In Vitro Cell.Dev.Biol.—Plant (2015) 51:294–302
DOI 10.1007/s11627-015-9682-0

MICROPROPAGATION

Direct and indirect *in vitro* regeneration of *Miscanthus* × *giganteus* cultivar Freedom: effects of explant type and medium on regeneration efficiency

Dinum Perera[1,4] · **Daniel J. Barnes**[2] · **Brian S. Baldwin**[1] · **Nancy A. Reichert**[3]

Received: 8 August 2014 / Accepted: 25 February 2015 / Published online: 20 March 2015 / Editor: Baochum Li
© The Society for In Vitro Biology 2015

Abstract *Miscanthus* × *giganteus* (giant miscanthus; Mxg) is a seed-sterile, perennial bioenergy crop with the potential to produce liquid fuel from lignocellulosic biomass. A new cultivar, Freedom, is being commercially grown in the USA on increasing acreage. To determine this genotype's regeneration responses in tissue culture, three explant sources were screened on media proven successful for other genotypes. Four callus induction media contained 13.6–22.6 μM 2,4-dichlorophenoxyacetic acid (2,4-D) alone or with 0.44–4.4 μM 6-benzyladenine (BA). Callus induction percentages for all explants ranged from 93 to 97%. Media yielding the greatest percentages of explants producing regenerable calli for shoot apices (from *in vitro* and greenhouse plant sources) were media containing either 13.6 μM 2,4-D plus 0.44 μM BA or 22.6 μM 2,4-D plus 0.44 μM BA. After culture on a regeneration medium containing 22 μM BA plus 1.3 μM naphthaleneacetic acid (NAA), 3.59–3.74 regenerants were obtained per explant. Immature inflorescence explants (from field-maintained plants) gave up to 77% regenerable calli and 6.99 regenerants per explant. Direct regenerants (shoots) arose from immature inflorescence explants on a medium containing 9.0 μM 2,4-D. Intact plants could be generated within 16–18 wk after culture initiation. Extensive visual assessments, and molecular assessments via inter-simple sequence repeat (ISSR) PCR analysis using 21 different primers, did not reveal distinguishable somaclonal variation among regenerants or when compared to rhizome-propagated transplants under field conditions. We believe that this is the first extensive *in vitro* and *ex vitro* analysis on a commercially grown Mxg genotype.

Keywords *Miscanthus* × *giganteus* · Bioenergy crop · Plant regeneration · Field screenings · ISSR analysis

✉ Dinum Perera
hnp21@msstate.edu

[1] Department of Plant and Soil Sciences, Mississippi State University, Mississippi State, MS 39762, USA

[2] Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA

[3] Department of Biological Sciences, Mississippi State University, Mississippi State, MS 39762, USA

[4] Present address: Institute for Genomics, Biocomputing, and Biotechnology, Mississippi State University, 650 Stone Blvd., 101 Pace Seed Laboratory, Mississippi State, MS 39762, USA

Introduction

With increased attention on environmental, economic, and sustainability issues surrounding use of fossil fuels, the US government set a target to replace 30% of petroleum with renewable resources by 2030 (Jessup 2009). The continuous increase in global energy consumption has also sparked interest in renewable energy (Dhugga 2007).

Giant miscanthus (*Miscanthus* × *giganteus*; formerly known as *Miscanthus* × *ogiformis* Honda 'Giganteus'; hereafter abbreviated Mxg) is a promising bioenergy crop based on high dry matter yield; low input requirements (fertilizer and pesticides); and high use efficiencies for solar radiation, water, and nitrogen (Lewandowski *et al.* 2000, 2003; Heaton *et al.* 2008; Zub and Brancourt-Hulmel 2010). A member of the Poaceae, Mxg, is a C4 perennial grass believed to originate from East Asia (Venturi *et al.* 1998). This sterile triploid ($2n=3x=57$) was likely derived from a rare, natural hybridization between *Miscanthus sacchariflorus* ($2n=4x=76$) and *Miscanthus sinensis* ($2n=2x=38$) (reviewed by Heaton *et al.* 2010). Freedom (MSU-MFL1) is a distinct, high-yielding

Mxg cultivar developed by Mississippi State University primarily for growth in the southeastern USA (Baldwin 2013). Cultivar Freedom was selected from PI 295762 (obtained from the Plant Materials Repository, Miami, FL) through serial selection of rhizomes/tillers for vigorous growth. PI 295672, labeled *Miscanthus floridulus*, was later determined and confirmed to be *M.* × *giganteus* (Chouvarine *et al.* 2012; Głowacka *et al.* 2014). Cultivar Freedom is distinct from cv. Illinois, the genotype primarily grown in the USA (Baldwin 2013). The Illinois genotype has also been referred to as both a clone (Heaton *et al.* 2010) and a line (Williams and Douglas 2011) but will be referred to as a cultivar here. Both Freedom and Illinois can be grown under the US Department of Agriculture Farm Service Agency Biomass Crop Assistance Program (USDA 2012).

Being a seed-sterile triploid, Mxg can be asexually propagated using either rhizomes or micropropagation (Lewandowski 1998; Atkinson 2009). Mutations have arisen leading to the generation of different genotypes/cultivars distinguished by inter-simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), single-nucleotide polymorphism (SNP), and simple sequence repeat (SSR) (Chouvarine *et al.* 2012; Baldwin 2013; Cichorz *et al.* 2014; Głowacka *et al.* 2014). Based on SSR analysis, Głowacka *et al.* (2014) concluded that all "legacy" Mxg accessions, such as cvs. Freedom and Illinois, originated from a single genotype via vegetative propagation. Therefore, indirect regeneration procedures might enable generation of additional desired mutations (Perera *et al.* 2015) and ploidy manipulations (Yu *et al.* 2009), leading to new cultivar development.

