Mutagenesis of *in vitro* cultures of *Miscanthus x giganteus* cultivar Freedom and detecting polymorphisms of regenerated plants using ISSR markers

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Abbreviations: Mxg *Miscanthus x giganteus*; EMS Ethyl methanesulfonate; DMSO Dimethyl sulfoxide; ISSR Inter simple sequence repeat; 2,4-D 2,4-dichlorophenoxyacetic acid; BA 6-benzylaminopurine; NAA α -naphthaleneacetic acid; RP Recovery percentage

Abstract

Giant miscanthus (*Miscanthus x giganteus*; Mxg) is a key candidate energy crop for use in biomass to liquid fuel production. It is a naturally seed sterile triploid lacking genetic variation, the basis for selection. Therefore, induced mutation techniques are particularly important in Mxg. The objective of this research was to induce variation in Freedom, an elite cultivar, through *in vitro* chemical mutagenesis. A previously-optimized *in vitro* propagation protocol for Freedom was used following the *in vitro* mutagenesis procedure. Immature inflorescence explants (2-3 mm) and calli (2-3 mm³) were treated with five mutagenic dose treatments [0.01%, 0.1%, 0.5%, 1%, and 3% (v/v) of ethyl methanesulfonate (EMS) for 90 min] along with 2% (v/v) dimethyl sulfoxide (DMSO) as a carrier agent to determine the optimum mutagen dosage. The dose at which 50% of the calli/explants recovered (RP₅₀) after the mutagen treatment was used as the optimum EMS dose. Calli and explants of Mxg were treated with RP₅₀, 2 x RP₅₀, and 3 x RP₅₀ EMS dosages, and subsequent regenerants that arose from explants/calli were transferred to soil. Inter simple sequence repeat (ISSR) markers were used to identify variants in the regenerated plants. Results showed variability among regenerants originating from EMS mutagenic treatments. Putative mutants of Mxg may be useful for bioenergy research and functional genomics.

Keywords: biofuel crop, crop improvement, genetic variation, microsatellite, *in vitro* culture

1 Introduction

Giant miscanthus (*Miscanthus x giganteus*; Mxg), a C₄ perennial grass, is a key candidate energy crop for use in biomass to liquid fuel production underlying positive agronomic characteristics such as great dry matter yield, low input requirements (fertilizer and pesticides), and high use efficiencies for solar radiation, water, and nitrogen (Heaton et al., 2008; Lewandowski et al., 2000; Lewandowski et al., 2003; Zub and Brancourt-Hulmel, 2010). Freedom (MSU-MFL1), resulting from selection of individual rhizomes/tillers for the most vigorous growth (serial propagation), is an elite cultivar of Mxg developed by Mississippi State University (Baldwin, 2013). The new distinct cultivar which was observed to have exceptionally vigorous growth accounts for approximately 9% of the Mxg acreage and has excellent applicability as an efficient biomass grass (Baldwin, 2013; Głowacka et al., 2014). It is a vegetatively propagated, naturally seed sterile triploid ($2n=3x=57$) lacking genetic variation, the basis for selection (Cichorz et al., 2014; Karp and Shield, 2008; Zub and Brancourt-Hulmel, 2010). Due to sterility and lack of genetic variation, progress of Mxg improvement by conventional breeding methods is hindered, thereby increasing reliance on spontaneous mutations. Exploiting induced genetic diversity through mutagenesis to create novel variation is a proven strategy in the improvement of crops with restricted genetic variability (Parry et al., 2009; van Harten, 1998).

Induced mutations have played a pivotal role in improving crops with economically important traits such as high yield, biotic and abiotic stress resistance, and early/late maturity etc. (Ahloowalia and Maluszynski, 2001; Maluszynski et al., 1995). To date, greater than 3000 mutant varieties including cereals, legumes, ornamental plants, as well as fiber, edible oil, and fruit species have been officially released, either as direct mutants selected from mutated populations or indirectly derived through crosses with induced mutants (Ahloowalia et al., 2004;

