

## PHOTOSYNTHETIC AND CARBOHYDRATE STATUS OF EASY- AND DIFFICULT-TO-ACCLIMATIZE SEA OATS (*UNIOLA PANICULATA* L.) GENOTYPES DURING *IN VITRO* CULTURE AND *EX VITRO* ACCLIMATIZATION

CARMEN VALERO-ARACAMA<sup>1</sup>, MICHAEL E. KANE<sup>1\*</sup>, SANDRA B. WILSON<sup>2</sup>, JOSEPH C. VU<sup>3</sup>,  
JOAN ANDERSON<sup>3</sup>, AND NANCY L. PHILMAN<sup>1</sup>

<sup>1</sup>*Department of Environmental Horticulture, Institute of Food and Agricultural Sciences,  
University of Florida, Gainesville, FL 32611*

<sup>2</sup>*Department of Environmental Horticulture, Institute of Food and Agricultural Sciences,  
University of Florida, IRREC, Fort Pierce, FL 34945*

<sup>3</sup>*Department of Agronomy, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32611*

(Received 17 January 2006; accepted 21 August 2006; editor B. M. Reed)

### SUMMARY

The photosynthetic and carbohydrate status of an easy-to-acclimatize (EK 16-3) and a difficult-to-acclimatize (EK 11-1) genotype of *Uniola paniculata* L. (sea oats), a native dune species of the southeastern US, were evaluated during *in vitro* culture and *ex vitro* acclimatization. Net photosynthetic rate was eight times greater for EK 16-3 than EK 11-1 plantlets after *ex vitro* transfer. *In vitro*-produced leaves were morphologically similar to *ex vitro*-produced leaves and exhibited similar photosynthetic competence. EK 11-1 plantlets exhibited greater transpiration rates at the time of *ex vitro* transfer than EK 16-3 plantlets. However, the small magnitude of this difference, although significant, indicated that control of water loss was probably not the main cause for poor acclimatization of EK 11-1 plantlets. Carbohydrate analysis *in vitro* revealed that EK 16-3 plantlets utilized leaf starch reserves more rapidly than EK 11-1 plantlets. Starch utilization correlated with the development of leaves with expanded leaf blades during *in vitro* rooting in EK 16-3 plantlets. After *ex vitro* transfer, both genotypes exhibited significant decreases of starch and soluble sugar content in shoots and roots. However, the higher photosynthetic ability of shoots in EK 16-3 resulted in greater accumulation of shoot soluble sugars than EK 11-1 after 2-wk *ex vitro* culture. After 6-wk *in vitro* rooting, there were significantly higher chlorophyll and soluble protein contents, ribulose 1,5-bisphosphate carboxylase (rubisco) and phosphoenolpyruvate carboxylase activities in EK 16-3 than EK 11-1 shoots. These differences also correlated with the development of anatomical and morphological leaf features in EK 16-3 similar to those of greenhouse-produced leaves.

*Key words:* micropropagation; phosphoenolpyruvate carboxylase; photosynthesis; rubisco.

### INTRODUCTION

Micropropagation has been used extensively 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 problematic. One of the most important problems 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 the 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; Arigita et al., 2002), low light and inadequate CO<sub>2</sub> supply (Kozai and Iwanami, 1988; De et al., 1993), and poor control of water loss caused by high relative humidity (RH) within the vessel (Desjardins, 1995; Estrada-Luna et al., 2001). These conditions

can ultimately influence plant development and photosynthetic performance *ex vitro* (Kozai, 1991b; Preece and Sutter, 1991).

*In vitro* culture conditions frequently result in alterations of mesophyll development as well as chloroplast structure, namely grana development (Wetzstein and Sommer, 1982), which may affect the photosynthetic ability of plants. 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) are often encountered in C<sub>3</sub> species. These conditions also contribute to low photosynthetic activity (Sinha et al., 2002).

During acclimatization, *in vitro*-cultured plants must go through a transition from a heterotrophic or a photomixotrophic mode to a fully photoautotrophic mode in the greenhouse. A common strategy of plants during *ex vitro* acclimatization is to use the carbohydrate storage of *in vitro*-produced leaves to cover metabolic demands of growing tissues (Piqueras et al., 1998; Van Huylenbroeck et al., 1998; Fuentes et al., 2005). However, the function of *in vitro*-produced leaves during acclimatization varies depending upon

\*Author to whom correspondence should be addressed: Email micropro@ufl.edu

plant species. In some plant species, such as *Calathea* (*Calathea loiusae* Gagnep. 'Maui Queen'), *in vitro*-produced leaves have limited photosynthetic ability and function as storage organs whose energy reserves are consumed during the initial days of acclimatization (Van Huylenbroeck et al., 1998). Furthermore, cauliflower (*Brassica oleracea* L.) or strawberry (*Fragaria* × *ananassa* Duch. 'Kent') leaves exhibit greater respiration than photosynthetic rates and their *in vitro*-produced leaves senesce rapidly after transplantation *ex vitro* (Grout and Aston, 1978; Grout and Millam, 1985). 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 (Van Huylenbroeck et al., 1998).

Sea oats (*Uniola paniculata* L.) is a perennial C<sub>4</sub> grass native to the southeastern US 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 of the natural sources of plants and seeds (Hester and Mendelsohn, 1987; Bachman and Whitwell, 1995; Burgess et al., 2002, 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). When this protocol was applied to multiple sea oats genotypes, microcuttings of different genotypes transferred *ex vitro* displayed differing capacities for acclimatization.

Understanding the reasons for the low acclimatization capacity of some sea oats genotypes is needed to produce a wide range of sea oats genotypes efficiently using micropropagation. In the present study, the photosynthetic ability and carbohydrate status, as well as the activity of some key enzymes in the carbon fixation process of C<sub>4</sub> plants, were compared between an easy-to-acclimatize sea oats genotype and a difficult-to-acclimatize sea oats genotype.

