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ORIGINAL PAPER

Shoot organogenesis in three *Miscanthus* species and evaluation for genetic uniformity using AFLP analysis

Caroline Rambaud · Stéphanie Arnoult · Aurélie Bluteau · Marie Chantal Mansard · Christelle Blassiau · Maryse Brancourt-Hulmel

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Abstract A simple, efficient protocol for direct in vitro shoot organogenesis and regeneration was established for three species of *Miscanthus* including two clones of *Miscanthus x giganteus*, one clone of *M. sinensis* and one clone of *M. sacchariflorus*. Shoots were induced from the axillary nodes of both *M. x giganteus* and *M. sacchariflorus* and from apical meristems of both *M. sinensis* and *M. sacchariflorus*. A tillering method was used to accelerate shoot proliferation. Shoots were rooted in a wet perlite substrate in pots in the greenhouse. Subsequently, rooted plants were transferred to the field. The genetic uniformity of regenerated plants was evaluated using amplified fragment length polymorphism analysis and compared to that of rhizome-propagated plants. A total of 33,443 fragments were generated, representing 869 markers. There were 21 fragments (0.06 % of the fragments) or 19 markers (2.19 % of the markers) that were polymorphic, and almost all of these were singletons. The three species showed similar polymorphisms. Genetic variability was also found in the rhizome-propagated plants, sometimes at a higher rate than in the in vitro culture, indicating that the genetic uniformity was not altered by the protocol. This protocol may help breeders produce new clones of *Miscanthus* in the future.

Keywords Micropropagation · AFLP analysis · Genetic uniformity · Breeding

C. Rambaud (✉) · A. Bluteau · C. Blassiau
INRA/USTL UMR 1281 SADV, Bât SN2, Université de Lille 1, 59655 Villeneuve d'Ascq Cedex, France
e-mail: caroline.rambaud@univ-lille1.fr

S. Arnoult · M. C. Mansard
INRA GCIE Picardie UE 0972, Estrées-Mons, BP 50136, 80203 Péronne Cedex, France

M. Brancourt-Hulmel
INRA AgroImpact UR 1158, Estrées-Mons, BP 50136, 80203 Péronne Cedex, France
e-mail: brancourt@mons.inra.fr

Introduction

Miscanthus sp. is a perennial of the *Poaceae* family with increasing potential as a renewable biomass feedstock (Heaton et al. 2008; Hastings et al. 2009). The genus *Miscanthus* contains more than 20 species that inhabit a broad geographic range in Asia, including both sub-tropic and sub-arctic areas (Numata 1974, cited by Clifton-Brown and Lewandowski 2002). In Europe, there are three species of interest for biomass production: *Miscanthus sinensis*, *M. sacchariflorus* and *M. x giganteus* (the hybrid of *M. sacchariflorus* and *M. sinensis*). Varieties of *M. x giganteus* are used for the cultivation of *Miscanthus*, whereas both the other species are used to synthesize new inter-species hybrids of the *M. x giganteus* type (Zub et al. 2011).

Cultivated *Miscanthus* are clones and can be propagated using either macro- or micropropagation methods. In macropropagation, small rhizome sections containing up to 4–5 buds are mechanically divided from the mother rhizome and planted. However, this process is time consuming and insufficient to supply the increasing demand for the current commercial development of *Miscanthus*. In micropropagation, the plantlets are generated via tissue culture and then established in the field. Other fertile genotypes, such as *M. sinensis*, can be propagated either vegetatively or by seed.

Two techniques have been described for the micropropagation of *Miscanthus*: a direct method called in vitro

tillering (Lewandowski 1997) and an indirect method. The direct method consists of direct bud development from the axillary nodes and apical meristems (Nielsen et al. 1993, 1995; Lewandowski 1997); this method is interesting for breeding purposes, as it is expected to preserve the genetic uniformity, though this has not been evaluated to date. The indirect method involves the callus culture of immature inflorescence explants through somatic embryogenesis (Holme and Petersen 1996; Holme et al. 1997; Glowacka et al. 2010; Kim et al. 2010; Lewandowski 1997; Petersen 1997; Plazek and Dubert 2010) and has also been studied for use in switchgrass (*Panicum virgatum* L.), another important biomass crop (Burris et al. 2009). This second method has been investigated more frequently because it is more appropriate for genetic transformation.

The direct method has been investigated only for *M. x giganteus* (Lewandowski 1997; Gubisova et al. 2013), whereas the indirect method has been applied to *M. sinensis* and *M. x giganteus*. The micropropagation of *M. sinensis* via the callus induction of immature inflorescences and the regeneration of *M. x giganteus* from shoots and somatic embryos have been reported (Glowacka et al. 2010). *M. sinensis* was also tested for the callus induction of in vitro-germinated seedlings and somatic embryo regeneration (Zhang et al. 2011; Wang et al. 2011). However, callus culture is a source of somaclonal variation, which was first described by Larkin and Scowcroft (1981). A disorganized growth phase in tissue culture, the use of growth regulators, the number and duration of subcultures, stress and the genotype are all factors that enhance somaclonal variation; in contrast, the direct formation of buds from tissue culture without any intermediate callus phase minimizes the chance of instability (Bairu et al. 2011). Therefore, the direct method is preferred for breeding when genetic uniformity is absolutely essential from one generation to the next. The genetic conformity of in vitro-propagated progeny can be analyzed using simple morphological observations. However, DNA marker assays, such as those for random amplified polymorphic DNA (RAPD) (Mishra et al. 2011), inter simple sequence repeats (ISSR) (Liu et al. 2011; Rai et al. 2012) or amplified fragment length polymorphism (AFLP) (Aversano et al. 2011), are efficient screens for in vitro shoot organogenesis-induced mutations because these markers are not affected by environmental factors and present more reliable and reproducible results. AFLP is an advanced technique (Saker et al. 2006; Smykal et al. 2007), as it combines the reliability of restriction fragment length polymorphism (RFLP) with the efficiency of RAPD. AFLP combines restriction digestion and PCR amplification to detect point mutations at restriction sites or deletions and insertions (Vos et al. 1995). AFLP markers were detected in the entire genome, although they often form clusters in some specific genomic regions as centromeres or possibly telomeres (Qi et al. 1998). Moreover, restriction enzyme used as *Eco*R1, insensitive to CpNpG methylation, promotes clustering in hypermethylated regions with low recombination rates, such as centromeres (Young et al. 1999).

