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Developing Best Practices for the Propagation of Spartina alterniflora for use in Salt Marsh Restortaion

Justin Hinson

DEVELOPING BEST PRACTICES FOR THE PROPAGATION OF *SPARTINA ALTERNIFLORA* FOR USE IN SALT MARSH RESTORATION

by

JUSTIN HINSON

(Under the Direction of Heather Joesting)

ABSTRACT

Coastal salt marshes are valuable ecosystems under threat from climate change and sea level rise. Living shorelines offer a promising solution, often incorporating the foundational salt marsh species *Spartina alterniflora* due to its ability to tolerate natural stressors and maintain sediment stability. However, research suggests that seed-based propagation protocols should be developed on a local scale due to the genetic heterogeneity within and between *S. alterniflora* populations. Here, we attempt to contribute to the development of one such protocol for coastal Georgia *S. alterniflora*.

In fall 2021, seeds were collected bi-monthly from four marshes of varying ocean proximity and stratified at 4 °C for 9-20 weeks in several types of storage vessels. Following this, seed viability was assessed, and seeds from spikelets with >10% viability were placed under germination trials. Plants were then moved to a greenhouse and grown for 10 weeks in a factorial combination of saltwater/freshwater x potting soil/salt marsh inoculated soil. During this growth period, plants were measured weekly for stem height, diameter, and number of ramets, and every three weeks for leaf chlorophyll content. At the end of the experiment, all plants were harvested, measured for root length, and dried at 65°C for at least 48 hours for determination of aboveground and belowground biomass.

Variation in seed set, viability, and germination rate was observed across sites, with the lower salinity site striking an optimal balance between seed set, viability, and germination. Across collection dates, variation was only observed in seed set and germination rate, with the November collection periods having the greatest seed set and germination rate. There was an effect to storage vessel on post-stratification viability, but there was no relationship between measured seed viability and seed germination. Plants grew best in inoculated soils and when watered with fresh water, growing faster, taller, developing greater biomass, and maintaining greater leaf chlorophyll concentrations. In summation, we recommend that seeds be collected from low salinity marshes and be stratified in spacious containers over the winter. While optimal conditions for germination remain elusive, plants grow best in freshwater and with a greater proportion of natural soil microbiomes.

INDEX WORDS: *Spartina alterniflora*, Living shorelines, Salt marsh, Plant ecology, Restoration, Propagation.

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DEDICATION

I'd like to dedicate the work presented here to the friends, family, and educators that played an integral role in guiding me to this point in my academic career; to all the future graduate students, may needed change come swiftly; and to the ecosystems we lack the wisdom to cherish, may all works like this become obsolete some day in the best possible way.

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I'd like to take the opportunity to acknowledge the contributions of:

All the family and friends that helped guide me to this point in my academic journey. Every effort is deeply valued: dragging me to Chem 1310 study sessions, loudly swearing at Physics 2 homework assignments, moving the same Ikea furniture over and over again, having each other's back through illness and a global pandemic, and grinding away school stress though hours of the most stressful games mankind has ever created.

My graduate advisor, Dr. Heather Joesting, for having the patience to guide me through the academic process, helping me adapt to changing research and university conditions, countless hours of editing and statistics double-checking, and giving me the space I needed to learn from my many mistakes along the way. Similarly, I'd like to acknowledge the other educators who put more than their fair share of time into helping me achieve my goals and grow as a person over the years. This project stands as a testament to the impact you've had on the world.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

DEVELOPING BEST PRACTICES FOR SEED COLLECTION AND GERMINATION OF *SPARTINA ALTERNIFLORA*

Introduction

Across the East and Gulf Coasts of the United States, salt marshes fringe the coastal landscape. In the state of Georgia specifically, naturally occurring salt marshes make up approximately 368,000 acres of coastal habitat (Coastal Resource Division n.d.). These important ecosystems are of tremendous economic, cultural, and ecological value. Not only do they serve as centers of high primary and secondary productivity, but salt marshes also serve as nutrient sinks for both carbon and nitrogen, protect coastal regions from erosion and flooding, maintain commercial fish and shellfish populations, provide key stopover sites for migratory birds, and provide space for tourism and recreation (Barbier et al. 2011; McFarlin et al. 2008; NOAA 2022; Smith et al. 2020).

Despite their myriad functions and high productivity, salt marshes exhibit relatively low floral diversity (Barbier et al. 2011). Along the Georgia coast, salt marshes are largely dominated by a single species of grass: *Spartina alterniflora*. An estimated 90% of Georgia's salt marshes are covered with a monoculture of this grass (Sapelo Island NERR 2022). As such, it is often considered a foundational species due to both its high abundance and the critical roles it plays in maintaining the ecosystem. From an ecosystem maintenance perspective, both the herbivores and detritivores in marshes are reliant on the primary productivity of *S. alterniflora*, and evidence suggests that *S. alterniflora* individuals may exert a level of control over the diversity of their rhizospheric microbial communities (Lin et al. 2018; Parker et al. 2008). Regarding abiotic features, *S. alterniflora* maintains marsh elevation by promoting sediment deposition, as dense

stands inhibit the movement of water during inundation and act as a physical obstruction for sediment particles (Gleason et al. 1979). Along tidal creek banks and mudflats, *S. alterniflora* has been shown to modify soil characteristics in such a way that limits erosion and promotes sediment stability (Feagin et al. 2009).

Unfortunately, due to changes in the global climate and land usage, salt marshes are in decline globally (Duarte et al. 2008). An estimated 1-2% of the world's salt marshes are lost each year due to numerous factors (Duarte et al. 2008). Human activities on and around coastlines has been shown to negatively impact coastal ecosystems in a number of ways, and only an estimated 15.5% of coastlines experience little or no anthropogenic impact (Gedan et al. 2009; Williams et al. 2021; Windom and Palmer 2022). Ultimately, though, the greatest threats to marshes are global climate change and the associated rise in sea levels (Fagherazzi et al. 2019; Gedan et al. 2009). While natural landscapes dominated by *S. alterniflora* should be able to resist rising sea levels through vertical accretion, it is possible that rates of sea level rise will outpace the accretion rates of marshes (Windom and Palmer 2022; Roman 2017). Furthermore, shifts in both temperature and rainfall associated with climate change have already been linked to overall declines in *S. alterniflora* populations along the East and Gulf Coasts of the United States (e.g., salt marsh dieback events), and erosion of a marsh's seaward edge may decrease the spatial extent a marsh can occupy (Hughes et al. 2012; Leonardi et al. 2016; Mckee et al.2004).

To combat these threats – particularly those associated with marsh loss via shoreline erosion- marshes are typically stabilized using some form of hard structure, such as a seawall or bulkhead. However, the usage of these structures is far from a perfect solution. According to information gathered by NOAA, typical seawalls and bulkheads are among the most expensive shoreline stabilization options in terms of both installation and maintenance (NOAA 2015). Hard

structures promote erosion in adjacent areas under normal conditions and fail to adequately protect their own shorelines when they are needed most: during storms (Polk and Eulie, 2018; Smith et al. 2018). Smith et al. (2018) found that out of four bulkhead sites observed in North Carolina, three were significantly damaged by Hurricane Matthew and all failed to maintain marsh elevation (Smith et al. 2018). A much more viable solution to erosion, climate change, and sea level rise in marshes is the implementation of living shorelines.

Living shorelines are a form of green infrastructure, which the 2019 Water Infrastructure Improvement Act defines as a range of measures that use plant or soil systems to alter the movement of stormwater (Sec 5a). Within the context of coastal salt marshes, a living shoreline is a man-made structure that mimics nature, the goal of which is to reduce erosion due to wave action and storms, enhance shoreline resilience, create habitat for endemic species, maintain terrestrial-marine connectivity, and restore ecological processes (DNREC n.d.; NOAA 2015). These structures typically combine native vegetation with natural physical structures like rocks, logs, or oyster shells (Davis et al. 2015; Polk and Eulie, 2018). Though they may vary slightly in design from place to place, a common living shoreline near a Georgia marsh edge or riverbank might consist of an arrangement of mesh bags filled with oyster shells stacked like sandbags along the face of an eroding embankment (Georgia Department of Natural Resources 2013). Along the top and behind the embankment, a native marsh plant, like *S. alterniflora*, is planted to trap suspended sediment and stabilize deposited sediment. Arrangements like these have already been shown as sediment accumulators - a critical component of maintaining marsh elevation in the face of stronger storms and rising sea levels predicted to occur as the climate changes (Polk and Eulie 2018). Even in extreme conditions, such as those created by Hurricane Matthew in 2016, living shorelines were able to withstand storm wind and wave energy and even accumulate

sediment while more traditional structures like bulkheads were severely damaged and experienced significant erosion (Smith et al. 2018). Furthermore, healthy natural marshes are noted for their high carbon sequestration rates, and living shorelines mimic these environments given enough time (Davis et al. 2015). Their implementation has been shown to improve the carbon sequestration rates of a degraded marsh immediately by limiting erosive loss and over long periods of time by sequestering carbon belowground (Davis et al. 2015). The integration of natural structures and critical native flora create what appears to be a relatively low maintenance and highly effective solution to many threats facing coastal salt marshes.

Although *S. alterniflora* makes an ideal candidate for use in living shorelines, a few critical issues arise with respect to its implementation. Firstly, *S. alterniflora* is a highly diverse species on multiple scales. Along the East and Gulf Coasts of the US, four unique cpDNA and random polymorphic site-based haplogroups have been identified (Blum et al. 2007; O'Brien and Freshwater, 1999). These haplogroups exhibit unique traits (e.g., biomass distribution, stem density, carbohydrate reserves) that help them thrive in their specific environments. Furthermore, these traits are maintained even when transplanted in a similar environment, demonstrating a lack of short-term adaptability in *S. alterniflora* (Seliskar et al. 2002). This presents an issue when it comes to restoration, as non-local varieties of *S. alterniflora* show a decline in overall fitness when grown outside of their native environment (Travis & Grace 2010). Travis and Grace (2010) recommend that transplanted individuals be taken from no further than 100 km away from their transplant site in order to minimize this non-adaptive stress.

