

**SEASHORE MALLOW (*KOSTELETZKYA PENTACARPOS*) AS AN  
ECOSYSTEM ENGINEER: COMMUNITY DYNAMICS AND GROWTH**

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Marine Studies

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This manuscript is dedicated:

To my mother, Beverly, to honor her inspiring perseverance.

And in memory of my father, Stavros Voutsinas, whose compassion lives on.

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## ABSTRACT

Planning ahead for climate change is urgent and essential if we are to moderate its impacts. Salt marshes and coastal farmlands will be directly impacted by sea level rise, particularly through salt-water intrusion, increased storm surges and inundation. Planting the halophytic crop *Kosteletzkya pentacarpos*, seashore mallow, could extend the economic viability of salt-affected working land, while it is still dry enough to mechanically harvest, and simultaneously maximize the ecological potential of its eventual transition to salt-marsh. The purpose of this thesis is to study the eco-physiology of *K. pentacarpos*. Firstly, we examined its interactions with other halophytes and soil properties, and secondly, we investigated the role of temperature in its growth and development.

We hypothesized that the presence of *K. pentacarpos* would facilitate the colonization of desirable marsh plants, such as *Spartina patens*, and assist in the evolution of soil conditions from working land to natural habitat. We examined these hypotheses through a multi-year field experiment. Communities initiated with *K. pentacarpos* were richer in species than those initiated directly with *S. patens* and more productive than control communities. The presence of *K. pentacarpos* enhanced the natural colonization of *S. patens* and *Baccharis halimifolia*. Although no effect of treatment was detected in major soil nutrients, an increase in organic matter and leaf litter was associated with *K. pentacarpos* treatments. Our results support the use of *K. pentacarpos* as a low-cost and efficient nurse crop in degraded agro-ecosystems.

Temperature is suspected to play an important role in the life cycle of *K. pentacarpos*. To test this hypothesis, seeds from two locations within the species range, Delaware and Georgia, were grown at five temperature treatments from 15°-35° C. Germination was not very temperature-sensitive but the development of secondary structures, such as leaves, occurred sooner at higher temperatures. Delaware seeds germinated sooner and seedlings grew larger than Georgia seedlings. These findings inform selection of optimum early growth conditions and reveal promising prospects for crop improvement, through the exploitation of trait variation found throughout the species' spatial range.

## Chapter 1

### GENERAL INTRODUCTION

Preparing for sea level rise is essential to alleviate its impacts.

*Kosteletzkya pentacarpos* is being developed as a salt-tolerant biofuel crop for salt-affected coastal land. Its cultivation could prolong economic viability of the land while simultaneously preparing it for an ecologically-sound transition to a natural ecosystem, after land becomes too wet to use. The purpose of this thesis was to investigate the eco-physiology and community ecology of *K. pentacarpos*, and how it interacts with its environment and other marsh species.

#### 1.1 Wetlands and Sea Level Rise

Wetland ecosystems are of high ecological value and are now at risk due to climate change. They have high productivity; serve as nurseries for numerous species; buffer the coast from storms, flooding and erosion; filter pollutants; and add to the scenic and therefore economic value of the coast. Nowadays, their ecological role is even more important due to the fact that they actively sequester carbon. However, severe weather events and sea level rise will cause great losses to wetlands. The IPCC report (Nicholls et al. 2007) estimates up to 59cm sea level rise by the end of the 21<sup>st</sup> century accompanied by an increase in annual severe weather events. Various models predict that a 38cm to 100cm sea level rise, by 2080, would inundate 22% to 46% of global salt marshes respectively (Nicholls et al. 1999; McFadden et al.

2007; Nicholls et al. 2007). Losses to wetlands will also vary on local scale due to parameters such as direct human disturbances.

There is already evidence that climate change is influencing salt marsh vertical zonation in low vulnerable regions (Warren and Niering 1993). Wetlands can tolerate sea level rise through two processes. Either, salt marsh accretion and vegetation growth rates must match local sea level rise rates to avoid inundation, or salt marshes must migrate inland where migration barriers, such as manmade structures, are not a physical limitation (Reed 1995). Marsh restoration and management techniques, such as supplementing sediment supply or carefully planning retreat pathways for migration, could mitigate future losses and should be applied now (Nicholls 1999).

## **1.2 Coastal Agriculture and Sea Level Rise**

Coastal agricultural land lies in the path of inland marsh migration. These working lands also face change due to climate change. Saltwater invasion is already occurring and will impact coastal agriculture tremendously since most common crops are not salt-tolerant. Affected farmland will become barren and could be colonized by invasive species such as the common reed, *Phragmites australis* (Cav.) Trin. ex Steud. (Figure 1). *P. australis* has successfully spread throughout this region, assisted by extensive coastal development. Chambers et al. (2008) showed that the presence of *P. australis* in the Chesapeake Bay is positively correlated with coastal agriculture. It can compete with the native marsh species in low to medium salinity soils and have a devastating effect on the vegetative succession of a new or recovering salt marsh (Wijte and Gallagher 1996a; Wijte and Gallagher 1996b). *P. australis* invasion results

in monocultures of little ecological value which are very expensive and difficult to eliminate.

Re-vegetation of salt-contaminated farmlands with desirable perennials can potentially revitalize the land (Barrett-Lennard 2002). Planting a perennial salt-tolerant crop during saltwater invasion would allow farmers continued productivity and also keep the land from becoming barren, and therefore exposed to invasive species. Such a crop would help transition the soil to a more natural state, through storing organic matter underground, thus facilitating the colonization of migrating vegetation.

### **1.3 Facilitation and Nurse Plants**

Preexisting vegetation, in a degraded habitat for which abiotic stresses may be considerable, has a significant impact on establishment of other species through competition or facilitation (Gomez-Aparicio 2009). The presence of one plant species can facilitate the growth and survival of another via improving soil properties, providing physical shelter and ameliorating environmental severity (Hacker and Bertness 1995; Callaway and Walker 1997; Armas and Pugnaire 2005; Brooker et al. 2008; Butterfield 2009). Egerova et al. (2003) showed that *Spartina alterniflora* acts as a nurse plant for *Baccharis halimifolia*, by physically trapping seeds and improving the success of seedlings, during the early stages of salt marsh creation.

In addition, vegetation can directly alter the physical properties of the microenvironment with which it interacts. Plants at the soil surface create drainage, evaporation, and temperature changes, while roots allow for oxygen and nutrients to infiltrate the soil (Jones et al. 1994). Hacker and Bertness (1995) showed that the

rush, *Juncus gerardi*, controlled salt accumulation and increased soil oxygen, and thus facilitated the growth of the salt marsh shrub *Iva frutescens*, which is not as salt-tolerant as the rush. Nurse plant roots can improve the soil by stimulating soil aggregation, releasing exudates, providing organic material, removing soluble nutrients, solubilizing nutrients from soil minerals, oxygenating soil, and providing resources for microflora and microfauna (Brady et al. 1996).

#### **1.4 *Kosteletzkya pentacarpos***

*Kosteletzkya pentacarpos* (L.) Ledebour (Blanchard 2008) has been proposed as a salt-tolerant oil-seed crop for saline coastal farmlands that can no longer produce common crops, and has also been used to re-vegetate salinized land in China (Somers 1979; Gallagher 1985; He et al. 2003; Ruan et al. 2008). We hypothesize that the presence of *K. pentacarpos* will assist in the establishment of other potential colonizers in salinized coastal land.

*K. pentacarpos* is a perennial dicot in the family Malvaceae. It is a facultative halophyte, which means that it does not require salt to grow; however it can tolerate salinity of up to 25ppt using both cellular and whole plant level mechanisms (Blits and Gallagher 1990a; Blits and Gallagher 1990b; Blits et al. 1993; Gallagher 1995; Li et al. 2006). *K. pentacarpos* is native to the Atlantic and Gulf coasts of the United States (Radford et al 1968) and is not an invasive species. The perennial's deep roots interact with the soil year round and also maintain the plant through periods of abiotic stress, such as drought or waterlogged conditions (Halchak et al. 2011). The flower of *K. pentacarpos* self-pollinates at midday, with minimal inbreeding loss, so the absence of pollinators does not pose a threat to reproductive success (Ruan et al.

2009). The seeds remain viable for periods up to 20 years without significant loss of germination success (Poljakoff-Mayber et al. 1992; Poljakoff-Mayber et al. 1994). In addition, *K. pentacarpos* has also been successfully grown as callus tissue culture (Cook et al 1989; Li et al. 2006) and could potentially be manipulated to produce desired characteristics to suit the functional need of a particular project (Li and Gallagher 1996), as has been demonstrated with other halophytes (Seliskar and Gallagher 2000).

*K. pentacarpos* has multiple products currently under development. Seeds contain oil similar in composition to that of cottonseed and with similar biodiesel product options. The seed is also high in protein but does not contain the toxic compound gossypol found in cottonseed (*Gossypium sp.*) and okra (*Abelmoschus esculentus*), making it a good candidate as an additive to animal feed (Islam et al. 1982). *K. pentacarpos* seeds and stems can be used to produce mucilage and cellulosic ethanol, respectively. Since it is a perennial, its cultivation has low energy costs because it does not need to be replanted yearly, and it can be harvested with common agricultural machinery, thereby avoiding the additional costs of equipment development and purchase. Each year the plant produces more stems than previous years, thus increasing its yield (Gallagher 1985; Halchak 2009). In our lab we have demonstrated the successful growth and harvest of this plant in a pilot project. Over the past five years, *K. pentacarpos* has been cultivated on three acres of land on the Freeman Farm in Lewes, Delaware, allowing for extensive study of the agricultural aspects of this halophyte (Halchak et al. 2011). However, not much is known about the ecosystem engineering qualities of this plant and how it interacts with other

species. We hypothesize that *K. pentacarpus* will facilitate the establishment of native marsh plants; acting as a nurse plant.

### 1.5 Growth and Temperature

Additional information about the growth and development of this perennial is necessary to further its domestication. Temperature is a defining environmental factor in plant life via its effect on plant processes and growth. Downton & Slatyer (1972) showed that net photosynthesis and CO<sub>2</sub> uptake is highest in upland cotton (*Gossypium hirsutum*) at its preferred growth temperature. For this crop, the heat units, or degree day (DDs), concept has been developed to predict when cotton will transition from one life stage to the next, therefore improving crop management. The heat unit formula describes the cumulative daily effect of temperature on cotton growth, with  $F_{\max}$ ,  $F_{\min}$  representing the daily maximum and minimum temperatures and where the numerical value 60 is the base temperature, below which cotton will not grow:  $HU = [(Daily\ max\ temperature + Daily\ min\ temperature) / 2] - 60$ . However, there are various criticisms of the heat unit concept. This because it is based on certain assumptions, such as that the relationship between growth and temperature is linear and that the number of DD units required to advance growth is constant over time, locations, and climate (Supak 1984). Even so, this method creates useful estimates for cotton crop managers.

*K. pentacarpus* is a close relative to cotton and may also exhibit a developmental rate driven by temperature instead of photoperiod. Such a growth estimate method could be very useful in the domestication of this halophyte. Greenhouse and field observations suggest that temperature is indeed a driving factor

in the development of *K. pentacarpos*; however no studies have been completed to test it. There are multiple levels of this relationship that could be examined. In addition to how temperature influences the growth of a plant, it would be useful to study this relationship at the sub-organismal level and as a local adaptation in ecotypes within the natural range of *K. pentacarpos*.

For a study of temperature effects on the plant cell, tissue culture methods can be used. Due to the nature of the process, tissue cultures have various applications. When in callus, cells are traumatized due to isolation and this stress will frequently induce genetic variation, known as somaclonal variation (Larkin and Scowcroft 1981; Jain 2001). The ability to select regenerates with desirable traits is a very useful tool for wetland creation and restoration and crop improvement (Wang et al. 2007; Wang et al, 2006; Wang et al. 2003; Jain 2001; Seliskar and Gallagher 2000). Tissue cultures of *K. pentacarpos* have also been used to study the physiology and chemical tolerance of seashore mallow (Cook et al. 1989). Further development of the culturing method for this plant would be useful in the process of its domestication, as desirable crop qualities could be selected for, and in this case, for the study of how the plant cells respond to various temperatures.

A study of variation in temperature requirements amongst ecotypes of *K. pentacarpos* would be interesting. A difference in heat requirements would illustrate genetic variation within the species' range, whereas similarity would indicate a high plasticity for the species since it is able to colonize multiple temperature regimes. Subtle differences can be noted in *K. pentacarpos* ecotypes along the East coast. The identification of a difference in heat requirements and then the development of a heat

unit method could be very useful in selecting an appropriate ecotype of *K. pentacarpos* for a particular agricultural or restoration project.

## **1.6 Research Objectives**

The purpose of this study was to further our understanding of the ecology and physiology of *K. pentacarpos*. Therefore, this project had two separate objectives, each of which intended to answer two to three related research questions. The first objective of this study was to evaluate *K. pentacarpos*' potential as a nurse plant. We sought to answer two questions. Firstly, how does *K. pentacarpos* interact with native vegetation and would its presence promote its establishment in a potential colonization site? And secondly, how does the perennial *K. pentacarpos* affect soil properties?

The second objective of this study was to further explore the role of temperature in the eco-physiology of *K. pentacarpos*' growth and development. The following questions sought to understand this relationship in depth, with an exploration of the response to temperature of the entire plant unit, the cellular unit, and as a comparison of two distant seed sources. Firstly, does temperature control growth and development of *K. pentacarpos*? Secondly, what is the effect of temperature on cellular growth rate? Finally, we made a comparison of two East coast *K. pentacarpos* ecotypes and asked whether plant communities along the coast of the USA show local adaptation to varying regional temperature regimes?

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## Chapter 2

### ***KOSTELETZKYA PENTACARPOS AS AN ECOSYSTEM ENGINEER***

#### **2.1 Abstract**

Rising sea level will increase soil salinity and flooding frequency, directly impacting low-lying coastal farmlands. Once productivity for traditional crops diminishes, management will be required to transition these working lands back to native status. Seashore mallow, *Kosteletzkya pentacarpos*, is being developed as a biofuel crop to prolong economic value in such cases; however its potential to facilitate establishment of wetland species has not been examined. We hypothesized that seashore mallow presence would act as a nurse crop and direct soil evolution through this transitional period. Four treatments were planted at an upland field site adjacent to a salt marsh. The control, *S.patens*, *K. pentacarpos*, and combined treatments were laid out in a complete randomized block design with replication. These were sampled for species percent vegetative cover, morphological traits, and above-ground biomass. In addition, soil was tested for major nutrients, pH, Cation Exchange Capacity, salinity, and organic matter. Results suggest that the presence of seashore mallow enhanced *S.patens* recruitment but did not significantly impact growth of planted *S.patens* individuals. Communities established around *K. pentacarpos* were productive and diverse. Although we did not detect an effect of *K. pentacarpos* on soil nutrients, leaf litter and 0-5 cm organic matter soil content

increased with its presence. Our findings thus far suggest the potential for use of *K. pentacarpos* as a low-cost and efficient nurse crop in degraded agro-ecosystems.

## **2.2 Introduction**

Accelerating sea-level rise is a serious threat to wetlands globally (Nicholls et al. 1999; Nicholls et al. 2007; Craft et al. 2009). Changes in vegetation and greater inundation rates have already been noted, particularly when combined with adjacent land use and increased frequency of severe weather events (Warren and Niering 1993; Donnelly and Bertness 2001; Hartig et al. 2002; Elsey-Quirk et al. 2011). Wetlands are very valuable as they protect the coast from storms, flooding, and erosion; act as nurseries and habitats for wildlife; filter pollutants and contribute to nutrient cycles; are highly productive and sequester carbon; and beautify coastal areas, thereby increasing their economic value. The value of these services is estimated at approximately \$14,785 per hectare per year (Costanza et al. 1997). In order to survive sea-level rise, marsh sedimentation rates must match sea-level rise or species must migrate inland (Reed 1995). Migration will be limited in many areas due to extensive coastal development; therefore planning ahead for space into which wetland species can migrate could mitigate future losses and ensure their vital ecosystem services are maintained (Nicholls et al. 1999; Stralberg et al. 2011).

Coastal agricultural land is also susceptible to the impacts of sea-level rise. Increase in frequency of severe weather events and higher tidal ranges will progressively introduce salt to working land and contaminate freshwater aquifers (IPCC 2007). Patchy areas of growth will become increasingly common, as traditional crops are not salt-tolerant. Figure 2.1 (a) captures the effect of salinization on a Delaware (Kent County) coastal farmland. Sea-level rise projections indicate that

many coastal agricultural areas will eventually be too saline and too wet to cultivate and therefore salt-water exclusion practices, such as building dikes, could be used as an increasingly expensive and temporary solution to preserving agricultural land. Many of these agricultural sites could be suitable upland sites into which marsh vegetation could migrate. This could be an opportunity to turn the unfortunate loss of one service into a positive gain of another.

However, if left unmanaged not only is coastal agricultural land at risk of losing productivity but it may also provide a poor habitat for migrating marshes. Specifically, one concern is that agricultural land and salt marshes differ too much for species establishment to succeed. Agricultural soil has been shaped by the prolonged cultivation of annuals, whose shallow roots and required maintenance diminish soil productivity (Lal 2004; Glover et al. 2007). Another concern is the highly invasive *Phragmites australis*, which is a costly problem on the East coast of North America. Figure 2.1 (b) shows an example of the early stages of such an invasion in a salinized area of a Delaware coastal farm. *P. australis* is capable of outcompeting native marsh species such as *Spartina alterniflora* at low salinities (Wijte and Gallagher 1996a; Wijte and Gallagher 1996b). Its spread has been shown to be linked to agricultural activities on the coast of the Chesapeake Bay (Chambers et al. 2008) and the potential for successful invasion increases with habitat disturbance (Silliman and Bertness 2004) and the presence of bare soil (Baldwin and Mendelsohn 1998). The risk is especially high in areas where *P. australis* is already established because viable seed production is important to the species ability to colonize of pristine sites and increases with genetic diversity (Baldwin et al. 2010; Kettenring et al. 2011).

A carefully chosen salt-tolerant crop could be a cost-effective tool to mitigate such impacts. The cultivation of a perennial may improve soil conditions through nutrient storage, carbon sequestration, increasing exchange of water and air between soil layers, and reducing erosion (Glover et al. 2007). Simultaneously, it would maintain a physical barrier and could reduce the risk of invasion. Therefore, by creating a more suitable environment and reducing bare soil surface, a transitional salt-tolerant crop could maximize marsh migration potential and prevent invasion while simultaneously prolonging economic yield for farmers.

Such species are referred to as ecosystem engineers and are used to effectively create a habitat through their impact on their surroundings (Jones et al. 1994; Byers et al. 2006). For plants, these species are also known as nurse plants and the value of their facilitative interactions with other species has been studied in recent years. Facilitation amongst plants can occur alongside competition, and may vary according to a stress gradient, a plant's life stage, or the species involved (Callaway and Walker 1997). Nurse plants can improve the potential for productivity in their immediate environment by ameliorating stressful conditions for seedlings, such as direct radiation, fluctuation of air and soil temperatures, salinity or drought, hence creating a microclimate that is ideal for germination and growth (Bertness 1991; Hacker and Bertness 1995; Armas and Pugnaire 2005; See Padilla and Pugnaire 2006 for review). They can improve soil properties, nutrients and texture, and enhance microbial activity (Armas and Pugnaire 2005; Padilla and Pugnaire 2006) while their physical presence can trap seeds (Egerova et al. 2003), protect from grazers, and attract pollinators (Padilla and Pugnaire 2006).

*Kosteletzkya pentacarpos* (L.) Ledebour (Blanchard 2008) is a perennial dicot (Malvaceae) that has been proposed for use as a halophytic crop (Sommers 1979; Gallagher 1985; Gallagher and Seliskar 1993; Gallagher 1995; He et al. 2003). It yields multiple products currently under development; specifically, it could be grown for biofuel, cellulosic ethanol, or animal feed among other options (Islam et al. 1982; Ruan et al. 2008). Its cultivation would require low energy expenditures since it does not need to be replanted annually and it can be harvested with common agricultural machinery, thereby avoiding the additional costs of equipment development and purchase. Although it can cross-pollinate, the flower of *K. pentacarpos* self-pollinates at midday with minimal inbreeding loss, therefore the absence of pollinators is not a threat to its yield (Ruan et al. 2009a). Seeds remain viable for periods up to 20 years without significant loss of germination success (Poljakoff-Mayber et al. 1992; Poljakoff-Mayber et al. 1994). In addition, *K. pentacarpos* has also been grown as callus tissue culture (Cook et al. 1989; Li et al. 2006) and thus desirable traits could be advanced (Li and Gallagher 1996; Ruan et al. 2009b), as has been demonstrated with other species (Seliskar and Gallagher 2000). Multiyear pilot projects have successfully cultivated and harvested *K. pentacarpos* in the United States of America (Halchak et al. 2011) and in China (He et al. 2003; Ruan et al. 2008). This halophyte has the properties necessary for becoming a salt-tolerant crop and for prolonging the productivity of coastal agricultural land.

Several qualities of *K. pentacarpos* theoretically could also make it an excellent ecosystem engineer. It is native to brackish marshes of the Atlantic and Gulf coasts of the United States of America (Radford et al. 1968) and does not exhibit invasive character. Making it a good candidate to survive unstable environmental

conditions that may arise during the transition, the perennial's deep roots maintain the plant through periods of abiotic stress, such as drought or waterlogged conditions (Halchak et al. 2011), while simultaneously investing nutrients and organic matter into the soil. Each year the plant produces more stems than previous years (Gallagher 1985; Halchak 2009), thus it will likely provide a dense physical barrier against invasion and a habitat for wildlife. It is a facultative halophyte, known to tolerate salinity of 25ppt (Gallagher 1985; Blits and Gallagher 1990a; Blits and Gallagher 1990b; Blits et al. 1993; Li 2006), and in its natural habitat is usually found intermittently dispersed amongst the dominant upper marsh species; therefore it is not likely to compete with or hinder the progress of migrating wetland halophytes when salinity and waterlogging increase.

In this study we sought to better understand the ecology *K. pentacarpos* and to investigate whether its qualities could be applied as a tool for promoting wetland habitat development in upland locations during sea-level rise. We hypothesize it would improve agricultural soil and we anticipate that it would act as a nurse plant and improve the potential for naturalization of salt-affected land. In order to evaluate the suitability of *K. pentacarpos* as an ecosystem engineer we asked four questions. (1) What is the impact of *K. pentacarpos* cultivation on soil properties and nutrient content? (2) What species naturally colonize a plant community started around *K. pentacarpos*? (3) What is the impact of *K. pentacarpos* on the species diversity and productivity of its surrounding community? (4) How does *K. pentacarpos* interact with the common coastal colonizer *Spartina patens* (Aiton) Muhl (S)? The goal of these questions was to determine whether the cultivation of *K.*

*pentacarpos* provides more benefits than allowing sea-level rise to change the land without further management and what these benefits are.

