

What Sets the Latitudinal Limit of the Mangrove Habit?

A Thesis

Presented to the Department of Biology

Harvard University

By Stephanie Stuart

In Partial Fulfillment of the Requirements

For the Degree of

Bachelor of Arts

March 2003

Thesis Director: Professor N. Michele Holbrook

“I would rather go without a shirt... through the whole of the Florida swamps in mosquito time than labor as I have... with the pen.”

—John James Audubon, **Letter to J. Bachman**, 1834

“...why, having reached this point of absurdity, you need have gone and painted the lily and adorned the rose, by being such a colossal ass as to come fooling about in mangrove swamps.”

—Mary Kingsley, **Travels in West Africa**, 1897

Table of Contents

Abstract.....	1-1
Chapter 1: Introduction.....	1-1
Why Are There No Temperate Mangroves?.....	1-1
Ecosystem Boundaries.....	1-3
Cold and Freezing.....	1-4
Boundaries to the Mangal Ecosystem.....	1-6
Mangroves as Stress Tolerators.....	1-7
Stress and Disturbance in Mangroves.....	1-9
Tension, Wood, and Freeze-Induced Cavitation.....	1-10
Hypothesis and Focus of the Study.....	1-12
Chapter 2: Materials and Method.....	2-1
Design of the Study.....	2-1
Manipulating Xylem Tension.....	2-1
Quantifying the Effects of Freezing.....	2-2
Hydraulic Conductivity.....	2-2
Experimental Approaches.....	2-4
Plant Species and Research Sites.....	2-7
Australia.....	2-7
North America/United States.....	2-9
Ion Content of Xylem Vessels and Living Cells.....	2-10
Xylem Structure and Anatomy.....	2-13
Hydraulic Conductivity.....	2-14
Sample Preparation and Measurement.....	2-16
Australia.....	2-16
United States.....	2-17
Freezing Protocol.....	2-18
Freezing Apparatus.....	2-19
Australia.....	2-19
United States.....	2-20
Freezing in situ	2-20
Leaf Freeze Tolerance Assay.....	2-21
Chapter 3: Results.....	3-1
Ion Content of Xylem Vessels and Living Cells.....	3-1
Vessel Anatomy.....	3-2
Response of Hydraulic Conductivity to Freezing.....	3-3
Effects of Freezing In-Situ.....	3-6
Freeze Assay.....	3-7
Chapter 4: Discussion.....	4-1
Freeze-Induced Cavitation Critically Impairs Xylem Function in Mangroves.....	4-1
Leaves or Stems?: Frost Damage to Living Tissue.....	4-3
Limits to mangrove physiology.....	4-4
Implications.....	4-5
Acknowledgements.....	i
References.....	ii

Abstract

Mangroves dominate tropical coastlines but are replaced by herbaceous salt marshes in temperate areas. This transition is examined here as a possible example of a disturbance paradigm of grassland/forest boundaries. I propose that freezing provides the disturbance that keeps mangroves from expanding into temperate latitudes. Mangroves, as woody halophytes, endure high xylem tensions that may increase the likelihood of bubble nucleation and embolism formation during a freeze-thaw cycle. I tested the hypothesis that physiological properties make this habit uniquely vulnerable to freezing, and show that interactions of xylem tension and freezing can cause xylem failure under frost conditions. Implications of physical limits restricting mangroves to tropical areas are discussed, and contrasted with possibilities for biological adaptation.

Chapter 1: Introduction

Why Are There No Temperate Mangroves?

Mangroves occur widely in the tropics but are never found in temperate regions (Duke *et al.* 1998, Spalding *et al.* 1997, Tomlinson 1994). An estimated 181,077 km² of coast between 32°N and 38°S can be classified as mangal (Spalding *et al.* 1997). Much attention has been given to the ‘mangrove biodiversity anomaly,’ the higher species richness of mangroves on east coasts and in the old-world tropics (Ellison *et al.* 1999, Duke *et al.* 1998, Tomlinson

1994), but effects of latitude are rarely addressed beyond a discussion of air and water temperatures. Since mangroves are often considered tropical by definition, (Tomlinson 1994) few have questioned why they do not spread to temperate areas.

Salt habitats alone are not inconsistent with cold temperatures; well-developed salt marsh communities replace mangroves and dominate tidal plains and estuaries above 25°N and below 25°S, and extend well into the arctic (Mitsch and Gosselink 1986). The feature that distinguishes the salt marsh from the mangal is the lack of woody, arborescent plants.

Salt marshes are characterized by herbaceous or succulent plants and grasses. While winter dieback is common and well-documented at high latitudes, grasses which resprout from rhizomes tend to dominate (Chapman 1940). The process of fall storage for spring growth has been particularly well studied in ***Spartina alterniflora***, (Ellison **et al.** 1996, Wijte and Gallagher 1991, Gallagher 1983) which dominates marshes from the Bay of Fundy (Mitsch and Gosselink 1986) to Northern Florida (Montague and Wiegert 1990). Patterns of latitudinal growth suggest that salt marsh plants do not have special seasonal adaptations but simply the ability to dieback in winter and resprout in spring. ***Spartina alterniflora*** may wait two years before senescing in Georgia, but follows a seasonal pattern of winter dieback and spring regrowth in Rhode Island (Gallager 1983).

Mangroves are an ecological assemblage rather than a natural group in the phylogenetic sense (Tomlinson 1994; Duke **et al.** 1998; Hanagata **et al.** 1999). This assemblage consists of widely disparate families and represents at least 5 major and many more minor independent evolutions of salt-tolerant, anoxia-tolerant, woody plants. Vivipary, breathing

roots, and salt glands have evolved multiple times within this ecological classification (Duke *et al.* 1998) and suggest strong, convergent selective pressures. With so many invasions of the swamp, it is surprising that in evolutionary time, no solution has been found to the problem of wood + cold + salt.

Ecosystem Boundaries

Forest/non-forest boundaries occur throughout the world (coniferous forest/tundra, broadleaf forest/steppe, tropical forest/savannah) and attract attention because their dominant species are markedly different (Longman and Jeník 1992). These boundaries are a striking example of competition between two very different strategies: the long-term investment of slow growing trees and the short-term investment, high-reproductive strategy of grasses. Successional theory suggests that in long-term competition for light and resources, trees become the dominant life form (Bazzaz 1996). However, succession must start over after every disturbance, so where disturbance occurs regularly, taxa that can regenerate rapidly, such as grasses, are the dominant life form (Bazzaz 1996). Some commonly-discussed disturbances include fire, grazing and freezing (Longman and Jeník 1992). Rainfall, nutrient availability, soil composition, and temperature can also be important (Longman and Jeník 1992).

The mangrove/salt marsh boundary has rarely been considered within the group of forest/non-forest boundaries, yet these two ecosystems clearly replace one another with changes in latitude. Frosts may act as the disturbance that prevents mangroves from succeeding salt marsh grasses at high latitudes.

Cold and Freezing

Chilling and freezing frequently limit the extent of plant species. Frost has been shown to set the tree line in northern ecosystems, and the transition from broadleaf to conifer forest (Krebs 2001). Cold damages plants in two ways. The plasma membranes in living tissue can be damaged either by phase transitions at low temperatures or by dehydration as freezing in extracellular spaces draws water out of the protoplast through the membrane (Wolfe and Bryant 1999). The water transport tissue, which must supply the leaves with a constant source of water, is also vulnerable to freezing. Air forced out of solution during ice formation may block this transport pathway upon thawing.

Membranes become acclimated to cold when the proportion of unsaturated lipids in the phospholipid bilayer is increased. Acclimation occurs in a seasonal cycle, increasing slowly in the fall as temperatures drop and disappearing rapidly in the spring, so that late frosts may cause disproportionate damage (Taiz and Zeiger 1998).

The water transport system, which consists primarily of dead cells forming conduits from the roots to the leaves, is also vulnerable to freezing. Water in the xylem is pulled from the roots to the leaves by evapotranspiration. When water evaporates from the leaf surface, tension or negative pressure is generated in the water column between the roots and the canopy. Like supercooled or superheated water, it is in a metastable state: pressures are low enough that only the high tensile strength of water, a result of hydrogen bonding, prevents a phase transition to water vapor. Because gasses have a lower solubility in ice than in water, freezing forces dissolved gas out of the sap, causing bubbles to form (cavitation). As the sap

thaws, tension in the water column can cause these bubbles expand, fill conduits and block water supply to the leaves (embolism).

Vulnerability to freeze-induced cavitation depends on conduit diameter and xylem tension (Davis **et al.** 1999). A bubble in the xylem will always expand when

$$P_x \leq P_{ww} - (2T/R)$$

where P_x represents the pressure (or tension) in the xylem, P_{ww} represents the vapor pressure of water, T represents the surface tension of the bubble and R represents the radius of the bubble. The size of the bubble is limited by the diameter of xylem vessels, which provides a direct link between xylem architecture and likelihood of cavitation. As a result, vulnerability to freeze-induced cavitation is often treated as a function of vessel diameter (Davis **et al.** 1999). Plants adapt to freeze-thaw pressures primarily by reducing the diameter of xylem conduits, which is correlated with the likelihood of freeze-induced embolism (Davis **et al.** 1999) or through a deciduous life cycle, abandoning blocked conduits in the fall and building new vessels in the spring (Davis **et al.** 1999). In at least one case, freeze-thaw cavitation has been linked to secondary loss of vessels (Feild **et al.** 2002) and interactions of vessel diameter and freezing limit the range of many species (Sperry **et al.** 1994, Pockman and Sperry 1997, Langan **et al.** 1997, Cavender-Bares and Holbrook 2001).

In the field, climate interacts with the physics and biology of freezing. If temperatures drop quickly, there is little time for membrane acclimation and even a relatively mild frost may cause significant damage (Larcher 1995). A mild climate subject to epochal killing frosts, as in Florida, USA (Harry *et al.*) may be more difficult to adapt to than a temperate climate with a gradual autumn (Larcher 1995). Multiple freeze-thaw cycles cause greater impairment than sustained low temperatures (Sperry *et al.* 1994). Discussion of the limiting effects of freeze damage should consider the range of conditions and interactions particular to species and site.

Boundaries to the Mangal Ecosystem

Climate is strongly correlated with limits to the mangroves' range. Limits to the mangrove ecosystem correlate well with coldest-month minimum average air temperatures of 20°C, especially where the seasonal range does not exceed 10°C (Duke, Ball and Ellison 1998) and with winter sea isotherms below 20° (Duke, Ball & Ellison 1998). The extreme outliers occur in Victoria, Australia (38° 45' S); Japan (31° N); and Florida, United States (29° 57' N). These boundaries are variable, since at the edge of their range mangroves establish briefly only to die in periodic frosts (Sherrod *et al.* 1986, Sherrod and McMillan 1985, Lugo and Zucca 1977). In general, subtropical mangals exist only where a warm current from a mangrove-rich region provides a renewable source of propagules (Tomlinson 1994).

