2013

Physiological and Biochemical Responses of Bald Cypress to Salt Stress

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Physiological and Biochemical Responses of Bald Cypress to Salt Stress

by

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A thesis submitted to the department of Biology in partial fulfillment of the requirements

for the degree of

Master of Science in Biology

UNIVERSITY OF NORTH FLORIDA

COLLEGE OF ARTS AND SCIENCES

July, 2013

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Table of Contents:

List of Tables and Figures

Abstract

Bald Cypress (*Taxodium distichum* (L.) Rich.) is native to freshwater wetlands of Florida. The vitality of cypress within coastal freshwater wetlands is threatened by saltwater intrusion. Biomarkers to detect sub-lethal salinity stress were developed using a controlled greenhouse study. Cypress saplings maintained at elevated salinities of 4 and 8‰ exhibited a decrease in maximum quantum yield (MQY) and an increase in non-photochemical quenching (NPQ). Cypress leaves exhibited an increase in Na^+, H_2O_2, and free proline content compared to plants maintained in freshwater. These biomarkers were used to detect salinity stress within a population of cypress associated with the lower St. Johns River where saltwater intrusion is occurring. Cypress in a basin swamp exhibited signs of salinity stress with low MQY and elevated NPQ values compared to Cypress at other sites. Cypress leaves at the basin swamp also had the highest Na^+, lipid peroxidation, and proline content compared to plants at other sites. Detached Cypress leaf experiments were conducted to explore the mechanisms of salt tolerance. Detached cypress leaves were first exposed to elevated NaCl concentrations for 24, 48, or 72 hours. Elevated salinity caused a decrease in leaf transpiration for all times tested. Total peroxidase activity exhibited an increase in response to salt stress after 48 hours. Enhanced peroxidase activity was found to be associated with the induction of a ~37 kDa peroxidase isoform. Treatment of leaves with clofibrate caused an increase in activity of the ~37 kDa peroxidase. Pre-treatment of leaves with brefeldin A (BFA) blocked the induction of the ~37 kDa peroxidase associated with salt stress. Pre-treatment of Cypress leaves with diphenyliodonium (DPI) blocked the decrease in transpiration associated with

salt stress, suggesting that H_2O_2 is enzymatically produced within the stomata in response to salt stress.

Chapter 1: General Introduction

1.1 Bald Cypress and Sea Level Rise in the St. Johns River

Bald Cypress (*Taxodium distichum* (L.) Rich.) is a large deciduous softwood tree native to the Southeastern United States and are often dominant in coastal freshwater wetlands where they create important structural habitats for numerous organisms (Bonner, 1974; Harris & Mulholland, 1983; Brightman, 1984; McMahan & Davis, 1984; Harris & Vickers 1984; Wilhite & Toliver, 1990). They provide a number of important ecosystem services which make them assets to coastal freshwater habitats. For example, cypress swamps are capable of retaining large quantities of water which aids in flood control (Ewel, 1985). In addition, cypress are incredibly windfirm and are able to protect surrounding areas from winds up to hurricane force (Wilhite & Toliver, 1990). Finally, Cypress have been documented to improve water quality by serving as biological filters removing excess nutrients and pollutants from porewater (Wharton, 1977; Dierberg & Brezonik, 1983). These aforementioned characteristics make Bald Cypress an important member of coastal freshwater wetland communities and an important candidate for conservation and protection.

The vitality of coastal freshwater wetlands is currently being threatened by saltwater intrusion associated with both global and local occurrences. Globally, sea level is predicted to rise by 30 cm within the next 100 years (IPCC, 2007). Locally,

anthropogenic activities such as dredging and surface water withdrawal are predicted to contribute to saltwater intrusion within the lower St. Johns River. As a result, the freshwater/saltwater boundary in estuaries is beginning to shift upstream. This phenomenon places environmental stress on coastal freshwater wetlands and ultimately causes a shift in ecosystems from freshwater wetlands into oligohaline marshes.

The St. Johns River is located in Northeast Florida and enters the Atlantic Ocean near Jacksonville, FL. The lower reach of the river is characterized by a limited elevational gradient and slow freshwater flow. The tidal amplitude at the mouth of the river is approximately 1.5 m. These characteristics influence the pronounced tidal signatures which can be detected up to 200 km from the mouth of the river (DeMort, 1991). As a result, salinities as high as 6‰ have been observed twice in a 10 year period (Figure 1.1). Sea level rise and anthropogenic effects are expected to increase the tidal range of the St. Johns River estuary (SJRWMD, 2008). An increase in the tidal range will ultimately cause saltwater intrusion events to be more frequent and severe than past events.

Freshwater plants have limited tolerance to the synergistic stressors associated with saltwater intrusion including: elevated water salinity, chronic flooding, soil hypoxia, and the accumulation of hydrogen sulfide in the soil (Allen et al. 1998; Hackney et al. 2007). The stressors associated with saltwater intrusion have been reported to cause adverse effects on the overall metabolism and survival of wetland plants (DeLaune et al. 1987). Saltwater intrusion has been shown to convert coastal freshwater wetlands into ‘ghost swamps’ containing stressed or recently dead trees (including cypress) and a groundcover of oligohaline marsh plants (Figure 1.2; Allen et al. 1998; Hackney et al.

2007). Prior to death, freshwater wetland plants can exhibit stress responses that include stomatal closure, a decrease in net photosynthesis, a drop in net carbon assimilation, and decreased biomass (Pezeshki et al. 1989; Kozlowski, 2000). Interestingly, Bald Cypress exhibits the greatest degree of tolerance compared to other plants within the same freshwater community. Cypress is known to persist for years after a freshwater wetland has been impacted by saltwater intrusion (Figure 1.2; Allen et al. 1998).

In Bald Cypress, chronic salinity stress has been linked to increased mortality. However, prior to death, cypress exhibit the same physiological responses observed in other freshwater wetland plants. The responses include stomatal closure (Pezeshki et al. 1987; Allen et al. 1997), a downregulation of photosynthesis (Pezeshki et al. 1995; Allen et al. 1997), and a negative correlation between leaf Na^+ content and net photosynthesis (Pezeshki et al. 1988). Cypress appear to lower their overall metabolism in response to elevated salinity which can lead to a decrease in biomass and sapflow compared to plants maintained in freshwater (Allen et al. 1994; Pezeshki et al. 1995; Allen et al. 1997; Krauss & Duberstein, 2010). It has also been shown that the combination of flooding and salinity stress exerts a greater stress on cypress than either stressor alone (Allen et al. 1996; Effler & Goyer, 2006). Some populations of cypress within coastal habitats have a greater degree of salt tolerance than cypress from freshwater sources (Allen et al. 1994; Allen et al. 1997; Conner & Inabinette, 2005).

The most comprehensive study of salinity tolerance in Bald Cypress to date was conducted by Allen et al. (1997) where one year old seedlings from 15 open pollinated families collected from freshwater and brackish sites were exposed to salinities of 0, 2, 4, 6, and 8‰ for 4 months. Physiological parameters such as biomass, stomatal

conductance, net photosynthesis, leaf water potential, and tissue ion content were assayed. It was found that all seedlings could tolerate the low salinity (2‰) treatment remarkably well. However, significant intraspecific variation became evident at higher salinities (6 & 8‰). Furthermore, cypress seedlings maintained at an elevated salinity of 8‰ exhibited a decrease in biomass, stomatal conductance, net photosynthesis, and leaf water potential. Populations with the greatest degree of salt tolerance had lower Na^+ and Cl^- content within leaf tissue. The authors suggested that salt tolerance was linked to the plant's ability to extrude these ions from their tissue. However, the underlying cellular mechanisms that lead to the adaptive response of Bald Cypress remain unexplored.

1.2 Physiological and Cellular Responses of Plants to Elevated Salinity

The physiological mechanisms for salt tolerance have been studied for a number of plants including model organisms (e.g. *Arabidopsis thaliana*) and agriculturally relevant species such as *Lycopersicon* pennelli, *Lotus japonicus*, and *Brassica oleracea* (Mittova et al. 2004; Ndimba et al. 2005; Jiang et al. 2007; Rubio et al. 2009; Hernandez et al. 2010). The physiological responses of these plants to salt stress are strikingly similar to the responses observed in freshwater wetland plants. Physiological responses include: a reduction in net photosynthesis, reduction in leaf transpiration, stomatal closure, a reduction in carbon assimilation, and a decrease in biomass (Ashraf, 2004; Mahajan & Tuteja, 2005). The downregulation of photosynthesis appears to be a common physiological mechanism to increase water use efficiency as well as to cope with the 'physiological drought' imposed by salinity stress (Mahajan & Tutejia, 2005).

On the cellular level, salinity stress imposes both an ionic and osmotic stress on plants (Yokoi et al. 2002). Sodium ions are toxic to plant cells because they interfere with

normal metabolism and enzyme function (Kalir et al. 1984; Niu et al. 1995). Salt tolerance is linked to a plant's ability to cope with Na^+ ions within tissue (Blumwald et al. 2000; Apse & Blumwald, 2007). Some salt tolerance mechanisms include vacuolar sequestration of Na^+ ions within leaves and roots as well as extrusion of Na^+ ions from tissue. However, both of these mechanisms require a tremendous amount of energy because they require active transport of ions across membranes (Blumwald et al. 2000; Apse & Blumwald, 2007). Many plants also display less metabolically costly mechanisms to cope with salinity stress such as leaf senescence. Some deciduous plants respond to salinity stress by selectively transporting Na^+ ions into older basal leaves along the base of the stem. These act as 'sacrificial leaves' that fall off the plant, thus reducing the number of Na^+ ions that enter younger leaf tissue (van der Moezel et al. 1988; Wolf et al. 1991). Plants respond to osmotic stress by synthesizing and accumulating osmolytes within the cytoplasm. These osmolytes include simple sugars, complex sugars, free amino acids, and betaines (Ewing et al. 1995; Yokoi et al. 2002; Sairam et al. 2002; Molinari et al. 2007). Individual plant species may not display all of the mechanisms mentioned above, but it is becoming increasingly apparent that more than one mechanism alone is employed by plants to tolerate elevated salinity.

On the sub-cellular and biochemical level, an upregulation of reactive oxygen species (ROS) has been associated with salt stress (Mittova et al. 2004; Gómez et al. 2004; Leshem et al. 2007; Hernandez et al. 2010). It was previously thought that ROS were a toxic byproduct of metabolism resulting from abiotic stress, such as elevated salinity. This hypothesis was supported by the fact that an increase in production of ROS is generally associated with oxidative damage to biomolecules such as lipids, proteins,

and nucleic acids (Møller et al. 2007). However, over the past decade, an overwhelming number of reports demonstrate that ROS play an important role for cell signaling in response to stress. It is now becoming clear that ROS are essential for plants to perceive and respond to abiotic stress (Mittler, 2002; Neill et al. 2002a; Neill et al. 2002b; Apel & Hirt, 2004; Jaspers & Kangasjärvi, 2010). For example, ROS have been shown to play a role in signal transduction by activating MAP kinase pathways (Neill et al. 2002b). Second, ROS can oxidize and change the activity of proteins. The oxidation of transcription factors by ROS have been shown to alter DNA binding properties and ultimately control the expression of specific genes (Tron et al. 2002). Third, ROS have been shown to play an essential role in regulating the activity of guard cells. Researchers have demonstrated that ROS are enzymatically produced within guard cells in response to the hormone abscisic acid, resulting in stomatal closure (Pei et al. 2000; Zhang et al. 2001; Desikan et al. 2004). Fourth, ROS have been shown to play a key role in the signaling events that lead to apoptosis as well as leaf senescence (del Río et al. 1998; Neill et al. 2002b). Finally ROS have been shown to increase within root tissue in response to salt stress (Leshem et al. 2007; Hernandez et al. 2010). Interestingly, it has been shown that ROS were enzymatically produced which led to the hypothesis that ROS may play a role in the plant's adaptive response to salt stress (Leshem et al. 2007; Hernandez et al. 2010). The increased production of ROS is clearly an important step for plants to perceive and respond to salt stress in both root and leaf tissue.

Although ROS appear to have an overall positive role for plants under stress conditions, their presence within cells is still deleterious if not kept in balance. Plants are capable of maintaining homeostasis under stressful conditions by altering levels of

numerous antioxidant enzymes that degrade ROS and maintain them within safe levels (Noctor & Foyer, 1998; Mittler, 2002; Mittler et al. 2004; Abogadallah 2010). Reactive oxygen species are continuously generated and destroyed within plant cells (for reviews see Mittler, 2002; Jaspers & Kangasjärvi, 2010; Sharma et al. 2012). These molecules can be produced enzymatically by NADPH-oxidase, apoplastic peroxidases, and amine oxidases. Non-enzymatic sources include chloroplasts and mitochondria (Orozco-Cárdenas et al. 2001; Tiwari et al. 2002; Leshem et al. 2007; Abogadallah, 2010). Both enzymatic and non-enzymatic processes result in the generation of the highly toxic superoxide anion (O_2^-). Superoxide can be converted into the less toxic hydrogen peroxide (H_2O_2) by a class of enzymes known as superoxide dismutase (SOD). A number of reports have demonstrated that SOD activity increases in response to acute salt stress (Sreenivasulu et al. 2000; Sairam et al. 2002; Meloni et al. 2003; Gómez et al. 2004; Mittova et al. 2004; Rubio et al. 2009). Hydrogen peroxide is considered to be the most important cell signaling molecule in plants because it is the least toxic, yet most stable form of ROS (Møller et al. 2007; Sharma et al. 2012). Hydrogen peroxide can be converted into the non-toxic products of water and molecular oxygen by a class of enzymes known as peroxidase (POX), including catalase (CAT) which is a peroxidase found specifically on peroxisomes. Some reports indicate an increase in POX activity in response to salt stress (Sairam et al. 2002; Meloni et al. 2003; Mittova et al. 2004). However, others report a decrease in response to salt stress (del Río et al. 1996; Shim et al. 2003; Hernandez et al. 2010). These conflicting results may be because of differences between species, intensity of treatment, or timing of sampling. Nonetheless, the alteration

of SOD and POX activity appears to be an important step in a plant's adaptive response to salt stress.

Salt stress and leaf senescence are reported to elicit the proliferation of peroxisomes within plant cells (Corpas et al. 1993; del Río et al. 1998; Lopez-Huertas et al. 2000; Mitsuya et al. 2010). Peroxisomes are very dynamic organelles because they are responsible for a number of metabolic roles within the cell including: the β oxidation of lipids, synthesis of jasmonic acid, participation in photorespiration, and the recycling of nucleic acids (León, 2008; Hu et al. 2012). Hydrogen peroxide is produced as a byproduct of these metabolic processes and some have suggested that peroxisomes may act as a significant source of ROS-mediated cell signaling within plant cells (del Río et al. 1996; Corpas et al. 2001; Mitsuya et al. 2010). The *de novo* synthesis of peroxisomes is an area of ongoing research and several models have been proposed. Currently, it is hypothesized that new peroxisomes are synthesized from the endoplasmic reticulum and the division of currently existing peroxisomes (Hu et al. 2012). Interestingly, others have reported that salt stress induced endocytosis and the production of endosomes (Mazel et al. 2004; Leshem et al. 2007; König et al. 2008). The newly produced endosomes have been reported to produce ROS and the authors hypothesized that this phenomenon plays an important role in the signaling pathways to respond to salt stress (Leshem et al. 2007). Remarkably, no clear links have been made between endocytosis and the proliferation of peroxisomes in response to salt stress. Thus, investigating a link between the *de novo* synthesis of peroxisomes and endocytosis remains an intriguing, yet unexplored area of research.

1.3 The Utilization of Biomarkers to Detect Salt Stress in Plants

An emerging field of research involves the utilization of 'biomarkers' to detect sub-lethal stress within organisms. The general concept is to identify a reliable indicator of stress within an organism to monitor the health of a population or ecosystem (Downs et al. 2000; Ferrat et al. 2003; Ashraf, 2009). The biomarkers could potentially be used as a tool for ecosystem monitoring or environmental impact studies. Biomarkers have been successfully developed in corals, aquatic vegetation, and wetland plants (Ewing et al.1995; Downs et al. 2000; Ferrat et al. 2003). However, the application of biomarkers on organisms *in situ* is limited (Ewing et al. 1997). The development of biomarkers for salt stress in plants could potentially be an exciting and useful area of research because the physiological and cellular responses of a plant to salt stress have been well studied. The upregulation of ROS, the subsequent alteration of antioxidant enzymes, and the accumulation of osmolites may provide useful insight into the physiological status of a plant (Ewing et al. 1997; Ashraf, 2009).

1.4 Objectives of Thesis

A substantial amount of literature has described the general physiological responses of Bald Cypress to elevated salinity at the organismal level; however, the underlying cellular and biochemical responses remain unexplored. The work presented herein represents the first report on the involvement of ROS and antioxidant enzymes within cypress tissue in response to elevated salinity. Furthermore, the work represents the first report utilizing biomarkers to detect salt stress within cypress populations *in situ*.

The research presented in Chapter 2 describes the development and utilization of biomarkers for sub-lethal salt stress in Bald Cypress. In a greenhouse study, one year old cypress seedlings were subjected to elevated salinity for 10 weeks. Physiological and biochemical responses of root and leaf tissue were assayed. At the end of the experiment salt stress caused an increase in H_2O_2 content in both leaf and root tissue. Furthermore, leaf tissue exhibited an increase in free proline content but a decrease in POX activity and photosynthetic efficiency in response to salt stress. Leaf tissue on adult cypress plants were then sampled *in situ* along a salinity gradient within a tidal creek associated with the lower St. Johns River. Cypress in a basin swamp near a salt-impacted wetland exhibited physiological and biochemical indicators of sub-lethal stress. Cypress at the basin swamp exhibited a decrease in photosynthetic efficiency, but had an increase in leaf free proline and lipid peroxidation compared to cypress at other sites.

The research presented in Chapter 3 investigated the cellular and biochemical responses of cypress leaves to salt stress. In a series of experiments, excised leaves were exposed to elevated NaCl for up to 72 h. Leaves were assayed for chlorophyll fluorescence, leaf transpiration, and POX activity. Leaves were then pre-treated with

pharmacological drugs to tease apart the cellular responses to salt stress. Salt stress caused leaves to increase non-photochemical quenching and decrease transpiration. Interestingly, salt stress caused an initial increase followed by a decrease in total POX activity after prolonged exposure to NaCl. The change in total POX was attributed to a low molecular weight peroxidase that could be induced by the peroxisome proliferating agent, clofibrate. The induction of this peroxidase in response to salt stress was blocked by the fungal toxin brefeldin A and the suicide inhibitor of NADPH-oxidase, DPI. The response of the low molecular weight peroxidase to the treatment of leaves with clofibrate and brefeldin A suggests that salt stress induces the proliferation of peroxisomes. The response of leaves treated with DPI suggests that the enzymatic production of ROS by NADPH-oxidase is associated with the induction of the low molecular weight peroxidase. DPI also blocked the decreased transpiration associated with salt stress, suggesting that the production of ROS by NADPH-oxidase also plays a role in stomatal closure.

