SALINITY EFFECTS ON GERMINATION IN PERENNIAL RYEGRASS

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A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Brunswick

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Plant Biology

Written under the direction of

Stacy A. Bonos

And approved by

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New Brunswick, New Jersey

May, 2015

ABSTRACT OF THE THESIS

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` As potable water restrictions continue to tighten and there is an increase in effluent water use, it is necessary to identify turfgrasses with improved salinity tolerance. High salinity levels can cause injury to turfgrass plants resulting in poor or unusable turfgrass sites. As more turfgrass sites become salt affected, there is a need for the development of salt tolerant turfgrass. Because salinity tolerance is a complex quantitative trait, the development of salt tolerant cultivars has been slow. Additionally, most screenings for salinity tolerant germplasm is conducted on mature plants. It has been observed that a different set of genes control salt tolerance of germinating seedlings when compared to mature plant tolerance. Past screening procedures for germination under saline conditions have been conducted *in vitro* on blotter paper, agar, and in hydroponic solutions. The goals of the first two chapters of this thesis were to develop novel screening techniques that would mimic realistic soil properties for germinating perennial ryegrass seeds.

To achieve these goals, a greenhouse and a growth chamber experiment were conducted using native soil and topdressing sand respectively. A diverse range of perennial ryegrass cultivars were used and numerous measurements were utilized to quantify salinity stress during germination. Significant differences were observed based on the salinity level and cultivar used. Overall, salinity delayed germination of the perennial ryegrass seeds as salinity levels increased. Interestingly, there was no cultivar x treatment interaction indicating that cultivars that performed well under saline conditions, also performed well in the untreated control.

Additional salinity research was conducted on perennial ryegrass to further understand the endophytic fungi that lives between the plant cells. The *Neotyphodium* endophyte in perennial ryegrass has been shown to convey resistance to various abiotic and biotic stresses but the study of salinity-endophyte interactions has been lacking in turfgrass. To further understand this interaction, a study was developed using perennial ryegrass clones both containing and not containing the endophytic fungi. The objective of this chapter of the thesis was to determine whether salinity tolerance is genotype-endophyte specific, or whether there is an overall endophyte effect on salinity tolerance in perennial ryegrass. Data obtained from this study showed a specific endophyte-host interaction where some plant genotypes perform better with the endophyte while other genotypes perform worse. Due to the complexity of salinity tolerance, evaluating aspects of this trait during plant growth and germination has been difficult.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Stacy A Bonos for her guidance and support throughout my graduate studies. I would also like to thank my other committee members Dr. William A. Meyer, Dr. Bingru Huang, and Dr. Richard Hurley. I greatly appreciate the helping hands of Austin A. Grimshaw, Eric N. Weibel, Lindsey Hoffman, and my brother, Matthew Koch.

My sincerest appreciation goes to the Rutgers Center for Turfgrass Science, the New Jersey Landscape Contractors Association, The Ralph Geiger Scholarship Fund, and the Peter S. Loft Memorial Scholarship for their financial support throughout my graduate school career.

I would like to thank my family for always supporting me and providing guidance throughout my career. Finally, I would like to thank my amazing fiancé, Allison, for always assisting me with all facets of my graduate career and providing me with a loving environment every day of my life.

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Introduction

Salinity is a growing concern as three hectares of arable land is lost every minute due to soil salinization (Abrol et al., 1998). In the United States alone, it has been estimated that 68,500 sq miles of land is salt affected (Carrow and Duncan, 1998). There are a variety of ways that land can become salt affected including dissolution of minerals, salts in irrigation water (ie: wastewater and effluent water)(Duncan et al., 2000), use of fertilizers, saltwater intrusion (Todd, 1974), application of ice melting salts to roadways (Marcum, 1994), and saltwater spray (Marcum, 1994; Humphreys, 1982)

Turfgrass sites are prime candidates for using alternative water sources and in fact, some localities have been mandated to use effluent water to alleviate the use of valuable potable water (California State Water Resources Control Board, 1993; Duncan et al., 2009; Arizona Department of Water Resources, 1995). Turfgrass sites have been switching to alternative water sources due to the large quantity of water required to maintain attractive turfgrass sites (Mancino and Peepper 2004). It has been estimated that an average golf course uses 250,000 to 1,000,000 gallons of irrigation water daily to maintain an 18-hole golf course (Huck et al., 2000). This quantity of daily water use can increase greatly due to environmental factors present in arid climates around the world. Alternative water comes with an array of challenges but provides an economic

opportunity for turfgrass managers because of its associated lower cost and increased availability (Harivandi, 2000). According to estimations by Huck et al. (2000), wastewater costs 80% less than the fresh water equivalent.

Wastewater (reclaimed water) can be defined as treated or semi-treated water from a water treatment plant that has been remediated through physical and chemical means (Lazarova, 2005). One process of remediation involves the addition of salts to the water that remain after the treatment. The constant irrigation with this salt-containing effluent water will eventually lead to a salt-affected turfgrass site. Salt affected sites have been associated with many detrimental features including decreased soil permeability due to the accumulation of Na ions within the soil. These ions dominate the CEC (cation exchange capactiy) sites of soil particles and cause the destruction of larger pore spaces. This type of soil destruction will cause a decrease in water infiltration, percolation, and drainage (Carrow and Duncan, 1998). The process of the physical change in the soil is caused by the clay particles repelling one another in a process known as dispersion (Bauder and Brock, 2001; Frenkel et al., 1978). As these clay particles disperse, they clog pore spaces and thus decrease water flow through the soil profile.

Turfgrass sites irrigated with alternative water sources with high levels of salts will also cause detrimental effects to the plants leading to poor turf quality and decrease usability of the site. Some of the first symptoms of salt stress are a reduction in growth in addition to the leaves becoming darker green. Prolonged salinity stress will lead to the wilting and burning of the leaves. If management practices are not introduced, the

turfgrass stand will start to thin and can eventually die (Horst, 1991). The injury of turfgrasses can be separated into two main causes: a rapid water (osmotic) stress and a slower ion toxicity phase (Munns and Tester, 2008). As the soil becomes more saline, the water potential decreases in comparison to the plant cells. This difference in water potential causes the soil to retain water needed for plant growth and survival leading to drought-like conditions (Marcum, 1994). Continued exposure to salinity will also cause the turfgrass plant to absorb toxic ions such as Cl^-, B, HCO_3-, and SO_4-. These ions have been known to cause massive disruptions in enzymes and other proteins essential for plant growth and development (Carrow and Duncan, 1998). In addition to salinity ions, a process known as lipid peroxidation can be very detrimental to plant growth.

Lipid Peroxidation

High levels of salinity have been shown to increase the production of reactive oxygen species ([ROS] such as O^{2-},H_2O_2, OH^-) due to the disruption of photosynthesis and an increase in photorespiration (Miller et al., 2009, Asada and Takahashi, 1987). These reactive oxygen species can be damaging to cell membrane stability due to a process known as lipid peroxidation. This process starts when a free radical pulls an electron from the lipid in the cell membrane. This lipid radical then combines with O_2 which will cause a transformation into lipid peroxide. Lipid peroxidation occurs more readily with polyunsaturated fatty acids because the double bonds within these lipids contain highly reactive hydrogen atoms. To protect cells from oxidative injury, the plant

uses ROS scavenging mechanisms such as the production of antioxidant enzymes (Smirnoff, 1993) which will bind free radicals.

Chawla et al (2011) showed that salinity increases oxidative stress in all tested varieties of rice (*Oryza sativa* L.), but the increase was much more pronounced in salt sensitive varieties. Additionally, antioxidants levels were significantly increased in salt tolerant varieties when compared to salt sensitive varieties indicating these antioxidants importance for increasing salinity tolerance. Positive correlations between drought and antioxidant production have also been found in tall fescue (*Lolium arundinacea* [Schreb]), creeping bentgrass (*Agrostis stolonifera* L.), and Kentucky bluegrass (*Poa pratensis*) (Zhang and Schmidt, 1999). The productions of antioxidants are therefore very important for salt tolerance and drought in that they reduce lipid peroxidation in a broad range of species.

Veerasamy and Huang (2013) conducted an experiment using 7 species of cool-season turfgrass (perennial ryegrass [*Lolium perenne* L.], creeping bentgrass, colonial bentgrass (*Agrostis capillaris* L.), velvet bentgrass (*Agrostis canina* L.), Kentucky bluegrass, tall fescue, and alkaligrass [*Puccinella* spp.]) to determine whether variation in salinity tolerance among species were associated with changes in antioxidant activities. All species were irrigated with 12 dS m^{-1} solution in a growth chamber. Results showed that alkaligrass and tall fescue were the most tolerant among tested species, while colonial and velvet bentgrass were the most sensitive to salinity stress. Additionally, salinity reduced membrane stability and caused lipid peroxidation. Tall fescue and

alkaligrass were able to maintain higher activities of antioxidant enzymes such as superoxide dismutase, catalase, and ascorbate peroxidase when compared among the other species utilized in this experiment. These results indicate that species with higher salinity tolerance are associated with higher production of antioxidant enzymes, which in turn protects the cellular membranes from lipid peroxidation.

In addition to drought, ion toxicity, and lipid peroxidation application of saline water directly to the leaf blades can lead to foliar burn. Salts applied overhead can be absorbed directly through the stomates leading to a fertilizer-type burn (Harivandi, 2004). This type of burn causes symptoms such as dieback and necrosis of leaf tips due to the high salt index of quick release fertilizers such as urea and ammonium nitrate (Landschoot, 2003). It has been found that the overhead application of saltwater irrigation causes significantly more damage to plants when compared with irrigation of the roots alone (Wu et al., 1999; Koch and Bonos, 2011).

Salinity Tolerance in Plants

Plants have two different mechanisms of salt tolerance. The first is the ability to keep salt concentrations in the cytoplasm low, and the second mechanism is to maintain functionality of cytoplasm components when salts enter the plant cell (Kramer, 1984). Turfgrass have evolved mechanisms to survive many different stresses including salinity stress. The mechanisms that are relevant to C3 grasses include salt exclusion, osmotic adjustment, production of compatible solutes, and salt compartmentalization. Glandular

ion secretion is another mechanism of turfgrasses, but is more relevant in C4 grasses (Marcum, 2008).

Salt Exclusion

One mechanism that has been shown to increase salt tolerance in turfgrass is salt exclusion**.** Munns (2014) claims that "a plant can grow or survive in a saline soil only if it can both continue to uptake water and exclude a large proportion of the salt in the soil solution". Exclusion of salt ions by the roots of the turfgrass plant ensures that damaging Na^+ and Cl^- ions will not accumulate to toxic concentrations within the leaf tissue (Munns, 2002). These damaging ions are deposited in the leaves due to the upward movement of water for transpiration purposes and no efficient way for the plant to relocate these ions to the roots. To prevent salt from building up over time in the shoots, the roots must exclude over 90 percent of the salt in soil, and allow only 10 percent to be transported into the xylem to the shoots (Munns, 2014). Failure to exclude these ions creates a toxic effect days or weeks after initial exposure to high levels of salinity (Munns and Tester, 2008). Excluding ions has been associated with salinity tolerance in many different C3 and C4 turfgrass species including alkaligrass, red fescue (*Festuca rubra* L.), Kentucky bluegrass, seashore paspalum (*Paspalum vaginatum*), and Bermudagrass (*Cynodon dactylon* (L.) Pers. Var. *C. dactylon* x C. *transvaalensis* Burtt-Davy) (Torello and Rice, 1986; Qian et al., 2001; Marcum and Murdock, 1994).

In all studies, increased levels of Na^+ in plant leaves were associated with plants that were more susceptible to high levels of salinity. Alkaligrass (a very salt tolerant species) showed very little Na^+ ions in leaf tissues which may be the important property of this species ability to tolerate salinity (Torella and Rice, 1986). In creeping bentgrass, Ahmed et al. (1981) showed that ion exclusion is a main mechanism for salinity tolerance of creeping bentgrass by analyzing salt tolerant plants isolated from salt marshes in comparison to those plants isolated inland. The high-affinity K^+ transporter (HKT) families may also provide insight on how the plant uptakes Na^+. The wheat TaHKT2:1 protein functions as a Na^+/K^+ symporter. Down regulation of this symporter in plants reduces root Na^+ accumulation and leads to improved growth in saline conditions (Laurie et al., 2002). In rice, OsHKT2 is rapidly downregulated to reduce potential Na^+ influx in the roots (Horie et al., 2007). The exclusion of damaging ions from entering and being transported to the shoots is one of the most important mechanisms for salt tolerance in plants.

Ion Homeostasis

Another mechanism that has been shown to relieve salinity and drought tolerance is ion balance/homeostasis. High levels of Na+ ions can lead to improper enzyme functions and denaturing of proteins within the cytoplasm. Turfgrass plants are able to keep the levels of Na^+ low in the cytoplasm by actively pumping Na^+ ions out of the cell and pumping K^+ ions into the cell. Potassium and sodium are known to compete for

binding sites which can cause a deficiency of potassium within the cell (Maathius and Amtmann, 1999). The competition of sodium inhibits metabolic processes that depend on potassium; therefore the accumulation of K^+ ions is important for salinity tolerance. Ion balance has been shown to be important in the development of salt tolerant C4 turfgrass species (Marcum and Murdoch, 1990a). For C3 turfgrass species, Qian et al. (2001) showed the difference between two Kentucky bluegrass varieties by analyzing the root/shoot growth, turgor pressure, and the K^+/Na^+ ratio. A high K^+/Na^+ ratio and a more positive turgor was found in the more tolerant varieties. Analyzing the K^+/Na^+ ratio is a useful way to verify salt tolerant genotypes.

The pathway for ion homeostasis has been studied extensively in Arabidopsis (*Arabidopsis thaliana*) using molecular techniques to create salt overly sensitive (sos) mutants (Zhu, 2003). The sos pathway starts with the sensing of high salinity levels by the cell membrane. This causes an increase in Ca^{2+} signals which is recognized by the protein sos3, which then activates protein kinase sos2. Sos 1 is the antiporter protein that is phosphorylated by sos2 which leads to Na^+ being pumped out of the cell cytoplasm. Interestingly, the expression of sos1 is stronger in the epidermal cells surrounding the root tip and in the parenchyma cells bordering the xylem (Chinnusamy et al., 2006). Oh et al. (2009) used a different approach to understand how sos1 interacts with salinity tolerance. These authors used RNA interference (RNAi) lines of the halophytic aribidopsis relative *Thellungiella salsuginea* to suppress sos1 and then expose these transgenic plants to high levels of salts. It was found that the reduction in sos1 expression caused *Thellungiella* (that can normally grow in seawater-strength NaCl

solutions) to become as salt sensitive as arabidopsis. These sos genes may be useful in the future for the use in quantitative trait loci analyses.

The genes from the sos pathway were recently incorporated into tall fescue plants by Ma et al. (2013). The co-overexpression of *Arabidopsis thaliana* sos1, sos2, and sos3 genes enhanced the salt tolerance of tall fescue. After being exposed to 350 mM NaCl transgenic plants displayed superior growth and accumulated less Na^+ and more K^+ in the roots. Additionally, Na^+ efflux, K^+ influx, and Ca^{2+} influx were higher in transgenic plants. Transgenic plants had a significant increase in superoxide dismutase, peroxidase, catalase, and proline content. The results from this study indicate that the co-expression of sos1, sos2, and sos3 genes are extremely important in creating transgenic plants that are able to tolerate high levels of salt. The incorporation of these genes into a salt sensitive species (such as colonial or velvet bentgrass) may be beneficial in understanding why creeping bentgrass is the more salt tolerant of the bentgrasses.

This salt tolerance pathway was again validated by Jannesar et al. (2014) in a C4 grass known as mangrove grass (*Aeluropus lagopoides*). This creeping perennial grass is distributed in regions with intermediate salinity and semi-arid desert climates such as the Iranian plateau (Bodla et al., 1995). After exposure to various salinity treatments (including exogenous abscisic acid [ABA] application), expression analysis for salt overly sensitive genes (sos1, sos2, and sos3) was conducted by semi-quantitative RT-PCR. The sos ESTs were isolated, cloned, and sequenced. The results showed an up regulation of sos3 expression in almost all salinity treatments in the shoots. Additionally,

ABA regulated the pathway by enhancing sos2 and sos3 expression in roots and shoots respectively indicating that ABA plays an important role in up regulating the sos pathway.

Yadav et al. (2012) isolated and cloned the Sbsos1 gene from *Salicornia brachiate*. This succulent halophyte occurs along marshes, on beaches, and among mangrove trees (Rao, 2014). The Sbsos1 gene was incorporated and overexpressed in tobacco which conferred high salt tolerance, increased seed germination, shoot length and root length, leaf area, fresh weight, dry weight, relative water content, K^+/Na^+ ratio, membrane stability, soluble sugar, proline, and amino acid content when compared to wild type plants. Additionally, transgenic plants exhibited reductions in electrolyte leakage, and reactive oxygen species when under salt stress. Similar to Chinnusamy et al. (2006), Sbsos1 showed a greater level of constitutive expression in roots when compared to the shoots.

Compatible solutes

Production of compatible solutes is a way that the plant can reduce water potential and increase the osmolarity within the cell cytoplasm to reduce water loss under saline conditions (osmotic adjustment). This reduction in water potential draws water into the cell cytoplasm. Evolutionarily, the production of compatible solutes has distinct advantages over accumulation of cheaper non-compatible perturbing solutes, such as Na^+ and Cl^-. The accumulation of perturbing solutes would require the evolution of many

salt-tolerant enzymes, which would require changes in the protein amino acid composition to offset the destabilization caused by Na^+ and Cl^- (Hochachka and Somero, 1984). Therefore, the accumulation of compatible solutes requires far fewer changes to the plant physiology and salinity tolerance can be obtained by modifications to the regulatory mechanisms controlling the biosynthesis of osmoregulants (Rhodes et al., 2002; Hochachka and Semero, 1984). There has been increased activity to identify genes encoding the key enzymes of compatible solute synthesis as these genes have the potential to improve salinity tolerance (and drought tolerance) in crop species (Jain and Selvarej, 1997; McNeil et al., 1999; Nucio et al., 1999).

Compatible solutes in plants include glycine betaine, proline, polyols, and trigonelline (Gorham 1996). Glycine betaine has been associated with salinity tolerance in many C4 turfgrass species (Marcum and Murdoch, 1994; Marcum 1999) and has been found to be a major osmoregulant that controls osmotic adjustment for plants under drought stress (Nilsen and Orcutt, 1996). Glycine betaine has also been shown to act as an osmoprotectant by stabilizing protein structures and preventing membrane instability (Chinnusamy et al., 2005). Exogenous applications of glycine betaine on creeping bentgrass and Kentucky bluegrass has been found to strengthen antioxidant defenses in plants under drought and salinity stress (Yang et al., 2012). Marcum and Murdoch (1994) showed that in all C4 grasses used (except centipedegrass [*Eremochloa ophiuroides*] which has poor salt tolerance ranging from 3-8 dSm^{-1}), glycine betaine and proline levels increased with increasing levels of salinity, indicating that C4 grasses utilize antioxidants to overcome salinity stress.

Qian et al. (2001) analyzed the effects of compatible solutes on two Kentucky bluegrass varieties and found that no glycine betaine was detected in either Kentucky bluegrass variety and only minimal amounts of proline were found in both varieties. This indicates that compatible solute accumulation plays little role in salinity tolerance in Kentucky bluegrass which helps to further understand this specie's poor salinity tolerance. Torello and Rice (1986) found low levels of proline in red fescue, and Kentucky bluegrass varieties. Interestingly, in the same experiment there were high levels of proline found in alkaligrass which led to a greater level of salt tolerance. The productions of glycinebetaine and proline are therefore more important for the tolerance of C4 species when compared to C3 species (with the exception of the extremely salt tolerant species alkaligrass).

Biotechnological approaches have been used to get a better understanding of compatible solutes. Redwine (2000) used a biolistic approach to transform creeping bentgrass (Cv Penncross) with mannitol-1-phosphate dehydrogenase (mtlD) to evaluate its ability to withstand drought and salinity conditions. The mtlD enzyme causes an increase in mannitol accumulation, a compatible solute sugar alcohol that has an increased NAPD-NADPH turnover compared with plants that do not produce any sugar alcohols. Although the transformed plants contained the mtlD gene, there was no expression of the gene due to post transcriptional gene silencing. Successful mtlD transformations have been made in the past as described by Tarczynski et al. (1992) who were successful in transforming tobacco (*Nicotiana tabacum L.*) with the mtlD gene. The tobacco transformants exhibited increased levels of salinity tolerance when compared

with nontransformed plants. The introduction of the mtlD gene may be useful for the creation of compatible solutes which causes increased salinity tolerance of some species.

Scientists are consistently searching for genes that convey salinity tolerance in a broad range of species. Chen et al. (1999) filed a patent for incorporation (via biolistic delivery) of the BADH (betaine aldehyde dehydrogenase) gene into turfgrass species. This gene was isolated from mountain spinach (*Atriplex hortensis*) which grows on the shore of a salt lake in western China. The BADH gene creates a higher accumulation of glycinebetaine in plants and makes them more salt tolerant due to the process of osmoregulation. By incorporating the BADH gene into cool season turfgrass callus, the authors found that 1.5% NaCl stress caused a 75% increase in glycinebetaine production when compared to nontransgenic callus. Along with salinity stress, these transgenic plants caused an increase in the plants ability to tolerate severe water deficits. The BADH gene was also studied by Jia et al. (2002) who transformed tomato callus for increased salinity tolerance. Transformed tomato callus tissue with the BADH genes was found to increase salinity tolerance up to 120 mM NaCl. Because cultivated tomato varieties produce small quantities of glycinebetaine, the incorporation of this gene has the potential to increase tomato production.

Compartmentalization

Another method turfgrasses and other plants use to manage high levels of salt is to accumulate damaging salt ions in the vacuole of the cell. By compartmentalizing salt

ions, damage to cell functionality can be prevented (Flowers et al., 1977). Gaxiola et al. (2001) used transgenic *aribidopsis* plants with a gene to overexpress the vacuolar H^+-pyrophosphatase which showed more Na^+ and K^+ accumulation in the vacuole when compared to the wild type. These transgenic plants also maintained a higher turgor pressure in the cells and had higher relative water content in the leaves under salinity stress in comparison to the wild type plants. In rice, the over expression of the gene NHX1 has also been shown to compartmentalize salt ions within the cell vacuole (Fukuda et al., 1999; Chinnusamy et al., 2006). The NHX1 gene encodes for the tonoplast Na^+/H^+ antiporter which actively pumps H^+ out of the vacuole to allow for the influx of Na^+. By compartmentalizing Na^+, the cell is able to protect important functions within the cytoplasm. Compartmentalization of damaging ions within the vacuole is an important mechanism for coping with increased levels of salinity.

