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MICROPROPAGATION

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

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Abstract Net photosynthetic rates (P_n) of easy (EK 16-3) and difficult-to-acclimatize (EK 11-1) sea oats genotypes were examined under the following culture conditions: (1) photoautotrophic [sugar-free medium, high photosynthetic photon flux (PPF), high vessel ventilation rates and CO_2 enrichment, (PA)]; (2) modified photomixotrophic [sugar-containing medium diluted with sugar-free medium over time, high PPF, and high vessel ventilation rates (PM)]; (3) modified photomixotrophic enriched [same as PM with CO_2 enrichment, (PME)]; or (4) conventional photomixotrophic [sugar-containing medium, low PPF, and low vessel ventilation rates (control)]. Regardless of genotype, plantlets cultured under PA conditions died within 2 wk, whereas under PM and PME conditions, plantlets increased their P_n. After 6 wk, P_n per gram dry weight was 1.7 times greater in EK 16-3 than EK 11-1 plantlets cultured under PME conditions. *In vitro*-produced leaves of EK 16-3 plantlets were elongated with expanded blades, whereas EK 11-1 produced short leaves without expanded blades, especially under control conditions. After *in vitro* culture, EK 16-3 PME plantlets exhibited the highest dry weights among treatments. EK 16-3 PME and EK 16-3 PM had similarly high survivability, shoot and root dry weights and leaf lengths *ex vitro* compared to EK 16-3 control and EK 11-1 PM and PME plantlets. *Ex vitro* growth, survivability and P_n per leaf area of either genotype were not affected by CO_2 enrichment under modified photomixotrophic conditions. These results suggest that growth and survivability of sea oats genotypes with different acclimatization capacities can be enhanced by optimizing culture conditions.

Keywords CO_2 enrichment · Environmental control · Photoautotrophic · Photomixotrophic · Net photosynthetic rate · Sucrose · Micropropagation

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Introduction

The coastal beach and dune systems of the southeastern United States are frequently eroded by severe storms and hurricanes. Sea oats (*Uniola paniculata* L.) is the dominant native dune grass used for dune restoration and stabilization in the southeastern United States (Woodhouse, 1982). Sea oats are 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 rate 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., 1991; Seon et al., 2000; Mosaleeyanon et al., 2004). 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 *in vitro* 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, 1991; 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, 1991). 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 (*Coffea arabusta* Capot & Aké Assi) (Nguyen et al., 1999).

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 threefold greater, and the CO_2 compensation point and CO_2 saturation point are lower (<10 and <1,000 μmol mol^{-1}, respectively) when both are exposed to high light levels and warm temperatures at ambient CO_2 levels (Salisbury and Ross, 1998). 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 on plants 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 (1) 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 (2) 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 *in vitro* (Philman and Kane, 1994) and genotyped using randomized amplified polymorphic DNA (RAPD) genetic analyses (Ranamukhaarachchi, 2000), 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} TC™ agar (*Phyto*Technology Laboratories, Shawnee Mission, KS). All media were adjusted to pH 5.7 with 0.1N KOH before the addition of agar and autoclaving at 117.7 kPa and 121°C for 21 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 μmol m^{-2} s^{-1} PPF as measured at culture level. After 8 wk, 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 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 μM thiamine-HCl, and 10 μM α-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} TC™ agar. Each treatment consisted of nine replicate Magenta GA7 vessels, each containing eight single microcuttings.

Environmental conditions of all treatments are described in Table 1. A 16-h photoperiod was provided by cool-white fluorescent lamps (F72T12/CW/VHO, Osram Sylvania,

Table 1. Environmental conditions during stage III rooting for EK 11-1 and EK 16-3 sea oats genotypes

Treatment	Photosynthetic photon flux ($\mu mol\ m^{-2}\ s^{-1}$)			Relative humidity (%)	Air temperature (°C)	CO_2 concentration ($\mu mol\ mol^{-1}$)	Number air exchanges (h^{-1})	
	Days 1–3	Days 4–10	Days 11–42				Days 1–3	Days 4–42
PA[z]	200	300	400	80±5	25±1	1,500	4.4	8.1
PME	200	300	400	80±5	25±1	1,500	4.4	8.1
PM	200	300	400	80±5	25±1	400	4.4	8.1
Control	100	100	100	50±5	22±2	400	0.2	0.2

[z] *PA* Photoautotrophic, *PME* modified photomixotrophic enriched, *PM* modified photomixotrophic, *control* conventional photomixotrophic.

