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Substitution of benzyladenine with *meta*-topolin during shoot multiplication increases acclimatization of difficult- and easy-to-acclimatize sea oats (*Uniola paniculata* L.) genotypes

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Abstract Benzyladenine (BA) is the only cytokinin to effectively induce shoot multiplication in vitro between genotypes of the important dune grass species *Uniola paniculata* (sea oats). However, a significant genotype-specific negative carryover effect of BA on ex vitro acclimatization has been observed. In the present study, the effects of multiplication media supplemented with *meta*-topolin (*m*T), a BA-analog, BA or no plant growth regulator, were compared on in vitro multiplication, rooting and ex vitro acclimatization using easy- and difficult-to-acclimatize sea oats genotypes. Both genotypes exhibited similar in vitro shoot dry weight, number of harvestable shoots and percent rooting when cultured under standard conditions (with 2.2 μM BA) or with an equimolar concentration of *m*T. In addition, both genotypes exhibited similar ex vitro leaf length and shoot production under these two culture conditions. However, ex vitro acclimatization of rooted microcuttings of the difficult-to-acclimatize genotype significantly increased when produced on shoot multiplication medium containing *m*T rather than BA. *Meta*-topolin concentrations 10 μM or greater were inhibitory to in vitro rooting and acclimatization ex vitro of both genotypes. Nevertheless, survival of the difficult-to-acclimatize genotype was significantly greater when cultured in the presence of 2.2 μM–30 μM *m*T, compared to 2.2 μM

BA. Therefore, a potential solution to overcome the detrimental BA carryover effect on ex vitro survival in sea oats is the substitution of BA with 2.2 μM *m*T for Stage II shoot multiplication. Use of *m*T may provide an efficient method to ensure in vitro propagation of a large number of diverse sea oats genotypes for dune restoration.

Keywords Cytokinin · Micropropagation · Dune restoration · BA carryover effect

Introduction

The coastal dunes of the southeastern United States of America are frequently eroded by tropical storms and hurricanes. Sea oats (*Uniola paniculata* L.), a dune species native to the southeastern United States of America, is commonly used for dune stabilization and restoration (Woodhouse 1982). Sea oats is commercially propagated from field-collected seed from donor populations. However, this perennial grass species is often not a prolific seed producer (Burgess et al. 2002) and recent destruction of seed donor populations from hurricanes and tropical storms has limited seed sources. A sea oats micropropagation protocol was developed to supplement seed propagation (Philman and Kane 1994). However, this protocol was optimized using only a single genotype. When attempts were made to acclimatize unrooted or rooted microcuttings of numerous genotypes to ex vitro conditions, significant variability in survival rates among genotypes was encountered (Kane et al. 2006). For the ecological purpose of maintaining genetic diversity and achieving cost-effective commercial micropropagation, the efficient in vitro production and ex vitro acclimatization of a large number of sea oats genotypes is essential.

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Benzyladenine (BA), the most widely used cytokinin incorporated into culture media to promote axillary shoot production, often has deleterious effects on subsequent rooting and ex vitro acclimatization (Pospíšilová et al. 1992; Werbrouck et al. 1995). Currently, BA is the only effective cytokinin that promotes in vitro shoot production of sea oats (Philman and Kane 1994). Werbrouck et al. (1995) correlated reductions in rooting and acclimatization of Spathiphyllum floribundum (peace lily) shoots produced on BA-supplemented medium with the accumulation of the BA metabolite 9-B-glucopyransosyl-benzyladenine (9G-BA) at the base of plantlets in vitro. Hydroxylated analogs of BA have been proposed as alternative cytokinin substitutes to BA in plant tissue systems (Werbrouck et al. 1996; Strnad 1997). Shoot multiplication, rooting and acclimatization can be increased by substituting BA with N^6-(3-hydroxybenzyl)adenine (meta-topolin, mT), a naturally-occurring BA analog in which the primary mT metabolite, although also deleterious, degrades more rapidly during acclimatization (Werbrouck et al. 1996; Strnad et al. 1997). More recently, mT has been shown to be the preferred cytokinin over BA for shoot multiplication, enhanced rooting, reduced hyperhydricity, and enhanced acclimatization ex vitro of Aloe polyphylla (Bairu et al. 2007). Given these reports, the possibility that mT might be an acceptable BA substitute to ensure reliable in vitro multiplication and ex vitro acclimatization of sea oats genotypes was explored.