Edaphic conditions and geomorphology are also important. Within the tropics, mangroves do not grow where conditions are incompatible with their habit, such as rocky or

arid coastlines. On local scales, these may simply act as barriers to dispersal between two areas of appropriate habitat (Duke *et al.* 1998, Spalding *et al.* 1997).

Mangroves as Stress Tolerators

Mangroves' unique habit makes it possible to suggest their range is limited by physiological factors. Mangroves display many of the characteristics of species classified as 'stress-tolerators' (Grime 1974), including slow growth, long lifespans, and strong herbivore defenses. Mangroves experience root anoxia, salt, and a low availability of water, all of which are considered extremely stressful for most plants (Larcher 1995). The adaptations that allow them to cope with these stresses appear to be energetically costly. Mangroves are not obligate halophytes but are limited to salty swamps because less encumbered species outcompete them in more hospitable environments (Tomlinson 1994).

High levels of salt are toxic for mangroves and concentrations above 0.125M limit growth (Burchett *et al.* 1984, Ball 1988, Takemura *et al.* 2000). Even mangroves that excrete salt through leaf glands exclude at least 90% of salt at the root (Tomlinson 1994). The mechanism of salt exclusion remains poorly understood, especially at the cellular level (Tomlinson 1994). Most salt is excluded at the root (Tomlinson 1994, Ball 1988, see also results presented in Chapter 3). Pioneering experiments by Scholander (1968) suggested that the root acts as an ultrafilter, with negative pressures in the xylem pulling pure water out of the soil against the osmotic gradient. Cold and metabolic poisons did not limit the ability to desalinate water, so this was presumed to be a passive process. However, a high level of root respiration, compatible with involvement of active transport in desalination, is essential to

the health of the tree. Mangroves invest a large proportion of carbon in root tissue (Ball 1988) and in tissue providing air to the roots—pneumatophores, root knees and stilt roots with lenticels above the mean high tide mark consist primarily of aerenchyma (Tomlinson 1994). Oxygen diffuses through this air-filled tissue down a partial pressure gradient (Tomlinson 1994) and covering them with an occlusive substance (Vaseline or wax) kills the roots (Tomlinson 1994).

The high osmotic potential of mangal soils causes increased tension on the xylem. Sea water has an osmotic potential of 2.5MPa; negative tensions in the mangrove xylem must balance this pressure before any water can be extracted from the soil. This subjects mangroves to tensions as low as -2.5MPa at night and as low as -6MPa while transpiring (Scholander 1968, Tyree and Sperry 1988, Tomlinson 1994, Melcher 2001). Water potentials of less than -2.5MPa are considered to indicate water stress in many plants (Taiz and Zeiger 1998); tensions in mangrove xylem are typical of plants growing in dry areas.

Low water availability limits transpiration, which sets the limits for photosynthesis. Although there has been some controversy over the rate of transpiration in mangroves, most modern estimates indicate low transpiration (Ball 1988, Tomlinson 1994, Hanagata **et al.** 1999). Even though they operate at very high water use efficiencies (Ball **et al.** 1988) the difficulty of extracting fresh water ultimately limits the ability of the plant to assimilate carbon (Ball 1988).

Mangroves grow slowly (Hogarth 1999) and are evergreen. They appear to be particularly vulnerable to defoliation. Species in the Rhizophoraceae, a principal mangrove

family, lack reserve meristems and cannot resprout after damage. Hurricane damage may limit the structural complexity of mangrove forests (Adam 1992). Herbivory may also be an important limit to growth (Ellison and Farnsworth 2001, Farnsworth and Ellison 1991) and, typical of stress tolerators, selective pressure may have favored herbivore defenses if the cost of defoliation was death.

Stress and Disturbance in Mangroves

Grime (1979) theorized that plant competition was governed by interaction between stress, competition, and disturbance. Plants can tolerate either high levels of stress or high levels of disturbance, but not both. Mangroves are so well-adapted to stress that disturbance often proves catastrophic. It is possible to suggest several ways that cold stress might affect mangroves physiologically. For physical reasons, mangroves have particular difficulty recovering from leaf mortality, root damage, and freeze-thaw cavitation.

Leaves are typically the most exposed plant organs; with a high surface area to volume ratio, they cool and freeze quickly; photoinhibition may also damage leaves when high light conditions coincide with temperatures too cold for the photochemistry to function (Hällgren and Öquist 1990). Thus leaves are easily lost to frost damage. Mangroves may be especially sensitive to loss of their leaves, since many lack reserve meristems, and almost all lack large energy reserves (Hogarth 1999). It is possible that if freezing kills mangrove, the plant simply does not have enough stored energy to regenerate leaves.

Although roots, underground and submerged in salt water, are not expected to freeze under conditions mangroves normally encounter, chilling has been shown to damage roots (Hällgren and Öquist 1990) and salt exclusion might be impaired if phospholipid membranes in the roots undergo phase changes at low temperatures and lesions, causing ion leaks, occur. In addition, membrane proteins associated with ion uptake may undergo conformational change at low temperatures (Hällgren and Öquist 1990). High levels of sodium leaking into the xylem would almost certainly overwhelm the plant; high levels of sodium cause stress at a cellular level by upsetting ion ratios (Blumwald 2000). For example, sodium ions may compete with other cations to impair protein synthesis (Blumwald 2000).

Xylem vulnerability to freeze-thaw cavitation may be significantly greater in mangroves because of the tensions they experience. Extra tension in the xylem leads to greater vulnerability to freeze-induced cavitation, which can lead to catastrophic xylem failure. In mangroves, tension is never relaxed; even at night it must approach -2.5MPa , the balance pressure for the osmotic potential of seawater.

Tension, Wood, and Freeze-Induced Cavitation

The combination of wood and desalination is a character set unique to mangroves. Among the hypotheses discussed above, the suggestion that high tension makes the xylem vulnerable to freeze-induced cavitation directly addresses this combination. Mangroves are an excellent system for exploring the interactions of tension and freezing, since these experiments are ecologically appropriate as well as theoretically interesting. The vulnerability of the xylem to freeze-induced cavitation brings together a great deal of information about

the plant. It can be used to relate xylem structure to vegetative phenology, gas exchange capability, distributional limits imposed by frost, and the range of environmental conditions to which a plant can adjust (Davis, Sperry and Hacke 1999).

Freeze-thaw embolism as a limit to climatic range is usually discussed in the context of the relationship between conduit diameter and the likelihood of bubble expansion on thawing (Sperry *et al.* 1994, Pockman and Sperry 1997, Langan *et al.* 1997, Cavender-Bares and Holbrook 2001). Conduit diameters below 30 μ m generally suffer very little freeze-induced cavitation (Davis *et al.* 1999). Most mangrove species have small vessel diameters consistent with their low water potentials (Tomlinson 1994); it may be for this reason that freeze-induced embolism in mangroves has been overlooked. A return to the bubble equation shows that expansion occurs when $P_x \leq P_{wv} - (2T/R)$; if pressure in the xylem (P_x) is very negative, then outweighs diameter and even small bubbles will expand.

As described, very negative xylem pressures are inescapable in mangroves. Other plants with narrow vessels may avoid bubble expansion because freezing typically occurs at nighttime; since transpiration typically occurs only in the day, at night tension relaxes as the xylem comes into equilibrium with the soil. In well-watered soils, the water potential is essentially zero. When mangroves come into equilibrium with the soil, tension in the xylem still must be at least -2.5MPa to balance the osmotic pressure of the saline substrate. Similar conditions are found in cold deserts (Pockman and Sperry 1997).

Greater degrees of blockage lead to runaway embolism and catastrophic xylem failure (Tyree and Sperry 1988). If embolism cannot be repaired, dieback results as distal

leaves and branches are sacrificed (Zimmerman 1983). Impairment to flow can be measured as the stem's resistance to the flow of water, or hydraulic conductivity.

Hypothesis and Focus of the Study

The combination of high xylem tension and expensive investment in wood that cannot easily be repaired or replaced makes mangroves uniquely vulnerable to frost. In this thesis I test the hypothesis that freeze-induced cavitation severely reduces xylem function in these plants by measuring the relative reduction in hydraulic conductivity in excised branches after freezing in two species of mangrove in southern New South Wales, Australia and two species in northern Florida, United States. Destructive experiments were complemented with **in situ** observation of branches frozen while attached to the tree as well as with assays of freeze resistance in living leaf tissue.

Many factors contribute to the death of mangroves after frost at the edge of their range. In addition to discovering what kills mangroves, we are interested in understanding what prevents them from adapting to and avoiding these causes of death. In this context, freeze-induced cavitation may set an absolute limit on growth at latitudes where freezing is common

Chapter 2: Materials and Method

Design of the Study

Manipulating Xylem Tension

To test the hypothesis that interactions of tension with freezing fatally impair water transport in mangroves, it is necessary to control the tension in the xylem of freezing branches. The fact that xylem vessels are of finite length, separated by end walls and bordered pits, makes it possible to compare the effects of freezing in branches with and without tension. Native tension, the tension present in a branch *in vivo*, is preserved in any uncut vessels when branches are cut in air. Xylem sap retracts to the endwall of the severed vessels, but tension in the xylem sap of the intermediate vessels is maintained as water is drawn into the living cells. Because volume drawn into living cells is a negligible fraction of the water in the system, vessels beyond the endwall retain a tension indistinguishable from that experienced in the intact tree. Freezing branches in this state mimics the effect of freezing at natural tension. This is true as long as precautions are taken to prevent further drying, which can increase tension (Cochard *et al.* 1992). Control (unfrozen) branches are cut in air and stored in sealed, humid bags in a cold room, and retain the native tension as long as no drying or adsorption of water takes place.

The role of tension during freezing can be assayed by reducing tension before freezing. Tension can be relieved in fully hydrated branches. The water column is relaxed by cutting branches under water or an artificial sap solution, and water is drawn into vessel

lumens when the water column is broken and retracts. By incubating branches with cut ends in solution until the living cells reach full turgor and xylem pressure equilibrates with the hydrating solution and atmospheric pressure, tension can be fully relieved. Control branches are cut under solution and stored with trimmed ends in solution in a cold room.

Cold room storage prevents deacclimation of living cells, and is unlikely to cause adverse chilling effects as temperatures of 4°C are common at night at both sites studied (McMillian 1975).