Jain and Suprasanna, 2011; Maluszynski, 2000; Maluszynski et al., 1995). Mutations are induced by physical (ionizing radiation such as gamma ray, X-ray, fast neutron etc.) and chemical [ethyl methane sulfonate (EMS), sodium azides etc.] mutagen treatments of both seed and vegetatively propagated crops (Pathirana, 2011; van Harten, 1998). Chemical mutagenesis is preferred over ionizing radiation for several reasons. Chemical mutagens have been demonstrated to produce more gene mutations (point mutations) with fewer chromosomal disruptions and can be applied relatively easily (Gottschalk and Wolff, 1983; Predieri and Di Virgilio, 2007). EMS, a chemical mutagen, is an alkylating agent which has been successfully used to introduce point mutations inducing C-to-T changes resulting in C/G to T/A substitutions [C-cytosine, T-thymine, G-guanine and A-adenine] (Pathirana, 2011; Sega, 1984).

In vitro mutagenesis, which couples a mutagenic treatment with a regeneration procedure, has several advantages over *in vivo* mutagenesis; it can: offer a wide variety of plant materials for mutagenesis, enable the generation of large mutant populations in a relatively short period of time, and provide an opportunity to use small explants and continuous subculture which minimizes the chances of producing chimeras (Jain et al., 2010; Pathirana, 2011). While the number of reports on cultivars derived from *in vivo* mutagenesis has increased, relatively few economically important cultivars have been released via *in vitro* mutagenesis (Ahloowalia and Maluszynski, 2001; Maluszynski, 2000; Xu et al., 2012). These include *in vitro* mutagenesis protocols developed for clonally propagated crops such as potato, sweet potato, cassava, date palm, sugarcane, and banana using physical and chemical mutagens (Ahloowalia and Maluszynski, 2001; Bhagwat and Duncan, 1998; Bidabadi et al., 2012; Chen et al., 2013; Jain, 2010a; Jain et al., 2011; Jankowicz-Cieslak et al., 2012; Xu et al., 2012).

Callus cultures of soybean (*Glycine max*), groundnut (*Arachis hypogaea*), sweetpotato (*Ipomoea batatas*), date palm (*Phoenix dactylifera*), and sugarcane (*Saccharum officinarum*), a close relative of Mxg, treated with varying EMS concentrations (0.01%-1%) and mutagenic treatment times (30 min-4 hrs) resulted in successful mutagenesis protocols (Hofmann et al., 2004; Koch et al., 2012; Luan et al., 2007; Mahlanza et al., 2013; Muthusamy et al., 2007; Omar and Novak, 1990). Besides the EMS concentration and treatment time, the type of the solvent [e.g. dimethyl sulfoxide (DMSO)] used in EMS solution affected overall mutagenic effects (Bhatia, 1967; Jain, 2010b). Efficient mutagen (EMS) uptake in date palm somatic embryos was significantly enhanced by use of 4% DMSO as a carrier agent (Omar and Novak, 1990).

Mutagenesis of banana, primarily used for improvement of triploid banana (*Musa* spp., AAA group), resulted in development of some of the leading cultivars with enhanced traits including early ripening, better flavor, and disease resistance (e.g. black sigatoka and fusarium wilt) (Ahloowalia and Maluszynski, 2001; Jain, 2010a). *In vitro* mutagenesis of banana using shoot tip cultures and micro-cross-sections of pseudostems treated with varying EMS concentrations (0.5%-4%) and treatment times (30 min-120 min) generated successful mutagenesis protocols (Bhagwat and Duncan, 1998; Bidabadi et al., 2012; Chen et al., 2013; Jain, 2010a). According to Bidabadi et al. (2012), the average number of shoots per explant, survival percentage, and fresh weight of treated shoot tips were significantly influenced by EMS treatments. Further, cultivar effects were noted; the EMS treatment that yielded reduced growth and proliferation varied by cultivar.