Materials and methods

Stage II shoot multiplication comparison

Five-year-old stabilized and indexed in vitro shoot cultures of sea oats (Uniola paniculata L.) genotypes, originally established from surface sterilized tillers collected from individual mature plants from Egmont Key, Florida, were clonally propagated at 4 week subculture intervals. Magenta GA7 vessels (Magenta Corp., Chicago, IL) were used, containing 80 ml sterile medium consisting of Murashige and Skoog (MS) inorganic salts (Murashige and Skoog 1962), 87.6 mM sucrose, 0.56 mM myo-inositol, and 1.2 μM, and thiamine-HCl, supplemented with 2.2 μM N^6-benzyladenine (BA). Each donor plant was genotyped using random amplified polymorphic DNA (RAPD) genetic analyses (Ranamukhaarachchi 2000) prior to in vitro establishment. Two genotypes, previously characterized as easy- and difficult-to-acclimatize (EK 16-3 and EK 11-1, respectively), were used (Valero-Aracama et al. 2007). To minimize carryover effects, shoot clusters of both genotypes were cultured on basal MS medium without plant growth regulators (Stage II medium without BA) for

6 weeks prior to experimentation. 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 shoot multiplication media. These media consisted of MS inorganic salts, supplemented with 87.6 mM sucrose, 0.56 mM myo-inositol, 1.2 μM thiamine-HCl, and six levels of plant growth regulators (PGR): 0 μM PGR, 2.2 μM N^6-benzyladenine (BA), and 2.2, 10, 20 and 30 μM 6-(3-hydroxybenzylamino)purine (meta-topolin; mT). All media were adjusted to pH 5.7 with 0.1 N KOH prior to the addition of 8 g L^{-1} TC^{TM} agar (PhytoTechnology Laboratories, Shawnee Mission, KS) and autoclaving at 117.7 kPa and 121°C for 20 min.

Cultures were maintained for 4 weeks in a growth chamber at 24 ± 1°C, 58 ± 5% relative humidity (RH), 16-h photoperiod was provided by cool-white fluorescent lamps (General Electric F20WT12·CW) at 40 ± 5 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF) as measured at culture level. Leaf number and shoot cluster dry weight from 5 replicate vessels per genotype were measured after each culture period. Based on previous data (not shown), production of rootable microcuttings ≥30 mm long were considered as harvestable shoots.

Stage III rooting and ex vitro acclimatization

In a concurrent experiment, shoot clusters of EK 11-1 and EK 16-3 genotypes were cultured in Stage II for 4 weeks under the same treatment conditions described above. Subsequently, the shoot clusters of each genotype were subdivided into single microcuttings and transferred into GA7 culture vessels containing 80 ml sterile Stage III rooting medium consisting of half-strength MS inorganic salts, 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 solidified with 8 g l^{-1} TC^{TM} agar. For both genotypes, five replicate culture vessels, each containing 8 single microcuttings 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. Rooting percentage data were recorded after 6 weeks Stage III culture.

After 6 weeks, rooted microcuttings were rinsed to remove residual media and then transplanted into coarse vermiculite contained in five replicate six-celled blocks consisting of 4 × 6 × 5.5 cm cells (T.O. Plastics, Inc., Clearwater, MN) before being transferred to Stage IV conditions. Plantlets were hand watered as needed, and fertilized weekly at 150 mg N l^{-1} using 20 N-8.7P-16.7 K liquid fertilizer (Peters; The Scotts Company, Marysville, OH). Greenhouse set points for cooling and heating were 24

and 22°C, respectively, and natural solar PPF ranged from 900-1200 μmol m^{-2} s^{-1} at noon. Survival percentage was monitored weekly, and after 6 week of 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* tests for significant differences among or between means were made using the Waller-Duncan procedure at $P \leq 0.05$.

Results

In Vitro shoot multiplication comparisons

During Stage II multiplication, shoot dry weights varied among treatments and between genotypes (Fig. 1). Lowest shoot dry weight accumulation was observed in shoot clusters cultured in medium containing no PGR followed by 30 μM *m*T. 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 μM *m*T, which were similar between genotypes.

