PHYSIOLOGICAL AND ANATOMICAL BASIS FOR DIFFERENCES IN GROWTH PERFORMANCE DURING IN VITRO AND EX VITRO CULTURE OF SEA OATS
(Uniola paniculata L.) GENOTYPES

By
CARMEN VALERO ARACAMA

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by

Carmen Valero Aracama
This dissertation is dedicated to my parents, for their unconditional love and encouragement.
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PHYSIOLOGICAL AND ANATOMICAL BASIS FOR DIFFERENCES IN GROWTH PERFORMANCE DURING IN VITRO AND EX VITRO CULTURE OF SEA OATS
(Uniola paniculata L.) GENOTYPES

By
Carmen Valero Aracama

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Chair: Michael E. Kane
Cochair: Sandra B. Wilson
Major Department: Environmental Horticulture

Sea oats (Uniola paniculata L.), a dune species native to the southeastern U.S., is commonly used for dune stabilization and restoration in Florida. A micropropagation protocol was developed for sea oats commercial production. However, significant variability in shoot production, rooting and ex vitro survival among sea oats genotypes was observed. Understanding the morphological, anatomical, and physiological basis for differences among genotypes would allow development of efficient micropropagation protocols to produce diverse sea oats genotypes for dune stabilization. Growth and development of two sea oats genotypes with differing acclimatization capacities were compared at morphological and anatomical levels as a function of the duration of multiplication and rooting stages using light and electron microscopy. During in vitro and ex vitro stages, changes in photosynthetic rates were monitored, and carbohydrate levels and photosynthetic enzymes activities were measured. Additionally, sea oats
photosynthetic capacity in vitro and ex vitro and during acclimatization was evaluated using in vitro photoautotrophic and photomixotrophic culture conditions. During the rooting stage, the easy-to-acclimatize genotype (EK 16-3) developed short but numerous roots and “grass-like” leaves with fully expanded blades. Conversely, the difficult-to-acclimatize genotype (EK 11-1) developed few long roots and short and thick “lance-like” leaves without expanded blades. During in vitro development of “grass-like” leaves, EK 16-3 plantlets exhibited increases in activities of the two photosynthetic enzymes phosphoenolpyruvate carboxylase (PEPC) and ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco), and chlorophyll and total soluble protein content. These increases were correlated with higher net photosynthetic rates in EK 16-3 than EK 11-1 plantlets after ex vitro transfer. Both genotypes accumulated carbohydrate and starch reserves during in vitro rooting, which were depleted during the transition from photomixotrophic to photoautotrophic mode of nutrition. However, due to the lack of production of photosynthetically competent leaves on EK 11-1 plants coupled with rapid exhaustion of carbohydrate reserves, acclimatization and survival ex vitro for this genotype were low. Conversely, rapid production of photosynthetically competent leaves resulted in 100% survival ex vitro in EK 16-3 plantlets. In conclusion, the major cause for low acclimatization was the in vitro formation of leaves with abnormal anatomy correlated with limited photosynthetic capacity.
CHAPTER 1

LITERATURE REVIEW

Introduction and Rationale

Florida’s beach and dune systems have significant economic and environmental value. Seventy-five percent of Florida’s population resides in the coastal counties where beach related tourism alone contributes more than $19 billion to the state’s economy annually. Besides providing a unique wildlife habitat, Florida’s coastal sand dunes serve as a natural defense to mitigate the destructive action associated with tropical storms, hurricanes and human activity (Crewz, 1987; Dahl and Woodard, 1977; Woodhouse, 1982). During the 2004 hurricane season, nearly all of the state’s sandy beach shoreline was adversely affected. According to the Florida Department of Environmental Protection, it was estimated in 2005 that about 365 of Florida’s 825 miles of sandy beaches were in a critical state of erosion. Destabilization and erosion of coastal beach and dune vegetation systems by natural forces or man-made activities have increased the risk of catastrophic ecological as well as economic damage following storm events. Continued protection and restoration of dune areas and high-energy beaches are necessary to prevent additional losses.

Control of coastal erosion is accomplished in Florida by installing mechanical structures such as fences, or by dredging sand from the offshore zone for beach re-nourishment. However, dune stabilization by planting bare areas with native or introduced dune species is the most cost-effective accepted practice to control erosion. The root and rhizome system of those species holds particles of sand in place, while the above ground
vegetation retards wind and water driven erosion and promotes sand accretion
(Woodhouse, 1982).

The most effective dune species used for beach and dune stabilization are perennial
grasses, including American beachgrass (*Ammophila breviligulata* Fern.) and sea oats
(*Uniola paniculata* L.). In the southeastern United States, sea oats is the primary native
dune grass used for beach and dune stabilization and restoration because it dominates the
foredune zone in the dune-strand ecosystems of the South and Atlantic and Gulf coasts
(Wagner, 1964; Brown and Smith, 1974). Additionally, sea oats has the ability to rapidly
colonize and establish, and exhibits high tolerance to heat, drought, salt spray and
occasional inundation by salt water (Wagner, 1964; Woodhouse, 1982). Growth of sea
oats is stimulated by sand burial, which occurs frequently on the dune. The linear grass
blades of sea oats help trap sand on the dune by raising the laminar boundary layer of the
wind velocity profile, causing sand deposition. Regrowth occurs even after rapid
deposition of sand up to 1 m thick. Plant reproduction occurs by seed and by rhizome
extension, which allows rapid plant distribution to help stabilize the surface of the dune.

Due to repeated coastal erosion, demand for sea oats planting materials has
significantly increased. Currently, the planting of a one-mile-long twenty-foot-wide
length of eroded beach in Florida requires 22,000 sea oats plants at a total cost of $40,000
(O. Bundy, pers. comm.). An estimated 24-36 million sea oats would be required to
revegetate the entire beach coastline of Florida (Sylvia et al., 2003). Sea oats is
propagated in commercial nurseries from field-collected seeds. However, sea oats is not
regarded as a prolific seed producer (Hester and Mendelssohn, 1987; Bachman and
Whitwell, 1995; Burgess et al., 2002; Burgess et al., 2005). Of the six to eight fertile
florets in each spikelet, most have embryos that have aborted and only a few florets actually set seeds (Wagner, 1964; Burgess et al., 2002). Consequently, dwindling natural stands have made it necessary for the State of Florida to impose restrictions on field harvesting of sea oats plants and seeds. These restrictions significantly limit the natural sources of sea oats and the ability of Florida's native plant nurseries to meet the demand for plant materials. Concerns regarding the potential introduction of un-adapted ecotypes at revegetation sites have also limited the use of seed material obtained from distant geographic sources. In Florida, this second issue has been addressed by restricting the collection of plant source materials to within populations local to the targeted planting site. Therefore, alternative propagation methods are needed for mass production of diverse sea oats genotypes.

Vegetatively, sea oats is a rhizomatous, herbaceous perennial. Buds at the base of the vegetative shoot can become tillers (offshoots) or rhizomes (underground stems) that eventually will develop aboveground shoots (Wagner, 1964). Miller et al. (2003) developed a system to induce tiller formation from rhizome fragments collected from the beach after a hurricane takes place. For this system, rhizome fragments needed to be collected immediately after the storm event, and replanted within 7 days to obtain 45% transplant success after rhizome reburial. Difficult access to the damaged areas and short-term viability of transplants were limiting factors to the use of this revegetation system.

Micropropagation, the rapid in vitro production of plants on a defined sterile medium in culture vessels, has many advantages over conventional plant propagation methods in horticulture, agronomy and forestry fields (Debergh and Zimmerman, 1991;
Jeong et al., 1995; Hartmann et al., 2002). The use of this technology for multiple applications is currently expanding worldwide (Conner and Thomas, 1981). Micropropagation has also been used for commercial production of plants used for habitat restoration (Kane et al., 1993; Kane and Philman, 1997; Seliskar and Gallagher, 2000), and therefore, it could be utilized for sea oats propagation. This technology also provides the opportunity to select and rapidly produce diverse ecotypes with ecologically valuable characteristics, particularly from localized sites.

In 1997, a project funded by the U.S. Department of Commerce to Dr. Michael Kane, Environmental Horticulture Department/University of Florida, was initiated to 1) examine genetic diversity and population structure in four Florida sea oats populations using genetic markers; 2) use micropropagation technology to clonally multiply representative genotypes from populations at these four sites; and 3) conduct reciprocal transplant studies at the sites using micropropagated genotypes to determine effects of genotype and geographical source on survival and growth following outplanting. As a consequence of that project, more than 28 sea oats genotypes in culture had been established (M.E. Kane, unpublished data). These genotypes were specifically collected from four sites in Florida: two Atlantic (Anastasia State Recreation Area [AN] and Sebastian Inlet State Recreation Area [SI]), and two Gulf coast populations (Egmont Key National Wildlife Refuge [EK] and St. George Island State Park [SG]).

The micropropagation shoot culture protocol was initially developed using a single sea oats genotype (Philman and Kane, 1994). However, significant problems were encountered when attempting to acclimatize Stage II unrooted microcuttings or Stage III rooted microcuttings to ex vitro conditions (Stage IV). Leaves produced during Stage II
were not expanded but more cylindrical in cross section. Survival of microcuttings under ex vitro conditions was very low. Microcuttings that rooted for 28 days in vitro (Stage III) exhibited only slightly higher survival ex vitro. When transferred to ex vitro conditions, these rooted plantlets began to exhibit new root and leaf development, but quickly stopped growing and died.

In subsequent studies, the severity of this problem varied when applied to different genotypes. In most genotypes, survival of rooted microcuttings ex vitro was significantly increased when microcuttings were rooted on Stage III medium for at least 6 to 8 weeks. During this extended culture period, morphological and anatomical changes of sea oats shoots and roots occurred during in vitro growth and development, and a greater root mass and more “grass-like” leaves with expanded blades typically developed. Although the physiological basis for increased survival is unknown, it is conceivable that these “grass-like” leaves were more photosynthetically competent than the “lance-like” leaves present after only 3 weeks in Stage III culture. Furthermore, carbohydrate reserves in sea oats possibly became depleted during Stage IV acclimatization.

For the purpose of maintaining genetic diversity, it is critical to be able to micropropagate a wide range of sea oats genotypes. However, commercial utilization of sea oats micropropagation could be limited by the poor survival rates of certain genotypes during acclimatization. Despite the extraordinary potential of micropropagation and all of its advantages, many problems still exist (Desjardins et al., 1995). The stimulation of vegetative growth in vitro, which is desirable in terms of production efficiency, induces several anatomical, morphological and physiological changes that seriously affect the performance of plantlets after ex vitro transfer.
Understanding the causes of aberrant morphology and physiology in vitro is necessary to increase growth and survival of plantlets during ex vitro acclimatization.

**Literature Review**

**Micropropagation for Habitat Restoration**

Multiple studies have been published on the feasibility of applying micropropagation techniques to commercial production of aquatic, dune, and marsh species for habitat restoration (Straub et al., 1988; Cook et al., 1989; Li and Gallagher, 1996; Kane and Philman, 1997; Rogers et al., 1998; Kane et al., 1999; Seliskar and Gallagher, 2000). Yet, few studies have been focused on applying micropropagation techniques for sea oats commercial production, especially production of diverse genotypes. Hovanesian and Torres (1986) reported indirect shoot regeneration from callus derived from in vitro germinated sea oats seeds. However, micropropagation systems based upon adventitious shoot production from callus are inherently more unreliable, both in terms of genetic stability and shoot regeneration rate, than shoot multiplication from axillary meristems (Pierik, 1987). Philman and Kane (1994) developed a micropropagation protocol for sea oats using tillers explants that induced axillary shoot regeneration for in vitro multiplication. However, significant variability in growth and development (especially during acclimatization) was observed when applied to 28 different sea oats genotypes collected from four populations sampled in Florida’s Gulf and Atlantic coasts.

Random amplified polymorphic DNA (RAPD) genetic analyses of adult sea oats plants and seedlings from those four populations indicated significant genetic variations between the two coastal communities, with the most variation occurring within the Gulf coast genotypes (Ranamukhaarachchi et al., 1999). These genetic differences were
attributed to the increased environmental stresses of the Gulf coast. Florida’s Gulf coast experiences a greater frequency of storms and hurricanes than does its Atlantic coast. Dune overwash and waves have created low-relief foredunes at St. George (SG) and a severely eroded foredune at Egmont Key (EK). Variations in seed weight, temperature requirements for seed production and germination, and seedling growth between the Gulf of Mexico and Atlantic populations further support genetic as well as phenotypic divergences (Seneca, 1972; Colosi, 1979; Hester and Mendelssohn, 1987).

Preliminary in vitro observations using sea oats genotypes collected from the Gulf coast indicate that genotypes collected from areas closer to the shoreline exhibit lower survival rates during ex vitro acclimatization than those collected further away from the shoreline. Ranamukhaarachchi et al. (1999) were reluctant to extrapolate ecotypic differences from the genetic analysis due to an absence of reciprocal transplant studies. Other possible contributions to low survival rates of sea oats plantlets during ex vitro acclimatization include morphological, anatomical, biochemical and physiological changes due to the tissue culture environment.

**In Vitro Culture Effects in Plant Anatomy**

Plants that develop in vitro acclimate to the environment in which they grow. Starting from the aerial environment of the vessel headspace, this system is characterized by a saturating atmosphere with water vapor, with very low vapor pressure deficit (Brainerd and Fuchigami, 1981; Fujiwara and Kozai, 1995), relatively low light intensities (photosynthetic photon flux, PPF; 12-70 µmol m⁻² s⁻¹), relatively high and constant temperature (25 ± 3 °C) (Fujiwara and Kozai, 1995), low CO₂ concentration during photoperiod (90 µmol mol⁻¹) (Pospíšilová et al., 1988), high CO₂ concentration
during dark period (3000-9000 µmol mol\(^{-1}\)) (De Proft et al., 1985), and high C\(_2\)H\(_4\) concentration (1.2 µmol mol\(^{-1}\)) (Kozai and Kubota, 2005). These characteristics, except for the light intensity, are largely due to the low number of air exchanges of the vessels and the relatively small air volume of the vessel headspace.

High relative humidity and low CO\(_2\) concentration in the vessel limit plant gas exchange (Pospíšilová et al., 1992). Leaves produced in vitro have a thin cuticle layer and abnormal stomatal function (stomata remain open even when subjected to sudden environmental changes; Sutter et al., 1992), which may exacerbate water stress during acclimatization to ex vitro conditions, and structural changes that may affect CO\(_2\) fixation mechanisms and light harvesting apparatus (alterations in the chloroplast ultrastructure). Despite the self-evident importance of the gaseous environment for tissue cultures, it is an often neglected component during the design of a micropropagation system. This shortcoming can have unacceptable consequences for culture performance because of the strong physiological impact of the gases involved, notably O\(_2\), CO\(_2\) and C\(_2\)H\(_4\), on plant growth and development (Jackson et al., 1994).

Significant changes in leaf morphology and anatomy, especially in epidermal characteristics caused by in vitro culture conditions, have been reported (Pospíšilová et al., 1999). Sweetgum (*Liquidambar styraciflua* L.) plantlets cultured in vitro had a less developed cuticle, as compared to the well developed cuticle in leaves of transplanted and field grown plants (Wetzstein and Sommer, 1982). Gilly et al. (1997), in studying the cuticle formation of ivy (*Hedera helix* L.) plants in vitro and after transferring to ex vitro conditions observed a progressive activation of cuticle biosynthesis as the plants adapted to the ex vitro environmental conditions.
Cuticle and epicuticular wax biosynthesis plays an important role in preventing water loss when plants are transferred to ex vitro conditions (Sutter and Langhans, 1982). In vitro light levels and relative humidity appear to be critical during the development of epicuticular waxes, stomata and epidermal cells (Capellades et al., 1990), although Gilly et al. (1997) also observed that cuticle formation is not exclusively dependent on stress conditions, but is also genetically programmed.

As a result of the tissue culture environment, in-vitro produced shoots and plantlets are also reduced in size compared to greenhouse-produced plants (Donnelly and Vidaver, 1984a). The relatively high concentration of cytokinins during the multiplication stage tends to inhibit apical dominance (Murashige, 1974). Additionally, in some species, vascular connections are reduced, thin, and poorly structured (Leshem, 1983). Oftentimes, the low light environment in vitro produces leaves that resemble shade leaves or hydrophytic leaves ex vitro (Lee et al., 1988). Also, leaves produced in vitro usually have low chlorophyll content (Grout and Aston, 1977), low percent dry matter or hyperhydrated shoots (Ziv, 1990), restricted leaf area expansion (Kozai et al., 1992), and poorly structured spongy and palisade tissues (Donnelly et al., 1985). These morphological and anatomical characteristics result in low photosynthetic ability associated with low activities of the photosynthetic enzyme rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) and increased activity of PEPC (phosphoenolpyruvate carboxylase) (Hdider, 1994; Hdider and Desjardins, 1994) and abnormal chlorophyll fluorescence responses (Hdider and Desjardins, 1994).

In sweetgum, highbush blueberry (Vaccinium corymbosum L.) and tobacco (Nicotiana tabacum L.), stomatal density decreased in newly developed leaves after ex
vitro transplant (Wetzstein and Sommer, 1983; Noé and Bonini, 1996; Tichá et al., 1999). The main reason for this was the enormous enlargement in leaf area after transfer. Conversely, tissue cultured plants exhibited greater stomatal densities than acclimated plants. However, stomatal indices, which depict the ratio of stomata number to total epidermal cells and stomata per unit area, were not calculated in these studies. Hence, the influence of leaf expansion on resultant stomata density cannot be evaluated.

In vitro sweetgum leaves contained numerous superficial and circular stomata as opposed to the ellipsoid, depressed and less numerous stomata observed on acclimated plants (Wardle et al., 1983). The general consensus has been that stomata developed in vitro are poorly functional with an inability to close in conditions otherwise leading to closing (Brainerd and Fuchigami, 1981; Wetzstein and Sommer, 1983; Conner and Conner, 1984; Ziv et al., 1987; Marin et al., 1988; Sutter, 1988; Preece and Sutter, 1991; Majada et al., 2001). Afreen (2005) concluded that the absence or reduction in leaf epicuticular and cuticular waxes, combined with non-functional stomata under conditions of low relative humidity and high light intensity, leads to abnormally high transpiration rates during acclimatization, which decreases plant survival. Additionally, plants may also guttate copiously, demonstrating their inability to control water loss (Donnelly and Tisdall, 1993). Apart from reduced or nonexistent stomatal control and poor epicuticular and cuticular wax formation, poor control of water loss could result from reduced trichome numbers (Donnelly and Vidaver, 1984a; Sutter, 1985).

**Influence of Exogenous Sugars**

Sucrose must be included in tissue culture media in order to promote adequate shoot regeneration, growth and development under low light conditions. The supply of sucrose in the media is also possibly the factor most significantly affecting net
photosynthetic rate \( (P_n) \) of plants in vitro. Studies with micropropagated rose (\textit{Rosa multiflora} L. ‘Montse’) showed that plantlet photosynthesis was influenced by the levels of sucrose in the culture medium; the lowest levels of sucrose resulted in the highest plantlet \( P_n \) values (Capellades et al., 1991).

Sucrose is the form of sugar that is most often transported within a plant in vivo. Culture medium and sugar levels decrease during the culture period. Isoforms of the enzyme invertase naturally occur in cell walls, vacuoles and cytoplasm. Microcuttings secrete invertase into the culture media in response to wounding (De la Viña et al., 1999; Sturm, 1999). This extracellular invertase hydrolyzes sucrose in the media into glucose and fructose, but the degree of hydrolysis depends on the amount of invertase secreted, which differs with each plant. Partial hydrolysis also occurs during autoclaving of the media, when 10-15% of sucrose is converted to glucose and fructose (George, 1993).

Accumulation of starch and soluble sugars following glucose uptake inhibited photosynthesis in leaves of spinach (\textit{Spinacea oleracea} L.) (Krapp et al., 1991) and rose (Capellades et al., 1991). High sugar accumulation in leaves influences photosynthesis by feedback inhibition of photosynthetic enzymes (Azcón-Bieto, 1983; Schäfer et al., 1992), and by down-regulation of expression of genes encoding photosynthetic enzymes (Sheen, 1994; Jones et al., 1996; Jang and Sheen, 1997; Sheen et al., 1999; Smeekens, 2000). The concept of sugars having an integrated role in the adjustment of cellular activity of the entire plant system provides a new framework for analysis. According to Koch’s feast/famine hypothesis, the accumulation of carbohydrates results in decreased photosynthesis and a long-term reduction in the expression of photosynthetic genes (Koch, 1996).


**Photoautotrophic Culture**

Kozai et al. (1997) showed that most chlorophyllous plantlets/microcuttings in vitro have the ability to grow photoautotrophically, in a medium without sugars, provided that the environmental conditions are favorable for photosynthesis. Plantlets grown in conventional culture vessels are characterized by low net photosynthetic rates caused by low CO$_2$ concentrations in the vessels during the photoperiod and low light intensities typical of culture rooms (Heo and Kozai, 1999). Amâncio et al. (1999) found that higher light intensities increased photosynthetic competence and subsequently improved survival of grape (*Vitis vinifera* L.) plantlets during acclimatization. Similar results have also been observed with elevated CO$_2$ concentration (Seon et al., 2000). Additionally, several studies report that addition of sucrose in the medium inhibits photosynthesis of in vitro formed leaves (Serret and Trillas, 2000; Seon et al., 2000; Van Huylensbroeck and Debergh, 1996; Lees et al., 1991).

Successful photoautotrophic systems have been developed for enhancing photosynthesis and growth of the plantlets by increasing CO$_2$ concentration and light intensity in the culture vessel (Kozai, 1988). Furthermore, to enhance acclimatization potential, Nguyen et al. (1999a) recommend the use of porous supporting material in addition to liquid medium instead of conventionally-used gelling agents to allow the formation of roots in vitro with higher vascular system development. Plantlets grown in these systems have been well characterized for many growth and developmental parameters including fresh and dry weight biomass accumulation (Cristea et al., 1999), net photosynthetic rate (Heo et al., 2001; Valero-Aracama et al., 2001), cuticular development and stomatal functioning (Zobayed et al., 1999), and improved carbohydrate
status (Wilson et al., 2001). A similar system could be used to enhance sea oats growth during in vitro culture and ex vitro acclimatization.

**Carbon Status during Acclimatization**

As previously mentioned, it is known that higher sugar concentrations in the medium suppress photosynthesis of in vitro plantlets. In vitro plantlets are mixotrophic in their mode of nutrition; they alternate between carbohydrates used from the medium and \( \text{CO}_2 \) fixation. Mixotrophy contributes to the recycling of respiratory and photosynthetic products and affects photosynthetic carbon metabolism. Kozai (1988) suggests heterotrophic/photomixotrophic growth influenced by the tissue culture environment contributes to low survivability of plantlets during acclimatization. Yet, Wilson et al. (2000) assert that appropriate higher levels of carbohydrates can favor plantlet survival upon transfer to ex vitro conditions, improve acclimatization and facilitate physiological adaptations to ex vitro conditions.