Roger and Lin (1983) used collections of creeping bentgrass from a seashore population and compared ion distribution within the plant following growth with 0, 100, or 200 mM NaCl. Differences were observed among genotypes especially when tolerance was measured by analysis of root elongation. Significant ion concentrations were also found when comparing plant organs where expanding leaves had the lowest concentrations of Na^+, Na^+/K^+ and Cl^-. In one of the most tolerant clones, ion concentration was highest in the leaf sheath. This data supports the hypotheses that ion compartmentalization is associated with a plants ability to resist salt and that the older plant vegetation, such as the leaf sheath, can compartmentalize more detrimental ions when compared to new growth.

Esechie and Rodriguez (2008) used both a salt sensitive and salt tolerant variety of alfafa to further understand ion compartmentalization. These authors found a pattern in the salt sensitive variety where the plant had the most Na^+ and Cl^- in the root, followed by the stem, and finally the leaf. The salt tolerant variety showed a different pattern in which the stem contained the most ions, followed by the leaf, then by the root. This research indicates that compartmentalization of detrimental ions is important in alfafa and the roots of the plant are avoided for the accumulation of detrimental salt ions.

Salinity Tolerance of Various Turfgrass Species

Salinity tolerance varies across cool-season and warm-season turfgrass species. An innovative way to measure the variation in salinity tolerance in turfgrasses is the estimation of salt concentrations that result in a 50% reduction in shoot growth. The variation in salinity tolerance can be seen in the different bentgrass species. Creeping bentgrass is considered moderately salt tolerant and can tolerate salinity levels ranging from 8 to 10 dS m^{-1} before exhibiting a 50% reduction in shoot growth. (Carrow and Duncan, 1998; Madison, 1971; Harivandi et al., 1992). Younger et al. (1967) found that creeping bentgrass salinity tolerance ranged from 9 to 26 dS m^{-1}. Other species such as velvet bentgrass and colonial bentgrass, which are used for the same application, have been characterized as being very salt sensitive. These species can only tolerate salinity levels that are less than 4 dS m^{-1} (Harivandi, 1988; Horst and Beard, 1977).

Marcum (2001) studied the salinity tolerance of 33 creeping bentgrass varieties and one variety of colonial and velvet bentgrass. Cultivars that had the highest level of salinity tolerance were Mariner, Grand Prix, Seaside, and Seaside 2. Penncross, Putter and Penn-G-6. The author found a substantial range in salinity tolerance indicating a great range of genetic diversity within the bentgrasses. Furthermore, the cultivars that performed best under the salt treatments were collected from areas with high salinity levels. Koch and Bonos (2011) also found variation in the salinity tolerance of bentgrasses using an overhead irrigation field screening technique. These researchers found that under saline irrigation Declaration, Kingpin, and 007 (all creeping bentgrass) performed best among cultivars, while EBM comp (colonial bentgrass), SR7200 (velvet bentgrass) and Tiger II (colonial bentgrass) performed poorly among tested cultivars.

Other cool-season turfgrass species have different salt tolerances as well. Tall fescue is known to be salt tolerant and has a 50% growth reduction range of 8-12 dS m^{-1} (Carrow and Duncan, 1998; Butler et al., 1985). Sometimes used for a similar application as tall fescue, Kentucky bluegrass is salt sensitive and can only tolerate salinity levels less than 4 dS m^{-1} (Marcum, 2000; Beard, 1973; Carrow and Duncan, 1998). Rose-Fricker and Wipff (2001) studied the effect of salinity on 64 varieties of Kentucky bluegrass. These authors used hydroponics to expose the bluegrass varieties to a 15 dS m^{-1} salinity solution for 8 weeks. It was found that cultivars such as Northstar, Ascot, and Moonlight performed best under salinity, while Kenblue, Livingston, and P-105 performed poorly among tested cultivars. Koch et al. (2011) used overhead irrigation under varying salinity regimes (3, 6, and 9 dS m^{-1}) to analyze the differences

between Kentucky bluegrass cultivars. It was found that Eagleton, Moonshadow, Fairfax, Cabernet, and Liberator performed best under salt treatments, while Baron, A03-85 and A03-TB246 were the most salt sensitive when compared among tested cultivars. Other researchers (Marcum, 2008; Ahti et al., 1980) found a low amount of variability in salinity tolerance within the Kentucky bluegrass species indicating the difficulty for selecting and breeding salt tolerant varieties.

Perennial Ryegrass is another cool-season turfgrass species that is used extensively on home lawns, athletic fields, and for overseeding golf courses on southern United States golf courses. Perennial ryegrass is considered to be moderately salt tolerant and is able to withstand salt levels ranging from 4-8 dS m^{-1}(Harivandi, 1988; Horst and Beard, 1977) or 6-10 dS m^{-1} (Harivandi et al, 1992). An interesting study was conducted by Gibeault et al. (1977) where the authors seeded seven perennial ryegrass cultivars into a salt affected (salinity levels =11.4 dS m^{-1}) golf course. Visual quality ratings were taken throughout the growing season. This data indicated that cultivars Pelo, Manhattan and NK-100 were the most salt tolerant varieties while K9-124 was the most salt sensitive.

There have also been numerous screening studies conducted on mature salinity tolerance in perennial ryegrass. Rose-Fricker and Wipff (2001) studied 45 cultivars for salinity tolerance after being exposed to salinity concentrations of 26 dS m^{-1}. These researchers found that at these salinity levels, only certain cultivars such as Brightstar SLT, PST-2SLW and Manhattan 3 were able to maintain their green leaf color. Cultivars

such as Allsport, Buccaneer, and MP-107 performed poorly under salinity when compared among tested cultivars. Koch and Bonos (2010) used an overhead irrigation screening technique and found that Brightstar SLT performed poorly under a salinity level of 15 dS m^{-1} while Palmer III performed well.

Perennial ryegrass is one of the species that may contain the endophytic fungus in the genus *Neotyphodium* that grows intercellulary between cells of the plant. The fungal endophyte presence results in the synthesis of alkaloids that protect the plant from above-ground feeding insects (Breen 1994) while the plant provides nutrients for the fungus to survive. This mutualistic relationship has been studied extensively in previous research (Clay, 1988). In addition to a reduction in herbivory, the endophyte has been shown to convey tolerance to abiotic and biotic stresses including drought tolerance (West 1994, Arachevaleta et al., 1989), phosphorus utilization (Malinowski and Belesky, 1999a), aluminum tolerance (Malinowski and Belesky, 1999b, Zaurov et al., 2001), and disease resistance (Clarke et al., 2000).

One of the most salt tolerant species of turfgrass is alkali grass which can maintain green turf cover and growth in saline conditions ranging from 20-30 dS m^{-1}(Marcum, 2000; Harivandi et al., 1992). At these salinity levels, establishment of other cool season turfgrass species is not possible. Alkaligrass can tolerate irrigation with salt levels equivalent to seawater as shown by Harivandi et al. (1982). These authors found that weeping alkaligrass (*Puccinellia distans* L. Parl.) showed higher levels of germination than Lemmon alkaligrass (*Puccinellia lemmoni* (Vasey) Scribn.) when sea

water was added to both germination pads and in sand. Butler et al (1974) observed alkali grass growing in areas in Illinois where salt deicing had destroyed all other vegetation. Soil tests from those areas indicated that total soluble salts were at levels over 30,000 ppm (46 EC). As a turfgrass, alkaligrass has reasonable turfgrass quality during the spring, but summer heat seems to be a primary limiting factor (Johnson and Bushman, 2014). Heat tolerant varieties must therefore be selected to increase the range of use of this species. A specific variety of alkaligrass, Fults, has been shown to be extremely salt tolerant due to its rapid accumulation of proline (Torello and Rice, 1986).

Because of its high salinity tolerance, alkaligrass has been studied physiologically for its increased levels of compatible solutes (such as proline) and production of antioxidant enzymes (Torello and Rice, 1986). It has been shown that alkali grass creates significantly higher levels of antioxidant enzymes such as superoxide dismutase, catalase, and ascorbate peroxidase when compared to other salt sensitive species. These antioxidants protect the cellular membrane from oxidative damage induced by high levels of salinity (Veerasamy and Huang, 2013).

Alkali grass is also very well known for growing in alkaline soils. Increasing Na^+ leads to sodic soils which contain a high alkaline pH. The pH of sodic soil is 8.5 or higher and can be very damaging to most turfgrass species (Carrow and Duncan, 1998). In salt-affected sites that have become alkaline, there has been research conducted to understand the effects of livestock consuming alkaligrass over time (Wang et al., 2011). This research concluded that feeding alkaligrass to livestock can be beneficial for

increasing feed intake and increasing meat production when compared to other forage crops. Using alkaligrass as a forage source will be useful in the future as more land becomes salt-affected. In conclusion, alkali grass possesses many important traits to persist in salt affected sites which may make it a suitable turfgrass selection for many sites in the future.

Saltgrass (*Distichlis spicata* L.) is a warm-season grass species that is known for its high level of salt tolerance been (50% growth reduction at 35 dS m^{-1}) (Marcum, 2000). It is low growing and spreads by rhizomes and sometimes stolons. This grass has been used extensively for wetland restoration where the plant acts as a guard between the ocean and the shore, protecting the land from pollutants and chemicals such as salts. Saltgrass has been utilized successfully in coastal restoration projects (Shadow, 2014). Additionally, saltgrass's tolerance levels to salinity are utilized for pastures that are watered with saline water (Skaradek and Miller, 2010). Salt grass grows in a wide range of soil types, pH levels (6.4-10.5[Shadow, 2014]), and salt levels, making it one of the most common and widespread halophyte species in the United States (Ungar, 1974). Along with salinity tolerance, saltgrass has excellent wear capabilities, compaction tolerance, and drought tolerance (Kopec and Marcum, 2001).

Qian et al. (2007) used 14 varieties of saltgrass to analyze the variability within the species. It was found that all varieties saw a 25% clipping yield reduction at a salinity range of 21.2 to 29.9 dS m^{-1}. This data indicates that saltgrass is one of the most salt tolerant species that can be used for a turfgrass site. Marcum et al. (2005) evaluated 21

desert saltgrass accessions and concluded that all tested accessions were more tolerant than bermudagrass. Pessarakli et al. (2006) used nitrogen-15 to enrich saltgrass plants and analyze nitrogen absorption in harvested plants after being exposed to varying levels of salinity. These researchers showed that shoot fresh/dry weights, shoot succulence, root dry weights, and nitrogen-15 content increased significantly under a salinity concentration of 200 mM when compared to the control. Elevating salinity levels higher than 200 mM, caused a decrease in all measurements. This research indicates that low levels of salinity benefit the growth of saltgrass which is the polar opposite of how other turfgrass species interact with salinity. This finding was further validated nn a pasture study (Pasternak et al., 1993) where brackish water was used to irrigate different forage crops (rhodes grass [*Chloris gayana* Kunth], bermudagrass, kallar grass [*Leptochloa fusca* L. Kunth], and saltgrass) and salt grass was the only species to maintain normal yields during second season growth.

Important weaknesses of saltgrass are its seed viability and its low germination percentage. Due to these factors it is recommended to propagate saltgrass by vegetative means (Shadow, 2014). Shaba et al. (2008) used various chemical treatments to increase the level of germination in saltgrass when under salinity stress (15 dS m^{-1}, 30 dS m^{-1}). It was found that Proxy (Bayer Environmental Science), thiourea, fusicoccin, ethephon, and kinetin increased germination of saltgrass seeds. This research may be useful in the future to increase seedling viability in many different turf species while under salinity stress. Additionally, work is being conducted at Colorado State University and The University

of Arizona to develop and breed genotypes of seed and vegatatively-propogated turf-type saltgrass for use in areas where soil and water salinities are high (Qian et al., 2013).

Salinity tolerance in the literature can vary depending on cultivars used in experiments and the methods in which the experiment was conducted. Marcum (2008) and Carrow and Duncan (1998) compiled tables summarizing the ranking of various turfgrass species for their ability to tolerate salinity and cause a reduction of 50% shoot growth. Extrapolating data from these two sources as well as other literature (Harivandi et al., 1992; Marcum, 2000; Madison, 1971; Horst and Beard, 1977) will be useful in creating a more accurate representation of salinity tolerance in turfgrass species (Table 1).

Germination of Turfgrass Seeds and Salinity

Germination begins with the imbibition of water by the seed and ends with the start of the elongation by the embryonic axis (radicle). The seed undergoes many events such as protein hydration, subcellular structural changes, respiration, and cell elongation which all contribute in turning a quiescent seed into a vigorous plant. For a seed to start germinating, the seed needs suitable temperature, oxygen, and water (Khan, 1977).

A very important part of establishment of a seedling is the mobilization of stored energy reserves. This is considered a postgerminative event although some mobilization can occur before germination finishes. The energy-rich starch reserves are converted into

forms that are readily available to be transported to sites where they are required. An important enzyme that converts amylose and amylopectin (components in starch) by hydrolysis is α-amylase. α-amylase converts these molecules into maltose and glucose which provide energy to a new seedling for rapidly metabolizing and growing organs (Bewley and Black, 1994). The production of α-amylase decreases when seeds are introduced into a salt environment (Oliveira-Neto et al, 1998). This may be an important contributing factor for the reduction in germination rate of seeds that are established in a salt affected site.

Additionally, giberellic acid (GA) plays an important role in germination. The production of GA from the scutellum causes the aleurone layer to create α-amylase enzyme. Because GA has such an important impact on germination, the introduction of salinity may pose a negative effect on GA production. Raeber and Lee (1991) used desert beardtongue (*Penstemon parryi* Gray) to show that a 24-hour pre-treatment with 500 ppm GA caused a significant increase in germination when compared to no pre-treatment when under all salinity treatments. At 3000 ppm NaCl, GA pre-soaked seeds exhibited 70% germination whereas non-presoaked seeds displayed 0% germination. Similar findings have been found in milk thistle (*Silybum marianum*) and sugar beets (*Beta vulgaris*) (Sedghi et al, 2010, Kandil et al, 2014). Ghodrat and Rousta (2012) conducted a similar study in corn (*Zea mays* L.) using presoaked seeds with GA to analyze the effects of salinity. Theses authors found that priming with GA had no effect on seed germination but some concentrations increased shoot length, root length, dry weight, fresh weight, and tissue water content.

Ashraf et al. (2002) conducted an experiment to assess whether gibberellic acid (GA_3) could remove the adverse effects of salinity stress in two spring wheat cultivars (*Triticum aestivum* L.); one salt tolerant cultivar and one salt sensitive cultivar. Plants that were 3 weeks old were subjected to 0, 100, and 200 mol m^{-3} of NaCl for 3 weeks followed by half of the plants receiving an exogenous application of 100 mg of GA_3. Plants were harvested three weeks after GA_3 application and fresh/dry weights of the roots and shoots were obtained. Unsurprisingly, fresh/dry weights of roots and shoots, plant height, and leaf area were found to decrease with increasing levels of salinity. Interestingly, the GA_3 treatment was successful in removing these negative effects in both the salt sensitive and salt tolerant cultivar. Net CO_2 assimilation rate was decreased by increasing salinity treatment, but exogenous application of GA_3 removed the negative effects of this measurement. The GA_3 treatment was successful in stimulating vegetative growth in wheat cultivars, although this growth hormone caused a slight decrease in grain yield.

Kabar (1989) used seven species of *Gramineae* including three cultivars of wheat, two cultivars of barley (*Hordeum vulgare* L.), rye (*Secale cereal* L.), and oat (*Avena sativa* L.) to understand the effect GA_3 and kinetin has on germinating seedlings under salinity stress. Salinity treatments were created using NaCl solutions having predetermined water potentials. Exogenous GA_3 had more of an effect than kinetin in alleviating salt stress on all species tested. In the wheat cultivars, the GA_3 treatment with salinity stress caused a 14 fold increase in germination compared to salinity treatment (-20.5 bars) only. In both barley and oat, GA_3 enabled 14% germination of seedlings at -

18.2 bars of salinity stress, while salinity stress alone maintained no germination. The author goes on to conclude that kinetin had little effect on *Gramineae* species while exogenous GA_3 benefits germination under salinity stress.

Molecular work has been conducted on Arabidopsis to analyze the GA-signaling pathway. Kim et al, (2008) demonstrated that a membrane-bound NAC transcription factor, NTL8, mediates salt regulation of seed germination via the GA pathway, which is independent from the hormone abscisic acid. NTL8 is induced by high levels of salinity. High salinity also represses the GA_3 oxidase 1 (GA3ox1) gene which supports the hypothesis that salt signals inhibit germination by repressing GA biosynthesis. Furthermore, this regulatory scheme may provide an evolutionary feature within the seed, which delays seed germination under high levels of salinity. The inhibition of GA may provide additional traits to analyze when breeding for salinity tolerance of cool-season grasses during germination.

After analyzing how exogenous GA_3 reduces NaCl-induced growth inhibition in rice, Wen et al. (2010) used a comparative proteomic approach to understand the mechanism of GA_3 activity in plants under salt stress. Seedlings were grown for 5 days and treated with salt and GA_3 while other treatments consisted of H_2O and salt. A total of 11 proteins were found to be regulated by salt and GA_3 which included proteins that are involved in photosynthesis and glycolysis. Additionally, some proteins identified were found to be novel proteins involved in response to salt in rice. The GA_3 caused a

significant up regulation of some salt-related proteins. The use of exogenous GA_3 to reduce salinity stress may have practical applications in the future.

Other studies have analyzed the effects of Trinexapac-ethyl ([TE] a popular growth regulator that inhibits the production of gibberellic acid and thus leaf elongation) applications of turfgrass grown and established on salt affected sites. Arghavani et al. (2012) used Kentucky bluegrass under five salinity regimes (0, 20, 40, 60, and 80 mM NaCl) and applied TE twice at 4 week intervals. Under low levels of salinity, TE increased turf quality, leaf total non-structural carbohydrates, and chlorophyll content. In high salinity treatments, the application of TE caused a decline in antioxidant enzyme activities, chlorophyll content, and leaf total non-structural carbohydrates when compared to the salinity treatment alone. The application of TE may be useful on sites with a low level of salinity, but using this product on high saline areas could be extremely detrimental.

Other important hormones such as auxin, cytokinin, and abscicic acid have crucial functions in growth and develop. Some examples include promoting protein accumulation, formation of new endosperm cells, triggering important enzymes, and many other essential operations (Bewley and Black, 1994). Xu et al (2011) conducted an experiment to investigate how levels of gibberellic acid (GA), abscicic acid (ABA), and indole-3-acetic acid (IAA) fluctuate in soybean (*Glycine max* (L.) Merrill). Two cultivars (salt-tolerant Lee68 and salt-sensitive N2899) were germinated on filter paper with a salt solution of 9.1 dS m^{-1}. They found that compared to controls, ABA content increased

and GA decreased in the plant, while IAA increased in Lee68, but did not change in N2899. The final germination percentage was not affected by salt, although the germination rate of Lee68 and N2899 was delayed by 0.3 and 1.0 day respectively. The researchers conclude that the increased IAA in salt-tolerant Lee68 might help seed germinate under salt stress. The GA content in this study was significantly reduced by salinity stress which caused a delay in germination. ABA content in both cultivars increased but Lee68 (salt-tolerant) exhibited a much higher level than N2899. ABA is important in a plant for stress tolerance and the authors claim that ABA may enhance salt tolerance in soybean.

Wang et al. (2001) used different stages of *Iris hexagona* to study the level of phytohormones within the plant. Salinity levels varied from 10 dS m^{-1} to 40 dS m^{-1} over a time period of 8 days. The short term effects of salinity were measured after 24 hours by isolating each phytohormone individually. ABA content in leaves was found to range from 0.2 to 1.2 µg/g DW (dry weight) and increased in response to salinity level and duration of salinity treatment. JA (jasmonic acid) increased in response to salinity concentration and duration of exposure from 0.2 to ~2 µg/g DW. IAA had the reverse impact, and rapidly declined 3-fold in the young leaves when exposed to salinity. SA (salicylic acid) also decreased from 2.8 to 1.7 µg/g DW. This data suggests that salinity has significant effects on these four growth regulators at a young growth stage. Similar to other studies, rising ABA content seems to be a defining hormone when studying salinity.

Techniques for Germination in Salinity

There are three main techniques for germinating seeds in a controlled setting (Zhang et al, 2011). These three techniques are germination in agar medium (Peacock and Dudeck, 1989; Dai et al, 2009), on germination paper (Zhang et al, 2011; Serena et al, 2012) and in a hydroponic system (Brilman and Sardar, 2010; Richardson and McCalla, 2008). Seed coating as well as media selection can impact both germination rate and germination percentages (Serena et al, 2012). In addition to controlled media, there are other methods to germinate seeds utilizing sterilized soil (Dewey, 1962) or sand (Harivandi et al, 1982). Choosing the correct media on which to germinate seeds plays a significant role on germination and salt ion accumulation during an experiment.

Agar

Peacock and Dudeck (1989) took some of the first steps in germinating turfgrass seeds on agar. In this study, warm season turfgrass seeds were placed on a 1% agar media salinized with synthetic sea salt at rates of 0, 1300, 2500, 3700, 4800, and 5800 mg L^{-1}. The agar solution was poured into petri dishes and placed in a germinator for the duration of the experiment (28 days). Seedling counts were taken at different intervals depending on the turfgrass species. A germinated seedling was defined as having a visible shoot when viewed at 2x magnification. This definition of a germinated seed has

been used in additional studies also using agar media (Serena et al, 2012; Dai et al, 2009). Results from this (Peacock and Dudeck, 1989) study showed that Bermudagrass germinated the fastest (13% per day) and bahiagrass (*Paspalum notatum*) was the slowest (5% per day). Bermudagrass also had the highest final germination (92%) followed closely by carpetgrass (*Axonopus affinis*) (90%) and centipedegrass (88%). One of the major results obtained from this study was the finding that at the highest salinity treatment, final germination was unaffected in both bermudagrass and centipedegrass, and only slightly reduced (8%) in bahiagrass. In warmer climates, bermudagrass may be a more suitable choice for establishing turf in a salt affected site. In this study, centipedegrass showed no reduction in final germination percentage, but this species should not be used in areas of high salinity because of its low level of salinity tolerance during mature growth which only ranges from 3-8 dSm^{-1} (Marcum, 2000).