Quantifying the Effects of Freezing

These two treatments (native xylem tensions and relieved tensions) were used to compare the effect of freezing against unfrozen ‘control’ branches and the interaction of freezing and tension in blocking the xylem. Direct comparison of hydraulic conductivity in matched segments was used in the first part of the study and percent loss of conductivity (PLC) was used in the second part of the study.

Hydraulic Conductivity

The ability to supply water to the leaves (Brodribb and Feild 2000, Nardini and Salleo 2000) and to continue such supply under stress (Tyree and Sperry 1989, Sperry *et al.* 1993, Cavender-Bares and Holbrook 2001, Cordero and Nilsen 2002) are both functions of

hydraulic conductivity. Hydraulic conductivity can be measured either directly or as a proportion of the maximum possible conductivity.

Hydraulic conductivity (K_h) can be calculated from:

$$K_h = (J_v L / \Delta P)$$

Where J_v equals mass flow rate (kg s^{-1}) along a length of stem L (m) for a given pressure difference (ΔP) in MPa (Tyree and Zimmerman, 2002). Conductivity can also be related to cross-sectional conductive area of the stem (A_s) in m^2 as stem-specific conductivity:

$$K_s = K_h / A_s$$

or the leaf area (A_L) in m^2 supplied by the segment under measurement as leaf specific conductivity:

$$K_L = K_h / A_L$$

Measurements can also be made of the degree of conductivity lost in response to a particular stress. If a stem is measured, flushed to remove emboli, and measured again the initial conductivity (K_i) can be expressed as a percentage of the maximum conductivity (K_{max}): $K_i / K_{\text{max}} \times 100$. Subtracting this from 100 percent gives the percentage of conductivity that was blocked before flushing, or the Percent Loss of Conductivity (PLC) (Sperry **et al.** 1988).

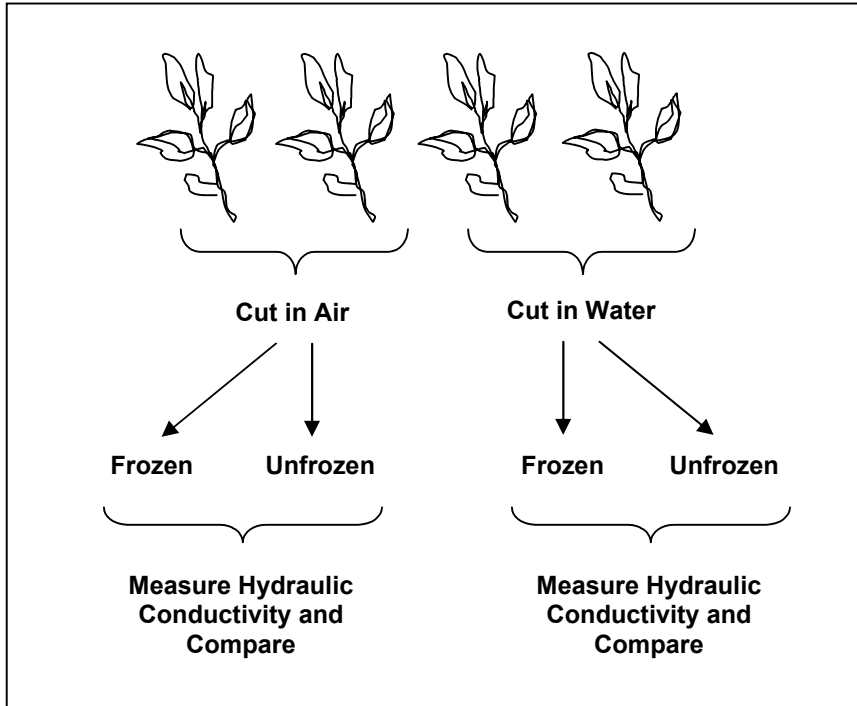


Figure 2.1 Matched Branch Design Four matched branches similar in age, length, and position on the tree were harvested from a single individual for each replicate. Two of these branches were cut in air, to preserve native tension as described above. The other two were cut under artificial sap solution and allowed to hydrate in order to relieve tension. This design allows comparison of the reactions of a single individual to all four treatments.

Experimental Approaches

The matched segment approach compares two branches of similar age, diameter and location that are assumed to have similar hydraulic conductivity (stem specific conductivity, K_s , $\text{kg s}^{-1} \text{MPa}^{-1} \text{m}^{-1}$). Four similar branches were selected, giving one control and one treatment branch for each of the tension protocols (Figure 2.1). Sampling at sites with differing salinity profiles allowed comparison of the effects of growing under differing tension conditions.

Matched segment comparison allows a single measurement on each segment, minimizing the chance for artifact. Paring eliminates the need to flush stems to estimate their original conductivity. Flushing times needed to reach a stable maximum conductivity are often 30 minutes or longer, (Sperry *et al.* 1988) and clogging may occur during this period. Long segments can be used, an advantage since they include pit membranes, the primary

contributors to xylem resistance in angiosperms (Zimmerman 1981; 1983) and give a more accurate measurement of the resistance of the stem.

Paring depends on the intrinsic similarity of match branches as a control. If this condition is not met, an alternative is to use the stem's maximum conductivity as an internal control by measuring PLC. A PLC design (Figure 2.2) was used to compare xylem impairment in branches frozen at native tension and branches fully hydrated before freezing with controls in the second part of the study.

Since PLC is internally normalized, segments shorter than vessels can be used. Segments must be handled carefully to avoid introducing air during preparation, and measured at low pressures in order to avoid flushing emboli from open vessels. Given this, however, the amount of emboli removed from a short segment is representative of the degree of blockage in the rest of the stem (Cochard 2002). For short segments, flushing times can be as short as 5 minutes or less (Brodribb *et al.* 2003) and associated errors are significantly reduced.

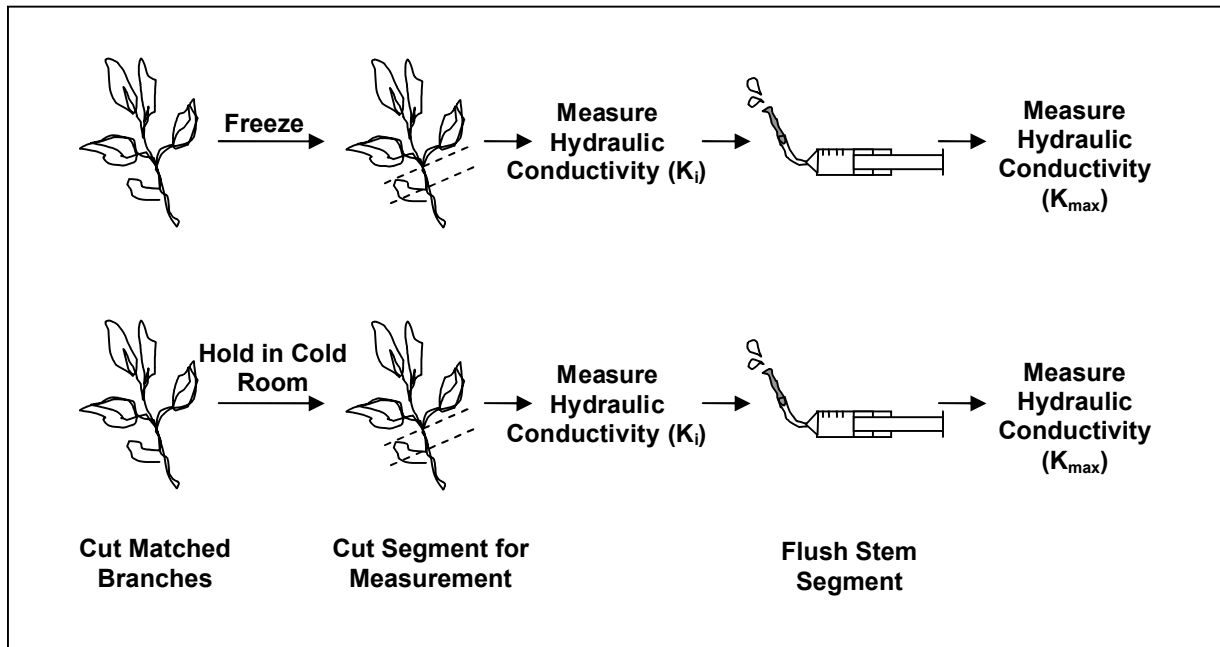


Figure 2.2 PLC Protocol Branches are flushed after an initial measurement and re-measured. The difference between maximum conductivity and initial conductivity represents the degree of xylem blockage and is expressed as the percent lost, in this case to freezing.

Destructive methods such as those described above should be complemented with **in situ** experiments when working with a system of inherent instability like the xylem. To confirm the results of the hydraulic conductivity experiments, branches were frozen on the tree with dry ice, and the effects of freezing were observed over time. This isolates effects of freezing on the xylem from effects on the leaves. It also allows observation of the effects of osmotic pressure exerted by the substrate during thawing which cannot be seen when the branch is detached from the roots.

Plant Species and Research Sites

Two sites were studied, one representing the northern and one representing southern latitudinal extremes of mangrove habitat. Mangroves extend to latitudes higher than either site, as mentioned in introduction: to Victoria, Australia (38°45'S) and Japan (31°N). Nonetheless, the two sites chosen, in southern New South Wales (NSW), Australia, and northern Florida, USA, represent outlier subtropical mangrove populations.

Australia

Mangroves are found on both the west and east coast of Australia, with over 38 species occurring in these regions (Spalding **et al.** 1997). More species are found on the east coast than on the west due to cold currents and dry coastlines (Tomlinson 1994). Species richness decreases from south to north; although 38 species are found in the northeast, only seven are reported in the southeast. Species of **Rhizophora** disappear around Brisbane, 27°30'S (Spalding **et al.** 1997), but **Aegiceras corniculatum** is found in New South Wales and **Avicennia marina** continues to Corner Inlet, Victoria, reaching the highest latitude of any mangrove at 38°45' (Tomlinson 1994).

Avicennia marina (Forsk.) Vierh, var. **australasica** (Walp.) Moldenke and **Aegiceras corniculatum** (L.) Blanco 1837 were studied in New South Wales, Australia along the Clyde River at Bateman's Bay (35°42'30"S, 150°12'00"E) and Nelligen (35°39'00"S, 150°8'30"E).

The two sites were selected for their positions along the river associated with decreasing substrate salinity.

Substrate salinity was measured directly by sampling water at a depth of approximately 35 cm. Samples collected were allowed to settle for a minimum of 8 hours before salinity in parts per thousand was measured with a portable refractometer (A366ATC, Vista, China). The Bateman's Bay site is located at the outlet to the sea, with an average salinity of 37 ppt (equivalent to 0.63M NaCl, if all solutes are assumed to be NaCl, approximately -3.1MPa osmotic pressure). The Nelligen site was approximately 13 km upstream with an average porewater salinity of 27 ppt (equivalent to 0.47M NaCl, or approximately -2.3MPa osmotic pressure).