To further complicate matters, *S. alterniflora* exhibits high clonal diversity within a marsh. Although capable of clonally reproducing through belowground rhizomes at a much lower energetic cost, *S. alterniflora* reproduces sexually through seeds unexpectedly often for a

plant found in such a stressful habitat, thus leading to the formation of genetic patchwork landscapes (Richards et al. 2004). Genetically identical patches typically cover an area of less than 10 m, indicating that while rhizomatous clonal reproduction does occur, sexual reproduction still occurs on significant scales (Richards et al. 2004). Bearing this in mind, it has been recommended that transplant individuals come from a genetically heterogenous stock in order to maintain levels of genetic diversity present in degraded marshes (Gaynor et al. 2019; Richards et al. 2004). Finally, seed production itself is variable within a given marsh, with plants that flower earlier in a season producing fewer seeds (Fang et al. 2004). Given all this information, an ideal *S. alterniflora* transplantation process would require the identification of a degraded site, identification of a high seed set marsh within 100 km, collection of seeds from this marsh during an ideal time, and germination and growth of seeds in optimal conditions for their environment.

Despite the aforementioned difficulties, the Georgia Department of Natural Resources acknowledges that living shorelines are one of the most effective means of improving the natural and economic health of Georgia's coastlines (Georgia Department of Natural Resources 2013). Already, a number of living shorelines have been constructed by the DNR for the express purpose of study, while others have been constructed by the University of Georgia (Georgia Department of Natural Resources 2013; Marine Extension and Georgia Sea Grant n.d.). However, currently no protocol exists for the cultivation of native *S. alterniflora* for coastal Georgia. The purpose of this research was to contribute to the development a readily accessible set of guidelines for the collection, germination, and growth of *S. alterniflora* to facilitate the nursery production of this species for marsh restoration in coastal Georgia. Specifically, this research aimed to determine ideal marsh conditions for maximal viable seed yield, identify optimal conditions and duration of overwintering storage to break dormancy, and ideal

conditions for seed germination. We hypothesized the following: 1) marshes further from the ocean would provide the greatest seed yields and average seed viability; 2) no significant differences between different methods of overwintering storage would be observed, but shorter overwintering duration would yield greater germination success; and 3) germination rates would be greatest in constantly elevated temperatures.

Methods

Study Species

Spartina alterniflora Loisel. is a perennial C4 grass native to the Gulf of Mexico and East Coast of North America. This salt-tolerant species often grows in dense monocultures in frequently inundated areas of coastal salt marshes, both along tidal creek banks and in the mid- to high marsh. In these two environments, *S. alterniflora* grows in two forms: tall-form and short-form. Tall-form *S. alterniflora* dominates at lower elevations along creek banks, typically growing up to 2.5 m tall. In contrast, the short-form fills the higher elevation mid-marsh with stems up to 40 cm tall (Walkup 1991). The driving mechanism behind growth form determination is yet to be fully understood, but current research indicates that a combination of genetic and environmental factors plays a role (Valiela et al. 1978; Gallagher et al. 1988; Wilson et al. 2015).

Plants typically flower in the late summer and shed their seeds in the fall, though the exact timing of each event varies along a latitudinal gradient (Crosby et al. 2015; Fang et al. 2004). In the state of Georgia, flowering has been observed to occur during August and September, followed by seed development during September and October and seed shedding during October and November (H. Joesting, personal communication). These timings may not hold true for populations in other regions, though. For example, individuals in Massachusetts and Rhode Island flower earlier in the year due to that region's shorter growing season (Crosby et al. 2015). When they develop, flowers are typically small and whitish in color. They occupy the upper 25-30 cm of the stem in a series of 5-10 cm spikelets and are pollinated by wind (Bush and Houck 2008). After pollination, flowers develop into light brown seeds typically 1 cm in length (Biber et al. n.d.)

Study Sites

S. alterniflora seeds were collected from four salt marshes distributed throughout Chatham County, Georgia, United States: (1) Lazaretto Creek Boat Ramp (LCBR, 32°00'56.9"N 80°53'25.8"W); (2) Priests Landing (PL, 31°57'49.6"N 81°00'50.1"W); (3) Rodney Hall Boat Ramp (RHBR, 31°56'47.8"N 81°04'04.5"W); and (4) Halcyon Bluff Community House (HBCH, 31°58'57.4"N 81°06'40.3"W) (Figure 1-1). These sites were selected based on both relative proximity to the ocean and ease of access via road and foot, and each varied in a number of other conditions (Table 1-2). LCBR is a roadside marsh featuring a moderately-sized parking lot and single 3-lane public boat ramp (Table 1-2). Near the bank of LCBR, a fringe of tall-form *S. alterniflora* dominates, and extending back behind this fringe to the roadside is a short-form *S. alterniflora* meadow (Table 1-2). Water salinity at LCBR ranged from 24-27 ppt throughout the study period (Table 1-1). PL is largely dominated by short-form *S. alterniflora*, with only a few select patches of tall-form near the banks of the Wilmington River (Table 1-2). Though the marsh is publicly accessible and there is a hiking trail nearby, there is no boat ramp or paved parking lot (Table 1-2). Throughout the study period, water salinity at PL ranged from 25-35 ppt (Table 1-1). RHBR consisted of an entirely tall-form fringe along the Skidaway River (Table 1-2). Two large public boat ramps are accessed via a large parking lot that frames the study site and the area is frequented by visitors (Table 1-2). Throughout the study, water salinity at RHBR ranged from 22-23 ppt (Table 1-1). HBCH is a largely tall-form marsh located along a tidal creek of the Vernon River (Table 1-2). The area is not open to the public but does feature a single concrete boat ramp for residents of the nearby neighborhood (Table 1-2). During the study period, the water salinity at HBCH ranged from 9-14 ppt (Table 1-1). In terms of proximity to

the ocean, LCBR was located closest to the Atlantic Ocean, PL and RHBR were located midway within the coastal landscape, and HBCH was the furthest removed from the ocean. All salinities were measured from a nearby waterway at low tide immediately before collection began.

Figure 1-1. Map of Chatham County, GA, showing the locations of field sites. Sites include Halcyon Bluff Community House (HBCH), Rodney J Hall Boat Ramp (RHBR), Priests Landing (PL), and Lazaretto Creek Boat Ramp (LCBR). Each site varied in proximity to the ocean and salinity ranges during the seed collection period.

Table 1-1. Salinity (ppt) measured at each field site during each collection period. LCBR lacks a salinity measurement during collection period 1 because no seeds were ready to be collected at that site during that time frame.

Field Site	Collection 1 Early Oct 10/4 - 10/9	Collection 2 Late Oct 10/16 - 10/19	Collection 3 Early Nov 11/1 - 11/7	Collection 4 Late Nov 11/16 - 11/19	Mean Salinity
HBCH	9	13	14	13	12.25
RHBR	22	23	22	23	22.50
PL	35	27	27	27	29.00
LCBR	-	24	24	25	24.33

Table 1-2. Observed differences in field sites. Dominant growth form indicates which growth form of *S. alterniflora* grew at the site in highest density. Waterway classification is determined by the name of the primary waterway near which samples were collected. Note that while HBCH is along the Vernon River, the waterway more closely resembles Lazaretto Creek (LCBR) where samples were collected. Frequency of human use is estimated here based on local site popularity followed by the number of collection periods (out of 4) in which people were encountered at the site. Upstream disturbance potential is an estimated likelihood of impactful upstream runoff, pollution, etc.

Field Site	Dominant Growth Form	Waterway Classification	Frequency of Human Use	Upstream Disturbance Potential
HBCH	Tall	Creek	Low (1)	High
RHBR	Tall	River	High (4)	Moderate
PL	Short	River	Very Low (0)	Low
LCBR	Short	Creek	High (4)	Very Low

Experimental Methods

Seeds were collected from each site bi-monthly from October 4th to November 18th, 2021, for a total of four collection periods. Collection period 1 ranged from Oct. 4 - Oct. 9, period 2 ranged from Oct. 16 - Oct. 19, period 3 ranged from Nov. 1 - Nov. 7, and period 4 ranged from Nov. 16 - 19. During sampling, 21 mature spikelets were collected from tall-form *S. alterniflora* plants first, and if none were present or accessible, short-form spikelets were taken. Spikelet maturity was determined in the field by physically examining each plant's floral stem (peduncle) color and readiness with which seeds were shed, with more mature spikelets generally with less green peduncles and seed shedding with gentle disturbance. Each spikelet contained between roughly 20 to over 650 seeds, with a mean seed count of 137 seeds. Seeds were processed within 5 days of collection, during which the total number of seeds per spikelet was counted and seeds were prepared for overwinter storage (cold stratification) following Biber et al. (n.d.). To prepare for overwinter storage, spikelets were randomly assigned to one of three storage vessels: sealable quart-sized plastic bags (PB), 50 mL plastic centrifuge tubes (CT), and 25 mL glass scintillation vials (SV). These vessels were selected for their low cost, ease of acquisition, and variation in internal volume. Seeds were then stored in water at 4°C (following Biber et al. n.d.) for between 9 and 20 weeks, a range of time spanning both the minimum needed to break dormancy (Biber et al. n.d.) and the time between the dormancy period's end and viability processing.