## 2.3 Methods

### 2.3.1 Experimental Design and Maintenance

The experiment was a complete randomized block design with replication conducted on the grounds of the University of Delaware H. R. Sharp campus in Lewes, Delaware (38°46'46.68 N, 75° 9'48.11 W). The site is shown in Figure 2.2 and was situated upland to a natural marsh, simulating an agricultural field adjacent to wetland. Deer and rabbit fences were erected around the study site to eliminate the impact of abundant local populations. Four treatments were replicated three times within each of two blocks (See Figure 2.3). The location of each treatment replicate plot was randomly assigned within the block and replicate plots were 3m x 4m in size. The treatments consisted of the following: (1) fallow/untreated control (C), (2) planted with *Kosteletzkya pentacarpos* (K), (3) planted with *Spartina patens* (S), and (4) both planted in combination (KS). With these treatments we sought to evaluate the potential of *K. pentacarpos* as a nurse crop for the common coastal colonizer *S. patens* and observe natural colonization as it occurred.

*K. pentacarpos* seed originating from Delaware (Sussex County, 38°44'01 N, 75°07'22 W) was planted in the K and KS plots in May of 2009 (Year 0) and was allowed to grow for a year prior to the experiment in order to simulate a salinized coastal agricultural setting where *K. pentacarpos* was grown in place of traditional crops. The site was maintained clear of other halophytes. In the spring of 2010 (Year

1), *S. patens* was planted in the S and KS treatments in the form of plugs purchased from Environmental Concern Nursery and originated from seed collected in Talbot County, Maryland (38°46'49 N, 76°07'55 W). Nine plugs were planted evenly within each plot and their positioning allowed for nine square quadrats of 50 cm by 50 cm to be flagged for observation within each replicate plot. Similar quadrats were marked in C and K plots. These nine permanent quadrats were established as an efficient way to monitor vegetation in the greater replicate plot area and their mean value was used to represent that plot in statistical analysis. A total of two hundred and sixteen quadrats were monitored throughout this experiment.

During the growing season, the plots were flood irrigated twice weekly from the neighboring tidal creek to simulate salt-water intrusion. The irrigation system was also linked to a freshwater source which allowed for salinity control. Specifically, the creek water salinity is approximately 28 during the summer; however the desired salinity for this project was approximately 22. Adjustments were made as necessary based on pore water salinity which was monitored frequently throughout the growing season. For this purpose, six PVC pipes ending in a porous porcelain cup were installed to a depth of 15 cm. Stoppers were used to seal the top of the pipes. Water samples were extracted using a syringe and read with a refractometer. Two HOBO temperature data loggers were installed at the site amongst the plants and monitored air and soil temperatures throughout the experiment. The soil probe was buried at approximately 15 cm depth and the air probe was positioned at 15cm height from the soil surface, and shielded from direct sunlight. A rain gage was installed on site and checked throughout the growing season and additional local rainfall data were provided by Joseph R. Scudlark (personal communication, University of Delaware), as

collected for the National Atmospheric Deposition Program – Atmospheric Integrated Research Monitoring Network (NOAA).

### **2.3.2 Species Measurements**

Natural colonization was allowed to occur, beginning in Year 1. Species and percent vegetative cover surveys were conducted regularly during the growing season for two consecutive years. Percent vegetative cover was calculated using a modified Daubenmire method (Mueller- Dombois 1974) whereby species presence and percent cover, within 50 cm by 50 cm fixed frame, were recorded and photographed.

Establishment of planted *S. patens* was monitored as described above. Additionally, morphological characteristics of planted *S.patens* clones were recorded at the beginning and end of the first growing season. Traits monitored were shoot height, number of shoots per clone, and clone circumference. Clone circumference included two measurements: an inner circumference which was defined as the circumference around the central clump of the plant where highest shoot density occurred, and an outer measurement which accounted for the distance around rhizome growth spread and areas of low shoot densities. Concurrently, morphological characteristics were also taken for *K. pentacarpos*. Stem number and height was recorded for each quadrat. Change in morphological traits was defined as the difference between the June and September value for that trait and used for further analysis.

At the end of the second growing season, above-ground biomass was harvested. In order to preserve the study for future work, only one representative

quadrat from each plot was harvested. The quadrat was carefully selected, based on recent vegetative cover data, as the most representative quadrat in the plot. Specifically, selection criteria required the plot to be characterized by (1) the presence of the three most dominant species of the replicate plot overall, (2) in quantities nearest to their mean vegetative covers for that replicate plot, and have (3) the highest representation of non-dominant species. Species present in the replicate plot but not in the quadrat selected for harvest, were sampled independently from another quadrat. The samples were stored at 4°C until processing. Samples were then sorted by species, dried to constant weight at 60°C and weighed. In this manner, a biomass measurement was matched to the recent vegetative cover measurement for the samples harvested and a biomass weight per unit of vegetative cover was calculated. This unit was then used to assign a biomass weight to each vegetative cover measurement of that species in the particular plot for quadrats not harvested. For example, if within the harvested quadrat of a replicate plot we found that *B. halimifolia*'s vegetative cover was 20% and its dry weight 5g, then for every one percent of vegetative cover *B. halimifolia* weighed 0.25g, and in another quadrat of the same plot for which *B. halimifolia* had vegetative cover of 15%, we could assign that it would have a biomass of approximately 3.75g. The process, described in the example above, was completed for all plots independently to maximize accuracy.

In order to evaluate the effect of the treatments on community diversity, the Shannon-Wiener diversity index was calculated using both species above-ground biomass and vegetative cover. Species counts were used to evaluate changes in community richness.

### 2.3.3 Soil Measurements

To address potential impact of *K. pentacarpos* on soil conditions, a fifteen centimeter depth core was taken randomly from each plot in the fall of Year 0 and Year 1. The location where the core was taken was determined by the blind toss of an object into the plot. Cores were cut in half lengthwise. The one half was processed by the University of Delaware Soil Testing Laboratory. Samples were analyzed for soluble salt content, pH, phosphorus, potassium, calcium, magnesium, manganese, zinc, copper, iron, boron, sulfur, aluminum, phosphorus saturation ratio, total carbon and total nitrogen, cation exchange capacity at pH 7, and particle size. In Year 1, we completed additional testing for cation exchange capacity of calcium, potassium, magnesium and sodium specifically. Soil soluble salt content was measured as the electrical conductivity of a soil solution (in mmhos/cm). Nutrients were analyzed by Mehlich-3 extraction. Total carbon and total nitrogen were evaluated via combustion. The remaining half core was cut in three 5 cm segments: 0-5 cm, 5-10 cm and 10-15 cm depth from the soil surface; organic matter for each depth section was evaluated through loss on ignition. We used an 1100° C Box Furnace, distributed by Lindberg/Blue. Samples were dried at 460° C for at least eight hours, and then weighed.

A single-ring infiltrometer was used to examine the impact of *K. pentacarpos* on soil infiltration rates, modified from Bertrand (1965). In August of Year 2, a short PVC ring of 15 cm diameter was driven to 5 cm depth in each of the C and K treatments with a mallet and core driver. Rings were leveled and marked so that water height at the same spot could be measured repeatedly. The plots were then flooded with fresh water and rings were checked for leaking before being filled by bucket. The distance of the water to the top of the ring was measured immediately and

at four known time intervals thereafter. Infiltration rate was calculated as the change in water height in mm over time in hours.

Soil surface leaf litter was measured in two ways during the second growing season (Year 2). A percent litter cover was estimated during vegetative cover measurements and litter was collected during biomass sampling and processed as previously described.

### **2.3.4 Statistical Analysis**

Soil soluble salt content and pH were analyzed as a change in that quality from Year 0 to Year 1. Morphological traits of plants were also processed as the change in height, circumference, or number of stems over the duration of the growing season (June to September). Variables for which quadrats were sampled, including vegetative cover, morphological traits, above-ground biomass, and number of species present; the mean value of the nine quadrats was considered the most accurate description of the particular replicate plot and used in statistical analysis.

Mixed model analysis of variance (ANOVA) was used to test differences ( $\alpha \leq 0.05$ ) between treatments, blocks, and time, for variables sampled. Treatment and time (year or months, when applicable) were treated as fixed factors. Blocks were treated as a random factor. Two-way mixed model ANOVA comparisons tested significance of the effect of treatments and blocks, and their interaction (for an example and model details see Appendix A, Table A1). For variables that were examined over a time factor, three-way mixed model ANOVA comparisons tested significance of the effect of treatments, blocks, time (year or month), and possible

interactions (for an example and model details see Appendix A, Table A2). All data were examined for normality and appropriately transformed when necessary to meet ANOVA assumptions. Tukey's HSD test was used for further comparison when appropriate.

All statistical analyses and graphs were completed in R, version 2.13.2 (R Development Core Team 2011). Scripts created for mixed model ANOVAs were tested for errors against example data sets.

## **2.4 Results**

### **2.4.1 Soil Properties**

The soil at our site was classified as sandy loam, with 73% sand, 18.6% silt, and 8% clay particles. Mean infiltration rates were measured to be 31 mm/hr in C plots and 43 mm/hr in K plots. In Year 0, before the experiment began, the pH of the soil was slightly basic averaging 8 and dropping to approximately 6 in Year 1. The change in soil pH was significantly different between blocks ( $p=0.03$ , 2-way mixed ANOVA). Block B had a greater increase in soil acidity; particularly in treatments C and S (see Figure 2.4).

With the initiation of the experiment in the summer of Year 1, we began salt-water irrigation and therefore an increase in soil salt content was expected. However, the change in soluble salts was significantly higher in Block B ( $p=0.01$ , 2-way mixed ANOVA) (See Figure 2.5). Mean pore water salinity remained fairly constant between the two growing seasons, and appeared to be determined primarily

by recent irrigation salinity. Mean pore water salinity across the growing season was 18 in Year 1 and slightly lower in Year 2 at 16.

Overall, cation exchange capacity at pH 7 of our site rated low (4-5 meq/100 g) compared to heavier inland agricultural loamy soil (>10 meq/100 g). In Year 1, when salt-water irrigation began, we completed specific cation exchange capacity tests and found that treatments and block were not different in soil retention of calcium, potassium, or magnesium. However, the mean quantity of sodium cations in Block A soil was 1.64 meq/100 g (SD  $\pm$  0.22) versus 2.27 meq/100 g (SD  $\pm$  0.74) for Block B. In this case, Block B soil was significantly higher in its sodium cation retention of sodium cations ( $p=0.019$ , 2-way mixed ANOVA) than Block A.

Precipitation was higher in Year 1 with an annual total rainfall of 98 cm, whereas Year 2 only received 91cm and was a dry year. Soil temperatures were similar throughout the two growing seasons, with an average daily maximum of 26° C in Year 1 and 27° C in Year 2, and common mean minimum daily temperature of 22.5° C. Over the winter season (December Year 1 through February Year 2), there was little variation in minimum and maximum daily temperatures and remained near freezing.

#### **2.4.2 Nutrients and Energy Cycling**

Treatments did not differ statistically in soil nutrient content (Table 2.1). In terms of primary nutrients: total nitrogen (%), soil phosphorus, and phosphorus saturation rate were similar between treatments, blocks, and years. Potassium increased significantly in Year 1 ( $p=0.0009$ , 3-way mixed ANOVA). In terms of secondary nutrients: calcium was significantly higher in Block A ( $p=0.03$ , 3-way

mixed ANOVA), sulfur ( $\log(y-1)$  transformed) was significantly higher in Year 1 ( $p=0.01$ , 3-way mixed ANOVA) and block B ( $p=0.001$ ), and magnesium levels showed no statistical difference. In terms of micronutrients: manganese levels (square-root transformed) were significantly higher in Block A ( $p=5.79E-07$ , 3-way mixed ANOVA) and in Year 0 ( $p=0.006$ ), boron (square-root transformed) was low at the beginning of the study but increased significantly to a high-medium rating in Year 1 ( $p=0.05$ , 3-way mixed ANOVA), and aluminum (square-root transformed), zinc, and copper levels did not vary significantly. Iron had a significant interaction between treatments and blocks ( $p=0.027$ , 3-way mixed ANOVA), in which treatments K and KS were higher in Block B than in block A, and was significantly higher in Year 1 ( $p=0.008$ ) and in Block B ( $p=0.006$ ).

Total carbon (%) of 15 cm cores did not differ between treatments, blocks, or years. For the core segments (0-5 cm, 5-10 cm, and 10-15 cm from the soil surface) tested separately for loss on ignition, organic matter ranged from 0.84 – 5.6%. Significant differences were only detected at the first depth, 0-5 cm from the soil surface, where treatment K had greater percent organic matter than other treatments ( $p=0.007$ , 3-way mixed ANOVA). This was confirmed with a Tukey's test. In addition, Block A was significantly higher than Block B ( $p=0.01$ ). However, we did not detect a difference between Year 0 and Year 1 (See Figure 2.6).

Percent cover of leaf litter (square-root transformed), sampled in August of Year 2, was different between treatments ( $p=0.009$ , 2-way mixed ANOVA) and blocks ( $p=0.0006$ ). A Tukey's test showed that treatments containing *K. pentacarpos* contained the highest leaf litter cover. Block A was higher than Block B. Litter biomass showed the same result. Treatments ( $p=0.005$ , 2-way mixed ANOVA) and

blocks ( $p=0.0002$ ) were significantly different, with greatest leaf litter biomass in treatments containing *K. pentacarpos* and Block A (See Figure 2.7).

### 2.4.3 Vegetation Dynamics

*K. pentacarpos* vegetative cover (arcsine transformed) in August was highest in Block A ( $p=0.0003$ , 3-way mixed ANOVA) and Year 1 ( $p=0.03$ ).

#### 2.4.3.1 Colonization

In order of decreasing occurrence, the experiment was colonized by: *Atriplex triangularis*, *Juncus gerardi*, *Spergularia salina*, *Baccharis halimifolia*, *Spartina patens*, *Phragmites australis*, *Aster tenuifolius*, *Distichlis spicata*, and a few seasonal weeds (See June in Figure 2.8 and August in Figure 2.9).

*A. triangularis*, an annual early-marsh colonizer, was found in all treatments. Its cover was higher in Block A and in Year 1. In Year 2, *A. triangularis*' increase in cover over the growing season appeared to be limited by high *S. patens* presence. *J. gerardi* had highest vegetative cover in the C treatment and peaked in early summer, which is consistent with the species early growing season (Bertness and Ellison 1987). Cover increased in the second year, except in the presence of *S. patens*. *B. halimifolia* was a rare occurrence in Year 1 but germinated broadly during the second summer and establishment was noted particularly in treatment K. Cover was higher in early summer and in Block A. *S. salina* was highest in Block B and in the first year. It had highest vegetative cover in early summer, which is consistent with the species flowering season, and was mostly litter by August. *P. australis*, an

invasive of particular interest, was found in a few replicate plots and its presence may have been linked to previous experiments at the site and therefore will not be explored further in this study. *A. tenuifolius* was found in C and K treatments primarily and only in the second summer (Year 2) (included under “Other” label in figure). Its cover was lower in the presence of *S. patens*. *D. spicata* was found in a few small clumps, primarily in open spaces with sparse cover (included under “Other” label in figure). Certain common weeds were observed but remained low overall due to salinity.

In order to determine the effect of *K. pentacarpos* on *S. patens* recruitment, we closely monitored naturally colonizing *S. patens* vegetative cover in C and K treatments during Year 1. Mean percent vegetative cover can be seen in Figure 2.10 and the specific locations, where *S. patens* clumps were identified, were cross-checked and consistent throughout the dataset. A significant interaction ( $p = 0.05$ ) of the main effects, treatment and month, was identified in a 3-way mixed ANOVA completed for naturally occurring *S. patens* cover in Year 1 overall, and to explore this relationship further analysis was completed for each month. The presence of *K. pentacarpos* was significantly related to higher colonization of *S. patens* in July ( $p < 0.0001$ , 2-way mixed ANOVA), August ( $p = 0.03$ ), and September ( $p = 0.009$ ), but not in June ( $p = 0.6$ ).

Throughout the growing season of Year 1, field observations indicated that the presence of *K. pentacarpos* may restrict the growth of the planted *S. patens* seedlings (See Figure 2.11). However, analysis showed only a nearly significant effect of treatment ( $p = 0.08$ , 2-way mixed ANOVA) on *S. patens* vegetative cover in September (Year 1), the time when maximum cover occurred. In Year 2, the plants continued to grow and expand over their second growing season and the effect of

treatment was significant ( $p=0.04$ , 3-way mixed ANOVA) in Year 2, with plants of greater cover in the SP treatment. However, the change in vegetative cover from June to August (Year 2) was not significantly different between treatments.

#### **2.4.3.2 Morphology**

Change in *K. pentacarpos* mean stem height from June to September (Year 1) was higher in Block A than in Block B ( $p=0.01$ , 2-way mixed ANOVA). The change in mean number of stems present was not different between treatments or blocks. Mean shoot height of *S. patens* was not statistically significant, however plants growing alongside *K. pentacarpos* were overall taller (See Figure 2.12). The effect of treatment on maximum shoot height was not significant ( $p=0.07$ , 2-way mixed ANOVA), with taller plants in the KS treatment in this case also. Inner (square-root transformed) and outer circumference (square-root transformed) was greater in Block B and the effect was particularly obvious in treatment S. There was a significant interaction of treatment and block for the inner circumference ( $p=0.026$ , 2-way mixed ANOVA), which can be attributed to the variation in degree of amplitude of the difference between the effect of treatments in the two Blocks. The increase in the number of shoots per clump was not statistically significant.

#### **2.4.3.3 Biomass**

Overall above-ground biomass results are shown in Table 2.2. *K. pentacarpos* biomass was significantly less in Block B than in Block A ( $p=0.035$ , 2-way mixed ANOVA). Above-ground biomass of naturally colonizing *S. patens* was

higher in the presence of *K. pentacarpos* than in control treatments ( $p=0.05$ , 2-way mixed ANOVA) and in Block B than in Block A ( $p=0.03$ ) (see Figure 2.13). Above-ground biomass of planted second-year *S. patens* was not significantly different among treatments. After removing the biomass of treatment species *K. pentacarpos* from K sums, treatments C and K still did not vary significantly in total community above-ground biomass productivity.

#### **2.4.3.4 Community Diversity and Richness**

Above-ground biomass was also used to calculate the Shannon-Wiener diversity index, (see Figure 2.14). The highest diversity index was associated with treatment K, with S and KS being the least diverse. Analysis revealed an interaction between treatments and blocks ( $p=0.004$ , 2-way mixed ANOVA) which is due to the increase in degree of difference between treatments in Block B. The diversity index analysis was repeated using percent vegetative cover data and yielded the same results, with treatment K and C being the most diverse. Species richness also followed the same pattern. The greatest increase in number of species present occurred in C and K treatments, whereas species richness in plots containing *S. patens* did not increase.

## **2.5 Discussion**

Our findings suggest that the use of *K. pentacarpos* as a transitional crop in salt-affected land has several benefits. *K. pentacarpos* did not negatively impact community diversity, richness, or productivity. The presence of *K. pentacarpos* improved natural recruitment of native halophytes *S. patens* and *B. halimifolia*. In

addition, we did not detect a significant negative effect on planted *S. patens* plugs in the immediate growing season or a year later. *K. pentacarpos* established leaf litter on the soil surface, and although there was no detectable change in soil nutrient content in this short time, an increase in soil organic matter in the upper layer was detectable after just one year of its growth at the site.

### 2.5.1 Interactions of Key Species

Our results indicate that *K. pentacarpos* had negligible impact on the growth of planted *S. patens* seedlings. In the first growing season, the change in both mean clone height and maximum clone height were greater when growing alongside *K. pentacarpos* and the increase in inner and outer clone circumference was greater in plants growing without *K. pentacarpos* (See Figure 2.12). Even though not all morphological variables sampled were significantly affected by treatment, the matching trends suggest that *S. patens* growing alongside *K. pentacarpos* allocated more energy toward vertical growth than *S. patens* growing alone. Dubinski (1987) found a similar pattern in *K. pentacarpos* growing in thick canopy and attributed the response to competition for light resources. Vegetative cover was also higher when *S. patens* was growing alone but was not significantly different when growing alongside the treatment species (See Figure 2.11). However, during the second growing season, planted *S. patens* under both treatments emerged larger and a mixed ANOVA (treatment, block, and month) showed that *S. patens* cover was greater in the S treatment ( $p=0.04$ ). This result can likely be explained by the insignificant, yet present, difference in growth of seedlings during the first year. In Figure 2.11, we note that planted *S. patens* growing alone are larger by September, although not

significantly, than those growing alongside *K. pentacarpos*. This small advantage of seedlings in the S treatment most likely translated to a higher cumulative reserve of energy and a significant head-start in size by the following June. However, the change in vegetative cover over the course of the second growing season was not different and neither was the final above-ground biomass sampled at the end of the second growing season (See *S. patens* biomass of S and KS treatment in Table 2.2), suggesting that any difference in growth is overall small and insignificant to the survival and productivity of the planted seedlings.

*S. patens* did not seem to impact the growth of *K. pentacarpos* either. *K. pentacarpos* is not a dominant species and is found scattered throughout brackish wetlands and salt-affected lands. Although the vegetative cover of *K. pentacarpos* declined significantly in Year 2, paralleling strong new spring growth by second year *S. patens* plants, *K. pentacarpos* cover was not greater when growing alone. This overall decline in cover could be attributed to various environmental factors and patchy spring regrowth was also observed at a second experimental site that year (8 km from our study site). The reason behind this occurrence is not clear; however prolonged freezing of the soil is one possible explanation (See Appendix Figure A1 and A2 for recorded winter air and soil temperatures). Alternatively, an accumulation of salt in the soil over the course of our study could reduce *K. pentacarpos* growth. It is a facultative halophyte that can tolerate approximately 25. Although flower and seed production is increased by moderate levels of salinity (Dubinski 1987), plants tend to grow larger in fresher conditions (communication with J.L. Gallagher). *K. pentacarpos* cover was also significantly lower in Block B than in Block A. The differences between the two blocks, only obvious in hindsight, have had an effect on

the halophyte comparable to that of increased salinity. During our study, plots were flood irrigated twice weekly with water pumped from the nearby tidal creek and after several hours drained to remove standing water. Block B did not drain as readily as Block A, and was therefore likely subject to greater evaporation rates leading to higher salt accumulation. Our soil analysis in Year 1 supports this possibility as soluble salt content (See Figure 2.5) and sodium cation retention capacity (1.64 meq/100 g in Block A and 2.27 meq/100 g in Block B) were significantly higher in Block B. Based on our understanding of *K. pentacarpus* ecophysiology, this evidence could explain the stunted vegetative cover, biomass, and stem height detected in Block B and in Year 2.

