

CRYOPRESERVATION OF SEA OATS (*Uniola paniculata* L.) SHOOT TIPS BY
VITRIFICATION AND ENCAPSULATION-DEHYDRATION:
A NOVEL APPROACH FOR PRESERVING FLORIDA'S COASTLINE

By

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To my family

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
BA	Benzyladenine
BFJ	Baby food jar
CA	Cold acclimation/acclimated
DMRT	Duncan's Multiple Range Test
DMSO	Dimethylsulfoxide
GA	Gibberellic acid
GDP	Gross Domestic Product
HR	Hour(s)
LN	Liquid nitrogen (-196 °C) exposure
MC	Bead moisture content
MIN	Minute(s)
MS	Full-strength Murashige and Skoog basal mineral salt mixture
PPF	Photosynthetic Photon Flux
PVS	Plant vitrification solution
PVS2	Plant Vitrification Solution 2
PVS3	Plant Vitrification Solution 3
SMM	Sea oats shoot multiplication medium

Abstract of Thesis Presented to the Graduate School
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Florida's coastal beach and dune systems provide unique wildlife habitat, tourism-based revenue, and a natural defense against the erosive forces of high winds and wave action generated by hurricanes and storms. Beach sand renourishment followed by planting native dune species is the most effective method for dune restoration. *Uniola paniculata* (sea oats) is the dominant sand-binding species in coastal dune systems in the southeastern United States. Nursery-grown sea oats plugs are the most reliable transplant source. This source is supplemented through the micropropagation of diverse genotypes. However, dwindling seed donor populations, short-term seed viability, limited availability of site-specific genotypes, and high micropropagation production costs limit the availability of plant materials.

The feasibility of using the cryopreservation protocols of vitrification and encapsulation-dehydration for efficient ultra-low temperature storage of excised sea oats shoot tips from *in vitro* cultures and germinated seedlings was examined. Shoot tips exhibited different cryostorage survival depending on population source and cryopreservation method.

Freezing tolerance of nine sea oats genotypes, representing three populations, were screened using a standardized cryopreservation protocol. Gamble Rogers (8.2%) and Delnor-Wiggins Pass (16.7%) genotypes displayed significantly lower regrowth than Perdido Key (47.8%). Cryopreservation step modification did not increase cryostorage survival of shoot tips from Delnor-Wiggins Pass, a cryostorage-sensitive population. The higher cryostorage tolerances observed in the more northern Perdido Key genotypes may reflect ecotypic differentiation and account for the inability to increase post-freezing regrowth in genotypes from the southern Delnor-Wiggins Pass population. Cold acclimation pretreatment or post-cryostorage dark culture did not influence survival following cryopreservation.

CHAPTER 1 LITERATURE REVIEW

Introduction and Rationale

Under the threat of destructive storms and sea level rise, Florida's coastal dune systems must be maintained to protect the economic and environmental values associated with the coastal environment. Demographically, over 75% of the Florida population is located in coastal counties with 82% of Florida's GDP derived from coastal communities (Wilson and Fischetti 2010; Kildow 2008). Hence, these important areas must be protected from the negative effects of devastating storms and hurricanes by minimizing beach sand erosion. However, storm surges from a rising sea level and intensifying storms will inevitably have a greater impact on the natural environment and human communities, particularly in coastal regions (Noss 2011). Currently, coastal dunes planted with native dune species function as the initial line of defense against the destructive impacts of hurricanes and storms that threaten coastal communities. In particular, the aerial shoots of coastal dune grasses intercept wind-borne sand particles resulting in sand accumulation. This accumulation promotes coastal dune building while an extensive root system maintains stability and prevents subsequent erosion (Wagner 1964).

The most commonly planted dune species in the southeastern United States is sea oats (*Uniola paniculata*; Poaceae) (Figure 1-1a). Ranging between Virginia and Mexico and surrounding the Caribbean islands, sea oats play a critical role in coastal ecosystems by forming and maintaining the coastal dune systems and serving as a habitat for the coastal ecosystem (Wagner 1964). Although beach habitats are subjected to harsh environmental extremes of wind, salt spray, sand, precipitation,

temperature, and soil moisture, sea oats thrive and function to stabilize the coastal geomorphology of the dunes from erosion (Wagner 1964; Lonard et al. 2011). Sea oats vegetatively propagate by axillary buds from rhizomes and sexually by seed production from wind pollination (Bachman and Whitwell 1995; Miller et al. 2003). However, the primary natural method of propagation is axillary bud production from rhizomes (Wagner 1964; Bachman and Whitwell 1995). Given the role that sea oats play in maintaining and building dunes, it is critical that methods be developed to maintain sea oats populations and provide ecologically-sound sources of plant material for replanting.

Sexual reproduction in sea oats can be limited. Approximately 70% of fertilized sea oats ovules abort for no apparent reason resulting in decreased seed production (Wagner 1964). A proposed rationale for such a high abortion rate was the incidence of fungal infection promoted during periods of high humidity and rainfall when florets were open for 24 hours (Bachman and Whitwell 1995; Burgess et al. 2002). For the seeds formed (<3 seeds per spikelet), germination can be inhibited by mechanical resistance of the testa and a physiological block within the embryo (Wagner 1964). Later, Westra and Loomis (1966) confirmed that sea oats embryos were not dormant by excising embryos and obtaining 80% germination in 12 days. Higher germination (90% - 100%) was obtained by cutting the testa and exposing the endosperm, further suggesting a diffusible inhibitor that is removed by disrupting the testa (Westra and Loomis 1966). Additionally, these forms of dormancy are overcome during cold layering in the winter (Wagner 1964). Finally, even if seeds are viable and dormancy is alleviated, threats of predation or extreme sand burial (i.e., $\geq 15\text{cm}$) are still present (Miller et al. 2003). Therefore, sea oats reestablishment by seed under field conditions presents major

challenges; thus, landscape managers must rely on vegetative propagation for sustaining sea oats populations.

Sea oats is a wind-pollinated outcrossing grass, suggesting high gene flow throughout its distribution (Hamrick et al. 1979). However, variations in germination requirements (i.e., cold stratification temperatures) between populations describe genetic differentiation, potentially due to local environmental conditions, between the two Florida coasts (Seneca 1972). Three major population groupings have been previously determined by Seneca (1972) studying sea oats' response to temperature-related physiology (i.e., germination): 1) Virginia and North Carolina population; 2) Atlantic Coast Florida population; and 3) Gulf Coast population. Differences in germination observed between sea oats populations may be a consequence of ecotypic differentiation. Distinct environmental conditions within a species' range, such as temperature, photoperiod, soil types, and biotic factors have been reported to create different ecotypes within the species' geographic range (Bischoff et al. 2006; Hufford and Mazer 2009). As described by Gregor (1946), an ecotype is a population of a species that has undergone genetic differentiation due to varying environmental conditions within that species' range. Because Florida is split between two major population groupings, it is vital to maintain current ecotypic differentiation observed in Seneca (1972) to preserve sea oats' resilience to natural and human impacts in the coastal ecosystem. Reed and Frankham (2003) found that genetic variation and diversity were positively correlated with a plant species' fitness and that population size was significantly correlated with heterozygosity. Therefore, greater genetic diversity

provides a larger capacity for sea oats to respond to the harsh fluctuating coastal environment (Huenneke 1991).

Genetic studies of sea oats have shown that clonal diversity and structure can vary greatly between different populations (Franks et al. 2004). Using amplified fragment length polymorphism (AFLP), Ranamukhaarachchi et al. (2000) found relatively lower genetic differentiation between populations on the same coast, especially the Atlantic Coast. Franks et al. (2004) used the same procedure and reported similarly high genetic differences between coasts and a positive relationship between genetic distance and geographic distance in sea oats. These similar discoveries suggest that the geographical distance between the Atlantic and Gulf Coasts on the Florida peninsula is a major agent for genetic divergence in sea oats. Using the AFLP technique, Subudhi et al. (2005) extended the genetic study of sea oats and reported the genetic diversity from eight different states. A coordinate analysis, using 703 AFLP markers, divided sea oats into a distinct geographic pattern (Subudhi et al. 2005). Populations from the same state were more genetically similar when compared to populations from different states. Therefore, this study appears to support the positive relationship between genetic and geographic distance. Florida sea oats populations presented the greatest degree of genetic diversity compared to the seven other states, however only Florida Gulf Coast populations were considered in the study. This high degree of genetic diversity within Florida populations could be attributed to the great number of hurricanes that impact the Florida panhandle. Genetic differentiation observed in the studied populations has been proposed to be an effect of the linear and fragmented distribution of sea oats.

Sea Oats Preservation in Light of Climate Change

Global sea levels are rising and the coastal area surrounding Florida has been identified as a region vulnerable to flooding (Nicholls and Cazenave 2010). More specifically, sea level rise is identified as the most immediate impact of climate change in low-lying regions like Florida (Noss 2011). Consequently, Florida's coastal areas will be threatened with an average sea level increase of 30 centimeters and a large-scale flood event every 10 years by 2100, resulting in coastal erosion (Saha et al. 2011; Wieting and Montanio 2010). To compound rising sea levels, continual human population growth and urban development in coastal areas will result in further landscape fragmentation of key coastal species, including sea oats (CELCP 2012). Historically, as sea levels have risen Florida coasts and coastal ecosystems have shifted inland. However, urban development has created barriers, such as sea walls, housing, and commerce, that prevent coastal ecosystems from shifting inward and are projected to be inundated over the next several decades (Nicholls and Cazenave 2010; Noss 2011).

To supplement the natural adaptive response of coastal species hindered by barriers to higher sea levels, humans should prevent local extinctions by enhancing ecosystem resilience, or the capacity of a system to recover from a disturbance before the system contains permanently redefined processes and structures (Noss 2011; DeAngelis 1980). One strategy proposed to enhance ecosystem resilience is to maintain or restore ecosystems by increasing biodiversity, landscape connectivity, and natural disturbance and hydrologic regimes (Noss 2011). Due to sea oats' linear and fragmented distribution, this species is susceptible to decreased resilience. As sea oats populations are destroyed by human and hurricane activity, biodiversity can be restored,

but only if seeds are available. Despite problems associated with sea oats seed production, landscape managers should maintain sea oats biodiversity and help connect populations to increase gene flow.

Noss (2011) offered another strategy for responding to disturbances in Florida's coastal ecosystems that called for the creation of germplasm libraries of species threatened by sea level rise. In response to this strategy, a sea oats collection, such as an *in vitro* germplasm library, should be created with genotypes that could be used to re-establish the biodiversity of a sea oats population if it declines or is destroyed. Careful efforts should be made to ensure genotypes are planted in the most suitable habitat where climate change has modified the local original habitat's abiotic and biotic factors (Hufford and Mazer 2003). Climate change-facilitated sea level rise compounds the threat of sea oats population decline by storms and urban development and should be considered a major driver for their preservation for coastal dune re-establishment.

Sea Oats Used for Restoration

Although established regulations help protect sea oats from damage by human activity, such as pedestrian traffic and illegal harvesting, the occurrence of natural catastrophic events will continue to adversely affect our coastline. As a consequence of the active 2004-2005 hurricane seasons, it was reported that over 50% (365 miles) of Florida beaches were "critically eroded" (FDEP 2006). Beach sand renourishment followed by the planting of native dune species, including sea oats, is required to effectively restore damaged dunes. Sand renourishment is accomplished by pumping offshore sand back onto the eroded beaches. Renourished beaches and dunes are stabilized by planting sea oats originating from local populations. Direct seeding of sea oats is not an effective means of revegetation due to dormancy mechanisms discussed

earlier, low viability, and sand movement affecting the planting depth (Bachman and Whitwell 1995). Additionally, direct seeding requires freshly harvested seeds (Westra and Loomis 1966; Hester and Mendelssohn 1987; Bachman and Whitwell 1995). Seed germination immediately after harvest was 50% following a presoak in 100 mgL^{-1} GA₄ for 36 hours, but decreased to about 20% after less than a month post-harvest (Westra and Loomis 1966). This loss of germinability suggests a decrease in viability or increased dormancy over time, requiring immediate use of seeds after harvest (Bachman and Whitwell 1995). Another ineffective method of dune restoration is the replanting of uprooted sea oats rhizome fragments. The probability of tiller emergence decreased drastically as exposure time of the uprooted rhizomes to the air increased (Miller et al. 2003). Fragmented and exposed rhizomes could only survive 1-3 days if not kept adequately moist (i.e., more than 68 mm of rain before or after exposure) (Miller et al. 2003). Therefore, it is not economically feasible to consider direct seeding, replanting fragmented sea oats rhizomes following a storm, or the relocation of established plants as effective methods for replanting.

Currently, commercial nurseries produce sea oats plugs from seedlings to avoid the inefficiencies of direct seeding and replanting (Bachman and Whitwell 1995; Miller et al. 2003) (Figure 1-1b). However, the low availability of nursery-grown plugs from site-specific populations limits the large-scale application for restoration projects. Nurseries are unable to maintain large quantities of genetically diverse and site-specific sea oats plants and must propagate them in response to market demand, such as following storm damage (Miller et al. 2003). Additionally, nursery attempts to rapidly propagate plants vegetatively to meet demand for many sea oats may result in reduced genetic

diversity of the plants produced (Bertrand-Garcia et al. 2012). Although nurseries are a source of plant material for revegetation, nurseries must obtain seeds from local populations. Sea oats populations on federal lands and state parks may only be re-established with plants that originated from that specific location to prevent planting of maladapted genotypes (Fla. Admin. Code 2013). Therefore, if local populations are severely damaged or destroyed, there may not be sufficient seed for collection, resulting in the inability to plant appropriate genotypes and restore the original biodiversity. Thus, alternative sources of sea oats that retain local genetic diversity are necessary. Certainly, augmenting natural and nursery seed propagation with local genotypes produced by *in vitro* propagation could offer an effective supplementary source to generate genetically diverse sea oats of local origin.

Sea Oats Micropropagation

Micropropagation is the rapid clonal production of plants on defined culture media under controlled conditions of light and temperature in a sterile environment (Kane 2011). Initially, a preliminary sea oats micropropagation protocol was established by Hovanesian and Torres (1986) and then modified by Philman and Kane (1994). Currently, there are several reported micropropagation protocols for sea oats (Valero-Aracama et al. 2008, 2010; Jasinski 2011). In these studies *in vitro* growth responses and *ex vitro* greenhouse survival of various genotypes were examined. Because sea oats are used for coastal dune community restoration, it is vital to maintain genetic diversity and not to reintroduce only a limited number of clones into a population. Consequently, creation of a “genotypic library” would be appropriate to achieve this goal (Noss 2011). This valuable resource can be maintained under optimal conditions and could be available for landscape managers regardless of season. This *in vitro* library

could provide a supplementary source of sea oats and reduce dependence upon specific donor sites for seed collection.

Sea oats micropropagation involves a series of sequential stages beginning with seed collection, continuing with clonal propagation of distinct genotypes, and culminating with the reintroduction of multiple genotypes to the original site (Figure 1-2). An efficient sea oats micropropagation protocol begins with the collection of seeds (Stage 0). At this time, seeds are pretreated with a 100 mgL^{-1} gibberellic acid (GA) solution for 45 hours (Bachman and Whitwell 1995). This GA concentration displayed the highest germination (56%) in sea oats compared to 500 mgL^{-1} GA (40%) and other common germination techniques such as scarification, distilled water soaking, or a combination of these treatments (8%-28%) (Bachman and Whitwell 1995). After GA soaking, the seeds are surface sterilized using 50% (v/v) ethanol and a 3% (v/v) sodium hypochlorite solution containing a surfactant to reduce contaminants such as bacteria and fungi on the testa. After rinsing with sterile water, seeds with 1 mm of the distal end removed are sown in a sterile culture vessel containing a mineral salts medium supplemented with sucrose and vitamins, and solidified with agar (Stage I). During Stage I, seedlings begin to physiologically stabilize to growing *in vitro* (Norton and Norton 1986). After repeated subculture, branched seedlings are then transferred to sterile culture vessels containing a sea oats shoot multiplication medium (SMM) consisting of a cytokinin, usually benzyladenine (Stage II). Cytokinins are a plant growth regulator class responsible for responses such as increased cellular division, disrupted apical dominance, and promoted organ regeneration (Choi and Hwang 2007; Ferreira and Kieber 2005; Hutchison and Kieber 2002). Thus in Stage II, lateral shoot

proliferation is promoted from sea oats basal meristems resulting in the formation of shoot clusters (Figure 1-1c). Stage II cultures are subcultured every 28 days by subdividing the shoot clusters and transferring the explants onto fresh medium. This process maintains actively growing cultures *in vitro* and increases the total number of plantlets. After a sufficient number of plantlets are generated, single-shoot microcuttings are rooted in a Stage III rooting medium supplemented with an auxin, usually α -naphthaleneacetic acid (NAA). Auxins are another plant growth regulator class that promotes rooting (De Klerk et al. 1997). Once the microcuttings have rooted, the sea oats are removed from the sterile environment and residual medium is rinsed off. The sea oats are then planted into plug trays containing vermiculite, and acclimatized in the greenhouse under increased light levels and decreased humidity for six weeks (Stage IV). Finally, acclimatized sea oats of multiple genotypes are reintroduced to a predetermined site for coastal dune stabilization.

Micropropagation Challenges

Maintenance of many actively growing shoot cultures of diverse sea oats genotypes *in vitro* from many Florida populations can be labor-intensive, require extensive space, and pose a continued risk of contamination and somaclonal variation. Actively growing sea oats cultures require constant subculture onto fresh medium every 28 days. This subculture must be done manually and therefore is very labor-intensive to maintain (Pence 2011). Additionally, environmentally controlled rooms to maintain optimal light and temperature levels have limited culture capacity and utility costs can be high. Furthermore, contamination by insects or microbial contaminants (i.e., bacteria and fungi) is always a risk especially during monthly subcultures. Between subcultures, insects such as mites can infiltrate vessels causing major contamination problems.

Finally, somaclonal variation is a potential threat to repeatedly subcultured germplasm libraries maintained *in vitro* (Volk 2010). Somaclonal variation occurs randomly when repeatedly subcultured shoots become genetically different from the original tissue (Evans 1989). All of these challenges pose risks to the application of micropropagation for the production of unlimited genotypes from many site-specific Florida populations. Short-term low temperature (2 °C - 10 °C) storage can be utilized to extend time between subcultures, but still requires continued subculture to prevent plant losses (Kartha 1985; Pence 2011). Therefore, a long-term conservation method, such as cryopreservation, the storage of tissue in liquid nitrogen (-196 °C), could be employed to help alleviate micropropagation challenges to ensure ecologically sound production of many site-specific sea oats genotypes reliably and efficiently.

Cryopreservation as a Conservation Tool

Although micropropagation can decrease the problems of plants grown under nursery conditions, such as space, environmental effects, pests, and diseases, these *in vitro* growing collections still pose challenges. *In vitro* collections are subjected to hazards such as diseases, pests, and equipment failure (Kaczmarczyk et al. 2011; Engelmann 2004). Additionally, long-term maintenance of actively growing cultures can be time-consuming, expensive, and space-limited (Pence 2011). Technically trained personnel and periodic transfers, even with low temperature storage, are required to maintain living collections (Van Den Houwe et al. 1998). To alleviate continued maintenance of living germplasm libraries, laboratories, such as the USDA-ARS National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon and the National Center for Genetic Resources Preservation in Ft. Collins, Colorado, are employing

cryopreservation for the long-term storage and conservation of plant germplasm of genera such as *Ribes*, *Pyrus*, and *Rubus* (Reed et al. 2005; Golmirzaie and Panta 1998; Pennycooke and Towill 2000).

Cryopreservation is the ultra-low storage of plant tissues within liquid nitrogen (LN; -196 °C). At this temperature, physiological processes are theoretically reduced for extended periods. Using cryostorage following micropropagation has been characterized as conservation biotechnology, and a method to help preserve living resources (Reed 2001). This method offers advantages to the *in vitro* storage challenges previously described (Turner et al. 2001; Hirai et al. 1998; Golmirzaie and Panta 1998). These advantages include a decrease in maintenance costs, storage space requirements, losses by disease and contamination, and somaclonal variation.

Ultra-low temperature storage of many genotypes helps offset biodiversity loss, especially for those plants such as *Uniola paniculata*, that provide vital ecological services for human communities like coastal protection and erosion control (Benson 2008; Barbier et al. 2011). Actively growing collections of plant cultures should be maintained concurrently with cryopreserved germplasm to ensure long-term conservation if one germplasm source should be lost, such as from a building fire or power outage (Reed 2001). These active collections can be placed into cryostorage, but must be routinely thawed and subcultured. Active cultures must be distinguished to protect long-term base collections from distribution and disturbances, such as accidentally removing from LN (Reed et al. 1998b; Benson 2008). Base collections are reserves of actively growing collections (Reed et al. 1998a) and should be comprised of

enough genetically diverse plant material of a single species to aid in biodiversity conservation (Touchell 1998).

Cryopreservation of conserved plants can readily provide material in response to changing needs (Kaczmarczyk et al. 2011). Therefore, genetically stable plant material can be conserved and serve as a source of micropropagation propagules to react more quickly to unanticipated environmental events, such as hurricanes and storms, or to biodiversity loss accompanying anthropogenic impacts.

An attractive advantage for the cryopreservation of plant materials over extended periods is the cost-effectiveness of the technology (Engelmann 2004; Reed et al. 2004; Pence 2011). However, this can only be accomplished when the optimal procedure is developed and utilized for particular species or genotypes. Plant survival following cryostorage can be compromised with suboptimal procedures (Reed 1996). Therefore, extensive preliminary research is required when establishing an optimized cryopreservation procedure for a species or genotype (Kaczmarczyk et al. 2011). The goal of this research must be to optimize the critical points in a cryopreservation procedure to attain the highest initial survival and regrowth following cryostorage (Benson 2008). Once an optimized protocol is developed, the *in vitro* cryogenic germplasm library can be developed. Little maintenance is necessary once the plant samples are in cryostorage. Collections only require occasional monitoring to ensure the LN level does not drop below the cryovials (Kaczmarczyk et al. 2011; Pence 2011).

A number of reports illustrate the potential cost savings when using cryopreservation. For example, fruit tree *in vitro* germplasm maintenance costs at the USDA-ARS NCGR lab decreased from approximately \$50-\$75 per cultivar per year, not

considering labor costs, to about \$1 per cultivar when cryopreserved (Reed et al. 2004). Additionally, it was projected that cryopreservation could decrease costs by 25% compared to field collections for clonal archives of *Ulmus* species in Europe (Harvengt et al. 2004). *Solanum* species storage space and labor costs have been reduced by more than 50% by using cryopreservation (Golmirzaie and Panta 1998). Finally, Pence (2011) estimated that the cost to maintain 200 genotypes for 20 years in tissue culture was \$102,000 compared to \$62,000 if the plant samples were cryopreserved, which represents a 39.2% savings. Therefore, it is apparent that cryopreservation can be a space-saving and cost-effective tool for laboratories to consider.

Although cryopreservation provides many advantages, such as decreased production space and costs, it should not replace *in situ* and active *in vitro* conservation. Cryopreservation is a tool to improve long-term conservation success, but should not be the sole solution for protecting a species or biodiversity (Engelmann 2004; Touchell 1998). Using cryopreservation requires constant LN refilling of cryotanks and electricity requirements, in some cases, which could put cryopreserved samples in jeopardy if not properly monitored and maintained.

Cryopreservation Fundamentals

Cryopreserved tissues are maintained at ultra-low temperatures (-196 °C) that arrest metabolic and biochemical processes and halt normal cell growth to achieve long-term storage (Moges et al. 2004). Theoretically described with the Arrhenius formula, $k=Ae^{-E/RT}$, ultra-low temperatures significantly decrease enzymatic activity and cell division but not to the point of death (Laidler 1984; Engelmann 2004). To describe this equation, k is the overall process rate, A is the pre-exponential frequency factor for

the particular reaction, E is the activation energy, R is the gas constant, and T is the temperature in Kelvin (Laidler 1984). Therefore, when the sample temperature decreases, the rate of metabolic and biochemical processes decreases exponentially until the rate is virtually zero. For example, germplasm exposure time to LN did not significantly affect *Solanum* species survival (46% after 1 day LN and 40% after 3 months LN) or *Pisum sativum* (68% after 1 week LN and 61% after 26 weeks) (Golmirzaie and Panta 1998; Kartha et al. 1979). With alginate-coated *Malus domestica*, cv. Fuji shoot tips, survival did not decrease between those recovered 3 days (73%) and 5 months (73%) after LN (Niino and Sakai 1992). These examples help provide evidence that critical biochemical processes are arrested during cryostorage without significant loss in survival over time. However, fresh *Lactuca sativa* seeds cryogenically stored in LN were anticipated to have a storage half-life of 3,400 years (Walters et al. 2004). Therefore, cryopreserved tissues are not indefinitely stored, but do have an extended storage period.

High survival following cryopreservation is achieved by rapidly transitioning intracellular water from a mobile phase to a glassy state in plant tissues thus minimizing ice crystallization (Volk and Walters 2006). Crystallization of cellular water is detrimental to cryopreservation protocol success because of cell wall, membrane, and tissue destruction (Pearce 2001). Therefore, the intracellular water concentration is decreased prior to cryopreservation through evaporative or osmotic dehydration and by pretreating the donor plant material (Reed 1996; Niino et al. 1992). The decrease in intracellular water and increase in the cellular solute concentration decreases the freezing temperature and increases the tissue's freeze-tolerance. Additionally, these solutes

prevent the mobility and connectivity of the remaining water molecules, thereby obstructing ice crystal formation and protecting membranes from lethal phase changes that occur during freezing (Benson 2008; Volk 2010).

Using cryopreservation protocols, the viability of seeds, pollen, dormant buds, and meristems can be maintained for extended periods (Engelmann 2004; Reed et al. 1998b). However, meristems are preferred when the conservation of genetic material is important (Turner et al. 2001), such as with specific sea oats genotypes. Shoot tips are suitable tissues for cryopreservation because they are composed of small, actively dividing cells with few small vacuoles and a high nucleo-cytoplasmic ratio (Engelmann 2004; Turner et al. 2001). These characteristics suggest that there is already a low intracellular water concentration in these cells than in other plant tissues leading to a higher chance of survival following cryostorage.

When using cryopreservation, three factors that influence optimal survival following storage are: 1) donor plant conditions, 2) cryogenic steps, and 3) the initial survival and regrowth processes following LN (Pennycooke and Towill 2000). Considering these three components during the development of a cryopreservation protocol can facilitate the desired outcomes of higher genetic stability and survival. Cryopreservation should be performed on tissues harvested from vigorously growing plants in order to achieve the highest tolerance to dehydration or cooling (Reed 1990, 1996). The conditions for efficient survival following cryogenic storage depends on the cryopreservation procedure used (i.e., vitrification, encapsulation-dehydration, or controlled-rate cooling). Ultimately, normal plant growth and development should resume after LN storage. These procedures must lead to true-to-type plants regrown

after cryostorage. A common minimum criterion to define a successful cryopreservation protocol is survival greater than 40% of the original cryopreserved samples (Reed et al. 1998b). This benchmark acts as a guide for laboratories to develop a protocol that would potentially have better large-scale application success.

Cryopreservation Pre-Dehydration Steps

As mentioned previously, only optimal procedures that result in higher survival (>40%) of genetically stable plants following cryostorage should be utilized. Therefore, protocol optimization experiments are required, and accomplishing this requires extensive time and plant material. Common variables examined include varying photoperiod, temperature, sugar levels, exposure durations, culture media, and cryoprotectants during cryopreservation steps (Figure 1-3). In addition to these variables, the type of cryopreservation method (e.g., vitrification, encapsulation-dehydration) chosen can be significant to the subsequent survival of the shoot tips.

Plant selection

Problems such as suboptimal culture conditions, endophytic bacteria, oxidative stress, and deterioration of genetic stability can lead to low survival rates following cryogenic storage (Benson 2008). Additionally, genotypic differences could lead to varying survival rates after cryopreservation (Matsumoto et al. 1994). Therefore, the growing and donor plant conditions can affect overall cryostorage success. Reed (1990) suggested that a major factor that affected cryopreserved *Pyrus* meristems survival was the *in vitro* vigor of the donor plants. She proposed that a lack of vigor due to decreased growth on suboptimal medium could contribute to lower survival rates following dehydration or freezing. For example, survival of *Anigozanthos viridis* ssp. *viridis* decreased from 28% to 5% when the medium was supplemented with 2.5 μ M BA

compared to 0.5 μM BA (Turner et al. 2000). A suboptimal culture medium was attributed to the poor survival between these two treatments. Consequently, conditions that should be considered when selecting the plant material include plant physiological state and growth (Kaczmarczyk et al. 2011).

Donor plant pretreatments

Tissues obtained from intact donor plants that have undergone various pretreatments prior to cryopreservation exhibit enhanced survival post-cryostorage. Pretreatments are often required to produce adequate post-cryostorage survival (Reed 1996; Turner et al. 2001). The four common types of donor plant pretreatments are cold acclimation, culture under short photoperiods, desiccation, and exogenous ABA application (Chang and Reed 2000). Overall, these treatments up-regulate cold-response genes that help prevent viability loss during the desiccation and freezing steps of cryopreservation (Thomashow 1999; Welling et al. 2002; Agarwal et al. 2006; Shinozaki and Yamaguchi-Shinozaki 2007; Guy 1990).

During cold acclimation (CA), *in vitro* plants or excised shoot tips are subjected to either exposure to a constant low temperature or an oscillating high/low temperature regime. Recent elucidation of the genetic pathways associated with cold acclimation and freezing tolerance suggest roles of key cold-regulated genes in the acquisition of cold tolerance in plant tissues (Volk 2010). This pretreatment causes changes in plant gene expression by up-regulating cold-defense genes and down-regulating primary metabolism (Benson 2008; Reed et al. 2005; Guy et al. 1985). Therefore, plants grow slowly during this pretreatment and exhibit changes in protein expression involved in membrane fluidity, protein conformation, rigidity, and dehydration response (Volk 2010; Kaczmarczyk et al. 2011). During cold acclimation, it was reported that the cytosolic

calcium ion concentration sharply increased within minutes after cold shock (Sung et al. 2003). This increase appeared to be linked to a plant's capacity to tolerate temperature stresses, such as cold acclimation. An increase in cytosolic calcium ions was proposed to transduce protein kinase cascades in response to temperature extremes (Sung et al. 2003). A common plant cellular response to cold temperatures is an increase in solute concentration, which serves as a natural protection against possible ice crystallization. Additionally, low temperature responses facilitate protein and membrane stabilization by increasing the levels of unsaturated fatty acids, altering lipid and protein composition, and increasing the phospholipid concentration to stabilize membranes and prevent lethal phase changes (Kaczmarczyk et al. 2011; Sung et al. 2003; Volk 2010).