Although the direct method of in vitro culture is an interesting technique in *Miscanthus* breeding, this method has been performed only on *M. x giganteus*, and no information is available for *M. sinensis* and *M. x sacchariflorus*. In addition, the growth regulators used in previous trials (Lewandowski 1997) could have jeopardized the genetic uniformity of the plants regenerated using this procedure. Within this context, the aims of the present study were as follows: (1) to adapt the *M. x giganteus* direct micropropagation technique for use in *M. sinensis* and *M. sacchariflorus* and (2) to assess the genetic conformity using AFLP and to compare the genetic variability associated with the classical propagation from rhizomes with in vitro propagation. We hypothesized that the reaction to culture conditions and way how the explants respond are species-dependent, requiring adaptations for each species considered. Although our method of micropropagation is direct, we also hypothesized that the in vitro culture propagation would alter the genetic uniformity of the plants but to a lesser extent as an indirect method.

Materials and methods

Plant material

The experiments were conducted on the following *Miscanthus* species: *M. sinensis* (var. Goliath) (Gol, 2n = 3x = 57), *M. x giganteus*, with one clone originating from Denmark (GigD, 2n = 4x = 76), and the cultivar Floridulus (Flo, 2n = 3x = 57) and *M. sacchariflorus* (Sac, 2n = 2x = 38) described by Zub et al. (2012). Rhizome cutting is typically used to propagate all of these species vegetatively. For the AFLP analysis, six plants of *M. x giganteus* (GigD), two plants of *M. x giganteus* (Flo), three plants of *M. sinensis* (Gol) and two plants of *M. sacchariflorus* (Sac) were used (Table 1). All of the plants originated from divided rhizomes are cultivated in the field nursery of INRA of Estrées-Mons (France).

The in vitro propagation regenerated many plants from these 13 mother plants, and five plants from each mother plant were randomly selected in different rounds of subculture, which included the third and the sixth rounds, respectively short-term (ST) culture and long-term culture (LT). These plants are listed in Table 1.

Table 1 Plants used in the AFLP analysis

Species	Mother plants	Plants from short-term (ST) in vitro culture	Plants from long-term (LT) in vitro culture
Miscanthus x giganteus (Gig D)	GigD1.1	ST GigD1.1	LT GigD1.1
	GigD1.2	ST GigD1.2	LT GigD1.2
	GigD2.1	ST GigD2.1	LT GigD2.1
	GigD2.2	ST GigD2.2	LT GigD2.2
	GigD3.1	ST GigD3.1	LT GigD3.1
	GigD3.1	ST GigD3.2	LT GigD3.2
Miscanthus x giganteus cv Floridulus (Flo)	Flo1.1	ST Flo1.1	LT Flo1.1
	Flo1.2	ST Flo1.2	LT Flo1.2
Miscanthus sinensis var. Goliath (Gol)	Gol1	STGol1	LTGol1
	Gol2	STGol2	LTGol2
	Gol3	STGol3	LTGol3
Miscanthus sacchariflorus (Sac)	Sac1	ST Sac1	LT Sac1
	Sac2	ST Sac2	LT Sac2

GigD1.1 and GigD1.2 mother plants originated from the division of the rhizome of a 3-year-old plant of one clone of *M. x giganteus* (GigD1). GigD2.1 and GigD2.2 originated from the division of GigD2; GigD3.1 and GigD3.2 originated from the division of GigD3. GigD1, GigD2 and GigD3 are three plants of the same clone. The Gol1, Gol2 and Gol3 mother plants are 3 independent 3-year-old plants of one clone of *M. sinensis* var Goliath. The Flo1.1 and Flo1.2 mother plants originated from the division of a 3-year-old plant of one clone of *M. x giganteus* cv. Floridulus. The Sac1 and Sac2 mother plants are 2 independent 3-year-old plants of one clone of *M. sacchariflorus*. Short-term (ST) culture corresponds to three in vitro subcultures, and long-term (LT) culture corresponds to six subcultures

Shoot organogenesis from axillary and apical buds

The pre-inflorescence apical meristems were removed from 1 to 20 cm shoots grown from plants that were 2 or 3 years old and cultivated in soil in pots in the greenhouse. The nodes were collected from greenhouse-grown plants on shoots that were one to two m in height.

The shoots and nodes were washed with tap water and sterilized for 15 min with 80 g l^{-1} calcium hypochloride (60 % active chlorine) supplemented with a drop of Mercryl foam solution (Menarini, France). The nodes were then washed three times with sterile distilled water, and the young shoots were washed once with sterile distilled water. The outermost leaf of the young shoots was removed, and a second sterilization with 40 g l^{-1} calcium hypochloride (60 % active chlorine) was performed for 10 min, followed by three washes in sterile water.

The apical meristems were dissected by removing the outermost leaves until the remaining shoot apices were approximately 1–5 mm, with the basal fragments 2–3 mm. The nodes were dissected by removing the leaves to reveal the axillary shoots and by cutting at 5 mm below and above the node.

The explants were cultured in Petri dishes on agar-solidified Murashige and Skoog (1962) (MS) medium (mineral salts and vitamins). The medium was prepared with 50 mg l^{-1} L-cysteine, as recommended by Lewandowski (1997), 30 g l^{-1} sucrose and 5 mg l^{-1} BAP and was adjusted to pH 5.5 before autoclaving at 115 °C for 25 min. The explants were grown at 24 °C under a 16 h light photoperiod provided by cool-white fluorescent lamps (40 mmol m^{-2} s^{-1}).

