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Miscanthus × *giganteus*: Regeneration system with assessment of genetic and epigenetic stability in long-term *in vitro* culture

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ABSTRACT

Species of *Miscanthus* Anderss., C_4 perennial rhizomatous grasses with low input requirements, have become representative crops for lignocellulosic bioenergy and material resources in temperate climates. Most of the commercial cultivars appropriate for cropping within Europe and North America are based on *Miscanthus* × *giganteus* species. Because of their triploid hybrid nature, micropropagation of these species using *in vitro* cultures is a common method both of plant multiplication on a commercial scale and of long-term preservation of their valuable genotypes. The somaclonal variation that can occur during *in vitro* culture may be useful for strain improvement during plant breeding, but is undesirable for long-term genotype preservation; therefore, the genetic and epigenetic stability of regenerated plants needs to be assessed. The objectives of our study were (1) to develop an effective system for *Miscanthus* × *giganteus* plantlet production, starting from callus induction, and shoot and root regeneration, and ending with plant acclimatization; and (2) to evaluate any genetic and epigenetic instability induced by long periods of *in vitro* cultivation using ISSR, RAPD and MS-ISSR molecular markers on selected *M.* × *giganteus* clones. We present an efficient and stable method of *M.* × *giganteus* regeneration and preservation by long-term *in vitro* culture. Both morphological and molecular marker analyses revealed no major genetic or epigenetic changes among clonally propagated *M.* × *giganteus* that had been maintained by long-term shoot culture. However, when screening for genetic variation, particular attention should be paid to genomic regions associated with GACA, GATA, AG and CTC repeats.

1. Introduction

Bioenergy has significant potential to ensure local energy supply security and to contribute to climate change mitigation. Over the past 25 years, perennial species selected as dedicated biomass crops have received much attention as potential renewable feedstocks for bioenergy and material usage (Clifton-Brown et al., 2017). Species of *Miscanthus* Anderss. are C_4 perennial rhizomatous grasses with low input requirements that originate from diverse climates ranging from tropical Africa and South-East Asia up to Siberia. They have become representative crops for lignocellulosic bioenergy and material resources in temperate climates (Robson et al., 2013). Although results depend on location and climate conditions, field trials in Europe have demonstrated that *Miscanthus* × *giganteus* (Greef et Deu. Ex Hodkinson et Renvoize) ($2n = 3x = 57$) is suitable for combustion in terms of feedstock supply, quality, conversion efficiency and ecological benefits (Clifton-Brown et al., 2008; Heaton et al., 2010; Robson et al., 2013; Iqbal et al., 2015; Clifton-Brown et al., 2017). In the climate of north-eastern Poland, *M.* × *giganteus* is more energy-efficient than maize, Amur silver grass, sweet sorghum and alfalfa or timothy grass (Jankowski et al., 2016).

This allotriploid hybrid appeared as a natural cross between two *Miscanthus* species, diploid *M. sinensis* ($2n = 2x = 38$) and allotetraploid *M. sacchariflorus* ($2n = 4x = 76$). Abnormal development of both male and female gametophytes and a strong postzygotic barrier attributed to total or partial sterility of triploid *M.* × *giganteus* that results in a very low frequency of seed production (Linde-Laursen, 1993; Lafferty and Lelley, 1994; Słomka et al., 2012; Tamura et al., 2016).

Abbreviations: ABA, abscisic acid; BAP, 6 benzylaminopurine; CPA, chlorophenoxyacetic acid; DIC, dicamba 3,6-dichloro-o-anisic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; 2-IP, 2-isopentenyl adenine; ISSR, inter simple sequence repeat; KIN, kinetin; MS-ISSR, methylation-sensitive inter simple sequence repeat; MS, Murashige and Skoog medium; NAA, α-naphthalene acetic acid; PGR(s), plant growth regulator(s); PMS, proliferation MS; RAPD, random amplified polymorphic DNA; RMS, rooting MS; RP, recovery percentage; TIBA, 2,3,5-triiodobenzoic acid; TDZ, thidiazuron

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The first attempts to develop seed-based interspecies *Miscanthus* hybrids adapted to European and American environments were undertaken by Clifton-Brown and colleagues (reviewed in Clifton-Brown et al., 2017), but at present the agronomy costs are still comparable to *M.* × *giganteus* rhizome propagation. Current plant propagation approaches comprise *in vitro* cultures and rhizome or stem-cutting production systems (Atkinson, 2009).