Cold acclimation has been found to be beneficial to most plant species tested for high cryopreservation survival. Niino et al. (1992) reported that *Malus domestica* cv. Fuji shoot meristems exhibited the highest regeneration (80%) when plants were cold acclimated for 3 weeks at 5 °C as compared to those that were not (10%). A similar trend was found with four different *Pyrus* varieties where meristems that underwent CA had higher average regrowth (32%) than those without CA (5%) (Reed 1990). A commonly employed CA oscillating cycle is an 8-hr photoperiod at 22 °C and a 0 °C night (Reed et al. 1998a). This CA cycle was studied with eight *Pyrus* varieties. Cold acclimated *Pyrus* shoot tips had higher regrowth (50-80%) compared to those without cold acclimation (0-45%) (Reed et al. 1998a). A similar CA cycle with an 8-hr photoperiod at 23 °C and a 5 °C night was studied with *Beta vulgaris* shoot tips. Cryopreserved shoot tips increased from 37% survival to 70% survival with one week of this oscillating temperature regime compared to no CA (Vandenbussche and De Proft

1998). After only one week in the oscillating CA cycle, four *Pyrus* genotypes showed significant increases in regrowth compared to those shoot tips not CA, increasing from 0-18% to 62-79% (Chang and Reed 2000). Reed et al. (2006) noted that non-CA *Cynodon* shoot tips had significantly lower regrowth (<21%) compared to those receiving at least one week CA (>65%). In *Malus x domestica* Borkh., only CA shoot tips survived LN (80%) (Paul et al. 2000). These studies suggest that CA is an important component of cryopreservation pretreatments for high survival post-cryostorage.

To determine if a high and low temperature fluctuation was important, Chang and Reed (2000) compared two pear varieties under: constant low temperature (4 °C) in the dark; constant low temperature (4 °C) under an 8-hr photoperiod cycle; constant warm temperature (25 °C) under an 8-hr photoperiod cycle; and fluctuating 22 °C with 8-hr photoperiod and -1 °C dark cycle. The authors subjected the *Pyrus* varieties to 3 or 5 weeks cold acclimation and reported that the temperature fluctuation (22 °C 8-hr light and -1 °C dark) during CA had the highest post-cryostorage growth (80-100%) compared to a constant low temperature (<40%). Regrowth of cryopreserved *Mentha* shoot tips that had undergone a constant temperature (25 °C) or two alternating-temperature regimes (25/15 °C and 25/-1 °C) displayed higher survival under a 25/-1 °C regime (91%) than the others (28% - 38%) (Senula et al. 2007). A study of alternating-temperature CA with *Dioscorea* species found that three weeks of incubation was necessary to achieve the highest shoot recovery (48%) compared to a constant temperature of 26 °C (30%) (Leunufna and Keller 2005). *Solanum* shoot tips pretreated with an alternating temperature regime (22/8 °C) had consistently higher regrowth, with an average of 91.6%, than a constant temperature (22 °C), with an overall average of

88.7% (Kaczmarczyk et al. 2008). The authors proposed that alternating temperatures may be beneficial to subtropical and tropical plant species because excessive cold temperature exposure may be detrimental to donor plant growth and vigor.

In addition to CA treatment duration, Pennycooke and Towill (2000) reported highest shoot tip survival when excised immediately following the dark period of cold acclimation (65%) compared to shoot tips excised during the light period (<40%), but could not provide an explanation for this. Since sugars are converted to starch in leaves at night to increase the cellular solute concentration, it was proposed by Pennycooke and Towill (2000) a similar response was achieved but could not determine if this was the case for the meristematic tissue they used. Overall, CA effectiveness is usually dependent upon optimizing the duration of the treatment (Benson 2008).

Sea oats *in situ* from North Carolina displayed seasonal fluctuations in cellular soluble sugar concentration with increases in the fall and winter (Harper and Seneca 1974). The total soluble sugars, comprised of starch, carbohydrates, and other sugars, composed a higher dry weight percentage in rhizomes (33% soluble sugars; 53% carbohydrates) and leaves (13% soluble sugars; 17% carbohydrates) in the fall and winter months than the dry weight percentage in rhizomes (10% soluble sugars; 11% carbohydrates) and leaves (1% soluble sugars; 1% carbohydrates) during the summer months (Harper and Seneca 1974). Therefore, sea oats may undergo a natural CA in response to seasonal temperature and day length decreases, which increases cellular soluble sugar concentrations potentially to protect plants against possible freezing temperatures. Consequently, it would be assumed that sea oats would respond similarly *in vitro*, leading to high solute concentrations following *in vitro* cold acclimation.

Cold acclimation is not always necessary or effective with certain species (Sakai et al. 2008). Osmotic hardening, using a medium with a high sugar concentration, can result in the same physiological condition following CA (Uragami et al. 1990). This pretreatment dehydrates plant cells by producing a lower water potential in the medium. Culture of the Australian endangered species, *Anigozanthos viridis*, on a 0.4 M sorbitol supplemented medium with no CA treatment increased survival from 0% with no pretreatment to over 40% after only one day of pretreatment (Turner et al. 2001). However, a seven day pretreatment resulted in a significant decrease in survival post-LN (<20%). *Chrysanthemum cinerariaefolium* shoot tips cultured on 0.35 M, 0.45 M, 0.55 M, or 0.65 M sucrose media for 1, 3, 5, 7, and 10 days displayed increased survival as sucrose concentration increased (Hitmi et al. 2000). However, meristems cultured on medium supplemented with 0.65 M sucrose exhibited lower survival than those cultured on a 0.45 M sucrose medium. The highest survival (49%) was observed in the presence of 0.55 M sucrose for 3 days (Hitmi et al. 2000). Adding 5%, 7.5%, or 10% DMSO and 2 μ M, 4 μ M, 6 μ M, or 8 μ M ABA to the preculture medium increased the survival of *C. cinerariaefolium* shoot tips. Using the optimal 0.55 M sucrose concentration found previously, shoot tips exhibited an even higher initial survival with 7.5% DMSO (62%) and 4 μ M ABA (75%) (Hitmi et al. 2000). These results suggest that a combination of pretreatments can be more effective than a single method.

A newer technique, which decreases cellular water content, is the extension of time between culture transfers onto fresh medium to increase tissue desiccation. Extended culture time between transfers improved the regrowth for eight *Pyrus* species genotypes (Chang and Reed 2000). Water content in *Pyrus cordata* leaves and stems

significantly decreased as weeks between subculture increased, decreasing from 79% to 69% for leaves and 83% to 73% for stems after 4 weeks and 12 weeks culture, respectively. Higher regrowth and shoot tip freezing tolerance was obtained sooner with a 12-week subculture interval compared to a 3-week subculture interval for two CA *Pyrus* species (Chang and Reed 2000). For example, *Pyrus pashia* stock cultures maintained on a 12-week culture cycle exhibited 100% regrowth following LN storage with only one CA week, but required 15 weeks CA to achieve 100% regrowth with a 3-week culture interval (Chang and Reed 2000).

Cryopreservation Dehydration Methods

As previously mentioned, there are three major cryopreservation methods: controlled-rate cooling, vitrification, and encapsulation-dehydration. Additional methods have been developed by combining these three methods, such as encapsulation-vitrification and droplet vitrification (Benson 2008). Although these methods vary in their procedures, the ultimate goal is to remove intracellular water via dehydration. Dehydration can be through osmotic or evaporative conditions with the purpose to prevent ice crystallization and loss due to dehydration during cryopreservation procedures.

Controlled-rate cooling

The oldest, but currently the least commonly used cryopreservation procedure, is controlled-rate cooling (Benson 1999, 2008). Plant samples are slowly cooled to a specific intermediate temperature and then plunged into LN for freezing (Reed and Uchendu 2008). To control this cooling rate, specialized equipment, such as large programmable cooling chambers, slowly cool the plant samples to a temperature below the ice nucleation phase and above the glass transition phase (Kantha et al. 1979).

Tissues are then transferred to LN. Although it requires expensive specialized equipment, Reed et al. (1998a) noted that controlled-rate cooling was the best technique because it worked for most genotypes, was easy to use with a large sample number, and the cryoprotectants used were less toxic than with vitrification. However, the same author found that protocol success between labs was influenced by the type of cryopreservation equipment used (Reed et al. 2001). Facilities without expensive and specific equipment to slowly cool plant samples electronically had lower survival (0-10%) than those with specialized equipment as found at the USDA-ARS NCGR laboratory (30-40%).

A common way to accomplish controlled-rate cooling without large programmable freezers is with a Nalgene “Mr. Frosty” ® (Figure 1-4) (Benson 2008). This slow-freezing unit exploits the cooling properties of isopropanol, which cools at a rate of $1.0\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ in a $-80\text{ }^{\circ}\text{C}$ freezer. Laboratories without expensive equipment to modify the cooling rate can use these units to accomplish controlled-rate cooling, but are not able to adjust the cooling rate.

Cooling rate significantly affected the subsequent regrowth of *Pisum sativum* shoot tips. Maximum regrowth (73%) occurred using a $0.6\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ cooling rate, but decreased to 35% following a $1.0\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ cooling rate (Kartha et al. 1979). Likewise, all four *Pyrus* genotypes tested had higher regrowth with $0.1\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ (55% - 95%) cooling rate compared to $0.3\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$, $0.5\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$, and $0.8\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ (5% - 82%) (Reed 1990). Chen et al. (1985) screened various cooling rates ($0.3\text{-}5.0\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$) and then direct immersion into LN for *Triticum aestivum* L. cv. Norstar suspension cultures and found that $0.5\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ displayed 40% survival, whereas faster cooling rates sharply

decreased survival. For this procedure, cryoprotectants, the cooling rate, and the final temperature before LN immersion must be optimized.

Vitrification

Vitrification is a common cryopreservation procedure that requires plant tissues to be osmotically dehydrated prior to LN. The term vitrification refers to the process where liquid water directly transitions into an amorphous or glass-like state upon freezing, while avoiding ice crystallization (Engelmann 2004). With this procedure, intracellular water is replaced in plant tissues, usually shoot tips, by exposure to solutions with varying concentrations of sugars and cryoprotectants (Golmirzaie and Panta 1998). Following dehydration, the shoot tips are directly immersed into LN to freeze rapidly at $-200\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ (Pennycooke and Towill 2000; Matsumoto et al. 1994). Due to the high viscosity of the vitrified cytoplasm at this ultra-low temperature, cellular processes are arrested (Reed 2001). Vitrification-based procedures usually allow for freezing larger tissue samples (0.5-3 mm) because of the intracellular water replacement with solutes (Engelmann 2004). Using larger tissues helps eliminate the unnecessary time needed for acquiring small fragile meristems. Vitrification also eliminates the need for any specialized equipment, such as programmable freezers for controlled-rate cooling, because it is based solely on the osmotic dehydration of the tissue using various solutions at various exposure durations (Benson et al. 2006; Matsumoto et al. 1994). When compared to controlled-rate cooling, the time needed to complete the process decreased from 2-3 hours to 30-40 min using vitrification and the survival of *Asparagus officinalis* embryogenic cells increased from 81.9% to 86% (Nishizawa et al. 1993). Therefore, vitrification can be a very cost-effective and efficient procedure.

The sequential dehydration steps in the vitrification method are critical for successful sample survival post-cryostorage and must be optimized to decrease dehydration stress (Engelmann 2004; Nishizawa et al. 1993). The cryoprotectants used to dehydrate and replace the intracellular water before LN exposure must be able to penetrate the cells and be non-toxic at the required concentration (Sakai et al. 2008). Organic compounds commonly used to pretreat plant cell tissues for vitrification include glycerol, dimethylsulfoxide (DMSO), and small molar mass glycols, such as ethylene glycol. Volk and Walters (2006) reported that shoot tips of the garlic cultivar 'German Extra Hardy' and *Mentha x piperita* L. cultivar 'Todd's Mitcham Peppermint' lost the same water mass as shoot tips gained in cryoprotectant (0.5 mgL^{-1} for garlic and 0.25 mgL^{-1} *Mentha*). This study helped to confirm theories that these cryoprotectants are dehydrating cells by replacing the intracellular water. In addition to the effect that increased solute concentration has on decreasing the cellular freezing point, these cryoprotectants help protect and stabilize membranes and proteins from damage due to dehydration and ice formation (Reed 1996; Volk 2010).

The most common dehydrating cryoprotectant used for vitrification is Plant Vitrification Solution 2, or PVS2 (Niino et al. 1992). This solution is a mixture of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO in a full-strength Murashige and Skoog basal mineral salt mixture (MS) supplemented with 0.4 M sucrose (Turner et al. 2001). This cryoprotectant solution alters the freezing behavior of the remaining water in the cell when plunged into LN by allowing the tissue to supercool to $-100 \text{ }^{\circ}\text{C}$ before solidifying to a glassy state at $-115 \text{ }^{\circ}\text{C}$ (Benson 2008). This small gap

between the temperature of the tissue and the freezing temperature does not allow for ice crystallization, which produces the vitrified glass state.

The cryoprotectant's dehydration duration should be optimized based on the species or genotype because of the negative effect on survival when samples are kept in a plant vitrification solution (PVS) for too long (Pennycooke and Towill 2000; Moges et al. 2004). *Ipomoea batatas* meristems exposed to PVS2 for 16 min resulted in a post-cryostorage survival of 66%, whereas meristems exposed for 26 min only displayed 20% survival (Pennycooke and Towill 2000). Exposure to PVS2 at 0 °C did not decrease regrowth for *Wasabia japonica* apical meristems (>90%) up to 60 min, but when exposed to PVS2 at 25 °C were more sensitive with regrowth decreasing to <50% after only 30 min (Matsumoto et al. 1994). Furthermore, *Trifolium repens* apical meristems exposed to PVS2 at 0 °C had higher survival (83%) than meristems exposed to PVS2 at 25 °C (75%) but required 10 min more of exposure time (Yamada et al. 1991). Therefore, PVS2 toxicity to meristems decreased when the vitrification solution was cooled to 0 °C. Volk and Walters (2006) and McGann (1978) suggested the decrease in PVS2 toxicity with lower temperatures was due to the PVS2 membrane penetration reaction slowing down; therefore, the longer exposure time was required.

For increased survival, other plant vitrification solutions, such as PVS3, have been developed. Nishizawa et al. (1993) reported that using PVS3 (50% (w/v) glycerol and 50% (w/v) sucrose solution) was the most suitable vitrification solution for *Asparagus officinalis* embryogenic cells (86.1% survival) compared to solutions with various glycerol and sucrose concentrations (e.g., 50% glycerol/40% sucrose – 75% survival; 45% glycerol/60% sucrose – 77.2% survival). However, using PVS3 and other

non-DMSO containing cryoprotectants displayed significantly lower survival (<20%) for the endangered *Anigozanthos viridis* ssp. *terraspectans* compared to those that contained DMSO (>60%) (Turner et al. 2001). Nevertheless, direct exposure of the meristems to a plant vitrification solution leads to osmotic stress and chemical toxicity (Matsumoto et al. 1994). Therefore, the effectiveness of various vitrification solutions must be evaluated to increase post-cryogenic survival.

One method to protect meristems from osmotic stress and increase survival post-cryostorage is to use a loading solution, or osmoprotectant (Turner et al. 2001). The loading phase has been shown to be effective in increasing dehydration tolerance when followed by exposure to dehydrating plant vitrification solutions, but its mode of action is not well understood (Langis and Steponkus 1990; Turner et al. 2001). Osmoprotectants usually contain sugars, glycerol-based compounds, and occasionally DMSO, to begin the slow replacement of intracellular water from the plant tissue, instead of a rapid replacement with phytotoxic dehydrating solutions. Shoot tip survival increased with this incremental dehydration instead of direct PVS2 exposure (Moges et al. 2004). In *Saintpaulia ionantha*, a 2 M glycerol/0.4 M sucrose osmoprotectant was used to determine if exposure time affected regrowth after cryopreservation (Moges et al. 2004). Highest regrowth (55%) was observed when *S. ionantha* meristems were exposed to osmoprotectant for 20 min before dehydration with PVS2. Additionally, maximum survival was obtained in *Citrus sinensis* var. *brasiliensis* Tanaka callus and *Wasabia japonica* meristems following a 20-min exposure to an osmoprotectant consisting of 2 M glycerol and 0.4 M sucrose (Matsumoto et al. 1994). *Wasabia japonica* apical meristems pretreated with a 2.0 M glycerol and 0.4 M sucrose loading solution exhibited

87.1% shoot formation following cryostorage compared to using no loading solution (12.5%) or to using other DMSO-containing solutions, such as 1.5 M glycerol with 0.4 M sucrose and 5% DMSO (54.8%) and 20% PVS2 (65%) (Matsumoto et al. 1994). Additionally, *Asparagus officinalis* embryogenic cells treated with a 2.0 M glycerol and 0.4 M sucrose loading solution displayed a higher shoot formation (85.7%) compared to no loading solution (37.1%) (Nishizawa et al. 1993). Furthermore, loading solutions containing DMSO (62.6% maximum survival – 2 M DMSO/0.4 M sucrose) or ethylene glycol (53.4% maximum survival – 1.7 M ethylene glycol/0.4 M sucrose) displayed lower shoot formation than the glycerol and sucrose solution. Loading solution composition and PVS2 exposure time should be optimized to ensure that sufficient time is given for the solution to penetrate into the cells (Moges et al. 2004).

Encapsulation-dehydration

Another common cryopreservation protocol, encapsulation-dehydration, involves encapsulating plant tissues prior to evaporative dehydration and LN. Shoot tips containing actively growing meristems are encapsulated in a small gelatinous bead, similar to synthetic seed (Ara et al. 2000). This bead is formed by placing growing tips in a 3% sodium-alginate solution and dropping these alginate-coated shoot tips into a 100 mM CaCl₂ solution (Shatnawi et al. 2004). Ionic bonding leads to a solid spherical matrix of calcium alginate. The bead can contain sugar or any other organic compound that enhances shoot tip survival following cryostorage. Once the tissue is encapsulated, the bead and the tissue are osmotically desiccated in a high sucrose pretreatment solution for a certain period before being dehydrated in a laminar flow hood or over silica gel via evaporation (Shatnawi et al. 2004). Dehydration of encapsulated shoot tips reduces cellular water without permanently wilting the tissue (Reed et al. 2006). In

addition to evaporation, sucrose in the alginate bead dehydrates the tissue and increases intracellular solute concentration, similar to vitrification (Shatnawi and Johnson 2004). *Malus domestica*, cv Fuji shoot tips encapsulated in alginate beads supplemented with 0.5 M sucrose displayed significantly higher regrowth with and without a sucrose solution pretreatment (73% and 47%, respectively) than alginate beads without the 0.5 M sucrose (64% and 7%) (Niino and Sakai 1992). The high solute concentration achieved during sucrose pretreatment and evaporation prevents the remaining water molecules from crystallizing when immersed into LN and increases post-cryostorage survival by stabilizing the cellular membranes, proteins, and cytoskeleton (Reed 1996; Volk 2010).

Encapsulated tissues are pretreated in a sucrose solution prior to evaporative dehydration to increase solute concentration thus preventing ice crystal formation and protecting membrane stability during desiccation, freezing, and thawing (Moges et al. 2004; Volk 2010; Reed 1996). Encapsulated *Malus domestica* cv. Fuji shoot tips with a 1.0 M sucrose pretreatment displayed significantly higher regrowth (73%) compared to beads without a sucrose pretreatment (47%) (Niino and Sakai 1992). *Saintpaulia ionantha* shoot tips pretreated with sucrose concentrations from 0.1 M to 0.3 M increased regrowth from 60% following 2 hours dehydration to 75% with 4 hours dehydration in a laminar flow hood (Moges et al. 2004). However, regrowth decreased to 45% with pretreatment in 0.5 M and 0.75 M sucrose with 4 hours dehydration. Encapsulated *Humulus lupulus* shoot tips had consistently lower daily regrowth when pretreated in 1.0 M sucrose than 0.75 M sucrose following 1-7 days pretreatment (Martinez et al. 1999). The maximum regrowth of shoot tips pretreated with 1.0 M

sucrose (43%) was following 4 days exposure and was only higher than the lowest regrowth of shoot tips pretreated with 0.75 M sucrose (32%) with only 1 day exposure. The highest regrowth (80%) was attained following 0.75 M sucrose pretreatment for 3 days and dehydration in a laminar flow hood for 4 hours. These examples suggest that the dehydration controlled by the sucrose concentration can affect initial survival and regrowth post-LN.

A 20% final bead moisture content (MC) was found to be most effective in attaining the highest survival following laminar air flow desiccation (Niino et al. 1992; Martinez et al. 1999; Wang et al. 2005; Scottez et al. 1992). Therefore, fresh weight and dry weight should be recorded and the following equation should be utilized to determine the optimal MC for cryopreservation (Reed et al. 2006).

$$\text{Moisture Content (MC)} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100\%$$

Successful cryostorage protocols for *Ceratopetalum gummiferum* and *Cynodon* species have been developed using encapsulation-dehydration. *Ceratopetalum gummiferum* shoot tips exhibited greatest regrowth (61.4%) following desiccation to 24.3% MC prior to LN compared to 0-3 hours dehydration (84% to 50% MC) with 0% regrowth (Shatnawi and Johnson 2004). *Cynodon* species shoot tips displayed greatest regrowth (91%) following desiccation to 19-23% MC compared to 0% regrowth with no dehydration (74% MC) (Reed et al. 2006). These examples, plus many others, such as *Malus domestica* cv. Fuji (80%), *Malus x domestica* Borkh. (85%), *Humulus lupulus* (80%), *Rubus idaeus* (65%), *Pyrus* species (80%), *Saccharum* species (60%), and *Saintpaulia ionantha* (75%) follow the same pattern of maximum survival following dehydration to 20% MC (Niino and Sakai 1992; Paul et al. 2000; Martinez et al. 1999;

Wang et al. 2005; Scottez et al. 1992; Barraco et al. 2011a; Moges et al. 2004).

However, caution should be taken when determining the dehydration period. *Syzygium francissi* survival decreased after 6 hours dehydration in a laminar flow hood. In this case survival was reduced from almost 60% regrowth to 0% after 9 hours dehydration even though the beads had 20% MC (Shatnawi et al. 2004). A similar trend was observed with *Ceratopetalum gummiferum* where regrowth decreased from 61.6% at 24.3% MC to 8.3% at 17% MC (Shatnawi and Johnson 2004). Therefore, longer dehydration times, which could induce fatal dehydration stress, do not necessarily lead to higher regrowth following cryostorage.

Instead of using filtered airflow for desiccation, silica gel can also be used to dehydrate encapsulated beads. The use of silica gel instead of a laminar flow hood is advantageous for high humidity climates or laboratories without sterile air flow (Sherlock et al. 2005). Although *Saintpaulia ionantha* shoot tips dehydrated for 4 hours with filtered airflow displayed 75% regrowth, shoot tips dehydrated for only 1 hour over 17 g of silica gel exhibited 80% regrowth, but shoot tips dehydrated over silica gel for 3 hours had no regrowth (Moges et al. 2004). The researchers proposed that excessive dehydration occurred after 3 hours with silica gel.

Encapsulation-dehydration has been preferred for shoot tip cryopreservation due to its capacity to provide nutrients directly to post-cryostored samples (Benson 2008). Furthermore, this method, unlike vitrification and controlled-rate cooling, eliminates the need for specialized freezers and phytotoxic cryoprotectants and allows more shoot tips to be processed at one time (Martinez et al. 1999; Wang et al. 2005). However, this procedure is time-consuming and may not be cost-effective if large sample numbers are

required. Additionally, air drying requires the species to be desiccation tolerant for successful cryopreservation survival (Niino et al. 1992). For this procedure, evaporation duration, alginate composition, and sucrose solution concentration and duration must be optimized.

Post-Cryostorage Recovery and Growth

Every cryopreservation procedure requires the recovery and subsequent growth of the cryogenically stored plant tissues. Therefore, cryopreservation protocol optimization research should not end at cryopreservation, but continue on to what is ultimately recovered from cryostorage (Benson 2008). In this study, initial survival was recorded as the greening and elongation of the shoot tips on the initial survival medium 4 weeks following cryostorage (Figure 2-4). All recovered plant material, including those perceived as dead, were placed on SMM. Regrowth was recorded as the continued elongation of green recovered shoot tips following 4 weeks on SMM (8 weeks post-cryostorage). After each 4-week interval, percent survival was measured. Additionally, regrowth is a measure of how many shoot tips have regrown into true-to-type plants. This step indicates if the plants will likely recover from freezing to become anatomically, morphologically, and physiologically the same as non-cryopreserved plants.

Once immersed into LN, the vitrified state of water in the plant tissue is metastable and becomes unstable during rewarming (Volk and Walters 2006; Niino et al. 1992). Care should be taken to avoid devitrification while rewarming vitrified shoot tips. Devitrification could lead to cracking and ice recrystallization if water molecules are able to connect if the entire tissue sample is not rewarmed at the same rate. As demonstrated with *Asparagus officinalis*, the quicker the warming rate the higher the survival, increasing from 46.9% with slow warming at room temperature (25 °C) to

81.1% with rapid warming in a 40 °C water bath (Nishizawa et al. 1993). Clover meristems held in a -70 °C bath for 15 seconds exhibited higher survival than those kept at 60 seconds before being warmed to 25 °C (Niino et al. 1992). This suggests that intracellular crystallization that causes cell death may occur more frequently during slow warming. Therefore, rewarming of tissues is usually done in a water bath at least 40 °C with a 250 °C·min⁻¹ warming rate (Matsumoto et al. 1994). This warming rate promotes thawing without devitrification. Once thawed, any solution in the cryovial is removed and a rehydrating sucrose solution is added (Matsumoto et al. 1994). In encapsulation-dehydration, a solution is not utilized for freezing; therefore, thawed beads are soaked in a sterile liquid MS solution for rehydration. Initial survival and regrowth are often considered successful when the shoot tips do not appear to be damaged (e.g., brown or completely white) and are elongating, or developing, into normally functional plants (Golmirzaie and Panta 1998).

Initial survival and regrowth are rarely the same with regrowth usually smaller than initial survival (Wang et al. 2005). This has been attributed to osmotic shock from rehydration or partial damage from ice crystallization during rewarming (Moges et al. 2004). Therefore, rapid warming and optimal rehydrating solutions can prevent initial survival and regrowth from varying (Nishizawa et al. 1993). Another factor that could affect differences seen between initial survival and regrowth is damage by photo-oxidation during initial survival (Benson and Noronha-Dutra 1988). Following desiccation or freezing stresses, reactive oxygen species, such as superoxide radical, hydrogen peroxide, hydroxyl radical, and singlet oxygen, can be found in damaged cells (Johnston et al. 2007). When present, reactive oxygen species could lead to lipid

peroxidation, protein denaturation, alterations in nucleic acids, membrane disruption, and severe cellular disorder (Uchendu et al. 2010). *Brassica napus* shoot tips showed increased singlet oxygen activity during early recovery and exposure to light (Benson 1990). Sugarcane shoot tips were maintained in the dark for 7 days and then transferred to standard culture conditions to minimize damage from reactive oxygen species (Barraco et al. 2011b). Additionally, recovered sweet potato shoot tips were maintained in darkness for 2 days and then gradually brought back into the light over an additional 3 days (Pennycooke and Towill 2000).

Callus formation and genetic instability are factors that must be considered and suppressed when recovering plant material from cryostorage. For effective survival, post-cryostorage protocols should promote rapid and direct shoot formation from shoot tips to ensure that somaclonal variation does not occur (Touchell 1998; Paul et al. 2000; Hirai et al. 1998; Uragami et al. 1990). Callus formation is usually observed with a protocol not optimized for shoot culture for that species (Engelmann 2004). In that case, further medium optimization to achieve better post-cryostorage results is necessary.

Verification of the trueness-to-type of the cryopreserved plant material to non-cryopreserved plants should be accomplished (Nishizawa et al. 1993; Engelmann 2004; Van Den Houwe et al. 1998). *Asparagus officinalis* L. embryogenic cells were verified to maintain the same embryogenic potential by comparing untreated, unfrozen controls (63.2%) to vitrified, frozen cells (61.3%) (Nishizawa et al. 1993). Upon analysis, the regeneration efficiencies between cryopreserved and non-cryopreserved samples were not significantly different. The initial survival and regrowth stages usually requires the

optimization of the rehydrating solution, initial survival medium, and reducing harm from reactive oxygen species.

Genotypic Responses to Cryostorage

Cryopreservation protocol optimization is usually conducted using a single genotype to eliminate the inherent variability between genotypes (Padro et al. 2012; Tanaka et al. 2011; Nishizawa et al. 1993; Sakai et al. 1990). However, it is understood that genotypic differences must be considered before the large-scale application of a cryopreservation protocol with an entire species, especially when ecological restoration is an objective (Benson 2008; Golmirzaie and Panta 1998). Cryopreservation protocols are not universal because of inherent genotypic variability that exists in a genebank that preserves biodiversity. A specific genotype's survival could be higher or lower than expected from a previously optimized protocol due to its capacity to survive severe desiccation and freezing stress (Benson 2008). Therefore, some plant genotypes may be able to survive better than others using the same cryopreservation procedure.

Studies involving direct comparisons of the cryostorage tolerance of excised shoot meristems of *different genotypes of the same species* are limited. In horticulturally important crops, differences in cryostorage survival between cultivars and even species within a genus make relevant comparisons difficult. Various *Wasabia japonica* cultivars varied in their shoot formation response (78.5% to 92.2%) when a previously optimized cryopreservation protocol for one cultivar was applied (Matsumoto et al. 1994). When five cryostored genetic lines were regrown, shoot formation ranged from 45% for a *Malus paradisiaca* genotype to 77.5% for a *Malus domestica* hybrid (Niino et al. 1992). In the same study, a slightly different protocol was applied to eight *Pyrus* cultivars. When recovered, shoot formation ranged between 40% for *Pyrus pyrifolia* cv. Yoshino

and 72.5% for *Pyrus communis* cv. Beurre d' Amanlis (Niino et al. 1992). Genotypic differences in regrowth (60% - 80%) were observed among 10 strawberry genotypes (Hao et al. 2002). Regrowth for five cryopreserved *Castanea sativa* genotypes ranged from 37.5% to 54.4% (Vidal et al. 2005). *Rubus* cultivars also demonstrated cultivar-specific responses to cryopreservation, showing that cold acclimation significantly increased the survival of three of the four cultivars by at least 50% (Reed 1988).

Application of Cryopreservation for Long-Term Sea Oats Storage

As previously described, sea oats genetics are variable based on population and geographic distance. Therefore, we must consider the long-term conservation of as many genotypes and populations as possible. It is anticipated that genotype will affect survival. The selection that occurs during the dehydration and freezing steps of cryopreservation will determine which genotypes and/or populations are better suited for cryostorage and long-term conservation.