Map redacted, paper copy available upon request to home institution

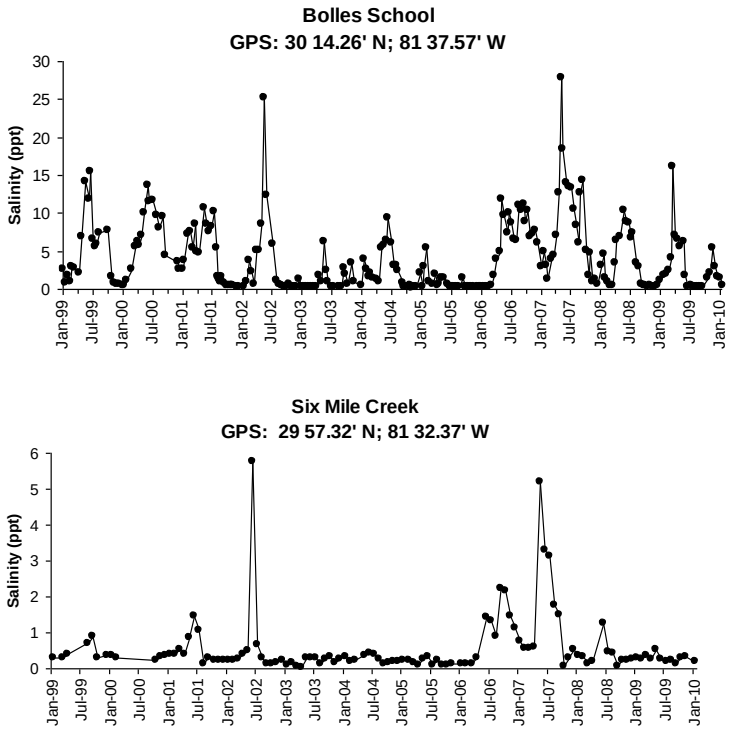

Figure 1.1 Historical salinity data for a 10 year period at two sites within the lower St. Johns River. Bolles School and Six Mile Creek are approximately 50 and 80 km from the mouth of the river, respectively.

Figure 1.2 “Ghost Swamp” associated with the Goodby’s Creek tributary within the lower St. Johns River.

Chapter 2: The development and utilization of biomarkers to detect sub-lethal salinity stress within Bald Cypress (*Taxodium distichum* (L.) Rich.).

Abstract:

Coastal freshwater wetlands are changing into oligohaline marshes via saltwater intrusion associated with anthropogenic effects and sea level rise. Plants in freshwater wetlands are particularly vulnerable to saltwater intrusion. In the current study, biomarkers were developed to detect sub-lethal salinity stress in Bald Cypress (*Taxodium distichum* (L.) Rich.). In a controlled greenhouse study, cypress saplings exhibited a quantifiable, tissue-dependent change in metabolism following a 10 week exposure to elevated salinity. In root tissue, elevated salinity was linked to an increase in intracellular Na^+ and H_2O_2 content. In leaf tissue, elevated salinity induced a significant decrease in maximum quantum yield (MQY; Fv/Fm) and an increase in non-photochemical quenching (NPQ; Fm-Fm'/Fm'). Additionally, leaf tissue of salt stressed plants exhibited an increase in Na^+, H_2O_2, and free proline content compared to plants maintained in freshwater. Leaf H_2O_2 was localized to the vascular tissue and guard cells of stressed plants. Furthermore, a strong negative correlation between MQY and leaf H_2O_2 content was observed, suggesting that an upregulation of H_2O_2 in response to salt stress may play a key role in downregulating photochemical efficiency.

These biomarkers were used to detect salinity stress within a population of cypress associated with the lower St. Johns River. Cypress in a basin swamp exhibited

sub-lethal signs of salinity stress based on low MQY and elevated NPQ values compared to plants at other sites. Leaf tissue of cypress at the basin swamp also had the highest Na^+, lipid peroxidation, and proline content compared to plants at other sites. Utilization of these biomarkers could serve as a tool to detect sub-lethal stress in cypress, thus allowing resource managers to identify habitat alterations in coastal wetlands.

2.1 Introduction

Coastal freshwater wetlands are valuable assets to estuarine ecosystems because of their ability to trap nutrients, stabilize sediment, cleanse water, and protect surrounding areas from flooding and high winds (Wharton, 1977; Dierberg & Brezonik, 1983; Wilhite & Toliver, 1990). However, the vitality of coastal freshwater wetlands is threatened by saltwater intrusion because of both global and local scale phenomena. Globally, sea level is predicted to rise by at least 30 cm within the next 100 years (IPCC, 2007). Locally, anthropogenic activities such as dredging and surface water withdraw are predicted to enhance saltwater intrusion within the lower St. Johns River (SJRWMD, 2008). As a result, the freshwater-saltwater boundary for estuaries is shifting further upstream. This phenomenon places an environmental stress on freshwater wetlands and ultimately causes a shift of the ecosystem into oligohaline marshes (Hackney et al. 2007).

The lower St. Johns River is located in Northeast Florida and empties into the Atlantic Ocean. The lower reach of the river is characterized by a limited elevational gradient and slow freshwater flow. Furthermore, the tidal amplitude is significant (1.5 m) at the mouth of the river. These aforementioned characteristics influence the pronounced tidal signature which can be detected up to 200 km from the mouth of the river (DeMort 1991). Salinities of up to 6‰ have been reported as far as 80 km from the mouth of the

river. Both sea level rise and anthropogenic activities are expected to increase the tidal range of the river. An increase in the tidal range will ultimately cause saltwater intrusion events to be more frequent and severe compared to previous events.

Plants within freshwater wetland communities are particularly vulnerable to saltwater intrusion because they have limited tolerance to the combined stressors of elevated salinity, chronic flooding, soil hypoxia and the accumulation of hydrogen sulfide in the soil (Allen et al. 1998; Hackney et al. 2007). Saltwater intrusion has been reported to convert coastal freshwater wetlands into 'ghost swamps' containing stressed or deceased freshwater trees with an understory of oligohaline marsh plants (Allen et al. 1998; Hackney et al. 2007). However, prior to death, freshwater wetland plants exhibit a stress response that includes stomatal closure as well as a decrease in net photosynthesis, net carbon assimilation, and total biomass (DeLaune et al. 1987; Pezeshki et al. 1989; Kozlowski, 2000). This stress response is thought to aid in salinity tolerance by reducing the pull of saline water into the plant by transpiration. This reduces the accumulation of toxic Na^+ ions within leaf tissue and help plants cope with the 'physiological drought' imposed by salinity stress (Allen et al. 1998; Ashraf, 2004; Mahajan & Tutejia, 2005). Freshwater wetland plants are also known to accumulate free proline within leaf tissue in response to salt stress potentially reducing osmotic stress (Ewing et al. 1995; Yokoi et al. 2002).

Bald Cypress (*Taxodium distichum* (L.) Rich.) is a large deciduous softwood plant native to the Southeastern United States. This species is often dominant in coastal freshwater wetlands and exhibits the greatest degree of tolerance to saltwater intrusion compared to other plants within the same community. Cypress is known to persist for

years after a freshwater wetland has been impacted by saltwater intrusion (Allen et al. 1998). However, Bald Cypress is not immune to this stress. Cypress exhibit similar physiological responses to saltwater intrusion as are reported in other freshwater wetland plants. The responses to elevated salinity include stomatal closure as well as a decrease in net photosynthesis, sap flow, growth, and biomass (Allen et al. 1994; Pezeshki et al. 1995; Allen et al. 1997; Krauss et al. 2000; Effler et al. 2006; Krauss et al. 2009; Krauss & Duberstein, 2010). Furthermore, a negative correlation between leaf Na^+ content and net photosynthesis has been reported (Pezeshki et al. 1988). The salt tolerance of cypress has been linked to the plant's ability to exclude Na^+ ions from the tissue (Allen et al. 1997). However, to date, the underlying cellular mechanisms that lead to the adaptive response of Bald Cypress to elevated salinity remain unexplored.

Studies on model plants and agriculturally relevant species have demonstrated that salt stress induces an increased production of reactive oxygen species (ROS) within tissue (Mittova et al. 2004; Gómez et al. 2004; Leshem et al. 2007; Hernandez et al. 2010). It was previously thought that ROS were produced as a toxic byproduct of metabolism because of abiotic stress. However, over the past decade, an overwhelming number of reports demonstrate that ROS play an important role for a plant's response to stress on the biochemical, cellular, and physiological level. For example, ROS have been shown to activate MAP kinase pathways and oxidize and modify the activity of key enzymes in plant metabolism (Neill et al. 2002b). For example, the oxidation of enzymes in the Calvin cycle by ROS was reported to cause a decrease in the activity of enzymes responsible for carbon fixation (Kaiser, 1979; Badger et al. 1980). The oxidation of transcription factors by ROS was reported to alter the DNA binding properties of plant

homeodomain transcription factors (Tron et al. 2002). Reactive oxygen species have also been shown to play an essential role in regulating the activity of guard cells. It has been reported that ROS are enzymatically produced within these cells in response to the hormone abscisic acid, resulting in stomatal closure (Pei et al. 2000; Zhang et al. 2001; Desikan et al. 2004). Finally, ROS have been shown to be enzymatically produced within root tissue in response to salt stress (Leshem et al. 2007; Hernandez et al. 2010). It is now becoming clear that increased ROS production is a ubiquitous and essential step for plants to perceive and respond to salt stress (Mittler, 2002; Neill et al. 2002a; Neill et al. 2002b; Apel & Hirt, 2004; Jaspers & Kangasjärvi, 2010).

Although ROS appear to have an overall positive role for plants under stress conditions, their presence within cells is still deleterious if not kept in balance. On the cellular level, an upregulation of ROS is associated with oxidative damage to biomolecules such as lipids, proteins, and nucleic acids (Møller et al. 2007; Sharma et al. 2012). Plants are capable of maintaining homeostasis under stressful conditions by altering levels of numerous antioxidant enzymes and metabolites that degrade ROS and maintain them within safe levels (Noctor & Foyer, 1998; Mittler, 2002; Mittler et al. 2004; Abogadallah, 2010). Both enzymatic and non-enzymatic processes result in the generation of the highly toxic superoxide anion (O_2^-). Superoxide can be converted into the less toxic hydrogen peroxide (H_2O_2) by a class of enzymes known as superoxide dismutases (SOD). Hydrogen peroxide can then be converted into the non-toxic products of water and molecular oxygen by a class of enzymes known as peroxidase (POX). The alteration of both SOD and POX activity appears to be an important step in a plant's

adaptive response to salt stress (Sreenivasulu et al. 2000; Sairam et al. 2002; Meloni et al. 2003; Shim et al. 2003; Gómez et al. 2004; Hernandez et al. 2010).

An emerging field of research involves the utilization of 'biomarkers' to detect sub-lethal stress within organisms. The general concept is to identify a reliable indicator of stress to monitor the health of a population or ecosystem (Downs et al. 2000; Ferrat et al. 2003; Ashraf, 2009). Biomarkers have been successfully developed to detect stress in corals, aquatic vegetation, and wetland plants (Ewing et al. 1995; Downs et al. 2000; Ferrat et al. 2003; Lauer et al. 2011). In order for biomarkers to be successful, the physiological parameters assayed must be well understood. It is well known that most plants exhibit a similar response to salt stress that includes increased ROS production, oxidative damage to biomolecules, the alteration of antioxidant enzyme activity, and the accumulation of free proline in leaf tissue. By quantifying these responses, useful insight into the physiological status of a plant experiencing salt stress may be obtained (Ewing et al. 1997; Ashraf, 2009). The objectives of this study were to (1) investigate the role of ROS in the physiological and biochemical responses of Bald Cypress to salt stress, (2) develop biomarkers to detect sub-lethal salinity stress within tissue, and (3) apply these biomarkers to Bald Cypress *in situ* for trees located within freshwater wetlands associated with the lower St. Johns River.

2.2 Materials and Methods

2.2.1 Greenhouse experiment

To evaluate the impacts of elevated salinity on the cellular stress response of *T. distichum*, a controlled greenhouse study was conducted. One year old bare root, dormant cypress seedlings were purchased from the Florida Division of Forestry (Chiefland, FL). Seedlings were grown for 6 months in plastic pots filled with potting soil and fertilized with fish emulsion fertilizer once a month prior to treatment. Plants were transported to the UNF greenhouse facilities, placed in 20 L mesocosms, and inundated 10 cm above the soil surface with fresh water. After a 20 day acclimation to flooding, seedlings were randomly assigned to salinity treatments of 0, 4 or 8‰ for 10 weeks (n=12). Saline water was prepared by diluting filtered seawater (35‰) with D.I. water. Water was changed every two weeks throughout the experiment.

Chlorophyll fluorescence

Immediately prior to harvest, chlorophyll fluorescence parameters were assayed on plants using a Diving Pulse Amplitude Modulated (PAM) fluorometer (Heinz-Walz GmbH ©,Effeltrich, Germany). Samples were held in place using a dark leaf clip (DIVING-LC) with the fiber optic cable 5 mm from the leaf. PAM settings were as follows: measuring intensity=5, gain=3, saturation intensity=2 and damp=2. Effective quantum yield (EQY= (Fm'-F)/Fm') was measured on each seedling. Individual leaves were then wrapped in aluminum foil and dark adapted for 10 to 20 minutes to open PSII reaction centers, oxidize the plastoquinone pool, and relax the xanthophyll cycle (Rascher et al. 2000; Ralph & Gademann, 2005). After dark adaptation, maximum quantum yield

(MQY= (Fm-Fo)/Fm) was measured on each seedling. From both EQY and MQY measurements, non-photochemical quenching (NPQ = (Fm-Fm')/Fm) was calculated.

Root respiration

To evaluate the effects of elevated salinity on the respiratory demand of cypress roots, oxygen consumption rates were determined on live tissue at the end of the experiment. Approximately 500 mg of living root tissue was assayed for oxygen consumption using an Oxygraph system fitted with a DW3 liquid phase electrode chamber (Clark type polarographic sensor; Hansatech, Norfolk, UK). Prior to measurements, it was determined that blank runs of O_2 saturated water alone did not exhibit any changes in oxygen consumption. Individual samples were weighed and placed in 10 mL of O_2 saturated water. Oxygen consumption was measured for 20 minutes and respiration was calculated as nanomoles of O_2 consumed per minute per gram of tissue.

Localization of ROS in leaf tissue

Reactive oxygen species were localized within living leaf tissue by utilizing the ROS-specific fluorescent probe 2,7-dichlorofluorescein-diacetate (DCFH-DA; Invitrogen). Only salinity treatments of 0 and 8‰ were examined. Excised leaves were incubated in 5 μM DCFH-DA for 15 minutes and then washed three times with distilled water to remove any unbound probe. Low power fluorescent imaging was performed on a Leica MC10F fluorescent microscope (ex 488/ em 525). High power imaging was performed on and Olympus BX60 fluorescent microscope with a similar filter set.

Tissue hydrogen peroxide content

Hydrogen peroxide was quantified within tissue by measuring H_2O_2 from biochemical extracts. A small amount of leaf and root tissue was harvested from selected plants (n=4 per treatment) and immediately flash frozen in liquid nitrogen as suggested by Cheeseman (2006). Tissue was transported to the lab in liquid nitrogen and H_2O_2 content was assayed on the same day by placing 100 mg of leaf or root tissue in a 2 mL screw cap microfuge tube containing five 2.0 mm zirconium disruptor beads. Tissue was homogenized with 1 mL of ice-cold 25 mM Tris-HCl buffer (pH = 7.8) supplemented with 5 mM NaCN to inhibit antioxidant enzymes that may degrade H_2O_2 within the extract (Cheeseman, 2006). Tissue was homogenized in a Fast-Prep-24 Tissue Disrupter (MP Biomedical, Solon, OH) for 30 sec at a speed of 6.0 m/sec. Samples were subsequently centrifuged at 13,300 x g for 15 min at 4 °C. All tissue extracts were then homogenized and centrifuged using the above protocol. Total H_2O_2 in the supernatant was assayed using the quantitative peroxide assay kit (Pierce, Rockford, IL, USA). Remaining leaf and root tissue from plants were harvested and stored at -80 °C until further analysis.

Leaf free proline content

Proline is an amino acid that is synthesized within leaf tissue of some plants in response to salt stress. Free proline acts as an osmolyte to combat osmotic stress as well as a molecular chaperone to stabilize proteins and membranes under oxidative stress (Yokoi et al. 2002; Molinari et al. 2007). Leaf free proline content was quantified according to the methods of Boctor (1971) with modification. Approximately 250 mg of

previously frozen leaf tissue was extracted with 1 mL of 20% EtOH. Isatin solution was prepared by adding 500 mg isatin (Sigma Aldrich, St. Louis, MO, USA) into 50 mL of warm MeOH containing 1.25 mL glacial acetic acid. Leaf extract (50 μL) was combined with 100 μL of isatin solution. Samples were then incubated at 75 °C for 5 min and immediately read at 595 nm using a BioTek PowerWave XS spectrophotometric microplate reader (BioTek, Winooski, VT, USA).

Activities of tissue SOD & POX

The specific activities of SOD and POX were assayed on crude extracts from previously frozen tissue. Approximately 250 mg of leaf or root tissue was homogenized with 1 mL of ice cold CelLytic P plant cell lysis/extraction reagent containing 1% (w/v) PVPP and 0.1% (w/v) of a protease inhibitor cocktail (Sigma Aldrich). Samples were kept on ice prior to being assayed. Total protein was quantified using the Bio-Rad Bradford assay (Bio-Rad, Hercules, CA, USA). Samples were then diluted 1:100 in 25 mM Tris-HCl buffer (pH = 7.8). Superoxide dismutase activity was quantified using a SOD assay kit (Cayman Chemical, Ann Arbor, MI, USA). Peroxidase activity was quantified using an Amplex Red POX assay kit (Invitrogen, Carlsbad, CA, USA). Leaf peroxidase isozymes were separated using non-denaturing SDS-PAGE on NuPAGE 4-12% bis-tris gels (Invitrogen). Electrophoresis was performed at 4 °C. All wells were loaded with 5 μg of total protein. In order to eliminate variability of staining between gels, each salinity treatment had three replicate samples loaded on the same gel. Peroxidase isozyme activity was detected by placing gels in a staining solution of 1 mM o-dianisidine and 0.5 mM H_2O_2 in acetate buffer (pH= 5.6). Pixel intensity of individual isozyme bands was quantified using Image J software (National Institute of Heath,

Bethesda, MD, USA). Individual isozyme pixel intensity was averaged for each treatment and then divided by the average pixel intensity of control values. Molecular weight values adjacent to isozymes are approximate because the proteins were separated in native form.

Quantification of tissue sodium content

Fifty micrograms of Sodium Green tetraacetate (Invitrogen) was reconstituted in 20 µL of DMSO containing 10% (w/v) pluronic f127. The reconstituted indicator was diluted to a final concentration of 5 µM in 25 mM Tris-HCl buffer (pH= 7.8) containing 20 U/mL of esterase. The diluted indicator was incubated at 37 °C for 30 minutes prior to the assay. Diluted indicator (50 µL) was added to 10 µL of leaf or root extract. Tissue sodium content was assayed from the same extracts that SOD & POX activities were assayed. The mixture was incubated at 37 °C for 1 hour and read on a BioTek FLx 800 fluorescent microplate reader (BioTek) (ex 485/em 528). Units were expressed as mg Na^+ per gram of tissue.

2.2.2 *In situ* sampling of *Taxodium distichum*

Study sites

Three wetlands containing adult cypress were sampled within Goodby's Creek, a tidal creek that empties into the lower St Johns River (Figure 2.1). The first site (Impacted Wetland; 30 12.907'N, 81 36.772'W) was closest to a source of saltwater and clearly impacted by saltwater intrusion. The site was characterized by a canopy of *T. distichum* and deceased *Fraxinus caroliniana*. The understory was invaded by oligohaline marsh plants such as: *Fimbristylis spathacea, Juncus effuses, Scripus robustus,* & *Spartina bakeri*. The second site (Basin Swamp; 30 13.088'N, 81 36.711'W)

was flooded at the time of sampling and characterized by a canopy of *T. distichum*. The understory contained plants only on elevated areas that were not affected by flooding. These plants included: *Myrica cerifera*, *Sabal minor*, & *Panicum repens*. The third site was furthest away from the primary source of saline water (Freshwater Wetland; 30 13.276'N, 81 36.532'W) and contained a canopy of *T. distichum, Acer rubrum, F. caroliniana,* & *Magnolia virginiana. Nymphaea mexicana,* a plant that is sensitive to salt stress, was present within shallow areas of the water.

