COMPARATIVE *IN VITRO* SHOOT MULTIPLICATION, ROOTING AND *EX VITRO* ACCLIMATIZATION OF FLORIDA SEA OATS (*Uniola paniculata* L.) GENOTYPES

By

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To Natalie and Isabella… I love you so much

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Uniola paniculata L. (Poaceae), Sea Oats, an ecologically important dune species in the southeastern United States, plays an integral role in coastal dune stabilization and construction. Sea oats is typically nursery propagated using field collected seed for restoration. It is not a prolific seed producer and seed donor sites in Florida have been diminished due to recent hurricane activity. As an alternative propagation method, micropropagation has the potential to supplement seed propagation of a wide range of sea oats genotypes. A micropropagation protocol was originally optimized using a single sea oats genotype utilizing 2.2 µM benzyladenine for Stage II shoot multiplication which has been shown to produce negative carry-over effects in certain species. Further studies indicated that when using this protocol, *in vitro* multiplication, rooting, and *ex vitro* acclimatization (Stages II – IV) varied widely among several genotypes collected from four differing populations. To assess genotypic responses from populations along the coast of Florida, forty-three genotypes from thirteen geographically diverse populations were micropropagated using this “optimized” protocol. Stage II, Stage III, and Stage IV growth responses differed significantly within and between sea oats populations and individual genotypes. Of the 43 genotypes

screened twenty-seven (62.7%) were shown to be moderately-difficult or difficult-to-acclimatize resulting in survival rates of 69.7% or less. Similarities in responses between genotypes within the same population were also observed. Significantly different genotypic responses observed in Stages II-IV reveal the importance of taking into consideration the influence of genotype when designing and optimizing micropropagation protocols.

CHAPTER 1
LITERATURE REVIEW

Introduction and Rationale

Florida's 1200 mile long coastline is comprised of over 600 miles of sandy beaches that are exposed to constant erosion by wind and wave action throughout the year. Storm activity can have a dramatic impact on Florida's beaches and coastal dune systems. The dune systems act as a first line of defense against damaging winds of tropical storms and hurricanes. A single severe storm can erode as much as 3 meters of sand at the base of a dune (Lewis and Bunce, 1980) creating a less stable shoreline. This subsequently results in a loss of natural wildlife habitat and can cost hundreds of millions of dollars in property damage along the coast. After the destructive 2004 – 2005 hurricane seasons, nearly half of Florida's beaches were classified as "critically eroded" (FDEP, 2005). In addition to heavy storm activity, animal grazing and foot and vehicle traffic on the beach also negatively impact dune vegetation (Lewis and Bunce, 1980). The financial impact that this extensive coastal damage has on Florida's $41.6 billion beach tourism economy (Murley et al., 2003) is significant. The need for constant beach and dune restoration and stabilization is critical.

Effective dune restoration involves beach sand renourishment and subsequent planting and establishment of native dune species for dune stabilization and building. The most long-term and sustainable approach to beach renourishment begins with pumping offshore sand from the ocean floor shallows onto the eroded beach. To decrease wind erosion, dunes must be stabilized by planting with native dune plant species (Lewis and Bunce, 1980). The grass sea oats (*Uniola paniculata* L.) is a major native dune species in the southeastern United States. It is used extensively for dune

stabilization and building as it is well adapted to protect against the damaging winds that can affect the southern coastline. Successful planting of sea oats and other dune species has been shown to result in the production of foredunes nearly six meters high within six years (Dahl and Woodard, 1977). Sea oats comprise a relatively small portion of a restoration budget but are an integral facet of successful dune restoration. The planting of a one-mile-long, twenty-five foot-wide length of eroded beach in Florida requires 88,000 sea oat plants at a total cost of about $63,000 including installation costs (G. Sharell, pers. comm.).

Sea oats typically dominate the foredune systems, a typically harsh environment that few other dune species can tolerate. Leaves of sea oats are highly tolerant to windblown salt spray from the ocean (Woodhouse et al., 1968; Oosting and Billings, 1942) while their shoot and root systems are conducive to stabilizing dunes. The deep root systems of sea oats bind sand particles together while their shoots and leaves decrease wind velocity resulting in the deposition of wind borne sand grains (Woodhouse, 1982). Even after sea oats have been uprooted from a major storm event, the dead root tissue and other vegetative material aggregate to form “bundles” that continue to collect sand (Stopp and O’Neal, 1996). Over time these deposited sand grains eventually develop into sand dunes until the bundle is covered by sand.

Given the frequent erosion damage to coastlines and the need for replanting, the market for sea oats has significantly increased. There are over thirty-five commercial nurseries in Florida (Sea Grant Florida, 2003) that supply sea oat plantlets for restoration. Sea oats are propagated under nursery conditions using field collected seed. Although sea oat seed is not difficult to germinate (Burgess et al., 2005), seed

availability is often limited by several factors. Despite producing six to eight fertile florets per spikelet, feeding insects (Wagner, 1964) and high humidity make embryos susceptible to fungal contamination with subsequent abortion occurring (Burgess et al., 2002). Typically, less than two viable seeds are produced per spikelet. These seeds will not withstand flooding conditions in sea water and are susceptible to temperature fluctuations. The small seeds are windblown, sand-blasted, or simply dry out when directly seeded onto a newly created beach. The seedlings require three to five years to reach flowering maturity in natural conditions (Woodhouse et al., 1968). Given these limitations, direct seeding is not a viable method for revegetation. It has also been observed that sea oats rely mainly upon rhizome reproduction to promote expansion (Hester and Mendelssohn, 1991). In addition to these natural barriers to seed availability and germination, the active 2004-2005 hurricane seasons have significantly reduced seed donor populations, especially in the Florida panhandle.

The limitation of sea oats seed is further complicated by ecosystem management policies established in state and federal parks. These policies serve to protect the long-term viability of local native plant communities by minimizing human interference with evolving genetic diversity and adaptation. This is achieved by requiring that native plant restoration be performed using plants of local provenance. Uncertainty and dissent exists among restoration scientists as how to distinguish local provenance and the specific need to maintain local genetic diversity (Kaye, 2001; Wilkinson, 2001). However, implementation of these landscape management policies has been prompted by concerns that: 1) non-local genotypes may be significantly disadvantaged when genetic differences between provenance result from local adaptation; 2) poor adaptation

may be transferred from introduced non-local plants to local populations with resultant outbreeding depression in subsequent generations and; 3) the potential of genetic swapping of the local gene pool by introduction of a non-local gene pool might lead to loss of biodiversity (Kaye, 2001). Barbour et al. (1987) had noted that southern Florida and the Florida panhandle differ significantly in environment due to hurricane disturbance, climate, sand type, and species differentiation. These differing environments play a role in determining what types of adaptations and characteristics are exhibited by local sea oats populations. To minimize the chance of introducing non-adapted genotypes, seed donor populations have therefore been restricted to within a fifty-mile radius of the targeted planting site or even limited to populations found within individual state park boundaries. However, this restriction has been imposed with no supportive scientific data.

Approaches to restoring the beach with uprooted sea oats rhizomes immediately following severe weather conditions have been explored. Miller et al. (2003) conducted a study to determine the effectiveness of replanting uprooted sea oats rhizomes after hurricane activity. The authors reported that rhizome moisture played a key role in tiller emergence, with rhizomes losing bud viability after 3 to 5 days unless fresh water was subsequently applied. Tillers replanted within 5 days following uprooting by storm activity exhibited 45% tiller regrowth. Tillers could be replanted after 11 days if rainfall exceeded 100 mm. Rainfall in excess of 100 mm during this time allows for rhizomes to be rinsed after being uprooted and salt to be leached from the soil before being naturally reburied. However, if 11 days passed prior to replanting, only 5-20% of tillers exhibited regrowth. Reliance upon this restoration technique is impractical due to the

need to sustain high moisture conditions following storm activity. In addition, replanting uprooted sea oats tillers in such a short timeframe after a severe storm is often unfeasible. Alternative sources of sea oats that represent a wide range of local genotypes are needed. Indeed, supplementing sparse seed producing populations with local ecotypes produced by *in vitro* propagation could provide an effective way to compliment nursery propagation by seed and rapidly increase ecologically sound production of genetically diverse sea oats of local origin.

Micropropagation Can Enhance Sea Oats Availability for Restoration

Micropropagation is a technique used for the rapid *in vitro* production of plants on a defined sterile medium in culture vessels (Kane, 2011). Numerous studies have been published on the feasibility of applying micropropagation techniques to commercial production of aquatic, dune, and marsh species for habitat restoration (Straub et al., 1988; Cook et al., 1989; Li and Gallagher 1996; Kane and Philman, 1997; Rogers et al., 1998; Seliskar and Gallagher, 2000). Efficient production of sea oats plantlets by micropropagation generates a source of sea oats plantlets to supplement seed production, thus decreasing the reliance upon donor sites for seed collection. In addition, micropropagation allows for year-round sea oats production. Establishment of a sea oats “genotype library” from major populations would create an invaluable resource for land management personnel to use when local sources of genotypes are not available for restoration.

The general sea oats micropropagation process is divided up into five sequential stages (Figure 1-1). Development of a viable micropropagation protocol begins with the selection of plant material and excision of the explant to be sterilized (Stage 0). At this point, the selected plant material can be genetically analyzed for further analysis.

Explants such as shoot meristems or seed are then surface sterilized in sodium hypochlorite containing a surfactant to remove surface microorganisms such as fungi and bacteria. After rinsing the tissue with sterile water, explants are then inoculated into sterile culture vessels (Stage I) containing an agar-solidified culture medium consisting of mineral salts, sucrose, vitamins and one or more plant growth regulators. Stage I cultures are allowed to physiologically adapt to *in vitro* conditions and then are indexed for contaminants. Cultures are then transferred onto a Stage II medium containing higher levels of cytokinins to promote shoot production and the proliferation of plant tissue. When propagating sea oats *in vitro*, Stage II medium is supplemented with cytokinins such as benzyladenine (BA) to disrupt apical dominance and induce lateral shoot formation. Sea oats produce a compact cluster of shoots within 28 days which are then subdivided to increase the total number of cultures. These shoot clusters are then divided into single-shoots (microcuttings) after 28 days and transferred onto a Stage III medium containing the auxin α-napthaleneacetic acid (NAA) which promotes adventitious rooting *in vitro*. This step is often critical for successful transfer of sea oats from an *in vitro* heterotrophic mode of nutrition to a photoautotrophic state required for successful acclimatization *ex vitro* (Stage IV). Following root formation, the rooted microcuttings are removed from the culture vessels, rinsed to remove residual medium, and placed into plug trays containing vermiculite in the greenhouse. Greenhouse set points for cooling and heating are set to 26 and 18°C, respectively, with natural noon solar photosynthetic photon flux (PPF) ranging from 900-1250 µmol m^{-2} s^{-1}. Sea oats are then watered daily and fertilized weekly at 150 mg N L^{-1} using 20-20-20; N-P-K

liquid fertilizer. Plants attain a sufficient size for outplanting after 6 weeks in greenhouse culture.

In vitro culture conditions provide plants with an environment characterized by relatively low light levels, high relative humidity, high sucrose concentration, and low CO_2 concentration which limits plant gas exchange and photosynthetic capacity (Pospíšilová et al., 1992, Valero-Aracama et al., 2007). These conditions alter plant morphology, anatomy, and physiology on plantlets cultured *in vitro* leading to decreased photosynthetic capacity, poor control of water relations and subsequent decreased survival *in vitro (*Pospíšilová et al., 1999; Valero-Aracama et al., 2007; Valero-Aracama et al., 2008). However, those abnormalities that contribute to a decrease in photosynthetic capacity can be overcome in some species by culturing in the presence of higher sugar concentration *in vitro* that the plants utilize as a carbohydrate reserve during acclimatization (Debergh et al., 2000).

Genetic Diversity in Dune and Marsh Plant Populations

Initially, Philman and Kane (1994) optimized an *in vitro* propagation protocol using a single sea oats genotype but later found that when applied across a wide range of genotypes, many genotypes exhibited variable shoot production, inconsistent rooting, and very low survival rates during *ex vitro* acclimatization. Kane et al. (2006) later established more than fifteen genotypes *in vitro* collected from four Florida populations on the Atlantic (Anastasia State Recreation Area [AN] and Sebastian Inlet State Recreation Area [SI]) and Gulf coasts (Egmont Key National Wildlife Refuge [EK] and St. George Island State Park [SG]). DNA marker analysis has demonstrated that genotypic variation exists among these sea oat populations due possibly to “intense

selection pressure" (Ranamukhaarachchi, 2000), and especially the frequency of hurricane and tropical storm events.

Random Amplified Polymorphic DNA (RAPD) genetic analyses of sea oats adult plants and seedlings from the four populations sampled indicated significant genetic variations between the Gulf and Atlantic coasts, with the most variation occurring within the Gulf coast populations (EK and SG) (Ranamukhaarachchi et al., 2000). Ranamukhaarachchi et al. (2000) attributed differences in genetic variation to the increased environmental disturbances in the Florida panhandle where more frequent storm and hurricane activity occurred over the past 120 years. However, the specific relationship between these genotypic differences and local adaptation remain unclear. Subudhi et al. (2005) reported the genetic diversity present among nineteen different *U. paniculata* accessions from eight different states including Florida using Amplified Fragment Length Polymorphism (AFLP) generated markers. Not surprisingly, populations originating from the same state had a higher degree of genetic similarity compared to those populations from different states. Of all accessions studied, Florida populations exhibited the highest degree of genetic variability. Depending on where the sea oats accessions were obtained, overall genetic patterns revealed a separation between Gulf or Atlantic and northern and southern sea oats.

Other dune and marsh plant species have also been found to possess a high degree of genetic diversity within their own natural populations and have been shown to be genetically distant to their neighboring populations due to differing localized environmental conditions, which perhaps results in ecotypic differentiation. O'Brian and Freshwater (1999) sampled *Spartina alterniflora* populations from five geographic

locations along the Atlantic and Gulf coasts. Using RAPD analysis, it was reported that genetic distance was positively correlated and highly significant with respect to geographic distance. Pezeshki and Delaune (1991) reported that *Spartina patens* plants collected from areas of higher salinity levels were able to grow better at higher salinity levels than those plants taken from low salinity environments suggesting differing environmental conditions play a major role in influencing plant growth and adaptability. Richards et al. (2004) examined *S. alterniflora* and *Borrichia frutescens* populations residing in salt marshes along the coast of Georgia using genetic markers to test for clonal diversity. These two populations were traditionally thought to have a high degree of clonal reproduction due to the harsh saline environments in which these populations resided. Despite clonal spread being extensive in these species, it was reported that a high degree of clonal diversity was present depending on where tissue samples were collected. In addition, the authors explored if the clonal structure in the two species was a result of clonal foraging or natural selection. However, there seemed to be no association between plant genotypes and microhabitat. These findings suggest that the factors impacting the genetic diversity in a given population may be much more complicated than simply examining a population's surrounding environment.