Ecologically sound cryopreservation will require the efficient storage of many diverse sea oats genotypes. Development of an optimal cryogenic storage protocol will allow for the long-term storage of site-specific sea oats genotypes for commercial use. Therefore, the goal of this thesis research was to optimize cryopreservation protocols for sea oats by comparing and quantifying the post-cryostorage survival following vitrification and encapsulation-dehydration.

To accomplish the goal of optimized cryopreservation protocols for sea oats, the following objectives define this thesis research:

- Determine the cryostorage success of select sea oats populations and genotypes
- Evaluate the necessary steps and solutions for an optimized vitrification procedure

- Examine the concentration of sucrose pretreatment and desiccation period for an optimized encapsulation-dehydration procedure

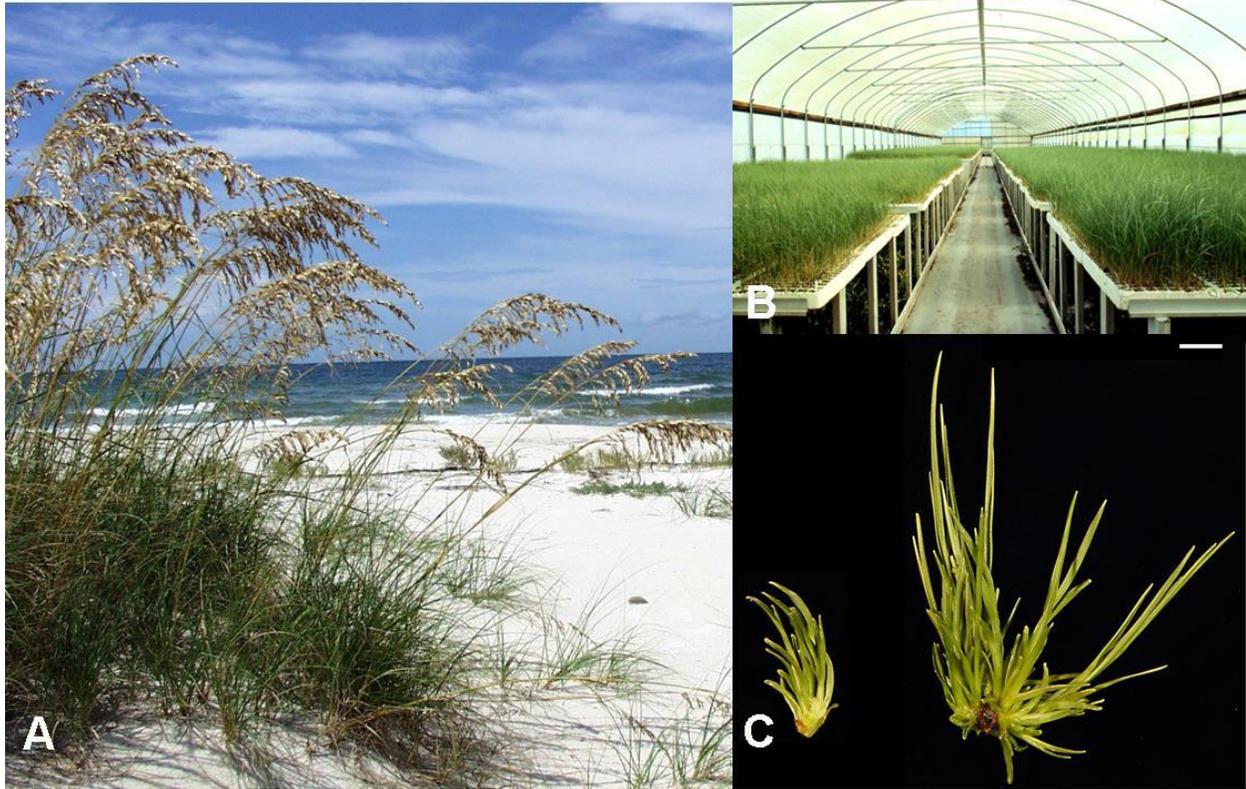


Figure 1-1. Sea oats (*Uniola paniculata*) is a native dune grass species in the southeastern United States. A) Sea oats are planted to stabilize and grow dunes following beach sand renourishment. B) Sea oats are grown from seed at nurseries to provide plugs for land managers. C) Sea oats shoot cluster explants at subculture and after 28 days culture on sea oats Stage II shoot multiplication medium for *in vitro* propagation of plants. Photos courtesy of Dr. Michael Kane.

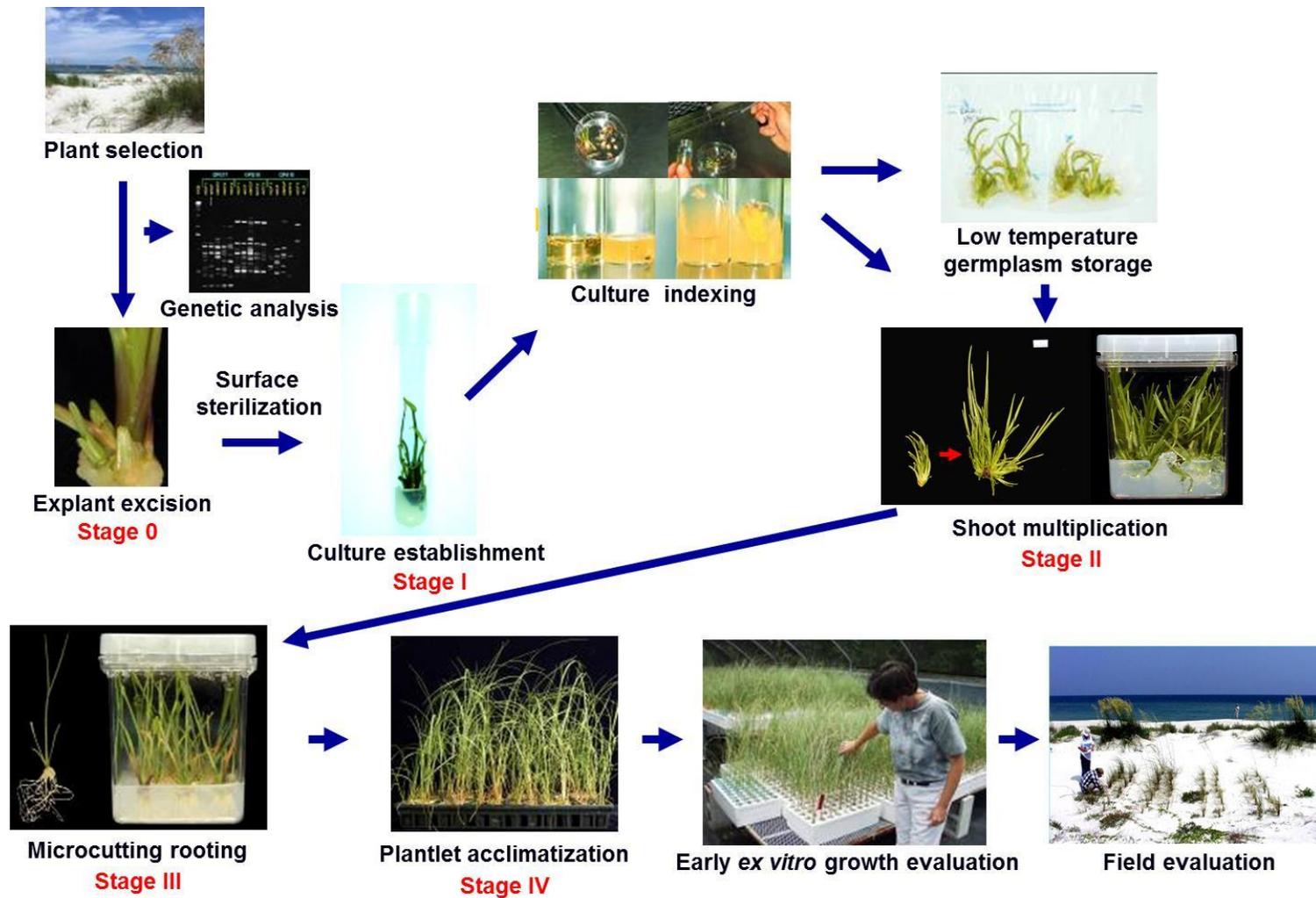


Figure 1-2. Step-wise protocol for the *in vitro* micropropagation, storage, and field evaluation of *Uniola paniculata*. Figure courtesy of Jonathan Jasinski (Jasinski 2011).

Vitrification

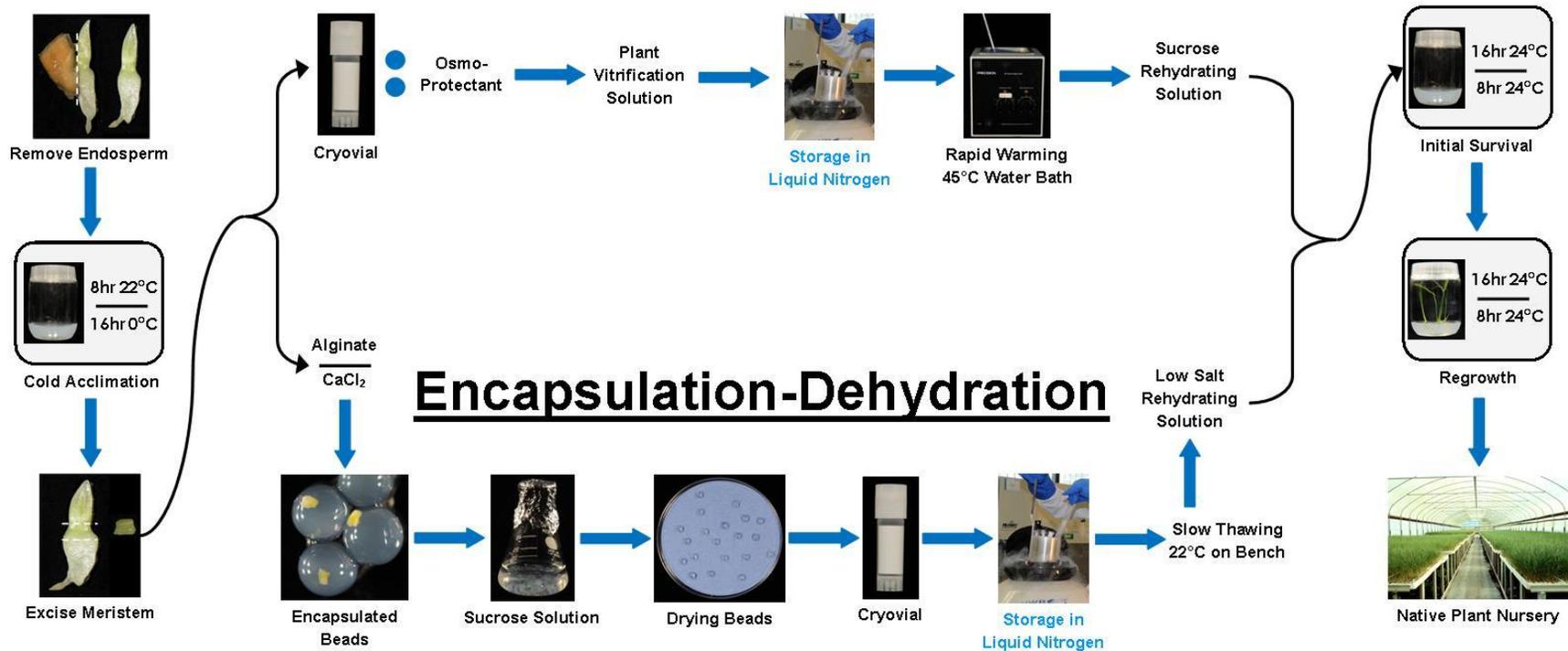


Figure 1-3. Two cryopreservation methods to cryopreserve sea oats shoot tips. Vitrification is the osmotic dehydration of shoot tips using a plant vitrification solution prior to LN. Encapsulation-dehydration is the evaporative dehydration of shoot tips encased in an alginate bead and dried in a laminar flow hood prior to LN. Both procedures use cold acclimated seedlings and follow similar post-cryostorage procedures. Photos courtesy of James Sadler.



Figure 1-4. Nalgene “Mr. Frosty”[®] used for controlled-rate cooling cryopreservation of plant tissues. This method is an alternative to using expensive programmable freezers prior to liquid nitrogen exposure. Photo courtesy of James Sadler.

CHAPTER 2 CRYOPRESERVATION OF SEA OATS (*Uniola paniculata* L.) SHOOT TIPS FROM GENOTYPES ESTABLISHED IN VITRO

Florida possesses 825 miles of coastal beaches with 75% of the population living within the 35 coastal counties. Moreover, Florida's coastal communities account for more than \$9 billion in beach-related tourism and 82% of the state's GDP (Wilson and Fischetti 2010; Kildow 2008). Simultaneously, Florida coasts are particularly vulnerable to the destructive impacts of tropical storms, hurricanes, and sea level rise. Currently, coastal dunes planted with native species function as the initial line of defense against the destructive impacts of hurricanes and storms that threaten coastal communities. Therefore, these areas must be protected from storm and hurricane damage to minimize beach sand erosion. However, it is predicted that storm surges from rising sea levels and intensifying storm frequencies will inevitably have a greater impact on the natural environment and coastal communities (Noss 2011).

Although established regulations help protect coastal dunes from damage by human activity, severe natural damage cannot be prevented. Global sea levels are rising and the coastal areas surrounding Florida have been identified as regions vulnerable to flooding (Nicholls and Cazenave 2010). More specifically, sea level rise is identified as the immediate negative impact of climate change for Florida (Noss 2011). Continual human population growth and urban development in coastal areas compound damage due to sea level rise. These factors also result in species fragmentation of key coastal ecosystems (CELCP 2012). Over 50% (about 365 miles) of Florida beaches were critically eroded as a result of the active 2004-2005 hurricane seasons (FDEP 2006). Effective restoration of damaged dunes requires beach sand renourishment followed by the planting of local native species for dune stabilization and subsequent building.

The most commonly planted dune species in the southeastern United States is sea oats (*Uniola paniculata*; Poaceae). Sea oats range between Virginia and Mexico and play a critical role in the creation, maintenance and building of coastal dune ecosystems (Wagner 1964). Sea oats thrive in the harsh environmental extremes of wind, salt spray, sand, precipitation, temperature, and soil moisture (Wagner 1964; Lonard et al. 2011). Furthermore, the plants help stabilize coastal dunes from erosion. However, sexual reproduction in sea oats can be limited. Approximately 70% of fertilized sea oats ovules abort resulting in decreased seed production (Wagner 1964). A proposed rationale for such a high proportion of aborted ovules focuses on the incidence of fungal infection promoted during periods of high humidity and rainfall (Bachman and Whitwell 1995; Burgess et al. 2002).

For the seeds which develop (<3 seeds per spikelet), germination can be inhibited by mechanical resistance of the testa and a physiological block within the embryo (Wagner 1964; Westra and Loomis 1966). Viable seeds must also contend with threats of predation or extreme sand burial (i.e., $\geq 15\text{cm}$) (Miller et al. 2003). Therefore, sea oats establishment by direct seeding under field conditions presents a major limitation. Likewise, reports of low survival of sea oats transplants and rhizome fragments suggest that these methods may not be viable for revegetation (Woodhouse et al. 1968; Miller et al. 2003). Clearly, methods need to be developed to preserve, conserve, and restore sea oats populations and provide genetically diverse and ecologically-sound sources of plant material for replanting.

The most reliable source of transplants has been the commercial nursery production of sea oats as plugs or containerized plants, established from seed collected

from donor populations (Barnett and Crewz 1991; Bachman and Whitwell 1995) (Figure 1-1b). However, the low availability of nursery-grown plugs from site-specific populations limits the large-scale application for restoration projects. Nurseries are unable to maintain large quantities of genetically diverse and site-specific sea oats plants and must propagate them in response to changing market demand, such as following storm damage (Miller et al. 2003). Additionally, attempts to rapidly propagate plants vegetatively to meet demand for many sea oats may result in reduced genetic diversity of the plants produced (Bertrand-Garcia et al. 2012). Moreover, nurseries are often required to obtain seeds from local populations. For example, sea oats populations on federal lands and state parks may only be re-established with plants that originated from the specific location to prevent planting of maladapted genotypes (Fla. Admin. Code 2013). Therefore, if local populations are severely damaged or destroyed, there may not be sufficient seeds for collection and propagation. This results in the inability to produce appropriate genotypes and restore the original biodiversity. Thus, alternative sources of sea oats that retain local genetic diversity are necessary.

Based on results of population genetics studies, Florida sea oats are divided between Gulf and Atlantic Coast populations (Ranamukhaarachchi et al. 2000; Franks et al. 2004). Based on genetic studies using amplified fragment length polymorphism (AFLP), lower genetic differentiation between sea oats populations on the same coast and a positive relationship between genetic distance and geographic distance have been reported (Ranamukhaarachchi et al. 2000; Franks et al. 2004). Additionally, variations in germination requirements (i.e., cold stratification temperatures) between

Florida sea oats populations described fragmented gene flow between the two Florida coasts (Seneca 1972).

It is vital to maintain local ecotypic differentiation to preserve population resilience to natural and human impacts in the coastal ecosystem since Florida sea oats are divided between two major sea oats groupings. Genetic variation and diversity have been positively correlated with a plant species' capacity to thrive (Reed and Frankham 2003). Therefore, greater genetic diversity provides a larger capacity for sea oats to respond to the harsh fluctuating coastal environment if the ecotypes are adapted to fit the current microclimate (Huenneke 1991).

A strategy offered to respond to disturbances in Florida's coastal ecosystems is the creation of germplasm libraries of species threatened by sea level rise (Noss 2011). In response to this strategy, a sea oats *in vitro* germplasm library could augment natural and nursery seed propagation. Micropropagation, the rapid aseptic clonal propagation of plants on a defined culture medium under controlled conditions of light and temperature, has been applied for sea oats production (Kane 2011). However, there are few micropropagation protocols reported which allow efficient production of diverse sea oats genotypes (Philman and Kane 1994; Valero-Aracama et al. 2008, 2010; Jasinski 2011). It is vital to maintain genetic diversity of regionally adapted genotypes and not to reintroduce only a limited number of genotype clones into a population since the goal of planting sea oats is ecologically-sound coastal dune restoration. Therefore, maintenance of actively growing *in vitro* shoot cultures of diverse sea oats genotypes from many Florida populations is required. Long-term maintenance of a large number of actively growing genotypes *in vitro* can be labor-intensive, require extensive space, lead

to somaclonal variation, and pose a continued risk of culture contamination (Pence 2011). Long-term conservation methods, such as cryopreservation, the long-term ultra-low temperature storage of tissue in liquid nitrogen (LN; -196 °C) with limited loss of viability, could be employed to help alleviate the high costs associated with maintaining a germplasm library consisting of actively growing cultures (Kaczmarczyk et al. 2011). Conceivably, cryostorage could provide a reliable, efficient, and ecologically-sound method for storage and preservation of many site-specific sea oats genotypes. The goals of the current study were twofold: 1) determine whether cryopreservation of sea oats was possible using shoot tips excised from established *in vitro* cultures; and 2) examine the influence of genotype and geographic source on recovery of shoot tips from cryostorage.

Materials and Methods

In Vitro Culture of Sea Oats Genotypes

Nine sea oats genotypes, GR 6-2, GR 6-3, GR 6-7, DWP 8-3, DWP 8-13, DWP 8-17, PK 2-2, PK 4-3, and PK 8-5, representing three Florida populations, Gamble Rogers Memorial State Recreation Area, Flagler Beach (GR), Delnor-Wiggins Pass State Park, Naples (DWP), and Perdido Key State Park, Pensacola (PK), were chosen from an *in vitro* germplasm library established June 2007 (Figure 2-1). This germplasm library consisted of actively proliferating shoot culture lines that were originally established from single seedlings (genotypes). Individual genotypes were sustained by subculturing shoot clusters every 28 days on fresh Stage II sea oats shoot multiplication medium (SMM). Sea oats SMM is a full-strength Murashige and Skoog basal salt mixture (MS) (Cat No. M-524 *PhytoTechnology* Laboratories, Shawnee Mission, KS)

with 30 gL⁻¹ sucrose, 100 mgL⁻¹ myo-inositol, 0.4 mgL⁻¹ thiamine-HCl, and 0.25 mgL⁻¹ benzyladenine and solidified with 7 gL⁻¹ high-grade TC agar (Cat. No. Agar A-175 *PhytoTechnology* Laboratories, Shawnee Mission, KS). The medium was adjusted to pH 5.7 using 0.1 M KOH before adding the agar. The medium was dispensed into 12 mL volumes in 150 x 25 mm glass culture tubes and autoclaved at 117.7 kPa for 20 min at 121 °C. Sea oats cultures were placed under a 16-hr photoperiod provided by cool white fluorescent lamps (General Electric F96T12·CW·WM) at 45 μmol·m⁻²·s⁻¹ PFF at 24 °C.

Genotypic Effect on Cryopreservation Survival

Multiple cultures of each genotype were placed under cold acclimation (CA) conditions, 22 °C day and 0 °C night, 28 days after the previous subculture. An 8-hr photoperiod was supplied by General Electric cool white fluorescent lights (F96T12·CW·WM·ECO) at 70 μmol·m⁻²·s⁻¹ PPF. Following two weeks CA, shoot tips (1–1.5 mm) were excised from established sea oats cultures (Figure 2-2) and subjected to a standard vitrification cryopreservation protocol developed based on the compilation of successful published cryopreservation studies (Figure 2-3).

Efficiency of the standard cryopreservation protocol was evaluated using excised CA shoot tips placed into 2 mL sterile cryovials (5 shoot tips/cryovial) (Cat. No. 10-500-26 Fisherbrand Cryogenic Storage Vial) containing 1 mL osmoprotectant at 0 °C for 25 min in a -15 °C freezer (Appendix B). This osmoprotectant solution consisted of a MS basal salt solution containing 2 M glycerol and 0.4 M sucrose. The osmoprotectant was removed using a sterile pipette and 1 mL Plant Vitrification Solution 2 (PVS2) at 0 °C was added for 5 min. PVS2 consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethylsulfoxide (DMSO) in a liquid MS solution with 0.4 M sucrose

(Sakai et al. 1990). When the PVS2 was removed, 1 mL fresh PVS2 solution was added and the cryovials were placed in a -15 °C freezer for 40 min. After osmotic dehydration in PVS2, cryovials were plunged into LN for 60 min. After freezing, the cryovials were rapidly warmed in a 45 °C water bath for 1.5 min. The thawed PVS2 solution was removed and replaced with 1 mL room temperature (≈ 22 °C) rehydrating solution, consisting of a MS solution supplemented with 1.2 M sucrose, for 20 min before being replaced with fresh rehydrating solution for an additional 20 min. All solutions were filter sterilized (0.2 μm pore size; Cat. No. 195-2520 Thermo Scientific) before use.

The rehydrating solution was removed and the five shoot tips were transferred into baby food jars (BFJ) containing 30 mL initial survival medium. This medium consisted of Gamborg B-5 basal medium (Cat. No. G-398 *PhytoTechnology* Laboratories, Shawnee Mission, KS) supplemented with 30 g L^{-1} sucrose and 0.25 mg L^{-1} benzyladenine and solidified with 6 g L^{-1} TC Agar (Appendix A). Cultures were maintained in an incubator for 4 weeks under a 16-hr photoperiod supplied by General Electric cool white fluorescent lights (F96T12-CW-WM-ECO) at 45 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF at 24 °C, henceforth referred to as standard growing conditions. All plant material was transferred into BFJ containing 30 mL SMM and placed in an incubator under standard growing conditions for an additional 4 weeks.

In this study, initial survival was recorded as the greening and elongation of the shoot tips on the initial survival medium 4 weeks following cryostorage (Figure 2-4). All recovered plant material, including those perceived as dead, were placed on SMM. Regrowth was recorded as the continued elongation of green recovered shoot tips

following 4 weeks on SMM (8 weeks post-cryostorage). Percent survival was measured after each 4-week interval.

Experimental Design and Statistical Analysis

Each treatment consisted of eight replicate cryovials each containing five shoot tips. Greening and shoot tip elongation were both required for samples to qualify as being fully recovered from cryopreservation. Statistical differences between data were determined using analysis of variance (ANOVA) using the generalized linear model procedures. The data are presented as percentages. Means separation was performed via Duncan's Multiple Range Test (DMRT) $\alpha = 0.05$ for initial survival and regrowth separately (SAS Institute 2009).

Results

Most non-frozen (-LN, control) sea oats genotypes exhibited minimal sensitivity to the cryopreservation preparative steps (initial survival: 72 - 92% and regrowth: 10.5 - 73.5%) (Figure 2-5). However, while all sea oats genotypes exhibited at least some tolerance to cryostorage (+LN) using the standard protocol, significant differences in survival and regrowth percentages were observed between genotypes (Figure 2-5). When the same cryopreservation procedure was applied, the three PK genotypes displayed significantly higher regrowth (41.1 – 52%) compared to the genotypes screened from the other locations (2.2 – 22.5%) (Figure 2-5). When individual genotype responses from the same population were pooled and analyzed (Figure 2-6), the PK population, as a group, exhibited a significantly higher regrowth percentage (47.8%) compared to either GR (8.2%) or DWP (16.7%). However, when individual genotype responses were pooled by population, no differences were noted among initial survival (75.8% - 85.1%) and regrowth (47.2% - 56.6%) of shoot meristems that were only

dehydrated (control) and not frozen (Figure 2-6). These results suggest the interesting possibility of population-specific differences in tolerance to cryostorage in sea oats.

Discussion

Application of micropropagation to create an *in vitro* germplasm library offers the potential to store and produce site-specific genotypes that may supplement current sea oats seedling plug production used for dune restoration. However, the ecological necessity to maintain genetic diversity in the outplantings requires continual maintenance of many actively growing shoot cultures of diverse sea oats genotypes, which potentially could make the approach both expensive and eventually unmanageable. Therefore, the capacity to cryostore excised sea oats shoot tips then thaw and recover them when needed would alleviate these limitations.

The results presented here indicate that sea oats shoot tips excised from established shoot cultures can tolerate cryostorage in LN. However, significant genotypic differences in initial survival and regrowth were observed following cryopreservation using the standard protocol (Figure 2-5). Some reductions in shoot tip viability were observed between initial survival and regrowth across genotypes even in the absence of freezing. This suggests a negative effect of the vitrification procedures used. The specific physiological processes underlying shoot tip survival and subsequent regrowth are poorly understood. Differences in initial survival of shoot tips between genotypes and further losses during regrowth post-cryostorage could be due to differing capacities to survive severe desiccation and/or freezing stress (Benson 2008) and cellular damage, particularly that occurring from production of free radicals.

These results also suggest that tolerance to cryopreservation of *in vitro* derived sea oats shoot meristems is influenced by genotype. Of the nine genotypes screened

only three (33%) displayed sufficient tolerance (>40% regrowth) to cryostorage. Genotypic differences in sea oats *in vitro* growth and development have also been observed. Significant differences in *in vitro* shoot proliferation, rooting, and capacity for *ex vitro* acclimatization occurred between sea oats clonal lines established from both seedlings (Jasinski 2011) and excised shoot tips from genetically characterized adult donor plants (Valero-Aracama et al. 2008, 2010).

Based on extensive experience with many horticultural species and cultivars, Reed (2008) concluded that development of a truly universal plant cryopreservation protocol might not be possible because of the genetic variability that exists in a germplasm bank established to preserve biodiversity. A cryopreservation protocol must enable successful cryostorage and regrowth of many genotypes of a species for the dual purposes of habitat restoration and achieving genetic diversity. However, one primary knowledge gap is the lack of a sound understanding as to why some genotypes are more tolerant to cryostorage than others (Volk 2010). It would be critical to understand the physiological and genetic basis for genotypic differences in freezing tolerance in order to develop an optimal cryopreservation protocol. This could be achieved by examining physiological differences between cryostorage intolerant (e.g., GR 6-2) and tolerant (e.g., PK 4-3) sea oats genotypes. A similar approach was used to successfully determine the physiological and anatomical differences between easy- and difficult-to-acclimatize sea oats genotypes (Valero-Aracama et al. 2008) which subsequently allowed development of a more efficient sea oats micropropagation protocol (Valero-Aracama et al. 2010). Given that successful acclimatization of plantlets to the *ex vitro* environment requires the capacity to survive freezing stressors,

correlating genotypic capacity for acclimatization with freezing tolerance may provide a simple screening method to select genotypes with the potential for greater tolerance to freezing stress. Additionally, marked differences in morphology and physiology between sea oats have been reported for easy- and difficult-to-acclimatize genotypes (Valero-Aracama et al. 2006). A significant difference that could relate to the increased freezing tolerance of some sea oats genotypes, and subsequently populations (i.e., Perdido Key), is the differences observed in shoot starch and soluble sugars during acclimatization. The easy-to-acclimatize genotype maintained higher carbohydrate levels than the difficult-to-acclimatize genotype, which might instill greater freeze tolerance.

Recent elucidation of the genetic pathways associated with cold acclimation and freezing tolerance suggest roles of key cold regulated genes in the acquisition of cold tolerance in plant tissues (Volk 2010). Volk (2010) has advocated combining physiological and genomic methods to elucidate the specific mechanisms through which cells are protected from freezing stress. Sea oats might provide an excellent model for future studies in this area.

Studies involving direct comparisons of the cryostorage tolerance of excised shoot tips of *different genotypes of the same species* are limited. In horticulturally important crops, differences in cryostorage survival between cultivars and even species within a genus make relevant comparisons difficult. For example, in *Wasabia japonica*, shoot formation (78.5 – 92.2%) was significantly different between cultivars when the same cryopreservation procedure was applied (Matsumoto et al. 1994). When five cryopreserved genetic lines of apple were regrown, shoot formation ranged from 45% for

a *Malus paradisiaca* cultivar to 77.5% for a *Malus domestica* hybrid (Niino et al. 1992). In the same study, a slightly modified protocol was applied to eight *Pyrus* cultivars. Shoot formation ranged between 40% for *Pyrus pyrifolia* cv. Yoshino and 72.5% for *Pyrus communis* cv. Beurre d' Amanlis (Niino et al. 1992). Surviving genotypes of seven cryopreserved species or natural hybrids of *Solanum* species ranged from 71% for *S. stenotomum* to 80% for *S. goniocalyx* x *S. stenotomum* (Golmirzaie and Panta 1998). Genotypic differences in regrowth were observed among 10 strawberry genotypes with the lowest displaying 60% regrowth while the highest displayed 80.7% regrowth (Hao et al. 2002). Regrowth for five cryopreserved *Castanea sativa* genotypes ranged from 37.5% to 54.4% (Vidal et al. 2005). Therefore, some plant genotypes may be able to survive better than others using the same cryopreservation procedure.