Field sampling

Only leaf tissue was sampled because of the difficulty associated with obtaining root samples from plants in the field. Approximately 50 g of fully expanded leaf tissue was excised from adult cypress for analysis. A total of 5 replicate trees were sampled at each site. Both maximum quantum yield and non-photochemical quenching values could not be assayed as described above because a 20 minute dark adaptation is difficult to achieve in field conditions (Rascher et al. 2000). Therefore, chlorophyll fluorescence parameters (MQY, EQY, and NPQ) were simultaneously measured using rapid light curves as described by Ralph & Gademann (2005). Maximum quantum yield was taken on leaves that were dark adapted for 10 s to achieve a 'quazi-darkness' state (Ralph & Gademann, 2005). Effective quantum yield was assayed after leaves were illuminated with actinic light (38 μmol photons $m^{-2}s^{-1}$) for 10s. Non-photochemical quenching (NPQ) was calculated from both MQY and EQY values as described above. Samples were held in place using a dark leaf clip (DIVING-LC) with the fiber optic cable 5 mm from the leaf. PAM settings were as follows: LC-INT: 1, LC-WIDTH: 0:10s. Samples were then placed on dry ice, transported to UNF, and stored at -80 °C until analysis.

Leaf proline, SOD, and POX activity were assayed as described above. However, lipid peroxidation was assayed instead of leaf H_2O_2 because H_2O_2 is labile and degrades quickly within plant tissue if not stored in liquid nitrogen (Cheeseman, 2006). Upon oxidative stress, ROS damage polyunsaturated fatty acids and produce malondialdehyde (MDA). It is generally accepted that the accumulation of MDA is an indication of oxidative damage to lipids caused by an upregulation of ROS. The amount of MDA was quantified within cypress leaves by the thiobarbituric acid assay according to the methods of Jambunathan (2010) with slight modification. Approximately 250 mg of leaf tissue was homogenized with 1 mL of 0.1% trichloroacetic acid (TCA). The supernatant (500 μL) was combined with 1 mL of 20% TCA and 1 mL of 0.5% thiobarbituric acid. The mixture was heated at 95 °C for 30 min and then cooled on ice. Samples were read at 532 and 600 nm on a BioTek PowerWave XS spectrophotometric microplate reader. The nonspecific absorbance at 600 nm was subtracted from the A_{532} values. The amount of MDA was calculated using the extinction coefficient of 155 $mM^{-1}cm^{-1}$. Units were expressed as nanomoles of MDA per gram of tissue.

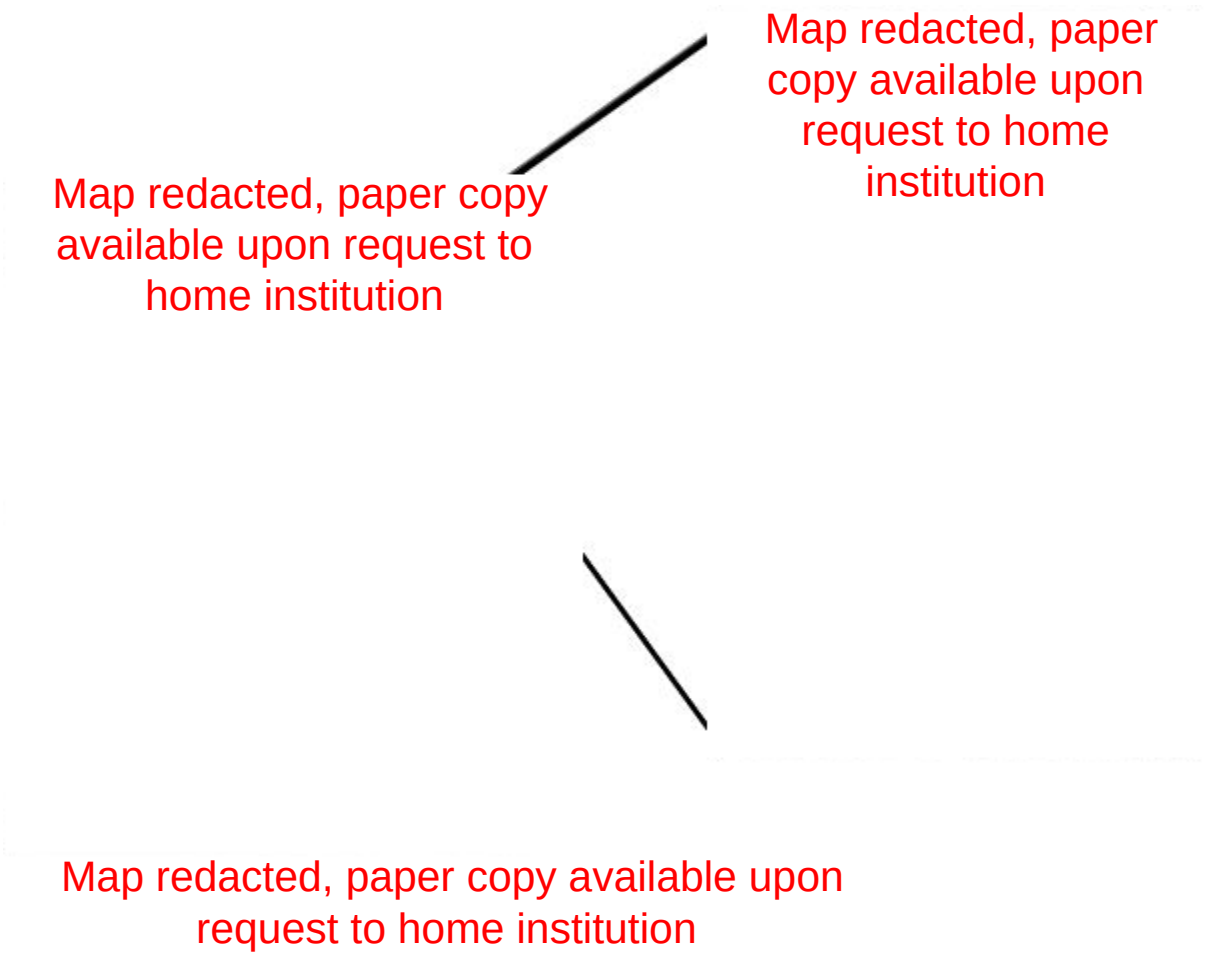

Figure 2.1 Location of field sampling sites.

2.2.3 Statistical analysis

Chlorophyll fluorescence values, root respiration, tissue H_2O_2 content, leaf proline content, tissue SOD & POX specific activity, tissue sodium, and leaf lipid peroxidation were analyzed using a one-way ANOVA. All data met the homogeneity of variance assumption of ANOVA. Outliers were detected by a studentized residual test and removed from datasets prior to analysis. Significant p values ($p < 0.05$) are highlighted in bold whereas marginally significant values ($0.1 < p < 0.05$) are italicized within figures. Significant differences among treatments are indicated by letters according to Tukey's HSD post-hoc comparison of means. Correlations between MQY and leaf H_2O_2 from the greenhouse study as well as the specific activity of leaf SOD and POX from the field study were tested using a one-tailed Pearson's correlation. Significant p values ($p < 0.05$) and strong correlation coefficients ($r > |0.5|$) are highlighted in bold within figures. All statistical analyses were performed on SYSTAT 11 (Systat software, Chicago, IL, USA).

2.3 Results

2.3.1 Greenhouse experiment

<u>Root sodium content, hydrogen peroxide content, and respiration</u>

Salinity treatments of 4 and 8‰ for 10 weeks showed a significant increase in root sodium content compared to control cypress maintained in freshwater (Figure 2.2). Root hydrogen peroxide content increased by 23% and 49 % for plants maintained at 4 and 8‰, respectively, compared to controls; however, results were not significantly different (Figure 2.3). Salinity did not cause changes in root respiration (Figure 2.4).

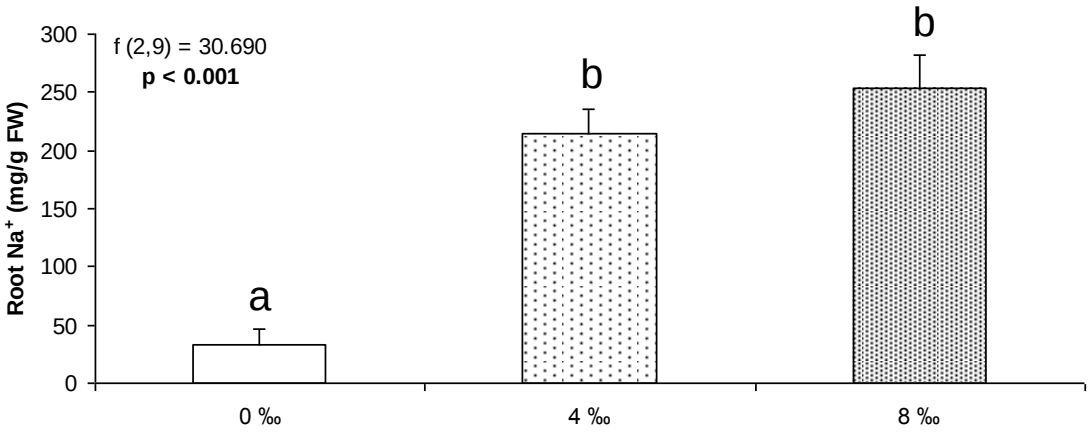

Figure 2.2 Root sodium content of *Taxodium distichum* after a 10 week greenhouse experiment. Plants maintained at higher salinities exhibited a significant increase in root sodium content. Values represent mean + SEM (n= 4).

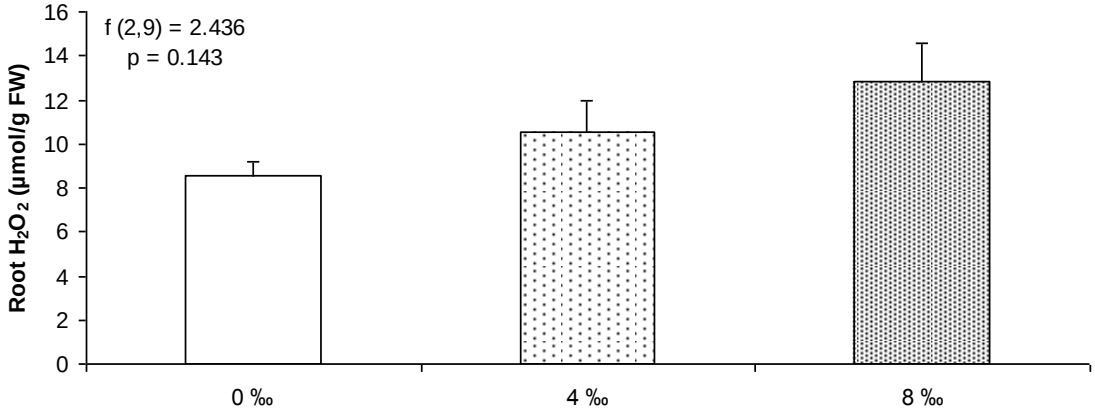

Figure 2.3 Root H_2O_2 content after a 10 week greenhouse experiment. Roots exhibited a 23% and 49% increase in H_2O_2 content for plants maintained at 4 and 8‰ respectively. Values represent mean + SEM (n= 4).

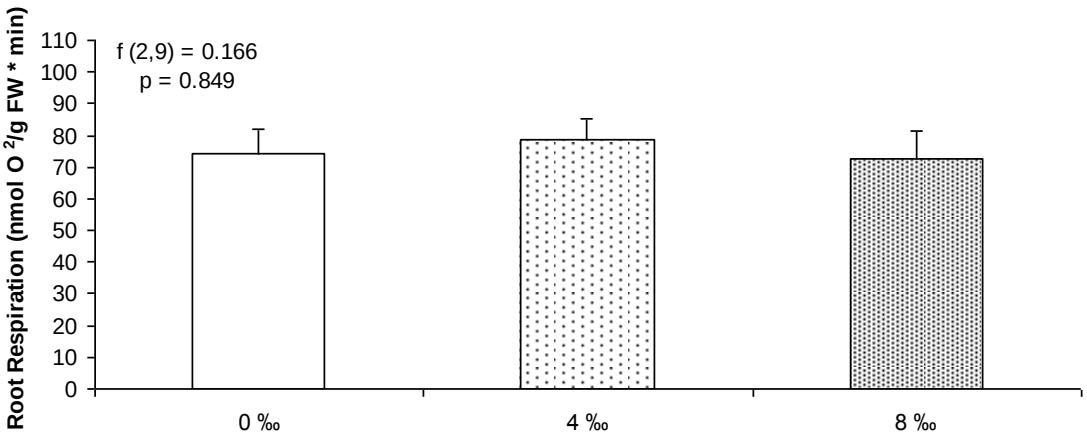

Figure 2.4 Root respiration after a 10 week greenhouse experiment. Root respiration was not affected by salinity. Values represent mean + SEM (n= 4).

Leaf sodium content, MQY, and NPQ

No sodium was detected in the leaf tissue of plants maintained in freshwater; however, plants maintained at 4 and 8‰ exhibited a significant increase in leaf sodium content. Leaf tissue of plants maintained at 8‰ had approximately three times the amount of sodium compared to plants at 4‰ (Figure 2.5). The MQY for plants maintained at 8‰ exhibited a significant drop from control values of 0.791 to 0.758 (Figure 2.6). The NPQ for plants maintained at 8‰ exhibited an increase from control values of 0.284 to 0.592. The results were marginally significant (Figure 2.7).

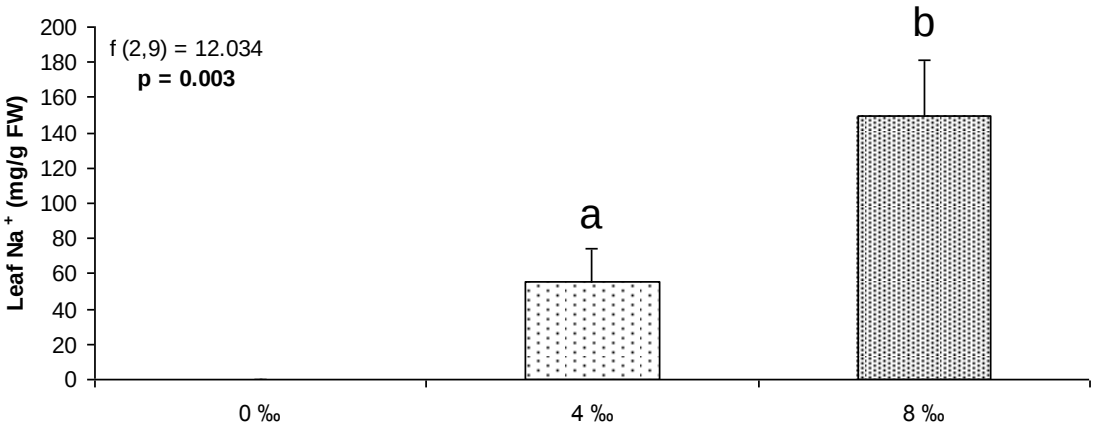

Figure 2.5 Leaf sodium content after a 10 week greenhouse experiment. No sodium was detected in the leaf tissue of control plants. Plants maintained at 8‰ exhibited a significant increase in leaf sodium content compared to plants maintained at 4‰. Values represent mean + SEM (n= 4).

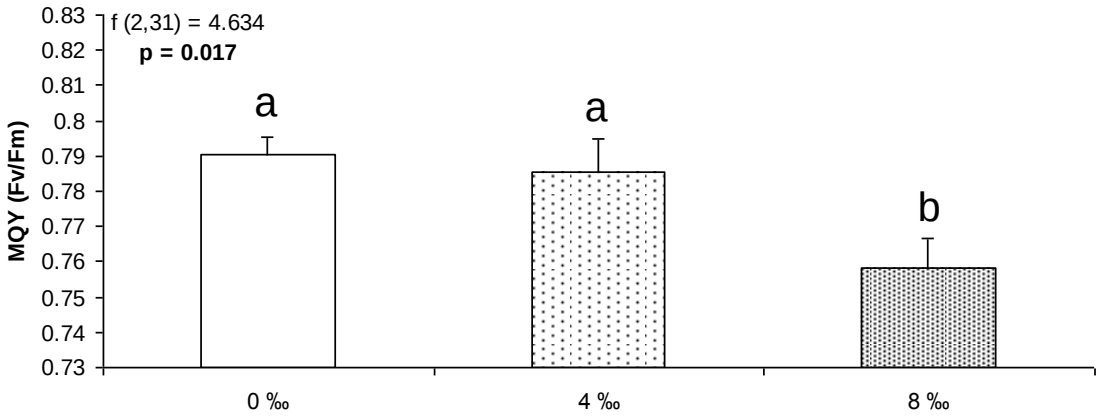

Figure 2.6 Maximum quantum yield (MQY) after a 10 week greenhouse experiment. Plants maintained at 8‰ exhibited a significant drop in MQY. Values represent mean + SEM (n= 10 or 12).

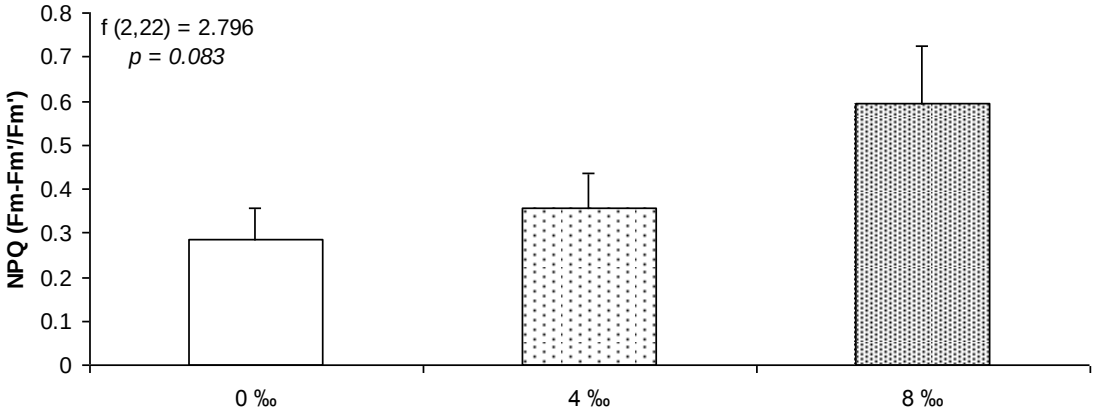

Figure 2.7 Non-photochemical quenching (NPQ) after a 10 week greenhouse experiment. Plants maintained at 8‰ exhibited a marginally significant increase in NPQ. Values represent mean + SEM (n= 7 or 9).

Leaf H_2O_2 content, free proline, and localization of ROS within tissue

Leaf H_2O_2 content exhibited a marginally significant increase of 36% for plants maintained at 8‰ compared to plants maintained in freshwater (Figure 2.8). Free proline content within leaves of plants maintained at 8‰ exhibited a significant increase compared to other treatments (Figure 2.9). Virtually no ROS were detected within leaf tissue of plants maintained in freshwater; however, ROS were localized within the vascular tissue as well as guard cells of plants maintained at 8‰ (Figure 2.10). A strong negative correlation existed between leaf H_2O_2 content and MQY (Figure 2.11).

