



Improving callus regeneration of *Miscanthus × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize 'M161' callus by inhibition of the phenylpropanoid biosynthetic pathway

Cassandra Doll Downey¹ · Jerzy Zoń² · Andrew Maxwell Phineas Jones¹

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Abstract

In previous studies, the regeneration rates of *Miscanthus × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize from callus tissue cultured on semi-solid media significantly declined after 4 mo of culture, which presents problems with germplasm conservation and use as an alternative propagation system. Due to the species' lignocellulosic nature, it was hypothesized that the accumulation of phenolic compounds in the callus may be responsible for inhibiting regeneration. The current study aimed to optimize regeneration of *M. × giganteus* callus by culturing the callus tissue in the presence of 2-aminoindan-2-phosphonic acid (AIP), a competitive inhibitor of phenylalanine ammonia lyase (PAL), to reduce the biosynthesis of phenolics. Embryogenic callus was cultured on media supplemented with 9.0- or 11.3- μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0-, 1-, 10-, 100-, or 1000- μM AIP. Every 28 d for 7 mo, the callus tissue was visually classified based on morphology and regeneration rate. Over the duration of the study, regeneration of shoots was consistently highest in callus cultured on 11.3- μM 2,4-D supplemented with 10- and 100- μM AIP (13–58.3%), and *in vitro* plantlet development from callus cultured on all concentrations of AIP demonstrated tillering and rooting. Total soluble phenolic content of the callus decreased in a dose-dependent manner from 2242.34- $\mu\text{g g}^{-1}$ dry weight in the control to 1569.71- $\mu\text{g g}^{-1}$ dry weight in AIP-treated callus. These data indicate that inhibiting PAL in *M. × giganteus* cultures increased the percentage of calluses exhibiting regeneration over time.

Keywords *Miscanthus × giganteus* · Phenylpropanoid biosynthetic pathway · 2-Aminoindan-2-phosphonic acid · 2,4-Dichlorophenoxyacetic acid · Micropropagation

Introduction

Miscanthus × giganteus J.M.Greef, Deuter ex Hodk., Renvoize is a fast-growing, temperate-adapted member of the Poaceae (Hodkinson *et al.* 2002). The potential of *M. × giganteus* as a sustainable, non-food, lignocellulosic feedstock

for bioethanol (Lewandowski 1998), and value-added product development (Engbers and Deen 2013) has been previously demonstrated. Its perennial nature allows for high yields of aboveground biomass for 20–25 yr depending on location and agronomic practices (Xue *et al.* 2015). After 3 yr of initial establishment, annual biomass yields can range between 31 and 61 t ha⁻¹, which is significantly greater than other biomass crops, such as maize (*Zea mays* L.) or switchgrass (*Panicum virgatum* L.) (Heaton *et al.* 2008). Net profit for yields can amount to as much as \$2900 (USD) ha⁻¹ annually 10 yr after initial establishment (Heaton *et al.* 2004).

Despite these advantages, adoption of *M. × giganteus* for commercial production is limited, in large part due to establishment costs. *M. × giganteus* is a seedless triploid ($3x = 57$) that can be propagated using direct rhizome planting (DRP) or micropropagation with estimated costs of approximately \$3835.39 (USD) and \$7181.99 (USD) ha⁻¹, respectively (Xue *et al.* 2015). While micropropagation using current

✉ Andrew Maxwell Phineas Jones
amjones@uoguelph.ca

¹ Gosling Research Institute for Plant Preservation, Department of Plant Agriculture, 50 Stone Road East, University of Guelph, Guelph, ON N1G 2W1, Canada

² Department of Thermodynamics, Theory of Machine and Thermal Systems, Faculty of Mechanical and Power Engineering, Wrocław University of Science and Technology, 27 Wybrzeże Wyspiańskiego St., 50-370 Wrocław, Poland

techniques is more expensive than DRP, plantlets derived from somatic embryos (embryoid plants—EP) exhibited greater shoot lengths and tiller number per plant (51 and 19.1 for DRP; 57 and 47.1 for EP, respectively), in the first year of field establishment in Germany (Lewandowski 1998). Morphological characteristics, such as the number of branches over 15-cm long and number of bamboo-like shoots, were greater in EP (7.0 and 11.6, respectively) than DRP (3.9 and 5.5, respectively; Lewandowski 1998). In addition to these characteristics, EP produced twice the shoot biomass and the EP rhizomes that were 135% heavier than DRP in the first year of planting (Lewandowski 1998). Furthermore, *in vitro* technologies can facilitate genetic improvement of this sterile species through mutagenesis or genome editing (Mehrotra *et al.* 2007), production of certified disease-free germplasm for national and international exchange (Taşkın *et al.* 2013), rapid multiplication of “elite” cultivars that exhibit genetic integrity (Rambaud *et al.* 2013), and aid in the study of plant processes without interference from unwanted artifacts (Vreugdenhil *et al.* 1998). As such, *in vitro* technologies offer many advantages for *M. × giganteus*, but additional research is needed to improve the technology.

Indirect regeneration of various species of *Miscanthus* has been successfully developed and has mainly focused on somatic embryogenesis from immature inflorescences (Lewandowski 1997; Petersen 1997; Kim *et al.* 2012; Gubišová *et al.* 2013; Perera *et al.* 2015; Ślusarkiewicz-Jarzina *et al.* 2017). Somatic embryo development in *M. × giganteus* is typically induced with 2,4-dichlorophenoxyacetic acid (2,4-D; Kim *et al.* 2012) and low levels of 6-benzylaminopurine (BAP; Lewandowski 1998). A major challenge in *M. × giganteus* micropropagation is the loss of competence during prolonged callus culture. Kim *et al.* (2010) attempted to improve regeneration of *M. × giganteus* plantlets from immature inflorescence cultures. Six weeks after initiation of callus, the majority were classified as yellow/white compact with shoots ($41 \pm 4\%$), followed by K2 ($22 \pm 2.1\%$), and K3 ($37 \pm 3.7\%$) callus tissue. The regeneration frequency of the callus was greatest for shoot-forming and embryogenic-like callus after 1 (0.93) and 4 mo (0.9) of culture on solid callus medium, respectively. However, regeneration was not achieved from callus maintained for more than 4 mo (Kim *et al.* 2010).