Contaminations can be detected by visual observations. Bacterial and fungal colonies are detected around the tissue on the surface or into the medium.

In vitro tillering

The shoots obtained in the induction stage were transferred to a modified tillering medium (Lewandowski 1997) in 240 × 24 mm glass tubes with transparent plastic covers. The medium consisted of MS salts, 100 mg l^{-1} myo-inositol, 750 mg l^{-1} $MgCl_2$ (recommended by Petersen 1997), 50 mg l^{-1} L-cysteine, 30 g l^{-1} sucrose, 3 mg l^{-1} BAP and 0.45 mg l^{-1} IAA. Each glass tube contained 20 ml of medium supplemented with 100 mg of perlite to support the young regenerated shoots (Fig. 1D). Every 6 weeks, the clusters were divided into single, double or triple shoot bundles and transferred to subculture under the same conditions of light and temperature as the induction phase.

Rooting of shoots and transfer of plantlets to field conditions

The rooting was performed in the greenhouse: clusters with two or three shoots were planted directly in hydrated perlite and covered for 1 week. After this period, the covers were removed during the day and replaced at night for another

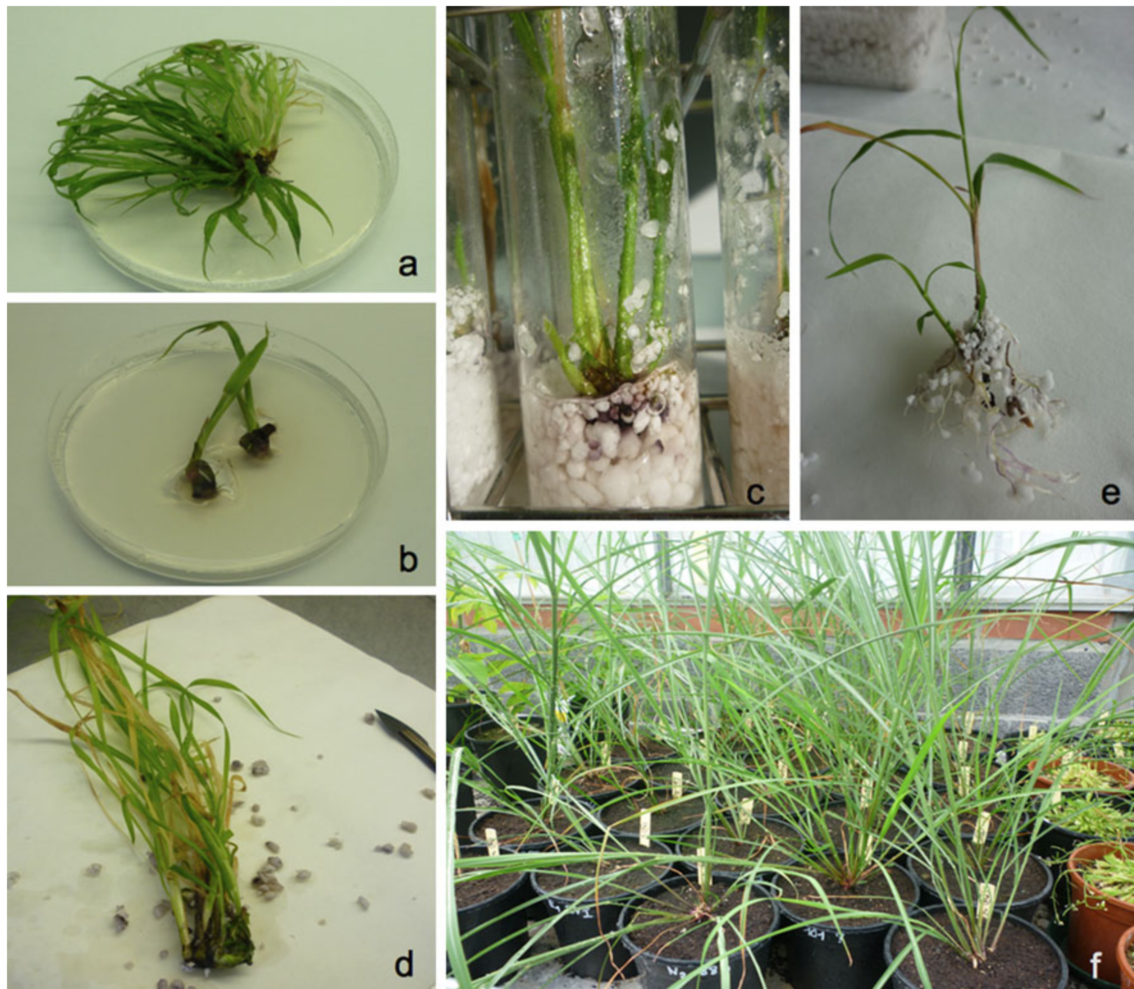

Fig. 1 The shoot induction, in vitro tillering and regeneration of *Miscanthus* species: development of numerous shoots from an explant from an apical meristem of a young greenhouse-grown shoot of *Miscanthus sacchariflorus* (Sac) in induction medium (**a**); the development of two shoots from two nodal fragments of a mature greenhouse-grown shoot of *M. x giganteus* (GigD) in induction medium (**b**); the in vitro tillering of GigD in liquid tillering medium supplemented with perlite (**c**); a cluster of shoots of GigD, from the tillering phase, after 6 weeks of culture (**d**); a GigD plant forming roots in water supplemented with perlite under greenhouse conditions (**e**); and regenerated *Miscanthus* sp. plants transferred to soil (**f**)

week before the plants were permanently uncovered. The shoots were kept at a day/night temperature of 24/18 °C and illuminated for 16 h using halogen lamps or daylight. After rooting, the young plants were transferred to soil in pots and in the field at 2 months, 6 months or 1 year after the end of the in vitro culture. One and a half years later, total vegetative height, overall plant height, number of shoots and diameter of the shoots were measured for both the GigD and Gol genotypes. The total vegetative height corresponds to the height of the canopy for its vegetative part. It is estimated as the distance from the soil surface to the horizontal level of the last ligulate leaf and the overall plant height is estimated as the distance from the soil surface to the horizontal level of the panicle end.

