, halophytes have two main problems. First, they need to tolerate direct toxicity effects of salt, and secondly, they need to absorb water from soil solution with low water potential. To maintain water uptake, halophytes need to maintain water potential that is more negative than in the soil solution. This could be achieved by an accumulation of inorganic ions that can be easily taken up from the soil (Kosova et al. 2013). In addition, halophytes have evolved different adaptations such as maintaining low water potential and water saving capacity in order to maintain root water uptake from high salinity soils (Casas et al. 1991).

2.4.4.1. Water potential maintenance

Under salinity stress, halophytes, just like all other plants, need to maintain water potential gradient in order to absorb water through their root system. In addition, if water potential of the soil is lower than water potential in plant, water may be lost from the roots to the soil. In this case dephosphorylation of root aquaporins can reduce root hydraulic conductivity and minimize water loss (Ishitani et al. 2000). Moreover, water uptake by halophytes, strongly depends on cell-to-cell pathway, which helps regulate water loss during the period of salinity conditions. Halophytes have developed different adaptations to maintain low root water potential and to function under water deficit stress conditions. Some of these adaptations include accumulation of organic and inorganic osmolytes and cavitation-resistant hydraulic anatomy (Ford et al. 2007).

2.4.4.2. Accumulation of organic and inorganic osmolytes

Organic and inorganic osmolytes play a crucial role in regulating and maintaining water uptake under salinity conditions and in regulating ionic charges in the cytoplasm. Studies reported that halophytes can utilize ions as solutes to reduce water potential in cells (Schroeder et al. 2013). It was observed that increasing soil salinity increases the concentration of solutes in the roots, leaves and stems (Schroeder et al. 2013). However,

many studies have demonstrated that the concentration of Na^+ inside the cells is different than that in the soil and varies with different environmental factors (Melcher et al. 2001).

In plants exposed to high salt concentrations, the contribution of ions to osmotic potential of the shoots is not sufficient to create favorable water potential gradient with the soil. Therefore, it is obvious that under these conditions, other factors are required to reduce water potential. Organic compounds such as mannitol, proline, glycinebetaine and triterpenoids play an important role in balancing osmotic potential in cytoplasm and help maintain enzyme activity under salinity condition. Since the cytoplasm constitutes about 10% of the cell volume, the presence of inorganic ions can be considered as the main factor in many plants that is responsible for a decrease in leaf water potentials of plnts exposed to salt stress (Marcum and Murdoch. 1992).

2.4.4.3. Water saving adaptations in halophytes

Since water uptake indirectly requires a lot of energy in plants growing in saline habitats, some halophytic plants have developed a different kind of adaptation that enables them to use water efficiently during the photosynthesis in the daytime and decreases water loss to the soil at night. Halophytes also have evolved other kinds of adaptations such as leaf temperature regulation and using non stomatal drive CO_2 for efficient use of water under salinity conditions (Flower and Colmer 2015).

Many halophytic plants have developed different anatomical characteristics to reduce water loss. One of the adaptations is the position of stomata which are located in some halophytes on the abaxial leaf surface inside the crypts. This adaptation helps plants to reduce transpiration while not affecting CO_2 uptake, by significantly increasing the humidity and reducing the leaf-to-air vapor pressure deficit around the stomatal pore (Glenn et al. 1997).

Another factor for saving water in plants under salinity stress is the position of leaves. Studies have shown that in some plants, leaves are held in a vertical position when they are exposed to full sunlight which is similar to a projected area and can be as low as 10% of that in the horizontal leaves (Kosova et al. 2013). In addition to leaf anatomical features, the leaf angle of sun-exposed leaf can also be an important factor

that affects the distribution of species under salinity stress. A lower leaf display area results in a reduction of direct radiation, which allows the leaf to remain at a temperature that is suitable for photosynthesis with requiring minimal evaporative cooling (Hamdy1996).

Leaf size is another important water-saving factor in halophytes. Plants exposed to high concentration of salt usually have smaller leaves in order to maintain the leaf temperature more effectively and keep the photosynthesis rate as high as possible. However, many studies have shown a negative correlation between leaf size and leaf sap osmolarity. In some plants, leaves have a high water content per unit area (salt succulence), which increases with salinity. The high water content can increase leaf heat capacity; therefore, the need for evaporative cooling is reduced (Melcher et al 2001). One of the water saving adaptations is supplying CO_2 for photosynthesis from the non leaf tissues which reduces lower water cost (Liu et al. 2000).

The ability of some halophytes to tolerate high concentration of salt can also be related to their ability to acquire or utilize less saline water sources trough the rhizosphere. For example, in plants growing in foggy coastal areas, fog can supply about 40% of the leaf water content, leading to the reverse movement from the leaf to the root (Hasanuzzaman et al. 2013).

Leaf cuticle structure can also play an important role in reducing water loss by creating hydrophobic barrier on leaf surfaces. It has been reported that cuticle thickness can be significantly increased in plants under salinity conditions (Bi et al. 2017).

2.5. Biology of studied plant species

2.5.1. *Poa pratensis*

Kentucky bluegrass (*Poa pratensis* L.) is the most important cold season grass which is planted as turf. Kentucky bluegrass is a sod forming rhizomatous (C3) perennial grass and the height of plants can vary from 7-90 cm. This grass has culms that are slender and flattened. Leaf blades are flat or folded with prow shape on tips similar to

most other *Poa* species. The sheath is rounded or keel-shaped (Bushman et al. 2016). This plant has a perennial root system and the maximum root growth happens one or two years after planting. Old roots usually stay alive while new roots grow during spring (Bushman et al. 2016).

Kentucky bluegrass is is very different in term of chromosome numbers and it can range from 2n = 28 to 154. Kentucky bluegrass can be reproduced from both seed and rhizomes. It does hybridize, but generally produces seed apomictically. Apomixis is asexual reproduction that seed forms from cells in the ovary wall of the flowers, therefore, the progeny are similar to the parent plant (Niu et al. 2017).

Generally, Kentucky grass is considered to be a stress-tolerant glycophytic plant and it made sod production possible especially in northern climate due to its resistance (Kanneganti and Kaffka 1995). These plants are very well adapted to different climates, also those with cold winters and short growing seasons (Kanneganti and Kaffka 1995). Seed germination happens during fall and all harvested seesd need a cold treatment about 5° to 15°C for 10-14 days in order to germinate (Duell 1985). Kentucky bluegrass is mostly found in meadows, open woodlands, and prairies outside of Alaska and also in disturbed sites throughout the world. In the western parts of the U.S.A, this plant mainly grows as understory dominant species with other plants such as aspen, pine and sagebrush (Uchytil 1993).

Kentachy bluegrass is considered to be relatively tolerant, however, when it comes to salinity stress, this plant is considered to be salt sensitive. Additionally, during the summer, high temperature can enhace the effect of salinity stress. However, certain cultivars can perform better under salt stress than others (Harivandi et al. 1992). This plant does not grow in pure sands unless is it used as a turf grass. Its rhizomatous habits let it to grow and penetrate between other plants (Dernoeden 1998).

2.5.2. *Poa juncifolia* (alkali bluegrass)

Poa juncilfolia with common name alkali bluegrass belongs to the Poaceae family. This grass usually grows during spring and become dormant during summer. This

perennial grass is considered to be native to California, but it is widely distributed and also grows in western North America. The leaves of this grass have general bluegrass characteristics and have prow-shaped tips that are folded, or rounded. The seeds are usually soft except the short crisp hairs on the lower part of the lemmas. The flowers are in a small panicle and seeds spread during anthesis and can contain more than 100 spikelets (Halvorson 2011).

The plants are usually short. Similarly, to other bluegrasses, *Poa juncifolia* has a very deep, complex and coarse fibrous root system that enables them to be resistant to grazing and trampling and tolerate harsh environmental condition, especially drought conditions (Arnow 1981).

2.5.3. *Puccenellia nuttalliana*

Puccenellia nuttalianna is a member of the Poacae family (subfamily: Poideae and tribe Poeae (Davis 1983) with the common names such as Nuttall's alkali grass or Nuttall's salt meadow. *Puccinellia nuttalliana* is also a perennial grass with different sizes that range from 25 to 80 cm. The leaf is about 1-15-cm tall with 0.8 – 2.60 mm width (Tarasoff et al. 2007). The inflorescence part is an open structure of about 7-cm in length and consists of a few thin branches. There are usually two to seven florets per spikelet and the spikelet is often covered by hairs. *Puccinellia nuttalliana* is known as a salt-tolerant and cool-season grass specie and is native to North America (Tarasoff et al. 2007). Because of its stress resistance, this plant is highly distributed in the areas where the soil pH is very high or in the areas poor soil conditions. This plant can grow along the road sides due to its salt tolerance (Huff et al. 2003).

There is still no agreement about the classification of *Puccinellia* species since these plants show a lot of differences in the floral parts that makes some different morphological characteristics similar to other member of the Poaceae family (Hitchcock et al. 1969).

Similarly, to the most of the caespitoes species, these plants are considered self-incompatible and cross-fertilizing that experience high level of introgression or hybridization (Davis and Manos 1991). Therefore, the high level of heterozygosity is

expected because of the hybridization. Genetic studies of *P. nuttaliana* populations has also demonstrated high level of heterogeneity of populations in this species (Liu et al. 2000). Similar genotypes in plants belonging to different populations suggest that there should be a high level of gene introgression among the population that can occur through the pollen or seed spread (Consaul et al. 2008).

More than half of the population of *Puccinellia* are polyploid (Davis and Consaul 2007). However, some researches suggest that *P. nuttalianna* is mainly an octoploid species (Davis and Manos 1991). The use of *P. nuttalliana* is presently limited in western Canada due to the limited availability of adapted seed sources.

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3 Materials and methods

3.1. Seed sterilization and germination

Several repeated experiments were conducted in the controlled-environment growth rooms at the University of Alberta, Edmonton, Canada. Plant species used in this study were relatively salt-sensitive (*Poa pratensis*) and salt-tolerant (*Poa juncifolia* and *Puccinellia nuttalliana*) grasses as demonstarted in an earlier study (Liu et al. 2009). The data shown in this thesis are from the last experiment that was carried out to take a whole set of measurements.

Seeds of *Poa pratensis*, *Puccinellia nuttalliana*, and *Poa juncifolia* were collected in 2006 at Vegreville, AB, Canada, and provided by the Alberta Innovates -Technology Futures.

The seeds were sterilized in 70% ethanol for 2 min followed by 5% commercial sodium hypochlorite bleach for 5 min. Then, the seeds were washed 6 times (each time for one minute) by autoclaved distilled water.

Sterilized seeds were transfered onto plates containing half strength Murashige & Skoog (MS) medium (Murashige and Skoog 1962) with no added sugar or hormones (Diet-MS) (Grant et al. 2017). The media were autoclaved at 121°C and 103 kPa for 25 min before being poured into 9-cm in diameter Petri dishes. The dishes with seeds were sealed with parafilm and placed at room temperature of about 25°C (day/night) under natural light. Seed germination was monitored daily for two weeks and germination rate was determined by the number of seeds with an emerging radicle divided by the total number of seeds on the plate.