Like other grass species, such as *Sorghum bicolor* (L.) Moench (sorghum), the regenerative capacity of callus maintained on callus maintenance medium over long periods of time may be hindered by an accumulation or change in phenolic acid content (Liu *et al.* 2015), which leads to recalcitrance and/or toxic effects to plant tissues (Zaid 1987), modification of endogenous phytohormone levels (Březinová *et al.* 1996), control of dormancy in somatic embryos, and influence over organogenesis (Cvikrová *et al.* 1998). Changes in phenolic content may be due to an upregulation of the phenylpropanoid

biosynthetic pathway or degradation of lignin macrostructures in response to wounding (Zawadzki and Ragauskas 1999). In *M. × giganteus* stems, Le Ngoc Huyen *et al.* (2010) demonstrated that cell walls were largely composed of a 3:1 ratio of ester-linked *p*-coumaric and ferulic acids. Regeneration of plantlets from *M. × giganteus* callus culture could be hindered due to formation of premature lignin from these soluble phenolic compounds in callus cells and/or somatic embryos.

Phenol and lignin biosynthesis has been shown to be significantly reduced in *in vitro* American elm (*Ulmus americana* L.) suspension cultures, sugar maple (*Acer saccharum* Marshall) callus, and *Artemisia annua* L. callus cultures by the addition of 2-aminoindan-2-phosphonic acid (AIP) to the culture media (Jones *et al.* 2012; Jones and Saxena 2013). 2-Aminoindan-2-phosphonic acid competitively inhibits phenylalanine ammonia lyase (PAL), the enzyme responsible for the first committed step of the phenylpropanoid biosynthetic pathway that converts phenylalanine to ammonia and trans-cinnamic acid (MacDonald and D’Cunha 2007). In *A. annua* callus cultures, it was found through both visual inspection and measurement of total phenolic content that tissue browning decreased in a dose-dependent manner up to 10- μ M AIP, and that 100- μ M AIP significantly reduced total tissue phenolics (Jones and Saxena 2013). In addition, total soluble phenolic content has been demonstrated to be correlated with the amount of lignin found in somatic embryos and callus *in vitro* (Cvikrová *et al.* 2003), and embryo growth and development were negatively correlated with total phenolic content (Malá *et al.* 2000). Similar results have been observed in a number of species *in vitro*, including maize, sessile oak (*Quercus petraea* Liebl.), and alfalfa (*Medicago sativa* L.) (Lozovaya *et al.* 1996; Cvikrová *et al.* 1998, 1999, 2003; Hrubcová *et al.* 2000).

The objective of the current study was to optimize the regeneration capacity of *M. × giganteus* callus after prolonged storage on semi-solid medium. It was hypothesized that the application of AIP would reduce the total soluble phenolic content in callus and subsequently improve regeneration potential and timeframe. The findings of this research may help to improve germplasm conservation, maintain disease-free stocks, and create a foundation for future *in vitro* breeding technologies.

Materials and Methods

Plant material Immature inflorescences (5.0–15.0 cm in length), were collected from field-grown *M. × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize ‘Illinois’ (‘M161’) at All Weather Farming Incorporated (Port Ryerse, Ontario, Canada) (42° 47' N 80° 12' W) on September 15, 2015. Through visual inspection using a modified Biologische Bundesantalt, Bundessortenamt, and Chemische Industrie (BBCH) scale, the majority of plants grown at this location were in principal growth stage 4: booting; specifically, varying between stages 43 to 47 (Tejera and Heaton

2017). Stems were cut beneath the most distal (youngest) node with the sheath intact. Segments were wrapped in moist paper towels and kept in darkness at 4°C for 5 d. Thereafter, all but two to three of the surrounding leaves were removed from the immature inflorescences. Immature inflorescences were rinsed under running tap water for 5 min, soaked in 70% (v/v) ethanol for 1 min, and rinsed with sterile, deionized water three times. Immature inflorescences were then immersed in 20% (v/v) commercial bleach (12.5% sodium hypochlorite; Clorox®; The Clorox Company, Oakland, CA), with approximately 0.1% (v/v) Tween® 20 (Thermo Fisher Scientific® Company, Ottawa, Canada) for 20 min with gentle shaking, followed by rinsing five times with sterile, deionized water. Spikelets (each approximately 5.0 mm in length) were aseptically dissected from the rachis, then inoculated individually for callus induction.

Callus induction and multiplication Callus induction and maintenance medium consisted of Murashige and Skoog (MS; Murashige and Skoog 1962) basal salts and vitamins (PhytoTechnology Laboratories®, Shawnee Mission, KS), supplemented with 30-g L⁻¹ sucrose, 11.3-μM 2,4-D, 2.2 μM BAP, 1000-mg L⁻¹ L-proline, 300-mg L⁻¹ casein hydrolysate, and 7-g L⁻¹ agar (Kim *et al.* 2012). All plant growth regulators and L-proline were purchased from Sigma-Aldrich® (St. Louis, MO); casein hydrolysate from PhytoTechnology Laboratories®; and Select Agar™, powder from Thermo Fisher Scientific®. Callus induction medium was adjusted to pH 5.7 using 1-N NaOH and 1-N HCl (Sigma-Aldrich®) before autoclaving at 121°C and 144.8 kPa for 20 min. Ten explants were placed on 20 mL of medium in 100 × 15-mm Petri dishes (Thermo Fisher Scientific®). The calluses were incubated in the dark in a growth room at 24 ± 2°C for the duration of the experiment. Callus tissue was subcultured and multiplied monthly (approximately every 28 d) for 6 mo. In the fifth month of culture, calluses measuring 3.0 to 8.0 mm in diameter were inoculated on one of five callus maintenance media. Media were composed as described previously and supplemented with either 0-, 1-, 10-, 100-, or 1000-μM AIP. In the sixth month of culture, half of the calluses from each of these treatments were placed on callus maintenance media modified with 9.0-μM 2,4-D and 0-, 1-, 10-, 100-, or 1000-μM AIP. At each subculture time point, visibly necrotic and/or atrophied tissue was gently removed from callus.

Callus morphology assessment At each subculture (beginning after the initial 6 mo of culture initiation and multiplication), calluses were visually assessed for morphology using the following rating system: (1) “compact white” callus (K2), characterized by a smooth, white surface (Petersen 1997), generally accompanied by the appearance of “embryogenic-like” structures; (2) “yellow/green” callus (K1), previously described as nodular and moderately soft; (3) “friable” callus (K3), identified by a soft, watery appearance (Kim *et al.* 2010) usually lacking embryogenic capacity (Morrish *et al.*

1987); and (4) “browning” callus. Representations of different callus morphologies may be found in Fig. 1a–d.