Plant genotype has been shown to influence *in vitro* growth responses. However, the number of published studies examining this relationship is limited. Gil-Humanes et al. (2011) examined *in vitro* shoot regeneration of fifty-one *Brassica carinata* genotypes established from seed. Highly significant differences were found amongst genotypes with respect to percent callus formation, swelling, onset of blackening in the petiole, and number of shoots produced from callus or direct regeneration. Kane et al. (2006)

reported significant *in vitro* phenotypic differences between sea oats genotypes originally collected from Florida Gulf and Atlantic populations. Differences included capacity for Stage II shoot regeneration, variable leaf morphology, Stage III rooting and leaf development and Stage IV *ex vitro* survival and acclimatization. Genotypes from these different locations were categorized as “easy- and difficult-to-acclimatize” with respect to survival when *in vitro* propagated sea oat plantlets were transferred to an *ex vitro* environment.

Ensuring a reliable supply of diverse sea oats genotypes from diverse geographical sources is critical for ecologically sound dune restoration and stabilization. Maintaining high genotypic diversity as opposed to a plant monoculture at a restoration site allows a greater degree of adaptability to environmental factors such as disease and invasive species competition (Travis et al., 2002) and may play a role in the long-term sustainability of a population (Richards et al., 2004). Since sea oats is an out-crossing species, there is concern that a non-local genotype monoculture could dilute the gene pool of locally adapted ecotypes through outbreeding depression or greatly increased expression of deleterious recessive mutations through selfing. Consequently, development of an *in vitro* propagation protocol to ensure production of a wide range of sea oats genotypes is necessary.

Negative Carry-Over Effects Caused by Benzyladenine

Optimizing *in vitro* culture conditions for propagating multiple sea oats genotypes with differing acclimatization capacities is a prerequisite for increased growth, survivability, and efficient production (Valero-Aracama et al., 2007). However, to accomplish this, the physiological cause for these genotypic differences needed to be determined. In a series of studies, Valero-Aracama et al. (2006, 2007; 2008) examined

the anatomical and physiological basis for differences in *in vitro* and *ex vitro* growth responses between a difficult and easy to acclimatize sea oats genotype.

Benzyladenine (BA) is the most widely used cytokinin incorporated into Stage II culture medium to promote axillary shoot production. In sea oats, BA was reported to be the only effective cytokinin that promoted axillary shoot multiplication (Philman and Kane, 1994).

Certain cytokinins, especially BA, used in Stage II shoot multiplication media can have deleterious carryover effects on subsequent growth, rooting, and acclimatization (Pospíšilová et al., 1992; Werbrouck et al., 1995; Valero-Aracama et al., 2007). Experimental evidence suggests that BA inhibits rooting and other growth parameters. Werbrouck et al. (1995) correlated BA accumulation at the base of *Spathiphyllum floribundum* plantlets with root inhibition and chloroplast ultra-structural changes. Removal of basal callus containing the highest BA concentration decreased the inhibition of roots. Werbrouck et al. (1996) later reported that significantly reduced rooting was due to the presence of the breakdown product of the BA metabolite, N^6-benzyladenine-9-glucoside ([9G]BA), which accumulated at the base of *Spathiphyllum* plantlets and remained for more than 6 weeks.

A genotype specific negative BA carryover effect on *ex vitro* acclimatization of sea oats was reported by Valero-Aracama et al. (2007, 2008). The sea oats genotype EK 11-1 was *in vitro* propagated on BA-supplemented Stage II medium and characterized as “difficult-to-acclimatize”. This genotype possessed abnormal anatomical features including “lance-like” leaves, late sclerenchyma tissue development, blocked stomata, and disorganized mesophyll and vascular bundles compared to EK 16-3, an “easy-to

acclimatize' genotype. All of these features impact photosynthetic capacity. Valero-Aracama et al. (2007) measured net photosynthetic rates of both easy and difficult-to-acclimatize genotypes under four different types of culture conditions: photoautotrophic, photomixotrophic with sugar-containing medium diluted with sugar-free medium over time, photomixotrophic with CO_2 enrichment, and conventional photomixotrophic conditions. After 6 weeks culture, the difficult –to-acclimatize genotype exhibited a negative net photosynthetic rate *in vitro* and the lowest survival (25%) *ex vitro* of all treatments observed. Conversely, the easy-to-acclimatize genotype exhibited both a significantly higher net photosynthetic rate *in vitro* and 100% ex *vitro* survival. It was concluded that the difficult to acclimatize genotype was unable to survive *ex vitro* due to their low photosynthetic capacity under greenhouse conditions. It has been shown that use of BA in sea oats Stage II medium may contribute to the variation in growth, rooting, and *ex vitro* survival of sea oats genotypes observed during acclimatization (Valero-Aracama et al., 2010). However, the extent to which BA negatively impacts *ex vitro* survival has not been screened across a wide range of sea oats genotypes.

***Meta*-Topolin to Alleviate Negative BA Carryover Effects**

Strnad et al. (1997) first reported the existence of N6 – (3-hydroxybenzyl) adenine or *meta*-topolin (mT), a naturally occurring BA-analog derived from poplar leaves (*Populus x canadensis* Moench. CV. robusta). Werbrouck et al. (1996) found that explants cultured on mT effectively regenerated *Spathiphyllum* shoots, with better rooting *in vitro* than those produced on medium containing equimolar concentrations of BA. This is thought to be due to the fact that mT contains a hydroxyl group on the benzyl ring making O-glucosylation possible. Naturally occurring beta-glucosidases more rapidly degrade the mT derivative, N6-(3-O-β-D-glucopyranosyl) benzyladenine-9-

riboside ([9R]mT) despite accumulation at comparable levels of [9G]BA (Werbrouck, et al., 1996). Bairu et al. (2011) evaluated the changes in a wide range of cytokinin profiles in micropropagated *Harpagophytum procumbens* [(Burch.) DC. ex Meisn.] tissues. The authors suggested that the hydroxyl group in topolins allows for greater accumulation of *O*-glucosides in topolin-treated explants allowing for active cytokinin to be available when needed by the plant. Valero-Aracama (2010) reported that, compared to BA, addition of mT to Stage II media overcame the negative BA carry-over effects previously observed in sea oats and further promoted shoot multiplication of a difficult-to-acclimatize *Uniola* genotype without inhibiting root formation or acclimatization. It was reported that both difficult-and easy-to-acclimatize genotypes exhibited the same *in vitro* shoot dry weight, number of shoots, and subsequent percent rooting when cultured on Stage II medium supplemented with either equal concentrations 2.2 µM of BA or mT.

The list of plant species of which aromatic topolins, such as mT, enhance *in vitro* propagation is rapidly increasing (Amoo et al., 2010; Aremu et al., 2011). Amoo et al. (2010) compared the use of mT to BA on adventitious shoot production and resulting abnormalities such as hyperhydric shoots and shoot-tip necrosis of *Barleria greenii*. An “abnormality index” (ratio of hyperhydric shoots and shoots with shoot-tip necrosis to normal shoots) was calculated after 6 weeks culture. When cultured in the presence of BA, *B. greenii* exhibited an abnormality index ranging from 1.7 to six times that of equimolar concentrations of mT. In addition, adventitious shoot production in the presence of equimolar BA and mT concentrations were not statistically different. Bairu et al. (2007) screened mT supplemented medium and its effects on *Aloe polyphylla*, a species that is typically cultured on medium containing BA or zeatin and normally

exhibits a high incidence of hyperhydricity. Plants cultured in the presence of mT exhibited a lower percentage of hyperhydric shoots at all concentrations screened than those cultured in the presence of equimolar BA. At the optimum cytokinin concentration for both mT and BA, explants cultured on mT supplemented medium produced more shoots than on BA medium with no evidence of shoot hyperhydricity. In contrast, explants cultured on BA supplemented medium produced fewer shoots with 21.8% of shoots displaying hyperhydricity.

Ecologically sound dune restoration will require the efficient micropropagation of many diverse sea oats genotypes. The efficacy of using mT as a BA substitute for the micropropagation of “BA-sensitive” genotypes has not been determined. However, before mT efficacy screening can be accomplished, a pool of sea oats genotypes representing a range of easy to difficult-to-acclimatize lines must be selected. Consequently, the goal of this thesis research was to compare and quantify differences in Stage II shoot production, Stage III rooting and leaf development, and categorize Stage IV acclimatization type of geographically diverse sea oats genotypes produced utilizing the BA based sea oats micropropagation protocol originally developed for a single sea oats genotype.

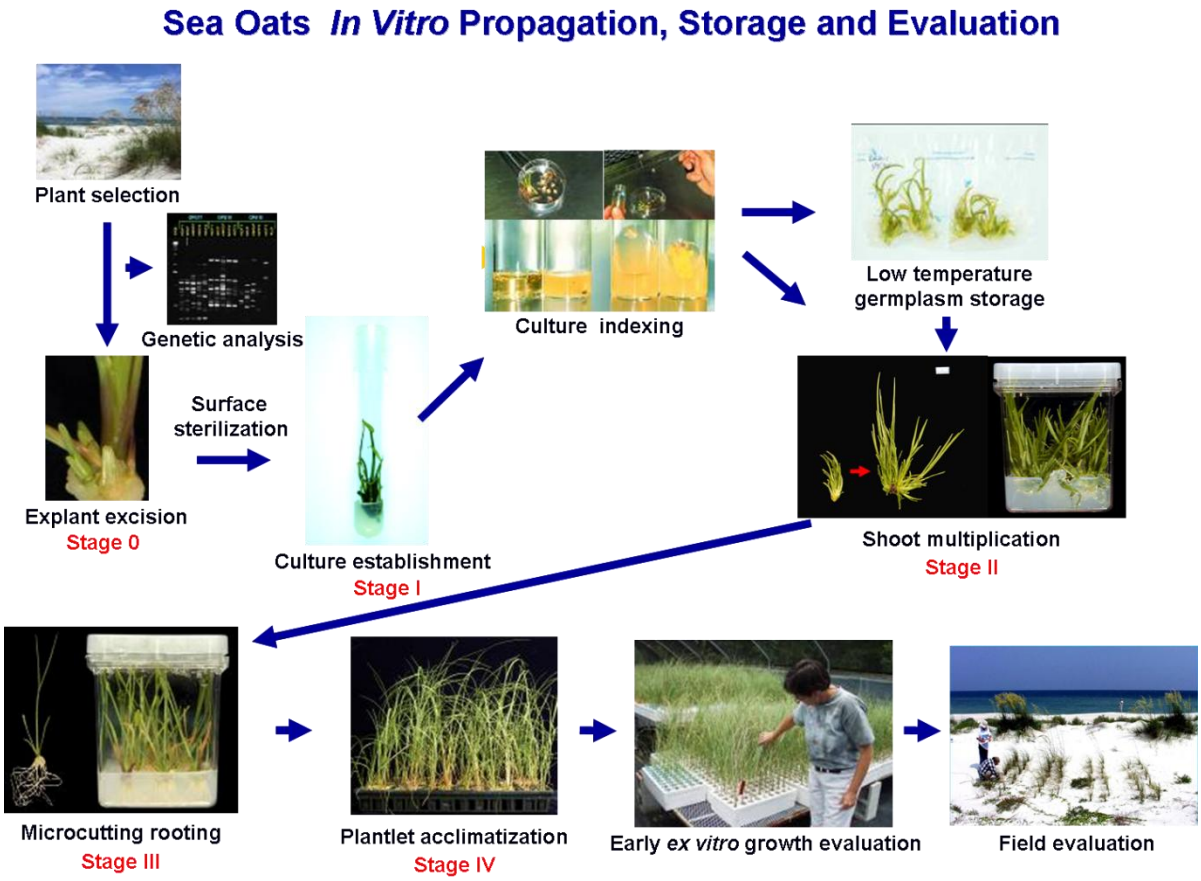

Figure 1-1. The step-wise sequential process for *U. paniculata in vitro* propagation, storage, and evaluation.

CHAPTER 2
COMPARATIVE *IN VITRO* SHOOT MULTIPLICATION, ROOTING AND *EX VITRO* ACCLIMATIZATION OF FLORIDA SEA OATS (*UNIOLA PANICULATA* L.) GENOTYPES

Florida's beach and dune systems are of significant economic and environmental importance. Eighty percent of Florida's population resides in the thirty-five coastal counties where beach related tourism, alone, contributes more than $41.6 billion to the state economy annually (Murley et al., 2003). Besides providing unique wildlife habitat, Florida's coastal dune system also serves as a natural defense system against erosion resulting from hurricanes and human activity. The 2004 hurricane season, with one tropical storm and four major hurricanes making landfall along Florida's coastline, was the most active storm season in Florida since record keeping began in 1851 (FDEP, 2004). Of the 825 miles of the sandy beach shoreline impacted, 365 miles of beaches have now been assessed as critically eroded (FDEP, 2005). The degraded condition of these beaches and dunes has significantly increased the risk of catastrophic economic and environmental loss to upland development, recreation, wildlife habitat and other cultural impacts following future storm events. Consequently, many of Florida's damaged areas require restoration ranging from natural recovery to dune restoration requiring beach sand re-nourishment (FDEP, 2005). The financial impact that this extensive coastal damage has on Florida's beach tourism economy is significant. In 2004, $68 million state and $116 million federal funding was appropriated for restoration of impacted coastal dunes and beaches.

Beach sand re-nourishment followed by planting of native dune species has proven to be the most cost-effective practice to stabilize and build dunes. The cost of beach re-nourishment alone averages $1.5 million per linear mile. Although structural

methods have been employed for enhanced stabilization following re-nourishment, dune stabilization by planting is the most cost-effective practice to control erosion. Stabilization is usually accomplished by planting bare areas with native or introduced dune species. The root and rhizome systems of these species hold sand particles together, while the vegetation above ground retards wind and water driven erosion and facilitates sand deposition (Woodhouse, 1982). The most effective species planted for dune stabilization are perennial grasses including American beachgrass (*Ammophila breviligulata* Fern.) and sea oats (*Uniola paniculata* L.).

In the southeastern United States, sea oats is the dominant sand-binding native species in the exposed pioneer/frontal zone of coastal primary dunes (Wagner, 1964; Woodhouse, 1982). Due to its high tolerance to heat, drought and salinity, sea oats is the primary species used in beach restoration and stabilization projects. Consequently, due to repeated coastal erosion, demand for planting materials of this species has significantly increased. As of 2011, planting a one-mile-long, 25 foot wide length of eroded beach in Florida requires 88,000 sea oats plants at a total cost of about $63,000 installed (G. Sharell, pers. comm).

Commercial nursery propagation of sea oats as liners or containerized plants, established from seed collected from donor populations, has proven to be the most reliable source for transplants (Barnett and Crewz, 1991; Bachman and Whitwell, 1995). Survival of transplanted sea oats from dune-derived rhizome divisions is low (Woodhouse et al., 1968). The potential use of storm-uprooted sea oats rhizomes as a plant material source has been examined but the time period for bud viability is extremely limited (Miller et al., 2003). Sea oats seed germination is low under natural

conditions and establishment by direct seeding is ineffective because of seed dormancy, sand movement or seedling vulnerability to insect and disease damage (Westra and Loomis, 1966) and lack of initial moisture control during establishment. Unfortunately, the massive damage which occurred during the 2004 and 2005 hurricane seasons has significantly reduced, even destroyed, some of the major sea oats seed donor populations, particularly along the Florida panhandle.