## MATERIALS AND METHODS

**Culture conditions.** Established and indexed *in vitro* shoot cultures of two sea oats (*U. paniculata* L.) genotypes, collected from Egmont Key on the Florida Gulf Coast were used. These plants were genotyped using random amplified polymorphic DNA genetic analyses (Ranamukhaarachchi, 2000), and characterized as easy-to-acclimatize and difficult-to-acclimatize (EK 16-3 and EK 11-1, respectively). 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, USA) containing 80 ml sterile Stage II medium. Stage II multiplication 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<sup>6</sup>-benzyladenine, and gelled with 8 g l<sup>-1</sup> TC™ agar (PhytoTechnology Laboratories, Shawnee Mission, KS, USA). All media were adjusted to pH 5.7 with 0.1 N KOH prior to the addition of agar and autoclaving at 117.7 kPa and 121°C for 20 min.

Cultures were maintained for 8 wk in a growth chamber at 24 ± 1°C, 58 ± 5% RH, 16-h photoperiod provided by cool-white fluorescent lamps (General Electric F20WT12-CW), and at 40 ± 5 μmol m<sup>-2</sup> s<sup>-1</sup> 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-naphthaleneacetic acid, gelled with 8 g l<sup>-1</sup> TC™ agar and 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<sup>-2</sup> s<sup>-1</sup> PPF, as measured at culture level.

After 6 wk, rooted microcuttings were placed individually in 48-cell plug trays (eight six-celled blocks, each cell 4 × 6 × 5.5 cm; T.O. Plastics, Inc., Clearwater, MN, USA) containing coarse vermiculite as supporting material and transferred to Stage IV greenhouse conditions. Plantlets were watered as needed and Peters 20N-20P-20K liquid fertilizer (150 mg N l<sup>-1</sup>; The Scotts Company, Marysville, OH, USA) 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<sup>-2</sup> s<sup>-1</sup> at noon.

**Photosynthesis studies.** Shoot clusters of the EK 11-1 and EK 16-3 sea oats genotypes were cultured for 8 wk under Stage II conditions as previously described. Subsequently, single microcuttings were transferred to Stage III conditions for 6 wk, with 10 replicate GA7 vessels per genotype. All rooted microcuttings were transferred in 48-cell plug trays (eight six-celled blocks, each cell 4 × 6 × 5.5 cm; Summit Plastics Inc., Clearwater, OH, USA) to a greenhouse under natural solar PPF ranging between 900 and 1200 μmol m<sup>-2</sup> s<sup>-1</sup> during the measurements and day/night temperatures of 25/22°C, respectively.

Net photosynthetic rates per leaf area ( $P_n$ ) were determined with a PP System Model Ciras-1 (PP System Co., Ltd, UK) without a supplemental light source and inlet CO<sub>2</sub> concentration fixed at 400 ± 10 μmol mol<sup>-1</sup>. Measurements were taken in full sun near midday (10 a.m. to 12 p.m.) on newly formed and 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 wk in GA7 vessels under Stage II conditions as previously described. There were four replicate vessels per genotype, each vessel containing five plants. Concurrently, another set of sea oats shoot clusters were cultured for 8 wk under the same conditions for shoot multiplication prior to transfer to Stage III conditions for 3, 6, or 9 wk. Each treatment in Stage III consisted of four replicate vessels per genotype, each vessel containing eight microcuttings.

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

For enzymatic extraction, powdered shoot tissue (*c.* 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<sub>2</sub>, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), 10 mM isoascorbate, 2% PVP-40 (w/v), and 0.1% TX-100 (w/v). Leaf tissue was ground with extraction buffer on ice for 2 min, transferred into two 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<sub>2</sub> plus 0.011 ml 200 mM NaHCO<sub>3</sub> 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 the start of extractions procedure with a Hitachi Model U-2000 Double-Beam UV/VIS Spectrophotometer (Hitachi Instruments, Inc., Danbury, CT, USA).

PEPC was assayed spectrophotometrically at 340 nm by following the reduction of oxaloacetic acid (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<sub>2</sub>, 0.1 mM EDTA, 10 mM NaHCO<sub>3</sub>, 5 mM DTT, 2.5 U MDH, and 0.2 mM NADH. After the addition of 0.01 ml extract, a steady baseline was established and the reaction was initiated by the addition of phosphoenolpyruvate 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 of 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 sulfate precipitates. Sulfate was removed prior to use either by desalting 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<sub>2</sub>, 1 mM EDTA, 20 mM NaCl, 10 mM NaHCO<sub>3</sub>, 5 mM DTT, 2.5 mM ATP, 5 mM phosphocreatine, 5 U creatine phosphokinase, 5 U of 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 0.01 ml of activated extract. The linear decrease in absorbance resulting from the 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 two microfuge tubes, each containing 0.1 ml of water. Subsequently, 0.8 ml of 100% acetone was added in each tube and incubated in the dark for at least 30 min in ice. After 3 min of centrifugation, 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 c. 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 d. Leaf tissues were then rinsed three times in phosphate buffer (pH 7.2), post-fixed in a 1% buffered osmium tetroxide solution and then rinsed in phosphate buffer, rinsed three times in distilled water, and dehydrated in a five-step ascending ethyl alcohol series (25, 50, 75, 95, and 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 using a Leica Ultracut ultramicrotome R (Leica Microscopy and Scientific Instruments, Deerfield, IL, USA), 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) at 75 kV. Digital micrographs were taken on a BioScan/Digital Micrograph 2.5 (Gatan Inc., Pleasanton, CA, USA) 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, USA).