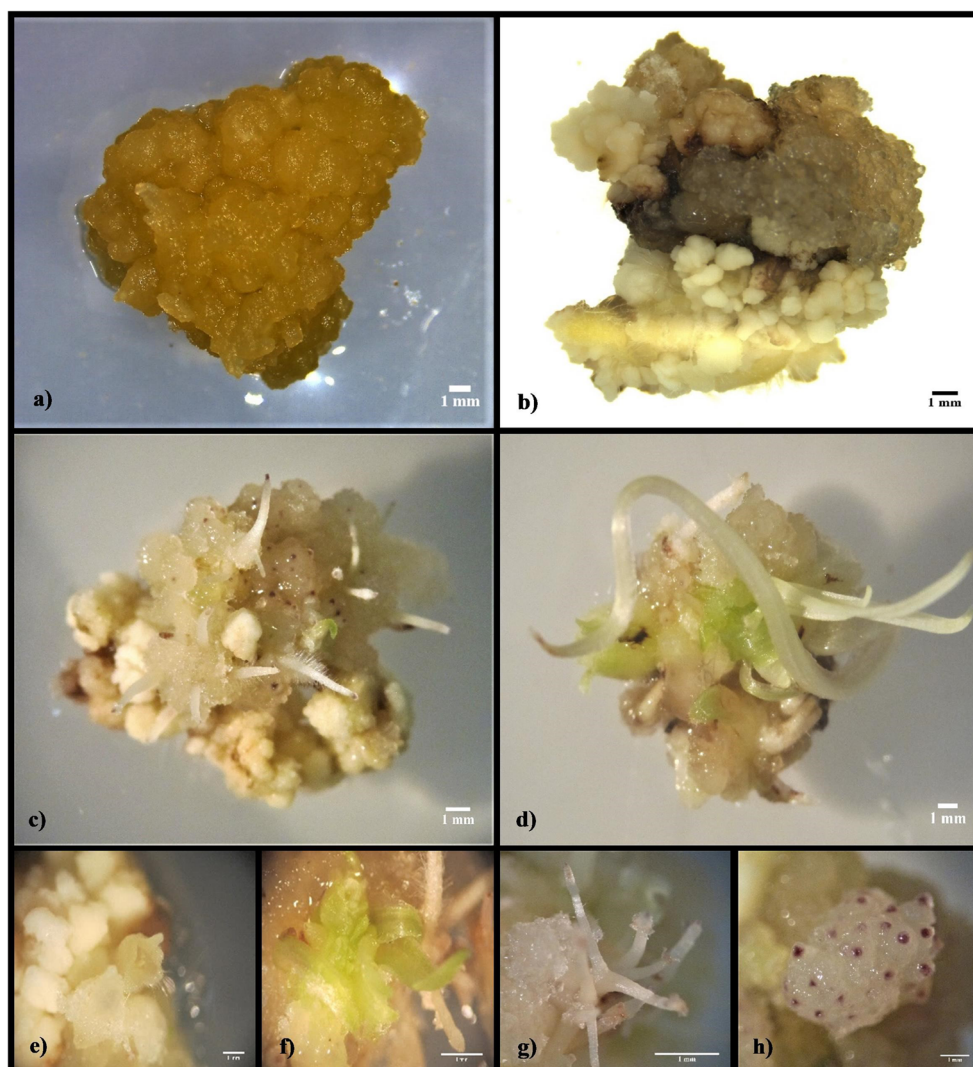
Development of differentiated and embryo-like structures In addition to callus morphology, the following characteristics were recorded: the development of “shoot-like” and “root-like” structures (herein referred to as “shoots” and “roots,” respectively) and the formation of “embryo-like” structures (herein referred to as “somatic embryos”) (representations may be found in Fig. 1e–h). The frequency of each callus type and accompanied structures was calculated as callus type or structure (%) = (number of each callus type or structure ÷ total number of calluses plate⁻¹) × 100 (Kim *et al.* 2010).

Shoot, root, and somatic embryo counts were conducted using a dissecting microscope. Analyses of differentiated structures were conducted between 9 and 13 mo of culture, while somatic embryo counts began at 11 mo of culture. Individual leaves on the callus constituted distinct shoots. Since somatic embryos typically developed in clusters and were quantified without destruction of the callus, conservative counts were conducted from the visible surface of the callus. For the duration of the AIP treatment period, six calluses were placed on each medium with five replications each. Care was taken to group calluses of similar morphologies on the same plate.

Soluble phenolic content The soluble phenolic content of calluses from each treatment was measured after 2 mo on AIP-supplemented media, with sampling occurring monthly for 5 mo. At each subculture, one callus (with necrotic and/or atrophied tissue removed) was removed from each treatment replicate (five replicates per treatment) and pooled in a 15-mL centrifuge tube (Thermo Fisher Scientific®). The sample g fresh weight (FW) from each treatment was recorded before flash-freezing in liquid nitrogen, followed by lyophilisation using a FreeZone™ 4.5 freeze dryer (Labconco®, Kansas City, MO). After lyophilization, the sample g dry weight (DW) was recorded, total moisture content (MC) (%) was calculated [(FW-DW) ÷ FW] × 100, and samples were finely ground. Extraction solvent was added to each tube (80% methanol, v/v), with a 1:10 tissue to solvent ratio (w/v). The tubes were vortexed and placed in a sonicating water bath (Branson 3510, Danbury, CT) for 30 min. The tubes were then removed and centrifuged for 10 min at 21.1 × g using a Fisherbrand™ AccuSpin™ Micro 17 (Thermo Fisher Scientific®). The supernatant from each sample was transferred into a new microcentrifuge tube.