Following the overwintering period, 25% of seeds were haphazardly selected from each spikelet x storage vessel combination and tested for viability using both a fluorescent light box (following Fang et al. 2004) and a TZ test - a commonly used method of biochemically determining seed viability. We chose to examine the effectiveness of both methods because

although the TZ test is widely used and accepted, it may present practical problems for use in plant nurseries. While more reliable, the TZ test requires the use of costly and potentially hazardous chemicals, requires a 48-hour waiting period, and leads to seed mortality. In contrast, the less widely used light box is comparatively cheaper, requires no special chemicals, provides instantaneous results, and does not lead to seed mortality. Seeds were first placed on the fluorescent light box where they were visually examined. Darker seeds were considered filled and therefore viable, whereas lighter seeds were considered empty and unviable. All seeds examined using the light box (both filled and unfilled) were then transferred to a glass petri dish and submerged in a 1% tetrazolium (TZ) chloride solution under dark conditions for at least 48 hours. Following this, seeds were visually examined for red and/or pink coloration, indicating metabolic activity. As the effectiveness of the light box was under investigation, only the TZ test results were used to determine a viability percentage of each spikelet.

Once viability percentages were determined for each spikelet, the remaining seeds for spikelets with greater than 10% viability underwent a total of four germination trials. For each germination trial, a random selection of 12 spikelets (determined using a random number generator) were removed from overwinter storage, and the seeds from each were separated equally into three glass culture dishes. Based on Biber et al. (n.d.), each dish was filled roughly halfway with tap water and placed into one of three germination conditions: (1) a room temperature grow-cart, (2) a growth chamber set at a constant temperature of 32℃, and (3) a growth chamber set to a 12:12 day/night temperature cycle with a daytime temperature of 32℃ and a nighttime temperature of 24℃. Hourly temperatures for each treatment were monitored using Hobo Pendant MX Temperature Data Logger (Onset, Bourne, MA). Germination was observed for 14 days, during which additional tap water was added as needed to ensure

submergence of seeds. Throughout each 14-day trial, germinated seeds were removed once the initial shoot reached an approximate length of 1.3 cm, placed into a peat pellet, and sub-irrigated in its experimental conditions for the remainder of the trial (following Biber et al. n.d.). Following the conclusion of each trial, seedlings were potted in a plastic 4.5 in x 4.5 in square pot filled with a 2:1 mixture of topsoil:sand. These pots were sub-irrigated and maintained under greenhouse conditions for use in the experiment described in Chapter 2.

Statistical Analyses

Prior to statistical analyses, all data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's tests, respectively. All variables (seed count, seed viability, and germination rate) could not be transformed to meet these assumptions. Thus, to determine the effect of site and collection period on the number of seeds per spikelet, data was analyzed using a Kruskal-Wallis test, with Steel-Dwass Post-Hoc test and significance at $p<0.025$ (Bonferroni corrected, $\alpha/2$ where $\alpha=0.05$). To determine the significance of collection site, collection date, overwintering dormancy period, and overwintering storage vessel on seed viability, a series of Kruskal-Wallis tests were performed followed by Steel-Dwass tests for multiple comparisons, with $p<0.0125$ (Bonferroni corrected, $\alpha/4$ where $\alpha=0.05$). To determine if there was a significant difference between the seed viability predictions between the light box and the TZ test, a Wilcoxon signed-rank test was performed with significance at $p<0.050$ given that the assumptions for a Matched Pairs T-test could not be met. To evaluate the significance of collection site, germination trial, germination treatment, overwintering dormancy period, storage vessel, and collection dates on seed germination rates, a series of Kruskal-Wallis tests were performed followed by Steel-Dwass tests for multiple comparisons, with $p<0.008$ (Bonferroni corrected, $\alpha/6$ where $\alpha=0.05$). The relationship between seed viability rate per spikelet and seed

germination rate per spikelet were compared using a linear regression, with significance at $p<0.050$.

Results

Spatial and Temporal Patterns

There was a significant effect of site (χ^2_3=30.658, p<0.001) on the number of seeds per spikelet. Of the four sample sites, HBCH produced significantly more seeds per spikelet on average than PL (p<0.001), RHBR (p<0.001), and LCBR (p<0.001) (Figure 1-2). There was little variation across collection dates, with a significant difference existing only between collection periods 3 (11/1 - 11/7) and 4 (11/16 - 11/19) (Table 1-3).

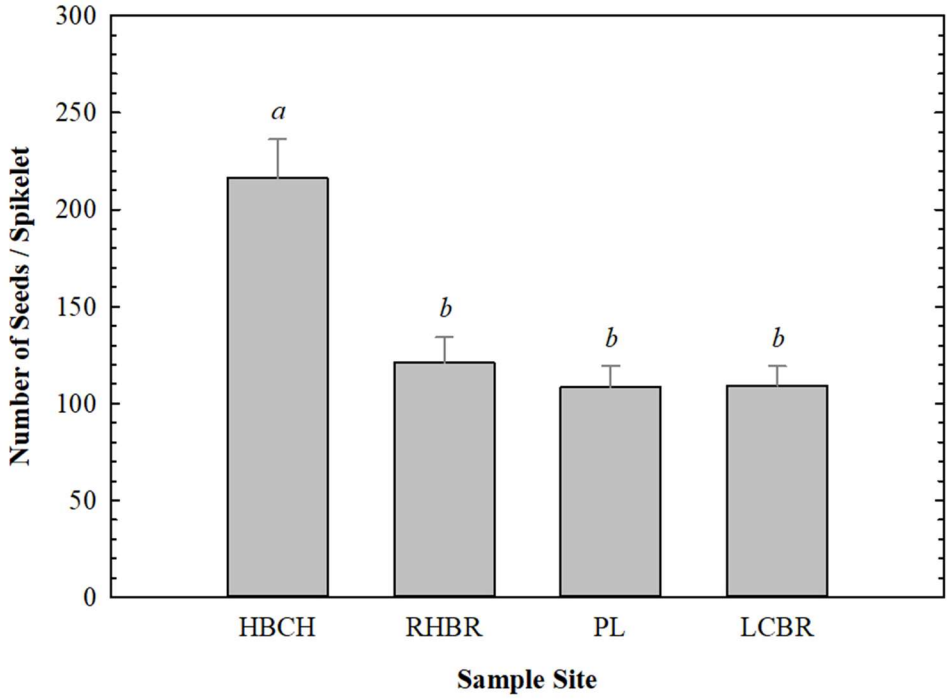

Figure 1-2. Mean number of seeds per spikelet from four marshes of varying proximity to the ocean. Error bars represent standard error and lettering indicates statistical significance at p<0.025.

Table 1-3. Mean ± standard error, test-statistic (χ^2_{df}), and p-value for number of seeds per spikelet, seed viability (as determined by the TZ tests), and seed germination rates for *S. alterniflora* sampled across four collection periods. χ^2 and p-value determined by Kruskal-Wallis with Steel-Dwass post-hoc, and lettering indicates significance determined at $p<0.025$.

Collection Dates	Collection 1 Early Oct 10/4-10/9	Collection 2 Late Oct 10/16 - 10/19	Collection 3 Early Nov 11/1 - 11/7	Collection 4 Late Nov 11/16 - 11/19	χ^2	p-value
Seed Count	129 ± 11.2^{ab}	150 ± 10.7^{ab}	113 ± 8.1^{b}	158 ± 14.2^{a}	$\chi^2_3=12.65$	p=0.006
Seed Viability	0.20 ± 0.034^{a}	0.24 ± 0.039^{a}	0.28 ± 0.042^{a}	0.27 ± 0.038^{a}	$\chi^2_3=2.90$	p=0.407
Germination Rate	0.01 ± 0.005^{b}	0.03 ± 0.007^{b}	0.07 ± 0.011^{a}	0.04 ± 0.009^{ab}	$\chi^2_3=13.46$	p=0.004

A significant effect of collection site was observed for seed viability (χ^2_3=12.2614, p=0.007), with LCBR having greater seed viability, but differing significantly only from RHBR (p=0.006; Figure 1-2A). There was no significant difference in seed viability across collection dates (p=0.407; Table 1-3) or duration of the overwintering period (χ^2_{10}=15.2638, p=0.123). However, there was a significant effect of storage vessel on seed viability (χ^2_3=17.6026, p<0.001; Figure 1-2B). Spikelets that overwintered in PB had significantly greater mean seed viability (37%) compared to those that overwintered in CT (16%, p<0.001; Figure 1-3B) and SV (22%, p=0.014; Figure 1-3B). CT and SV did not differ from each other (p=0.369). Finally, there was a significant difference in seed viability predictions between the light box and the TZ test (S_{164}=-1254.5, p=0.041). The light box and TZ test had mean predicted viability of approximately 27% and 24%, respectively.

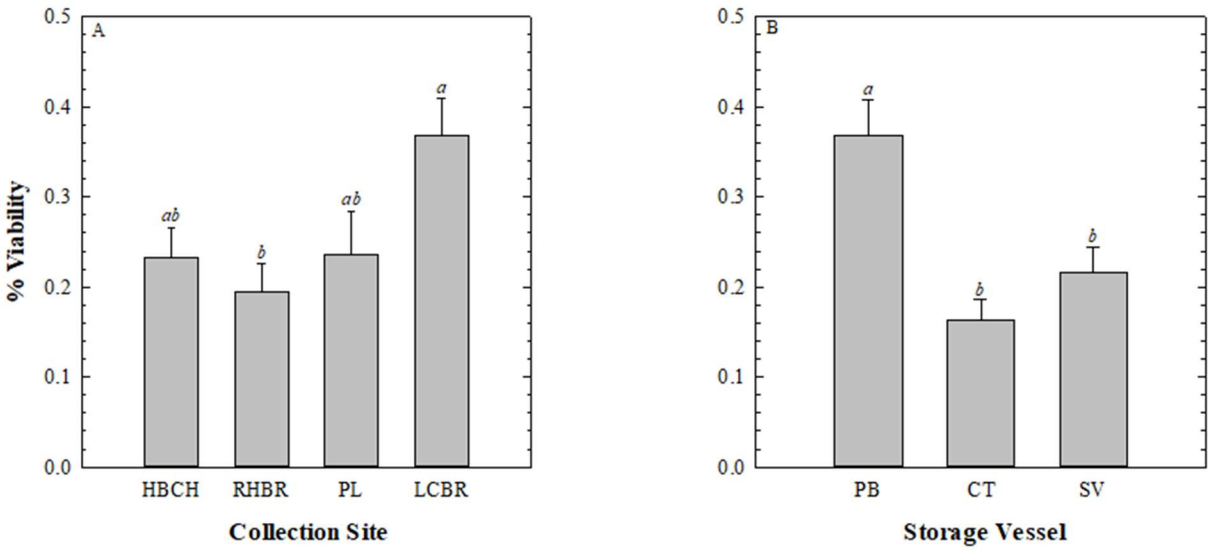

Figure 1-3. Mean seed viability among collection sites (A) and overwintering storage vessels (B). Error bars indicate mean standard error; and letters indicate statistically significant groupings at p<0.0125. Abbreviations as stated in text.