Shoot production in both genotypes was similar in medium supplemented with either 2.2 μM BA or *m*T (Figs. 2, 3a, b). Shoot production was significantly higher
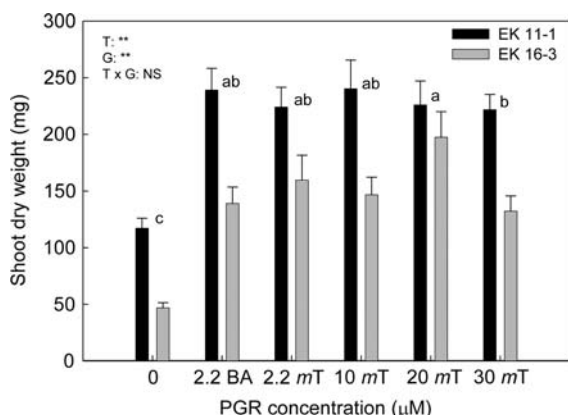

Fig. 2 Effect of cytokinin type and concentration after 4 weeks Stage II culture on number of harvestable shoots (≥ 30 mm) of EK 11-1 (difficult-to-acclimatize) and EK 16-3 (easy-to-acclimatize) sea oats genotypes. *Error bars* indicate SE ($n = 5$). BA: N^6-benzyladenine; *m*T: 6-(3-hydroxybenzylamino)purine. ANOVA analysis is shown on top *left* corner of each graph; *T* Treatment, *G* Genotype, NS, **: Non-significant or significant at $P \leq 0.01$, respectively. *Different letters* on top of *histobars* are significantly different according to Waller-Duncan test at $P \leq 0.05$
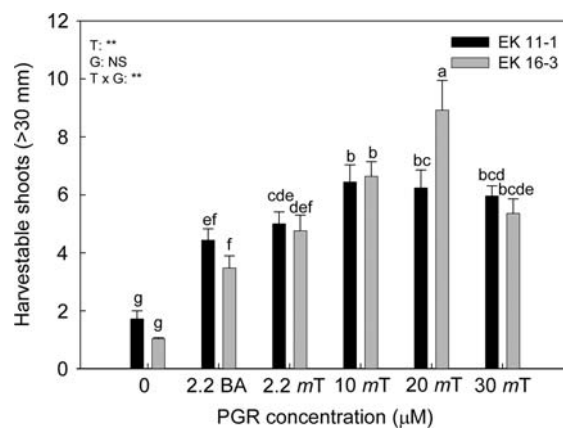
in medium supplemented with greater than 2.2 μM *m*T. Greatest harvestable shoot production was observed on EK 16-3 plantlets in the presence of 20 μM *m*T. Regardless of genotype, the lowest number of harvestable shoots was obtained when no PGR was supplemented to the medium.

Carryover effects on in vitro rooting

A negative carryover effect of *m*T concentration during Stage II multiplication on subsequent Stage III microcutting rooting was only observed when microcuttings of either genotype had been previously cultured with concentrations greater than 2.2 μM *m*T (Figs. 4, 3c, d). In contrast, high Stage III rooting percentages were observed when microcuttings were previously generated on Stage II medium without PGR or with either 2.2 μM BA or 2.2 μM *m*T (Figs. 4, 3c, d).

Carryover effects on ex vitro acclimatization

Rooted microcuttings of both genotypes propagated on Stage II medium devoid of BA or *m*T, exhibited the highest ex vitro acclimatization after 6 weeks transfer to the greenhouse (Figs. 5a, b, 6a). The EK 11-1 genotype exhibited the greatest negative carryover effect of BA: lowest ex vitro survival of rooted microcuttings (30%) was observed in EK 11-1 plantlets when 2.2 μM BA was used for Stage II multiplication (Fig. 5a, 6b). Survival improved significantly if 2.2 or 10 μM *m*T were used for Stage II multiplication (Figs. 5, 6c, d). However, higher