The ability of Florida's native plant nurseries to meet increased demand for site-specific planting materials is further limited by ecosystem management policies in both state and national parks, which serve to protect the long-term viability of local native plant communities by preserving evolving genetic diversity and adaptation. This is achieved by requiring that native plant restoration be performed using plants of local provenance. Implementation of these landscape management policies has been prompted by concerns that: 1) non-local genotypes may be significantly disadvantaged when genetic differences between provenance result from local adaptation; 2) poor adaptation may be sexually transferred from introduced non-local plants to local populations with resultant outbreeding depression in subsequent generations; and 3) the potential of genetic swamping of the local gene pool by introduction of a non-local gene pool might lead to loss of biodiversity (Kaye, 2001; Krauss and Koch, 2004). Clearly, additional approaches are necessary to preserve local population-specific genetic diversity.

One solution to alleviate restrictions in sea oats plant availability from local populations, especially those with inherently low seed production and/or destruction from storm-related damage, is to develop alternative propagation methods to

supplement seed propagation. One such method is micropropagation, the rapid *in vitro* propagation of plants (Kane, 2011). The feasibility of applying micropropagation technology to commercial production of native aquatic, dune, and marsh species (Straub et al., 1988; Cook et al., 1989) for habitat restoration has been reported (Li and Gallagher, 1996; Kane and Philman, 1997; Rogers et al., 1998; Seliskar and Gallagher, 2000).

Ecologically sound applications of vegetative propagation techniques, including micropropagation, will require production of a large number of genotypes of local origin for planting sites. Initially, Philman and Kane (1994) optimized an *in vitro* propagation protocol using a single sea oat genotype but later (Kane et al., 2006) observed that when the protocol was applied to propagate fifteen additional genotypes, variable shoot production, inconsistent rooting, and variable survival occurred. However, the extent to which genotype influences the micropropagation efficiency of sea oats remains unknown. In the current study, the *in vitro* growth performance and *ex vitro* survival was assessed in forty-three sea oats genotypic lines from thirteen Florida populations.

Materials and Methods

Seed Collection

In October 2006, seed was harvested from the following thirteen Florida State Parks and Recreation Areas: Perdido Key, Navarre Beach, Henderson Beach, St. George Island, Little Talbot Island, Anastasia, Gamble Rogers, Honeymoon Island, Sebastian Inlet, John D. MacArthur, Delnor-Wiggins Pass, John U. Lloyd, and Bill Baggs Cape Florida (Figure 2-1). Park managers and biologists were consulted prior to collection to assure that seed was harvested only from areas that had not been replanted. Seed production varied significantly between populations. Fifty

infructesences were collected from different plants within each population. Upon return to the laboratory, the seed was cleaned, pooled by population, and stored dry at room temperature (22°C) in 20 ml glass scintillation vials for two months before germination.

Seed Surface Sterilization and *In Vitro* Germination

To establish seedlings *in vitro* under sterile conditions, fifty randomly selected seed from each of the thirteen sea oats populations (Figure 2-1) were first presoaked in 20 ml scintillation vials containing 5 ml of an aqueous solution of 100 mg/liter gibberellic acid (GA_3) for forty-five hours in darkness at 11°C in a refrigerator. The GA_3 solution was decanted and the seeds were then transferred to sterile scintillation vials and rinsed in 50% (v/v) ethanol for thirty seconds. The seeds were then agitated in 3% (v/v) sodium hypochlorite plus several drops of Tween 20 (surfactant) for 12 minutes followed by three rinses of sterile distilled deionized water. Approximately 1 mm of the distal end of each seed was removed with a sterile scalpel before inoculating into 150 x 25 mm glass culture tubes containing 12 ml medium consisting of half strength Murashige & Skoog mineral salts (Murashige and Skoog, 1962), 100 mg/liter myo-inositol, 0.4 mg/liter thiamine, and 30 g/liter sucrose and solidified with 8 g/liter TC® Agar (A-296 *Phyto*Technology Laboratories, Shawnee Mission, KS). Medium pH was adjusted to 5.7 with 0.1N KOH before the addition of agar and autoclaving at 117.7 kPa for 20 minutes at 121°C. Seeds were oriented horizontally on the surface of the medium with one seed inoculated per culture tube. Each culture was assigned a population and individual genotype code. Culture tubes inoculated with individual seed were placed on heating mats providing a constant temperature ranging from 36 – 39°C under a 16-hour photoperiod provided by cool-white florescent lamps at 45 µmol m^{-2} s^{-1}.

Establishment of Sea Oats Germplasm Library

A germplasm library consisting of actively proliferating shoot cultures, originating from each germinated seedling (genotype), was established by cloning in 150 x 25 mm glass culture tubes containing 12 ml Stage II shoot multiplication medium (SMM) consisting of sterile full-strength Murashige & Skoog mineral salts, 100 mg/liter myo-inositol, 0.4 mg/liter thiamine, and 30 g/liter sucrose, 2.2 µM benzyladenine (BA) and solidified with 7 g/liter TC® Agar. By 16 weeks, most shoot cultures exhibited stable shoot regeneration rates and were subsequently subcultured at 4 week intervals at 22°C under a 16-hour photoperiod as provided by cool white fluorescent lamps (General Electric F96T12·CW·WM) at 45 µmol m^{-2} s^{-1} PPF as measured at culture level. After approximately nine months, stabilized shoot cultures of all genotypes were maintained in the dark in cold storage (9°C) prior to use.

Comparative Stage II Shoot Production of Sea Oats Genotypes

Stabilized and indexed shoot cultures of forty-three randomly selected genotypes chosen from the thirteen sea oats populations were removed from cold storage (stored at 9°C in dark for six months) and clonally propagated for at least two 4-week subculture intervals in GA7 vessels (Magenta Corp., Chicago, IL) containing 80 mL SMM solidified with 8 g/liter TC™ agar. For the comparative study, five multiple shoot clusters of each genotype, each consisting of three shoots cut to 30 mm in length (Figure 2-2A) were transferred into each of eight, GA7 vessels sealed with one layer of Nescofilm (Karlan Research Products Corp, Cottonwood, AZ). Cultures were maintained for 4 weeks at 22°C under a 16-hour photoperiod provided by cool white fluorescent lamps (General Electric F96T12·CW·WM) at 45 µmol m^{-2} s^{-1} PPF as measured at culture level. Shoot

and leaf number and shoot cluster dry weight was measured from eight replicate vessels per genotype after the 4 week culture period.

Comparative Stage III Rooting and Stage IV Acclimatization of Sea Oats Genotypes

Stage II shoot clusters of each genotype were then subdivided into sixty-four single shoot microcuttings cut to 30 mm in length (Figure 2-2B) after 4 weeks culture on SMM and transferred into eight GA7 vessels sealed with a single layer of Nescofilm. Each vessel was inoculated with eight microcuttings containing 80 mL sterile Stage III rooting medium consisting of full strength MS inorganic salts, 0.56 mM myo-inositol, 1.2 µM thiamine-HCL, 87.6 mM sucrose, and 10 µM α-napthaleneacetic acid (NAA) solidified with 8 g/liter TC^{TM} agar. Culture vessels were maintained for 6 weeks at 22°C under a 16-hour photoperiod provided by cool white fluorescent lamps (General Electric F96T12·CW·WM) at 45 µmol m^{-2} s^{-1} PPF. Two microcuttings from each vessel were randomly selected and measured for root number and root length after 6 weeks Stage III culture. Microcutting rooting percentage data were recorded for all replicate vessels after 6 weeks culture.

After 6 weeks culture, forty-eight microcuttings from each genotype were rinsed to remove residual media and then transplanted into coarse vermiculite contained in eight replicate six celled blocks consisting of 4 x 6 x 5.5 cm cells (T.O. Plastics, Inc., Clearwater, MN) before being transferred to Stage IV conditions (Figure 2-2C). Plantlets were watered as needed and after 3 weeks *ex vitro*, were fertilized weekly at 150 mg N l^{-1} using 20N-20P-20K liquid fertilizer (Peters; The Scotts Company, Marysville, OH). Greenhouse set points for cooling and heating were 26.7 and 18.3°C, respectively. Natural solar PPF in the greenhouse ranged from 900 to 1250 µmol m^{-2} s^{-1} at noon.

Plantlet survival percentages were monitored weekly and after 6 weeks Stage IV culture, shoot number and longest leaf lengths were determined. Shoot production and leaf lengths were calculated on the basis of per responsive plantlet. Genotypes were categorized based upon their survival rate in the greenhouse as: 1) easy-to acclimatize (>70 to100%), moderately-difficult-to-acclimatize (36 to ≤ 70%), and difficult-to-acclimatize (0 to 35%) (Table 2-1).

Experimental Design and Statistical Analyses

Experiments were completely randomized designs and repeated once. Where appropriate, data were analyzed using analysis of variance (general linear model) procedures of SAS Institute (2003), LS means and multivariate analyses were performed using JMP (2007).

Results

Stage II *In Vitro* Genotype Comparisons

Sea oats genotypes displayed marked differences in growth and development patterns when cultured *in vitro*. Proliferation of basal shoots occurred from three-shoot clusters cultured in the presence of BA (Figure 2-2A). None of the forty-three genotypes clusters exhibited signs of callus formation or rooting during Stage II culture. Stage II shoot multiplication, length, dry weight, and overall morphology differed significantly within and between sea oats populations (Figures 2-3, 2-4, 2-5, and 2-6). Similarities in responses between genotypes within the same population were also observed (Figures 2-4, 2-5, and 2-6). Genotypes producing shoots of longer length (Figure 2-5) typically exhibited fewer numbers of shoots (Figure 2-4). Populations with genotypes exhibiting the lowest shoot production included Henderson Beach, Honeymoon Island, and Gamble Rodgers. Maximum shoot multiplication was observed in Navarre Beach and

Delnor-Wiggins Pass genotypes (Figures 2-3 and 2-4). When the genotypes of all populations were pooled into North versus South groupings (Figure 2-1), no significant differences in shoot production were noted (Table 2-3). However, as a group, Gulf coast populations exhibited significantly greater *in vitro* shoot multiplication rates compared to those on the Atlantic coast.

Stage III *In Vitro* Genotype Comparisons

Similar to that of Stage II culture, sea oats displayed variable rooting responses dependent upon genotype when grown on Stage III medium. Growth characteristics varied significantly within and between populations (Figures 2-7, 2-8, 2-9, 2-10, and 2-11). Microcuttings displayed moderate callus development prior to the development of adventitious roots (Figure 2-2B). Similarities in rooting responses between genotypes from the same population were also observed. Percent rooting ranged from a low of 33.6% (GR 8-9) to 99.3% (NB 8-1) (Figure 2-8). The average overall rooting response across all genotypes was 78.4%. John D MacArthur State Park genotypes collectively performed the poorest in terms of microcutting rooting. Percent rooting was higher in Gulf (84.9%) vs. Atlantic (73.9%) coast populations as well as being higher in North (83.3%) vs. South (73.2%) populations. Microcutting rooting percentages were highest for genotypes from the Perdido Key and Navarre Beach populations (Figure 2-8). Northern populations exhibited a significantly higher rooting percentage than southern populations (Table 2-3). However, there were no significant differences in microcutting root number between north and south populations. In contrast, populations on the Gulf coast, especially those in the Florida Panhandle region, exhibited higher percent rooting than Atlantic populations (Table 2-3).

Stage IV *In Vitro* Genotype Comparisons

Stage IV *ex vitro* survival rates after 6 weeks were highly variable depending on genotype and ranged from 0-95% (Figures 2-12 and 2-13). Twenty-three of the forty-three genotypes (53%) exhibited a survival rate of 60% or less. After 6 weeks growth *ex vitro*, St. George Island, Perdido Key and Navarre Beach genotypes displayed the highest percent survival of all genotypes. Gamble Rogers and John D. MacArthur genotypes exhibited the lowest percent survival. Populations in the Florida panhandle collectively had a higher survival rate than other Florida populations. Overall, the Gulf coast populations exhibited the most genotypes with survival rates exceeding 60% or greater. Based on these results, individual genotypes were categorized as difficult, moderately-difficult, and easy-to-acclimatize (Table 2-1).

Multivariate Correlation of *In Vitro* Growth Responses with *Ex Vitro* Survival

Multivariate correlation analysis was completed to infer possible functional relationships between individual *in vitro* growth responses and *ex vitro* percent survival of the pooled sea oats genotypes from the same population (Table 2-2). Stage II shoot dry weight, shoot number per explant, and shoot length were not highly correlated with *ex vitro* survival in any of the populations studied. Similarly, Stage II rooting percentage, root length and leaf production were not strong predictors of Stage IV percent survival in any of the genotypes screened. Similar results were observed when populations were pooled (Table 2-2).

Discussion

Our survey of forty-three cloned genotypes, derived from *in vitro* germinated seedlings from thirteen Florida sea oats populations, clearly indicates that marked differences in *in vitro* shoot production, morphology, rooting and *ex vitro* acclimatization

exist between these sea oats genotypes. Of the forty-three genotypes screened, sixteen (37%) were characterized as easy-to-acclimatize, sixteen (37%) as moderately difficult-to -acclimatize and eleven (26%) as difficult-to-acclimatize. Consequently, approximately 63% of the genotypes produced using the "optimized" BA-based sea oats micro-propagation protocol appear to exhibit reduced capacities for *ex vitro* acclimatization. Genotypic limitations on *ex vitro* acclimatization could have profound effects on production costs and the ability to produce the large number of diverse sea oats genotypes required using micropropagation technology.

Due to its low cost and high effectiveness, the cytokinin 6- benzyladene (BA) is one of the most widely used plant growth regulators for *in vitro* plant propagation (Bairu et al., 2007). However, negative carryover effects on the production, rooting and/or acclimatization of shoots produced on BA-supplemented media have been described for a rapidly increasing number of species (Werbrouck et al., 1995; Bairu et al., 2007; Amoo et al., 2011; Aremu et al., 2011). Werbrouck et al. (1995) correlated BA accumulation at the base of *Spathiphyllum floribundum* plantlets with root inhibition and chloroplast ultra-structural changes. Removal of basal callus containing the highest endogenous BA concentration decreased the inhibition of rooting. Werbrouck et al. (1996) later reported that significantly reduced rooting was correlated with the presence of the BA metabolite, N^6-benzyladenine-9-glucoside ([9G]BA), which accumulated at the base of *Spathiphyllum* plantlets and remained for more than 6 weeks.