Most of the seeds germinated about 10 days after placing them in the Petri dishes. The germinants were transferred into plastic pots (17-cm in diameter, 20 cm height with four holes at the bottom for drainage) filled with about 5 kg of horticultural soil .The growth room was set at 22/18^{o}C (day/night) temperature, 65 ± 10% relative humidity (RH), and 16-h photoperiod with 300 ($\mu mol.m^{-1}.s^{-1}$) photosynthetic photon flux density

(PPFD) at the top of the seedlings provided by the full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada) for about 8 weeks. Plants were fertilized once a week with half strength modified Hoagland's solution (Hoagland and Arnon 1950) and were watered twice weekly to runoff.

3.2. Experimental set-up

After 8 weeks, roots of seedlings were washed and placed in aerated mineral solution culture containing 50% Hoagland's solution in a controlled-environment growth room. Environmental conditions in the growth room were maintained at 22/18^{o}C (day/night) temperature, 65±10% RH, and 16-h photoperiod with 300 μmol m^{-2} s^{-1} PPFD at the top of the seedlings provided by the full-spectrum Philips fluorescent bulbs.

The solution culture set-up consisted of twelve 11 L plastic containers with Styrofoam lids. Into each lid, 9 x 3.8 cm holes were cut, so that seedling roots could be slipped into the nutrient solution through the lid. There were 3 seedlings per species in each container for a total of 9 plants per treatment. Foam plugs were fitted around the stems, and inserted into the holes to hold the stems in place while the roots were immersed in solution, with the stems protruding through the lids. All tubs were filled with 50% modified Hoagland’s solution. The containesr were connected to two air pumps to provide aeration.

3.3. Treatments

After one-week of acclimation to hydroponic conditions, three NaCl treatments were applied to each container. Control included only 50% Hoagland's solution and salt stress was induced with 50, 150 and 300 mM NaCl for 10 days. The measurements were carried out after 3, 6, and 9 days of treatments.

3.4 Measurements

3.4.1. Net photosynthesis (Pn) and transpiration (E) rates

Net photosynthesis (A) and transpiration rate (E) were measured with the aid of infrared gas analyzer (LCA- 4, Analytical Development Company Ltd., Hertfordshire, UK) with an auxiliary LED bulb (400 μmol m^{-2} s^{-1} PPFD) as previously described (Nguyen et al. 2006).

Six seedling of each species (n = 6) were randomly taken from each treatment for the measurements of Pn and E. For the measurements, three fully expanded leaves were inserted in the leaf chamber of the infrared gas analyzer. Reference CO_2 concentration was 400 μmoL and the flow rate was 250 μmoL s^{-1} in the leaf chamber. The measurements were carried out three, six and nine days after treatment application. To determine leaf areas, the leaves that were inserted in the leaf chamber were marked, excised after each measurement with scissors and scanned. The leaf areas were calculated following scanning using the Sigmascan Pro 5.0 computer software (Systat Software, San Jose, CA, USA).

3.4.2. Shoot Water Potential Measurements

Shoot water potentials were measured nine days after NaCl application in the three species. Shoot water potential (ψ_w) measurements were conducted using a Scholander-type pressure chamber (PMS instruments, Corvallis, OR, USA) in the leaf as previously described (Wan et al. 1999). A fully expanded leaf located in the middle of stem was detached and sealed in the pressure chamber with the end of the stem protruding through the chamber. Chamber pressure was increased to the point when xylem sap was released from the excised stem and balance pressure was recorded. The leaf water potential measured by this method is an average of the total potential throughout the whole shoot (Reviewed by Tunner 1988). In order to meet the assumption of ANOVA, data were transformed with a log10 function.

3.4.3. Measurements of fresh weights, dry weights and water content

Water content (WC) of leaves of control and NaCl- treated plants was measured following the method of Barrs and Weatherley (1962). Leaf fresh weight (FW) was recorded immediately after sampling. Leaves were then placed in the oven at 70°C for 72 h. Then, leaf dry weight (DW) was determined. The leaf water content (WC) was calculated using following equation:

$$\mathbf{WC\ (\%) = (FW- DW) / FW) \times 100}$$

3.4.4. Shoot and root dry weights

Shoot and root dry weights were determined for all seedlings from the each NaCl treatment and control (n = 9). Ten days after salt application, plants were harvested. Then, the separated shoots and roots were transferred and placed in the oven at 70^{o} C for 72 h. Nine plants were taken for each treatment.

3.4.5. Cell hydraulic conductivity

Water relation parameters such as half-time of water exchange ($T_{1/2}$), turgor pressure (Pt), and cell elasticity (ε) of individual cells were measured using the cell pressure probe (CPP) technique. To make the cell pressure probes, microcapillary were pulled to a fine point using a pipette puller (Kopf Vertical puller, Model 72, Tujunga, California, USA) and subsequently ground to openings ranging from 8-10 µm. The microcapillary was filled with silicone oil (Type AS4, Wacker, Munchen, Germany). A distal root segment was attached to a metal sledge, covered with paper towel and bathed in half-strength Hogland's solution (Epstein 1972).

The probe was inserted 20 mm from the root apex into the third to fifth cortical layer in eight to ten weeks old plants. The distance of exposed microcapillary was subtracted from the total distance of the previously marked reference point on the

microcapillary to determine to what depth the capillary was inserted into the root. Once the cell is punched, a meniscus was appeared in the capillary and the meniscus position could be adjusted through pressurization and depressurization of the probe. As a result, the hydraulic parameters of the cells could be measured as previously described (Zimmermann et al. 2000).

Turgor pressure was recorded after eight minutes once it became stable. Root cross sections were taken 20 mm from the root apex so that mean dimensions of each cortical layer could be determined.

Osmotic pressure of the cell was determined from the relationship $\pi = P_t + \pi^0$ where the π^0 is equal to the external osmotic pressure of the growth solution (Azaizeh et al. 1992). Since π^0 acquiring small values ranging between 0.02 and 0.04 MPa (Lee et al. 2010), it can be assumed that $P_t = \pi$. Cell elastic modulus was estimated from V and changes in V caused by pressurization of the probe, so that:

$$\varepsilon = V * \Delta P_t / \Delta V$$

Four to six plants per treatment were used for all measurements and eight week old plants were used for the measurements.

3.4.6. Tissue elemental analyses

Elemental analyses were carried out on 25mg washed and oven-dried root and shoot tissues. Leaf and roots were milled to powder. Na, K, Mg, P, Ca and Fe concentration in shoots and roots of individulat plants were determined in the Natural Resources Analytical of the University of Alberta, Edmonton, Alberta, Canada. Cation concentrations were measured using the Inductivley Coupled Plasma – Optical Emission Specterometry (iCap 6000, Thermo Fisher Scientific Inc, Waltham, MA, USA) (Calvo-Polanco et al. 2009).

3.5. Statistical analysis

Statistical analysis of the data was carried out using the R software for statistical analysis (version 3.2.4, R Development Core Team, Vienna, VA, Austria). A one-way ANOVA was used with species and NaCl level as the main factors. The data that did not meet the ANOVA assumptions of normality of distribution and homogeneity of variance were transformed with a log10 function. Comparisons between different treatment means were conducted by the Tukey test (paired t-test for L_p data for short-term responses to NaCl) using the Sigma Plot v13.0 statistical software (Systat Software Inc., Chicago, IL, USA).

4 Results

4.1. Growth rate and plant biomass

The morphology of the shoots and roots of *Poa pratensis*, *Puccinellia nuttalliana*, and *Poa juncifolia* plants before and after 10 days of NaCl treatments are shown in Figs. 4.1 and Fig. 4.2. *P. juncifolia* plants of the same age as the other two species were visibly smaller compared with the other plants both before (Fig. 4.1) and after NaCl treatments (Fig. 4.2). When subjected to NaCl treatments for 10 days, *P. pratensis* showed a clear decline in growth and leaf necrosis, while *P. juncifolia* did not exhibit visible signs of injury in 50 mM NaCl and 150 mM NaCl treatments (Fig. 4.2). *P. nuttalliana* did not appear to suffer from injury when subjected to NaCl concentrations as high as 300 mM (Fig. 4.2).

Figure 4.1. Experimental plants before NaCl treatments.

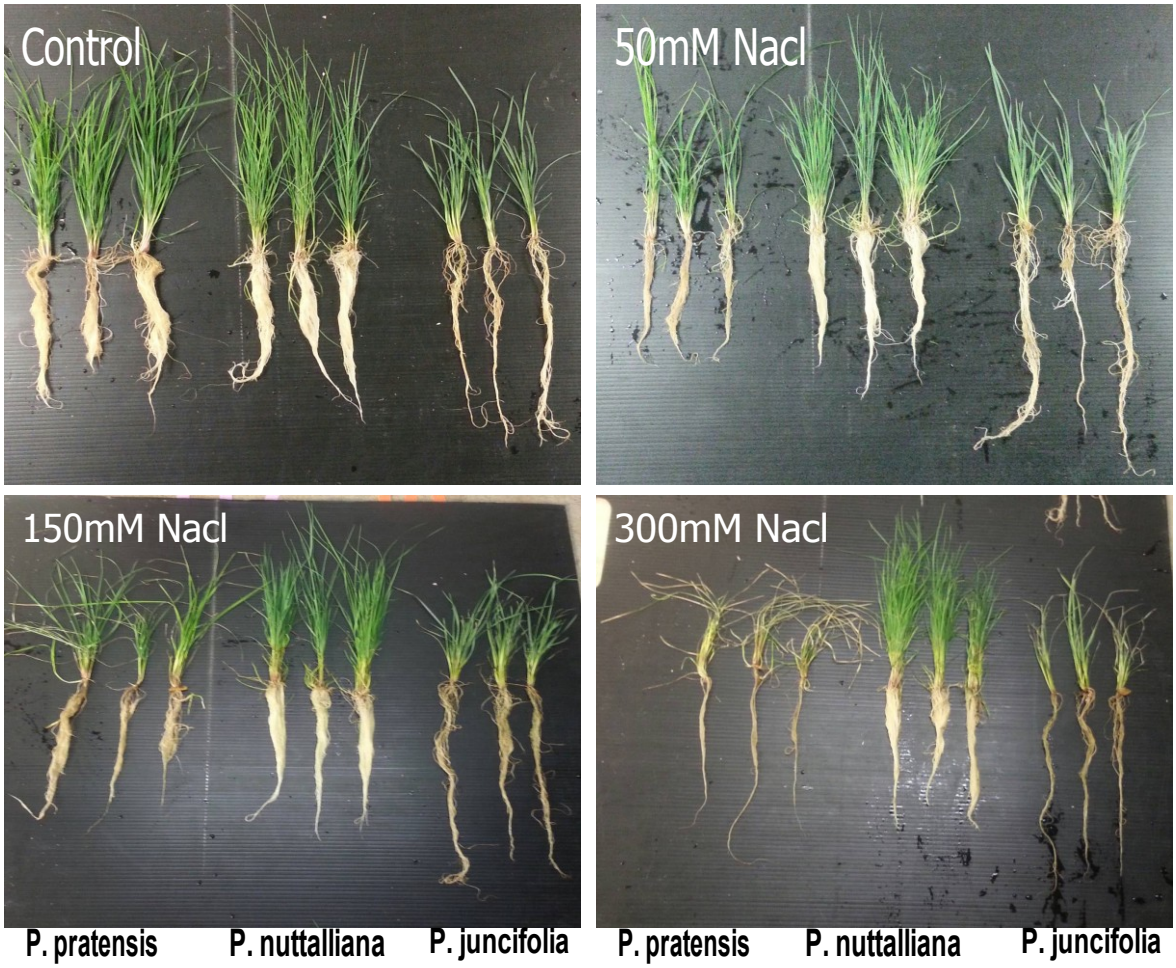


Figure 4.2. Experimental plants after 10 days of treatments with different NaCl concentrations.