In addition to individual genotypic differences, significant population differences were observed when genotype data were pooled based on sea oats population (Figure 2-6). PK was significantly higher for initial survival and regrowth (74%, 47.8%, respectively) after LN compared to GR (43.4%, 8.2%, respectively) and DWP (33.2%, 16.7%, respectively). No current published results were found to demonstrate population based differences in cryostorage tolerance within a species. Kaczmarczyk et al. (2011) also emphasized the need for additional studies to elucidate the underlying causes for limited survival of some genotypes. Given that only three sea oats populations each with three representative genotypes were screened, additional populations and genotypes would need to be screened to accurately determine the relationship between source population and tolerance to cryostorage. Given the time and labor required to establish shoot cultures of individual genotypes *in vitro* and then

excise shoot tips, it may be more efficient to determine this relationship by comparing the post-LN germination of seed from different source populations. However, differences in freezing tolerance between excised meristems and intact seed may exist considering dissimilarities in morphological structure

Genotypic differences to cryopreservation observed between sea oats populations may be a consequence of ecotypic differentiation. Distinct environmental conditions within a species' range have been reported to influence different ecotypes within the species (Gregor 1946). As described by Gregor (1946), an ecotype is a population of a species that has undergone genetic differentiation due to varying environmental conditions within that species' range. Ecotypic differentiation has been studied with *Impatiens pallida* and *Carex aquatilis* using genetic and reciprocal transplant studies (Bennington and McGraw 1995; Chapin and Chapin 1981). When *I. pallida* plants from two different environments were reciprocally transplanted, differences in flowering timing, morphological traits, and seed production were noted (Bennington and McGraw 1995). Common garden studies of *C. aquatilis* plants from five different populations displayed a highly significant effect of population on tiller and plant morphology, shoot and root masses, and ratios of roots and shoots indicating ecotypic differentiation among the populations (Chapin and Chapin 1981). Similar findings were reported with micropropagated *Sagittaria latifolia* ecotypes deriving from the species' range from Rhode Island to south Florida (Kane et al. 2002). When grown under the same conditions, significant differences in shoot, leaf, and corm production and in flowering and senescence patterns were observed between *S. latifolia* ecotypes.

The *in vitro* germination of the terrestrial orchid *Calopogon tuberosus* var. *tuberosus* from populations ranging from Michigan to south Florida indicated ecotypic differentiation when optimal temperature and chilling requirements varied between populations (Kauth et al. 2011). Although screening for ecotypic differentiation through variable environmental conditions has been found to affect many growth responses in many species, the influence geographical source on cryostorage survival in sea oats has not been studied. Variations in sea oats temperature-related germination requirements (i.e., cold stratification) between populations have previously determined three major sea oats ecotypic groupings: 1) Virginia and North Carolina population; 2) Atlantic Coast Florida population; and 3) Gulf Coast population (Seneca 1972). Furthermore, genetic studies of sea oats have shown that clonal diversity and structure can vary greatly between different populations (Franks et al. 2004). Consequently, differences in survival following cryostorage maybe the result of ecotypic differentiation of sea oats from different latitudinal sources, thus different temperature ranges. Therefore, genotypes originating from GR and DWP populations may be genetically predisposed not to respond well to cryostorage following the standard protocol. These populations could be more sensitive to the extreme desiccation, extreme freezing, or excision wounding during the cryopreservation procedure. Hence, more extensive studies are warranted to further develop an optimized protocol that would increase regrowth for these sea oats populations and to screen other sea oats populations for cryopreservation success across the geographical range. Additionally, the underlying mechanism(s) for freezing tolerance should be studied to determine what allows PK genotypes to survive cryostorage better than DWP and GR genotypes.

Conclusions

This study provides novel information on the capacity to cryopreserve *Uniola paniculata* excised shoot tips from established *in vitro* cultures. Cryopreservation using the standard protocol was effective in preparing tissues for survival with PK genotype regrowth surpassing the 40% minimum established by Reed et al. (1998b) (Figure 2-5). Survival following cryostorage was significantly affected by genotype and source population with GR (8.2%) and DWP (16.7%) shoot tip regrowth being significantly lower than PK (47.8%) (Figure 2-6). Ecotypic differentiation has been shown to affect various plant growth responses, but its role in affecting tolerance to cryostorage has not been examined. Based on the results reported here, differences in cryopreservation success between genotypes may be a reflection of ecotypic differentiation resulting from environmental differences (i.e., temperature ranges) at source populations. Studies focused on optimizing procedures to increase survival following cryopreservation should be conducted on low surviving genotypes such as those originating from the DWP or GR populations.



Figure 2-1. Origins of sea oats genotypes from the four Florida populations used in this thesis. Photo courtesy of James Sadler.

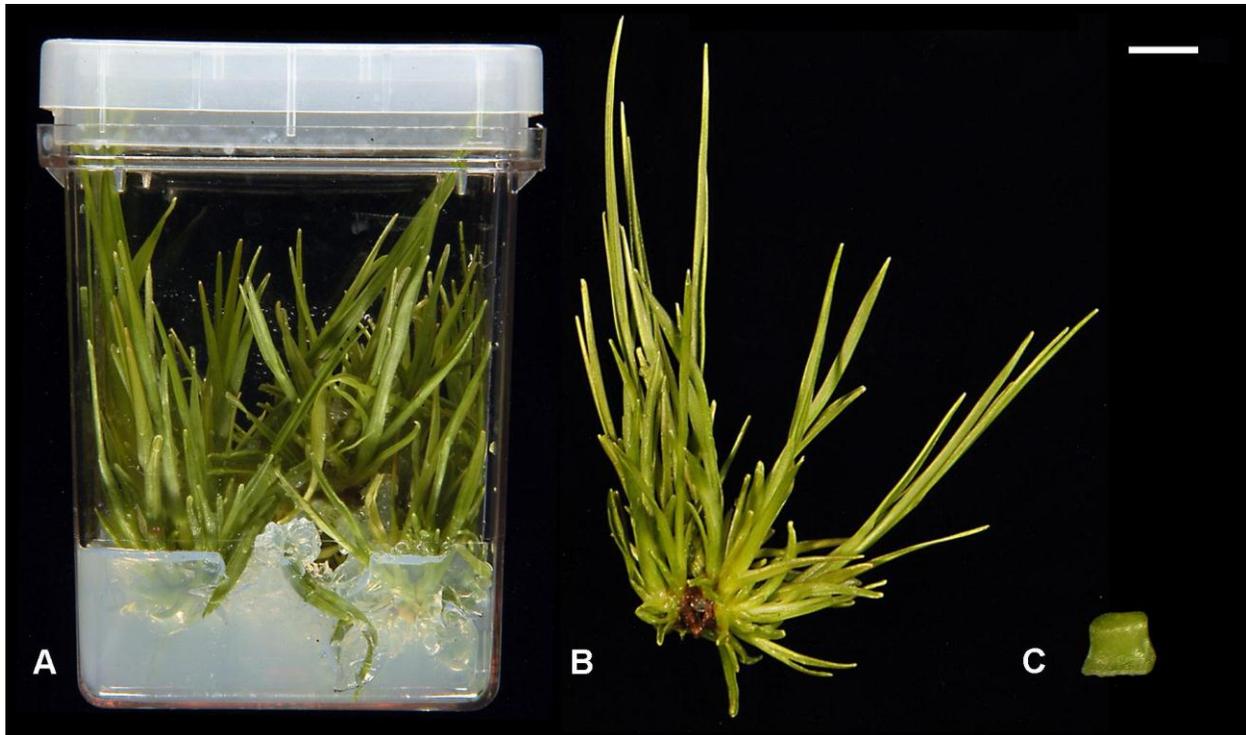


Figure 2-2. Example of an *in vitro* propagated sea oats shoot cluster that provides shoot tips for excision. A) Sea oats grown in a culture vessel for 28 days develop clusters of shoots. B) Shoot clusters are individually removed from cultures vessels in sterile laminar flow hoods. C) Many shoot tips are excised from shoot clusters. Scale bar = 1 cm (A-B) and 1 mm (C). Photos courtesy of James Sadler.

Standard Vitrification Protocol

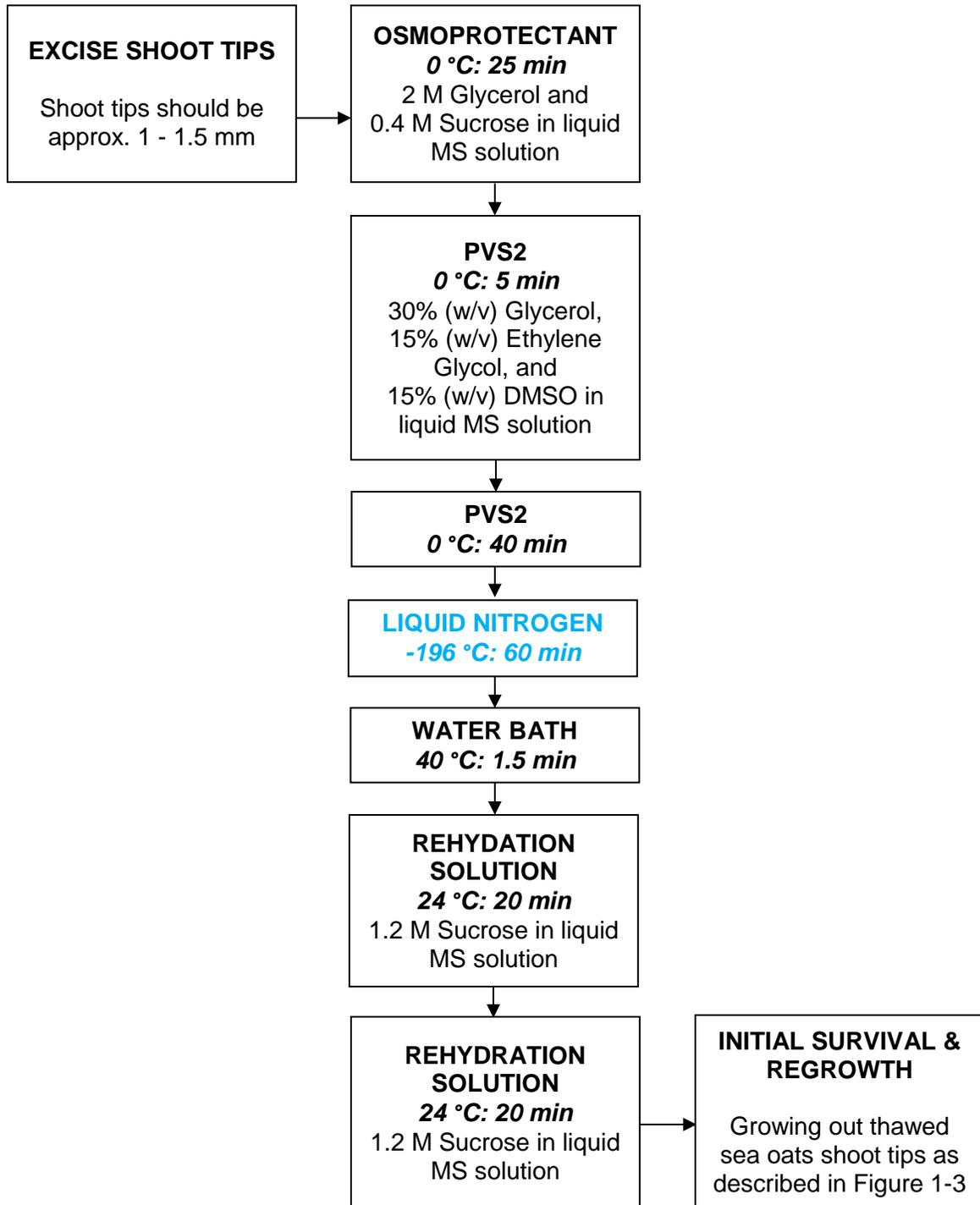


Figure 2-3. Standard vitrification protocol synthesized based on successful published cryopreservation studies. This standard protocol served as the basis for systematic modification to cryopreserve excised sea oats seedling shoot tips.

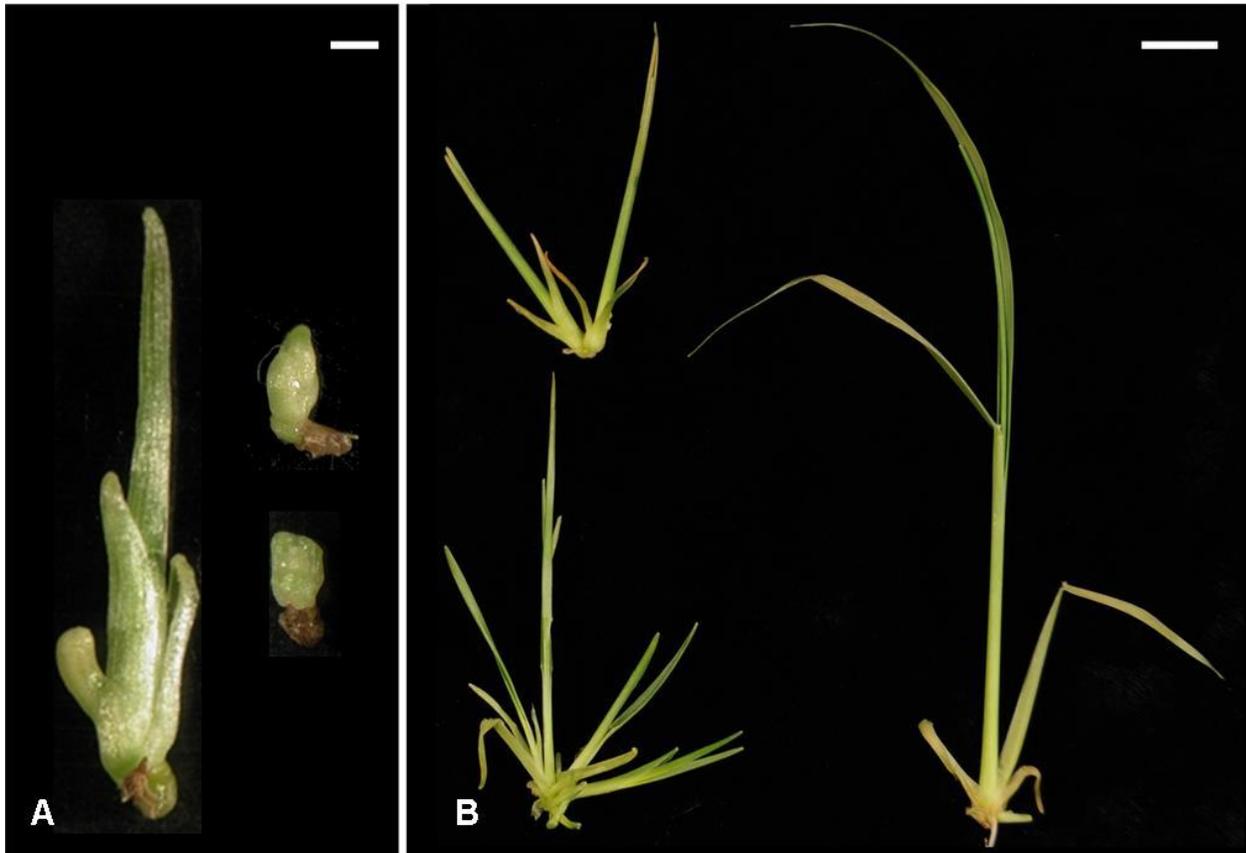


Figure 2-4. Growth responses of sea oats shoot tips following cryopreservation. A) Green and elongating shoot tips during initial 4-week survival period. B) Green and elongating shoot tips regrowth after an additional 4-week interval on shoot multiplication medium. Scale bar = 1 mm (A) and 1 cm (B). Photos courtesy of James Sadler.

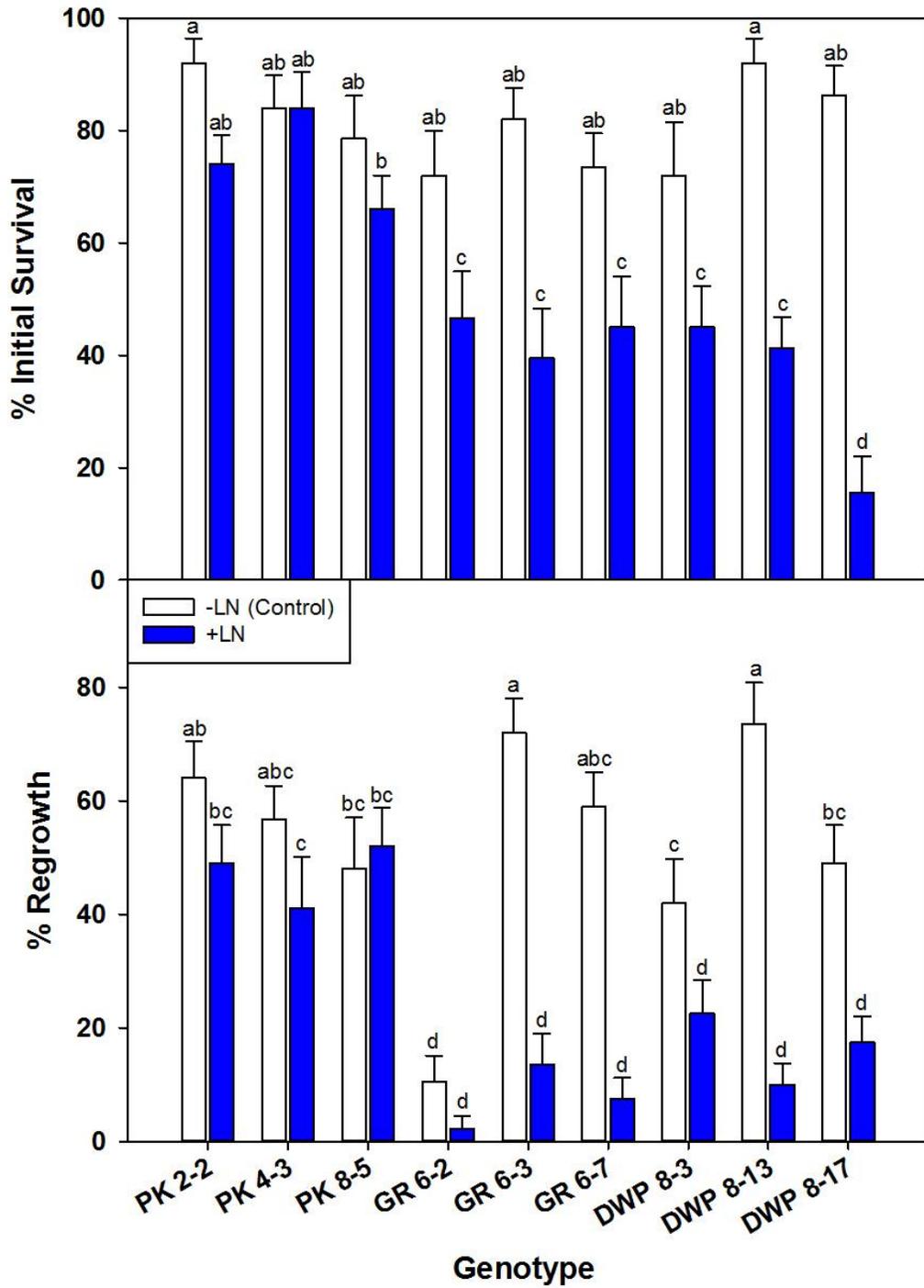


Figure 2-5. Comparative effects of genotype on cryopreservation survival of *U. paniculata* shoot tips. Initial survival and regrowth are presented following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Bars (\pm SE) marked by different letters are significantly different within each graph according to DMRT at $\alpha = 0.05$.

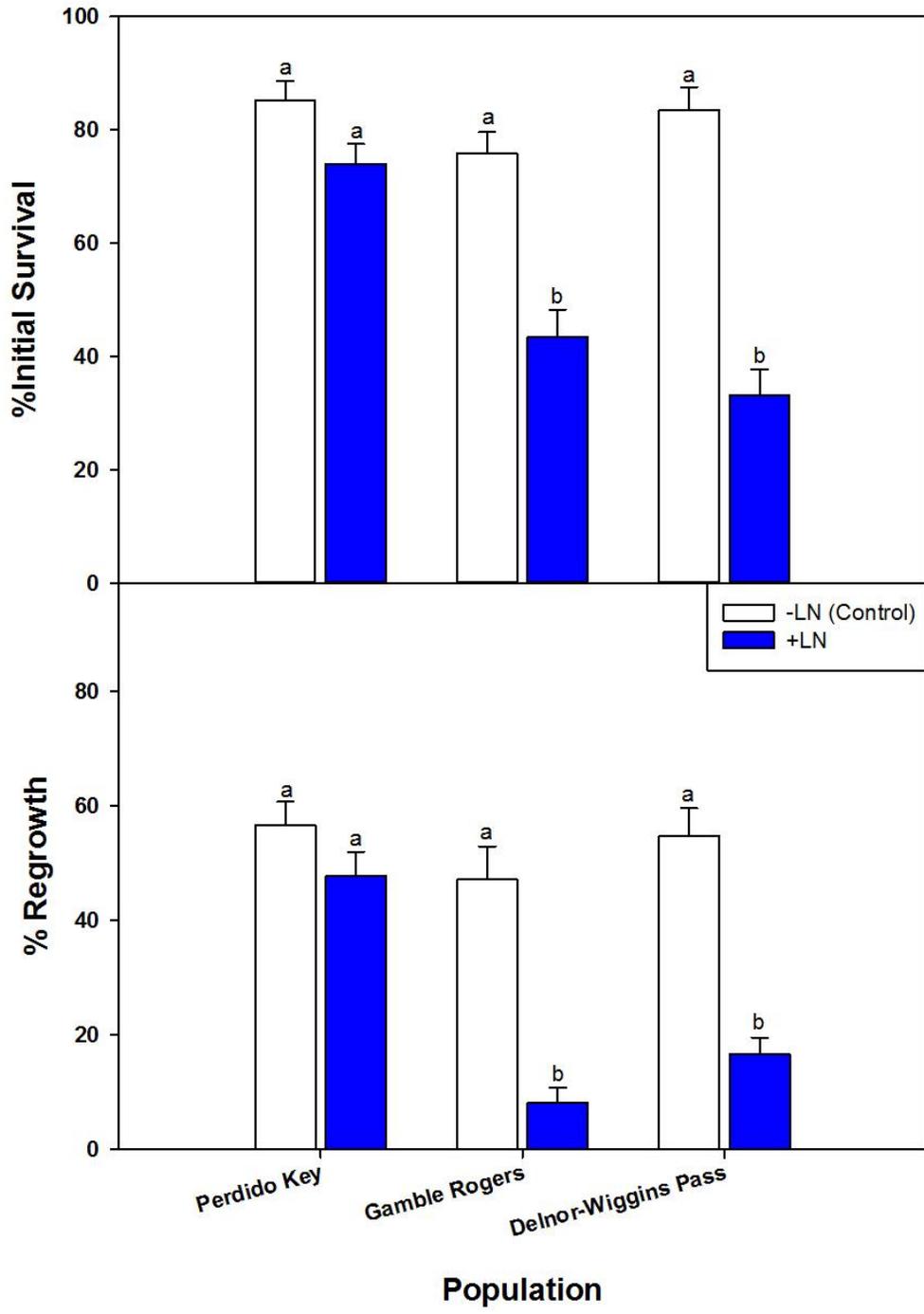


Figure 2-6. Comparative effects of population on cryopreservation survival of *U. paniculata* shoot tips originating from three Florida populations. Initial survival and regrowth are presented following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Bars (\pm SE) marked by different letters are significantly different within each graph according to DMRT at $\alpha = 0.05$.

CHAPTER 3
OPTIMIZING THE CRYOPRESERVATION PROTOCOL OF SEA OATS (*Uniola
paniculata* L.) SHOOT TIPS USING VITRIFICATION AND ENCAPSULATION-
DEHYDRATION

Florida is particularly vulnerable to the destructive impacts of tropical storms, hurricanes, and sea level rise. Currently, coastal dunes planted with native species function as the initial line of defense against the destructive impacts of hurricanes and storms that threaten coastal communities. Because of the active 2004-2005 hurricane seasons, it was reported that over 50% (about 365 miles) of Florida beaches were critically eroded (FDEP 2006). Effective restoration of damaged dunes requires beach sand renourishment followed by the planting of local native dune species for dune stabilization and subsequent building.

The most commonly planted dune species in the southeastern United States is sea oats (*Uniola paniculata*; Poaceae). Sea oats thrive in the harsh environmental extremes of wind, salt spray, sand, precipitation, temperature, and soil moisture (Wagner 1964; Lonard et al. 2011). Given the role that sea oats play in maintaining and building dunes, it is critical that methods are developed to maintain sea oats populations and provide ecologically sound sources of material for replanting.

A strategy offered to respond to disturbances in Florida's coastal ecosystems was the creation of germplasm libraries of species threatened by sea level rise (Noss 2011). In response to this strategy, a sea oats *in vitro* germplasm library using cryopreservation could provide a means to preserve many site-specific sea oats genotypes to re-establish the biodiversity of a population if it declines or is destroyed. Long-term conservation methods, such as cryopreservation in liquid nitrogen (LN; -196

°C), help alleviate the high costs associated with maintaining a germplasm collection of actively growing plants *in vitro* or *ex vitro* (Pence 2011).

Many species have been successfully placed into cryostorage (Engelmann 2004; Benson 2008). Survival of cryopreservation in plant tissues is achieved by controlling the phase transition of intracellular water from a liquid to a glassy state thereby minimizing lethal ice crystallization (Volk and Walters 2006; Sakai et al. 2008). Crystallization is detrimental to cryopreservation protocol success because of cell wall, membrane, and tissue damage (Pearce 2001). Therefore, the intracellular water concentration of donor plant material is decreased prior to cryopreservation through pretreatments and by laminar air flow or osmotic dehydration (Reed 1996; Niino et al. 1992). Two major cryopreservation methods are vitrification and encapsulation-dehydration. Vitrification requires the intracellular water in plant tissues to be replaced by sugars and cryoprotectants (Sakai et al. 2008). Encapsulation-dehydration requires plant tissues to be encapsulated by an alginate bead and dehydrated in a laminar flow hood (Engelmann et al. 2008). Although these methods vary in their procedures, the ultimate aim is to remove intracellular water to prevent ice crystallization and promote membrane stability when placed into liquid nitrogen.

A significant decrease in post-cryostorage survival of sea oats shoot tips from Delnor-Wiggins Pass (DWP) and Gamble Rogers (GR) compared to genotypes from Perdido Key (PK) was shown (Figures 2-5 and 2-6). These differences in cryopreservation survival could be the consequence of ecotypic differentiation or genetic differences in sensitivity to cryostorage procedures leading to reduced regrowth. The objective of this study was to attempt to increase the cryopreservation success of

DWP sea oats shoot tips using vitrification and encapsulation-dehydration cryopreservation methods. These techniques have been shown to increase survival post-cryostorage with other species.

Materials and Methods

Plant Material

Seed source

Harvesting shoot tips excised from *in vitro* established shoot cultures of specific genotypes (Chapter 2) is very time consuming. Therefore, *in vitro* germinated seeds collected from specific populations were used to more efficiently provide a large supply of easily harvestable shoot tips. In October 2011, mature sea oats (*Uniola paniculata* L.) infructescences were harvested from Delnor-Wiggins Pass State Park, Naples, Florida (DWP) and Little Talbot Island State Park, Jacksonville, Florida (LTI) (Figure 2-1). Seeds were dried for 1.5 weeks, mechanically cleaned by SeedClean (Sarasota, Florida), and stored in darkness in vacuumed sealed bags at 9 °C until needed. Seeds used in this study originated from DWP unless otherwise noted.

Seed surface sterilization and *in vitro* germination

Seeds were presoaked in 20 mL scintillation vials containing 15 mL aqueous 100 mgL⁻¹ gibberellic acid (GA₃) solution for 45 hr at 9 °C in a dark refrigerator. A 0.1% (v/v) solution of Plant Preservative Mixture (Plant Cell Technology Inc., Washington, D.C.) was added to the presoaking solution due to observations of bacterial contamination. Seeds were removed from GA₃ and placed in sterile scintillation vials containing 50% (v/v) ethanol for 0.5 min. The seeds were then agitated in 3% (v/v) sodium hypochlorite containing 2 drops Tween 20 (per a 150-200 mL volume) for 13 min. Following sterilization, seeds were rinsed three times with sterile water and then approximately 1

mm of the distal end of each seed was removed with a sterile scalpel (Figure 3-1b). Seeds were inoculated horizontally into 100 mm x 15 mm Petri plates containing sea oats germination medium, consisting of ½ strength Murashige and Skoog basal mineral salts (Cat No. M-524 *PhytoTechnology* Laboratories, Shawnee Mission, KS), 30 gL⁻¹ sucrose, 100 mgL⁻¹ myo-inositol, and 0.4 mgL⁻¹ thiamine-HCl, and solidified with 7 gL⁻¹ Plant TC Agar (Cat. No. A-111 *PhytoTechnology* Laboratories, Shawnee Mission, KS). The medium was adjusted to pH 5.7 using 0.1 M KOH before adding the agar. Medium was autoclaved in 1 L volumes in 2 L Erlenmeyer flasks at 117.7 kPa for 40 min at 121 °C. The medium was allowed to cool for 30 min (≈40 °C). Approximately 25 mL of medium was poured into each Petri plate. Seeds were germinated for 4 days in an incubator set at 35 °C with constant light supplied by Philips fluorescent lights (F17T8/TL741 Alto) at 65 μmol·m⁻²·s⁻¹ PPF.

Seedling preculture

Germinated seedlings (Figure 3-1c) were separated from the testa and endosperm with a sterile scalpel (Figure 3-1d) then transferred into baby food jars (BFJ) containing 30 mL preculture medium. This medium consisted of 0.3 M sucrose solidified with 8 gL⁻¹ high-grade TC Agar (Cat. No. A-175 *PhytoTechnology* Laboratories, Shawnee Mission, KS). Seedlings were cold acclimated in an incubator under an 8-hr photoperiod supplied by General Electric cool white fluorescent lights (F96T12·CW·WM·ECO) at 70 μmol·m⁻²·s⁻¹ PPF with 22 °C day and 0 °C night for 2 weeks. The shoot tips (1 mm – 1.5 mm) were excised from each cold acclimated seedling (Figure 3-1e) (Appendix A).

Cryopreservation Procedures

Vitrification

Cryogenic protocol optimization. To determine if certain steps from the standard protocol used in Chapter 2 were affecting post-cryostorage survival, steps were systematically eliminated and percent survival was compared. The osmoprotectant, PVS2, one rehydration step, and both rehydration steps were each eliminated from the standard protocol. Initial survival and regrowth evaluation procedures were the same as the standard protocol. Based on the results found, one 20-min rehydration step was eliminated from the standard protocol for the remaining vitrification experiments.

Effect of PVS2 exposure time. PVS2 exposure times following the initial 5 min ranged between 10 and 120 min to determine how long shoot tips must be exposed to PVS2 for optimal post-cryostorage survival. A treatment without any PVS2 exposure was also included. Initial survival and regrowth procedures were the same as the standard protocol.