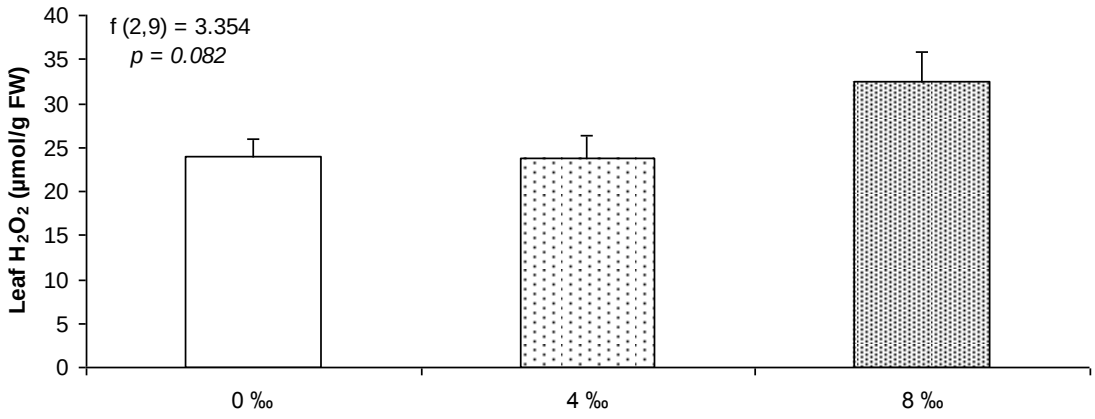

Figure 2.8 Leaf H_2O_2 content after a 10 week greenhouse experiment. Leaves of plants maintained at 8‰ exhibited a marginally significant increase of 36% compared to plants maintained in freshwater. Values represent mean + SEM (n= 4).

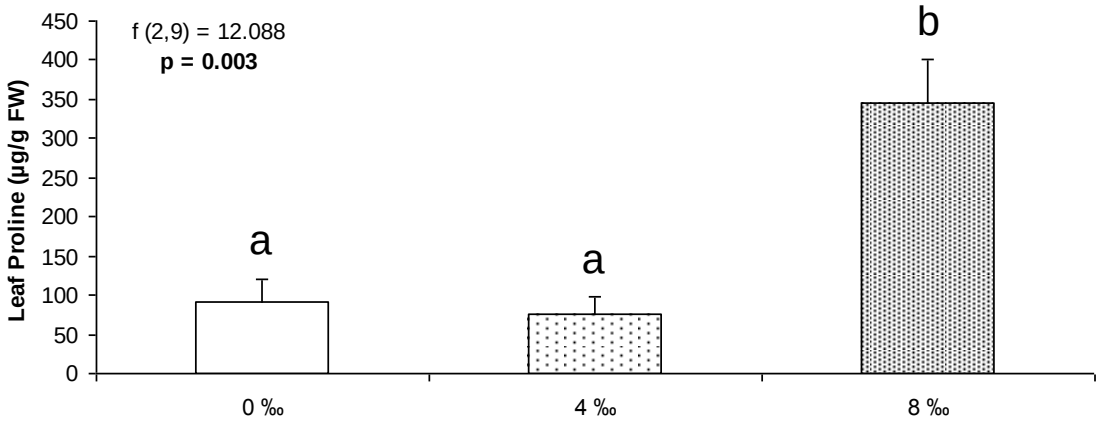

Figure 2.9 Leaf free proline content after a 10 week greenhouse experiment. Leaves of plants maintained at 8‰ exhibited a significant increase in free proline compared to other treatments. Values represent mean + SEM (n= 4).

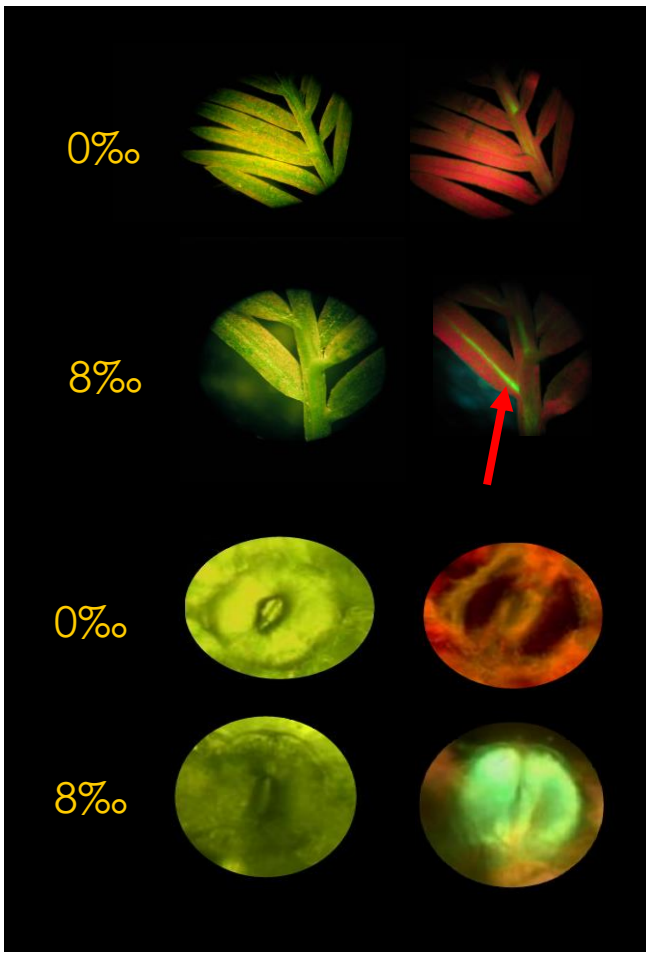

Figure 2.10 Localization of ROS within leaf tissue after a 10 week greenhouse experiment using fluorescent microscopy. Light microscopy images of leaves and guard cells are shown on the left whereas fluorescent microscopy images are on the right. The red color of tissue under fluorescent microscopy is caused by the autofluorescence of chlorophyll. The presence of ROS was not detected in the leaves of plants maintained at 0‰; however, ROS was detected within the vascular tissue (red arrow) and guard cells within leaves of plants maintained at 8‰.

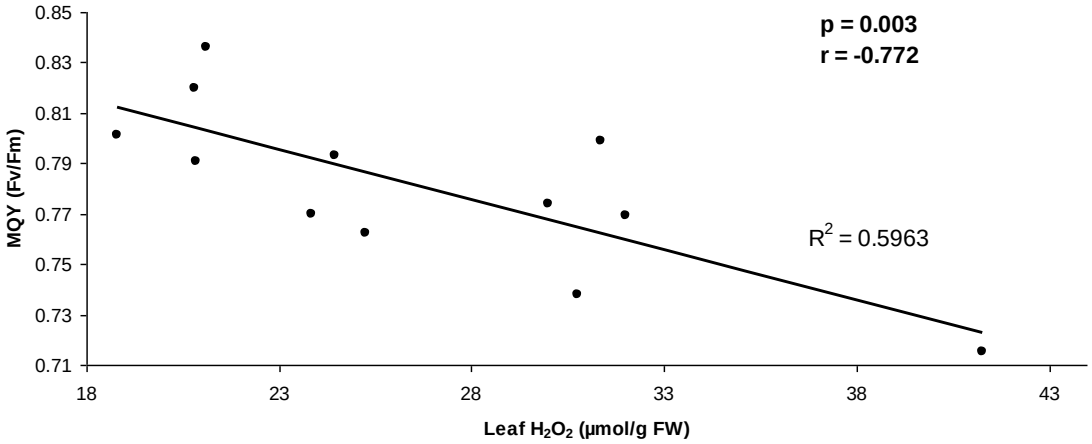

Figure 2.11 The relationship between leaf H_2O_2 content and MQY after a 10 week greenhouse experiment. A strong negative correlation was observed between leaf H_2O_2 content and MQY.

Tissue SOD & POX, isozyme analysis of leaf POX

The specific activity of SOD and POX within root tissue exhibited no trends or significant differences between treatments (data not shown). Any putative trends in the specific activity of leaf SOD would likely be masked by the large amount of variance found within cypress leaves maintained at 8‰ (Figure 2.12). Plants maintained at 8‰ exhibited a 42% decrease in leaf POX compared to plants maintained in freshwater; however, results were not significant (Figure 2.13). After in-gel detection of leaf POX isozymes, the pixel intensity of the ~100 kDa isozyme for plants maintained at 8‰ dropped by 14% compared to the pixel intensity of plants maintained in freshwater. However, the pixel intensity of the ~37 kDa isozyme for plants maintained at 8‰ dropped by 65% compared to plants maintained in freshwater (Figure 2.14).

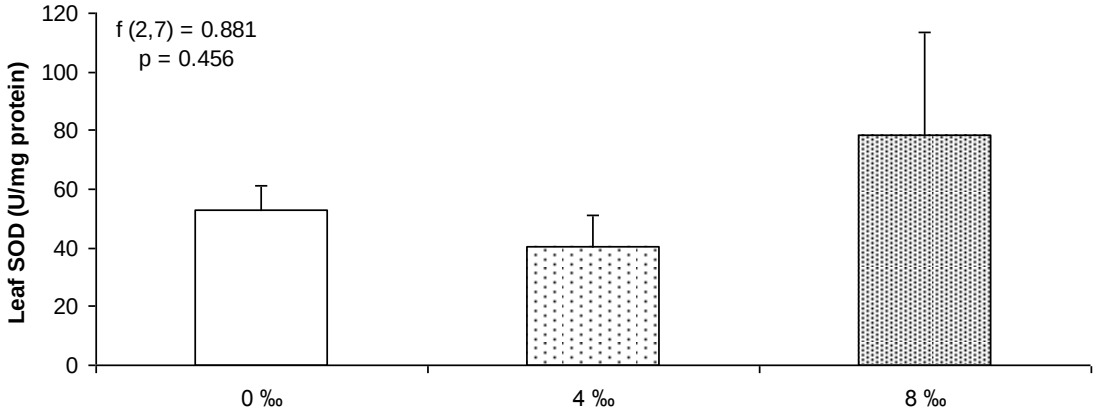

Figure 2.12 Specific activity leaf superoxide dismutase after a 10 week greenhouse experiment. Values represent mean + SEM (n= 3 or 4).

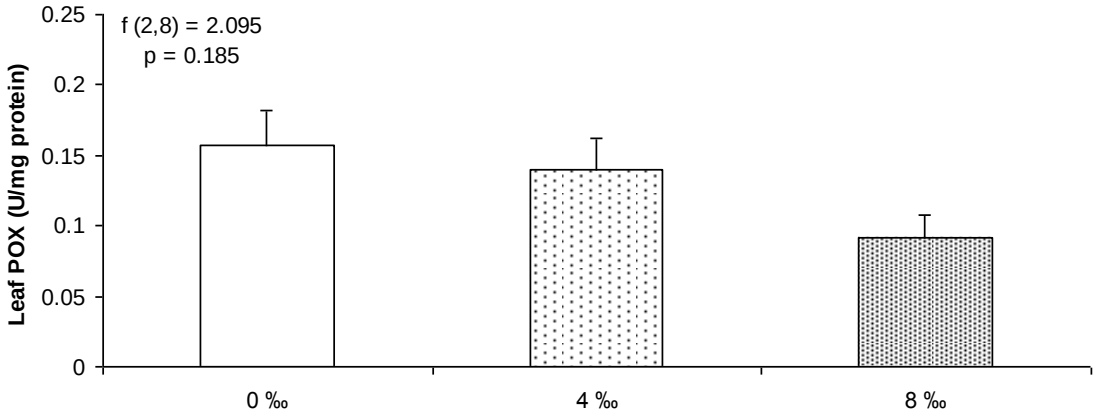

Figure 2.13 Specific activity leaf peroxidase after a 10 week greenhouse experiment. Leaves of plants maintained at 8‰ exhibited a 42% decrease compared to plants maintained in freshwater; however, results were not significant. Values represent mean + SEM (n= 3 or 4).

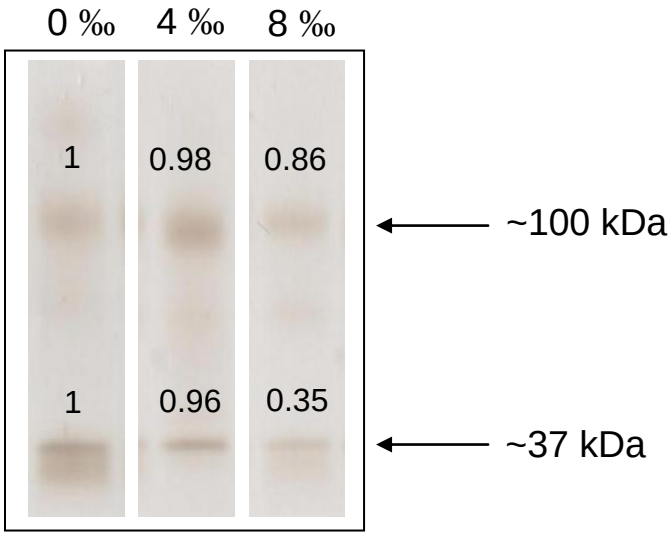

Figure 2.14 In-gel analysis of leaf POX isozymes. The pixel intensity of the ~37 kDa isozyme for plants maintained at 8‰ dropped by 65% compared to plants maintained in freshwater. All wells were loaded with 5 μg of total protein and each salinity treatment had three replicate samples. Molecular weight values adjacent to isozymes are approximate.

2.3.2 *In situ* sampling of *Taxodium distichum*

Leaf sodium content, MQY, and NPQ

No significant differences in leaf sodium content were observed between sites. However, it was noted that leaf sodium content for cypress at the basin swamp was 27% higher compared to cypress at the freshwater wetland (Figure 2.15). The MQY for cypress at the freshwater wetland had the highest value at 0.782 whereas the basin swamp had the lowest value at 0.738. The results were marginally significant (Figure 2.16). The basin swamp site had the highest NPQ compared to other sites; however, results were not significant (Figure 2.17).

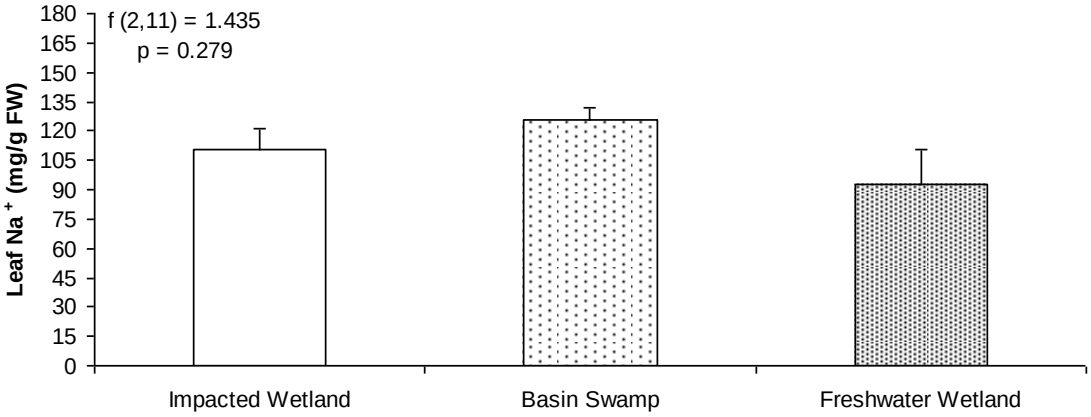

Figure 2.15 Leaf sodium content of adult cypress sampled from three wetland sites within Goodby's Creek. Cypress at the basin swamp had the highest leaf sodium content; however, results were not significant. Values represent mean + SEM (n= 4 or 5).

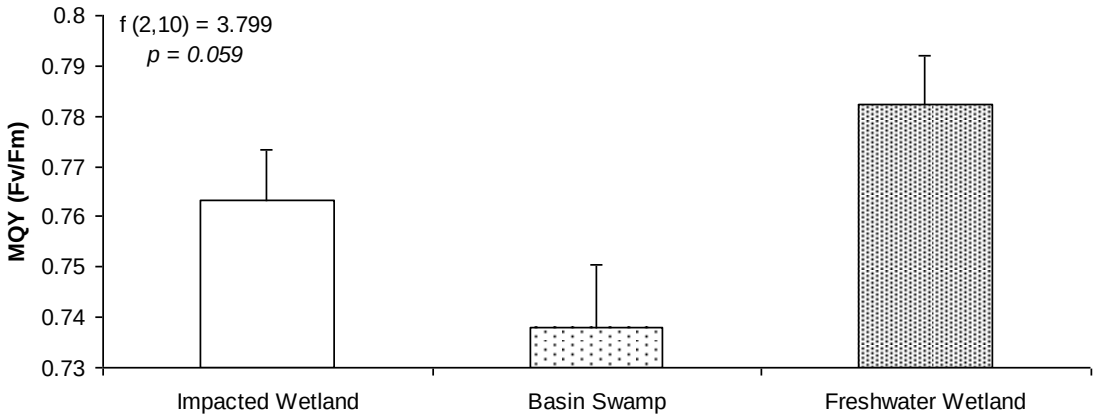

Figure 2.16 Maximum quantum yield (MQY) of adult cypress sampled from three wetland sites within Goodby's Creek. Values represent mean + SEM (n= 4).

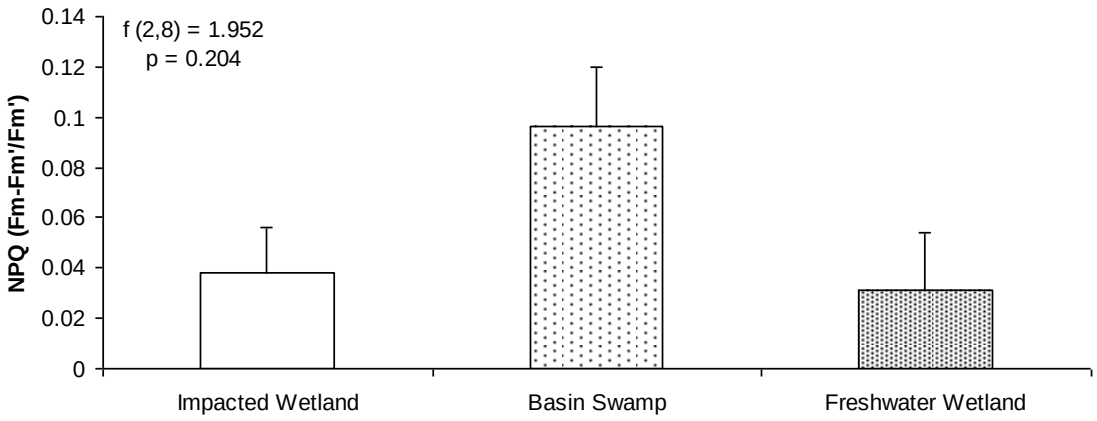

Figure 2.17 Non-photochemical quenching (NPQ) of adult cypress sampled from three wetland sites within Goodby's Creek. Values represent mean + SEM (n= 3 or 4).

Leaf lipid peroxidation and free proline

Lipid peroxidation was significantly higher within leaf tissue of plants at the basin swamp (Figure 2.18). A similar trend was observed for leaf free proline content where cypress at the basin swamp had a significantly higher amount of free proline compared to cypress at other sites (Figure 2.19).

Leaf SOD & POX

A similar trend was observed for the specific activity of both leaf SOD & POX for cypress at each site. The specific activity of leaf SOD for cypress at the freshwater wetland was 82% higher than that of the impacted wetland; however, results were not significant (Figure 2.20). The specific activity of leaf POX for plants at the freshwater wetland was 71% higher than that of the impacted wetland and results were marginally significant (Figure 2.21). A strong positive correlation was observed between the specific activities of leaf SOD & POX (Figure 2.21).