After transfer to ex vitro conditions, micropropagated plantlets are very susceptible to various stresses because their physiological and morphological status may restrict them from allocating sufficient energy resources (Chaves, 1994). Moreover, plants respond differently to the removal of exogenous sugars in the culture medium. Some plants have no difficulty undergoing the transition from a heterotrophic or photomixotrophic to a photoautotrophic condition. For example, broadleaf arrowhead (*Sagittaria latifolia* Willd.) exhibited high survival rates when unrooted microcuttings, multiplied with low levels of \( \text{N}^6 \)-benzyladenine (BA) in Stage II, were transferred to the greenhouse (Lane, 1999). Peace lily (*Spathiphyllum floribundum* Schott. ‘Petite’) leaves formed in vitro also exhibited the capacity to photosynthesize and maintain a positive carbon balance ex vitro (Van Huylenbroeck et al., 1998). Stage II unrooted microcuttings of other species
survive when transferred to the greenhouse, but the leaves formed in vitro die, giving rise to new, more photosynthetically capable leaves. These leaves demonstrate a cotyledonary effect, described by Kane (2000) as a “lifeboat” in which old leaves become the source of carbohydrate reserves to shoot meristems. This phenomenon has been observed in plants such as strawberry (*Fragaria x ananassa* Duch. ‘Kent’), grape, and *Calathea loiusae* Gagnep. ‘Maui Queen’ (Hdider and Desjardins, 1995; Amâncio et al., 1999; Van Huylenbroeck et al., 1998). Grout and Millam (1985) make a distinction between these two groups of leaves formed in vitro: those that are photosynthetically competent, and those that are photosynthetically non-competent.

**Photosynthetic Rates during Acclimatization**

Net photosynthetic rates of potato (*Solanum tuberosum* L.) and peace lily plants decreased during the first week after transplanting to greenhouse conditions (Baroja Fernández, 1993; Baroja Fernández et al., 1995; Van Hyulenbroeck and Debergh, 1996). While *Calathea* leaves formed in vitro were not able to photosynthesize during the first days after transfer, and peace lily leaves formed in vitro were photosynthetically competent ex vitro, both plant species exhibited substantial photosynthetic activity after new leaves were fully developed (Van Huylenbroeck et al., 1998).

**Cytokinin Carryover Effects on Plantlet Acclimatization**

Plant growth regulators incorporated into shoot multiplication culture media, especially cytokinins, can have deleterious carryover effects on in vitro and ex vitro growth and development (Pospišilová et al., 1992; Werbrouck et al., 1995). **N**\(^6\)-benzyladenine (BA) is the most widely used cytokinin for shoot multiplication in tissue culture (Werbrouck et al., 1996), and this cytokinin is required for sea oats micropropagation (Philman and Kane, 1994). Experimental evidence indicates that
reduction in rooting and acclimatization, observed in some plants produced on BA-supplemented medium, may result from production of an inhibitory BA metabolite, [9G]BA. This metabolite accumulates at the base of plantlets in vitro and remains for more than 6 weeks (Werbrouck et al., 1995; Werbrouck et al., 1996). Moncaleán et al. (2001) found that kiwi (Actidinia deliciosa Chev. ‘Hayward’) explants cultured over 2 days in medium containing BA followed by BA-free medium performed better during rooting and acclimatization stages than those cultured in BA-containing medium for longer periods. In vitro culture duration in medium containing BA appears to have an effect on ex vitro acclimatization. Strnad et al. (1997) reported that meta-topolin, a naturally occurring BA analog, produces a deleterious metabolite with a shorter half-life and is less inhibitory than BA. Werbrouck et al. (1996) found that meta-topolin effectively multiplies peace lily shoots, with better rooting in vitro than equimolar concentrations of BA. Conceivably, meta-topolin may be an acceptable BA substitute for sea oats micropropagation, which may enhance rooting and Stage IV survival.

C₄ Photosynthesis

Sea oats is a C₄ plant and it exhibits Kranz anatomy (Brown and Gracen, 1972; Brown and Smith, 1974). Kranz anatomy is considered an evolutionary advancement because it facilitates two mechanisms of CO₂ fixation (Keeley, 1998) and it allows greater water-, carbon-, and nitrogen-use efficiencies (Zelitch, 1982; Robichaux and Pearey, 1984). In vitro culture conditions of low irradiance, high humidity, low CO₂ concentration, and high exogenous sugar levels in the medium are an atypical environment for a C₄ plant. These conditions could lead to limitations in growth and photosynthetic rates of plantlets in vitro. Changes in the activity of PEPC have been observed under these conditions. Furbank et al. (1997) observed a substantial increase in
PEPC activity in smelter’s bush (*Flaveria bidentis* [L.] Kuntze) seedlings, a C₄ plant, when grown in the presence of exogenous sucrose. In the same treatment, rubisco activity was markedly decreased. Phosphoenolpyruvate carboxylase has a higher affinity for CO₂, but requires twice the energy of ATP (Salisbury and Ross, 1992). Additionally, C₄ plants have adapted to high heat and light intensity, and a large investment in ATP may render them disadvantaged in the tissue culture environment. Sugarcane (*Saccharum officinarum* L.), also a C₄ species, can be micropropagated in vitro through various culture techniques (Sauvaire and Galzy, 1978; Barba et al., 1978; Ho and Vasil, 1983; Chengalrayan and Gallo-Meagher, 2001). Enhancement of growth and photosynthetic rates of sugarcane in vitro can be achieved by manipulating the environmental culture conditions (Erturk and Walker, 2000; Xiao et al., 2003). Rubisco and PEPC activities are possibly also affected by the changes of the environmental conditions.

**CAM Photosynthesis**

Abnormal physiological stomatal responses in plantlets may result from the tissue culture environment or changes in the Kranz anatomy of sea oats in vitro. Malda et al. (1999) observed similar adaptations in nellie cory cactus (*Coryphantha minima* Baird). Under high humidity in vitro, this cactus performed crassulacean acid metabolism (CAM) photosynthesis in the light. During acclimatization, carbon fixation via the CAM pathway reverted back to operating only in the dark. In sea oats, high humidity conditions could prevent stomatal closure throughout the day and night periods. This would result in increased transpiration rates after ex vitro transfer and subsequent water stress.
Research Objectives

It is likely that the in vitro environment is influencing the anatomy, morphology and physiology of sea oats resulting in the low survival observed in some sea oats genotypes during acclimatization. Modifications of the culture environment and comparison between genotypes with differing responses during ex vitro acclimatization provide the opportunity to better understand sea oats physiology in vitro. Consequently, in the present study, anatomical and physiological basis for poor acclimatization of selected sea oats genotypes were examined.

To examine the physiological and anatomical basis for sea oats genotypic differences in acclimatization capacity, genotypes were selected from plants established in vitro from Egmont Key, in the Gulf Coast: EK 16-3 and EK 11-1, an easy- and difficult-to-acclimatize. The main objective of the present research was to correlate morphological, anatomical and physiological characteristics between the two genotypes with survival rates ex vitro.

This dissertation research has been organized in three chapters:

Comparative Morphology and Anatomy of In Vitro and Ex Vitro Cultured Sea Oats Genotypes

Changes in the in vitro culture environment, including vessel headspace gas composition, culture medium mineral salt composition, plant growth regulators (PGR) levels and sugars, occur with time. Conceivably, these changes could influence growth and development of in vitro cultures and affect the acclimatization capacity of shoots. In vitro time course experiments were designed to examine this further. The morphological and anatomical characterizations of both sea oats genotypes cultured in vitro and ex vitro were characterized by evaluating in vitro multiplication, rooting and survival ex vitro as a
function of Stage II and Stage III culture duration. Furthermore, leaf samples were collected at different periods during multiplication, rooting, and after acclimatization ex vitro to determine anatomical and morphological changes. To accomplish this, samples were processed, observed and photographed using optical light, scanning electron and transmission electron microscopy. These techniques facilitated making anatomical comparisons at tissue, cellular and organelle levels.

**Photosynthetic and Carbohydrate Status of Sea Oats Genotypes during In Vitro and Ex Vitro Culture Conditions**

During the first days of acclimatization, photosynthetic capacity of leaves and carbohydrate status of plants become critical for survival ex vitro. Therefore, the effects of in vitro culture on photosynthetic capacity of sea oats genotypes were studied by measuring photosynthetic rates during ex vitro acclimatization. Subsequently, a study was conducted to determine the carbohydrate status of sea oats genotypes during in vitro rooting and ex vitro acclimatization. This was correlated with the analyses of the enzymatic activities of rubisco and PEPC during in vitro multiplication and rooting, ex vitro acclimatization, and after establishment to greenhouse conditions.

**Influence of In Vitro Growth Conditions on In Vitro and Ex Vitro Photosynthetic Rates of Sea Oats Genotypes**

In this section, the effects of environmental conditions during micropropagation of sea oats genotypes on survival ex vitro were studied. A system that induced photoautotrophy in vitro was designed for sea oats genotypes. Furthermore, effects of CO₂ enrichment during in vitro micropropagation on in vitro and ex vitro growth of plantlets were evaluated. In this experiment the photosynthetic capacity of plantlets during in vitro and ex vitro culture under different environmental culture conditions was compared.
CHAPTER 2
COMPARATIVE GROWTH, MORPHOLOGY AND ANATOMY OF IN VITRO AND
EX VITRO CULTURED EASY- AND DIFFICULT-TO-ACCLIMATIZE SEA OATS
(Uniola paniculata L.) GENOTYPES

Introduction

Sea oats (Uniola paniculata L.), is a perennial dune grass native to the southeast U.S. This species is commonly used for beach restoration and dune stabilization in Florida after dune systems are damaged or destroyed by tropical storms, hurricanes or human activity (Bachman and Whitwell, 1995). Sea oats has the ability to rapidly colonize and establish, and exhibits high tolerance to heat, drought, and salinity (Wagner, 1964; Woodhouse, 1982). The root and rhizome systems hold sand particles together, while the vegetation above ground retards wind and water driven erosion and promotes sand deposition (Woodhouse, 1982).

Sea oats is propagated under nursery conditions from field-collected seed. However, seed sources are limited due to increased beach erosion. Moreover, concerns regarding the introduction of un-adapted ecotypes to revegetation sites have also limited the use of seeds and or plant materials obtained from distant geographic sources. Consequently, alternative propagation methods, including micropropagation, have been developed for mass production of sea oats plants.

A micropropagation protocol for sea oats was developed by Philman and Kane (1994). Additionally, 28 different sea oats genotypes were established, multiplied, and rooted using this protocol. However, significant differences in survival and acclimatization capacity were observed between genotypes when plants were transferred
to ex vitro conditions. Similarly, many other horticultural plants species are readily micropropagated in vitro but exhibit poor acclimatization and subsequent survival ex vitro (Debergh and Zimmerman, 1991). Alterations in the morphological and anatomical characteristics of plants during and after in vitro culture appear to play a critical role in successful acclimatization to greenhouse conditions (Donnelly and Vidaver; 1984a; Serret and Trillas, 2000; Wetzstein and Sommer, 1982). In sea oats, a better understanding of the morphological and anatomical differences between genotypes is required to develop more efficient micropropagation procedures.

In vitro plantlets produced under high humidity conditions and transferred ex vitro to the soil are usually very susceptible to desiccation (Wetzstein and Sommer, 1983). Typically, low light intensities in vitro result in the production of leaves that resemble shade or hydrophytic leaves ex vitro (Lee et al., 1988). These leaves often have little epicuticular and cuticular wax formation (Grout, 1975) and malfunctioning stomata (Brainerd and Fuchigami, 1981). Leaves produced in vitro also have low chlorophyll content (Grout and Aston, 1977), restricted leaf blade expansion (Kozai et al., 1992), low stomatal density (Ziv, 1995), poorly differentiated spongy and palisade tissues (Donnelly et al., 1985), low percent dry matter, and/or hyperhydrated shoots (Ziv, 1991). All of these characteristics negatively impact the potential for ex vitro acclimatization.

Some researchers have attributed hyperhydration to the effect of certain plant growth regulators in the medium, such as N6-benzyladenine (BA) (Ziv, 1991; Khan et al., 2002). Other medium components, such as sucrose, also affect the anatomy of plantletts. Some in vitro plants contain chloroplastic starch granules while others have little or none
depending upon sucrose concentration (Dhawan and Bhojwani, 1987; Lee et al., 1985; Queralt, 1989).

These morphological and anatomical characteristics result in insufficient photosynthetic capacity to achieve a positive carbon balance (Grout and Aston, 1978). The degree to which plants are affected by the in vitro environment depends on the plant species. Furthermore, genotypic differences during in vitro propagation have also been reported (Llorente and Apóstolo, 1998). The extent of the differences between sea oats genotypes needs further investigation.

The only effective cytokinin for shoot multiplication of sea oats is BA. Werbrouck et al. (1995) identified a negative carryover effect of BA during ex vitro acclimatization. The extent of this carryover effect appeared to depend upon the formation of BA derivatives that accumulated in the base of plantlets and negatively affected rooting and survival ex vitro. Furthermore, duration of in vitro incubation with BA appeared to affect survival and acclimatization ex vitro (Moncaleán et al., 2001). Preliminary studies with sea oats genotypes indicated that there is similar negative BA carryover effect after ex vitro transfer (Valero-Aracama et al, 2003). The extent of the effects of BA on multiplication, rooting and survival ex vitro of sea oats genotypes needs further investigation.

Additionally, other in vitro culture conditions, such as nutrient, sugar or plant growth regulator concentration in the medium and gas composition in the vessel headspace, change with culture duration. Understanding the effects of these changing conditions with time on the anatomy and morphology of sea oats genotypes and
consequently on acclimatization and survival ex vitro will help improve the sea oats micropropagation protocol.

In the present study, we compared the morphology of easy- and difficult-to-acclimatize sea oats genotypes as affected by in vitro multiplication and rooting conditions. Comparative analysis of leaf anatomy, stomatal formation in leaf surfaces and chloroplast ultrastructure in mesophyll cells and bundle sheath cells of in vitro and ex vitro leaf samples were conducted. Ultimately, we investigated the relationship between morphological and anatomical development of sea oats genotypes and their differing capacity for acclimatization.

**Materials and Methods**

**Culture Conditions**

Two established, stabilized and indexed sea oats genotypes (*Uniola paniculata* L.), genotyped using random amplified polymorphic DNA (RAPD) genetic analyses (Ranamukhaarachchi, 2000), previously characterized as easy- and difficult-to-acclimatize (EK 16-3 and EK 11-1, respectively) were used in this study. Five sea oats shoot clusters (each consisting of three shoots, 25-mm long) of EK 16-3 and EK 11-1 genotypes were subcultured in 80 mL sterile multiplication medium (Stage II) into separate Magenta GA7 vessels (Magenta Corp., Chicago, IL). Culture medium consisted of Murashige and Skoog (MS) inorganic salts (Murashige and Skoog, 1962), supplemented with 87.6 mM sucrose, 0.56 mM myo-inositol, 1.2 \( \mu \)M thiamine-HCl, 2.2 \( \mu \)M N\(^6\)-benzyladenine (BA), and solidified with 8 g L\(^{-1}\) TCTM agar (*PhytoTechnology* Laboratories, Shawnee Mission, KS). The medium was adjusted to pH 5.7 with 0.1 N KOH prior to the addition of agar and autoclaving at 1.2 kg cm\(^{-2}\) and 121 °C for 20 min.
Cultures were maintained for 8 weeks in a growth chamber at 24 ± 1 °C, 58 ± 5% relative humidity (RH), and 16-h photoperiod provided by cool-white fluorescent lamps (General Electric F20WT12-CW), at a 40 ± 5 μmol m⁻² s⁻¹ photosynthetic photon flux (PPF) as measured at culture level. Subsequently, 25-mm long single shoots from each genotype were excised and transferred to rooting medium (Stage III).

Stage III rooting basal medium consisted of 80 mL sterile half-strength MS medium, supplemented with 0.56 mM myo-inositol, 1.2 μM thiamine-HCl, 87.6 mM sucrose, and 10 μM α-naphthalene acetic acid (NAA), and adjusted to pH 5.7 with 0.1 N KOH. Medium was solidified with 8 g L⁻¹ TC™ agar and autoclaved at 1.2 kg cm⁻² and 121 °C for 20 min. Each GA7 culture vessel contained 8 single microcuttings, and were maintained in a culture room at 22 ± 2 °C air temperature, 16-h photoperiod provided by cool-white fluorescent lamps (General Electric F96T12-CW-WM), and 100 ± 5 μmol m⁻² s⁻¹ PPF as measured at culture level.

Microcuttings were transferred to acclimatization conditions (Stage IV) into 48-cell plug trays (8 six-celled blocks, each cell 4 x 6 x 5.5 cm; T.O. Plastics, Inc., Clearwater, MN) containing coarse vermiculite as supporting material. Plug trays were placed in a greenhouse under controlled environmental conditions. Plantlets were hand watered as needed, and Peters 20N-20P-20K liquid fertilizer (150 mg N L⁻¹; The Scotts Company, Marysville, OH) was applied weekly.

**Description of Treatments**

**Effect of Stage II duration on in vitro rooting and ex vitro survival of sea oats genotypes**

Plantlets of both genotypes were cultured for 4, 8 and 12 weeks in Stage II, and subsequently, 48 unrooted microcuttings were directly transferred to Stage IV conditions.
Percent survival was determined after 4 weeks ex vitro. Another set of 64 microcuttings per genotype, were concurrently transferred to Stage III rooting medium in vessels arranged in a completely randomized design. Percent rooting was evaluated after 6 weeks in Stage III. Subsequently, 48 microcuttings were transferred to Stage IV conditions and percent survival was determined after 4 weeks ex vitro culture. Arcsine transformation was applied to the data where appropriate. Microcuttings were maintained at 25/22.5 °C day/night temperature from August 8 until December 12, 2002 in a greenhouse in Gainesville, FL with light intensities ranging 700-1200 µmol m\(^{-2}\) s\(^{-1}\).

This experiment was replicated once in time.

**Comparative Stage II shoot multiplication and growth of sea oats genotypes**

Plantlets from EK 11-1 and EK 16-3 consisting of 3 shoot clusters were cultured for 4, 8, and 12 weeks under Stage II conditions. Number of shoots, number of leaves per shoot, leaf length, and dry weight of plantlets from 5 replicate vessels per genotype were measured after each culture period. Production of rootable microcuttings ≥ 25 mm long were considered as harvestable microcuttings. A completely randomized design was used. This experiment was replicated once in time.

**Effect of Stage III duration on in vitro rooting, growth and development and ex vitro survival of sea oats genotypes**

EK 11-1 and EK 16-3 plantlets were cultured for 8 weeks under Stage II conditions. Subsequently, single shoots of each genotype were excised and transferred to Stage III conditions for 3, 6 or 9 weeks in 8 replicate vessels per treatment and time interval. After Stage III culture, 2 rooted plantlets per vessel were selected to measure shoot number, leaf number per plant, leaf length, root number, root length, and dry weight of shoots and roots per plantlet. The remaining 6 plantlets per vessel were
transferred to Stage IV conditions into the greenhouse. The same growth measurements of 2 plants per six-celled pack were obtained after four weeks ex vitro culture. Microcuttings were maintained at 25/22.5 °C day/night temperature from June 12 until July 24, 2003 in a greenhouse in Gainesville, FL with light intensities ranging 700-1000 µmol m⁻² s⁻¹. This experiment was replicated once in time.

**Comparative anatomy of sea oats genotypes during Stage II, Stage III and Stage IV culture**

Leaf histological cross sections collected from EK 11-1 and EK 16-3 microcuttings cultured in vitro in Stage II and Stage III and from greenhouse-produced leaves were made. For optical light microscopy (OLM) and transmission electron microscopy (TEM), leaf sections approximately 2 mm from the center of the leaf blade were fixed in Trumps fixative solution (McDowell and Trump, 1976). Fixative infiltration was achieved under vacuum for 2 days. Leaf tissues were then rinsed 3 times in phosphate buffer (pH 7.2), post-fixed in a 1% buffered osmium tetroxide solution and then rinsed in phosphate buffer, 3 times in distilled water, and dehydrated in a five-step ascending ethyl alcohol series (25, 50, 75, 95, 100%) followed by dehydration in 100% acetone. An enbloc stain of 2% uranyl acetate was applied between the 75 and 95% steps of the ethyl alcohol dehydration series. Leaf sections were then embedded in Spurr resin (Spurr, 1969). For OLM, thick leaf sections (500 nm) were obtained with a Leica Ultracut ultramicrotome R (Leica Microscopy and Scientific Instruments, Deerfield, IL) and then collected on glass slides. Sections were stained with 0.2% toludine blue and examined using an Olympus BH-2 Epifluorescent Microscope (Olympus America Inc., Melville, NY). Photographs were taken using a Pixera 120C digital camera attachment. For TEM, ultrathin leaf sections (70 nm) were cut from the center part of the leaf blade with a Leica
Ultracut ultramicrotome R, collected on 0.35% form-var coated copper grids, stained with methanolic uranyl acetate and lead citrate (Reynolds, 1963). Sections were viewed on a Hitachi H7000 transmission electron microscope (Hitachi Scientific Instruments, Danbury, CT) at 75 kV. Digital micrographs were taken on a BioScan/Digital Micrograph 2.5 (Gatan Inc., Pleasanton, CA) at an exposure level optimized for viewing the outer layer and processed with MEGA View III/AnalySIS 3.1 (Soft Imaging System Corp., Lakewood, CO).

For scanning electron microscopy (SEM), leaf sections of approximately 5 mm from the center of the leaf blade were immersed in 100% methanol. Leaf tissues were collected from cultures 2 h after photoperiod started and from cultures 5 h after dark period started. Sections were lyophilized using a Bal-Tec 030 critical point drier (ICMAS Inc., Alcoa, TN) with liquid CO$_2$, sputter coated with gold-palladium using a Denton Vacuum Desk II (Denton Vacuum, Moorestown, NJ) for approximately 50 s and viewed with a Hitachi S-4000 FS scanning electron microscope (Hitachi Scientific Instruments, Danbury, CT) operating at 6 kV. Digital images were processed using SEMages 16 software (Advance Database Systems, Inc., Denver, CO).

**Statistical Analyses**

Percent data was transformed using the arc sine transformation, and the significant differences among means were determined by two-way analysis of variance (ANOVA) using the GLM procedure of SAS (SAS institute Inc., 1999). Interactions among genotypes and time, where appropriate, are shown in the tables and graphs. Separate *a posteriori* tests for significant differences among or between means were analyzed using the Waller-Duncan procedure at $P \leq 0.05$. 
Results

Effect of Stage II Duration on In Vitro Rooting and Ex Vitro Survival of Sea Oats Genotypes

In vitro rooting

All Stage III EK 16-3 plantlets rooted regardless of Stage II duration. Rooting of EK 16-3 plantlets was significantly greater ($P = 0.0136$) than EK 11-1 plantlets, with 93, 86 and 99% rooting at 4, 8 and 12 weeks Stage II, respectively. Significant differences in root architecture and morphology were observed between the two genotypes after 4, 8 and 12 weeks Stage II culture following 6 weeks Stage III culture (Figure 2-1). The EK 11-1 root system consisted of a few thick elongated roots with small lateral branching without visible root hairs after 8 and 12 weeks Stage II. The EK 16-3 root system exhibited large root numbers that were shorter than EK 11-1 roots regardless of Stage II duration. Visual assessment of shoot development during Stage III, revealed that EK 16-3 plantlets had greater leaf length, leaf expansion and shoot thickness than EK 11-1 plantlets (Figure 2-1). However, increasing Stage II duration from 8 to 12 weeks resulted in decreased shoot and root development in EK 16-3 plantlets after 6 weeks Stage III. This decrease in plantlet growth was attributed to the quality of harvestable microcuttings obtained after 12 weeks Stage II, since older shoots had died and the remaining harvestable microcuttings were smaller than after 4 or 8 weeks culture. During Stage II, visual assessments demonstrated that EK 16-3 produced longer leaves than EK 11-1.