In the current study, reduced *ex vitro* acclimatization capacities in 63% of sea oats genotypes screened may be the result of a negative carry-over effect of BA supplemented Stage II medium. Earlier, Valero-Aracama et al. (2006; 2007; 2008)

attributed the anatomical, morphological and physiological differences between a difficult- and easy-to- acclimatize sea oats genotype as a consequence of the *in vitro* propagation on BA-supplemented media. These differences included an eight-fold decrease in photosynthetic capacity associated with a concomitant decrease in photosynthetic enzymatic activity and abnormal leaf anatomy and chloroplast ultrastructure in the difficult-to-acclimatize genotype after 6 weeks *in vitro* rooting immediately before *ex vitro* acclimatization (Valero-Aracama et al., 2006; 2008).

Two additional observations further supported the presence of a negative carryover effect of BA on the *ex vitro* acclimatization of sea oats plantlets. Although Stage II shoot multiplication was minimal, *ex vitro* survival of the difficult-to-acclimatized genotype was significantly increased when plantlets were cultured first on medium without BA before Stage III rooting or by substituting BA with the benzyladenine (BA) analog N^6 – (3-hydroxybenzyl) adenine or *meta*-topolin (mT) for Stage II shoot multiplication (Valero-Aracama et al., 2010). Further increases *in ex vitro* survival of the easy-to-acclimatize sea oats genotype were also observed.

Strnad et al. (1997) first reported the existence of mT, a naturally occurring BA-analog derived from poplar leaves (*Populus x canadensis* Moench. CV. robusta). Werbrouck et al. (1996) reported that *Spathiphyllum* shoots multiplied on mT supplemented medium exhibited increased *in vitro* rooting over those produced on medium containing equimolar BA concentrations. It has been proposed that mT mediated increases in rooting result from the presence of a hydroxyl group on the benzyl ring of the mT molecule which makes O-glucosylation possible. Naturally occurring beta-glucosidases more rapidly degrade the mT derivative, N^6-(3-O-β-D-

glucopyranosyl) benzyladenine-9-riboside ([9R]mT) despite accumulation at comparable levels of [9G]BA (Werbrouck et al., 1996). Since the discovery of mT, a number of naturally occurring aromatic cytokinins classified as topolins have been evaluated as alternatives to BA and other cytokinins (Aremu et al., 2011). Bairu et al. (2011) evaluated the changes in a wide range of cytokinin profiles in micropropagated *Harpagophytum procumbens* [(Burch.) DC. ex Meisn.] tissues. The authors suggested that the hydroxyl group in topolins allows for greater accumulation of *O*-glucosides in topolin-treated explants allowing for active cytokinin to be available to the plant. The advantages of using topolins for *in vitro* propagation has been demonstrated in an increasing number of species (Amoo et al., 2011; Aremu, et al., 2011).

Conceivably, enhanced acclimatization may be a common response exhibited by difficult, moderately difficult and easy-to-acclimatize sea oats genotypes following *in vitro* propagation in the presence of mT. Since ecologically sound dune restoration will require the efficient micropropagation of diverse sea oats genotypes, the efficacy of using mT or other aromatic cytokinins for efficient micropropagation of many genotypes must to be further evaluated. The library of forty-three acclimatization characterized sea oats genotypes generated in the current study provides the opportunity to further explore this.

With the exception of previous sea oats research (Kane et al., 2006) and the expanded comprehensive work reported here, there have been very few comparative studies of the effect of genotype on *in vitro* growth responses in other native plant species used for habitat restoration. Genotypic differences in both *in vitro* and *ex vitro* growth responses of the wetland species *Pontederia cordata* L. have been observed

(Zurinsky, 1995; Kane et al., 1997). Interestingly, significant genotypic differences in the effects of Stage II cytokinin type on the requirement for *in vitro* rooting for increased Stage IV acclimatization were observed. (Zurinsky, 1995). Likewise, significant differences in shoot growth, corm formation and flowering of micropropagated *Sagittaria latifolia* L. genotypes have also been reported (Kane et al., 2000; 2003). Although these studies focused only on the *ex vitro* growth responses of micropropagated genotypes in this species, *S. latifolia* exhibited a high sensitivity to relatively low BA levels *in vitro*. Repeated subculture on medium supplemented with > 2.5 µM BA resulted in increased mortality, reduction in multiplication, or production of dormant corms (Lane, 1999).

Environmental conditions vary depending on the geographical locations sea oats population location (Williams, 2007). Differences in substrate type, wave action, storm frequency, and seasonal temperature fluctuations all may influence local ecotypic differentiation. Ecotypic differentiation with changing latitude has been reported for sea oats seed production and germination (Wagner, 1964; Seneca, 1972). Florida's Gulf and Atlantic coasts are characterized by extensive quartz sand beaches and strong wave action while south Florida beaches are composed of mostly calcium carbonate sand (Williams, 2007). From Naples northward to Tarpon Springs, quartzite sand beaches are prominent. The sea oats genotypes established from the Florida Panhandle populations consistently displayed higher survival when transferred to greenhouse conditions (Figure 2-13). The basis for this increased survival among these populations is unclear. However, examination of location and frequency of tropical storm and hurricane landfall events over the past 110 years indicates that the Florida Panhandle has been more frequently impacted by storm events. The relatively

increased instability of these dunes may, over time, have resulted in selection for more vigorous genotypes. However, care must be taken when interpreting *in vitro* growth responses as selection pressures may be over simplified since *in vitro* and *in situ* conditions differ greatly (Kauth et al. 2011). An assessment of a significantly greater number of genotypes from each population would be required to develop clearer relationships between population specific responses.

Of the Stages II and III culture responses measured, there was no single growth characteristic for any population that could be correlated to poor *ex vitro* performance (Table 2-2). This might be related to the fact that Valero-Aracama et al. (2006) reported that low *ex vitro* survival in a difficult-to-acclimatize genotype was a consequence of low photosynthetic capacity. Consequently, correlation of *in vitro* growth responses on media with sugar with ease of acclimatization may not be possible. Given these results, it would be very difficult to reliably predict which genotypes would be easy or difficult-to-acclimatize without actual transfer to greenhouse conditions.

Conclusion

For micropropagation to be considered a practical and ecologically-sound method for supplementing sea oats seedling production for dune restoration, it is critical that a large number of diverse genotypes from many geographic sources be efficiently propagated *in vitro*. Results of the current study clearly demonstrate that sea oats genotypes display significant differences in Stage II shoot production and elongation, Stage III rooting, and Stage IV acclimatization when cloned using a BA-based micropropagation protocol. Of the 43 sea oats genotypes examined, 62.7% were characterized as moderately- to difficult-to-acclimatize with *ex vitro* survival rates ranging from 0 – 69.7%. Clearly a more efficient micropropagation protocol resulting in

higher acclimatization rates for a greater number of genotypes is required. Currently, the effects of using a BA-analog, *meta*-Topolin, on Stage II shoot multiplication, rooting and acclimatization of difficult-to-acclimatized sea oats genotypes are being examined.

Table 2-1. Categorization of sea oats genotypes as easy-, moderately-difficult, and difficult-to-acclimatize based on percent *ex vitro* survival performance after 6 weeks.

Easy-to-Acclimatize[1]	Percent Survival	Moderately-Difficult-to-Acclimatize[2]	Percent Survival	Difficult-to-Acclimatize[3]	Percent Survival
SG 5-11	95.8	PK 4-1	69.7	JDM 8-6	33.3
PK 8-13	94.8	JUL 8-20	68.6	DWP 8-23	30.3
HRA 5-2	93.7	PK 8-16	63.1	AN 7-4	28.1
SG 5-8	93.5	AN 7-7	62.4	JDM 8-8	27.3
NB 8-13	89.5	JUL 8-4	59.4	DWP 8-26	20.3
NB 8-1	87.4	HI 8-12	59.1	DWP 8-25	19.1
HRA 7-16	83.3	SI 8-1	57.7	JDM 8-3	15.7
LTI 8-1	82.3	DWP 8-1	56.6	JDM 8-5	13.6
LTI 8-8	82.1	SI 8-16	55.9	GR 8-2	10.5
PK 8-12	80.1	LTI 1-4	53.4	GR 8-9	10.1
NB 3-4-3-2	78.6	LTI 8-9	53.1	JUL 8-23	0.00
HRA 7-12	77.3	AN 7-17	51.1		
HRA 7-8	72.8	JUL 8-11	51.1		
BB 8-13	71.9	AN 7-21	48.9		
HI 8-4	70.8	HI 1-2	48.4		
HI 1-3	70.8	GR 8-1	36.9		

[1]Easy-to-Acclimatize survival rates range from > 70 to100%.
[2]Moderatley-Difficult-to-Acclimatize survival rates range from 36 to ≤ 70%.
[3]Difficult-to-Acclimatize survival rates range from 0 to 35%.

Table 2-2. Multivariate correlation of *in vitro* growth responses with *ex vitro* survival of sea oats populations.

Response	PK	NB	HRA	SG	HI	DWP	BB	JUL	JDM	SI	GR	AN	LTI	Combined Populations
Stage II														
Shoot Dry Weight	-0.3833	-0.4078	0.1613	0.1552	-0.09	0.4191	0.0709	-0.0486	-0.0957	-0.09	-0.3696	-0.2525	0.0602	0.0669
Shoot Number/Cluster	-0.3309	0.0899	-0.0172	0.1418	0.2223	0.1203	-0.1544	-0.2813	-0.1895	0.2223	0.2111	-0.2284	-0.0228	0.1548
Shoot Length/Cluster	-0.0261	-0.3207	0.1016	0.1596	-0.2215	-0.1267	-0.0418	0.2206	0.2616	-0.2215	-0.4093	-0.0712	0.0415	0.1373
Stage III														
Root Number/Microcutting	0.0657	-0.1519	-0.1381	-5E-04	-0.4418	-0.0748	0.0672	-0.2101	0.0207	-0.4418	0.0337	0.2484	-0.0446	-0.0357
Leaf Number/Microcutting	-0.1061	0.1378	-0.1561	0.2138	0.0069	-0.2912	-0.1578	0.3944	-0.0547	0.0069	0.2416	-0.1078	-0.0176	-0.0286
Root Length/Microcutting	-0.2602	-0.0434	0.2086	0.2514	-0.2789	0.1835	0.0615	0.0778	0.1338	-0.2789	-0.0633	-0.0332	0.1619	0.0599
% Microcutting Rooting	0.1913	-0.0827	0.045	-0.072	-0.2208	-0.1245	-0.1131	0.2092	-0.0503	-0.2208	-0.249	0.0963	0.0293	0.122

Table 2-3. ANOVA analysis of pooled growth responses of northern/southern and Atlantic/Gulf coast sea oats populations (refer to Figure 2-1).

	Stage II - Total Shoot Number		Stage III - Root Number		Stage III - Percent Rooting		Stage IV - Percent Survival	
	F	p	F	p	F	p	F	p
North x South	11.664	0.4485	15.0521	0.3027	1.492221	<0.0001	8.40949	<0.0001
Atlantic x Gulf	451.471	<0.0001	67.182	0.0292	1.765705	<0.0001	10.64508	<0.0001

Figure 2-1. Collection sites for *Uniola paniculata* seed with delineation of northern and southern populations (red horizontal line).

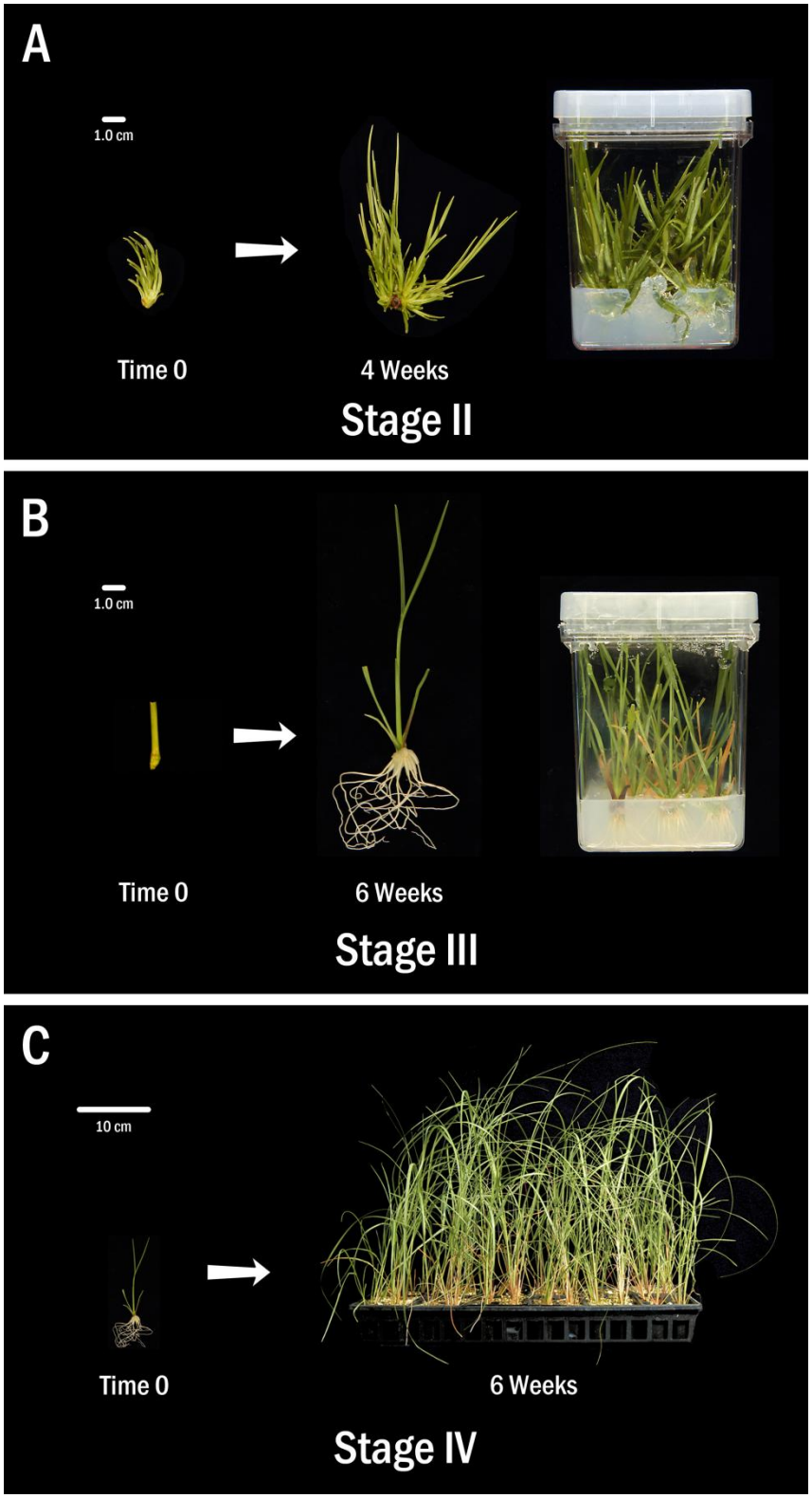

Figure 2-2. Growth and developmental sequence of Stage II shoot multiplication (A), Stage III microcutting rooting (B), and Stage IV acclimatization in plug trays (C).