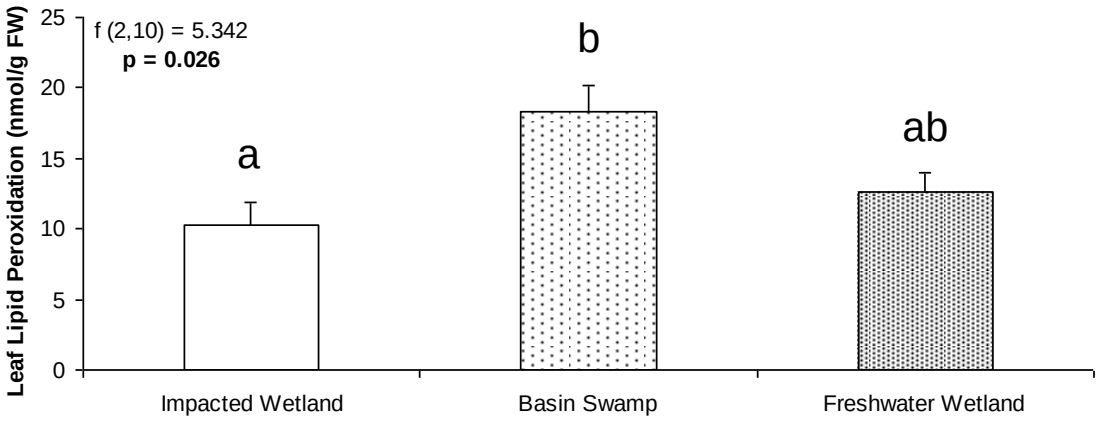

Figure 2.18 Lipid peroxidation of adult cypress sampled from three wetland sites within Goodby's Creek. Cypress at the basin swamp had a significantly higher amount of leaf lipid peroxidation. Values represent mean + SEM (n= 4 or 5).

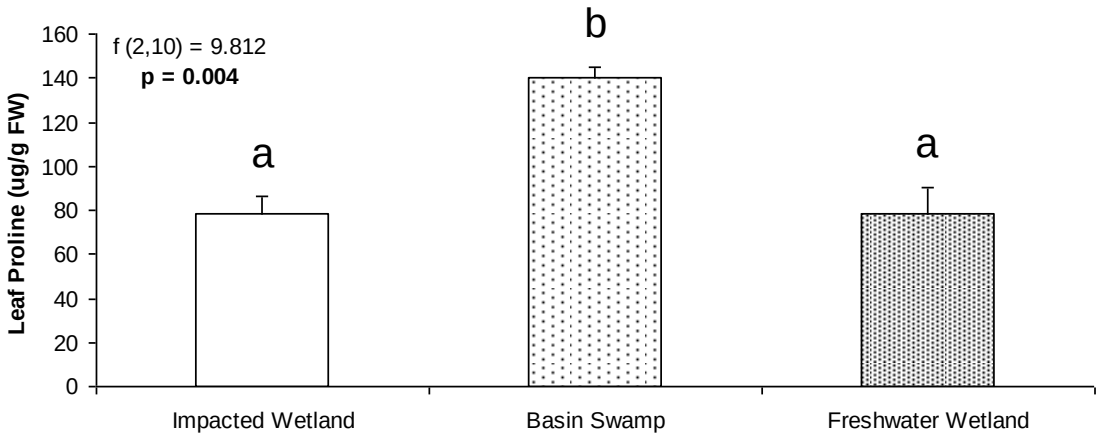

Figure 2.19 Free proline content of adult cypress sampled from three wetland sites within Goodby's Creek. Cypress at the basin swamp had a significantly higher amount of leaf free proline. Values represent mean + SEM (n= 4 or 5).

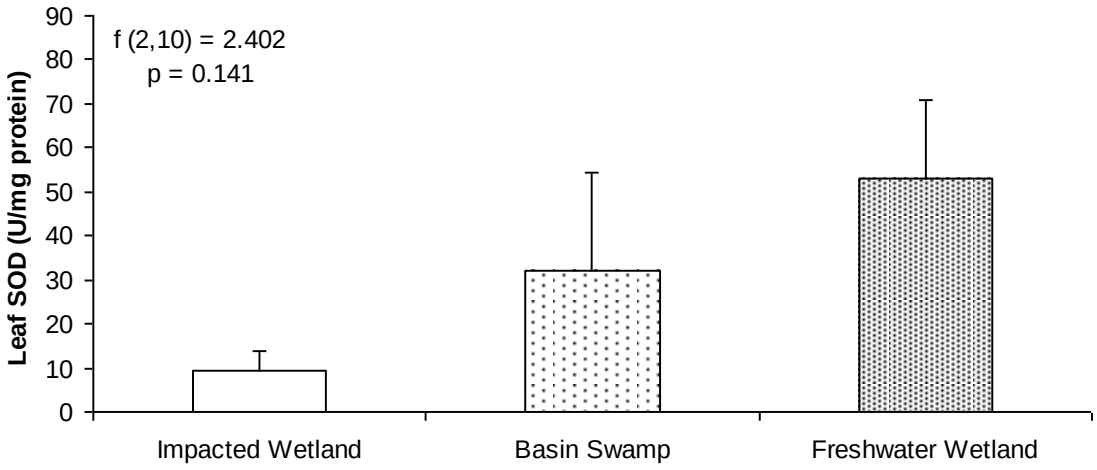

Figure 2.20 Specific activity of leaf superoxide dismutase (SOD) on adult cypress sampled from three wetland sites within Goodby's Creek. Values represent mean + SEM (n= 4 or 5).

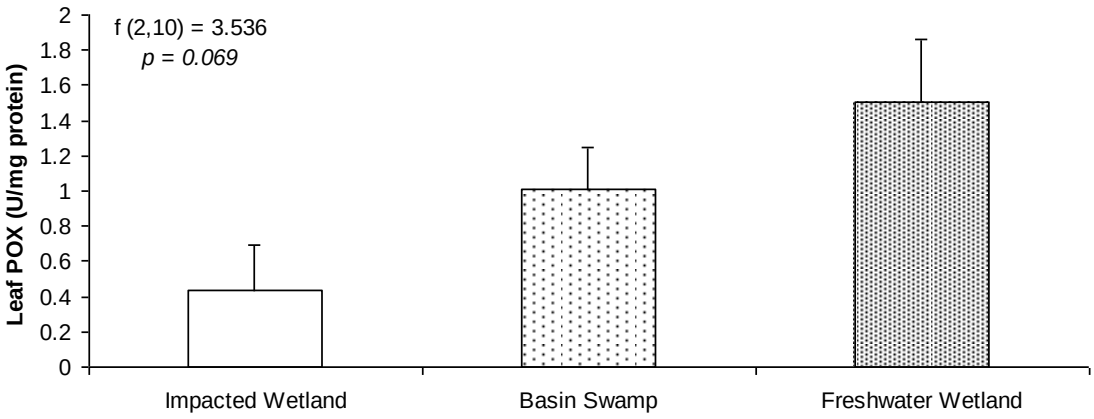

Figure 2.21 Specific activity of leaf peroxidase (POX) on adult cypress sampled from three wetland sites within Goodby's Creek. Values represent mean + SEM (n= 4 or 5).

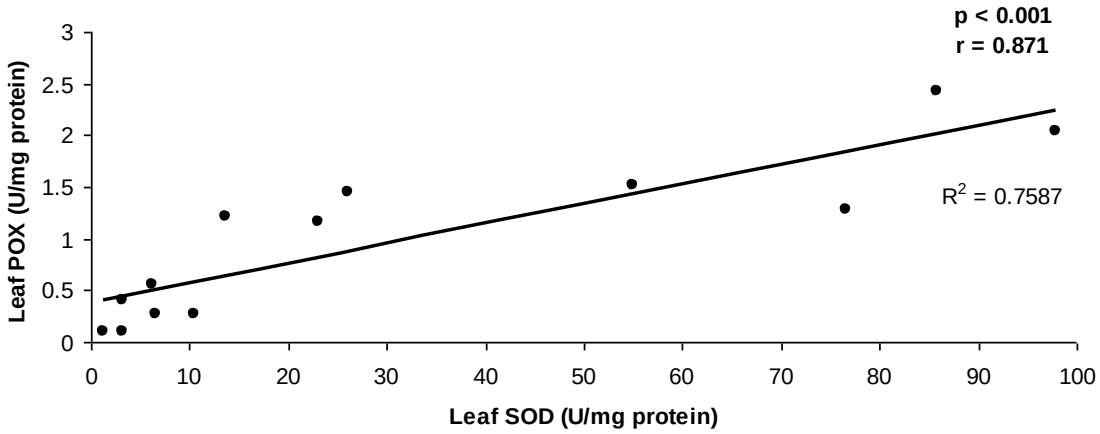

Figure 2.22 Relationship between the specific activity of leaf superoxide dismutase and leaf peroxidase within adult cypress sampled from three wetland sites within Goodby's Creek. A strong positive correlation was observed between the specific activities of SOD and POX.

2.4 Discussion

2.4.1 Greenhouse experiment

Elevated salinity causes an increase in root H_2O_2 content but no change in O_2 consumption.

Cypress roots exhibited an increase in both Na^+ and H_2O_2 content in response to salt stress. Similar observations have been reported in glycophytes including *Brassica oleracea*, *Arabidopsis thaliana*, and *Populus euphratica* (Hernandez et al. 2010; Leshem et al. 2007; Zhang et al. 2007). Root growth enhances salt tolerance in plants by increasing the surface area of belowground tissue to increase a plant's ability to obtain freshwater (Shannon et al. 1993). In other plant species, root growth appears to be regulated by H_2O_2 in other species because it has been localized in the apical meristem and tip of growing roots (Dunand et al. 2006; Jones et al. 2007). Allen et al. (1997) found that biomass was partitioned to root tissue of cypress seedlings maintained at salinity levels of 2 & 4‰ for 16 weeks. The authors suggested that low doses of salinity may stimulate root growth and aid in salt tolerance of cypress. It is possible that the increased production of H_2O_2 may stimulate root growth in cypress experiencing low levels of salt stress.

Many plants display active transport mechanisms in response to increased salinity. Plants are able to extrude Na^+ ions from tissue or sequester them within vacuoles. (Blumwald et al. 2000; Apse & Blumwald, 2007). The aforementioned responses are energetically costly and require a tremendous amount of ATP. Lauer et al. (2011) reported that dark-adapted respiration increased in blades of the aquatic grass *V. americana* exposed to elevated salinity. The authors hypothesized that the increase in respiration was a result of ion extrusion or vacuole sequestration of Na^+ ions. In the

present study, respiration of cypress roots was not affected by salinity treatment. This suggests that the active transport mechanisms of ion extrusion or vacuole sequestration either may not be occurring or may not place a significant respiratory demand on roots of salt stressed cypress.

Elevated salinity causes a decrease in maximum quantum yield and an increase in non-photochemical quenching.

Plants maintained at 8‰ exhibited a significant drop in MQY. Maximum quantum yield is a chlorophyll fluorescence parameter that is sensitive to many types of environmental stress, including salt stress. Numerous reports demonstrate that salt stress elicits a drop in MQY in a variety of plant species (Brugnoli & Bjorkman, 1992; Shim et al. 2003; Biber, 2006; Stępień & Kłbus, 2006). A drop in MQY is considered to reflect an overall downregulation of photosynthesis (Logan et al 2007). Within cypress, salt stress is well known to cause a decrease in net photosynthesis (Pezeshki et al. 1987; Pezeshki et al. 1995; Allen et al. 1997). Thus, the drop in MQY in this study is considered a reflection of the overall downregulation of photosynthesis of cypress upon salt stress.

Plants maintained at 8‰ exhibited a marginally significant increase in NPQ. Salt stress has been reported to elicit an increase in NPQ for a variety of plant species (Brugnoli & Bjorkman, 1992; Dionisio-Sese & Tobita, 2000; Stępień & Kłbus, 2006). Increased NPQ values reflect an increase in non-photochemical quenching mechanisms of leaf tissue. Non-photochemical quenching aids plants experiencing salt stress by dissipating excess light energy as heat. This process occurs primarily from actions of the xanthophyll cycle and is viewed to be a form of photoprotection (Logan et al. 2007). From this study, it appears that cypress respond to salt stress by increasing non-photochemical quenching mechanisms within leaves.

Elevated salinity causes an increase in leaf H_2O_2 which was localized within vascular tissue and guard cells of leaves.

Plants maintained at 8‰ exhibited a marginally significant increase in leaf H_2O_2 content. The accumulation of ROS was localized within vascular tissue and guard cells of salt stressed cypress leaves. The increase in ROS within cypress leaves could be responsible for the activation of defense genes, stomatal closure, and the downregulation of photosynthesis in response to salt stress.

An increase in ROS within the vascular tissue of leaves has been reported in other plant species in response to an array of elicitors, including salt stress. Miller et al. (2009) demonstrated that mechanical wounding, temperature extremes, and salt stress elicited a rapid and systemic accumulation of H_2O_2 within the vascular tissue of *Arabidopsis thaliana*. In a similar study, Orozco-Cárdenas et al. (2001) demonstrated that H_2O_2 accumulated within the vascular tissue of tomato leaves after mechanical wounding. In both reports, H_2O_2 was enzymatically produced within the vascular tissue. The upregulation of H_2O_2 then elicited the activation of defense genes. It is possible that the accumulation of ROS within the vascular tissue of cypress leaves may result in the activation of defense genes responsible for salt tolerance.

An upregulation of H_2O_2 within guard cells is known to elicit stomatal closure in plants. Others have reported that the exogenous application of H_2O_2 will cause stomatal closure (Pei et al. 2000; Zhang et al. 2001). It appears that a similar mechanism is responsible for stomatal closure in cypress because an accumulation of ROS was observed within guard cells of salt stressed cypress. Salinity stress induces stomatal closure in cypress (Pezeshki et al. 1987; Allen et al. 1997; Pezeshki et al. 1995). This

physiological response is thought to aid cypress by reducing transpiration and thus, the amount of Na^+ ions being pulled into the plant from the roots. This response to elevated salinity is an important physiological adaptation to not only cypress, but many other plant species (Mahajan & Tutejia, 2005).

Increased H_2O_2 has been linked to a downregulation of carbon fixing processes of photosynthesis. Badger et al. (1980) demonstrated that the exogenous application of H_2O_2 to Rubisco caused an inhibition of carboxylase activity. Furthermore, Kaiser (1979) reported that the application of exogenous H_2O_2 to intact chloroplasts caused a 90% inhibition of CO_2 fixation. In the current study, a strong negative correlation between leaf H_2O_2 content and MQY was observed. Elevated salinity is well known to cause a drop in net photosynthesis and carbon fixation within cypress (Pezeshki et al. 1995; Allen et al. 1996; Allen et al. 1997). Thus, the upregulation of H_2O_2 within leaf tissue of cypress may be responsible for the downregulation of photosynthesis observed in response to salt stress.

Elevated salinity causes an increase in leaf free proline content.

An accumulation of proline within leaf tissue is a common response to salt stress for many plant species (Yokoi et al. 2002). From this study, it appears that Bald Cypress also responds in this way because plants maintained at 8‰ exhibited a significant increase in free proline content of leaves. Free proline was originally thought to act as an osmolyte to alleviate the osmotic component associated with salt stress (Hong et al. 2000; Verbruggen & Hermans, 2008). However, other reports demonstrate that free proline acts as a molecular chaperone to stabilize proteins and cell membranes under oxidative stress

(Samuel et al. 2000; Molinari et al. 2007). The accumulation of leaf proline in response to salt stress is likely protecting cypress from osmotic stress as well as oxidative stress.

Elevated salinity causes a decrease in a low molecular weight peroxidase within leaf tissue.

Peroxidases (POX) are a class of enzymes that convert H_2O_2 into water and oxygen within plant tissue. Some have reported that total POX activity will increase in response to salt stress (Sairam et al. 2002; Meloni et al. 2003; Mittova et al. 2004); however, others have reported the opposite trend (del Río et al. 1996; Shim et al. 2003; Hernandez et al. 2010). The conflicting results may result from differences between species, intensity of salinity treatment, type of tissue sampled, timing of sampling, or the activation/deactivation of specific isozymes. In this study, the specific activity of total POX within leaf tissue decreased in response to salt stress. After isozyme separation, a distinct drop in intensity of the ~37 kDa POX isozyme was observed. This suggests that the decrease in total POX activity was caused by a drop in the ~37 kDa POX isozyme. The ~37 kDa POX isozyme was found in the pellet proteins upon differential centrifugation suggesting that this isozyme was associated with an organelle (data not shown). A common response of plants to environmental stress is the proliferation of peroxisomes within cells, but the inhibition of catalase, a peroxidase found specifically on peroxisomes (del Río et al. 1996). It is known that peroxisomes produce H_2O_2 which then diffuses into the cytoplasm and then plays an important role in stress tolerance of plants (Corpas et al. 2001). It is possible that the ~37 kDa POX isozyme is localized within peroxisomes of cypress leaves. However, more study is needed to (1) verify that the ~37 kDa POX isozyme is from a peroxisome and (2) that peroxisome proliferation occurs within cypress leaves in response to salt stress.

2.4.2 *In situ* sampling of *Taxodium distichum*

The greenhouse study found that cypress responded to salt stress in a predictable physiological manor. It was hypothesized that cypress sampled *in situ* would exhibit a similar physiological response to elevated salinity. Cypress sampled from the basin swamp exhibited the most signs of salinity stress because these plants had the lowest MQY and highest NPQ values compared to plants at other sites. Leaf tissue at the basin swamp also had the highest Na^+, lipid peroxidation, and proline content compared to plants at other sites. At the time of sampling, it was noted that the cypress population at the basin swamp was affected by chronic flooding. This is important because Allen et al. (1996) reported that both flooding and salinity together exert a greater stress on cypress than either stressor alone. It is likely that cypress at the basin swamp exhibited the most evident signs of salinity stress because they experienced both elevated salinity and chronic flooding.

It was noted that the specific activity of both SOD and POX was the lowest at the impacted wetland, but increased in cypress further upstream. A strong positive correlation between SOD and POX suggests that the activity of these two enzymes is tightly linked. This trend may be explained by leaf senescence. Many deciduous plants respond to salinity stress by selectively transporting Na^+ ions into older leaves along the base of the stem. These leaves then senesce to act as 'sacrificial leaves' that fall off the plant, thus reducing the amount Na^+ ions that enter younger leaf tissue (van der Moezel et al. 1988; Wolf et al. 1991). In the process of leaf senescence, the specific activity of both SOD and POX has been reported to drop significantly (Dhindsa et al. 1981; Kanazawa et al. 2000; Prochazkova et al. 2001). Allen et al. (1994) reported that cypress seedlings

maintained at 8‰ for three months exhibited signs of leaf senescence and suggested that leaf senescence may play an important role in salinity tolerance of cypress. The impacted wetland was closest to a source of saline water from the St. Johns River, however; cypress further downstream at the basin swamp were most affected by salt stress. An increase in leaf senescence of cypress at the impacted wetland could explain why cypress have the lowest activities of SOD and POX, but appear to be less affected by elevated salinity compared to cypress at the basin swamp.

A number of researchers utilize chlorophyll fluorescence to detect sub-lethal stress for plants *in situ*. However, these measurements have an inherent diurnal variation which may lead to erroneous interpretation of data. Logan et al. (2007) suggested that chlorophyll fluorescence measurements be complemented with other parameters such as photosynthetic pigment content or leaf gas exchange. In a field study, Trevathan-Tackett et al. (2013) coupled chlorophyll fluorescence with tissue Cl^- and $SO4^{-2}$ content to detect salinity stress in the tropical seagrass *Thalassia testudinum*. The authors identified a site near Key West, FL where *Thalassia* exhibited an increase in tissue Cl^- and $SO4^{-2}$ content and a decreased MQY compared to plants at other sites. In the current study, salinity stress was detected in cypress by coupling chlorophyll fluorescence parameters with leaf Na^+ content as well as lipid peroxidation and free proline.

The ability to detect sub-lethal stress in plants could be useful for restoration projects, ecological studies, agriculture, or management agencies. Biomarkers coupled with chlorophyll fluorescence could be a tool to detect sub-lethal stress within plants. However, a great amount of care must be taken in order to obtain accurate results. Before field sampling, a thorough greenhouse study should be conducted in order to understand

how plants respond to the specific stressors. In the field, samples should be placed on dry ice no less than 3 minutes after harvest from the plant. This will reduce the degradation of tissue as well as limit any treatment effect caused by harvesting.