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

At week 8 of Stage II conditions, clusters of plantlets obtained from five vessels were taken out of the medium, washed, and packaged in aluminum foil envelopes. Every package contained three plantlets (sub-samples), which were collected from the same vessel. Samples were frozen in liquid N<sub>2</sub> and stored at -80°C prior to drying in a 10-MR-TR freeze drier (The Virtis Company, Gardiner, NY, USA) for 7 d. Dry weights of each sub-sample were then recorded. At weeks 3, 6, and 9 of Stage III conditions, all plantlets from five 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 from EK 11-1 and EK 16-3 sea oats genotypes were cultured for 8 wk under Stage II conditions, followed by 6 wk Stage III conditions and then transferred to Stage IV conditions for 4 wk. Five replicate samples consisting of three shoots or roots per genotype were collected at weeks 2 and 4 of Stage IV and processed as previously described.

Procedures for total soluble sugar (TSS) extraction were modified following the description by Boersig and Negrin (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

microliters of mannitol (10 mg ml<sup>-1</sup>) were added as an internal standard to each sample. One hour extractions were repeated two times 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 4000 rpm. The aqueous phase was removed and applied to polyethylene columns containing 3 ml of a 1:1 methanol:water (MW) solution (v/v) and cation and anion exchange resin (1 ml Amberlite IRA-67 layered with 1 ml Dowex 50-W, Sigma-Aldrich Co., St Louis, MO, USA). Soluble sugars were eluted and rinsed two times with 1:1 MW prior to complete evaporation using a RapidVap vacuum evaporator system (Labconco Corp., Kansas City, MO, USA). The dry residue was resuspended in 1 ml high-pressure liquid chromatography (HPLC)-grade water and filtered through a 0.45 µm membrane prior to HPLC injection. TSSs (sucrose, glucose, and fructose) were analyzed using a Waters 2695 High-Pressure Liquid Chromatograph (Waters Technological Corporation, Milford, MA, USA) equipped with a Waters 2414 refractive index detector (Waters Technological Corporation) and two connected BioRad Aminex HPX-87C columns (BioRad Laboratories, Hercules, CA, USA). Column and detector temperatures were maintained at 80 and 50°C, respectively. HPLC-grade water was used as the mobile phase, at a flow rate of 0.6 ml min<sup>-1</sup>.

For starch analysis, procedures were modified as described by Haissig and Dickson (1979) and Miller and Langhans (1989). The tissue residue (remaining in pipettes after soluble sugar extraction) was oven-dried overnight at 50°C, suspended in 4 ml Na-acetate buffer (100 mM, pH 4.5), and kept in a boiling water bath (90°C) for 30 min. After cooling to room temperature, 1.0 ml amyloglucosidase solution (from *Rhizopus* mold, Sigma-Aldrich Co.) [50 U per 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°C 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 U ml<sup>-1</sup>) and peroxidase (200 U ml<sup>-1</sup>). 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, USA) and starch content was calculated based on the regression equation of the glucose calibration line (0.0–1.0 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 TSS 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 general linear model procedures developed by Statistical Analysis System (SAS Institute Inc., 1999) and mean separation was evaluated using Waller–Duncan's test 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 wk, whereas EK 11-1 plants started senescing after 2-wk acclimatization, resulting in 29% survival after 6-wk *ex vitro* culture (Fig. 1A). Significant differences in  $P_n$  were also observed during initial plant growth *ex vitro*, with  $P_n$  values being greater for EK 16-3 than EK 11-1 plants (Fig. 1B). At week 0, *ex vitro*  $P_n$  for EK 11-1 was 1.9 µmol m<sup>-2</sup> s<sup>-1</sup>, which was significantly lower than  $P_n$  of EK 16-3 plants. Surviving EK 11-1 plants exhibited a significant increase of  $P_n$  over time that was not significantly different to the  $P_n$  of EK 16-3 plants after 5 wk *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 1-wk *ex vitro* culture, transpiration rates in both genotypes increased until after week 3, when they decreased (Fig. 1C).

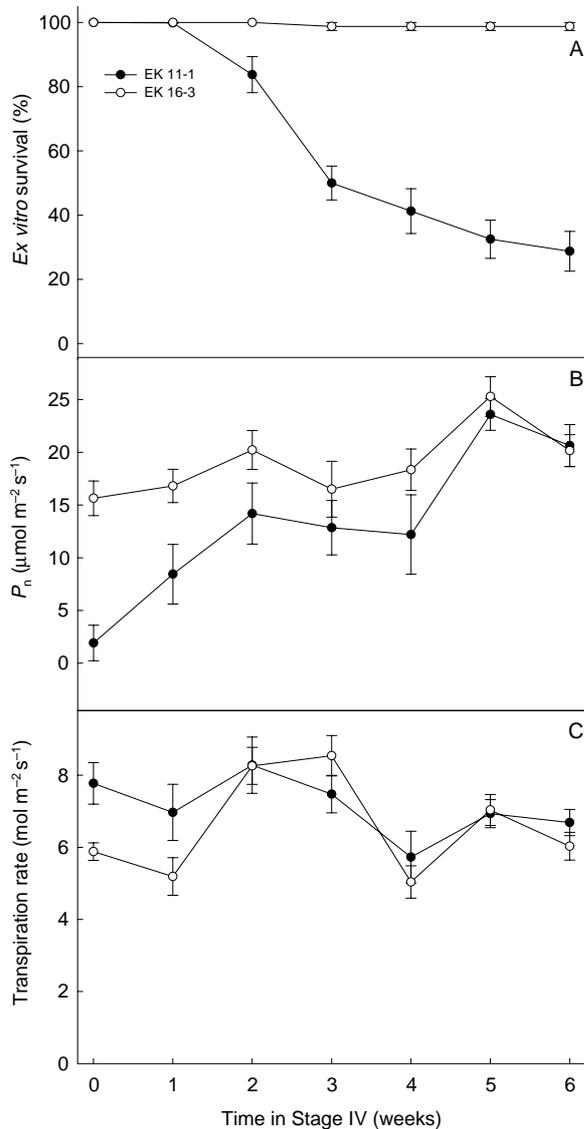


FIG. 1. Effect of *in vitro* culture conditions on A, *ex vitro* survival; B, *ex vitro* net photosynthetic rate per leaf area ( $P_n$ ); and C, *ex vitro* transpiration rate per leaf area, of EK 11-1 and EK 16-3 genotypes during Stage IV culture. Means  $\pm$  SE are shown ( $n = 10$ ).