Indirect Mxg regeneration procedures have been successfully developed for shoot apices, leaf sections, and immature inflorescences (Lewandowski and Kahnt 1993; Holme and Petersen 1996; Holme *et al.* 1997; Petersen 1997; Petersen *et al.* 1999; Głowacka *et al.* 2010; Kim *et al.* 2010, 2012; Gubišová *et al.* 2013; Zhao *et al.* 2013). Immature inflorescence explants were most successfully regenerated (Holme and Petersen 1996; Holme *et al.* 1997; Petersen *et al.* 1999). Callus induction was obtained on media containing 4.5–31.7 μM 2,4-dichlorophenoxyacetic acid (2,4-D) either alone or in combination with 0.44–4.4 μM 6-benzyladenine (BA). Regeneration could be achieved via organogenesis and somatic embryogenesis, with intervening calli usually categorized into four distinct types: shoot-forming, embryogenic, root-forming, and non-morphogenic (Holme and Petersen 1996; Lewandowski 1997; Petersen 1997). Głowacka *et al.* (2010) labeled these callus types K1 to K4, respectfully. Callus induction and the types of calli generated depended on the explant (type, size, and developmental stage), medium composition, and culture duration. Addition of 12–50 μM proline improved explant response (Holme *et al.* 1997).

In addition to indirect regeneration, a few direct regeneration procedures have been reported via axillary shoot tillering from shoot apices (Lewandowski 1997) and nodal sections (Gubišová *et al.* 2013; Rambaud *et al.* 2013) on initial media containing either 4.4–22.2 μM BA alone or with 2.6 μM indole-3-acetic acid (IAA).

There are multiple Mxg genotypes/cultivars, and genotype can be important in developing a successful regeneration procedure, as was recently demonstrated for tissue culture responses of three distinct *M. sinensis* genotypes (Głowacka *et al.* 2010). However, the genotypes/cultivars utilized were not often identified; Mxg genotypes were provided by Głowacka *et al.* (2010; cv. MG1) and Rambaud *et al.* (2013; cv. Floridulus). One goal of this research was to determine if cv. Freedom could regenerate via indirect and direct regeneration procedures previously described. Additional goals included determining the most efficient regeneration procedures by comparing explants and medium compositions and by developing ISSR PCR analysis to determine the presence of somaclonal variation among regenerants.

Materials and Methods

Plant material and culture conditions. Mxg cv. Freedom was used in all experiments. Shoot apices (5–7 mm in length) harvested from plants grown in the greenhouse for 4 mo were surface disinfested in 2% sodium hypochlorite (NaOCl) for 15 min, rinsed three times with sterile deionized water, and then trimmed to 2–3 mm in length. Shoot apices (2–3 mm in length) were also harvested from *in vitro* plantlets (propagation method described by Nielsen *et al.* 1993) that originated from nodal segments of field-grown Freedom. Immature inflorescences (1–6 cm in length) harvested from field-grown plants were surface disinfested as intact stems (covered with the innermost leaf sheaths) in 6% NaOCl for 15 min and rinsed, and then, immature inflorescences were dissected out (2–3 mm in length).

Callus induction. Explants were cultured on five different callus induction media (M1–M5). The media were based on Murashige and Skoog (MS) basal medium (mineral salts and vitamins; Murashige and Skoog 1962), containing sucrose (30 g L^{-1}) and $MgCl_2 \cdot 6H_2O$ (750 mg L^{-1}), and various combinations and concentrations of plant growth regulators (PGRs; 2,4-D and BA) ± proline were added prior to autoclaving (Table 1). The following are the initial references for PGR combinations used in this report: M1 (Petersen 1997), M2 (Głowacka *et al.* 2010), M3 (Lewandowski 1997), M4 (Holme and Petersen 1996), and M5 (Holme *et al.* 1997). All media (pH 5.5) were solidified with 2 g L^{-1} Phytagel™ (Sigma-Aldrich, St. Louis, MO). Five explants were placed per culture dish (60 × 15 mm), and each treatment consisted of 15 dishes. All explants/cultures were incubated in growth chambers at 27±2/25±2°C day/night temperatures under dark

Table 1. Callus induction medium compositions used for Mxg cv. Freedom

Medium	Plant growth regulator (μM)		Proline (mM)
	2,4-D	BA	
M1	13.6	0.44	25
M2	22.6	0.44	25
M3	22.6	4.40	25
M4	13.6	–	–
M5	9.0	–	–

conditions (explants on media M1–M4) or a 16-h photoperiod (125 μmol m^{-2} s^{-1} illumination with warm white fluorescent bulbs; explants on M5) and arranged in a completely randomized design within each growth chamber (dark and light). Explants were subcultured once per week for the first 2 wk and then at 2-wk intervals for 4 wk. At 6 wk after culture initiation, the numbers of explants forming calli were scored, and different callus types were categorized according to visual appearance, following Głowacka *et al.* (2010) descriptions/labels: K1=shoot-forming (compact, white callus with translucent shoot-like structures), K2=embryogenic (compact, opaque white, or cream-colored), K3=root-forming (nodular, semisoft cream-colored callus sometimes containing anthocyanin), and K4=non-morphogenic (soft, watery, and translucent white). Callus induction percentages were calculated according to the following formula: (number of explants with calli/total number of explants)×100. Callus type percentages (for M1–M4 media) were calculated according to the following formula: (number of calli in each callus category/total number of calli)×100.

Plant regeneration and rooting. Six-week-old K1 and K2 callus pieces (2–3 mm^3) were separated from calli originating on M1–M4 media and transferred onto a regeneration medium composed of MS basal salts and vitamins supplemented with 20 g L^{-1} sucrose, 22 μM BA, and 1.3 μM 1-naphthaleneacetic acid (NAA; Kim *et al.* 2010) and solidified with 0.7% agar. Four callus pieces were transferred to each vessel (Magenta box, 3 × 3×4 inches) with 15 vessels per treatment group (initial explant and medium type) and subcultured at 2-wk intervals for 2 mo. Cultures were incubated in a growth chamber under a 16-h photoperiod (same illumination indicated above) at 25±2/22±2°C day/night temperatures. Calli and direct regenerants originating on M5 medium were subcultured on the basal medium described above but lacking PGRs since the cultures had already formed shoots. Regeneration rates (numbers of shoots/plantlets arising from each initial explant) were determined after 2 mo in culture.