Torello and Symington (1984) conducted research on five Kentucky bluegrass cultivars using agar media with increasing salinity concentrations of sodium chloride. Salinity tolerance was quantified based upon the reduction in root and leaf length. In addition to the bluegrass cultivars, two cultivars of creeping red fescue and one cultivar of alkaligrass were tested under the same salinity regimes. The creeping red fescue and alkaligrass were determined to have higher salinity tolerance when compared to all of the bluegrass cultivars. Among the bluegrasses, Adelphi and Ram I showed high tolerance to salinity across all measurements, while Baron performed poorly when exposed to the salinity stress.

Dai et al (2009) also used 1% agar solutions to compare greens type *Poa annua* L. to other cool-season species under salinity stress. When analyzing final germination percentage and germination rate, it was found that salinity stress had a significant impact on germination rate. Salinity levels of 5 dS m^{-1} had a significant effect on germination rate but not on final germination percentage. It was also found that Charger II (perennial ryegrass) and Mariner (creeping bentgrass) required the highest salinity levels to reduce germination rates by 50% (average of 13.8 dS m^{-1}). The other bentgrass cultivars (Seaside II and Penncross) and most annual bluegrass (*Poa annua)* lines showed intermediate levels of salinity tolerance. Kentucky bluegrass cultivars (Moonlight and Northstar) showed the lowest level of salinity tolerance. These results follow previous reports of variation in levels of salinity tolerance in mature turfgrass species (Turgeon, 2008; Carrow and Duncan, 1998). Further studies should be conducted with more cultivars to screen a larger population of a particular species.

Germination Paper/Blotter Paper

Camberto and Martin (2004) germinated fifty rough bluegrass (*Poa trivialis*) seeds of each cultivar (33 total cultivars used) on pre-moistened germination paper in petri dishes. The solution used to moisten the germination paper contained different levels of salinity. The petri dishes were then sealed with parafilm and analyzed at 2 day intervals for 25 days in a growth chamber. Results from this study concluded that germination rates vary considerably among cultivars of rough bluegrass. Findings also

were in agreement with other studies, that rough bluegrass final germination percentages did not vary much between cultivars.

When using germination paper it is extremely important to use parafilm to seal the petri dishes. If evaporation occurs while an experiment is underway, salt concentrations will increase. Addition of any concentration of solution will yield different salinities than the concentration implemented at the start of the study. Dudeck et al. (1986) stated that blotter paper loses water more quickly than agar media which may lead to the seed experiencing higher levels of salinity than originally intended. Serena et al (2012) also had lower levels of germination of turfgrass seeds on germination paper which can be attributed to the rewetting of germination paper as water evaporated throughout the experiment. Because of this methodology, levels of salinity were higher at the end of the experiment when compared to the beginning of the experiment.

Hydroponic

Zhang et al (2011) compared three germination media (Agar medium, germination paper, and hydroponics) to screen turfgrass for salinity tolerance. Solutions were made of NaCl at levels ranging from 0-20,000 parts per million. Thirty non-sterilized seeds were placed in cells and were floated on a double layer of plastic screens. The plastic screens were used to allow the roots of germinating seedlings to penetrate down into the solution below. Deionized water was replaced with the same concentration solution every 4 days and an aerator was utilized to supply oxygen to the roots. The

authors observed that there were no significant differences in salt tolerance among Kentucky bluegrass, tall fescue, and creeping bentgrass when analyzing leaf area and final germination percentage. The authors propose that analyzing germination rate is the most important feature to study and that agar mediums may function more similarly to a soil system when compared to germination paper and a hydroponic system. Using a hydroponic system requires more management (replacing solutions), but allows for better seedling growth because of the amount of space available for root growth (Dudeck et al, 1986).

Richardson and McCalla (2008) conducted a hydroponic study with varying salinity levels of 12, 16, 20, and 24 dS m^{-1}. The hydroponic system used a foam insulation board with 12 holes and a nylon screen affixed to the bottoms of the holes. The insulation floated on the surface of the solution so the nylon screens were submerged. Twenty-five seeds of meadow fescue (*Festuca pratensis*) and perennial ryegrass were placed in the cells and monitored for germination daily for 14 days. Germinated seedlings were counted when both radicle and coleoptile emerged and were removed from solution. Results indicated that by day 14, 80% of all ryegrass emerged in all solutions except 20 and 24 dS m^{-1}. At 20 and 24 dS m^{-1}, germination was reduced by 60% and 45% respectively. These results indicate that high levels of salinity may be used to screen perennial ryegrass seedlings when using a hydroponic solution.

Selection for Kentucky bluegrass varieties during germination and early growth by Horst and Taylor (1983) were conducted using mats floating on tanks with salt

solutions. A total of 44 cultivars were used and measurements included total germination, days to initial germination, leaf blade length, and leaf bladé fresh weights. Salinity slowed germination rates and caused a negative effect on all measurements taken in 70% of the tested cultivars. Cultivars Nugget, Arista, Prato, Baron, Delta, and Park were among the most salt tolerant cultivars tested.

Ashraf et al. (1986) analyzed root growth of seven grass species including perennial ryegrass, creeping bentgrass, colonial bentgrass, tufted hair grass (*Holcus lanatus* L.), creeping red fescue, orchard grass (*Dactylis glomerata* L.), and weeping alkaligrass. These species were then selected for root growth during germination under saline conditions. Selection started at a germination stage on rafts of black alkathene beads, where seeds with the longest radicle were selected as a high tolerance group, and seeds with a radicle less than 0.5 cm were selected as a low tolerance group. These individuals were grown in neutral conditions until they reached maturity. At maturity, plants were again subjected to varying levels of salinity for 14 days and root lengths were measured. It was reported that salinity inhibited germination in all species, but only a very small number of individuals within the salt tolerant species were able to root at the highest salinity levels (250 mM NaCl). An example of a species that was affected greatly by salinity levels was perennial ryegrass which had 1 seedling germinate and root while 99 seeds failed to germinate. Additionally, selection of seedlings that had the longest root at germination translated into plants that had longer roots at maturity while the opposite was true for seedlings selected for small roots. The variability in salinity tolerance for these seven species indicates that selection for this trait may be useful in

plant breeding programs. Selection for root growth at a germination stage may be useful in creating roots that are vigorous and deep at maturity.

Soil

Dewey (1962) germinated crested wheatgrass (*Agropyron desertorum*) in soil to determine the effect of salinity on germination. Using three different salinity solutions (9, 18.75, and 28.125 dS m^{-1}) and 4 different cultivars, it was found that soil salinity levels were much higher at the end of the 15 day experiment. At the highest salinity, soil conductivity was 18.4 dS m^{-1} at the beginning of the experiment and 22.7 dS m^{-1} at the end of the experiment. This research proves that salts accumulate in the soil and can negatively impact seedlings. The author also found that average germination percentages of the cultivars at 9, 18.75, and 28.125 dS m^{-1} were 87.6, 58.4, and 22.9%, respectively. The author states that there is sufficient genetic variability within crested wheatgrass with regards to salinity tolerance germinability, to make selection procedures effective in developing salt-tolerant strains of this particular species.

Germination Mechanisms

It has been reported that the mechanism for seedling germination under saline conditions is different than mature plant resistance (Maas, 1985). This mechanism is not well understood and many researchers are currently analyzing changes in the seed during

germination. Researchers have indicated that the main reason for germination failure is due to the inhibition of water uptake by the seed caused by the high level of salinity in the environment (Coons et al., 1990). Other researchers have contributed the lack of growth to ion toxicity and salt accumulation within the newly emerging seed (Atak et al, 2006). This contrast has been the central dilemma of salinity damage in mature plants as well as germinating seedlings (Greenway and Munns, 1980). Many studies have been conducted to further understand the effects that may inhibit germination of seeds in a saline environment.

Water Relations of Seeds

Osmotic adjustment is an important feature to analyze during germination because water is essential for healthy seed germination. Hadas (1977) showed that a decrease in germination percentage and germination rate was related to a reduction in water absorption into the seeds at the imbibition stage in leguminous seeds. Werner and Finkelstein (1995) used *Aribidopsis thaliana* mutants that expressed genes to reduce sensitivity to salt and osmotic stress during germination and found that salinity decreased the amount of water absorbed by seeds and also reduced growth of roots and shoots. Literature has suggested that osmotic effects on seeds vary greatly across different plant species therefore further analysis of each individual species should be investigated to understand the effects of osmotic stress (Atak et al, 2006).

Kaymakanova (2009) conducted an experiment to analyze the effects of water relations on bean (*Phaseolus vulgaris L.*) seeds. Seeds were germinated in a 100mM solution of NaCl on filter paper. Water uptake was recorded for 12 hours by first measuring initial weights and then measuring weights after water absorption. Results indicated that water uptake is reduced in salt-treated seeds in comparison to distilled water. A reduction of 17% moisture was lost in a particular cultivar when observed in saline conditions. Collecting and analyzing seed weights to understand how a seed imbibes water under salinity stress may be a feasible option to further understand seed water relations.

Atak et al (2006) used Triticale (*x Triticosecale Wittmack*) to show the effects of water relations on seeds. The experiment was conducted on filter paper with varying levels of salinity ranging from 2.4 to 13.2 dS m^{-1}. Seeds were removed at 6, 12, and 24 hours and were weighed after surface water was removed. Dry seed weights were then compared to seeds that had undergone water absorption. The results from this analysis revealed that increasing NaCl levels did not cause water uptake to differ statistically among cultivars but salinity caused a decrease in water uptake when cultivars were averaged within treatments. In this study, a monocot was used which may be more comparable to a turfgrass species. These results differed from the results of Kaymakanova (2009) where a dicot species was utilized to study salinity tolerance. The study of water uptake should be explored using various turfgrass species.

Ion Accumulation in Seeds

Because the source of salt sensitivity during germination is not fully understood, research has been aimed at the analyses of ion accumulations that are causing ion toxicity within the seed and emerging radicle (Leopold and Willing, 1986). It has been observed that plants exposed to saline environments take up high amounts of Na^+, and the uptake of K^+ and Ca^{2+} is significantly reduced. It is well known that K^+ and Ca^{2+} are required by plants to maintain the integrity and functionality of membranes (Marschner, 1995).

Atak et al (2006), not only studied seed weights due to water absorption, but also examined the toxic effects of NaCl on seed germination in *Triticale*. Using salinity solutions ranging from 2.4 to 13.2 dS m^{-1}, the researchers germinated three *Triticale* cultivars on filter paper. It was discovered that salinity increased the accumulation of Na^+ and decreased the accumulation of K^+ ions in the roots and shoots. The accumulation of Na^+ and Cl^- gradually increased in seeds while K^+ diminished. It was concluded that the delay in germination was due to higher Na^+ accumulation and not due to osmotic stress in all *Triticale* cultivars.

Ashraf and Orooj (2006) conducted a greenhouse study with ajawai (*Trachyspermum ammi* L.) to analyze ion accumulations within the glycophyte (a plant that will only grow healthily in soils with low salinity levels). They evaluated the roots and shoots of this species for ions including Na^+, Cl^-, K^+, and Ca^{2+}. Their results found that Na^+ and Cl^- increased in both roots and shoots, while K^+ and Ca^{2+} decreased consistently with the progressive increase in salt level in the growth media (sand). It was

also found that the Ca^{2+} /Na^{+} ratio in the shoots was significantly higher than 1, which was determined to be the level suggested for normal functioning plants under saline conditions (Wyn Jones, 1981). Therefore, analyses of the Ca^{2+} /Na^{+} ratio may be an important aspect of salinity tolerance. This study was conducted on 67 day old plants, but ion accumulation can occur immediately during seedling emergence.

Breeding for Salinity Tolerance

The goal of a plant breeder is to develop new germplasm and varieties that are resistant/tolerance of abiotic and biotic stresses, however breeding for salt tolerance has been slow and difficult (Rose-Fricker and Wipff, 2001). The reasons for the slow progress in breeding salt tolerant plants include: 1) incomplete knowledge of the effects of salinity on plants; 2) poor means of detecting and measuring salinity; 3) selection methods that are ineffective; 4) the complexity of interactions between salinity and the environment; 5) lack of ability to capture the vague effects besides growth responses; 6) the interactions of ionic and osmotic properties of salts on plants; 7) the changing salt tolerance at varying stages of growth; 8) the large number of plant physiological parameters that contribute to salt tolerance; 9) salinity tolerance is a quantitative trait that is controlled by multiple genes (Rose-Freicker and Wipff, 2001). Development of salt tolerant germplasm is difficult but successful gains have been made in the past years using multiple methods to create salt tolerant cultivars.

Heritability

A very important feature of plant breeding is the understanding of the inheritance of particular traits. Knowledge of inheritance allows for efficient selection of quantitative traits such as salinity tolerance. Understanding the inheritance of salinity tolerance is an important tool when breeding for such a complex trait (Winicov, 1994). Heritability estimates are used by plant breeders to predict the expected improvements after cycles of selection (Nyquist, 1991). Heritability is defined as the proportion of the variation that is observed in a progeny that is inherited (Poehlman and Sleper, 1995). The main objective of estimating heritability and the genetic parameters that compose the heritability estimate is to compare the expected gains from selection based on alternative selection strategies (Holland et al, 2003) Heritability estimates are useful for comparing the gain from selection under different experimental designs; using the heritability estimates with other important factors such as costs of additional replications within each macroenvironment, amount of evaluation years, and additional locations for each evaluation will allow for the creation of an effective breeding strategy (Milligan et al. 1990). There are two important heritability estimates used by plant breeders; broad-sense and narrow-sense (Fehr, 1987). Estimating heritability is based on partitioning variation of a quantitative characteristic into genetic and environmental components. Selection is most effective when there is high genetic variation in relation to environmental variation (Poehlman and Sleper, 1995).

Broad-Sense Heritability

Broad-sense heritability estimates heritability on the basis of all genetic effects (Poehlman and Sleper, 1995). Additive, dominance, and epistasis genetic variance are all incorporated into broad-sense heritability and if the proportion of the genotype and environment are high this would be an indication that the phenotype is determined by the genotype. Broad-sense heritability can be useful as a measurement in any species, but it is most useful in crops that reproduce vegetatively or apomictically for commercial crop production (Nyquist, 1991).

Horst and Taylor (1983) evaluated salinity tolerance in Kentucky bluegrass. They germinated seeds from 44 cultivars on floating mats using a hydroponic system. Three different salinity levels were used (7500, 12,500, 15,000 ppm) with four replications arranged in a randomized complete block. Germination percentage and germination rate were both found to be highly significant. This study also showed that broad-sense heritability estimates for germination percentage and germination rate could be valuable selection criteria for use in screening Kentucky bluegrass cultivars for increased salinity tolerance at the germination stage. Broad-sense heritability estimates were found to be 0.44 for percent germination and 0.31 for germination rate. Because these heritability ratios include both additive and non additive genetic effects, the author states that studying parent-progeny relationships would be important to estimate genetic progress that could be expected in progeny from selected parents.

Using a hydroponic system, broad-sense heritability estimates were also determined for salinity tolerance parameters such as relative leaf firing (indication of salt

injury), root and shoot growth, and sodium and potassium content (Qian et al., 2000). Twenty-nine zoysiagrass cultivars and experimental lines were exposed to gradually increasing salt solutions for 25 days with a final concentration reaching 42.5 dS m^{-1}. This concentration was then held constant for an additional 21 days. Broad-sense heritability estimates were moderate to high (leaf firing = 0.67, shoot growth = 0.50, root growth = 0.41, sodium accumulation = 0.54, and potassium content = 0.40) and indicate that genetic progress may be made to develop salt tolerant zoysiagrass in a conventional breeding program.

Broad-sense heritability estimates for salinity tolerance have also been studied in other plant species. Gregorio and Senadhira (1993) determined the broad-sense heritability in a rice population grown under salinity stress. Two-week old seedlings were grown hydroponically in salt concentrations at 12 dS m^{-1} for 19 days. Salinity tolerance in rice has been characterized by its ability to exclude Na^+ ions and increase absorption of K^+ ions. To explore this physiological mechanism, plant shoots were analyzed for Na-K ion balance. Broad-sense heritability estimates were equal to 0.37 when analyzing this particular trait in the rice population which indicated that this mechanism is greatly affected by environmental factors.

From the cited literature it is clear that the inheritance of germination under saline conditions is controlled somewhat by genetic effects but that there is also a significant environmental component. For breeding it will be important to determine what types of

environmental influences most affect germination in order to efficiently select genotypes with improved germination under saline conditions.

Narrow-Sense Heritability

Although broad-sense heritability estimates are useful for determining the variation due to genetic effects, narrow-sense heritability is much more useful for plant breeding. Broad-sense heritability estimates include both dominance and epistatic gene effects. These genetic effects can be predicted in clonally propagated crops and crops exhibiting apomixes but for an out-crossing species, both dominance and epistatic genetic effects cannot be accurately predicted (Poehlman and Sleper, 1995). Narrow-sense heritability estimates measure the portion of additive genetic effects compared to the total observed variation (Nyquist, 1991). Because the most effective breeding design (recurrent/mass selection) maximizes the use of additive variance, it is important to understand the ratio of the additive genetic variance to the total phenotypic variance.

Gragorio and Sanadhira (1993) were able to not only estimate broad-sense heritability, but were able to generate narrow-sense heritability estimates of a rice population. Their findings indicate that the genes controlling the Na-K ratio had a narrow-sense heritability of 0.20. The authors go on to say that the low NA-K ratio is governed by both additive and dominance gene effects and because of the low inheritance of this trait, selection must be done at a later generation and under controlled conditions in order to minimize environmental effects.

Seedling stage salinity tolerance was analyzed using forage rape (*Brassica napus* L.), berseem clover (*Trifolium alexandrinum* L.), alfalfa (*Medicago sativa* L.), and red clover (*Trifolium pratense* L.). Using a hydroponic system, ten-thousand seeds of each species were screened for shoot growth at high NaCl concentrations with a selection intensity of < 1% (Ashraf et al., 1987). Narrow-sense heritabilities from parent-progeny regression were 0.74 for forage rape, 0.50 for berseem clover, 0.52 for alfalfa, and 0.98 for red clover. Because of the high heritability estimates, the authors concluded that a recurrent selection breeding program is an effective and efficient way to screen for salinity tolerance within these particular species due to the high proportion of additive gene effects associated with this trait.

Divergent selection

Another selection process that has been used for determining the efficiency for screening of a specific trait is divergent selection. By analyzing a specific trait over multiple generations, it is possible to calculate a gain from selection. Divergent selection occurs in plant populations readily due to environmental changes and selection pressures in two populations that are isolated from one another. By selecting this trait at both optimal and suboptimal levels and breeding in both directions, it is possible to see both the genetic increase in this trait and the genetic decrease in this trait (Mithen et al, 1995). This technique will be useful in understanding the realized heritability of salinity tolerance during germination.

Venuprasad et al. (2008) used divergent selection on four rice populations to determine the yield advantage of stress selected lines in comparison to nonstress selected lines. Two cycles of selection occurred under drought stress in upland or under nonstress conditions in lowland environments. Averaged across populations and environments, it was determined that the stress-selected lines had a yield advantage of 25 and 37% over the nonstress-selected and random lines, respectively. The authors conclude that direct selection for grain yield under stress is effective and does not reduce yield potential. The authors go on to say that selection under managed drought stress in the dry season can result in yield gains.

Using selection in one direction is also very useful to find gain from selection for a specific trait or a combination of traits. Bonos et al. (2004) used recurrent selection to determine the feasibility of simultaneously selecting plants with low-shoot-to-high-root ratios and increased root mass in a sand profile using flexible tubes in a greenhouse setting. The top 2 to 4% of the population were selected for low clipping weights and high root weight. After 2 cycles of screening, gain from selection was approximately 41% in the narrow germplasm base population and 81% in the broad germplasm base population in tall fescue. Perennial ryegrass exhibited a 130% gain from selection in a turf-type and 367% in a forage-type population. The authors conclude that this breeding approach should be very effective in developing turfgrasses with improved rooting characteristics.

Jensen et al. (2005) studied the LD_{50} of wheatgrass (*Elymus hoffmanii*) by irrigating plants with increasing levels of salinity in a greenhouse. The solution used to irrigate was increased by 6 dS m^{-1} every 1 to 2 weeks until the EC reached 42 dS m^{-1}. When this level of salinity was reached, it was maintained until plant mortality occurred. Using divergent selection, it was found that the largest single gain was found from cycle-0 to cycle-1 which required an additional 145 ECdays to reach an LD_{50} level. The author states that smaller gains were made as selection cycles continued in the following years and that field evaluation is necessary to validate the application of the novel screening method.

Somaclonal variation

Increasing the amount of variation in a population will allow for the selection of dominant genotypes to use in future generations of breeding. Somaclonal variation is a way to obtain variations in the properties of plants *in vitro*. Recently, somaclonal variation has been used as another means for the creation of salt tolerant germplasm. The process uses tissue culture on media with chemical modifications to regenerate callus tissue and can lead to point mutations, loss of genes, change in chromosome numbers, change in chromosome structure, and chromosomal rearrangements. Somaclonal variation is another source of germplasm for plant breeders, but can also lead to undesirable mutations. Variants must undergo extensive and extended field trials to fully understand how the genotype will perform under normal field conditions (Larkin and

Scowcroft, 1981). In species where hybridization is difficult (such as Kentucky bluegrass), somaclonal variation is an important tool for creating new genetic variation.

Zhang et al. (2003) used somaclonal variation for the production of salt tolerant Kentucky bluegrass mutants. The authors created 30 independent regenerated lines from embryonic callus of the cultivar Shamrock. The authors found ten regeneration lines that showed significant salinity tolerance improvements in the greenhouse at 1.0-1.2% NaCl concentration. Eight of the lines also showed a significant improvement for drought resistance. The findings prove that the use of somaclonal variation is therefore very useful for creating variation especially in facultative apomicts such as Kentucky bluegrass.