Ex vitro survival

Survival of microcuttings transferred ex vitro directly from Stage II cultures was 0 (EK 11-1) and less than 17% (EK 16-3) (Figure 2-2A). There was no significant effect of Stage II duration on survival of unrooted microcuttings within each genotype. EK 16-3
Figure 2-1. Comparative morphological differences in shoot and root development of EK 11-1 (left) and EK 16-3 (right) sea oats genotypes after 4, 8 and 12 weeks Stage II culture (from top to bottom, respectively) followed by 6 weeks Stage III culture. Scale = 1 cm.
Stage III microcuttings that rooted for 6 weeks Stage III, exhibited nearly 100% ex vitro survival regardless of Stage II duration. In contrast, survivability of EK 11-1 rooted microcuttings was 30, 54, and 64% respectively for 4, 8 and 12 weeks Stage II culture (Figure 2-2B). Even though survival was higher in EK 11-1 plantlets cultured for 12 weeks rather than 8 weeks Stage II, we determined that the optimal Stage II culture duration was 8 weeks, since the microcutting quality was higher at 8 weeks than at 12 weeks Stage II.

**Comparative Shoot Multiplication and Growth of Sea Oats Genotypes during Stage II Culture**

Visual observations indicated that EK 11-1 and EK 16-3 sea oats genotypes exhibited shoot multiplication and subsequent elongation of leaves after 4 weeks culture (Figure 2-3; Table 2-1). However, unlike EK 11-1 leaves, EK 16-3 leaves continued elongating after 8 weeks culture. At week 8 Stage II, both genotypes exhibited browning of shoots and leaves, which continued until week 12 Stage II culture. At that time, most of the longest shoots were brown and only the newly developed shoots remained green in either genotype.

Shoot dry weights in both genotypes were greatest after four weeks culture (Figure 2-4). Browning and dying of tissue steadily increased with time (Figure 2-3). Shoot number per plantlet (Table 2-1) of both genotypes increased from weeks 4 to 8 during Stage II but decreased from weeks 8 to 12. Similarly, leaf number decreased during Stage II culture in both genotypes due to leaf death (Table 2-1). Leaf length was significantly higher for EK 16-3 than EK 11-1 plantlets throughout Stage II. After four weeks culture, leaf length decreased in both genotypes (Table 2-1). The longest leaves,
Figure 2-2. Comparative survival percentage after 4 weeks under Stage IV culture of A: Stage II unrooted, and B: Stage III rooted sea oats microcuttings after 4, 8 or 12 weeks Stage II culture. Error bars indicate SE (n = 16). ANOVA is shown on top left corner of each graph; T: Time, G: Genotype, NS, **: Non-significantly or significantly different at $P = 0.01$, respectively. Different letters on top of histobars are significantly different according to Waller-Duncan test at $P \leq 0.05$. 

<table>
<thead>
<tr>
<th>Time in Stage II (weeks)</th>
<th>EK 11-1</th>
<th>EK 16-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>8</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>12</td>
<td>c</td>
<td></td>
</tr>
</tbody>
</table>

Ex vitro survival (%) of unrooted microcuttings

Ex vitro survival (%) of rooted microcuttings

T: NS
G: **
T x G: NS

T: **
G: **
T x G: **
Figure 2-3. Comparative morphological differences in shoot multiplication of EK 11-1 (left) and EK 16-3 (right) sea oats genotypes after 4, 8 and 12 weeks Stage II culture (from top to bottom, respectively). Scale = 1 cm.
Figure 2-4. Comparative Stage II shoot dry weights of EK 11-1 and EK 16-3 sea oats genotypes after 4, 8 and 12 weeks culture. Error bars indicate SE (n = 16). ANOVA is shown on top left corner; T: Time, G: Genotype, NS, **: Non-significantly or significantly different at $P = 0.01$, respectively. Different letters on top of histobars are significantly different according to Waller-Duncan test at $P \leq 0.05$. 
Table 2-1. Comparative shoot number, leaf number and leaf length of EK 11-1 and EK 16-3 sea oats genotypes after 4, 8 and 12 weeks Stage II culture.

<table>
<thead>
<tr>
<th>Stage II Duration (weeks)</th>
<th>Shoot Number</th>
<th>Leaf Number</th>
<th>Leaf Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EK 11-1</td>
<td>EK 16-3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>12.4 ± 0.5</td>
<td>14.7 ± 0.7</td>
<td>10.2 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>13.6 ± 0.6b</td>
<td>10.8 ± 4.5</td>
<td>10.4 ± 4.2a</td>
</tr>
<tr>
<td></td>
<td>18.1 ± 0.7b</td>
<td>25.5 ± 0.9a</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>16.2 ± 0.9</td>
<td>17.3 ± 1.1</td>
<td>92.4 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>16.8 ± 1.0a</td>
<td>81.7 ± 9.4</td>
<td>87.1 ± 7.3b</td>
</tr>
<tr>
<td></td>
<td>8.8 ± 0.3d</td>
<td>16.5 ± 0.7b</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>13.9 ± 1.0</td>
<td>15.7 ± 1.3</td>
<td>80.7 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>14.8 ± 1.2b</td>
<td>69.7 ± 6.0</td>
<td>75.2 ± 5.9c</td>
</tr>
<tr>
<td></td>
<td>9.4 ± 0.8d</td>
<td>11.4 ± 0.8c</td>
<td></td>
</tr>
<tr>
<td>( \bar{\chi} )</td>
<td>14.2 ± 0.8B</td>
<td>15.9 ± 1.0A</td>
<td></td>
</tr>
</tbody>
</table>

Analysis of variance\(^*\)

<table>
<thead>
<tr>
<th></th>
<th>Genotype (G)</th>
<th>Time (T)</th>
<th>G * T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*</td>
<td>**</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

\(^\text{z}\) Means ± SE followed by different letters are significantly different according to Waller-Duncan test at \( P \leq 0.05 \).

\(^\text{y}\) When no interaction was observed, means followed by lowercase letters indicate significant differences within Stage II duration.

Means followed by uppercase letters indicate significant differences between genotypes.

\(^*\), **: Significant at \( P \leq 0.05 \) or 0.01, respectively \((n = 16)\).
which were also the oldest, died and the newly formed leaves were considerably shorter.

Relative leaf length frequencies indicated a greater occurrence of longer leaves in EK 16-3 than EK 11-1 plantlets throughout Stage II culture (Figure 2-5).

Figure 2-5. Comparative relative leaf length frequency per plantlet of sea oats genotypes after A: week 4, B: week 8, and C: week 12 Stage II culture.
Effect of Stage III Duration on In Vitro Rooting and Growth and Ex Vitro Survival and Acclimatization of Sea Oats Genotypes

In vitro rooting and growth

Shoot and root dry weights of both genotypes increased throughout Stage III culture (Figure 2-6). At week 3, shoot dry weights of EK 16-3 plantlets were lower than those of EK 11-1 plantlets, but at weeks 6 and 9, EK 16-3 shoot dry weights were higher than those of EK 11-1 plantlets. Conversely, root dry weights were similar at week 3 but higher for EK 11-1 than for EK 16-3 plantlets at weeks 6 and 9. Shoot to root dry weight ratios were not significantly different between genotypes regardless of Stage III culture duration (data not shown).

Shoot number increased with time in EK 11-1 plantlets and it was not significantly different from weeks 6 to 9 in EK 16-3 plantlets (Figure 2-7; Table 2-2). Significantly higher numbers of leaves were produced by EK 11-1 plantlets as the duration of Stage III increased to nine weeks. However Stage III duration did not affect leaf number of EK 16-3 plantlets (Table 2-2). Conversely, by week 3, leaves were 2-fold longer in EK 16-3 than EK 11-1 plantlets and elongated rapidly, becoming 6.5-fold longer in EK 16-3 than EK 11-1 plantlets by week 9 Stage III. Leaf length distributions (Figure 2-8) indicated that leaf elongation was significantly inhibited in EK 11-1. Ninety-five percent of the EK 11-1 leaves were ≤ 15 mm long by week 9, in contrast with EK 16-3, with 50% of the leaves ≥ 16 mm long.

Stage III root development differed significantly between genotypes. Root production, at each culture interval, was higher in EK 16-3 than in EK 11-1 plantlets (Table 2-3). Conversely, EK 11-1 roots were longer than EK 16-3 roots throughout Stage III culture (Figure 2-7; Table 2-3). Root length distributions (Figure 2-9) indicated that
EK 11-1 plantlets produced a greater percentage of longer roots than EK 16-3 plantlets from week 3, and continued elongating until 50% of the roots ranged from 243-550 mm in length.

Figure 2-6. Comparative A: shoot dry weights, and B: root dry weights of sea oats genotypes after weeks 3, 6 and 9 Stage III culture. Error bars indicate SE (n = 32). ANOVA is shown on top left corner of each graph; T: Time, G: Genotype, *, **: Significantly different at $P \leq 0.05$ or 0.01, respectively. Different letters on top of histobars are significantly different according to Waller-Duncan test at $P \leq 0.05$. 
Figure 2-7. Comparative morphological differences in rooting and shoot multiplication of EK 11-1 (left) and EK 16-3 (right) sea oats genotypes after 8 weeks Stage II followed by 3, 6 and 9 weeks Stage III culture (from top to bottom, respectively). Scale = 1 cm.
Table 2-2. Comparative shoot number, leaf number and leaf length of EK 11-1 and EK 16-3 sea oats genotypes after 3, 6 and 9 weeks Stage III culture.

<table>
<thead>
<tr>
<th>Stage III Duration (weeks)</th>
<th>Shoot Number</th>
<th></th>
<th></th>
<th>Leaf Number</th>
<th></th>
<th></th>
<th>Leaf Length (mm)</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>EK 11-1</td>
<td>EK 16-3</td>
<td>EK 11-1</td>
<td>EK 16-3</td>
<td>EK 11-1</td>
<td>EK 16-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4.5 ± 0.3cd</td>
<td>4.3 ± 0.3d</td>
<td>25.6 ± 0.9c</td>
<td>24.3 ± 1.2c</td>
<td>3.8 ± 0.2c</td>
<td>7.8 ± 0.4c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.7 ± 0.3b</td>
<td>5.3 ± 0.3bc</td>
<td>39.2 ± 1.8b</td>
<td>29.0 ± 1.7c</td>
<td>5.6 ± 0.5c</td>
<td>23.2 ± 1.7b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>7.4 ± 0.5a</td>
<td>4.6 ± 0.5cd</td>
<td>45.5 ± 2.6a</td>
<td>24.0 ± 1.5c</td>
<td>6.9 ± 0.6c</td>
<td>44.9 ± 3.3a</td>
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Analysis of variance<sup>y</sup>

<table>
<thead>
<tr>
<th></th>
<th>Genotype (G)</th>
<th>Time (T)</th>
<th>G * T</th>
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</table>

<sup>x</sup>Means ± SE followed by different letters are significantly different according to Waller-Duncan test at $P \leq 0.05$.

<sup>y</sup>**: Significantly different at $P \leq 0.01$ ($n = 32$).
Table 2-3. Comparative root number and root length of EK 11-1 and EK 16-3 sea oats genotypes after 3, 6 and 9 weeks Stage III culture.

<table>
<thead>
<tr>
<th>Stage III Duration (weeks)</th>
<th>Root Number</th>
<th>Root Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EK 11-1</td>
<td>EK 16-3</td>
</tr>
<tr>
<td>3</td>
<td>5.1 ± 0.5d</td>
<td>12.9 ± 1.3c</td>
</tr>
<tr>
<td>6</td>
<td>6.5 ± 0.5d</td>
<td>21.1 ± 1.5b</td>
</tr>
<tr>
<td>9</td>
<td>6.8 ± 0.7d</td>
<td>26.0 ± 1.8a</td>
</tr>
</tbody>
</table>

Analysis of variance\(^{\text{y}}\)

<table>
<thead>
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<th></th>
<th>**</th>
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</thead>
<tbody>
<tr>
<td>Genotype (G)</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Time (T)</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>G * T</td>
<td>**</td>
<td>**</td>
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</tbody>
</table>

\(^{\text{x}}\) Means ± SE followed by different letters are significantly different according to Waller-Duncan test at \(P \leq 0.05\).

\(^{\text{y}}\)**: Significantly different at \(P \leq 0.05\) \((n = 32)\).
Figure 2-8. Comparative relative leaf length frequency per plantlet of sea oats genotypes after A: week 3, B: week 6, and C: week 9 Stage III culture.
Figure 2-9. Comparative relative root length frequency per plantlet of sea oats genotypes after A: week 3, B: week 6, and C: week 9 Stage III culture.
Ex vitro survival and acclimatization

Stage III duration had a significant effect on ex vitro survival of sea oats genotypes especially in EK 11-1. After 4 weeks Stage IV, survivability was 79, 94 and 94% for EK 16-3, and 1, 20 and 26% for EK 11-1 at weeks 3, 6 and 9 Stage III culture, respectively. Shoot and root dry weights after four weeks Stage IV, were significantly greater for EK 16-3 than EK 11-1 plantlets regardless of Stage III duration (Figure 2-10A, B). Shoot and root dry weights increased in both genotypes with duration. Comparative ex vitro survival and growth of rooted microcuttings of both genotypes previously cultured for eight weeks in Stage II and six weeks in Stage III, are shown at weeks 0, 3 and 6 after transfer to Stage IV in Figure 2-11.

Anatomical and Ultrastructural Comparisons

Optical light microscopy (OLM)

Leaf sections collected from greenhouse-produced leaves of plants established ex vitro (Figure 2-12) served as reference for leaf development comparisons of plantlets in vitro (Figures 2-13 and 2-14). The anatomy of ex vitro sea oats plants was typical of \( \text{C}_4 \) plants, and was very similar between EK 11-1 and EK 16-3 genotypes (Figure 2-12). Both genotypes possessed a compacted mesophyll composed of few large cells with most visible chloroplasts present in cells surrounding the vascular bundle. Both genotypes possessed extensive vascular bundles that were oval and occupied most of the cross section of the leaves (Figure 2-12). Bundle sheath cells of greenhouse-produced leaves were similar in shape between genotypes, contained larger chloroplasts than those of in vitro plantlets (Figures 2-13 and 2-14) and were arranged centripetally in the bundle sheath.
Figure 2-10. Comparative ex vitro A: shoot dry weights, and B: root dry weights of sea oats genotypes after weeks 3, 6 and 9 Stage III culture followed by 4 weeks Stage IV culture. Error bars indicate SE (n = 1, 14 and 16 for EK 11-1 at 3, 6 and 9 weeks, respectively and n = 32 for EK 16-3 during Stage III). ANOVA is shown on top left corner of each graph; T: Time, G: Genotype, NS, **: Non-significantly or significantly different at P ≤ 0.01, respectively. Different letters on top of histobars are significantly different with time according to Waller-Duncan test at P ≤ 0.05.
Figure 2-11. Comparative morphological differences in shoot multiplication of EK 11-1 (left) and EK 16-3 (right) sea oats genotypes previously cultured for 8 weeks in Stage II and 6 weeks in Stage III, at weeks 0, 3, and 6 Stage IV culture (from top to bottom, respectively).
Schlerenchyma tissue was clearly present between the epidermal layers and the vascular bundles. A visible cuticle was present over the abaxial epidermis of both genotypes. The adaxial epidermis of leaves contained bulliform cells. These cells were responsible for the epidermal foldings which formed the characteristic adaxial ribs of the leaf blades. Multicellular glands were observed on both abaxial and adaxial surfaces.

Early in Stage II (Figure 2-13A, D), large intercellular spaces in the mesophyll tissue of both genotypes were present. Both genotypes comprised mesophyll cells of varied sizes and shapes. At week 8 Stage II, these intercellular spaces were still present in EK 11-1 plantlets whereas mesophyll of EK 16-3 leaves were more compact with fewer intercellular spaces. A compact mesophyll was present in both genotypes by week 12 Stage II. Production of adaxial sinuses was only observed in EK 11-1 by week 12 Stage II. Adaxial sinuses were present in EK 16-3 plantlets by week 4 Stage II. Conversely, EK 11-1 plantlets lacked adaxial sinuses during weeks 4 and 8, and possessed round rather than oval vascular bundles. In greenhouse-produced leaves (Figure 2-12) both genotypes exhibited a visible cuticle in the adaxial epidermis. However, the abaxial epidermis of both genotypes lacked a visible cuticle during Stage II (Figure 2-13). Throughout Stage II culture, schlerenchyma tissue was present between the epidermal layers and the vascular bundles of EK 16-3 plantlets, whereas initial development of schlerenchyma tissue was only present at week 12 culture in EK 11-1 plantlets. Based on visual observations, mesophyll and bundle sheath cells of either genotype contained fewer numbers of chloroplasts than greenhouse-grown plants.

During Stage III culture, there were visible changes in leaf development in both genotypes (Figure 2-14). At weeks 6 and 9 Stage III culture, EK 16-3 plantlets exhibited
anatomical features similar to greenhouse-grown plants, whereas differences in tissue organization and anatomical features were observed between greenhouse-produced leaves and leaves of Stage III EK 11-1 plantlets. Intercellular spaces appeared to be as frequent during Stage III as during Stage II. EK 16-3 leaves were comprised of a more compacted mesophyll than EK 11-1 plantlets, and larger intercellular spaces. Furthermore, the mesophyll tissue in leaves of both genotypes was comprised of numerous small cells as compared to that of greenhouse-produced leaves (Figure 2-12). Both genotypes lacked adaxial sinuses at week 3 Stage III culture and exhibited large adaxial sinuses at weeks 6 and 9 (Figure 2-14B, C, E, F). Vascular bundles were round at week 3 (Figure 2-14A, D) but became oval with time. At week 6, mesophyll and vascular bundles of EK 16-3 plantlets (Figure 2-14E) were highly structured and similar to those of greenhouse-produced leaves (Figure 2-12). At that time, EK 16-3 vascular bundles were oval and completely occupied the leaf cross section and filled the adaxial leaf sinuses. However, mesophyll and vascular bundles were comparably disrupted and unorganized in EK 11-1 plantlets throughout Stage III (Figure 2-14A-C). EK 16-3 plantlets developed a visible abaxial epidermal cuticle by week 6 (Figure 2-14E, F). In contrast, EK 11-1 plantlets exhibited minimal cuticle development during Stage III (Figure 2-14A-C). Schlerenchyma tissue between the epidermal layers and the vascular bundles was visible in EK 16-3 plantlets throughout Stage III (Figure 2-14D-F), whereas it was less apparent in EK 11-1 plantlets and only visible after six weeks Stage III (Figure 2-14B-C).
Figure 2-12. Comparative histological leaf sections of A: EK 11-1, and B: EK 16-3 sea oats genotypes in greenhouse-produced leaves. (b: bundle sheath cell, c: cuticle, m: mesophyll cell, s: stoma). Scale = 100 µm.
Figure 2-13. Comparative histological leaf sections of EK 11-1 (A, B, C) and EK 16-3 (D, E, F) genotypes at week 4 (A, D), week 8 (B, E) and week 12 (C, F) Stage II culture. (b: bundle sheath cell, i: intercellular space, m: mesophyll cell, s: stoma). Scale = 100 µm.
Figure 2-14. Comparative histological leaf sections of EK 11-1 (A, B, C) and EK 16-3 (D, E, F) genotypes at week 3 (A, D), week 6 (B, E) and week 9 (C, F) Stage III culture. (b: bundle sheath cell, c: cuticle, i: intercellular space, m: mesophyll cell, s: stoma). Scale = 100 µm.
Scanning electron microscopy (SEM)

Leaf sections collected from greenhouse-grown plants (Figures 2-15 and 2-16) served as a reference for leaf surface and stomata comparisons with in vitro produced plantlets (Figures 2-17 and 2-18). Adaxial epidermal surface features and stomate morphology of greenhouse-produced leaves were similar between both genotypes (Figures 2-15 and 2-17). Both genotypes possessed glands on the adaxial ribs (Figure 2-15). Stomata were found arranged in rows inside the invaginations of the adaxial leaf surfaces. Each row had 1 or 2 epidermal cells between each stomate in either genotype. Epidermal cells were raised, convex and rectangular. A closer image of the stomata (Figure 2-16) of both genotypes revealed significant epicuticular wax layer arranged in a homogeneous layer and bearing a crystalline structure. Stomata were characteristic of grass species, with elongated guard cells surrounded by two large subsidiary cells, and very small stomatal apertures. Leaves were amphistomatous (with stomata in both epidermal surfaces), and based on visual assessments, stomatal density was greater on the adaxial surface. Yet, because stomata were present in the invaginations of the adaxial epidermises, stomatal density could not be accurately quantified. Stomata were arranged in longitudinal rows of cells throughout the leaf blade length.

Similarly to that observed in histological sections, leaves produced in Stage II exhibited no or relatively low wax deposition in both genotypes (Figures 2-17A-D). Wax clusters were observed randomly over the leaf surfaces, instead of in a homogeneous layer as in greenhouse-grown plants. Stomata apertures were fused or blocked with wax depositions regardless of being collected during light or dark period (images not shown) during Stage II culture. Both genotypes exhibited similar stomatal structure and epidermal surface features regardless of Stage II duration.
During Stage III culture (Figures 2-18A-D), increased wax deposition was observed on the leaf surfaces of both sea oats genotypes, especially on EK 16-3 leaves after nine weeks culture. Abaxial cuticle development was minimal at the OLM level in EK 11-1 plantlets (Figure 2-18C). Wax deposition was more uniform in EK 16-3 than in EK 11-1 leaf surfaces (Figure 2-18F). While EK 11-1 stomatal apertures appeared blocked in most cases, clearly defined stomatal apertures without blockage were observed in EK 16-3 leaves. The stomatal structure was similar between genotypes regardless of Stage III duration.

Figure 2-15. Comparative SEM of adaxial epidermis of A: EK 11-1, and B: EK 16-3 genotypes in greenhouse-produced leaves. (g: gland, s: stomate). Scale = 75 µm.
Figure 2-16. Comparative SEM of stomata on adaxial epidermis of A: EK 11-1, B: EK 16-3 genotypes in greenhouse-produced leaves. Scale = 10 µm.
Figure 2-17. Comparative SEM of stomata on adaxial epidermis of A-C: EK 11-1, and D-F: EK 16-3 genotypes at week 4 (A, D), week 8 (B, E) and week 12 (C, F) Stage II culture. Scale = 10 μm.
Figure 2-18. Comparative SEM of stomata on adaxial epidermis of A-C: EK 11-1, and D-F: EK 16-3 genotypes at week 3 (A, D), week 6 (B, E), and week 9 (C, F) Stage III culture. Scale = 10 µm.
Transmission electron microscopy (TEM)

Ultra-thin section examination of samples collected from EK 11-1 and EK 16-3 plants revealed additional details, such as differences in chloroplast ultrastructure in both mesophyll and bundle sheath cells. Chloroplast ultrastructure in acclimatized plants was very different in mesophyll cells compared to bundle sheath cells (Figure 2-19). Ultrastructural characteristics of ex vitro leaves were similar between genotypes. Mesophyll cell chloroplasts (Figures 2-19A, C) consisted of a highly compacted thylakoid membrane system with minimal stroma. A compact thylakoid membrane system was similarly observed in bundle sheath chloroplasts (Figures 2-19B, D) and included large starch granules. Mesophyll cell chloroplasts were smaller and randomly arranged within the cells, whereas those in bundle sheath cells were larger and arranged centripetally within the bundle sheath.