Molecular markers are widely used as tools to detect and characterize somaclonal variations and induced mutations at the genome level (Agarwal et al., 2008; Barakat and El-Sammak, 2011; Khan et al., 2007; Khan et al., 2009). Of the available markers, inter simple

sequence repeat (ISSR) markers have been noted to be more reliable and reproducible than random amplified polymorphic DNA (RAPD) markers which have been used more frequently (Hodkinson et al., 2002; Reddy et al., 2002). Furthermore, RAPD markers have been found to be very sensitive to experimental conditions due to use of short primers and low annealing temperatures, and laboratory-to-laboratory variation tends to limit the utility of RAPD markers (Reddy et al., 2002; Vermerris, 2008). ISSR molecular markers rely on the detection of variation in the number of simple sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome and are good targets for PCR-based fingerprinting techniques (Agarwal et al., 2008; Qian et al., 2001). As a result, the ISSR technique is a powerful method to identify genetic diversity among individuals. Researchers have used ISSR procedures in Mxg to characterize accessions, and in related species of Mxg such as sorghum (*Sorghum bicolor*) and sugarcane to differentiate somaclones and induced mutants (Cichorz et al., 2014; da Costa et al., 2011; Hodkinson et al., 2002; Smith et al., 2000; Varshney et al., 2005).

There are no previously reported studies of EMS-induced mutations in Mxg. The goal of the present investigation was to induce mutations in Mxg cv. Freedom through *in vitro* mutagenesis by treating Mxg explants and callus with EMS, then applying ISSR analysis for the detection of genetic polymorphisms among Mxg mutants compared to untreated including their mother plants (i.e. rhizome-propagated plants). Variability was noted among regenerants arising from EMS mutagenic treatments, and was confirmed by ISSR marker analysis.

2 Materials and Methods

2.1 Callus induction

Mxg cultivar Freedom was used in all experiments. Our previously-optimized *in vitro* regeneration protocol developed for Freedom was incorporated into this *in vitro* mutagenesis

procedure. Immature inflorescence explants (2-3 mm in length) from field-grown Mxg plants were utilized during the callus induction procedure; immature inflorescences (0.5-6 cm in length) were surface disinfested as intact stems (covered with the innermost leaf sheaths) in 6% sodium hypochlorite (NaClO) for 15 min, rinsed three times with sterile deionized water, then immature inflorescences were dissected out (2-3 mm in length). The explants were cultured on a callus induction medium based on Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium (mineral salts and vitamins), and contained sucrose (30 g L^{-1}), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (750 mg L^{-1}), proline (2878 mg L^{-1}), 3 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg L^{-1} 6-benzylaminopurine (BA). The medium (pH 5.5) was solidified with 2 g L^{-1} Phytigel™ (Sigma-Aldrich, St. Louis, MO) and autoclaved at 121°C at 15 psi for 25 min. Cultures were incubated in growth chambers (CU-32L, Percival Scientific Inc., Boone, Iowa) under dark conditions at $27 \pm 2^\circ\text{C}/25 \pm 2^\circ\text{C}$ day/night temperatures and subcultured every week for the first two weeks and then at two-week intervals for four weeks.

2.2 Dosage optimization of EMS for Mxg explants and calli

Immature inflorescence explants (2-3 mm in length) and callus ($2\text{-}3 \text{ mm}^3$) from six-week old cultures (calli capable of organogenic and somatic embryogenic regeneration) were transferred to aqueous solutions containing 0.01%, 0.1%, 0.5%, 1%, and 3% (v/v) EMS plus contained 2% (v/v) dimethyl sulfoxide (DMSO) as a carrier agent, and shaken on an orbital shaker (75 rpm at 25°C) 90 min in order to optimize mutagenic dosage. An aqueous solution of 2% DMSO without EMS was used as a control. After EMS treatment, explants and calli were rinsed three times in sterilized distilled water and placed on callus induction and shoot regeneration media, respectively. Four explants and calli were placed per Petri dish (60x15 mm) and Magenta box (3x3x4 inches), respectively. Each treatment consisted of 15 vessels (i.e. Petri