P. pratensis had higher dry shoot and root weights compared with the plants of the other two species (Fig. 4.3A, B). After 10 days of 50 mM treatments, root, shoot and total dry weights of *Poa pratensis* were about two-fold lower compared with untreated control and the dry weights declined with increasing NaCl treatment concentration (Fig. 4.3 A , B , C). In *P. nuttalliana*, root dry weights were higher in all NaCl treatments and those of *P. juncifolia* were higher in the 50 mM NaCl treatment compared with control (Fig. 4.3A). Shoot dry weights were not affected by the NaCl treatments in *P. juncifolia*, but were higher in the 50 mM and 150 mM NaCl treatments compared with control (Fig. 4.3B). The total dry weights were higher in *P. juncifolia* treated with 50 mM and in *P. nuttalliana* in all NaCl treatments compared with control plants (Fig. 4.3C). Since root dry weights in *P. pratensis* were affected somewhat more than shoot dry weights, the shoot to root ratios in these plants showed an increasing trend with increasing NaCl treatment concentrations (Fig. 4.3D). The opposite was observed in *P. nuttalliana* (Fig. 4.3D).

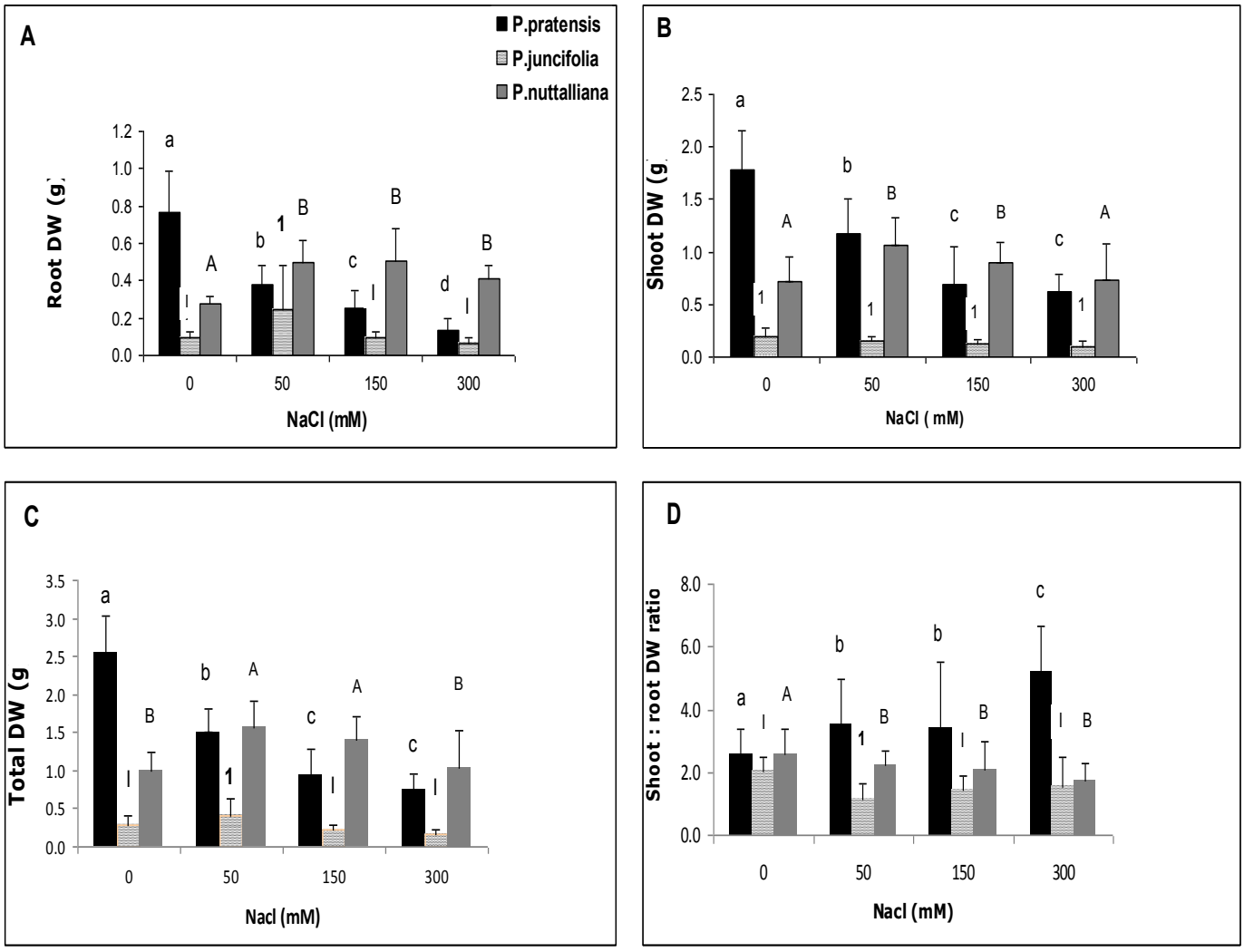


Figure 4.3. Root dry weights (g)**(A)**, shoot dry weights (g) (B), total dry weights (C) and shoot to root ratios (g)(D) in *Poa pratensis*, *Poa juncifolia* and *Puccinellia nuttalliana* treated for 10 days with different concentrations of NaCl. Different letters or numbers above the bars indicate significant differences ($p \leq 0.05$) between treatments within each plant species as determined by the Tukey's test. Means ($n = 6$) ± SE are shown.

4.2. Net photosynthesis and transpiration

Net photosynthetic rates (Pn) in *P. pratensis* significantly decreased with increasing NaCl concentrations 3 days after the treatments (Fig. 4.4A). A decrease in Pn was also observed after 3 days of treatments in *P. juncifolia* treated with 150 and 300 mM NaCl, however, none of the applied NaCl treatments had an effect on Pn in *P. nuttalliana* (Fig. 4.4A). Similar effects of NaCl treatments as those after 3 days of treatments were observed in plants treated with NaCl for 6 days with the exception of the 50 mM NaCl treatment which had no effect on Pn in *P. pratensis* and resulted in higher Pn values in *P. juncifolia* compared with control (Fig. 4.4B). The increase in Pn by the 50 mM NaCl treatment in *P. juncifolia* was not present after 9 days of treatment and a small decrease of Pn compared with control was observed in *P. nuttalliana* treated with 300 mM NaCl (Fig. 4.4 C).

Transpiration rates (E) showed a similar trend to Pn in response to NaCl treatments (Fig. 4.5A, B,C). After 3 days of treatments, most pronounced decreases in E were observed in *P. pratensis* and *P. juncifolia* treated with 150 and 300 mM NaCl and only the highest; 300 mM NaCl treatment resulted in a decrease of E in *P. nuttalliana* (Fig. 4.5A). Similar trends were observed after 6 and 9 days of treatments (Fig. 4.5 B,C) except for the 50 mM NaCl treatment after 9 days which resulted in a decrease of E in *P. pratensis* and *P. juncifolia* and the 300 mM treatment which reduced E in all plant species including *P. nuttalliana* (Fig. 4.5D).

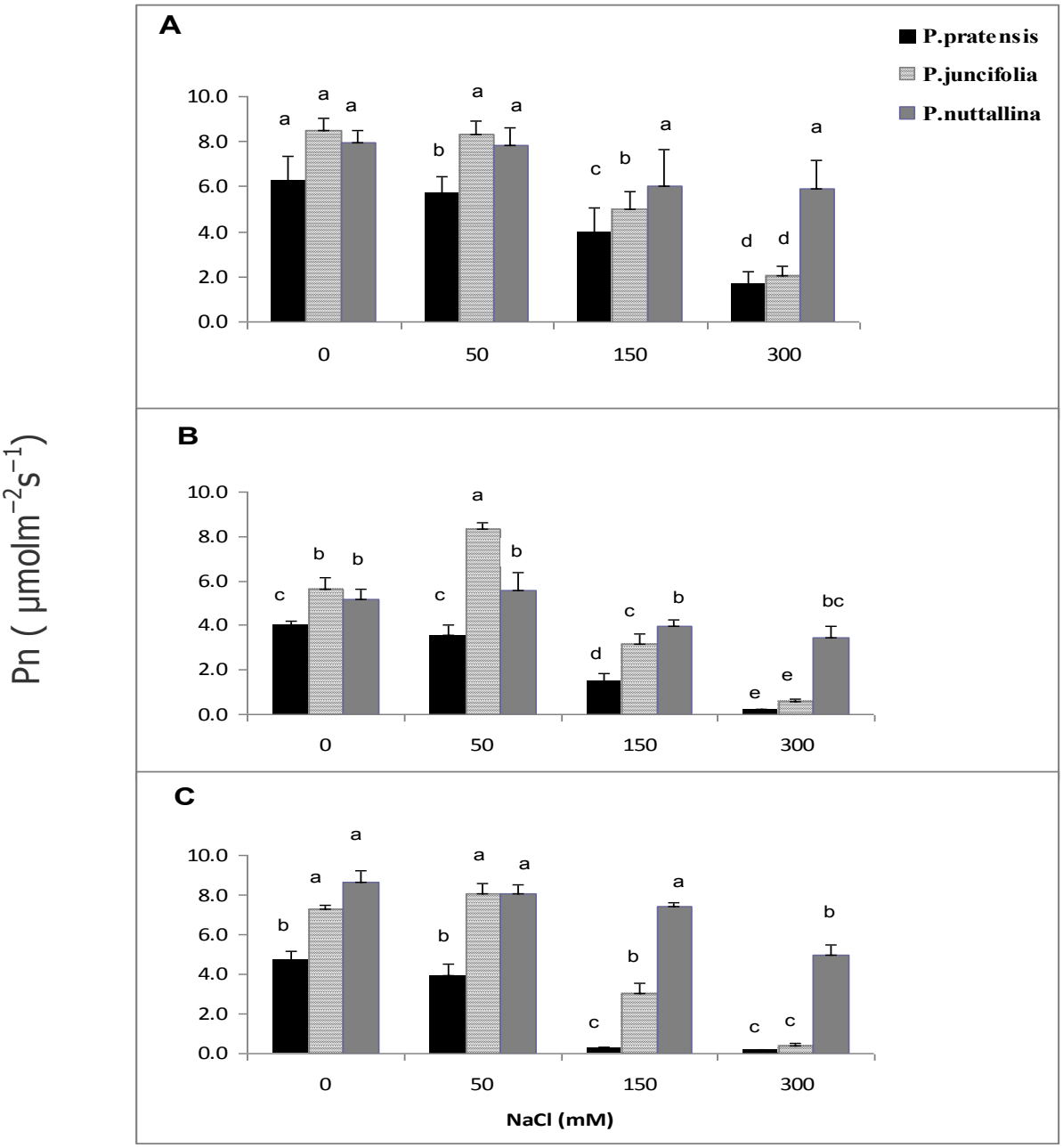


Figure 4.4. Net photosynthesis (Pn) in *Poa pratensis*, *Poa juncifolia* and *Puccinellia nuttalliana* after three (A), six (B), and nine (C) days of treatments with different concentrations of NaCl. Different letters above the bars indicate significant differences ($p \leq 0.05$) between treatments within each plant species as determined by the Tukey's test. Means ($n = 6$) ± SE are shown.