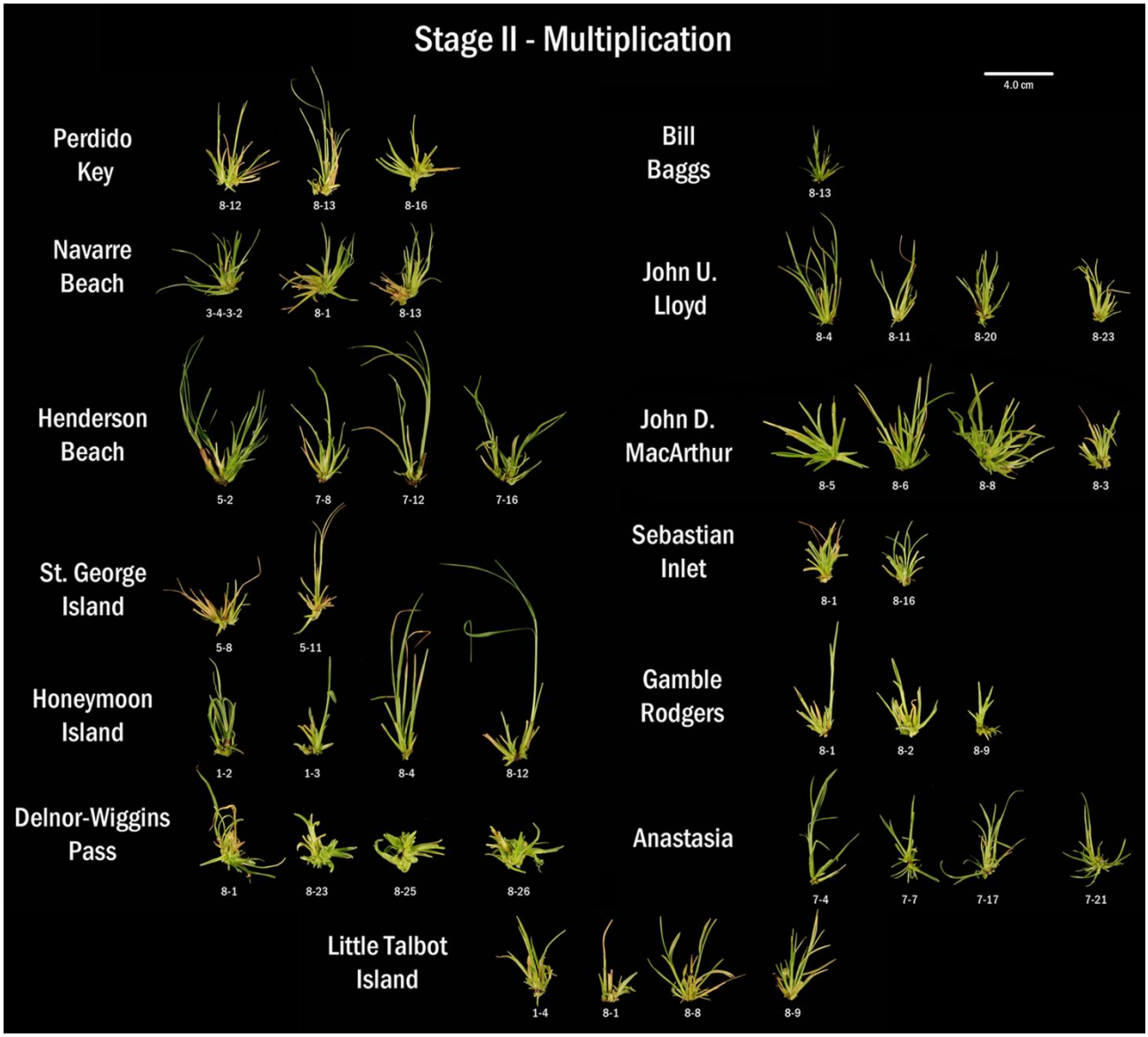

Figure 2-3. Comparative Stage II *in vitro* shoot multiplication and morphological responses of forty-two *Uniola paniculata* genotypes after 4 weeks culture.

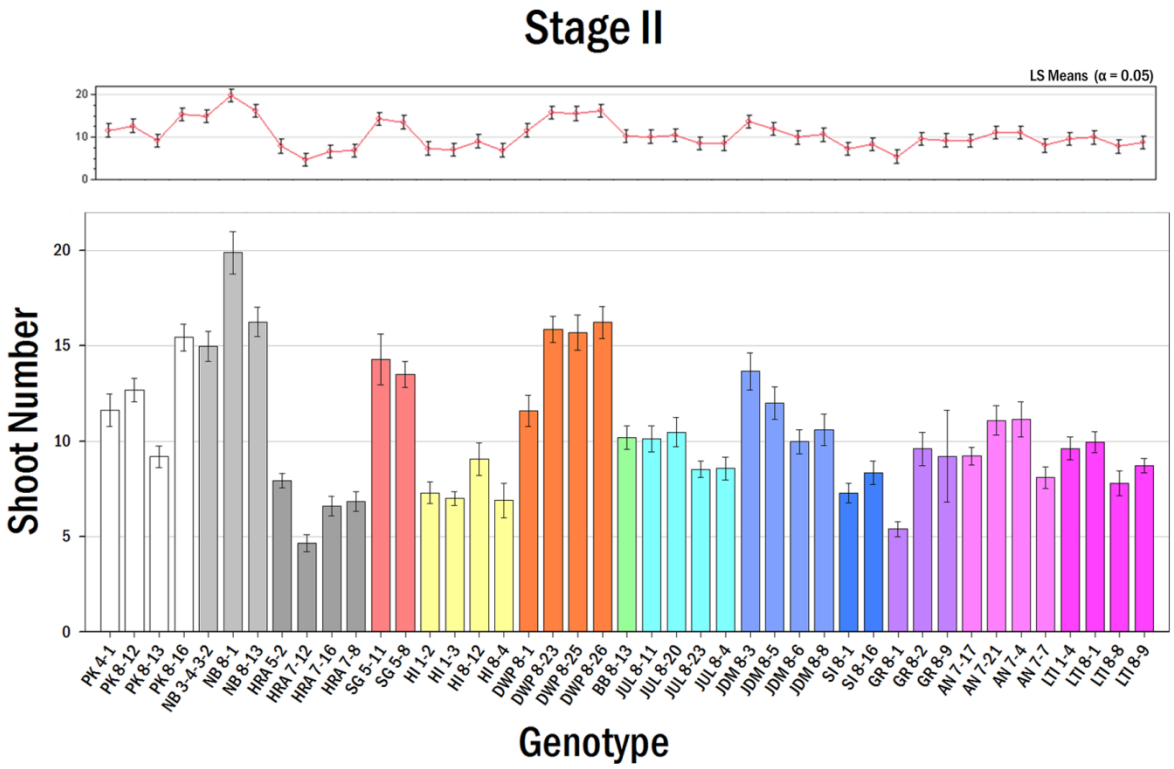

Figure 2-4. Comparative Stage II shoot multiplication of *Uniola paniculata* genotypes after 4 weeks culture. The LS mean value (P< 0.05) for each response is shown.

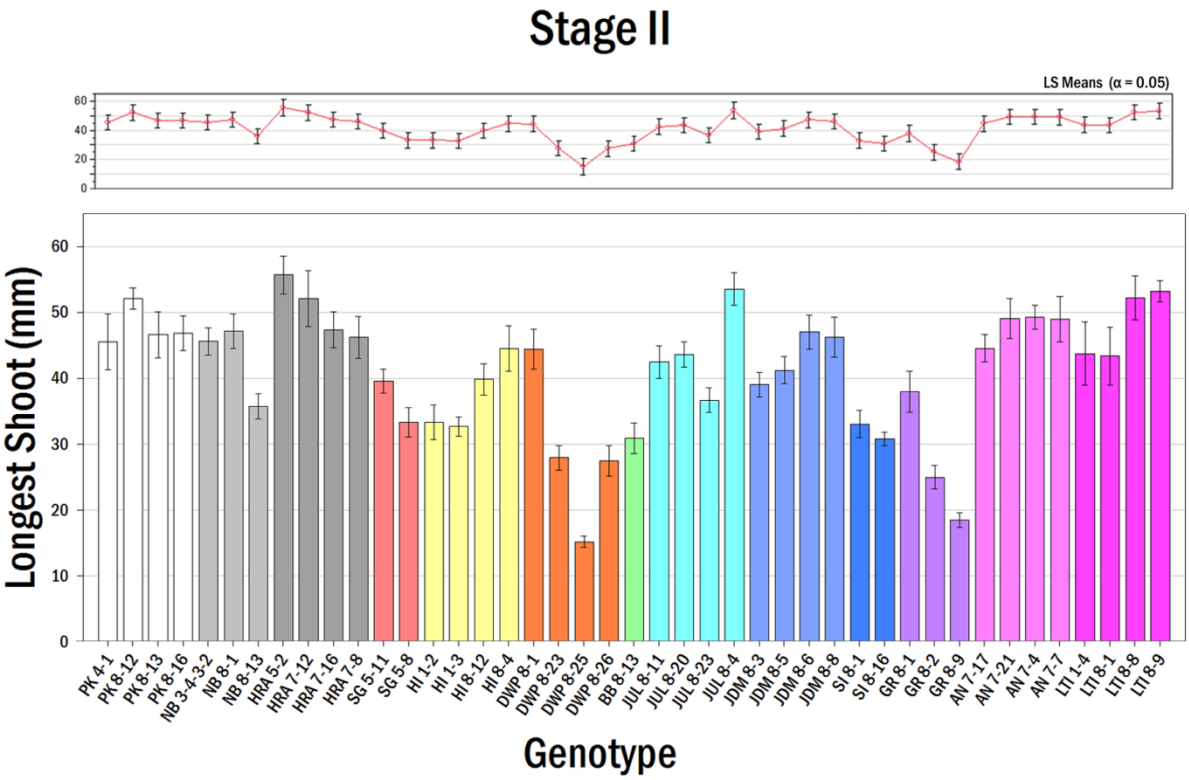

Figure 2-5. Comparative Stage II shoot length of *Uniola paniculata* genotypes after 4 weeks culture. The LS mean value (P< 0.05) for each response is shown.

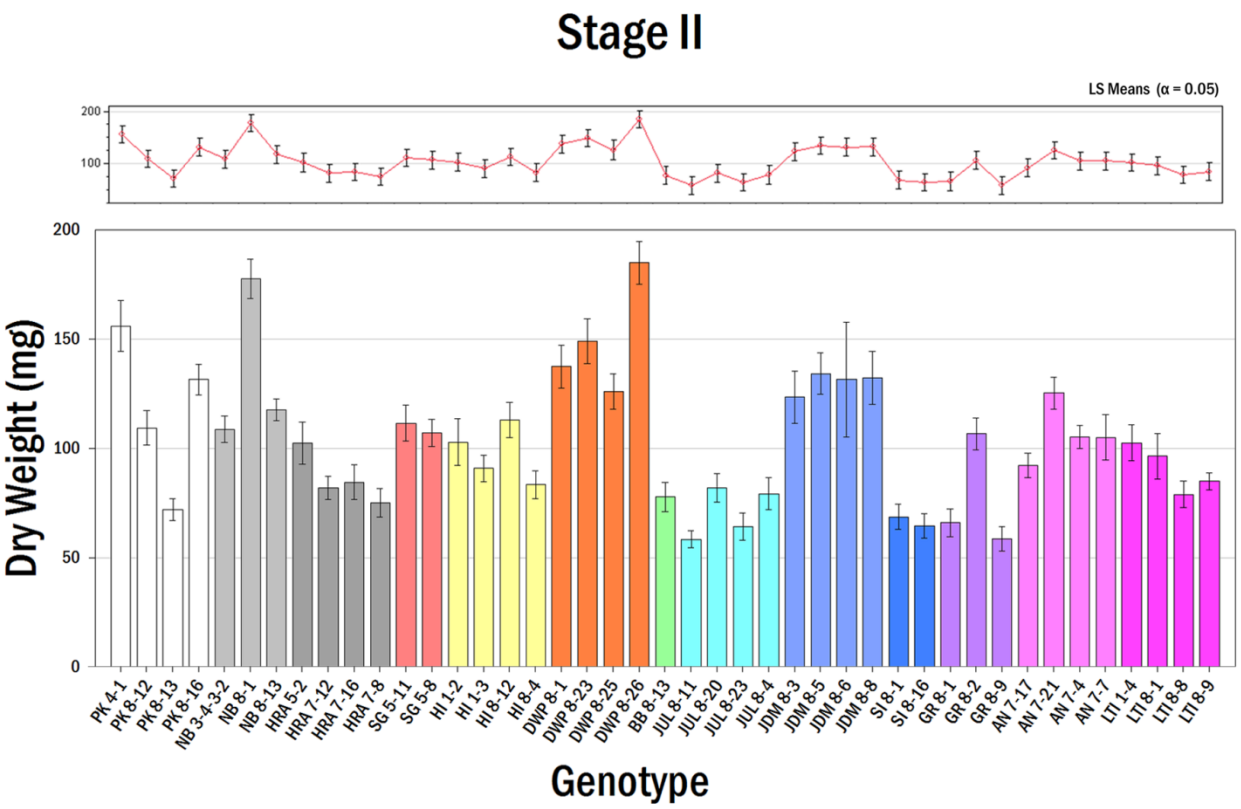

Figure 2-6. Comparative Stage II biomass of *Uniola paniculata* genotypes after 4 weeks culture. The LS mean value (P< 0.05) for each response is shown.