Lu et al. (2007) used somaclonal variation to produce 3 new variant lines from cultured calli of triploid bermudagrass cv. Tifeagle. Triploid bermudagrass is a prime candidate for somaclonal variation because it is sterile and will not reproduce sexually. The variants produced in this study showed higher relative growth and less injury than Tifeagle under salt stress. Additionally, after exposing these variants to drought conditions they had higher relative water content and lower electrolyte leakage than Tifeagle indicating that the selection under salt stress may be useful for selecting drought tolerant varieties. Under no salinity concentration all three variant lines also showed higher proline content. Using somaclonal variation for producing salt and drought tolerant cultivars in sterile plant species is extremely beneficial in creating new germplasm.

Conclusions

Salinity is a growing problem in agriculture due to the increase of salt affected sites and the decrease in availability of potable water sources. Turfgrasses are prime candidates for the use of wastewater irrigation but this alternative water source has an increased level of salinity. Irrigation with saline water can be detrimental to germination of turfgrass seedlings as well as mature turf. Previous research has been successful in creating screening techniques to demonstrate significant difference in salinity tolerance of cool-season turfgrass species for both germination and mature growth. Using overhead irrigation techniques and other methods to further mimic field conditions may be more useful in screening perennial ryegrass cultivars for increased salinity tolerance.

Goal of This Thesis: The goal of this thesis was to develop two novel salinity screening procedures for perennial ryegrass seedlings in a soil media. These screening techniques can be used in the future to efficiently select cultivars that have increased levels of salt tolerance. Additionally, this thesis investigates the endophyte-host interaction of mature perennial ryegrass plants to evaluate what role (if any) the endophyte plays when grasses are under salinity stress.

This thesis reports the following:

1. Screening and evaluation of perennial ryegrass cultivars in native soil media under varying levels of salinity
2. A novel growth chamber screening technique for evaluating perennial ryegrass seedlings under salt stress
3. The endophyte effects on salinity stress in mature perennial ryegrass plants

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Table 1. Rankings of species based upon estimation using current literature Sources: Harivandi et al., 1992; Marcum, 2000; Madison, 1971; Horst and Beard, 1977.

Common name	Scientific name	50% Growth Reduction Average (dS m^{-1})	Grass Type (cool/warm-season)
Saltgrass	*Distichlis spicata sp. Stricta*	35	warm
Seashore paspalum	*Paspalum vaginatum*	25	warm
Alkaligrass	*Puccinella* spp.	25-30	cool
St. Augustine	*Stenotaphrum secundatum*	25	warm
Bermudagrass	*Cynodon dactylon*	18-22	warm
Western wheatgrass	*Agropyron smithii*	14	cool
Zoysiagrass	*Zoysia japonica*	14	warm
Kikuyu	*Pennisetum clandestinum*	12	warm
Slender creeping red fescue	*Festuca rubra* L. spp. *trichophylla*	10	cool
Tall fescue	*Festuca Arundinacea*	8-12	cool
Creeping bentgrass	*Agrostis stolonifera*	8-10	cool
Perennial ryegrass	*Lolium perenne*	4-8	cool
Fairway wheatgrass	*Agropyron cristatum*	7	cool
Buffalograss	*Buchloe dactyloides*	7-11	warm
Blue grama	*Bouteloua gracilis*	5	warm
Hard fescue	*Festuca longiflora*	4	cool
Centipedegrass	*Eremochloa ophiuroides*	3-8	warm
Creeping red fescue	*Festuca rubra* L. spp. *Rubra*	3-7	cool
Carpetgrass	*Axonopus* spp.	3	warm
Bahiagrass	*Paspalum notatum*	2-3	warm
Annual bluegrass	*Poa annua*	0-2	cool
Colonial bentgrass	*Agrostis tenuis*	0-2	cool
Velvet bentgrass	*Agrostis canina* L.	0-4	cool
Rough bluegrass	*Poa trivialis*	0-3	cool
Kentucky bluegrass	*Poa pratensis*	0-4	cool

CHAPTER 2

Germination and Early Establishment of Perennial Ryegrass Cultivars Under Varying Levels of Salinity

Abstract. Perennial ryegrass (*Lolium perenne* L.) is a cool-season grass known for its quick establishment making this species popular for winter overseeding warm-season turfgrasses on golf courses and sports fields. Ryegrass has moderate salinity tolerance which has caused germination issues especially on sites that are mandated to use effluent or waste water. Wastewater can contain soluble salts, which if applied as irrigation can damage seedlings directly and can also accumulate in the soil which can also influence germination and establishment of young seedlings. The objectives of this study were 1) to determine what levels of saline irrigation water cause reductions in germination and early establishment of perennial ryegrass in a sandy loam soil; 2) how much of a reduction in germination and early establishment was observed with each salinity level; and 3) to determine the variability in salinity tolerance among eight perennial ryegrass cultivars. Eight perennial ryegrass cultivars were seeded into a sterilized sandy loam soil in pots and irrigated overhead with seven saline water levels ranging from 0.25 dS m^{-1} to 6.0 dS m^{-1} under greenhouse conditions. Saline treatments of 3 dS m^{-1} had a significant reduction of 34% while 6 dS m^{-1} caused a 58% (Run 1) and 75% (Run 2) reduction in percent germination compared to control pots (0.25 dS m^{-1}). The highest salinity treatment (6 dS m^{-1}) caused a significant reduction in percent germination. Cultivars Linn, Paragon GLR, and Zoom had the highest seedling vigor

regardless of salt stress. There was no cultivar by treatment interactions for any measurements indicating that cultivars germinate similarly in different salt water treatments suggesting that germination under salinity stress may be more affected by environmental conditions than genetic differences.

INTRODUCTION

Salt affected turfgrass sites are becoming more common as usable land becomes salinized (Abrol et al., 1988). Turfgrass sites can become salt affected through the use of reclaimed waste water (Duncan et al., 2000), saltwater intrusion into groundwater (Newport, 1997), saltwater spray, or ice melting salt runoff (Marcum, 1994). Arid and semi-arid regions have a higher potential to accumulate salts due to insufficient potable water or rain to leach salts from the soil (Carrow and Duncan, 1998).

Wastewater is defined as treated or semi-treated water from a water treatment plant that has been remediated through physical and chemical means (Lazarova and Bahri, 2005). During the treatment process, the additions of salts are used to treat incoming wastewater. Due to the additional expenses needed for removal, these salts remain in the water after the treatment process (Gross, 2008). Increasing numbers of turfgrass sites have potential to use alternative water sources for irrigation since these are not agricultural land used for food production.

Salinity can cause wilt and leaf burn on established turfgrass, and in extreme cases death (Horst, 1991). Salinity delays germination in many grass species such as Kentucky bluegrass (*Poa pratensis* L.), creeping bentgrass (*Agrostis stolonifera* L.), and perennial ryegrass (*Lolium perenne* L.) (Harivandi et al., 1982, Dai et al., 2009). Dai et al. (2009) found that a salinity level of 14.07 dS m^{-1} reduced the germination rate of perennial ryegrass by 50% while Kentucky bluegrass varieties only required 4-7 dS m^{-1} for the same reduction percentage. Perennial ryegrass germinates more quickly than other cool-season species and is used to overseed dormant bermudagrass [*Cynodon dactylon* (L.) Pers.] for winter play on golf courses and sports turf. Therefore, identifying acceptable salinity levels required for perennial ryegrass cultivars to germinate successfully will be important to provide a quick, high quality turf cover in salt-affected sites using effluent water.

It is well known that perennial ryegrass is moderately salt tolerant and is able to withstand salt levels of 6 to 10 dS/m (Harivandi et al, 1992). Using these salinity levels *in vitro* has been shown to slow germination and only disrupt final germination percentage slightly (Peacock and Dudeck. 1989). Rose-Fricker and Wipff (2001) found that at a salinity treatment of 15.625 dS m^{-1}, Charger II maintained an 84% germination rate while Manhattan II dropped to 57% germination. Dai et al. (2009) found similar reductions in Charger II for germination rate but also found that a salinity level of 15.25 dS m^{-1} only caused a reduction of 25% for final germination percentage.

An important aspect for the study of salt tolerance is the media in which plants are grown. Many studies have analyzed seed germination under salinity using soil less media such as blotter paper (Camberato and Martin, 2004, Johnson et al., 2007), agar (Serena et al., 2012), and hydroponics (Marcum, 2000). Zhang et al., (2011) and Serena et al. (2012) determined that germination method (agar, germination paper, or hydroponic) was a factor in the evaluation of salinity tolerance in turfgrass and should be a consideration when evaluating turfgrasses for salinity tolerance. These controlled methods allow for consistent salinity levels due to the fact that one can either check salinity levels throughout the study (hydroponics) or control water loss due to evaporation (sealing petri dishes with agar and blotter paper). In soils the results may be more variable. Constant irrigation with wastewater will cause the accumulation of salts in soil. Different soils have varying CEC (Cation Exchange Capacity) sites which can cause the excess absorption of sodium ions. As sodium dominates CEC sites of soil particles, larger pore spaces become destroyed which can affect soil permeability. This can cause surface sealing and reduction of oxygen to plant roots (Carrow and Duncan, 1998, U.S. Salinity Laboratory. 1954). Therefore, analyzing the effects of irrigation with varying levels of salt on germination in native soils may yield more realistic results for germination and establishment under salinity stress.

The objectives of this study were 1) to determine what levels of saline irrigation water cause reductions in germination and early establishment of perennial ryegrass in a sandy loam soil; 2) to determine the reduction in germination and early establishment

observed within each salinity level; and 3) to determine the variability in salinity tolerance among eight perennial ryegrass cultivars.

MATERIAL AND METHODS

Seed of eight perennial ryegrass cultivars (Apple GL, KSA, Palmer III, Zoom, Linn, Paragon GLR, ESP, and RKS) were obtained from various seed companies. A Freehold sandy loam soil (Fine-loamy, mixed, active, mesic Typic Hapludults) was collected from the Rutgers Plant Biology Research and Extension Farm in Freehold, NJ and sterilized using a soil sterilizer (Famco Inc. Medina, Ohio). Equal quantities of sterilized soil were placed into 10.16x10.16 cm plastic horticulture pots (Griffin Greenhouse & Nursery Supplies Tewksbury, MA) to a weight of 0.55kg.

Pots were placed in a greenhouse with temperatures maintained between 17 and 24°C. Four hundred Watt high pressure sodium bulbs provided supplemental lighting under 14-hour day lengths which provided photosynthetically active radiation in the range of 90-105 µmol $m^{-2}s^{-1}$. Pots were seeded at a rate of 5.43g/m^2 and granular fertilizer (10-10-10) was applied to each pot at a rate of 1.48 g N/m^2 at the time of seeding. The experiment was set up as a completely random design with 3 replications. Salinity treatments were made using equal quantities of NaCl and $CaCl_2$ mixed with tap water. Seven treatments were applied to each cultivar: (control): 0.25 dS m^{-1}, 1.0 dS m^{-1}, 2.0 dS m^{-1}, 3.0 dS m^{-1}, 4.0 dS m^{-1}, 5.0 dS m^{-1}, 6.0 dS m^{-1}. These salinity levels capture the range of realistic effluent water that is being used across the country (Huck et al.,

2000). Salinity treatments were applied to the top of the pots using a trigger sprayer (Delta Industries, King of Prussia, PA) to simulate overhead irrigation. Four hundred ml/m^2 of each saline water treatment were applied to each pot daily in the beginning of the study to keep seeds fully hydrated. Fourteen days after seedling emergence, irrigation was reduced to three times a week.

Two types of visual ratings (percent green and emergence) were collected weekly. Digital images were also collected weekly and analyzed using SigmaScan Pro 5 to quantify variability between cultivars and treatments (Richardson et al., 2001). Percent green visual ratings were based on the percent green tissue in the 10x10 cm pot and ranged from 0 to 100 and indicated establishment of turfgrass stand. Percent green ratings were collected weekly starting two weeks after planting and continued weekly until the end of the six week study. Seedling emergence ratings were collected by assigning a number value (1=poor emergence 9=highest emergence) to each pot that indicated the level of germination within that pot. Emergence ratings were collected weekly for a total of three weeks after planting. A seedling was deemed germinated when the presence of a leaf extended past the coleoptile. This study was replicated 2 times- in the winter of 2010 and the spring of 2011. Soil electrical conductivity and pH were collected for three replicates per treatment. Soil within each treatment was amalgamated before samples were taken. The soluble salts by 1:2 (V:V) soil:water extract method was used to analyze soil electrical conductivity (Dellavalle, 1992). pH of each soil sample was measured using the 1:1 (V:V) soil:water method (Miller and Kissel, 2010).

Statistical Analysis

Ratings and digital images were subjected to Analysis of Variance (ANOVA) using SAS version 9.3 and were presented as a percentage of control to eliminate genetic differences and seed lot effects between cultivars. Correlation analysis between visual percent green ratings and digital images were conducted using Proc CORR in SAS version 9.3.

RESULTS AND DISCUSSION

Emergence

Our results indicated that there was a significant run effect (Table 1) which could be attributed to increased daylength, higher temperatures, and increased evaporation that may have led to increased salinity stress during the second run of the study which took place in the late spring (average greenhouse temperature 22.2 °C) compared to the winter when Run 1 was conducted (average greenhouse temperature 18.6 °C). There were significant differences between treatments (averaged across all cultivars) and between cultivars (averaged across all treatments) for percent green, seedling emergence, and digital image analysis (Table 1). However, there were no significant differences between cultivars within each salinity treatment (data not shown) and no interactions between cultivar and treatments (Table 1). A significant run x cultivar interaction was observed for the majority of the weeks, therefore data is presented separately for each run. This interaction could have been due to differences in cultivar responses under different

environmental conditions of the two runs which been observed in previous studies (Koch and Bonos, 2010),

Emergence levels after three weeks remained unchanged. Significant differences were observed in emergence between salinity treatments when all cultivars were averaged together (Fig. 1a and 1b). The highest salinity treatment (6.0 dS m^{-1}) caused a 58% reduction in emergence rate in run 1 (2010) and a 75% reduction in run 2 (2011) when compared to the control treatment (0.25 dS m-1). The 5 dS m^{-1} treatment resulted in a 41% (run 1) and 65% (run 2) reduction in emergence. The 4 dS m^{-1} resulted in a 32% (Run 1) and 52% (Run 2) reduction in emergence; 3 dS m^{-1} reduced germination by 25% (Run 1) and 40% (Run 2); The 2 dS m^{-1} treatment resulted in only a 10% (Run 1) and 25% (Run 2) reduction; 1 dS m^{-1}, the lowest salinity treatment only reduced germination by 7% (Run 1) and 13% (Run 2). The salinity level at which a 50% reduction in emergence was observed was 5.604 dS m^{-1} in run 1 and 3.921 dS m^{-1} in run 2. This was similar to the results observed by Camberto et al. (2000) for rough bluegrass (*Poa trivialis*) where there was a linear decrease in germination rate as salinity levels increased and a salinity concentration of 5.0 dS m^{-1} caused a 50% reduction in emergence rate. This negative trend has been shown in other turfgrass species in addition to perennial ryegrass (McCarty and Dudeck, 1993; Zhang et al., 2011, Peacock and Dudeck. 1989).

Significant differences were also observed between perennial ryegrass cultivars when averaged across all treatments (Fig. 2a and 2b) however it was variable across runs (Table 1). Paragon GLR and Zoom had among the highest seedling emergence when

averaged across all salinity treatments. The results for these two cultivars were consistent across runs however most cultivars responded differently depending on the runs. For example, Apple GL had high seedling emergence in run 1 but among the lowest in run 2. There were no cultivars with consistently low emergence ratings across runs. These results indicate that environment plays a significant role in the salinity tolerance observed among germinating turfgrass cultivars. McCarty and Dudeck (1993) and Camberto et al., (2000) found that salinity was also influenced by seed lot, a further indication that factors other than genetic (seed quality, growing media etc.) influence germination in perennial ryegrass. Additionally, there were no cultivar by treatment interactions for emergence rate on any of the rating dates. These data indicate that cultivars emerged similarly under varying levels of salinity stress. These findings concur with data from Johnson et al. (2007) who also did not find any significant salinity by cultivar interactions in tall fescue [*Lolium arundinacea* (Schreb.)] and perennial ryegrass. Due to confounding environmental effects, it is difficult to determine whether cultivar differences observed in this study were due to genetics or to the environment.

Analysis of percent green visual ratings to estimate early establishment

Visual percent green ratings collected throughout the six week study were correlated ($r^2 = 0.73$; $p = 0.04$) with digital images, therefore data from DIA was not shown. Differences in percent green visual ratings were observed among salinity treatments when cultivars were averaged across salinity treatments (Fig. 3a and 3b). The salinity level at which a 50% reduction in establishment was observed was 5.881 dS m^{-1}

in run 1 and 3.662 dS m^{-1} in run 2. The highest salinity treatment (6 dS m^{-}) caused a 65.67% reduction in percent green ratings in Run 1 (2010) and an 88.40% reduction in Run 2 (2011). At 2 dS m^{-1} salinity caused reductions in percent green by 8% in Run 1 and 21% in Run 2 indicating that EC levels of 2.0 dS m^{-1} do not have a large impact on germinating perennial ryegrass cultivars. This is particularly evident in Run 1. The temperature of Run 2 was higher than Run 1 and caused more dramatic reductions in establishment however, our data does support prior research by Harivandi (2004) suggesting irrigation water greater than 3.0 dS m^{-1} will cause severe salt stress. At 3.0 dS m^{-1} in our study, we saw an average reduction of 29% in Run 1 and 39% in Run 2. Our data contrasts with Johnson et al. (Johnson, 2007) who reported that salinity levels of 3.0 dS m^{-1} caused no negative affect on germination. This was most likely due to the different methods that irrigation was applied in both studies. Soil (used in our study) absorbs and retains salt, which may have resulted in more significant reductions in plant growth at lower EC levels when compared to using saturated blotter paper (as used by Johnson et al., 2007). This demonstrates the importance of conducting these types of studies to most simulate field conditions. Further reductions were observed with higher salinity levels (Fig. 3a and 3b). The recommendation based on this study is to keep salinity levels of irrigation water below 3 dS m^{-1} when working with a sandy loam soil.

Visual percent green ratings between cultivars were significant on weeks 2, 3, 5, and 6 (Fig. 4a and 4b) although differences in Run 2 were less pronouced. In both runs, Zoom, KSA, Paragon GLR and Linn exhibited good establishment under salinity stress while ESP, RKS, and Palmer III had low percent green ratings (and therefore poor

establishment) under salinity stress. These results were more consistent across runs than emergence ratings, but were somewhat related in that cultivars with high emergence tended to also have good establishment. Interestingly, RKS exhibited high salinity tolerance as a mature plant (Koch and Bonos, 2011) but poor establishment tolerance in our study. These findings concur with previous claims that different mechanisms may be involved with seed germination under salinity and mature plant salt tolerance (Rose-Fricker and Wipff, 2001). Researchers hypothesize that the main explanation for germination reduction under salinity is due to inhibition of water uptake by the seed, or because of the accumulation of salt and ion toxicity within the germinating seed (Coons et al., 1990, Atak et al., 2006).

Soil analysis

Soil pH was determined by using the 1:1 (V:V) soil:water method. Analysis of soluble salts was determined by using the 1:2 (V:V) soil:water extract method to analyze soil electrical conductivity (EC). Soil pH ranged from 5.22 to 5.83 but no significant differences were seen across treatments. Significant differences were observed for soil electrical conductivity where the control (0.25 dS m^{-1}) had a final EC of 0.17 in run1 and 0.19 in run 2, and the highest salinity treatment (6 dS m^{-1}) had a final EC of 0.99 in run1 and 1.62 in run 2 (Table 2). As irrigation water EC increased, final soil EC increased significantly (Table 2). Run 2 was conducted in spring which had increased daylength, higher temperatures, and increased evaporation in comparison to run 1, which was conducted in winter. Salt accumulation in the soil of run 2 was much more detrimental to

germinating perennial ryegrass seedlings as evidenced by the higher reductions in germination and establishment ratings in Run 2 compared to Run 1 (Figs. 2a and b and 4a and b). This data concurs with other research showing that salt accumulation can occur more prevalently with increasing amounts of total soluble salts in irrigation water and higher levels of evapotranspiration (Carrow and Duncan, 1998, Rahayu et al, 2011, Miyamoto, 2013). This data also shows high accumulation of salt in the soil compared to conducting a study in sand (Koch and Bonos, 2010) which has lower CEC sites in the soil. Interestingly, cultivars performed similarly in establishment across runs indicating that those cultivars with good establishment under low soil salinity stress will also establish better under higher soil salinity stress.

CONCLUSION

Our results indicate that irrigation with salinity levels of 3.0 dS m^{-1}causes a significant decrease in emergence (32%) and early establishment (33%) of perennial ryegrass cultivars when planted in a soil substrate. When irrigated with higher levels of salinity (6.0 dS m-1), perennial ryegrass stands exhibited a reduction in emergence of 67% and a reduction in early establishment by 74%. This indicates that irrigation with effluent water will decrease turfgrass stands as some effluent water contains salinity levels of 2 to10 dS m^{-1}. Frequent irrigation with effluent water can cause a buildup of salt within the soil if proper management techniques are not applied as our soil tests have shown.

Our research has shown that Linn, Paragon GLR, and Zoom exhibited higher seedling vigor under salt stress than other cultivars tested although these results could be affected by other factors including seed lot which was not evaluated in this study. For all ratings and measurements in our study, treatment by cultivar was not significant indicating that cultivars that perform well in control conditions also perform well under saline irrigation. This also suggests that other factors such as seed quality or environmental elements play a significant role in establishment of turfgrasses in saline conditions. Future research needs to address what the most efficient and effective selection method is to develop salt tolerant perennial ryegrass seedlings.

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Table 1. Analysis of variance of salinity tolerance measurements (seedling emergence ratings, percent green ratings, digital image analysis) evaluated in a greenhouse using overhead irrigation at six salinity treatments (2, 3, 4, 5, 6, or 7 dS m^{-1})

	Seedling Emergence Ratings			% Green Ratings					Digital Image Analaysis				
	Weeks			Weeks					Weeks				
	1	2	3	2	3	4	5	6	2	3	4	5	6
Run	***	***	***	***	***	***	***	***	***	***	***	***	***
Treatment	***	***	***	***	***	***	***	***	***	***	***	***	***
Run x Treatment	**	NS	NS	NS	*	NS	**	**	**	***	***	***	**
Cultivar	**	**	**	***	***	**	***	***	**	***	**	***	**
Run x Cultivar	NS	***	***	**	**	NS	**	**	**	**	**	**	***
Treatment x Cultivar	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Run x Treatment x Cultivar	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the 0.001 probability level
†NS, not significant.