Root initiation, when needed, briefly employed use of the medium described by Nielsen *et al.* (1993) since a preliminary study determined that rooting was inhibited if cv. Freedom was continuously cultured on this medium (data not shown). Shoots were placed on half-strength MS containing 5.4 μM NAA (Nielsen *et al.* 1993) for 3 d and then transferred to half-strength MS containing 0.2% activated charcoal for 2 wk. Rooted plantlets were transplanted into autoclaved soil and acclimatized as described by Perera *et al.* (2015), and maintained in the greenhouse prior to being transplanted into the field.

Phenologic, phenotypic, and genotypic evaluation of field-grown regenerants. Regenerants arising from direct and indirect regeneration were maintained in the greenhouse for 2 mo and then transplanted into two planting blocks at the Mississippi State University Henry H. Leveck Animal Research Farm, Starkville, Mississippi, in May 2011. Rhizome-propagated plants, grown in the greenhouse for 2 mo prior to transplant, were used as control plants. In total, 454 plants were transplanted into the field (103 direct regenerants, 276 indirect regenerants, 75 rhizome-propagated plants). Regenerants were evaluated based on phenology (onset of flowering) in the fall of 2011 and 2012; flowering dates were recorded when the panicle appeared from the shoot apex. In 2012, regenerants were assessed for several morphological parameters (leaf color, stem color, plant height, stalk diameter, and leaf angle) at the flowering stage. Leaf and stem color were determined based on the Munsell color system (Munsell color charts for plant tissues, 2011, Kollmorgen Instruments, Baltimore, MD). Plant height was measured from the surface of the soil to the top leaf tip with a height stick, and stem diameter was measured at 1 m from the surface of the soil with a digital vernier caliper. Leaf angle was measured from above the node to the upper surface of the leaf with a protractor.

To investigate potential genotypic variations, total genomic DNA was extracted from leaf samples taken from 30 field-grown transplants using a DNeasy Plant Mini kit (Qiagen, Valencia, CA). Twenty-one primers (Table 2) previously screened in *Miscanthus* species and *Saccharum officinarum* (sugarcane) via ISSR PCR analysis (Hodkinson *et al.* 2002; da Costa *et al.* 2011) were used to detect polymorphisms. Amplifications were performed using 100 ng template DNA, 100 μM of each dNTP, and 2 mM $MgCl_2$ in a 50-μl reaction mix using 0.5 units of *Taq* polymerase as described by Perera *et al.* (2015). Reaction products were separated on 2% MetaPhor Agarose (Lonza, Rockland, ME) gels, which were then stained with ethidium bromide and scanned using a VersaDoc imager (Bio-Rad, Hercules, CA).

Statistical analysis. Each experiment was performed three times. Least square means and analysis of variance (ANOVA; general linear model procedure) were performed using Statistical Analysis System (SAS Version 9.1.2., SAS

Table 2. List of inter-simple sequence repeat (ISSR) primers used during PCR analysis of DNA extracted from leaves of Mxg cv. Freedom

Primer name	Primer (5′–3′ sequence)[z]	Annealing temp (°C)
Mxg 1	GACAGACAGACAGACA	50
Mxg 2	CACACACACACACACARG	50
Mxg 3	AGAGAGAGAGAGAGAGYT	53
Mxg 4	ACACACACACACACACYT	52
Mxg 5	GAGAGAGAGAGAGAGAA	52
Mxg 6	TGTGTGTGTGTGTGTGA	53
Mxg 7	CTCTTCTCTCTCTCTCTG	54
Mxg 8	ACACACACACACACACYG	54
Mxg 9	GAGAGAGAGAGAGAGAT	50
Mxg 10	GAGAGAGAGAGAGAGAC	53
Mxg 11	DVHCACACACACACACA	54
Mxg 12	TCTCTCTCTCTCTCTCC	52
Mxg 13	AGAGAGAGAGAGAGAGT	52
Mxg 14	ACACACACACACACACT	48
Mxg 15	TGTGTGTGTGTGTGTGC	48
Mxg 16	CTCCTCCTCCTCCTC	48
Mxg 17	AGAGAGAGAGAGAGAGA	48
Mxg 18	AGAGAGAGAGAGAGACC	48
Mxg 19	CAGCAGCAGCAGCAGT	48
Mxg 20	CAGCAGCAGCAGCAGG	48
Mxg 21	GCACACACACACACACA	48

[z] R=(A,G); Y=(C,T); D=(A,G,T); V=(A,C,G); H=(A,C,T)

Inst., Cary, NC). Treatment means were separated by Fisher's protected least significant difference (LSD) method at $\alpha < 0.05$.

Results

Callus induction. Calli were visible after 8–10 d of culture initiation on M4 medium, whereas explants took 14–16 d to form visible callus on media M1–M3. Callus induction percentages were not significantly ($P<0.05$) influenced by either medium or explant type and ranged from 93 to 97% (data not shown). Browning of the explants and surrounding media was visible 2–3 d after explants were initially transferred to media regardless of medium composition.

The formation of different callus types (K1–K4) was significantly ($P<0.05$) affected by both explant type and medium composition. Shoot apices from both *in vitro* and greenhouse sources yielded a higher percentage of K2 calli (embryogenic) than K1 calli (shoot-forming), regardless of medium used (Figs. 1 and 2). For immature inflorescence explants, however, K2 was the predominant regenerable callus type on only two media (M3 and M4), with K1 the predominant regenerable callus type on M1 and M2 (Fig. 3). Serially subculturing