The soluble phenolic content was assessed using a modified Folin and Ciocalteu (F-C) colorimetric assay. A standard curve was constructed using gallic acid (Sigma-Aldrich®) to obtain gallic acid equivalent (GAE) values at concentrations of 31.25, 62.5, 125, 250, 500, and 1000 μg mL⁻¹ (Folin and Ciocalteu 1927). In brief, 10-μL aliquots of sample extracts, standards, or sample blanks were added to each well of a 96-

Figure 1 Representative *Miscanthus × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize ‘M161’ callus morphologies. Calluses were assessed using a dissecting microscope and were putatively classified as: (a) compact yellow/green (K1); (b) somatic embryo forming and friable (K2 and K3, respectively, occurring in the same sample); (c) root forming; and (d) shoot forming. Leaf primordia (e) and differentiated shoot-like structures (f) formed on calluses. The root-caps of regenerated roots were identifiable by the production of red/purple pigmentation and lack of root hairs (g), and anthocyanin spots were noticeable on the remainder of the callus (h). Scale bars represent 1 mm.

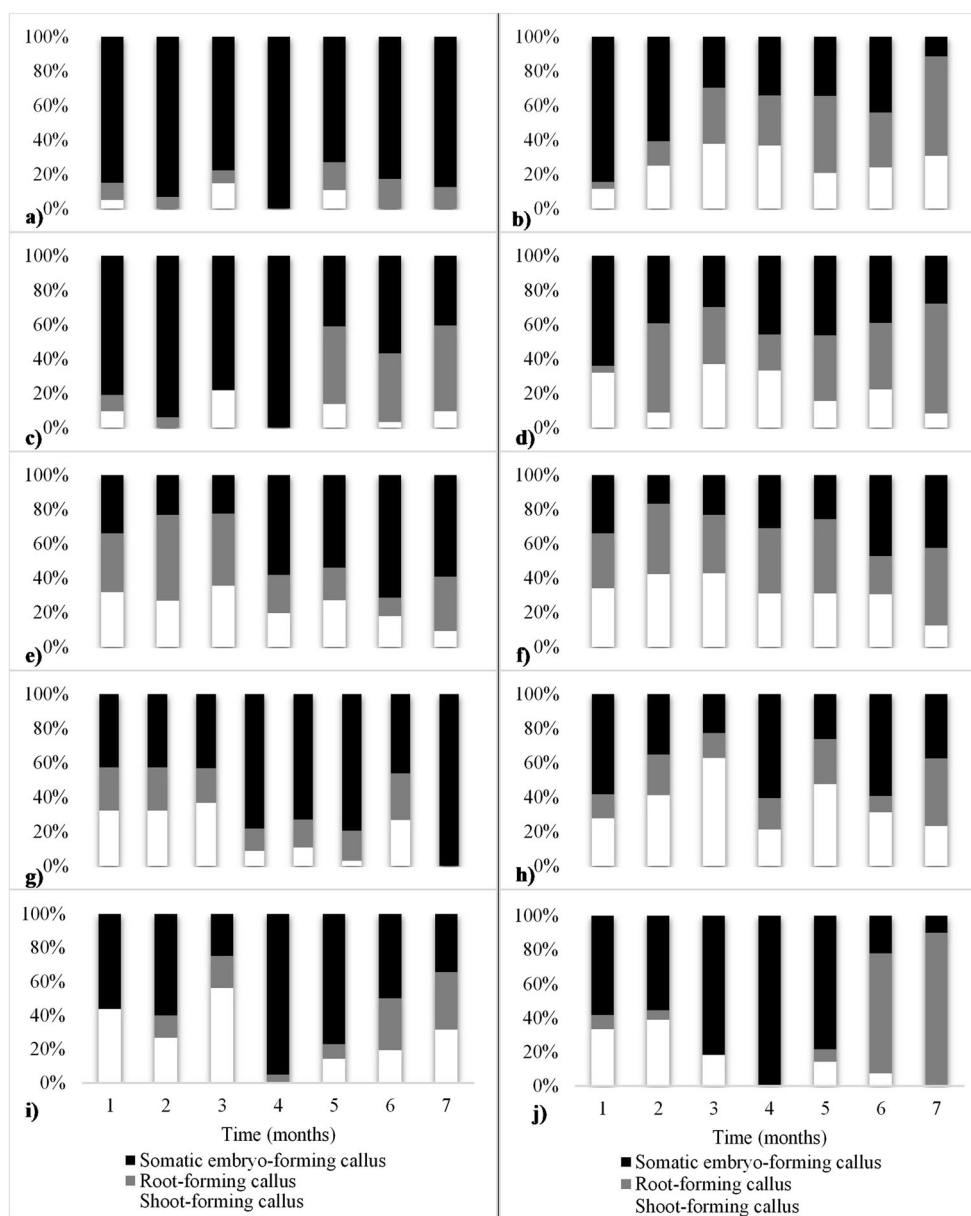


well flat bottom microplate (Corning, Corning, NY). A volume of 100 μL of 1:10 water: F-C phenol reagent (MP Biomedicals, Santa Ana, CA) was added to each well, and the plate was incubated for 5 min before adding 80 μL of aqueous 0.25 M Na_2CO_3 (Sigma-Aldrich®). The plate was then incubated in the dark for 1 h before absorbance values at 725 nm were measured with a Synergy™ H1 microplate reader (Biotek, Winooski, VT). All sample and standard readings were corrected with blanks, and all samples, standards, and blanks were replicated in triplicate.

Regeneration and *in vitro* plantlet development At 6 mo of culture, excess shoot-forming callus from each 11.3- μM 2,4-D treatment measuring 3.0–8.0 mm in diameter were placed on regeneration medium described by Kim *et al.* (2012). Regeneration medium consisted of MS basal salts and vitamins supplemented with 30-g L^{-1} sucrose, 5-mg L^{-1} BAP, 1-mg L^{-1} 2,4-D, 1000-mg L^{-1} L-proline,

300-mg L^{-1} casein hydrolysate, and 7-g L^{-1} Select Agar™, powder. Regeneration medium was adjusted to pH 5.8 using 1-N NaOH and 1-N HCl before autoclaving at 121°C at 144.8 kPa for 20 min. Six to seven calluses were placed on each plate and incubated in the dark for 1 mo. After regeneration, calluses with shoots were transferred to either semi-solid root induction [MS basal salts with 30-g L^{-1} sucrose, 0.1% (w/v) activated charcoal (Sigma-Aldrich®), and 7-g L^{-1} Select Agar™, powder, pH 5.8], or liquid tillering medium [MS basal salts with vitamins, 30-g L^{-1} sucrose, 5-mg L^{-1} BAP, 0.1-mg L^{-1} indole-3-butyric acid (IBA; Sigma-Aldrich®), and 0.45-mg L^{-1} indole-3-acetic acid (IAA; Sigma-Aldrich®), pH 5.7] for plantlet development (Kim *et al.* 2012). The cultures were incubated in a growth room at a temperature of $24 \pm 2^\circ\text{C}$, under a 16-h photoperiod with light intensity of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool-white fluorescent bulbs (Philips® Canada, Scarborough, Canada). All chemicals