Effect of plant vitrification solution type. Due to the phytotoxic effects of dimethylsulfoxide (DMSO), alternative plant vitrification solutions (PVSs) have been developed as potential replacements for PVS2 (Turner et al. 2001; Nishizawa et al. 1993). Three other plant vitrification formulations, PVS3, A9, and B5, were compared for post-cryostorage survival. Each solution replaced PVS2 in the modified standard protocol. PVS3 contains 50% (w/v) glycerol and 50% (w/v) sucrose in a liquid MS solution. A9 contains 30% (w/v) glycerol, 20% (w/v) DMSO, 20% (w/v) ethylene glycol and 15% (w/v) sucrose in a liquid MS solution. B5 contains 40% (w/v) glycerol and 40%

(w/v) sucrose in a liquid MS solution. Initial survival and regrowth procedures were the same as the standard protocol.

Effect of dimethylsulfoxide concentration. The post-cryostorage survival of shoot tips pretreated in modified PVS2 solutions with various concentrations (0%, 5%, 7.5%, 10%, and 15%) of DMSO was examined. Initial survival and regrowth procedures were the same as the standard protocol.

Effect of a dark culture period following cryostorage. Recovered meristems in BFJ were placed in a constant dark incubator set at 24 °C for 0, 3, 7, or 12 days. After the dark period, BFJs were transferred to an incubator to complete 4 weeks of initial survival under a 16-hr photoperiod provided by General Electric cool white fluorescent lamps (F96T12·CW·WM) at $45 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PFF at 24 °C, henceforth referred to as standard growing conditions. Following the transfer to standard growing conditions, initial survival and regrowth procedures were the same as the standard protocol.

Effect of cold acclimation pretreatment time with two populations. Two sea oats populations, LTI (north Florida) and DWP (south Florida), were chosen to study the effect cold acclimation period had on shoot tip survival following cryostorage. Sea oats seedlings with the endosperm removed were cold acclimated for 0 to 4 weeks. Initial survival and regrowth procedures were the same as the standard protocol.

Encapsulation-dehydration

An alternative approach to using the often phytotoxic dehydrating solutions used in the vitrification protocol is to dehydrate alginate encapsulated shoot tips via evaporation. Therefore, encapsulation-dehydration cryostorage procedures were followed as described with bermudagrass (Reed et al. 2006; Figure 1-3). Excised shoot

tips were placed onto sterile alginate gel in 100 mm x 15 mm Petri plates. The alginate gel consisted of a modified MS solution lacking calcium and supplemented with 0.75 M sucrose and 30 gL⁻¹ low-viscosity alginic acid (Cat. No. A0682, Sigma Chemical Company). Using a sterile plastic pipette, excised meristems were slowly drawn up with the alginate and dropped into a MS solution supplemented with 100 mM CaCl₂ and 0.75 M sucrose after which the alginate bead was allowed to polymerize for 20 min.

Effect of dehydration time. Encapsulated shoot tips (4-5 mm diameter) were transferred to a MS solution supplemented with 0.75M sucrose for 20 hr on a rotary shaker (50 rpm). Excess sucrose solution was decanted and beads were dehydrated on sterile germination paper (Cat. No. SD3.5 Anchor Paper, Saint Paul, MN) on an uncovered Petri plate top in a laminar flow hood (0.46 m·s⁻¹ airflow) at ambient room temperature (≈22 °C). At various dehydration durations (0, 2, 4, 8, 12 hr), beads containing 1 or 2 shoot tips each were transferred into 1.2 mL cryovials (5 beads/cryovial) and plunged into LN for 60 min. Encapsulated shoot tips in cryovials were thawed at room temperature (≈22 °C) for 20 min. One mL of filter-sterilized liquid MS was added for 10 min. The solution was removed and the beads were transferred to BFJ containing 30 mL initial survival medium (Appendix A) and maintained in standard growing conditions for 4 weeks. All shoot tips were removed from the beads and transferred to BFJ containing 30 mL Stage II sea oats shoot multiplication medium (SMM). This medium consisted of MS with 30 gL⁻¹ sucrose, 100 mgL⁻¹ myo-inositol, 0.4 mgL⁻¹ thiamine-HCl, and 0.25 mgL⁻¹ benzyladenine and solidified with 7 gL⁻¹ high-grade TC agar. The medium was adjusted to pH 5.7 using 0.1 M KOH before adding the agar and autoclaved in 30 mL volumes in BFJ at 117.7 kPa for 20 min at 121 °C. Shoot tips

were maintained in an incubator under standard growing conditions for an additional 4 weeks. Percent survival was recorded after each 4-week interval.

At each dehydration interval, 20 beads without shoot tips were weighed (fresh weight), dried at 110 °C for at least 16 hr, and weighed again (dry weight). Bead moisture content (MC) was determined using the following formula.

$$\text{Moisture Content (MC)} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100\%$$

Effect of sucrose pretreatment concentration and dehydration time.

Encapsulated shoot tips (4-5 mm diameter) were transferred to a MS solution supplemented with various sucrose concentrations (0.5 M, 0.75 M, 1.25 M) for 20 hr on a rotary shaker (50 rpm). Excess sucrose solution was decanted and beads were dehydrated on sterile germination paper (Cat. No. SD3.5 Anchor Paper, Saint Paul, MN) in an uncovered Petri plate top in a laminar flow hood (0.46 m·s⁻¹ air flow) at ambient room temperature (≈22 °C). At various dehydration durations (0, 4, 8, 12 hr), beads containing 1 or 2 shoot tips each were transferred into 1.2 mL cryovials (5 beads/cryovial) and plunged into LN for 60 min. Recovery, regrowth, and MC procedures were carried out as previously described.

Experimental Design and Statistical Analysis

Each treatment consisted of eight replicate cryovials each containing five shoot tips or beads. Greening and shoot tip elongation were both required for samples to qualify as being fully recovered from cryopreservation. Experiments were repeated once. Statistical differences between data were analyzed using analysis of variance (ANOVA) using the generalized linear model procedures. The data are presented as percentages with means separation using Duncan's Multiple Range Test (DMRT) for all

experiments except the final encapsulation-dehydration factorial experiment, where Tukey-Kramer's Range Test $\alpha = 0.05$ was utilized (SAS Institute 2009).

Results

Excised shoot tips from germinated seedlings were used to study the effects of modifications to the standard vitrification and encapsulation-dehydration methods in an effort to optimize specific cryopreservation steps (Figure 3-1). Use of seedlings probably reflects the general response of the population to cryostorage with 40 genotypes per treatment. Shoot tips were more easily excised from germinated seedlings than from shoots of established *in vitro* cultures. The results generated from the multiple experiments in this study revealed that survival following vitrification and encapsulation-dehydration cryopreservation methods is possible using sea oats seedling shoot tips; however, survival was low regardless of protocol modification. Shoots developed from the excised seedling shoot tips without intermediate callus formation (Figure 2-4). Initial survival and regrowth are presented to provide a progression of sea oats' survival following cryopreservation with an overall trend of regrowth being the same or lower than initial survival.

Vitrification Cryopreservation Protocol Development

Cryogenic step optimization

When a standard protocol (Figure 2-3) was applied to cryostored excised seedling shoot tips, 20% initial survival and 11.7% regrowth was observed (Figure 3-2). Sea oats shoot tips displayed significantly different survival responses when steps were systematically eliminated from the standard protocol. No initial survival and regrowth of shoot tips following LN was observed when the PVS2 step was eliminated (Figure 3-2). Initial survival and regrowth following cryostorage was significantly lower than the

standard protocol when both rehydration steps were eliminated (2.7% and 1.9%, respectively). However, when the PVS2 step was eliminated, initial survival (80%) and regrowth (60%) without LN (control) was significantly higher than all other non-frozen or frozen shoot tips. No significant difference from the standard protocol was observed in initial survival or regrowth when the osmoprotectant step (15% and 8.3%, respectively) or when only one rehydration step (23% and 11.1%) was eliminated. Based on these results, PVS2 and at least one rehydration step are necessary to maintain higher survival rates after LN. However, for a more succinct protocol, one rehydration or the osmoprotectant step could be eliminated without significant loss in survival (Figure 3-11).

PVS2 exposure time

Following the results from the previous experiment, the second rehydration step of the standard protocol was eliminated for all subsequent experiments. Since the elimination of PVS2 resulted in no survival following LN, the time of PVS2 exposure, and therefore the dehydration time, was further studied. PVS2 exposure times of 40 min and 60 min, prior to LN, yielded the highest initial survival (26.7% and 35.6%, respectively) and regrowth rates (22.9% and 25.8%, respectively) (Figure 3-3). Furthermore, an exposure time of 120 min significantly decreased shoot tip initial survival (6.7%) and regrowth (4.4%). A significant difference in regrowth was observed between no PVS2 exposure and only 10 min exposure, decreasing from 69.2% regrowth to 41.1% when not cryopreserved. Survival without LN decreased as PVS2 exposure time increased, showing sensitivity to PVS2. However, survival with LN increased up to 60-min exposure to PVS2. Based on these data, a 40- to 60-min PVS2 exposure should be used before cryostorage of sea oats shoot tips (Figure 3-11).

Effect of plant vitrification solution

Shoot tip regrowth was significantly affected by the type of plant vitrification solution used. When non-DMSO containing vitrification solutions (PVS3 and B5) were used, almost no survival was observed following LN after a 40-min dehydration period (Figure 3-4). Initial survival and regrowth of shoot tips pretreated with PVS3 and B5 were both significantly lower than PVS2 and A9. Shoot tips pretreated with PVS3 and B5, plant vitrification solutions containing only sucrose and glycerol, displayed 2.5% and 1.1% regrowth following LN, respectively. Additionally, when recovered from LN, shoot tips treated with PVS3 and B5 required approximately one more minute of rapid re-warming compared to PVS2 and A9. Shoot tips pretreated with vitrification solutions containing DMSO, PVS2 and A9, displayed statistically similar and relatively higher regrowth (23.8% and 29.2%, respectively). Initial survival was different among the vitrification solutions screened, but regrowth was statistically the same, ranging between 23.6% and 34.2% when shoot tips were not exposed to LN (control). Results for the regrown control shoot tips suggested that the differing cryoprotectants alone had no effect on the survivability of the shoot tips. It is recommended that PVS2 or A9 should be used as the vitrification solution to ensure survivability, albeit survival of shoot tips remained low (Figure 3-11).

Effect of dimethylsulfoxide concentration

Based on the previous experiment, DMSO appears to be advantageous for effective plant tissue desiccation and post-LN survival. Therefore, the effects of DMSO concentration in the plant vitrification solution were studied further. Non-frozen shoot tips (-LN) exhibited similar initial survival (51.4% - 64.1%) and regrowth (14.3% - 24.6%) (Figure 3-5). A significant increase in post-LN regrowth of shoot tips was observed

when the vitrification solution contained 15% DMSO (19.5%) compared to those treated with $\geq 7.5\%$ DMSO. A similar pattern of increased survivability with increasing DMSO concentrations was observed for initial survival and regrowth. These results suggest that a DMSO concentration of at least 15% should be used to ensure higher survivability and regrowth following cryostorage.

Effect of a dark period following cryostorage

A dark period was applied during the culturing of shoot tips on initial survival medium in an attempt to increase regrowth post-LN. The trend of initial survival results was similar to the trend exhibited by regrowth (Figure 3-6). Twelve days dark culture did not result in significantly higher initial survival (48.8%) than 0 days (35%) for shoot tips exposed to LN. Likewise, there were no significant differences in post-LN regrowth (10% - 28.1%) regardless of dark treatment. Based on these results, maintaining thawed (post-LN) sea oats shoot tips in the dark before incubation under standard growing conditions provides no advantage for regrowth.

Effect of cold acclimation period with two populations

Preliminary studies demonstrated that one week of CA did not result in higher regrowth of sea oats shoot tips compared to those without CA (Appendix B). Therefore, seedling shoot tips from two populations, DWP and LTI, were subjected to up to four weeks CA to determine if latitudinal source had an effect on CA effectiveness. South Florida DWP shoot tip initial survival was highest after 1 week (70%) and 2 weeks (71.1%) CA relative to the other treatments when not frozen (-LN) (Figure 3-7). However, control DWP shoot tips exhibited similar regrowth (28.9% - 35%). When cryopreserved, DWP shoot tip initial survival (11.3% - 29.3%) and regrowth (11.9% - 18.6%) were not significantly different among the CA durations (Figure 3-7).

Similar survival results were observed with excised seedling shoot tips from the north Florida LTI population (Figure 3-8). LTI initial shoot tip survival was higher for 1 week (58.2%), 2 weeks (77.8%), and 3 weeks (64.9%) CA than the other treatments when not frozen (-LN) (Figure 3-8). However, control LTI shoot tips exhibited significantly similar regrowth (38.3% - 60%). When cryopreserved, LTI shoot tip initial survival (20% - 38%) and regrowth (18.7% - 32.6%) were not significantly different among the CA durations. Therefore, cold acclimation did not improve regrowth of cryopreserved sea oats seedling shoot tips from either population since statistically similar regrowth was observed.

Encapsulation-Dehydration Cryopreservation Protocol Development

Effect of dehydration time

Encapsulated shoot tips were not able to survive LN without air flow dehydration (Figure 3-9). Unfrozen encapsulated shoot tips (-LN) displayed high initial survival following dehydration up to 4 hr (57% - 59.6%) but survival significantly decreased following longer dehydration periods. Regrowth of unfrozen shoot tips was similar (20% - 37.2%) except following 8 hr dehydration, which decreased to 15%. Cryopreserved shoot tips dehydrated to a bead moisture content (MC) between 19.6% - 27.2% showed a similar trend for initial survival (34.6% - 41.2%) and regrowth (27% - 32.9%). Therefore, at least 4 hours of dehydration, to approximately a 20% bead moisture content, was necessary for shoot tips to recover from cryostorage.

Effect of sucrose pretreatment concentration and dehydration time

There was a significant interaction of sucrose concentration and dehydration time on shoot tip regrowth with and without LN (Table 3-1) when using the encapsulation-

dehydration method. Bead moisture content followed a similar trend with a significant decrease from 0 hr (63.9% - 89.6%) to 4 hr (24.5% - 25.8%) dehydration (Figure 3-10). A general trend observed was that as dehydration duration increased at all sucrose concentrations, unfrozen (-LN) shoot tip initial survival and regrowth decreased (Figure 3-10).

Similar trends between initial survival and regrowth were observed in cryopreserved shoot tips. Higher initial survival was observed with the following combinations: 0.75 M sucrose pretreatment and dried for 4 hours (38.6%) and 1.25 M sucrose pretreatment and dried for 4 hours (33.3%). Higher shoot tip regrowth was observed following 0.25 M sucrose pretreatment and drying for 8 hr (20.5%), 0.75 M sucrose pretreatment and dried for 4 hours (28.6%) and 8 hours (19.9%), and 1.25 M sucrose pretreatment and dried for 4 hours (19.9%) (Figure 3-10). These combinations of sucrose pretreatment and dehydration time correlated with MC ranging between 16.5% and 25.8%. Regardless of sucrose concentrations and bead drying time, shoot tip recovery could not be increased over the low survival observed when using the standard vitrification method with *in vitro* derived shoot tips (Chapter 2).

Discussion

Excised seedling shoot tips from the cryo-sensitive population Delnor-Wiggins Pass were screened using various substitutions and modifications to the standard protocol in order to improve cryopreservation success for sea oats (Figure 2-3). An earlier study of sea oats shoot tip cryopreservation displayed marked differences between genotypes and source population (Figures 2-5 and 2-6). Shoot tips from Gamble Rogers were not used in this study because of the low seed germination rates in a preliminary study (data not shown). The use of germinated seedling shoot tips

rather than those derived from *in vitro* propagated clones took advantage of the genetic variability of that population for cryopreservation studies.

Vitrification Cryopreservation Protocol Development

Cryogenic protocol optimization

A standard protocol was developed by compiling successful cryopreservation protocols from the literature (Figure 2-3). Modifications to this standard protocol were applied to determine if all steps were necessary to maintain or improve the survival following cryostorage. Elimination of the PVS2 step (the dehydration step) was completely detrimental to the survival of shoot tips following LN (Figure 3-2). This was expected since shoot tip intracellular water must be sufficiently removed and replaced by small molecules such as glycerol, DMSO, and glycols to avoid lethal freezing during rapid cooling (Pegg 2007). Similarly, when not exposed to a dehydration step, embryogenic cell suspensions of *Asparagus officinalis* exhibited no survival following thawing, but survival significantly increased to 60% after 5 min exposure to a dehydration solution (Nishizawa et al. 1993). This response was likely due to decreased intracellular water and increased cryoprotectant infiltration. Similarly, when not exposed to PVS2, *Malus domestica* cv. Fuji shoot tips exhibited no shoot formation, but this increased to 20% after a 30-min exposure (Niino et al. 1992). Therefore, a dehydration step is required for post-cryostorage survival. In the current study, the highest initial survival (80%) and regrowth (60%) were observed when the dehydration step was eliminated and the shoot tips were not placed into LN. With no regrowth following LN and high regrowth without LN (control), it is suggested that the shoot tips were not adequately dehydrated to survive rapid cooling (B. Reed, personal communication).

As anticipated, elimination of all rehydration steps following LN led to a significant loss in seedling shoot tip regrowth (1.9%) compared to the standard protocol (11.7%) (Figure 3-2). A significant loss was also observed without LN with regrowth decreasing from 28.8% to 11.2%. Many, if not all, vitrification protocols require a rehydration step following LN (Barraco et al. 2011b; Moges et al. 2004; Reed 2008; Vujović et al. 2011). The loss in survival by cryopreserved and non-cryopreserved sea oats shoot tips could be attributed to the cells inability to survive without direct rehydration following the osmotic dehydration resulting from PVS2 exposure. There are no published studies looking at the mechanism that direct rehydration has on recovery, but Pegg (2007) contends that cryoprotectants are not removed if tissues are not exposed to a rehydrating solution. Therefore, the phytotoxic compound DMSO could remain in the cells throughout the recovery process resulting in further damage (Reed and Uchendu 2008).

PVS2 exposure time

Since the elimination of the PVS2 step resulted in the complete loss of survival following LN, the exposure time to PVS2 was further studied. Regrowth gradually increased with increased PVS2 exposure time with maximum regrowth observed after a 40- and 60-min exposure (22.9%, 25.8%, respectively) (Figure 3-3). However, a significant drop in regrowth (4.4%) was observed after a 120-min exposure time. Exposure time of plant tissues to PVS2 has a significant impact on survival following cryostorage (Sakai et al. 2008). Following PVS2 exposure, Volk and Walters (2006) reported that shoot tips of the garlic cultivar “German Extra Hardy” and *Mentha x piperita* L. cultivar “Todd’s Mitcham Peppermint” lost the same water mass as the shoot tips gained in cryoprotectant (0.5 mgL⁻¹ for garlic and 0.25 mgL⁻¹ *Mentha*). Therefore,

cryoprotectants dehydrate cells by replacing intracellular water. Longer exposure times would then result in further tissue dehydration but would lead to a higher probability of injury by chemical toxicity or over-dehydration (Niino et al. 1992; Pennycooke and Towill 2000).

A similar decrease in survival with longer PVS2 exposure durations was reported in *Malus domestica* cv. Fuji (Niino et al. 1992). Shoot tip regrowth increased steadily with increased PVS2 exposure up to a maximum regrowth (~80%), but decreased with longer durations. Additionally, survival of *Ipomoea batatas* shoot tips significantly increased from 10% to 66% when the exposure time to PVS2 increased from 10 min to 16 min, but decreased from 66% to 20% after a 26-min exposure (Pennycooke and Towill 2000).

Although no survival was observed without PVS2 exposure, it is interesting that there was a significant decrease in survival of control shoot tips between 0 min and 10 min durations. Once exposed to PVS2, control shoot tips displayed the same survival for initial survival and regrowth. If longer exposure durations result in higher injury, this would probably have been apparent in the controls, but it was not. A decrease with longer PVS2 exposure durations was seen following cryostorage, but not with the controls (-LN). Thus, the difference between shoot tips exposed to LN after longer PVS2 exposure times should be studied. Nonetheless, adequate exposure to PVS2 must occur before optimal survival rates are achieved, but an over-exposure could cause widespread irreparable damage prior to LN leading to a decrease in survival (Niino et al. 1992). Therefore, tissue sample exposure time to PVS2 must be precisely timed to avoid potential under- or over-dehydration. Utilizing sea oats genotypes with high (PK

genotypes) and low tolerance (DWP genotypes) to cryopreservation may prove useful in better elucidating the influence of dehydration.

Plant vitrification solution type

Some plant species are sensitive to the phytotoxic compound DMSO in PVS2, and display higher survival following pretreatment with other vitrification solutions using other cryoprotectants (Nishizawa et al. 1993; Vujović et al. 2011). In an attempt to use a less toxic vitrification solution, Nishizawa et al. (1993) used PVS3 and reported 80% survival following LN. Vujović et al. (2011) reported higher regrowth (70%) with PVS3 than with a slightly modified PVS2 solution. However, sea oats seedling shoot tips pretreated with plant vitrification solutions containing DMSO prior to LN displayed significantly higher regrowth (23.8% – 29.2%) than those without DMSO (1.1% – 2.5%) (Figure 3-4). When developing a vitrification protocol for *Anigozanthos viridis* ssp. *terraspectans*, PVS3 pretreated shoot tips resulted in significantly lower survival (15%) than PVS2 (75%) (Turner et al. 2001). Shoot tips pretreated with the four types of plant vitrification solutions exhibited the same regrowth without LN (control) in this study. This suggests that all of the solutions resulted in a similar ultimate decrease in regrowth, but that DMSO and/or ethylene glycol, found in PVS2 and A9, could provide advantages when placed into LN. The mechanism by which different vitrification solutions protect cells from damage is poorly understood (Fuller 2004).

Studies are limited on DMSO effects on plant cells. However, Delmer (1979) reported that DMSO increased the permeability of plasma membranes in tobacco cells to small molecular weight molecules. This increased permeability may allow cryoprotectants to replace intracellular water and increase the solute concentration in the cells. Increased solute concentration has been shown to inhibit ice crystallization

and to stabilize membranes during freezing (Volk 2010). Consequently, the inclusion of DMSO, as in PVS2 and A9, may be required to increase permeability of plasma membranes in sea oats cells to facilitate cryoprotectants to replace intracellular water to suppress lethal ice crystallization and plasma membrane phase changes.

The significant decrease in regrowth (<40%) of all non-cryopreserved shoot tips (-LN) was unexpected. In an eloquent study, Volk et al. (2006) treated *Mentha x piperita* shoot tips with one-, two-, three-, or four-component solutions containing glycerol, ethylene glycol, DMSO, and/or sucrose (all components of PVS2). It was noted that any solution containing glycerol would significantly decrease shoot tip survival following cryopreservation. Besides sucrose, glycerol was a common cryoprotectant in all tested solutions. Therefore, a step-wise examination of the individual effects and combinations of cryoprotectants on shoot tip survival of sea oats shoot tips is warranted.

Effect of dimethylsulfoxide concentration

PVS DMSO concentration on cryostorage survival was further studied given that sea oats shoot tips pretreated only with DMSO-containing solutions survived cryostorage and the conflicting reports that DMSO often has a phytotoxic effect. Following cryostorage, shoot tips exhibited an increase in survival from 1.4% regrowth with 5% DMSO to 19.5% regrowth with PVS2 [15% DMSO] (Figure 3-5). However, this increase was not observed when *Chrysanthemum cinerariaefolium* shoot tips pretreated with 7.5% DMSO exhibited the highest survival (61%) whereas tissue treatment with 10% DMSO resulted in the lowest survival (Hitmi et al. 2000). Similarly, shoot tips of *Saintpaulia ionantha* exhibited decreased post-LN regrowth (80% to 65%) when 30% DMSO was used for the dehydration solution compared to PVS2 containing 15% DMSO (Moges et al. 2004).

DMSO, a penetrating compound, allows non-penetrating sugar alcohols, such as glycerol, to move into cells for the stabilization and protection of membranes and cytoskeletons and to decrease the cellular freezing point (Reed 1996). Shoot tips pretreated in A9 solution (20% DMSO) displayed the highest regrowth (29.2%) when compared to the other vitrification solutions described earlier (Figure 3-4). Tobacco cells displayed higher levels of released intracellular molecules with an increase in DMSO concentration due to the increased permeability of the plasma membranes (Delmer 1979). The increasing DMSO concentration could allow a higher concentration of cryoprotectant to enter the plant cells and result in more effective membrane and cytoskeleton stabilization and protection (Volk 2010). Therefore, sea oats may require higher DMSO concentrations for the cryoprotectants to effectively penetrate and protect shoot tip cells prior to rapid freezing, but not so much as to negatively affect the survival.

Dark culture period following cryostorage

Attempts to increase regrowth following LN by including a dark period during recovery from LN were unsuccessful (Figure 3-6). No significant difference was observed in regrowth of sea oats shoot tips following any dark culture period up to 12 days. Successful protocols including a dark culture period after post-LN thawing have been reported for sweet potato (Pennycooke and Towill 2000), *Vitis vinifera* (Wang et al. 2002), sugarcane (Paulet et al. 1993; Barraco et al. 2011) and orchid cell suspensions (Tsukazaki et al. 2000). However, no explanation is provided in any of these papers as to why recovered plant tissues were placed in the dark for the first few days post-cryostorage. Engelmann et al. (2008) stated that a dark period post-warming is beneficial for organized tissues such as meristems and shoot tips. Some authors

suggest that dark culture during recovery may improve shoot tip survival by limiting lethal photo-oxidative effects resulting from osmotic and freezing stresses (Benson 1990; Paulet et al. 1993). *Brassica napus* shoot tips showed increased singlet oxygen activity during early recovery, which was stimulated by exposure to light (Benson 1990). Lynch et al. (1994) cites this rationale for conducting all recovery manipulations in the dark for rice cryopreservation. However, sea oats shoot tips cultured in the dark for up to 12 days did not display any difference in regrowth compared to shoot tips directly placed into standard growing conditions with a 16-hr photoperiod (Figure 3-6). Sea oats shoot tips may not need this dark period to survive, or could require a longer period in the dark to further limit photo-oxidation while the shoot tips recover from desiccation and freezing stresses.

Johnston et al. (2007) considered antioxidant status of a genotype and species to determine cryogenic stress tolerance and ultimate survival. The authors studied two types of *Ribes* species, a cryo-sensitive and a cryo-tolerant variety, and reported that the cryo-tolerant variety accumulated higher levels of antioxidants during a sucrose-stimulated cold acclimation period than the cryo-sensitive variety. Cryogenic survival was increased from 40% and 50% to 90% when exogenous antioxidants (i.e., Vitamin C and D) were successfully applied to *Rubus* genotypes (Uchendu et al. 2010). If reactive oxygen species are a limiting factor for sea oats genotypes and/or populations to survive cryostorage, studying the variability of antioxidant accumulation between a cryo-tolerant and a cryo-sensitive population may provide some evidence for specific procedural manipulations, such as the addition of antioxidants.

Cold acclimation pretreatment time with two populations

Cold acclimation has been shown effective, and most times necessary, to increase post-cryostorage survival in many species (Reed 2008; Benson 2008). However, inclusion of a CA regime of a 22 °C 8-hr photoperiod and 0 °C dark cycle did not significantly increase sea oats shoot tip regrowth post-LN (Figures 3-7 and 3-8). After up to four weeks of CA, no significant difference was observed between seedlings that were exposed to CA and those that were not exposed to CA. Two populations (north and south Florida) were screened to determine if local climate factors could result in differing CA requirements, but no significant difference was observed within each population. Although in the minority, protocols have been developed without incorporating a cold acclimation step. These include cryopreservation protocols for *Limonium* sp. (Barraco et al. 2011b), *Wasabia japonica* (Matsumoto et al. 1994), and *Saintpaulia ionantha* (Moges et al. 2004).

Cold acclimation acts as a natural protection against possible ice crystallization through an increased cellular osmolyte concentration and facilitated protein and membrane stabilization (Kaczmarczyk et al. 2011; Sung et al. 2003). Increased cellular solute concentration decreases the freezing temperature of the cell, but does not provide sufficient defense from ultra-low cryogenic temperatures alone (Volk 2010). In response to cold temperatures, the phospholipid and unsaturated fatty acid concentration increases in the plasma membrane to prevent an undesirable membrane phase change common at low temperatures (Uemura et al. 2006). An increase in phospholipids creates a more fluid membrane that can survive the severe desiccation cells experience during the cryopreservation procedure.

In this study, sea oats shoot tips that underwent CA did not exhibit higher rates of survival compared to those that did not. Many factors must be considered to interpret these unexpected results. Sea oats may not require a CA pretreatment to provide additional cryoprotection if the cryoprotectants are sufficient. Or, sea oats may require more than four weeks of CA to display significant gains in survival. *Pyrus cordata* meristems increased regrowth from 19% after 4 weeks to 42% after 8 weeks CA (Chang and Reed 2000). Scottez et al. (1992) reported that *Pyrus communis* cv. Beurre Hardy shoot tips required 12 weeks CA to attain greatest shoot recovery (82%). Moreover, a rapid increase of recovery was observed after increasing CA from 0 weeks (2%) to 2 weeks (44%).

The CA regime used in this study may not be optimal for sea oats to initiate low temperature responses like increased cellular solute concentrations and membrane stabilization. The oscillating regime chosen for this study was shown to be suitable for many plant species such as pear (Chang and Reed 2000); *Zoysia* sp. and *Lolium* sp. (Chang et al. 2000); and yams (Leunufna and Keller 2005). In the future, a constant low temperature should be tested as the CA pretreatment on sea oats seedlings instead of the oscillating regime chosen for the current study. This constant low temperature, usually around 5 °C, has been shown to be suitable for species such as strawberry (Hirai et al. 1998); apple and pear (Niino et al. 1992); and *Trifolium repens* (Yamada et al. 1991). Sea oats may respond differently if a constant low temperature was used for cold acclimation. Finally, Reed (1996) stated that CA is most effective for temperate species and could be completely ineffective for tropical species. Therefore, sea oats, a sub-tropical grass (depending on population source), may not be responsive to CA

treatments intended to improve post-cryogenic survival. Comparison of physiological differences with more cryostorage-tolerant populations such as PK may be productive.

Encapsulation-Dehydration Cryopreservation Protocol Development

Encapsulation-dehydration cryopreservation methods deviate from vitrification methods by first encapsulating the tissue in alginate gel followed by evaporative desiccation before plunging into LN (Engelmann et al. 2008). Cryopreservation of sea oats shoot tips was recorded using encapsulation-dehydration. The highest regrowth observed was 32.9% following 12 hours dehydration coupled with 0.75 M sucrose pretreatment. This pretreatment yielded a 19.6% MC prior to LN (Figure 3-9).