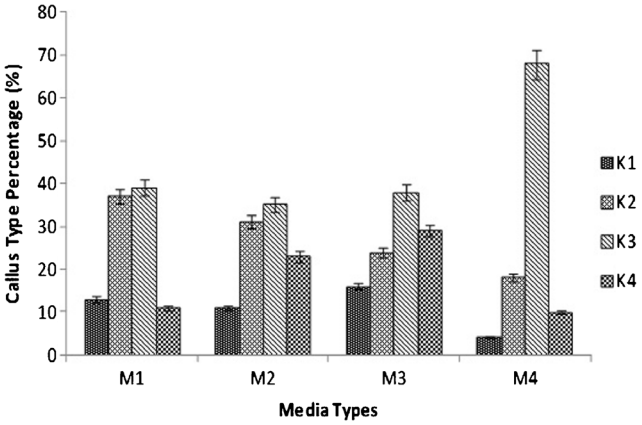

Figure 1. Effect of medium composition on callus type percentages for Mxg cv. Freedom shoot apex explants (*in vitro*) cultured on callus induction media for 6 wk. Callus type percentages [per medium: (number of calli in each callus category/total calli)×100] were scored based on visual appearance. Media are as described in Table 1. Callus morphologies: K1=shoot-forming, K2=embryogenic, K3=root-forming, K4=non-morphogenic. M5 medium was not included since it resulted in morphologically different calli than described above and yielded direct shoot clusters. Two-factor analysis of variance was performed; *error bars* represent the standard deviation.

calli onto the same initial media formulations (M1–M4) for greater than 3 mo resulted in development of primarily non-morphogenic calli and some root-forming calli, irrespective of medium or initial explant.

Plant regeneration and rooting. Shoot/plant regeneration was significantly ($P<0.05$) affected by both explant type and medium composition (Tables 3 and 4). Among the explant

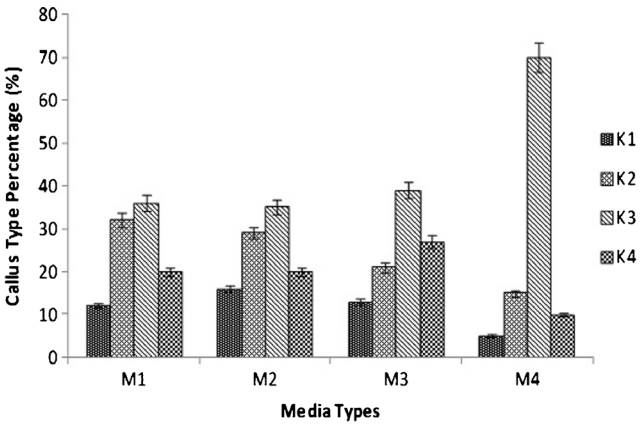

Figure 2. Effect of medium composition on callus type percentages for Mxg cv. Freedom shoot apex explants (greenhouse) cultured on callus induction media for 6 wk. Callus type percentages [per medium: (number of calli in each callus category/total calli)×100] were scored based on visual appearance. Media are as described in Table 1; callus types are as described in the text and legend to Fig. 1. M5 medium was not included since it resulted in morphologically different calli than described above and yielded direct shoot clusters. Two-factor analysis of variance was performed; *error bars* represent the standard deviation.

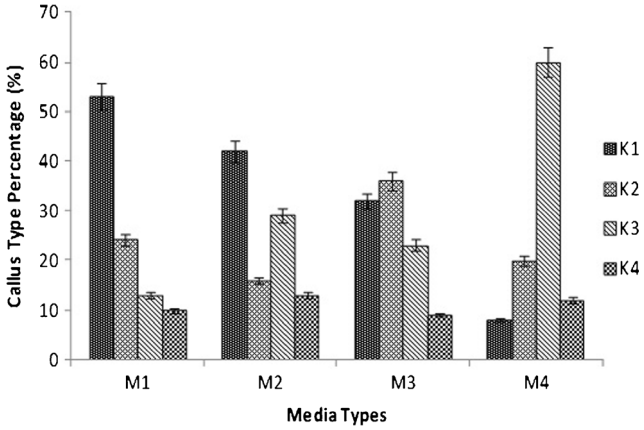

Figure 3. Effect of medium composition on callus type percentages for Mxg cv. Freedom immature inflorescence explants cultured on callus induction media for 6 wk. Callus type percentages [per medium: (number of calli in each callus category/total calli)×100] were scored based on visual appearance. Media are as described in Table 1; callus types are as described in the text and legend to Fig. 1. M5 medium was not included since it resulted in morphologically different calli than described above and yielded direct shoot clusters. Two-factor analysis of variance was performed; *error bars* represent the standard deviation.

sources, immature inflorescence explants yielded the greatest number of regenerants on M1 medium; when the frequencies of the two regenerant types (shoots and somatic embryos) are combined, medium M1 generated approximately seven (6.99) regenerants per inflorescence explant. Within each medium (M1–M4), this explant source yielded significantly greater numbers of regenerants than shoot apices (except in the case of embryogenic regeneration on medium M2; see Table 4). Shoot apices responded the best on media M1 and M2, yielding 3.59–3.74 regenerants per explant. A greater numbers of

Table 3. Effect of medium and explant type on number of shoots generated per explant in Mxg cv. Freedom

Initial medium[z]	Explant type		
	Shoot apex (*in vitro*)	Shoot apex (greenhouse)	Immature inflorescence
M1	1.96de[y]	2.01d	4.98a
M2	1.92e	1.95de	4.02b
M3	1.77f	1.81f	2.73c
M4	1.27h	1.25h	1.39g
M5	0.99i	1.01i	0.75j

Data were taken after 2 mo on regeneration medium (22 μM BA plus 1.3 μM NAA); cultures arising on M5 produced morphologically different calli than K1–K4 types and were already producing shoots, so transfer was onto a medium lacking PGRs

[z] Media are as described in Table 1

[y] Two-factor analysis of variance; numbers of shoots generated per initial explant. Treatment means followed by the same *letter* are not significantly different ($P<0.05$)

Table 4. Effect of medium and explant type on number of somatic embryos generated per explant in Mxg cv. Freedom

Initial medium[z]	Explant type		
	Shoot apex (*in vitro*)	Shoot apex (greenhouse)	Immature inflorescence
M1	1.69cd[y]	1.73bc	2.01a
M2	1.67cd	1.70bcd	1.74d
M3	1.64d	1.67cd	1.78b
M4	1.37f	1.34f	1.47e

Data were taken after 2 mo on regeneration medium (22 μM BA plus 1.3 μM NAA); M5 was not included because it did not generate embryogenic callus

[z] Media are as described in Table 1

[y] Two-factor analysis of variance; numbers of somatic embryos generated per initial explant. Treatment means followed by the same *letter* are not significantly different ($P<0.05$)

regenerants arose from shoot-forming calli than from embryogenic calli for all explants initially cultured on M1 or M2 (Tables 3 and 4).