Figure 2 Proportion of *Miscanthus × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize ‘M161’ calluses exhibiting shoots, roots-, and somatic embryos over time. Data are presented by 0 (a and b), 1 (c and d), 10 (e and f), 100 (g and h), and 1000 (i and j) μM 2-aminoindan-2-phosphonic acid (AIP) treatments grouped by 9.0 (a, c, e, g, and i) and 11.3 (b, d, f, h, and j) μM 2,4-dichlorophenoxyacetic acid (2,4-D). Each bar represents the mean of 30 samples.



listed above that are not followed by a supplier were purchased from the same companies as those described in “*Callus induction and multiplication.*”

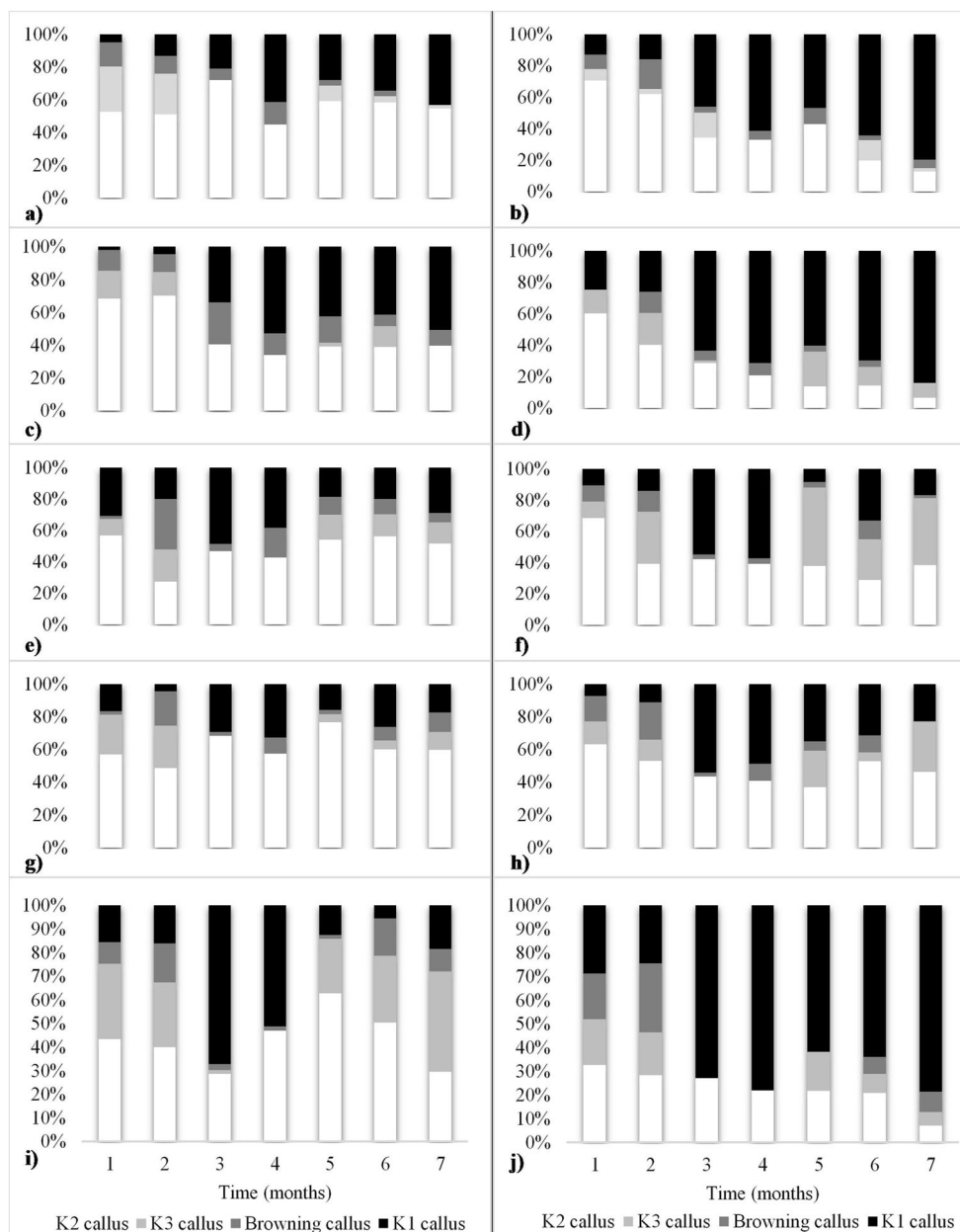
Experimental design and statistical analysis Data obtained from non-destructive visual assessments [“callus morphology frequencies” (7 mo), “number of structures,” “phenolic content,” and “total MC” (5 mo), and “number of embryo-like structures” (3 mo)] were subject to variance analysis conducted using a mixed-model repeated measures analysis of variance (ANOVA), using PROC GLIMMEX in SAS® 9.4 (SAS Institute Inc., Cary, NC). The experiment was constructed in a factorial design and arranged in a completely randomized design (CRD). Each treatment was replicated over five plates, each containing six samples. A logit link function with beta distribution

was used for assessing callus morphology frequencies, and an identity link with lognormal distribution was used to analyze the number of differentiated structures and somatic embryos. Correlation coefficients (R^2) regarding the occurrence of differentiated structures and somatic embryos on different callus types were calculated. For phenolic content and total MC, each analyzed treatment was composed of five individual calluses from five replicate plates. A type I error rate of $\alpha = 0.05$ was assigned for all analyses.