Fig. 1 Effect of cytokinin type and concentration after 4 weeks Stage II culture on shoot cluster dry weight of EK 11-1 (difficult-to-acclimatize) and EK 16-3 (easy-to-acclimatize) sea oats genotypes. *Error bars* indicate SE ($n = 5$). BA: N^6-benzyladenine; *m*T: 6-(3-hydroxybenzylamino)purine. ANOVA analysis is shown on top *left* corner of each graph; *T* Treatment, *G* Genotype, NS, **: Non-significant or significant at $P \leq 0.01$, respectively. *Different letters* on top of *histobars* are significantly different according to Waller-Duncan test at $P \leq 0.05$
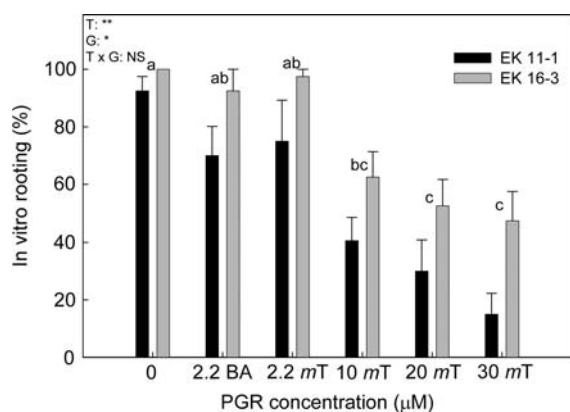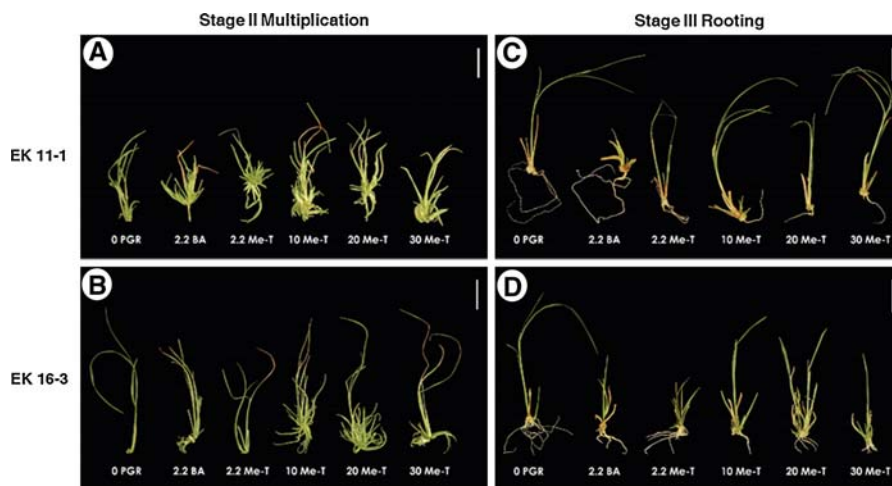
Fig. 4 Carryover effect of Stage II cytokinin type and concentration
followed by 6 weeks Stage III culture on rooting of EK 11-1
(difficult-to-acclimatize) and EK 16-3 (easy-to-acclimatize) sea oats
genotypes. Error bars indicate SE ($n = 5$). BA: N[6]-benzyladenine;
*m*T: 6-(3-hydroxybenzylamino)purine. ANOVA analysis is shown on
top *left* corner of each graph; *T* Treatment, *G* Genotype, NS, *, **:
Non-significant or significant at $P \le 0.05$ or 0.01, respectively.
Different letters on top of *histobars* are significantly different
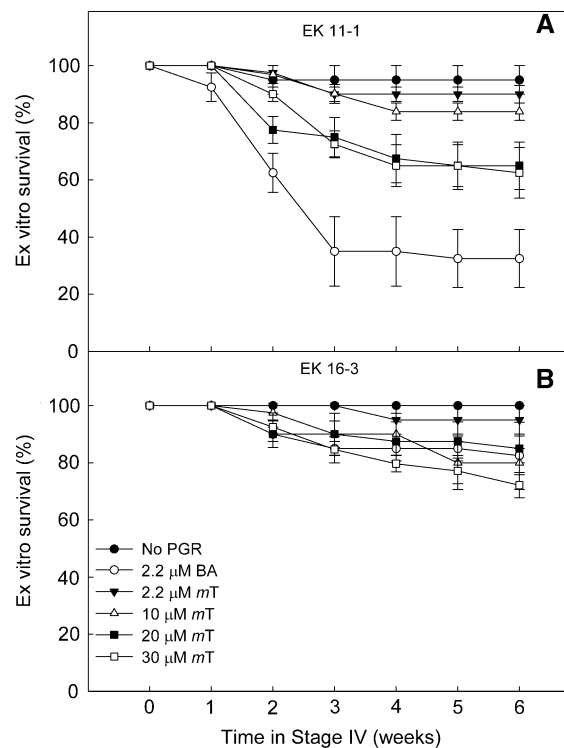according to Waller-Duncan test at $P \le 0.05$