Most of the commercial cultivars appropriate for cropping within Europe and North America are based on one particular *M.* × *giganteus* clone, which raises concerns about limited variability in the available gene pool (Cichorz et al., 2014; Clark et al., 2016). Current efforts to address the issue of limited genetic variability in *M.* × *giganteus* are focused on new crosses between the parental species or direct germplasm collection from the wild, especially natural triploid interspecific hybrids, which have been reported in East Asia during recent expeditions (Nishiwaki et al., 2011; Tamura et al., 2016). Therefore, micropropagation via *in vitro* cultures is still frequently used to increase the production efficiency of valuable plant materials or is used in biotechnology together with transformation systems (Takahashi et al., 2017).

Although *in vitro* clonal propagation enables the efficient multiplication of large numbers of elite genotypes, genotypic instability is commonly observed in plants derived from tissue culture; this is known as somaclonal variation (Larkin and Scowcroft, 1981; Smýkal et al., 2007). The molecular mechanisms of somaclonal variation are not yet known, but both genetic and epigenetic explanations have been proposed (Guo et al., 2007). Thus, differentiation and tissue culture conditions have been observed to induce genetic variation in sugarcane (Tawar et al., 2016). The somaclonal variation phenomenon may be useful in a plant breeding context for strain improvement, but it is undesirable in long-term genotype preservation. Therefore, it is important to assess the genetic and epigenetic stabilities of regenerated plants (Smýkal et al., 2007).

Different molecular techniques have been developed to study either the global level of methylated cytosine or methylation at specific gene sequences. One method for determining DNA methylation changes and the presence of anonymous methylated CCGG sites is methylation-sensitive ISSR (MS-ISSR), where DNA is digested with *Hpa*II or *Msp*I and then analysed by ISSR PCR. *Hpa*II and *Msp*I are two restriction enzymes that recognize the same DNA sequence, 5′-CCGG-3′, i.e. they are isoschizomers, but they have different sensitivities to DNA methylation at the restriction site. Both enzymes will cut unmethylated CCGG sites, but *Hpa*II will not cut if either the external or internal cytosine is fully methylated (i.e. methylated on both strands of DNA). In contrast, *Msp*I will cut when the internal cytosine is methylated, although not if the external cytosine is fully or hemimethylated (i.e. methylated on a single DNA strand) (Linacero et al., 2011).

As Guo et al. (2007) have pointed out, it is worth underlining that the use of *Hpa*II and *Msp*I has some limitations, because several methylated forms of their restriction site cannot be distinguished, which potentially leads to underestimation of the numbers of methylated CCGG sites. For example, it is impossible to distinguish between a CCGG site whose sequence has been mutated and a CCGG site that is fully methylated on its external cytosine, because both situations block cleavage of these isoschisomers. Moreover, unmethylated CCGG gives the same pattern as CCGG that is hemimethylated on its internal cytosine, as both enzymes digest both sites. Otherwise, *Hpa*II and *Msp*I enable reliable and efficient estimation of total relative methylation percentages, including full methylation of the internal cytosine and hemimethylation of the external cytosine. In comparison with another frequently used approach, the methylation-sensitive amplification polymorphism (MSAP) technique, MS-ISSR represents a simpler method for studying methylation and has been used in plant species such as *Oryza sativa*, *Secale cereale, Zizania latifolia*, *Spinacia oleracea* and *Solanum* hybrids (Wang et al., 2004; Linacero et al., 2011; Shan et al., 2012; Gao et al., 2014; Tiwari et al., 2015). This method was used to reveal the effect of long-term nodal tissue culture on epigenetic variation in potato somatic hybrid regenerants, while in rye MS-ISSR patterns showed epigenetic modifications in a large proportion of somatic embryo-derived plants (Wang et al., 2004; Linacero et al., 2011; Shan et al., 2012; Gao et al., 2014; Tiwari et al., 2015).