Germination Trials

There was a significant effect of site on seed germination (χ^2_3=26.43, p<0.001; Figure 1-4A). Specifically, the germination rate for LCBR was significantly different from both PL (p<0.001) and HBCH (p=0.001), and RHBR was different from PL (p=0.009). The duration of the overwintering period had an effect as well (χ^2_{10}=27.09, p=0.003), though no clear pattern was observed. Only dormancy weeks 12 and 15 were significantly different from each other (p=0.008). There was also a significant difference among seed collection dates (p=0.004; Table 1-3), with collection periods 1 and 3 significantly different from each other (p=0.005). There was no significant effect of germination temperature treatments (χ^2_2=4.63, p=0.010; Figure 1-4B), overwintering storage vessel (χ^2_2=4.64, p=0.010; Figure 1-4C), or germination trial (χ^2_3=3.63, p=0.304; Figure 1-4D) on germination rate.

Table 1-4. Mean ± standard deviation of germination rates and sample sizes across dormancy weeks. Sample sizes refer to the number of spikelets stored for a given length of time.

Dormancy Weeks	N	Mean Germination Rate
9	3	0 ± 0
10	11	0.05 ± 0.09
11	19	0.03 ± 0.04
12	20	0.09 ± 0.08
13	16	0.07 ± 0.08
14	20	0.02 ± 0.03
15	20	0.01 ± 0.02
16	9	0.04 ± 0.06
17	16	0.03 ± 0.05
18	0	-
19	3	0 ± 0
20	1	0.07 ± -

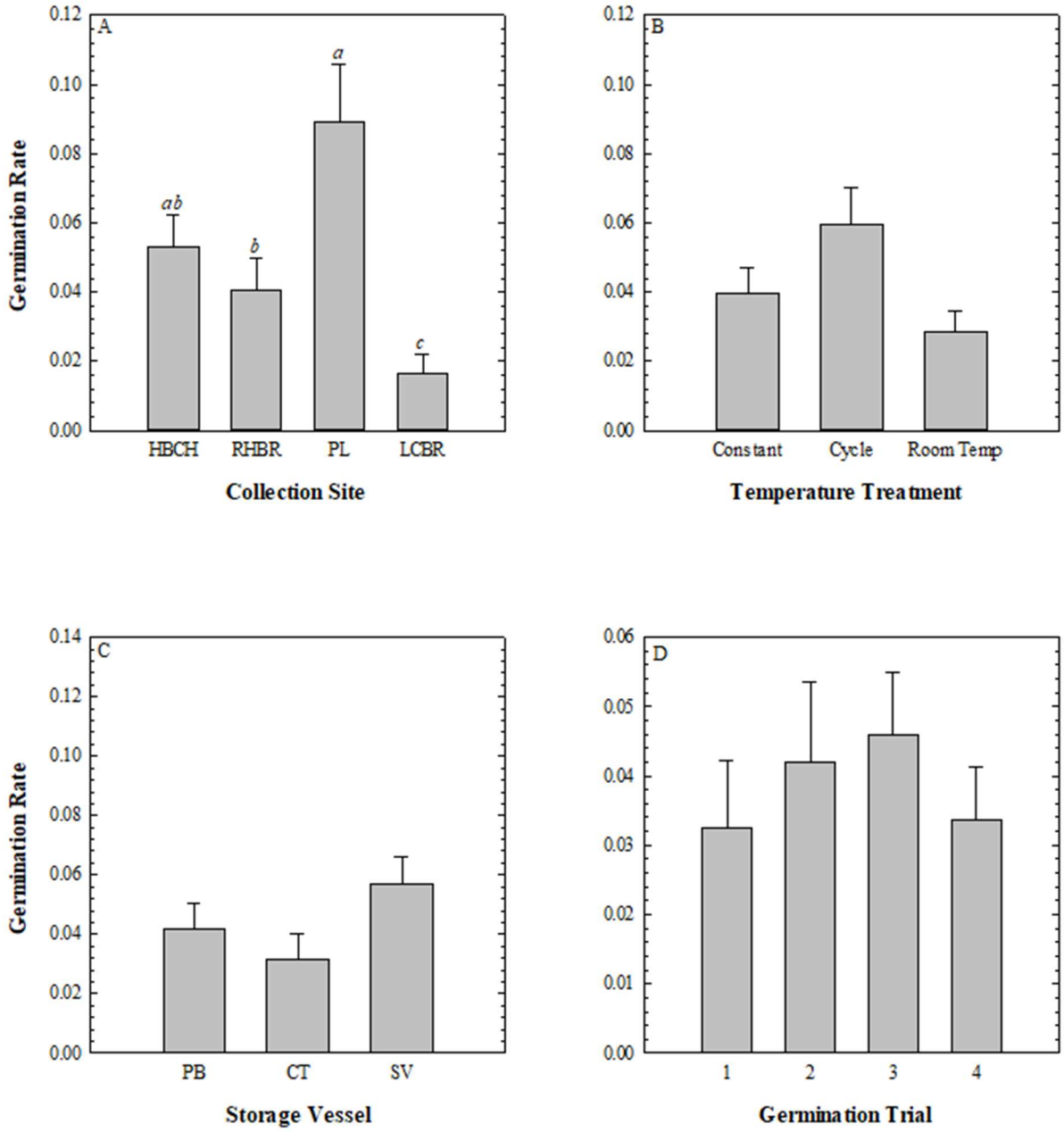

Figure 1-4. Mean germination rate among collection sites (A), germination temperature treatment (B), overwintering storage vessel (C), and germination trial (D). Error bars represent mean standard error; letters indicate statistically significant groupings at $p<0.008$.

Discussion

Results revealed both spatial and temporal patterns in fecundity in *S. alterniflora* populations occupying multiple marshes in coastal Georgia. Across collection sites, seed set was greatest at the site furthest from marine influence (HBCH), seed viability was greatest at the site closest to the ocean (LCBR), followed by HBCH and PL, and germination rate was greatest at one of the sites with moderate proximity to the ocean (PL). Additionally, there was an effect of collection period on fecundity, with the lowest seed set but greatest germination rate observed during Collection 3 and greatest seed set during Collection 4. Together, these results suggest that plants occupying marsh sites further from the marine influence may strike the most optimal balance between seed set, viability, and germination, suggesting that seeds should be collected from these sites later in the season to maximize seedling production.

Furthermore, there was an effect of storage vessel on seed viability, with the greatest viability found for seeds stored in plastic bags overwintering period. However, there was no effect of germination temperature on germination rate. Thus, although it is suggested that seeds be overwintered in plastic bags, there is no optimal temperature within the range measured that promotes germination.

Spatial and Temporal Patterns in Fecundity

Results suggested spatial patterns in fecundity among *S. alterniflora* populations in coastal Georgia. In the current study, one marsh site (HBCH) produced significantly more seeds per spikelet than the other sites. Spikelets at HBCH contained a mean of 216 seeds compared to the next highest yield at RHBR, which produced a mean of 120 seeds per spikelet. LCBR and PL had mean seed counts of 109 and 108, respectively. Combined, all sites collectively yielded a mean seed count of approximately 145 seeds per spikelet. This result offers some support for our

hypothesis that marshes further from the ocean - and therefore less influenced by tidal cycles and resulting salinity - would produce the greatest yield. Due to the scope of this project, though, only a single gradient of sites was able to be sampled (i.e. only one inland site, only one near-coast site, etc.). Further investigation of more sites is required before a more reliable conclusion can be drawn, especially considering the wide range of factors that may play a role in seed set and viability in the field.

Under stressful conditions, plants are often unable to dedicate energy to reproduction, instead allocating resources to stress tolerance and cell maintenance (Zhang et al., 2020). Therefore, plants growing in marshes closer to the ocean may produce fewer seeds due to greater resource allocation to tolerating salt stress. It should be noted, though, that while the other sites (i.e., RHBR, PL, and LCBR) varied in salinity ranges (Table 1-1) and proximity to the ocean (Figure 1-1), they did not vary significantly with respect to seed count, suggesting that other factors, like nutrient availability, may play a role in *S. alterniflora* seed set. HBCH's high seed yield may be linked to the upstream course of the creek flowing through the site. It, in contrast to other sites, winds near multiple neighborhoods and through a golf course, both of which may increase the nitrogen content of the waterway (Table 1-2). Though we gathered no data on this variable, nutrient-rich water has been shown to increase the ground coverage of *S. alterniflora* in natural marshes, indicative of greater reproduction (McFarlin et al. 2008). Additionally, a host of other factors could play a role in influencing the observed seed set among sites, including those discussed below with respect to other variables.

Seed viability varied among sites, though the only significant difference was between RHBR and LCBR (Figure 1-3). While salinity may have played a role in this, it is more likely that additional factors influenced viability in this study. LCBR may have had significantly

greater seed viability compared to RHBR due to a large portion of its spikelets being harvested from short form *S. alterniflora* plants (Table 1-2). Persistent differences have been observed between both short and tall growth forms, though the relationship of growth forms and both seed set and viability are not well documented in the literature (Gallagher et al. 1988). While efforts were made to collect only from tall form plants, conditions in the field limited the availability of such individuals at LCBR. In the future, investigation into reproductive differences between the two growth forms may yield insight into this potential driver.