The plants were established in a nursery at the INRA experimental unit in Mons (49°53 N, 3°00E), Northern France. The experimental field is characterized as a deep loam soil (Ortic luvisol, FAO, classification). The clones were planted by hand in 2010 at a density of 2 plants m^{-2} in rows of 10 plants. Each row was 5 m long and the distance between the rows was 80 cm. During the first year, the plots were irrigated 1 month after planting. No fertilization was applied during the 2 years of the experiment, and residual nutrients into the soil were estimated each year by soil sampling to verify that the crop did not suffer from any deficiencies. These in vitro plants were compared to rhizome-propagated plants, which were established in the same field at a same density (see Zub et al. 2011, for the description of the corresponding trial).

AFLP analysis

AFLP analysis is commonly used to assess the genetic conformity of plants regenerated by in vitro culture

(Aversano et al. 2011; Mehta et al. 2011) or to detect somaclonal variations (Mo et al. 2009; Perez et al. 2012). An analysis was performed on the rhizome-propagated plants, short-term culture (ST) in vitro-propagated plants ("vitro-plants") and long-term culture (LT) in vitro-propagated plants. For the rhizome-propagated plants, the DNA was extracted from three tiller leaves from each of the 13 mother plants originating from the four clones (Table 1), for a total of 39 samples. For the micropropagated plants, the leaves were collected after rooting in the greenhouse. The samples included 10 in vitro-propagated progenies from each mother plant, five "vitroplants" after three subcultures (ST) and five "vitroplants" after six subcultures (LT), which altogether resulted in 130 DNA samples. The plants are listed in Table 1. Three technical repetitions were performed for DNA samples from three mother plants.

The cellular DNA was extracted from the leaves using the NucleoSpin plant II Kit (Macherey–Nagel, Germany) following the manufacturer's protocol, with the following modifications: the RNase incubation was extended from 10 to 30 min, the DNA was eluted in 70 ml rather than 50 ml of buffer PE, and the samples were incubated at room temperature rather than at 70 °C. The DNA concentration was estimated using a Biophotometer (Eppendorf, Germany), and was diluted with sterile water to a final concentration of 500 ng/19.5 ml. The AFLP reactions were performed according to the description in Vos et al. (1995), as modified by Myburg et al. (2000). The DNA was digested with *Eco*RI and *Mse*I and ligated to the corresponding adapters. The adapter-ligated DNA was pre-amplified with primers containing sequences that were complementary to the adapter sequences, with an additional selective nucleotide at the 3′ end (*Eco*R1 + A and *Mse*I + C). Subsequently, selective amplifications were conducted using primers carrying two additional selective nucleotides. For the selective amplification, five combinations of primers were used (Table 5). These primers were selected based on the maximum number of polymorphisms detected between the different species (Zub 2010). The PCR reactions were resolved using an ABI3130XL genetic analyzer (Applied Biosystems). The data generated by the capillary electrophoresis were collected and analyzed using GENEMAPPER (Applied Biosystems) software. All of the reactions were performed twice, and only the consistently reproducible peaks were considered.

The corresponding results were first analyzed according to the marker polymorphism previously performed (Saker et al. 2006; de la Puente et al. 2008; Perez et al. 2009). Due to the variable number of plants in each species group, we added the fragment polymorphism analysis because its results were independent of the number of samples.

Results

Shoot induction

For the development of axillary buds, both nodes and young, recently emerged shoots have been used as explants; thus, we distinguished the results obtained with these two types of explants. The young shoots that began to develop in the soil were difficult to sterilize compared to the nodal fragments that were aerial explants. By pooling the results of the four clones, we obtained an average of 60.6 % aseptic young shoots and 94.6 % aseptic nodal fragments (Table 2).

However, the three species had different shoot development. When the young shoots were used as the explants, only *M. sinensis* and *M. sacchariflorus* were able to produce one or many shoots (Fig. 1a). No viable shoots were obtained from the apical explants of *M. x giganteus*; when explants from these clones were excised and cultured in vitro, they turned brown, purple or black, after 1 or 2 weeks of culture, due to the release of numerous oxidized phenolic compounds. In contrast, shoots could not be induced from the *M. sinensis* explants when the nodal fragments were used as the initial fragments. All of the non-contaminated fragments of GigD and 81.8 % of Flo

Table 2 The clone and explant type effects on contamination rates, and number of developing axillary shoots after 4 or 9 weeks of culture

Miscanthus clones	Nature of cultured explant	% sterile explants (nb of cultured explants)	% sterile explants developing shoots	Nb of shoots by 4 weeks regenerating explants	Nb of shoots by 9 weeks regenerating explants
GigD	Young shoots	26.6 (15)	0	0	0
	Nodes	100 (19)	100	1	1
Flo	Young shoots	56.2 (16)	0	0.2 ± 0.5	0.2 ± 0.5
	Nodes	78.5 (14)	81.8	1	1
Gol	Young shoots	72.9 (37)	85.1	1.6 ± 0.9	6.4 ± 3.7
	Nodes	100 (10)	0	0	0
Sac	Young shoots	86.6 (30)	100	4.0 ± 1.7	10.3 ± 3.1
	Nodes	100 (16)	100	2	5

developed only a single shoot in culture. When the culture was extended, no multiplication of the shoots was observed (Fig. 1b). Conversely, all of the aseptic fragments of *M. sacchariflorus* developed numerous shoots, with two per fragment after 4 weeks and five per fragment after 9 weeks (Table 2). After 1 month of culture, the brown tissue was removed from the bottom of the shoots originating from the young shoots or nodal fragments, and the new shoots were then transferred to the second stage for the in vitro tillering.