Fig. 5 Carryover effect of Stage II cytokinin type and concentration
followed by 6 weeks Stage III on ex vitro survival of **a** EK 11-1
(difficult-to-acclimatize), and **b** EK 16-3 (easy-to-acclimatize) sea
oats genotypes. BA: N[6]-benzyladenine; *m*T: 6-(3-hydroxybenzylami-
no)purine. Means ± SE are shown ($n = 5$)

concentrations (20 or 30 μM *m*T) appeared to cause a
negative carryover effect on survival (Figs. 5a, 6e, f).
Although the carryover effect of BA during Stage II was
relatively less in EK 16-3 than EK 11-1, survival of EK
16-3 was also greater in shoot cultures multiplied in the
presence of 2.2 μM *m*T (Fig. 5b). Shoot cultures main-
tained in the absence of BA or *m*T exhibited the greatest
leaf elongation after 6 weeks ex vitro culture (Fig. 7),
followed by EK16-3 plantlets cultured with 2.2 μM BA,
2.2 μM *m*T, 10 μM *m*T with similar leaf length to EK
11-1 plantlets cultured with 2.2 μM *m*T. The shortest
leaves among treatments after 6 week ex vitro culture,
was observed on EK 11-1 plantlets cultured in multipli-
cation medium containing 10, 20 and 30 μM *m*T and EK
16-3 plantlets cultured in 30 μM *m*T.

Ex vitro shoot number was also affected by in vitro
treatment and genotype (Fig. 8): it ranged from 1.5 to 2.5
after 6 weeks ex vitro culture. Greater ex vitro shoot
multiplication was observed in EK 16-3 plantlets previ-
ously cultured in Stage II medium containing 0, 10, 20, or
30 μM *m*T (Figs. 8, 6d–f) and in EK 11-1 plantlets cultured
in the presence of 30 μM *m*T (Fig. 8).