Mesophyll cells of EK 11-1 Stage II plantlets contained chloroplasts with similar thylakoid membrane distribution at weeks 4 and 8 (Figures 2-20A, B), however signs of senescence, including thylakoid disruption, were observed by week 12 Stage II (Figure 2-20C). Similar signs of senescence were present at week 12 Stage II culture in EK 16-3 chloroplasts (Figure 2-20I). Bundle sheath cells of either genotype contained chloroplasts that exhibited compacted thylakoid membrane throughout Stage II culture. While no visible starch granules were observed in chloroplasts of EK 11-1 plantlets, regardless culture duration, EK 16-3 chloroplasts of either cell type contained starch granules at week 8 Stage II.

During Stage III culture, EK 11-1 mesophyll cell chloroplasts contained swollen thylakoids at weeks 6 and 9 (Figure 2-21B, C), whereas typical compacted thylakoid membranes were observed in EK 16-3 chloroplasts (Figure 2-21H, I). While large starch
granules were only observed in the early weeks of Stage III in either genotype, smaller starch granules were observed at week 6 Stage III culture (Figure 2-21D, E, J).

Figure 2-19. Comparative TEM of chloroplasts of A-B: EK 11-1, and C-D: EK 16-3 genotypes after Stage IV acclimatization in the greenhouse. Mesophyll (A, C) and bundle sheath cell (B, D) chloroplasts are shown. Scale = 2 µm. (ch: chloroplast, s: starch granule, t: thylakoid membranes)
Figure 2-20. Comparative TEM of chloroplasts of EK 11-1 (A-F) and EK 16-3 (G-L) genotypes at week 4 (A, D, G, J), week 8 (B, E, H, K), and week 12 (C, F, I, L) Stage II culture. Mesophyll (A-C and G-I) and bundle sheath cell (D-F and J-L) chloroplasts are shown. Scale = 2 μm. (s: starch granule, t: thylakoid membranes)
Figure 2-21. Comparative TEM of chloroplasts of EK 11-1 (A-F) and EK 16-3 (G-L) genotypes at week 3 (A, D, G, J), week 6 (B, E, H, K), and week 9 (C, F, I, L) Stage II culture. Mesophyll (A-C and G-I) and bundle sheath cell (D-F and J-L) chloroplasts are shown. Scale = 2 µm. (s: starch granule, t: thylakoid membranes)
Discussion

We observed differing responses between the two genotypes tested in vitro, and especially during ex vitro acclimatization. Anatomical and morphological differences among plants are largely attributed to genotypic differences and phenotypical plasticity, but also to different responses to these conditions (Majada et al., 2000). The culture duration during multiplication and rooting stages also had differing effects on acclimatization and survival of both genotypes to ex vitro conditions.

During in vitro multiplication and rooting conditions, the rate of leaf differentiation and development differed between genotypes. In EK 11-1 development of grass-like leaves with expanded blades was significantly suppressed. Therefore, the developmental stages of leaves of both genotypes differed when the anatomical leaf comparisons were made. However, our main interest was to evaluate the anatomical state of leaves at time of transfer to rooting conditions and to ex vitro conditions. This information is helpful to understand the differences in growth and survival ex vitro between both genotypes.

Histological observations indicated that unorganized tissues and abnormal anatomical features, such as lack of cuticle, intercellular spaces in the mesophyll, blocked stomata, or disrupted chloroplast ultrastructure, were common during Stage II for both genotypes, especially in the difficult-to-acclimatize genotype, EK 11-1. Additionally, SEM micrographs showed that both genotypes similarly lacked a homogeneous epicuticular wax layer and exhibited fused or blocked stomata during Stage II.

Transmission electron micrographs showed chloroplast disruption in both genotypes throughout Stage II. Several authors have described similar chloroplast disruption as a characteristic of hyperhydric leaves (Wetzstein and Sommer, 1982; Ziv et al., 1983; Lee et al., 1985; Capellades et al., 1991) and leaves produced under low light
intensities (Lee et al., 1988). Furthermore, like hyperhydric leaves, Stage II sea oats leaves had fewer chloroplasts with reduced thylakoid stacking compared to greenhouse-produced leaves (Drennan and van Staden, 1986; Jones et al., 1993).

Low light levels during culture result in swollen thylakoids in chloroplasts of certain plant species (Queralt, 1989). Similarly, under the low light intensity characteristic of Stage II, swollen thylakoids appeared more frequently in sea oats genotypes than when they were cultured under higher light intensity during Stage III. Frequently, swollen thylakoids occur after starch granules have accumulated within the chloroplast (Queralt, 1989). When plants utilize their stored reserves, starch is used but the thylakoid membranes remain swollen. Anatomical modifications caused by the tissue culture environment have direct impact on diffusion of CO₂ inside the in vitro produced leaves and thus in photosynthesis in vitro (Desjardins, 1995). Alterations of the chloroplast ultrastructure are associated with disorganization of the light harvesting pigments. As a result, the photosynthetic capacity of developing leaves in vitro is low (Lee et al, 1985), causing poor acclimatization and survival ex vitro of Stage II plantlets. This state may contribute to the low ex vitro survival of sea oats Stage II microcuttings.

Another possible cause of mortality during acclimatization is poor control of water loss (Brainerd and Fuchigami, 1982). In angiosperms, this phenomenon has been related to poor stomatal functioning (Brainerd and Fuchigami, 1982) and reduced or abnormal structure of epicuticular wax (Grout and Aston, 1977, Dhawan and Bhojwani, 1987, Sutter, 1988). These characteristics were common in Stage II sea oats leaves. Therefore, Stage II unrooted sea oats microcuttings of either genotype transferred directly ex vitro probably exhibited poor control of water loss during acclimatization. Additionally, Stage
II unrooted microcuttings did not root after ex vitro transfer, possibly because the energy resources of shoots were not sufficient to initiate root development or to continue shoot growth. Unrooted microcuttings subsequently had minimal capacity to uptake water from the substrate. Water uptake by roots, together with reduced water loss from shoots, is critical for maintenance of water balance during acclimatization of in vitro produced plants (Fila et al., 1998).

For Stage II sea oats shoots multiplication, only the cytokinin BA is effective (Philman and Kane, 1994). The negative BA carry over effect of Stage II on ex vitro survival of the difficult-to-acclimatize genotype was observed. However, EK 11-1 survival increased by increasing Stage II culture duration. This may, in part, be due to a detrimental effect of using BA for Stage II multiplication. Moncaleán et al. (2001) observed decreased ex vitro survival of kiwi (Actidinia deliciosa Chev. ‘Hayward’) explants with increasing Stage II incubation with BA. However, these authors used 35-day subculture periods consisting of shorter BA incubation periods (from 30 min to 2 days) followed by incubation in plant growth regulator-free medium. Werbrouck et al. (1995) observed a rapid accumulation of BA derivatives one day after incubation of peace lily (Spathiphyllum floribundum Schott ‘Petite’) plantlets on BA containing medium. One of these derivatives ([9G]BA) accumulated at the base of the plantlets, did not seem to be transported, and had longer half-life than BA. It was concluded that [9G]BA accumulation was detrimental to root formation and to ex vitro survival of plantlets. The increased survival in EK 11-1 with increasing Stage II duration was possibly caused by the degradation of [9G]BA with time. These results are in agreement with those reported by Moncaleán et al. (2001), because longer incubation periods with
BA would result in higher accumulation of [9G]BA at the base of the plantlets, consequently resulting in lower survival ex vitro.

To evaluate multiplication rates, we considered microcutting yield along with quality of shoots, including shoot biomass, leaf number, leaf length and visual quality assessments. Our observations indicated that eight weeks Stage II culture yielded the highest multiplication rates of both genotypes. At week 8, multiplication rates decreased mainly because leaf senescence was extensive, possibly a consequence of the translocation of nutrients and energy reserves to newly developing tissues (Thomas and Sadras, 2001). During senescence, at the molecular level chloroplasts and chlorophyll are degraded. Proteins and lipids are also degraded in the form of amino acids and sugars. The various culture conditions that change with time, such as BA concentration, secondary metabolite production, sugar content, and gas composition inside the vessel headspace, could cause the senescence of older leaves and the translocation of resources to new shoots or storage areas (Buchanan-Wollaston et al., 2003). Therefore, in a multiplication stage longer than eight weeks, medium nutrient and sugar depletion could be limiting to the growth and multiplication of sea oats genotypes and induce leaf senescence.

During Stage III culture, shoot and root growth increased in both genotypes, but clear differences in plantlet morphology were observed between genotypes. At week 6 Stage III, EK 16-3 plantlets had greater shoot but lower root biomass than EK 11-1. While energy sources were directed mainly to root growth in EK 11-1, EK 16-3 plantlets predominantly utilized their energy sources towards growth of elongated leaves with expanded leaf blades. These differences in developmental patterns are important during
the transition from in vitro photomixotrophic or heterotrophic conditions to the ex vitro photoautotrophic conditions. During this transition, plantlets must adjust from using medium sucrose or their stored reserves to producing their own photoassimilates to continue growth and development ex vitro (Piqueras et al., 1998). Although the importance of photosynthetic capacity during acclimatization has been emphasized in many studies, Van Huylenbroeck and Debergh (1996) concluded that the photosynthetic ability at time of ex vitro transfer is of secondary importance; the primary requirement are carbohydrate reserves large enough to overcome the transition to ex vitro conditions. However, in sea oats, photosynthetic capacity is also critical during acclimatization since in previous studies we observed low ex vitro survival of EK 11-1 plants that exhibited high starch reserves at time of ex vitro transfer (Valero-Aracama et al., 2004b). These starch reserves are rapidly depleted during the first 2 weeks of ex vitro acclimatization (Chapter 3). Leaves in these plants were also short, thick, and without expanded blades, and plants also exhibited large root systems.

One significant difference between the easy- and difficult-to-acclimatize genotype is the ability of the former to produce leaves during Stage III of similar morphology and anatomical features as ex vitro produced leaves (Valero-Aracama et al., 2003). These features may be reflected in normal photosynthetic capacity when transferred ex vitro. Optical light micrographs of leaves from EK 16-3 during Stage III revealed similar leaf tissue organization between plantlets cultured for six weeks in Stage III and after being acclimatized to greenhouse conditions. At weeks 6 and 9 Stage III, tissues were highly organized in the easy-to-acclimatize genotype compared to the difficult-to-acclimatize genotype. EK 11-1 leaf sections exhibited disorganized mesophyll tissue throughout
Stage III. This has also been reported by other authors (Brainerd et al., 1981; Donnelly and Vidaver, 1984a; Johansson et al., 1992, Tichá and Kutík, 1992; Dami and Hughes, 1995; Noé and Bonini, 1996). Likewise, whole leaf morphology, especially expanded blades, was similar between acclimatized plantlets and EK 16-3 plantlets cultured for six or nine weeks in Stage III. Conversely, EK 11-1 plantlets produced small thin leaves throughout Stage III culture. Several studies have indicated that in vitro produced leaves of some species were both functionally and structurally anomalous (Sutter, 1981).

Leaves of cauliflower (Brassica oleracea L.) (Wardle et al., 1979), and apple (Malus pumila Mill.) (Brainerd and Fuchigami, 1981) had reduced stomatal functioning as well as reduced photosynthetic capacity (Grout and Aston, 1977) during in vitro culture. These leaves would be more likely injured under the stress conditions characteristic during the change from in vitro to ex vitro culture.

A decreased biomass would imply a loss of photosynthetic capacity during acclimatization. Loss in shoot dry weight (from 81 mg to 71 mg) was observed when EK 11-1 plantlets were compared from week 6 Stage III (time 0 Stage IV) to week 4 Stage IV. In contrast, EK 16-3 plantlets exhibited a 2.7-fold increase in shoot biomass (from 94 mg to 261 mg) for the same culture periods. After transfer ex vitro, a positive carbon balance is required for plantlet acclimatization and continued growth (Grout and Ashton, 1978). A positive carbon balance was not attained in EK 11-1 plantlets. The anomalous anatomy in EK 11-1 limited photosynthesis ex vitro resulting in decreased survival and initially limiting growth of those that survived. Additionally, stored carbohydrate reserves were likely depleted before plants could produce sufficient photoassimilates to overcome the energy demands for growth (Chapter 3).
Although adventitious root formation was necessary to increase ex vitro survival of both sea oats genotypes, excessive root biomass may have rendered EK 11-1 plants disadvantaged during acclimatization ex vitro. In sea oats, large root systems are sources of starch reserves and carbohydrates accumulated in vitro (Valero-Aracama et al., 2004b). However, being composed of heterotrophic tissue, the root often has high energy demands.

In vitro produced roots of sea oats genotypes differed significantly in architecture and morphology. Several investigations have indicated that adventitious roots of various plant species formed in vitro display particular anatomical and morphological features induced by the physical characteristics of the gelled culture medium (Mohammed and Vidaver, 1988; McClelland and Smith, 1988; McClelland et al., 1990). Therefore, the roots produced in vitro may promote growth in vitro but may not be functional after ex vitro transfer (McClelland et al., 1990; Bonal and Monteuuis, 1997).

During the initial acclimatization period, the fraction of dry matter allocated to roots has been reported to be small or unchanged (Fila et al., 1998). When comparing in vitro and ex vitro root dry weights of plants cultured for six weeks in Stage III and four weeks in Stage IV, we observed a 38% decrease in root biomass of EK 11-1 whereas there was a 57% increase in that of EK 16-3 plantlets. The decrease of EK 11-1 root biomass indicated that those roots partially or totally died ex vitro and either a portion of the roots were still functional or newly developed roots were formed. Conversely, the increase of EK 16-3 root biomass ex vitro indicated that those roots formed in vitro were possibly functional and continued growing after ex vitro transfer. Possibly, high energy
demands by roots, limited root functionality, and limited photosynthetic capacity of
shoots in EK 11-1 plants, contributed to death of roots.

These morphological and anatomical differences between sea oats genotypes may
influence photosynthetic capacity of plants. This should be verified by comparative
determination of photosynthetic capacity in vitro and ex vitro in both genotypes.
Additionally, the carbohydrate status of both genotypes during the same culture periods
should be investigated.

Conclusions

The abnormal anatomy and morphology of the difficult-to-acclimatize sea oats
genotype in vitro correlated with poor survival and acclimatization ex vitro. In vitro
produced sea oats plantlets require the formation of elongated leaves with expanded
lamina, and highly organized leaf tissues for successful acclimatization. These
anatomical and morphological characteristics possibly facilitate the transition from
photomixotrophic or heterotrophic conditions to photoautotrophic conditions by
improving the control of water loss and the photosynthetic capacity ex vitro.
Additionally, formation of roots in vitro appeared critical for survival ex vitro due to: 1)
increased water demands ex vitro, and 2) reduced energy demands to produce roots ex
vitro because this energy is supplied in vitro (Stage III). Furthermore, the Stage II
duration should be long enough to overcome the negative ex vitro carryover effect of BA.
Yet, to increase production efficiency, it would be beneficial to culture sea oats
genotypes for eight weeks rather than twelve weeks Stage II to limit leaf senescence and
to obtain higher shoot multiplication rate and quality of microcuttings in vitro.

During Stage III, sea oats plantlets require at least six weeks culture to produce
elongated shoots and roots. This stage is critical for the development of photosynthetic
competence and to accumulate carbohydrate reserves. The high survival observed in the easy-to-acclimatize genotype indicated that its carbon balance was positive after acclimatization. Conversely, the difficult-to-acclimatize genotype exhibited poor development of shoots and an extensive heterotrophic root system that likely resulted in a negative carbon balance ex vitro, thus leading to low survival.

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CHAPTER 3
PHOTOSYNTHETIC AND CARBOHYDRATE STATUS OF EASY- AND DIFFICULT-TO-ACCLIMATIZE SEA OATS (Uniola paniculata L.) GENOTYPES DURING IN VITRO CULTURE AND EX VITRO ACCLIMATIZATION

Introduction

Micropropagation has been extensively used for the rapid production of many plant species and cultivars (Debergh and Zimmerman, 1991; Jeong et al., 1995; Hartmann et al., 2002). However, despite its extraordinary potential, this technology is still confronted with many problems. Among these, one of the most important is the poor survival of plantlets following ex vitro transfer, during acclimatization to greenhouse or field conditions (Pospíšilová et al., 1999). This problem originates from poor development of photosynthetic capacity in vitro, which has been attributed to the presence of sugar in the medium (Kozai, 1991a; Pospíšilová et al., 1992), low light and inadequate CO₂ supply (Kozai and Iwanami, 1988; De et al., 1993), and poor control of water loss caused by high relative humidity within the vessel (Desjardins, 1995; Estrada-Luna et al., 2001). These conditions can ultimately influence plant development and photosynthetic performance (Kozai, 1991b; Preece and Sutter, 1991).

Kozai et al. (1997) demonstrated that most chlorophyllous plantlets/microcuttings in vitro have photosynthetic ability provided that the environmental conditions are favorable for photosynthesis. However, low ventilation rates, characteristic of conventional culture vessels, limit CO₂ availability during almost the entire photoperiod (Kadleček et al., 2001). Consequently, plantlets commonly exhibit low net
photosynthetic rates caused by low CO$_2$ concentrations in the vessels during the photoperiod and low light intensities typical of culture rooms (Heo and Kozai, 1999).

In vitro culture conditions frequently result in alterations in mesophyll development as well as chloroplast structure, namely grana development (Wetzstein and Sommer, 1982). At the biochemical level, low ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) activity (Grout, 1988) and high phosphoenolpyruvate carboxylase (PEPC) activity (Triques et al, 1997) is often encountered in C$_3$ species. These conditions also contribute to low photosynthetic activity.

Acclimatization, the transition period from in vitro to ex vitro conditions, is critical because this is when developmental and physiological abnormalities of plants produced in vitro need to be corrected to ensure survival and continued plant growth (Debergh and Zimmerman, 1991; Preece and Sutter, 1991). During acclimatization, in vitro cultured plants must go through a transition from a heterotrophic or photomixotrophic mode to a fully photoautotrophic mode in the greenhouse. It has been frequently reported that high sugar concentration in the culture medium results in feedback inhibition of photosynthesis in C$_3$ plants (Hdider and Desjardins, 1994). Also, several investigations indicate that high sugar concentration decreases rubisco activity (Hdider, 1994; Hdider and Desjardins, 1994). The activity of this enzyme and the presence of starch granules in chloroplasts, which is common in in-vitro cultured plantlets, are known to affect the regeneration of ribulose 1,5-bisphosphate (RuBP), the substrate for rubisco (Desjardins, 1995). It has also been reported that low light levels, typical of in vitro culture, inhibit rubisco activity by reduced activation of rubisco activase (Potris, 1992). During the
initial days of acclimatization, typically low rubisco activity steadily increases to levels common in greenhouse-grown plants (Desjardins, 1990).

During initial stages of acclimatization, in vitro leaves of certain plant species serve as carbohydrate storage organs to cover metabolic demands of growing tissues (Van Huylenbroeck et al., 1998; Piqueras et al., 1998). However, the function of in vitro produced leaves during acclimatization varies depending upon plant species. Van Huylenbroeck et al. (1998) concluded that in some plant species such as *Calathea* (*Calathea loiusae* Gagnep. ‘Maui Queen’), in vitro formed leaves can function as storage organs, whose energy reserves are consumed during the first days of acclimatization. These types of leaves have limited photosynthetic ability. However, in other plant species such as peace lily (*Spathiphyllum floribundum* Schott. ‘Petite’), in vitro leaves are photosynthetically competent and function similarly to greenhouse-produced leaves. Furthermore, cauliflower (*Brassica oleracea* L.) or strawberry (*Fragaria x ananassa* Duch. ‘Kent’) leaves are net respirers, and their in vitro produced leaves senesce rapidly after transplantation ex vitro (Grout and Aston, 1978; Grout and Millam, 1985).

Sea oats (*Uniola paniculata* L.) is a perennial C₄ grass, native to the southeastern U.S., and commonly used for beach and dune restoration and stabilization (Wagner, 1964; Brown and Smith, 1974). This species is usually propagated by seed. However, alternative vegetative propagation methods are necessary because of the limitation on the natural sources of plants and seeds (Hester and Mendelssohn, 1987; Bachman and Whitwell, 1995; Burgess et al., 2002; Burgess et al., 2005). Furthermore, concerns regarding the use of unadapted ecotypes collected from distant locations have also limited the collection of plant materials. Consequently, a micropropagation protocol was
developed to mass produce sea oats genotypes from localized sites (Philman and Kane, 1994). This protocol was defined using a single genotype. However, for the purpose of maintaining genetic diversity, it is critical to be able to micropropagate a wide range of sea oats genotypes. When this protocol was applied to multiple sea oats genotypes, microcuttings of different genotypes transferred ex vitro displayed differing acclimatization capacities.

Understanding the reasons for low acclimatization capacity of some sea oats genotypes is needed to efficiently produce a wide range of sea oats genotypes using micropropagation. The difference in acclimatization capacity between sea oats genotypes could be the result of differing photosynthetic and carbohydrate status during in vitro culture. Furthermore, the physiological changes occurring during the initial period of acclimatization appear critical during ex vitro establishment. The objective of the present study was to compare the photosynthetic characteristics, photosynthetic enzymatic activity and carbohydrate status of in vitro and ex vitro sea oats genotypes to correlate this with their differing capacity for acclimatization.

**Materials and Methods**

**Culture Conditions**

Established and indexed in vitro shoot cultures of two sea oats (*Uniola paniculata* L.) genotypes, collected from Egmont Key, on the Florida Gulf coast, genotyped using random amplified polymorphic DNA (RAPD) genetic analyses, and previously characterized as easy- and difficult-to-acclimatize (EK 16-3 and EK 11-1, respectively) were used. Five sea oats shoot clusters each consisting of three shoots, 25 mm long of EK 16-3 and EK 11-1 genotypes, were subcultured into separate Magenta GA7 vessels (Magenta Corp., Chicago, IL) containing 80 mL sterile Stage II medium. Stage II
medium consisted of Murashige and Skoog (MS) inorganic salts (Murashige and Skoog, 1962), supplemented with 87.6 mM sucrose, 0.56 mM myo-inositol, 1.2 µM thiamine-HCl, 2.2 µM N\textsuperscript{6}-benzyladenine (BA), and solidified with 8 g L\textsuperscript{-1} TCT\textsuperscript{TM} agar (PhytoTechnology Laboratories, Shawnee Mission, KS). All media were adjusted to pH 5.7 with 0.1 N KOH prior to the addition of agar and autoclaving at 1.2 kg cm\textsuperscript{-2} and 121 °C for 20 min.