Climate is generally mild. At Morya Heads, the closest weather station at 22km south of Bateman's Bay, weather records from 1875 to 2001 show a mean minimum temperature for the three coldest months (June, July and August) of 6.5°C and a lowest recorded minimum of 0°C (Commonwealth Bureau of Meteorology 2001). Temperatures may be cooler inland; one frost reported to have been -6°C occurred during the study at Nelligen. Minimums of 0°C and -3°C were recorded at the Bateman's Bay and Nelligen field sites, respectively, by the experimenters during the study.

North America/United States

The north-most extent of principal mangrove genera, **Rhizophora** and **Avicennia**, occurs in northern Florida, USA, near the city of St. Augustine (Odum and McIvor, 1990). Mangroves also occur in Texas and Louisiana (Sherrod and McMillan 1985; McMillan and Sherrod 1986). Florida mangroves may represent a continuum through the Caribbean, rather than along the continental coast (Sherrod and McMillan 1985) and mangrove forests in Florida reach their greatest structural complexity in the southwest tip of the state (Odum and McIvor, 1990).

Avicennia germinans (L.) Stearn 1958 was studied in Marineland, Florida, USA (29°40'00"N, 81°12'W) and **Rhizophora mangle** L. 1753 was collected from Ponce Inlet, FL, USA (29° 4' 35" N, -80° 55' 30" W). **Rhizophora mangle** occurs at Marineland; four seedlings were observed during exploration of the site. As abundances did not permit sampling, **Rhizophora mangle** was collected from Ponce Inlet, 84 km south, currently the northmost well-established mangal in Florida (Odum and McIvor, 1990).

Florida's climate is mild but shows a great deal of microclimatic variation (Henry **et al.** 1994). Although the average winter minimums are high, 8.2°C in Jacksonville, near the Marineland site, and 9.5°C at Daytona Beach, near the Ponce Inlet site, minimum recorded temperatures were -10°C and -9.4°C (21 and 30 year records, respectively, NOAA-CIRES 2002). Hard freezes occur at least once a decade and are well-documented because of their economic importance. Frost in Florida may be considered a disturbance, since it is a rare but

regular event. Since 1950, severe freezes have occurred 9 times: in 1957, 1958, 1962, 1970, 1971, 1977, 1983, 1985 and twice (February and December) in 1989 (Henry *et al.* 1994).

Ion Content of Xylem Vessels and Living Cells

Ion contents of xylem vessels were measured to determine an appropriate perfusing solution for use in hydraulic measurements. Physiologically relevant measurements of hydraulic conductivity are best made with a perfusing solution matched to the ion content of the xylem sap *in vivo* (Zwieniecki *et al.* 2001). The ion content of the perfusing solution can markedly affect measured conductivity, believed to be a result of swelling in pit membrane pectins (Zwieniecki *et al.* 2001). Hydrogen ions (protons) are drawn into the negatively charged pectins, causing them to swell and increasing resistance across the pit membrane. Adding ions shields the negative pectins and prevents disassociation of protons from water, causing pectins to shrink and decreasing resistance (Zwieniecki *et al.* 2001). Matching the ion content of the perfusing solution to that of the living plant approximates state and resistance of the pit membranes pectins *in situ*. Ion effects increase with the number of vessel endings in the segment being measured, since the solution will be forced through pit membranes. Hydrating plants to relieve tension also requires an appropriate solution. Branches of *Avicennia germinans* initially incubated with ends in deionized water did not reach leaf water potentials below 1MPa after over an hour's incubation; when the branches were switched to 30mM NaCl 5mM KCl, they reached water potentials of 0.15MPa in one hour.

Accurate measurements of xylem sap content are difficult to obtain because sap is under tension and retracts when cuts made for sampling break the cohesion of the water column (Schurr 1998). This is a particular problem in mangroves since xylem tension is never fully relaxed. In addition, contamination from ion-rich live cells may confound measurements, both during cutting and extraction of sap. Since all methods of extraction rely on either positive pressure or vacuum to overcome the retraction of sap, content from living cells is almost always pushed or pulled in to xylem conduits (Schurr 1998). Even a small amount of contamination from living cells can dramatically change apparent ion concentrations in the ion-poor xylem sap.

The ion content of mangrove sap has long been a subject of interest due to its halophytic habit and apparent exclusion of salt (Tomlinson 1994, Scholander 1968). Measurements from mangroves species with the ability to excrete salt on the leaves ('secretors') generally have higher concentrations of ions in the xylem sap than those without salt glands, which exclude all salt at the root ('excluders') (Tomlinson 1994). This terminology is misleading since all species exclude most salt. Secretor species are generally thought to exclude 90% of salt, and excluder species 99% (Tomlinson 1994). The two species found at the Clyde river site that supplied samples for analysis, **Avicennia marina** and **Aegiceras corniculatum**, are secretor species and can be expected to show higher ion concentrations than excluder genera such as **Rhizophora**.

Electron Diffusive X-ray, or EDX, can measure concentration of ions in ice in volumes as small as $1 \mu\text{m}^3$. When plant tissue is frozen **in situ** with liquid nitrogen, freezing proceeds rapidly, with little cell dehydration or associated membrane damage. Water is

frozen in its physiological location, preventing retraction of the water column into living cells and preserving ion content of different compartments (McCully 1998; 2000). Material is planed while frozen so that there is little chance of contamination from neighboring cells, and maintained at liquid nitrogen temperature during measurement so that evaporation does not change ion concentration. The effectiveness of EDX technique for quantitative analysis ion concentration of xylem sap has been established by McCully *et al.* (2000). An EDX with a cryostage was not available in the United States so measurements were made in Australia only.

Contents of anatomical compartments were examined using EDX in three individuals from Nelligen, NSW, Australia, and three individuals from Bateman's Bay, NSW, Australia. Replicates were rapidly frozen by submersion of intact, attached branches in liquid nitrogen (-196°C). After removal from the tree, five segments were cut from each branch and transported the lab in a cryo shipper at -170°C.

Samples were planed with a diamond knife at -80°C (McCully *et al.* 1998; 2000) and transferred to a cryo-stage in a scanning electron microscope (Oxford CT 1500, Oxford Instruments, Eynsham, Oxford, UK). Samples were coated with aluminum and viewed through a beryllium window. Ion concentration in 6 – 12 filled xylem conduits and 5 – 6 living cambium or pith cells was examined using EDX as described in McCully *et al.* (2000). Figure 2.3 shows a sample micrograph with anatomical compartments labeled.

This technique may result in a slight bias to overestimate the concentration of Na^+ , as the limit of resolution for Na^+ is approximately 30mM with the equipment used. However, the limits of resolution for K^+ and Cl^- are lower, 1 and 7mM respectively (Cheng Huang, personal communication). The combined concentration of Na^+ and K^+ measured generally approximates the concentration of Cl^- . Assuming an anion/cation balance, the actual Na^+ content will be close to that observed.

Xylem Structure and Anatomy

Knowledge of the physical structure of the xylem, particularly vessel length and diameter, is essential to avoid artifacts in measuring hydraulic conductivity. In order to choose an appropriate buffer length for branches cut in air, and avoid introduction of embolism into the segments being measured, it is necessary to measure the vessel length.

Maximum vessel length was estimated by perfusing stems with air from a syringe at 1 atm at the proximal end and trimming from the distal end, which is held underwater to visualize bubbles. Air will not pass through wet pit membranes, but passes easily through vessels, so the first appearance of bubbles from the distal end is assumed to represent the longest vessel (Zimmerman and Jeje 1981). Trimming from the proximal end ensures an appropriate buffer length for an experimental segment distal to a cut.

The diameter of vessels has been shown to be correlated with the likelihood of catastrophic freeze-induced embolism; vessels greater than 30nm in diameter a typically most vulnerable (Davis *et al.* 1999, Sperry *et al.* 1994). Vessel diameter also dictates the amount of

pressure needed to move a meniscus along a tube and as a result, the pressure drop it is possible to use during measurement without flushing emboli and confounding the effect of treatments that impair conductivity (Holbrook **et al.**, **submitted**). The pressure that will push a bubble out of a blocked vessel can be calculated using the capillarity equation

$$\Delta P = 2\tau \cos(\theta) / r$$

where ΔP is the pressure difference across the segment, τ is the surface tension of water, θ is the contact angle of water with the xylem wall, and r is the (assumed circular) radius of the vessel (Holbrook **et al.**, **submitted**).

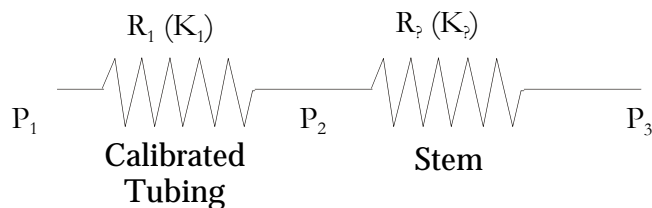
Average vessel diameter was estimated from images captured from the scanning electron microscope (SEM). In Australia, image capture was done on an Oxford CT 1500 (Oxford Instruments, Eynsham, Oxford, UK) and in the United States, on an FEI Quanta 200 Scanning Electron Microscope and Environmental Scanning Electron Microscope (FEI, Hillsboro, Oregon, USA). Image analysis was done using Scion Image (Scion Corporation, Frederick, Maryland, USA). Each vessel was measured as an ellipse with a major and minor axis; diameters were calculated as the diameter of a circle of equivalent area.

Hydraulic Conductivity

Hydraulic conductivity measurements were made using a Steady-State Flow Meter (SSFm) equipped with a pressure transducer amplification circuit as described in Holbrook **et al.** (**submitted**). The pressure transducer used was Omega PX26 (5 psi, Omega, Stamford,

Connecticut, USA) and a digital multimeter (Test Bench 390A, B+K Precision, Yorba Linda, CA, USA) gave a digital readout of pressure measurements in volts.

The SSFM consists of a gravity feed and a calibrated capillary tube of known resistance (the resistor) in series with a branch of unknown resistance (Figure 2.4). Pressure drop across these resistors, calculated as the difference between measured pressures at three points in the system, is analogous to voltage drop across resistors in a circuit. The known resistance of the capillary tube (R_1) can be used to calculate the unknown resistance of the branch (R_2).