Fig. 6 Comparative effects of Stage II cytokinin type and concentration followed by 6 weeks Stage III and 6 weeks Stage IV acclimatization on ex vitro growth of EK 11-1 (difficult-to-acclimatize) and EK 16-3 (easy-to-acclimatize) sea oats genotypes. BA: N[6]-benzyladenine; *m*T: 6-(3-hydroxybenzylamino)purine. *Scale bars* = 5 cm
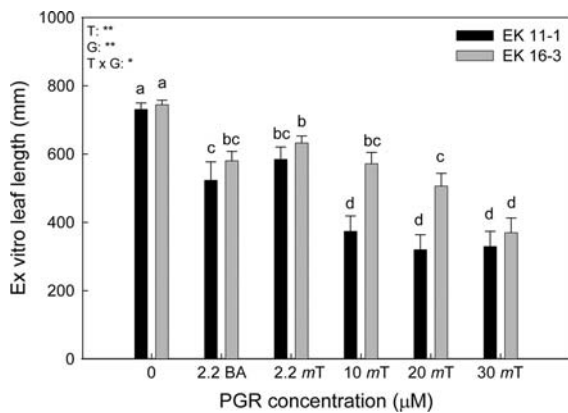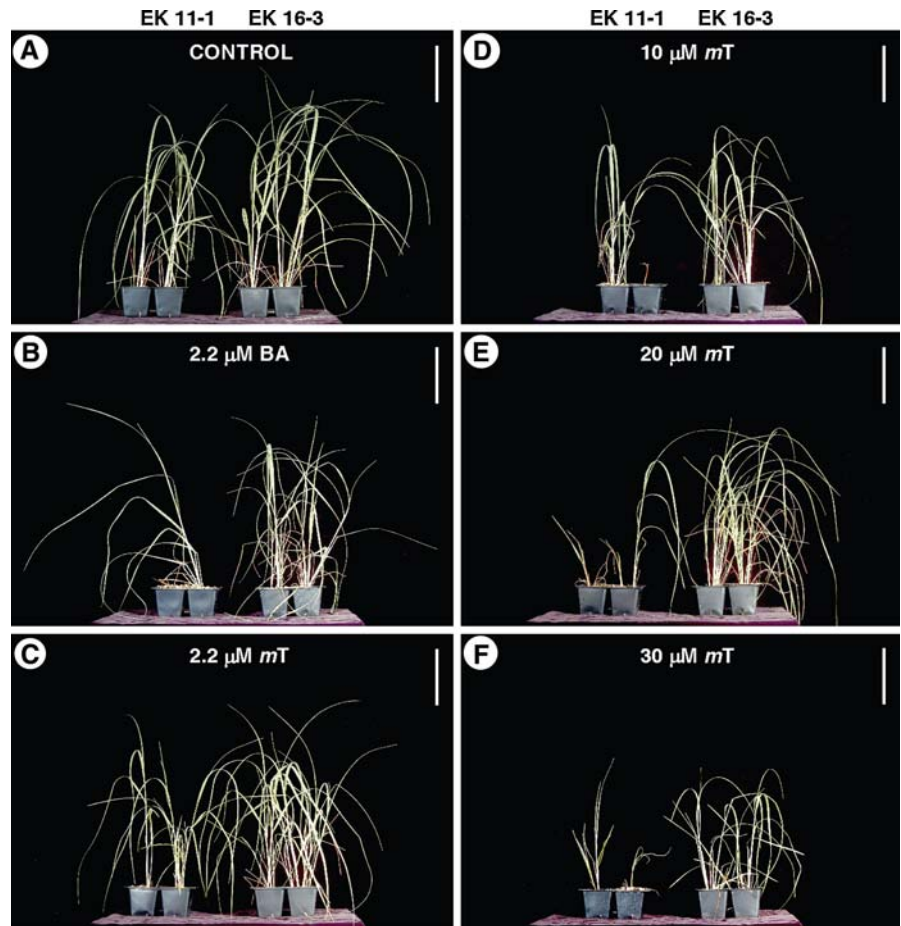

Fig. 7 Carryover effect of Stage II cytokinin type and concentration following 6 weeks Stage III rooting and 6 weeks Stage IV acclimatization on ex vitro leaf length of EK 11-1 (difficult-to-acclimatize) and EK 16-3 (easy-to-acclimatize) sea oats genotypes. Error bars indicate SE ($n = 5$). BA: N[6]-benzyladenine; *m*T: 6-(3-hydroxybenzylamino)purine. ANOVA analysis is shown on top left corner of each graph; *T* Treatment, *G* Genotype, *, **: Significant at $P \le 0.05$ or 0.01, respectively. *Different letters* on top of *histobars* are significantly different according to Waller-Duncan test at $P \le 0.05$