In summary, a single common method for shoot induction could not be used for all of the species investigated. For successful regeneration, *M. sinensis* and *M. sacchariflorus* required young shoot explants, and *M. x giganteus* required nodal explants.

In vitro tillering

In a single, common tillering medium, the shoots of the four clones grew and produced numerous new shoots (Fig. 1c, d). The newly formed shoots were counted every week (Fig. 2), and the shoot number increased during the period of 8 weeks but with a slower rate after the fifth week. Therefore, 6 weeks was concluded to be the best duration for transferring the shoots to subculture and for counting the rate of tillering (corresponding to the number of shoots per cycle of culture).

After 6 weeks of culture (Table 3), the three clones GigD, Flo and Gol displayed approximately equivalent tillering rates of 4.6, 4.6, and 4.9 shoots per cycle, respectively. *M. sinensis* exhibited the highest variability in the number of formed shoots. *M. sacchariflorus* had the highest average tillering rate (7.1 shoots per cycle), but this species also displayed a high variability, with one shoot producing one to 16 new shoots after 6 weeks of culture. Most of these shoots could be transferred in small clusters of two or three shoots for the rooting stage.

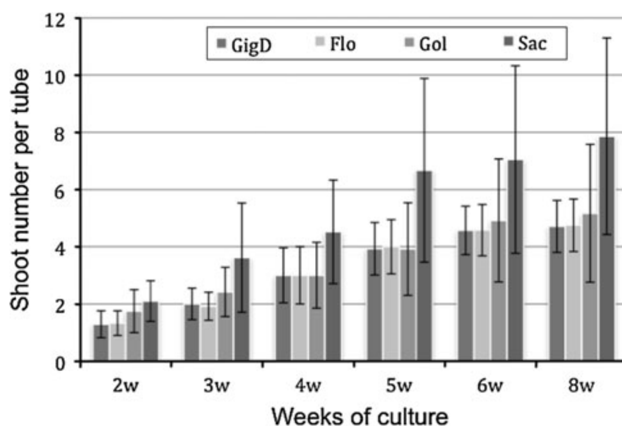

Fig. 2 Evolution of the tillering rate from the second week to the eighth week of culture for the four clones *M. x giganteus* (Gig D and Flo), *M. sinensis* var Goliath (Gol), and *M. sacchariflorus* (Sac)

Table 3 Tillering rates in the third subculture and the success of root generation for the four clones of *Miscanthus*

Miscanthus clones	Number of shoots in 6-week-old cultures	% of rooting after 1 month (nb of tested plants)
GigD	4.6 ± 0.9	84.44 (87)
Flo	4.6 ± 0.9	81.08 (37)
Gol	4.9 ± 2.2	91.95 (45)
Sac	7.1 ± 3.3	88.71 (62)

Lastly, all of the species were able to produce tillers. Although *M. sacchariflorus* demonstrated a greater ability for tillering than the two other species, this ability was much more variable.

Transfer to the field

The rooting of the shoots could be achieved in the greenhouse by transferring the shoots directly into water-saturated perlite. The small plants started forming roots after being transferred to the perlite, but for the two clones of *M. x giganteus*, some roots were occasionally observed in the tillering stage. The four clones had similar rooting percentages, ranging from 81.1 to 92.0 % after 1 month of testing (Table 3). As soon as the plantlets formed roots (Fig. 1e), they were transferred from perlite to soil in individual pots (Fig. 1f) and then to the field at least 2 months after the end of the in vitro culture. The duration of the complete regeneration process was approximately 13 weeks from initial nodal fragment or apical meristem to the rooted plantlets.

Almost all of the plants transferred to the field survived (108/109), in spite of the severe winter in 2010–2011, regardless of the time of planting after the end of the in vitro culture. The morphologies of the GigD and Gol genotype plants that were micropropagated or rhizome-propagated were compared after 1.5 yrs of culture (Table 4). In general, the micropropagated plants formed a bushier "tuft" than the rhizome-propagated plants and were characterized by more shoots per plant and thinner shoots than the rhizome-propagated plants. In addition, the micropropagated plants were smaller than the rhizome-propagated plants when the canopy height was measured; however, the opposite result was found when the overall plant height was measured. This difference was due to the panicle, which was much larger for the micropropagated plants than the rhizome-propagated plants.

AFLP analysis

Using five AFLP primer pairs to examine the effect of the direct in vitro regeneration on the four clones of *Miscanthus*, different profiles were obtained, confirming the differences between the species (Zub 2010), so they were

Table 4 Morphological characteristics of the two clones GigD and Gol propagated by rhizome or micropropagated after 2 years of culture in the field

	Rhizome propagated plants		Micropropagated plants	
	GigD	Gol	GigD	Gol
Total vegetative height (cm)	162 ± 24	143 ± 12	141 ± 35	114 ± 14
Overall plant height (cm)	208 ± 31	182 ± 16	218 ± 34	212 ± 23
Shoot number	19 ± 4	29 ± 3	36 ± 19	31 ± 14
Shoot diameter (mm)	8.9 ± 0.5	7 ± 0.2	6.3 ± 1	6.4 ± 0.7

Table 5 AFLP total fragments (TF), polymorphic fragments (PF) and percentage (%) of polymorphism in the four clones of *Miscanthus* (GigD, Flo, Gol and Sac) propagated by rhizome cuttings (17, 6, 9 and 6 samples, respectively, for GigD, Flo Gol and Sac) or in vitro micropropagation after 3 subcultures (ST) (30, 10, 15 and 9 samples, respectively, for GigD, Flo, Gol and Sac) or 6 subcultures (LT) (29, 10, 15 and 9 samples, respectively, for GigD, Flo, Gol and Sac)