Overall, we did not detect a negative interaction between mature *K. pentacarpus* and *S. patens*. Most importantly, *K. pentacarpus* did not hinder the growth or cause recorded mortality of planted *S. patens*, which is a desirable trait in a nurse crop proposed to improve conditions for establishment of wetlands. Indeed, by the second year of our study replicate plots in the KS combined treatment were beginning to mirror the natural hierarchy in which one would find these two species in a natural wetland. Additionally, the natural colonization of *S. patens* was significantly higher with *K. pentacarpus* than in the control treatment. Vegetative cover throughout the first growing season was greater in the K treatment (See Figure 2.10), as was the above-ground biomass harvested the following year, at the end of the study. Biomass of naturally occurring *S. patens* in the K treatment was 2.9 times higher for Block A and 1.4 times higher in Block B than in the control treatment C (See Table 2.2).

In addition to *S. patens*, our study area was successfully colonized by a variety of annuals and perennials. Area not cover by the planted treatment species in

Year 1 was occupied by the annual *A. triangularis* and *S. salina*. In Year 2, incidence increased of the perennial *B. halimifolia*, *J. gerardi*, and the annual *A. tenuifolius*. Control replicate plots were the least consistent in species composition and varied in terms of most dominant species. The robust emergence of planted *S. patens* individuals during the second growing season increased its dominance in S plots and species richness in these plots decreased (See Figure 2.9). Specifically, the vegetative cover of *A. triangularis*, *J. gerardi*, and *A. tenuifolius* appeared limited by the presence of *S. patens* in Year 2. This result is contrary to that in Bertness (1991) where *J. gerardi* was able to dominate over *S. patens* in the high marsh because of its earlier growing season (late February), therefore having competitive advantage of open space. However, Bertness (1991) also noted that *J. gerardi* is not very tolerant of environmental stress such as flooding and high salinity, both of which were regular in our experiment and are tolerated well by the early colonizer *S. patens*, which increased its dominance. On the other hand, *K. pentacarpos* plots were rich in *A. triangularis*, *B. halimifolia*, and *S. patens*.

Research has shown that successful establishment of wetland species is highly related to the availability of seeds, tying a newly created or restored site to neighboring wetlands (Erfanzadeh et al. 2010). Our site was upland to a natural marsh, although separated by upland hedgerow, and adjacent to other experimental plots. Therefore seeds introduced to this study could come from two main sources: (1) seeds that were carried in by irrigation water from the marsh creek, and (2) seeds that were dispersed by neighboring plots by the wind. We would expect that coastal agricultural land flooded regularly during sea-level rise would also be exposed to desirable seeds in a similar fashion. If this is true, our results suggest that cultivation

of *K. pentacarpos* during transition from working land to natural habitat will improve the recruitment and establishment of desirable wetland species.

### **2.5.2 Ecosystem Engineering Considerations**

For salinized agricultural land that can be reclaimed by native vegetation, some might argue for either a direct planting of the project site with desirable species or a hands-off management approach. Other than bypassing the opportunity to prolong the economic benefits to the farmer and allowing for an ecologically valuable long-term succession to occur, these strategies have certain risks. Coastal agricultural soil, with long-term application of fertilizers and cultivation of shallow-rooted annual species, differs from undisturbed land in a natural habitat. Garbutt et al. (2006) studied the development of an intertidal system in former agricultural land and identified properties of agricultural soil that were destructive to the establishment of planted wetland species. Firstly, the architecture of the soil created depressions in which standing water accumulated and killed off introduced plants via waterlogging. Secondly, the working soil's properties were not conducive to drainage of water through the water table. *K. pentacarpos* has an extensive root system, with a large number of fine roots, increasing its resilience to environmental stressors and reinforcing the soil against erosion (Halchak et al. 2011). Therefore, the cultivation of such a perennial would increase soil micro-channels thereby improving water infiltration. Our results showed this to be true; as infiltration rates were higher in *K. pentacarpos* replicate plots (43 mm/hr) than in control plots (31 mm/hr) after just two growing seasons. In addition, *K. pentacarpos*'s heavy leaf and stem litter would increase texture of soil surface and potentially encourage sedimentation by the

increasingly intruding tides. The application of a nurse crop provides many additional benefits to soil environment, including limiting soil temperature fluctuations, ameliorating salinity through hydraulic lift and reduction of evaporation, and improving the availability of nutrients (Padilla and Pugnaire 2006).

A second risk that may be avoided by the use of a transitional nurse crop is that of invasive species. Garbutt et al. (2006) determined that the introduction of desirable species was not necessary in the United Kingdom when there are adjacent marshes containing species by which the project site can be colonized. However, invasion by *Phragmites australis* is a costly problem in the eastern North American region. Agricultural land was shown to be highly associated with *P. australis* presence (Chambers et al. 2008) and disturbed land without canopy cover is most likely to become invaded by *P. australis* (Baldwin and Mendelsohn 1998). Figure 2.1 (b) shows recently salinized farmland due to tidal flooding during storm events in Kent County, Delaware, and *P. australis* invasion is evident at the edges of the farm field. The cultivation of a transition crop would be preferable over allowing salinized land to become bare. In addition, Wang et al. (2006) determined that the presence of a “multi-layered wall of plants” was an effective way to reduce *P. australis* growth. Ecosystems of higher diversity are less prone to invasion because they are more likely to already have various species exploiting a broad range of niches. In our study, replicate plots with *K. pentacarpos* had higher productivity (above-ground biomass) than control plots and less productivity than *S. patens* plots, however in plots containing *S. patens*, biomass was primarily composed of *S. patens*. K plots were more diverse than *S. patens*-containing plots and more than or equally as diverse as control plots. This means that a community started around *K. pentacarpos* would be

more diverse than a community started directly with *S. patens* and more productive than a community that developed unmanaged. Therefore, the use of *K. pentacarpos* as a transitional crop would increase diversity and canopy cover, the most desirable combination for a healthy community with minimized risk of invasion.

As previously mentioned, *P. australis* occurred in specific sites in our study and its growth may have been associated with previous experiments at this location, likely originating from pre-existing rhizome growth. Unfortunately, due to this possibility we cannot make any conclusive statements about the direct interaction between *K. pentacarpos* and *P. australis*. However, Wang et al. (2006) conducted a study employing vegetative blocking of *P. australis* in a newly restored marsh site, and noted that *Baccharis halimifolia* showed promising inhibition of *P. australis* growth, potentially due to shading by its thick canopy. And in our study, *Baccharis halimifolia* had the highest vegetative cover and biomass in the presence of *K. pentacarpos* (See Figure 2.9 and Table 2.2). In addition, leaf litter accumulation associated with *K. pentacarpos* could also potentially act as a barrier for the growth of invasive species. Spence (1982) reported that the interception of light by leaf litter can reduce the germination of freshwater *Phragmites* sp. It would be interesting to determine if the facilitation of *B. halimifolia* germination and establishment, and an increase in leaf litter could be indirect pathways of potential inhibition or reduction of *P. australis* invasion by *K. pentacarpos*.

Leaf litter cover and biomass were significantly higher in treatments containing *K. pentacarpos* (see Figure 2.7). The function of leaf litter in nutrient and energy cycling is very crucial (see Facelli and Pickett 1991 for review). In one year our study showed no impact of treatment on soil nutrient content, however it would

likely do so given a longer time frame. We did detect an increase in organic matter content associated with *K. pentacarpos* in the top five centimeters of soil (See Figure 2.6). Therefore, we would expect to see more nutrients cycling through this system than in control treatments, or an agricultural field which previously only hosted annual crops. Variation in soil nutrients may have been detectable in a study of longer duration and we expect to detect differences in future samplings of this site.

In our experiment, *K. pentacarpos* was planted at the location only one year prior to the experiment. In contrast, under working conditions we would expect that the biofuel crop would be cultivated for multiple years, specifically from the point when traditional crops can no longer be grown to the time when flooding is too regular for functional and efficient use of the land. In addition, our comparison only accounts for two years whereas we would expect a transition from coastal agricultural land to natural habitat would take many years of succession. Mitsch and Wilson's review (1996) on creation and restoration of project systems encourages managers to allow for the time required for the system to naturally develop and argue that large scale studies would greatly improve our understanding of ecosystem creation. Although expensive and difficult to conduct, long-term studies are ideal. However, this study simulated and tested many practical hypotheses and serves as a useful tool for the exploration of plant interactions that would occur under transitional conditions.

## **2.6 Conclusions**

Whether coastal agricultural fields that become unproductive during sea level rise have the potential to transition into functional wetlands depends on the specific character and hydrology of the site and the time scale of evaluation. The final

design of a created wetland system is really dependent on local characteristics. However, the growth of *K. pentacarpos* can supply an economic purpose to the land during its salinization and has the potential to increase recruitment of desirable native vegetation as it transitions to wetland, while reducing space available for invasion. Unless the wetland is allowed to migrate upward, this native vegetation is already at risk due to increased sea-level rise and coastal development. Therefore such a strategy is a very appropriate and positive use of a native halophytic nurse crop toward the engineering of opportunities for a better future for coastal ecosystems.

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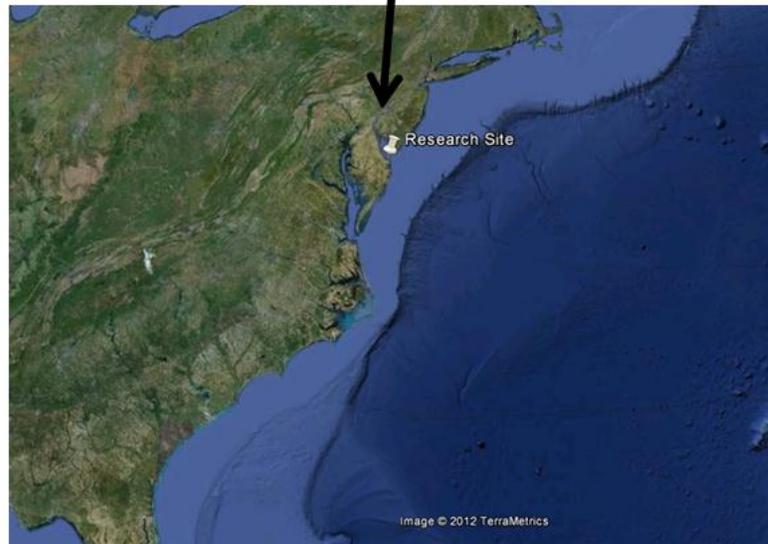


(a)

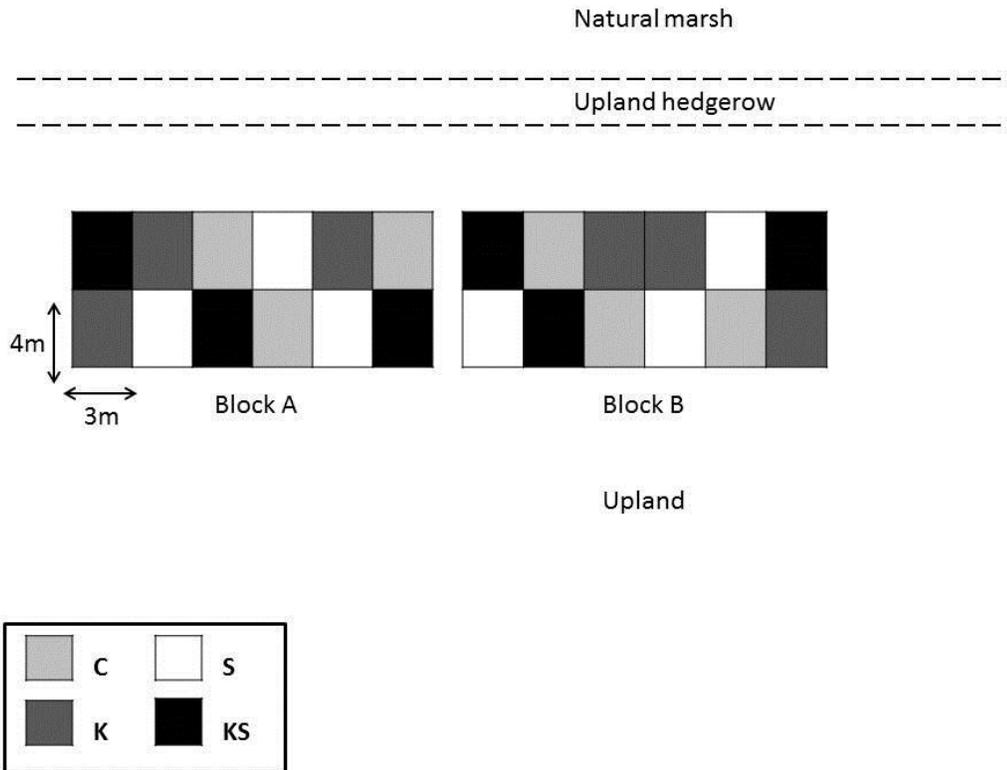


(b)

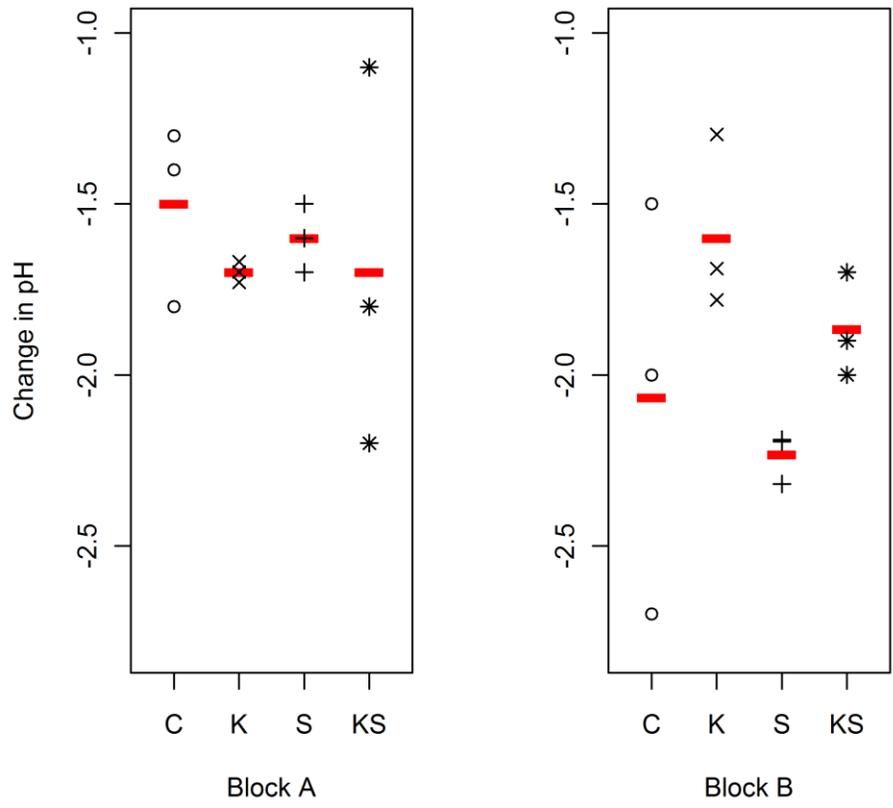
**Figure 2.1** Examples of coastal agricultural land in Delaware, U.S.A., impacted by: (a) salinization and (b) salinization and *P. australis* invasion.



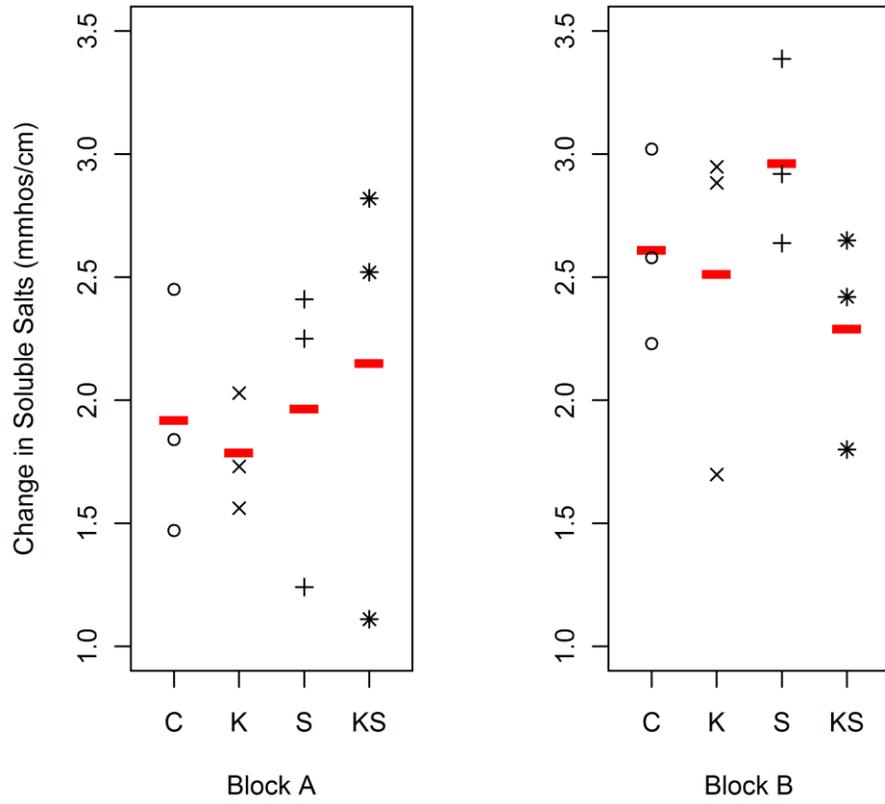
**Figure 2.2** The field site location for this project on the grounds of the University of Delaware’s H. R. Sharp campus in Lewes, Delaware. The experimental plots are upland to natural salt marsh (rectangle), and the irrigation system and water pump (circle) are also visible.



**Figure 2.3** The experimental design was a complete randomized block design with replication. The treatments were: (1) C, a control that was untreated and fallow, (2) K was planted with *K. pentacarpos*, (3) S was planted with *S. patens*, and (4) KS was planted with both species.



**Figure 2.4** Change in soil pH from the fall of Year 0 to the fall of Year 1 by treatment and block. Each point represents a replicate plot and the red bar signifies the mean.



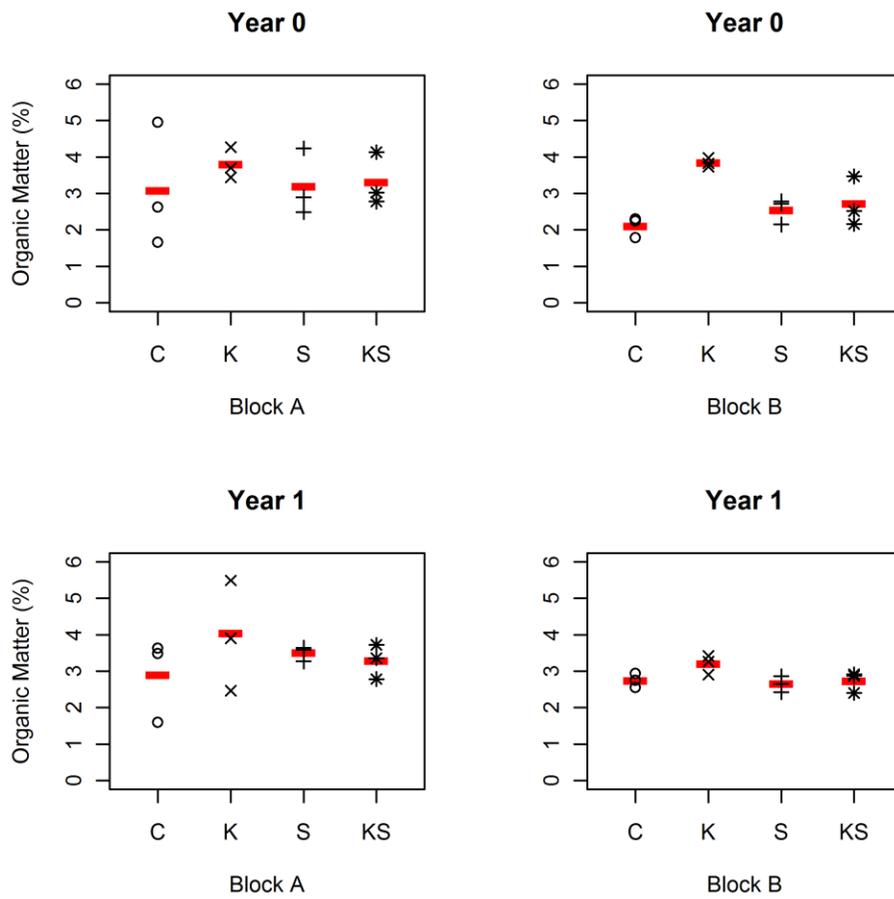
**Figure 2.5** Change in soluble salt content of the soil from the fall of Year 0 to the fall of Year 1. Each point represents a replicate plot and the red bar signifies the mean.