dishes/Magenta boxes). Immature inflorescence explants on callus induction media were incubated in the dark at $27\pm 2^{\circ}\text{C}/25\pm 2^{\circ}\text{C}$ day/night temperatures. The shoot regeneration medium, based on MS basal medium (mineral salts and vitamins), was supplemented with sucrose (20 g L^{-1}), 0.24 mg L^{-1} NAA, and 4.95 mg L^{-1} BA (Kim et al., 2010; Seong et al., 2010) and solidified with 0.7% agar (Sigma-Aldrich, St. Louis, MO). These cultures were incubated in a growth chamber under a 16 h photoperiod ($125\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ illumination with warm white fluorescent bulbs) at $25\pm 2^{\circ}\text{C}/22\pm 2^{\circ}\text{C}$ day/night temperatures. The Petri dishes and Magenta boxes were arranged in a completely randomized design within each growth chamber (dark and light). Four weeks after mutagen treatment, the number of recovered explants/calli was scored separately for each of the six treatments and culture type. A “recovered” explant/callus was one that displayed visual growth after four weeks post-mutagenic treatment. The recovery percentage (RP) of explants/calli for each treatment category was calculated: $\text{RP} = [\text{the number of explants or calli recovered (i.e. resumed growth) after the mutagen treatment} / \text{total number of explants or calli subjected to that mutagen treatment}] \times 100$; RP was determined four weeks post-treatment. The experiment was repeated three times.

2.3 Statistical analysis

The RP data were subjected to logistic regression analysis through a revised logistic function for dose response data ($p < 0.05$) using SlideWrite software (Advanced Graphics Software, Inc., Encinitas, CA). Mean RP of explants/calli versus EMS treatment concentration were graphed; the dose at which 50% of the explants or calli recovered after mutagen treatment (RP_{50}) was determined for each and represented their RP_{50} EMS dose.

2.4 Treating Mxg explants and callus with EMS

The explants and calli were treated with RP_{50} , 2 x RP_{50} , and 3 x RP_{50} EMS dosages. For the explants, the RP_{50} was 0.6% EMS whereas the RP_{50} for the callus was 1.2%. Therefore, the explants were treated with 0.6%, 1.2%, and 1.8% EMS in 2% DMSO for 90 min whereas the calli were treated with 1.2%, 2.4%, and 3.6% EMS in 2% DMSO for 90 min. Control explants and calli were treated with DMSO alone; rhizome-propagated control plants received no treatment.

2.5 Plant regeneration and rooting

After mutation treatment, explants/calli were transferred to callus induction/shoot regeneration media, respectively, and plants were regenerated as described above. Rooting was carried out in a two-step process: shoots were transferred to one-half strength (0.5X) MS medium supplemented with 1 mg L^{-1} NAA for three days then transferred to 0.5X PGR-free MS medium containing 0.2% activated charcoal for two weeks. Rooted plantlets were transferred to autoclaved soil (1 part Star Green Moisture MaxTM Potting Mix:1 part sand) and acclimatized for 3-4 days under low light ($60 \mu\text{mol m}^{-2} \text{ s}^{-1}$ illumination with warm white fluorescent bulbs) at room temperature (25°C), then transferred to the greenhouse. A total of 737 plantlets were recovered from all mutagenic treatments; of total, 127 plantlets were generated from explants and calli treated with optimum EMS concentrations (i.e. RP_{50} , 2 x RP_{50} , and 3 x RP_{50}). Sixteen out of 127 plantlets were selected for further molecular analysis on the basis of phenotyping through visual observation (early screening) where putative off-types were observed. Those included dwarf off-types (5.5%) and plantlets with chlorotic patches and stripes (3.9%) on their leaves.

2.6 ISSR PCR amplification

Total genomic DNA from individual field-grown regenerants was extracted from leaf samples stored at -80 °C using DNeasy Plant Mini kit (Valencia, CA). Inter simple sequence repeat (ISSR) PCR analysis was carried out using 21 primers which were previously screened in *Mxg* and some other closely related species (da Costa et al., 2011; Hodkinson et al., 2002). Amplifications were performed using 100 ng template DNA, 100 µM of each dNTP, 2 mM MgCl₂ in a 50 µl reaction mix using 0.5 units of *Taq* polymerase (Lucigen, Middleton, WI) in a Peltier thermal cycler (PTC-200, MJ Research, Waltham, MA). The thermal cycling program used included preliminary DNA denaturation for 6 min at 94°C, followed by 40 cycles, each with 20 s denaturation at 93°C, 1 min annealing at 48°C to 54°C (depending on the primer), 20 s elongation at 72°C, and final elongation of 6 min at 72°C. The reaction products (10 µl) were separated in 2% horizontal Metaphor agarose (Lonza, Rockland, ME) gels in TBE buffer with a voltage of 5 V/cm. A 100 bp DNA ladder (New England Biolabs, Beverly, MA) was used to compare the DNA band lengths of amplified products. The gels were stained with 0.5 µg/mL ethidium bromide and scanned under VersaDoc imager (Bio-Rad, Hercules, CA). The number of bands generated by each primer was scored, and the frequency of polymorphism was calculated.