Clone	Primer combination	Via rhizome			In vitro ST			In vitro LT		
		TF	PF	% of polymorphism	TF	PF	% of polymorphism	TF	PF	% of polymorphism
GigD	Eco-AAC/Mse-CAT	833	0	0	1,471	1	0.07	1,421	0	0
	Eco-AAG/Mse-CTT	867	0	0	1,530	0	0	1,478	1	0.07
	Eco-ACA/Mse-CTA	594	1	0.17	1,051	1	0.09	1,014	3	0.3
	Eco-ACC/Mse-CTA	391	0	0	690	0	0	667	0	0
	Eco-AGC/Mse-CAG	409	1	0.24	721	1	0.14	700	4	0.57
	Total	3,094	2	0.06	5,615	3	0.05	5,425	8	0.15
Flo	Eco-AAC/Mse-CAT	432	0	0	720	0	0	720	0	0
	Eco-AAG/Mse-CTT	372	0	0	620	0	0	620	0	0
	Eco-ACA/Mse-CTA	324	0	0	540	0	0	540	0	0
	Eco-ACC/Mse-CTA	270	0	0	450	0	0	450	0	0
	Eco-AGC/Mse-CAG	318	1	0.31	531	1	0.16	531	1	0.16
	Total	1,716	1	0.06	2,861	1	0.03	2,861	1	0.03
Gol	Eco-AAC/Mse-CAT	315	0	0	525	0	0	525	0	0
	Eco-AAG/Mse-CTT	189	0	0	315	0	0	315	0	0
	Eco-ACA/Mse-CTA	297	0	0	494	1	0.21	495	0	0
	Eco-ACC/Mse-CTA	288	0	0	481	1	0.21	481	1	0.21
	Eco-AGC/Mse-CAG	243	0	0	405	0	0	404	1	0.25
	Total	1,332	0	0	2,220	2	0.09	2,220	2	0.09
Sac	Eco-AAC/Mse-CAT	360	0	0	600	0	0	540	0	0
	Eco-AAG/Mse-CTT	336	0	0	560	0	0	503	1	0.2
	Eco-ACA/Mse-CTA	270	0	0	450	0	0	405	0	0
	Eco-ACC/Mse-CTA	240	0	0	400	0	0	360	0	0
	Eco-AGC/Mse-CAG	234	0	0	390	0	0	351	0	0
	Total	1,440	0	0	2,400	0	0	2,259	1	0.04

analyzed separately. Consistently reproducible profiles were generated by three technical repetitions performed using three mother plants.

To elucidate the polymorphisms observed by the AFLP analysis of the four clones, we examined the fragment polymorphisms (Table 5) and marker polymorphisms (Table 6). For the first clone, GigD, the assay generated a total of 14,134 fragments for all of the mother plants (via rhizome) and the ST and LT in vitro-regenerated plants (76 samples, Table 5). There were a total of 190 distinguishable genetic loci or markers (Table 6). Through the fragment polymorphism analysis, small polymorphisms were detected (Fig. 3) in all three categories at frequencies of 0.06, 0.05 and 0.15 % for the rhizome-propagated plants, ST in vitro-propagated plants and LT in vitro-regenerated plants, respectively (Table 5). The frequency was slightly higher when the marker polymorphisms were analyzed: the values shifted to 1.1 % for the rhizome-propagated plants, 1.6 % for the ST in vitro-propagated plants and 4.2 % for the LT in vitro-propagated plants.

For the second clone, Flo, a total of 7,438 fragments were generated, representing 26 samples (Table 5) and 289

Table 6 Number and percentage of polymorphic markers according to the genotypes and clones of *Miscanthus* propagated via rhizome (mother plants) or by in vitro propagation

Clones	Number of AFLP markers	Number of polymorphic markers (singletons)				% of polymorphic markers			
		Via rhizome	In vitro ST	In vitro LT	In vitro ST + LT	Via rhizome	In vitro ST	In vitro LT	In vitro ST + LT
GigD1.1	190	0	0	0	0	0	0	0	0
GigD1.2	190	0	0	0	0	0	0	0	0
GigD2.1	190	0	1	0	1	0	0.5	0	0.5
GigD2.2	190	2	0	3	3	1.1	0	1.6	1.6
GigD3.1	190	0	2	0	2	0	1.1	0	1.1
GigD3.2	190	0	0	5	5	0	0	2.6	2.6
GigD	190	2	3	8	11	1.1	1.6	4.2	5.8
Flo1.1	289	1	0	1	1	0.4	0	0.4	0.4
Flo1.2	289	0	1	0	1	0	0.4	0	0.4
Flo	289	1	1	1	2	0.4	0.4	0.4	0.7
Gol1	150	0	0	1	1	0	0	0.7	0.7
Gol2	150	0	0	1	1	0	0	0.7	0.7
Gol3	150	0	2	0	2	0	1.3	0	1.3
Gol	150	0	2	2	4	0	1.3	1.3	2.7
Sac1	240	0	0	0	0	0	0	0	0
Sac2	240	0	0	1	1	0	0	0.4	0.4
Sac	240	0	0	1	1	0	0	0.4	0.4

For each genotype, 3 samples of rhizome-propagated plants were analyzed; for the in vitro-propagated plants, 5 micropropagated plants from short-term (ST) or long-term culture (LT) or all 10 in vitro-propagated plants (ST + LT) were compared with the mother plants from which they were originated

markers (Table 6). As for GigD, polymorphism that corresponded to a single polymorphic fragment (singleton) was detected in all three types of plants. Polymorphisms were present in 0.06 % of the fragments generated from the rhizome-propagated plants and 0.03 % of those from both types of in vitro-regenerated plants. The marker polymorphism was 0.4 % for all of the plants.