Table 2. Soil electrical conductivity (EC) measurements from a greenhouse study sampled at the end of each run from amalgamated soil within each treatment. Treatments within columns represent significant differences at the 0.05 probability level using Fischer's protected least significant difference means separation test.

Treatment	**Run 1**	**Run 2**
0.25 dS m^{-1}	0.17 A	0.19 a
1 dS m^{-1}	0.36 B	0.52 a
2 dS m^{-1}	0.46 BC	0.94 b
3 dS m^{-1}	0.58 CD	0.94 b
4 dS m^{-1}	0.69 D	1.10 b
5 dS m^{-1}	0.89 E	1.46 c
6 dS m^{-1}	0.99 E	1.62 c

Figure 1. Weekly visual emergence ratings among treatments averaged across all cultivars for run 1 (a) and run 2 (b) of a greenhouse salinity screening study of perennial ryegrass (*Lolium perenne* L.) seedlings. Emergence is presented as a percentage of control. Treatments with different letters represent significant differences at the 0.05 probability level using Fischer's protected least significant difference means separation test.

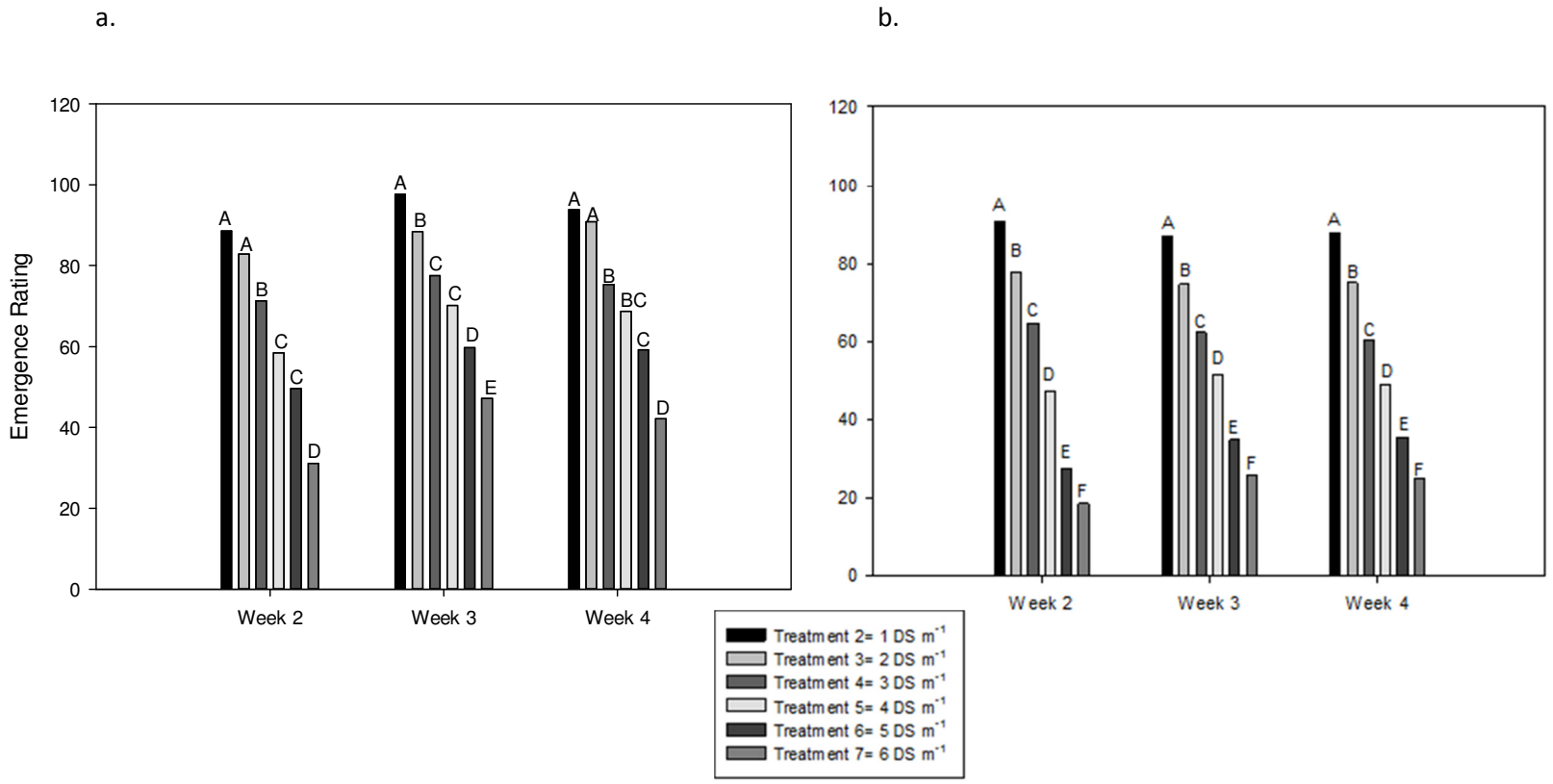


Figure 2. Weekly visual emergence ratings of individual cultivars averaged across all treatments for run 1 (a) and run 2 (b) of a greenhouse salinity screening study of perennial ryegrass (*Lolium perenne* L.) seedlings. Emergence ratings were presented as a percentage of control. Error bars indicate significant differences between cultivars at the 0.05 probability level on dates where significance cultivar difference were observed.

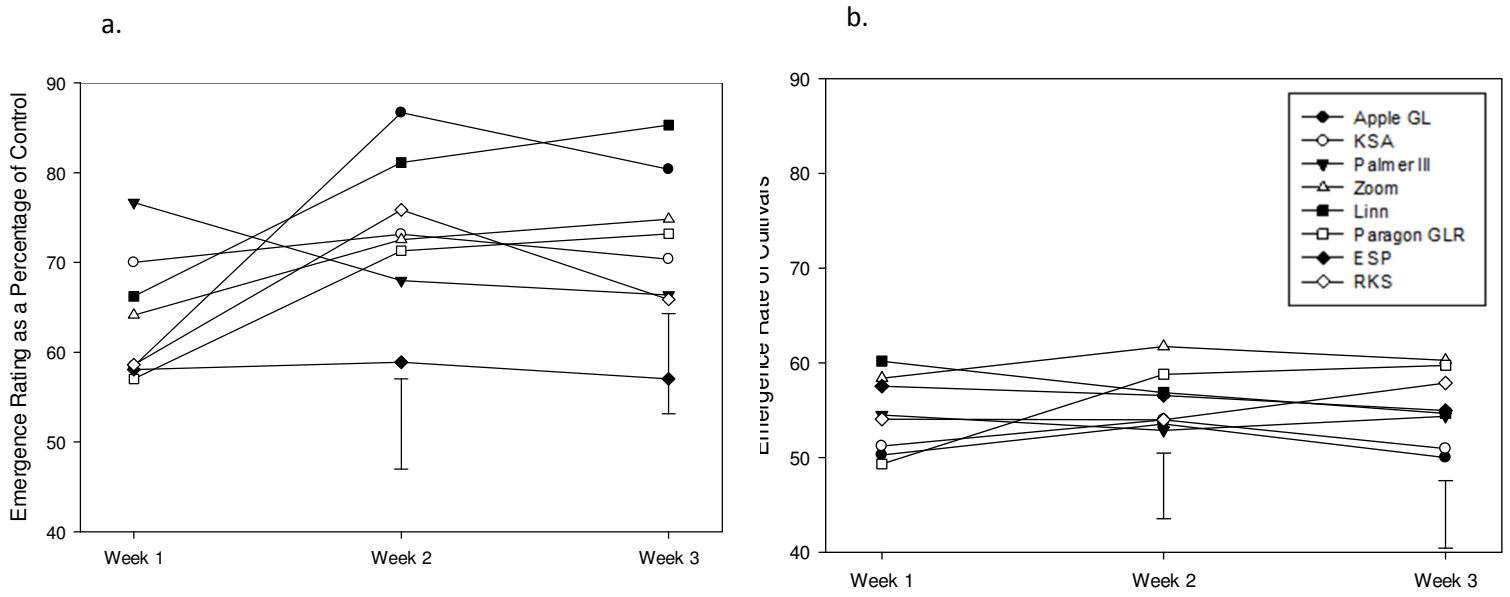


Figure 3.

Weekly percent green visual ratings among treatments averaged across all cultivars for run 1 (a) and run 2 (b) of a greenhouse salinity screening study of perennial ryegrass seedlings. Percent green ratings are presented as a percentage of control. Treatments with different upper-case letters represent significant differences at the 0.05 probability level using Fischer's protected least significant difference means separation test.

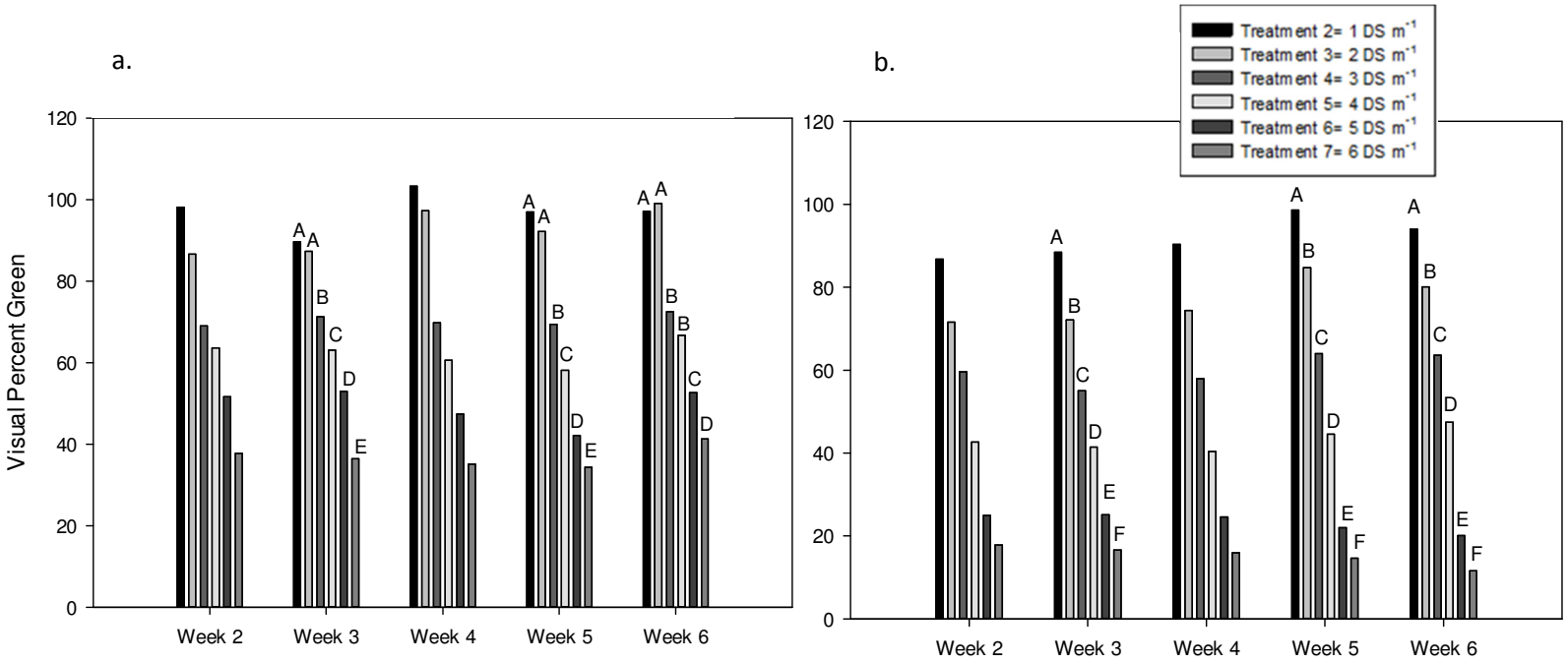
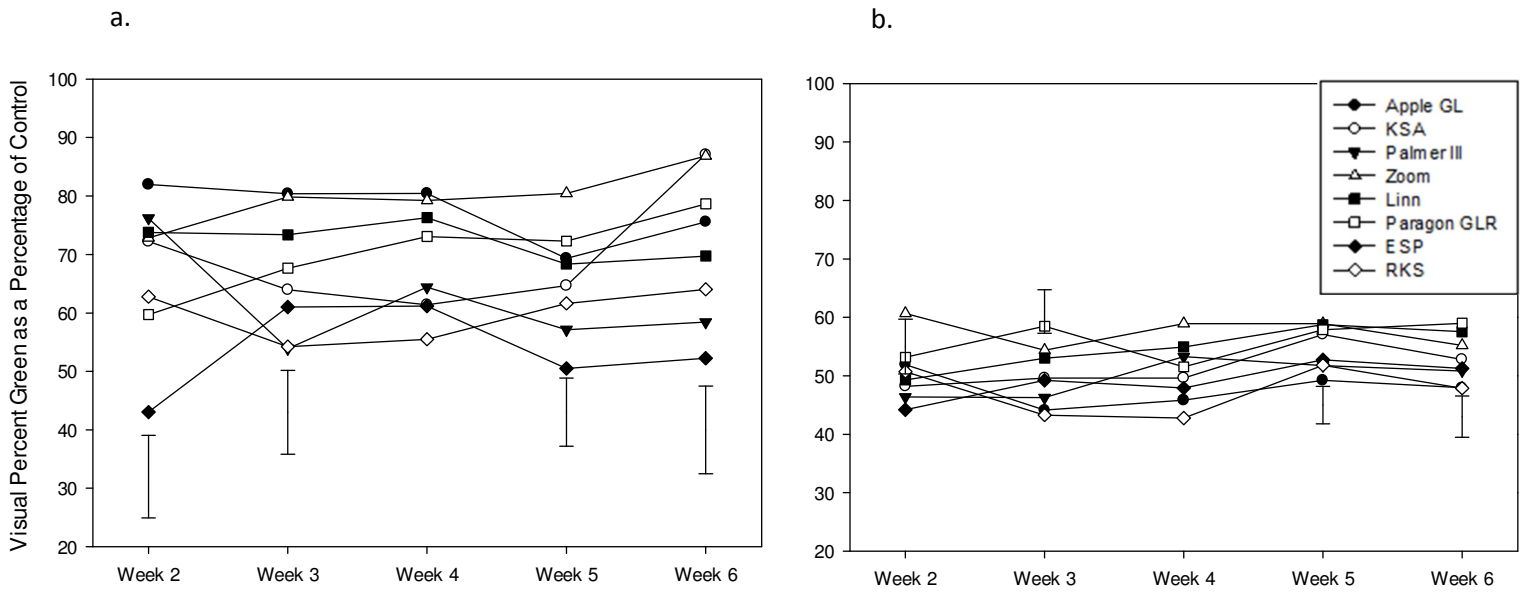


Figure 4. Weekly visual percent green ratings of individual cultivars averaged across all treatments for run 1 (a) and run 2 (b) of a greenhouse salinity screening study of perennial ryegrass (*Lolium perenne* L.) seedlings. All cultivars are represented as a percentage of control. Error bars indicate significant differences between cultivars at the 0.05 probability level on dates where significance cultivar difference were observed.



CHAPTER 3

Quantifying the Effects of Salinity Stress on Germinating Perennial Ryegrass

Abstract. Southern golf courses using bermudagrass (*Cynodon* sp.) are often overseeded annually with perennial ryegrass (*Lolium perenne* L.) in arid regions around the United States due to this species fast germination and rapid tillering. Many golf courses in these regions are required to use effluent water as an irrigation source which can lead to an accumulation of salts in the soil. Salinity in the soil can often lead to damage of turfgrass stands especially at germination and early establishment stages when turf is weakest. The objectives of this study were 1) to determine the variability in salinity tolerance among ten perennial ryegrass cultivars; and 2) to evaluate physiological growth features for future selection of salt tolerance during germination. Ten perennial ryegrass cultivars were seeded into topdressing sand in a pot and irrigated using an ebb and flow technique under two salinity treatments; 0.25 dS m^{-1} (control) and 10.0 dSm^{-1}. The goal was to simulate the conditions that might occur on a golf course during overseeding with effluent water. Individual seedlings were quantified for parameters such as germination rate, final germination percentage, shoot length/dry weight, and root length/dry weight. The salinity treatment caused a significant decrease in germination rate (21.5%), final germination percentage (11.2%), shoot length (47.6%), shoot dry weight (61%), and root length (39.4%) when averaged across all cultivars. On average, Phenom and Soprano had the highest germination while Affirmed and Paragon GLR had the lowest.

Interestingly, there was no treatment by cultivar interaction for germination rate indicating that cultivars that germinated quickly under control conditions also germinated quickly under saline conditions. This lack of interaction suggests that germination rate may be more affected by environmental conditions than by inherent genetic differences.

INTRODUCTION

Due to water shortages, water conservation has become a necessary practice in high water-using landscapes and golf courses. The use of lower quality saline water, such as effluent (waste) water, is on the rise in certain areas of the United States such as the arid Midwest as a solution to relieve potable water shortages (Arizona Department of Water Resources, 1995, Carrow and Duncan, 1998). Effluent water is wastewater that has been treated in a water treatment plant and has been remediated through physical and chemical means (Lazarova and Bahri, 2005, Asano and Pettygrove, 1987) such as the additions of salt. Due to the additional expenses needed for removal, these salts remain in the water after the treatment process. Consistent use of effluent water will lead to a salt affected turfgrass site (Duncan et al., 2000). Other ways turfgrass sites can become salt affected are by saltwater intrusion into groundwater (Newport, 1997), saltwater spray near ocean fronts (Humphreys, 1982), or ice melting salt runoff (Marcum, 1994).

Plant growth in salt affected sites negatively impacts plant growth and development (Carrow et al., 2001). Salinity can cause wilt and leaf burn on established

turfgrass, and in extreme cases cause death (Horst, 1991). Seeds are especially impacted by soil salinity because seeds are near the surface where saline conditions can be greater due to evaporation and capillary action (Almansouri et al., 2001). Cool-season turfgrass species such as Kentucky bluegrass (*Poa pratensis* L), rough bluegrass (*Poa trivialis* L.) and creeping bentgrass (*Agrostis stolonifera* L), are particularly sensitive to salinity stress during germination and show a reduction in germination rates when exposed to high levels of salinity (Harivandi et al., 1982, Dai et al., 2009, Camberato and Martin, 2004). Additionally, previous findings have shown that different mechanisms may be involved with seed germination under salinity when compared to mature salt tolerance (Rose-Fricker and Wipff, 2001) indicating the need for continual evaluation of all growth stages of the turfgrass plant.

Perennial ryegrass (*Lolium perenne* L.) is a cool-season turfgrass species that is moderately salt tolerant and is able to withstand salt levels of 6 to 10 dS m^{-1} (Harivandi et al, 1992, Horst and Beard, 1977). Due to its quick germination rates, perennial ryegrass is used to overseed dormant warm-season turfgrass which allows for year-round play in a golf course setting. These sites using this technique are usually the ones that have water restrictions and are required to use effluent water which results in saline soils. Identification of genotypes that are salt tolerant at a germination stage have been studied extensively using *in vitro* methods. These methods have shown that high levels of salinity can slow germination in turfgrasses but salinity does not disrupt final germination percentage (Horst and Dunning, 1989, Peacock and Dudek, 1989, Dai et al., 2009). Horst and Dunning (1989) used floating mats to test perennial ryegrass cultivars and found that

salinity levels up to 23.4 dS m^{-1} caused no statistical reduction in final germination percentage.

Screening for salt tolerance has been conducted in many different growing medias such as agar (Serena et al., 2012, Peacock and Dudeck, 1989), hydroponics (Horst and Dunning, 1989), and blotter paper (Camberato and Martin, 2004, Johnson et al., 2007). These methods are artificial and allow for consistent water availability with the absence of important natural soil features such as surface dry down and accumulation of salt ions (Carrow and Duncan, 1998, Dewey, 1962). Differences in germination rates and final germination percentage when comparing cultivars have been observed in many turfgrass species but the analysis of other growing features such as shoot length, shoot dry weight, root length, and root dry weight are not available for current commercially available cultivars (Horst and Dunning, 1989, Dai et al., 2009, Serena et al.,2012) These growing features may reveal important factors to consider while selecting for salinity tolerant cultivars in early stages of growth.

The objectives of this study were 1) to determine the variability in salinity tolerance among ten perennial ryegrass cultivars; and 2) to evaluate physiological growth features for future selection of salt tolerance during germination.

MATERIALS AND METHODS

Ten commercially available perennial ryegrass cultivars (Phenom, Apple GL, Zoom, SR 4600, Fiesta 4, Soprano, Stellar GL, Gator 3, Paragon GLR, and Affirmed)

were obtained from various seed companies (from the same harvest year) and were evaluated at two salinity treatments; control, 0.5 dSm^{-1} and a salinity treatment of 10 dSm^{-1}. Topdressing sand obtained from US Silica was weighed and equal quantities were put into 10.16x10.16 cm plastic horticulture pots (Griffin Greenhouse & Nursery Supplies, Ewing, NJ) to a weight of 0.8kg. Filter paper was used to line the bottom of the pots to restrict loss of sand throughout the experiment. Fifty seeds of each cultivar were counted, selected at random, and placed into the individual pot to represent a single cultivar. Twenty-five grams of topdressing sand was evenly distributed over the seeds to ensure proper seed to sand contact of all seeds.

The saline water was made using Instant Ocean (Spectrum Brands Inc. Atlanta, GA) mixed with tap water. The control treatment was tap water only. Solutions were placed in 37.85 litre water coolers (Rubbermaid, High Point, NC) until irrigation was needed. Half-strength Hoagland's solution was added to coolers to provide nutrients to emerging seedlings. Solutions were replaced once, 7 days after seeding, to ensure consistent nutrient availability. Three replicates of each cultivar within each salinity treatment were arranged in a completely random design. Irrigation was provided by submerging the pots daily into 6 cm's of solution for 5 minutes to achieve field capacity. Seeded pots were placed in a growth chamber for the extent of the study under the recommendations from the Association of Official Seed Analysts (25°C 8-hour days and 15°C 16-hour nights)(AOSA, 2009). Relative humidity was maintained at 75% and lighting was set to 30 $\mu E\ m^{-2}\ s^{-1}$.