Since resistance is the reciprocal of conductance, it is possible to calculate hydraulic conductivity directly. Conductance of the branch is calculated as flow rate through the stem ($J_{v\text{Stem}}$) divided by pressure drop across the stem (ΔP_{Stem}). In steady state, there is a single flow rate through the whole system (J_v), and flow rate through the stem is equal to flow rate through the capillary resistor. J_v is given by the pressure drop across the capillary resistor ($\Delta P_{\text{res}} = P_1 - P_2$) multiplied by the known conductance of the resistor, which is established through calibration. Hydraulic conductivity is conductance normalized by the length of the sample.

In the SSFM used in Australia, resistors were attached to a machined acrylic block; this design was prone to leaks and work by others in the Holbook lab substantially improved it before the second half of the study. The SSFM used in the United States consisted of resistors attached directly to the supply manifold with soft tubing.

Sample Preparation and Measurement

Australia

Experiments in Australia used the matched branch design. Four paired branches growing in full sun at an average height of 1.5 m were taken from five individuals between 23 July and 17 August of 2002 at each site ($n=10$). Collections were made at both sites during each day of sampling and branch collection times varied from 8:30 am to 1:45 pm. A buffer of 20 cm was left beyond the 20 cm section reserved for measurements. Two branches were cut in air and transported in sealed plastic bags with a moist paper towel, and two were cut under artificial sap solution (30mM NaCl 5mM KCl) solution and transported with ends in this solution. Solution matched measurements of xylem ion content in intact branches; see Ion Content of Xylem Vessels and Living Cells, above. All hydrated branches were incubated with ends in water for at least two hours before beginning the freezing treatment.

After treatment, frozen and control branches were trimmed under 30mM NaCl 5mM KCl solution; leaves were trimmed and cut leaf ends were sealed with superglue (Tarzan's Grip Super Glue, Padstow, Australia). Stems were perfused with 30mM NaCl 5mM KCl and 0.01M Oxalic Acid to prevent clogging due to wounding. Measurements were taken after 20

minutes equilibration. Conductive area was measured with digital calipers after 10 minutes perfusion with dye at approximately 0.1MPa. Dye was prepared by dissolving 0.5 grams safranin in 5mL Ethanol and 40mL deionized water and diluted to 10% with artificial sap solution. Leaf area was measured with a LI-COR leaf area meter (LI-3000).

United States

In the United States, the Percent Loss of Conductivity (PLC) protocol was used. Branch segments of 3.5 to 8.5 cm were excised in a solution matched to typical ion concentrations in mangrove sap (30mM NaCl 5mM KCl) and trimmed with a fresh razorblade. Flow rate was measured using a steady-state flowmeter and each segment was flushed for less than five minutes (Brodrribb *et al.* 2003) with filtered solution using a syringe and caulking gun to apply pressure until no more bubbles emerged from the cut end. Each segment was then re-measured. Percent lost conductivity is expressed as a percentage of total conductivity following flushing.

The effect of freezing both at native nighttime xylem tension and after hydration to reduce xylem tension was measured in *Avicennia germinans*. For the native tension experiments, ten paired branches were collected after 10 pm on 5 January 2003 and 6 January 2003 at Marineland, FL for a total of $n=20$ branches. Branches were cut in air and double-bagged with a moist paper towel and allowed to equilibrate for .5 to 1 hours. The water potential of 5 branches was measured before beginning freezing treatment. Control branches were placed in a 4°C cold room in sealed plastic bags with moist paper towel. Percent loss of conductivity was measured the following morning.

In the hydration experiments, ten paired branches were cut after 10 pm under 30mM NaCl 5mM KCl on 7 January 2003. Branches were then hydrated for one hour in 30mM NaCl 5mM KCl at 4°C to an average water potential of 0.15MPa. One branch from each pair was frozen with the end attached to a supply of 30mM NaCl 5mM KCl at slight positive pressure (5cm gravity head, 0.5kPa). The other member of the pair was kept at 4°C, a temperature comparable to the night air temperature of the site, in a humid environment, with the cut end of each branch submerged in 30mM NaCl 5mM KCl. Hydraulic conductivity was measured the following morning.

Rhizophora mangle was also sampled as a comparison to **Avicennia germinans**. Ten paired branches from **Rhizophora mangle** were collected after 8 pm on 8 January 2003 and 9 January 2003 from Ponce Inlet, FL for a total of $n=20$ branches. Branches were cut in air and double-bagged with moist paper towel and transported to the laboratory at Marineland, FL (1.5 hour travel time). Water potential was measured for one branch from each of five individuals before freezing overnight. Five control branches were stored in a 4°C cold room in sealed bags kept humid with moist paper towel. PLC in each treatment group was measured as described.

Freezing Protocol

Rate of freezing and thawing affect blockage of the xylem by embolism (Langan *et al.* 1997; Cordero and Nilsen 2002). Slow freezing at -2°C/hr and fast thawing at 1°C/minute, reflects the conditions commonly encountered in the study sites in nature, where freezing progresses at night until sunrise when thawing is rapid.

The minimum temperatures of -6°C in Australia and -10°C in the United States represent a balance between the need to ensure freezing and temperatures encountered at the sites during frosts. Both temperatures are within the range of historical frosts. The temperature of at least one branch in the freezing setup was tracked during each freeze with a thermocouple inserted into xylem tissue. Freezing exotherms were observed in every case and occurred between -4°C and -8°C (Figure 2.5). Tests of the effect of leaves on branch supercooling showed that branches frozen with leaves intact consistently froze one degree warmer than branches with leaves removed (Figure 2.6).

Freezing Apparatus

Australia

In Australia, freezing was controlled by a programmable water bath (Julabo Labortechnik, Seelbach, Germany) circulating through a larger insulated bath. The water bath was equipped with an external temperature control (platinum resistance thermometer) which was placed in the larger bath to control its temperature directly. Branches were frozen sealed in plastic bags submerged in the bath solution. Branches were brought from 4°C to 0°C in five minutes, and held at 0°C for 30 min before freezing at a rate of -2°C per hour to -6°C . Each trial included a sentinel branch with a thermocouple inserted into the xylem tissue, and a freezing exotherm was observed in each case (Figure 2.6 A). Branches were held at -6°C for 1 hour, then thawed at 1°C per minute to 10°C .

United States

In the United States, branches were frozen in an insulated box 1.5m x 0.33m x 0.5m. The freezing setup consisted of a flow-through chiller connected to an insulated temperature-controlled programmable bath filled with 70% propylene glycol (Prestone LowTox Antifreeze.) Propylene glycol solution was pumped from the temperature-controlled bath through a heat exchanger (auto oil transmission cooler) inside the large insulated box. Mixing was accomplished with two fans blowing across the heat exchanger for efficient exchange of heat. The freezing apparatus was placed outside and all freezing took place at night between 11 pm and 7 am. Samples were cooled at -2°C per hour and thawed at 1°C per minute to a minimum temperature of -10°C , where they were held for 1 hour.

Freezing *in situ*

Five paired branches were selected at the Marineland site. Experiments were carried out on the night of 10 January 2003. One branch in each pair was phloem girdled; the other was fitted with a Styrofoam cup approximately 4 cm in diameter. The cup was filled with dry ice (-78.5°C) and covered with aluminum foil. The branches were checked and photographed for discoloration after four and thirteen days on 14 January 2003 and 23 January 2003.

Leaf Freeze Tolerance Assay

The effect of freezing on membranes and photopigments in leaf tissue was investigated at the Australian site. The vulnerability of leaf tissue to freeze damage is drastically affected by acclimation. For this reason, experiments were performed during July, midwinter and typically the coldest month, in New South Wales. Freeze tolerance was assessed using maximal dark adapted chlorophyll fluorescence (F_v/F_m) measured with a Plant Efficiency Analyser (PEA, Hansatech, Kings Lynne, UK) and electrolyte leakage. Five mature leaves growing in full sun were collected from *Avicennia marina* and *Aegiceras corniculatum* at each site. They were transported to the lab in Canberra, ACT, Australia (approx. 2 hours transport time) in an insulated container with moist paper towel. Experiments were performed the day after collection and leaves were stored overnight at 4°C to prevent deacclimation.

Leaves were dark adapted for 30 mins at 4°C and chlorophyll fluorescence was measured. Ten 8 mm disks were cut from each leaf, and one disk from each leaf was assigned to each temperature treatment. Each disk was placed in a 10mL test tube in a programmable water bath (Julabo Labortechnik, Seelbach, Germany) at 5°C for 30 minutes. The bath was then cooled to -1°C over 30 minutes. After the samples had been held at -1°C for 30 minutes a small amount of ice was placed in contact with the cut edge of the leaf disk to cause ice nucleation and avoid supercooling. The leaves were then chilled at -2°C/hr to nadir temperatures from -2° to -10°C. One disk from each leaf was also kept in the cold room at 4°C.

Leaf disks were equilibrated for 30 minutes at each nadir temperature. At the end of that time, one disk from each leaf was removed and allowed to thaw 10 minutes on the benchtop. Loose electrolytes were captured by adding 4mL distilled water the test tube containing each leaf disk. Disks were removed and dark adapted in leaf clips for 30 minutes at 4°C before measuring chlorophyll florescence. Leaf disks were returned to test tubes and stored overnight at 4°C to allow electrolytes to diffuse out of ruptured cells.

Disks were frozen in liquid nitrogen to completely destabilize membranes and cause total electrolyte leakage. A further 3mL deionized water was added to the test tube to provide enough volume for measurement with a conductivity electrode (TPS LC81, TPS, Springwood, Australia).

Leaf disks were again incubated overnight at 4°C to obtain baseline total electrolyte leakage. Conductivity of the solution was measured the next day by the previously described method. Initial electrolyte leakage at each temperature is reported as a percent of total leakage from each disk.

Chapter 3: Results

Ion Content of Xylem Vessels and Living Cells

EDX analysis of *Avicennia marina* averaged 32.5mM Na⁺, 5.8mM K⁺ and 24.8mM Cl⁻ in xylem conduits. *Aegiceras corniculatum* xylem averaged 36.56mM Na⁺, 9.03mM K⁺ and 44.01mM Cl⁻. Mg, P, S, and Ca were only rarely above the limit of detection in xylem conduits. Xylem conduits of plants growing at the higher salinity coastal site were 2-6mM lower in Na⁺, K⁺, and Cl⁻ in *Avicennia marina* and 3-25mM lower in *Aegiceras corniculatum*, but the difference between the two sites was not significant in most cases. *Avicennia marina* on average took up more ions than *Aegiceras corniculatum*, but there was not a significant difference between species (Figure 3.1).