Explants cultured on M5 displayed morphologically different calli than the types previously described (K1–K4) and could yield direct shoot clusters. Shoot apices on M5 produced light-green callus that later developed into shoot primordia and some non-morphogenic callus; immature inflorescence explants on M5 primarily produced direct shoot primordia that developed into shoot clusters (Fig. 4*A*).

Overall, generation of rooted plantlets (Fig. 4*B*) took 16–18 wk from the time that explants were initially cultured on M1–M4. Plantlets were generated at least 2 wk faster from explants cultured on M5 and the fastest from immature inflorescences cultured on that medium.

Variation among field-transplanted regenerants. The initiation of flowering for all 454 field transplants evaluated (Fig. 4*C*) occurred within a 2- to 3-wk time period; approximately 1% of the plants arising from each regeneration pathway (direct and indirect *in vitro* regenerants, rhizome-propagated plants) flowered at the beginning of the flowering period with the remainder flowering within 2–3 wk after that. Leaf color (Munsell color value: 5GY 4/4) and stem color (Munsell color value: 5GY 4/8) were the same for all plants regardless of regeneration pathway. Plant height was significantly ($P<0.05$) different between indirect regenerants and rhizome-propagated plants (indirect regenerants being taller), whereas the stem diameter and the leaf angle were similar (Table 5). Analysis of 30 field transplants using 21 ISSR primers in PCR produced clear, reproducible banding patterns on gels, with no polymorphisms detected (data shown for primer Mxg 7; Fig. 5).

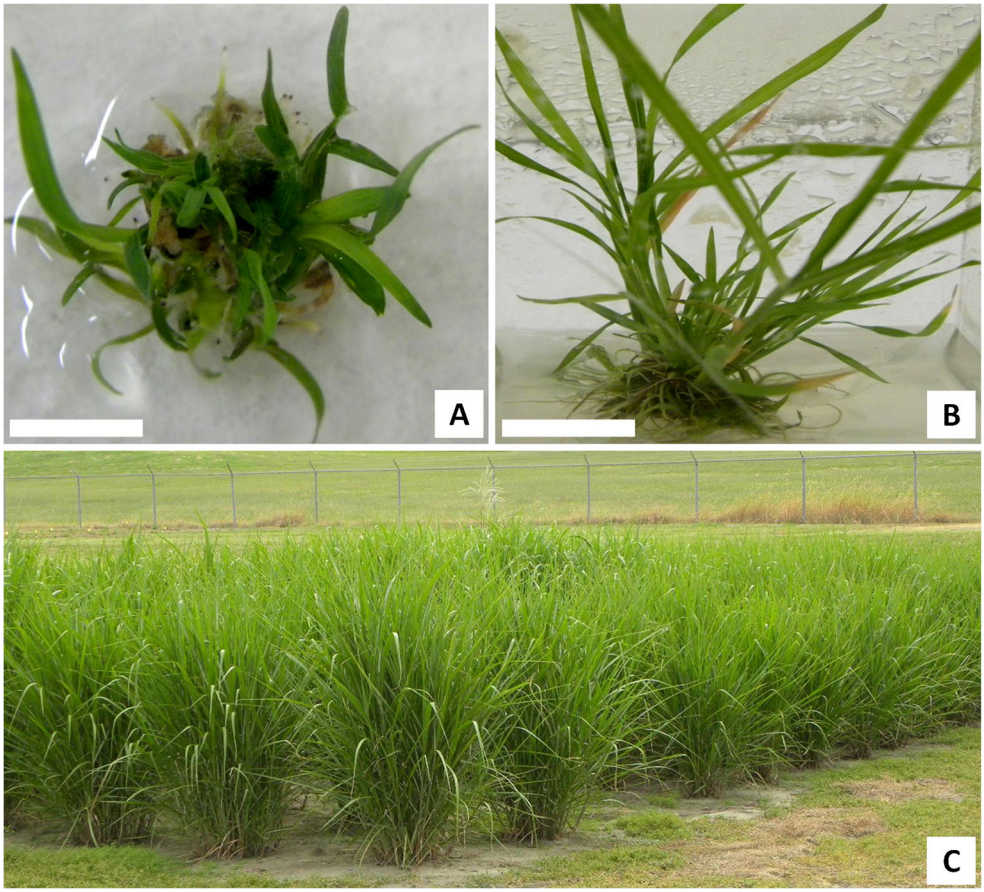

Figure 4. Mxg cv. Freedom *in vitro* cultures and regenerants. (*A*) Immature inflorescence explant on M5 medium 6 wk after initiation, (*B*) rooted plantlet cluster on rooting medium after 18 wk (total) in culture, and (*C*) regenerated plants in the field in 2012. *Bars* equal 1 mm (*A*) and 1 cm (*B*).

Discussion

The seed-sterile nature of Mxg poses challenges in regard to plant multiplication and genetic enhancement. Freedom, a new Mxg cultivar (Baldwin 2013), has demonstrated yearly biomass harvests that can exceed other cultivars analyzed (cvs. Illinois, Nagara; White *et al.* 2014). Exploring alternate propagation methods (micropropagation) as well as potential future genetic enhancements through biotechnology-based strategies could assist commercial growers as well as the bioenergy industry.