A sucrose pretreatment is applied prior to evaporative dehydration to accumulate sucrose inside the bead and shoot tip to increase dehydration tolerance (Al-Ababneh et al. 2002). This osmotic dehydration and accumulation of solutes result in the maintenance of cell membrane structure during evaporative dehydration and rapid freezing. Quicker osmotic dehydration is apparent with significant drops in MC with increasing sucrose concentrations without laminar flow hood dehydration (89.6% for 0.25 M, 76.4% for 0.75 M, and 63.9% for 1.25 M) (Figure 3-10). Similar decreases in MC following an increase in sucrose concentration were reported by Al-Ababneh et al. (2002). MC dropped from 85.8% (0.3 M) to 82.4% (0.5 M) to 74.9% (0.75 M) demonstrating that more osmotic dehydration, and a larger concentration of solutes in the bead, was apparent with higher sucrose pretreatment concentrations.

Encapsulated sea oats shoot tips pretreated with the lowest sucrose concentration, 0.25 M, required a longer dehydration time (8 hr) than those pretreated with the highest concentration (4 hr), 1.25 M, to achieve similar levels of regrowth (19.9% to 20.5%) (Figure 3-10). When pretreated with 0.1 M sucrose, encapsulated

pear shoot tips attained the highest recovery following 5 hours of dehydration (8%), but reached the highest level of recovery (35%) after only 2 hours of dehydration when pretreated with 2.0 M sucrose (Scottez et al. 1992). Encapsulated *Artemisia herba-alba* shoot tips pretreated with 0.5 M sucrose achieved highest regrowth after 4 hours of evaporative dehydration, but required less than 2 hours when pretreated with 1.0 M sucrose (Sharaf et al. 2012). Therefore, the results found in the current study are similar to others where lower sucrose pretreatment concentrations required longer periods of laminar air flow dehydration to achieve similar shoot tip survival.

Considering both cryopreserved (+LN) and control shoot tips (-LN), longer dehydration periods (over-dehydration) resulted in a decrease in initial survival and regrowth (Figure 3-10). Encapsulated pear shoot tips pretreated in 0.75 M sucrose resulted in 80% shoot recovery, but decreased to 60% and 20% shoot recovery using 1.0 M and 2.0 M sucrose pretreatments, respectively (Scottez et al. 1992). Similarly, encapsulated *Ceratopetalum gummiferum* shoot tips exposed to 6 hr dehydration displayed 54.6% regrowth. However, exposure to 7 hr dehydration decreased regrowth to 31.6% (Shatnawi and Johnson 2004).

Similar to over-dehydration, under-dehydration resulted in lower regrowth. When under-dehydrated, no regrowth of sea oats shoot tips was observed. More than 2 hours dehydration was required for regrowth post-LN (Figures 3-9 and 3-10). Similarly, no regrowth was reported for *C. gummiferum* shoot tips until after 3 hours of dehydration (Shatnawi and Johnson 2004). There was a significant interaction effect of sucrose concentration and dehydration time on the survival of sea oats shoot tips following

cryostorage (Table 3-1). This interaction effect has been reported with other encapsulation-dehydration studies (Al-Ababneh et al. 2002; Sharaf et al. 2012).

Determination of the bead moisture content prior to LN is an important step to encapsulation-dehydration cryopreservation. Similar to vitrification, the reason for desiccating the encapsulated shoot tips is to eliminate intracellular water that could potentially lead to lethal ice crystallization during freezing. Bead moisture content decreases as dehydration duration and sucrose pretreatment concentration increases (Martinez et al. 1999; Shatnawi and Johnson 2004). A MC of about 20% has been shown to be optimal for many plant species as exemplified by *Citrus aurantium* with 47% regrowth at 18% MC (Al-Ababneh et al. 2002), *Humulus lupulus* with 80% regrowth at 16% MC (Martinez et al. 1999), *Syzygium francissi* with 58.3% regrowth at 20% MC (Shatnawi et al. 2004), and *Rubus idaeus* with 65% regrowth at 17.2% MC (Wang et al. 2005). Maximum regrowth was similarly achieved with encapsulated sea oats shoot tips with 16.5% and 25.8% MC (Figure 3-10). This range appears to be consistent with published literature describing optimal MC values.

Surprisingly, some shoot tips with MC found within this optimal range had lower regrowth, suggesting that MC may not be the only factor affecting regrowth in sea oats. Scottez et al. (1992) reported MC of about 20% for all encapsulated pear shoot tips, but found shoot recovery ranging between 20% and 80%. Therefore, it could be suggested that the sucrose pretreatment concentration is a possible factor in shoot tip survival following cryopreservation. Therefore, with the precise combination of sucrose pretreatment concentration and dehydration time, regrowth for sea oats could be achieved.

Cryopreservation of excised shoot tips from germinated sea oats seedlings was possible using two different methods: vitrification and encapsulation-dehydration. Overall, it was demonstrated that sea oats seedling shoot tips could be cryopreserved with up to 32.9% regrowth, a relatively low regrowth. Conversely, *Wasabia japonica* apical meristems resulted in up to 100% shoot formation following vitrification protocol optimization with LN (Matsumoto et al. 1994). Moges et al. (2004) achieved 75% post-cryostorage shoot tip regrowth for encapsulation-dehydration and 90% regrowth for vitrification protocol optimization using *Saintpaulia ionantha*. Niino et al. (1992) reported up to 80% shoot formation following LN in an effort to determine optimal cold acclimation periods for *Malus domestica* cv. Fuji shoot tips. Therefore, further studies are warranted to achieve higher survival rates following cryostorage in sea oats.

In initial cryostorage experiments using the standard vitrification protocol (Chapter 2), excised shoot tips from *in vitro* shoot cultures of three DWP genotypes displayed significantly lower regrowth (Figures 2-5 and 2-6) than those of PK genotypes. Consequently, considerable effort was directed toward increasing post-LN regrowth of seedling shoot tips originating from the DWP sea oats population by attempting to systematically optimize or eliminate the sequential steps of the standard protocol (Figure 2-3). Use of seedling derived shoot tips not only increased the ease at which shoot tips could be excised, it also increased the number of genotypes screened (40/treatment) which may enable a more accurate representation of the freezing tolerance of the DWP population. The use of different genotypes could be a reason for the high variability observed in some experiments and the differences observed between regrowth with encapsulation-dehydration when pretreating with 0.75 M sucrose

(Figures 3-9 and 3-10). However, the 40% regrowth established by Reed et al. (1998b) as the minimum for a successful cryopreservation protocol was not achieved with DWP.

The importance of looking at initial survival and regrowth is to ensure cryopreserved plants develop into normally functioning plants without a callus phase to maintain genetic integrity (Golmirzaie and Panta 1998). For both cryopreservation methods, regrowth was generally lower than initial survival for each treatment, as seen with other studies (Moges et al. 2004; Wang et al. 2005). Encapsulated *Artemisia herba-alba* shoot tips had significant decreases from initial survival (6% to 45%) to regrowth (0% to 6%) (Sharaf et al. 2012). Some treatments even decreased from 45% initial survival to 0% regrowth. For sea oats, some shoot tips that were green and elongating during the initial survival phase turned brown during the regrowth phase. Mortality of shoot tips between the time initial survival percentages and regrowth percentages are collected could be attributed to many possible explanations. Previously, the differences between initial survival and regrowth have been attributed to severe osmotic shock from dehydration, rehydration, or partial damage from ice crystallization during rewarming (Moges et al. 2004). Therefore, rapid warming and optimal rehydrating solutions could prevent initial survival and regrowth variability, if this was the sole underlying reason (Nishizawa et al. 1993). Another factor described that could affect differences seen between initial survival and regrowth rates is damage by photo-oxidation during initial survival (Benson and Noronha-Dutra 1988). Although not addressed in this study, longer dark culture periods and the screening of antioxidant accumulation should be considered. Volk (2010) acknowledges that our understanding

of the process of tissue recovery following cryostorage, especially protection from free radical damage, is still poorly understood.

Further studies should be directed toward optimizing the initial medium for cryopreserved shoot tips because cryopreserved shoot tips may respond differently than non-cryopreserved shoot tips. Excised *Castanea sativa* shoot tips that were 0.5 mm (36%) and 1 mm (25%) had significantly higher regrowth than 2 mm shoot tips (5%) due to the homogenous collection of small, actively growing cells in the meristem that make smaller shoot tips more tolerant to dehydration and cryostorage (Vidal et al. 2005). Conversely, 2 mm control shoot tips cultured on medium would have a better probability of elongating and surviving than smaller shoot tips. Likewise, Volk and Caspersen's (2007) observation that meristem cells of *Mentha x piperita* were the smallest and least plasmolyzed than larger cells in shoot tips when cryopreserved further support this. Larger cells in the excised sea oats shoot tips surrounding the meristem could be plasmolyzed. If too many dead cells surround surviving meristem cells, regeneration may be inhibited (Volk and Caspersen 2007).

Future studies should look at the duration recovered shoot tips are exposed to the initial survival medium before transferring onto SMM to assess regrowth. This aspect of recovery is rarely addressed by the published literature and could have profound effects on the ultimate survival of shoot tips. The 28-day culture period on the initial survival medium was selected as the time period to assess survival because it was the standard culture interval for sea oats in our laboratory. Time intervals for reporting survival vary greatly. Barraco et al. (2011b) chose 7 days following rewarming to assess initial survival and 8 weeks to determine regrowth. Niino et al. (1992) reported

two survival collection times with initial survival 20 days following thawing and regrowth after an additional 20 days. However, Hirai et al. (1998) chose 2 weeks following the removal from cryostorage to record shoot formation and did not have a second collection of survival results. Similarly, Matsumoto et al. (1994) decided to record shoot formation 21 days after plating thawed shoot tips without a second collection. Therefore, no standardized procedures exist for when to record shoot tip survival data and probably must be considered for each species depending on its capacity to recover.

Sea oats cryopreservation represents a new approach to storing sea oats genotypes for population-specific plant material for dune restoration. Although the maximum regrowth attained for DWP was low relative to other published research and another sea oats population (Perdido Key), cryostorage is still a possible long-term preservation method. Although, more shoot tips from DWP would need to be placed into cryostorage initially to ensure higher numbers of recovered shoot tips when thawed, it is very important to note that many shoots could be reliably propagated from each successfully recovered shoot tip using micropropagation. A more comprehensive screening of seedling shoot tips from populations across the geographic range of sea oats as far north as North Carolina is necessary to assess the occurrence of ecotypic differentiation for freezing tolerance. In addition, it would be beneficial to examine the relationship between freezing tolerance and field performance of sea oats genotypes. Such information is critical for addressing the selection of planting materials in consideration of climate change and the potential selection pressure of cryopreservation.

Finally, the results of this thesis research offer some exciting avenues for further studies to elucidate the fundamental physiological and molecular mechanisms controlling tolerance to cryostorage. The observation that sea oats genotypes, such as those from Perdido Key, display high post-LN recovery rates (Chapter 2) while others like DWP do not, offer contrasting freezing tolerance phenotypes to probe the mechanisms for tolerance to desiccation and freezing stress by combining physiological and genomic approaches (Volk 2010). The knowledge generated could provide novel and practical procedures to enhance recovery of typically freezing intolerant genotypes following cryostorage.

Cryopreservation of Sea Oats Seeds

Sea oats seed cryopreservation was also studied to compare two methods of storing genetic material. Although DWP sea oats seedling shoot tips had an overall low survival following cryostorage (<32.9% regrowth), preliminary studies show that intact DWP seeds exhibited high tolerance to survive cryostorage with little loss of germination (>80%) (Appendix C). With high germination percentages achieved with DWP seeds spanning a wide range of relative humidities (0.5% - 91%), sea oats seed cryopreservation is a viable and efficient method of preserving sea oats genetic diversity.

Although the process to cryopreserve seed requires less labor and displays higher survival post-cryostorage, seed cryopreservation should not be the sole option for long-term preservation of sea oats genetic diversity. Each preserved seed represents a distinct genotype. Meristems from an established *in vitro* germplasm library can be derived from select genotypes that have been screened for desirable phenotypic

characteristics such as shoot growth and branching, rhizome production, flowering, and seed production responses under both nursery and dune conditions. A more horticultural approach encompassing selection and propagation of superior sea oats lines with enhanced performance for coastal dune restoration is being evaluated (Bertrand-Garcia et al. 2012). This may be a more effective approach since planting sea oats for dune stabilization and building might be more accurately considered a *bioengineering solution* than true habitat restoration. This approach is further supported considering the limited species diversity established on restored dunes. Therefore, it is important to develop a sea oats germplasm library consisting of both cryopreserved seeds as well as excised shoot tips of phenotypically characterized genotypes that would be subsequently propagated *in vitro*.

Conclusions

This study provides novel information on the effects of modifying or substituting procedural steps to the standard cryopreservation protocols in an attempt to increase cryostorage survival of low freezing tolerant DWP sea oats using excised seedling shoot tips. Successful regrowth post-LN of sea oats shoot tips did not require a second rehydration step but required at least one for any survival to occur. PVS2 exposure times should be between 40 and 60 min to ensure sufficient time for the vitrification solution to penetrate the cells, but not long enough to cause irreparable damage. Additionally, A9, a modified PVS2 solution with 20% DMSO, can replace PVS2 without further loss of regrowth following cryostorage. Given these responses, further studies should examine DMSO concentrations higher than 20%. Surprisingly, neither a cold acclimation treatment up to 4 weeks pre-LN nor a dark culture treatment post-LN had any effect on post-LN initial survival and regrowth. Encapsulation-dehydration proved to

be a successful cryopreservation method producing the highest regrowth (32.9%) in this study.

Regrowth was lower than expected following cryopreservation by vitrification and encapsulation-dehydration, which necessitates further optimization experiments. It is probable that the treatments modified in this study may be effective under different experimental conditions such as longer or shorter exposure periods, but these aspects were not investigated.

Following repeated attempts to increase cryopreservation regrowth for sea oats originating from the DWP population, higher regrowth was not achieved. When the highest regrowth results from this study (<32.9%) are compared to DWP genotype results (<22.5%) using shoot tips excised from *in vitro* shoot cultures, regrowth was similarly low (Figures 2-5 and 3-9). Ecotypic differentiation could be a possible explanation for the inability to increase regrowth even following substantial modifications to the standard protocol and using another cryopreservation method.

Table 3-1. Factorial ANOVA analysis of the main effects and interactions of regrowth responses following sucrose pretreatment and dehydration of DWP sea oats seedling shoot tips (refer to Figure 3-10).

Source	F-value	p-value
Sucrose Concentration	1.65	0.1933
Dehydration Time	12.22	<0.0001
Liquid Nitrogen Exposure	25.08	<0.0001
Sucrose Concentration * Liquid Nitrogen Exposure	8.00	0.0004
Dehydration Time * Liquid Nitrogen Exposure	9.19	<0.0001

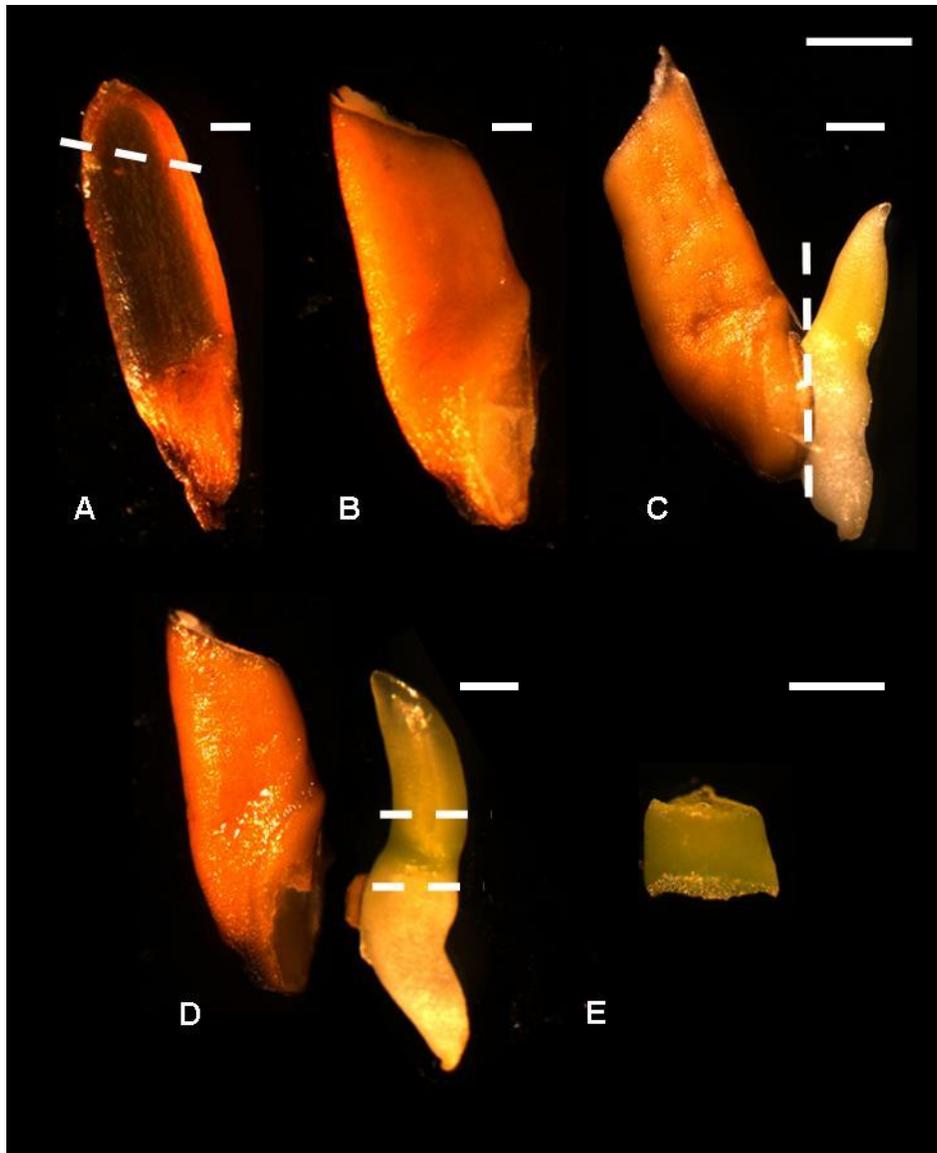


Figure 3-1. Isolation of excised seedling shoot tip from germinated sea oats seed. A) Sea oats seeds are sterilized using a 3% sodium hypochlorite solution. B) The distal end of each seed was removed prior to sowing on germination medium. C) After germination, the endosperm was removed to yield an excised seedling. D) Cold acclimated seedlings have shoot tips excised for all experiments E) A 1-1.5 mm shoot tip was used in all experiments. Scale bar = 2 mm (A-D) and 1 mm (E). Photos courtesy of James Sadler.

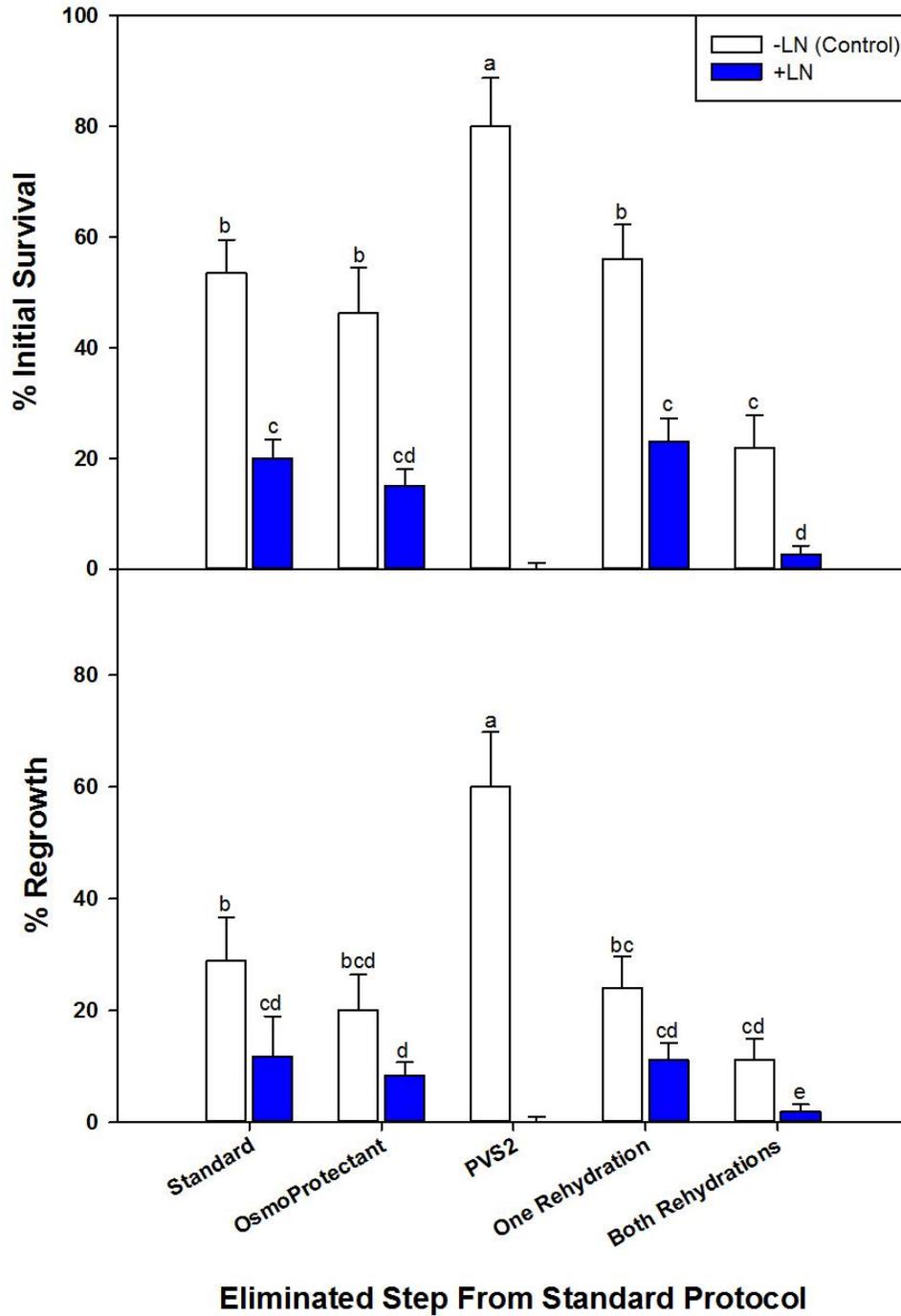


Figure 3-2. Comparative effects of standard vitrification protocol modifications on the survival of *U. paniculata* DWP seedling shoot tips. Initial survival and regrowth are presented following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Bars (\pm SE) marked by different letters within each graph are significantly different according to DMRT at $\alpha=0.05$.

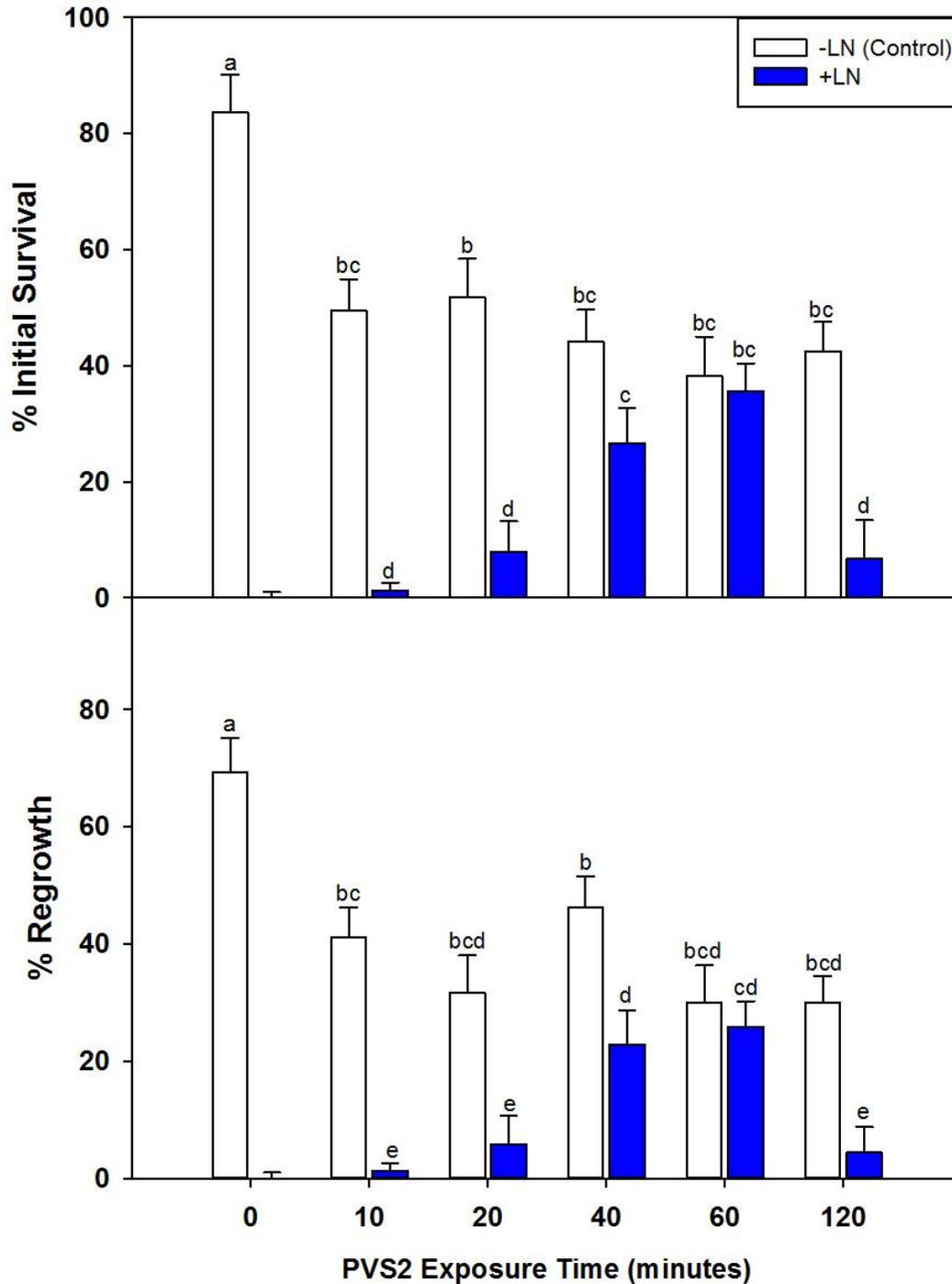


Figure 3-3. Comparative effects of PVS2 exposure duration on the survival of *U. paniculata* DWP seedling shoot tips. Initial survival and regrowth are presented following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Bars (\pm SE) marked by different letters within each graph are significantly different according to DMRT at $\alpha=0.05$.

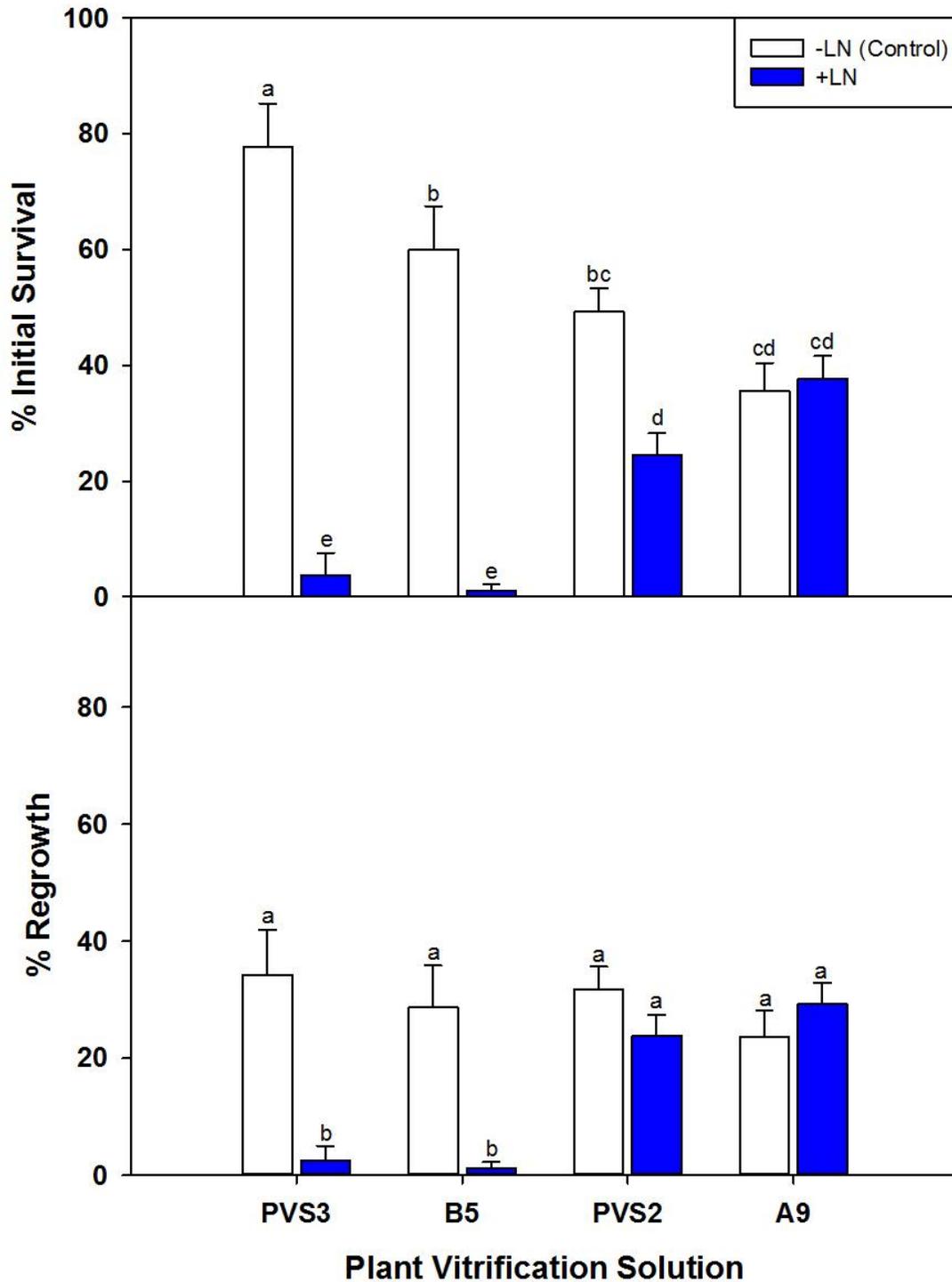


Figure 3-4. Comparative effects of different types of vitrification solution on cryopreservation survival of *U. paniculata* DWP seedling shoot tips. Initial survival and regrowth are presented following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Bars (\pm SE) marked by different letters within each graph are significantly different according to DMRT at $\alpha=0.05$.