**Carbohydrate status in vitro and ex vitro.** During weeks 3–9 of Stage III culture, 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 (Fig. 2A). After 6 wk in Stage III, both genotypes exhibited a remarkable decrease in starch content when transferred *ex vitro* to the greenhouse. By week 4 in 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 (Fig. 3) of bundle sheath cell chloroplasts indicated differences in the size and the distribution of starch grain chloroplasts with time and between genotypes. While large starch granules were found after 3 wk in Stage III, small starch grains were observed after 6 and 9 wk in Stage III. Chloroplasts in EK 11-1 plantlets after 3 wk in Stage III (Fig. 3A) exhibited larger numbers of plastoglobuli than in EK 16-3 (Fig. 3D). Furthermore

at the same time, thylakoid membranes of EK 11-1 chloroplasts were separated and appeared disrupted (Fig. 3A).

At 6 wk in Stage III, root starch content (Fig. 2B) was lower in both genotypes compared with shoot starch content (Fig. 2A), and it was not significantly different between genotypes at 6 and 9 wk in Stage III culture. Root starch content also decreased from 6 to 9 wk under Stage III culture conditions. Throughout Stage IV acclimatization, root starch content was similar among genotypes. At 2 wk in 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 weeks 0 to 3 and then gradually decreased from weeks 3 to 9 (Fig. 4A–C). At 0 wk in Stage III (after 8 wk in Stage II), there was significantly higher shoot sucrose content per dry weight in EK 16-3 than 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 wk in Stage III, at 6 wk, sucrose content was 21% greater in EK 11-1 shoots than EK 16-3 shoots. During Stage IV (after 6 wk in Stage III), shoot soluble sugars decreased from weeks 0 to 2, and then remained steady from weeks 2 to 4 (Fig. 4A–C). Furthermore, EK 16-3 plants exhibited significantly higher hexose and TSS contents than EK 11-1 plants after 2 and 4 wk *ex vitro*.

During Stage III, root soluble sugars (sucrose and hexose) decreased similarly among genotypes between weeks 6 and 9 (Fig. 5A–C). Sucrose, hexose, and TSS contents in roots were significantly higher for EK 11-1 than EK 16-3 plantlets, and decreased after 9 wk in Stage III in both genotypes (Fig. 5A–C). During Stage IV (after 6 wk in Stage III), root sucrose and hexose of both genotypes dramatically decreased within 2 wk, being nearly depleted during acclimatization.

**Chlorophyll and soluble protein contents.** Total chlorophyll content was relatively low ( $0.2 \text{ mg g}^{-1} \text{ FW}$ ) throughout Stage III (EK 11-1) and for the first 3 wk of Stage III culture (EK 16-3). Chlorophyll content of EK 16-3 then increased significantly after 6 and 9 wk in Stage III (Fig. 6A). During Stage IV (after 6 wk in 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 wk in Stage IV). Chlorophyll content was significantly greater for EK 16-3 than EK 11-1 plantlets after 2 and 4 wk in Stage IV. Conversely, EK 11-1-acclimatized plants (>6 wk 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 EK 11-1 plantlets after 6 and 9 wk *in vitro* (Fig. 6B). A rapid increase in TSP was observed in EK 16-3 plantlets during the first 4 wk after transfer to *ex vitro* conditions, with a significant decrease after plantlets were established *ex vitro* (>6 wk). 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 gram fresh weight over time and between genotypes (Fig. 7A). EK 16-3 plantlets exhibited higher activity of PEPC with time under Stage III conditions that was also significantly higher

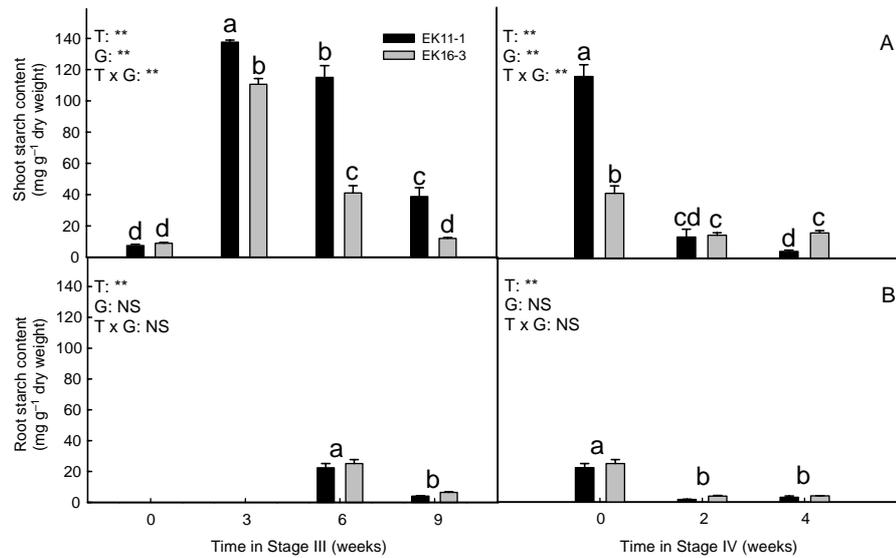


FIG. 2. A, Shoot starch content and B, root starch content of EK 11-1 and EK 16-3 genotypes during *in vitro* Stage III (left) and after microcuttings were rooted for 6 wk, Stage III and transferred to Stage IV (right). Error bars indicate SE ( $n = 10$ ). ANOVA analysis is shown on top left corner of each graph: T, time; G, genotype; NS, non-significant; \*\* significant at  $P \leq 0.01$ . Different letters on top of histobars within each culture stage are significantly different according to the Waller–Duncan test at  $P \leq 0.05$ .