Table 5. Phenotypic variation of Mxg cv. Freedom plants arising from direct and indirect regeneration and traditional rhizome propagation

Plant source	Plant height (m)	Stem diameter (mm)	Leaf angle (°)
Indirect regenerants	2.24 a[z]	0.66 a	30.0 a
Direct regenerants	2.19 ab	0.65 a	29.0 a
Rhizome-propagated	2.14b	0.64a	29.5a

Indirect regenerants arose from shoot-forming and embryogenic calli on M1–M4 media and calli from shoot apices on M5; direct regenerants arose from immature inflorescences cultured on M5

[z] Analysis of variance; treatment means within a *column* followed by the same *letter* are not significantly different ($P < 0.05$)

The PGR formulations for our initial media were obtained from previous research (Holme and Petersen 1996; Holme *et al.* 1997; Lewandowski 1997; Petersen 1997; Głowacka *et al.* 2010). In our study, the explants tested included shoot apices harvested from *in vitro* and greenhouse plants, immature inflorescence explants, and leaf explants (leaves proximal to the shoot apex from *in vitro* and greenhouse plants). Data on leaf explants initially cultured on M1–M5 media were not included due to no callus formation or regeneration from these explants, although these media included PGR formulations that were successful for this explant in other genotypes (Holme and Petersen 1996; Holme *et al.* 1997; Petersen 1997; Petersen *et al.* 1999). The cv. Freedom genotype was unresponsive on this medium, but direct regeneration was obtained from immature inflorescence explants initially cultured on a medium containing 2,4-D alone (medium M5), which had not been noted in previous studies.

The explant source and medium composition had no effect on callus induction; immature inflorescence explants and shoot apex explants were both successfully used to generate callus on four of the media tested (M1–M4). However, callus morphologies differed, as first reported by Holme and

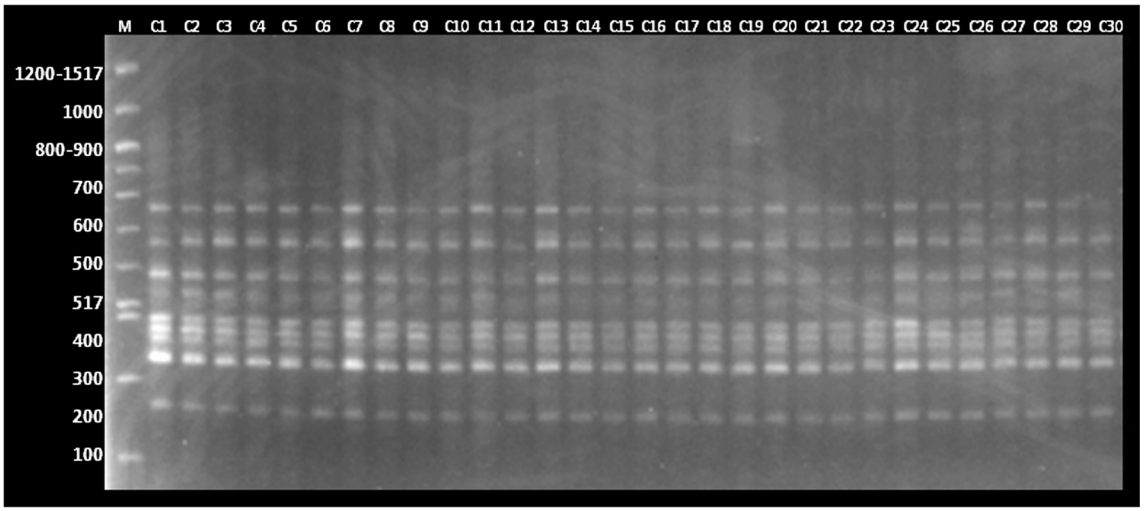

Figure 5. Gel electrophoresis pattern of inter-simple sequence repeat (ISSR) products using primer Mxg 7. DNA was extracted from leaves of Mxg cv. Freedom indirect and direct regenerants and from traditional rhizome-propagated plants. *M*, marker (100-bp ladder); *C1–C13*, direct regenerants from immature inflorescence explants on M5 medium; *C14–C23*, indirect regenerants from shoot-forming calli on M1; *C24–C27*, indirect regenerants from embryogenic calli on M1; *C28–C30*, rhizome-propagated plants.

Petersen (1996). Four distinct callus types typically generated by Mxg on callus induction media have been described previously (Holme and Petersen 1996; Lewandowski 1997; Petersen 1997; Głowacka *et al.* 2010); we adhered to the labels Głowacka *et al.* (2010) assigned to the four callus types. This study noted callus induction percentages (percentages of explants capable of generating calli) of 93–97% which was comparable to those that used callus induction media containing 13.6 μM 2,4-D±0.44 μM BA (Holme and Petersen 1996, 85–90%; Kim *et al.* 2010, 78%). Media supplemented with both auxin and cytokinin enhanced callus induction on Mxg explants compared to media containing auxin alone (Lewandowski and Kahnt 1993; Petersen 1997). This difference was not noted in our experiments although higher concentrations of 2,4-D alone yielded more K3 and K4 calli (data not shown). However, callus induction media containing both 2,4-D and BA yielded greater percentages of regenerable calli (K1 and K2 types), which was consistent with previous studies.

In our study, the choice of medium, explant type, and subsequent callus type strongly influenced regeneration rates. The most common callus type produced by shoot apices on media M1–M4 was K3, which did not yield regenerants (Figs. 1 and 2). In contrast, K3 was the predominant callus type generated on immature inflorescence explants only when tissues were cultured on M4 (Fig. 3). These immature inflorescence explants yielded 58–77% regenerable calli (K1 and K2 types) on M1–M3. The greatest percentage was noted on M1 (13.6 μM 2,4-D plus 0.44 μM BA), which yielded 6.99 regenerants (somatic embryos or shoots) per explant (Tables 3 and 4). The K1 (shoot-forming) callus type generated on M1 yielded a greater number of regenerants (4.98 per explant; Table 3) than the K2 (embryogenic) callus type (2.01 regenerants per explant; Table 4). This reinforced the importance of callus type in regeneration efficiency of Mxg, which is in agreement with previous studies showing that young shoot-forming callus cultures were more regenerable than other callus types, including embryogenic callus (Głowacka *et al.* 2010; Kim *et al.* 2010). Głowacka *et al.* (2010) noted generation of 25% shoot-forming calli (K1) in cv. MG1 on a medium containing our M2 PGR combination, whereas we noted 42% K1-type calli on that medium (Fig. 3). Kim *et al.* (2010) noted that 41% shoot-forming calli of another genotype were generated on a medium containing the PGR combination used in our medium M1, whereas we noted 53% K1-type calli on that medium (Fig. 3). Differences noted in shoot-forming callus percentages could be due to genotype differences.