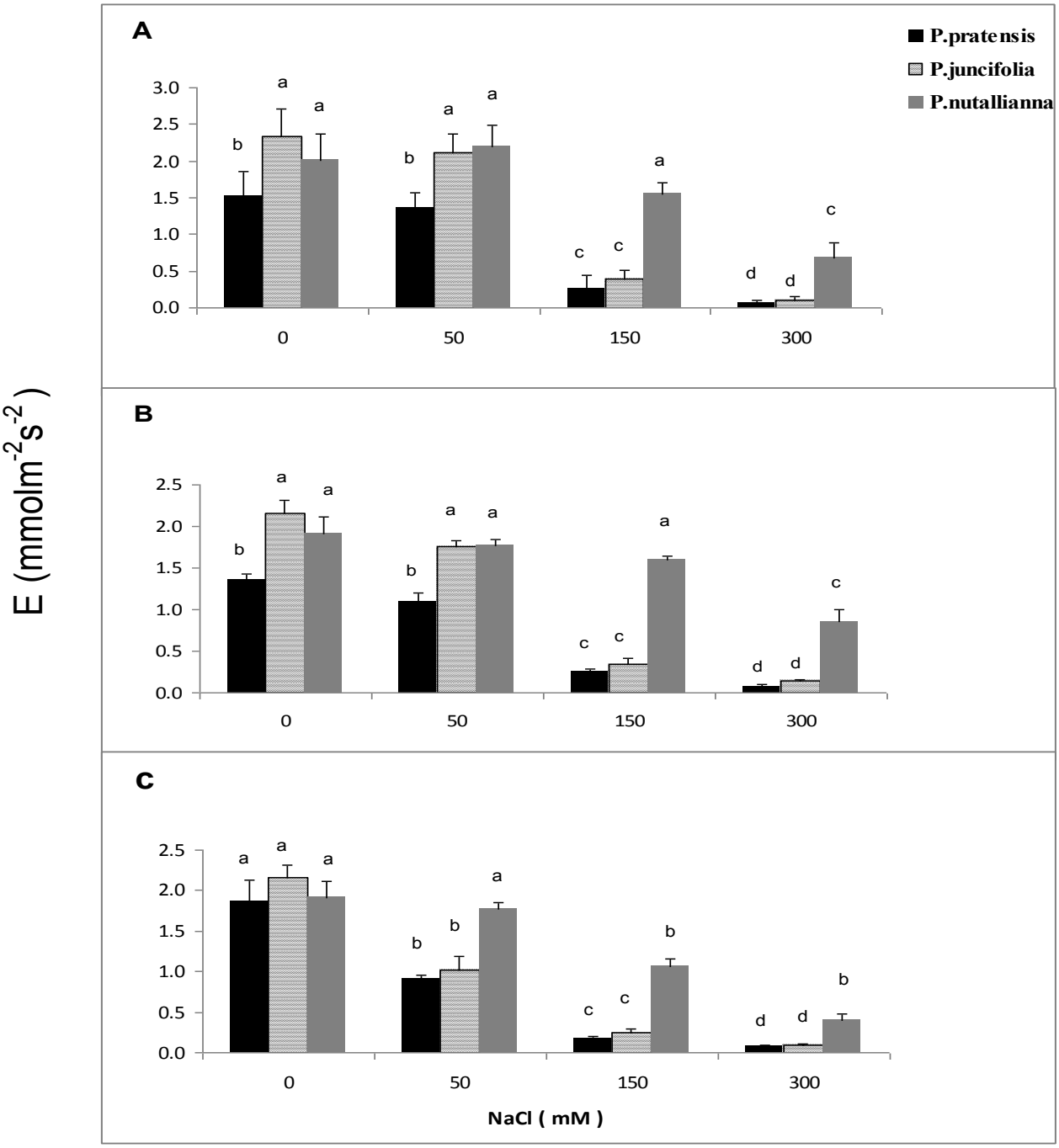


Figure 4.5. Transpiration rates (E) in *Poa pratensis*, *Poa juncifolia* and *Puccinellia nuttalliana* after three(A), 6(B), and 9(C) days of treatments with different concentrations of NaCl. Different letters above the bars indicate significant differences ($p \leq 0.05$) between treatments within each plant species as determined by the Tukey's test. Means ($n = 6$) ± SE are shown.

4.3. Water potential and water content

After 9 days of 50 mM NaCl treatments, shoot water potentials were not affected in any of the three studies species (Fig. 4.6). Shoot water potential were decreased by the 150 mM NaCl in *P. pratensis* and *P. juncifolia*, but only the highest, 300 mM NaCl treatment resulted in a decrease of shoot water potentials in *P. nuttalliana* (Fig. 4.6).

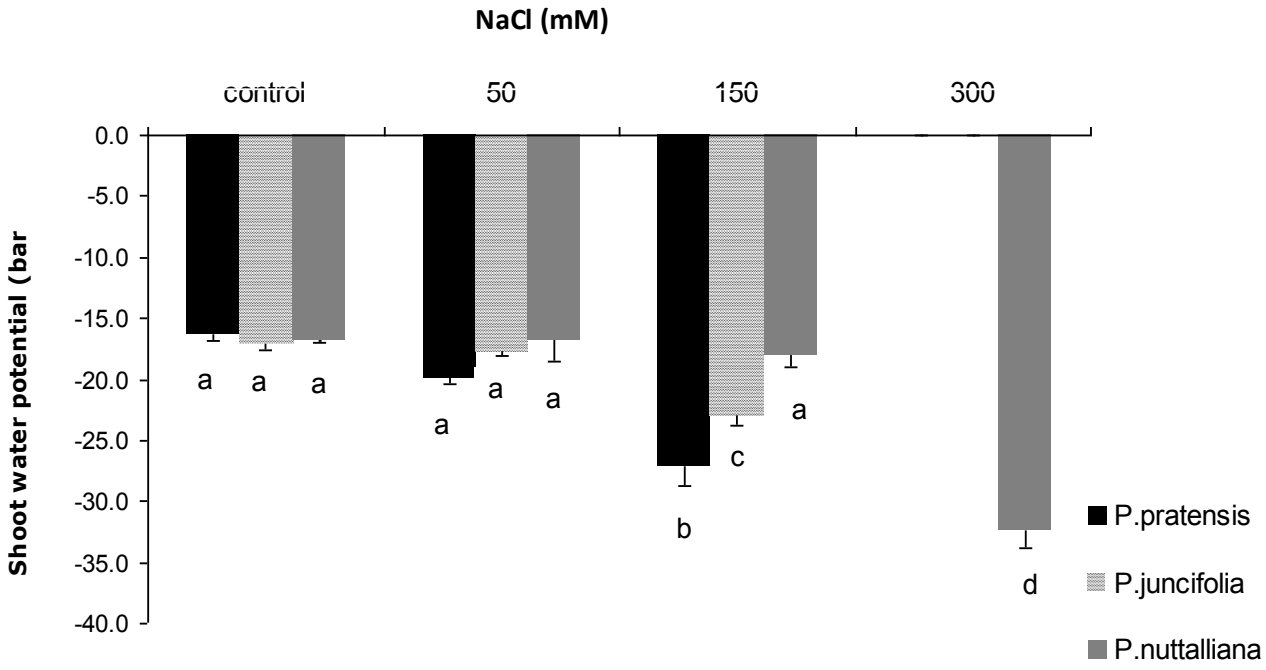


Figure 4.6. Shoot water potentials in *Poa pratensis*, *Poa juncifolia* and *Puccinellia nuttalliana* treated for 9 days with different concentrations of NaCl. Different letters above the bars indicate significant differences ($p \leq 0.05$) between treatments within each plant species as determined by the Tukey's test. Means ($n = 6$) ± SE are shown. Missing values for 300 mM NaCl treatment are due to high plant mortality.

Shoot water content of *P. pratensis* decreased in all examined NaCl concentrations and in *P. juncifolia* in 150 and 300 mM NaCl treatments (Fig. 4.7). There was no effect of any of the examined NaCl concentrations on shoot water potentials in *P. nuttalliana* (Fig. 4.7).

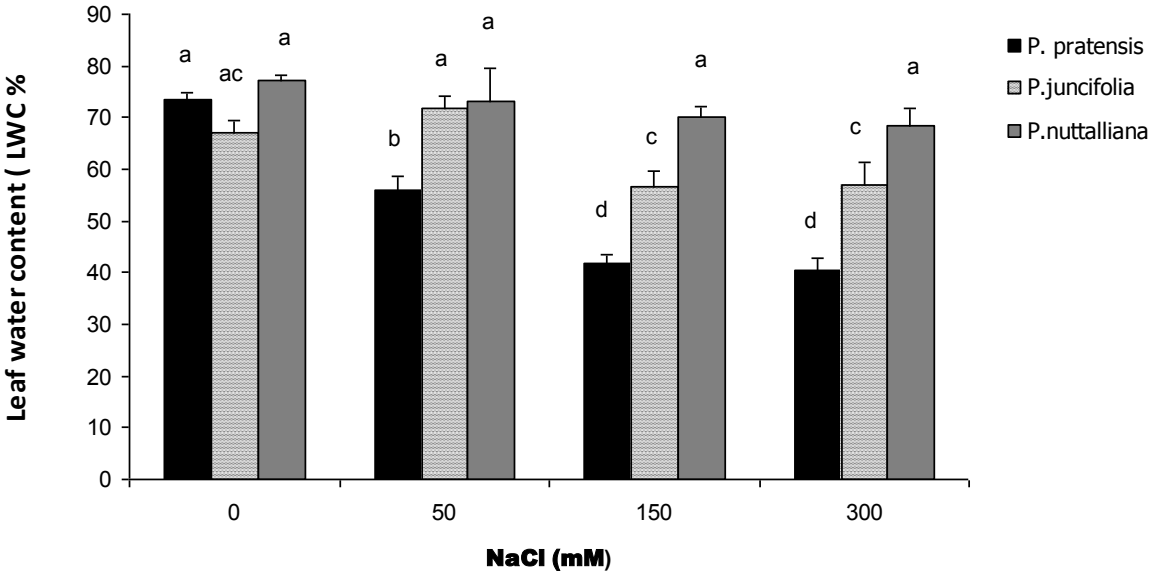


Figure 4.7. Leaf water content (LWC) in *Poa pratensis*, *Poa juncifolia* and *Puccinellia nuttalliana* treated for 9 days with different concentrations of NaCl. Different letters above the bars indicate significant differences ($p \leq 0.05$) between treatments within each plant species as determined by the Tukey's test. Means ($n = 6$) ± SE are shown.

4.4. Cell hydraulic conductivity (L_p)

Representative cell pressure probe traces show an almost three-fold increase in the $T_{1/2}$ value in *P. pratensis* after the addition of 50 mM NaCl to the roots (Fig. 4.8). There was no effect of 50 mM NaCl on the $T_{1/2}$ value in *P. juncifolia* and the $T_{1/2}$ value in *P. nuttalliana* decreased following the 50 mM NaCl treatment (Fig. 4.8). The $T_{1/2}$ values were several-fold lower in *P. juncifolia* and *P. nuttalliana* compared with *P. pratensis* (Fig. 4.8).