Another factor that may have played a role in overall seed set and viability was anthropogenic disturbance. While all four sample sites had boat ramps or docks within roughly 100 m, RHBR plants were taken from a busy public access point. This area features a large parking lot, two double-lane boat ramps, and dock, all of which are frequently used by county residents (Table 1-2). It is possible that disturbance in the form of boat wake and boat-related pollution may have been a stressor to the plants, thereby affecting the amount of energy allocated to reproduction (Zhang et al. 2020). A study of wave tolerance of *S. alterniflora* in Alabama found a correlation between waves over 0.13 m and declines in plant density (Roland and Douglass 2005). Similarly, a study on the effects of chronic diesel exposure linked petroleum pollution to decreases in N_2 fixation from *S. alterniflora* associated microbial epiphytes (Piehler et al. 1997).

Results also suggest temporal (seasonal) patterns in fecundity within *S. alterniflora* populations. With respect to the timing of seed collection, there does appear to be an optimal time for seed collection. The greatest seed set was observed near the end of November (11/16 - 11/19). Although there was a significant difference between the number of seeds collected in early and late November, expanding the dataset to include multiple years eliminates this

difference (H. Joesting, personal communication). Seed collection timing did not appear to have any significant impact on seed viability, but there was a significant effect on germination. Collection period 3, which took place during the first week of November, had the greatest rate of germination. In contrast, collection period 1 during the first week of October had the lowest germination rate. Based on this, it would appear that seeds released early in the growing season may not be as well developed as those shed later.

Based on the data gathered, it is recommended that seeds be gathered from HBCH to increase seedling production. While other sites showed higher rates of viability and germination, seeds from HBCH showed the greatest overall performance (i.e., combination of seed set, viability, and germination). Furthermore, seeds should be collected later in the reproductive season (November for coastal Georgia) in order to maximize the production of seedlings.

Seed Storage and Germination

In the current study, resealable plastic bags maintained the highest seed viability among the three overwintering storage vessels. This could be due to the water volume of each storage vessel. Compared to scintillation vials and centrifuge tubes, there was a marked difference in volume capacity for water. The plastic bags used held approximately 650 mL compared to approximately 50 mL and 25 mL in the centrifuge tubes and scintillation vials, respectively. Additionally, due to their flexibility, plastic bags allowed for the formation of a wide air-water interface upon which most seeds were able to float. Given these two details, the larger interior space and greater volumes of both air and water may have allowed more seeds to disperse along the surface of the water, thereby gaining environmental access to both oxygen and water. In contrast, smaller containers filled with seeds may have limited the availability of air, forcing some seeds to spend prolonged periods of time fully submerged. Access to both air and water are

key requirements for many seeds, as oxygen is needed for seeds to break down their energy reserves through cellular respiration, and water maintains cellular functions that contribute to the eventual breakage of the seed coat (Australian Academy of Science 2016). Furthermore, contact with air may allow seeds immersed in water to transport water across cell membranes and maintain optimal turgor pressure through a lower pressure potential on the air-exposed side of the seed (Reece et al. 2014). Given that spikelets were assigned to their containers without regard to their seed set, spikelets with fewer seeds may have had a competitive edge over those with more seeds given their lower metabolic requirements and spatial consumption. Future investigations may yield greater insight into the effects of seed density on overwinter viability maintenance.

When determining the viability of seeds in preparation for germination, the widely used TZ method did outperformed the fluorescent light box. The fluorescent light box tended to overestimate viability, which is expected given its assumption that all filled seeds must be metabolically active. However, it should be noted that viability, regardless of how it is determined, was not a good predictor of overall germination. Across all germination treatments, far fewer seeds germinated compared to those that were deemed viable. These results suggest that it may be more efficient and economical to skip an assessment of seed viability in the nursery production of *S. alterniflora*.

Overall, a low germination rate was observed throughout the study and across all variables. On average, fewer than 10% of seeds were successfully germinated, regardless of the variables being examined. The only variable that appeared to have any significant effect on the rate of germination was the original sample site, though a clear pattern is yet to emerge from the results. The low germination rates observed in the current study may have been due to

overwintering storage and germination conditions that were not assessed in this research. For example, the overwintering temperature and use of freshwater (~0 ppt) were utilized based on a previous protocol for *S. alterniflora* seed propagation in Mississippi (Biber et al. n.d.). However, there are environmental differences between coastal Mississippi and Georgia salt marsh systems, and thus these conditions may not be representative of local Georgia marshes. The soil of Mississippi salt marshes trend towards lower salinity, with interstitial salinities ranging from 12-15.5 ppt compared to Georgia marshes, which can range from 20-35 ppt (Eleuterius and Caldwell 1985; Nestler 1977). Furthermore, throughout the study period, none of the field sites were observed to have a salinity at or near zero. Although saltwater has been assumed to be a stressor for *S. alterniflora* seeds, it may provide some sort of unanticipated metabolic or microbial benefit to germination.

Additionally, the temperature used in this study (4℃) may have been below optimal temperatures for Georgia *S. alterniflora* seed germination. Based on climate data gathered from WeatherSpark, average winter low temperatures approach 5.5℃ and average winter high temperatures approach 15.5℃ (WeatherSpark 2023). Additionally, the University of Georgia reports that 2-inch soil temperatures fluctuate between approximately 19℃ and 10℃ between October 2022 and March 2023 (University of Georgia Weather Network 2023). Given this, the methods presented here may have maintained seeds at an uncharacteristically low temperature for an extended period of time, which may have impacted not only the germination rates, but also the viability of the seeds. More research should be conducted to determine the optimal conditions for seed germination in *S. alterniflora*, including storage and germination salinity and temperatures that better represent Georgia marshes.

Conclusion

Due to the crucial role of *S. alterniflora* in maintaining coastal salt marshes and the genetic heterogeneity within natural populations, the development of propagation protocols that emphasize genetic diversity must happen on a local scale. The overall goal of the present research is to develop a seed-based propagation protocol for *S. alterniflora* local to coastal Georgia. Based on our observations, seeds should be collected from more inland marshes with relatively lower marine influence, and collections should occur later in the reproductive season (November) to optimize the number of seeds/spikelets, seed viability, and germination rates. Collected seeds should then be placed in resealable plastic bags filled with water for cold stratification for up to 12 weeks. Not only do plastic bags maintain higher seed viability, but have the added benefits of being relatively cheap, easy to acquire, and reusable. Once this overwintering period is complete, seed viability may be optionally determined using either a fluorescent light box or a TZ test, but this does not necessarily correlate to germination rate. Optimal conditions for germination remain elusive, and it is recommended that future work focuses on determining what abiotic and/or biotic variables play a significant role in germination for *S. alterniflora* seeds in coastal Georgia.

CHAPTER 2

EXAMINING THE ROLE OF SALINITY AND MICROBIAL DIVERSITY IN THE GROWTH OF *SPARTINA ALTERNIFLORA*

Introduction

As presented in chapter 1, coastal salt marshes dominate the East and Gulf coasts of the US. These important and productive ecosystems protect coastal regions from flooding and storm surges, maintain fish, shellfish, and migratory bird populations, sequester large amounts carbon and nitrogen, and provide aesthetically pleasing spaces for human recreation (Barbier et al. 2011; McFarlin et al. 2008; NOAA 2022; Smith et al. 2020). In these florally homogenous marshes, *Spartina alterniflora* Loisel is a critical component of the ecosystem. It forms the foundation of both the herbivore and detritivore food web, influences edaphic microbial communities, limits erosion through dense belowground root and rhizosphere networks, and promotes accretion by trapping suspended sediments in dense culms (Feagin et al. 2009; Gleason et al. 1979; Lin et al. 2018; Parker et al. 2008).

Due to its key role in maintaining important marsh ecosystems, *S. alterniflora* is often incorporated into restoration and stabilization projects, including the construction of living shorelines. Living shorelines are alternatives to more traditional marsh edge stabilization structures like bulkheads and seawalls that aim to not only limit erosion but preserve the marine-terrestrial ecosystem interface and their respective functions (DNREC n.d.; NOAA 2015). The inclusion of *S. alterniflora* in such structures is difficult, though, as the species is highly diverse across its native range, adapting to local conditions slowly over generations and experiencing a decrease in fitness until these adaptations arise (Blum et al. 2007; O'Brien and Freshwater 1999; Seliskar et al. 2002; Travis and Grace 2010). Furthermore, populations within a single marsh or

landscape exhibit high genetic diversity, making it beneficial for any restoration efforts to use genetically unique individuals rather than clones of highly successful individuals (Gaynor et al. 2019; Richards et al. 2004).

Due to the local specificity of *S. alterniflora*, a universal propagation guide is unrealistic. Nursery practices vary from region to region to reflect local conditions, with no overall consensus on ideal germination and growth conditions. For example, seed germination substrate ranges from sand, peat, soil composition mixtures, or pure tap water (Biber et al. n.d.; Materne et al. 2022; Woodhouse et al. 1976). Beyond germination, greenhouse growth conditions vary between regional sources as well. A Mississippi based propagation guide recommends planting seedlings in a 2:1 mixture of topsoil and sand, a North Carolina based propagation guide recommends utilizing peat, and a Rhode Island based project utilized a 1:1 ratio of sand and peat (Biber et al. n.d.; Walker 2015; Woodhouse et al. 1976). The only commonality among these propagation methods is the use of freshwater, an environmental variable which the salt tolerant *S. alterniflora* is not likely to encounter in its natural habitat.

Along the Georgia coast, the semidiurnal tides are often among the highest in the southeast (6-9 ft), with average high tides often double or triple those in areas to the north or south (National Park Service 2021). These frequent high tides combined with high sediment loads result in extensive salt marsh networks (Coastal Resources Division n.d). *S. alterniflora* dominates these extensive marshes, forming widespread monocultures despite the salinity and frequent inundation (Coastal Resources Division n.d.). These plants are able to separate dissolved salt from the water they uptake and expel it through salt glands in the leaves. Due to the energetic demands of this, salt is still considered a stressor to *S. alterniflora* and growth in freshwater is still widely implemented (Biber et al. n.d.; Coastal Resources Division n.d).