Danvers, MA) for all treatments. Photoautotrophic and PME vessels were placed in a CO_2-enriched growth chamber (1,500 μmol mol^{-1}), whereas control and PM vessels were placed in ambient CO_2 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 wk as needed with sucrose-free medium.

At the end of Stage III culture, a high pressure liquid chromatograph (HPLC) (Waters 2695, Waters Technological Corporation, Milford, MA) equipped with a Waters 2414 refractive index detector and a BioRad Aminex HPX-87C column (BioRad Laboratories, Hercules, CA) was used to measure the final concentration of sucrose in the medium, to determine whether plantlets were photoautotrophic before *ex vitro* transfer. Column and detector temperatures were maintained at 80°C and 50°C, 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 wk in 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 two gas samples per vessel obtained from five 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 2 wk starting the day after inoculation using a sanitized syringe from a hole in the vessel lid. CO_2 concentrations (μmol mol^{-1}) inside and outside the vessels (C_{in} and C_{out}, respectively) were determined and P_{np} was calculated using the equation described by Fujiwara et al. (1987):

$$P_{np} = k\,E\,V\,(C_{in} - C_{out})n^{-1}$$

where k is a conversion factor of CO_2 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 four vessels per treatment to calculate photosynthetic rates, and dry weights were obtained to calculate photosynthetic rates per gram dry weight (P_{nw}).

Ex vitro greenhouse conditions, growth, survival, photosynthetic rates and transpiration rates. After 6 wk in Stage III, plantlets cultured in sucrose containing media (PM, PME and control) had rooted (Fig. 1*B*, *C*, *D*) and were subsequently transferred to the greenhouse to measure photosynthetic rates during acclimatization. Plantlets obtained from five vessels per treatment were transferred *ex vitro* into eight-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 PPF of 1,000± 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_{nl}) were taken from 10 replicate plantlets per treatment every 3 wk at midday starting the day after establishment *ex vitro* on newly formed and fully expanded leaves. Measurements were taken using a portable photosynthesis measuring system (CIRAS-1, PP System Co., Ltd., UK) with inlet CO_2 concentration fixed at 400±10 μmol mol^{-1} and no supplemental light source. After 6 wk, the longest leaves of surviving plants were

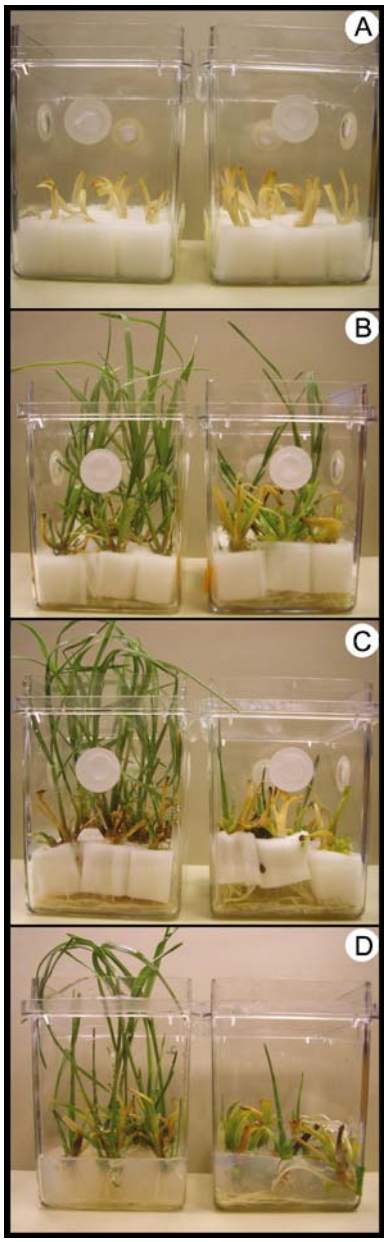

Figure 1. Six-week-old *in vitro* cultures of EK 16-3 (*left*) and EK 11-1 (*right*) sea oats genotypes under (*A*) photoautotrophic (PA), (*B*) modified photomixotrophic enriched (PME), (*C*) modified photomixotrophic (PM), and (*D*) conventional photomixotrophic (control) culture conditions.

measured, plants were destructively harvested, divided into shoots and roots, and dried at 70°C for 1 wk.

Experimental design and statistical analysis. The *in vitro* growth chamber study was a split plot design, with cultural conditions as the whole plot (four levels) and genotypes as the subplots (two levels). The *ex vitro* greenhouse study was a complete randomized 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 (Steel and Torrie, 1981) at $P \leq 0.05$.