than EK 11-1 plantlets. Both genotypes increased their PEPC activities following *ex vitro* transfer. Once the plantlets became established *ex vitro* (>6 wk), 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 (Fig. 7B). EK 11-1 plantlets exhibited a lower rubisco activity per gram fresh weight than EK 16-3 plantlets, which increased after 6 and 9 wk under Stage III conditions. After transferring plantlets to *ex vitro* conditions (following 6 wk Stage III), rubisco activity did not significantly increase in EK 11-1

plantlets until they were established *ex vitro* (>6 wk). Conversely, rubisco activity significantly increased in EK 16-3 plantlets after 2 wk *ex vitro* but it decreased after plants were established (>6 wk).

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* (Fig. 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 wk *in vitro*, but

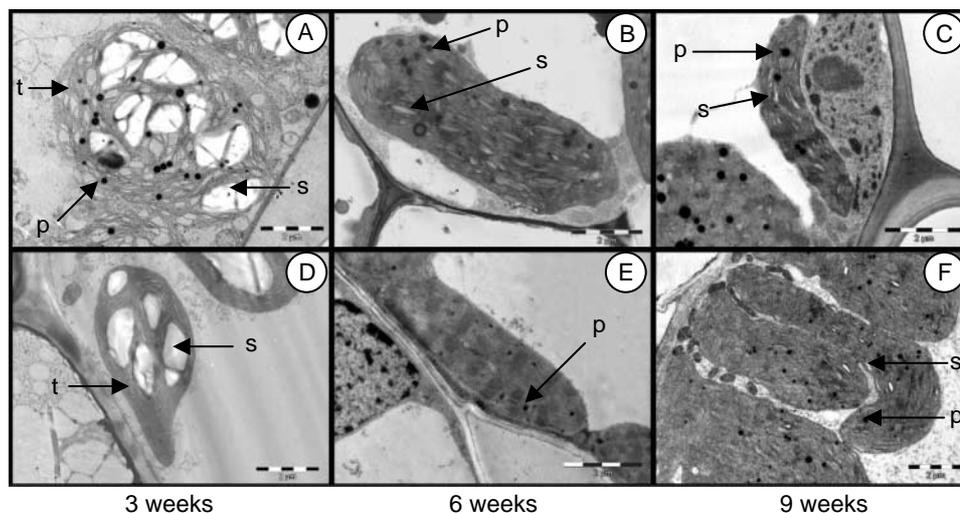


FIG. 3. Comparative TEM of chloroplasts of A–C, EK 11-1 and D–F, EK 16-3 genotypes after (A, D) 3 wk, (B, E) 6 wk, and (C, F) 9 wk Stage III culture conditions in bundle sheath cells. Bar = 2 μm. p, Plastoglobuli; s, starch granule; and t, thylakoid membranes.

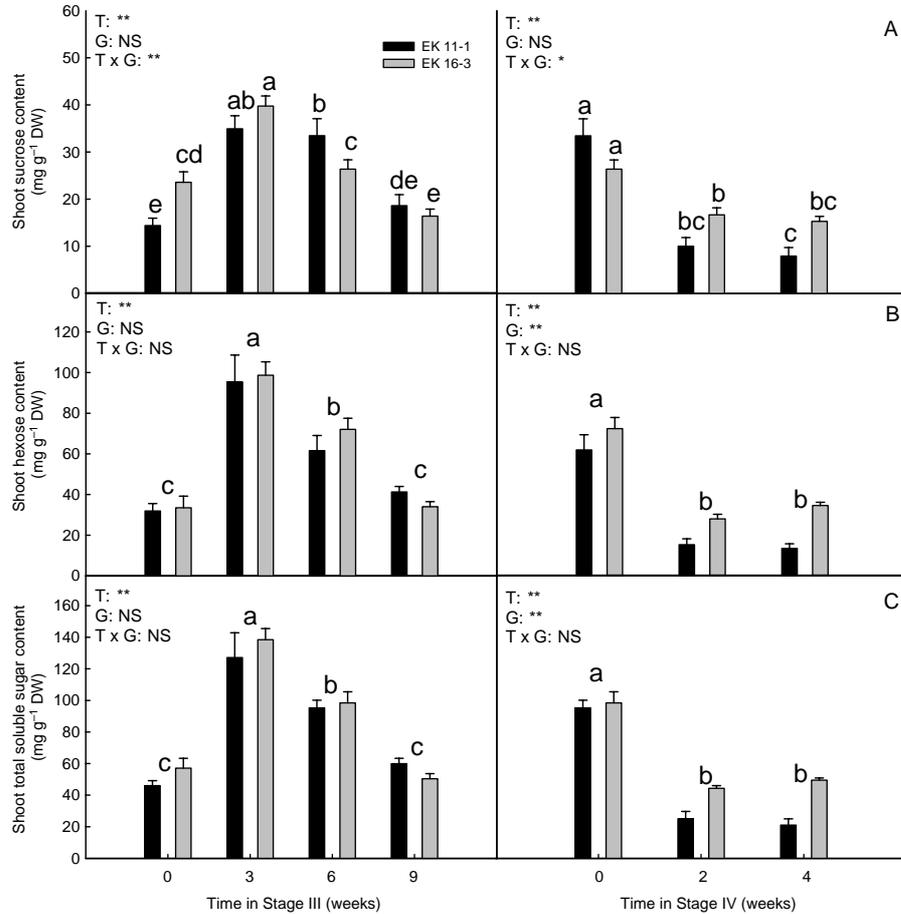


FIG. 4. A, Shoot sucrose content; B, shoot hexose content; and C, shoot total soluble sugar content of EK 11-1 and EK 16-3 genotypes during *in vitro* Stage III (left) and after microcuttings were rooted for 6 wk, Stage III and transferred to Stage IV (right). Error bars indicate SE ( $n = 10$ ). ANOVA analysis is shown on top left corner of each graph: T, time; G, genotype; NS, non-significant; \*,\*\* significant at  $P \leq 0.05$ , or 0.01, respectively. Different letters on top of histograms within each culture stage are significantly different according to the Waller–Duncan test at  $P \leq 0.05$ .