Despite this, the USDA specifically notes the difficulty in establishing *S. alterniflora* stands in freshwater environments, particularly in field trials (Materne et al. 2022). Thus, Materne et al. (2022) recommends establishing plants in field sites with salinities of 8-33 ppt and given the aforementioned frequency and amplitude of inundation in Georgia marshes and the local specificity of *S. alterniflora*, plants from this region may benefit from being grown under brackish conditions (National Park Service 2021; Seliskar et al. 2002; Travis and Grace 2010).

In addition, typical propagation methods may not account for the unique soil conditions in which *S. alterniflora* naturally grows. Once again, the USDA notes that plants grow best in heavy, dense soils like clays, silts, and fine sands with pH ranging from 3.7 - 7.9 (Materne et al. 2022). *S. alterniflora* has been observed to struggle in soils high in organic matter like peat, which it traditionally used in many nursery protocols (Materne et al. 2022). Marsh soils are also noted for their high microbial activity which, when coupled with abiotic factors, render most marsh soils anoxic a few inches below the surface (Zedler et al. 2008). These anoxic conditions lend themselves to reduction-based microbial metabolisms that can significantly influence soil conditions. For example, sulfide reduction is widespread in many salt marshes and contributes to both the characteristic marsh smell and the harsh conditions for plants (Lamers et al. 2013; NOAA 2022). Despite this, *S. alterniflora* clearly thrives in salt marshes, and it may do so by directly influencing and receiving benefits from its rhizospheric microbial community. Previous studies examining the microbial communities of Georgia *S. alterniflora* rhizopheres observed that a combination of plant phenotype and soil chemistry influenced the diversity of microorganisms. Specifically, plants selected microorganisms that beneficially altered the redox potential of soil and oxidized potential sulfide phytotoxins through the secretion of photosynthates (Berg and Smalla 2009; Kolton et al. 2019; Lamers et al. 2013).

A plant's ability to influence its rhizospheric microbial communities is a developing field, but the basis of the interaction is generally understood (Berendsen et al. 2012). By secreting microbially beneficial compounds from their roots and, in the case of *S. alterniflora*, aerating soils through their root aerenchyma, plants are able to select for specifically beneficial microbes from a soil's existing microbial stock (Berendsen et al. 2012; Granse et al. 2022). These beneficial microbes flourish near a plant's roots, while other detrimental microbes are suppressed (Berendsen et al. 2012). For example, evidence suggests that individual *S. alterniflora* plants can select for different nitrification-denitrification associated microbes based on their environments. Plants in N-limited environments may recruit more microflora associated with reducing N loss compared to those less limited by the availability of N (Lin et al. 2018). Traditional nursery practices for *S. alterniflora* often ignore the natural edaphic microbiome of salt marshes, and therefore plants grown in nursery conditions may be unable to cultivate the microbial community necessary to thrive once transplanted into salt marshes. Woodhouse et al. (1976) note that field-grown *S. alterniflora* plants exhibit increased growth and clonal spread compared to greenhouse-grown individuals when it comes to transplantation.

The purpose of this study was to examine the impact of both salinity and natural salt marsh soil on the growth and productivity of local *S. alterniflora* to create growth guidelines for native plant nurseries. We hypothesize that the introduction of both salt and naturally occurring marsh soil - along with its native microbial community - will have a positive impact on the growth of *S. alterniflora* in a greenhouse setting.

Methods

Experimental Design

Salt marsh soil was collected from Halcyon Bluff Community House (HBCH) from multiple locations to minimize the environmental disturbance of sampling. Samples were gathered by scraping the top ~10 cm of soil with a shovel near stands of *S. alterniflora*, though soil was taken so as to avoid the inclusion of plant material, such as *S. alterniflora* rhizomes and ramets. These samples were then homogenized in a bucket, sealed with a lid, and stored at ambient temperature for 48 hours until used.

168 *S. alterniflora* seedlings gathered from all four sample sites and across all four collection periods (Table 2-1) propagated in Chapter 1 were randomly assigned to one of four treatment combinations: (1) potting soil (i.e., 2:1 topoil:sand mix) x freshwater (PS + FW), (2) potting soil x saltwater (13 ppt) (PS + SW), (3) marsh soil x freshwater (SMS + FW), and (4) marsh soil x saltwater (SMS + SW). A salt concentration of 13 ppt was selected for this experiment because it was the approximate mean salinity for HBCH (Table 1-1) during the seed collection period presented in Chapter 1. Each seedling was transplanted into 15 cm diameter round plastic pots and filled with either a 2:1 mixture of topsoil:sand (i.e., potting soil) or the same mixture with approximately 100 mL natural marsh soil added in a layer roughly in the center of each pot. To add the salt marsh soil, each pot was filled halfway with the 2:1 topsoil:sand and the natural marsh soil was added. Seedlings were placed on top of this layer, and any remaining space in the pot was filled in with the original 2:1 topsoil:sand mixture.

Table 2-1. Greenhouse plant composition across origins. Sample site abbreviations and collection period numbering defined in chapter 1.

Sample Origin	Greenhouse Percentage (%)
Sample Site	
HBCH	48.2
RHBR	15.5
PL	30.4
LCBR	3.0
Unknown	3.0
Collection Period	
1	4.9
2	4.9
3	50.9
4	39.3

Six seedlings were then placed in plastic plant trays, and seven trays (42 individuals) were placed into each of the four treatment groups (n=42 individuals x 4 treatments, N=168). Treatment groups were separated onto greenhouse tables by treatment combination, and trays were sub irrigated with either freshwater or saltwater. Throughout the 10-week experiment, plants were watered in this way as needed and maintained under uniform greenhouse conditions. It should be noted that given the proximity of the marsh soil to each plant's roots, plant roots were visibly able to grow through the marsh soil layer throughout the growth period.

Plant Measurements

Each week, all plants were measured for growth and productivity. The number of individual ramets was counted for each pot, and diameter and stem height were assessed for the original ramet. Weekly growth rate for each plant was determined by calculating the mean change in height from week to week. Additionally, leaf chlorophyll concentration (proxy for photosynthesis) was measured every three weeks using an MC-100 Chlorophyll Concentration Meter (Apogee Instruments. Logan, UT). Three measurements were taken from each plant on a single mature, healthy leaf at multiple locations.

At the conclusion of the 10-week experimental period, plants were removed from their pots and roots were rinsed to remove soil. Longest root length was assessed for each pot, biomass was sorted into total aboveground and belowground portions, and then dried at 65°C for at least 48 hours for measurement of dry aboveground and belowground biomass.

Statistical Analyses

Prior to statistical analyses, all data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's tests, respectively. Aboveground biomass was log10 transformed, leaf chlorophyll concentration and mean growth rate was log transformed, and belowground biomass and total biomass was cube-root transformed to meet assumptions. The effect of each treatment combination for these variables was analyzed using an ANOVA with a significance at $p<0.050$, followed by a Tukey's HSD to determine statistical differences between groups. Since plants were not rotated throughout the growth period, the effect of tray by treatment combination was also examined using an ANOVA with a significance at $p<0.050$.

The number of ramets, final stem height, final stem diameter, root length, and root:shoot ratio could not be transformed to meet normality and homogeneity of variance assumptions.

Thus, the effect of each treatment combination was analyzed using a Wilcoxon followed by a Steel-Dwass post hoc test with a significance at $p<0.05$. Similarly to the parametric tests, the effect of tray by treatment combination was also examined using Wilcoxon test with a significance at $p<0.05$.

Each growth variable was also examined across parent plant origin sites. Variable transformations were maintained for this analysis, with normal variables being examined using an ANOVA with a significance at $p<0.050$, followed by a Tukey's HSD to determine statistical differences between field sites. Non-normal variables were examined nonparametrically using a Wilcoxon followed by a Steel-Dwass post hoc test with a significance at $p<0.05$.

Results

Effect of Soil and Salinity Treatments

Plants grown in a combination of salt marsh soil and freshwater (SMS + FW) outperformed their counterparts in all metrics in which there was a significant difference: stem height ($p=0.003$; Table 2-2), growth rate ($p<0.001$; Table 2-2), number of ramets ($p=0.022$, Table 2-2), root:shoot ratio ($p=0.031$, Table 2-2), chlorophyll concentration ($p<0.001$, Table 2-2), aboveground biomass ($p<0.001$; Table 2-2), belowground biomass ($p=0.002$; Table 2-2) and total biomass ($p=0.007$; Table 2-2). Plants in SMS + FW grew taller than plants grown in both PS + FW ($p=0.012$; Figure 2-1) and PS + SW ($p=0.005$; Figure 2-1) treatments but were not different from those grown in SMS + SW ($p=0.0674$; Figure 2-1). This trend of separation between soil components and salinity components of each treatment combination grew more distinct over time (Figure 2-3). SMS + FW plants grew faster than plants grown in PS + FW ($p=0.006$; Figure 2-1), PS + SW ($p=0.002$; Figure 2-1), and SMS + SW ($p=0.009$; Figure 2-1). SMS + FW only developed a greater number of ramets and had a significantly different root:shoot ratio than their direct opposite group: PS + SW ($p=0.014$ and $p=0.041$, respectively; Figure 2-1). SMS + FW plants had greater mean leaf chlorophyll concentrations than PS + FW plants ($p=0.010$; Figure 2-1) and PS + SW plants ($p<0.001$; Figure 2-1). Aboveground biomass varied across treatment groups, with SMS + FW plants developing greater aboveground biomasses than PS + FW plants ($p=0.010$; Figure 2-1) and PS + SW plants ($p<0.001$, Figure 2-1). Belowground biomass followed a similar trend, with SMS + FW plants developing greater biomasses than PS + FW plants ($p=0.001$; Figure 2-1) and PS + SW plants ($p=0.025$; Figure 2-1). There was only a difference in total biomass observed between SMS + FW plants and PS + FW plants ($p=0.003$; Figure 2-1).