**Table 2.1 Mean nutrient levels ( $\pm$  standard error of the mean) in Year 0 and Year 1 for treatments and blocks. Transformation and significance of three-way mixed model ANOVA are noted on the left for block (B) and year (Y). No significant effect of treatment was detected. Significance is noted as: ' = 0.05, \* < 0.05, \*\*  $\leq$  0.01, \*\*\*  $\leq$  0.001, \*\*\*\* < 0.0001.**

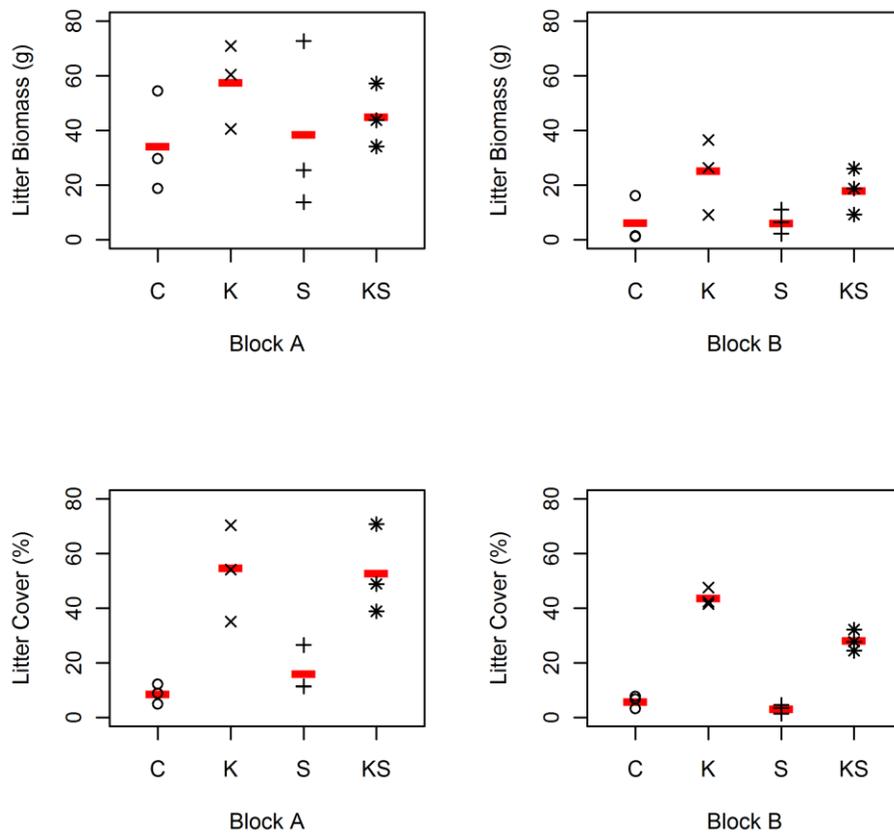
Nutrient		Treatment					
			C	K	S	KS	MEAN
<b>Phosphorus</b>	A	Year 0	175.59 ( $\pm$ 23.36)	171.21 ( $\pm$ 22.93)	173.61 ( $\pm$ 21.73)	136.43 ( $\pm$ 25.74)	164.21
		Year 1	194.81 ( $\pm$ 36.32)	205.96 ( $\pm$ 15.48)	199.24 ( $\pm$ 26.06)	160.58 ( $\pm$ 13.31)	190.15
	B	Year 0	128.43 ( $\pm$ 31.76)	138.20 ( $\pm$ 20.56)	142.50 ( $\pm$ 28.59)	194.86 ( $\pm$ 27.75)	151.00
		Year 1	157.89 ( $\pm$ 30.82)	175.65 ( $\pm$ 21.31)	168.73 ( $\pm$ 22.25)	184.19 ( $\pm$ 18.70)	171.62
<b>Potassium</b>	A	Year 0	108.37 ( $\pm$ 11.49)	97.52 ( $\pm$ 12.78)	107.37 ( $\pm$ 6.13)	111.64 ( $\pm$ 11.60)	106.22
		Year 1	125.01 ( $\pm$ 10.36)	124.60 ( $\pm$ 1.24)	117.61 ( $\pm$ 5.47)	110.11 ( $\pm$ 3.46)	119.33
	B	Year 0	98.39 ( $\pm$ 6.72)	106.35 ( $\pm$ 9.18)	89.88 ( $\pm$ 4.13)	116.06 ( $\pm$ 9.64)	102.67
		Year 1	116.42 ( $\pm$ 3.51)	123.42 ( $\pm$ 3.56)	112.32 ( $\pm$ 8.92)	110.79 ( $\pm$ 5.08)	115.74
<b>Calcium</b>	A	Year 0	231.95 ( $\pm$ 82.86)	210.86 ( $\pm$ 7.84)	198.59 ( $\pm$ 9.36)	192.53 ( $\pm$ 16.60)	208.49
		Year 1	194.58 ( $\pm$ 29.19)	201.16 ( $\pm$ 13.11)	181.92 ( $\pm$ 10.80)	161.25 ( $\pm$ 6.23)	184.73
	B*	Year 0	154.84 ( $\pm$ 10.75)	203.28 ( $\pm$ 27.99)	174.10 ( $\pm$ 16.35)	169.08 ( $\pm$ 21.51)	175.33
		Year 1	147.46 ( $\pm$ 2.84)	190.95 ( $\pm$ 0.20)	155.87 ( $\pm$ 18.11)	155.10 ( $\pm$ 17.29)	162.35
<b>Sulfur</b>	A	Year 0	15.69 ( $\pm$ 1.44)	14.99 ( $\pm$ 1.81)	16.11 ( $\pm$ 2.04)	13.76 ( $\pm$ 0.46)	15.14
		Year 1	70.26 ( $\pm$ 4.98)	71.04 ( $\pm$ 2.35)	69.62 ( $\pm$ 13.35)	59.89 ( $\pm$ 12.03)	67.70
	B	Year 0	20.05 ( $\pm$ 2.37)	17.03 ( $\pm$ 0.83)	18.44 ( $\pm$ 3.74)	21.25 ( $\pm$ 1.08)	19.19
		Year 1	92.05 ( $\pm$ 15.88)	100.08 ( $\pm$ 19.96)	98.28 ( $\pm$ 27.25)	93.27 ( $\pm$ 19.63)	95.92
<b>Magnesium</b>	A	Year 0	170.94 ( $\pm$ 44.14)	162.00 ( $\pm$ 15.24)	156.05 ( $\pm$ 6.79)	134.02 ( $\pm$ 13.18)	155.75
		Year 1	223.22 ( $\pm$ 21.77)	196.18 ( $\pm$ 2.47)	215.40 ( $\pm$ 3.28)	174.80 ( $\pm$ 9.75)	202.40
	B	Year 0	130.71 ( $\pm$ 13.72)	142.78 ( $\pm$ 15.77)	147.31 ( $\pm$ 23.11)	132.39 ( $\pm$ 21.59)	138.30
		Year 1	192.54 ( $\pm$ 7.79)	223.43 ( $\pm$ 17.79)	215.13 ( $\pm$ 14.79)	186.56 ( $\pm$ 24.90)	204.41

**Table 2.1 Continued.**

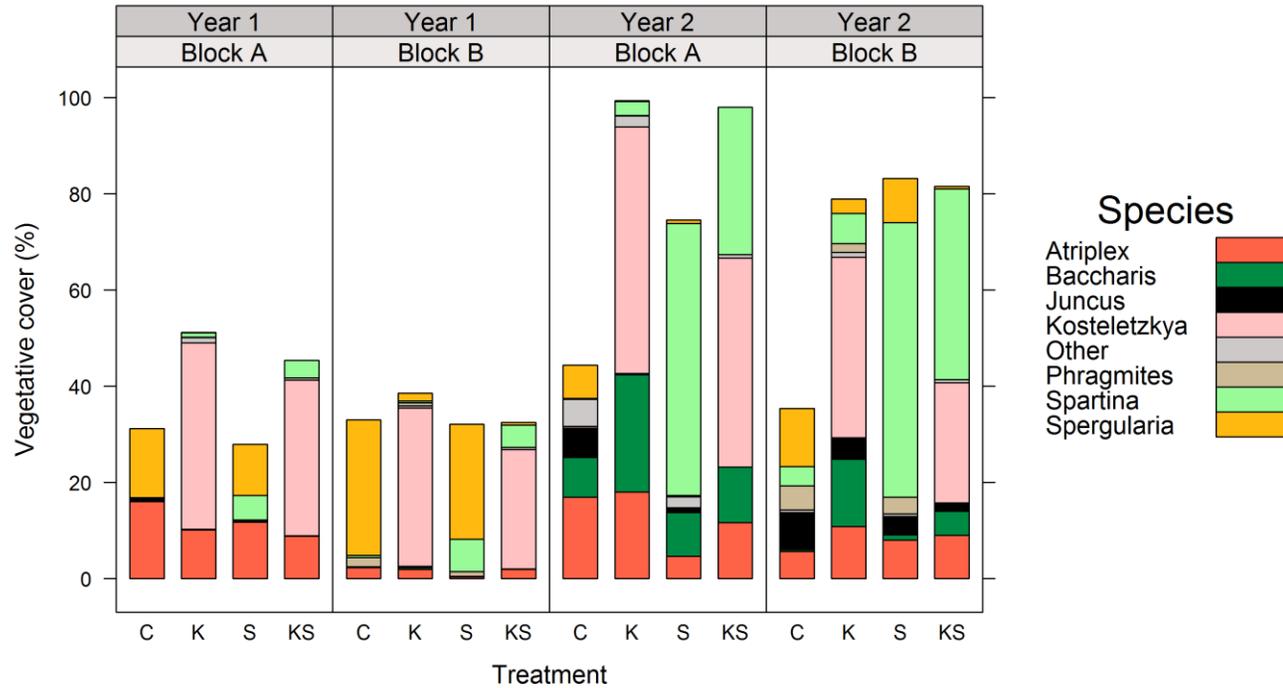
			C	K	S	KS	MEAN
<b>Manganese</b>	A	Year 0	8.03 ( $\pm 1.67$ )	15.27 ( $\pm 5.89$ )	9.40 ( $\pm 2.81$ )	18.84 ( $\pm 3.87$ )	12.89
		Y**	Year 1	8.54 ( $\pm 2.28$ )	10.82 ( $\pm 4.32$ )	8.32 ( $\pm 4.37$ )	8.27 ( $\pm 0.96$ )
	B	Year 0	6.09 ( $\pm 1.78$ )	4.92 ( $\pm 1.22$ )	3.39 ( $\pm 0.97$ )	4.08 ( $\pm 1.58$ )	4.62
		Year 1	2.76 ( $\pm 0.96$ )	3.12 ( $\pm 1.20$ )	2.42 ( $\pm 0.82$ )	1.48 ( $\pm 0.21$ )	2.45
<b>Boron</b> sq. root	A	Year 0	0.74 ( $\pm 0.12$ )	0.60 ( $\pm 0.03$ )	0.59 ( $\pm 0.04$ )	0.58 ( $\pm 0.09$ )	0.63
		Y'	Year 1	1.22 ( $\pm 0.12$ )	1.08 ( $\pm 0.09$ )	1.15 ( $\pm 0.04$ )	0.95 ( $\pm 0.04$ )
	B	Year 0	0.54 ( $\pm 0.06$ )	0.53 ( $\pm 0.05$ )	0.54 ( $\pm 0.07$ )	0.62 ( $\pm 0.12$ )	0.56
		Year 1	1.07 ( $\pm 0.03$ )	1.20 ( $\pm 0.03$ )	1.12 ( $\pm 0.13$ )	1.20 ( $\pm 0.16$ )	1.10
<b>Iron</b>	A	Year 0	264.19 ( $\pm 44.57$ )	211.79 ( $\pm 30.64$ )	167.86 ( $\pm 21.90$ )	198.92 ( $\pm 13.33$ )	210.69
		Y**	Year 1	318.58 ( $\pm 43.46$ )	282.59 ( $\pm 20.46$ )	308.56 ( $\pm 21.21$ )	262.09 ( $\pm 7.42$ )
	B	Year 0	234.73 ( $\pm 40.50$ )	258.67 ( $\pm 31.38$ )	186.48 ( $\pm 15.43$ )	345.81 ( $\pm 45.62$ )	256.42
		T:B*	Year 1	343.68 ( $\pm 36.82$ )	372.86 ( $\pm 32.34$ )	285.96 ( $\pm 12.61$ )	343.63 ( $\pm 32.27$ )
<b>Zinc</b>	A	Year 0	6.31 ( $\pm 0.46$ )	5.56 ( $\pm 0.67$ )	5.36 ( $\pm 0.72$ )	5.75 ( $\pm 0.60$ )	5.75
		Year 1	9.26 ( $\pm 0.96$ )	10.00 ( $\pm 1.17$ )	8.07 ( $\pm 0.53$ )	9.12 ( $\pm 0.63$ )	9.11
	B	Year 0	5.36 ( $\pm 0.59$ )	8.06 ( $\pm 1.38$ )	5.09 ( $\pm 0.88$ )	5.80 ( $\pm 0.95$ )	6.08
		Year 1	9.10 ( $\pm 0.33$ )	9.04 ( $\pm 0.46$ )	7.82 ( $\pm 1.22$ )	7.31 ( $\pm 0.26$ )	8.32
<b>Aluminum</b> sq. root	A	Year 0	1324.88 ( $\pm 53.76$ )	1150.77 ( $\pm 194.32$ )	1026.00 ( $\pm 6.5$ )	1529.79 ( $\pm 185.32$ )	1257.9
		Year 1	1247.42 ( $\pm 33.99$ )	1265.97 ( $\pm 30.90$ )	1229.98 ( $\pm 30.5$ )	1199.66 ( $\pm 11.99$ )	1235.8
	B	Year 0	1622.14 ( $\pm 154.2$ )	1157.43 ( $\pm 199.05$ )	1013.56 ( $\pm 88.3$ )	1346.75 ( $\pm 44.60$ )	1285.0
		Year 1	1162.31 ( $\pm 68.44$ )	1176.82 ( $\pm 12.83$ )	1136.06 ( $\pm 69.3$ )	1154.24 ( $\pm 51.25$ )	1157.4
<b>Copper</b>	A	Year 0	10.09 ( $\pm 1.08$ )	8.67 ( $\pm 1.00$ )	9.38 ( $\pm 1.40$ )	9.62 ( $\pm 1.32$ )	9.44
		Year 1	15.58 ( $\pm 2.34$ )	17.00 ( $\pm 1.61$ )	13.45 ( $\pm 1.52$ )	16.10 ( $\pm 1.63$ )	15.53
	B	Year 0	9.27 ( $\pm 1.26$ )	11.69 ( $\pm 1.79$ )	7.49 ( $\pm 1.61$ )	7.79 ( $\pm 0.37$ )	9.06
		Year 1	15.04 ( $\pm 0.51$ )	14.42 ( $\pm 1.19$ )	13.28 ( $\pm 2.63$ )	12.08 ( $\pm 0.20$ )	13.71



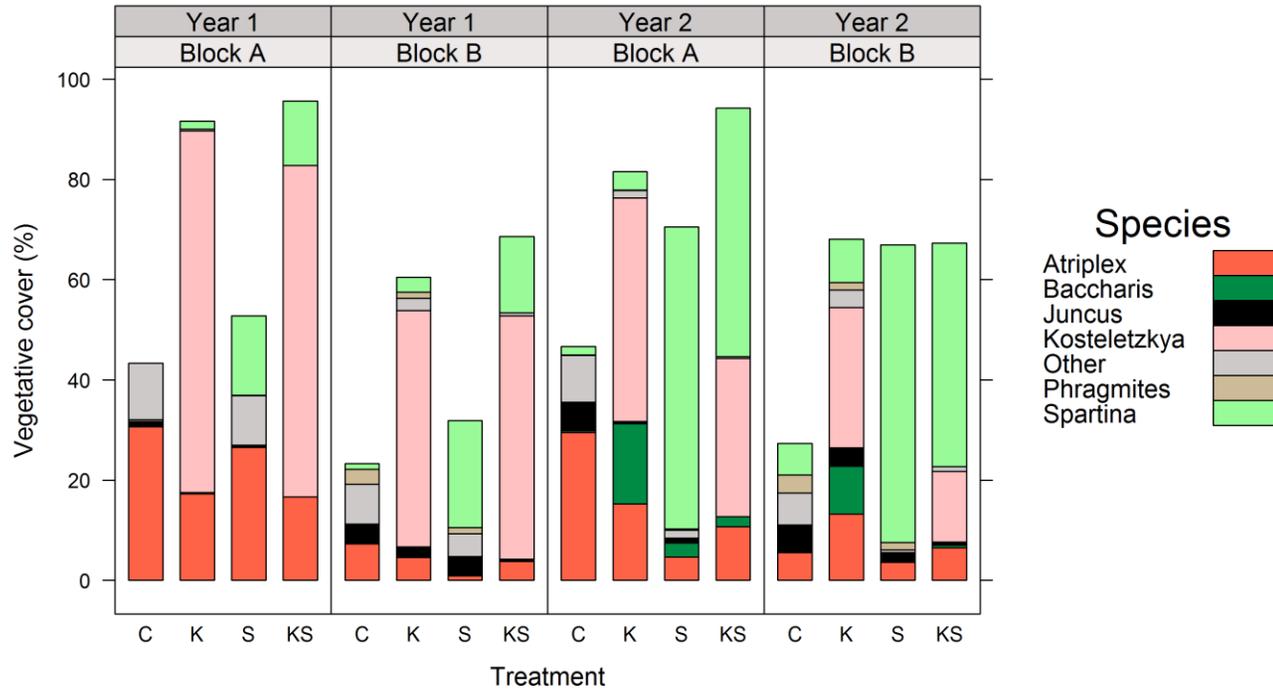
**Figure 2.6** Organic matter content (%) of the top 0-5 cm depth of soil in treatments and blocks from the fall of Year 0 and Year 1. Each point represents a replicate plot and the red bar signifies the mean.



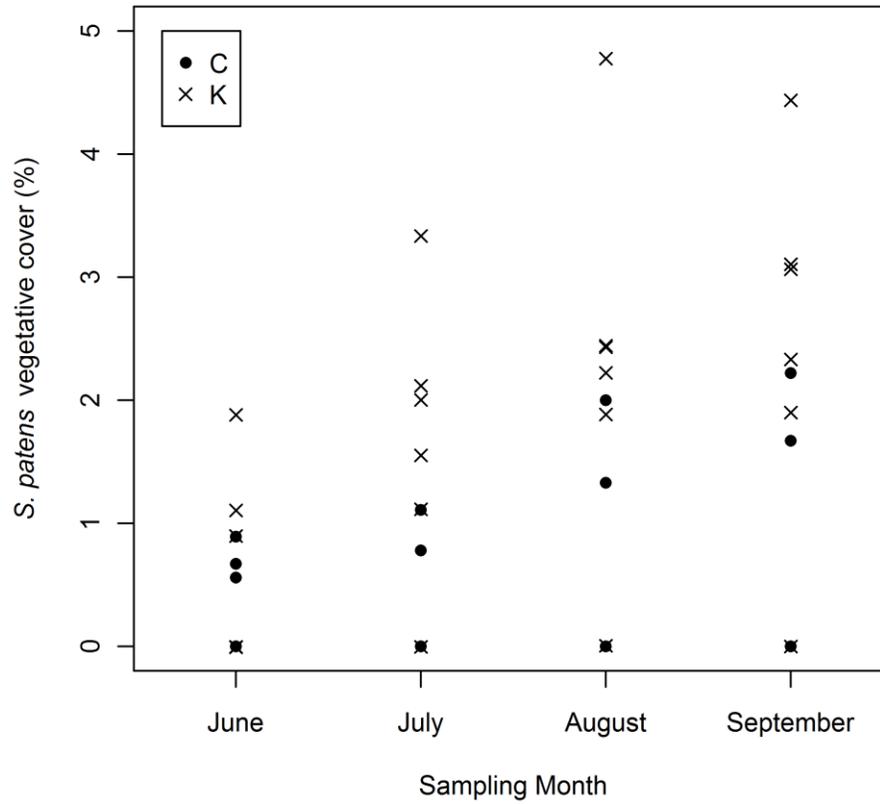
**Figure 2.7** Leaf litter biomass (g) and cover (%) as measured in August of Year 2 for treatments and blocks. Each point represents a replicate plot and the red bar signifies the mean.



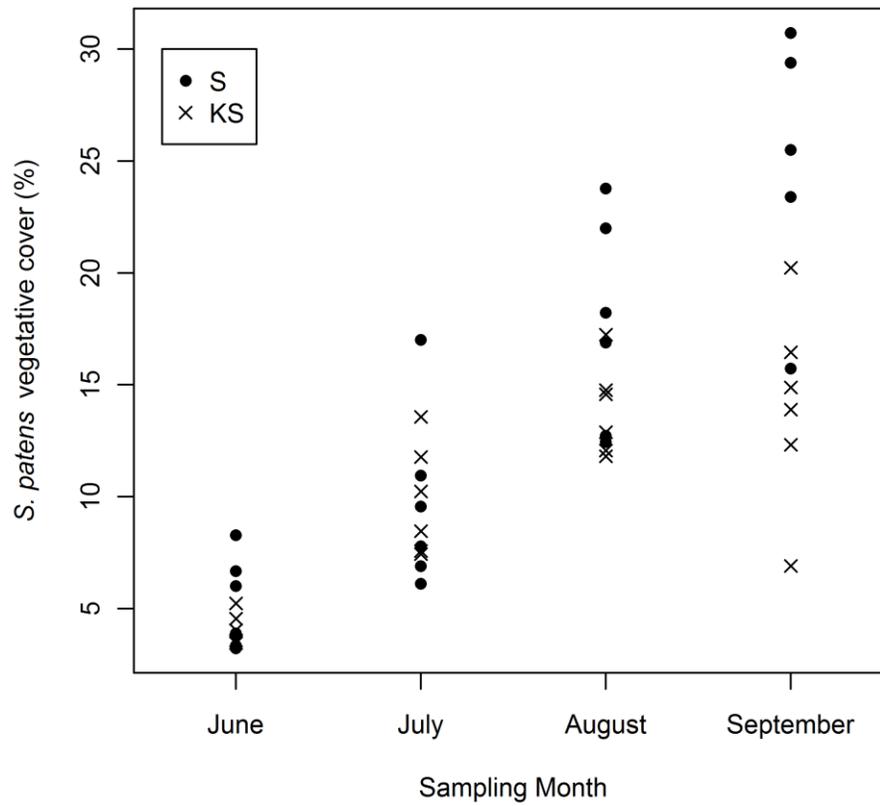
**Figure 2.8** Species composition (% vegetative cover) of treatments and block in June of Year 1 and Year 2. For each species, the specified stalked bar represent the mean percent vegetative cover of three replicate plots. The label 'Other' denotes less common species: *A. tenuifolius*, *D. spicata*, *Rumex crispus* L., *Solidago odora* Aiton, and *Cyperus esculentus* L.



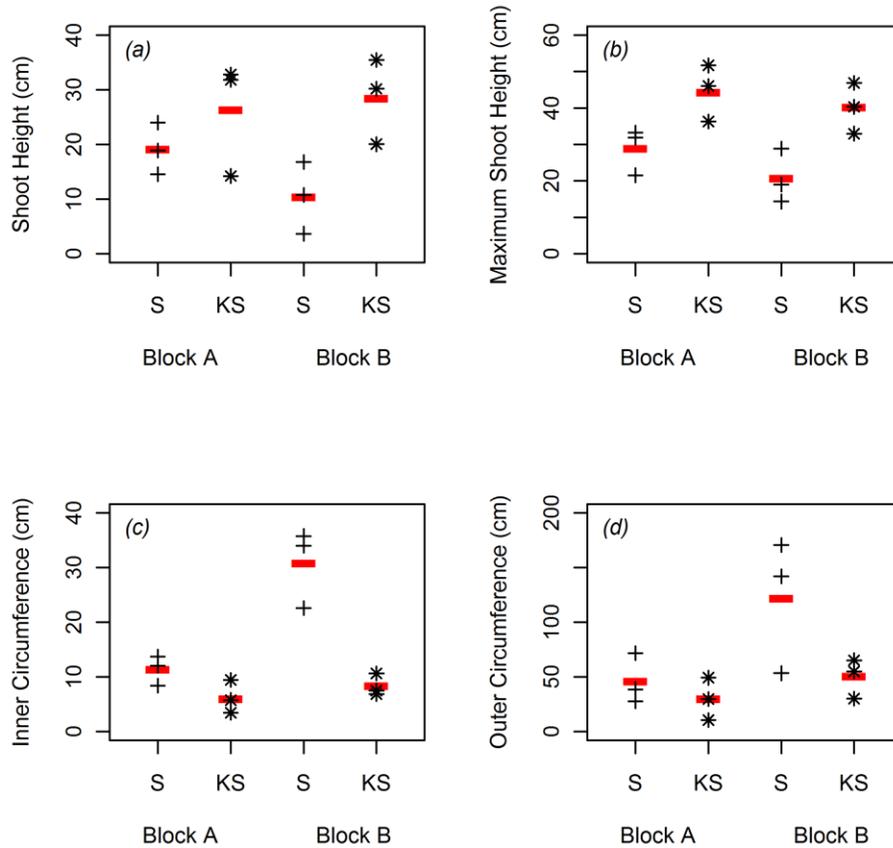
**Figure 2.9** Species composition (% vegetative cover) of treatments and blocks in August of Year 1 and Year 2. For each species, the specified stalked bar represent the mean percent vegetative cover of three replicate plots. The label ‘Other’ denotes less common species: *A. tenuifolius*, *D. spicata*, *R. crispus*, *S. odora*, and *C. esculentus*.