Cultures were maintained for eight weeks in a growth chamber at 24 ± 1 °C, 58 ± 5% relative humidity (RH), 16-h photoperiod provided by cool-white fluorescent lamps (General Electric F20WT12-CW), and at 40 ± 5 µmol m\textsuperscript{-2} s\textsuperscript{-1} photosynthetic photon flux (PPF) as measured at culture level. Subsequently, the shoot clusters of each genotype were subdivided into single shoots and transferred to Stage III rooting medium.

Stage III rooting medium consisted of 80 mL sterile half-strength MS medium, supplemented with 0.56 mM myo-inositol, 1.2 µM thiamine-HCl, 87.6 mM sucrose, and 10 µM α-naphthalene acetic acid (NAA), contained in GA7 vessels. Culture vessels contained eight single microcuttings each, and were maintained in a culture room at 22 ± 2 °C, under a 16-h photoperiod provided by cool-white fluorescent lamps (General Electric F96T12-CW·WM), at 100 ± 5 µmol m\textsuperscript{-2} s\textsuperscript{-1} PPF, as measured at culture level.

After six weeks, rooted microcuttings were placed in 48-cell plug trays (8 six-celled blocks, each cell 4 x 6 x 5.5 cm; T.O. Plastics, Inc., Clearwater, MN) containing coarse vermiculite as supporting material and transferred to Stage IV conditions. Plantlets were watered as needed, and Peters 20N-20P-20K liquid fertilizer (150 mg N L\textsuperscript{-1}; The Scotts Company, Marysville, OH) was applied weekly. Greenhouse set points
for cooling and heating were 24 and 22°C, respectively, and natural solar PPF ranged 900-1200 µmol m⁻² s⁻¹ at noon.

**Photosynthesis Studies**

Shoot clusters of the EK 11-1 and EK 16-3 sea oats genotypes were cultured for eight weeks under Stage II conditions as previously described. Subsequently, single microcuttings were transferred to Stage III conditions for six weeks, with 10 replicate GA7 vessels per genotype. All rooted microcuttings were transferred in 48-cell plug trays (6 eight-celled blocks, each cell 4 x 6 x 5.5 cm; Summit Plastics Inc., Clearwater, OH) to a greenhouse under natural solar PPF ranging 900-1200 µmol m⁻² s⁻¹ during the measurements and day/night temperatures of 25/22 °C, respectively.

Net photosynthetic rates per leaf area ($P_{nl}$) were determined with a PP System Model Ciras-1 (PP System Co., Ltd., UK) without a supplemental light source and inlet CO₂ concentration fixed at 400 ± 10 µmol mol⁻¹. Measurements were taken in full sun near midday (10 am to 12 pm) on newly formed with fully expanded leaves every week beginning the day after establishment ex vitro. Percent survival was scored every week during Stage IV. Two plants per plug tray were measured, compiling data from 20 plants per genotype. Data were collected weekly for 7 consecutive weeks.

**Photosynthesis Enzymatic Studies**

Shoot clusters from EK 11-1 and EK 16-3 were cultured for 8 weeks in GA7 vessels under Stage II conditions as previously described. There were 4 replicate vessels per genotype, each vessel containing 5 plants. Concurrently, another set of sea oats shoot clusters were cultured for 8 weeks under the same conditions for shoot multiplication.
prior to transfer to Stage III conditions for 3, 6 or 9 weeks. Each treatment in Stage III consisted of 4 replicate vessels per genotype each containing 8 microcuttings.

Shoots of EK 11-1 and EK 16-3 cultures from Stage II and Stage III in vitro and Stage IV ex vitro conditions were harvested 3 h after the beginning of photoperiod for rubisco and PEPC analyses. Each replicate collected from in vitro conditions consisted of all 5 shoot clusters per vessel from Stage II or all 8 clusters per vessel from Stage III, which immediately were placed in liquid N\textsubscript{2}. During ex vitro Stage IV, each replicate contained 3 plants, which were placed in liquid N\textsubscript{2} in the greenhouse. Subsequently, all replicates were ground to a fine powder in a mortar cooled with liquid N\textsubscript{2} and placed in plastic vials in a -80 °C freezer before analyses.

For enzymatic extraction, powdered shoot tissue (~225 mg per sample) was placed in a glass mortar with 1.8 mL extraction buffer containing 100 mM bicine (pH 8.0 at 25 °C), 10 mM MgCl\textsubscript{2}, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), 10 mM isocitrate, 2% PVP-40 (w/v) and 0.1% TX-100 (w/v). Leaf tissue was ground with extraction buffer on ice for up to 2 min, transferred into 2 microfuge tubes, and centrifuged for 45 s. A 0.2 mL aliquot of the supernatant was incubated (on ice for a minimum of 5 min) with 0.01 mL 500 mM MgCl\textsubscript{2} plus 0.011 mL 200 mM NaHCO\textsubscript{3} to obtain fully-carbamylated rubisco (“activated extract”) prior to assay of rubisco activity. A separate aliquot of the supernatant was held at room temperature for immediate assay for PEPC. Both enzymes were assayed in total volumes of 0.5 mL at 25 °C, in triplicate, and assays were completed within 30 min from start of extractions procedure with a Hitachi Model U-2000 Double-Beam UV/VIS Spectrophotometer (Hitachi Instruments, Inc., Danbury, CT).
Phosphoenolpyruvate carboxylase was assayed spectrophotometrically at 340 nm by following the reduction of OAA by NADH in the presence of excess malate dehydrogenase [MDH] (Ashton et al., 1990). The reaction mixture contained 100 mM bicine (pH 8.0), 10 mM MgCl$_2$, 0.1 mM EDTA, 10 mM NaHCO$_3$, 5 mM DTT, 2.5 units MDH, and 0.2 mM NADH. After addition of 0.01 mL extract, a steady baseline was established and the reaction was initiated by addition of PEP to a final concentration of 5 mM. The linear decrease in absorbance was recorded over a period of 150 s.

Total rubisco activity (fully activated rubisco) was assayed spectrophotometrically, based on the method by Lilley and Walker (1974). This enzyme-linked assay couples the activity of rubisco with the oxidation of NADH using 3-phosphoglyceric phosphokinase and glyceraldehyde 3-phosphate dehydrogenase extracted from rabbit muscle (linking enzymes). The linking enzymes were purchased as ammonium sulphate precipitates. Sulphate was removed prior to use either by de-salting solubilized precipitates or by dissolving the precipitates in 20% glycerol solution in buffer (Sharkey et al., 1991). The reaction mixture for measuring rubisco activity contained 100 mM bicine (pH 8.0), 20 mM MgCl$_2$, 1 mM EDTA, 20 mM NaCl, 10 mM NaHCO$_3$, 5 mM DTT, 2.5 mM ATP, 5 mM phosphocreatine, 5 units creatine phosphokinase, 5 units each of the linking enzymes, 0.2 mM NADH and 0.6 mM ribulose 1,5-bisphosphate. After a steady baseline absorbance at 340 nm was established, the reaction was initiated with 0.011 mL of activated extract. The linear decrease in absorbance resulting from oxidation of NADH was recorded over a period of 150 s.

Chlorophyll content was determined using the method described by Arnon (1949). From each tube containing the crude extract after grinding, an aliquot of 0.1 mL was
transferred to 2 microfuge tubes, each containing 0.1 mL water. Subsequently, 0.8 mL 100% acetone were added in each tube, and incubated in the dark for at least 30 min in ice. After 3 min centrifuging, the supernatant was collected and absorbance was measured at 645 and 663 nm for total chlorophyll determination. Total soluble protein (TSP) in the extracts was quantified by the dye-binding assay of Bradford (1976) using bovine serum albumin as standard.

**Transmission Electron Micrograph Studies**

Leaf histological cross sections from EK 11-1 and EK 16-3 microcuttings cultured in vitro in Stage III were obtained to compare bundle sheath chloroplast ultrastructure. Transmission electron microscopy (TEM) was used, for which leaf sections approximately 2 mm from the center of the leaf blade were fixed in Trump's fixative solution (McDowell and Trump, 1976). Fixative infiltration was achieved under vacuum for 2 days. Leaf tissues were then rinsed 3 times in phosphate buffer (pH 7.2), post-fixed in a 1% buffered osmium tetroxide solution and then rinsed in phosphate buffer, 3 times in distilled water, and dehydrated in a five-step ascending ethyl alcohol series (25, 50, 75, 95, 100%) followed by dehydration in 100% acetone. An en bloc stain of 2% uranyl acetate was applied between the 75 and 95% steps of the ethyl alcohol dehydration series. Leaf sections were then embedded in Spurr resin (Spurr, 1969). Ultrathin leaf sections (70 nm) were cut from the center part of the leaf blade with a Leica Ultracut ultramicrotome R (Leica Microscopy and Scientific Instruments, Deerfield, IL), collected on 0.35% form-var coated copper grids, stained with methanolic uranyl acetate and lead citrate (Reynolds, 1963). Sections were viewed on a Hitachi H7000 transmission electron microscope (Hitachi Scientific Instruments, Danbury, CT) at 75 kV. Digital micrographs were taken on a BioScan/Digital Micrograph 2.5 (Gatan Inc., Pleasanton,
CA) at an exposure level optimized for viewing the outer layer and processed with MEGA View III/AnalySIS 3.1 (Soft Imaging System Corp., Lakewood, CO).

**Carbohydrate Studies**

Plantlets from EK 11-1 and EK 16-3 sea oats genotypes were cultured for 8 weeks under Stage II conditions, with 20 replicate vessels per genotype, each containing 5 shoot clusters. Subsequently, single shoots from each genotype were cultured under Stage III conditions for 3, 6 or 9 weeks. Each Stage-III duration treatment consisted of 5 replicate vessels per genotype, each containing 8 single shoot microcuttings.

At week 8 Stage II conditions, clusters of plantlets obtained from 5 vessels were taken out of the medium, washed, and packaged in aluminum foil envelopes. Every package contained 3 plantlets (sub-samples), which were collected from the same vessel. Samples were frozen in liquid N\textsubscript{2} and stored at -80\textdegree C prior to drying in a 10-MR-TR freeze drier (The Virtis Company, Gardiner, NY) for 7 days. Dry weights of each sub-sample were then recorded. At weeks 3, 6 and 9 of Stage III conditions, all plantlets from 5 vessels were collected and processed in the same way, this time separating roots from shoots.

A parallel study was completed to analyze the carbohydrate status of both genotypes during Stage IV duration. Plantlets form EK 11-1 and EK 16-3 sea oats genotypes were cultured for 8 weeks under Stage II conditions, followed by 6 weeks Stage III conditions and then transferred to Stage IV conditions for 4 weeks. Five replicate samples consisting of 3 shoots or roots per genotype were collected at weeks 2 and 4 Stage IV and processed as previously described.

Procedures for total soluble sugar extraction were modified following the description by Boersig and Negm (1985) and Miller and Langhans (1989). Glass Pasteur
pipettes with glass wool plugs were loaded with 50 mg of each sample. Soluble sugars were extracted with 1.5 mL of methanol:chloroform:water (MCW) (12:5:3 v:v:v) overnight. One hundred µL mannitol (10 mg mL⁻¹) was added as an internal standard to each sample. One hour extractions were repeated twice with 1.5 mL MCW followed by two additional MCW (1.5 mL) rinses. Nanopure water (3.5 mL) was added to extract prior to 20-min centrifugation at 4,000 rpm. The aqueous phase was removed and applied to polyethylene columns containing 3 mL 1 methanol:1 water (v:v, MW) and cation and anion exchange resin (1 mL Amberlite IRA-67 layered with 1 mL Dowex 50-W, Sigma-Aldrich Co., St. Louis, MO). Soluble sugars were eluted and rinsed twice with methanol: water (MW) (1:1 v:v) prior to complete evaporation using a RapidVap vacuum evaporator system (Labconco Corp., Kansas City, MO). The dry residue was re-suspended in 1 mL HPLC-grade water and filtered through a 0.45 µ membrane prior to HPLC injection. Total soluble sugars (TSS; sucrose, glucose and fructose) were analyzed using a Waters 2695 High Pressure Liquid Chromatograph (Waters Technological Corporation, Milford, MA) equipped with a Waters 2414 refractive index detector (Waters Technological Corporation, Milford, MA) and two connected BioRad Aminex HPX-87C columns (BioRad Laboratories, Hercules, CA). Column and detector temperatures were maintained at 80 and 50 ºC, respectively. High pressure liquid chromatography grade water was used as the mobile phase, at a flow rate of 0.6 mL min⁻¹.

For starch analysis, procedures were modified as described by Haissig and Dickson (1979) and Miller and Langhans (1989). The tissue residue (remaining in pipets after soluble sugar extraction) was oven dried overnight at 50 ºC, suspended in 4 mL Na-
acetate buffer (100 mM, pH 4.5), and placed in a boiling water bath (90 ºC) for 30 min. After cooling to room temperature, 1.0 mL amyloglucosidase solution (from Rhizopus mold, Sigma-Aldrich Co., St. Louis, MO) (50 units/assay in 0.1 M pH 4.5 Na-acetate buffer) was added to each test tube to hydrolyze starch to glucose. Samples were incubated for 48 h at 55 ºC with occasional agitation. An aliquot (100-µL) of each sample (glucose hydrolyzate) was transferred to a clean test tube and subjected to an enzyme assay containing glucose oxidase (5 units mL⁻¹) and peroxidase (200 units mL⁻¹). After the addition of 2.2 N HCl (1.0 mL), absorbance at 450 nm was determined using a Beckman DU-64 spectrophotometer (Beckman Coulter Inc., Fullerton, CA) and starch content was calculated based on the regression equation of the glucose calibration line (0.0 to 1.0 µmol).

For ex vitro analyses, two replicate sub-samples were taken from each tissue sample per genotype. These sub-samples were averaged to estimate starch and total soluble sugar contents.

**Experimental Designs and Statistical Analyses**

All experiments were arranged in completely randomized designs. For photosynthesis measurements, each plant per genotype was considered a replication. For carbohydrate and enzymatic analyses all shoots or roots obtained from each vessel were considered a replication. Main treatment effects and interactions were evaluated using the general linear model (GLM) procedures developed by Statistical Analysis System (SAS Institute Inc., 1999) and mean separation was evaluated using Waller-Duncan at \( P \leq 0.05 \).
Results

Photosynthetic and Transpiration Status Ex Vitro

The ability of plantlets to acclimatize to ex vitro conditions differed significantly between genotypes. EK 16-3 plants exhibited nearly 100% survival after 6 weeks, whereas EK 11-1 plants started senescing after 2 weeks acclimatization, resulting in 29% survival after 6 weeks ex vitro culture (Figure 3-1A). Significant differences in $P_{\text{nl}}$ were also observed during initial plant growth ex vitro, with $P_{\text{nl}}$ values being greater for EK 16-3 than for EK 11-1 plants (Figure 3-1B). At week 0, ex vitro $P_{\text{nl}}$ for EK 11-1 was 1.9 $\mu$mol m$^{-2}$ s$^{-1}$, which was significantly lower than $P_{\text{nl}}$ of EK 16-3 plants. Surviving EK 11-1 plants exhibited a significant increase of $P_{\text{nl}}$ over time that was not significantly different to the $P_{\text{nl}}$ of EK 16-3 plants after 5 weeks ex vitro culture. Transpiration rates of plants at initial ex vitro transfer were significantly higher for EK 11-1 than EK 16-3 plantlets. After one week ex vitro culture, transpiration rates in both genotypes increased until after week 3 when they decreased (Figure 3-1C).

Carbohydrate Status in Vitro and Ex Vitro

During weeks 3 to 9 of Stage III, starch content in shoots was greater in EK 11-1 than EK 16-3 plantlets and both genotypes exhibited a steady decrease of shoot starch with time (Figure 3-2A). After 6 weeks in Stage III, both genotypes exhibited a remarkable decrease in starch content when transferred ex vitro to the greenhouse. By week 4 Stage IV, shoot starch content of EK 11-1 plantlets was 3.8 times lower than that of EK 16-3 plantlets. Transmission electron micrographs (Figure 3-3) of bundle sheath cell chloroplasts indicated differences in the size and distribution of starch grain chloroplasts with time and between genotypes. While large starch granules were found after 3 weeks in Stage III, smaller starch grains were observed after 6 and 9 weeks.
Figure 3-1. Effect of in vitro culture conditions on A: ex vitro survival B: ex vitro transpiration rate per leaf area, and C: ex vitro net photosynthetic rate per leaf area ($P_{nl}$) of EK 11-1 and EK 16-3 genotypes during Stage IV culture. Means ± SE are shown ($n = 10$).
Stage III. Chloroplasts in EK 11-1 plantlets after 3 weeks Stage III (Figure 3-3A) exhibited larger numbers of plastoglobuli than in EK 16-3 (Figure 3-3D). Furthermore, at the same time, thylakoid membranes of EK 11-1 chloroplasts were separated and appeared disrupted (Figure 3-3A).

At 6 weeks Stage III, root starch content (Figure 3-2B) was lower in both genotypes compared to shoot starch content (Figure 3-2A), and it was not significantly different between genotypes at 6 and 9 weeks Stage III culture. Root starch content also decreased from 6 to 9 weeks under Stage III culture conditions. Throughout Stage IV acclimatization, root starch content was similar among genotypes. At 2 weeks Stage IV, root starch content was nearly depleted (13.2 and 6.5 times lower than that of EK 11-1 and EK 16-3, respectively, at week 0).

During Stage III, shoot soluble sugars (sucrose and hexose) increased from week 0 to 3 and then gradually decreased from weeks 3 to 9 (Figure 3-4 A-C). At 0 weeks Stage III (after 8 weeks in Stage II), there was significantly higher shoot sucrose content per dry weight in EK 16-3 than in EK 11-1 plantlets, whereas hexose and TSS were non-significantly different between genotypes. Although shoot sucrose, hexose and TSS were similar among genotypes at 3 and 9 weeks Stage III, at 6 weeks, sucrose content was 21% greater in EK 11-1 shoots than in EK 16-3 shoots. During Stage IV (after 6 weeks in Stage III), shoot soluble sugars decreased from week 0 to 2, and then remained steady from weeks 2 to 4 (Figure 3-4 A-C). Furthermore, EK 16-3 plants exhibited significantly higher hexose and TSS contents than EK 11-1 plants after 2 and 4 weeks ex vitro.

During Stage III, root soluble sugars (sucrose and hexose) decreased similarly among genotypes between weeks 6 and 9 (Figure 3-5 A-C). Sucrose, hexose and TSS
contents in roots were significantly higher for EK 11-1 than EK16-3 plantlets, and decreased after 9 weeks Stage III in both genotypes (Figure 3-5A-C). During Stage IV (after 6 weeks in Stage III) root sucrose and hexose of both genotypes dramatically decreased within two weeks, being nearly depleted during acclimatization.

Figure 3-2. Comparative A: shoot starch content and B: root starch content of EK 11-1 and EK 16-3 genotypes during in vitro Stage III (left) and after microcuttings were rooted for 6 weeks Stage III and transferred to Stage IV (right). Error bars indicate SE (n = 10). ANOVA analysis is shown on top left corner of each graph; T: Time, G: Genotype, NS, **: Non-significant or significant at P \leq 0.01, respectively. Different letters on top of histobars within each culture stage are significantly different according to Waller-Duncan test at P \leq 0.05.
Figure 3-3. Comparative TEM of chloroplasts of A-C: EK 11-1, and D-F EK 16-3 genotypes after 3 weeks (A, D), 6 weeks (B, E) and 9 weeks (C, F) Stage III culture conditions in bundle sheath cells. Scale = 2 µm. (p: plastoglobuli, s: starch granule, t: thylakoid membranes).
Figure 3-4. Comparative A: shoot sucrose content, B: shoot hexose content, and C: shoot total soluble sugar content of EK 11-1 and EK 16-3 genotypes during in vitro Stage III (left) and after microcuttings were rooted for 6 weeks Stage III and transferred to Stage IV (right). Error bars indicate SE (n = 10). ANOVA analysis is shown on top left corner of each graph; T: Time, G: Genotype; NS, **: Non-significant or significant at P ≤ 0.01, respectively. Different letters on top of histobars within each culture stage are significantly different according to Waller-Duncan test at P ≤ 0.05.
Figure 3-5. Comparative A: root sucrose content, B: root hexose content, and C: root total soluble sugar content of EK 11-1 and EK 16-3 sea oats genotypes during in vitro Stage III (left) and after microcuttings were rooted for 6 weeks Stage III and transferred to Stage IV (right). Error bars indicate SE (n = 10). ANOVA analysis is shown on top left corner of each graph; T: Time, G: Genotype; NS, *, **: Non-significant or significant at $P \leq 0.05$ or 0.01, respectively. Different letters on top of histobars within each culture stage are significantly different according to Waller-Duncan test at $P \leq 0.05$. 
Chlorophyll and Soluble Protein Contents

Total chlorophyll content was relatively low (0.2 mg g\(^{-1}\) FW) throughout Stage III (EK 11-1) and for the first 3 weeks of Stage III culture (EK 16-3). Chlorophyll content of EK 16-3 then increased significantly after 6 and 9 weeks Stage III (Figure 3-8A). During Stage IV (after 6 weeks Stage III), chlorophyll levels of EK 16-3 plantlets generally increased with time. In EK 11-1 plantlets the increase in chlorophyll was delayed until after plantlets were established to greenhouse conditions (after 4 weeks Stage IV). Chlorophyll content was significantly greater for EK 16-3 than EK 11-1 plantlets after 2 and 4 weeks Stage IV. Conversely, EK 11-1 acclimatized plants (> 6 weeks in the greenhouse) exhibited higher chlorophyll levels than EK 16-3 plants.

Total soluble protein (TSP) content in shoots of sea oats genotypes was lowest at the beginning of Stage III for both genotypes and increased with time, being significantly higher for EK 16-3 than for EK 11-1 plantlets after 6 and 9 weeks in vitro (Figure 3-8B). A rapid increase in TSP was observed in EK 16-3 plantlets during the first 4 weeks after transfer to ex vitro conditions, with a significant decrease after plantlets were established ex vitro (> 6 weeks). EK 11-1 plantlets also increased their TSP content with time, being higher after plants had acclimatized ex vitro.

Photosynthetic Enzyme Status in Vitro and Ex Vitro

During Stages III and IV there were significant differences in PEPC activity per g fresh weight over time and between genotypes (Figure 3-7A). EK 16-3 plantlets exhibited higher activity of PEPC with time under Stage III conditions that was also significantly higher than in EK 11-1 plantlets. Both genotypes increased their PEPC activities following ex vitro transfer. Once the plantlets became established ex vitro (> 6 weeks) both genotypes had similar PEPC activity.
Differences in rubisco activity were also observed over time and between genotypes during Stage III and after ex vitro transfer to Stage IV (Figure 3-7B). EK 11-1 plantlets exhibited a lower rubisco activity per g fresh weight than EK 16-3 plantlets, which increased after 6 and 9 weeks under Stage III conditions. After transferring plantlets to ex vitro conditions (following 6 weeks Stage III), rubisco activity did not significantly increase in EK 11-1 plantlets until they were established ex vitro (> 6 weeks). Conversely, rubisco activity significantly increased in EK 16-3 plantlets after 2 weeks ex vitro but it decreased after plants were established (> 6 weeks).