For the third clone, Gol, the assay generated a total of 5,772 fragments from 39 samples (Table 5), which represented 150 markers (Table 6). As expected, polymorphisms were not detected for the rhizome-propagated plants. Although there were similar rates of fragment polymorphism (0.09 %) and marker polymorphism (1.33 %) in the in vitro-regenerated plants (ST and LT), the observed polymorphisms were present in different markers in the ST and LT groups.

For the last clone, Sac, 6,099 fragments were generated (Table 5), which represented a total of 240 markers (Table 6). Polymorphisms were detected only for the in vitro LT-regenerated plants, and the frequencies of the fragment and marker polymorphism were estimated to be 0.04 and 0.42 %, respectively.

A comparison of the four clones revealed very low but similar fragment and marker polymorphisms for all of the species. GigD had more marker polymorphisms than the other species, but this result could have been due to the greater number of plants analyzed. The observed polymorphism was due to singletons, and, the marker polymorphism, which is dependent of the plant number, was higher than the fragment polymorphism. Altogether, we generated 33,443 fragments, representing 869 markers with the following properties: 17 singletons or AFLP fragments that were present or absent in just one plant (0.05 % of the fragments or 1.96 % of the markers); 10 amplified singletons (0.03 % of the fragments or 1.15 % of the markers) and 7 non-amplified singletons (0.02 % of the fragments or 0.81 % of the markers). Two markers (0.23 % of the markers) were polymorphic for two plants from the same cell line.

Lastly, a small degree of polymorphism was observed in both the vegetatively and in vitro-propagated plants. The LT plants showed a slightly higher polymorphism than the ST plants for the two clones GigD and *M. sacchariflorus*, but the degree of polymorphism was similar in the *M. sinensis* and Flo ST and LT plants. Therefore, the genetic uniformity of the in vitro-propagated plants was similar to that of the rhizome-propagated plants.

Discussion

We established a simple in vitro culture protocol for a highly efficient plant regeneration that preserves the

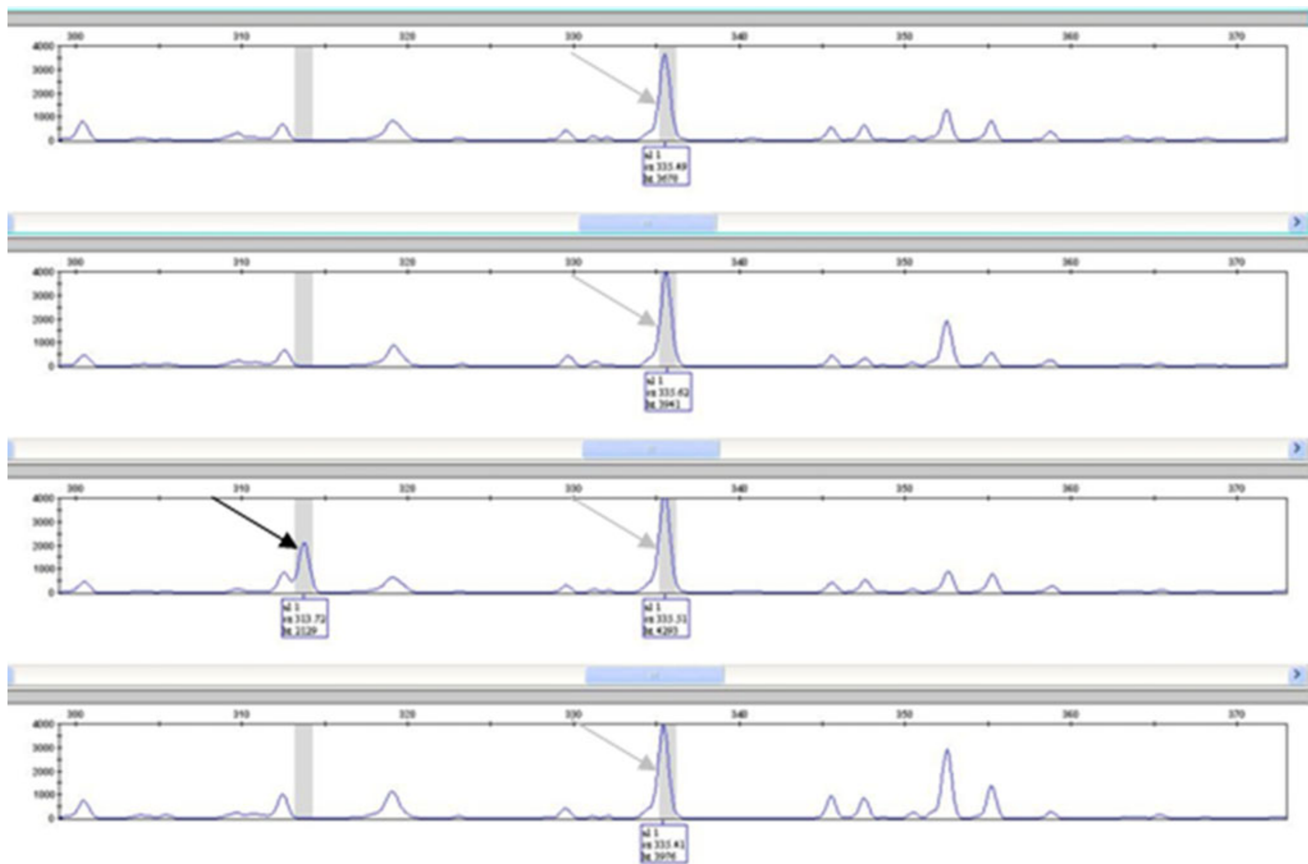

Fig. 3 Example of AFLP DNA fingerprints from some *Miscanthus x giganteus* (GigD2.2, coded as in Table 1) samples using Eco-AGC/Mse-CAG primer combination. A single polymorph peak (indicated with a *black arrow*) is present in one sample but not in others. Other peaks are monomorph (indicated with *grey arrows*) and are present in all the samples

genetic uniformity in three species, *M. x giganteus, M. sinensis* and *M. sacchariflorus*. We will discuss the three following points: (1) the response of the explants to culture conditions of shoot induction and tillering is species-dependent; (2) the preservation of the genetic conformity in the regenerated plants was demonstrated by AFLP; and (3) a low genetic variation was observed for the plants propagated from rhizomes.