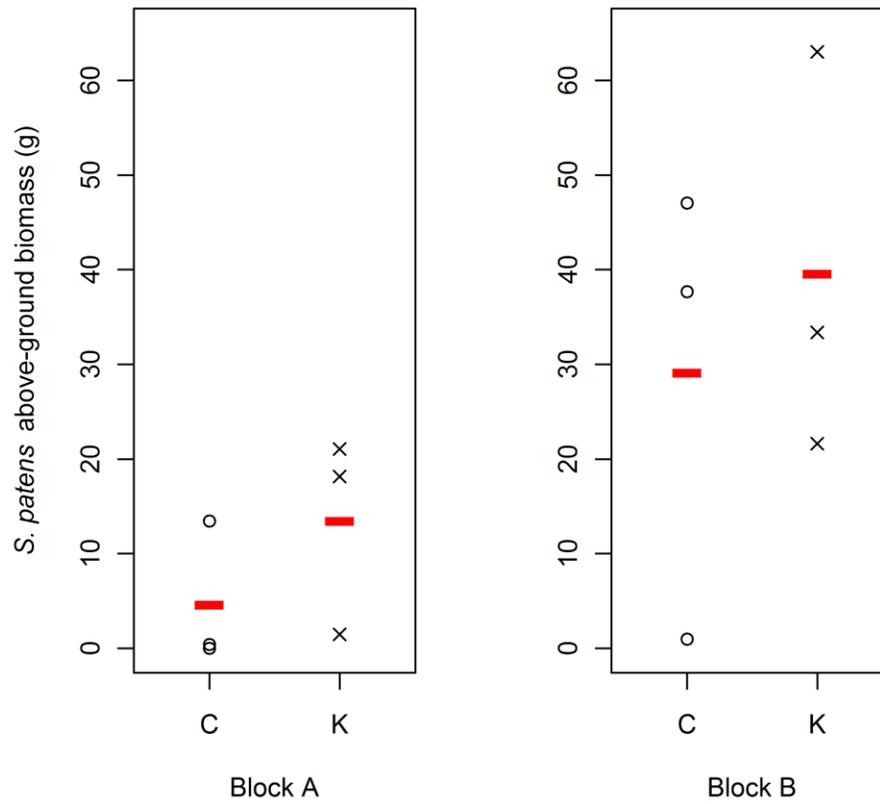
**Figure 2.10** Natural colonization of *S. patens* during the growing season of Year 1 in C (control) and K (*K. pentacarpos*) treatments. Points represent vegetative cover (%) for each of six replicate plots.



**Figure 2.11** Growth of planted *S. patens* during Year 1 of this study. *S. patens* were planted in May of Year 1: alone in the S treatment and alongside *K. pentacarpos* in KS. Points represent vegetative cover (%) for each of six replicate plots.



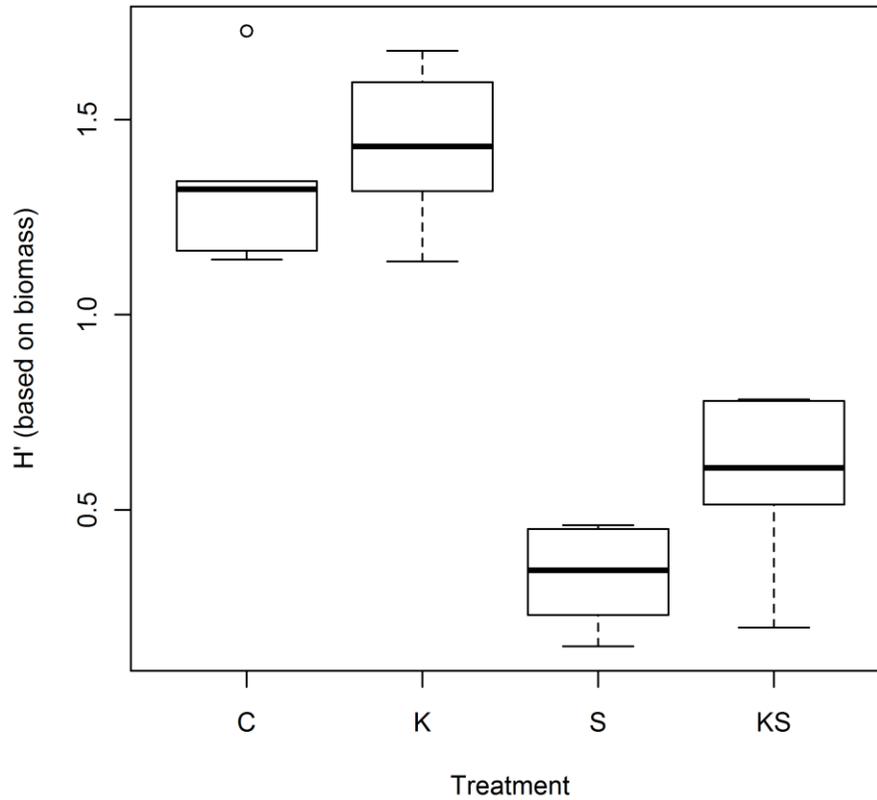
**Figure 2.12** Change in morphological traits of planted *S. patens* in treatments and blocks across the growing season of Year 1: (a) mean shoot height (cm), (b) maximum shoot height (cm), (c) inner clone circumference (cm), and (d) outer clone circumference (cm). Each point represents a replicate plot and red bars signify the mean.



**Figure 2.13** Above-ground biomass of naturally colonizing *S. patens* in C (control) and K (*K. pentacarpos*) treatments, as harvested at the end of the second growing season (Year 2). Each point represents one replicate plot and red bars signify the mean.

**Table 2.2** Above-ground biomass (g) for treatments and blocks, as harvested in early September of Year 2. Numbers are the mean of three replicate plots. *S. patens* in S and KS treatments and *K. pentacarpos* in K and KS treatments were planted. All other species occurrence is natural.

Species	C		K		S		KS	
	Block A	Block B						
<i>A. tenuifolius</i>	10.49	1.17	0.30	5.12	0.40	0.11	0.21	0.09
<i>A. triangularis</i>	24.54	8.41	8.27	4.53	0.25	0.85	5.00	1.85
<i>B. halimifolia</i>	0.11	-	5.28	2.88	0.18	-	0.11	0.08
<i>D. spicata</i>	1.20	-	0.14	-	2.83	-	-	-
<i>J. gerardi</i>	5.31	3.11	0.14	3.02	0.95	1.84	-	0.36
<i>K. pentacarpos</i>	0.20	-	34.35	19.40	-	-	31.96	11.36
<i>P. australis</i>	0.03	11.86	0.35	7.02	0.84	4.84	-	-
<i>S. patens</i>	4.61	29.11	13.42	39.56	311.06	270.74	261.17	305.69
<i>S. salina</i>	0.12	0.44	0.02	0.55	0.01	0.04	-	0.02
<i>C. esculentus</i>	1.37	-	-	-	-	-	-	-
Total	47.98	54.10	62.26	82.07	316.51	278.42	298.45	319.46



**Figure 2.14** The Shannon-Wiener diversity index (based on above-ground biomass) of the plant community present within each treatment at the end of Year 2. A higher  $H'$  is indicative of higher diversity in terms of species richness and evenness. Box plots represent six replicate plots and show: the sample median (dark line), lower and upper quartile (box), highest and lowest observations (limits), and outliers (points).

## Chapter 3

### THE ROLE OF TEMPERATURE IN THE DEVELOPMENT AND GROWTH OF *KOSTELETZKYA PENTACARPOS* SEEDLINGS

#### 3.1 Abstract

*Kosteletzkya pentacarpos* is being developed as a biofuel crop for salt-affected coastal farmland. A better understanding of key processes driving the growth and development of this halophyte will improve domestication and commercialization efforts. This study explores the role of temperature in seed germination and early development of *K. pentacarpos*. For this purpose, seedlings originating from two distant locations, Delaware and Georgia, were grown in a controlled-environment chamber at five temperature treatments: 15°, 20°, 25°, 30°, and 35° C. Days required to reach major developmental stages were recorded and seedlings were harvested, measured, and weighed at the time of fourth leaf development. In addition, *K. pentacarpos* callus tissue culture was also grown at these temperatures and tissue area growth was recorded and compared. Our results suggest that seed coat impermeability is the cause of seed dormancy for *K. pentacarpos* and that seedlings developed faster at higher temperatures. Optimum seedling growth rates were noted at temperatures between 25-30° C and lower/upper growth thresholds were near 15° C/35° C, under present experimental conditions. Delaware and Georgia seedlings differed in their response to temperature, in terms of reaching early developmental stages, growth rates, and seedling size, suggesting the existence of local adaptation within the species range.

Tissue cultures were not as tolerant of higher temperatures as whole plants in this study, with optimum growth at 20° C. This study informs decisions in the domestication and managements of this useful crop, through revealing temperatures for optimum seedling growth, and through demonstrating the existence of latitudinal variation.

### **3.2 Introduction**

Coastal areas are faced with the serious threat of sea level rise and its consequences in the near future. The development of strategic management plans is essential to sustain both their ecological and economic services. Low-lying agricultural land is particularly susceptible to salt-water intrusion, through progressively higher tides, storm surges, and freshwater aquifer contamination. Traditional crops are not salt-tolerant and land will increasingly become bare, and vulnerable to invasive species. As global population rises, demand for food and fuel are ever growing, as is the demand for arable land for their cultivation. A means to produce biofuel on salinized land would help relieve some of this demand. *Kosteletzkya pentacarpos* (L.) Ledebour (Blanchard 2008), seashore mallow, is being developed as a salt-tolerant biofuel crop to replace traditional crops in salinized coastal land (Sommers 1979; Gallagher 1985; Gallagher and Seliskar 1993; Gallagher 1995; He et al. 2003). The cultivation of perennials benefits the environment through storage of nutrients and organic matter in the soil, prevention of erosion and run-off, and providing a wildlife habit (Glover et al. 2007). Therefore, the cultivation of the halophytic perennial *K. pentacarpos* could prolong economic viability of land and simultaneously invest in the environment.

*K. pentacarpos* produces an oil seed containing approximately 20 % oil that is comparable to that of cotton and soybean oil (Islam 1982; Ruan et al. 2008). The seed is also high in protein (25%) but does not contain salt, or the undesirable compound gossypol found in cotton, therefore, the meal would be a suitable animal feed (Islam 1982). In addition, the plants' stems are woody and contain fiber, comparable to kenaf (*Hibiscus cannabinus*), that could be used to produce fiber-based products or cellulosic ethanol. Currently several pilot projects are already underway in the United States (Halchak et al. 2011) and China (He et al. 2003; Ruan et al. 2008). Yield is likely to be increased by a breeding program, which has been initiated at the University of Delaware, U.S.A. (Seliskar et al. 2011), with plants and seeds collected from various regions within the species U.S.A. range. In addition, the protocols have been developed to further desirable traits through growth and manipulation of *K. pentacarpos* callus tissue culture (Cook et al. 1989; Li et al. 2006; Ruan et al. 2009a), as has been demonstrated with other species (Seliskar and Gallagher 2000).

There are many features that recommend this species as a promising energy crop. *K. pentacarpos* is a perennial dicot of the Malvaceae family, native to brackish marshes of the Atlantic and Gulf coast of the United States (Radford et al. 1968) and is not invasive. Each plant lives approximately 10 years, therefore eliminating the energy expenditure of annual soil tillage and seeding, and each year *K. pentacarpos* regrows with a greater number of stems, thereby increasing its canopy cover and yield (Gallagher 1985; Halchak 2009). It is a facultative halophyte, known to tolerate salinities of 25, but does not require salt to grow (Gallagher 1985; Blits and Gallagher 1990a; Blits and Gallagher 1990b; Blits et al. 1993; Li et al. 2006). The plants' extensive perennial root system serves to protect it against drought and water-

logged conditions (Halchak et al. 2011), while in the long-term investing in the soil and minimizing erosion and run-off. *K. pentacarpos* has pink flowers that attract local pollinators; however, it is not dependent on pollinators as the flowers self-pollinate with minimum inbreeding loss at midday (Ruan et al. 2009b). Seed of *K. pentacarpos* is viable for long period of time, approximately 20 years (Poljakoff-Mayber et al. 1992, Poljakoff-Mayber et al. 1994), making the maintenance of seed reserves easy.

Further understanding of *K. pentacarpos* growth and development are needed to contribute to domestication and standardization of cultivation protocols. *K. pentacarpos* is related to various other commercialized Malvaceae species, such as cotton (*Gossypium sp.*), kenaf (*Hibiscus cannabinus* L.), and okra (*Abelmoschus esculentus* L. Moench). Cotton production, in particular, has a large global market and cotton growth has been extensively researched. The rate at which cotton plants' arrive at developmental stages is strongly related to temperature and the heat unit (HU) method is used to improve crop management (Supak 1984; Ritchie et al. 2007; Robertson et al. 2007). This method quantifies the cumulative effect of daily minimum and maximum temperature on a cotton plant and predicts the plants arrival at a given developmental stage based on cumulative heat units required to reach that stage. Although there are several criticisms of the heat unit method, most notably its oversimplification of the complex processes influencing plant life (Supak 1984), it is overall a very useful tool in agricultural management. The identification of similar relationships in the growth and development of *K. pentacarpos* would be valuable.

Based on field and greenhouse observations, we suspect that growth and development of *K. pentacarpos* may be similarly influenced by temperature (communication with J.L. Gallagher). The objectives of this study were to (1) evaluate

the role of temperature in early growth and development of *K. pentacarpos*, (2) explore whether this relationship is valid on a broad scale for more than one ecotype of *K. pentacarpos*, and (3) investigate the effect of temperature at the cellular level with *K. pentacarpos* callus tissue cultures. The information gathered in this study will further our understanding, and improve our current development decisions and cultivation approach, thereby accelerating the domestication of this valuable halophyte.

### **3.3 Methods**

#### **3.3.1 Germination and Seedling Response to Temperature**

To evaluate the above objectives, a controlled-environment growth chamber was employed. The experiment included five treatment temperatures (daily maximum/minimum): 15° (22.1°/15.3°) C, 20° (22.1°/20.9°) C, 25° (27.8°/26.8°) C, 30° (34.9°/26.7°) C, and 35° (39.5°/26.7°) C, and with seed from two ecotypes: Delaware and Georgia. For both ecotypes, the source of seeds was an individual plant germinated and grown, from seed collected in Delaware and Georgia accordingly, in our greenhouse at the Halophyte Biotechnology Center (University of Delaware). The Georgia seeds used had been collected the summer of 2010 and Delaware seeds used had been collected the summer of 2011. All seeds had been stored in a 5° C refrigerator prior to use in this study. At each temperature treatment the below described procedure was repeated.

Twenty five seeds from each ecotype were soaked in 10N sulfuric acid for ten minutes and then washed three times in autoclaved distilled water. The seeds were

then soaked in a solution of 20% bleach and three drops of Tween-20 surfactant for thirty minutes, and mixed frequently. This sterilization procedure is a modified version of the procedure used to sterilize *K. pentacarpos* seeds for tissue culture (Cook et al. 1989) and is necessary to completely sterilize its rough seed coat. In order to ensure that water could penetrate the seed coats and that imbibition could occur, seeds were lightly scarified with a sterile scalpel to create a small split. The seeds were then soaked in autoclaved distilled water for twenty-four hours to activate germination. Seeds were plated in upright large test tubes containing a clear media comprised of Gel Gro and Hoagland solution, and kept under cool white fluorescent bulbs ( $150 \mu\text{mol s}^{-1} \text{m}^{-2}$  as measured with a LI-1400 data logger, LICOR, Nebraska, U.S.A.) for 14 hr:10 hr (light:dark) days.

Over the following weeks, seedlings were monitored for the time they reached various stages of development. For each seedling we recorded the days after planting to emergence of a root radicle, the unfolding of cotyledons, the development of secondary (lateral) roots, and the first to fourth true leaves. Root radicle and secondary roots were considered present once the length of the root tip extended approximately one centimeter from the seed or primary root, respectively. Cotyledons were considered emerged when the two cotyledon leaves were unfolded and in their final open position. A leaf was considered present once it was 0.6 cm or larger in length from tip to base, not including the petiole. This size was selected based on preliminary trials in which it was informally determined that a phototropic response occurred at this size.

Each seedling was harvested twenty-four hours after a fourth leaf was determined as developed. During harvest, various size characteristics were measured,

specifically primary stem length, primary root length, stem biomass, and root biomass. Samples were then dried in a 60° C oven to constant weight.

### **3.3.2 Callus Tissue Growth and Temperature**

To investigate the effect of temperature on *K. pentacarpus* at the cellular level, callus tissue was initiated from Delaware *K. pentacarpus* seed and exposed to the previously specified treatment temperatures. *K. pentacarpus* seeds were sterilized in sulfuric acid, washed three times in autoclaved distilled water, and soaked for thirty minutes in a solution of 20% bleach with three drops of Tween-20 surfactant. Seeds were then scarred with a sterile scalpel blade and germinated in a sterile environment. Hypocotyls were cut and plated on 2I1K (2 mg/L IAA, 1 mg/L kinetin) agar-based media which has been previously used to induce *K. pentacarpus* callus tissue generation (Cook et al. 1989). The callus was successfully maintained for approximately one year by subculturing to new media bimonthly.

For this study, four pieces of callus with a diameter of approximately 0.5 cm and from the same original source were plated evenly within each of four small petri dishes of 2I1K media. A total of sixteen clumps of callus were each marked for identification. The samples were left for two days at original temperature (23° C) to acclimate to the new dish before initiating the study. Each callus formation was then photographed on the first day of the study. The camera was mounted on a 3.5 cm permanent frame and a ruler was positioned in each photograph for size reference. Dishes were kept on a test tube rack to maximize light availability and within a sterilized case in the previously described controlled-environment chamber. Cool white fluorescent bulbs were mounted vertically on the three closed-wall sides of the

case. Samples were photographed in the same way every third day for two weeks. Area cover of each callus sample and the change in area over time were calculated.

### **3.3.3 Statistical Analysis**

The comparison of days required for the development of root, cotyledon, and leaf organs; the size at harvesting of stem, roots, cotyledon leaves and true leaves; and the biomass of stem and root systems, for each temperature treatment and ecotype was analyzed with two-way analysis of variance (ANOVA). For a detailed example of the ANOVA models used, see Appendix B, Table B1. Radicle emergence, cotyledon unfolding, secondary root production, second and third leaf development, stem height growth rate, and root length growth rate were log<sub>10</sub> transformed to meet ANOVA assumptions. Ecotype and temperature treatments were regarded as fixed effects. Type III sums of squares calculations were used to account for unbalanced numbers of observations due to cases of plant mortality or abnormal growth. Interactions of the main effects were reported and described in depth as they are of biological interest. To support these descriptions, a simple main effects test was completed in each interaction case.

Due to the mortality of most individuals at 35° C before meeting harvesting requirements this level was excluded from all ANOVA comparisons, except in the case of radical and cotyledon emergence where the dataset was complete. For cotyledon unfolding and secondary structure development (leaves and roots), days after germination (DAG) and days after cotyledon emergence (DAE), respectively, were used for analysis in order to remove variation due to physical and physiological processes involved in earlier stages of germination and emergence.

Callus tissue culture images were analyzed using Image J software provided by the National Institute of Health (Abramoff et al. 2004). Average growth rate of callus area was calculated and the possible effect of size on growth rate was accounted for by dividing the observed change in callus size by the callus size at the earlier time point. As described previously, samples were grown in petri dishes of four and hence analyzed using a nested ANOVA (for the detailed ANOVA table see Appendix B, Table B2). Temperature was treated as a fixed factor.

Statistical analysis was completed in IBM SPSS Statistics (Version 19) software. Figures were made in R version 2.13.2 (R Development Core Team 2011).

### **3.4 Results**

#### **3.4.1 Germination and Seedling Response to Temperature**

In this study, germination rates were high: 96 – 100 %, with germination conventionally defined as root radicle emergence. Seedling mortality before the production of a fourth leaf, and subsequent harvest, was not common in most levels. However, it was exceptionally high at 35° C. Germination at this temperature was successful and seedlings began development of secondary structures, however these remained only partially formed or very small. Within thirty days at 35° C approximately fifty percent of seedlings withered. This level was therefore only included in the analysis of days to germination and days to cotyledon emergence since seedlings did not meet size criteria for completion of later stages of development. Seedling mortality was rare for other temperature treatments and most seedlings reached our size criterion of a fourth true leaf at 0.6 cm or larger, although the time required to this stage varied greatly (Table 3.1). The only exception was in the case of

Georgia seedlings grown at 15° C where after fifty days some seedlings had yet to produce a fourth leaf, and in some cases even a third leaf of 0.6 cm or larger, and did not seem to grow further. In comparison, seedlings grown at 25° C had reached harvest standards (fourth leaf greater than 0.6 cm) within 20 days. Overall, days after planting (DAP) measurements are shown in Figure 3.1.

Figure 3.2 shows the days required, after planting (DAP), for root radicle emergence (germination). Germination at 15° C lagged behind the other temperatures tested ( $p < 0.0005$ ) and this difference was confirmed as significant by a Tukey's test. DAP to germination at higher temperatures did not vary significantly and was shorter for Delaware than Georgia seeds ( $p < 0.0005$ ).

The lowest and highest temperatures required more days, after germination (DAG), for the cotyledons to unfold (Figure 3.3). The highest DAG noted was at 15° and lowered as temperature warmed with the fastest cotyledon emergence at 30° C ( $p < 0.0005$ ). Tukey's test showed that time to cotyledon unfolding at temperatures 20° and 35° C was not different. The cotyledons of Georgia seeds emerged later than Delaware seeds ( $p = 0.002$ ) and this difference was noticeable in lower temperatures.

Temperature was a significant factor ( $p < 0.0005$ ) in determining the time needed for the development of secondary root structures. Time in days, after cotyledon emergence (DAE), was highest at 15° C and decreased as temperatures increased. A Tukey's test confirmed that DAE required for seedlings to produce secondary roots differed significantly at each treatment temperature, with the exception of the two highest temperatures, 25° and 30° C, at which a comparable number of DAE was required. Figure 3.4 illustrates this pattern. The two ecotypes were not significantly different in terms of DAE to secondary root development.