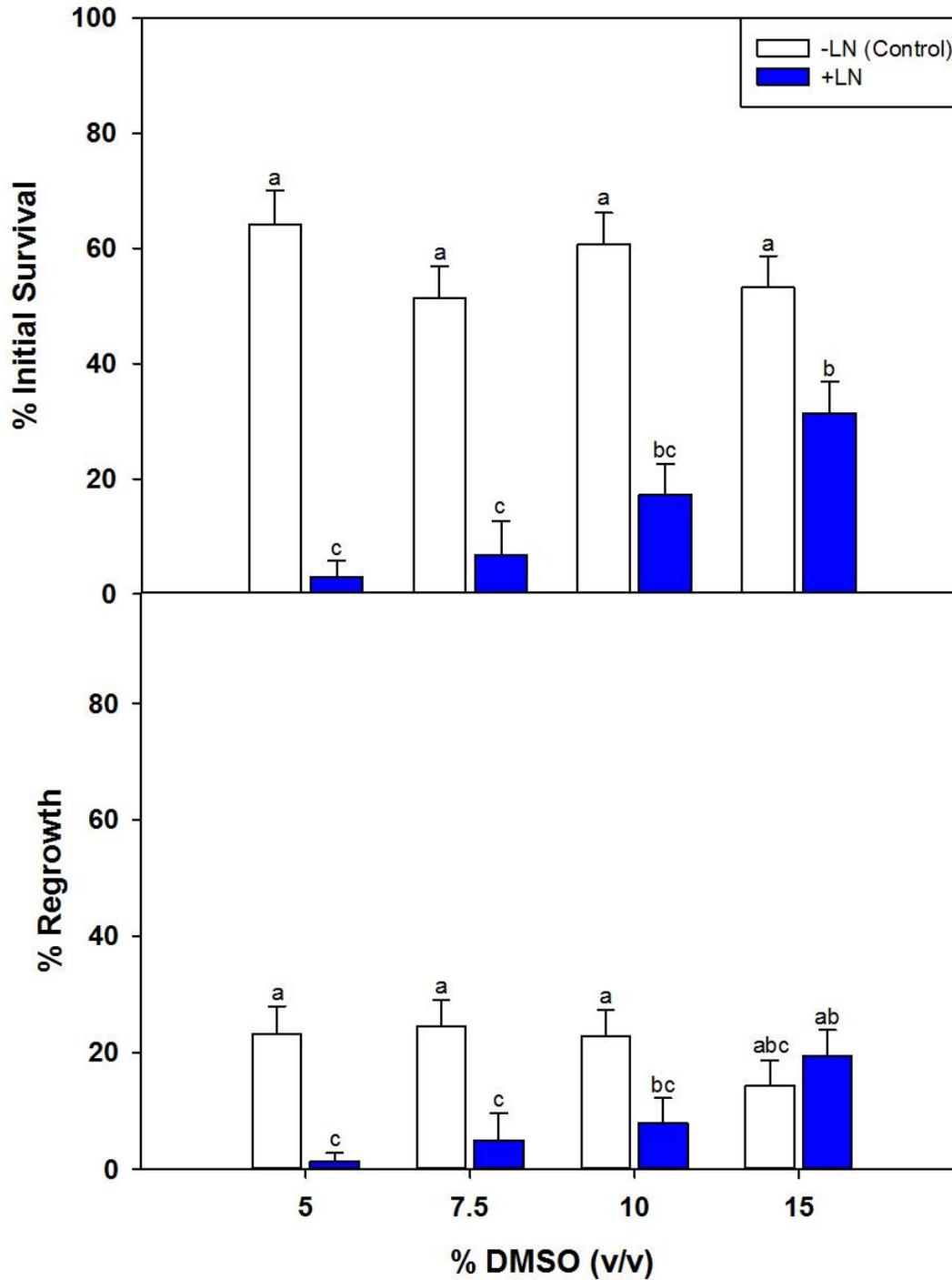


Figure 3-5. Comparative effects of PVS DMSO concentration on cryopreservation survival of *U. paniculata* DWP seedling shoot tips. Initial survival and regrowth are presented following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Bars (\pm SE) marked by different letters within each graph are significantly different according to DMRT at $\alpha=0.05$.

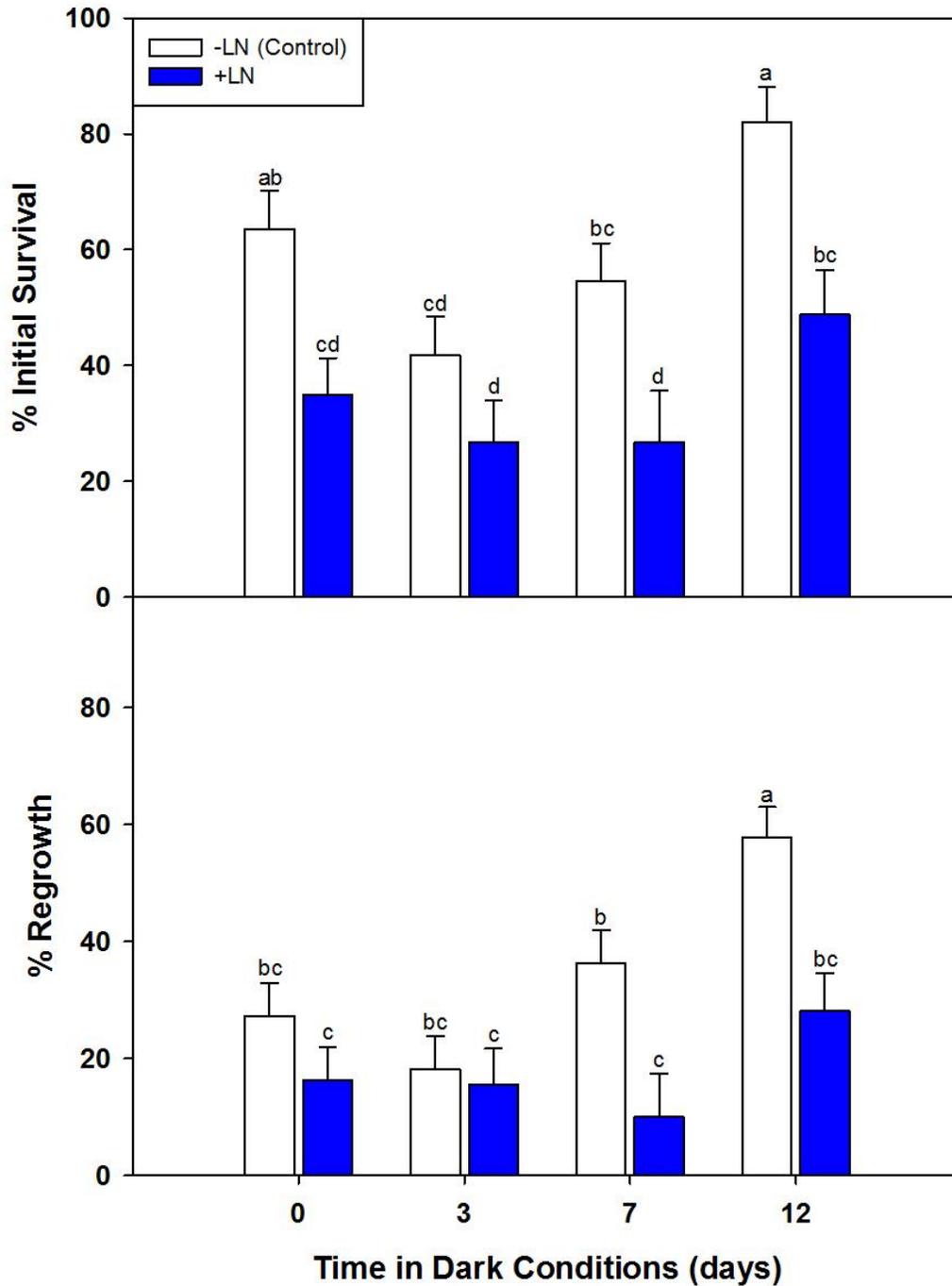


Figure 3-6. Comparative effects of a dark culture period after thawing on cryopreservation survival of *U. paniculata* DWP seedling shoot tips. Initial survival and regrowth are presented following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Bars (\pm SE) marked by different letters within each graph are significantly different according to DMRT at $\alpha=0.05$.

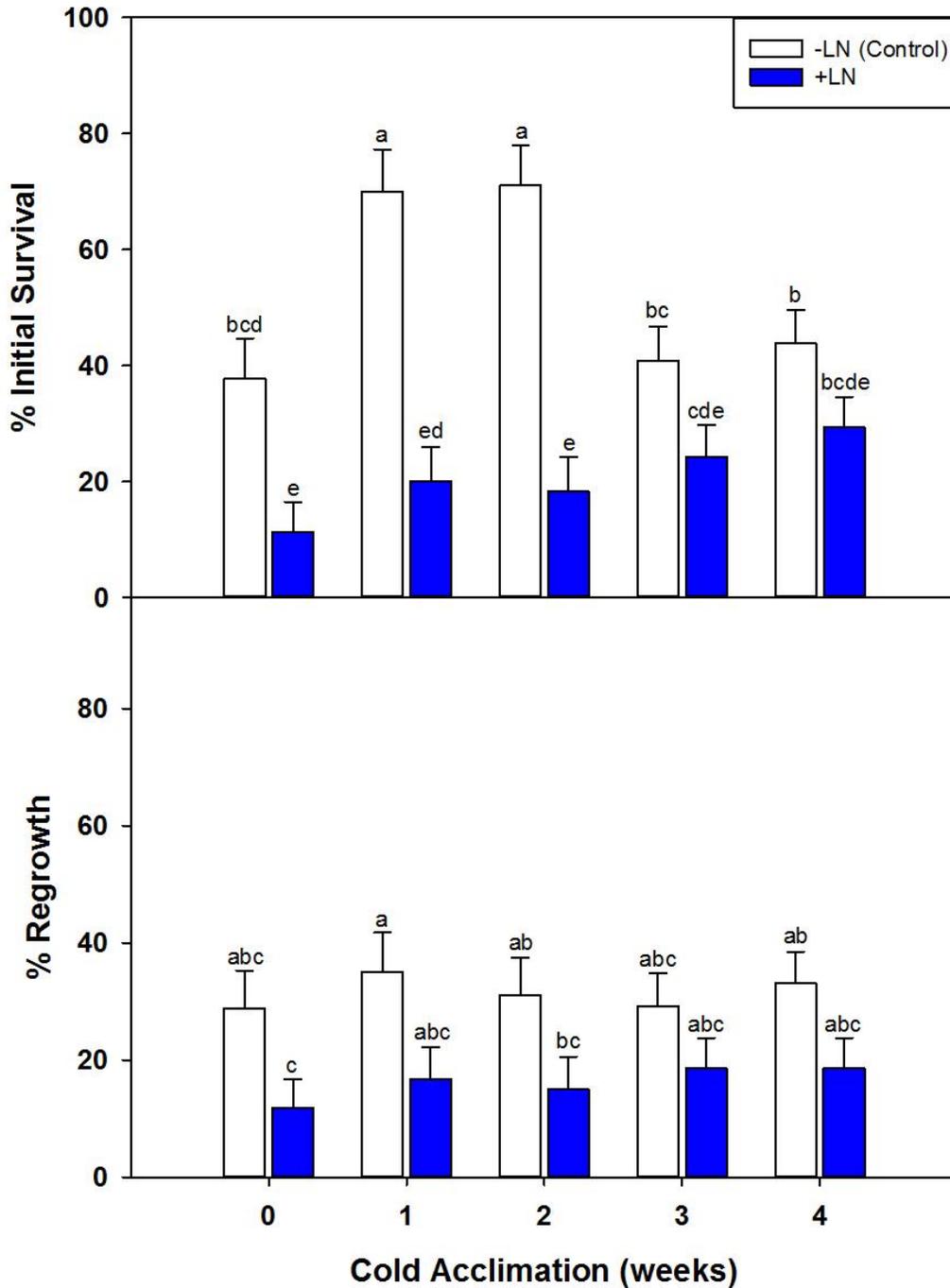


Figure 3-7. Comparative effects of cold acclimation on cryopreservation survival of *U. paniculata* DWP seedling shoot tips. Initial survival and regrowth are presented following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Bars (\pm SE) marked by different letters within each graph are significantly different according to DMRT at $\alpha=0.05$.

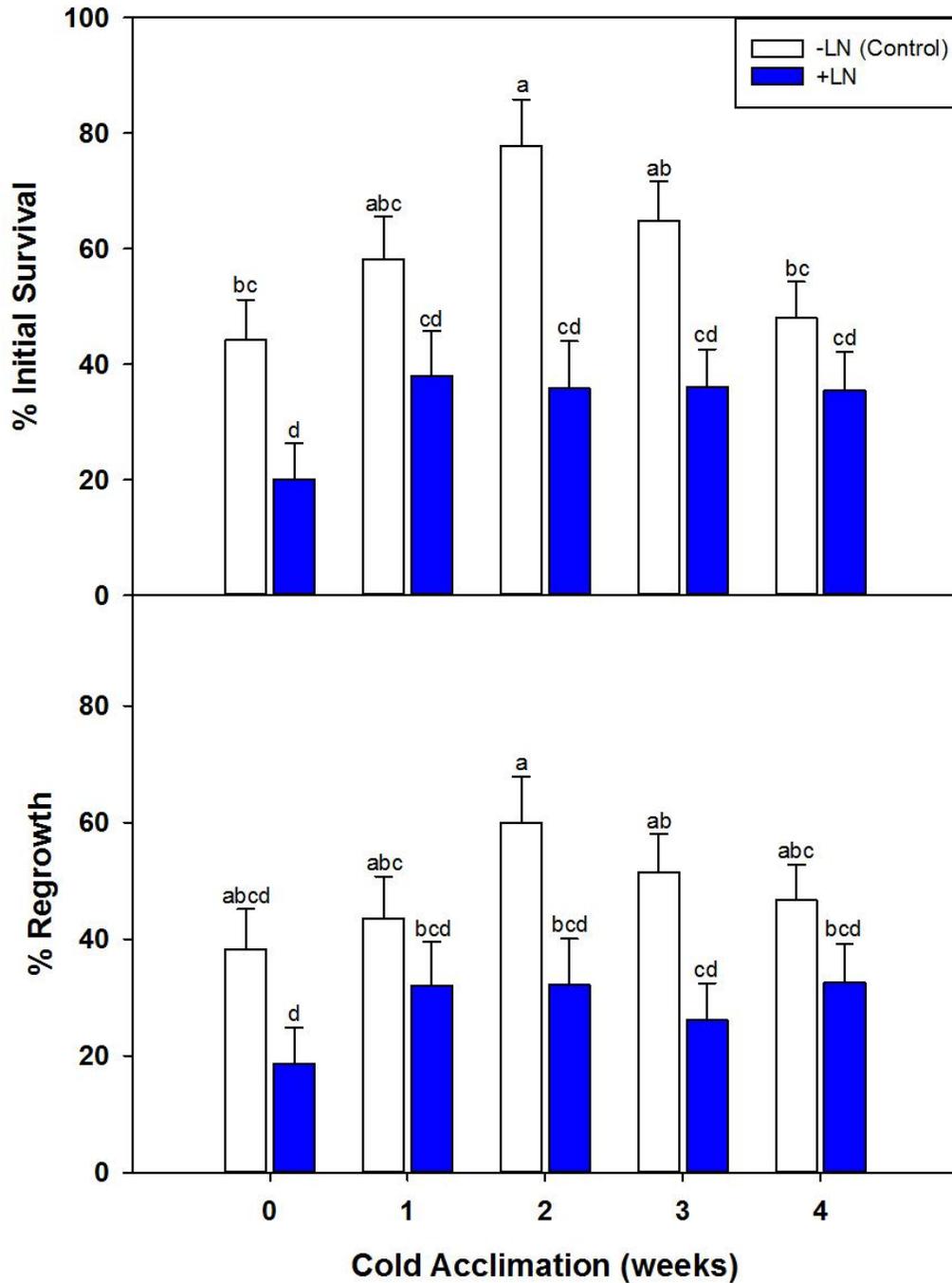


Figure 3-8. Comparative effects of cold acclimation on cryopreservation survival of *U. paniculata* LTI seedling shoot tips. Initial survival and regrowth are presented following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Bars (\pm SE) marked by different letters within each graph are significantly different according to DMRT at $\alpha=0.05$.

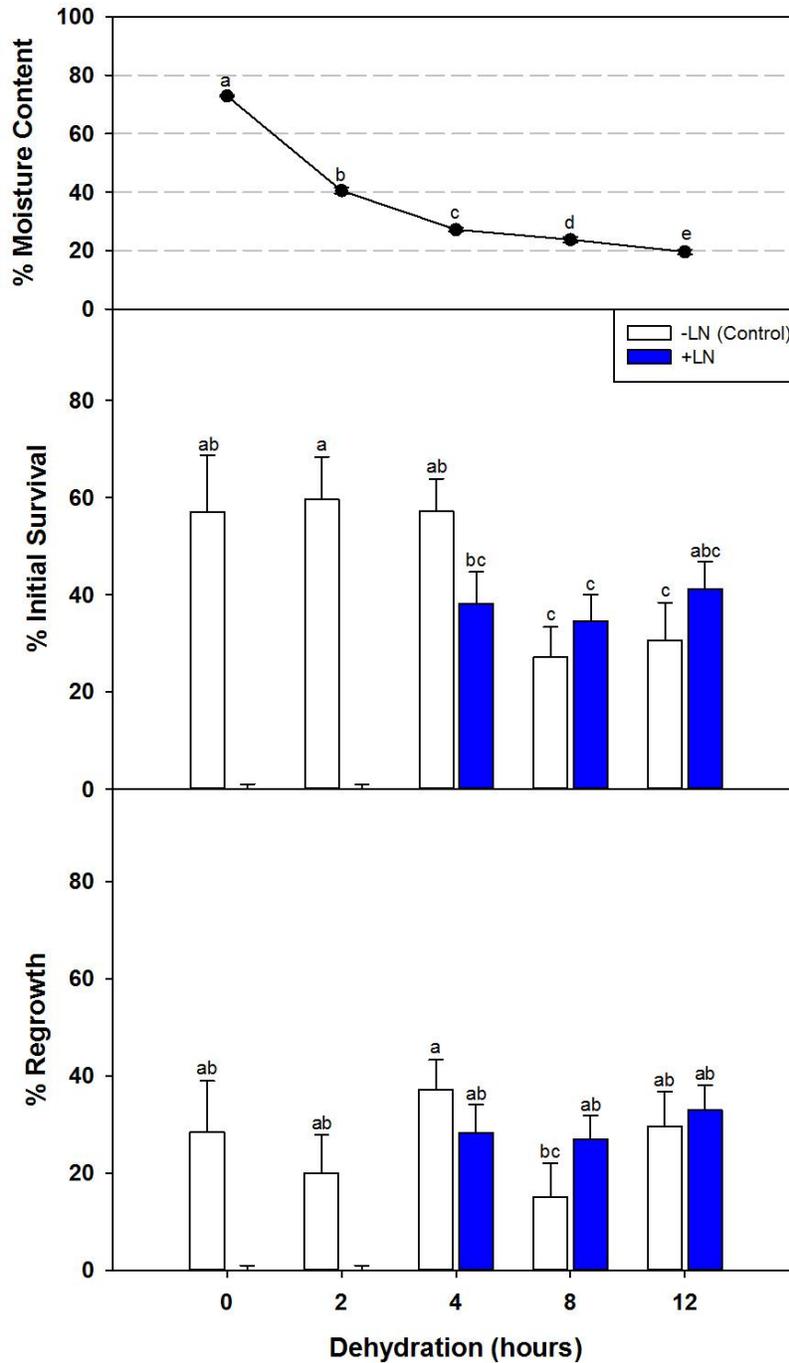


Figure 3-9. Comparative effects of bead dehydration on cryopreservation survival of *U. paniculata* DWP seedling shoot tips following a 0.75 M sucrose pretreatment. Initial survival and regrowth are presented following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Changes in bead moisture content are presented. Bars or plots (\pm SE) marked by different letters within each graph are significantly different according to DMRT at $\alpha=0.05$.

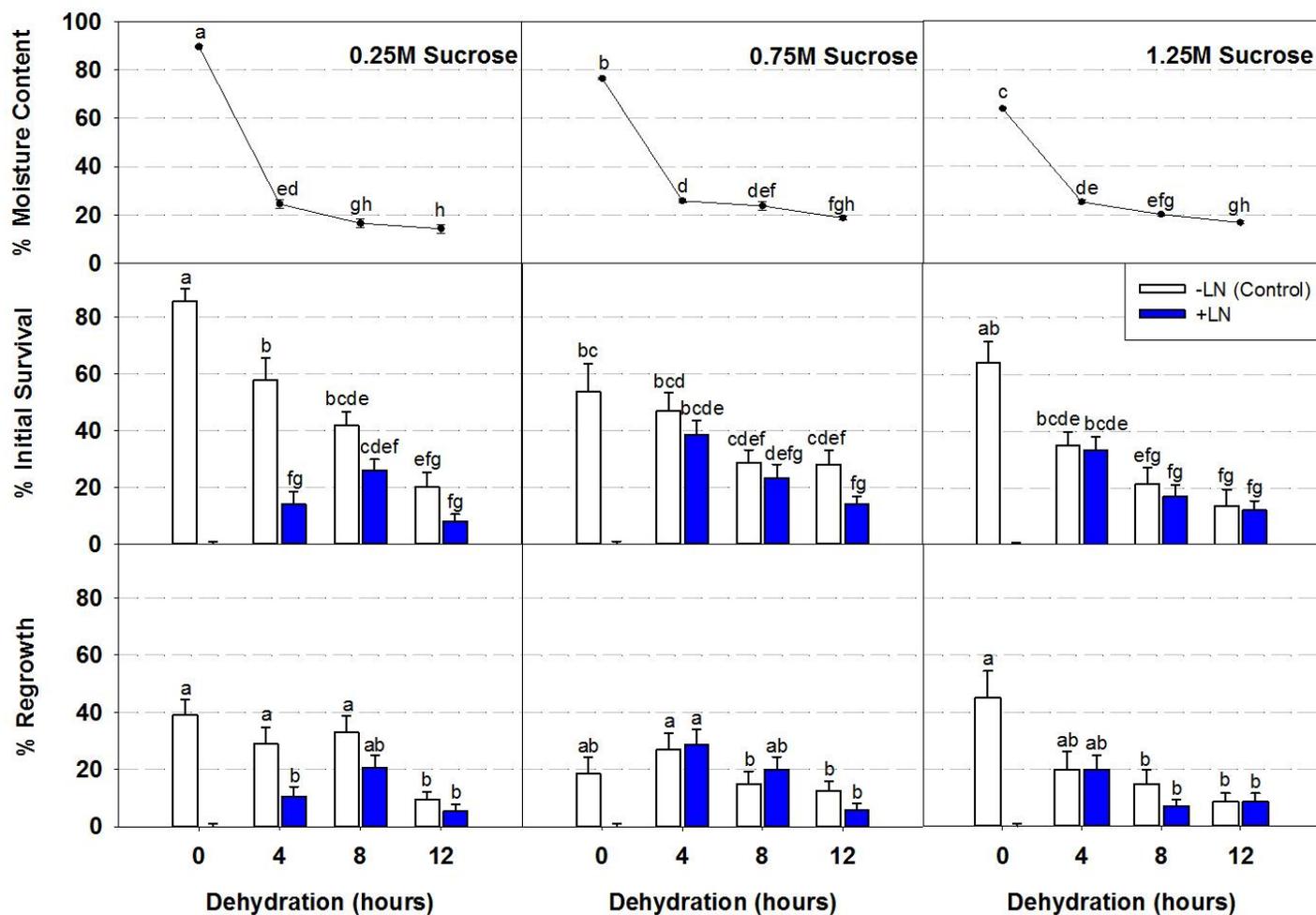


Figure 3-10. Comparative effects of sucrose concentration pretreatment and dehydration duration on cryopreservation survival of *U. paniculata* DWP seedling shoot tips. Initial survival and regrowth are presented following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Changes in bead moisture content are also presented. Bars or plots (\pm SE) marked by different letters in rows are significantly different according to Tukey-Kramer's Least Square Means Test at $\alpha=0.05$.

Proposed Optimized Vitrification Protocol

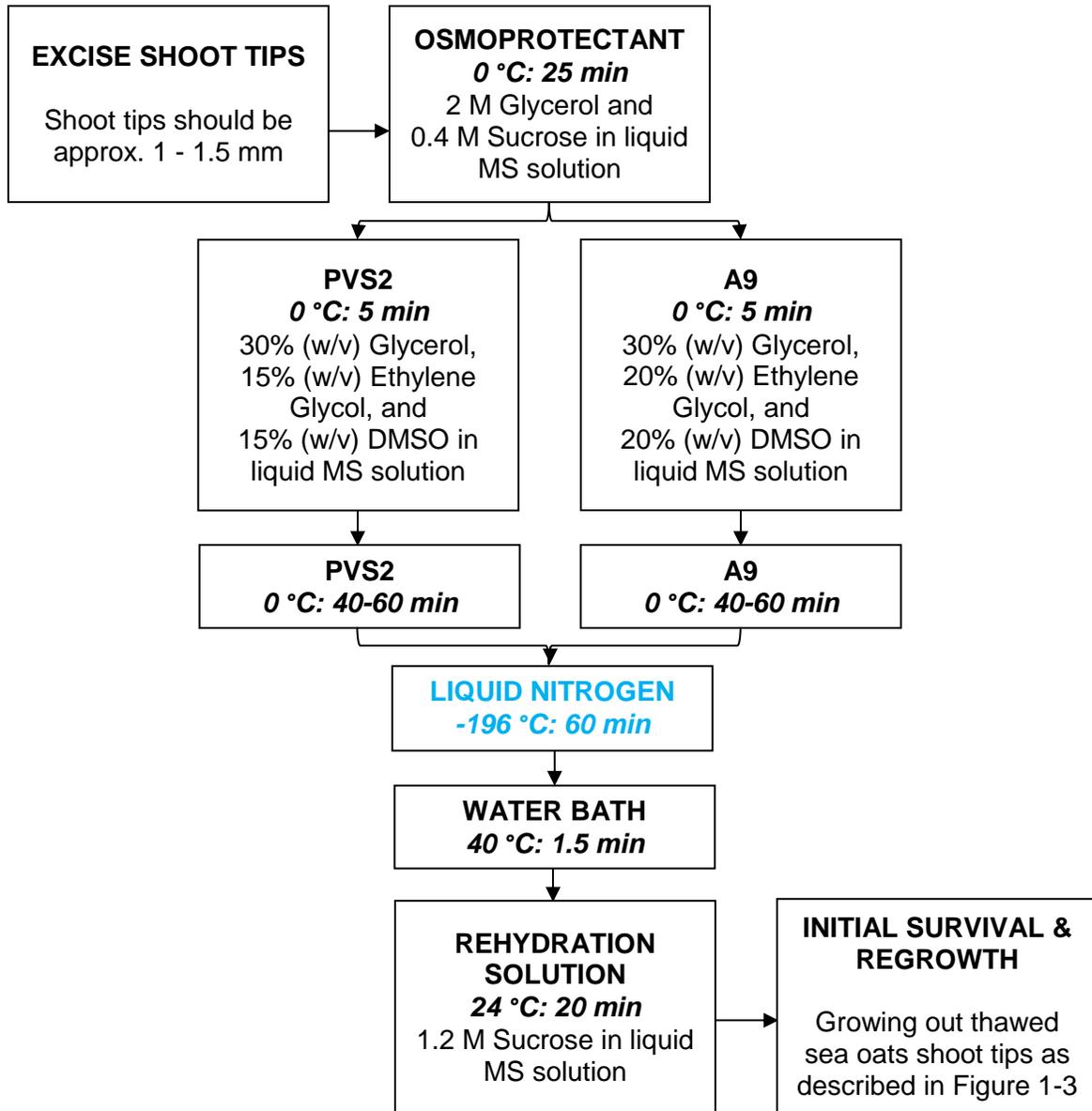


Figure 3-11. Proposed optimized vitrification protocol for sea oats shoot tips compiled by systematically modifying the standard vitrification protocol (Figure 2-3).

Proposed Optimized Cryopreservation Protocols

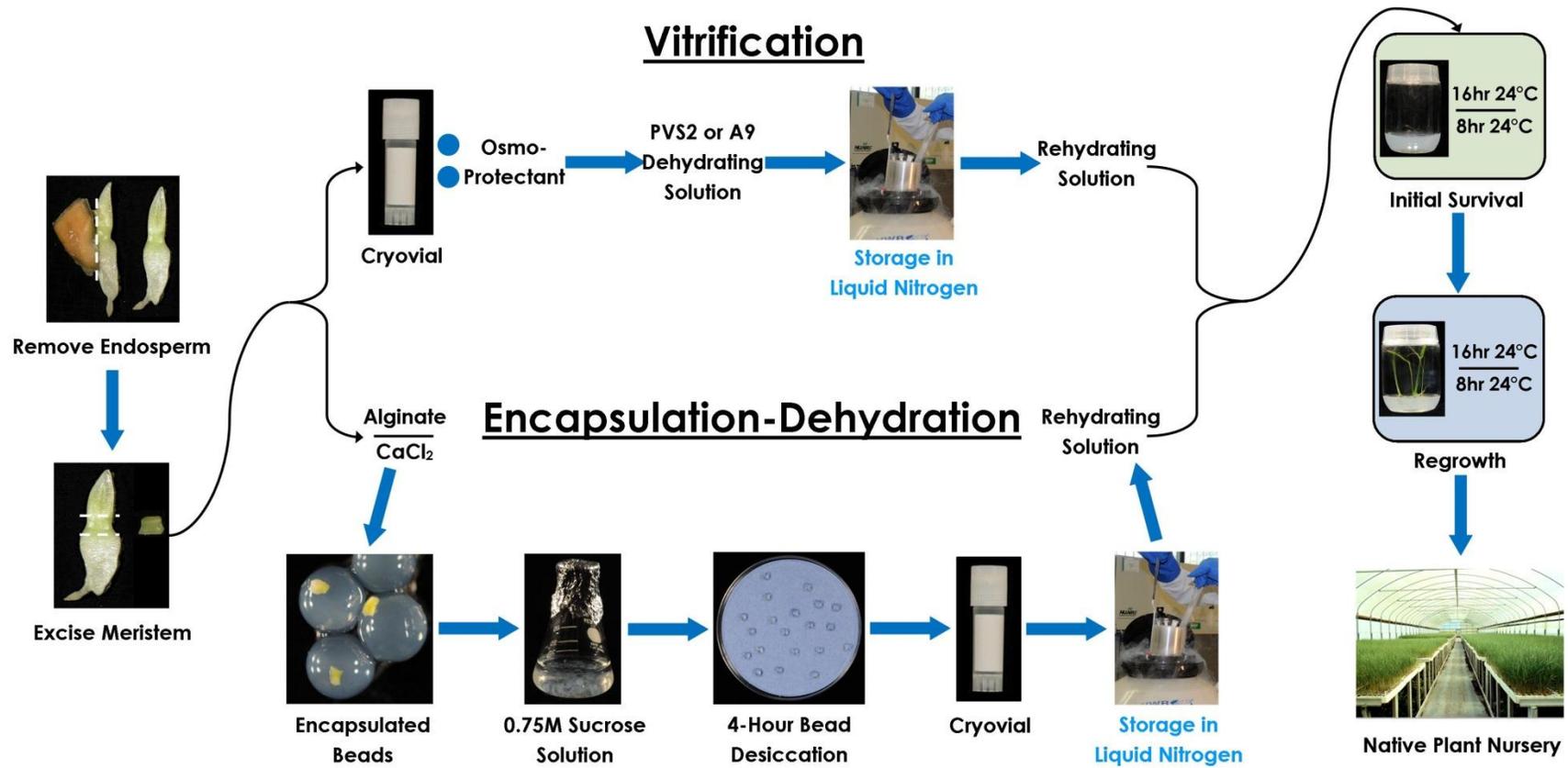


Figure 3-12. Proposed cryopreservation protocols for sea oats shoot tips using vitrification and encapsulation-dehydration. Photos courtesy of James Sadler.