To extend the propagation capacity for *Miscanthus*, the development of a micropropagation method that produces genetically homogenous progeny is essential (Atkinson 2009). The protocol must be applicable for the regeneration of all *Miscanthus* species that are utilized in the breeding of new varieties. In this study, we adapted a method of in vitro tillering (Lewandowski 1997) to several species, and we used suitable types of explants for each, i.e. young shoots for *M. sinensis* and *M. sacchariflorus* and young nodal fragments for the two clones of *M. x giganteus* and *M. sacchariflorus*. This simple protocol was efficient for plant regeneration, and it was applicable to the three species that are used in clonal trials for biomass production (Zub et al. 2011). Unlike *M. x giganteus*, *M. sacchariflorus* has not been assayed for regeneration, and only recent studies have reported *M. sinensis* regeneration (Glowacka et al. 2010; Zhang et al. 2011; Wang et al. 2011).

Genotypic effects have been observed for the four studied genotypes. First, due to the lack of axillary buds on the tillers, *M. sinensis* var Goliath is the only species that does not develop shoots from nodal fragments. Thus, young shoots must be used as the initial explants before the induction of inflorescences, which occurs early in *M. sinensis* var Goliath: this is a drawback for the micropropagation of this species because less starting material is available and the sampling period is reduced. However, compared to the two clones of *M. x giganteus*, the initial phase in *M. sinensis* provided more new shoots and, thus, compensated for the small number of primary explants. Moreover, *M. sacchariflorus* and *M. sinensis* multiplied quickly after 8 weeks of culture in the first phase, whereas the two clones of *M. x giganteus* produced only one shoot each. The apical dominance in these two clones was marked, which possibly explains why the tillering-phase multiplication rates of these clones were lower than those of *M. sinensis* and *M. sachariflorus*. With regard to

regeneration, significant genotypic differences between *M. x giganteus* and *M. sinensis* and within the varieties of *M. sinensis* were reported by Glowacka et al. (2010). This phenomenon has been reported for many species, including wheat (Zale et al. 2004) and sugarcane (Lakshmanan et al. 2006). Therefore, the in vitro culture protocol was efficient for the three species tested, which were able to regenerate plants from different types of explants: plant generation was induced from nodal explants of *M. x giganteus* and *M. sacchariflorus*, whereas *M. sinensis* required young shoots for the explants.

AFLP demonstrated that no major genetic variation occurred during the in vitro shoot regeneration through direct regeneration for the three species tested. Very few variations (0.05 % cumulative fragment polymorphisms) were found in the present analysis. Conversely, Mehta et al. (2011) found more polymorphism in bamboo (1.2 %) using AFLP analysis. In sugarcane, RAPD analysis has demonstrated very high polymorphism (14.2–41.3 %) between eight Brazilian varieties (Da Silva et al. 2008) and less significant variation (0.9 and 7.3 %) for two Indian varieties (Lal et al. 2008). These somaclonal variations occurred during meristem culture in sugarcane and caused marked morphological abnormalities and reductions in the yield (Burner and Grisham 1995).

The presence of genetic "hot spots" were not detected, indicating that the polymorphisms were different in each sample. In contrast, the molecular differences in *Humulus lupulus* occurred mainly in the same sequence, independent of the genotype, suggesting the presence of hypervariable DNA regions (hot spots) (Patzak 2003). This phenomenon was described by Linacero et al. (2000) in *Secale cereale* but has not been described for *Miscanthus*. In all cases, the modified peak was observed in only one plant, except for two cases in which a molecular difference occurred in two samples.

Lastly, the small genetic variation observed was of the same order as that observed for the rhizome-propagated plants, indicating that the genetic uniformity was not altered by the in vitro culture protocol. These variations were even much smaller than those observed in sugarcane.

Conventional rhizome propagation can induce genetic variation. In related *Saccharum* sp., the rhizome-propagated plants showed high degrees of genetic variation, from 12.1 to 28.9 %, by RAPD analysis (Da Silva et al. 2008). These variations were so high that the authors differentiated the variant rhizome-derived plants as genetically individual varieties. Similarly, genomic changes were observed for rhizome-propagated *Agave tequilana* by inverse sequence-tagged repeat (ISTR) molecular markers analyses (Torres-Moran et al. 2010) and in date palms in which similar percentages were detected in traditionally propagated plants and in tissue culture-derived plants (Saker et al. 2006). In contrast, AFLP analysis has shown that the mother plants of *Bambusa nutans* are genetically uniform (Mehta et al. 2011).

Therefore, the genetic variation observed in the plants propagated from rhizomes could correspond to somaclonal variation, which arisen from somatic mutations in the mother plant. These mutations could amplify the genetic variation observed among the in vitro-propagated plants, and some of these variations could be due to the initial samples from the mother plants.

Therefore, our new protocols of propagation are available for breeders and producers of *Miscanthus* to improve the efficiency of the propagation in a breeding scheme. The breeding scheme includes the evaluation of clones at the plant level and then at the crop level for the best individuals. Our protocols will allow the propagation of individuals for the evaluation at the crop level in microplots where 100–200 individuals are at least needed per clone and per trial.

Our protocols can be applied for the breeding of *M. sinensis* varieties but also for the synthesis of interspecific hybrids of *M. x giganteus* type, where protocols are needed not only for *M. x giganteus* but also for its both parents, *M. sinensis* and *M. sacchariflorus*.

It will allow the breeders and the producers to save time and to gain in efficiency.

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