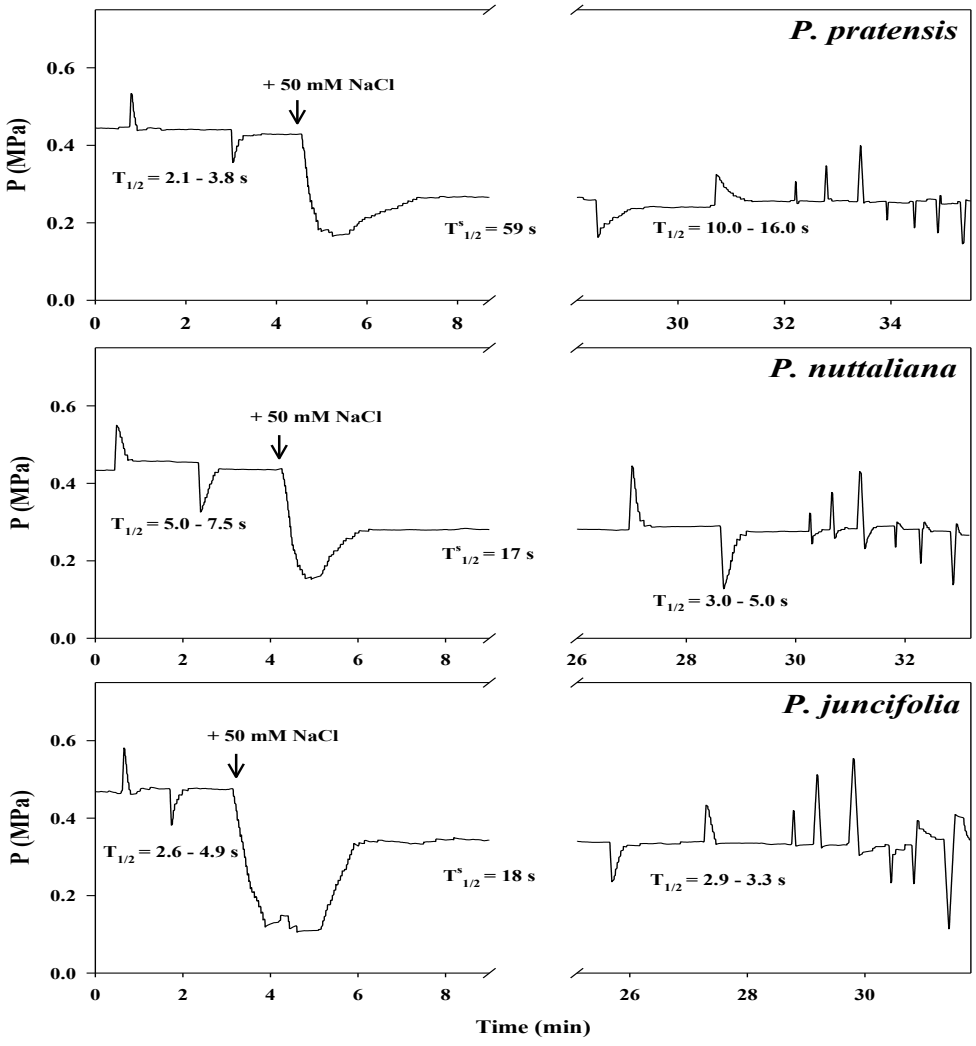


Figure 4.8. Examples of cell pressure probe traces showing hydrostatic half-time values of cell water exchange ($T_{1/2}$) and half times of solute permability ($T_{1/2}$) in *Poa pratensis*, *Poa juncifolia* and *Puccinellia nuttalliana* before and after the application of 50 mM NaCl.

Reflection coefficient (σ) was similar in control (untreated) plants of the three examined species, but solute permeability (P_s) values were two-fold higher in *P. juncifolia* and *P. nuttalliana* compared with *P. pratensis* (Table 4.1). After 20-30 min following the application of 50 mM NaCl, L_p decreased by about two-fold in *P. pratensis* (Fig. 4.9). The same, 50 mM NaCl, treatment had no effect on L_p in *P. juncifolia* and increased L_p by almost two-fold in *P. nuttalliana* (Fig. 4.9A). When the plants were subjected to 50 and 150 mM NaCl treatments in the soil and L_p was measured with the cell pressure probe, the L_p values declined by several-fold in *P. pratensis* (Fig. 4.9B). In *P. juncifolia*, 50 mM NaCl treatment had no effect on L_p, however, the L_p values declined by more than two-fold in plants treated with 150 mM NaCl (Fig. 4.9). In *P. nuttalliana*, L_p was approximately three-fold and two-fold higher in plants treated with 50 and 150 mM NaCl, respectively, compared with control plants (Fig. 4.9).

Table 4.1. Cell dimensions, permeability coefficient (P_s), and reflection coefficient (σ) of root cortical cells in *Poa pratensis*, *Poa juncifolia* and *Puccinellia nuttalliana* seedlings. The measurements were taken about 30 min after the application of 50 mM NaCl and in untreated control. Means ± SE (n = 6 cells from 6 plants) are shown.

Plant	Cell dimensions		$P_s \times 10^8$ ($m\ s^{-1}$)	σ [1]
	Length (μm)	Diameter (μm)		
P. pratensis	228 ± 12.5	27.8 ± 1.2	9.1 ± 3.9	0.82 ± 0.1
P.nuttalliana	121 ± 7.1	24.0 ± 1.5	18.7 ± 4.1	0.90 ± 0.1
P. juncifolia	210 ± 10.5	30.3 ± 1.6	21.9 ± 3.2	0.89 ± 0.1

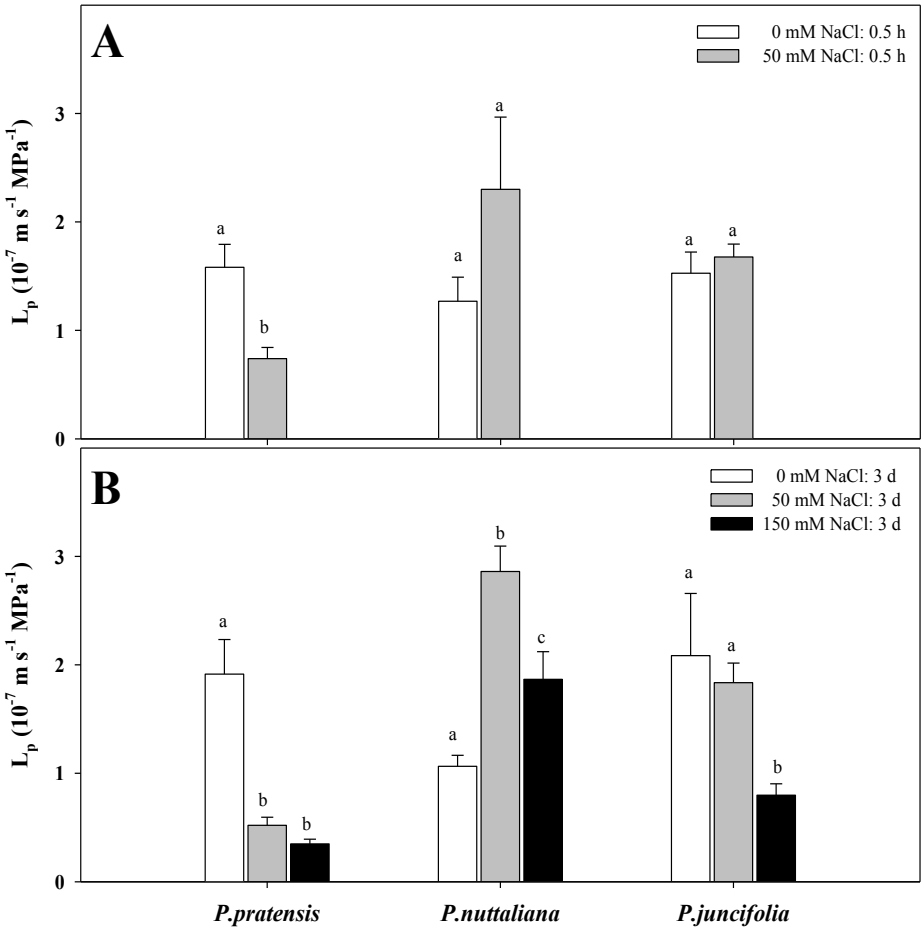


Figure 4.9. Effect of NaCl on cell hydraulic conductivity **(**L_p). (A) 50 mM NaCl was added to circulating medium for about30 min after performing control measurements. (B) The L_p was measured in the roots of plants that were subjected to 0, 50 and 150 mM NaCl for 3 days. Values are means ± SE (n=6). Different letters in each species indicate significant differences at $p \leq 0.05$ as determined by the paired t-test (A) and Tukey's test (B).

4.5. Root and shoot elemental analysis

Root Na concentrations increased in all three plant species as a result of NaCl treatments for 10 days (Fig.4.10A). However, there was relatively little difference in Na root concentrations between the 150 and 300 mM NaCl treatments (Fig. 4.10A). A progressive increase in shoot Na concentrations with increasing NaCl treatment concentrations was observed in all plant species, and the increase was of much greater magnitude in *P. pratensis* compared with the other two species (Fig. 4.10B).

Root Ca concentrations showed a slight decrease with increasing NaCl treatment concentrations in *P. nuttaliana* (Fig. 4.10 C). NaCl treatment had little effect on root Ca concentrations in *P. pratensis* and *P. juncifolia* (Fig. 4.10 C). A similar trend was observed for Ca concentration in the shoots (Fig. 4.10 D).