The objectives of our study were (1) to develop an effective system for *Miscanthus* × *giganteus* plantlet production, encompassing callus induction, shoot and root regeneration, and plant acclimatization; and (2) to evaluate any genetic and epigenetic instability resulting from long periods of *in vitro* cultivation using ISSR, RAPD and MS-ISSR molecular marker analysis of selected *M.* × *giganteus* clones. Despite the economic importance of *Miscanthus* species and the potential impact of tissue culture techniques on this crop, there are no published reports on genetic stability and methylation status of *M.* × *giganteus* plants derived from long-term tissue culture.

2. Material and methods

2.1. Plant material for in vitro cultures

The cultures were initiated from 5 to 30 mm fragments of immature inflorescences of *Miscanthus* × *giganteus* (2n = 3*x* = 57) clone 'Germany', which is probably the same as clone 'Hornum' or 'Aksel Olsen'. Explants were excised from a five-year-old field plantation at Plant Breeding and Acclimatization Institute − National Research Institute (PBAI-NRI). All external layers of the leaf shuck were removed, then surface sterilized with 70% (v/v) ethanol for 1 min, treated with 5% (w/v) sodium hypochlorite for 20 min and rinsed three times with sterile distilled water.

2.2. Callus induction and culture conditions

The sterilized explants were cut into 2 mm sections. Five or six of them were planted in a flask in MS medium with basal salts and vitamins at the following concentrations: 750 mg l^{-1} $MgCl_2 \cdot 6H_2O$, 30 g l^{-1} sucrose, 100 mg l^{-1} inositol, 0.4 mg l^{-1} thiamine, 0.5 mg l^{-1} nicotinic acid and 0.5 mg l^{-1} pyridoxine (Murashige and Skoog, 1962). The magnesium and sucrose were autoclaved together with other medium constituents; the other organic compounds were filter-sterilized and added after autoclaving. The pH of all media was adjusted to 5.8 and 7.5 g l^{-1} agar (Difco) was added prior to autoclaving (121 °C for 20 min). For callus induction, different concentrations and combinations of plant growth regulators, such as 6-benzylaminopurine (BAP) (0.1–5.0 mg l^{-1}), thidiazuron (TDZ) (1.0, 2.0, 5.0 mg l^{-1}), 2,3,5-triiodobenzoic acid (TIBA) (2.5, 5.0 mg l^{-1}), 2,4-dichlorophenoxyacetic acid (2,4-D) (2.5, 5.0 mg l^{-1}), chlorophenoxyacetic acid (CPA) (5.0 mg l^{-1}), dicamba (DIC) (5.0 mg l^{-1}) and abscisic acid (ABA) (2.5, 5.0 mg l^{-1}), were tested in separate experiments, each with a negative control (no growth regulators) in a total of forty different treatments with the particular number of cultured explants listed in Table 1. Cultures were incubated at 24 ± 1 °C under a 16/8 h (light/dark) photoperiod cycle (cool white fluorescent light, 40 µmol m^{-2} s^{-1}). Individual callus development was visible three weeks after culture initiation and during the following weeks embryogenic callus was formed. From six to eight weeks after culture initiation, the number of explants with white, compact (embryogenic-like) callus was recorded and for further analyses transferred to proliferation MS (PMS) medium described below. The embryogenic callus percentage was calculated as follows: (number of explants with embryogenic callus/the total number of explants) × 100. Analysis of variance (one-way ANOVA for the complete randomized design) and a corresponding Tukey-Kramer test (Kramer, 1956) at $P < 0.05$ were performed to determine significant differences between different medium combinations, which were replicated from 2 to 6 times. Minimal number of explants per each variant was 50 (25 per one replicate). Percentage data were transformed according to Bliss (1938) ($x' = \arcsin\sqrt{x}$). The Statistica 12.0 (StatSoft, Poland) software