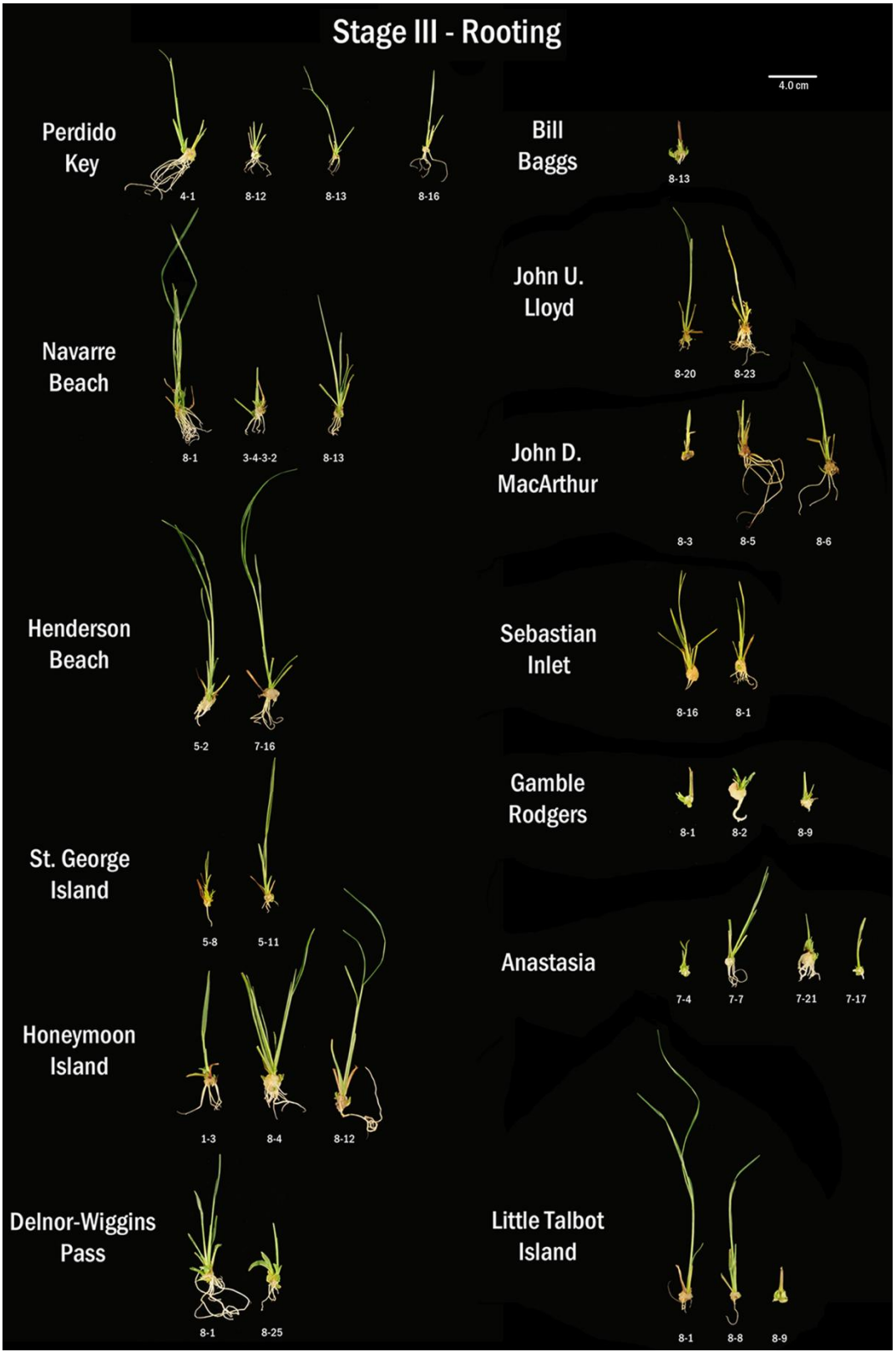

Figure 2-7. Comparative Stage III *in vitro* rooting of *Uniola paniculata* microcuttings after 6 weeks culture.

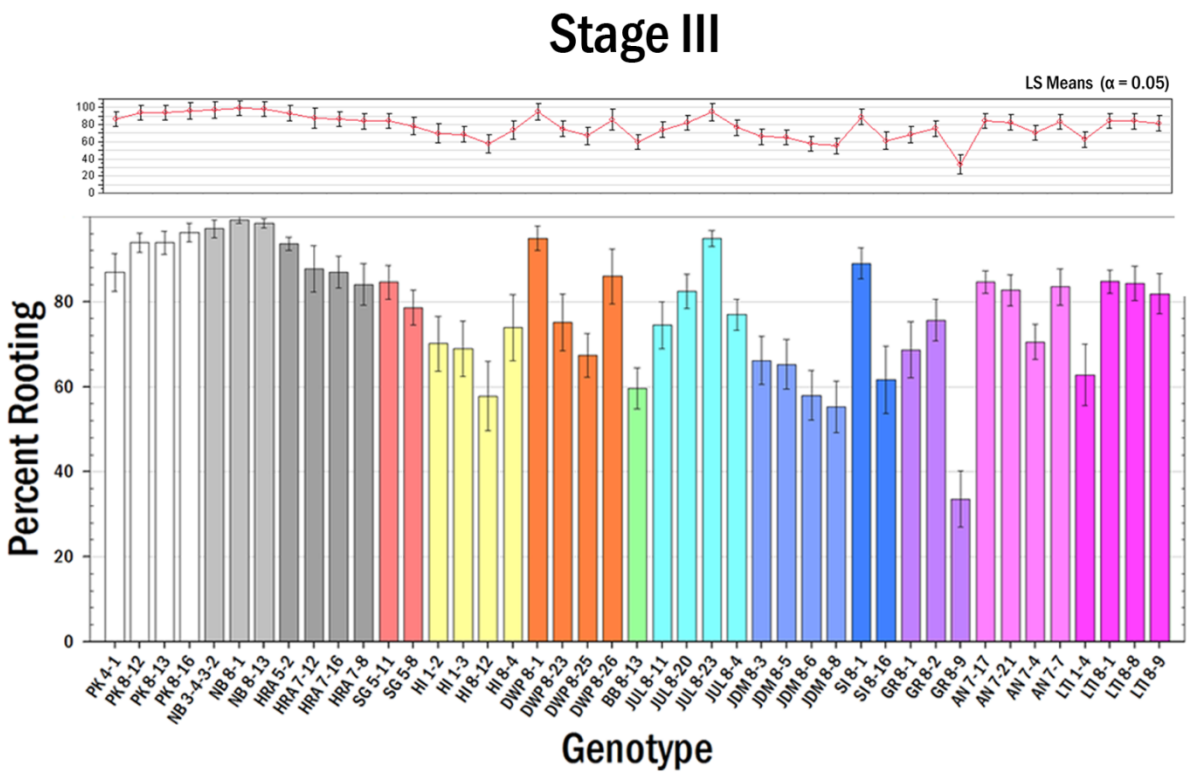

Figure 2-8. Comparative Stage III microcutting percent rooting of *Uniola paniculata* genotypes after 4 weeks culture. The LS mean value (P< 0.05) for each response is shown.

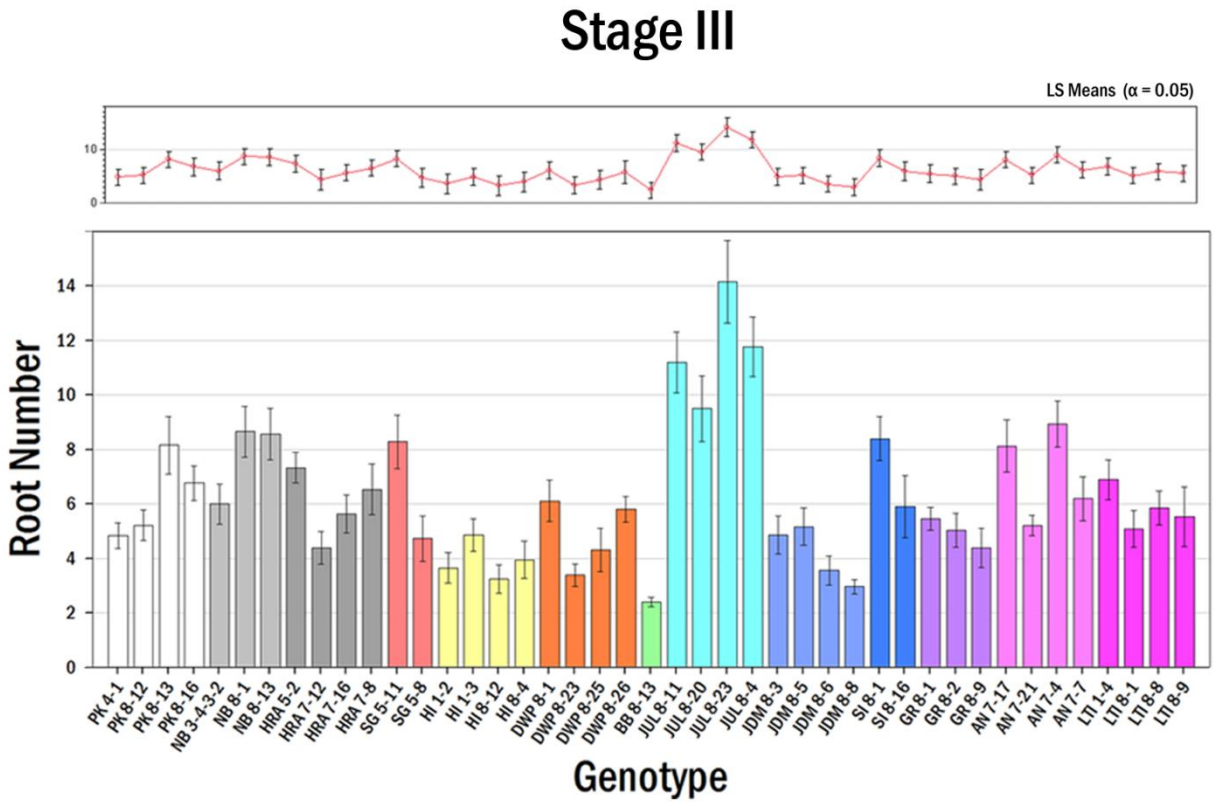

Figure 2-9. Comparative Stage III microcutting root number of Uniola paniculata genotypes after 4 weeks culture. The LS mean value (P< 0.05) for each response is shown.

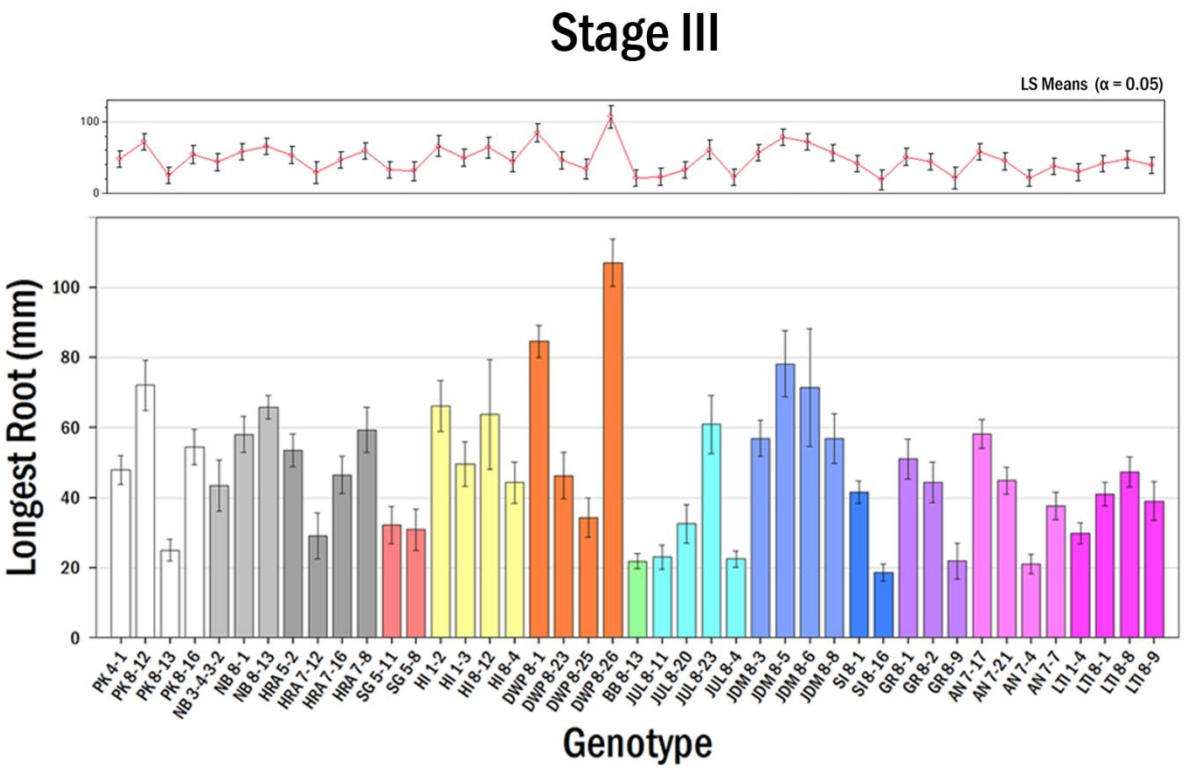

Figure 2-10. Comparative Stage III microcutting root length of *Uniola paniculata* genotypes after 4 weeks culture. The LS mean value (P< 0.05) for each response is shown.

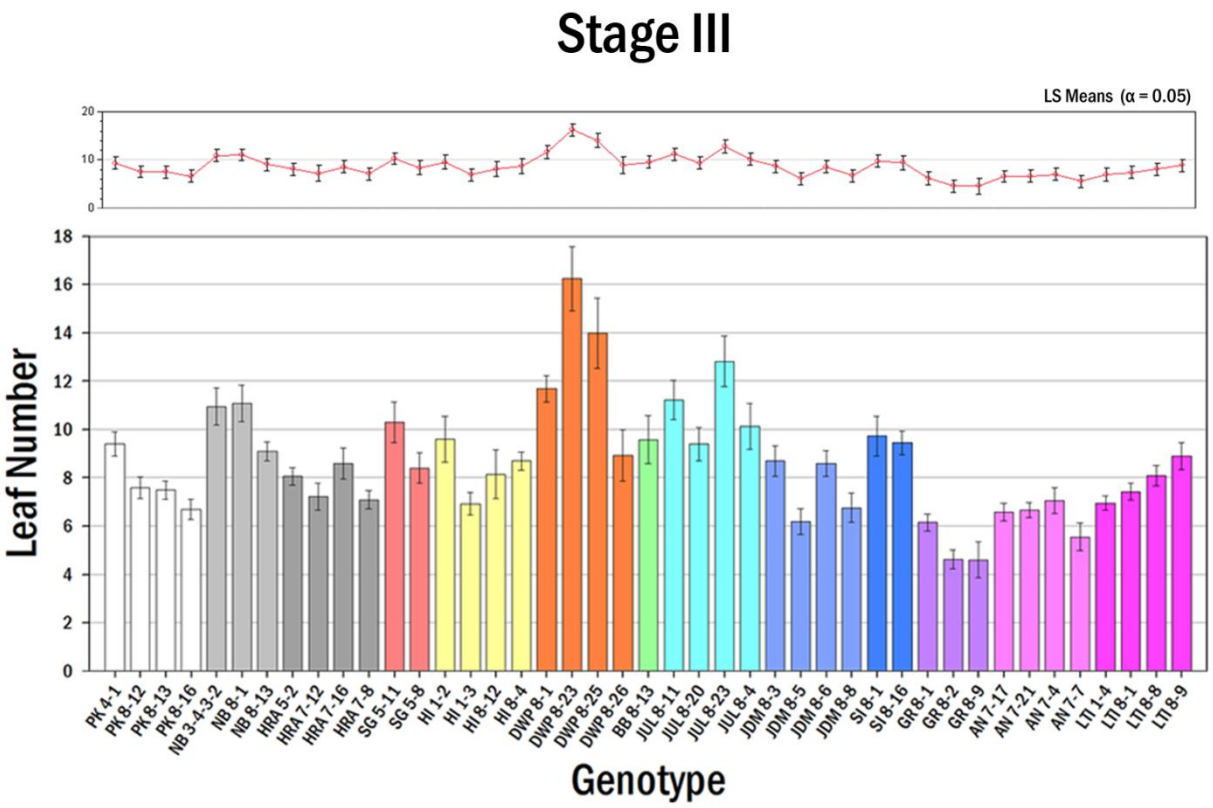

Figure 2-11. Comparative Stage III microcutting leaf number of *Uniola paniculata* genotypes after 4 weeks culture. The LS mean value (P< 0.05) for each response is shown.