2.7 Cluster analysis

Putative mutants regenerated through *in vitro* mutagenesis procedures were compared with each other using amplification profiles. Data were scored for statistical analysis on the basis of the presence of the amplified products for each primer. Bands that were unique to one or more of the putative mutants were scored. Profiles of DNA were scored as presence of each individual band (1) or absence of band (0) from ISSR amplification images; only clear, unambiguous, and reproducible bands were considered as “scorable” for the analysis. Bands that were present in all

lanes were not scored. The Jaccard coefficient was used to compute a similarity matrix and then to construct a dendrogram using a server running the program DendroUPGMA (<http://genomes.urv.cat/UPGMA/>) (Garcia-Vallvé et al., 1999). The dendrogram, based on these similarity coefficients, was constructed by using the unweighted pair group method of arithmetic means (UPGMA) (Mohammadi and Prasanna, 2003).

3 Results

3.1 Optimum EMS dosage for Mxg explants and callus

There were significant differences among recovery of explants/calli on their respective medium four weeks after mutagenic treatment. Recovery of the Mxg explant/callus cultures decreased significantly with the increased dosage of EMS. The mean recovery percentage (RP) of Mxg inflorescence explants of controls were 92%, then decreased from 80% (0.1% EMS) to 17% (3% EMS) (Figure 1). Moreover, the RP of control calli were 95%, then decreased from 85% (0.1% EMS) to 32% (3% EMS) (Figure 2). The RP_{50} , dose at which 50% of the explants/calli recovered after the mutagen treatment, for Mxg inflorescence explants and callus were 0.6% and 1.2%, respectively.

3.2 Molecular characterization of EMS induced Mxg putative mutants using ISSR PCR amplification

The genetic variability of sixteen (C1-C16) Mxg putative mutant regenerants (Table 1) arising from the three RP_{50} (RP_{50} , 2 x RP_{50} , and 3 x RP_{50}) EMS treatments along with two controls [C: *in vitro* regenerated plants from non-mutagen treated shoot-forming callus (DMSO treatment alone) and CR: traditional rhizome-propagated plant)] was assessed using ISSR markers. For example, Figure 3 illustrates the amplification profile generated by ISSR PCR using primer Mxg8 which resulted in five scorable bands (shown in arrows) noted to be

polymorphic among putative mutants compared to controls. Three out of the 21 tested primers detected polymorphisms among putative mutant regenerants arising from explants/calli subjected to the three RP₅₀ EMS treatments (Table 2). The number of amplified DNA fragments ranged from seven to eleven, depending on the primer and the DNA sample, with a mean value of nine bands per primer. A total of 28 fragments were produced by the three primers. Of these 28 amplified fragments, 12 (42.85 %) were polymorphic in one or more of the 16 individuals. The ISSR markers produced by three primers (Mxg3, Mxg7 and Mxg8; Table 2) were used to construct a similarity matrix where the Jaccard similarity estimates of the pairwise comparisons among the control and putative mutants were based on 12 polymorphic bands; rhizome propagated plant (CR) were not included in this analysis since this control continuously produced similar banding patterns (for each primer used) to the *in vitro* generated non-mutagen treated plant (C) (Table 3). The Jaccard similarity coefficients, ranging from 0.67 to 1.00, were helpful in generating clusters of the non-mutagen treated plants (i.e. control) and 16 putative mutants (Figure 4). Mainly, two clusters could be observed; the smaller cluster included C3 and C9 putative mutants while the larger cluster included the control (i.e. C: *in vitro* generated non-mutagen treated plant) and the remaining putative mutants.