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 in weeks 6 and 9 of Stage III in EK 16-3 plantlets (Fig. 8A). 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 wk in 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 wk *in vitro* and during *ex vitro* acclimatization than EK 11-1 plantlets (Fig. 8B).

#### 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 having short blades with minimal blade expansion. 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, 1984) 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  $P_n$  in EK 16-3 leaves at time of *ex vitro* transfer was eight times greater than 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

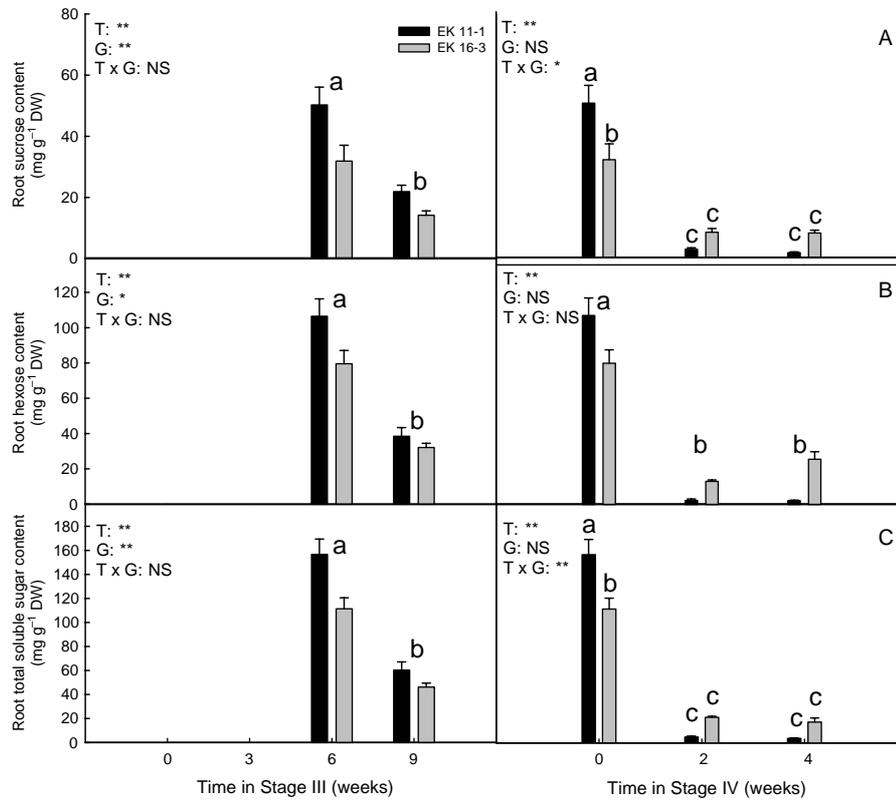


FIG. 5. A, Root sucrose content; B, root hexose content; and C, root total soluble sugar content of EK 11-1 and EK 16-3 sea oats genotypes during *in vitro* Stage III (left) and after microcuttings were rooted for 6 wk, Stage III and transferred to Stage IV (right). Error bars indicate SE ( $n = 10$ ). ANOVA analysis is shown on top left corner of each graph: T, time; G, genotype; NS, non-significant; \*,\*\* significant at  $P \leq 0.05$  or 0.01, respectively. Different letters on top of histograms within each culture stage are significantly different according to the Waller-Duncan test at  $P \leq 0.05$ .

system combined with the occurrence of abnormal stomate function. Transpiration rates measured after 6 wk *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*.

Starch accumulation and utilization in plant cells prior to the initiation and progression of developmental processes, such as shoot and root initiation, have been correlated (Swarnkar et al., 1986; Branca et al., 1994). The low initial starch levels in both sea oats genotypes, obtained from 8-wk-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 microcuttings were 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 wk *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 (Valero-Aracama, 2005).

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  $P_n$  in EK 11-1 plantlets during the time of transfer to *ex vitro* conditions.

Capellades et al. (1991) described an inverse relationship between starch content in shoot chloroplasts and photosynthetic