APPENDIX A
INITIAL DETERMINATION OF CULTURE MEDIUM AND EXPLANT SIZE ON
RECOVERY OF EXCISED SHOOT TIPS

Introduction

When optimizing cryopreservation procedures, three factors should be considered that influence optimal survival following cryostorage. These factors are the initial donor plant conditions, the cryogenic steps, and the recovery process following cryostorage (Pennycooke and Towill 2000). The goal of every cryopreservation procedure is the efficient recovery of cryogenically stored plant tissues. Therefore, cryopreservation protocol optimization research does not stop at the cryostorage stage, but must include an evaluation of tissue recovery following cryostorage (Benson 2008). Following cryopreservation, rehydrated plant material is cultured on an initial survival, or recovery, medium. The recovery medium must be optimized before extensive cryopreservation is to be completed to yield the highest survival. In this preliminary experiment, the recovery medium was optimized for the medium type, benzyladenine (BA) presence in the medium, and the size of excised shoot tips using established sea oats shoot cultures.

Materials and Methods

In Vitro Culture of Sea Oats Genotypes

Two sea oats genotypes, PK 8-15 (Perdido Key, Florida) and LTI 8-12 (Little Talbot Island, Florida), were selected from an *in vitro* germplasm library of actively proliferating shoot cultures established June 2007. Each genotype, originating from a single seedling, was established and sustained by subculturing shoot clusters every 28 days on fresh SMM and cultured in standard growing conditions.

Determination of Recovery Medium and Shoot Tip Size

Three media formulations, Gamborg B-5 (Cat No. G-398 *PhytoTechnology* Laboratories, Shawnee Mission, KS), Schenk and Hildebrandt (Cat No. S-813 *PhytoTechnology* Laboratories, Shawnee Mission, KS), and Murashige and Skoog basal salts (Cat No. M-524 *PhytoTechnology* Laboratories, Shawnee Mission, KS) supplemented with Linsmaier & Skoog vitamins (Linsmaier and Skoog 1965), were screened with each genotype. Each medium was supplemented with 30 gL⁻¹ sucrose and was adjusted to pH 5.7 using 0.1 M KOH before adding the agar, dispensing 30 mL per BFJ, and autoclaving at 117.7 kPa for 20 min at 121 °C. Half of the treatments had 0.25 mgL⁻¹ BA added to the medium before adjusting the pH. Additionally, the response of three shoot tip lengths, 0.5 mm, 1.0 mm, and 1.5 mm, were studied with each genotype and medium composition. Percent regrowth was scored 4 weeks after shoot tip culture on the various media.

Experimental Design and Statistical Analysis

Each treatment consisted of five BFJs each containing five shoot tips. Greening and shoot tip elongation were all required for samples to qualify as being fully recovered following cryopreservation. Statistical differences between data were determined by analysis of variance (ANOVA) using the generalized linear model procedures. The data were presented as percentages with means separation using Duncan's Multiple Range Test (DMRT) $\alpha = 0.05$ (SAS Institute 2009).

Results and Discussion

Media composition and shoot tip size had a significant difference on regrowth after four weeks culture (Figure A-1). Shoot tip regrowth between the two genotypes was not different between any treatments (data not shown). Shoot tips cultured on

Murashige and Skoog medium exhibited significantly lower percent regrowth (16 – 66%) compared to shoot tips cultured on the other two media (Figure A-1). Shoot tips cultured on Gamborg B-5 (83.3 – 98%) and Schenk and Hildebrandt (82 – 88%) media with BA present displayed high regrowth and were not significantly different from one another.

A possible explanation for the significant difference between shoot tips cultured on Murashige and Skoog medium compared to the other two could be differences in the basal salts. Murashige and Skoog medium contains a considerably higher total salt concentration than either Gamborg B-5 or Schenk and Hildebrandt (Gamborg et al. 1976). Furthermore, Gamborg B-5 and Schenk and Hildebrandt medium compositions are similar to one another. The higher medium salt concentration may limit water absorption through the osmotic components of salt stress as was found with delayed and inhibited *Triticum durum* seed germination from salt stress (Almansouri et al. 2001). No reports have been published describing the effects, such as salt stress from lower medium water potential, different basal medium compositions have on plant growth, but many reports show differential preferences among plant species (Linsmaier and Skoog 1965; Schenk and Hildebrandt 1972; Gamborg et al. 1968; Gamborg et al. 1976; Reed 1996).

The regrowth of excised shoot tips on both Gamborg B-5 and Schenk and Hildebrandt media were similar with the addition of BA. Gamborg B-5 medium supplemented with 0.25 mgL^{-1} BA using 1-1.5 mm shoot tips was chosen as the optimal recovery medium and shoot tip size class. Consequently, this medium was used in all experiments for initial survival described in this thesis.

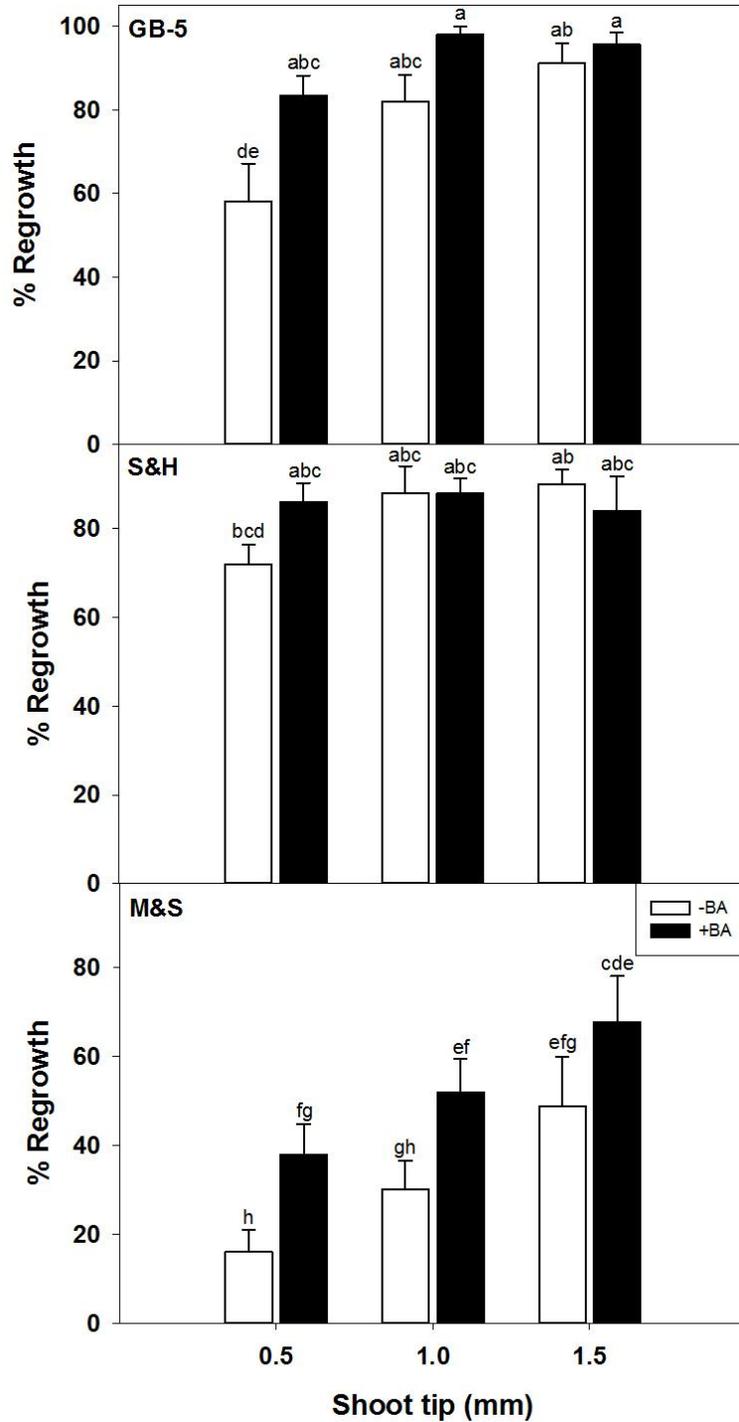


Figure A-1. Comparative effects of recovery medium composition and shoot tip size on regrowth of *U. paniculata* shoot tips. Shoot tips were excised from established *in vitro* plant cultures. Medium compositions were Gamborg B-5 (GB-5), Schenk & Hildebrandt (S&H), and Murashige & Skoog (M&S) medium with (+BA) and without (-BA) the presence of BA. Bars (\pm SE) marked by different letters are significantly different according to DMRT at $\alpha = 0.05$.

APPENDIX B INFLUENCE OF COLD ACCLIMATION AND OSMOPROTECTANT TEMPERATURE ON REGROWTH

Introduction

Various pretreatments to plants or excised shoot tips can be explored to increase post-cryostorage survival. Cold acclimation (CA) and using an osmoprotectant prior to PVS2 exposure are two such pretreatments. During CA, plants or excised shoot tips are subjected to a constant low temperature or an oscillating high/low temperature cycle. This pretreatment up-regulates cold-defense genes and down-regulates primary metabolism (Benson 2008; Reed et al. 2005; Guy et al. 1985). Common cellular responses to cold temperatures are an increase in solute and membrane phospholipid concentrations, which may serve as natural protection against possible ice crystallization and cellular membrane damage (Reed 1996; Volk 2010). This response facilitates protein and membrane stabilization (Kaczmarczyk et al. 2011; Sung et al. 2003). Cold temperature responses are exploited for cryopreservation success by initially beginning to decrease the intracellular water concentration by increasing the solute concentration.

Niino et al. (1992) reported that *Malus domestica* cv. Fuji shoot meristems exhibited highest regrowth (80%) when plants were cold acclimated for 3 weeks at 5 °C as compared to those that were not (10%). A similar trend was found with four different *Pyrus* varieties where CA meristems had higher regrowth (32%) than those without CA (5%) (Reed 1990). A study of CA with *Dioscorea* species found that shoot tips cultured with three weeks of incubation in an oscillating 25 °C / 5 °C 12-hr thermo-photoperiod exhibited the highest shoot recovery (48%) compared to those cultured at 26 °C (30%) (Leunufna and Keller 2005). Likewise, Chang and Reed (2000) determined that a 22 °C

/ -1 °C 8-hr day thermo-photoperiod resulted in the highest post-cryostorage growth (80 – 100%), compared to a constant low temperature of 4 °C (<40%).

Vitrification is the cryopreservation method through which intracellular water is replaced in shoot tips by exposure to solutions containing various sugar and cryoprotectant concentrations (Golmirzaie and Panta 1998). The most common dehydrating cryoprotectant used for vitrification is Plant Vitrification Solution 2, or PVS2 (Niino et al. 1992). However, PVS2 can be phytotoxic to shoot tips if dehydrated for too long. *Ipomoea batatas* meristems exposed to PVS2 for 16 min resulted in 66% post-cryostorage survival, whereas meristems exposed for only 26 min displayed 20% survival (Pennycooke and Towill 2000).

One method to protect meristems from osmotic stress from PVS2 dehydration is to use of a loading solution, or osmoprotectant (Turner et al. 2001). This loading phase has been shown to be effective in increasing PVS2 dehydration tolerance (Langis and Steponkus 1990; Turner et al. 2001). Highest survival was obtained in *Saintpaulia ionantha* shoot tips, *Citrus sinensis* var. *brasiliensis* Tanaka callus, and *Wasabia japonica* meristems following a 20-min exposure to an osmoprotectant consisting of 2 M glycerol and 0.4 M sucrose (Matsumoto et al. 1994; Moges et al. 2004). In these reports, osmoprotection was conducted at room temperature, but the temperature of PVS2 has been highly noted as optimal at 0 °C. In the current study, the requirements of cold acclimation exposure and the temperature of the osmoprotectant were evaluated.

Materials and Methods

Seed Germination and Shoot Tip Excision

Germination of sea oats seeds originating from Henderson Beach State Park, Destin, FL and shoot tip excision from the germinated seeds followed the protocol described in Chapter 2.

Determination of Cold Acclimation Requirement and Osmoprotectant Temperature

Excised seedling shoot tips (Figure 3-1) were transferred into BFJs containing 30 mL preculture medium, consisting of 0.3 M sucrose solidified with 8 gL⁻¹ high-grade TC Agar (Cat. No. A-175 *PhytoTechnology* Laboratories, Shawnee Mission, KS). The medium pH was adjusted to 5.7 prior to agar addition and autoclaving. Shoot tips were either cold acclimated (22 °C 8-hr days/0 °C nights) for one week or not cold acclimated. Additionally, half of the excised shoot tips were pretreated with osmoprotectant at 25 °C while the remainder was pretreated with osmoprotectant at 0 °C. When the osmoprotectant was removed, the procedure followed the standard protocol as described in Figure 2-3. Rehydrated shoot tips were placed on recovery medium for four weeks before initial survival data were collected. Four weeks after shoot tips were placed on the recovery medium, regrowth data were collected.

Experimental Design and Statistical Analysis

Each replicate consisted of eight cryovials with five shoot tips for each treatment plunged into LN and two cryovials with five shoot tips for all treatments not plunged into LN (controls). Greening and elongation from excised shoot tips were required criteria for samples to qualify as being fully recovered following cryopreservation. Statistical differences between data were determined using analysis of variance (ANOVA) using

the generalized linear model procedures. The data were presented as percentages with mean separation using Duncan's Multiple Range Test (DMRT) $\alpha = 0.05$ (SAS Institute 2009).

Results and Discussion

When cryopreserved, initial survival (24.1 – 44.4%) and regrowth (24 – 36.3%) for all treatments were not significantly different (Tables B-1 and B-2). Based on these data, one week CA did not provide any survival advantage compared to shoot tips without CA. Additionally, the temperature of the osmoprotectant did not significantly affect the initial survival or regrowth of cryopreserved shoot tips. In contrast, previous studies have shown that more than one week CA was necessary for significantly higher survival rates (Niino et al. 1992; Chang and Reed 2000; Leunufna and Keller 2005). Therefore, sea oats may require more than one week CA for survival following cryostorage to significantly increase cryostorage survival. An osmoprotectant solution temperature of 0 °C was selected for all subsequent experiments.

Table B-1. Comparative effects of cold acclimation and osmoprotectant temperature on initial survival following cryopreservation of *U. paniculata* shoot tips. Initial survival results marked by different letters are significantly different according to DMRT at $\alpha = 0.05$.

One week CA	Osmoprotectant (°C)	Liquid nitrogen	Initial survival
Yes	0 °C	Yes	40.6% a
Yes	0 °C	No	73.5% a
Yes	25 °C	Yes	43.0% a
Yes	25 °C	No	37.5% a
No	0 °C	Yes	44.4% a
No	0 °C	No	46.5% a
No	25 °C	Yes	24.1% a
No	25 °C	No	30.0% a

Table B-2. Comparative effects of cold acclimation and osmoprotectant temperature on regrowth following cryopreservation of *U. paniculata* shoot tips. Regrowth results marked by different letters are significantly different according to DMRT at $\alpha = 0.05$.

One week CA	Osmoprotectant (°C)	Liquid nitrogen	Regrowth	
Yes	0 °C	Yes	33.1%	a
Yes	0 °C	No	63.5%	a
Yes	25 °C	Yes	34.1%	a
Yes	25 °C	No	25.0%	a
No	0 °C	Yes	36.3%	a
No	0 °C	No	36.5%	a
No	25 °C	Yes	24.0%	a
No	25 °C	No	30.0%	a

APPENDIX C PRELIMINARY SEA OATS SEED CRYOPRESERVATION

Introduction

Seed banking, in addition to the cryostorage of excised shoot tips, could also be a possible option for the preservation of site-specific genetic diversity. Seed banking with cryopreservation has been successful with different species, such as *Salix* sp. (Wood et al. 2003) and *Gossypium hirsutum* (Gonzalez-Benito et al. 1998). Pritchard (2008) stated that successful seed cryopreservation is dependent on the seed water content prior to freezing and the rate that seeds are transferred to ultra-low temperatures, either with controlled-rate cooling or rapid freezing. Desiccation-tolerant (orthodox) seeds can survive desiccation pretreatments before immersion into liquid nitrogen (LN), but desiccation-sensitive (recalcitrant) seeds require careful study to determine optimal moisture content and freezing temperature (Engelmann 2011). No reports have been published on the seed storage physiology of sea oats to determine if the seed of this species is orthodox, recalcitrant, or intermediate.

Controlling seed moisture content is accomplished with the use of saturated salt solutions in small-enclosed spaces to produce specific relative humidity levels, which dry the seed to a specific water content (Winston and Bates 1960). Control of the relative humidity inside the container adjusts the seed water content to screen the cryostorage survival of various water contents. As described by Pritchard (1995), as relative humidity increased, seed moisture content also increased. In this preliminary study, sea oats seeds were equilibrated to specific water contents by controlling the relative humidity levels to determine the relationship of seed water content and optimal cryostorage survival as evaluated by seed germination post-LN.

Materials and Methods

In October 2011, mature sea oats (*Uniola paniculata* L.) infructescences were harvested from Delnor-Wiggins Pass State Park, Collier, Florida. Seeds were dried for 1.5 weeks, mechanically cleaned by SeedClean (Sarasota, Florida), and stored in darkness in vacuumed sealed bags at 9 °C until needed.

Twelve saturated salt solutions were chosen to produce a wide range of specific relative humidity levels (Table C-1). One hundred seeds were placed into enclosed desiccation jars and suspended over saturated solutions (Figure C-1a). Jars were placed on germination mats set at 25 °C. Seed fresh mass was recorded daily for 25 seeds until no changes in mass were detected (Figure C-1b).

Once seeds exhibited stable weights, 25 seeds were placed into 1.2 mL cryovials (5 seeds/cryovial) and plunged into LN for 1 min, 1 hr, and 1 day, depending on the experimental treatment. When removed from LN, seeds were allowed to re-warm at room temperature (≈ 22 °C) before being placed on damp germination paper (Cat. No. SD3.5 Anchor Paper, Saint Paul, MN) (Figure C-1c). Finally, seeds were placed in an incubator at 35 °C with constant light supplied by Philips fluorescent lights (F17T8/TL741 Alto) at $65 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF and monitored daily for germination or visible signs of fungal contamination for 28 days. The remaining 25 seeds were rehydrated on germination paper then placed in the incubator (control). Each seed was discarded when it exhibited signs of germination (appearance of a shoot and a root) or contamination (fungal hyphae protruding from the micropyle). After 28 days, seeds that did not germinate were discarded. Germination percentages were calculated based on the number of seeds that germinated and the total seeds that did not show signs of

contamination. The data were presented as percentages with means separation using Duncan's Multiple Range Test (DMRT) $\alpha = 0.05$ (SAS Institute 2009).

Results and Discussion

All published research reports on sea oats seed germination have focused on the influences of temperature and stratification (moist-pre-chilling). It is not known whether sea oats seeds show recalcitrant (desiccation-sensitive), intermediate, or orthodox (desiccation-tolerant) seed storage physiology (Ellis 1991). Therefore, it was not known how sea oats seeds would respond to cryopreservation. Based on these results, sea oats seeds appear desiccation-tolerant with minimal loss of germination (>80%) following cryopreservation even across a wide range of relative humidities (0.5% to 91%) (Figure C-2). Ellis (1991) stated that an indicative feature of recalcitrant seeds is their inability to survive dehydration. Conversely, orthodox seeds can survive considerable dehydration.

No differences were seen within relative humidity levels between cryopreserved seeds and seeds that were only dehydrated (-LN). Although overall germination was high for all treatments, one significant difference between humidity levels for cryopreserved seeds was noted. At a 0.5% relative humidity level generated by a saturated phosphorous pentoxide solution, germination was significantly lower (87.4%) than the highest germination rate with potassium hydroxide (95.4%). This suggests that severe desiccation does decrease sea oats germination to a limited degree. However, as previously noted, these germination percentages are still very high following LN. Seed moisture content at these extreme relative humidity levels should be investigated to determine what change occurs in a 0.5% relative humidity. These results indicate that cryopreservation may be a very efficient method for long-term storage of viable sea oats

seed from many populations. However, additional studies should be conducted to examine the freezing tolerance of sea oats seed from diverse populations.

Table C-1. Relative humidity levels generated by saturated salt solutions at 25 °C in sealed vessels (Winston and Bates 1960).

Salt	Relative humidity (%)
Phosphorous Pentoxide	0.5
Zinc Chloride	5.5
Potassium Hydroxide	8.0
Lithium Chloride	13.0
Potassium Acetate	25.0
Calcium Chloride	29.5
Magnesium Chloride	32.5
Sodium Iodide	38.0
Calcium Nitrate	50.5
Sodium Chloride	75.0
Potassium Chloride	85.0
Potassium Nitrate	91.0

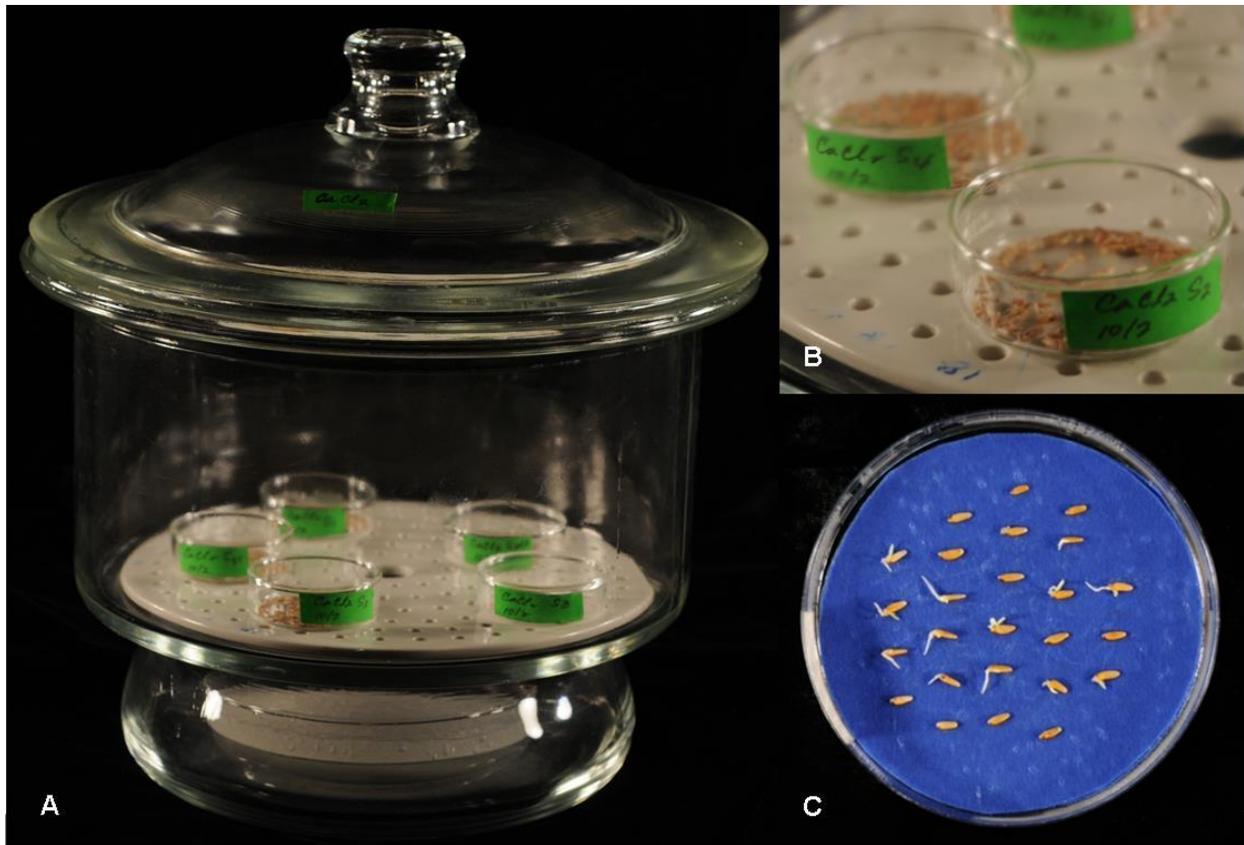


Figure C-1. Seed drying over saturated salts and recovery from cryostorage. A) Large sealed glass desiccator vessels contained saturated salt solutions. B) Sea oats seeds were placed in a single layer in glass petri plates prior to cryostorage. C) Seeds recovered from liquid nitrogen were rehydrated and germinated. Photos courtesy of Dr. Michael Kane.

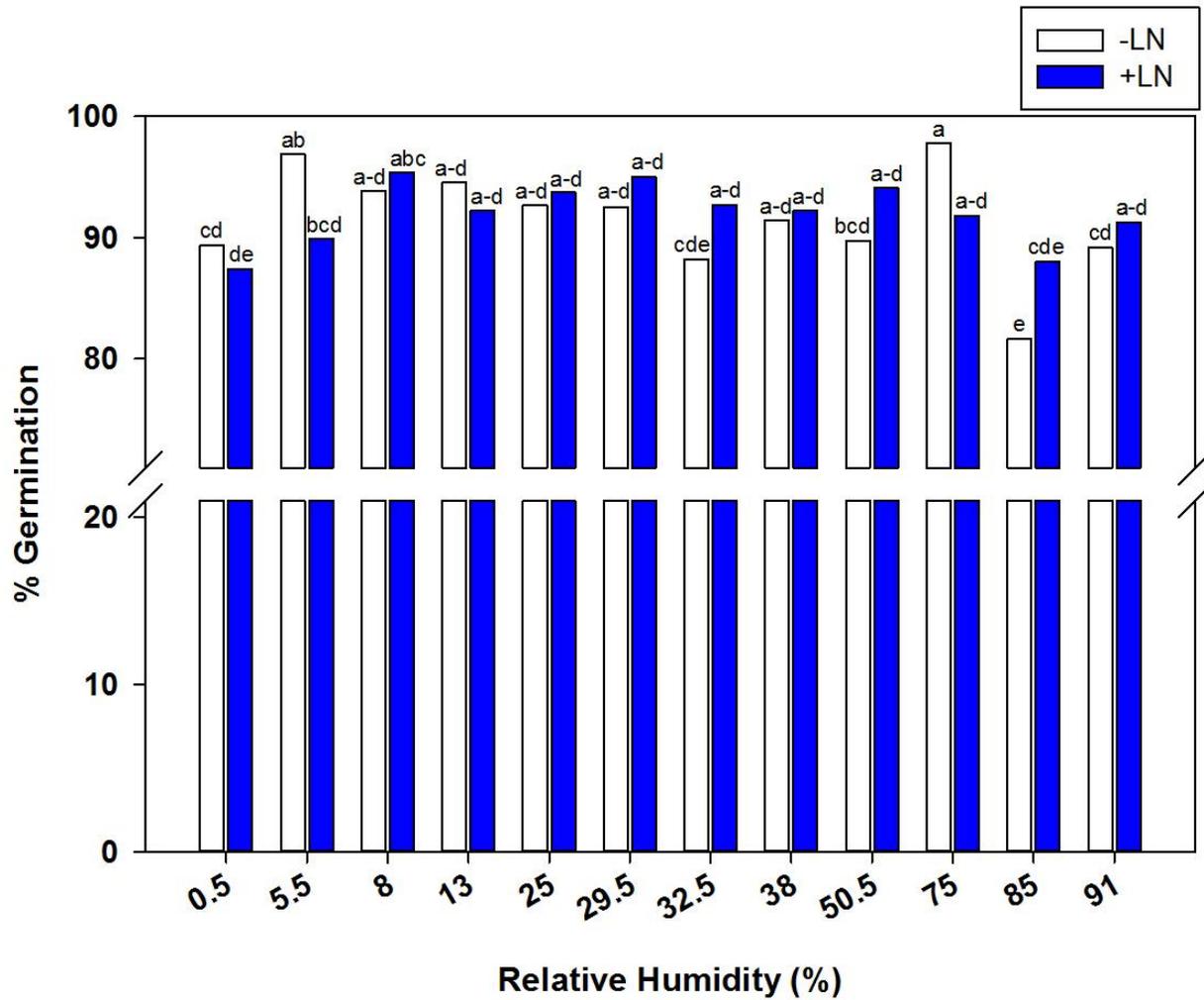


Figure C-2. Comparative effects of relative humidity levels on germination rates of cryopreserved (+LN) and non-cryopreserved (-LN) sea oats seeds originating from DWP. Bars marked by different letters are significantly different according to DMRT at $\alpha=0.05$.

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BIOGRAPHICAL SKETCH

As an Illinois native, James Sadler has begun to realize his appreciation for having four seasons instead of the usual Gainesville, FL climate. After graduating valedictorian of his high school class, James attended Illinois College, a private, liberal arts college in Jacksonville, IL where he obtained a Bachelor of Science in a biology/chemistry combined major after discovering his passion for biology from Dr. Lawrence Zettler and his love for chemistry from Dr. Clayton Spencer. During his time as a student researcher at Illinois College, James met Dr. Michael Kane, a professor in the Plant Restoration, Conservation, and Propagation Biotechnology Program in the Environmental Horticulture Department at the University of Florida in June 2008. After applying for graduate schools, James was accepted into Dr. Kane's program to pursue a Master of Science in August 2010. Upon graduating in 2013 with a Master of Science in horticultural science, James will pursue many options consisting of teaching, outreach, or higher education administration careers in the Midwest, all of which are his passion. In his spare time, James enjoys leading service-minded organizations, playing an active role in politics and activism, playing with his two guinea pigs, Gizmo and Biscuit, and relaxing with his partner, Eric.