4 Discussion

Although most induced mutations are recessive and deleterious, mutations have contributed to crop improvement worldwide, including development of enhanced food, feed, and fiber. Induced mutation techniques are particularly important in Mxg since it is a natural sterile triploid lacking genetic variation, the basis for selection (Maluszynski et al., 1995). The present investigation was designed to induce mutations in Mxg cv. Freedom through *in vitro* mutagenesis and investigate the efficiency of ISSR markers in accurately assessing genetic

differences between Mxg putative mutants and traditional rhizome-propagated plants. In mutation breeding, it is generally accepted that the frequency of mutations increase with an increased dosage which is a combination of mutagen concentration and treatment duration (Bhagwat and Duncan, 1998). However, the survival of mutagen-treated tissues and their ability to regenerate into plants decrease with an increased mutagen treatment, and the incidence of unfavorable mutations increases (Bhagwat and Duncan, 1998). Since Mxg is sterile, it would be impossible to separate/segregate unfavorable mutations by cross-breeding or back-crossing. Therefore, an important consideration in this study, as in other mutagenic studies, was to optimize EMS dosage in order to effectively induce mutations with the lowest doses needed. In general, mutagen dosage is optimized by using various mutagen concentrations or treatment durations or a combination of both. Longer treatment durations, such as 4 hrs, were employed in previous *in vitro* mutagenesis protocols using callus cultures of sweet potato, date palm, sugarcane, and soybean (Hofmann et al., 2004; Koch et al., 2012; Luan et al., 2007; Omar and Novak, 1990). However, longer treatment durations may not necessarily be the best criterion for enhanced mutagenic effects since effectiveness of EMS drastically decreases with time as EMS is oxidized to toxic hydrolytic compounds, leading to tissue destruction (Omar and Novak, 1990). Therefore, in our study, we treated Mxg explant and callus cultures for 90 min and instead, used higher EMS concentrations for overall greater mutagenic effect.

Analysis of variance (data not shown) revealed that the tested EMS dosages (0-3%) yielded highly significant variations in explant versus callus recovery percentages. The type of plant material (inflorescence explant or callus) had a significant effect on RP with inflorescence explants having a smaller RP than callus cultures at every EMS dosage tested. Therefore, the

1 RP₅₀ concentrations differed, further indicating the importance of optimizing mutagen dosage for
2 each plant material used during the *in vitro* mutagenesis procedure.

3 Theoretically, the greatest frequency of mutations could be expected from a mutagen
4 treatment killing about 50% of the treated material (LD₅₀), a value frequently used in physical
5 mutagenesis procedures (Jain, 2010a; van Harten, 1998). However, LD₅₀ may not be the best
6 criterion for determining optimum chemical mutagen dosage. Previous studies on *in vitro*
7 chemical mutagenesis used other criteria in determining the optimum EMS dosage, although all
8 studies were in agreement that performance of mutagen-treated tissues decreased with increased
9 mutagen dosage. For example, in several studies of banana where shoot tips were treated with
10 various EMS dosages, visual characters (reduced growth and morphogenic performance,
11 proliferation rate, and regeneration capacity) were scored; the concentrations noted to yield 50%
12 responses compared to untreated controls were utilized as optimum EMS dosages instead of
13 survival percentage (LD₅₀) (Bhagwat and Duncan, 1998; Bidabadi et al., 2012; Chen et al.,
14 2013). The use of these parameters might partly be due to the occurrence of quiescent (non-
15 responsive yet alive) cells/tissues which showed no further growth even after subculturing for six
16 months post-EMS treatment (Bidabadi et al., 2012). Our study is in agreement; post-
17 mutagenesis, we noted that some Mxg calli that remained quiescent even after continuous
18 subculturing. Therefore, we used recovery percentage (RP), which was the percentage of
19 explants/calli that resumed growth after EMS treatment, and calculated their respective RP₅₀
20 values to be our optimum EMS dosages.

21 Researchers have previously determined that DMSO can be successfully utilized as a
22 carrier agent during *in vitro* mutagenesis coupled with chemical mutagens. Uptake of EMS into
23 somatic embryos of date palm was significantly enhanced when 4% DMSO was incorporated

1 into the EMS solution (Omar and Novak, 1990). Nevertheless, the use of lower percentages of
2 DMSO is recommended to prevent browning of explants and to enhance mutagen absorption;
3 use of 1% DMSO, along with EMS, in banana shoot tip cultures resulted in successful generation
4 of mutants (Bidabadi et al., 2012; Omar and Novak, 1990). During our preliminary studies
5 (results not shown) on Mxg explants and calli using EMS solutions with and without DMSO,
6 DMSO treated explants/calli displayed the greatest damage indicating incorporation of DMSO
7 into EMS solutions may have enhanced the penetration of EMS into the tissues.