Chapter 3: Preliminary investigation into the cellular mechanism of salinity tolerance in Bald Cypress (*Taxodium distichum* (L.) Rich.).

Abstract:

Plant exposure to saline conditions may induce a series of metabolic dysfunctions including sodium toxicity, osmotic stress and ion imbalance. Salinity stress has been linked to an increased production of reactive oxygen species (ROS) within tissue and it is now widely accepted that these molecules act as cell signaling agents that play a key role in a plant's ability to perceive and respond to abiotic stress.

To better understand the relationship between salt stress and the mechanisms driving intracellular ROS accumulation, leaves of cypress seedlings were studied to evaluate the short-term effects of salinity exposure. Maximum quantum yield (MQY), peroxidase specific activity and leaf transpiration were assayed on detached leaves that were exposed to elevated NaCl concentrations for a 24, 48, or 72 h duration. There was a strong negative correlation between leaf Na^+ content and MQY. Furthermore, a similar correlation existed between leaf Na^+ content and the maximum fluorescence value (Fm), suggesting that the drop in MQY was attributed to a decrease in the number of available PSII reaction centers. Elevated salinity caused a decrease in leaf transpiration for all time points. Total peroxidase activity exhibited a significant increase in response to salt stress after 48 h; however, there was no difference at 72 h. Enhanced peroxidase activity at 48h

was found to be specifically associated with the induction of a ~37 kDa peroxidase isoform.

Treatment with clofibrate, a drug known to induce the proliferation of peroxisomes, caused an increase in total peroxidase as well as the activity of the ~37 kDa peroxidase. Pre-treatment of leaves with the fungal toxin brefeldin A (BFA) appeared to block the induction of the ~37 kDa peroxidase in response to salt stress. Neither drug affected rates of leaf transpiration. Pre-treatment of leaves with diphenyliodinium chloride (DPI), a suicide inhibitor of NADPH-oxidase, appeared to block the induction of the ~37 kDa peroxidase in response to elevated salinity. Furthermore, DPI blocked the decrease in transpiration associated with salt stress. These data suggest that the enzymatic production of ROS by NADPH-oxidase leads to a drop in leaf transpiration associated with salt stress in cypress.

3.1 Introduction

The exposure of plants to saline water imposes a significant stress. On the cellular level, Na^+ ions are toxic to plants because they interfere with normal metabolism and enzyme function (Kalir et al. 1984; Niu et al. 1995). Salt tolerance has been linked to a plant's ability to reduce the amount of Na^+ ions within cells (Blumwald et al. 2000; Apse & Blumwald, 2007). The majority of salt sensitive plants exhibit similar physiological responses to salt stress such as stomatal closure and a reduction in leaf transpiration, net photosynthesis, carbon assimilation, and biomass (Ashraf, 2004; Mahajan & Tuteja, 2005). Both stomatal closure and the downregulation of photosynthesis are perceived to aid in salinity tolerance by reducing water use and the loss of water through transpiration,

thus reducing the amount of saline water being pulled into the tissue from the roots and ultimately the amount of Na^+ ions which enter the plant (Allen et al. 1998, Ashraf, 2004).

Bald Cypress (*Taxodium distichum* (L.) Rich.) is a large, deciduous softwood plant native to the Southeastern United States. This species is dominant in coastal freshwater wetlands where salinity stress from periodic saltwater intrusion events occurs (Hackney et al. 2007; Krauss et al. 2007). Cypress exhibit a similar physiological response to salinity as reported in other plant species such as: stomatal closure and a decrease in net photosynthesis, sap flow, growth, and biomass (Pezeshki et al. 1987; Allen et al. 1994; Pezeshki et al. 1995; Allen et al. 1997; Effler & Goyer, 2006; Krauss et al. 2009; Krauss & Duberstein, 2010). Furthermore, a negative correlation between leaf Na^+ content and net photosynthesis has been reported (Pezeshki et al. 1988). However, nothing is known about how Bald Cypress perceives salinity stress on the cellular level.

In other plant species, salinity stress has been linked to an increase of reactive oxygen species (ROS) (Mittova et al. 2004; Gómez et al. 2004; Leshem et al. 2007; Hernandez et al. 2010). The increase in ROS appears to act as key cell signaling molecules that regulate the plant's ability to perceive and respond to abiotic stress, including elevated salinity (Mittler, 2002; Neill et al. 2002a; Neill et al. 2002b; Apel & Hirt, 2004; Jaspers & Kangasjärvi, 2010). For example, stomatal closure has been linked to the accumulation of ROS within guard cells (Pei et al. 2000; Zhang et al. 2001; Desikan et al. 2003). Furthermore, it has been demonstrated that elevated ROS elicit a downregulation of carbon fixation processes within chloroplasts, suggesting that elevated ROS may play a key role in reducing net photosynthesis (Kaiser, 1979; Badger et al. 1980). It is now becoming clear that the increase of ROS in response to abiotic stress on

the cellular level affects physiological processes necessary for adaptation and tolerance on the organismal level.

The increase of ROS in response to salt stress has been linked to enzymatic and non-enzymatic sources within plant tissue. Enzymatic sources of ROS may include plasma membrane-bound NADPH-oxidase and cell wall or apoplastic peroxidases (Bolwell et al. 1995; Bolwell et al. 1998; Torres and Dangle 2005; Sagi and Fluhr 2006). NADPH-oxidase has been shown to produce ROS within the vascular tissue of *Arabidopsis thaliana* in response to salt stress (Miller et al. 2009). Similarly, NADPH-oxidase has been shown to produce ROS within guard cells (Pei et al. 2000; Zhang et al. 2001; Desikan et al. 2003). However, a positive link between salt stress and the enzymatic production of ROS within guard cells to elicit stomatal closure is not established. Corpas et al. (2001) demonstrated that peroxisomes act as a significant source of ROS within plants under stress conditions. A common response of plants to environmental stress is the proliferation of peroxisomes within cells (del Río et al. 1996). Mitsuya et al. (2010) demonstrated that salt stress induced the proliferation of peroxisomes within root tissue of *Arabidopsis thaliana*. It appears that the upregulation of ROS in response to salt stress originates from the enzymatic production by NADPH-oxidase or from the non-enzymatic production by peroxisomes.

Previous work by the author investigated the role of ROS in relation to salinity stress in Bald Cypress. In a controlled greenhouse experiment, one year old saplings were exposed to salinities of 0, 4, or 8‰ for 10 weeks. It was found that plants exposed to a salinity of 8‰ exhibited a marginally significant increase in leaf H_2O_2 content compared to plants maintained at 0‰. The H_2O_2 was localized within the vascular tissue and guard

cells of plants. Furthermore, there was a strong negative correlation between leaf H_2O_2 content and the chlorophyll fluorescence parameter MQY, suggesting that an upregulation of H_2O_2 in response to salt stress may play a key role in downregulating photochemical efficiency. It was hypothesized that H_2O_2 was being enzymatically produced in guard cells in order to induce stomatal closure.

Interestingly, leaves of plants maintained at a salinity of 8‰ exhibited a non-significant decrease in the specific activity of peroxidase, an enzyme responsible for the degradation of H_2O_2, compared to leaves of plants maintained at a salinity of 0‰. Upon separation of isozymes, it was found that the activity of a ~37 kDa peroxidase decreased in leaf tissue in response to salt stress suggesting that this specific isozyme was localized within a peroxisome. It was also hypothesized that salt stress elicited the proliferation of peroxisomes within leaf tissue to act as an intracellular source of ROS.

The purpose of this study was to obtain preliminary insight into the mechanisms of salinity tolerance and the relationship to ROS in leaves of Bald Cypress. The specific goals were to:

1) Investigate the mechanisms behind ROS production in response to elevated salinity using pharmacological inhibitors and activators of specific cell processes.
2) Investigate the association between ROS production and leaf transpiration in response to salt stress.

3.2 Materials and Methods

3.2.1 Detached leaf experiment

Detached leaves were used to investigate the physiological and cellular responses of cypress leaves to salinity stress. A preliminary experiment exposed fully expanded leaves to salinities of 125 and 250 mM NaCl for 72 h. Maximum quantum yield (MQY), leaf water consumption, total POX activity, and activity of a ~37 kDa peroxidase isozyme were assayed on leaves every 24 h for three days. Leaf Na^+ content was assayed at the end of the experiment. Leaves, approximately 8 cm in length, were cut with a razor blade from cypress saplings and immediately placed in 1.5 mL microcentrifuge tubes containing D.I. water. Leaves were equilibrated for 24 h under continuous light at 32 μmol photons $m^{-2}s^{-1}$ prior to treatment. All experiments were subsequently run under continuous light (32 μmol photons $m^{-2}s^{-1}$) and temperature (23 °C).

Chlorophyll fluorescence parameters were assayed on leaves using a Diving Pulse Amplitude Modulated (PAM) fluorometer (Heinz-Walz GmbH ©,Effeltrich, Germany). Samples were held in place using a dark leaf clip (DIVING-LC) with the fiber optic cable 5 mm from the leaf. PAM settings were as follows: measuring intensity=5, gain=3, saturation intensity=2 and damp=2. Maximum quantum yield (MQY= (Fm-Fo)/Fm) was assayed on leaves that were dark adapted for 10 s to achieve 'quazi-darkness'. A 10 s dark adaptation allows for the re-oxidation of the primary PSII electron acceptor without significant relaxation of non-photochemical quenching mechanisms. This measurement is understood to represent the physiological state to which the plant has adapted to and is independent of light history (Ralph & Gademann, 2005).

For each sample, approximately 250 mg of leaf tissue was placed in a 2 mL screw cap microfuge tube containing five 2.0 mm zirconium disruptor beads. Tissue was homogenized with 1 mL of ice cold CelLytic P plant cell lysis/extraction reagent containing 1% (w/v) PVPP and 0.1% (w/v) of a protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Tissue was homogenized in a Fast-Prep-24 Tissue Disrupter (MP Biomedical, Solon, OH) for 30 s at a speed of 6.0 m/s. Samples were centrifuged at 13,300 x g for 15 min at 4 °C. Samples were kept on ice prior to assay. The supernatant was assayed for the specific activity of peroxidase (POX). Total protein was quantified using the Bio-Rad Bradford assay (Bio-Rad, Hercules, CA, USA). Samples were then diluted 1:100 in 25 mM Tris-HCl buffer (pH = 7.8). Peroxidase activity was quantified by the Amplex Red POX assay kit (Invitrogen, Carlsbad, CA, USA). Leaf peroxidase isozymes were separated using non-denaturing SDS-PAGE on NuPAGE 4-12% bis-tris gels (Invitrogen). Electrophoresis was performed at 4 °C. All wells were loaded with 5 µg of total protein. In order to eliminate variability of staining between gels, samples from each experiment were loaded on the same gel. Peroxidase isozyme activity was detected by placing gels in a staining solution of 1 mM o-dianisidine and 0.5 mM H_2O_2 in acetate buffer (pH= 5.6). Pixel intensity of individual isozyme bands was quantified using Image J software (National Institute of Heath, Bethesda, MD, USA). Individual isozyme pixel intensity was averaged for each treatment and then divided by the average pixel intensity of control values.

Leaf Na^+ content was assayed from the same extracts that POX activity at 72h was assayed. Fifty micrograms of Sodium Green tetraacetate (Invitrogen) was reconstituted in 20 µL of DMSO containing 10% (w/v) pluronic f127. The reconstituted

indicator was diluted to a final concentration of 5 μM in 25 mM Tris-HCl buffer (pH= 7.8) containing 20 U/mL of esterase. The diluted indicator was incubated at 37 °C for 30 min prior to the assay. Fifty microliters of the diluted indicator was added to 10 μL of leaf extract. The mixture was incubated at 37 °C for 1 h and read on a BioTek FLx 800 fluorescent microplate reader (BioTek, Winooski, VT, USA) (ex 485/em 528). Units were expressed as mg Na^+ per gram of tissue.

Salt stress will elicit stomatal closure in many plants, including cypress (Pezeshki et al. 1987; Allen et al. 1997; Mahajan & Tutejia, 2005). Stomatal closure results in decreased transpiration and water consumption. In the detached leaf experiments, transpiration was measured by gravimetrically determining the amount of water consumed by individual leaves according to the methods of Wilkinson et al. (1998). Values were normalized by leaf weight.

3.2.2 Detached leaf experiments pre-treated with pharmacological agents

In a series of additional experiments leaves were pre-treated with selected pharmacological compounds to gain insight into the cellular mechanisms of salinity tolerance in cypress leaves. Leaves were assayed for total POX, activity of the ~37 kDa peroxidase isozyme, and transpiration. Chlorophyll fluorescence values were not reported because treatment with pharmacological agents alone caused a drop in yield values (data not shown).

Clofibrate experiment

Clofibrate is a xenobiotic drug known to induce the *de novo* synthesis and proliferation of peroxisomes in plants (Palma et al. 1991; López-Huertas et al. 1995). Detached leaves were placed in D.I. water alone or D.I. water containing 10 or 20 mM

clofibrate (Sigma). Data presented were collected on leaves 72 h after treatment with clofibrate.

Brefeldin A experiment

Brefeldin A is a fungal toxin known to interfere with vesicle trafficking involved with the endomembrane system of plants (Samaj et al. 2004). A factorial design was used on leaves treated with 250 mM NaCl or BFA. Selected leaves were pre-treated with 37.5 mM brefeldin A (BFA) (Sigma) for 24 hours prior to salinity treatment. The data presented was collected on leaves 48 hours after salinity treatment.

Diphenyliodinium chloride experiment

Diphenyliodinium chloride is a suicide inhibitor of the ROS producing enzyme NADPH-oxidase (Orozco-Cárdenas et al. 2001). A factorial design was used on leaves treated with 250 mM NaCl or DPI. Selected leaves were pre-treated with 100 mM diphenyliodinium chloride (DPI) (Sigma) for 24 h prior to salinity treatment. Data presented were collected on leaves 48 h after salinity treatment.

3.2.3 Statistical analysis

For parametric data, differences among each response variable were analyzed by a one-way ANOVA. Significant differences among treatments are indicated by letters according to Tukey's HSD post-hoc comparison of means. All data analyzed using one-way ANOVA met the homogeneity of variance assumption of ANOVA. Non-parametric data was analyzed using a Kruskal-Wallis analysis followed by a Mann-Whitney U post-hoc test. For all data, outliers were detected by a studentized residual test and removed from datasets prior to analysis. Significant p values ($p < 0.05$) are highlighted in bold. Correlations between chlorophyll fluorescence parameters and leaf Na^+ content were

tested using a one-tailed Pearson's correlation. Significant p values ($p < 0.05$) and strong correlation coefficients ($r > |0.5|$) are highlighted in bold within figures. All statistical analyses were performed on SYSTAT 11 (Systat software, Chicago, IL, USA).

3.3 Results

3.3.1 Detached leaf experiment

Chlorophyll fluorescence

Maximum quantum yield exhibited a dose dependent drop in response to NaCl treatment. After 72 h leaves treated with NaCl exhibited a significant drop compared to leaves maintained in D.I. water. Leaves maintained in D.I. water alone exhibited no change in MQY throughout the course of the experiment, suggesting that detaching leaves did not affect MQY measurement (Figure 3.1). A strong negative correlation was observed between leaf Na^+ content and MQY (Figure 3.2). Initial (Fo) and maximum (Fm) chlorophyll fluorescence values were separated and plotted against leaf Na^+ content. A strong negative correlation existed between leaf Na^+ content and Fm; however, no correlation existed between leaf Na^+ content and Fo (Figures 3.3 and 3.4 respectively).

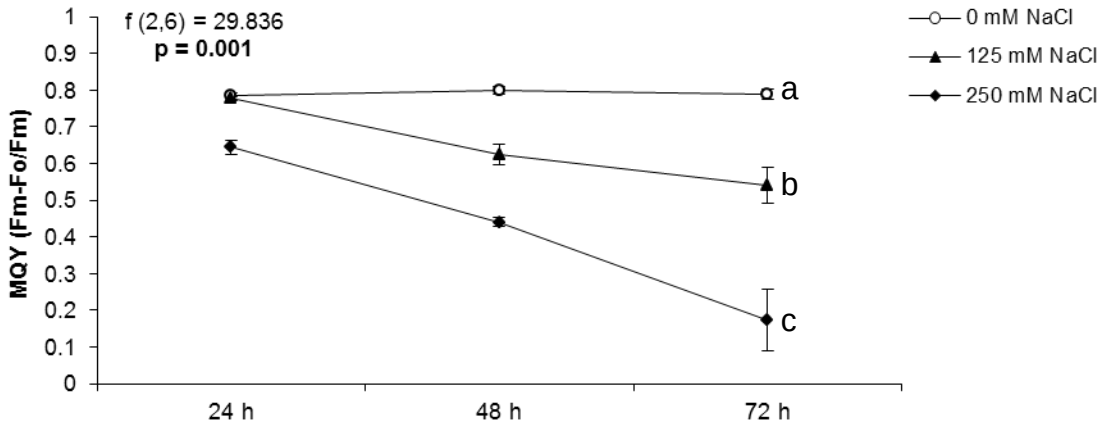

Figure 3.1 Maximum quantum yield (MQY) for detached leaves exposed to salinities of 0, 125, or 250 mM NaCl. Leaves exposed to elevated NaCl exhibited a significant decrease in MQY after 72 hours compared to leaves maintained in D.I. water. Values represent mean ± SEM (n= 3).

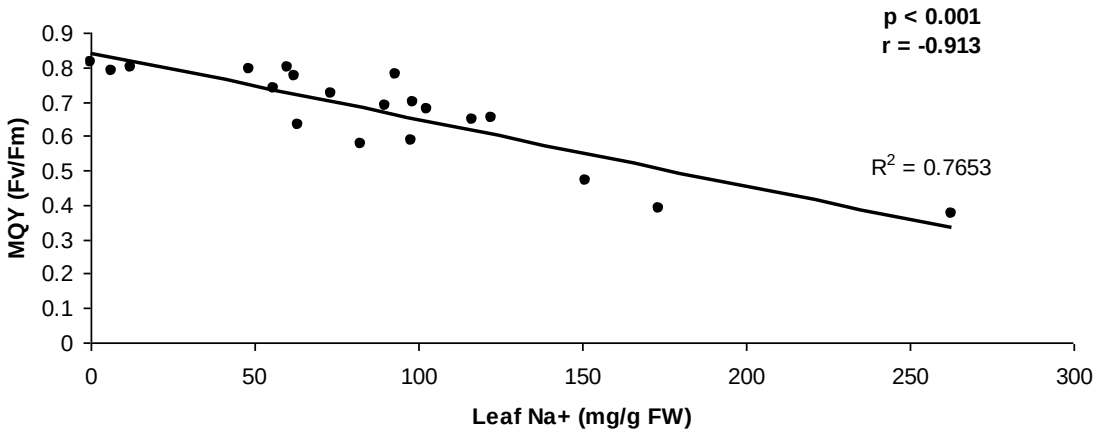

Figure 3.2 The relationship between leaf Na^+ content and MQY after detached leaves were exposed to NaCl concentrations of either 0,125, or 250 mM for 72 h. A strong negative correlation existed between leaf Na^+ content and MQY.