Small amounts of contamination from living cells high in osmolytes could significantly affect measurements of xylem composition made from cut stems. Concentrations of ions in the vacuoles of living pith and cambial cells were measured with this in mind. Living cells were consistently higher in solutes than xylem conduits by almost two orders of magnitude (Figure 3.2). Although other solutes were often detected in living cells, analysis focused on concentrations of Na⁺, K⁺ and Cl⁻. Average concentrations were 257.15mM Na⁺, 258.62mM K⁺ and 400.40mM Cl⁻ in *Avicennia marina* and 124.36mM Na⁺, 215.05mM K⁺ and 311.73mM Cl⁻ in *Aegiceras corniculatum*. Measurements were notable for their variability; standard deviations for all three ions in living cells in both species were between 240 and 272mM.

A perfusing solution with 30mM NaCl and 5mM KCl was selected for measurements of hydraulic conductivity based on typical xylem conduits contents for both species. These measurements agree with previously reported measurements of xylem sap content (Tomlinson 1994, Ball 1988) representing 92-94% exclusion of substrate salt. They are also consistent with reports of 24.4 to 36.6mM NaCl by Melcher **et al.** (2001) in **Rhizophora mangle**.

Vessel Anatomy

The average length of the longest vessel was 19 cm in **Avicennia marina**, 30 cm in **Aegiceras corniculatum**, 22 cm in **Avicennia germinans**, and greater than 40 cm in **Rhizophora mangle**. However, there were very few long vessels continuous from main to side branches in **Rhizophora mangle**, so side branches were used in conductivity measurements, with smaller amounts (~20 cm) of main branch as a buffer.

Aegiceras corniculatum had an average vessel diameter of $14.7\mu\text{m}(\pm 0.66)$, **Avicennia marina** had average vessel diameter of $13.6\mu\text{m}(\pm 0.61)$, **Avicennia germinans** had an average diameter of $29.9(\pm 0.72)\mu\text{m}$, and **Rhizophora mangle** had an average diameter of $28.4(\pm 4.87)\mu\text{m}$. Sample SEM micrographs of each species can be seen in Figure 3.3 and 3.4. The seemingly large difference between the vessel diameters may be attributable to the fact that younger and smaller branches were used in the Australia study, since they are better suited to cryoplaning. Vessel diameters often scale allometrically with branch diameter.

As discussed in the methods, ΔP for measurements of hydraulic conductivity in open vessels is dictated by vessel diameter. Generally, a hydraulic head of 55cm is permissible for these species (Table 3.1).

Table 3.1 Pressure Necessary to Move a Meniscus Along Vessels of Different Diameters

Species	Average vessel diameter (μm)	ΔP (kPa)	Hydraulic head (cm)
<i>Avicennia marina</i>	14.7(± 0.66)	11.36	113.6
<i>Aegiceras corniculatum</i>	13.6(± 0.61)	12.28	122.8
<i>Avicennia germinans</i>	29.9(± 0.72)	5.59	55.9
<i>Rhizophora mangle</i>	28.4(± 4.87)	5.88	58.8

Response of Hydraulic Conductivity to Freezing

Experiments to determine the sensitivity of mangroves to freeze-thaw induced cavitation were conducted at both research sites. There was no clear trend toward reduction of hydraulic conductivity due to either freezing or tension, in *Avicennia marina* at Bateman's Bay, New South Wales, Australia, (high salinity, 37 ppt NaCl, approximately -3.1MPa soil osmotic potential) and at Nelligen (lower salinity, 27.4 ppt NaCl, approximately -2.3MPa.) There was a weak reduction in conductivity in branches frozen at native tension from the Nelligen site, which were 22% ($\pm 26\%$) less conductive than control branches once measurements were normalized for length and conductive area (stem specific conductivity). However, at the higher salinity Bateman's Bay site, branches frozen at native tension showed on average 78% ($\pm 25\%$) greater conductivity than the corresponding controls (Figure 3.5). Although tensions in adjacent branches are likely to be similar, the fact that tensions in excised branches were not measured introduced another source of possible variation that was not accounted for.

On consideration of these results, potential problems appear in the methods used in Australia. Segments were not internally normalized, so differences between apparently similar segments not due to the treatment confounded the results. Variation in the data may also have resulted from error on the part of the experimenter, particularly failure to recognize leaks in the flow meter. The SSFM operates at very low flow rates and even small leaks obscure flow through the branch, especially when there is any blockage in the stem itself. It is also possible that there is no underlying trend toward loss of conductivity when frozen in these plants. The relative impact of these three factors cannot be resolved in this data

A PLC protocol was used in the second part of the study to address the problems encountered in the Australian data. An improved flow meter design, which reduced artifacts due to leaks, was also available during the second half of the study. Tension in the branches was standardized by harvesting at night, when the water potential in the leaves comes into equilibrium with the substrate, and leaf water potential was measured to confirm that only a small range of tensions was present.

Freezing at native tensions resulted in severe blockage to the xylem. **Avicennia germinans**, frozen at -2.7MPa ($\pm 0.5\text{MPa}$), had a PLC of 97.8% ($\pm 1.27\%$) conductivity. Control (unfrozen) branches -3.1MPa ($\pm 0.9\text{MPa}$) had PLC of 3.53% ($\pm 0.81\%$) conductivity (8 replicates) excluding two outliers that lost 63.7% and 54.2% . Including these controls lost 14.6% (± 8.13). Branches hydrated for 1 hour to -0.13MPa ($\pm 0.03\text{MPa}$) before freezing lost 49.2% (± 8.04); control (unfrozen) hydrated branches stored overnight with ends in water

had average leaf water potentials of -0.19MPa ($\pm 0.10\text{MPa}$) and lost on average 15.5% ($\pm 3.15\%$).

Apparently, although hydrating branches significantly reduced the degree of embolism, it did not eliminate it. Tension may not have been completely eliminated by hydration, or may have developed locally as a result of differential freezing rates in the leaves versus the stems (Marilyn Ball, personal communication).

Rhizophora mangle frozen at -2.58MPa ($\pm 0.20\text{MPa}$) lost on average 72.3% ($\pm 6.86\%$) conductivity; branches held in the cold room overnight had an average leaf water potential of -2.50MPa ($\pm 0.13\text{MPa}$) before measurement and lost on average 31.2% ($\pm 7.79\%$) conductivity. These results are summarized as a comparison between **Avicennia germinans** native and hydrated branches (Figure 3.6) and a comparison between **Avicennia germinans** and **Rhizophora mangle** (Figure 3.7).

Effects of Freezing In-Situ

In situ freezing of *Avicennia germinans* resulted in branch death in all cases as compared to phloem-girdled control branches. Leaves dried slowly, as has been described for this species (Lugo and Zucca 1977) and as was observed at the site—both branches detached by the experimenters and an uprooted tree were green for several days before showing signs of drying. After three days, only slight drying and discoloration was observed (Figure 3.8). After two weeks, however, there was marked evidence of catastrophic xylem failure. Leaves on frozen branches were brown, dry and brittle, but leaves on the phloem girdled branch and nearby untreated branches showed no discoloration.

No **in situ** experiments were performed in Australia, but observations were made of the results of a frost reported have reached -6°C at Nelligen. Leaves were initially discolored; leaves of both *Aegiceras corniculatum* and *Avicennia marina* suffered damage (Figure 3.9), but a greater proportion of *Aegiceras corniculatum* was affected (Figure 3.10). There was some evidence of patchy dieback in *Avicennia marina*. More striking effects were seen in *Aegiceras corniculatum*. Over three weeks, leaves initially damaged at the tips (as in Figure 3.9) dried and abscised from the stems, suggesting death of the stem itself as well as damage to the leaves.

Freeze Assay

Freezing may damage living tissue as well as cause xylem failure, and the degree of damage to living tissues can be very sensitive to minimum temperature. Minimum temperatures sufficient to cause critical damage were determined as indicators of leaf acclimation to freezing. The assay of freezing damage to leaf tissue consisted of two measurements, designed to address the degree of membrane damage and the resilience of the photosynthetic apparatus. Electrolyte leakage measures membrane damage by the percentage of conductive ions that leak out of the cells in a frozen sample. F_v/F_m is a measure of the efficiency of photosystem II and an indicator of photoinhibition (Critchley 1998).

A clear decline was seen in both **Avicennia marina** (Figure 3.10) and **Aegiceras corniculatum** (Figure 3.11) exposed to minimum temperatures from -2°C to -10°C . Minimum temperatures of -9°C were clearly sufficient for severe damage. Only small differences were observed between the high salinity and low salinity sites, and where one site showed greater damage, the trend often reversed as minimum temperature decreased. 50% membrane leakage, often considered a critical indicator of cold hardiness, occurred at -7°C in **Avicennia marina** and -6°C in **Aegiceras corniculatum**. **Avicennia marina** showed a sharper drop at a critical temperature, where **Aegiceras corniculatum** shows a gradual decline over the entire range of temperatures.

Photoinhibition increased at a rate consistent with electrolyte leakage. Normal values of F_v/F_m are 0.8 for sun leaves and 0.83 for shade leaves, and F_v/F_m below 0.725 is considered photoinhibition (Critchley 1998). Unfrozen leaves for both species are below this value, 0.634 (± 0.011) for *Avicennia marina* and 0.634 (± 0.007) for *Aegiceras corniculatum*, indicating that they may have been stressed in the natural environment. Photoinhibition can indicate either reversible photoprotection or irreversible photodamage as reactive species destroy the photosynthetic apparatus; (Critchley 1998) thus, it is not possible to isolate a single F_v/F_m ratio that indicates critical damage. It is clear, however, that leaves in both species suffered severe inhibition below -8°C

Chapter 4: Discussion

Freeze-Induced Cavitation Critically Impairs Xylem Function in Mangroves

In the introduction to this thesis, I suggested that while many factors may contribute to the death of mangroves after frost, their possible susceptibility to freeze-induced cavitation sets an absolute limit to their expansion. Results from this study show a high degree of xylem impairment after freezing, and *in situ* experiments and observations confirm that freezing of the xylem is sufficient to cause branch death.

Branches of *Avicennia germinans* showed almost complete loss of conductivity after freezing, while *Rhizophora mangle* showed PLC of 72%. Loss of water transport to the degree observed in *Avicennia germinans* is almost certain to result in death of living tissue distal to branch sections (Tyree and Sperry 1988). The only possibility for recovery lies in the refilling of vessels, a process that has not yet been conclusively demonstrated for xylem vessels remaining under significant tension (Holbrook and Zwieniecki 1999). That xylem freezing alone is sufficient to cause death of distal leaves is unambiguously demonstrated by the freezing of *Avicennia germinans* branches *in situ* with dry ice.