Emerged seedlings were counted daily and were defined as a green shoot protruding from the coleoptile (McCarty and Dudeck, 1993). Germination rate (GR, $\%d^{-1}$) was based on seedling counts taken daily, and final germination percentage (FGP%) was based on the total number of germinated seeds counted over 14 days. Final germination percentage is described by FGP% =

$$100\Sigma(\frac{n}{50})$$

And germination rate by GR ($\%d^{-1}$) =

$$\frac{100}{50}\Sigma(\frac{n}{D})$$

where n is the number of seeds that had germinated at each counting and D is the number of days accumulated up to the counting. The GR measurement provides information on how quickly seeds emerged during the experiment; higher values indicate faster emergence. Additionally, linear regression models were calculated for all cultivars as an estimation of days required to reach both 25% and 50% germination under salt stress.

Seedlings were removed 14 days after seeding and each plant within the pot was washed free of sand before root and shoot lengths were measured. All seeds were accounted for and seeds that did not germinate received a zero for shoot and root measurements. Roots were measure by laying down each seedling on a polypropylene tray and measuring the longest root. Seedlings were then cut below the coleoptile and separated into roots and shoots for each individual pot. These cuttings were dried at 65°C for 48 hours and weighed. Soil electrical conductivity and pH were collected for

three replicates per treatment. Soil within each treatment was amalgamated before samples were taken. The 1:2 (V:V) soil:water extract method was used to analyze soil electrical conductivity (Dellavalle, 1992). pH of each soil sample was measured using the 1:1 (V:V) soil:water method (Miller and Kissel, 2010).

Measurements were subjected to Analysis of Variance (ANOVA) using SAS version 9.3. Shoot length, shoot weight, root length, and root weight are presented as a percentage of control to eliminate genetic differences and seed lot effects between cultivars. A linear regression analysis was conducted on germination of seedlings in the salt solution within each cultivar and was averaged across both runs. An ANOVA was conducted on results for 25% and 50% germination to indicate significant difference between cultivars.

RESULTS AND DISCUSSION

The ANOVA revealed a treatment effect for all measurements taken with the exception of root weights. Although root weights have been shown to be impacted by salinity stress in other experiments, these experiments were conducted over a longer time period (Horst and Dunning, 1989, Torello and Symington, 1983).Our observations conclude that 14 days of growth was not enough time to see the detrimental effects of salinity on root growth. There was no significant replication effect with the exception of root lengths. When analyzing the cultivar effect, cultivars were significantly different for all measurements obtained. We observed a significant run effect for all measurements with

the exception of shoot weights. We believe this was due to the small amount of tissue that resulted after only 14 days of germination. The results from this study found a significant cultivar x run interaction was observed for germination rate which could have been caused by the seed storage in a freezer prior to the initiation of the second run. For the first run, seeds were removed from the freezer for 5 days prior to the initiation of the study. It has been report that continued ripening, decline in seed viability, and differences in seed lots may cause inconsistencies in germination studies (Camberato and Martin, 2004).

Germination Rate

Germination rate (GR) decreased under salinity by 24% in run 1 and 19% in run 2 when averaged across all cultivars (Figure 1a and 1b). Cultivars performed better during run 2 in both the salinity treatment and in the control. This could be due to the additional ripening of newly harvested seed acquired at the beginning of the experiment. When treated with salt, the cultivars Phenom and Soprano were among the top performers consistently across both runs while Affirmed and Paragon GLR were among the bottom performers (Figure 1a and 1b). When comparing the control to the salinity treatment, Paragon GLR saw the biggest decrease in GR of 31.718 $\%d^{-1}$ in run 1 and 19.298 $\%d^{-1}$ in run 2 (Figure 1a and 1b). On average across runs SR 4600 saw the lowest decrease in GR of 22.799 $\%d^{-1}$ in run 1 and 10.081 $\%d^{-1}$ in run 2 and maintained a higher rate of germination under salt stress when compared to all tested varieties.

Data from the linear regression analysis shows that Affirmed and Paragon GLR required 10.73 and 10.17 days, respectively, for 25% of seedlings to germinate in the salt solution while Phenom, Soprano, and SR4600 only required 7.33, 7.96, and 8.08 days respectively (table 2). Data estimates for 50% of seedlings to germinate followed a similar cultivar relationship where Affirmed and Paragon GLR required 17.22 and 16.23 days to reach 50% germination and Phenom, Soprano, and SR4600 required 10.70, 11.94, and 12.26 days respectively. These results contradict Serena et al. (2012) who found that salinity levels ranging from 0.6 to 12.5 dS m^{-1} did not affect GRs in perennial ryegrass, however, that study was done on agar and blotter paper. Similarly, Horst and Dunning (1989) used hydroponic floating mats and also found that germination rates were not reduced below 50%, even at a salinity concentration of 23.4 dS m^{-1}. Since our study was conducted on sand and resulted in at least a week long delay in germination under lower salinity levels, we hypothesize that water availability (or lack of available water in the sand media) could have contributed to the delay in germination when compared to other studies conducted on agar or in a hydroponic solution where water is not limited to germinating seedlings (Hadas, 1977; Werner and Finkelstein, 1995; Katembe et al., 1998). Lack of water availability has been identified as a major limiting factor of other species germinating under salinity stress (Atak et al., 2006). These results indicate that the soil physical properties could play a significant role in germination of turfgrass seedlings and studies using agar or hydroponics may not predict what will happen under field conditions. This is contrary to previous research conducted on mature plants that found that perennial ryegrass plants performed similarly regardless of the screening

method (Koch and Bonos, 2011). A potential reason for this discrepancy is that mature plants have the available resources to use mechanisms to cope with increased levels of salts, such as ion exclusion. Seeds cannot employ such mechanisms because seeds must uptake water (saline or potable) to successfully germinate. In other methods, such as hydroponics, ions may be moving freely in and out of the seed removing the stress of ion accumulation.

Final Germination Percentage

Final germination percentage was probably one of the least affected traits evaluated. For the best performing cultivars, final germination decreased significantly by 13.933% in run 1 and 8.4 % in run 2 when averaged across all cultivars. For the control treatment, there were no observed differences between cultivars in run 1, although there were slight differences between cultivars in run 2. Affirmed consistently performed poorly when compared to other tested cultivars. FGP decreased by 27.33% in run 1 and 9.333% in run 2 when compared to the control (Figure 2a and 2b). Phenom consistently performed well across runs and maintained a reduction of only 8% in run 1 and 8.667% in run 2 when compared to the control. This data contradicts research conducted by Serena et al (2011) who found no reduction in FGP in perennial ryegrass at salinity levels of 22.5 dS m^{-1} however, they used agar as a germination media. These authors also found that germination on blotter paper caused no significant difference in final germination in salinity treatments ranging from 0.6 to 12.5 dS m^{-1}. Other studies using agar and hydroponics have also found no, or minimal, reduction in FGP due to high

levels of salts (Peacock and Dudeck, 1989; Camberato and Martin, 2004; Hanslin and Eggen, 2005). Horst and Dunning's (1989) experiment on perennial ryegrass found that even at the highest salinity concentration, 23.5 dS m^{-1}, significant differences for total germination among tested cultivars were minimal with 98% of the seedlings germinating across all cultivars after a 3 week period. Dai et al. (2009) used agar plates to germinate a single cultivar of perennial ryegrass (Charger II) and found a reduction in FGP by 25% at 15.25 dS m^{-1}. These varying results in FGP's may not only be due to different salinity levels (and type of salts being used), but are also due to the variation in media used and the length of time given for seeds to germinate. This variability observed across experiments indicates that germination under salt stress is affected by soil/ media, the irrigation technique used, and seed characteristics. Even in sand, binding of salt ions to sand cation exchange capacity sites may impose a water stress on the seeds causing a reduction in the absorption of water. In our media, we were able to simulate the accumulation of salt ions over time and allow for the sand media to lose moisture between irrigation periods. These conditions are more applicable to a golf course scenario and may have provided more accurate results on FGP when compared to methods using hydroponics, agar, or blotter paper.

Shoot Length/Weight

Shoot length decreased significantly in run 1 (45.5%) and run 2 (49.75%) due to the salt treatment when averaged across all cultivars. Cultivar differences are presented as a percentage of control to account for inherent genetic differences in growth rate

between cultivars. Soprano and Phenom were among the top performers in both run 1 and run 2. Soprano maintained 52.5% in Run 1 and 59.5% in Run 2 and Phenom maintained 51.8% in Run 1 and 59.6% in Run 2 for shoot growth under salinity as a percentage of control (Figure 3). Affirmed and Paragon GLR exhibited the most decline in shoot growth. Affirmed only maintained 35.2% in Run 1 and 38.3% in Run 2 and Paragon GLR maintained 33.0% in Run 1 and 34.5% in Run 2 under the salt treatment.

Shoot weights also showed significant differences between cultivars whereas Soprano maintained 74.4% shoot weight while Paragon GLR only maintained 43.6% shoot weight as a percentage of the control (Figure 4). Because there were no run effects, run 1 and run 2 were combined. Interestingly, Gator 3 performed well and was able to maintain 70.6% growth even though it was among the slowest to germinate. Our data concurs with data from Horst and Dunning (1989) who also found a significant decrease in shoot weights of perennial ryegrass cultivars germinating on floating mats containing salts. These authors found a mean reduction in shoot dry weight of 15.4% at a salinity level of 11.6 dS m^{-1} indicating that the use of their germination media allowed for better shoot growth and less stress when compared to our sand media. In Kentucky bluegrass, similar reductions in blade fresh weights were found where 11.7 dS m^{-1} caused a 50% reduction in salt sensitive varieties (Horst and Taylor, 1983). Similar reductions in dried biomass weights influenced by salinity have been studied in other non-turfgrass species including wheat [(*Triticum aestivum* L.) Saboora et al., 2006] and canola [(*Brassica napus* L.) Bybordi and Tabatabaei, 2009].

Root Length/Weight. Root length decreased significantly in run 1 (39.86%) and run 2 (38.98%) due to the salt treatment when averaged across all cultivars. Unsurprisingly, Phenom maintained 70.3% (in run 1) and 71.4% (in run 2) and Soprano maintained 67.95% (in run 1) and 70.3% (in run 2) root length under salinity (Figure 5). As with the other measurements, Paragon GLR only maintained 45.6% (in run 1) and 47.1% (in run 2) and Affirmed maintained 43.2% (in run 1) and 46.8% (in run 2) and exhibited the most reduction in growth due to salinity stress when compared among other tested cultivars.

Root weights were not significant when comparing the control with the salt treated seedlings. Because this experiment finished after 14 days of growth, there may not have been enough time to identify statistical differences in root weights. Interestingly, significant differences were observed in root lengths. A higher salinity level may be required to show root weight reductions during early seedling growth. Other authors have found that turfgrass introduced to salinity can cause a positive or a negative effect on root formation over time. In mature plants, moderate salinity causes an increase in the root/shoot ratio and is a mechanism for the plant to maintain water uptake (Donovan and Gallagher, 1985; Dudeck et al., 1983). For germinating turfgrass plants, salinity had the opposite effect on roots in our study. This difference is most likely due to the fact that during germination, the seedling has a limited amount of resources required to produce a shoot and start photosynthesizing nutrients. Introduction of a stress such as salinity causes the seedling to use additional energy to exclude salt ions from the plant, thus reducing vegetative growth. Similarly, Horst and Dunning (1989) studied germination of various perennial ryegrass cultivars and found a reduction in root growth

of 40% after 21 days of exposure at a salinity level of 19.5 dS m^{-1}. Torello and Symington (1983) studied salinity tolerance of germinating seeds of Kentucky bluegrasses in agar. Salinity tolerance was quantified based upon the reduction in root and leaf length. Tolerant cultivars consistently showed higher root growth and higher tolerance to salts across all measurements.

Soil

Soil pH was determined by using the 1:1 (V:V) soil:water method (Miller and Kissel, 2010). Analysis of soluble salts was determined by using the 1:2 (V:V) soil:water extract method to analyze soil electrical conductivity (Dellavalle, 1992). Soil pH ranged from 7.82 to 8.0 but no significant differences were observed across treatments. Significant differences were observed for soil electrical conductivity where the control (0.25 dS m^{-1}) had an average final EC of 0.11 in both runs, and the salinity treatment (10 dS m^{-1}) had an average final EC of 0.55. Although sand has lower CEC sites than that of soil, the sand media showed more accumulation of salt ions and thus caused additional damage to the seedlings when compared to research using agar, germination paper, or hydroponics.

CONCLUSION

Our results indicate that a salinity level of 10 dS m^{-1} caused a significant decrease in germination rate (21.5%), final germination percentage (11.2%), shoot length (47.6%),

shoot dry weight (61%), and root length (39.4%). Using sand as the growing media caused a higher level of reduction in all measurements when compared to other research using hydroponics, agar, and blotter paper. This reduction in measurements may likely be due to the retention of salt ions within the sand growing media which was confirmed by soil tests conducted at the end of the experiment. Our study may be more relative to what is occurring on golf courses than the artificial media experiments. The variability observed across experiments indicates that germination under salinity stress is controlled by numerous factors and therefore predicting turfgrass salinity tolerance might be difficult due to confounding factors.

The cultivars Phenom and Soprano performed best among tested cultivars when averaged across all measurements, while Paragon GLR and Affirmed performed poorly. In this study, we were able to limit more confounding environmental effects than previous work due to the use of a growth chamber. Linear regression models demonstrated that Affirmed took 3 additional days to germinate 25% of seedlings under salt stress compared to Phenom. Germination rate and shoot length maintained the largest separation in cultivar differences and seemed to be the most important measurements for future selection of salt tolerant perennial ryegrass cultivars. The least important measurement taken was dry root weight and lack of a treatment difference is most likely due to the short time frame of our study; this measurement has proven useful in previous experiments (Torello and Symington, 1984). Additionally, we observed no treatment by cultivar interaction for germination rate indicating that cultivars that performed well in the control also performed well in the salt treatment. This data suggests that germination

rate may be more affected by environmental conditions than genetic differences. These results suggest that breeding for seedling salinity tolerance may be difficult to progress due to the lack of genetic effects and the confounding effects of the environment.

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Table 1. Analysis of variance of salinity tolerance measurements (germination rate, final germination percentage, shoot length, root length, shoot weight, and root weight) of perennial ryegrass evaluated in a growth chamber under salinity stress (10 dS m^{-1}).

	Germination Rate	Final Germination Percentage	Shoot Length	Root Length	Shoot Weight	Root weight
Replication	ns	ns	ns	***	ns	ns
Treatment	***	***	***	***	***	ns
Cultivar	***	***	***	***	***	***
Run	***	***	***	***	ns	***
Treatment*run	***	***	ns	ns	ns	ns
Treatment*Cultivar	ns	*	***	***	***	ns
Cultivar*Run	***	ns	***	ns	ns	ns
Treatment*Cultivar*Run	*	ns	ns	ns	ns	ns

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the 0.001 probability level
†NS, not significant.

Table 2. Linear regressions of 10 perennial ryegrass cultivars germinated in a salt solution of 10 dS m^{-1} to predict the days after seeding required for 25% and 50% of seedlings to germinate. Cultivars with different letters represent significant differences at the 0.05 probability level using Fischer's protected least significant difference means separation test.

	Regression	Days Until 25% Germination	Days Until 50% Germination
Affirmed	y=1.9421x-8.3516	10.7533 A	17.2233 A
Paragon GLR	y=2.1575x-9.4432	10.3100 A	16.2333 A
Gator 3	y=3.1011x-12.568	8.6367 B	13.1633 B
Fiesta 4	y=2.9905x-12.238	8.3500 B	12.6033 B
Stellar GL	y=2.8982x-11.641	8.3333 B	12.6467 B
SR 4600	y=3.1011x-12.568	8.1600 B	12.2567 B
Apple GL	y=3.2066x-13.121	8.0200 CB	11.9500 CB
Soprano	y=3.1853x-12.842	7.9867 CB	11.9467 CB
Zoom	y=3.2051x-13.015	7.9633 CB	11.8600 CB
Phenom	y=3.7238x-14.81	7.3400 C	10.7033 C

Figure 1. Daily germination rate of seedlings of perennial ryegrass (*Lolium perenne* L.) cultivars in a growth chamber salinity screening study for run 1(a) and run 2(b) . Cultivars with different letters represent significant differences at the 0.05 probability level using Fischer's protected least significant difference means separation test. Upper and lower case letters represent a statistical difference within the control and the treatment (10 dS m^{-1}) respectively.

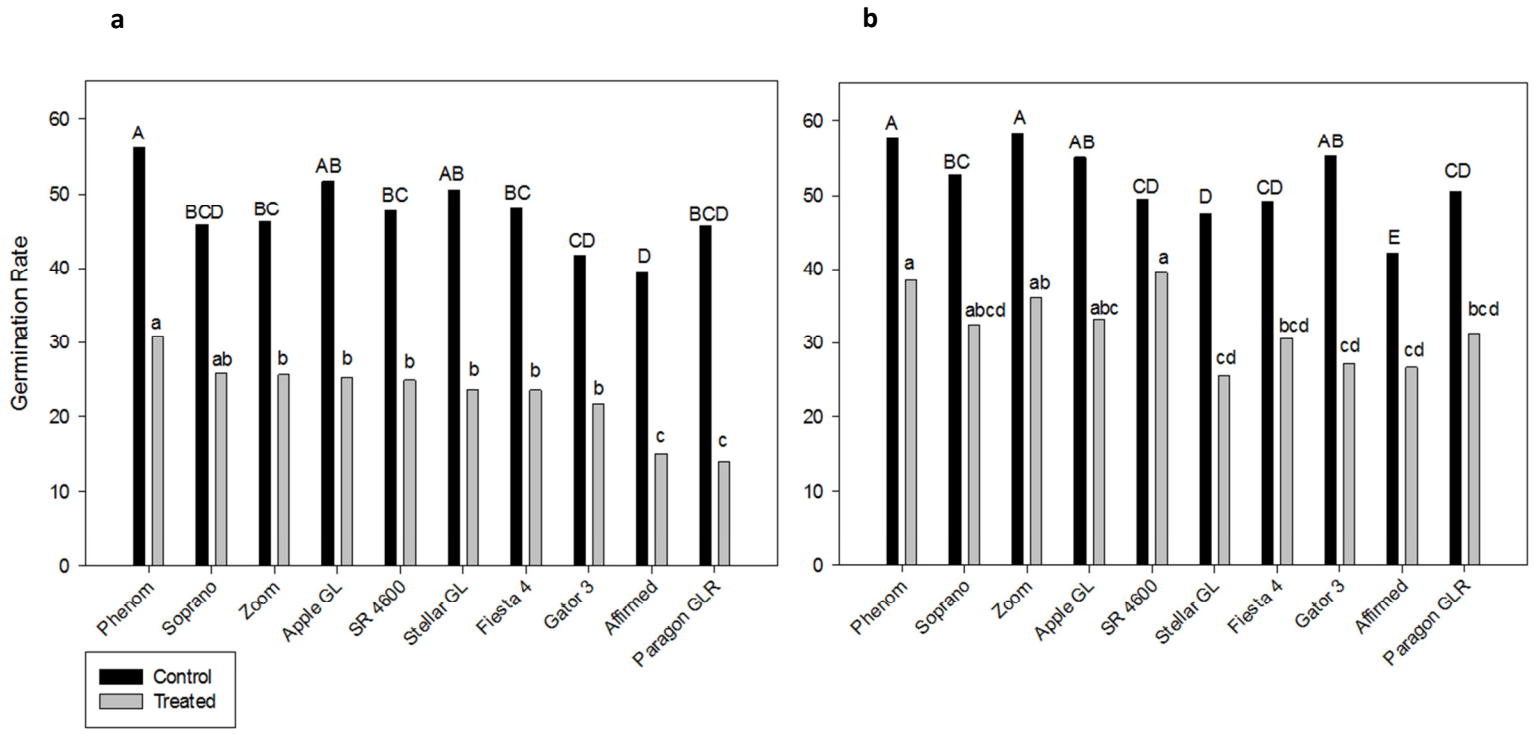


Figure 2. Final germination percentage of seedlings of perennial ryegrass (*Lolium perenne* L.) cultivars grown in a growth chamber salinity screening study for run 1 (a) and run 2 (b). Cultivars with different letters represent significant differences at the 0.05 probability level using Fischer's protected least significant difference means separation test. Upper and lower case letters represent a statistical difference within the control and the treatment (10 dS m^{-1}) respectively.