Results

Callus morphology frequencies The percentage of calluses producing shoots and roots were significantly affected by

Figure 3 Proportion of *Miscanthus × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize ‘M161’ compact yellow/green (K1), somatic embryo forming (K2), friable (K3), and browning callus over time. Data are presented by 0 (a and b), 1 (c and d), 10 (e and f), 100 (g and h), and 1000 (i and j) μM 2-aminoindan-2-phosphonic acid (AIP) treatments grouped by 9.0 (a, c, e, g, and i) and 11.3 (b, d, f, h, and j) μM 2,4-dichlorophenoxyacetic acid (2,4-D). Each bar represents the mean of 30 samples.



interactions between culture time and AIP concentration. For shoot-forming calluses, declines were observed as culture time progressed in all individual treatments (Fig. 2). Apart from media lacking AIP in the first month of treatment ($66.7 \pm 6.811\%$), the greatest frequency of calluses developing shoots was observed on media supplemented with 10- μM AIP (peaking in the third culture month at 58.3%). Regarding the proportion of calluses producing shoots compared to root and somatic embryos, 100- μM AIP supplemented with 11.3- μM 2,4-D exhibited the highest value by the third culture month (73.3%).

The highest frequency of rooting calluses was observed on medium supplemented with 10- μM AIP ($45.4 \pm 4.663\%$), followed by 1- and 100- μM treatments (24.5 ± 3.705 and $25.2 \pm 3.467\%$, respectively). Compared to calluses producing

other distinctive structures, 1000- μM AIP supplemented with 11.3- μM 2,4-D accounted for 60 to 63.3% root-forming calluses by the sixth and seventh culture mo.

Three-way interactions between AIP concentration, 2,4-D concentration, and culture time were observed with somatic embryo-forming calluses. When the proportion of somatic embryo calluses were compared to shoot- and root-forming calluses, higher values were generally observed in AIP treatments supplemented with 9.0 than 11.3- μM 2,4-D.

The frequencies of K1, K2, and K3 calluses, along with those which browned and could not be properly classified, are depicted in Fig. 3. Differentiated structures and somatic embryos were able to develop on every callus type; however, the occurrence of shoots on K2 and K3 calluses was significantly

Table 1 Correlations regarding the occurrence of structures (shoots, roots, and somatic embryos) on the following *Miscanthus × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize ‘M161’ callus types: compact yellow/green (K1), somatic embryo forming (K2), friable (K3), and browning callus. Sample correlations (R^2) followed by an asterisk (*) are significant at $\alpha = 0.05$

Structures	Callus type	Correlation coefficient (R^2)	p value
Shoots	K1	0.27	0.0577
	K2	0.7*	< 0.0001
	K3	0.56*	< 0.0001
	Browning	0.0033	0.982
Roots	K1	0.22	0.1164
	K2	-0.17	0.2267
	K3	0.075	0.6056
	Browning	0.13	0.3558
Somatic embryos	K1	-0.54*	< 0.0001
	K2	0.7*	< 0.0001
	K3	-0.56*	< 0.0001
	Browning	-0.17	0.2408

correlated (Table 1). Additionally, the development of somatic embryos was positively correlated with K2 calluses, while negatively correlated with K1 and K3 types.

Differentiated structure and somatic embryo counts AIP concentration significantly influenced the average number of shoots per callus, with 10 μM AIP exhibiting the highest counts per callus (4.2 ± 0.5464), followed by 100- and 1000- μM treatments (3.8 and 3.2, respectively) (refer to Fig. 4 for mean structure counts averaged over time and Fig. 5 for means at each mo).

The average number of roots demonstrated significant interactions between culture time and AIP concentration. The highest values for the average number of roots were observed in the fifth culture month in treatments 1- and 100- μM AIP and the fourth month in 1000- μM AIP (17.3 ± 2.444 , and 18.8 ± 3.5655 , respectively). Increasing trends for root development were observed in 1-, 100-, and 1000- μM AIP treatments as time progressed throughout 5 mo of culture, while 0- and 10- μM treatments remained relatively unchanged during this time span.

Although the average number of somatic embryos ranged from 14.9 (1000- μM AIP) to 33.7 (100- μM AIP), no observable trends were detected with these levels when analyzed over the culture period.

Soluble phenolic content Total soluble phenolic content ($\mu\text{g g}^{-1}$ DW) was negatively associated with increasing AIP concentrations, with the highest content being observed at 1 μM (2242.3 ± 149.03 - $\mu\text{g g}^{-1}$ DW), and the lowest at 1000- μM AIP (1569.7 - $\mu\text{g g}^{-1}$ DW) (Fig. 6).