In *Rhizophora mangle*, the case for complete dysfunction of xylem transport was less clear. It cannot be said with certainty that 72% PLC would result in leaf death. Melcher *et al.* (2001) showed that healthy *Rhizophora mangle* in Hawaii experienced, and partially recovered, up to 60% loss of conductive vessels. However, this data was obtained using the cryo-SEM

method of embolism detection, which has been brought into question by other researchers (Cochard *et al.* 2000, Kikuta and Richter 2003).

Given the high xylem tensions, it is likely that mangrove species may be susceptible to the “runaway embolism” process described by Tyree and Sperry (1989). This process involves negative feedback in which embolism increases resistance to flow, increasing the pressure gradient required to sustain transpiration and generating more embolism. Runaway embolism in **Rhizophora mangle** was modeled by Tyree and Sperry (1989), who calculated that 30% loss of conductivity would be sufficient to cause complete xylem dysfunction of distal branches in a patchy pattern across the crown.

Scalariform perforation plates, which are typical of mangrove members of the Rhizophoraceae, (tribe Rizophoreae) (Tomlinson 1994) of which **Rhizophora mangle** is a member, may play a role in the lower PLC observed here. Scalariform perforation plates are rare in tropical vegetation and their presence is correlated with growth at higher latitudes or environments frequently subjected to freezing.

Comparison of results for **Avicennia germinans** frozen at native tension and after hydration suggest that xylem impairment is strongly exacerbated by negative xylem pressure, confirming the suggestion that xylem tension can produce embolism even in narrow vessels. **In vivo**, this effect is likely to be magnified by the high osmotic potential of the mangrove substrate, which can be expected to exert strong, steady tension on the thawing water column.

Leaves or Stems?: Frost Damage to Living Tissue

Results from the leaf freeze assay suggest that leaves of these species are indeed quite vulnerable to frost, even during midwinter when some resistance due to acclimation might be expected. 50% electrolyte leakages indicates extensive membrane damage in the range of -6°C to -7°C . Loss of leaves initially seems like an appealing explanation for mangrove dieback, since mangroves seem to be particularly dependent on a constant supply of energy. This temperature range is possible in severe frosts; such conditions in the field would most likely result in severe damage to the canopy.

Although a slightly lower temperature is required to guarantee xylem freezing, freezing exotherms often occurred at -4 or -5°C , above the -6 to -7°C associated with collapse of the living cells. There is a small range of temperatures, -6 to -9°C , during which the leaves might suffer damage if they froze but the xylem might never freeze and would suffer no damage. However, in most cases the conditions sufficient to cause damage in each organ are similar. In the field, both sources of damage are likely to play a role in limiting mangrove survival.

Over evolutionary time, however, frost damage to leaves is less likely to limit plants with a mangrove habit to the tropics. Living tissues can be adapted to cold relatively easily. Changes in the phospholipid composition of membranes protect acclimated plants from membrane damage (Wolfe and Bryant 1999, Pearce 2001). Acquired tolerance to chilling has been observed in mangroves (McMillian, 1975; Markely *et al.* 1982). In addition, there is some evidence that selection for cold tolerance may take place at higher latitudes. *Avicennia*

marina from New South Wales, Australia and New Zealand survived chilling temperatures at a much higher rate than plants from Darwin and Guam and **Avicennia germinans** from Texas survived better than plants from Belize when grown in common garden greenhouse experiments (McMillian 1975). This suggests that it is possible not only for mangroves to undergo cold-hardening processes described in other plants but also to adapt under selection pressure for greater tolerance to cold.

Limits to mangrove physiology

Cavitation, however, remains an insurmountable obstacle. As discussed in the introduction, plants adapt to the selective pressure of freeze-thaw cavitation primarily by reducing vessel diameter. In general, the vessels in mangrove wood, with almost all diameters under 100 μm , are small relative to the range of vessel diameters that occur in nature, 50 μm to 250 μm (Tomlinson 1994). To decrease vessel diameter further has a high cost either in construction of wood or loss of transport. Since the conductivity of a capillary is proportional to the fourth power of its radius, conductivity drops quickly as vessel diameter decreases. Replacing a single conduit requires sixteen new capillaries half the diameter of the original to maintain the same amount of flow (Tomlinson 1994). Mangroves may not be able to make the energetic investment in large amounts of wood, or adjust to the reduction in flow rate. In any vessel, cavitations that form are limited by the diameter but typically much smaller (Davis *et al.* 1999); since the relationship between vessel diameter and bubble diameter is direct but not exact, it is difficult to be sure whether it is possible to create a xylem vessel narrow enough to resist bubble expansion when it thaws under -2.5MPa of negative pressure.

Given the apparent lower limit to vessel diameter, a number of species, for example ring-porous deciduous trees in temperate areas, circumvent winter loss of xylem function by manufacturing new conduits. This might lead one to ask why there are no deciduous mangroves. Mangroves grow slowly and have low CO₂ assimilation rates (Ball 1998) and would have difficulty producing new wood as an alternate water transport pathway. It would be equally difficult for them to store the energy necessary to regenerate an entire leaf canopy. Observations of the susceptibility of mangroves to defoliation in hurricanes (Adam 1992) and frost (Lugo and Zucca 1977) suggest that these trees generally do not generate new leaves. *Avicennia* and others (eg, *Excoecaria*, Adam 1992) can recover through coppicing, but mangrove members of the Rhizophoraceae, which do not have a resting bud state (Tomlinson 1994) are typically devastated. When leaves are lost to frost, blockage of the xylem also prevents leaf regeneration, since new leaves must generate turgor pressure in order to expand, a process dependent on ample amounts of water (Taiz and Zieger 1998).

Implications

The present distribution of the mangrove likely results from the interaction of a number of factors, including membrane damage, the difficulty of producing and expanding new leaves, a low transpiration rate which limits carbon assimilation and storage, and impairment to xylem conductivity, as well as geographical limitations and historical contingencies. However, loss of xylem conductivity, here shown to be significantly impaired by freezing, presents a physical limitation to the mangrove's range which cannot be overcome through adaptation.

Global climate change, and especially associated sea level rise, may expand the range of the mangrove (Ellison and Farnsworth 2001); in some locations the extent of mangal has been increasing (Ball 1980). However, global climate change has been associated not only with an average increase in temperature but also with erratic weather patterns, including both tropical storms (Ball and Sobrado 1998) and unseasonal frosts. These disturbances are important in limiting the structural complexity of these slow-growing forests. The absolute limit imposed on the mangrove by its physiology may keep it out of temperate climates, but only limits to disturbance keep this ecosystem healthy in the long term.

Acknowledgements

Senior theses have a reputation for being all-consuming—fail-safe in their ability to elicit understanding nods and explain distraction. This has been no exception. However, theses also have a reputation for being isolating. I have been fortunate that this has not been my case. The product you see here is the result of collaboration as much as individual effort and it would be difficult to do justice to the way the help I've received has enriched not only my study but my understanding of science. Thus some thanks are in order:

First of all, to Missy Holbrook, my advisor, who first made me wonder about mangroves and shared her love of weird plants in general, and who has made all of this possible, for her teaching, her energy and her belief in this project. Second to Marilyn Ball, whose guidance in the first part of this study was indispensable. To Peter Melcher, who taught me to use the SSFM and helped me pack for my field trip, each item accompanied by advice for the young scientist, and to Brendan Choat, for his collaboration in the field and tireless patience in answering questions and offering explanations. Brendan, Rachel Spicer and Tiffany Bloomfield also deserve thanks for their editing and proofreading of this manuscript. Wayne Phippen for his help in the field and Masha Boulina for her stunning digital photographs of the *in situ* freezing experiments. A great deal of thanks is also due to Cheng Huang for his help with cryoplaning and electron microscopy. Beth Loveys and Jack Egerton deserve acknowledgement for their help with the leaf freezing assay. While I'm at it, the Florida data almost certainly would not have been collected without the advice of a very friendly, knowledgeable guy from Home Depot known only as Dave. Finally, to all the members of the Holbrook Lab, who have given me so much attention and useful feedback, and especially Matt Thompson, who first introduced me to plant physiology.

References

- Adam, P. 1992. Mangroves. Pp 200-212 in **Australian Rainforests**. Clarendon Press; Oxford, UK.
- Ball, MC. 1988. Salinity tolerance in the mangroves *Aegiceras corniculatum* and *Avicennia marina* I. Water use in relation to growth, carbon partitioning, and salt balance. **Australian Journal of Plant Physiology** 15:447-464.
- Ball MC. 1980. Patterns of secondary succession in a mangrove forest of southern Florida. **Oecologia** 44:226-234.
- Ball, MC, IR Cowan and GD Farquhar. 1988. Maintenance of leaf temperature and the optimization of carbon gain in relation to water loss in a tropical mangrove forest. **Australian Journal of Plant Physiology** 15:263-276.
- Ball, MC, and MA Sobrado. 1998. Ecophysiology of mangroves: challenges in linking physiological process with patterns in forest structure. Pp 331-346 in: MC Press, JD Scholes and MG Barker, eds. **Plant Physiological Ecology The 39th Symposium of the British Ecological Society**. Blackwell Science; London, UK.
- Ball, MC, J Wolfe, M Canny, M Hofmann, AB Nicotra and D Hughes. 2002. Space and time dependence of temperature and freezing in leaves of snow gum seedlings (*Eucalyptus pauciflora*). **Functional Plant Biology** 29(11):1259-1272.
- Bazzaz, FA. 1996. **Plants in Changing Environments**. Cambridge University Press, Cambridge, UK.
- Blumwald, E. 2000. Sodium transport and salt tolerance in plants. **Current Opinion in Cell Biology** 12:431-434.
- Brodribb TJ, NM Holbrook, EJ Edwards and MV Gutiérrez. 2003. Relations between stomatal closure, leaf turgor and xylem vulnerability in eight tropical dry forest trees. **Plant, Cell & Environment** 26(3): 443-450.
- Burchett, MD, CD Field and A Pulkownik. 1984. Salinity, growth and root respiration in the grey mangrove, *Avicennia marina*. **Physiologia Plantarum** 60:113-118.
- Cavender-Bares, J, and NM Holbrook. 2001. Hydraulic properties and freezing induced cavitation in sympatric evergreen and deciduous oaks with contrasting habits. **Plant, Cell and Environment** 24:1243-1256.
- Chapman, VJ. 1940. Studies in salt-marsh ecology: sections VI and VII. Comparison with marshes on the east coast of North America. **Journal of Ecology** 28(1):118-152.