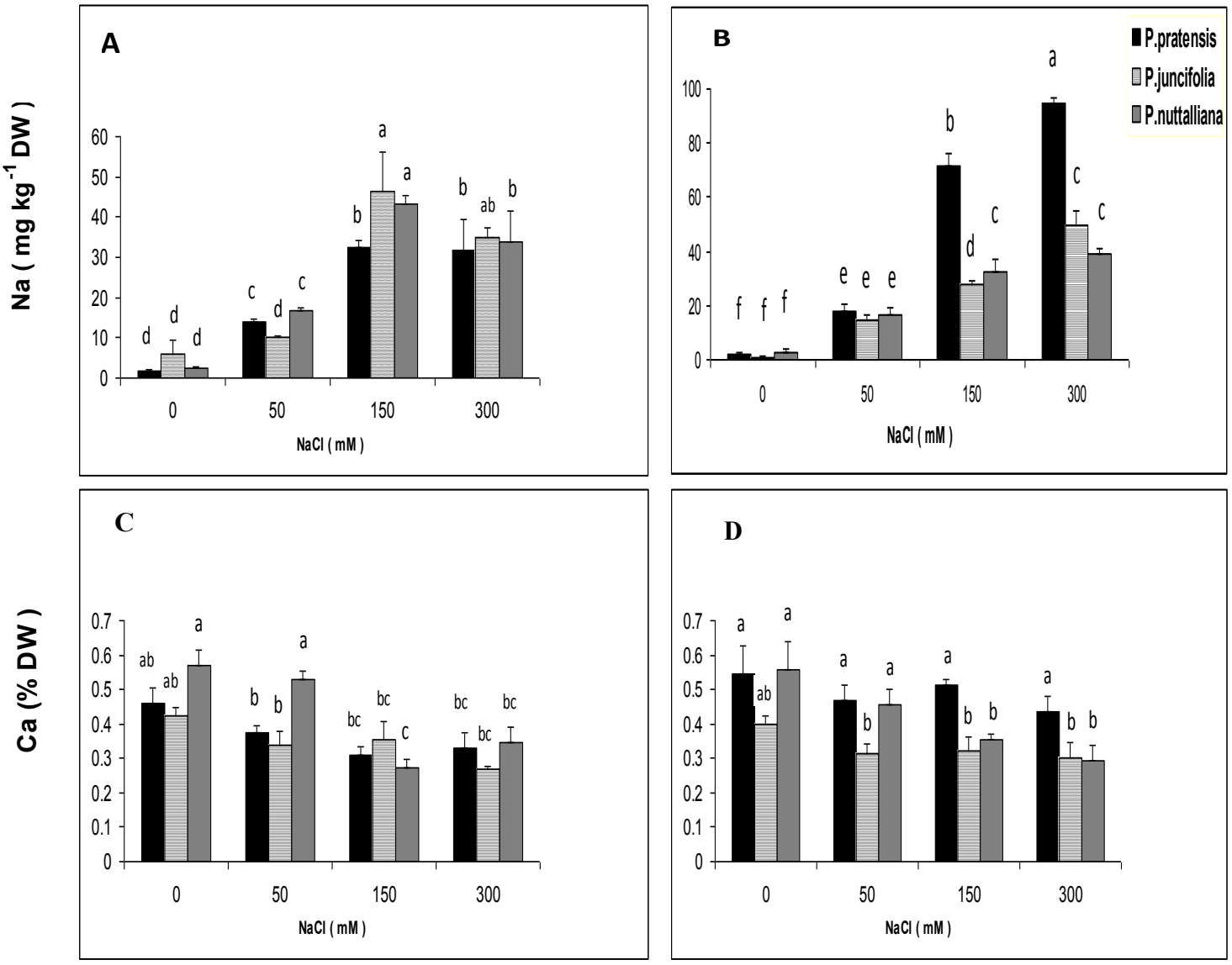


Figure 4.10 Sodium and calcium concentrations in roots (A, C) and shoots (B, D) of *Poa pratensis*, *Poa juncifolia* and *Puccinellia nuttalliana* after 10 days of treatments with different concentrations of NaCl. Different letters above the bars indicate significant differences ($p \leq 0.05$) between treatments within each plant species as determined by the Tukey's test. Means ($n = 6$) ± SE are shown.

The concentrations of K in roots of *P. pratensis* showed a drastic decrease with an increase in NaCl treatment concentrations (Fig. 4.11A) with no change observed in the shoots (Fig. 4.11B). No change was observed in the root and shoot K concentrations of *P. nuttalliana* as a result of NaCl treatments (Fig. 4.11A, B). In *P. juncifoila*, root K decreased compared with control only in the 300 mM NaCl treatment (4.11A) and shoot K concentrations decreased in the 150 mM NaCl treatment (Fig. 4.11B).

The concentration of Mg in roots (Fig. 4.11C) and shoots (4.11B) of *Poa pratensis* decreased compared with control in 150 and 300 mM NaCl treatments. In P. *juncifolia*, the decreases in Mg concentrations were measured in roots treated with 300 mM NaCl (Fig. 4.11C) and in shoots treated with 150 mM NaCl (4. 11D). In *P. nuttalliana*, there were relatively small decreases in Mg concentrations in roots (Fig. 4.11C) and roots (4.11D) of plants treated with 150 and 300 mM NaCl.

Root P concentrations were relatively little affected by NaCl treatments in all plants with the exception of the decrease of P compared with control of *P. juncifolia* in the 150 mM treatment (Fig. 4.12A). Decreases in P shoot concentrations were also observed in *P. juncifolia* treated with 150 mM NaCl and in *P. nuttalliana* treated with the 50, 150, and 300 mM NaCl (Fig. 4.12B).

Tissue concentrations of Fe were higher in roots of all plants compared with shoots (Fig. 4.12 C,D). Root Fe concentrations in *P. pratensis* were increased by the 150 and 300 mM NaCl treatments compared with control (Fig.4.12C). A similar increase in root P concentration was also observed in *P. nuttalliana* treated with 300 mM NaCl. In *P. juncifolia*, Fe shoot concentrations drastically decreased as a result of the 150 mM NaCl treatment (Fig. 4.12C). This decrease was accompanied by a drastic increase of Fe concentration in the shoots (Fig. 4.12D). With this exception, there were no significant changes in Fe shoot concentrations of the NaCl-treated plants in the three examined species (Fig. 4.12D).

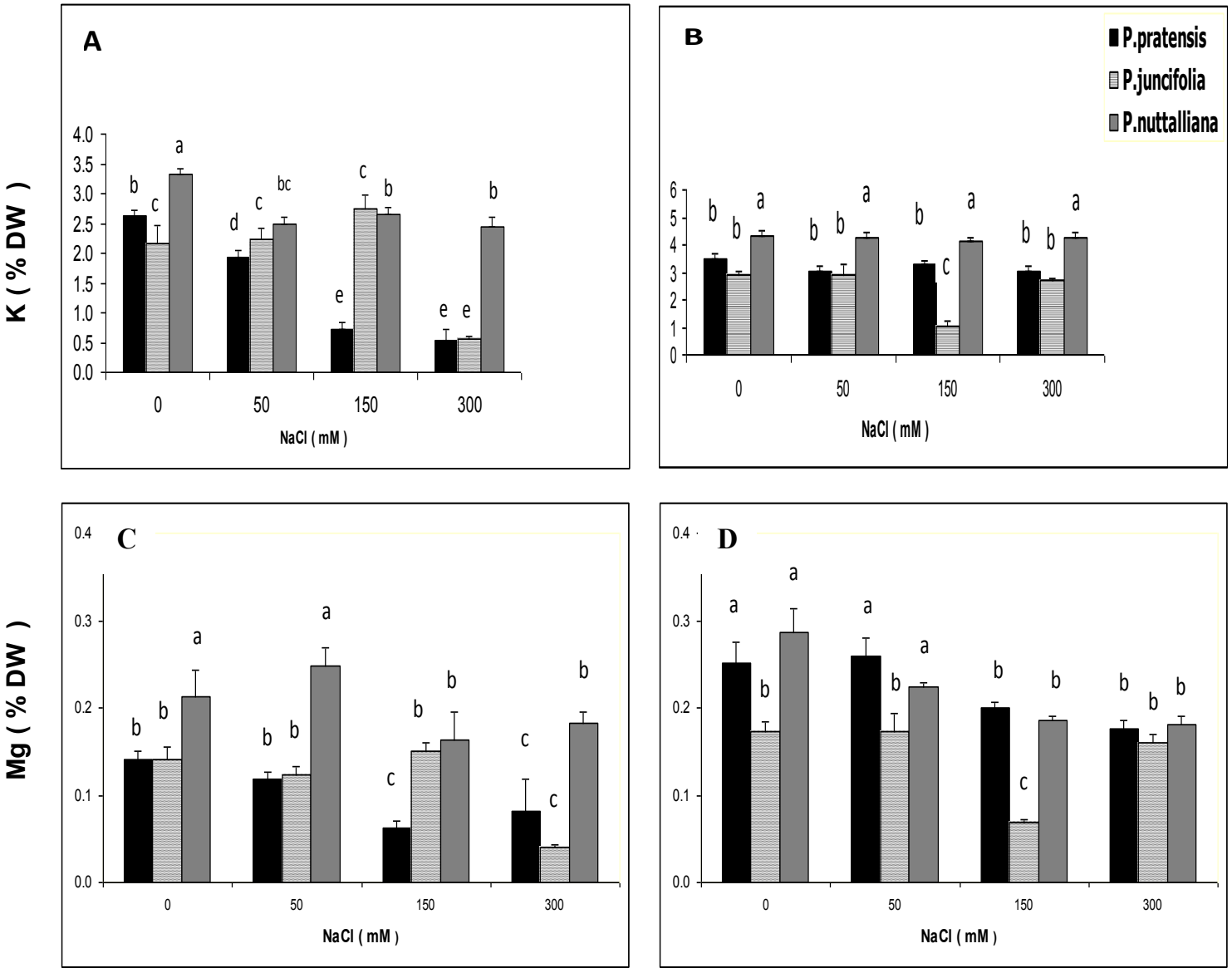
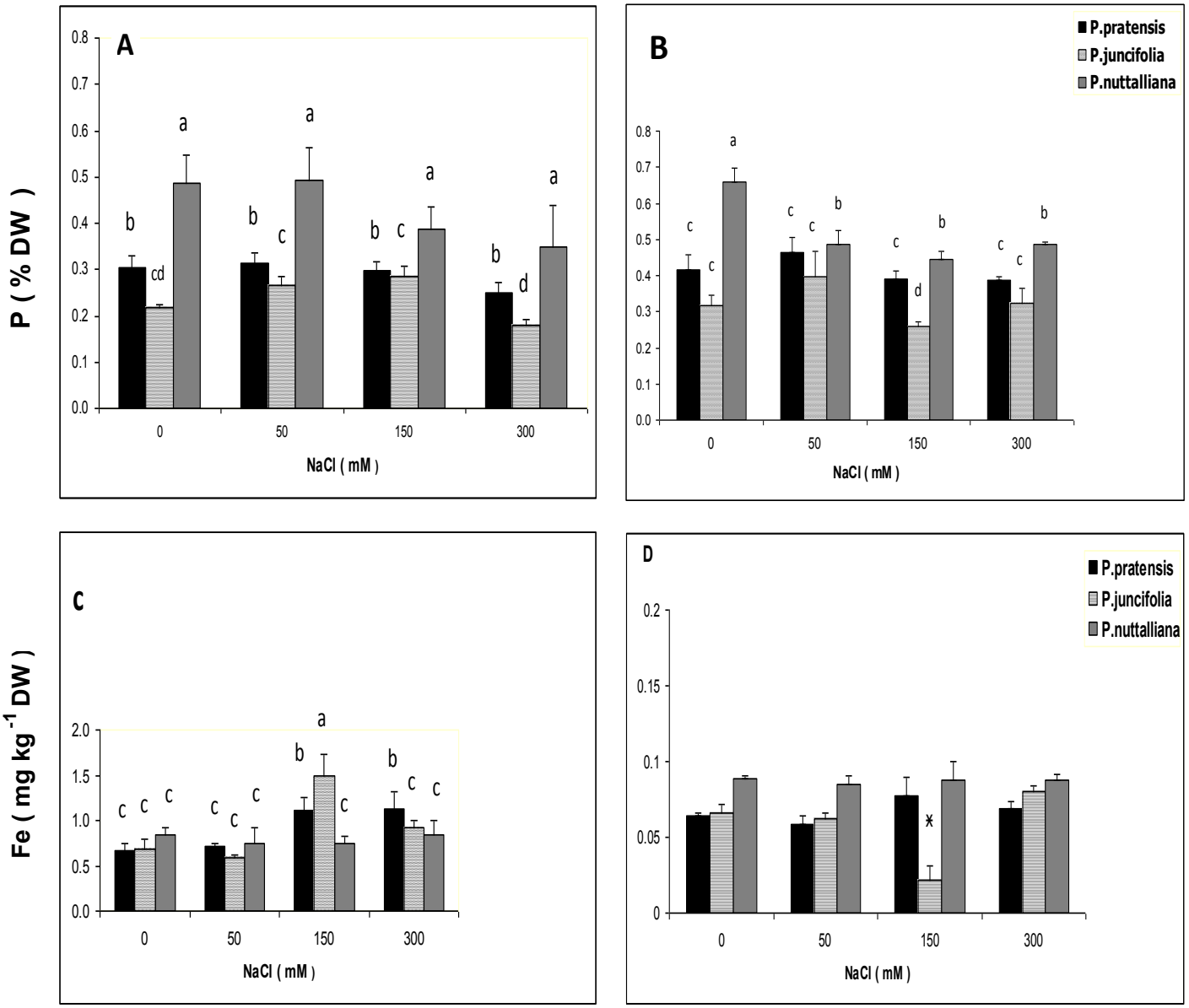


Figure 4.11. Potassium and magnesium concentrations in roots (A, C) and shoots (B, D) of *Poa pratensis*, *Poa juncifolia* and *Puccinellia nuttalliana* after 10 days of treatments with different concentrations of NaCl. Different letters above the bars indicate significant differences ($p \leq 0.05$) between treatments within each plant species as determined by the Tukey's test. Means ($n = 6$) ± SE are shown.