Immature inflorescence explants yielded the greatest number of regenerants via organogenesis and somatic embryogenesis. As noted above, immature inflorescence explants yielded the highest percentages of calli capable of regeneration; medium M1 yielded 77% regenerable callus types (K1 and K2). Single immature inflorescences could also yield numerous explants and were easier to disinfest than other explant sources requiring surface disinfestation.

Immature inflorescence explants also generated shoots directly on medium M5 (9.0 μM 2,4-D), the first time that direct regeneration has been reported for this explant on media containing 2,4-D alone. However, less than one plant was regenerated per explant (Table 3). Since this study suggested that somaclonal variation was absent in regenerants, this direct regeneration procedure might not be important to pursue in future studies, although plantlets were generated at least 2 wk faster than via indirect regeneration procedures.

K1 and K2 callus cultures were transferred to the regeneration medium reported by Kim *et al.* (2010; 22 μM BA plus

1.3 μM NAA). Rooting was achieved using a medium supplemented with 5.4 μM NAA, determined optimal in previous studies [Nielsen *et al.* 1993 (*M. sinensis*); Głowacka *et al.* 2010]. We adopted a two-step rooting process to induce more efficient rooting (3 d on NAA-containing medium followed by transfer to a medium containing 0.2% activated charcoal). This type of two-step procedure has commonly been used to enhance rooting efficiency in several species including *Eucalyptus*, which is hard to root *in vitro* (Fett-Neto *et al.* 2001).

Field-grown direct and indirect regenerants showed no morphological abnormalities compared to rhizome-propagated plants. The individuals were similar for all characteristics except plant height, which might have been attributed to environmental differences. This is consistent with the few Mxg regeneration studies that analyzed field-transplanted micropropagules, although their primary focus was on comparison to rhizome-propagated plants (Lewandowski 1998; Kim *et al.* 2010; Rambaud *et al.* 2013). We also conducted ISSR PCR analysis on field-transplanted regenerants, the first time that this procedure has been used to assess somaclonal variation among/between Mxg tissue culture and rhizome-propagated regenerants. Rambaud *et al.* (2013) conducted amplified fragment length polymorphism (AFLP) analysis on their Mxg cv. Floridulus regenerants and noted one polymorphic fragment in their regenerants, but that primer pair also yielded a polymorphic fragment in the rhizome-propagated plants. Screenings of 30 plants using 21 primers via ISSR PCR analysis did not identify any polymorphisms. Based on these results, the potential for somaclonal variation in cv. Freedom appears to be limited, indicating that both direct and indirect regeneration procedures could be used for micropropagation, but that addition of *in vitro* mutagenic approaches might be necessary to generate mutations. Polymorphisms were obtained in regenerants when an ethyl methanesulfonate (EMS) treatment was added prior to regeneration (Perera *et al.* 2015).

Conclusions

Mxg cv. Freedom was successfully regenerated *in vitro* and transferred to the field. Immature inflorescence explants were used for regeneration via indirect organogenesis and somatic embryogenesis as well as direct organogenesis. With this explant source, the combination of 13.6 μM 2,4-D plus 0.44 μM BA yielded the highest indirect regeneration rates; use of 9 μM 2,4-D alone yielded direct shoot regeneration, but at a lower frequency. Development of regeneration procedures that can be employed in micropropagation and in strategies for further genetic enhancement is integral to biotechnology approaches for maintenance and improvement of this elite, commercially grown bioenergy cultivar. ISSR PCR analysis coupled with extensive field evaluation of cv. Freedom regenerants lays a baseline for future improvement of this and other commercially grown cultivars.

Acknowledgments This research was funded by Repreve Renewables Inc., Soperton, GA, and underwritten by the US Department of Energy. The authors thank Dr. Karen Koefoed Petersen, Danish Institute of Plant and Soil Sciences, Denmark, for providing valuable expertise and advice. We also thank Dr. Richard Harkess, Department of Plant and Soil Sciences, and Dr. Maria Tomaso-Peterson, Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, both from Mississippi State University, for providing editorial comments on previous drafts.

References

Atkinson CJ (2009) Establishing perennial grass energy crops in the UK: a review of current propagation options for *Miscanthus*. Biomass Bioenergy 33:752–759

Baldwin BS (2013) Miscanthus plant named 'MSU-MFL1'. US Plant Patent 23:489

Chouvarine P, Cooksey AM, McCarthy FM, Ray DA, Baldwin BS, Burgess SC, Peterson DG (2012) Transcriptome-based differentiation of closely-related *Miscanthus* lines. PLoS One 7(1):e29850. doi:10.1371/journal/pone.0029850

Cichorz S, Gośka M, Litwiniec A (2014) *Miscanthus*: genetic diversity and genotype identification using ISSR and RAPD markers. Mol Biotechnol. doi:10.1007/s12033-014-9770-0

da Costa MLM, Amorim LLB, Onofre AVC, de Melo LJOT, de Oliveira MBM, de Carvalho R, Benko-Iseppon AM (2011) Assessment of genetic diversity in contrasting sugarcane varieties using inter-simple sequence repeat (ISSR) markers. Am J Plant Sci 2:425–432

Dhugga KS (2007) Maize biomass yield and composition for biofuels. Crop Sci 47:2211–2227

Fett-Neto AG, Fett JP, Goulart LWV, Pasquali G, Termignoni RR, Ferreira AG (2001) Distinct effects of auxin and light on adventitious root development in *Eucalyptus saligna* and *Eucalyptus globulus*. Tree Physiol 21:457–464

Głowacka K, Jeżowski S, Kaczmarek Z (2010) The effects of genotype, inflorescence developmental stage and induction medium on callus induction and plant regeneration in two *Miscanthus* species. Plant Cell Tissue Organ Cult 102:79–86