Results

In vitro survival and growth. All shoots initially cultured in media without sucrose (PA) died during the first 2 wk of the experiment (Fig. 1*A*). After 6 wk *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 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. In addition, 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 (Fig. 1).

In vitro net photosynthetic rate. At week 0, EK 11-1 and EK 16-3 control shoots and EK 11-1 PM shoots exhibited negative P_{nw} (Table 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 wk *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 (Fig. 2). At 2 wk, plantlets from both genotypes under non-enriched CO_2 conditions (PM and control), exhibited P_{np} near zero, which was significantly lower than P_{np} in PME plantlets. At 4 wk, PM and PME conditions increased P_{np} of plantlets in both genotypes. At 6 wk, EK 16-3 PME plantlets exhibited significantly higher P_{np} than the other treatments. Control plantlets of both genotypes continued to

Table 2. Growth of EK 11-1 and EK 16-3 sea oats genotypes after 6 wk *in vitro*

Treatment	Shoot dry weight (mg)		Root dry weight (mg)	
	EK 11-1	EK 16-3	EK 11-1	EK 16-3
PA[z]	–[a]	–	–	–
PME	136±7bc[b]	180±8a	50±4b	77±4a
PM	127±6cd	150±11b	52±4b	52±3b
Control	134±6bc	111±6d	33±5c	36±3c
Analysis of variance[c]				
Genotype (*G*)	*		**	
Treatment (*T*)	**		**	
G×*T*	**		**	

[z] *PA* photoautotrophic, *PME* modified photomixotrophic enriched with CO_2, *PM* modified photomixotrophic, *control* conventional photomixotrophic.
[a] Indicates that shoots died.
[b] Means±SE followed by different letters in each column are significantly different according to Waller–Duncan test at $P \leq 0.05$ ($n = 32$).
[c] *, **Significant at $P \leq 0.05$ or 0.01, respectively.

exhibit negative or near zero P_{np} throughout the *in vitro* culture period.

Ex vitro survival, transpiration, photosynthesis, and growth. EK 11-1 control plantlets had a negative P_{nw} after 6 wk *in vitro* (Table 3) and consequently exhibited the lowest survival (25%) among treatments after 6 wk *ex vitro* acclimatization (Fig. 3*A*). 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 (Fig. 3*B*). These differences in transpiration rates were less pronounced after 3 wk and were similar among treatments after 6 wk for both genotypes. EK 11-1 control plantlets exhibited the lowest P_{nl} among all measured treatments (Fig. 3*C*) at week 0 *ex vitro*. Three weeks later, P_{nl} increased in all treatments,

Table 3. Net photosynthetic rate per dry weight (P_{nw}) during *in vitro* rooting of EK 11-1 and EK 16-3 sea oats genotypes under four culture conditions

Treatment	P_{nw} (μmol h^{-1} g^{-1})				
	Week 0		$\bar{\chi}$	Week 6	
	EK 11-1	EK 16-3		EK 11-1	EK 16-3
PA[z]	70±9[a]	96±30	83±20a	–[b]	–
PME	20±9	66±7	43±8b	307±50b	532±45a
PM	−9±13	5±4	−2±9c	210±17c	240±25bc
Control	−38±9	−25±3	−31±6d	−14±1d	5±2d
$\bar{\chi}$	11±10B	36±11A			
Analysis of variance[c]					
Genotype (*G*)	*			**	
Treatment (*T*)	**			**	
G×*T*	NS			**	

[z] *PA* Photoautotrophic, *PME* modified photomixotrophic enriched with CO_2, *PM* modified photomixotrophic, *control* conventional photomixotrophic
[a] 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 $P \leq 0.05$ ($n = 4$).
[b] Indicates that plants died.
[c] NS, *, **Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively, within each measurement period (week 0 or 6).