8 The genetic variability created in Mxg through *in vitro* mutagenesis was efficiently
9 assessed by ISSR markers. Polymorphic fragments, revealed by three ISSR primers, among
10 Freedom regenerants arising from *in vitro* mutagenesis were most likely due to treatment with
11 the chemical mutagen, EMS. Besides alterations in template DNA, primary factors which aid in
12 determining the extent of polymorphism include nature (anchored/unanchored) of the primer and
13 sequence of the repeats in the primer (Reddy et al., 2002). In general, di-nucleotide repeats,
14 anchored either at 3' or 5' end reveal high polymorphism; this is in agreement with our results
15 where three primers (i.e. Mxg3, Mxg7, and Mxg8) that revealed polymorphic fragments were
16 anchored-di-nucleotide repeats. Polymorphisms noted in ISSR analysis indicated presence of
17 genetic differences among plants regenerated from EMS treated explants/calli of Mxg compared
18 to controls. Noted polymorphisms were likely due to mutations occurring in primer binding sites
19 resulting in an increase or decrease in the total number of primer binding sites, and consequently,
20 the number of amplified fragments, although mutations that occurred within the amplified
21 fragments wouldn't be detected (Hofmann et al., 2004; Reddy et al., 2002). The Jaccard
22 similarity coefficients reflected the genetic relationship between the Mxg regenerants. The
23 highest similarity (1.00) was observed between (C11, C12, and C14), (C15 and C16), and (C6

1 and C8) whereas the lowest similarity value (0.67) was observed between (C3 and C13), and (C4
2 and C13). The results of the UPGMA cluster, based on Jaccard similarity coefficients ranging
3 from 0.67 to 1.00, suggest a relatively narrow genetic base for these putative mutants.

4 **5 Conclusions**

5 ISSR markers are useful in detecting polymorphisms in Mxg Freedom regenerants
6 resulting from *in vitro* mutagenic treatments. These ISSR polymorphisms indicated presence of
7 genetic differences among the Freedom regenerants, although it is not yet known whether these
8 mutations will yield alterations in agronomically useful traits. These mutant plants may be useful
9 for bioenergy research and/or functional genomics of Mxg. Furthermore, this developed *in vitro*
10 mutagenic protocol, reported for the first time in Mxg, may be utilized for generation and
11 screening of mutants leading to the development of new cultivars.

13 **Acknowledgements**

14 This research was funded by Repreve Renewables Inc. and underwritten by the U.S.
15 Department of Energy. The authors thank Dr. Ranjith Pathirana, Plant and Food Research
16 Institute, Palmerston North, New Zealand for providing valuable expertise.

Table 1 Origin of sixteen *Miscanthus x giganteus* Freedom putative mutants resulting from EMS mutagenesis of explant and callus cultures along with the controls.

Mxg putative mutants	Origin	
	Plant material	EMS treatment (%)
C	Shoot-forming callus	None
C1	Inflorescence explant	1.20
C2	Embryogenic callus	1.20
C3	Embryogenic callus	2.40
C4	Shoot-forming callus	2.40
C5	Inflorescence explant	1.20
C6	Inflorescence explant	1.80
C7	Embryogenic callus	3.60
C8	Inflorescence explant	1.80
C9	Shoot-forming callus	3.60
C10	Shoot-forming callus	3.60
C11	Inflorescence explant	1.80
C12	Shoot-forming callus	2.40
C13	Embryogenic callus	2.40
C14	Embryogenic callus	1.20
C15	Shoot-forming callus	3.60
C16	Inflorescence explant	0.60
CR	Rhizome	None

Controls are C and CR [i.e. *in vitro* generated non-mutagen treated plants and mother (i.e. rhizome propagated) plants, respectively].

Putative mutants originated from callus (shoot-forming callus and embryogenic callus) were resulted from immature inflorescence explants grown on callus induction media prior to treatment; each was capable of both regeneration pathways so regenerants were listed according to how they arose.

1 Table 2 Number of amplification and polymorphic products, using three primers out of 21 tested in *Miscanthus x giganteus*
2 Freedom putative mutants.