Discussion

Negative carryover effects on acclimatization following shoot multiplication on BA supplemented media have been described for several species (Werbrouck et al. 1995, 1996; Moncaleán et al. 2001; Bairu et al. 2008). In the present study, a marked difference in sensitivity to BA at the time of ex vitro transfer was observed between the easy- and the difficult-to-acclimatize *U. paniculata* genotypes. EK 11-1 plantlets exhibited significantly lower ex vitro survival than EK 16-3 when multiplied on Stage II medium containing BA. The fact that both genotypes exhibited very high ex vitro survival rates when cultured in Stage II medium without BA, strongly suggests an inhibitory carryover effect of BA on subsequent ex vitro acclimatization, especially in EK 11-1. The significant decrease in ex vitro acclimatization observed in EK 11-1, even after 6 weeks Stage III rooting, indicates that the inhibitory carryover effects of BA are prolonged. We have observed that extending the Stage II culture duration on BA-supplemented medium from 4 to 12 weeks doubles the acclima-
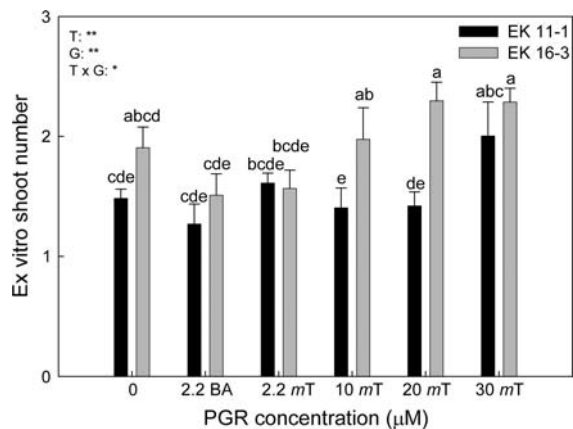
Fig. 8 Carryover effect of cytokinin type and concentration after 4 weeks Stage II culture followed by 6 weeks Stage III rooting and 6 weeks Stage IV acclimatization on ex vitro shoot number of EK 11-1 (difficult-to-acclimatize) and EK 16-3 (easy-to-acclimatize) sea oats genotypes. *Error bars* indicate SE ($n = 5$). BA: N^6-benzyladenine; mT: 6-(3-hydroxybenzylamino)purine. 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 $P \leq 0.05$

tization rate of rooted EK 11-1 microcuttings (Valero-Aracama et al. 2008). This improvement may be due to the eventual degradation of 9G-BA, or another inhibitory BA derivative, which has been shown to remain at high levels in plants even after a 9 week acclimatization period (Werbrouck et al. 1995).

In vitro culture in the *absence* of cytokinins does not provide a practical solution for efficient micropropagation of sea oats genotypes. Sea oats shoot cultures, maintained without PGR, or in the presence of other cytokinin types besides BA, exhibited very low shoot multiplication rates regardless of genotype (data not shown). However, when using mT, both genotypes exhibited similar growth and multiplication as when cultured in equimolar concentrations of BA-supplemented medium. Positive effects on in vitro shoot multiplication with mT have been described with other species (Werbrouck et al. 1996; Wojtania and Gabryszewska 2001; Bairu et al. 2007, 2008; Roels et al. 2005). Negative carryover effects on rooting at too high mT levels (13.3 μM, 22.2 μM) have similarly been observed (Bairu et al. 2008; Escalona et al. 2003). Increased ex vitro acclimatization, particularly in EK 11-1, may be the consequence of a more rapid turnover of the mT main derivative N^6-(3-O-β-D-glucopyranosyl)benzyladenine-9-riboside (Strnad et al. 1997). However, the direct role of this or other cytokinin derivatives on acclimatization is unclear and warrants further investigation. Utilizing BA-sensitive sea oats genotypes may prove useful in this endeavor.

Accumulation of a cytokinin metabolite may not fully explain the deleterious effects of BA during sea oats micropropagation. Compared to EK 16-3, in the difficult-to-acclimatize sea oats genotype EK 11-1, in vitro multiplication in the presence of BA induces a mosaic of detrimental biochemical, physiological and developmental characteristics leading to significantly reduced capacity for ex vitro acclimatization. These characteristics include lower photosynthetic enzyme activities, reduced net photosynthetic capacity at the time of ex vitro transfer, initially higher transpiration rates, and insufficient carbohydrate reserves (Valero-Aracama et al. 2006). Recently, comparative anatomical observations made using light and electron microscopy further revealed that poor ex vitro acclimation of sea oats produced on BA-supplemented media was also correlated with abnormal leaf organization and morphology, stomatal aperture blockage and chloroplast thylakoid membrane disruption (Valero-Aracama et al. 2008). Irreversible chloroplast disruption in the presence of BA or its metabolites in vitro has been suggested (Werbrouck et al. 1995).

Our results indicate that substituting 2.2 μM BA with equimolar mT did not compromise in vitro shoot multiplication and rooting; yet significantly enhanced ex vitro acclimatization of the difficult-to-acclimatize EK 11-1 sea oats genotype. Inclusion of mT in media may provide a method to ensure efficient commercial in vitro propagation of a large number of diverse sea oats genotypes. However, further screening of the efficacy of mT with a wide range of sea oats genotypes is required to confirm this broader application.

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