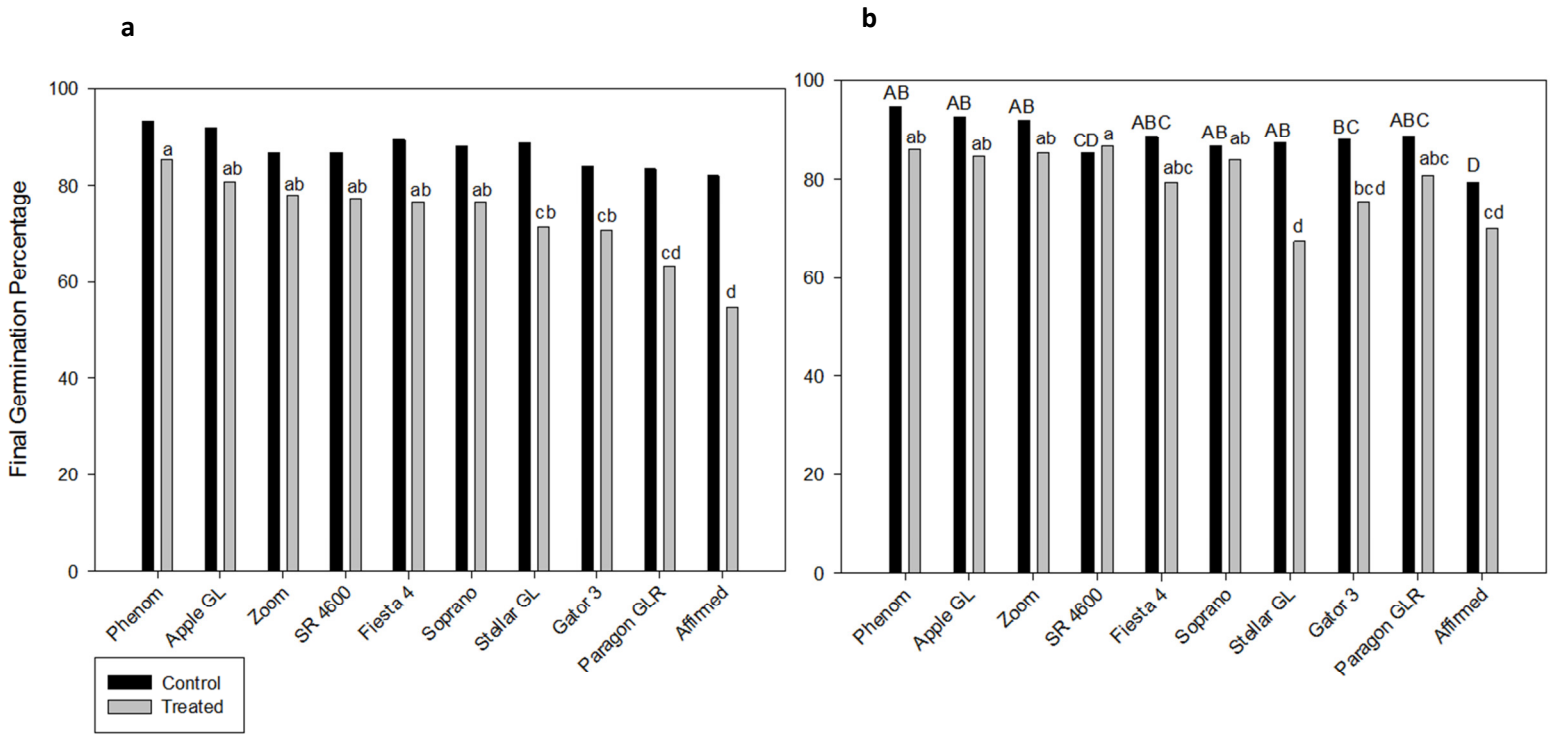


Figure 3. Shoot length of cultivars of perennial ryegrass (*Lolium perenne* L.) seedlings after 14 days of salt stress in a growth chamber salinity screening study. Shoot length is presented as a percentage of control. Cultivars with different letters represent significant differences at the 0.05 probability level using Fischer's protected least significant difference means separation test. Upper and lower case letters represent a statistical difference within run 1 and run 2 respectively.

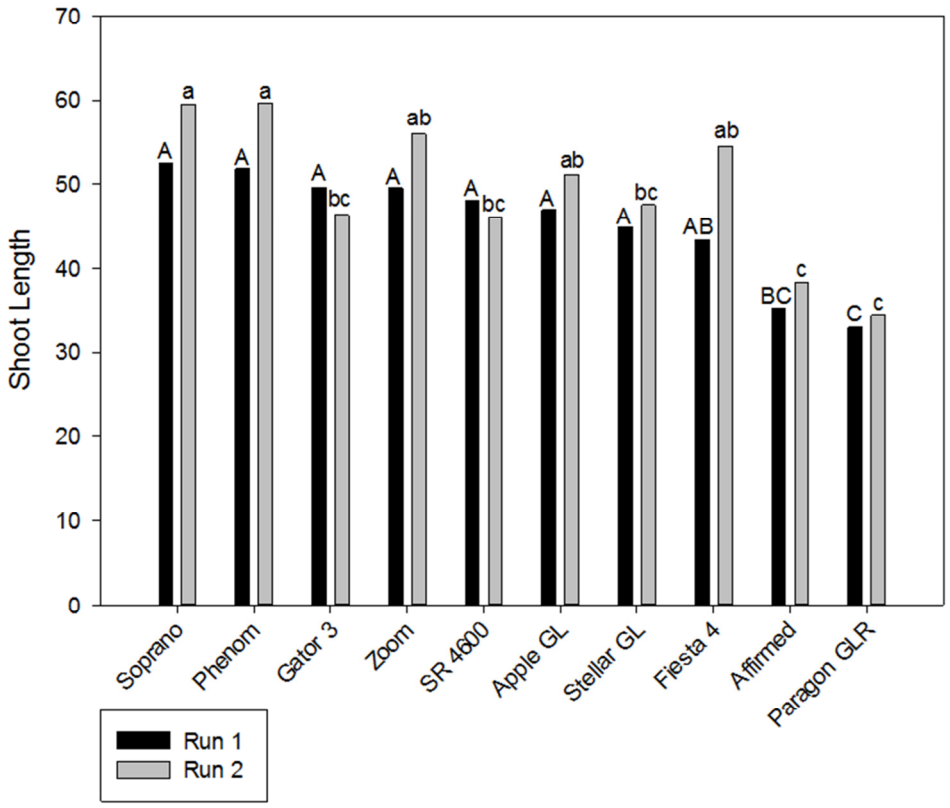


Figure 4. Shoot weights of perennial ryegrass (*Lolium perenne* L.) seedlings after 14 days of salt stress in a growth chamber salinity screening study. Shoot length is presented as a percentage of control. Cultivars with different letters represent significant differences at the 0.05 probability level using Fischer's protected least significant difference means separation test. Upper case letters represent a statistical difference when averaged across runs.

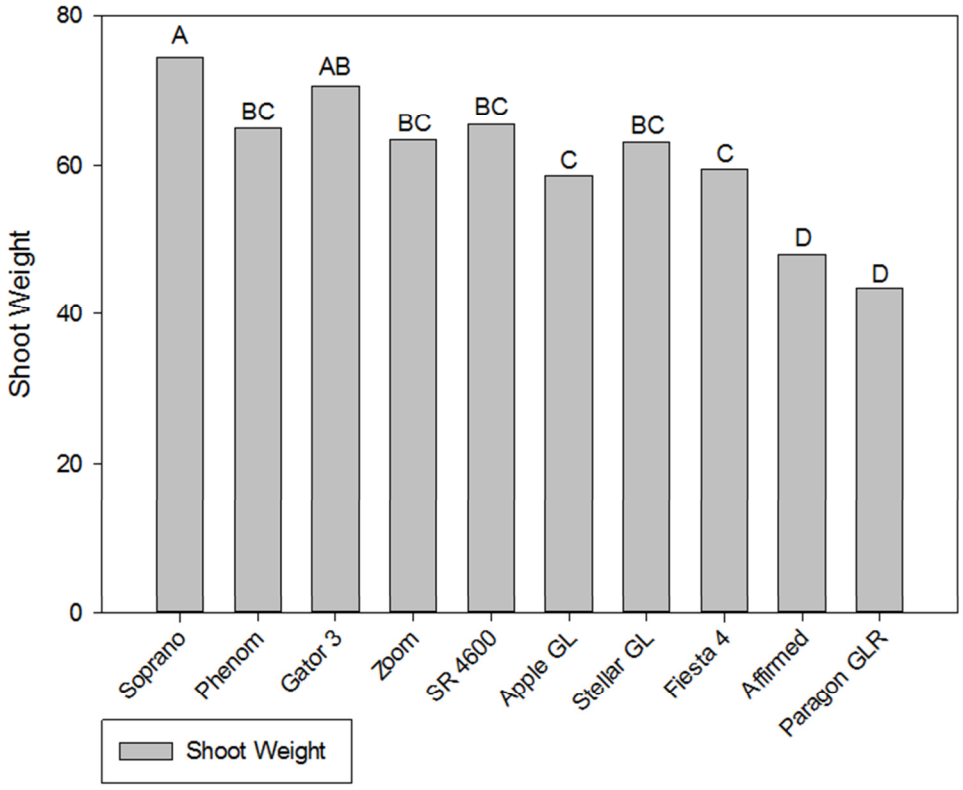
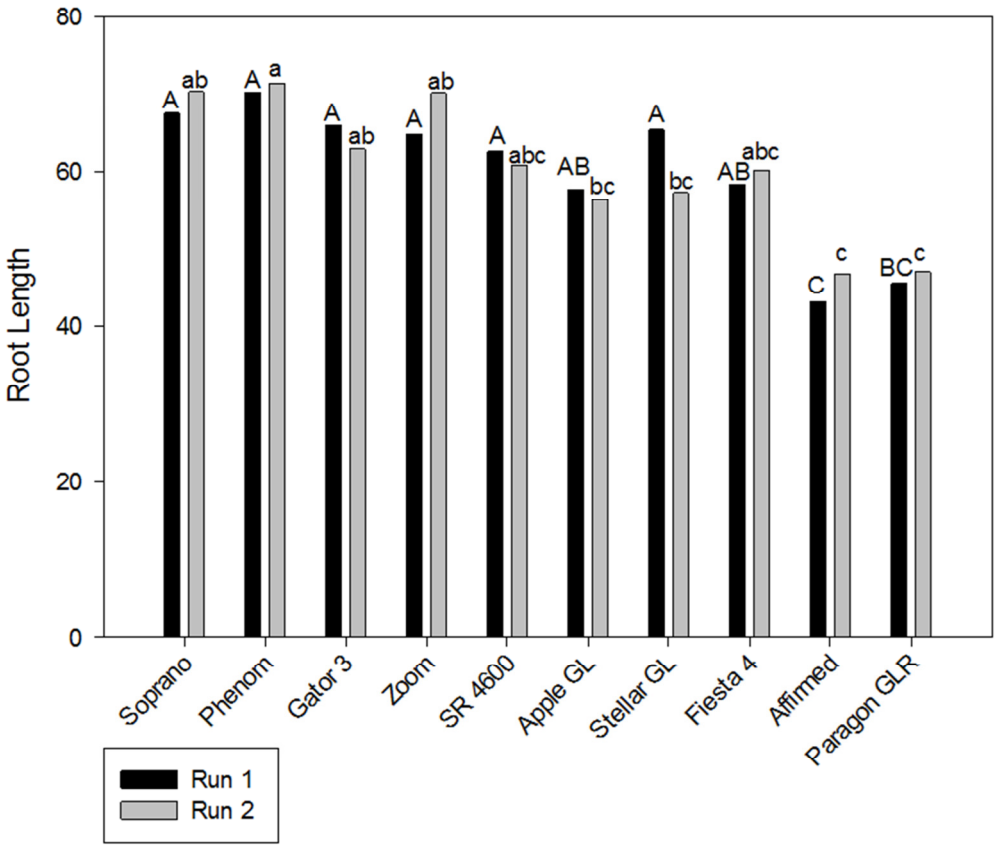


Figure 5. Root lengths of perennial ryegrass (*Lolium perenne* L.) seedlings after 14 days of salt stress in a growth chamber salinity screening study. Shoot length is presented as a percentage of control. Cultivars with different letters represent significant differences at the 0.05 probability level using Fischer's protected least significant difference means separation test. Upper and lower case letters represent a statistical difference within run 1 and run 2 respectively.



CHAPTER 4

Endophyte Effect on Salinity Tolerance in Perennial Ryegrass

Abstract. Perennial ryegrass is an important cool-season turfgrass species that is known to contain the fungal endophyte in the genus *Neotyphodium.* This species of turfgrass will be increasingly more important as water restrictions continue to tighten and alternative water sources (with increased salinity levels) become commonplace. The *Neotyphodium* endophyte in perennial ryegrass has been shown to convey resistance to various abiotic and biotic stresses but the study of salinity-endophyte interactions have been lacking in turfgrass. The objective of this study was to determine whether salinity tolerance is genotype-endophyte specific, or whether there is an overall endophyte effect on salinity tolerance in perennial ryegrass. Four perennial ryegrass clones (Brightstar SLT clone 5, Paragon GLR clone 4 [salt susceptible], 4501-7 and 4540-9 [salt tolerant]), with (E+) and without (E-) endophyte were grown in control (1 dS m^{-1}) and saline water conditions (15 dS m^{-1}). Control and saline water treatments were applied to the two-month old transplanted plants using overhead irrigation chambers in the greenhouse. Growth parameters were collected including visual percent green, clipping yields, shoot weight, and root weights. In PG4 the E- clone performed better than the E+ clone for all measurements when averaged across rating dates. Endophyte effects in the other clones were more evident in root and shoot weights compared to visual percent green ratings and clipping yields. For most of the clones, E- clones tended to be more tolerant than E+ clones except for 4540-9 which exhibited the opposite effect. Our data concurs with

previous research indicating that there may be an endophyte-host genotype dependent response when under salinity stress but there was not a strong positive effect of the endophyte on salinity tolerance among perennial ryegrass clones.

INTRODUCTION

As the population continues to grow, the use of alternative water resources for irrigating turfgrass has become more of a concern. However, these alternative water sources may contain a higher level of total soluble salts when compared to potable water which can result in salt injury and poor turf quality. Developing turfgrass cultivars that are more salt tolerant and maintain high turf quality with use of these alternative water sources may lead to the easy transition to non-potable water. One of the species currently being studied for salinity tolerance is perennial ryegrass (*Lolium perenne* L.). This bunch type cool-season species is used extensively in turfgrass situations due to its quick establishment and good wear tolerance. It is known to be moderately salt tolerant up to salinity levels of 6 to 10 dS m^{-1} (Harivandi et al, 1992). It also contains an endophytic fungus *(Neotyphodium.lolii)* that grows intercellulary between cells of the plant.

Other turfgrass species (including perennial ryegrass) are also hosts to the fungal endophytes of the genus *Neotyphodium* or the teleomorph genus *Epichloe* (Glen et al., 2001, Funk and White, 1997). The fungal endophyte presence results in the synthesis of alkaloids that protect the plant from above-ground feeding insects (Breen 1994) while the

plant provides nutrients for the fungus to survive. This mutualistic relationship has been studied extensively in previous research (Clay, 1988)

In addition to a reduction in herbivory, the endophyte has been shown to convey tolerance to abiotic and biotic stresses including drought tolerance (West 1994, Arachevaleta et al., 1989), phosphorus utilization (Malinowski and Belesky, 1999a), aluminum tolerance (Malinowski and Belesky, 1999b, Zaurov et al., 2001), and disease resistance (Bonos et al., 2005; Clarke et al., 1994). The mechanisms that result from these tolerances are not well understood. Lately, there have been studies to further understand the endophyte-host interaction when a plant is under stress. Zaurov et al. (2000) conducted a study on strong creeping red fescue (*Festuca rubra* ssp. *rubra*) and Chewings fescue (*Festuca rubra* ssp. *fallax*) inoculated with different fungal endophyte genotypes to study aluminum tolerance. These authors found a significant endophyte-host interaction. All turf genotypes did not perform better with or without endophyte infection, but rather certain combinations of endophyte and plant genotypes performed best. This type of endophyte-host interaction has also been observed in another study where certain endophyte-host combinations were compared for growth characteristics such as panicle emergence, horizontal spreading, post-harvest regrowth, and flag leaf length (Johnson-Cicalese et al., 2000). Cheplick et al. (2004) did not find an endophyte by host interaction. These authors conducted a drought study on perennial ryegrass plants and found that the plants with no endophyte had greater tiller numbers, greater leaf area, and greater mass when compared to the same genotype plant containing the endophytic fungi.

Studies on turfgrass-endophyte interactions under salinity stress have not been conducted in turfgrasses. In other species, Waller et al. (2005) found that *Piriformospora indica*, a plant-root-colonizing basidiomycete fungal endophyte, provided increased resistance to salinity in barley (*Hordeum vulgare* L.). There has been little research to determine if salinity tolerance in cool-season grasses is influenced by fungal endophytes. The objective of this study was to determine whether salinity tolerance is genotype-endophyte specific, or whether there is an overall endophyte effect on salinity tolerance in perennial ryegrass.

MATERIALS AND METHODS

Four perennial ryegrass clones were selected based on their susceptibility to salinity stress. For the salt susceptible germplasm, Paragon GLR clone 4 (PG4) and Brightstar SLT clone 5 (BS5) were chosen due to prior research conducted at Rutgers University which indicated their poor ability to tolerate salinity (Koch and Bonos, 2010). The salt tolerant germplasm, clones 4501-7 and 4540-9, were selections from a mature plant salinity screening nursery in a rain out shelter located at the Rutgers Plant Biology Research Extension Farm in Freehold, NJ. These plants were evaluated in the summer of 2012 and were top performers after multiple applications of salinity stress. All plants tested positive for the presence of the endophytic fungi using the Agrinostics Phytoscreen Immunoblot Kit (Agronostics Ltd. Co).

The four genotypes were vegetatively propogated and placed into two groups; 1) Plants to maintain their positive endophyte status and 2) plants that would be subjected to fungicide treatment to kill the endophyte. To remove the endophyte, propiconazole, Banner MAXX II (Syngenta) was used at a rate of 0.06g ai / m^2 and was sprayed 5 times on 1 week intervals. Endophyte presence was again checked using the immunoblot kit and all plants exposed to the fungicide regimen contained no endophyte. The presence of the endophyte was again checked 5 weeks into salinity treatments and again at week 10, before the extraction of plants. Plants exposed to the fungicide maintained no endophyte presence throughout the course of the experiment, while plants not exposed to the fungicide maintained their endophyte status.

The 8 entries (PG4 E+, PG4 E-, BS5 E+, BS5 E-, 4501-7 E+, 4501- E-, 4540-9 E+, 4540-9 E-) were vegetatively propagated and were separated into 6 individual clonal replicates each containing 1 tiller. These clones were established in Pro-Mix growing media (Premier Tech Ltd. Quebec, Canada) and were grown for 2 months in the greenhouse. All clones were again tested for presence or absence of the endophytic fungi. Plants were then removed from the Pro-mix and roots were washed and cut directly below the crown. Shoots were cut to 3.8 cm above the crown. One of the 6 replicate clones of each entry was planted randomly into one cell of a plastic 15 cm deep tray filled with topdressing sand from US Silica (Mauricetown, NJ). Each tray had 24 individual cells separated by plastic dividers to prevent roots from adjacent plants growing together. At the bottom of each cell, 2 holes 0.6 cm in diameter were made to allow irrigation water to drain through the root zone. Each tray contained 3 replications

of each entry for a total of two trays (1 tray for each treatment). Both trays were irrigated with half-strength Hoagland's solution (Hoagland and Arnon, 1950) every other day for a week to allow plants to establish in the sand media before the initiation of the salt treatment.

An overhead irrigation spray chamber system was designed to apply saline water directly onto the plants (Koch and Bonos, 2010). These chambers used recirculating saltwater solutions that were emitted through spray nozzles above the plants. The plants (in their plastic tray) were placed on a platform of plastic lattice above the holding tanks. The holding tanks were filled with 100 L of tap water with half-strength Hoagland's Solution.

The salt treatment was made using Instant Ocean (Spectrum Brands Inc. Atlanta, GA). Instant Ocean was added to the nutrient solution at a rate of 2 dS m^{-1} each irrigation day until the final concentration was reached. The final electrical conductivities (EC) of the solutions were: Treatment 1 (Control): 1 dS m^{-1} (this treatment contained Hoagland's solution therefore the EC did not equal 0), and Treatment 2: 15 dS m^{-1}. Electrical conductivities were checked and adjusted as needed on each irrigation day. Plants were irrigated every other day for 10 weeks after the final EC was reached in the salt treatment. The Hoagland's solution was replaced weekly to ensure that the stress observed was due to salinity, and not nutrient deficiency. Each irrigation cycle applied a total of 4.98 cm of each solution. Solution pH was adjusted to 6.5 with H_2SO_4 (acid) or

NaOH (base) before each irrigation cycle since this is the optimum pH for perennial ryegrass (Murphy and Mohr, 2002)

The experiment was conducted in a greenhouse with 400 Watt HPS supplemental lighting (Kavita Canada Inc. Niagara-on-the-Lake, Canada) which maintained 14-hour day lengths throughout both runs of the 10 week study (Run 1: average temperature = 21.6°C; SD = 0.89, Run 2: average temperature = 17.1°C; SD = 0.71) . Greenhouse temperatures were maintained between 17°C and 24°C. An Onset HOBO Series data logging temperature probe was used to monitor temperature and humidity on 15 minute intervals over the course of the study. Humidity was not controlled during the experiment.

Visual percent green ratings (0-100%) were taken weekly on all plants which indicated leaf senescence due to salinity stress. After the ratings were obtained, all plants were cut to a height of 3.8 cm. Clippings were collected every 2 weeks (every week for the final 3 weeks of the experiment to capture more data), dried at 65°C for 48 hours and weighed. Digital images were also taken of turfgrass plants to quantify the percentage of green tissue remaining after irrigation salinity regimes. Digital images (DIA) were taken using a Canon PowerShot SD1300 (Canon USA; Melville, NY) mounted at a 90° angle on a camera stand 1 m above turfgrass plants. Opaque black plastic was used to enclose the camera stand to prevent light from entering. The camera's flash was used when taking pictures. Digital images were measured for each plant using Sigmascan Pro 5.0 (Systat Software, Inc.; Point Richard, CA) and a software macro written to calculate

percent green tissue in turfgrass species (Karcher and Richardson, 2005). Relative percent green values for the digital images were then calculated by comparing the percent green of treated entries against the control plants to eliminate inherent genetic differences (Koch and Bonos, 2010).

After 10 weeks of salt treatment, all plants were extracted from the trays and the roots were washed free of all sand. Plants were then cut at the crown into two parts: roots and shoots. The roots and shoots were immediately dried at 65°C for 48 hours, and weighed. Soil electrical conductivity and pH were collected for three replicates per treatment. Soil within each treatment was amalgamated before samples were taken. The 1:2 (V:V) soil:water extract method was used to analyze soil electrical conductivity (Dellavalle, 1992). pH of each soil sample was measured using the 1:1 (V:V) soil:water method (Miller and Kissel, 2010).

The experimental design was a completely random design within each tray. The 10 week experiment was repeated twice; Run 1 was initiated in April 2013 and Run 2 in December 2013. All data was reported as a percentage of control in order to account for inherent genetic differences and growth patterns among genotypes of perennial ryegrass. All data was subjected to Analysis of Variance (ANOVA) using SAS Version 9.3 (SAS Institute, 1991). Correlations between measurements were conducted using Proc CORR in SAS. Contrast between E+/E- entries was conducted in SAS Proc GLM Contrast Statement.

RESULTS AND DISCUSSION

The ANOVA revealed a significant treatment effect for percent green ratings, clipping yields, and root and shoot weights (table 1) when the control was compared directly to the salt treatment. A significant run x entry interaction was observed for the majority of dates for percent green rating and clipping weights, therefore data for each run is presented separately for these two measurements. There was no significant run x entry interaction for shoot and root dry weights indicating that cultivars performed similarly across both runs for these measurements and therefore, data for both runs are presented together.