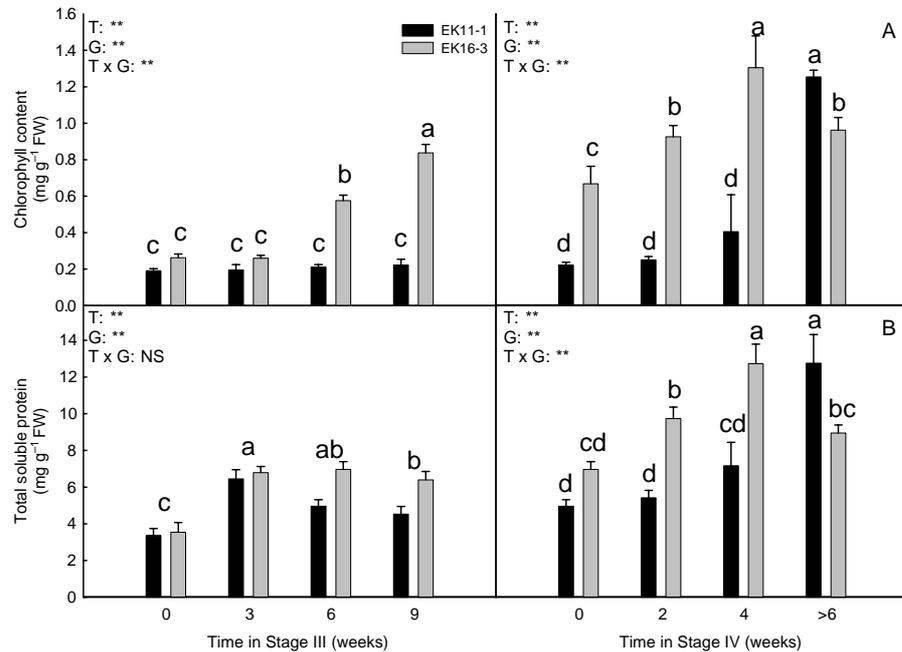


FIG. 6. A, Chlorophyll content per shoot gram fresh weight and B, TSP content per shoot gram fresh weight of EK 11-1 and EK 16-3 sea oats genotypes during *in vitro* Stage III (left) and after microcuttings were rooted for 6 wk, Stage III and transferred to Stage IV (right). Error bars indicate SE ( $n = 4$ ). ANOVA analysis is shown on top left corner of each graph: T, time; G, genotype; NS, non-significant; \*\*, significant at  $P \leq 0.01$ , respectively. Different letters on top of histograms and next to legend within each culture stage are significantly different according to the Waller–Duncan test at  $P \leq 0.05$ .

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 contents in leaves are high. At 3 wk in 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 Huylenbroeck and Debergh, 1996; Piqueras et al., 1998; Van Huylenbroeck et al., 1998; Fuentes et al., 2005). 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, the 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 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 those levels 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 wk *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  $\text{CO}_2$  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  $\text{C}_3$  species, such as strawberry (Hdider, 1994; Desjardins, 1995) and plantain (*Musa* AAB cv. CEMSA 3/4) (Aragón et al., 2005). 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,  $\text{C}_3$  plants exhibit increased respiration, resulting in increased PEPC activity (Hdider and Desjardins, 1994). Increased PEPC activity has also been reported in the  $\text{C}_4$  species *Flaveria bidentis*, when fed with

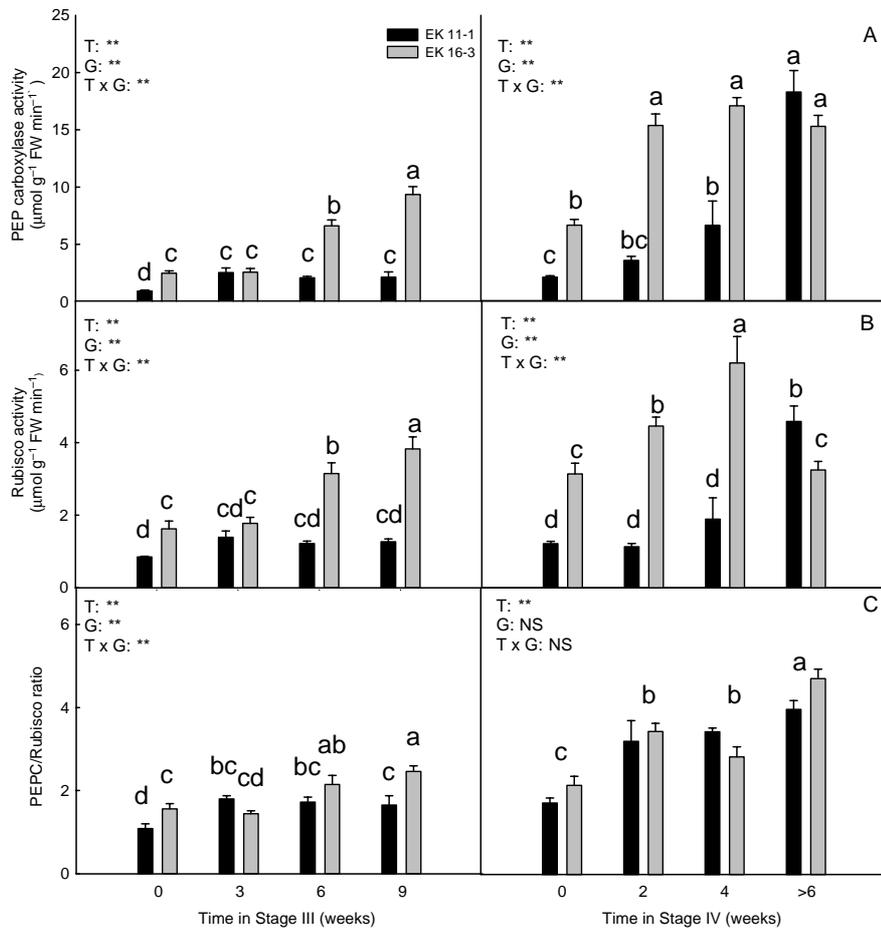


FIG. 7. A, Rubisco activity per shoot gram fresh weight; B, PEPC activity per shoot gram fresh weight; and C, PEPC/rubisco ratio of EK 11-1 and EK 16-3 sea oats genotypes during *in vitro* Stage III (left) and after microcuttings were rooted for 6 wk, Stage III and transferred to Stage IV (right). Error bars indicate SE ( $n = 4$ ). ANOVA analysis is shown on top left corner of each graph: T, time; G, genotype; NS, non-significant; \*\* significant at  $P \leq 0.01$ . Different letters on top of *histobars* within each culture stage are significantly different according to the Waller–Duncan test at  $P \leq 0.05$ .