The PEPC and rubisco activities of maize (Zea mays L.) plants cultured under greenhouse conditions were assayed following the identical analytical procedures used for sea oats, and a PEPC/rubisco (P/R) ratio of 3.7 was obtained. Similarly, P/R ratios ranging from 3 to 5 were observed in sea oats cultures ex vitro (Figure 3-7C). Conversely, lower P/R values were observed when sea oats were cultured under Stage III conditions. EK 16-3 plantlets had higher P/R ratios than EK 11-1 plantlets after 9 weeks in vitro, but there were no significant differences between genotypes after transfer to Stage IV conditions.

Photosynthetic enzyme analyses expressed on basis of TSP indicated that PEPC activity was not affected by Stage III duration in EK 11-1 plantlets but increased on weeks 6 and 9 of Stage III in EK 16-3 plantlets (Figure 3-7A). Plantlets of either genotype exhibited from 1.5 to 3.9 times greater PEPC activities after ex vitro acclimatization than at the time of initial transfer (week 0). Conversely, rubisco activity per TSP did not vary from 6 weeks Stage III or after ex vitro transfer in either genotype. Additionally, rubisco activity per TSP was significantly higher in EK 16-3 after 6 and 9
weeks in vitro and during ex vitro acclimatization than in EK 11-1 plantlets (Figure 3-7B).

Figure 3-6. Comparative A: chlorophyll content per shoot g fresh weight, and B: TSP content per shoot g fresh weight of EK 11-1 and EK 16-3 sea oats genotypes during in vitro Stage III (left) and after microcuttings were rooted for 6 weeks Stage III and transferred to Stage IV (right). Error bars indicate SE (n = 4). ANOVA analysis is shown on top left corner of each graph; T: Time, G: Genotype; NS, *, **: Non-significant or significant at $P \leq 0.01$, respectively. Different letters on top of histobars and next to legend within each culture stage are significantly different according to Waller test at $P \leq 0.05$. 
Figure 3-7. Comparative A: rubisco activity per shoot g fresh weight, B: PEPC activity per shoot g fresh weight, and C: PEPC/Rubisco ratio of EK 11-1 and EK 16-3 sea oats genotypes during in vitro Stage III (left) and after microcuttings were rooted for 6 weeks Stage III and transferred to Stage IV (right). Error bars indicate SE \( n = 4 \). ANOVA analysis is shown on top left corner of each graph; T: Time, G: Genotype; NS, **: Non-significant or significant at \( P \leq 0.01 \), respectively. Different letters on top of histobars within each culture stage are significantly different according to Waller-Duncan test at \( P \leq 0.05 \).
Figure 3-8. Comparative A: PEPC activity per shoot mg total soluble protein (TSP), B: rubisco activity per shoot mg TSP of EK 11-1 and EK 16-3 sea oats genotypes during in vitro Stage III (left) and after microcuttings were rooted for 6 weeks Stage III and transferred to Stage IV (right). Error bars indicate SE ($n = 4$). ANOVA analysis is shown on top left corner of each graph; T: Time, G: Genotype; NS, *, **: Non-significant or significant at $P \leq 0.05$ or 0.01, respectively. Different letters on top of histobars and next to legend within each culture stage are significantly different according to Waller-Duncan test at $P \leq 0.05$. 
Discussion

Differences in acclimatization capacities between sea oats genotypes involved remarkable and divergent developmental and physiological responses to in vitro culture conditions. Low ex vitro survival in the EK 11-1 genotype correlated to in vitro development of short leaves with minimal blades. In contrast, EK 16-3 cultures produced elongated leaves with expanded blades in Stage III by week 6. These leaves were morphologically similar to those produced ex vitro on acclimatized plants. Physiologically, these leaves, like those in red raspberry (Rubus idaeus L.) (Donnelly and Vidaver, 1984b) and Asian white birch [Betula platyphylla ‘Szechuanica’ (Schneid.) Rehd.] (Smith et al., 1986), did not deteriorate rapidly after transplantation and were photosynthetically competent ex vitro. The $P_{nl}$ in EK 16-3 leaves at time of ex vitro transfer was 8 times greater than in EK 11-1 leaves. Thus, there was a strong correlation between in vitro production of sea oats leaves morphologically similar to those produced ex vitro and photosynthetic competence.

Given that transpiration rates were significantly different at the time of ex vitro transfer, initial poor control of water loss ex vitro most likely contributed to lower EK 11-1 survival. Initial decreases in transpiration rates during week 1 in both genotypes possibly reflect restricted water uptake due to limited root hair development in vitro. After week 1, the increase in transpiration rates of both genotypes possibly reflects the impact of the greater functioning root system combined with the occurrence of abnormal stomate function. Transpiration rates measured after 6 weeks ex vitro transfer were similar in both genotypes and likely reflected measurements of surviving plants that had become acclimatized. However, due to the small magnitude, albeit significant, difference in transpiration rates between genotypes at time of ex vitro transfer, we conclude that
control of water loss was probably not the main cause for poor ex vitro acclimatization. Although high transpiration rates may exacerbate the transition to ex vitro conditions, other physiological limitations, including poor photosynthetic capacity or limited carbohydrate reserves during acclimatization, had a greater impact on survival of EK 11-1 ex vitro.

Several investigations have correlated starch accumulation and utilization in plant cells prior to the initiation and progression of developmental processes, such as shoot and root initiation (Swarnkar et al., 1986; Branca et al., 1994). The low initial starch levels in both sea oats genotypes, obtained from 8-week old Stage II cultures, indicated that the shoot clusters from which microcuttings were derived were starch-depleted at the end of the multiplication stage. Starch content increased rapidly after being transferred to sucrose-containing Stage III medium, prior to root emergence. Rapid accumulation of starch was also observed when begonia (Begonia rex) explants were transferred to medium containing 87.6 mM sucrose prior the formation of shoot-bud primordia (Mangat et al., 1990). Greater decline in starch reserves in EK 16-3 over EK 11-1 after 3 weeks in vitro may have resulted from the more rapid and earlier initiation and growth of EK 16-3 roots and shoots. This observation is supported by comparative growth measurements of both genotypes during Stage III culture (Chapter 2).

Our studies indicated that sea oats genotypes stored starch and contained soluble sugars in shoots and roots throughout Stage III. At the time of acclimatization, starch storage was greater in shoots of EK 11-1 than EK 16-3 plantlets, whereas shoot soluble sugar content was not significantly different between genotypes. During acclimatization ex vitro, shoot starch and soluble sugars in both genotypes were depleted, yet EK 16-3
maintained higher carbohydrate levels than EK 11-1 ex vitro. This was due, in part, to the higher net photosynthetic rates of EK 16-3 leaves at time of ex vitro transfer. During acclimatization, EK 16-3 utilized two carbon sources, namely stored carbohydrate reserves and photoassimilates. In contrast, EK 11-1 plants depended primarily on stored carbohydrate reserves. Given that starch levels were actually higher in shoots of the difficult-to-acclimatize sea oats genotype (EK 11-1) at the time of ex vitro transfer, low ex vitro survival probably resulted from limited production of photosynthetically competent leaves in vitro and subsequent rapid depletion of energy reserves ex vitro. This is supported by the very low $P_{nl}$ in EK 11-1 plantlets during the time of transfer to ex vitro conditions.

Capellades et al. (1991) established an inverse relationship between starch content in shoot chloroplasts and photosynthetic rates of in vitro rose (*Rosa multiflora* L. ‘Montse’) leaves. Additionally, low photosynthetic rates in vitro have also been attributed to carbohydrate accumulation in the leaves (Azcón-Bieto, 1983). A similar effect may occur during early stages of sea oats in vitro development, when starch and soluble sugar content in leaves are high. At 3 weeks Stage III, EK 11-1 chloroplasts also exhibited large number of plastoglobuli and separation of thylakoid membranes. These features are often observed in senescing leaves (Arntzen and Briantais, 1975). Plastoglobuli apparently function as extralamellar pools for membrane lipids, utilized for membrane biosynthesis or for deposit after membrane degradation.

In vitro leaves usually serve as storage organs to cover metabolic demands of growing tissues during the initial days of acclimatization (Capellades et al., 1991; Van Huylsenbroeck and Debergh, 1996; Van Huylsenbroeck et al, 1998; Piqueras et al., 1998).
Capellades et al. (1991) also concluded that during ex vitro acclimatization of rose, plants with leaves that exhibited low photosynthetic rates utilized starch reserves while re-establishing the photosynthetic capacity of leaves. However, in other species only leaves produced ex vitro are photosynthetically competent (Grout and Millam, 1985). Leaves in EK 11-1 plants exhibited low photosynthetic capacity and utilized stored starch reserves during acclimatization. The subsequent high ex vitro mortality indicated that, in most EK 11-1 plants, the respiratory demands were too high to allow restoration of photosynthetic capacity in in-vitro produced leaves or through production of new leaves. In these plants allocation of energy resources appears to be directed mostly towards maintenance of root biomass and non-leafy shoot production.

During Stage III culture, starch content in roots of both genotypes was lower than in shoots, and was similarly depleted ex vitro. Soluble sugar levels were higher in EK 11-1 than EK 16-3 roots during Stage III but decreased significantly after ex vitro transfer in both genotypes. Sucrose in the medium is hydrolyzed by the enzyme invertase that is released by microcuttings in response to wounding (De la Viña et al., 1999; Sturm, 1999). Then, roots uptake sucrose and hexose, which are translocated and stored as starch in shoots and roots in vitro. In contrast, under ex vitro conditions, photosynthetically active leaves are the sources of carbon, where soluble sugars are produced and, either stored as starch reserves, or translocated into other sink organs such as roots. Photoassimilates produced during acclimatization in either genotype were translocated and utilized for growth and development, rather than accumulated as starch. This was indicated by the maintenance of low starch levels after 2 and 4 weeks ex vitro.
The low photosynthetic capacity of EK 11-1 in-vitro produced leaves can be attributed to the alteration in the activities of the two primary enzymes of CO$_2$ fixation, namely PEPC and rubisco, during in vitro culture and initial ex vitro acclimatization. Rubisco activity in vitro has been reportedly reduced by medium supplementation with exogenous sucrose in strawberry plants, a C$_3$ species (Hdider, 1994; Desjardins, 1995). This may, in turn, cause feedback inhibition of photosynthesis because of low phosphate or high sugar-phosphate concentration in the cytosol, both known to affect the regeneration of the rubisco substrate RuBP. Additionally, C$_3$ plants exhibit increased respiration, resulting in increased PEPC activity (Hdider and Desjardins, 1994). Increased PEPC activity has also been reported in the C$_4$ species *Flaveria bidentis* when fed with 146.1 mM exogenous sucrose in the culture medium (Furbank et al. 1997). In contrast, rubisco activity per total soluble protein did not significantly change in either sea oats genotype after ex vitro transfer. Furthermore, decreased PEPC activity per total soluble protein caused by in vitro conditions was observed in both genotypes. Ex vitro P/R ratios for sea oats averaged 3.6, which was comparable to the P/R ratio we obtained with maize. However, in vitro P/R ratios were lowered possibly by the reduced PEPC activity measured.

During weeks 6 and 9 of Stage III culture, activities of PEPC and rubisco and contents of chlorophyll and total soluble protein were significantly greater in EK 16-3 than in EK 11-1. Histological sections of in vitro produced leaves of EK 11-1 and EK 16-3 (Chapter 2) indicated that after 6 weeks Stage III, EK 16-3 leaves exhibited more typical Krantz anatomy of C$_4$ plants than EK 11-1 leaves. After 6 weeks Stage III, the fixation of CO$_2$ by PEPC and the CO$_2$ concentrating mechanism in EK 16-3 might have
reduced some of the inefficacy of rubisco under the stress caused by in vitro conditions, enabling the maintenance of photosynthesis (Rodríguez et al. 2003). Likewise in sugarcane (*Saccharum officinarum* L.) plants, a typical C\textsubscript{4} species (Crafts-Brandner and Salvucci, 2002), photosynthesis was tolerant of relatively high environmental stress. Additionally, in vitro sugarcane leaves exhibited minimal Krantz anatomy compared to the more typical Krantz anatomy exhibited by ex vitro leaves (Rodríguez et al., 2003).

This increase in enzymatic activity, chlorophyll and soluble protein content after 6 weeks Stage III correlates with the initial decrease of starch and soluble carbohydrates in shoots of both genotypes at the same time period, and with formation of the first fully expanded leaves with typical Krantz anatomy in EK 16-3 (Chapter 2). These increased levels of chlorophyll and total soluble protein content, and increased enzymatic activities are indicative of the development of photosynthetic characteristics in EK 16-3 after 6 weeks Stage III. Furthermore, EK 16-3 plantlets transferred ex vitro after 6 weeks Stage III exhibited 100% survival. Conversely, EK 11-1 plants exhibited limited leaf elongation and expansion during Stage III culture (Chapter 2), which correlated with lower PEPC and rubisco activities, and low chlorophyll and total soluble protein contents in vitro compared to EK 16-3 plants. This limited development of photosynthetic features in vitro resulted in 30% survival after 6 weeks ex vitro conditions.

**Conclusions**

Lower photosynthetic capacity ($P_{nl}$) of EK 11-1 than EK 16-3 at time of transfer to ex vitro conditions was correlated with reduced survival. In addition to lower photosynthetic capacity ex vitro, other factors including initially higher transpiration rates and insufficient carbohydrate reserves to facilitate the transition from in vitro to ex vitro conditions further contributed to poor ex vitro survival in EK 11-1.
Carbohydrate analyses indicated rapid accumulation of starch and sugars in plantlets for the first three weeks following transfer to Stage III medium in both genotypes. These carbohydrates were used for root and shoot initiation and growth during latter Stage III culture period. Carbohydrate reserve utilization in EK 16-3 during the late Stage III was greater than EK 11-1, and resulted in lower starch and soluble sugars reserves than EK 11-1 at time of ex vitro transfer. After 6 weeks Stage III, there were significantly greater increases in PEPC and rubisco activities, chlorophyll and total soluble protein content in EK 16-3 than EK 11-1 plantlets. These differences correlated with the development of anatomical and morphological leaf features similar to those in greenhouse-produced leaves. Changes in leaf structure and morphology were described in Chapter 2.

Stage III in vitro culture conditions have different effects on growth and development of photosynthetic characteristics of sea oats genotypes. Those genotypes capable of developing photosynthetically competent leaves while storing carbohydrates in vitro, can overcome the energy demands to feed respiration requirements of cells, as well as growth requirements of different organs during acclimatization. Difficult-to-acclimatize sea oats genotypes could possibly benefit from modifications of the in vitro culture conditions, such as alteration of the environmental culture conditions or the medium components, for improved ex vitro acclimatization.

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CHAPTER 4
INFLUENCE OF IN VITRO GROWTH CONDITIONS ON IN VITRO AND EX VITRO PHOTOSYNTHETIC RATES OF EASY- AND DIFFICULT-TO-ACCLIMATIZE SEA OATS (*Uniola paniculata* L.) GENOTYPES

**Introduction**

The coastal beach and dune systems of southeastern U.S. are frequently eroded by storms and hurricanes. Sea oats (*Uniola paniculata* L.) is the dominant native dune grass used for dune restoration and stabilization in the southeastern U.S. (Woodhouse, 1982). Sea oats is commercially propagated from field collected seeds. However, this perennial C$_4$ grass is not a prolific viable seed producer (Burgess et al., 2002) and alternative propagation methods are necessary to meet the demands for sea oats in restoration projects.

Micropropagation has become an important technique for mass production of many plant species. Philman and Kane (1994) developed a micropropagation protocol for sea oats genotypes. However, when attempting to acclimatize unrooted or rooted microcuttings of various genotypes to ex vitro conditions significant variability in survival rates was encountered among genotypes. Low ex vitro survival as a result of the change from in vitro to ex vitro culture conditions was a limitation for the micropropagation in some sea oats genotypes. However, for the purpose of maintaining genetic diversity, it is critical to be able to micropropagate a wide range of sea oats genotypes.

Several investigations have linked low ex vitro survival directly or indirectly to the use of heterotrophic or photomixotrophic in vitro culture conditions (Capellades et al.,
In conventional micropropagation, sucrose is provided in the culture medium as an energy source, and culture conditions are characterized by low photosynthetic photon flux (PPF) and low CO$_2$ concentration in the culture vessels (Kozai et al., 1991). Consequently, in vitro plantlets have low photosynthetic rates regardless of their photosynthetic ability because they are induced to grow photomixotrophically with high sucrose levels and limited CO$_2$ in the vessel headspace (Kubota et al., 2002). In addition, the conventional use of poorly ventilated vessels creates high relative humidity (RH) within the plant cultures which adversely affects the development of photoautotrophy in vitro (Solárová, 1989; Kozai, 1991c; Kubota and Kozai, 1992; Hdider and Desjardins, 1994).

Leafy explants of numerous plant species can be micropropagated photoautotrophically using sugar-free medium, provided that environmental factors such as CO$_2$ concentration, light intensity and RH are controlled during plantlet in vitro growth and development (Kozai, 1991c). Photoautotrophic micropropagation has proven to induce growth and development of numerous C$_3$ plant species, such as tobacco (Nicotiana tabacum L.) (Mousseau, 1986), potato (Solanum tuberosum L.) (Kubota and Kozai, 1992), eucalyptus (Eucalyptus camaldulensis Dehnh.) (Kirdmanee et al., 1995), and coffee (Coffeea arabusta Capot & Aké Assi) (Nguyen et al., 1999b).

Most studies using photoautotrophic culture conditions have utilized C$_3$ plants. Photosynthesis in C$_4$ plants is different from C$_3$ plants (Black, 1971) in that the net photosynthetic rate is two- to three-fold greater, the CO$_2$ compensation point and the CO$_2$ saturation point are 10-fold and 2 to 3-fold lower, respectively, when both are exposed to high light levels and warm temperatures at ambient CO$_2$ levels (Salisbury and Ross,
Therefore, raising the CO$_2$ concentration above ambient partial pressure could have little or no effect on assimilation rates of C$_4$ plants under natural growth conditions. However, because in vitro environmental conditions differ significantly from natural field conditions, the effects of CO$_2$ enrichment may vary from those under natural conditions. Previous studies using photoautotrophic micropropagation of sugarcane (*Saccharum officinarum* L.) indicated that it is possible to enhance growth of C$_4$ plants under photoautotrophic conditions using CO$_2$ enrichment (Erturk and Walker, 2000; Xiao et al., 2003). Possibly, a similar system could be used to enhance sea oats growth during in vitro culture. The objectives of the present study were i) to determine the effects of in vitro culture conditions (photoautotrophic and photomixotrophic) on in vitro photosynthesis and growth and ex vitro acclimatization of easy- (EK 16-3) and difficult-to-acclimatize (EK 11-1) sea oats genotypes and ii) to develop in vitro culture procedures for efficient production of a wide range of sea oats genotypes.

**Materials and Methods**

**In Vitro Culture Conditions**

Five sea oats shoot clusters (each consisting of three shoots) of EK 16-3 and EK 11-1 genotypes previously established and indexed in vitro (Philman and Kane, 1994) and genotyped using random amplified polymorphic DNA (RAPD) genetic analyses, were subcultured in 80 mL sterile Stage II medium into separate Magenta GA7 vessels (Magenta Corp., Chicago, IL). The medium consisted of Murashige and Skoog (MS) inorganic salts (Murashige and Skoog, 1962), supplemented with 87.6 mM sucrose, 0.56 mM myo-inositol, 1.2 µM thiamine-HCl, 2.2 µM $N^6$-benzyladenine (BA) and solidified with 8 g L$^{-1}$ TCT™ agar (*Phyto*Technology Laboratories, Shawnee Mission, KS). All media were adjusted to pH 5.7 with 0.1 N KOH prior to the addition of agar and
autoclaving at 1.2 kg cm\(^{-2}\) and 121°C for 20 min. Cultures were maintained in a growth chamber at 22 ± 2 °C air temperature, 50 ± 5% RH, 16-h photoperiod provided by cool-white fluorescent lamps (F20W T12CW, General Electric Co., Cleveland, OH) at 45 ± 5 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PPF as measured at culture level. After 8 weeks, single shoots from each genotype were excised, transferred to Stage III rooting medium, and placed under four treatment conditions: photoautotrophic (PA), modified photomixotrophic with CO\(_2\) enrichment (PME), modified photomixotrophic with ambient CO\(_2\) concentration (PM), and conventional photomixotrophic (control) (Table 4-1).

For all treatments, Stage III rooting basal medium consisted of 80 mL sterile half-strength MS medium, supplemented with 0.56 mM myo-inositol, 1.2 \(\mu\)M thiamine-HCl, and 10 \(\mu\)M \(\alpha\)-naphthalene acetic acid (NAA) adjusted to pH 5.7 with 0.1 N KOH before autoclaving. Basal media in control, PME, and PM treatments were supplemented with 87.6 mM sucrose, whereas sucrose-free medium was used for the PA treatment. Liquid medium and cellulose supporting plugs (12 plugs per vessel; Sorbarod®, Baumgartner Papiers S.A., Switzerland) were used in PA, PM and PME treatments. The control medium was solidified with 0.8 g L\(^{-1}\) TCT™ agar. Each treatment consisted of 9 replicate Magenta GA7 vessels, each containing 8 single microcuttings.

Environmental conditions of all treatments are described in Table 4-1. Photoautotrophic, PME and PM treatment conditions included PPF of 200, 300, and 400 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) on days 1-3, 4-10, and 11-42, respectively; 16-h photoperiod provided by cool-white fluorescent lamps (F72T12/CW/VHO, Osram Sylvania, Danvers, MA), 80 ± 5% RH and 25 ± 1 °C air temperature. Control treatment conditions included the same photoperiod (16-h), but a lower PPF (100 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)), lower RH (50 ± 5%), and lower
air temperature (22 ± 2 ºC). Photoautotrophic and PME vessels were placed in a CO₂-enriched growth chamber (1500 µmol mol⁻¹), whereas control and PM vessels were placed in ambient CO₂ growth chambers (400 µmol mol⁻¹) (Conviron CMP4030 Model E15, Environments Ltd., Pembina, ND). Photoautotrophic, PM and PME vessels were ventilated by using four 10-mm diameter holes, one in each side wall, covered with gas-permeable microporous filter discs (pore size 0.5 µm; Milliseal, Millipore, Tokyo). By covering and uncovering the filter discs with tape, the air exchange rate (measured as described by Kozai et al., 1986) increased from 4.4 air exchanges h⁻¹ on days 1 to 3 to 8.1 air exchanges h⁻¹ on days 4 to 42. Control vessels had restricted ventilation (0.2 air exchanges h⁻¹). In PA, PM and PME treatments, the medium volume was replenished every 2 weeks as needed with sucrose-free medium.