Figur 4.12. Phosphorus and iron concentrations in roots (A, C) and shoots (B, D) of *Poa pratensis*, *Poa juncifolia* and *Puccinellia nuttalliana* after 10 days of treatments with different concentrations of NaCl. Different letters above the bars (and asterisk in D) indicate significant differences ($p \leq 0.05$) between treatments within each plant species as determined by the Tukey's test. Means ($n = 6$) ± SE are shown.

5 Discussion

5.1. Effect of NaCl on growth rate and gas exchange parameters

For this study, three related grass species were selected to examine their responses to salt stress. Poaceae family includes over 7500 species with a wide range of salinity tolerance varying from salt sensitive (e.g. *Poa annua*), moderately salt tolerant (e.g. *Agrostis stolonifera*), salt tolerant (e.g. *Cynodon* spp), to extremely salt tolerant or true halophytes (e.g. *Puccinellia* spp.) (Gould and Shaw 1983; Aronson and Whitehead 1989). The study was carried out to characterize the responses of these grasses to salt to provide a foundation for future research aimed at better understanding of the exact mechanisms of water transport regulation in halophytes. Halophytes are specifically adapted to the environments with high concentration of salt and their water transport characteristics show little sensitivity to salt, contrary to salt sensitive glycophytes in which root water transport that is mediated by aquaporins is very sensitive to salt (Lee and Zwiazek 2015). To understand the function of aquaporins in water relations of these plants under salinity conditions, their growth, gas exchange, and other physiological responses must be first understood.

In the present study, plants of the three grass species were of the same age, but were different in size at the beginning of the NaCl treatments. *P. pratensis* was relatively larger compared with the other two species and *P. juncifolia* plants were the smallest. When subjected to the NaCl treatments for 10 days, *P. pratensis* showed a clear decline in root and shoot growth, as evidenced by the differences in dry weights between the NaCl-treated plants and untreated control. However, NaCl treatments did not negatively affect shoot and root dry weights in *P. juncifolia* and *P. nuttalliana*. On the contrary, the dry weights of *P. juncifolia* were higher in the 50 mM NaCl treatment and those of *P. nuttalliana* were higher in all NaCl treatments compared with control. Both *P. nuttalliana* and *P. juncifolia* plants displayed a typical halophyte response to salt of increased growth under moderate salt levels (Alshammary 2102). Root and shoot dry weights in *Poa pratensis* were significantly decreased as the concentration of NaCl increased in the root

zone, which is a typical growth response in glycophytes (Alshammary 2012). Growth inhibition in salt-treated plants is due to a combination of several processes including a decrease in water uptake followed by photosynthetic reduction, hormonal imbalance, and tissue injury (Hasegawa et al. 2000; Apostol and Zwiazek 2003). These processes are mainly affected due to osmotic effects and direct ion toxicity of NaCl (Greenway and Munns 1980 ; Ashraf et al. 2010).

It is interesting that the root dry weights were affected more than shoot dry weights in NaCl-treated *P. pratensis*, which resulted in an increase in shoot: root dry weight ratios with increasing NaCl treatment concentrations. Shoot growth of glycophytes (salt-sensitive) plants and moderately salt tolerant plants (mesophytes) generally show a linear decrease in growth with increasing salinity level. However, depending on the species, there may be no growth decline at low concentrations of salt (Maas 1985). Unlike glycophytes, halophytic plants often respond to moderate salt levels with increased shoot growth and their growth declines only in the presence of very high concentrations of salt (Suplick et al. 2002). However, even in highly tolerant halophytes such as Salicornia species, increased biomass production has been shown to occur only in the range from 170 to 340 mM NaCl (Ungar 1991).

Increased root growth in halophytic turfgrasses is more common under moderate salinity stress than shoot growth stimulation (Uddin et al. 2012). As a result, similarly to my study, shoot:root ratio in plants decreases. Increasing root: shoot ratio is one of the salt tolerance mechanisms increases root absorptive area and water uptake (Gorham et al. 1985). Another common response to salt stress which also leads to the reduction in shoot: root ratio is a reduction of total leaf area. Indeed, decreased leaf growth is among the earliest response of glycophytes exposed to salt stress (Munns and Termaat 1986).

Increasing NaCl treatment concentrations aggravated the decrease of Pn over time. However the three plants species showed wide differences in Pn tolerance of NaCl. In *Poa pratensis*, a decrease in Pn was also observed already after 3 days of treatments with the lowest, 50 mM NaCl concentration. The other extreme was *P. nuttalliana* which showed a decrease in Pn only in the highest, 300 mM NaCl and only after 9 days of

treatment. *P. juncifolia* showed intermediate responses to NaCl compared with the other two species and its Pn was reduced by the 150 and 300 mM NaCl treatments after three, six, and nine days.

It has been frequently reported that there is a correlation between decline in growth rates in glycophytes exposed to salt and decrease in photosynthetic rates (Munns and Termaat 1986). Decline in photosynthesis rate under salinity condition can occur due to decreased CO_2 availability caused by diffusion limitations through the stomata and the mesophyll (Flexas et al. 2004), reductions in photosynthetic pigments especially chrophyll (Hussain Wani et al. 2013), and effects on the electron transport processes (Lawlor and Cornic 2002).

Halophytes have developed different mechanisms in order to cope with high salinity conditions and photosynthesis in halophytes is usually not affected by salinity or in some halophytic plants, photosynthesis rate even stimulated at low salt concentrations (Parida et al. 2004). For example, in some obligate halophytes, such as *Sesuvium portulacastrum* (Aizoaceae), which require salt to reach their optimum growth, it was observed that photosynthesis and stomatal conductance increased when NaCl concentration increased from 0 to 600 mM NaCl (Venkatesalu and Chellappan 1993). Salt tolerance mechanisms in plants can be related to their ability to maintain adequate photosynthesis, stomatal conductance and high chlorophyll content (Krishna Raj et al. 1993) during salinity conditions.

Although in this study leaf chlorophyll concentrations were not measured, reductions in photosynthetic pigment are often reported in plants subjected to salt stress. The effect depends on the severity of stress and low salt concentrations may increase leaf chlorophyll concentrations, especially in salt tolerant plants (Locy et al. 1996). Misra et al (1997) suggested that the salt-induced increase in the chlorophyll content in halophyte leaves could be due to an increase in the number of chloroplasts. However, high concentrations of salt strongly reduce leaf chlorophyll content (Malibari et al. 1993). Therefore, it can be expected that similarly to other studies (Reddy and Vora 1986), leaf

chlorophyll concentrations in the relatively salt-sensitive *P. pratensis* could be among the factors contributing to the sharp reduction of Pn.

In the present study, the gas exchange responses of the three examined grass species corroborate the growth results and indicate that *P. nuttalliana* is highly salt-tolerant since even the highest; 300 mM concentration of NaCl in the root zone did not affect the Pn in these plants. Both *P. juncifolia* and *P. nuttalliana* do appear to be facultative rather than obligate halophytes that require salt for growth and reproduction since Pn did not show a strong and consistent increase in response to NaCl. More studies need to be carried out to determine whether these plants can complete their life cycles in the absence of Na to consider Na as an essential element to these plants. However, it can be concluded that both of these plant species, especially *P. nuttalliana* are well adapted to salinity. Since *P. pratensis* showed significant reductions in growth rates, net photosynthesis and transpiration in response to NaCl concentrations higher than 50 mM NaCl, plants of this species grass can be regarded as relatively salt tolerant glycohytes.

5.2. Effect of NaCl on water relations

Shoot water content of *P. pratensis* decreased in all examined NaCl concentrations and in *P. juncifolia* in 150 and 300 mM NaCl treatments. There was no effect of any of the examined NaCl concentrations on leaf water content in *P. nuttalliana*. The leaf water content was not reflected by similar changes as shoot water content and the 50 mM NaCl treatment did not affect shoot water potentials in any of the three examined grasses. However, shoot water potential declined in response to 150 mM NaCl treatment in *P. pratensis* and *P. juncifolia* and remained unchanged in *P. nuttalliana*. One of the characteristics of all halophytes is the ability to adjust their tissue water potential to the lower level than water potential of the soil solution (Flower et al. 1977; Sultana et al. 1999). Shoot dehydration and loss of turgor are common responses of plants to salinity (Neumann et al. 1988). To avoid the toxic effect of excessive ion accumulation particularly Na , many halophytes dilute the ion concentration by increasing their

succulence (Flowers et al. 1977). Succulence is one of the mechanisms that halophytes utilize to deal with the high internal ion concentrations (Debez et al. 2004). It plays a key role in the survival and maintenance of halophytes under saline conditions by maintaining positive turgor (Khan et al. 1999). Therefore, it is plausible that increased water uptake by *P. nuttaliana* could have been the main factor explaining no effect of NaCl on leaf water contents and shoot water potentials. It cannot be discounted that a transient decline in water potential during the initial several days could have occurred and contributed to the increased water content measured nine days after NaCl treatments.

Maintenance of shoot water content is very important to plant growth and survival. Although the exact amount of water that is needed for tissue hydration depends on factors such as the type of tissue and age of plants, shoot water content level ranging from 50 – 75 % dry weight is lethal to most plants (Wyn Jones and Gorham 2002). Therefore it can be concluded that *P. pratenis* at higher NaCl concentrations was suffering from severe stress, the decrease in shoot water content and, likely, turgor of NaCl-treated *P. pratensis*, led to the profound shoot growth reduction since cell turgor and cell volume maintenance are essential for sustained growth and development (Greenway and Munns 1980).

Shoot water potential in *P. nuttalliana* significantly decreased in response to the 300 mM NaCl treatment. It was reported that water potential and osmotic potential in a other halophytes such as *Amphibolis griffithii* became more negative with increasing salinity level, suggesting that *A. griffithii* adjusts osmotically in response to increased salinity (Burnell et al. 2014). This could happen due to osmotic adjustment when excessive absorbed ions themselves contribute to lower the internal water potential in both halophytes and in glycophytes (Zhao and Harris 1992). Halophytes are distinguished by their capacity to produce high concentrations of compatible osmotica (Storey and Wyn Jones 1979). It was observed that exogenously applied glycinebetaine enhanced the salinity tolerance in *Oryza sativa* (Marcum 2008) and transformation of *Poa pratensis*, which lacks glycinebetaine with betaine aldehyde dehydrogenase gene (glycinebetaine synthesis gene) improved salinity tolerance (Meyer et al. 2000). In salt tolerant plants that successfully accumulate ions for osmotic adjustment under high salinity conditions, an

accumulation of organic solutes that are compatible with enzyme activity helps maitainig osmotic potential of cytoplasm (Wyn Jones 1984). Only few organic solutes such as glycinebetaine, proline, trigonelline, and certain polyols and cyclitols, can be accumulated in adequate concentrations to osmotically adjust the cytoplasm without inhibiting enzymes (Gorham 1995). Other studies suggested that these compound mainly accumulate in turf and other grasses (Marcum and Murdoch 1990) , however, in *Puccinellia distans* the opposite has been reported and proline appears to play an important role in salt tolerance of these plants (Torello and Rice 1986). Glycinebetaine also accumulates in the cytoplasm and in C4 turfgrasses its accumulation has been correlated with salinity tolerance (Marcum 1999).