- Cochard, H, P Cruiziat, MT Tyree. 1992. Use of positive pressures to establish vulnerability curves—further support for the air-seeding hypothesis and implications for pressure-volume analysis. **Plant Physiology** 100:205-209.
- Cochard, H, C Bodet, T Améglio and P Cruiziat. 2000 Cryo-scanning electron microscopy observations of vessel content during transpiration in walnut petioles. Facts or artifacts? **Plant Physiology** 124: 1191-1202.
- Cochard H, L Coll, X Le Roux and T Améglio. 2003. Unraveling the effects of plant hydraulics on stomatal closure during water stress in walnut. **Plant Physiology** 128:282-290.
- Commonwealth Bureau of Meteorology. 2001. What is the weather usually like? - Climate averages for Australian Sites - Averages for Moruya Heads pilot station. <http://www.bom.gov.au/climate/averages/tables/cw_069018.shtml> (cited 29 March 2003).
- Cordero, RA and ET Nilsen. 2002. Effects of summer drought and winter freezing in stem hydraulic conductivity of **Rhododendron** species from contrasting climates. **Tree Physiology** 22:919-928.
- Critchley, C. 1998. Photoinhibition. Pp 264-272 in **Photosynthesis: A Comprehensive Treatise**, AS Raghavendra ed. Cambridge University Press: Cambridge, UK.
- Davis, SD, JS Sperry and UG Hacke. 1999. The relationship between xylem conduit diameter and cavitation due to freezing. **American Journal of Botany** 86(10):1367-1372.
- Duke, NC, MC Ball and JC Ellison. 1998. Factors influencing biodiversity and distributional gradients in mangroves. **Global Ecology and Biogeography Letters** 7(1):27-47.
- Ellison, AM, MD Bertness, and . Miller. 1986. Seasonal patterns in the belowground biomass of *Spartina alterniflora* (Gramineae) across a tidal gradient. **American Journal of Botany** 73: 1548-1554.
- Ellison, AM and EJ Farnsworth. 2001. Mangrove communities. Pp 423-442 in: MD Bertness, S Gaines, and ME Hay, eds. **Marine Community Ecology**. Sinauer Press, Sunderland, MA, USA.
- Feild, TS and T Brodribb. 2001. Stem water transport and freeze-thaw xylem embolism in conifers and angiosperms in a Tasmanian treeline heath. **Oecologia** 127(3):314-320.
- Feild, TS, Brodribb T, and NM Holbrook. 2002. Hardly a relict: freezing and the evolution on vesselless wood in Winteraceae. **Evolution** 56(3):464-478.
- Gallagher JL. 1983. Seasonal patterns in recoverable underground reserves in *Spartina alterniflora* Loisel. **American Journal of Botany** 70 (2): 212-215.
- Grime, JP. 1974. Vegetation classification by reference to strategies. **Nature** 250:26-31.
- Grime, JP. 1979. **Plant Strategies and Vegetation Processes**. John Wiley; Chichester, UK.

- Hällgren, JE and G Öquist. 1990. Adaptations to low temperatures. Pp 265-293 in **Stress Responses in Plants: Adaptation and Acclimation Mechanisms**, RG Alscher and JR Cumming, eds. Wiley-Liss, Inc.; New York, NY, USA.
- Hanagata, N, T Takemura, I Karube and Z Dubinsky. 1999. Salt/water relationships in mangroves. **Israel Journal of Plant Sciences** 47:63-76.
- Henry, JA, KM Portier, and J Coyne. 1994. **The Climate and Weather of Florida**. Sarasota, Florida, USA: Pineapple Press.
- Hogarth, PJ. 1999. **The Biology of Mangroves**. Oxford University Press; Oxford, UK.
- Holbrook, NM, and Zwieniecki, MA. 1999. Embolism repair and tension: do we need a miracle? **Plant Physiology** 120:7-10.
- Holbrook, NM, MJ Burns, MA Zwieniecki, AR Cobb, L Sack, PJ Melcher and TJ Brodribb. 2002. Measurement of xylem hydraulic conductivity: field instrumentation and implementation. **Submitted**.
- Krebs, CJ. 2001. Factors that limit distributions: temperature, moisture and other physical-chemical factors. In **Ecology**, Addison Wesley Longman, Inc.; New York, NY, USA. pp86-105.
- Langan, SJ, FW Ewers and SD Davis. 1997. Xylem dysfunction caused by water stress and freezing in two species of co-occurring chaparral shrubs. **Plant, Cell and Environment** 20:425-437.
- Larcher, W. 1995. Plants under stress: natural environmental constraints. Pp 332-396 in **Plant Physiological Ecology**, trans. J Wieser. Springer-Verlag: Berlin, Germany.
- Lugo, AE and CP Zucca. 1977. Impact of low temperature stress on mangrove structure and growth. **Tropical Ecology** 18:149-161.
- Markley, JL, C McMillan and GA Thompson. 1982. Latitudinal differentiation in response to chilling temperatures among populations of three mangroves, **Avicennia germinans**, **Laguncularia racemosa**, and **Rhizophora mangle** from the western tropical Atlantic and Pacific Panama.
- McCully, ME, CX Huang and LEC Ling. 1998. Daily embolism and refilling of xylem vessels in the roots of field-grown maize. **New Phytologist** 138:327-342.
- McCully, ME, MW Shane, AN Baker, CX Huang, LEC Ling and MJ Canny. 2000. The reliability of cryoSEM for the observation and quantification of the xylem embolisms and quantitative analysis of xylem sap **in situ**. **Journal of Microscopy** 198:24-33.
- McMillan, C. 1971. Environmental factors affecting seedling establishment of the black mangrove on the central Texas coast. **Ecology** 53(5)927-930.

- McMillan, C. 1975. Adaptive differentiation to chilling in mangrove populations. Pp 62-68. In GE Walsh, SC Snedaker, and HJ Teas, eds. **Proceedings of the International Symposium on Biology and Management of Mangroves. Vol. 1** EPA:University of Florida, Gainesville.
- McMillan, C and CL Sherrod. 1986. The chilling tolerance of black mangrove, *Avicennia germinans*, from the gulf of Mexico coast of Texas, Louisiana, and Florida **Contributions in Marine Science** 29:9-16.
- Melcher, PJ, G Goldstein, FC Meinzer, DE Young, TJ Jones, NM Holbrook, CX Huang. 2001. Water relations of coastal and estuarine *Rhizophora mangle*: xylem pressure potential and dynamics of embolism formation and repair. **Oecologia** 126:182-192.
- Mitsch, WJ and JG Gosselink. 1986. **Wetlands**. New York, NY, USA: Van Nostrand Reinhold Company.
- Montague, CL and RG Wiegert. 1990. Salt Marshes. Pp 481-516 in **Ecosystems of Florida**, RL Myers and JJ Ewel, eds. University of Central Florida Press, University Presses of Florida, Gainesville, Florida, USA..
- Nardini, A, and Salleo, S. 2000. Limitation of stomatal conductance by hydraulic traits: sensing or preventing xylem cavitation? **Trees** 15:14-24.
- NOAA-CIRES Climate Diagnostic Center. 2002. Climatology for Jacksonville NAS FL; Climatology for Daytona Beach FL. <<http://www.cdc.noaa.gov/cgi-bin/USclimate/state.pl?lane=fast&state=FL>> (cited 29 March 2003).
- Odum, WE, and CC McIvor. 1990. Mangroves. Pp 517-548 in **Ecosystems of Florida**, RL Myers and JJ Ewel, eds. University of Central Florida Press, University Presses of Florida, Gainesville, Florida, USA.
- Pearce, RS. 2001. Plant Freezing and Damage. **Annals of Botany** 87:417-424.
- Pockman, WT and JS Sperry. 1997. Freezing-induced cavitation and the northern limit of *Larrea tridentata*. **Oecologia** 109:19-27.
- Schurr U. 1998. Xylem sap sampling - new approaches to an old topic. **Trends in Plant Science** 3(8):293-298.
- Scholander, PF 1968. How mangroves desalinate seawater. **Physiologia Plantarum** 21:251-261.
- Sherrod, CL, and C McMillan. 1985. The distributional history and ecology of mangrove vegetation along the northern Gulf of Mexico coastal region. **Contributions in Marine Science** 28:129-140.
- Sherrod, CL, DL Hockaday and C McMillan. 1986. Survival of the red mangrove, *Rhizophora mangle*, on the Gulf of Mexico coast of Texas **Contributions to Marine Science** 26:27-36.

- Spalding, M, F Blasco, C Field, eds. 1997. **World Mangrove Atlas**. Okinawa, Japan:International Society for Mangrove Ecosystems.
- Sperry, JS, JR Donnelly and MT Tyree. 1988. A method for measuring hydraulic conductivity and embolism in xylem. **Plant, Cell and Environment** 11:35-40.
- Sperry, JS, KL Nichols, JEM Sullivan and SE Eastlack. 1994. Xylem embolism in ring-porous, diffuse-porous, and coniferous trees of northern Utah and interior Alaska. **Ecology** 75(6):1736-1752.
- Stewart, GR and M Popp.1987. Mangrove ecophysiology. Pp 333-345 in RMM Crawford, ed. **Plant Life in Aquatic and Amphibious Habitats**. Blackwell Scientific Publications; Oxford, UK.
- Taiz, L, and E Zeiger. 1998. "Chapter 25: Stress Physiology" in **Plant Physiology**. Sunderland, MA, USA: Sinauer Associates.
- Tomlinson, PB. 1994. **The Botany of Mangroves**. Cambridge University Press, Cambridge, UK.
- Takemura, T, N Hanagata, K Sugihara, S Baba, I Karube and Z Dubinsky. 2000. Physiological and biochemical responses to salt stress in the mangrove *Bruguiera gymnorrhiza*. **Aquatic Botany** 68:15-28.
- Tyree, MT and JS Sperry. 1989. Vulnerability of xylem to cavitation and embolism. **Annual Review of Plant Physiology and Molecular Biology** 40:19-38.
- Tyree, MT and MH Zimmerman. 2002. **Xylem structure and the ascent of sap**. New York, NY, USA: Springer-Verlag Inc.
- Wijte AHBM and Gallagher JL. 1991. The importance of dead and young live shoots of *Spartina alterniflora* (Poaceae) in a midlatitude salt marsh for overwintering and recoverability of underground reserves. **Botanical Gazette** 152 (4): 509-513.
- Wolfe, J, and G Bryant. 1999. Freezing, drying, and/or vitrification of membrane-solute-water systems. **Cryobiology** 39:103-129.
- Zimmerman, MH and AA Jeje. 1981. Vessel length distribution in stems of some American woody plants. **Canadian Journal of Botany** 59(10):1882-1892.
- Zwieniecki, MA, PJ Melcher and NM Holbrook. 2001. Hydrogel control of xylem hydraulic resistance in plants. **Science** 291:1059-1062