At the end of Stage III culture, a high liquid pressure chromatograph (HPLC) (Waters 2695, Waters Technological Corporation, Milford, MA) equipped with a Waters 2414 refractive index detector and two connected BioRad Aminex HPX-87C columns (BioRad Laboratories, Hercules, CA) was used to measure the final concentration of sucrose in the medium, to confirm whether plantlets were photoautotrophic before ex vitro transfer. Column and detector temperatures were maintained at 80 ºC and 50 ºC, respectively. High pressure liquid chromatography grade water was used as the mobile phase, at a flow rate of 0.6 mL min⁻¹.

**In Vitro Growth and Net Photosynthetic Rates**

At 0 weeks Stage III, single shoot dry weights of both genotypes were obtained from 18 representative samples per genotype. During Stage III, net photosynthetic rate per plantlet ($P_{np}$) was monitored in situ from 2 gas samples per vessel obtained from 5
replicate vessels using a gas chromatograph (SRI model 8610, SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector (TCD) with helium as the carrier gas. Air headspace samples were obtained every two weeks starting the day after inoculation using a sanitized syringe from a hole in the vessel lid. CO₂ concentrations (µmol mol⁻¹) inside and outside the vessels (Cᵢₙ and Cₒᵤₜ, respectively) were determined and \( P_{np} \) was calculated using the equation described by Fujiwara et al. (1987):

\[
P_{np} = k E \frac{V}{n} (C_{in} - C_{out}) n^{-1}
\]

where \( k \) is a conversion factor of CO₂ from volume to moles (0.041 mol L⁻¹ at 25 °C); \( E \) is the number of air exchanges of the vessel (0.2, 4.4 or 8.1 h⁻¹); \( V \) is the air volume of the vessel (0.37 L), and \( n \) is the number of plantlets per culture vessel. At week 6, two gas samples per vessel were analyzed from 4 vessels per treatment to calculate photosynthetic rates, and dry weights were obtained to calculate photosynthetic rates per g dry weight (\( P_{nw} \)).

**Ex vitro Greenhouse Conditions, Growth, Survival, Photosynthetic Rates and Transpiration Rates**

After 6 weeks in Stage III, surviving plantlets (PME, PM and control; Figures 4-1B, 4-1C, 4-1D) had rooted and were transferred to the greenhouse to measure photosynthetic rates during acclimatization. Plantlets obtained from 5 vessels per treatment were transferred ex vitro into 8-pack cell plugs containing coarse vermiculite as the supporting material. Plantlets were watered as needed and Peters 20N-20P-20K liquid fertilizer (150 mg N L⁻¹; The Scotts Company, Marysville, OH) was applied weekly. Microcuttings were maintained at 28 ± 4 °C air temperature and natural solar PPF of 1000 ± 200 µmol m⁻² s⁻¹ from May until June 2004 in a greenhouse in Fort Pierce, FL.
Net photosynthetic rate and transpiration rate measurements per leaf area ($P_{\text{nl}}$) were taken from 10 replicate plantlets every 3 weeks at midday starting the day after establishment ex vitro on newly formed and fully expanded leaves. Measurements were taken using a PP System Model Ciras-1 (PP System Co., Ltd., UK) with inlet CO$_2$ concentration fixed at $400 \pm 10 \, \mu\text{mol mol}^{-1}$ and no supplemental light source. After 6 weeks, the longest leaves of surviving plants were measured, plants were destructively harvested, divided into shoots and roots and dried at 70 °C for 1 week.

**Experimental Design and Statistical Analysis**

The in vitro growth chamber study was as a split plot design, with cultural conditions as the whole plot (4 levels) and genotypes as the subplots (2 levels). The ex vitro greenhouse study was a randomized complete block design. In vitro and ex vitro data were analyzed using analysis of variance (mixed and general linear model procedures of SAS institute Inc., 1999, respectively). The general linear model procedure was also used to evaluate the effect of CO$_2$ enrichment on photosynthetic rates and growth in vitro and ex vitro of plantlets cultured under modified photomixotrophic conditions. Separate *a posteriori* test for significant differences among or between means were made using the Waller-Duncan procedure at $P \leq 0.05$. 
Table 4-1. Environmental conditions during Stage III rooting for EK 11-1 and EK 16-3 sea oats genotypes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Photosynthetic photon flux (µmol m(^{-2}) s(^{-1}))</th>
<th>Relative humidity (%)</th>
<th>Air temperature (°C)</th>
<th>CO(_2) concentration (µmol mol(^{-1}))</th>
<th>Number air exchanges (h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days 1-3</td>
<td>Days 4-10</td>
<td>Days 11-42</td>
<td>Days 1-3</td>
<td>Days 4-42</td>
</tr>
<tr>
<td>PA</td>
<td>200</td>
<td>300</td>
<td>400</td>
<td>80 ± 5</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>PME</td>
<td>200</td>
<td>300</td>
<td>400</td>
<td>80 ± 5</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>PM</td>
<td>200</td>
<td>300</td>
<td>400</td>
<td>80 ± 5</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>50 ± 5</td>
<td>22 ± 2</td>
</tr>
</tbody>
</table>

\(^{2}\)PA: photoautotrophic; PME: modified photomixotrophic enriched; PM: modified photomixotrophic; control: conventional photomixotrophic.
Results

In Vitro Survival and Growth

All plantlets initially cultured in media without sucrose (PA) died during the first 2 weeks of the experiment (Figure 4-1A). After 6 weeks in vitro, shoot dry weights of EK 11-1 plantlets were similar among all remaining treatments, whereas root dry weights were lower under control conditions (Table 4-2). EK 16-3 plantlets cultured under PME conditions had greater shoot and root dry weights than plantlets cultured under PM or control conditions. When comparing both genotypes, shoot dry weights were greater in EK 16-3 than EK 11-1 plantlets under PME and PM conditions. However, under control conditions, EK 11-1 plantlets had greater shoot dry weights than EK 16-3 plantlets. Root dry weights were greater in EK 16-3 than EK 11-1 plantlets under PME conditions but similar under PM or control conditions. Additionally, shoots in PME, PM and control EK 16-3 plantlets produced elongated leaves and fully expanded leaf blades, whereas shoots of EK 11-1 plantlets were reduced in size and lacking fully expanded leaf blades (Figure 4-1).

In Vitro Net Photosynthetic Rate

At week 0, EK 11-1 and EK 16-3 control plantlets and EK 11-1 PM plantlets exhibited negative $P_{nw}$ (Table 4-3). At week 6, control plantlets exhibited the lowest $P_{nw}$ among treatments, whereas PM and PME plantlets increased their $P_{nw}$ during in vitro culture, being the highest under PME conditions. Plantlets of both genotypes cultured under PME and PM conditions depleted all the sucrose from the media, as indicated by sucrose measurements taken after 6 weeks in vitro (data not presented). At week 0, plantlets under CO$_2$-enriched conditions (PA and PME) exhibited significantly greater $P_{np}$ than those under control or PM conditions, regardless of genotype (Figure 4-2).
Figure 4-1. Six-week old in vitro cultures of EK 16-3 (left) and EK 11-1 (right) sea oats genotypes under A: photoautotrophic [PA], B: modified photomixotrophic enriched [PME], C: modified photomixotrophic [PM] and D: conventional photomixotrophic [control] culture conditions.
Table 4-2. In vitro shoot and root dry weights of EK 11-1 and EK 16-3 sea oats genotypes. In vitro culture conditions were: PA (photoautotrophic), PM (modified photomixotrophic) PME (modified photomixotrophic with CO₂ enrichment), and control (conventional photomixotrophic). Data were collected after 6 weeks in vitro culture.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot dry weight (mg)</th>
<th>Root dry weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EK 11-1</td>
<td>EK 16-3</td>
</tr>
<tr>
<td>PA</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PME</td>
<td>136 ± 7bc(^y)</td>
<td>180 ± 8a</td>
</tr>
<tr>
<td>PM</td>
<td>127 ± 6cd</td>
<td>150 ± 11b</td>
</tr>
<tr>
<td>Control</td>
<td>134 ± 6bc</td>
<td>111 ± 6d</td>
</tr>
</tbody>
</table>

Analysis of variance\(^x\)

- Genotype (G): *
- Treatment (T): **
- G * T: **

\(^x\) -- indicates that plants died.

\(^y\) Means ± SE followed by different letters in each column are significantly different according to Waller-Duncan test at \(P \leq 0.05\) (\(n = 32\)).

\(^x\), **: Significant at \(P \leq 0.05\) or 0.01, respectively.
At 2 weeks, plantlets from both genotypes under non-enriched CO\textsubscript{2} conditions (PM and control), exhibited $P_{\text{np}}$ near 0 µmol h\textsuperscript{-1} plantlet\textsuperscript{-1}, which was significantly lower than $P_{\text{np}}$ in PME plantlets. At 4 weeks, PM and PME conditions increased $P_{\text{np}}$ of plantlets in both genotypes. At 6 weeks, EK 16-3 PME plantlets exhibited significantly higher $P_{\text{np}}$ than the other treatments. Control plantlets of both genotypes continued to exhibit negative $P_{\text{np}}$ throughout the in vitro culture period.

**Ex Vitro Survival, Transpiration, Photosynthesis and Growth**

EK 11-1 control plantlets had a negative $P_{\text{nw}}$ after 6 weeks in vitro (Table 4-3) and consequently exhibited the lowest survival (25%) among treatments after 6 weeks ex vitro acclimatization (Figure 4-3A). Significantly higher survival ex vitro was observed in EK 16-3 control plantlets (77.5%) and in EK 16-3 PME and PM plantlets (100%). At the beginning of ex vitro transfer, EK 16-3 plantlets exhibited lower transpiration rates than EK 11-1 plantlets regardless of treatment conditions (Figure 4-3B). These differences in transpiration rates were less pronounced after 3 weeks and were similar among treatments after 6 weeks for both genotypes. EK 11-1 control plantlets exhibited the lowest $P_{\text{nl}}$ among all measured treatments (Figure 4-3C) at week 0 ex vitro. Three weeks later, $P_{\text{nl}}$ increased in all treatments and EK 11-1 control plantlets had similar transpiration rates to EK 11-1 PME, EK 16-3 PM and EK 16-3 control plantlets. There were no significant differences in $P_{\text{nl}}$ among treatments after 6 weeks ex vitro. After 6 weeks ex vitro, shoot and root dry weights of plantlets cultured under PME and PM conditions were higher than that of plantlets cultured under control conditions for both genotypes (Table 4-4). Furthermore, EK 16-3 plantlets exhibited greater shoot and root dry weights than EK 11-1 plantlets regardless of treatment conditions. EK 16-3 PME and
PM plantlets, and EK 11-1 PM plantlets exhibited 1.2 to 1.7 times longer leaf lengths ex vitro than control plantlets of either genotype (Table 4-4).

Regardless of genotype, modification of photomixotrophic conditions with CO₂ enrichment in vitro did not significantly affect $P_{nl}$ and ex vitro survival. Furthermore, shoot and root dry weights and longest leaf lengths of both genotypes ex vitro were not significantly affected by in vitro CO₂ enrichment. Although in vitro CO₂ enrichment decreased transpiration rates of EK 16-3 plantlets after transfer, transpiration rates of EK 11-1 plantlets were not affected.

Table 4-3. Net photosynthetic rate per dry weight ($P_{nw}$) during in vitro rooting of EK 11-1 and EK 16-3 sea oats genotypes cultured under PA (photoautotrophic), PME (modified photomixotrophic with CO₂ enrichment), PM (modified photomixotrophic with ambient CO₂) and control (conventional photomixotrophic) conditions. Data were collected on weeks 0 and 6 of in vitro culture.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Week 0</th>
<th>Week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EK 11-1</td>
<td>EK 16-3</td>
</tr>
<tr>
<td>PA</td>
<td>70 ± 9 $^z$</td>
<td>96 ± 30</td>
</tr>
<tr>
<td>PME</td>
<td>20 ± 9</td>
<td>66 ± 7</td>
</tr>
<tr>
<td>PM</td>
<td>-9 ± 13</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>Control</td>
<td>-38 ± 9</td>
<td>-25 ± 3</td>
</tr>
<tr>
<td>$\chi$</td>
<td>11 ± 10B</td>
<td>36 ± 11A</td>
</tr>
</tbody>
</table>

Analysis of variance$^x$

<table>
<thead>
<tr>
<th>Genotype (G)</th>
<th>Treatment (T)</th>
<th>G * T</th>
</tr>
</thead>
<tbody>
<tr>
<td>*</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

$^x$ Means ± SE followed by different lowercase letters in each column are significantly different according to Waller-Duncan test at $P \leq 0.05$ ($n = 4$). Means ± SE followed by different uppercase letters in each row are significantly different according to Waller-Duncan test at $P \leq 0.05$ ($n = 4$).

$^y$ -- indicates that plants died.

$^x$ NS, *, **: Non-significant or significant at $P \leq 0.05$ or 0.01, respectively, within each measurement period (week 0 or week 6).
Figure 4-2. Effect of in vitro culture conditions on net photosynthetic rate per plant ($P_{np}$) of EK 11-1 and EK 16-3 genotypes during Stage III culture. Means ± SE are shown ($n = 5$) when larger than symbol. In vitro culture conditions were: photoautotrophic [PA], modified photomixotrophic enriched [PME], modified photomixotrophic [PM] and conventional photomixotrophic [control] culture conditions.
Figure 4-3. Effect of in vitro culture conditions on A: ex vitro survival \((n = 5)\); B: ex vitro transpiration rate per leaf area \((n = 10)\), and C: ex vitro net photosynthetic rate per leaf area \((P_{nl})\) \((n = 10)\) of EK 11-1 and EK 16-3 genotypes during Stage IV culture. Means ± SE are shown when larger than symbol. In vitro culture conditions were: modified photomixotrophic enriched [PME], modified photomixotrophic [PM] and conventional photomixotrophic [control] culture conditions.
Table 4-4. Ex vitro shoot and root dry weights and longest leaf length of EK 11-1 and EK 16-3 sea oats genotypes. In vitro culture conditions included: PA (photoautotrophic), PM (modified photomixotrophic) PME (modified photomixotrophic with CO$_2$ enrichment), and control (conventional photomixotrophic). Data were collected after 6 weeks ex vitro culture.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot dry weight (mg)</th>
<th>Root dry weight (mg)</th>
<th>Longest leaf length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EK 11-1</td>
<td>EK 16-3</td>
<td>$\bar{x}$</td>
</tr>
<tr>
<td>PA</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PME</td>
<td>477 ± 41$^y$</td>
<td>747 ± 30</td>
<td>653 ± 29a</td>
</tr>
<tr>
<td>PM</td>
<td>528 ± 34</td>
<td>750 ± 24</td>
<td>651 ± 24a</td>
</tr>
<tr>
<td>Control</td>
<td>147 ± 22</td>
<td>461 ± 39</td>
<td>384 ± 37b</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>451 ± 28B</td>
<td>669 ± 21A</td>
<td>128 ± 8B</td>
</tr>
</tbody>
</table>

Analysis of variance$^x$

| Genotype (G) | ** | ** | ** |
| Treatment (T) | ** | ** | ** |
| G * T | NS | NS | * |

$^z$ -- indicates that plants died.

$^y$ Means ± SE followed by different lowercase letters in each column are significantly different according to Waller-Duncan test at $P \leq 0.05$. Means ± SE followed by different uppercase letters in each row significantly different between genotypes according to Waller-Duncan test at $P \leq 0.05$ (n varied depending upon survival from 10-40).

$^x$ NS, *, **: Non-significant or significant at $P \leq 0.05$ or 0.01, respectively.
Discussion

In Vitro Survival, Growth and Photosynthetic Rates

Numerous investigations using C₃ plants have indicated that use of sucrose-free medium with high PPF and CO₂ enrichment enhances plant growth (Kozai et al., 1987; Cournac et al., 1991; Fujiwara et al., 1992; Buddenhof-Joosten and Woltering, 1996) and plant net photosynthetic rates (Kozai et al., 1991; Nakayama et al., 1991; Lian et al., 2002a; Lian et al., 2002b). Conversely, in our study, sea oats cultured in sucrose-free medium died after 2 weeks culture. Initial use of sucrose in the medium with subsequent dilutions of sucrose-free medium induced sea oats plantlets to become photoautotrophic in vitro under specific environmental conditions of high PPF and high ventilation. This indicates that sea oats plantlets require an initial source of carbon from the medium until they are capable of using CO₂ from the vessel headspace as their main carbon source. This is consistent with the findings of Arigita et al. (2002) with kiwi explants (Actidinia deliciosa Chev. Liang & Ferguson ‘Hayward’) under similar culture conditions.

During in vitro culture, the negative initial $P_{nw}$ in EK 11-1 PM plantlets and negative initial and final $P_{nw}$ in control plantlets of both genotypes indicated that these plantlets had higher rates of respiration than photosynthesis. Therefore, these plantlets were either photomixotrophic or heterotrophic. By the time of ex vitro transfer, plants under PM and PME conditions had completely depleted the sucrose from the media, and had become photoautotrophic, as indicated by the relatively high $P_{nw}$.

Net photosynthetic rate per plantlet of both genotypes cultured under PM and PME conditions increased after 2 weeks in vitro. Yet, EK 16-3 plantlets exhibited greater shoot elongation and leaf blade expansion and less root elongation than EK 11-1 plantlets, indicating that EK 16-3 plantlets used most of the sucrose for production of
photosynthetically competent leaves while EK 11-1 plantlets used it for root production. The increasing $P_{np}$ over time of EK 16-3 compared to EK 11-1 plantlets also indicates that EK 16-3 became photoautotrophic sooner than EK 11-1 plantlets. An inverse correlation between sucrose concentrations in the medium and $P_{np}$ has been observed using C$_3$ species such as rose ($Rosa$ $multiflora$ L. ‘Montse’) (Capellades et al., 1991), strawberry ($Fragaria$ $x$ $ananassa$ Duch. ‘Kent’) (Hdider and Desjardins, 1994), avocado ($Persea$ $americana$ Mill.) (De la Viña et al., 1999), and rain tree ($Samanea$ $saman$ Merr.) (Mosaleeyanon et al., 2004). The rapid increase of $P_{np}$ in EK 16-3 plantlets could thus be correlated with the decrease or total depletion of sucrose from the medium with time. Furthermore, the increase in $P_{np}$ is often proportional to the increase in shoot and root growth (Mosaleeyanon et al., 2002), which was also observed in EK 16-3 plantlets cultured in vitro (Table 4-2).

**Effect of In Vitro CO$_2$ Enrichment**

In vitro CO$_2$ enrichment increased $P_{nw}$ in both genotypes, however this increase was 1.5 times greater for EK16-3 than for EK 11-1 plantlets (Table 4-3). Furthermore, CO$_2$ enrichment only increased in vitro biomass of EK 16-3 plantlets (Table 4-2). In previous studies, CO$_2$ enrichment did not significantly affect growth of sugarcane plantlets when cultured in sugar-containing medium (Tay et al., 2000), whereas growth of sugarcane plantlets was enhanced under high PPF with CO$_2$ enrichment when cultured in a sugar-free medium (Erturk and Walker, 2000). In our study, it is likely that EK 16-3 plantlets depleted the sucrose from the medium earlier than EK 11-1 plantlets, and subsequently CO$_2$ enrichment enhanced growth earlier in EK 16-3 than EK 11-1 plantlets.
In vitro CO₂ enrichment resulted in a higher photosynthetic capacity increase in EK 16-3 than in EK 11-1 plantlets after 6 weeks in vitro (Table 4-3). The differences in photosynthetic capacity of these two sea oats genotypes may be related to their differing morphological and anatomical development in vitro. Valero-Aracama et al. (2004a) conducted anatomical studies of EK 16-3 and EK 11-1 sea oats genotypes during in vitro photomixotrophic culture and found similar leaf development between in vitro and acclimated EK 16-3 leaves. Conversely, in vitro EK 11-1 leaves exhibited significant disruption of mesophyll and vascular bundles and irregular size and shape of epidermal cells when compared to acclimated leaves. The higher similarity found between in vitro and ex vitro leaf tissues of EK 16-3 than EK 11-1 plantlets correlates with the higher in vitro photosynthetic capacity of EK 16-3 than EK 11-1 plantlets.

**Ex Vitro Acclimatization**

EK 16-3 control plantlets exhibited 77.5% survival after 6 weeks in the greenhouse even though $P_{nw}$ after 6 weeks in vitro was relatively low. Photosynthetic capacity of plants ex vitro is critical during acclimatization. However, control of water loss may also have played an important role during the first several days of acclimatization. EK 16-3 plantlets displayed lower transpiration rates than EK 11-1 plantlets, indicating better regulation of water loss during acclimatization. Comparative anatomical study of sea oats genotypes cultured in vitro showed greater cuticularization in EK 16-3 than EK 11-1 plantlets (Valero-Aracama et al., 2004a). Sutter and Langhans (1979) observed a lack of epicuticular wax formation in in-vitro produced carnation (*Dianthus caryophyllus* L.) leaves, and Grout and Aston (1978) observed high susceptibility to desiccation in in-vitro produced cauliflower (*Brassica oleracea* L.) leaves. Similarly, Desjardins et al. (1987) found that if transitional leaves of strawberry plants were produced after transfer to
greenhouse conditions, in-vitro produced leaves could not regulate water loss and would degenerate before new leaves became functional and supported growth. With sea oats genotypes such as EK 16-3, we observed that the development of elongated leaves with fully expanded blades produced in vitro was critical to shoot establishment ex vitro. If leaves were short and lacked fully expanded leaf blades after in vitro culture, they exhibited poor photosynthetic capacity and poor control of water loss (as observed in EK 11-1 control plantlets), and consequently, growth ceased and most plants died.

During early acclimatization, sufficient levels of carbohydrate reserves are required for plantlet survival, to improve acclimatization and to hasten physiological adaptations (Wilson et al., 2000). Valero-Aracama et al. (2004b) observed that the starch reserves in sea oats genotypes during conventional in vitro culture conditions and after transfer to ex vitro conditions were greater in leaves of EK 11-1 than EK 16-3 plantlets. However, after 2 weeks ex vitro, a 8.6- and 2.8-fold decrease in leaf starch content of EK 11-1 and EK 16-3 plants, respectively, was observed (Chapter 3). Given the lack of leaf production in EK 11-1, starch reserves clearly are not utilized for development of photosynthetic tissues ex vitro, therefore resulting in significantly lower survival.

EK 16-3 plantlets cultured in vitro under control conditions had greater photosynthetic capacity at the time of ex vitro transfer than EK 11-1 control plantlets as indicated by their initial $P_{\text{nl}}$ ex vitro. By using in vitro modified photomixotrophic conditions rather than conventional conditions, we were able to increase $P_{\text{nl}}$ in EK 11-1 plantlets and significantly increase survival ex vitro (Figure 4-3). Modified photomixotrophic conditions, including an increase in ventilation rates of vessels and CO₂ availability in the vessel headspace, increases gas exchange of plantlets (Pospíšilová
et al., 1992). These conditions possibly promoted the development of photosynthetically
cOMPETENT LEAVES WITH GREATER CUTICLE DEVELOPMENT, NORMAL STOMATAL FUNCTION AND
therefore, lower water stress of sea oats plants during acclimatization to ex vitro
conditions.