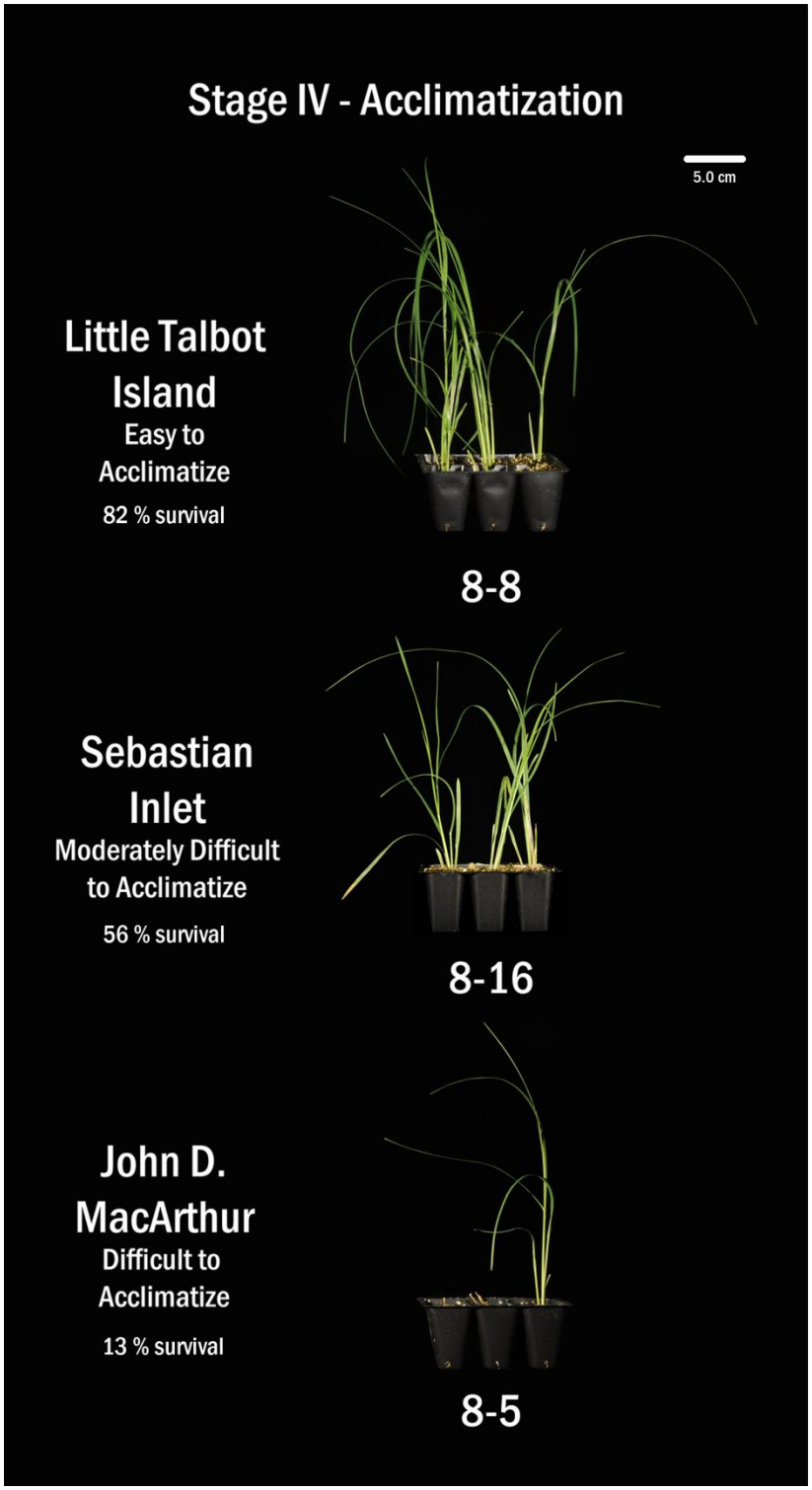

Figure 2-12. Examples of 6-week old Stage IV *ex vitro* acclimatized *Uniola paniculata* plantlets categorized as easy-, moderately-difficult, and difficult-to-acclimatize genotypes.

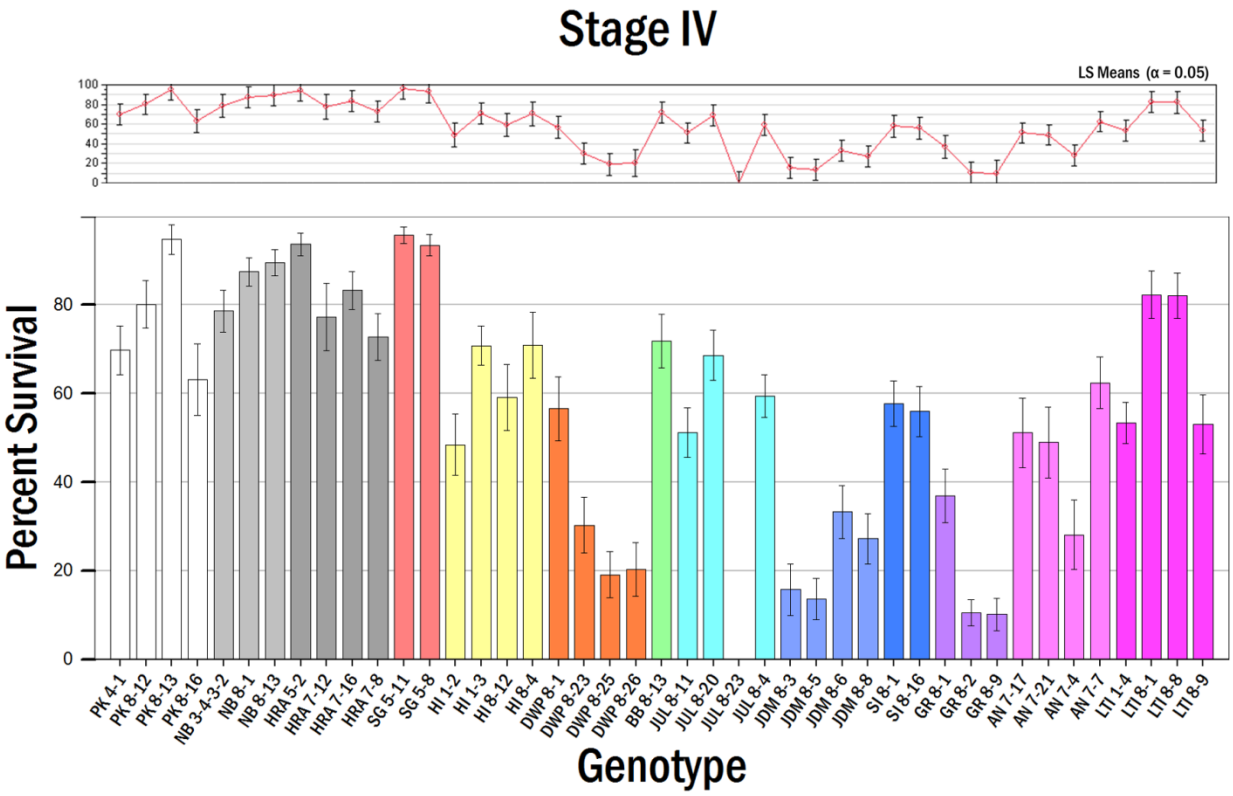

Figure 2-13. Comparative Stage IV percent survival of *Uniola panciulata* genotypes after 6 weeks *ex vitro* acclimatization. The LS mean value (P< 0.05) for each response is shown.

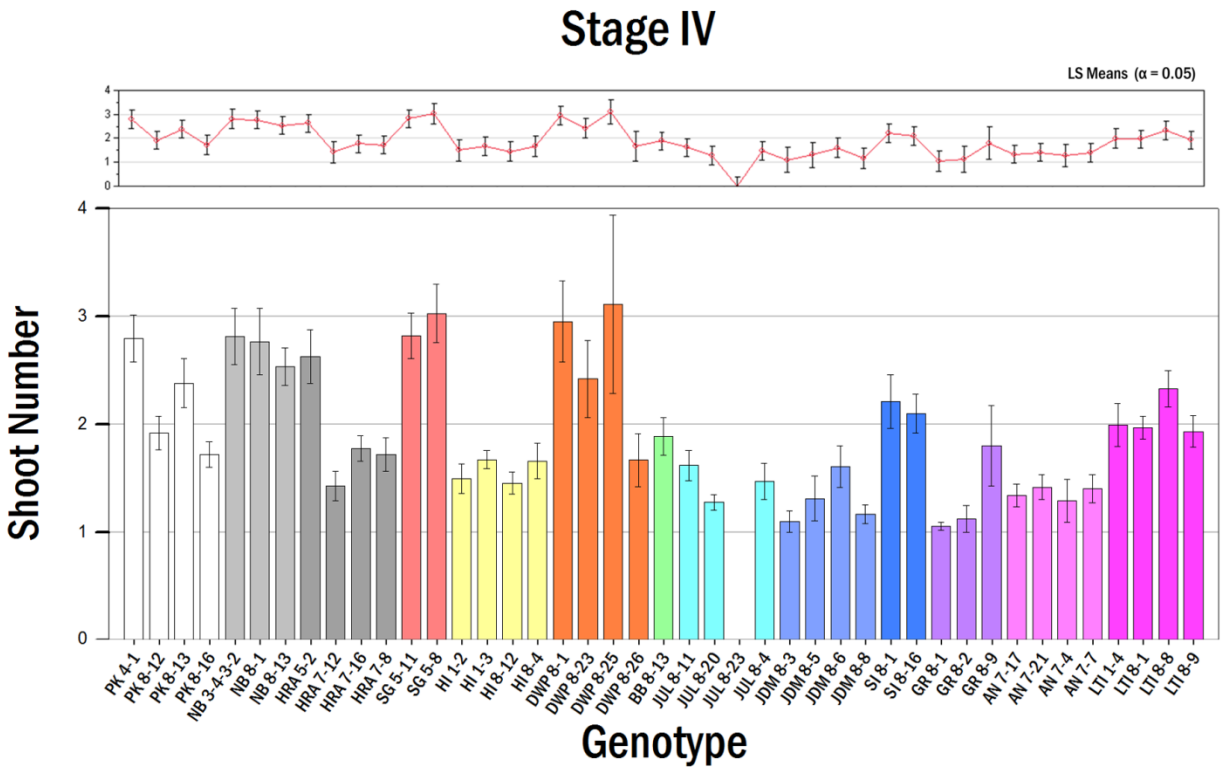

Figure 2-14. Comparative Stage IV shoot production of *Uniola panciulata* genotypes after 6 weeks *ex vitro* acclimatization. The LS mean value (P< 0.05) for each response is shown.

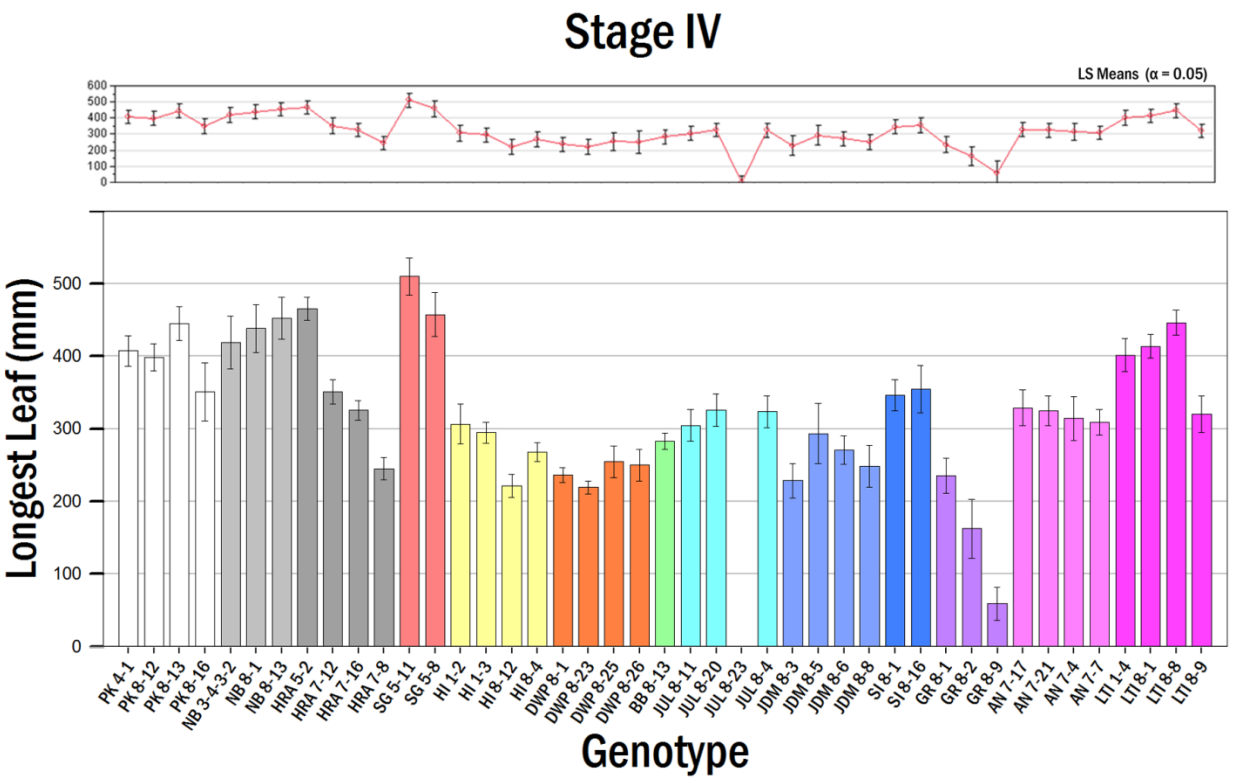

Figure 2-15. Comparative Stage IV leaf length of *Uniola panciulata* genotypes after 6 weeks *ex vitro* acclimatization. The LS mean value (P< 0.05) for each response is shown.

LIST OF REFERENCES

Amoo SO, Finnie, J.F., Van Staden, J., 2011. The role of *meta*-topolins in alleviating micropropagation problems. Plant Growth Regul. 63, 197-206.

Aremu, A.O., Bairu,M.W., Dolezal, K., Finnie, J.F. and Van Staden, J. 2011. Topolins: A panacea to plant tissue culture challenges? Plant Cell Tiss. Organ Cult. DOI 10.10007/s11240-011-0007-7.