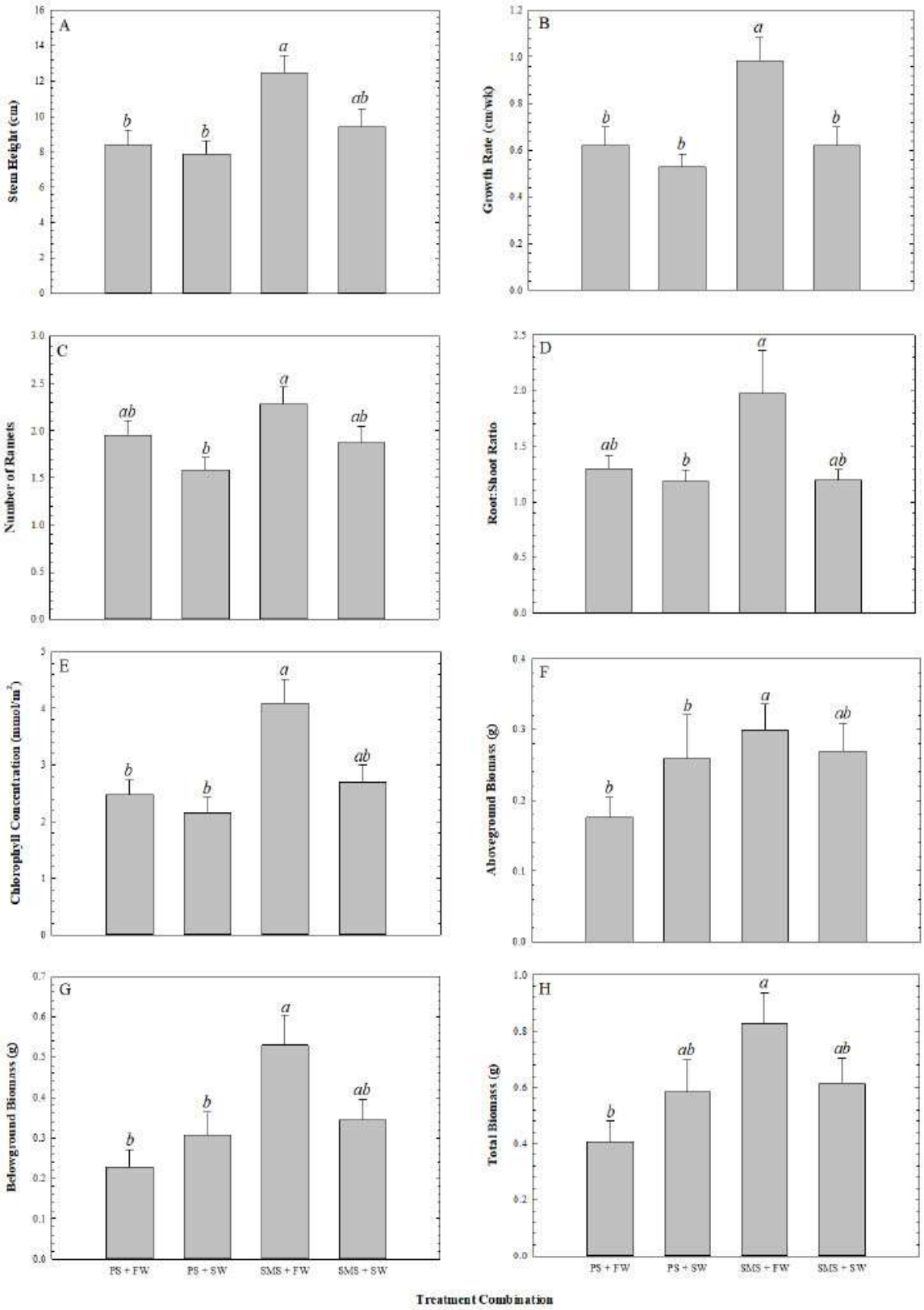
A
Stem Height (cm)
B
Growth Rate (cm/wk)
C
Number of Ramets
D
Root:Shoot Ratio
E
Chlorophyll Concentration (nmol/m²)
F
Aboveground Biomass (g)
G
Belowground Biomass (g)
H
Total Biomass (g)
PS + FW
PS + SW
SMS + FW
SMS + SW
Treatment Combination

Figure 2-1. Mean (A) stem height (cm), (B) growth rate (cm/wk), (C) number of ramets, (D) root:shoot ratio, (E) leaf chlorophyll concentration (mmol/m^2), (F) aboveground biomass (g), (G) belowground biomass (g), and (H) total biomass (g) for *S. alterniflora* plants grown in Experiment 2. Measurements were taken across all treatment combinations: potting soil x fresh water (PS + FW), potting soil x salt water (PS + SW), marsh soil x fresh water (SMS + FW), and marsh soil x salt water (SW) Error bars represent mean standard error and lettering indicates statistical grouping.

Table 2-2. Degrees of freedom (df), test statistic, and p-value for statistical analyses examining the effect of soil and salinity combination on S. *alterniflora* growth and productivity variables. Significance determined at $p<0.050$, and asterisks indicate significant effects. F-statistics, df, and p-values for parametric tests were determined via an ANOVA. χ^2-statistics, df, and p-values for nonparametric tests were determined via Kruskal-Wallis tests. Note that sample sizes indicated by df values vary as a result of plant mortality and sample loss.

Variable	df	Test Statistic	p - value
Chlorophyll Content	3, 158	$F = 6.09$	<0.001*
Mean Growth Rate	3, 161	$F = 5.95$	<0.001*
Aboveground Biomass	3, 158	$F = 6.09$	<0.001*
Belowground Biomass	3, 160	$F = 5.20$	0.002*
Total Biomass	3, 155	$F = 4.18$	0.007*
Number of Ramets	3	$\chi^2 = 9.67$	0.022*
Root Length	3	$\chi^2 = 1.38$	0.709
Root:Shoot Ratio	3	$\chi^2 = 8.86$	0.031*
Stem Diameter	3	$\chi^2 = 6.11$	0.106
Stem Height	3	$\chi^2 = 13.75$	0.003*

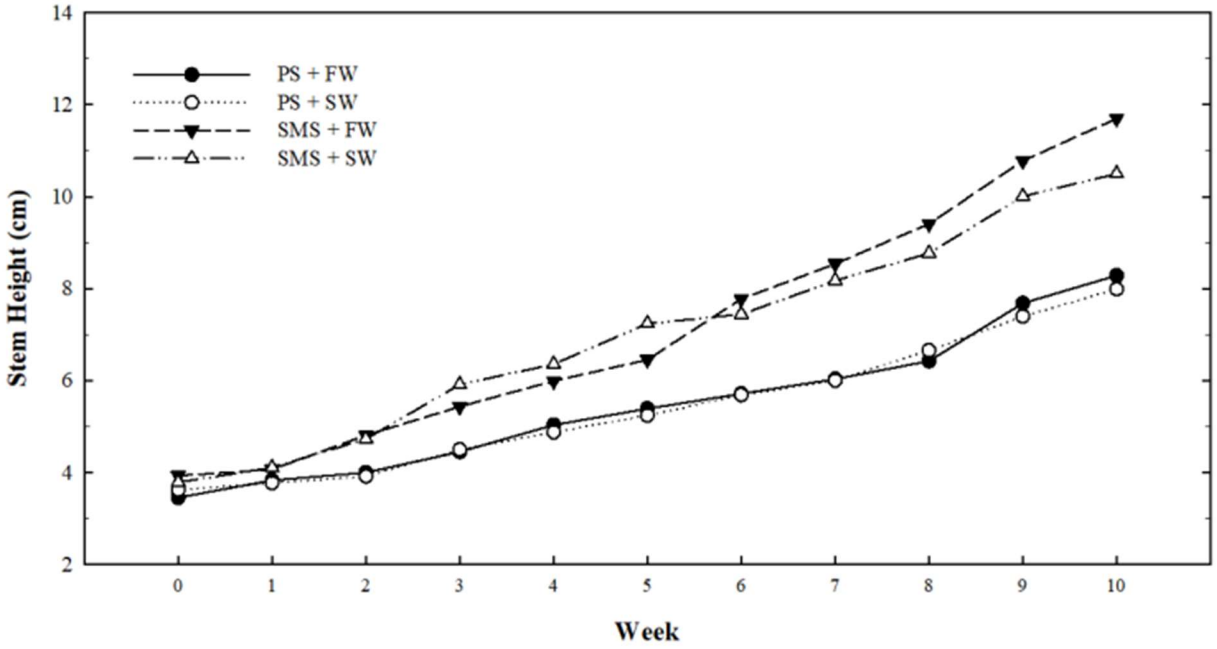

Figure 2-2. Change in stem height (cm) of experimental groups over time across the 10-week growth period. Shapes differentiate soil components while colors differentiate salinity components across treatment combinations.

Confounding Variables

In order to examine the significance of potentially confounding variables, analyses were run to examine differences in growth across both trays and parent plant sample site. Both were found to have significant effects on at least one growth variable. Significant differences across trays in each treatment combination are outlined in appendix Table A-1. Significant differences in parent plant origin site were found across growth variables, though no one site or set of site characteristics appears to correlate to better growth. These differences are outlined in appendix Table A-2. As an example, the impact of parent plant site on average growth is presented below. There was a significant difference across site ($p=0.0323$; Figure 2-4; Table A-2), with HBCH and PL having a significant difference between one another ($p=0.0415$; Table A-2).