In an earlier study, proline content in *P. nuttalliana* was much higher than in *P. pratensis* and it was suggested that this accumulation plays a crucial role in preventing salt injury in this plant species (Alshammary 2012). Proline tissue concentrations often increase in salt-stressed plants (Wyn Jones 1984) and its accumulation is interpreted as an indicator of an adaptive response to salt and drought stress conditions (Ashraf and Harris 2004).

Cell pressure probe measurements demonstrated a several-fold increase in the $T_{1/2}$ values and a similar increase of L_p in *P. pratensis* after the addition of 50 mM NaCl to the roots. There was no effect of 50 mM NaCl on the $T_{1/2}$ value in *P. juncifolia* and the $T_{1/2}$ value in *P. nuttalliana* decreased following the 50 mM NaCl treatment. When the plants were grown in hydroponics and NaCl was added to the roots for 10 days, both 50 and 150 mM NaCl treatments severely reduced L_p in *P. pratensis*, but increased L_p in *P. nuttalliana* by about two- to three-fold. In *P. juncifolia*, there was no effect of the 50 mM NaCl treatment on L_p and 150 mM naCl reduced L_p in treated plants.

Measurements of root hydraulic conductivity and cell pressure probe measurements of cell water relations have been commonly carried out with glycophytes and little is known about the responses of root water transport to salt in halophytes. Root hydraulic conductivity is among the initial responses of plants to NaCl (Boursiac et al. 2005 ; Lee et al. 2010 ; Sutka et al. 2011), likely due to altered function of root

aquaporins and consequent inhibition of cell hydraulic conductivity (Sutka et al. 2011). In *Arabidopsis thaliana*, root treatment with 10 mM NaCl within minutes reduced L_p ,but had no effect on L_p in plants over-expressing one of the main water-transporting aquaporins, demonstrating that the function of aquaporins is among the initial targets of NaCl in plants and that the effects of NaCl can be at least partly alleviated by maintaining the aquaporin-mediated water transport (Lee et al. 2015).

While in the present study, it was hypothesized that the function of root aquaporins in halophytes would be less affected by NaCl compared with the glycophyte *Poa pratensis*, the stimulation of cell hydraulic conductivity in roots of *P. nuttalliana* was very surprising. Specific targets of that have been identified in plants for the inhibition of the aquaporin-mediated water transport include transcriptional and post - translational changes, especially protein phosphorylation/dephosphorylation, as well as protein trafficking (Lee and Zwiazek 2015; Pou et al. 2016). Since posttranslational regulation may involve different amino-acid residues of the aquaporins, it would be essential to identify the aquaporin genes from *P. nuttalliana* and carry out the protein structure analysis to identify potential mutations which make the aquaporins respond so differently to NaCl. The analysis of aquaporin transcripts and protein analysis would help determine whether an increase in the production of aquaporins and their membrane abundance may partly explain this phenomenon and which aquaporins are involved in this process. It appears that the membrane permeability properties to solutes may also be different between the glycophytic *Poa pratensis* and halophytic *P. juncifolia* and *P. nuttalliana*, judging from the differences in solute permeability coefficient values. Clearly more research is needed to understand these differences and their functional significance for salt tolerance.

5.3. Tissue elemental analysis

In the present study, *P. nuttalliana* and *P. juncifolia* accumulated less Na in the shoot tissues at higher NaCl treatment concentrations compared with *P. pratensis*. Regulation of Na and Cl uptake from the soil and minimizing their transport from roots to shoots are important parts of salt tolerance mechanisms in both glycophytes and

halophytes (Cramer 1985). It has been commonly reported that all plant tissues exposed to salt stress decrease growth, but this growth decrease is mostly noticeable in the aerial parts of the plants. To provide an explanation, previous studies have shown that in addition to Na, an accumulation of Cl in the leaves of NaCl - stressed plants triggers 1-aminocyclopropane-1-carboxyllic acid (ACC) synthesis and its conversion to ethylene with high efficiency, releasing enough hormones to trigger leaf abscission in citrus leaves and other plants (Tudela and Primo-Millo 1992). Accumulation of Cl may also affect the ability of plants to restrict Na transport from roots to shoots (Franklin and Zwiazek 2004). Salt tolerant plants have certain mechanisms to restrict the excessive level of ions in their cytoplasm (Wyn Jones 1984). In salt-affected plants, they tend to compartmentalize them in the vacuoles, which typically make up 90 – 95% of a mature plant cell's volume (Marcum 2008).

In the present study, root Na concentrations increased in all three plant species after 10 days of NaCl treatments, however, the increase was of much greater magnitude in *P. pratensis* compared with the other two species. It has been suggested that plant roots have certain capacity to store Na (Franklin and Zwiazek 2004). This capacity varies between the species and may be affected by tissue metabolism including energy reserves and once this capacity is exceeded, excessive amounts of Na are transported to shoots causing shoot injury and reduced shoot growth (Franklin and Zwiazek 2004. Salt tolerance of *P. nuttalliana* and *P. juncifolia* can also be related to salt excretion glands that may be present in a number of salt-adapted species (Liphschitz et al. 1974). Compared to multicellular glands dicots, in Poaceae plants, these structures seem to be very unique. (Wyn Jones 1984). The accumulation of Na in the shoots was proposed to be general characteristics of halophytes, whereas glycophytes tend to exclude Na from the root tissues (Folta et al. 2003). However, this appears to be the opposite in the present study since NaCl-treated *P. pratensis* accumulated more Na in the shoots compared with *P. juncifolia* and *P. nuttalliana*.

Concentrations of K in the roots of *P. pratensis* showed a drastic decrease with an increase in NaCl treatment concentrations with no change observed in the shoots. No

change was observed in the root and shoot K concentrations of *P. nuttalliana* as a result of NaCl treatments. In *P. juncifoila*, root K decreased compared with control only in the 300 mM NaCl treatment and shoot K concentrations decreased in the 150 mM NaCl treatment. With increased NaCl concentrations in *P. nuttaliana*, K concentrations in shoots were maintained at the same level as control. Both *P. nuttalliana* and *P. juncifolia* maintained high root K concentrations compared with control suggesting that these halophytes can actively absorb K from the saline medium. Generally, K concentration in the cytoplasm and even in the chloroplasts and mitochondria must be retained relatively high in order to maintain low level of Na in cytoplasm (Leigh and Wyn Jones 1984 ;Wang et al. 2004). Previous studies have demonstrated by increasing the salinity level, the competition between Na and K can reduce the level of internal K. Increasing salinity decreased the K contents in some halophytes such as *Suaeda maritima* (Clipson 1987). It was also reported that K uptake was adversely affected by the NaCl treatment in *Hordeum vulgare* and *Salicornia europea* (Demiral et al. 2005). One of the characteristics of salt-tolerant plant cells is the ability to maintain high concentration of K (Trivedi et al. 1991) by increasing Na^+/H^+ selectivity and reducing Na flux through low affinity cation channel or indirectly regulating Na^+/ H^+ antiporters responsible for the Na flux from cells through the calcium sensor coded by SOS3 (Qiu et al. 2002).

Calcium concentration was relatively little affected by increasing salinity level in the three examined plant species. Root Ca concentrations showed a slight decrease with increasing NaCl treatment concentrations in *P. nuttalliana* and NaCl had little effect on root Ca concentrations in *P. pratensis* and *P. juncifolia*. A similar trend was observed for Ca concentration in the shoots (Fig. 3.10 D). It has been reported that NaCl treatment can decrease Ca and Mg levels in plants (Khan et al. 1999). However, in a halophyte *Suaeda nudiflora*, salinity caused no change in Ca content (Joshi and Iyengar 1987). Decreasing Ca and Mg concentration under salinity condition can be the result of reducing cellular osmolytes in the presence of Na (Wyn Jones et al. 1977). On the other hand, an increase in Ca content could be attributed to the overall performance of metabolic activity of plants treated with the salts up to the optimum concentrations. Calcium increases salt

tolerance in plants by protecting against membrane damage and plays a key role in the selective transport of K in the presence of excessive Na (Aslam et al. 2003).

Although NaCl does not usually have a major effect on Mg, P, and Fe in salt-tolerant plants (Flowers et al. 1977), their tissue concentrations may provide important clues concerning plant overall health and were also examined in this study. Relatively minor changes in these elements in the NaCl - treated plants suggest that they did not play a major role in salt responses of the three studied grass species.

6 Conclusions

6.1. Review of the results in relation to tested hypotheses

The present study has demonstrated superior NaCl tolerance of *Puccinellia nuttalliana* which can be regarded as facultative halophyte. *Poa juncifolia* can be regarded either as a relatively moderately salt-tolerant halophyte or a salt-tolerant glycophyte. Although *Poa pratensis* showed typical responses to NaCl of a glycophytic plant, it can be still considered as a relatively salt-tolerant glycophyte. Both *P. nuttalliana* and *P. juncifolia* should be considered for a revegetation of salt-affected areas. Salt tolerance of these two halophytes can be largely explained by their ability to maintain water balance allowing them to retain turgor, gas exchange, and growth when exposed to salt. This ability may be partly explained by restricting Na transport from roots to shoots and by the reduced sensitivity (*P. juncifolia*) and stimulation (*P. nuttalliana*) of root cell hydraulic conductivity when exposed to NaCl. Since cell hydraulic conductivity is controlled by the aquaporin-mediated water transport and aquaporins are highly sensitive to NaCl in glycophytes, this is the most exciting and novel aspect of the study that deserves further attention.

6.2. Recommendation for future research

To address the question of aquaporin function under salt conditions in halophytic grasses, future research should be carried out to characterize and compare the structure and function of aquaporins in *P. nuttalliana*, *P. juncifolia*, and *P. pratensis*. The gene expression of aquaporines should be determined in control (no NaCl) and NaCl-treated plants and either the *Xenopus laevis* or yeast expression systems should be used to over-express these genes and study the effects of their over-expression on water transport in the presence and absence of NaCl using the stopped-flow spectrometric methods. A complimentary study could be also carried out to knock down these genes and determine the effect on water transport properties with and without added NaCl. Protein analysis can be carried following electrophoretic separation and the localization of the aquaporins

in cells could be determined after producing appropriate antibodies. RNA nucleotide sequence of the aquaporins should be used to construct three dimensional models of the aquaporin proteins and the amino acid residues and three dimensional structures of aquaporins should be studied to reveal the sensitive locations in their structures that may provide clues concerning their functions. The above studies would help shed more light on the salt tolerance characteristics of aquaporins and, in consequence, on the salt tolerance of plants.

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