Days, after emergence (DAE), to the development of the seedlings first true leaf are shown in Figure 3.5. At each temperature, Georgia seedlings required more days to produce a first true leaf than Delaware seedlings ( $p < 0.0005$ ), although the pattern of their overall response to temperature was the same. The largest DAE was at 15° C and dropped greatly at 20° C. For both ecotypes, DAE required at 20° C and 30° C were the same according to Tukey's test but at 25° C there was an unexpected increase in DAE to first true leaf.

In Figure 3.6, the same pattern was noted in the DAE to second leaf development as with the first leaf. The highest time demand being at 15° C followed by a steep drop at 20° C, but as with the first true leaf, a slight increase of DAE was noted at 25° C. The interaction of the main effects was significant ( $p = 0.039$ ). Further analysis of this interaction suggested that Georgia seedlings required consistently more time than Delaware seedlings ( $p < 0.05$ ) and the effect of temperature was significant, except for Georgia seeds where DAE at 20° and 30° C were not different.

The interaction of the effects of temperature and ecotype was significant ( $p = 0.014$ ) in regards to the development of a third leaf also (Figure 3.7). DAE decreased in warmer temperatures and each temperature was significantly different from the other temperatures for both ecotypes. Ecotypes were not different from each other except at 30° C where Delaware seedlings had a lower DAE requirement. The analysis was also run without a high outlier in the Georgia seedlings grown at 15° C, but the results were not different. Similar trends were observed for the development of a fourth leaf (Figure 3.8) where the effect of temperature was always significant and ecotype response differed at all temperatures except at 25° C.

### 3.4.2 Post-harvest Seedling Size Comparison

We compared size and weight of stems and roots produced by *K. pentacarpus* seedlings from Delaware and from Georgia when grown at various temperatures. Seedlings were harvested twenty four hours after they had a fourth true leaf larger or equal to 0.6 cm in length. Note that the rate at which plants reached the harvest marker (fourth leaf) varied by temperature. For example, plants grown at 15° C required approximately twice as long as plants grown at 25° C to reach the harvest marker. To explore the relative growth of seedlings, an additional comparison was made using an average daily growth rate for each size variable. This value was equal to the measured length or dry weight divided by the number of days for which the plant grew. Plants that did not reach the harvest marker were not included in this analysis.

Figure 3.9 shows mean stem height (cm) of plants at each temperature when they produced a fourth leaf (temperature \* ecotype,  $p=0.045$ ). Delaware seedlings were taller than Georgia seedlings at each temperature however this difference was not significant at 25° C. Only Georgia plants grown at 15° and 25° C were statistically different from each other. Delaware plants grown at the various temperatures were not different from each other. The mean stem height growth rate also showed an interaction of main effects ( $p=0.003$ ). Growth rate of both ecotypes was lowest at 15° C and increased in warmer temperatures. Delaware stem height growth rate continued to increase through 30° C, whereas Georgia plants seemed to plateau beyond 20° C.

An interaction of the main effects was present in the examination of primary root length (temperature \* ecotype,  $p=0.015$ ). According to a simple main effects test, Delaware seedlings grown at 20° C differed significantly from those

grown at 25° and 30° C, and Georgia seedlings grown at 15° and 20° C differed significantly from those grown at 25° and 30° C (Figure 3.10). Seedlings from Georgia and Delaware did not vary statistically in terms of mean primary root length except at 30° C, where Delaware roots were longer. The mean root length growth rate also exhibited an interaction of the main effects ( $p < 0.0005$ ). Plants grown at 15° C had the slowest growing primary roots, however growth rate increased significantly at 20° C. Growth rate for both ecotypes appeared to plateau here with no increase at 25° C; however at 30° C Delaware plants again showed an increase in growth rate.

Mean stem biomass of seedlings, at the time when a fourth leaf was produced, decreased as temperatures warmed and Delaware plants always weighed more than Georgia plants (Figure 3.11). Stem biomass was highest at the lower temperatures than at the higher temperatures. A main effect interaction ( $p = 0.005$ ) was present due to the fact that stem biomass of Delaware plants varied significantly between 15° and 20° whereas Georgia seedlings did not. The mean growth rate of this variable was lowest at 15° C and increased sharply by 20° C. Growth rate of Georgia plants did not increase significantly beyond 20° C whereas the stem biomass growth rate of Delaware plants increased with temperature (temperature \* ecotype,  $p = 0.009$ ).

There was an interaction ( $p < 0.0005$ ) of temperature and ecotype on below-ground biomass produced by time of fourth leaf (Figure 3.12). For Delaware plants, root biomass decreased almost linearly with temperature, suggesting that seedlings at higher temperatures had smaller root systems at the time of fourth leaf production. For Georgia plants, variability between temperature treatments was very limited and root biomass stayed within a small range. Delaware roots were always heavier than Georgia plants; however at 30° C this difference was not significant. The mean root

biomass growth rate was lesser at 15° C and increased up sharply at 20°C, staying fairly consistent after that. The effect of temperature on root biomass growth rate was significant ( $p < 0.0005$ ) and Delaware seedlings increased the biomass of their roots quicker than Georgia plants at all temperatures tested ( $p < 0.0005$ ).

A root to shoot ratio (R:S) was calculated based on biomass allocated by the seedlings above and below ground and is shown in Table 3.2. Delaware seedlings had a higher R:S ratio overall, with the exception of 30°C where there was a noticeable drop in R:S to levels below that of Georgia seedlings (temperature \* ecotype,  $p = 0.015$ ).

### **3.4.3 Callus Tissue Growth Response to Temperature**

Figure 3.13 shows mean area growth rate for callus tissue which was highest at 20° C, with a mean of 0.079 mm<sup>2</sup> per day, and was lowest at 35° C, with a mean of 0.002 mm<sup>2</sup> per day. Within the first week at 35° C several samples began to brown and were not viable by the end of the experiment. A nested ANOVA showed a significant difference among dishes ( $p = 0.016$ ) and between temperatures ( $p < 0.0005$ ). The analysis was repeated without a particularly low outlier at 20° C but the results did not change.

## **3.5 Discussion**

### **3.5.1 Seed Dormancy in *K. pentacarpos***

The results of this study suggest that seed coat impermeability is the main cause of dormancy in *K. pentacarpos* seeds. In Poljakoff-Mayber et al. (1992) seeds of 2 years or less were found to have very low germination rates (less than 10%),

however seeds used in the present study were harvested just 1.5 (Georgia) and 0.5 (Delaware) years prior and both ecotypes germinated at 96% or higher (Table 3.1). In both studies, seeds were stored at 5° C before use and seed coats were physically scarified with a scalpel. However, in the present study scarified seeds were then soaked in water for twenty-four hours before being plated in a water-based gel media, whereas in the former study seeds were germinated on wet blotting paper with access to water only through capillary action. It seems likely that the abundant availability of water was central to the very high germination success noted in this study. Our results support the hypothesis that seed dormancy in *K. pentacarpos* may be attributed to seed coat impenetrability and not immaturity of embryos, as fresh seeds germinated readily once imbibition occurred. A similar conclusion has been reached about the nature of seed dormancy in other Malvaceae species, such as *Sida cordifolia* L. (Cardoso 1991), *Malva pusilla* Sm.(Makowski and Morrison 1989), *Hibiscus trionum* L. (Westra et al. 1996), and *A. esculentus* (Demir 1997).

### **3.5.2 Growth and Development According to Temperature**

Interpreting the role of temperature in germination and early seedling development is essential for the effective cultivation of plant crops and native species preservation. Poljakoff-Mayber (1992) determined that optimal germination temperature for Delaware seeds was 28-30° C. In the present study, the time required for radicle emergence was not temperature sensitive and did not vary greatly between 20-35° C. Germination at the lowest temperature tested (15° C) was significantly slower, however germination rates were still high (See Figure 3.2). The role of temperature appeared to be of increasing importance in later developmental stages,

such as secondary root and leaf production (See Figures 3.4, 3.7, and 3.8). This is in agreement with our understanding of the processes driving seed germination and early growth. While the rupture of the testa and emergence of the radicle are driven mainly by physical force, specifically turgor pressure created by water uptake in the seed, post-germination development is driven by metabolic processes, cell division and elongation, and is therefore increasingly affected by temperature (Bewley 1997; Weitbracht et al. 2011).

Cotyledon unfolding after germination occurred sooner at 25-30 °C than at other temperatures and the first two true leaves required a fairly constant time to develop between 20-30°C. An odd increase in DAE for the first two leaves at 25° C (Figures 3.5, 3.6) may have resulted from leaf measurement criteria which were too conservative (at 1cm leaf length), or due to slower growth of the leaf length dimension at this temperature for unknown reasons. In either case the difference in DAE between the 20°, 25° C, and 30° C treatments was small, 1-3 days, and did not seem to inhibit further development or have an effect on growth. Development of the seedlings' next two leaves, the third and fourth, required less time as temperatures increased and was the fastest at 30° C. The fact that temperature had a greater effect on the development of the second set of leaves may be due to the energy source used by the seedlings for their production. Stored reserves may still have been the seedlings' primary energy source during the development of the first two leaves, converting to mainly photosynthetic energy near the development time of the second set of leaves. Cooper and MacDonald (1970) found that photosynthetic activity was irrelevant for approximately the first ten days of *Zea mays* L. seedling growth and seedlings had two complete leaves at the time when photosynthesis became essential. Lane and Hesketh

(1977) noted a progressive decrease in respiration and increase in photosynthetic activity of cotton cotyledons during the first week after epigeal germination, illustrating the transition in the energetic role of cotyledons during early seedling life. A similar sequence of events is likely occurring in *K. pentacarpos* seedlings also, where potentially near the time of third leaf development, which was on average already 1 cm in length on the fifteenth day after emergence at 25° C, the seedlings transition to energy generated primarily by photosynthesis. As photosynthesis is affected by temperature, this could explain the amplified role of temperature in the third and fourth leaf development and growth.

Development and growth of *K. pentacarpos* seedlings under the present experimental conditions was adversely affected by the temperature of 35° C. At this temperature, following successful seed germination, tissue differentiation occurred in less than 50% of seedlings with the development of one or two true leaves and was followed by either slow or nonexistent growth of these organs. Seedlings were observed for fifty days before the study was terminated and many seedlings withered within this period. For this treatment, the diurnal temperature fluctuation under lights, which was greater than with any other treatment (approximately 12.8° C), and the maximum daily temperature of 39.5° C likely became inhibitory for seedling growth. Development and growth was noticeably slowed by the coldest temperature tested also. Seedlings grown at 15° C required the longest time to meet developmental markers, including radicle emergence, cotyledon unfolding, and secondary root and leaf development and exhibited the slowest observed mean daily growth rates. Georgia seedlings at 15° C grew particularly slowly and in some cases had not produced a third or fourth leaf by the fiftieth day of growth. Growth at this

temperature appeared to be suboptimal and near base temperature (lowest temperature at which any growth will occur) for *K. pentacarpos* emergent seedlings, particularly with the Georgia plants.

Optimum mean daily growth rates were generally at 25-30° C, near the photosynthetic optimum for C3 plants. Delaware seedlings had maximum daily growth rate at the 30° C treatment for all size variables examined: stem height, primary root length, stem biomass, and root biomass. For Georgia seedlings, mean daily growth rate did not vary greatly at temperatures above 20° C and the maximum daily growth rate was observed at 30° C for stem height and root biomass, but at 25° C for stem biomass and 20° C for primary root length. For *K. pentacarpos* tissue culture, optimal growth rate was determined to be 20° C. Warmer temperatures appeared to hinder tissue area growth, and tissue mortality occurred at 35°. These findings indicate that the temperature tolerance varies between the whole-plant and cellular levels, possibly due to the absence of advantageous whole-plant controls, such as evapotranspiration, and morphological features, such as plant hairs.

### **3.5.3 Considerations for *K. pentacarpos* Crop Development**

In this study, seedlings were allowed to grow and, instead of being harvested on the same date, were harvested at the same developmental stage, i.e. at fourth leaf. Seedlings grown at colder temperatures were larger at the time a fourth leaf was produced than seedlings grown at higher temperatures, which reached maturity in a shorter period of time. This observation has important implications for crop development of *K. pentacarpos* because it may offer a method to manipulate resource allocation depending on the product of interest. For example, if a grower is

interested in stem-based products, such as cellulosic ethanol, growing a plant under colder conditions, or sowing earlier in the growing season, could secure a larger amount of investment in vegetative growth and a delay in reproductive maturity, hence increasing stem yield. Further work is required to explore whether seedling growth, or growth overall, at colder temperatures can delay maturity through to later developmental stages.

Another means to explore agricultural potential is through the use and improvement of varying traits found in wild ecotypes. In this study, a variable response to an environmental factor of temperature confirms the existence of local adaptations within the species. Delaware plants required less time to arrive at several developmental stages (radicle emergence, cotyledon unfolding and first few true leaves), grew larger (stem height, stem biomass, and root biomass), and at greater daily rates than Georgia seedlings. It appears that Delaware plants may be adapted to development at a wider range of temperatures and hence use heat energy more efficiently than Georgia seedlings. However, time required in DAE to secondary root development was not different between the two ecotypes, as soil temperature tends to fluctuate less than air temperature, the two distant sites may indeed be more alike in this aspect. An understanding of the diverse characteristics and qualities that exist within the spatial range of the species can be used in the selection of desirable traits for a breeding program to maximize agricultural potential. For example, Delaware seedlings had higher R:S ratios indicating a greater investment in below-ground biomass. Plants from this ecotype could be a hardier crop or more appropriate for managers interested in improving soil properties of marginal land (See Chapter 2).

The effect of varying root and shoot growth temperatures on these ratios would be interesting to explore.

### 3.6 Conclusions

This study furthers our understanding of the relationship between temperature and *K. pentacarpus* early growth and development. We have created a basic framework which can improve our utilization of *K. pentacarpus* and within which additional knowledge of *K. pentacarpus* growth can be assimilated. This work showed that the key for successful germination of *K. pentacarpus* seeds is ensuring that imbibition occurs. In addition, our results direct the selection of sowing conditions by indicating that optimum temperatures for seedling growth are 25-30° C, but that seedlings tolerated 20° C well. The low temperature of 15° C and the high of 35° C (39.5° C daily max) appeared to suboptimal and therefore sowing at such temperatures may not be prudent.

Developmental stages were reached faster when seedlings were grown in warmer temperatures. Further work would be useful in order to identify whether this relationship reaches throughout the vegetative phase and to reproductive development. If this is true, influencing the plants' resource allocation to shoots or seed yield could be possible based on temperature of growth, or temperature of seeding.

Finally, it seems likely that local adaptation is present in *K. pentacarpus* ecotypes found at various latitudinal locations where temperatures, and other environmental conditions, vary. These differences could be exploited to alter *K. pentacarpus* growth and reproduction at different latitudes based on the product of interest. For example, if Delaware *K. pentacarpus* was grown in Georgia it may reach

reproductive maturity fast enough to set seed twice during one season, hence increasing its value. Overall, this conclusion suggests that great progress toward the domestication of this useful halophyte can be made through the establishment of a breeding program.

### 3.7 References

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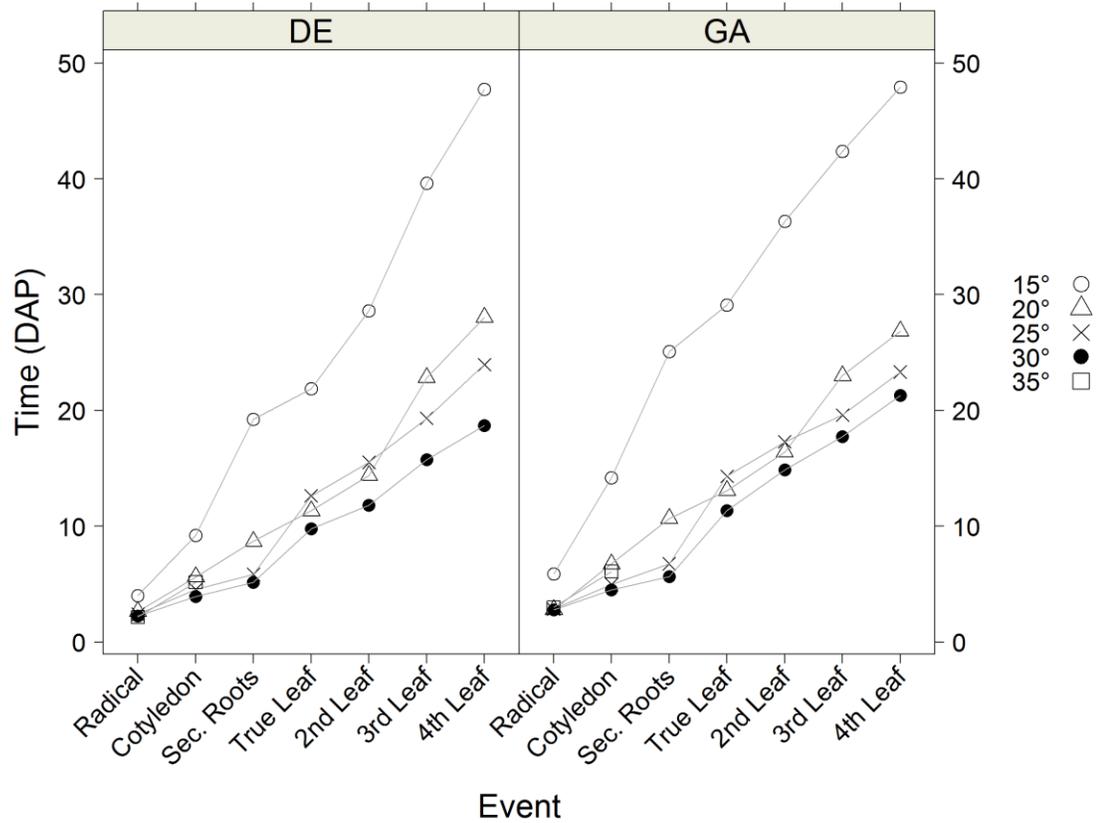
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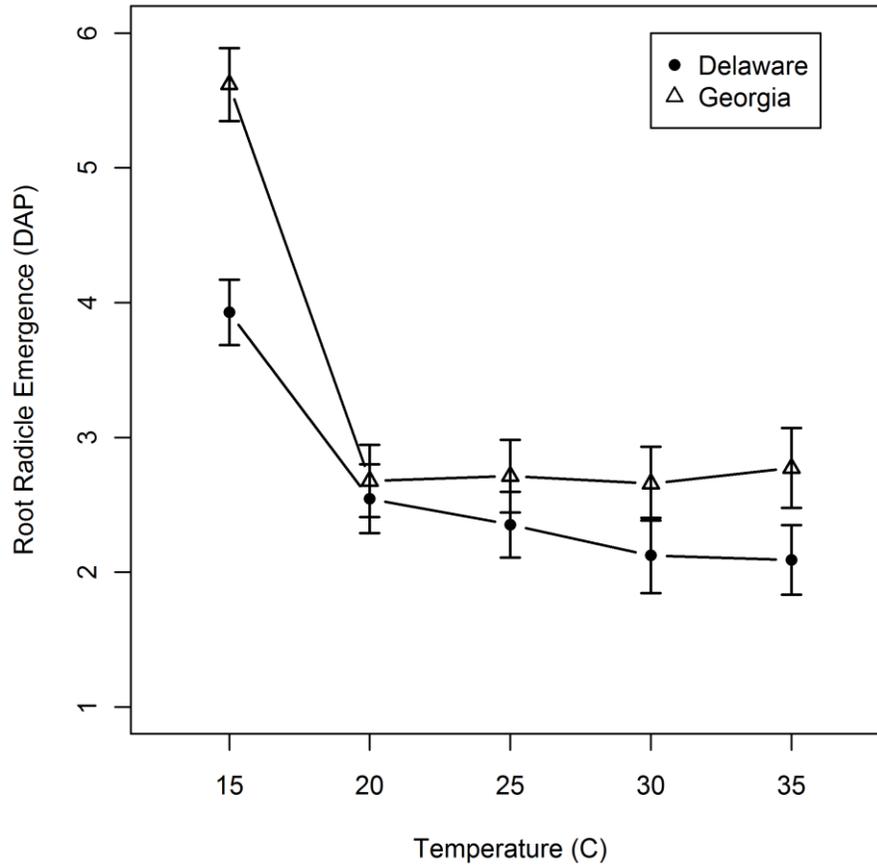
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**Table 3.1** For each temperature and ecotype, the number and percentage of seeds to successfully germinate (radicle emergence) and to reach harvest size (fourth leaf present and  $\geq 0.6$  cm in length) are shown. Sample size was 25 seeds.