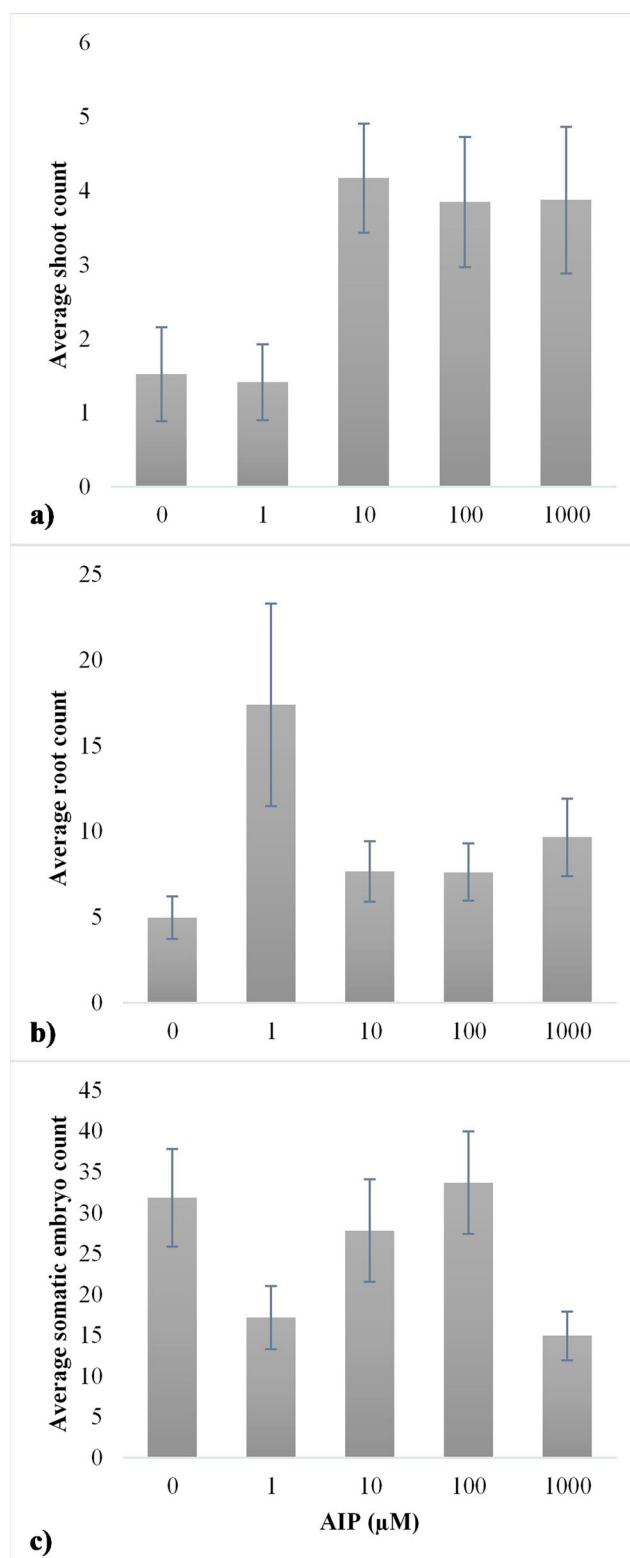
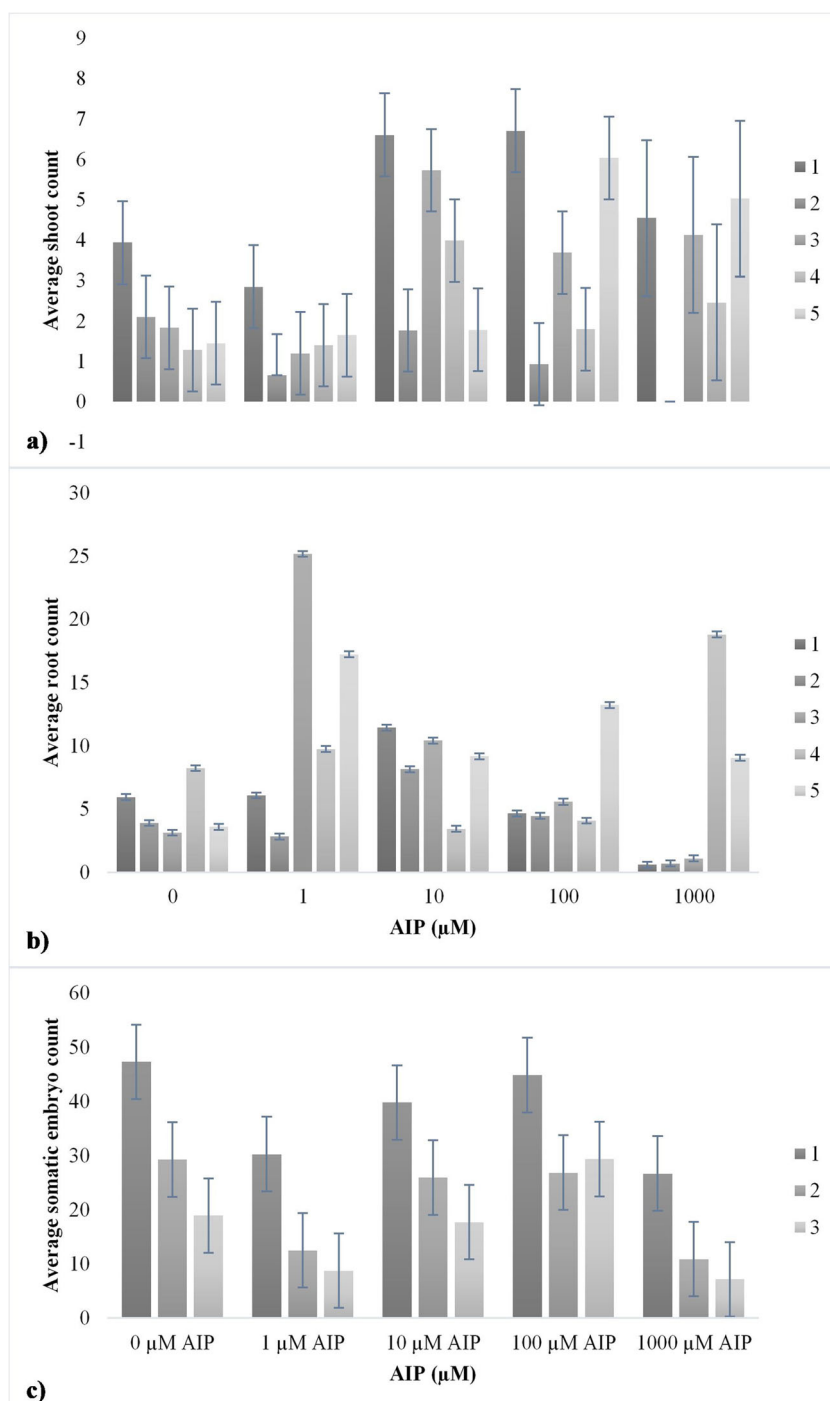


Figure 4 Average count of (a) shoots, (b) roots, and (c) somatic embryos developing on *Miscanthus × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize ‘M161’ calluses cultured on different levels of 2-aminoindan-2-phosphonic acid (AIP) and averaged over time. Each bar represents the mean of 300 (a and b) and 180 (c) samples \pm S.E.

Figure 5 Average count of (a) shoots, (b) roots, and (c) somatic embryos per *Miscanthus × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize 'M161' callus at specific culture times. The legends associated with each graph represent culture time (approximately 1 mo). Each bar represents the mean of 60 samples \pm S. E (pooled).



Total MC (%) was significantly affected by both 2,4-D and AIP concentrations; however, there were no interactions between these two factors ($p = 0.1893$). Total MC was generally greater in 11.3- μ M 2,4-D and 1000 μ M-AIP media compared to the remaining treatments (Table 2).

In vitro plantlet development After calluses were maintained on callus maintenance media for 6 mo, a portion of the shoot-

forming samples from each AIP treatment (cultured on media supplemented with 11.3- μ M 2,4-D) were transferred to regeneration medium. After 1 mo, regenerants were transferred to semi-solid rooting- or liquid tillering-media. Shoot-forming calluses from all AIP treatments were able to develop healthy roots and/or shoots *in vitro* (representations may be observed in Fig. 7). Shoot multiplication rates ranged from six to 18 per explant (data not shown).