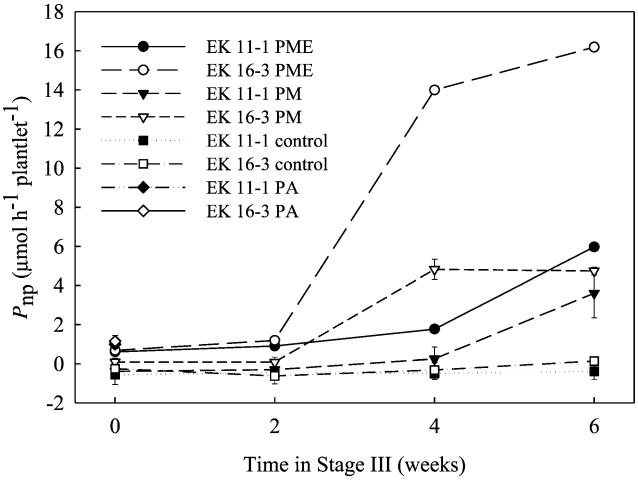

Figure 2. Effect of *in vitro* culture conditions on net photosynthetic rate per plant (P_{np}) of EK 11-1 and EK 16-3 genotypes during Stage III culture. Means±SE are shown (n = 5) when larger than the symbol. *In vitro* culture conditions were: photoautotrophic (PA), modified photomixotrophic enriched (PME), modified photomixotrophic (PM), and conventional photomixotrophic (control) culture conditions.

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_{nl} among treatments after 6 wk *ex vitro*.

After 6 wk *ex vitro*, shoot and root dry weights of plantlets cultured under PME and PM conditions were higher than under control conditions for both genotypes (Table 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 leaves *ex vitro* than control plantlets of either genotype.

Regardless of genotype, modification of photomixotrophic conditions with CO_2 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* CO_2 enrichment. Although *in vitro* CO_2 enrichment decreased transpiration rates of EK 16-3 plantlets after transfer, transpiration rates of EK 11-1 plantlets were not affected.

Discussion

In vitro survival, growth and photosynthetic rates. Numerous investigations using C_3 plants have indicated that use of sucrose-free medium with high PPF and CO_2 enrichment enhanced plant growth (Kozai et al., 1987; Cournac et al., 1991; Fujiwara et al., 1992; Buddenhorf-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 wk of 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 require an initial source of carbon from the

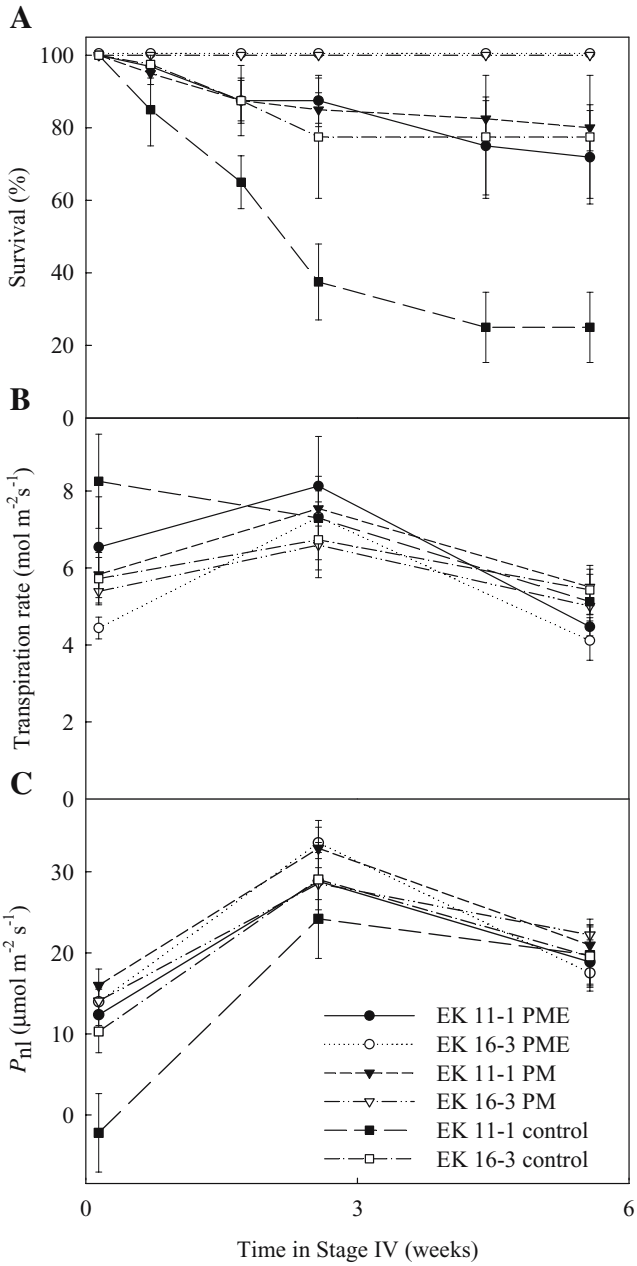

Figure 3. Effect of *in vitro* culture conditions on (*A*) *ex vitro* survival (n = 5); (*B*) *ex vitro* transpiration rate per leaf area (n = 10), and (*C*) *ex vitro* net photosynthetic rate per leaf area (P_{nl}) (n = 10) of EK 11-1 and EK 16-3 genotypes during Stage IV culture. Means±SE are shown when larger than the symbol. *In vitro* culture conditions were: modified photomixotrophic enriched (PME), modified photomixotrophic (PM), and conventional photomixotrophic (control) culture conditions.