146.1 mM exogenous sucrose in the culture medium (Furbank et al., 1997). In contrast, rubisco activity per TSP did not significantly change in either sea oats genotype after *ex vitro* transfer. Furthermore, decreased PEPC activity per TSP caused by *in vitro* conditions was observed in both genotypes. Similar results have been observed in plantain (Aragón et al., 2005). *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, the activities of PEPC and rubisco and the contents of chlorophyll and TSP were significantly greater in EK 16-3 than EK 11-1. Histological sections of *in vitro*-produced leaves of EK 11-1 and EK 16-3 (Valero-Aracama, 2005) indicated that after 6 wk in Stage III, EK 16-3 leaves exhibited more typical Kranz anatomy of  $C_4$  plants than EK 11-1 leaves. After 6 wk in 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 inefficiency 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  $C_4$  species

(Crafts-Brandner and Salvucci, 2002), photosynthesis was tolerant of relatively high environmental stress. Additionally, *in vitro* sugarcane leaves exhibited minimal Kranz anatomy compared with the more typical Kranz anatomy exhibited by *ex vitro* leaves (Rodríguez et al., 2003).

This increase in enzymatic activity, chlorophyll and soluble protein content after 6 wk in Stage III correlates with the initial decrease of starch and soluble carbohydrates in shoots of both genotypes during the same time period, and with the formation of the first fully expanded leaves with typical Kranz anatomy in EK 16-3 (Valero-Aracama, 2005). These increased levels of chlorophyll and TSP content and increased enzymatic activities are indicative of the development of photosynthetic characteristics in EK 16-3 after 6 wk in Stage III. Furthermore, EK 16-3 plantlets transferred *ex vitro* after 6 wk in Stage III exhibited 100% survival. Conversely, EK 11-1 plants exhibited limited leaf elongation and expansion during Stage III culture (Valero-Aracama, 2005), which correlated with lower PEPC and rubisco activities, and low chlorophyll and TSP contents *in vitro* compared to EK 16-3 plants. This limited development of photosynthetic features *in vitro* resulted in 30% survival after 6 wk *ex vitro* conditions.

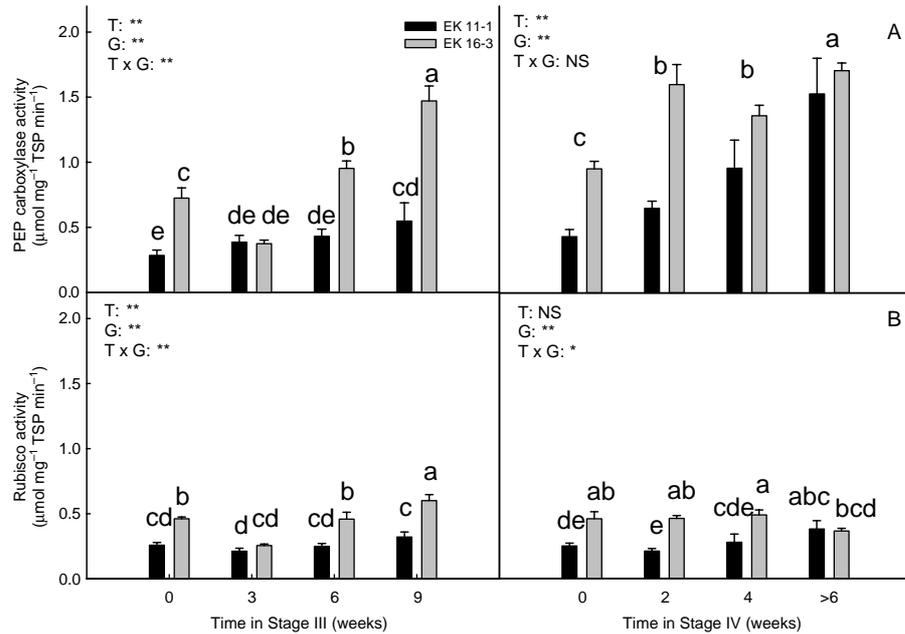


FIG. 8. A, PEP activity per shoot milligram total soluble protein (TSP) and B, rubisco activity per shoot milligram TSP of EK 11-1 and EK 16-3 sea oats genotypes during *in vitro* Stage III (left) and after microcuttings were rooted for 6 wk, Stage III and transferred to Stage IV (right). Error bars indicate SE ( $n = 4$ ). ANOVA analysis is shown on top left corner of each graph: T, time; G, genotype; NS, non-significant; \*,\*\* significant at  $P \leq 0.05$  or  $P \leq 0.01$ , respectively. Different letters on top of histograms and next to legend within each culture stage are significantly different according to the Waller-Duncan test at  $P \leq 0.05$ .

## CONCLUSIONS

Lower photosynthetic capacity ( $P_n$ ) 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 3 wk following transfer to Stage III medium in both genotypes. These carbohydrates were used for root and shoot initiation and growth during the later Stage III culture period. Carbohydrate reserve utilization in EK 16-3 during the late Stage III culture period was greater than EK 11-1, and resulted in lower starch and soluble sugar reserves than EK 11-1 at the time of *ex vitro* transfer. After 6 wk in Stage III, there were significantly greater increases in PEPC and rubisco activities, and in chlorophyll and TSP contents 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 the greenhouse-produced leaves (Valero-Aracama, 2005).

Stage III *in vitro* culture conditions differently affected the growth and development of photosynthetic capacity 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 to feed 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.

## ACKNOWLEDGMENTS

This project was developed under the guidance of the Florida Sea Grant College Program and funded from NOAA, Department of Commerce, Grant No. NA16RG-2195. The authors would like to thank Carolyn Bartuska for her statistical expertise, Chris Wilson for his HPLC instrumentation, and Karen Kelley and Fred Bennett in the Electron Microscopy Core Laboratory at the UF, for their guidance and assistance in histological studies.

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