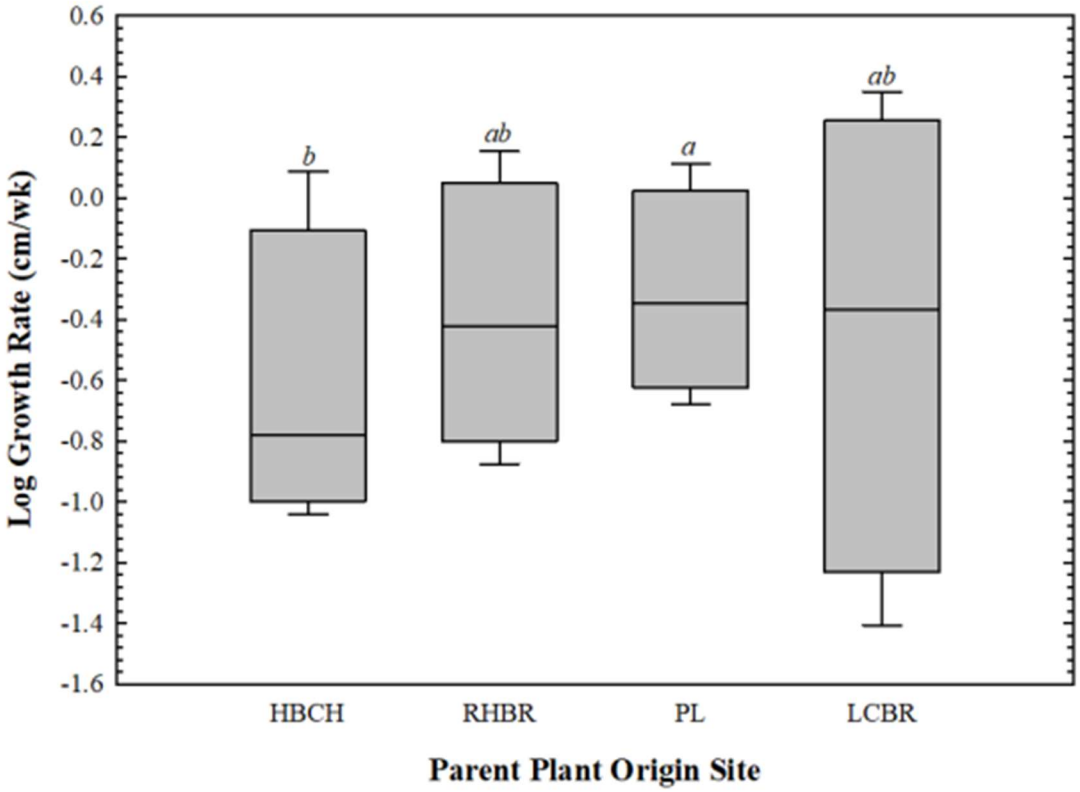

Figure 2-3: Log transformed growth rate (cm/wk) of plants separated by parent plant origin site. Lettering indicates statistical groupings between origin sites. Origin site abbreviations outlined in the text.

Discussion

There were clear effects of soil and water treatment combinations on the growth and productivity of *S. alterniflora*. Plants grown in soil inoculated with natural salt marsh soil tended to exhibit greater growth than their potting soil counterparts. In contrast, the salinity component of each treatment tended to have a less clear effect, with freshwater plants really only exhibiting greater growth when combined with salt marsh soil. Thus, these results suggest that the use of freshwater and the inclusion of natural marsh soil to growth media appear to promote the growth of tall, healthy *S. alterniflora* plants for use in marsh restoration initiatives.

Effect of Salinity

Based on the results presented here, *S. alterniflora* had better growth in freshwater compared to saltwater, though in many cases the inclusion of salt marsh soil minimized this difference. Plants grown in freshwater trended towards greater growth rates an overall greater mean leaf chlorophyll concentration (Figure 2-1), indicating greater photosynthetic potential (Palta 2009). These results partially support previously developed propagation trials, such as those presented by Biber et al. (n.d.) and refute our hypothesis that salt may positively influence the growth of Georgia *S. alterniflora*. To prepare plants for marsh transplant, Biber et al. (n.d.) suggest implementing a salt hardening regime in which saline water is slowly introduced during regular watering at least a month in advance of planting. Plants grown in PS + FW did tend to have lower aboveground and belowground biomasses than their PS + SW counterparts, but only their total biomasses varied significantly (Figure 2-1). Further research may be needed to more thoroughly examine the interaction between environmental salinity and biomass allocation.

It should be noted that the methods of watering used in the present study appeared to lead to relatively high soil salinity for plants in the saltwater treatment combinations. During the

growth period, saltwater was added to both plant pots and sub irrigation trays; however, trays were never flushed completely of saltwater prior to watering. This resulted in evaporation and evapotranspiration of water and subsequent accumulation of salts. The accumulation of salts in this manner may have contributed to the differences observed between plants grown in different trays, as it is unlikely that this process occurred uniformly.

Following the growth period and directly preceding plant harvest, data was gathered to examine the effect of treatments on edaphic conditions and microbial community composition. These data were collected by collaborators from Dr. Joel Kostka's Microbial Ecology Lab at the Georgia Institute of Technology, and although not ready to be reported at the time of writing, soil salinities approached 50 ppt in the saltwater treatments (data unpublished). These salinities are consistent with drought conditions in the marsh. As mentioned previously, the USDA notes that individuals grown in highly saline field environments tend to be shorter and less dense than those grown in less saline environments (Materne et al. 2022). Our results support these findings, again suggesting that the inclusion of salt is disadvantageous in the nursery production of *S. alterniflora*. However, the effect of salinity on *S. alterniflora* growth should be further studied under proper watering methods (i.e., complete flushing of sub-irrigated trays).

Effect of Marsh Soil Inoculation

Broader research in rhizospheric microbiomes has highlighted the important roles microbes play in influencing overall plant health, as well as plants' ability to influence soil conditions to promote the development of beneficial microbial communities (Berendsen et al. 2012). Previous work on marsh restoration using *S. alterniflora* suggests that plants grown in greenhouse settings are often less successful than those grown for transplant in natural marshes (Woodhouse et al. 1979). While a combination of factors likely influences transplantation

success, the presence of a well-developed rhizospheric microbial community prior to introduction into a new location may facilitate transplantation success. The rhizosphere of greenhouse grown plants using traditional potting soils may not have a broad enough diversity or appropriate composition of microorganisms present to adapt to salt marsh conditions when transplanted.

In this study, plants grown in soil mixed with natural marsh soil trended towards greater growth and productivity compared to those grown in potting soil, especially when combined watered with fresh water (Figure 2-1). Not only did these plants grow taller, but they cultivated greater aboveground and belowground biomasses (Figure 2-1). Furthermore, their leaves had greater leaf chlorophyll concentration, indicating greater potential photosynthetic carbon gain (Figure 2-1). It should be noted that plants grown in SMS + SW were often not statistically different from PS + FW and PS + SW plants, though non-significant differences were still observed across many measurements (Figure 2-1). Still, the results presented here suggest that the introduction of a broader microbial community - particularly one that includes microbes taken from their native environment – promoted *S. alterniflora* growth and productivity. When compared, the soil component of each treatment combination appeared to more impactful on the growth of *S. alterniflora* than the water component. Although differences were observed between plants grown in freshwater and salt water – as presented above – those differences are dwarfed by those present between soil treatments (Figure 2-2). Even after two weeks of growth, a greater difference was observed between soil types than water types (Figure 2-2). Furthermore, even in the unintentionally high salinity environment created in this study, the presence of a salt marsh soil microbiome greatly increased plant growth, suggesting that a healthy microbiome may promote greater salinity tolerance.

Additional data on the microbial diversity and community compositions of both soil treatments was also collected by research collaborators from Dr. Joel Kostka's Microbial Ecology Lab, but at the time of writing the analyses for these data in progress. However, an examination of the microbial community present in each treatment group may highlight important taxa correlated with increased plant growth. Regardless, the results of this experiment clearly support the inclusion of natural salt marsh soil in the cultivation of *S. alterniflora*.

Confounding Variables

During statistical analysis, two potential confounding variables for Experiment 2 were examined: differences across trays of plants and differences across parent plant sample site. Both appeared to have significant effects on some growth variables, but not all (Table A-1; Table A-2).

As previously mentioned, some of the differences across trays may have been the result of unevenly increasing salinity. In the future, we recommend flushing trays to avoid both increases in salinity over time and to reduce differences across trays. Furthermore, trays remained stationary throughout the growth period, which may have created a number of differences. Due to prolonged warm temperatures and heavy loads, the plastic greenhouse tables upon which trays were placed bowed throughout the growth period. This resulted in an uneven distribution of water within some trays, which may have reduced the available moisture for some plants. Additionally, small scale differences in greenhouse conditions from one table to the next may have had an impact on growth as well. While conditions were assumed to be constant between the trays spread across three tables, rotating plants may result in more even conditions in the future.

Parent plant field site also had a significant effect across some growth variables, but no consistent pattern has emerged. Given that this experiment was not designed to investigate differences across sites, better results could have been obtained by only growing plants whose parents were growing at a single marsh. However, due to the overall low viability and germination rates discussed in Experiment 1, plants sourced from multiple field sites had to be used in order to maintain an adequate sample size for Experiment 2.

Conclusions

The results of this research indicate that nursery growth in salt marsh soil increases growth and productivity and may facilitate transplantation success. While plants in the freshwater treatments trended towards greater growth, as suggested in current protocols (Biber et al. n.d.), the inclusion of naturally occurring salt marsh soil, including the soil microbial community, appears more impactful. Its inclusion increased plant growth and biomass and made plants more tolerant of salt-related stress. Thus, addition of marsh soil to nursery grown plants is recommended to enhance growth and transplant size. Future research aims to examine the differences between soil chemistry and microbial communities under the conditions presented here, as well as the benefits of growing marsh plants under a simulated tidal cycle, with salt water being flushed regularly. Furthermore, a comparison of transplantation success between plants grown with and without both saltwater and naturally occurring marsh microbial communities may provide an important look at the application of the results presented here.

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