Primer number	Nucleotide sequence (5'-3')	Total number of bands per primer	Total number of polymorphic bands per primer	Polymorphism percentage
Mxg3	AGAGAGAGAGAGAGAGYT	7	3	42.85
Mxg7	CTCTTCTCTCTCTCTG	11	4	36.36
Mxg8	ACACACACACACACACYG	10	5	50.00

3 Y = (C,T)

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Table 3 Jaccard similarity coefficients between *Miscanthus x giganteus* Freedom *in vitro* generated control plant (C) and putative mutants (C1-C16) were calculated based on inter simple sequence repeat amplification products using Mxg3, Mxg7, and Mxg8 primers.

C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16
C	1.00	0.95	0.95	0.86	0.76	0.82	0.78	0.82	0.70	0.95	0.83	0.83	0.78	0.83	0.86	0.86
C1		1.00	0.91	0.82	0.80	0.86	0.83	0.86	0.74	0.91	0.87	0.87	0.83	0.87	0.91	0.91
C2			1.00	0.81	0.72	0.75	0.77	0.74	0.73	0.90	0.78	0.78	0.82	0.78	0.82	0.82
C3				1.00	0.72	0.75	0.77	0.74	0.81	0.81	0.71	0.71	0.67	0.71	0.74	0.74
C4					1.00	0.96	0.83	0.88	0.72	0.72	0.70	0.70	0.67	0.70	0.73	0.73
C5						1.00	0.87	0.83	0.75	0.75	0.73	0.73	0.69	0.73	0.76	0.76
C6							1.00	1.00	0.86	0.86	0.83	0.83	0.78	0.83	0.86	0.86
C7								0.95	0.82	0.82	0.79	0.79	0.75	0.79	0.83	0.83
C8									1.00	0.86	0.83	0.83	0.78	0.83	0.86	0.86
C9										1.00	0.73	0.71	0.74	0.71	0.74	0.74
C10											1.00	0.86	0.82	0.86	0.91	0.91
C11												1.00	0.96	1.00	0.96	0.96
C12													1.00	1.00	0.96	0.96
C13														1.00	0.96	0.91
C14															1.00	0.96
C15																1.00
C16																1.00

CR (i.e. rhizome propagated plant) was not included in this analysis since it continuously produced similar banding patterns (for each primer used) to the *in vitro* generated non-mutagen treated plant (C). Numbers in bold indicate range of the Jaccard similarity coefficients.

Figure captions

Figure 1. Recovery percentage (RP) of *Miscanthus x giganteus* (Mxg) Freedom inflorescence explants (2-3 mm) on callus induction medium four weeks after 90 min EMS treatments. The dose at which 50% of the explants recovered after the mutagen treatment (RP₅₀) was used as the optimum EMS dose for Mxg inflorescence explants.

Figure 2. Recovery percentage (RP) of *Miscanthus x giganteus* (Mxg) Freedom callus (2-3 mm³) on shoot regeneration medium four weeks after 90 min EMS treatments. The dose at which 50% of the calli recovered after the mutagen treatment (RP₅₀) was used as the optimum EMS dose for Mxg callus cultures.

Figure 3. The amplification profiles, generated by primer Mxg8 indicating ISSR polymorphism in *Miscanthus x giganteus* (Mxg) Freedom putative mutants compared to *in vitro* generated non-mutagen treated plants and mother (i.e. rhizome-propagated) plants. M: Marker (100 bp ladder), C: *in vitro* generated non-mutagen treated plants, CR: Mxg rhizome propagated plant, C1-C16: Mxg putative mutants. Arrows indicate polymorphic bands. Dots indicate band placement within the gel, as a reference.

Figure 4. Phylogenic tree illustrating the relationship among the *Miscanthus x giganteus* Freedom *in vitro* generated control (C) and putative mutants developed from inter simple sequence repeat (ISSR) data using the unweighted pair group method of arithmetic means (UPGMA) to show DNA similarity. CR (i.e. rhizome-propagated plant) was not included in this analysis since this control continuously produced

similar banding patterns (for each primer used) to the *in vitro* generated non-mutagen treated plant (C).

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1 Figure 4

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Competing interests

The authors declare that they have no competing interests.

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