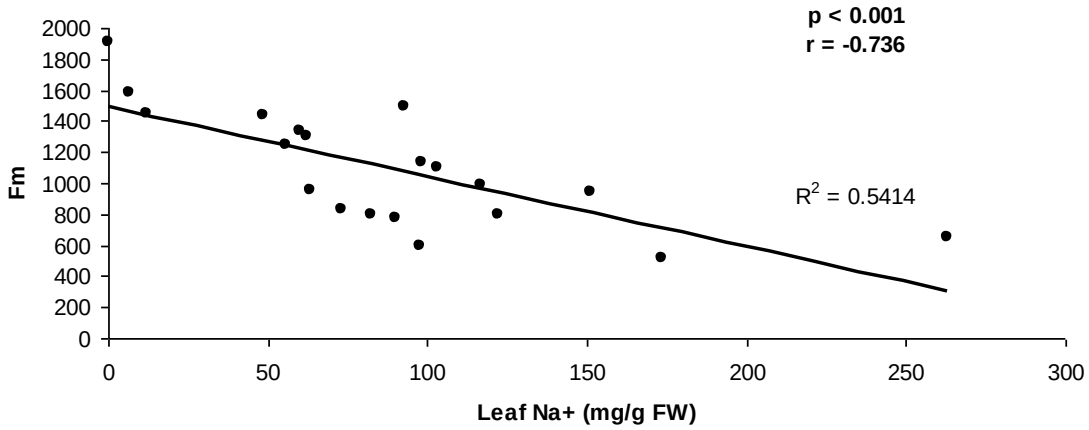

Figure 3.3 The relationship between leaf Na^+ content and maximum fluorescence (Fm) after detached leaves were exposed to NaCl concentrations of either 0,125, or 250 mM for 72 h. A strong negative correlation existed between leaf Na^+ content and Fm.

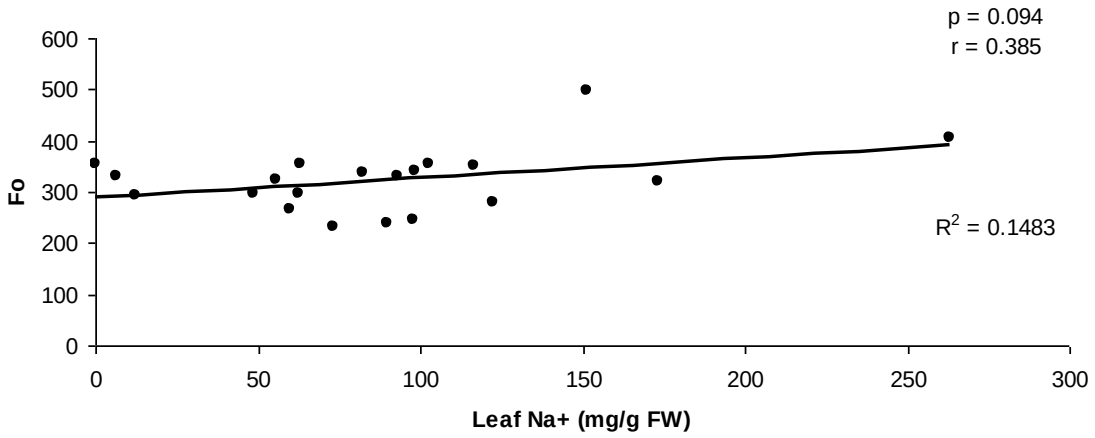

Figure 3.4 The relationship between leaf Na^+ content and initial fluorescence (Fo) after detached leaves were exposed to NaCl concentrations of either 0,125, or 250 mM for 72 h. No correlation existed between leaf Na^+ content and Fo.

Total leaf POX activity and activity of the ~37 kDa peroxidase isozyme

The specific activity of POX exhibited no changes in response to salinity treatment after a 24 h exposure to NaCl. After 48 h, a significant increase in the specific activity of POX in response to NaCl was observed. However, after 72 h, no significant differences were observed between treatments (Figure 3.5). Leaves maintained at 250 mM NaCl exhibited a 42% increase in activity compared to controls after 24 h relative to the activity of the ~37 kDa peroxidase. The activity of this band increased even more in response to salt stress after 48 h with the activity of leaves maintained at 250 mM NaCl increasing more than six fold compared to controls. Interestingly, after 72 h, the activity for leaves maintained at 250 mM NaCl was 43% lower than that of control leaves (Figure 3.6).

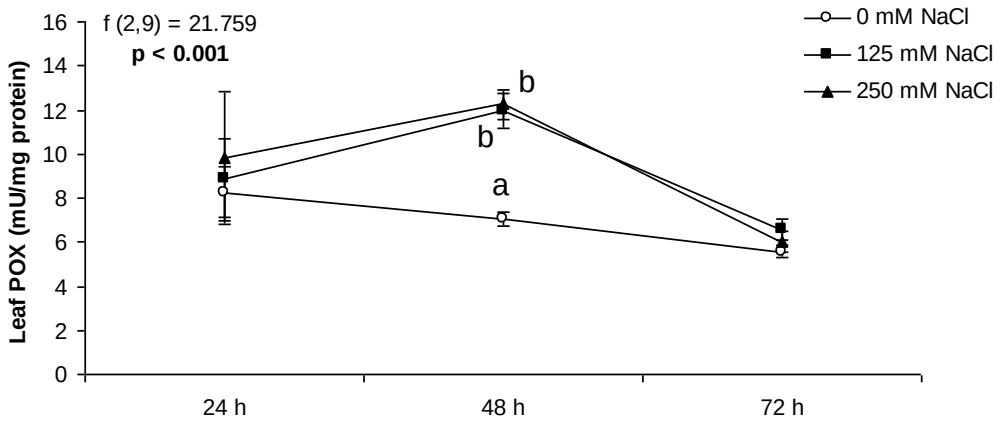

Figure 3.5 Specific activity of leaf peroxidase for 24, 48, and 72 hours. Leaves exposed to treatments of 125 or 250 mM NaCl exhibited a significant increase in total peroxidase after 48 h. However, no differences were observed at 24 and 72 hours. Values represent mean ± SEM (n= 4 or 5).

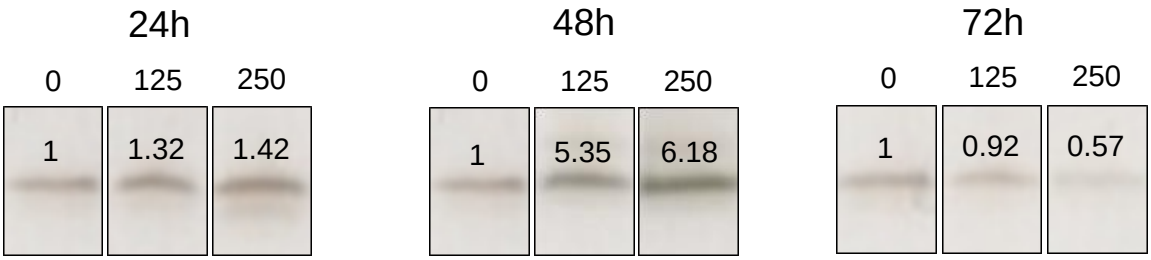

Figure 3.6 In-gel analysis of a ~37 kDa leaf peroxidase isozyme. After 24 h, leaves exposed to 250 mM NaCl exhibited a 42% increase in activity compared to controls. After 48 h, leaves maintained at 250 mM NaCl exhibited a six fold increase in activity compared to controls. After 72 h leaves maintained at 250 mM NaCl exhibited a 43% decrease in activity compared to controls. All wells were loaded with 5 μg of total protein and each treatment had four replicate samples.

Leaf transpiration

Transpiration was lower in leaves exposed to NaCl for all timepoints. After 72 h, detached leaves exposed to 125 and 250 mM NaCl exhibited a significant decrease in transpiration compared to leaves maintained in D.I. water (Figure 3.7).

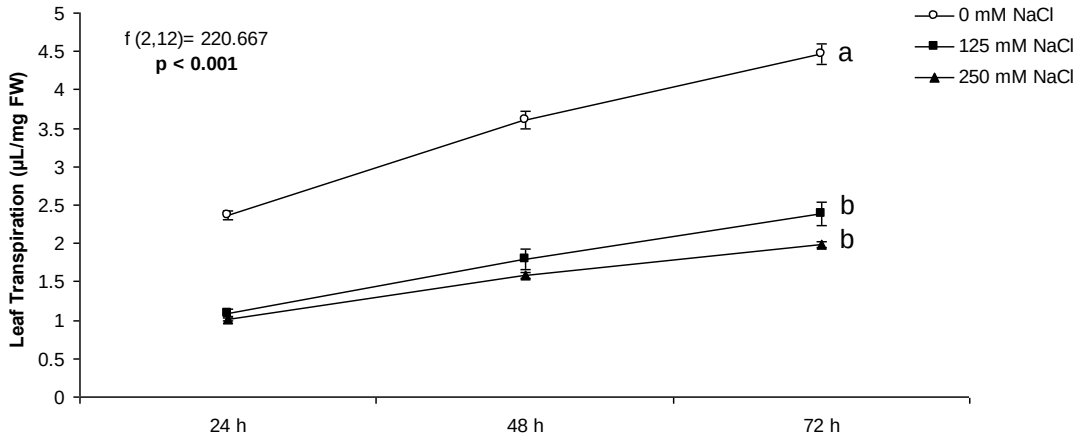

Figure 3.7 Transpiration for detached leaves exposed to salinities of 0, 125, or 250 mM NaCl. Leaves exposed to elevated NaCl exhibited a significant decrease in transpiration after 72 h compared to leaves maintained in D.I. water. Values represent mean ± SEM (n= 5).

3.3.2 Detached leaf experiments pre-treated with pharmacological agents

Treatment with clofibrate

Treatment of detached leaves with 10 and 20 mM clofibrate induced a significant increase in total peroxidase compared to leaves maintained in D.I. water alone (Figure 3.8). Furthermore, treatment of leaves with 20 mM clofibrate induced a 36% increase in activity of the ~37 kDa peroxidase compared to leaves maintained in D.I. water (Figure 3.9). Treatment of leaves with clofibrate did not cause changes in transpiration (Figure 3.10).

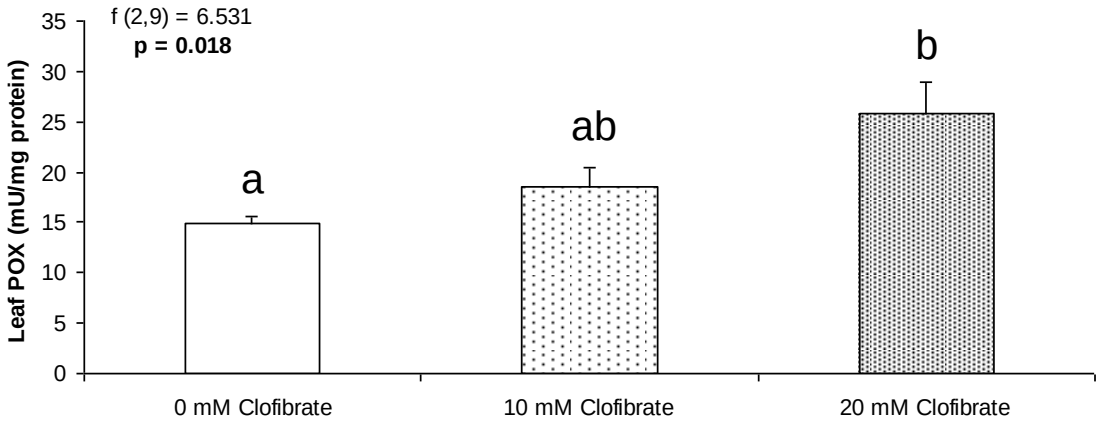

Figure 3.8 Specific activity of leaf peroxidase 72 h after treatment with clofibrate. Detached leaves treated with clofibrate exhibited a significant increase in total peroxidase compared to leaves maintained in D.I. water. Values represent mean + SEM (n= 4).

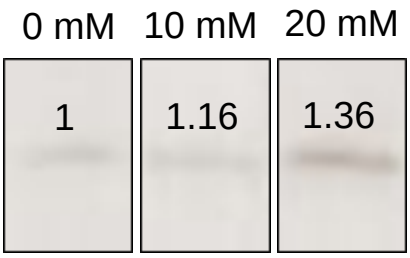

Figure 3.9 In-gel analysis of a ~37 kDa leaf peroxidase isozyme. Leaves treated with 10 mM clofibrate exhibited a 16% increase in activity whereas leaves treated with 20 mM clofibrate exhibited a 36% increase in activity compared to controls. All wells were loaded with 5 μg of total protein and each treatment had four replicate samples.

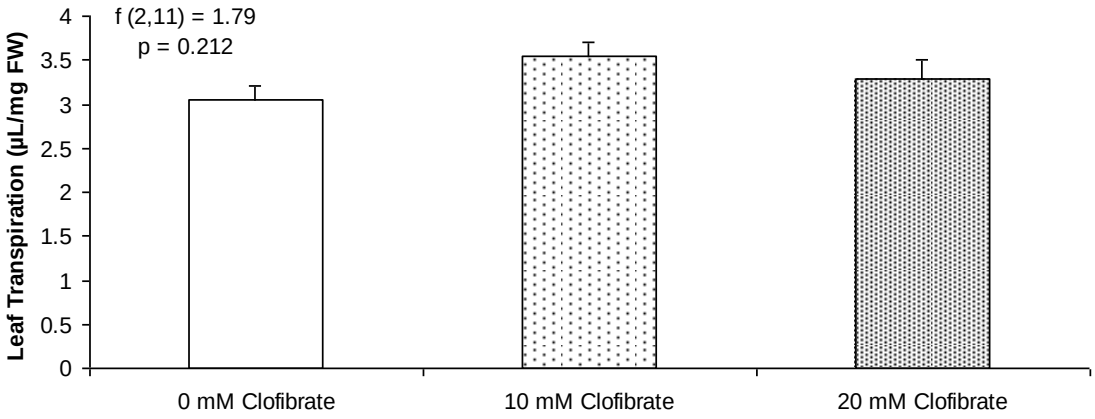

Figure 3.10 Transpiration for leaves treated with clofibrate after 72 h. Treatment with clofibrate did not elicit changes in transpiration. Values represent mean + SEM (n= 4 or 5).

Treatment with Brefeldin A

Pre-treatment of leaves with BFA appeared to block the increase in total peroxidase in response to 250 mM NaCl treatment. Leaves treated with 250 mM NaCl alone exhibited a 30% increase in total peroxidase compared to leaves maintained in D.I. water after 48 h. However, leaves pre-treated with BFA and then treated with 250 mM NaCl did not exhibit an increase in total peroxidase compared to controls (Figure 3.11). Treatment of leaves with 250 mM NaCl for 48 h caused a 14% increase in the activity of the ~37 kDa peroxidase compared to leaves maintained in D.I. water. However, leaves pre-treated with BFA and then exposed to 250 mM exhibited a 10% drop in the activity of the ~37 kDa peroxidase compared to leaves maintained in D.I. water (Figure 3.12). Treatment of leaves with BFA did not cause changes in transpiration. Leaves treated with BFA alone were not significantly different from leaves maintained in D.I. water. Furthermore, leaves pre-treated with BFA and then treated with 250 mM NaCl did not exhibit a significant difference in transpiration compared to leaves treated with 250 mM NaCl alone (Figure 3.13).

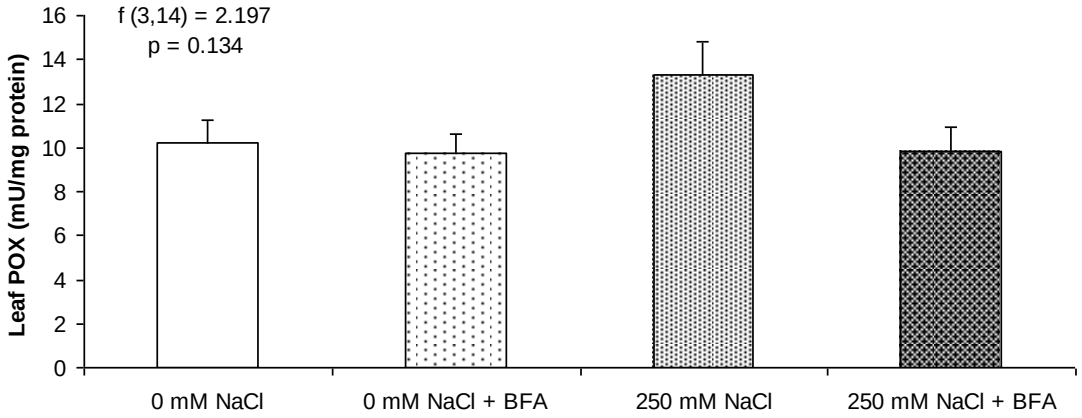

Figure 3.11 Specific activity of peroxidase after 48 h for leaves pre-treated with BFA and treated with 250 mM NaCl in a factorial design. Detached leaves treated with 250 mM NaCl exhibited a 30% increase in the specific activity of peroxidase compared to leaves maintained in D.I. water alone. Leaves pre-treated with BFA and then treated with 250 mM NaCl for 48 h exhibited values similar to those of leaves maintained in D.I. water alone. Values represent mean + SEM (n= 4 or 5).

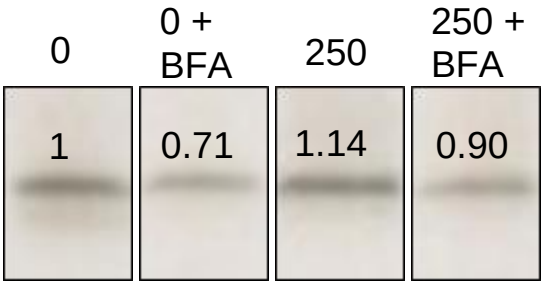

Figure 3.12 In-gel analysis of a ~37 kDa leaf peroxidase isozyme. Treatment of leaves with 250 mM NaCl for 48 h caused a 14% increase in the activity of the ~37 kDa peroxidase. However, leaves pre-treated with BFA and then exposed to 250 mM NaCl exhibited a 10% drop in activity compared to leaves maintained in D.I. water. All wells were loaded with 5 μg of total protein and each treatment had three replicate samples.

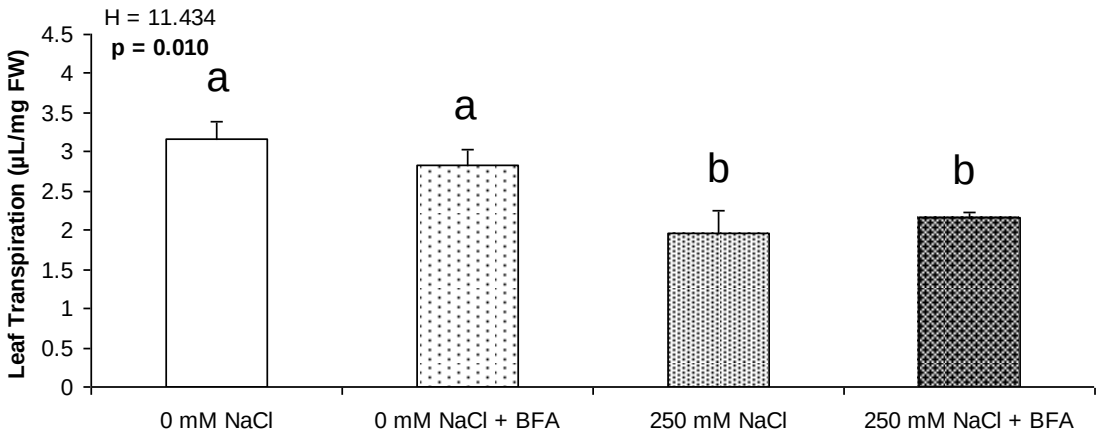

Figure 3.13 Transpiration after 48 h for leaves pre-treated with BFA and treated with 250 mM NaCl in a factorial design. Pre-treatment with BFA did not cause changes in transpiration. Values represent mean + SEM (n= 5).