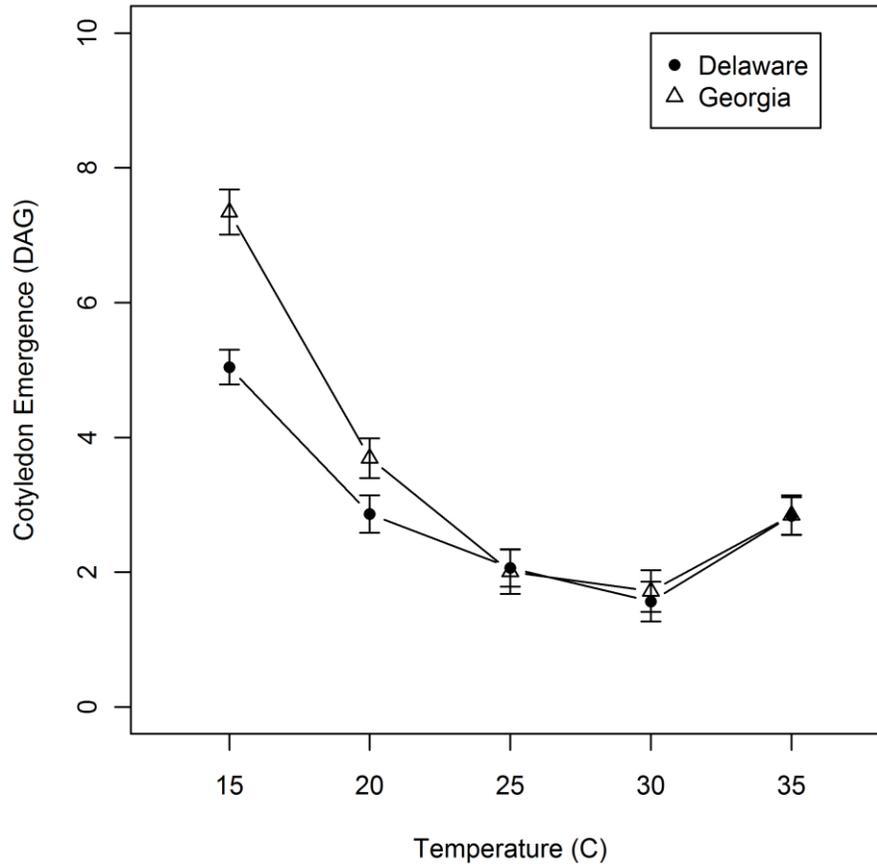
Temperature	Ecotype	Germination success	%	Fourth leaf and harvest	%
15°	DE	25/25	100	23/25	92
	GA	24/25	96	14/25	56
20°	DE	24/25	96	22/25	88
	GA	24/25	96	20/25	80
25°	DE	25/25	100	25/25	100
	GA	25/25	100	25/25	100
30°	DE	25/25	100	24/25	96
	GA	25/25	100	24/25	96
35°	DE	25/25	100	0/25	0
	GA	24/25	96	0/25	0



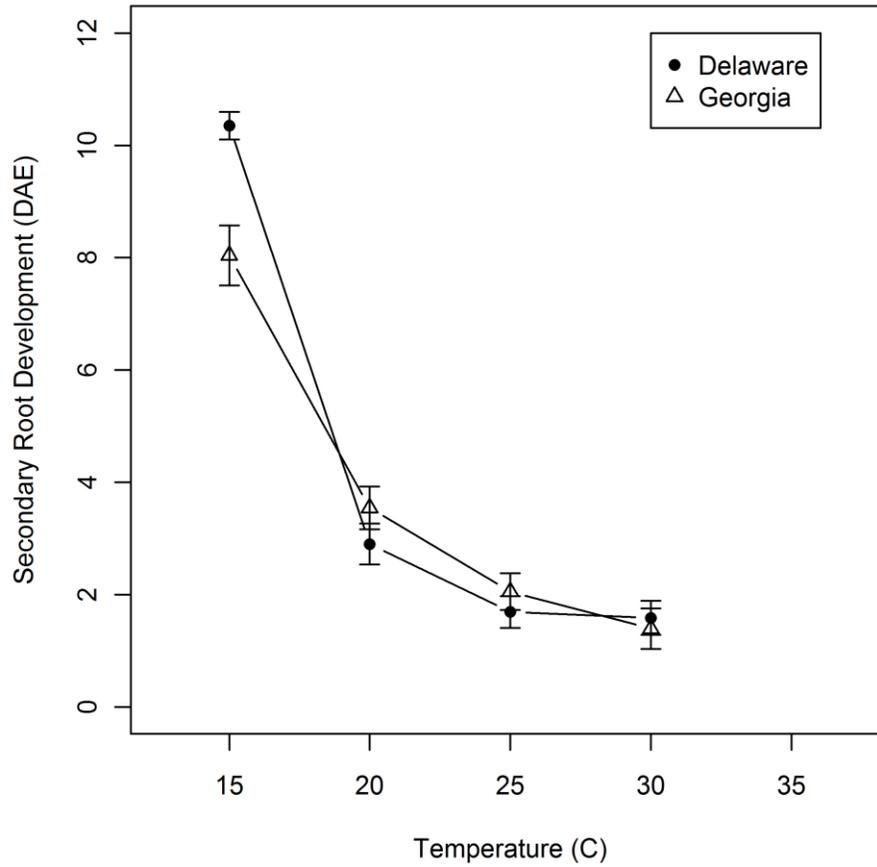
**Figure 3.1** Mean days, after planting (DAP), to indicated developmental stages by *K. pentacarpus* seedlings from Delaware (DE) and Georgia (GA) seed grown at five temperature treatments (°C). Data on seedlings growing at 35° C is only available for germination and cotyledon emergence due to seedling mortality or lack of growth.



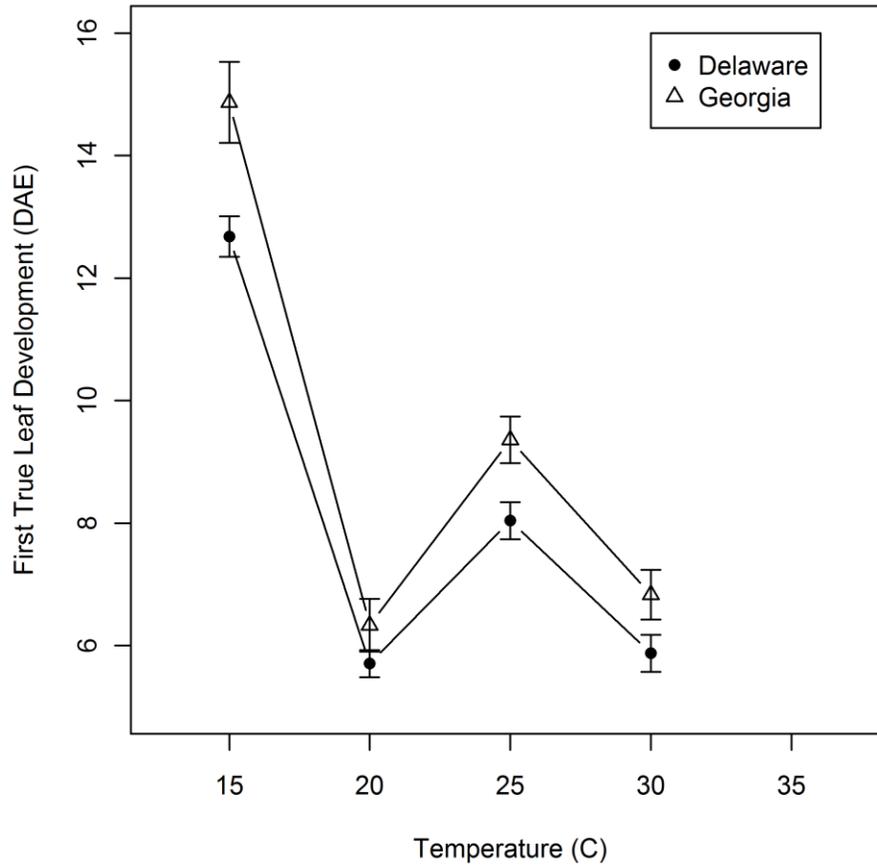
**Figure 3.2** Mean days, after planting (DAP), to root radicle emergence by *K. pentacarpos* seedlings from Delaware and Georgia seed grown at five temperature treatments. Error bars indicate the standard error of the mean.



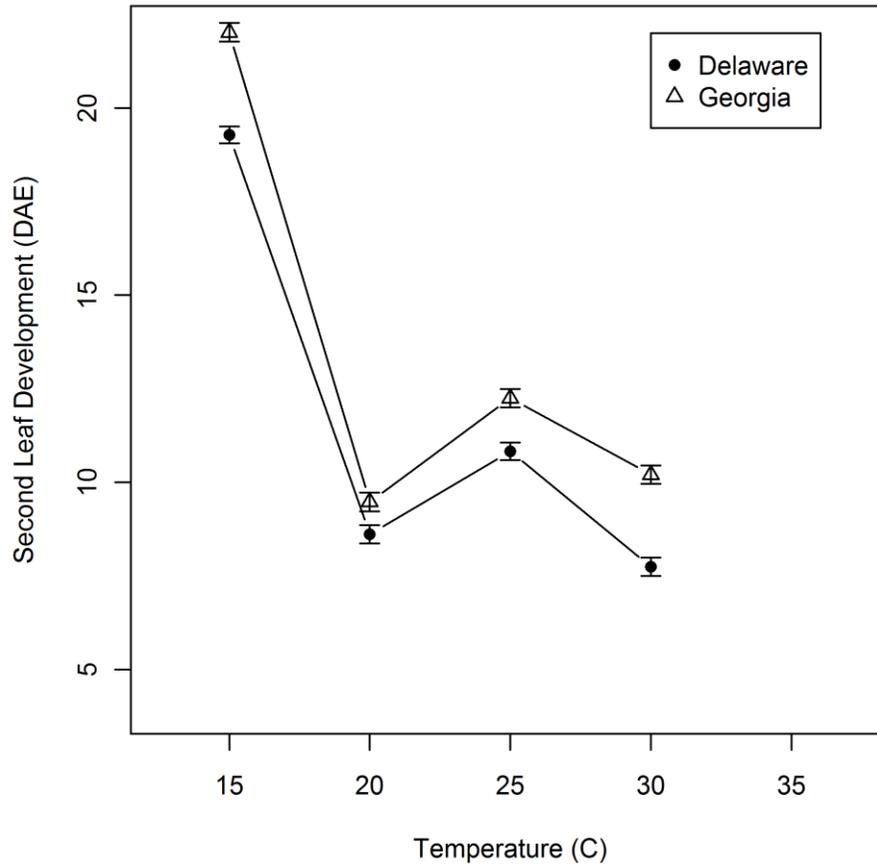
**Figure 3.3** Mean days, after germination (DAG), to cotyledon unfolding by *K. pentacarpos* seedlings from Delaware and Georgia seed grown at five temperatures treatments. Error bars indicate the standard error of the mean.



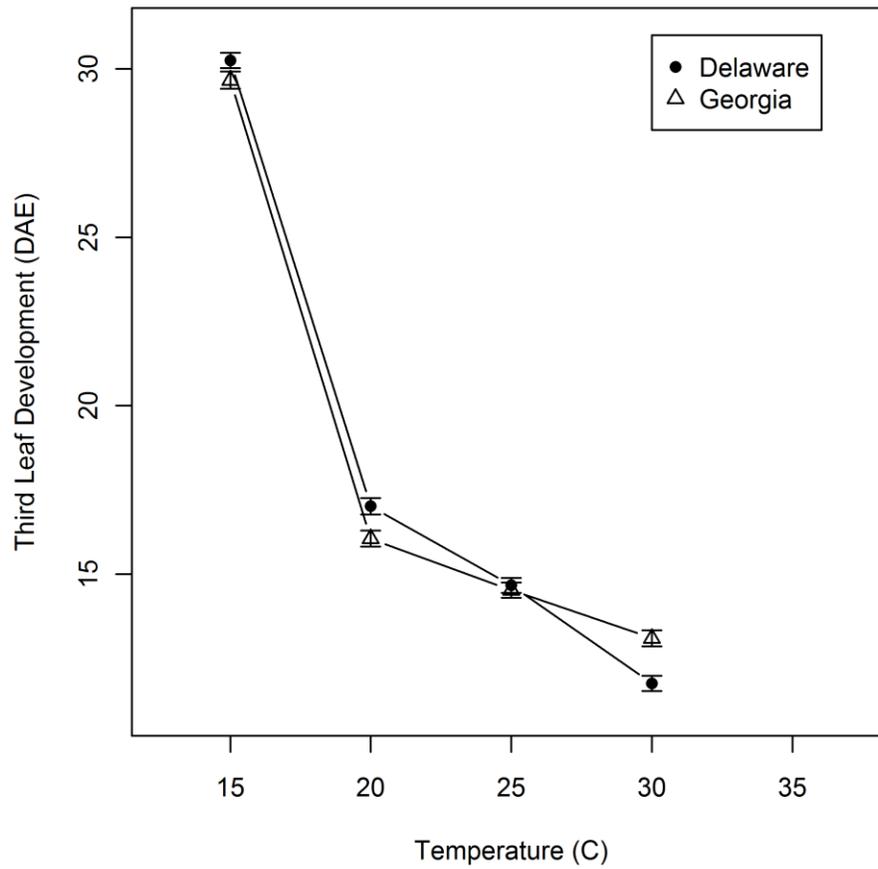
**Figure 3.4** Mean days, after cotyledon emergence (DAE), to development of secondary roots by *K. pentacarpos* seedlings from Delaware and Georgia seed grown at five temperature treatments. Seedlings at 35° C did not develop secondary roots. Error bars indicate the standard error of the mean.



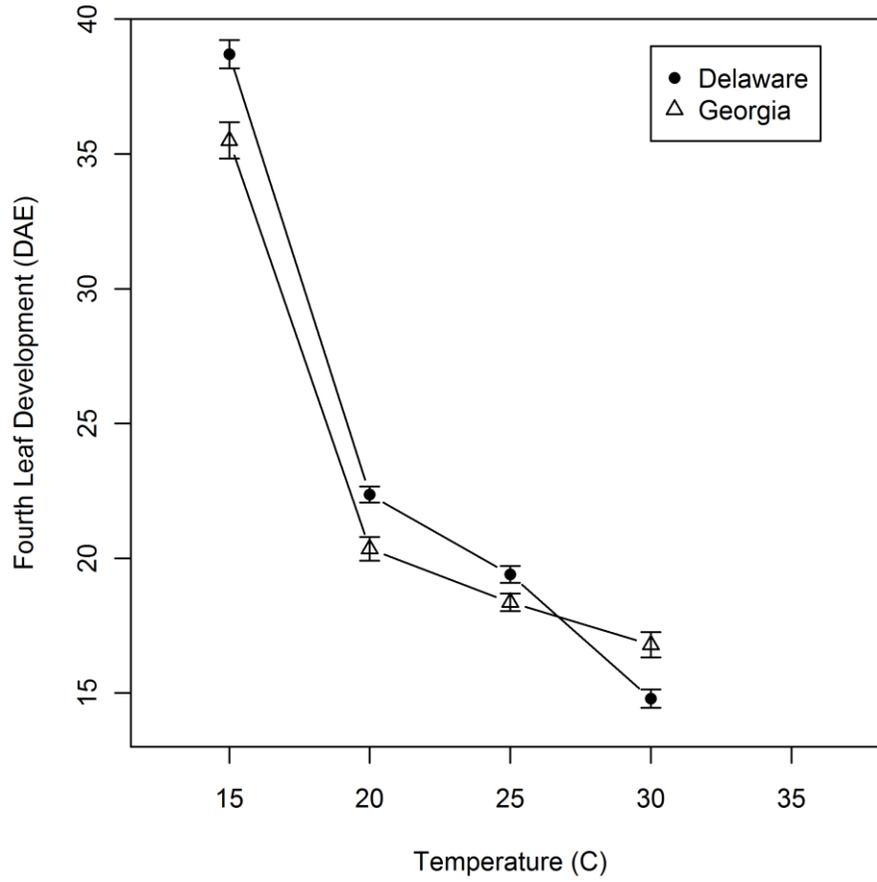
**Figure 3.5** Mean days, after cotyledon emergence (DAE), to first true leaf development by *K. pentacarpos* seedlings from Delaware and Georgia seed grown at five temperature treatments. Development of a first true leaf  $\geq 0.6$  cm in length did not occur at 35° C. Error bars indicate the standard error of the mean.



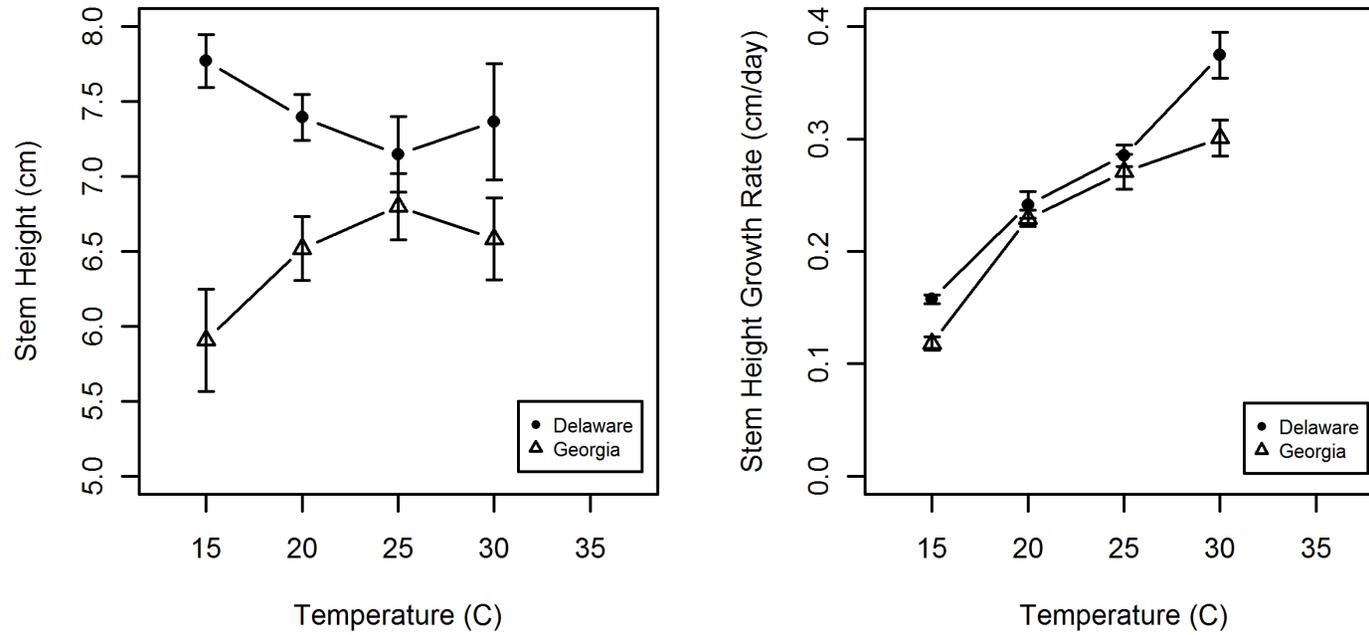
**Figure 3.6** Mean days, after cotyledon emergence (DAE), to second leaf development by *K. pentacarpos* seedlings from Delaware and Georgia seed grown at five temperature treatments. Development of a second leaf  $\geq 0.6$  cm in length did not occur at 35° C. Error bars indicate the standard error of the mean.



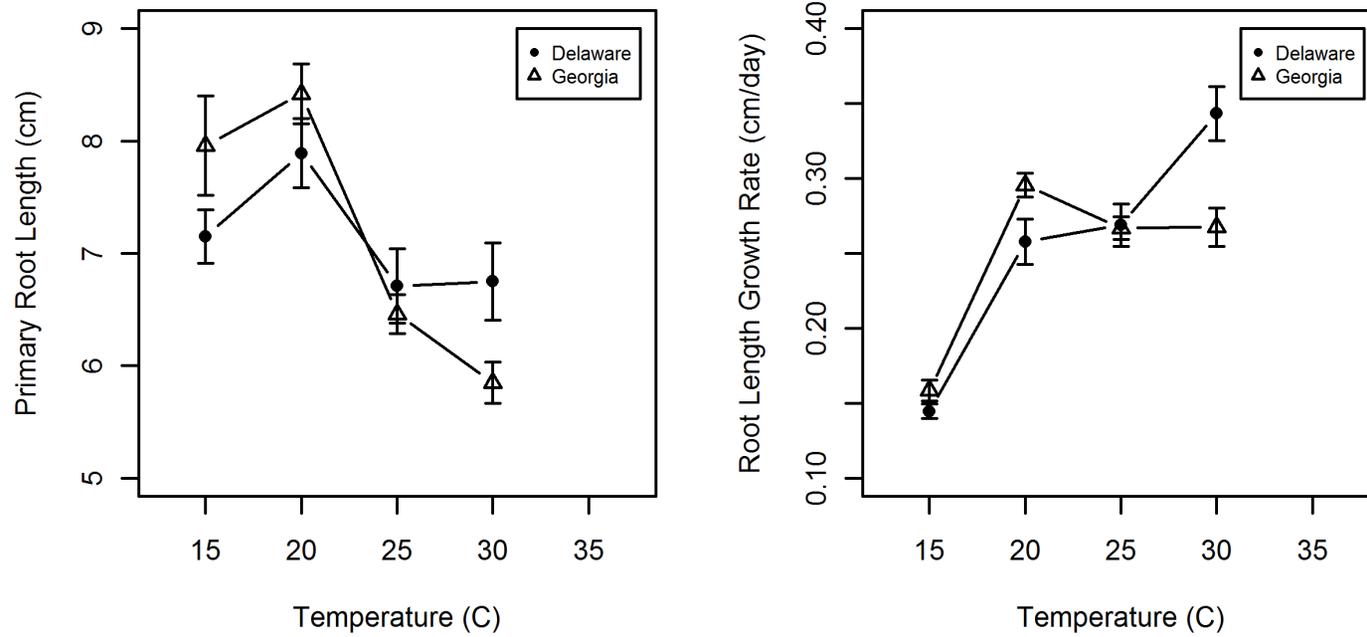
**Figure 3.7** Mean days, after cotyledon emergence (DAE), to the developments of a third leaf by *K. pentacarpos* seedlings from Delaware and Georgia seed at five temperature treatments. Development of a third leaf  $\geq 0.6$  cm in length did not occur at 35° C. Error bars indicate the standard error of the mean.



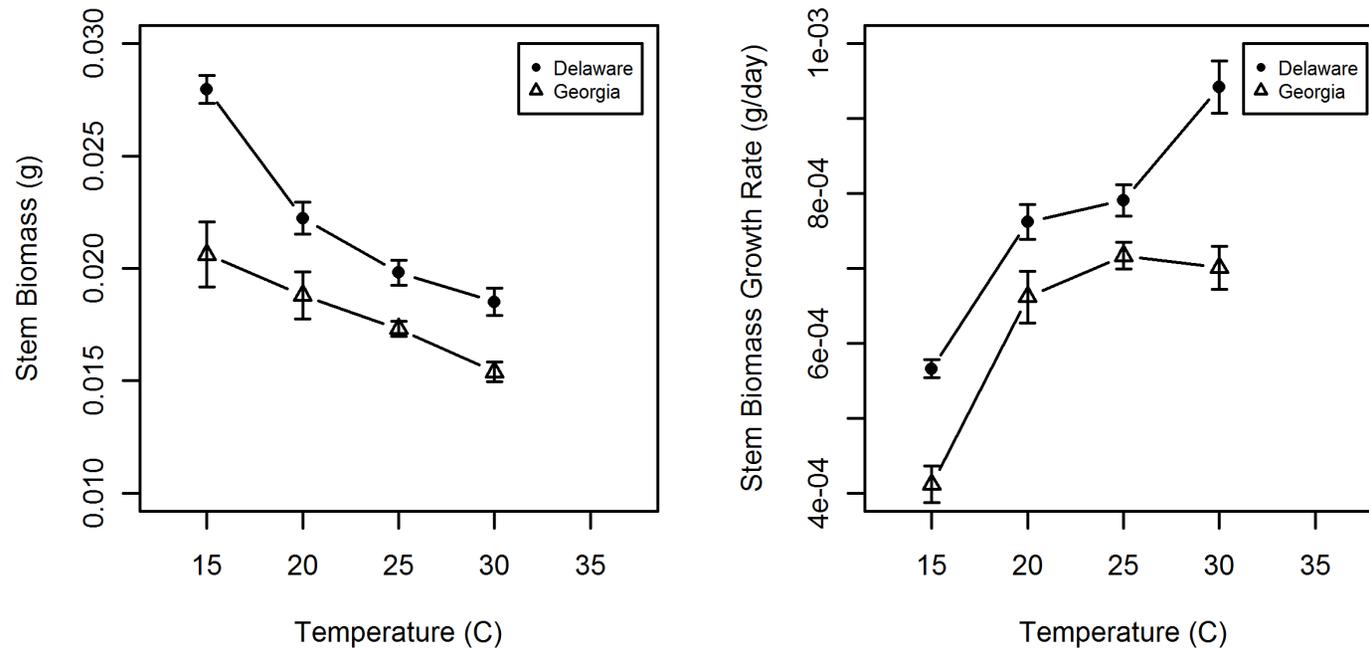
**Figure 3.8** Mean days, after cotyledon emergence (DAE), to the developments of a fourth leaf by *K. pentacarpos* seedlings from Delaware and Georgia seeds at five temperature treatments. Development of a fourth leaf  $\geq 0.6$  cm in length did not occur at 35° C. Error bars indicate the standard error of the mean.