Growth data after 6 weeks ex vitro indicated that acclimated EK 16-3 plants
produced significantly greater leaf and root biomass than acclimated EK 11-1 plants,
regardless of treatment conditions. These differences between genotypes were more
pronounced under control conditions than under PM or PME conditions. An increase in
leaf length was also observed in EK 16-3 compared to EK 11-1 plants. Also, PM and
PME conditions significantly enhanced leaf and root biomass and leaf length of plants
compared to conventional control conditions. Seon et al. (2000) found a similar increase
in growth of Chinese foxglove [Rehmannia glutinosa (Gaertn.) Steud.] plants cultured in
vitro under photoautotrophic conditions as compared to conventional photomixotrophic
conditions. Likewise, photoautotrophic conditions induced high photosynthetic rates in
eucalyptus resulting in significant leaf area increase and improved survivability
(Kirdmanee et al., 1995).

Although there was a positive effect under modified photomixotrophic conditions
of CO₂ enrichment on in vitro $P_{nw}$ of both genotypes and on in vitro growth of EK 16-3
plantlets, no residual effect of in vitro CO₂ enrichment was observed for ex vitro survival,
$P_{ni}$ or growth in either genotype. Natural ventilation under ambient CO₂ allowed sea oat
plants to develop photosynthetic competence to levels comparable to plants under CO₂
enriched conditions. Ambient levels of CO₂ during acclimatization, regardless of in vitro
CO₂ pre-treatment, allowed similar rates of growth and development in both genotypes.
It should be noted that experimental evidence indicates that reductions in rooting and acclimatization capacity of some plant species cultured in BA-supplemented medium may result from the production of the breakdown product [9G]BA, which accumulates at the base of plantlets and remains for more than 6 weeks (Werbrouck et al., 1995; Werbrouck et al., 1996). Similarly, Valero-Aracama et al. (2003) observed a detrimental carryover effect from Stage II culture conditions (BA-supplemented medium) on in vitro growth and rooting and ex vitro survival of certain sea oats genotypes. Unfortunately, in sea oats, BA is the only effective cytokinin for shoot multiplication. Modification of photomixotrophic conditions in the present study likely decreased the detrimental carryover effects of BA by increasing in vitro growth rates and the photosynthetic competence of EK 11-1 plants in vitro.

**Conclusions**

Our results suggest that growth and survivability of sea oats genotypes with different acclimatization capacities can be enhanced by optimizing culture conditions. In vitro photoautotrophic conditions can be used for sea oats only with adequate transition from photomixotrophic to photoautotrophic conditions by adding sugar to the medium and subsequent dilution with sugar-free medium over time. This method is suitable for obtaining quality transplants that can withstand transplantation, and ultimately grow ex vitro at higher growth rates than plantlets cultured under conventional photomixotrophic conditions.

Furthermore, CO₂ enrichment did not significantly enhance survival and growth of sea oats genotypes ex vitro, indicating that under modified photomixotrophic conditions without CO₂ enrichment, the increase in vessel ventilation rates allowed sufficient CO₂ diffusion around the sea oats plantlets to enhance growth and develop photoautotrophy in
vitro. Similar to many C$_3$ species, sea oats, a C$_4$ species, benefits from photoautotrophic micropropagation, in that plantlet growth and development of photoautotrophy are enhanced during in vitro culture. Yet, unlike many C$_3$ species, sea oats did not benefit from CO$_2$ enrichment in vitro to obtain high survival rates and comparable growth and development to that of in vitro plantlets exposed to ambient CO$_2$ conditions.

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CHAPTER 5
CONCLUSIONS

Micropropagation of sea oats genotypes is achieved through the in vitro establishment of explants, multiplication and rooting and subsequent ex vitro transfer of plantlets to greenhouse conditions. During in vitro culture, plantlets are typically grown in conditions of low ventilation, which results in high relative humidity, and lower irradiance levels than in conventional propagation. The low ventilation rates of vessels limit the inflow of CO$_2$ and the outflow of other gases such as O$_2$ or C$_2$H$_4$. The culture media is supplied with sugars as main carbon source, N$^6$-benzyladenine (BA) to promote shoot multiplication and α-naphthalene acetic acid (NAA) for root induction. These conditions can result in the formation of plantlets of abnormal morphology, anatomy and physiology. The effects of in vitro culture conditions on sea oats acclimatization and survival ex vitro have different effects depending upon genotypes. In this dissertation, in vitro and ex vitro culture of sea oats genotypes were compared to understand the basis for anatomical, morphological, and physiological differences in easy- and difficult-to-acclimatize genotypes (EK16-3 and EK 11-1, respectively).

Results indicated that abnormal anatomy and morphology of sea oats genotypes produced in vitro were correlated with poor survival and acclimatization ex vitro (Chapter 2). These abnormal characteristics consisted of leaves that were short, thick and without expanded leaf blades, and poorly differentiated mesophyll, vascular bundles and epidermis. Scanning electron micrographs revealed a lack of wax deposition and blocked or fused stomata on leaves produced during Stage II multiplication in both genotypes that
possibly affected the control of water loss at time of ex vitro transfer. Transmission
electron micrographs further revealed relatively more disruption of leaf chloroplast
 ultrastructure in EK 11-1 than in EK 16-3 plantlets. Poor acclimatization and high
mortality ex vitro of unrooted microcuttings transferred directly from Stage II to Stage IV
conditions indicated that pre-rooting prior to acclimatization was required in both
genotypes. This could be due to: 1) increased water demands ex vitro and/or 2) reduced
energy demands to produce roots ex vitro because this energy was supplied in vitro.
During Stage III, EK 11-1 plantlets exhibited poor development of shoots and an
extensive heterotrophic root system. Nevertheless, although rooted microcuttings
exhibited higher survival ex vitro than unrooted microcuttings, acclimatization of EK 11-
1 was still significantly lower than that of EK 16-3 plantlets.

Higher mortality of EK 11-1 plantlets was the consequence of a negative carbon
balance resulting from limited production of photosynthetic leaves and the high energy
demands from the heterotrophic root system. Analyses of phosphoenolpyruvate
carboxylase (PEPC) and ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco)
activities during in vitro culture and ex vitro acclimatization indicated that after 6 weeks
Stage III, EK 16-3 plantlets exhibited significant increases in activities of PEPC and
rubisco and levels of chlorophyll and total soluble protein, compared to EK 11-1 plantlets
(Chapter 3). These increases in EK 16-3 were correlated with in vitro development of
anatomical and morphological leaf features comparable to those of greenhouse-produced
leaves. In contrast to EK 11-1 plantlets, after 6 weeks Stage III, the enzymatic activity
levels in EK 16-3 were closer to the levels observed during or after acclimatization in
either genotype. Therefore, the capacity for carbon fixation of EK 16-3 plantlets after 6
weeks Stage III was comparable to that of plants that had been successfully acclimatized. This was supported by significantly different net photosynthetic rates ($P_{nl}$) observed between genotypes at the time of ex vitro transfer. EK 16-3 plantlets exhibited positive $P_{nl}$ during in vitro culture, whereas the negligible $P_{nl}$ observed in the EK 11-1 plantlets indicated that these plantlets were either net respirers or had minimal $P_{nl}$ rates (Chapter 4).

Carbohydrate analyses of in vitro plantlets indicated rapid accumulation of starch and sugars in plantlets following transfer to Stage III medium (Chapter 3). During in vitro shoot and root development, carbohydrate reserves were utilized at different rates between these two genotypes. As a result, EK 11-1 exhibited higher levels of starch reserves than EK 16-3 at the time of ex vitro transfer. Although rooted EK 11-1 plantlets had higher energy reserves than EK 16-3 at the onset of acclimatization, these reserves were not sufficient to allow transition from the in vitro heterotrophic/photomixotrophic mode to the ex vitro photoautotrophic mode. These physiological observations indicated that EK 16-3 plantlets were capable of developing photosynthetically competent leaves while storing carbohydrates in vitro. Consequently, these plants had a positive carbon balance that supported the energy requirements for respiration of cells, as well as growth requirements of different organs during acclimatization. The possible negative carry-over effect using BA for shoot multiplication cannot be ruled out.

A common problem of in vitro produced plants during acclimatization is the poor control of water loss after ex vitro transfer. Transpiration rates in fully expanded leaves of sea oats indicated significant differences between genotypes at time of ex vitro transfer, being higher for EK 11-1 than EK 16-3 plantlets until they became similar after
2 weeks ex vitro culture. However SEM observations indicated both genotypes exhibited similar wax deposition and similar stomatal appearance during Stage III. Given the small magnitude of the difference in transpiration rates, these results indicated that control of water loss was not the primary cause for low survival and poor acclimatization ex vitro. Nevertheless, lack of control of water loss in EK 11-1 may have been a contributing factor affecting survival and acclimatization.

Given that in vitro conditions limited the growth and development of photosynthetically competent leaves of EK 11-1 plantlets, the possibility that plantlets could benefit from modifications of the in vitro culture conditions which promote photoautotrophic growth in vitro prior to acclimatization was examined (Chapter 4). It was concluded that in vitro photoautotrophic conditions of limited sucrose, higher ventilation rates and higher light levels could be used to produce sea oats genotypes, provided that there was an initial sucrose supplementation in the medium subsequently diluted with sucrose-free medium throughout the remaining culture period. This method facilitated production of quality plants that could withstand transplantation and grow at higher rates than conventionally micropropagated plantlets.

The additional provision of CO₂ enrichment, however, did not significantly improve ex vitro survival or growth of sea oats genotypes. It was proposed that natural ventilation using filter discs on the vessels allowed sufficient CO₂ diffusion around the plantlets to enhance growth and develop photoautotrophy in vitro.

The morphological, anatomical and physiological studies shown in this dissertation contribute to the understanding of the effects of in vitro conditions on acclimatization of
diverse sea oats genotypes. Ultimately, this could be used to improve large-scale micropropagation systems of a wide range of sea oats genotypes.
APPENDIX
EFFECT OF META-TOPOLIN DURING MULTIPLICATION, ROOTING AND ACCLIMATIZATION OF EASY- AND DIFFICULT-TO-ACCLIMATIZE SEA OATS (*Uniola paniculata* L.) GENOTYPES

**Introduction**

Plant growth regulators incorporated into shoot multiplication culture media, especially cytokinins, can have deleterious effects on growth and development (Pospíšilová et al., 1992; Werbrouck et al., 1995). Benzyladenine (BA) is the most effective cytokinin for shoot multiplication in tissue culture (Werbrouck et al., 1996), and is the cytokinin required for sea oats micropropagation (Philman and Kane, 1994). Experimental evidence suggests that reductions in rooting and acclimatization, observed in some plants produced on BA-supplemented medium, may result from production of the breakdown product, [9G]BA, which accumulates at the base of plantlets in vitro and remains for more than 6 weeks (Werbrouck et al., 1995; Werbrouck et al., 1996). Strnad et al. (1997) reported the existence of meta-topolin, a naturally occurring BA analog that produces the deleterious metabolite with a shorter half-life. Werbrouck et al. (1996) found that meta-topolin effectively regenerates peace lily (*Spathiphyllum floribundum* Schott ‘Petite’) shoots, with better rooting in vitro than equimolar concentrations of BA. Consequently, the possibility that meta-topolin may be an acceptable BA substitute for sea oats micropropagation was explored.
Established and indexed in vitro shoot cultures of two sea oats (*Uniola paniculata* L.) genotypes, collected from Egmont Key, on the Florida Gulf coast, genotyped using random amplified polymorphic DNA (RAPD) genetic analyses (Ranamukhaarachchi, 2000) and previously characterized as easy- and difficult-to-acclimatize (EK 16-3 and EK 11-1, respectively) were used. Five sea oats shoot clusters each consisting of three shoots, 30 mm long of EK 16-3 and EK 11-1 genotypes, were subcultured into separate Magenta GA7 vessels (Magenta Corp., Chicago, IL) containing 80 mL sterile Stage II medium. Stage II medium consisted of Murashige and Skoog (MS) inorganic salts (Murashige and Skoog, 1962), supplemented with 87.6 mM sucrose, 0.56 mM myo-inositol, 1.2 µM thiamine-HCl, and 6 levels of plant growth regulators (PGR): 0 µM PGR, 2.2 µM N\textsubscript{6}-benzyladenine (BA), and 2.2, 10, 20 and 30 µM N\textsubscript{6}-(3-hydroxy benzyl) (meta-topolin; MT). All media were adjusted to pH 5.7 with 0.1 N KOH prior to the addition of 8 g L\textsuperscript{-1} TC™ agar (*Phyto*Technology Laboratories, Shawnee Mission, KS) and autoclaving at 1.2 kg cm\textsuperscript{-2} and 121 ºC for 20 min.

Cultures were maintained for 4 weeks in a growth chamber at 24 ± 1 ºC, 58 ± 5% relative humidity (RH), 16-h photoperiod provided by cool-white fluorescent lamps (General Electric F20WT12-CW), and at 40 ± 5 µmol m\textsuperscript{-2} s\textsuperscript{-1} photosynthetic photon flux (PPF) as measured at culture level. Number of leaves and dry weight of clusters from 5 replicate vessels per genotype were measured after each culture period. Production of rootable microcuttings ≥ 30 mm long were considered as harvestable shoots.

In a concurrent experiment, microcuttings of EK 11-1 and EK 16-3 genotypes were cultured in Stage II for 4 weeks as described above. Subsequently, the shoot clusters of
each genotype were subdivided into single shoots and transferred to Stage III rooting medium. Stage III rooting medium consisted of 80 mL sterile half-strength MS medium, supplemented with 0.56 mM myo-inositol, 1.2 µM thiamine-HCl, 87.6 mM sucrose, and 10 µM α-naphthalene acetic acid (NAA), contained in GA7 vessels. Culture vessels contained 8 single microcuttings each, and were maintained in a culture room at 22 ± 2 °C, under a 16-h photoperiod provided by cool-white fluorescent lamps (General Electric F96T12·CW·WM), at 100 ± 5 µmol m⁻² s⁻¹ PPF as measured at culture level. After 6 weeks Stage III, percent rooting was recorded.

After 6 weeks, microcuttings were placed in 5 replicate six-celled blocks, each cell 4 x 6 x 5.5 cm (T.O. Plastics, Inc., Clearwater, MN) containing coarse vermiculite as supporting material and transferred to Stage IV conditions. Plantlets were watered as needed, and Peters 20N-20P-20K liquid fertilizer (150 mg N L⁻¹; The Scotts Company, Marysville, OH) was applied weekly. Greenhouse set points for cooling and heating were 24 and 22°C, respectively, and natural solar PPF ranged 900-1200 µmol m⁻² s⁻¹ at noon. Survival percentage was recorded weekly, and after 6 week Stage IV culture, shoot number and longest leaf length were measured.

**Experimental Designs and Statistical Analyses**

Both experiments were completely randomized designs. Data were analyzed using analysis of variance (general linear model procedure of SAS institute Inc., 1999). Separate *a posteriori* test for significant differences among or between means were made using the Waller-Duncan procedure at \( P \leq 0.05 \).
Results

In Vitro Shoot Multiplication

During Stage II multiplication, shoot dry weights varied among treatments and between genotypes (Figure A-1). The lowest shoot dry weight accumulation was observed in shoot clusters cultured in medium containing no PGRs followed by 30 \text{ \mu M} meta-topolin (MT). EK 11-1 plantlets exhibited significantly higher shoot dry weight accumulation than EK 16-3 plantlets in all treatments except for those cultured in medium containing 20 \text{ \mu M} MT.

Leaf production was highest in EK 16-3 plantlets cultured in 20 \text{ \mu M} MT and EK 11-1 plantlets cultured in 30 \text{ \mu M} MT (Figure A-2). In all remaining treatments containing MT, leaf production was consistently higher in EK 11-1 than EK 16-3 plantlets, and number of leaves were non-significantly different between genotypes in treatments without PGR and with BA (control).

Shoot production in both genotypes was similar in medium supplemented with either 2.2 \text{ \mu M} BA (control) or MT (Figure A-3). Shoot production was significantly higher in medium supplemented with greater than 2.2 \text{ \mu M} MT. Highest harvestable shoot production was observed on EK 16-3 plantlets in the presence of 20 \text{ \mu M} MT. The lowest number of harvestable shoots was obtained when no PGR was supplemented to the medium.

Carry Over Effect on Vitro Rooting

A negative carry over effect of MT concentration during Stage II multiplication on Stage III rooting was only observed in concentrations greater than 10 \text{ \mu M} in EK 16-3 and
2.2 µM in EK 11-1 (Figure A-4). The highest rooting percentages were observed in plantlets cultured in medium without PGR, 2.2 µM BA and 2.2 µM MT.

**Carry Over Effects on Ex Vitro Acclimatization**

The EK 11-1 genotype exhibited the greatest negative carry over effect of BA. Lowest (30%) ex vitro survival 6 weeks after transfer to the greenhouse was observed in EK 11-1 plantlets when 2.2 µM BA was used for Stage II multiplication. In contrast, EK 11-1 plantlets produced in Stage II using either 2.2 or 10 µM MT exhibited significantly higher Stage IV survival. A negative carry over effect of MT concentration during Stage II on survival ex vitro was observed in EK 11-1 plantlets cultured in the presence of 20 or 30 µM MT (Figure A-5A). Although the carry over effect of BA during Stage II was relatively less in EK 16-3 than EK 11-1, survival of EK 16-3 was greater in plantlets multiplied in the presence of 2.2 µM MT (Figure A-5B). Treatments without PGRs exhibited the highest leaf length after 6 weeks ex vitro culture (Figure A-6). Leaf length was similar between EK16-3 plantlets cultured with BA, 2.2 µM MT, 10 µM MT and EK 11-1 plantlets cultured with 2.2 µM MT. EK 11-1 plantlets cultured in medium containing 10, 20 and 30 µM MT and EK 16-3 plantlets cultured in 30 µM MT resulted in the shortest leaves ex vitro among treatments.

Ex vitro shoot number was also affected by in vitro treatment and genotype (Figure A-7). Ex vitro shoot number ranged from 1.5 to 2.5 after 6 weeks ex vitro culture. The highest shoot number was obtained in EK 16-3 plantlets cultured in 10, 20, 30 µM MT and without PGRs and in EK 11-1 plantlets cultured in 30 µM MT.
Figure A-1. Effect of cytokinin type and concentration after 4 weeks Stage II culture on shoot cluster dry weight of EK 11-1 and EK 16-3 sea oats genotypes. Error bars indicate SE (n = 5). BA: N\textsuperscript{6}-benzyladenine; MT: N\textsuperscript{6}-(3-hydroxy benzyl). ANOVA analysis is shown on top left corner of each graph; T: Treatment, G: Genotype, NS, **: Non-significant or significant at P ≤ 0.01, respectively. Different letters on top of histobars are significantly different according to Waller-Duncan test at P ≤ 0.05.
Figure A-2. Effect of cytokinin type and concentration after 4 weeks Stage II culture on leaf number of EK 11-1 and EK 16-3 sea oats genotypes. Error bars indicate SE (n = 5). BA: N<sub>6</sub>-benzyladenine; MT: N<sub>6</sub>-(3-hydroxy benzyl). ANOVA analysis is shown on top left corner of each graph; T: Treatment, G: Genotype, **: Significant at P ≤ 0.01. Different letters on top of histobars are significantly different according to Waller-Duncan test at P ≤ 0.05.
Figure A-3. Effect of cytokinin type and concentration after 4 weeks Stage II culture on number of harvestable shoots (≥30 mm) of EK 11-1 and EK 16-3 sea oats genotypes. Error bars indicate SE (n = 5). BA: N\textsuperscript{6}-benzyladenine; MT: N\textsuperscript{6}-(3-hydroxy benzyl). ANOVA analysis is shown on top left corner of each graph; T: Treatment, G: Genotype, NS, **: Non-significant or significant at P ≤ 0.01, respectively. Different letters on top of histobars are significantly different according to Waller-Duncan test at P ≤ 0.05.
Figure A-4. Effect of cytokinin type and concentration after 4 weeks Stage II followed by 6 weeks Stage III culture on rooting of EK 11-1 and EK 16-3 sea oats genotypes. Error bars indicate SE (n = 5). BA: N⁶-benzyladenine; MT: N⁶-(3-hydroxy benzyl). ANOVA analysis is shown on top left corner of each graph; T: Treatment, G: Genotype, NS, *, **: Non-significant or significant at $P \leq 0.05$ or 0.01, respectively. Different letters on top of histobars are significantly different according to Waller-Duncan test at $P \leq 0.05$. 
Figure A-5. Effect of cytokinin type and concentration supplemented in Stage II (4 weeks) followed by 6 weeks Stage III on ex vitro survival of A: EK 11-1, and B: EK 16-3 sea oats genotypes. Means ± SE are shown (n = 5).
Figure A-6. Effect of cytokinin type and concentration after 4 weeks Stage II followed 6 weeks Stage III and 6 weeks Stage IV on ex vitro leaf length of EK 11-1 and EK 16-3 sea oats genotypes. Error bars indicate SE (n = 5). BA: N\textsuperscript{6}-benzyladenine; MT: N\textsuperscript{6}-(3-hydroxy benzyl). ANOVA analysis is shown on top left corner of each graph; T: Treatment, G: Genotype, *, **: Significant at \( P \leq 0.05 \) or 0.01, respectively. Different letters on top of histobars are significantly different according to Waller-Duncan test at \( P \leq 0.05 \).
Figure A-7. Effect of cytokinin type and concentration after 4 weeks Stage II followed 6 weeks Stage III and 6 weeks Stage IV on ex vitro shoot number of EK 11-1 and EK 16-3 sea oats genotypes. Error bars indicate SE ($n = 5$). BA: N$^6$-benzyladenine; MT: N$^6$-(3-hydroxy benzyl). ANOVA analysis is shown on top left corner of each graph; T: Treatment, G: Genotype, *, **: Significant at $P \leq 0.05$ or 0.01, respectively. Different letters on top of histobars are significantly different according to Waller-Duncan test at $P \leq 0.05$. 

**T**; **G**; **T x G**: *


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BIOGRAPHICAL SKETCH

Carmen Valero Aracama was born in Elda, Alicante, Spain. She completed her last year of high school at Roger Bacon High School, Cincinnati, OH, in May 1992. She received a Bachelor Degree of Science in biological sciences with specialization in environmental sciences in July 1996 from the Universidad de Alicante, Spain. She was awarded an Intercampus Scholarship by the Spanish Agency for International Cooperation to gain experience in ecological research for two months at the Laboratory of Coastal Plant Communities at the Fundaçao Universidade do Rio Grande, in Rio Grande, RS, Brazil. Subsequently she was awarded the Mombusho Scholarship by the Ministry of Education of Japan for research and education in Japan. She traveled to Chiba, Japan, in April 1998 to start her studies of Japanese and in October 1998, she joined the Laboratory of Environmental Control Engineering at Chiba University, Japan, where she started her horticultural studies to pursue a Master of Science degree in horticulture in March, 2001. Upon completion of the master’s degree, she was awarded the Fulbright Scholarship by the Fulbright Commission of the U.S. to continue her education in the Environmental Horticulture Department at the University of Florida. She was also awarded the Alumni Fellowship by the College of Agricultural and Life Sciences to complete her doctoral studies. Her dissertation topic was to determine physiological aspects of Florida’s sea oats genotypes, an important dune grass used for beach and dune restoration and to prevent coastal erosion.