This run x entry interaction could be caused by the fact that the two runs were conducted at different times of the year. The first run was conducted in the spring/summer which allowed for actively growing roots/shoots and the plants were exposed to increasing day lengths in the greenhouse prior to initiation of the experiment. The second run was conducted in the winter; the plants in this study were exposed to short day lengths and may have been allocating more nutrients and carbohydrates to storage rather than elongating shoots as cool-season turfgrasses prepare for winter dormancy (Turgeon, 2004). It has also been found that humidity levels and ambient light can interact with salinity tolerance of plants (Maas, 1986). This interaction can be validated for the relative clipping yields; whereas our salt tolerant clones (4501-7 E+/E- and 4540-9E+/E-) performed better during run 2 in comparison to run 1. Additionally,

our results showed a faster decrease in clipping weights during run 2 when compared with run 1.

The salinity irrigation treatment resulted in decreased relative percent green ratings (run 1 = 41.6%; run 2 = 47.59%), relative clipping yields (run 1 = 76%; run 2 = 79%), relative shoot weights (run 1 = 26.44%; run 2 = 32.11%), and relative root weights (run 1 = 63.67%; run 2 = 54.74%) by the end of the study when averaged across all entries. Similar results have been observed in other salinity studies which found that salinity caused reductions in many different growth parameters (Qian et al., 2001, Marcum, 2001, Gibeault et al., 1977, Greub et al., 1985) Additionally, the salinity irrigation resulted in significant differences in the electrical conductivity of the sand growing media. The control treatment (1 dS m^{-1}) maintained a soil EC of 0.09 dS m^{-1} (Run 1) and 0.08 dS m^{-1} (Run 2) while the salinity treatments soil EC was 1.38 dS m^{-1} (Run 1) and 1.88 dS m^{-1} (Run 2). The low EC measurements in the salt treated media are not surprising due to the fact that sand has limited cation exchange sites (Carrow and Duncan, 1998). All parts of the plant were exposed to the high EC irrigation water so the stress response was a combination of ions within the rooting media along with the frequent exposure of the plants to the salt solution.

For visual percent green ratings, significant differences were observed between entries starting on week 5 in both runs (Figure 1). Digital images were highly correlated with visual percent green ratings (r^2=0.91 $p<0.001$) (Table 2). Unsurprisingly, clones of BS5 (E+ and E-) performed poorly when compared to other plants due to its poor salinity

tolerance. Interestingly, PG4 E- performed well and maintained green color longer in both runs when compared to all entries (Figure 1). Furthermore, PG4 E- consistently performed better than PG4 E+ and outperformed our salt tolerant clones (4501-7 and 4540-9) in run 1. This interaction can be seen not only in visual percent green, but in all other measurements as well (as seen below). Endophyte presence resulted in a negative effect on visual percent green in PG4 for 2 rating dates in run 1 (week 8 and 9) and 5 rating dates in run 2 (week 5, 6, 7, 8, and 9). This clone (PG4) was the only clone that exhibited a consistent endophyte interaction for visual percent green across both runs. There was no total endophyte interaction when comparing all E+ to all E- clones for any rating date. This indicates that there may be an endophyte-host specificity for salinity tolerance in perennial ryegrass, the endophtye may have a negative effect in one genotype and a positive effect in another genotype. Endophyte by host interactions have been discovered in other abiotic stresses. Zaurov et al., 2001 conducted a study on aluminum tolerance in 2 species of fine fescue and found an endophyte-host interaction where some genotypes performed better with the endophyte and vice versa.

Relative clipping weights were also correlated to visual percent green ratings (r^2= 0.85 $p<0.01$) and digital images (r^2=0.81$p<0.05$) (Table 2). Clippings weights during Run 2 declined faster in Run 2 compared to run 1 which could have been due to the fact that run 2 was conducted in the winter and run 1 was conducted in the spring (as stated above) (Figure 2). Similar to visual percent green ratings, BS5 E+ and E- performed poorly when compared among all other entries. The entry PG4 E- was a top performer in run 1 and run 2 when compared to all other entries. PG4 E+ performed worse than PG4

E- for relative clipping weights throughout run 1 and run 2. A significant endophyte interaction was observed for 2 dates in run 1 (week 9 and 10) and 3 dates (week 2, 6, and 9) in run 2. Interestingly, 4540-9 E- was a top performer early in run 2 but 4540-9 E+ maintained its salinity tolerance throughout the duration of the experiment. This clone saw an endophyte interaction on week 8 of run 2 where 4540-9 E+ performed better than E-. Interestingly, 4540-9 E+ also performed better than E- for shoot and root weights (as seen below). This trend in salinity tolerance based on endophyte presence further strengthens the hypothesis of an endophyte-host interaction where depending on the genotype and the host the endophyte may have a positive effect or a negative effect.

Significant differences between entries were observed for both dried shoot and root weights. Both shoot (r^2=0.69 $p<0.05$) and root (r^2=0.78 $p<0.01$) weights were correlated with visual percent green ratings (table 2). Shoot and root weights have been successfully used in prior research to identify the effects of salinity on turfgrass growth (Marcum and Murdoch, 1990, Qian et al., 2001, Marcum et al., 2005, Koch and Bonos, 2010). Similar to data presented on relative clipping weights and visual present green measurements, PG4 E- performed significantly better than all other tested entries including PG4E+ (figure 3). Contrasts between E+ and E- clones revealed a significant endophyte interaction for PG4 where the E+ plants had significantly lower shoot and root weights than E-. Interestingly there was an opposite endophyte interaction for 4540-9 where the E+ clones performed better than the E-. For shoot weights, there was no difference between E+ and E- clones of BS5 and 4501-7 but for root weights, both BS5 E- and 4501-7 E- performed better than E+ clones. Interestingly, since 3 out of the 4

entries performed better without the endophyte, there was a total significant endophyte interaction where presence of the endophyte caused a significant reduction in root and shoot weights.

The hypothesis for this finding is that the fungal endophyte may be using vital nutrients and other components to survive while the plant is trying to adapt to the abiotic salinity stress. Additionally, some clones showed no consistent endophyte response across measurements (4501-7 and BS5) while 4540-9 E+ performed better than E- clones for dried shoot/root weights and relative clipping weights for week 8. The biggest endophyte interaction was observed when analyzing the dried root and shoot weights. Future research studying nutrient accumulation in roots and shoots should be conducted to analyze if the endophyte is reducing nutrient availability to the plant. This variability in endophyte presence and response to salinity stress concurs with prior research that also found a significant specific endophyte-host interaction (Zaurov et al., 2001, Belesky and Fedders, 1995, Johnson-Cicalese et al., 2000) that depended upon the particular genotype.

CONCLUSION

Using the overhead irrigation spray chamber method was successful in separating clones of perennial ryegrass with and without the fungal endophyte under salinity stress. Our data shows a significant reduction in all clones for relative percent green ratings, relative clipping weights, digital images, and dried root/shoot weights due to the salinity treatment. As expected, BS5 E+ and E- performed poorly for salinity tolerance when

averaged across all measurements among other clones. In prior research, BS5 was not salt tolerant at high salinity levels (Koch and Bonos 2010). The clone PG4 E- performed better than PG4 E+ in all measurements indicating that in this genotype, the endophyte has a negative effect on growth under salinity stress but in other clones like 4540-9, the opposite was true. The positive and negative influences of the endophyte depending on host genotypes is not new and has been observed for other abiotic traits. This research indicates that endophytes most likely do not have a strong effect on salinity tolerance in perennial ryegrass. Additional research on the specific genotype of the endophyte is needed to further understand this interaction as there is also potential of a genotype-host by genotype-endophyte interaction.

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Table 1. Analysis of variance of salinity tolerance measurements (relative visual percent green ratings, relative clipping yields, relative shoot weights, and relative root weights) of perennial ryegrass clones evaluated in a greenhouse using overhead irrigation with a salinity treatment of 15 dS m^{-1}.

	Relative Visual percent green										Relative Clipping Yields						Relative	Relative
	Weeks										Weeks						Shoot	Root
	1	2	3	4	5	6	7	8	9	10	2	4	6	8	9	10	Weight	Weight
Treatment	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Replication	ns	ns	*	*	*	ns	ns	ns	*	ns	ns	ns	ns	**	ns	ns	ns	ns
Entry	ns	ns	ns	**	***	***	***	***	**	**	*	ns	***	***	***	***	**	***
Run	ns	ns	ns	ns	ns	ns	**	**	ns	*	***	***	***	ns	ns	ns	ns	*
Entry*Run	ns	ns	ns	ns	**	**	**	*	*	***	ns	ns	ns	*	**	**	ns	ns
Replication* Entry*Run	ns	ns	ns	ns	**	**	*	**	**	**	ns	ns	*	*	*	**	ns	ns

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level
***Significant at the 0.001 probability level
NS, not significant

Table 2. Pearson correlation coefficients for growth parameters of perennial ryegrass clones exposed to salinity stress for 10 weeks under greenhouse conditions.

	Visual Ratings	Digital Images	Clipping Weights	Shoot Weight
Digital Images	0.91***			
Clipping Weights	0.85**	0.81*		
Shoot Weights	0.69*	0.54^{ns}	0.60^{ns}	
Root Weights	0.78**	0.84*	0.59^{ns}	0.78*

*Significant at the 0.05 probability level
**Significant at the 0.01 probability level
***Significant at the 0.001 probability level
NS, not significant
Correlation analysis was run on week 6 for all measurements

Figure 1. Relative percent green ratings of perennial ryegrass entries taken weekly for 10 weeks under a salinity stress of 15 dS m^1 using a greenhouse salinity screening technique. All data were represented as a percentage of the control plants that did not receive saline irrigation. Vertical bars denote LSD values at the 0.05 probability level and were present only when the differences between genotypes were present.

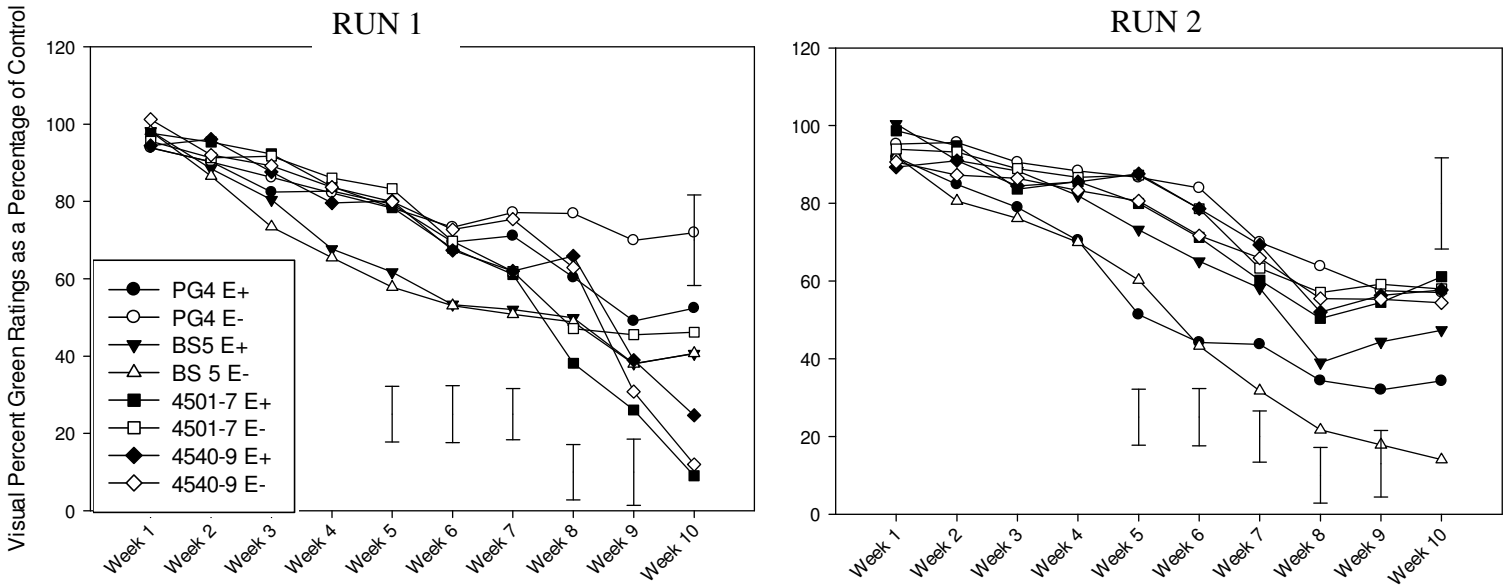


Figure 2. Relative clipping yields of perennial ryegrass entries taken biweekly (and weekly for the final 3 weeks) for 10 weeks under a salinity stress of 15 dS m^1 using a greenhouse salinity screening technique. All data were represented as a percentage of the control plants that did not receive saline irrigation. Vertical bars denote LSD values at the 0.05 probability level and were present only when the differences between genotypes were present.

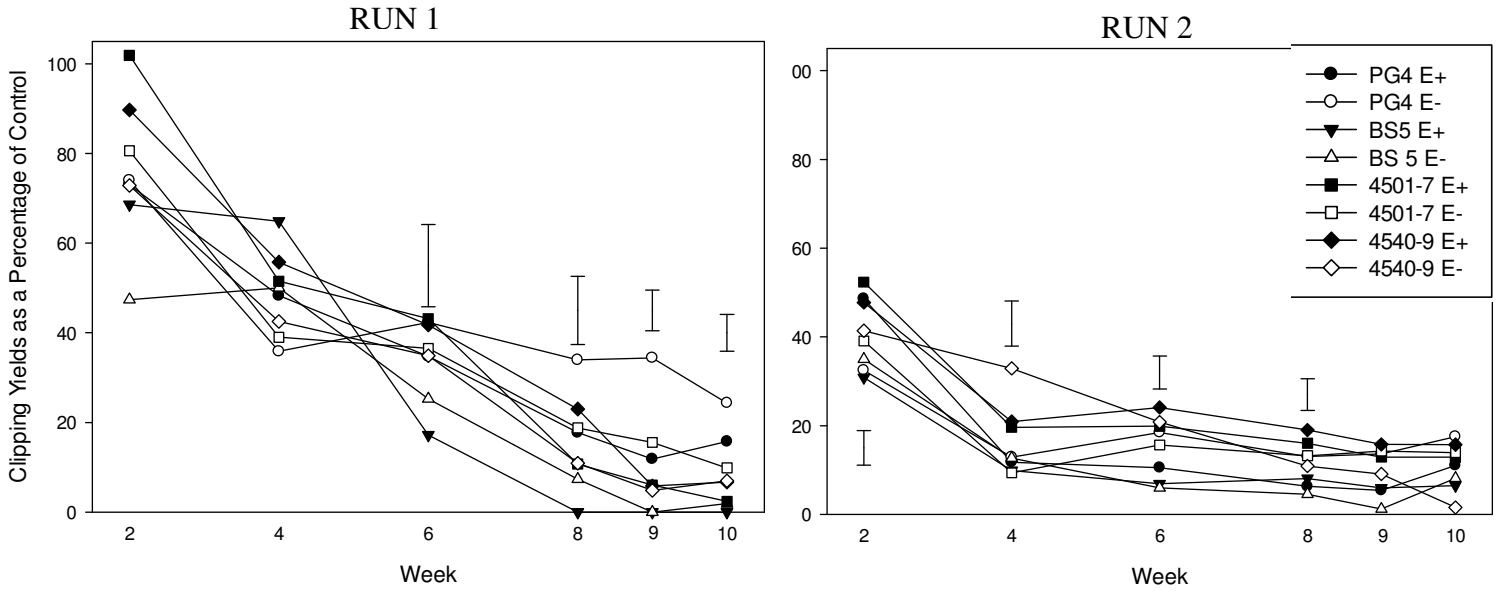
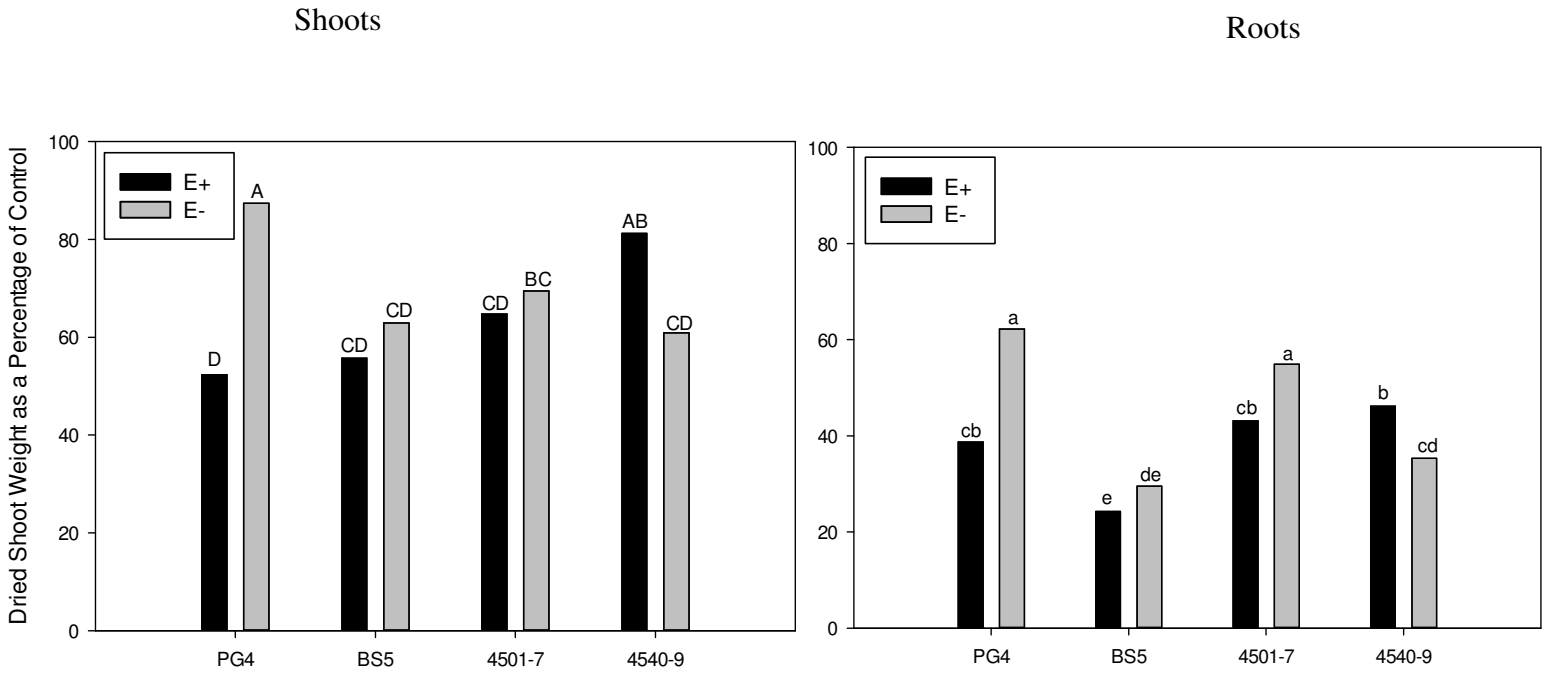


Figure 3. Relative shoot and root weights of perennial ryegrass entries taken after 10 weeks under a salinity stress of 15 dS m^1 using a greenhouse salinity screening technique. All data were represented as a percentage of the control plants that did not receive saline irrigation. Upper Case (shoots) and lower case (roots) letters denote LSD values at the 0.05 probability level.



CHAPTER 7

Thesis Conclusion

As the human population continues to increase the use of low quality irrigation will become more ubiquitous as potable water becomes increasingly scarce. Screening cool season turfgrass for increased levels of salinity tolerance is therefore important for the future irrigation of lawns, sports fields, and golf courses. Research on salinity tolerance during germination has been lacking thus far and the need for screening available cultivars for salinity tolerance is among the first steps in understanding this trait. Due to the nature of this complex quantitative trait, breeding for mature salinity tolerance of cool season grasses has been difficult. Through cycles of recurrent selection breeding, new cultivars are now becoming available displaying traits for increased salinity tolerance.

This research effectively ranked many currently available perennial ryegrass cultivars for salinity tolerance. Additionally, varying levels of salinity tolerance in native soil allowed for the accumulation of detrimental salt ions which led to a decrease in germination as salinity levels increased. One of the most important findings from this research was that there was consistently no interaction between cultivar and salinity treatment. This means that cultivars are performing similarly in the saline treatment as well as the control. Therefore, selection for seedlings that germinate quickly in potable water may translate to quick germination in saline conditions.

All of the measurements that were used to quantify salinity tolerance were useful but overall, emergence ratings and germination rate were the most informative measurements for determining a salt tolerant cultivar. Digital images to quantify early establishment were also useful and were highly correlated with visual percent green cover ratings. In the future, it may be possible to limit the analysis of individual pictures to increase efficiency in screening large quantities of available cultivars.

Another important finding from this research was the recurring variability between runs of the experiments. This variability can be attributed to confounding effects from the environment. From this study, we have found the environment may affect germination of perennial ryegrass more than genetic factors. A field study (data not presented) was conducted over 3 years where over 150 cultivars were evaluated for their salinity tolerance in seeded plots. This data was not included in this thesis because of the variability within replications and across years. Additionally, the seeded plots within the salt treatment lagged so far behind in germination time that comparing the treatment to the control plots was not feasible. In the future, selection for this complex trait needs to be conducted in controlled conditions to eliminate as many confounding environmental effects as possible. Salinity levels should also be increased during the selection process whereas only 1% of the seedlings survive and move forward into a mature salt tolerance screening.

The research on the endophytic fungi in perennial ryegrass revealed a significant endophyte-host interaction while under salt stress where certain host genotypes benefit

from containing the endophytic fungi while other genotypes do not. This finding has been observed with other abiotic stresses such as aluminum tolerance in fine and tall fescues. To further understand this interaction, nutrient analysis should be conducted on both the roots and the shoots of plants with/without the endophytic fungi. This future study would help to understand if the endophytic fungi are starving certain host genotypes of important nutrients when the plant is under salinity stress.

Salinity tolerance research has been studied extensively, but due to the quantitative nature of this trait, selection and breeding has been difficult. Complex interactions between salinity and the environment can further complicate salinity research. Further evaluation and selection at varying levels of growth will be required in the future to continue to improve varieties with increased salinity tolerance.