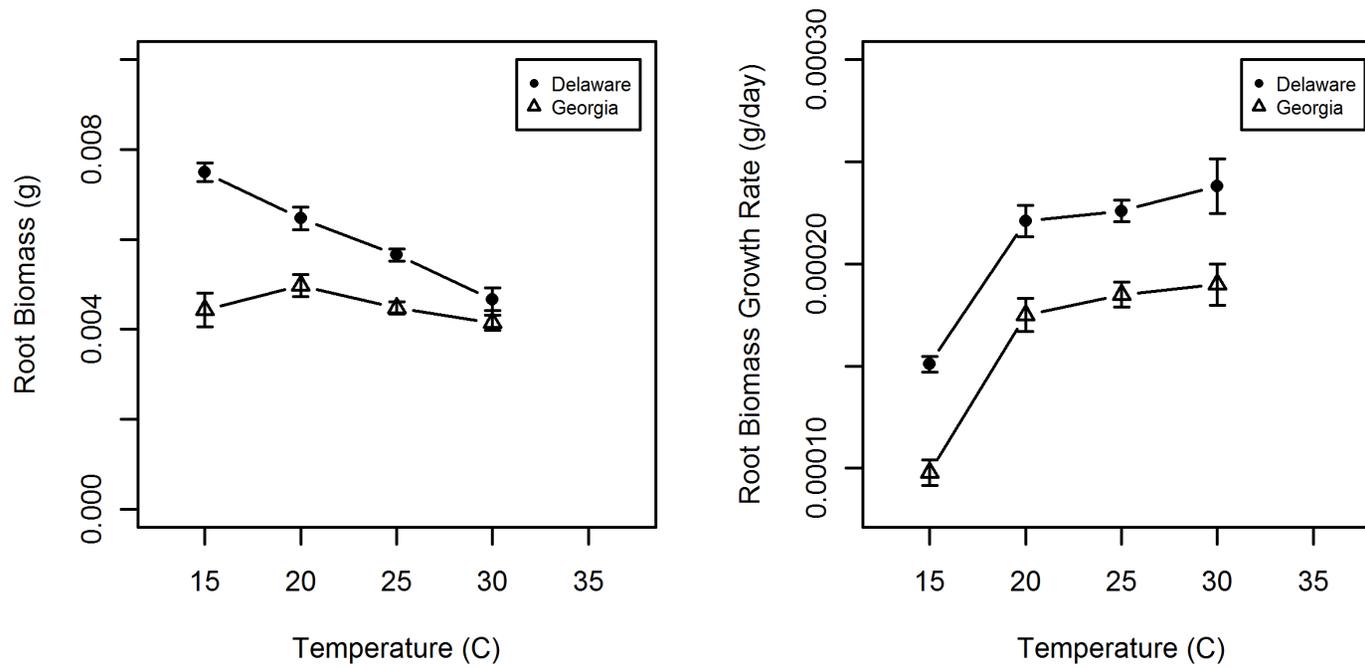
**Figure 3.9** Mean stem height (cm), at the time of fourth leaf development, and mean daily stem height growth rate (cm/day) of Delaware and Georgia *K. pentacarpos* seedlings grown at five temperature treatments. Seedlings grown at 35° C did not reach this developmental stage. Error bars indicate the standard error of the mean.



**Figure 3.10** Mean root length (cm), at the time of fourth leaf development, and mean daily root length growth rate (cm/day) of Delaware and Georgia *K. pentacarpos* seedlings grown at five temperature treatments. Seedlings grown at 35° C did not reach this developmental stage. Error bars indicate the standard error of the mean.



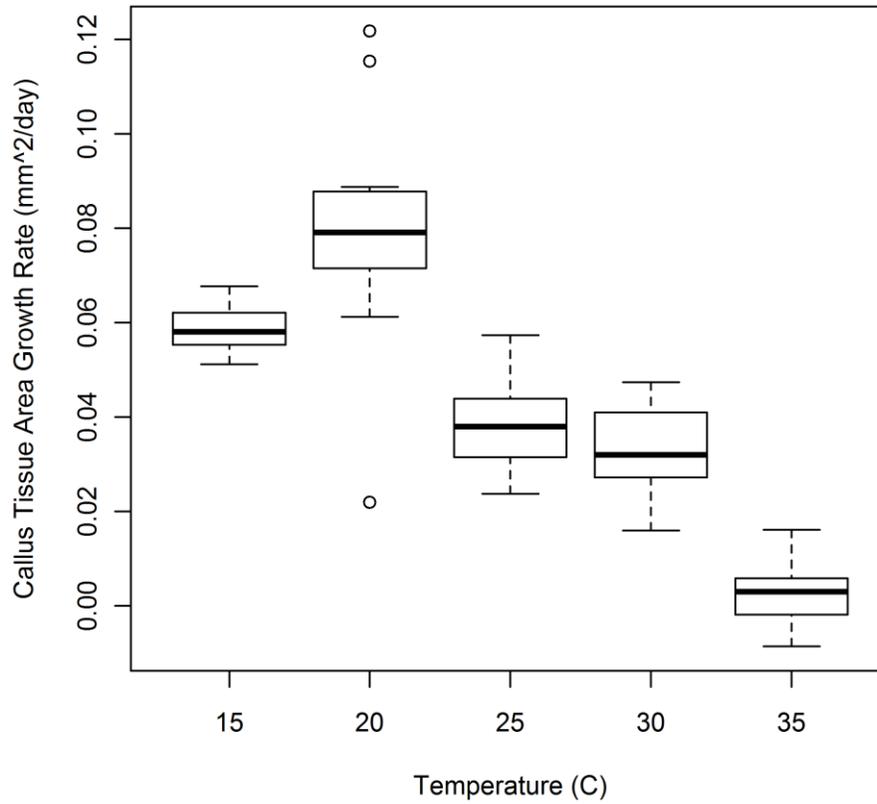
**Figure 3.11** Mean stem biomass (g), at the time of fourth leaf development, and mean daily stem biomass growth rate (g/day) of Delaware and Georgia *K. pentacarpos* seedlings grown at five temperature treatments. Seedlings grown at 35° C did not reach this developmental stage. Error bars indicate the standard error of the mean.



**Figure 3.12** Mean root biomass (g), at the time of fourth leaf development, and mean daily root biomass growth rate (g/day) of Delaware and Georgia *K. pentacarpos* seedlings grown at five temperature treatments. Seedlings grown at 35° C did not reach this developmental stage. Error bars indicate standard error of the mean.

**Table 3.2 Mean ( $\pm$  standard error of the mean) root:shoot ratio for Delaware and Georgia *K. pentacarpus* seedlings grown at five temperature treatments and harvested at the time of fourth leaf developments.**

	Delaware	Georgia
15°	0.27 ( $\pm$ 0.006)	0.24 ( $\pm$ 0.011)
20°	0.30 ( $\pm$ 0.012)	0.27 ( $\pm$ 0.008)
25°	0.29 ( $\pm$ 0.007)	0.26 ( $\pm$ 0.008)
30°	0.25 ( $\pm$ 0.010)	0.27 ( $\pm$ 0.011)



**Figure 3.13** The daily area growth rate ( $\text{mm}^2/\text{day}$ ) of Delaware *K. pentacarpos* callus tissue culture grown at five temperature treatments. Each box plot represents sixteen samples and show: median (dark line), lower and upper quartile (box), highest and lowest observation (limits), and potential outliers (points).

## Chapter 4

### CONCLUSIONS

Climate change and the circumstances which are expected to arise are frequently the main point of focus; however planning for these circumstances must also be prioritized. Innovative and sustainable strategies could greatly ameliorate the cost of climate change on a local scale. The cultivation of *Kosteletzkya pentacarpos* (L.) Ledebour in coastal agricultural land, from the time when brackish water is first introduced to the time when the land is too frequently flooded for efficient human exploitation, is such a plan and could provide more than just an economic buffer. Our research recommends this approach as a long-term investment in maintaining coastal environmental soundness through sea level rise.

Firstly, a newly salinized area is likely to lose plant productivity quickly, followed by a lag period with not plant cover. These conditions are ideal for invasive species, such as *Phragmites australis* (Cav.) Trin. ex Steud. Planting *K. pentacarpos* will create a native and stable presence in the salinized soil during transitional conditions. The reduction of bare soil is itself a benefit. *K. pentacarpos* creates an increasingly dense wall of plant stems, aids the growth of species that may block *P. australis*, and deposits high quantities of leaf litter onto the soil surface, qualities which could reduce *P. australis* success. Its direct interaction with *P. australis*, however, was not investigated during this study and calls for further questioning.

During this study, the cultivation of *K. pentacarpos* contributed to certain changes in soil properties. Its dense perennial roots increased soil infiltration rates,

suggesting that water drainage and soil aeration can be improved by its presence. An increase in percent organic matter at 0-5 cm soil depth was detectable within just one year of cultivation, which points to this perennial crop's potential to sequester carbon in the long term, a desirable function quite opposite to that of soil nutrient depletion and leaching that is associated with the cultivation of annual crops. Although changes in major soil nutrients were not detectable in the present, short-term investigation, leaf litter deposited onto the soil surface was significant under *K. pentacarpos* canopy suggesting that nutrient cycling is likely to reach detectable levels in the future. The cultivation of this perennial halophyte will be an investment in soil resources.

At a later stage, when exploitation of the *K. pentacarpos* crop is no longer efficient due to regular flooding, the occupation of the land by a *K. pentacarpos* community could improve the establishment of native wetland species. In our study, *K. pentacarpos* facilitated natural colonization of both *Spartina patens* (Aiton) Muhl. and *Baccharis halimifolia* L. In addition, *K. pentacarpos* did not negatively impact planted *S. patens* seedlings; instead in the second year the study site began to mirror a natural brackish marsh dominated by stands of *S. patens*. The communities that develop around *K. pentacarpos* were more diverse than those planted directly with *S. patens* and more productive than control communities, indicating that the plant communities that would arise around *K. pentacarpos* would have the highest probability of becoming ecologically sustainable. The long term succession that may occur in these communities is of particular interest and will continue to be monitored in the following years after the completion of this study.

The role of temperature was explored to further our understanding of its role in the growth and development of *K. pentacarpos*. Seed dormancy of *K.*

*pentacarpos* was readily broken once water imbibition occurred. Time required for germination was not particularly temperature-sensitive; however the effect of growth temperature on the time required for seedlings to reach later developmental stages, such as secondary root and leaf production, was significant. The increasing impact of temperature on *K. pentacarpos* development may be attributed to the increasing cellular growth component in the seedlings' growth process. In addition, temperature appeared to affect the development of the second set of leaves more clearly than that of the first set of leaves. An interesting question is developed here concerning the energetics involved in seedling growth and development. Specifically, when are the seedlings transitioning from using cotyledon-stored energy to photosynthesis? And is the impact of temperature on photosynthesis greater than its impact on the seedlings' ability to transfer stored energy from the cotyledons to its growing meristems? A variation in the effect of temperature on these processes, in combination with a transition from one energy source to another, would have implications for the role of temperature in seedling early life history.

Delaware and Georgia seedlings responded differently to temperature in terms of time required to reach developmental stages, final size and weight of roots and stems at the time of fourth leaf development, and daily growth rate attained at various temperatures. Delaware seedlings showed a higher tolerance for a greater range of temperatures and an ability to increase growth rates with temperature above that of Georgia seedlings. The lower growth threshold temperature (base temperature) for Georgia seedlings is near 15° C and neither Delaware nor Georgia seedlings tolerated temperatures of 35° C, with a daily maximum at 39.5° C. These limits should be considered for sowing and early *K. pentacarpos* growth.

Finally, seedlings grown at higher temperatures reached maturity at a smaller size. This quality could be useful in influencing the crops resource allocation, for greater vegetative or reproductive growth according to product interests, and should be explored further. Specifically, does this observation hold true throughout the plant's life cycle? Would *K. pentacarpos* grown at colder latitude, or sown earlier in the spring when temperatures are cooler, take longer to mature and hence produce greater stem biomass? Could we increase reproductive yield by growing a cold-adapted *K. pentacarpos*, a Delaware ecotype for example, at temperatures warmer than its natural location? Innovative explorations of the specific adaptations found in ecotypes throughout the species' natural range could contribute greatly in efforts for commercialization of this useful halophyte.

## Appendix A

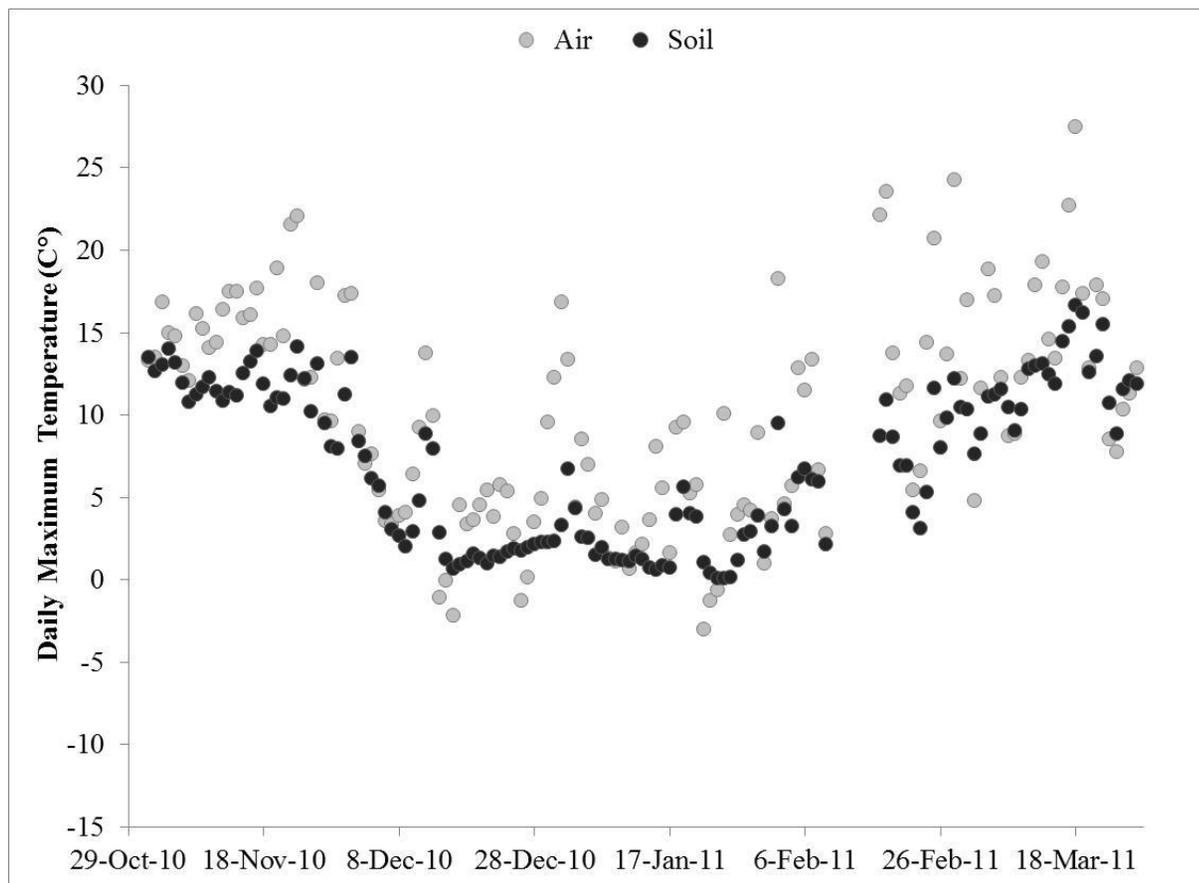
### CHAPTER 2 SUPPLEMENTARY MATERIAL

**Table A1** Two factor mixed model ANOVA table for the change in soil pH over the first year of this study. Treatment is a fixed factor and block is a random factor. Null hypotheses were: No difference in pH change between treatments. No difference in pH change between blocks. And no interaction between treatment and block, i.e. the effect of treatment on change in pH is independent of block and vice versa.

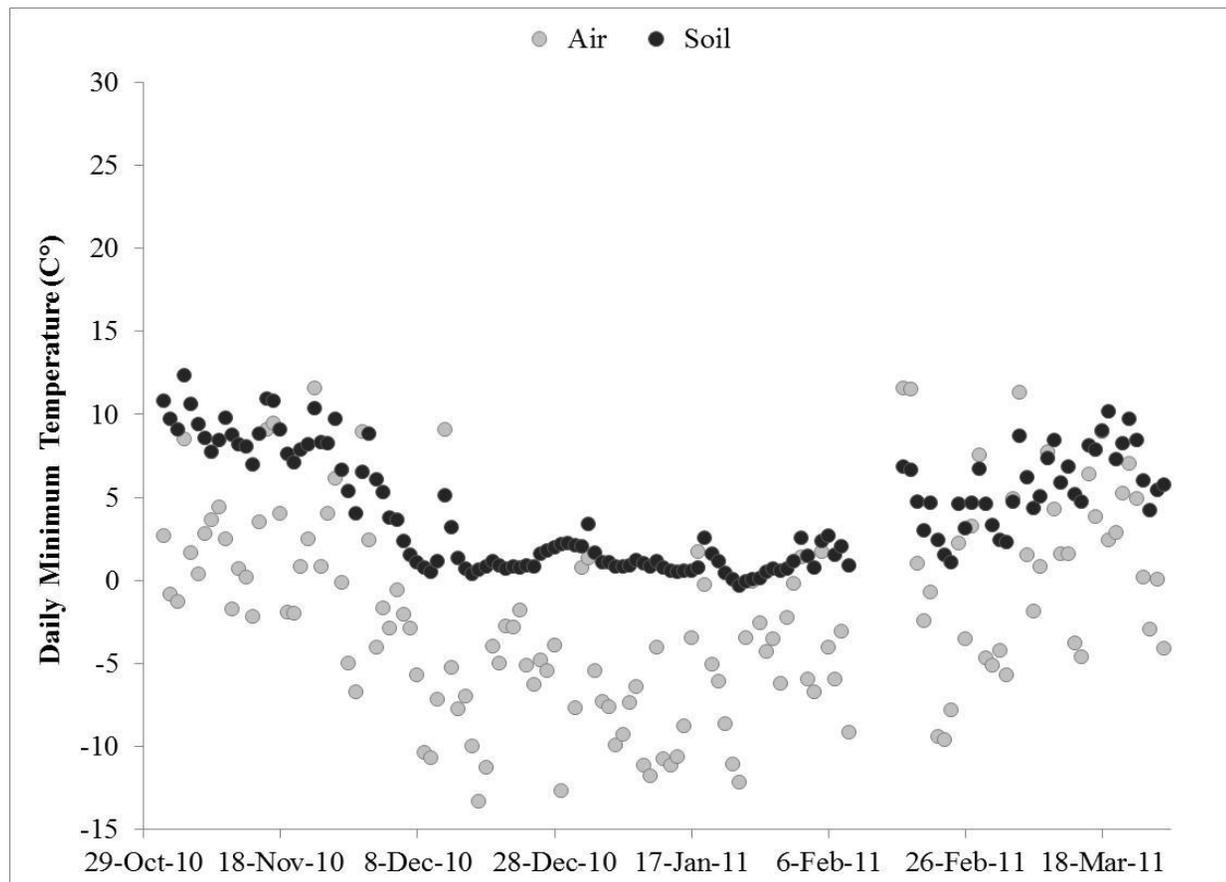
	df	MS	<i>F</i>	<i>P</i>
Treatment	3	0.0711	0.3963	0.7664
Block	1	0.6017	5.6628	0.0301
Treatment x Block	3	0.1794	1.6889	0.2094
Residual	16	0.1062		

**Table A2 Three factor mixed model ANOVA table for soil potassium concentrations in this study. Treatment and year were fixed factors and block was a random factor. Null hypotheses were: No difference in potassium concentrations between treatments. No difference in potassium concentrations between blocks. And no interaction between treatment, block and year in all combinations, i.e. the effect of treatment on potassium concentrations is independent of block and year and vice versa.**

	df	MS	<i>F</i>	<i>P</i>
Treatment	3	95.9473	0.5160	0.6998
Block	1	153.2960	0.8275	0.3698
Year	1	2055.3919	428206.6406	0.0010
Treatment x Block	3	185.9596	1.0038	0.4038
Treatment x Year	3	381.3553	5.7590	0.0922
Block x Year	1	0.0048	0.00003	0.9960
Treatment x Block x Year	3	66.2194	0.3574	0.7841
Residual	32	185.2599		



**Figure A1** Daily maximum air and soil temperatures, as recorded at the study site, during the cold months between the growing seasons sampled in this study.



**Figure A2** Daily minimum air and soil temperatures, as recorded at the study site, during the cold months between the growing seasons sampled in this study.

## Appendix B

### CHAPTER 3 SUPPLEMENTARY MATERIAL

**Table B1** Two factor fixed model ANOVA table for the days after planting required by seedlings root radicles to emerge from the seed coat. Temperature and ecotype were both fixed factors. Null hypotheses: No difference in days required for radicle emergence between temperature treatments. No difference in days required for radicle emergence between ecotypes. And no interaction between temperature and ecotype, i.e. the effect of temperature on the days required for radicle emergence is independent of ecotype and vice versa.

	df	MS	<i>F</i>	<i>P</i>
Temperature	4	0.0771	48.9790	> 0.000001
Ecotype	1	0.5180	32.9310	> 0.000001
Temperature x Ecotype	4	0.0330	2.0850	0.0830
Residual	236	0.0160		

**Table B2** Two factor nested ANOVA table for the growth rate of *K. pentacarpos* callus tissue culture at temperature treatments. Temperature was a fixed factor. Petri dishes contained four samples each and were the nested factor. Null hypotheses: No difference in the growth rate of callus tissue cultures between the temperature treatments. And no difference in the growth rate of callus tissue cultures between all dishes in any treatment.

	df	MS	<i>F</i>	<i>P</i>	% of total variance
Temperature	4	69.636	16.595	< 0.00001	(87.5)
Dish (temperature)	14	4.196	2.263	0.00003	12.5
Residual	57	1.854			