Treatment with diphenyliodinium chloride

Pre-treatment of leaves with DPI appeared to have a treatment effect on the specific activity of leaf peroxidase because both groups of leaves pre-treated with DPI had a significantly higher specific activity compared to leaves maintained in D.I. water. However, leaves maintained in 250 mM NaCl for 48 h exhibited the greatest increase in the specific activity of peroxidase compared to other treatments (Figure 3.14). All treatments exhibited an increase in the activity of the ~37 kDa peroxidase compared to leaves maintained in D.I. water alone. However, leaves maintained in 250 mM NaCl exhibited the greatest increase in the activity (Figure 3.15). Leaves pre-treated with DPI and then treated with 250 mM NaCl for 48 h exhibited a significant increase in transpiration compared to leaves maintained in 250 mM NaCl alone. Transpiration for leaves pre-treated with DPI and then treated with 250 mM NaCl was significantly lower than leaves not treated with NaCl (Figure 3.16).

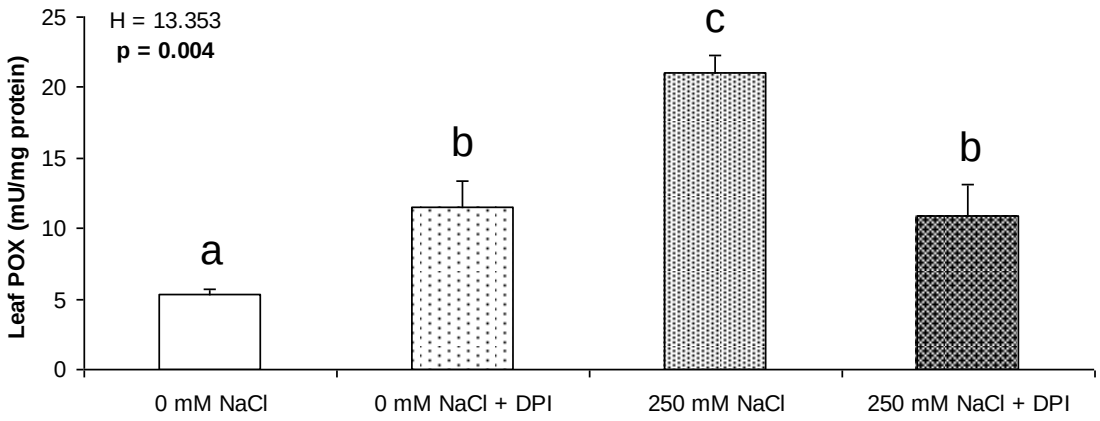

Figure 3.14 Specific activity of peroxidase after 48 h for leaves pre-treated with DPI and treated with 250 mM NaCl in a factorial design. Detached leaves treated with 250 mM NaCl exhibited a significant increase in the specific activity of peroxidase compared to leaves maintained in D.I. water alone. Leaves pre-treated with DPI exhibited values greater than leaves maintained in D.I. water alone but less than leaves maintained in 250 mM NaCl. Values represent mean + SEM (n= 5).

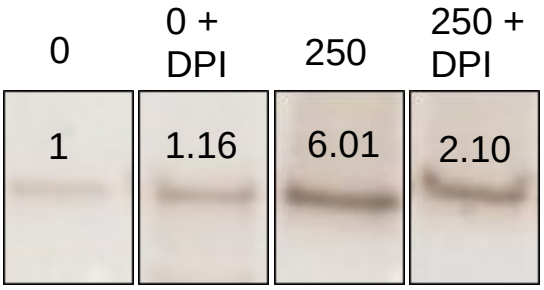

Figure 3.15 In-gel analysis of a ~37 kDa leaf peroxidase isozyme. Treatment of leaves with 250 mM NaCl for 48 h caused a six fold increase in the activity of the ~37 kDa peroxidase. However, leaves pre-treated with DPI and then exposed to 250 mM exhibited a two fold increase in the activity compared to leaves maintained in D.I. water. All wells were loaded with 5 µg of total protein and each treatment had three replicate samples.

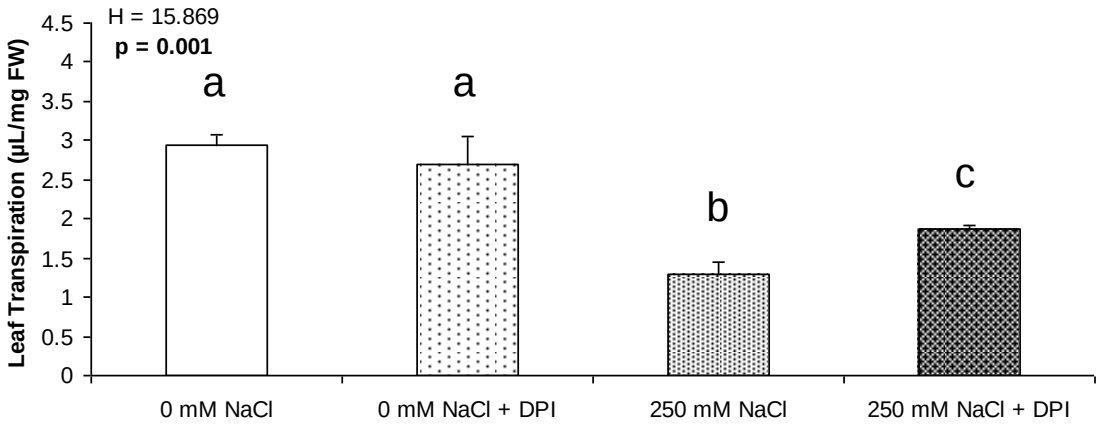

Figure 3.16 Transpiration after 48 h for leaves pre-treated with DPI and treated with 250 mM NaCl in a factorial design. Leaves pre-treated with DPI and then treated with 250 NaCl for 48 h exhibited a significantly greater transpiration than leaves treated with 250 mM NaCl alone. Pre-treatment with DPI alone did not cause changes in transpiration compared to control. Values represent mean + SEM (n= 5).

3.4 Discussion

3.4.1 Detached leaf experiment

NaCl induces a drop in MQY which is attributed to a drop in Fm.

A strong negative correlation existed between leaf Na^+ content and MQY. A similar trend existed between leaf Na^+ content and Fm, suggesting that the drop in MQY is attributed to a decrease in Fm. The decrease in Fm is likely an indication of fewer available PSII reaction centers.

The majority of chlorophyll fluorescence originates from the light harvesting complex and reaction centers of PSII (Govindjee, 2004; Lichtenthaler et al. 2005). Typically, the Fo fluorescence signal originates only from the PSII light harvesting complex and varies with chlorophyll content (Govindjee, 2004; Jeff Kay, personal communication). However, Fm measures fluorescence from both the light harvesting complex and reaction centers of PSII (Govindjee, 2004; Lichtenthaler et al. 2005). Thus, Fm is a reflection of available PSII reaction centers (Lichtenthaler et al. 2005; Jeff Kay, personal communication). The negative correlation between leaf Na^+ content and Fm suggests that salinity stress triggers a reduction in available PSII reaction centers. Interestingly, Nishiyama et al. (2006) proposed that elevated ROS may reduce the number of available PSII reaction centers; however, more work is needed to verify this phenomenon in cypress.

NaCl induces an increase in the ~37 kDa peroxidase after 48 hours but a decrease after 72 hours.

Detached leaves exhibited an increase in total peroxidase after 48 h in response to NaCl treatment. This trend was mirrored by an increase in the activity of the ~37 kDa peroxidase. No difference in total peroxidase were observed between treatments after 72

h. However, it was noted that the activity of the ~37 kDa peroxidase decreased at 72 h in response to NaCl treatment. These data suggest that NaCl treatment causes an initial increase followed by a decrease in the activity of the ~37 kDa peroxidase isozyme. del Río et al. (1996) stated that plant stress was associated with the proliferation of peroxisomes followed by a decrease in the activity of catalase, a peroxidase found specifically on peroxisomes. It was hypothesized that the trend observed in this study was a reflection of the *de novo* synthesis of peroxisomes followed by a decrease in the activity of catalase in response to salinity stress. The proliferation of peroxisomes has been observed in *Arabidopsis thaliana* in response to salinity stress (Mitsuya et al. 2010). The authors noted that peroxisome proliferation is a common response of plants to stress. However, they did not consider that the peroxisomes were producing ROS as signaling molecules as suggested by del Río et al. (1996) and Corpas et al. (2001).

The current paradigm states that new peroxisomes originate from vesicular budding of the endoplasmic reticulum or by fission of existing peroxisomes (Hu et al. 2012). However, work by Leshem et al. (2007) demonstrated that salt stress triggered plasma membrane internalization, resulting in the formation of endosomes which produce ROS within cells. Recent reports suggest that endocytosis represents an efficient signal dispersal mechanism within plant cells (Šamaj et al. 2004). Although no connection currently exists between endocytosis and the *de novo* synthesis of peroxisomes, it is hypothesized that peroxisomes are produced by endocytosis in response to salt stress. Pharmacological experiments in this study were an attempt to test this hypothesis.

NaCl induces a decrease in leaf transpiration.

Detached leaves exposed to NaCl treatments exhibited a decrease in transpiration compared to leaves maintained in D.I. water. This is likely a result of stomatal closure, which is a well documented response of cypress to elevated salinity (Pezeshki et al. 1987; Allen et al. 1997). The decrease in leaf transpiration because of stomatal closure is thought to aid the plant in tolerating elevated salinity by reducing the amount of Na^+ ions that enter the tissue (Allen et al. 1998, Ashraf, 2004).

3.4.2 Detached leaf experiments pre-treated with pharmacological agents

Treatment of leaves with clofibrate induces an increase in the ~37 kDa peroxidase after 72 hours but causes no change in transpiration.

Clofibrate caused a significant increase in the specific activity of total peroxidase and an increase in the activity of the ~37 kDa peroxidase after 72 h. There were no differences in total peroxidase or the activity of the ~37 kDa peroxidase after 24 or 48 h treatments (data not shown). Clofibrate is well known to induce the proliferation of peroxisomes within plants, but evidence for clofibrate treatment causing an effect on the activity of peroxidase is limited. Work by Nila et al. (2006) on tobacco demonstrated that clofibrate treatment induced the proliferation of peroxisomes with a concomitant increase in peroxidase activity. Clofibrate treatment did not cause changes in leaf transpiration, suggesting that this drug did not elicit stomatal closure. Mitsuya et al. (2010) noted that the artificial induction of peroxisomes did not improve NaCl tolerance of *Arabidopsis thaliana*. Data from this study suggests that the artificial induction of peroxisomes by clofibrate may not cause changes at the physiological level.

Pre-treatment of leaves with Brefeldin A inhibits the increase in the ~37 kDa peroxidase associated with NaCl treatment but causes no changes in transpiration

Pre-treatment of leaves with BFA appeared to inhibit the induction of total peroxidase and the activity of the ~37 kDa peroxidase after a 48 h exposure to 250 mM NaCl. This toxin is known to interfere with vesicle trafficking, including endocytosis, in plant cells (Šamj et al. 2004). Leshem et al. (2007) demonstrated that BFA inhibited endocytosis and the intracellular production of ROS in response to salt stress. It is hypothesized that BFA inhibits endocytosis and the *de novo* synthesis of peroxisomes in cypress. This phenomenon has not been reported in plants; however, BFA is known to interfere with peroxisomal protein sorting in yeast (Salomons et al. 1997). Treatment with BFA did not cause changes in leaf transpiration in response to salt stress, suggesting that this toxin does not interfere with stomatal closure.

Pre-treatment of leaves with diphenyliodinium chloride inhibits the increase in the ~37 kDa peroxidase and the decrease in transpiration associated with NaCl treatment.

Pre-treatment of leaves with DPI appeared to elicit a slight induction in total peroxidase activity. However, this induction was significantly less that treatment with 250 mM NaCl alone, suggesting that pre-treatment with DPI was causing a treatment effect. However, treatment with DPI appeared to inhibit the upregulation of total peroxidase in response to salt stress. Furthermore, pre-treatment with DPI blocked the decrease in transpiration associated with salt stress, suggesting that the activation of NADPH-oxidase is necessary to elicit stomatal closure in response to salt stress.

The activation of NADPH-oxidase and the enzymatic production of ROS appear to be an important step in a plant's adaptive response to environmental stress, including salt stress. For example, Miller et al. (2009) demonstrated that salt stress elicited the

activation of defense genes in *Arabidopsis thaliana*. However, this response could be blocked by pre-treating the plants with DPI. This could possibly explain why the activation of total peroxidase in response to salt stress was inhibited by DPI in this study. Zhang et al. (2001) demonstrated that reactive oxygen species were a necessary component to elicit stomatal closure. The authors were able to block stomatal closure by pre-treating guard cells with DPI. However, the authors did not demonstrate that this action would have physiological significance. In the current study, pre-treatment with DPI blocked the decrease in transpiration associated with NaCl stress. This study is the first report of its kind to demonstrate that blocking the enzymatic production of ROS affects a physiological process, such as transpiration.

Conclusions

Salinity stress caused an initial induction in the activity of a ~37 kDa peroxidase at 48 h followed by a decrease in the activity after 72 h. This enzyme was activated by clofibrate, suggesting that it is associated with a peroxisome. Furthermore, the induction of this enzyme in response to salinity stress was apparently blocked by BFA and DPI. This suggests that vesicle trafficking and the enzymatic production of ROS are needed to induce the activity of this enzyme in response to salt stress.

Salinity stress also caused a drop in transpiration compared to leaves maintained in D.I. water alone. Only pre-treatment with DPI blocked a drop in transpiration in response to salinity stress. This suggests that NaCl triggers the enzymatic production of ROS within guard cells to elicit stomatal closure and reduce transpiration.

More data is needed to verify the hypothesis that salinity stress induces the proliferation of peroxisomes in cypress leaves. First, the ~37 kDa peroxidase should be

sequenced by LC-MS/MS in order to identify the type of peroxidase and the predicted sub-cellular location of the enzyme. Second, peroxisomes within the leaves of salt stressed cypress should be visualized by microscopy.

Chapter 4: Conclusions and Future Direction

The objectives of this thesis were to investigate the role of reactive oxygen species in Bald Cypress in response to elevated salinity and to develop biomarkers to detect sub-lethal salinity stress for plants *in situ*.

From the greenhouse experiment, cypress exposed to elevated salinities for 10 weeks exhibited a predictable response to salt stress. Both Na^+ and H_2O_2 content increased within root tissue in response to salt stress. It was proposed that an upregulation of H_2O_2 contributed to root growth. Leaf tissue exhibited a response which included: an increase in foliar Na^+, H_2O_2, and free proline content. Salinity stress caused a drop in MQY but an increase in NPQ. In leaf tissue, an upregulation of H_2O_2 was proposed to activate defense genes, elicit stomatal closure, and cause a downregulation of carbon fixation. The aforementioned physiological responses would aid in the salinity tolerance of cypress by reducing the amount of Na^+ ions within tissue.

In the greenhouse experiment, the specific activity of total POX within leaf tissue decreased in response to salt stress. The decrease in total POX was attributed to a drop in a ~37 kDa POX isozyme. It was hypothesized that this isozyme was localized within peroxisomes and that these organelles would proliferate within cells in response to salt stress in order to produce H_2O_2.

Three populations of cypress were sampled along a tidal creek associated with the lower St. Johns River. A population within a basin swamp was identified to be experiencing salt stress because it had the most indicators of sub-lethal stress. Cypress at

the basin swamp exhibited the most signs of salinity stress because they had the lowest MQY and highest NPQ values compared to plants at other sites. Furthermore, leaf tissue of cypress at the basin swamp had the highest Na^+, lipid peroxidation, and proline content compared to plants at other sites. Cypress at the basin swamp were experiencing the combined stress of salinity and flooding. The specific activity of both SOD and POX was lowest at the site closest to a source of saltwater, but increased as cypress were sampled further upstream. This trend suggests that leaf senescence may have contributed to salt tolerance of the cypress population closest to a source of saltwater. It was proposed that chlorophyll fluorescence coupled with other biomarkers, such as leaf Na^+ content, lipid peroxidation, and free proline content could be used to detect sub-lethal stress for plants *in situ*.

The mechanism of salinity tolerance of Bald Cypress was further explored in detached leaf experiments. Detached leaves exposed to elevated salinity exhibited a drop in MQY. Strong negative correlations existed between leaf Na^+ content and both MQY and Fm. This suggests that the drop in MQY was attributed to a decrease in Fm. A drop in Fm was associated with less available PSII reaction centers. Total peroxidase of leaf tissue exposed to salt stress exhibited a significant increase after 48 h. This was attributed to the induction of a ~37 kDa peroxidase because the pixel intensity of this isozyme increased at least five fold compared to leaves maintained in D.I. water alone. It was hypothesized that the increase in this isozyme represented the *de novo* synthesis of peroxisomes. Leaf transpiration decreased in response to salt stress at all time points.

In other experiments, leaves were pre-treated with various pharmacological agents to tease apart the cellular mechanisms of salinity tolerance in cypress leaves. Leaves were

assayed for total peroxidase, the pixel intensity of the ~37 kDa peroxidase isozyme, and transpiration. Treatment with clofibrate, a drug known to induce the proliferation of peroxisomes, caused an increase in total peroxidase and the pixel intensity of the ~37 kDa peroxidase. However, no changes were observed in transpiration. Treatment with brefeldin A, a fungal toxin known to interfere with the endomembrane system of plants, appeared to block the induction of the ~37 kDa peroxidase in response to salt stress. However, no changes in transpiration in response to brefeldin A treatment were observed. Treatment with diphenylene iodonium, a suicide inhibitor of the ROS-producing enzyme NADPH-oxidase, appeared to block the induction of the ~37 kDa peroxidase in response to salt stress. Furthermore, this inhibitor blocked the decrease in transpiration associated with salt stress. This suggests that ROS are produced enzymatically within guard cells to elicit stomatal closure.

Perhaps induced the proliferation of peroxisomes within leaf tissue and the ~37 kDa peroxidase isozyme was localized within peroxisomes. However, more work is needed to verify this hypothesis. First, the ~37 kDa peroxidase should be sequenced by LC-MS/MS. This will reveal the identity of the protein and its sub-cellular location. Second, the proliferation of peroxisomes should be verified by microscopy. Conventionally, peroxisomes can be stained with 3,3'-Diaminobenzidine. However, preliminary work by the author has found that similar results can be obtained if tissue is stained with o-dianisidine (data not shown). Thus, peroxisomes will be visualized with the latter method. Finally, a clear link between endocytosis and the proliferation of peroxisomes within plant cells should be established. However, it may be advisable to perform this work on a model organism such as *Arabidopsis thaliana*.

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VITA

EDUCATION

University of North Florida

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Graduated April 2010
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M.S in Biology
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PUBLICATIONS

Lauer N., Yeager M., Kahn A., Dobberfhul D. & Ross C. The Effects of Short Term Salinity Exposure on the Sublethal Stress Response of *Vallisneria americana* Michx. (Hydrocharitaceae). 2011. Aquatic Botany. 95(3): 207-213.

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Trevathan-Tackett, S., Lauer, N**.,** Loucks, K., Rossi, A. & Ross, C. Correlating seagrass health and habitat with wasting disease prevalence: a snap shot in the Florida Keys. *In review for 'Journal of experimental marine biology'*

Williams, A., Lauer, N., & Hackney, C. The influence of saltwater and sulfate reduction on tidal wetlands: St. John's River, Florida. *In press for 'Wetlands'*

RESEARCH ASSANTSHIPS

5/2008 – 9/2008: Assistant under Anthony Rossi PhD.
-An Experimental Test of the 'Talking Trees Hypothesis'.

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-Salinity-Induced Stress Response of the Aquatic Macrophyte Vallisneria Americana.

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-Potential Nutrient Flux from Intertidal Wetland Sediments during Sea Level Rise.

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-Salinity-Induced Stress Response of the Aquatic Macrophyte Vallisneria Americana II.

TEACHING EXPERIENCE

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