Table 4. *Ex vitro* growth of EK 11-1 and EK 16-3 sea oats genotypes cultured for 6 wk under four *in vitro* culture conditions

Treatment	Shoot dry weight (mg)			Root dry weight (mg)			Longest leaf length (mm)	
	EK 11-1	EK 16-3	$\bar{\chi}$	EK 11-1	EK 16-3	$\bar{\chi}$	EK 11-1	EK 16-3
PA[z]	–[a]	–		–	–		–	–
PME	477±41[b]	747±30	653±29a	132 aB	257 aA	211±11a	326±18b	390±12a
PM	528±34	750±24	651±24a	152 aB	257 aA	210±10a	377±15a	399±9a
Control	147±22	461±39	384±37b	45 bB	131 bA	110±10b	232±25c	327±12b
$\bar{\chi}$	451±28B	669±21A						
Analysis of variance[c]								
Genotype (*G*)	**			**			**	
Treatment (*T*)	**			**			**	
G×*T*	NS			NS			*	

[z] *PA* Photoautotrophic, *PME* modified photomixotrophic enriched with CO_2, *PM* modified photomixotrophic, *control* conventional photomixotrophic.
[a] Indicates that plants died.
[b] 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 to 40).
[c] NS, *, **Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively.

medium until they are capable of using CO_2 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 and 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 wk *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 toward production of photosynthetically competent leaves, whereas EK 11-1 plantlets used it for root elongation. The increasing P_{np} over time of EK 16-3 plantlets 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} was observed using C_3 species such as Rosa (*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 and to the increase in shoot and root growth (Table 2), as described by Mosaleeyanon et al. (2004) with rain tree.

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 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., 2004). Furthermore, CO_2 enrichment only increased *in vitro* biomass of EK 16-3 plantlets (Table 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.

Ex vitro acclimatization. EK 16-3 control plantlets exhibited 77.5% survival after 6 wk in the greenhouse although P_{nw} after 6 wk *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 d of acclimatization. EK 16-3 plantlets displayed lower transpiration rates than EK 11-1 plantlets, indicating better regulation of water loss during acclimatization. The comparative anatomical study of sea oats genotypes cultured *in vitro* showed greater cuticle development in EK 16-3 than EK 11-1 plantlets (Valero-Aracama et al., 2004). Sutter and Langhans (1979) observed a lack of epicuticular wax formation on *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*. We have observed this response in previous studies (Valero-Aracama et al., 2006).

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_{nl} *ex vitro*. By using *in vitro*-modified photomixotrophic conditions rather than conventional conditions, we were able to increase P_{nl} in EK 11-1 plantlets and significantly increase survival *ex vitro* (Fig. 3). Modified photomixotrophic conditions, including an increase in ventilation rates of vessels and CO_2 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 wk *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 compared to 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 of CO_2 enrichment under PME conditions on *in vitro* P_{nw} of both genotypes and on *in vitro* growth of EK 16-3 plantlets, no residual effect of *in vitro* CO_2 enrichment was observed for *ex vitro* survival, P_{nl}, or growth in either genotype. Natural ventilation under ambient CO_2 allowed sea oats plants to develop photosynthetic competence to levels comparable to plants under CO_2-enriched conditions. Ambient levels of CO_2 during acclimatization, regardless of *in vitro* CO_2 pretreatment, allowed similar rates of growth and development in both genotypes.

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 wk (Werbrouk et al., 1995; Werbrouk 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, BA is the only effective cytokinin for sea oats 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*.

Conclusion

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, CO_2 enrichment did not significantly enhance survival and growth of sea oats genotypes *ex vitro*, indicating that under modified photomixotrophic conditions without CO_2 enrichment, the increase in vessel ventilation rates allowed sufficient CO_2 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) benefited from photoautotrophic micropropagation in that plantlet growth and development

of photoautotrophy were enhanced during *in vitro* culture. Yet, unlike C_3 species, sea oats did not require CO_2 enrichment *in vitro* to obtain high survival rates and similar growth and development to plants exposed to ambient CO_2 conditions *in vitro*.

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