Głowacka K, Clark LV, Adhikari S, Peng J, Stewart JR, Nishiwaki A, Yamada T, Jørgensen U, Hodkinson TR, Gifford J, Juvik JA, Sacks EJ (2014) Genetic variation in *Miscanthus* × *giganteus* and the importance of estimating genetic distance thresholds for differentiating clones. GCB Bioenergy. doi:10.1111/gcbb.12166

Gubišová M, Gubiš J, Žofajová A, Mihálik D, Kraic J (2013) Enhanced *in vitro* propagation of *Miscanthus* × *giganteus*. Ind Crop Prod 41: 279–282

Heaton EA, Dohleman FG, Long SP (2008) Meeting US biofuel goals with less land: the potential of Miscanthus. GCB Bioenergy 14: 2000–2014

Heaton EA, Dohleman FG, Miguez AF, Juvik JA, Lozovaya V, Widholm J, Zabotina OA, McIsaac GF, David MB, Voigt TB, Boersma NN, Long SP (2010) Miscanthus: a promising biomass crop. In: Kader JC, Delseny M (eds) Advances in botanical research, vol 56. Elsevier, Oxford, pp 76–137

Hodkinson TR, Chase MW, Renvoize SA (2002) Characterization of a genetic resource collection for *Miscanthus* (Saccharinae, Andropogoneae, Poaceae) using AFLP and ISSR PCR. Ann Bot 89:627–636

Holme IB, Petersen KK (1996) Callus induction and plant regeneration from different explant types of *Miscanthus* × *ogiformis* Honda 'Giganteus'. Plant Cell Tissue Organ Cult 45:43–52

Holme IB, Krogstrup P, Hansen J (1997) Embryogenic callus formation, growth and regeneration in callus and suspension cultures of *Miscanthus* × *ogiformis* Honda Giganteus' as affected by proline. Plant Cell Tissue Organ Cult 50:203–210

Jessup RW (2009) Development and status of dedicated energy crops in the United States. In Vitro Cell Dev Biol Plant 45:282–290

Kim HS, Zhang G, Juvik JA, Widholm JM (2010) *Miscanthus* × *giganteus* plant regeneration: effect of callus types, ages and culture methods on regeneration competence. GCB Bioenergy 2:192–200

Kim S, Da K, Mei C (2012) An efficient system for high-quality large-scale micropropagation of *Miscanthus* × *giganteus* plants. In Vitro Cell Dev Biol Plant 48:613–619

Lewandowski I (1997) Micropropagation of *Miscanthus* × *giganteus*. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 39, High-tech and micropropagation V. Springer, Berlin, pp 239–255

Lewandowski I (1998) Propagation method as an important factor in the growth and development of *Miscanthus* × *giganteus*. Ind Crop Prod 8:229–245

Lewandowski I, Kahnt G (1993) Development of a tissue culture system with unemerged inflorescences of *Miscanthus* '*Giganteus*' for the induction and regeneration of somatic embryoids. Beitr Biol Pflanzen 67:439–451

Lewandowski I, Clifton-Brown J, Scurlock J, Huisman W (2000) Miscanthus: European experience with a novel energy crop. Biomass Bioenergy 19:209–227

Lewandowski I, Scurlock JM, Lindvall E, Christou M (2003) The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe. Biomass Bioenergy 25:335–361

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Nielsen JM, Brandt K, Hansen J (1993) Long-term effects of thidiazuron are intermediate between benzyladenine, kinetin or isopentenyladenine in *Miscanthus sinensis*. Plant Cell Tissue Organ Cult 35:173–179

Perera D, Barnes DJ, Baldwin BS, Reichert NA (2015) Mutagenesis of *in vitro* cultures of *Miscanthus* × *giganteus* cultivar Freedom and detecting polymorphisms of regenerated plants using ISSR markers. Ind Crop Prod 65:110–116

Petersen KK (1997) Callus induction and plant regeneration in *Miscanthus* × *ogiformis* Honda 'Giganteus' as influenced by benzyladenine. Plant Cell Tissue Organ Cult 49:137–140

Petersen KK, Hansen J, Krogstrup P (1999) Significance of different carbon sources and sterilization methods on callus induction and plant regeneration of *Miscanthus* × *ogiformis* Honda 'Giganteus'. Plant Cell Tissue Organ Cult 58:189–197

Rambaud C, Arnoult S, Bluteau A, Mansard MC, Blassiau C, Brancourt-Hulmel M (2013) Shoot organogenesis in three *Miscanthus* species and evaluation for genetic uniformity using AFLP analysis. Plant Cell Tissue Organ Cult 113:437–448

USDA (2012) Farm Service Agency energy programs, Biomass Crop Assistance Program (BCAP) project areas listing, Beltsville, MD. Document3 http://www.fsa.usda.gov/FSA/webapp?area=home&subject=ener&topic=bcap-pjt-bloc. Cited 05 Jan 2015

Venturi P, Huisman W, Molenaar J (1998) Mechanization and costs of primary production chains for *Miscanthus* × *giganteus* in The Netherlands. J Agric Eng 69:209–215

White J, Lemus R, Rushing B, Johnson B, Saunders JR, Rivera, D, Slusher P (2014) Mississippi biomass feedstock variety trials, 2013. Info Sheet No 1363. Mississippi agricultural & forestry experiment station. http://msucares.com/pubs/infosheets_research/is1363.pdf

Williams MJ, Douglas J (2011) Planting and managing giant miscanthus as a biomass energy crop. Tech Note No 4. USDA Natural resources conservation service, plant materials program. http://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/16/stelprdb1044768.pdf

Yu CY, Kim HS, Rayburn AL, Widholm JA, Juvik JA (2009) Chromosome doubling of the bioenergy crop, *Miscanthus* × *giganteus*. GCB Bioenergy 1:409–412

Zhao L, Hu H, Zhan H, Diao Y, Jin S, Zhou F, Hu Z (2013) Plant regeneration from the embryogenic calli of five major *Miscanthus* species, the non-food biomass crops. In Vitro Cell Dev Biol Plant 49:383–387

Zub H, Brancourt-Hulmel M (2010) Agronomic and physiological performances of different species of *Miscanthus*, a major energy crop. A review. Agron Sustain Dev 30:201–214