Bachman, G.R., Whitwell, T., 1995. Nursery production of *Uniola paniculata* (southern sea oats). HortTechnology 5:295-298.

Bairu, M.W., Stirk, W.A., Dolezal, K., Van Staden, J., 2007. Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: Can *meta*- topolin and itsderivatives serve as replacement for benzyladenine and zeatin? Plant Cell Tiss.Organ Cult. 90, 15-23.

Bairu, M.W., Stirk, W.A., Dolezal, K., Van Staden, J., 2008. The role of topolins in micropropagation and somaclonal variation of banana cultivars 'Williams' and 'Grand Naine' (*Musa* spp. AAA). Plant Cell Tiss. Organ Cult. 95, 373-379.

Bairu, M.W., Novak, O., Dolezal, K., Van Staden, J., 2011. Changes in endogenous cytokinin profiles in micropropagated *Harpagophytum procumbens* in relation to shoot-tip necrosis and cytokinin treatments. Plant Growth Regul. 63, 105-114.

Barbour, M.G., Rejmanek, D.M., Johnson, A.F., Pavlik, B.M., 1987. Beach vegetation and plant distribution patterns along the northern Gulf of Mexico. Phytocoenologia 15, 201-233.

Barnett, M.R., Crewz, D.W., 1991. An introduction to planting and maintaining selected common coastal plants in Florida. Florida Sea Grant Report No. 97. 108 pp.

Burgess, T.L., Blazich, F.A., Nash, D.L., 2002. Seed germination of southern sea oats (*Uniola paniculata*) as influenced by stratification, temperature, and light. J. Environ. Hort. 20, 180-183.

Burgess, T.L., Blazich, F.A., Nash, D.L., Randall-Schadel, B., 2005. Influence of selected surface disinfectants, fungicides, and temperature on seed germination and initial growth of southern seaoats (*Uniola paniculata*). J. Environ. Hort. 23, 4-8.

Cook, D.A., Decker, D.A., Gallagher, J.L., 1989. Regeneration of *Kosteletzkya* (L.) Presl. (Seashore Mallow) from callus cultures. Plant Cell Tiss. Org. 17, 111-119.

Dahl, B.E., Woodard, D.W., 1977. Construction of Texas coastal foredunes with sea oats (*Uniola paniculata*) and bitter panicum (*Panicum amarum*). Int. J. Biometeor. 21, 267-275.

Debergh, P.C., Topoonyanont, N., Van Huylenbroeck, J., Moreira da Silva, H., Oyaert, E., 2000. Preparation of microplants for *ex vitro* establishment. Proceedings from the International Symposium on Methods and Markers For Quality Assurance In Micropropagation. Eds. Cassells, A. C., Doyle, B.M., Curry, R. F. Acta Hort. 530, 269-275.

Florida Dept. Environmental Protection. 2004. Hurricane Recovery Plan for Florida's Beach and Dune System. Division of Water Source Management, Bureau of Beaches and Coastal Systems, November 30, 2004. 65 pp.

Florida Department of Environmental Protection, 2005. Critically eroded beaches in Florida. Bureau of Beaches & Coastal Systems, Division of Water Resource Management, 75 pp.

Gil-Humanes, J., Martin, A., Barro, F., 2011. Characterization of a collection of *Brassica carinata* Genotypes for *in vitro* culture response and mode of shoot regeneration. Amer. J. of Plant Sci. 2, 27-34.

Hester, M.W., and Mendelssohn, I.A., 1991. Expansion patterns and soil physiochemical characterization of three Louisiana populations of *Uniola paniculata* (sea oats). J. Coastal Res. 7, 387-401.

Hovanesian, J.C., and Torres, K.C., 1986. Micropropagation of *Uniola paniculata* L. Plant Cell Rep. 5, 385-386.

JMP, Version 8. SAS Institute Inc., Cary, NC, 1989-2007.

Kane, M.E., Philman, N., 1997. *In vitro* propagation and selection of superior wetland plants for habitat restoration. Comb. Proc. Int. Plant Prop. Soc. 47, 556-560.

Kane. M.E., R. Gillis, Philman, N., Campbell, S., 2000. Seasonal differences in *ex vitro* growth and corm formation between two micropropagated *Sagittaria latifolia* ecotypes. Acta Horticulturae 520, 229-237.

Kane, M.E., Philman, N., and Emshousen, C., 2003. Wetland Plant Micropropagation: Comparative Growth and Reproduction of *Sagittaria latifolia* ecotypes. Combined Proceedings International Plant Propagators' Society. 52, 453-459.

Kane, M.E., Philman, N.L. , Kauth, P., Shen, X., Sleszynski, P., Stewart, S.L., Valero Aracama, C., 2006. Comparative *in vitro* shoot multiplication, rooting and *ex vitro* acclimatization of sea oats (*Uniola paniculata* L.) genotypes. In Vitro Cell. Dev. Biol. 42, 24A.

Kane, M.E., 2011. Micropropagation by Shoot Culture. *In*: R. N. Trigiano and D.J. Gray (eds.), Plant Tissue Culture, Development, and Biotechnology, Chapter 12, pp. 181-192, CRC Press, Boca Raton.

Kauth P.J., Kane, M.E., and Vendrame, W.A., 2011. Comparative *in vitro* germination ecology of *Calopogon tuberosus* var. *tuberosus* (Orchidaceae) across its geographic range, In Vitro Cell. Dev. Biol. -Plant 47,148-156.

Kaye, T.N., 2001. Common ground and controversy in native plant restoration: the SOMS debate, source distance, plant selections and restoration-oriented definition of nature, pp.5-12. *In*: D.L. Hasses and R. Rose (eds.), Proceedings of the Conference: Native Plant Propagation and Restoration Strategies, Nursery Technology Cooperative and Western Forestry and Conservation Assoc., Eugene, OR.

Krauss, S.L., J.M. Koch., 2004. Rapid genetic delineation of provenance for plant community restoration. J. Appl. Ecol. 41,1162-1173.

Lane, C.W., 1999. Micropropagation of *Sagittaria latifolia*. Masters Thesis, Univ. Florida, Gainesville, Florida, 83 pp.

Lewis, J.C., Bunce, E.W., 1980. Rehabilitation and creation of selected coastal habitats: Proceedings of a workshop. U.S. Fish and Wildlife Service, Biological Services Program, Washington, DC. FWS/OBS-80/27. 162 pp.

Li, X., Gallagher, J., 1996. Tissue culture and plant regeneration of big cordgrass, *Spartina cynosuroides*: implications for wetland restoration. Wetlands 16, 410-415.

Miller, D. L., Yager, L., Thetford, M., & Schneider, M., 2003. Potential use of *Uniola paniculata* rhizome fragments for dune restoration. Restoration Ecology 11, 359-369.

Murashige, T, Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

Murley, J.F., Alpert, L., Matthews, M.J., Bryk, C., Woods, B., Grooms, A., 2003. Economics of Florida's beaches: the impact of beach restoration. Catanese Center for Urban and Environmental Solutions at Florida Atlantic University. 24 pp.

O'Brien, D.L., Freshwater, D.W., 1999. Genetic diversity within tall form *Spartina alterniflora* Loisel. along the Atlantic and Gulf coasts of the United States. Wetlands 19, 352-358.

Oosting, H.J., Billings, W.D., 1942. Factors effecting vegetational zonation on coastal dunes. Ecology 23, 131-142.

Pezeshki S.R., Delaune, R.D., 1991. Ecophenic variations in Wiregrass (*Spartina patens*). J. Aquat. Plant Manage. 29, 99-102.

Philman, N.L., Kane, M.E., 1994. Micropropagation of *Uniola paniculata* L. (sea oats) from tiller explants. HortScience 29, 559 (abstract).

Pospíšilová, J., Solarova, J., Catsky, J., 1992. Photosynthetic responses to stresses during *in vitro* cultivation. Photosynthetica 26, 3-18.

Pospíšilová, J., Ticha, I., Kadlecek, P., Haisel, D., Plzakova, S., 1999. Acclimatization of micropropagated plants to *ex vitro* conditions. Biol. Plantarum 42, 481-497.

Ranamukhaarachchi, D.G., 2000. Molecular analysis of genetic diversity in Florida sea-oats (*Uniola paniculata*) populations: New approaches to generate and analyze molecular data. Ph.D. Dissertation. Univ. Florida, Gainesville, Florida, 142 pp.

Richards, C.L., Hamrick, J.L., Donovan, L.A., Mauricio R., 2004. Unexpectedly high clonal diversity of two salt marsh perennials across a severe environmental gradient. Ecology Letters 7, 1155-1162.

Rogers, S.D., Beech, J., Sarma, K.S., 1998. Shoot regeneration and plant acclimatization of the wetland monocot Cattail (*Typha latifolia*). Plant Cell Rep. 18, 71-75.

SAS Institute Inc. 2003. SAS version 9.1.3. SAS Institute, North Carolina.

Sea Grant Florida., 2003. *Commercial Suppliers of Sea Oats in Florida*. Sea Grant Extension Fact Sheet #150.

Seliskar, D.M., Gallagher, J.L., 2000. Exploiting wild population diversity and somaclonal variation in the salt marsh grass *Distchlis spicata* (Poaceae) for marsh creation and restoration. Amer. J. Bot. 87, 141-146.

Seneca, E.D. 1972. Germination and seedling response of Atlantic and Gulf coasts populations of *Uniola paniculata*. Amer. J. Bot. 59, 290-296.

Stopp, G.H., O'Neal, A.S., 1996. Initial effects of hurricane storm surge on barrier island vegetation. Technical Paper of the Sea Grant College Program, Florida, USA. 10pp.

Straub, P.F., Decker, D.M., Gallagher, J.L., 1988. Tissue culture and long term regeneration of *Phragmites austalis* (Cav.) Trin ex Steud. Plant Cell Tiss. Org. 15, 73-78.

Strnad, M., Hanus, J., Vanek, T., Kaminek, M., Ballantine, J., Fussell, B., Hanke, D., 1997. *Meta*-topolin, a highly active aromatic cytokinins from poplar leaves (*Populus* x *Canadensis* Moench. cv. *Robusta*). Phytochemistry 45, 213-218.

Subudhi, P.K., Parami, N.P., Harrison, S.A., Materne, M.D., Murphy, J.P., Nash D., 2005. An AFLP-based survey of genetic diversity among accessions of sea oats (*Uniola paniculata*, Poaceae) from the southeastern Atlantic and Gulf coast states of the United States. Theor. Appl. Genet. 111, 1632-1641.

Travis, S.E., Proffitt, C.E., Lowenfeld, R.C., Mitchell, T.W., 2002. A comparative assessment of genetic diversity among differently-aged populations of *Spartina alterniflora* on restored versus natural wetlands. Restoration Ecology 10, 37-42.

Valero-Aracama, C., M.E. Kane, S.B. Wilson, J.C. Vu, J. Anderson, and N.L. Philman. 2006. Photosynthetic and carbohydrate status of easy- and difficult-to-acclimatize sea oats (*Uniola paniculata* L.) genotypes during *in vitro* culture and *ex vitro* acclimatization. In Vitro Cell. Dev. Biol. 42, 572-583.

Valero-Aracama, C., Wilson, S.B., Kane, M.E., Philman, N.L., 2007. Influence of *in vitro* growth conditions on *in vitro* and *ex vitro* photosynthetic rates of easy- and difficult-to-acclimatize sea oats (*Uniola paniculata* L.) genotypes. In Vitro Cell. Dev. Biol. -Plant 43, 237-246.

Valero-Aracama, C., Kane, M.E., Wilson, S.B., Philman, N.L., 2008. Comparative growth, morphology, and anatomy of easy- and difficult-to-acclimatize sea oats (*Uniola paniculata*) genotypes during *in vitro* culture and *ex vitro* acclimatization. J. Amer. Soc. Hort. Sci. 133, 830-843.

Valero-Aracama, C., Kane, M.E., Wilson, S.B., Philman, N.L., 2010. Substitution of bezyladenine with *meta*-topolin during shoot multiplication increases acclimatization of difficult- and easy-to-acclimatize sea oats (*Uniola paniculata* L.) genotypes. Plant Growth Regul. 60, 43-49.

Wagner, R.H., 1964. The ecology of *Uniola paniculata* L. in the dune-strand habitat of North Carolina. Ecol. Monogr. 34, 79-96.

Werbrouck, S., Van der Jeugt, B., Dewitte, W., Prinsen, E., Van Onckelen H, Debergh P.C., 1995. The metabolism of benzyladenine in *Spathiphyllum floribundum* 'Schott Petite' in relation to acclimatization problems. Plant Cell Rep. 14, 662-665

Werbrouck, S., Strnad, M., Van Onckelen, H., Debergh, P., 1996. *Meta*-topolin, an alternative to benzyladenine in tissue culture? Physiol. Plantarum 98, 291-97.

Westra, R.N. and W.E. Loomis. 1966. Seed dormancy in *Uniola paniculata*. Amer. J. Bot. 53, 407-411.

Wilkinson, D.M., 2001. Is local provenance important in habitat creation? J. App. Ecol. 38, 1371-1373.

Williams, M.J. 2007. Native Plants for Coastal Restoration: What, When, and How for Florida. USDA, NRCS, Brooksville Plant Materials Center, Brooksville, FL. 51p.

Woodhouse, W.W., Seneca, E.D., Cooper, A.W., 1968. Use of sea oats for dune stabilization in the Southeast. Shore and Beach 35, 15-21.

Woodhouse Jr., W.W., 1982. Coastal sand dunes of the U.S. In: Lewis, R. (Ed.), Creation and Restoration of Coastal Plant Communities. CRC Press, Boca Raton, Florida, pp. 1-44.

Zurinsky, C.L., 1995. Micropropagation refinement and genotypic growth responses for *Pontederia cordata*. Masters Thesis, Univ. Florida, Gainesville, Florida, 74 pp.

BIOGRAPHICAL SKETCH

Although born in Tallahassee, FL, Jonathan Jasinski realized his mistake early in life and has spent the majority of his life in Gainesville, FL. After high school, Jonathan attended Santa Fe College and subsequently transferred to the University of Florida where he obtained a Bachelor of Science in plant science with an emphasis in plant pathology – biotechnology and a minor in horticultural sciences. He went on to work for the Walt Disney Company as an intern at the Biotechnology Laboratory in EPCOT where he developed a passion for plant science research. Jonathan went on to pursue graduate level education and joined the Plant Restoration, Conservation, and Propagation Biotechnology Program in the Environmental Horticulture Department at the University of Florida in August 2009. During his time as a master's student, Jonathan married his fiancée, Natalie and became a father, welcoming his new daughter Isabella in December 2009. Upon graduating in December 2011 with a Master of Science in horticultural science, Jonathan will pursue a doctoral degree with the Horticultural Sciences Department at the University of Florida. In his spare time, Jonathan enjoys Green Bay Packers football, the thought of shooting craps at the Palms in Las Vegas with good friends, and spending time with his loving wife and daughter.