◀ **Figure 6** Average gallic acid equivalent (GAE) ($\mu\text{g g}^{-1}$ dry weight (DW)) values of *Miscanthus × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize ‘M161’ calluses cultured on media supplemented with various 2-aminoindan-2-phosphonic acid (AIP) levels. Each bar represents the mean of 50 calluses over two 2,4-dichlorophenoxyacetic acid (2,4-D) levels (10 callus samples over five replications) \pm S.E. Bars labeled with the same letter are not significantly different ($p < 0.05$) according to Tukey’s honest significant difference (HSD) test.

Discussion

Regeneration of *M. × giganteus* through somatic embryogenesis and/or shoot organogenesis from immature inflorescences has been developed previously (Lewandowski 1997; Głowacka *et al.* 2010a; Kim *et al.* 2010; Kim *et al.* 2012; Gubišová *et al.* 2013). While this approach offers opportunities for genetic improvement and large-scale plant propagation of this sterile triploid, several problems have been encountered that limit its application. Specifically, callus has been reported to lose its regenerative capacity within 4 mo of culture (Kim *et al.* 2010), which presents issues for applying this technology. In the current study, AIP—a potent inhibitor of the phenylpropanoid pathway—was found to increase the percentage of calluses exhibiting regeneration of shoots over relatively-extended culture periods.

The capacity for shoot and somatic embryo development from *M. × giganteus* callus (the majority of these structures occurring on K2 callus) was achieved after prolonged culture (7 mo on AIP-supplemented media; 12 mo including the callus induction phase) on semi-solid callus maintenance media for both 2,4-D concentrations tested. The results presented here are an improvement from the findings reported by Kim *et al.* (2010), which showed complete loss of shoot-regenerative and embryogenic-development capacity after 4 mo of culture on semi-solid media. Though the current study recognized that the control treatment (0- μM AIP) was able to produce shoot-forming calluses exceeding 4 mo of culture, this could be attributed to a number of factors, including potential differences in genotype, immature inflorescence explant developmental stage used in callus induction (Głowacka *et al.* 2010b), and/or more care taken to remove necrotic/atrophied tissue at each subculture. Nonetheless, this system will allow for a relatively long term, low-maintenance, effective regeneration system for *M. × giganteus* ‘M161’, better known as the “Illinois” variety (Withers 2015).

The frequency of different callus types, differentiated structures, and somatic embryos was highly variable among and within treatments, and the proportions of each of these changed noticeably over the duration of the experiment. This phenomena have previously been observed in other grasses, such as *Saccharum* spp. (sugarcane) (Fitch and Moore 1990). Additionally, the presence of browning callus could have been classified as any of the typical callus morphologies reported in

Table 2 Total moisture content (MC) (%) of *Miscanthus × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize ‘M161’ calluses used for soluble phenolic content measurements. Analyses were conducted separately for 2-aminoindan-2-phosphonic acid (AIP) and 2,4-dichlorophenoxyacetic

acid (2,4-D). Each mean represents 50 and 100 calluses (10 and 25 callus samples over five replications, respectively). Means followed by the same letter are not significantly different ($p < 0.05$) according to Tukey’s honest significant difference (HSD) test

Concentration (μM)	AIP					2,4-D	
	0	1	10	100	1000	9.0	11.3
Mean (%)	84.6 ab	85 ab	84 b	84.9 ab	87.3 a	84.4 B	85.9 A
S. E.	0.7715					0.4879	

this study; however, tissue necrosis due to any number of stressors made its original classification indiscernible.

Somatic embryos were identified by a smooth, rounded architecture, which appeared either white or green in color. These structures varied in shape, with clusters of embryos represented by a conglomerate of spherical (globular)- and torpedo-shaped structures. While histological analysis to verify their bipolar nature was not conducted, previous studies using a similar system demonstrated that these structures exhibited a bipolar configuration with a discernable epidermis, which is characteristic of somatic embryos (Ślusarkiewicz-Jarzina *et al.* 2017).

The occurrence of differentiated structures on the various callus types reported in this study is generally in agreement with what has been documented in the literature. Shoots (determined by the presence of green buds or primordia and light green, translucent leaves) formed primarily on K2 (Gairi and Rashid 2004) calluses. Leaves could be ascertained by the

presence of trichomes observable on all parts of the tissue, including the leaf tip. Previously reported by Owaga (2015), who worked on the regeneration of Switchgrass (*P. virgatum* L.) cultures, two callus morphologies were described: type I, which was characterized as a compact, nodular, white to light-yellow callus and type II, being friable, semi-soft, and highly regenerable (Vasil and Vasil 1994). Based on these definitions, the K3 calluses described in this study fit the characteristics of type II callus, which may explain the significant correlation of shoot occurrence on K3 ($R^2 = 0.56$, $p < 0.0001$).

Calluses that developed roots were generally nodular and semi-soft in appearance (K1) and occurred alongside anthocyanin spots similar to what has been previously reported in *Miscanthus* (Petersen 1997). These structures were glabrous and displayed a red/purple pigmentation in the area of the calyptra. In previous grass culture studies, K1 calluses are generally root-forming (Morrish *et al.* 1987); however, the current study did not observe a strong correlation ($R^2 = 0.22$, $p = 0.1164$) between the occurrence of roots and K1 calluses.

The modified F-C assay (Singleton and Rossi 1965) used in this study is a rapid, non-specific colorimetric technique, which functions to quantify the amount of readily oxidized phenolic substances (Singleton *et al.* 1999) within biological material. In this study, soluble phenolic content was significantly affected by AIP levels, with the lowest and highest values detected in tissues cultured on media supplemented with 1000- and 1- μM AIP, respectively. Previous authors found that supplementing 10- μM AIP in the medium of alfalfa (*M. sativa* L.) cultures increased mitotic activity and prompted cellular division (Hrubcová *et al.* 2000). This has also been demonstrated in sessile oak cultures, in which increased total phenolic acid content positively correlated with non-converting embryos, and decreased phenolic content through application of AIP yielded embryos that more readily regenerated into plantlets (Cvikrová *et al.* 1998). In the current study, AIP failed to increase the capacity for regeneration in *M. × giganteus* over time compared to the control; however, the percentage of calluses exhibiting regenerated shoots increased significantly in 10- and 100- μM AIP. Regeneration of calluses at the highest concentration (1000- μM AIP) was lower than samples cultured on these two AIP treatments, and this was accompanied by a greater total MC

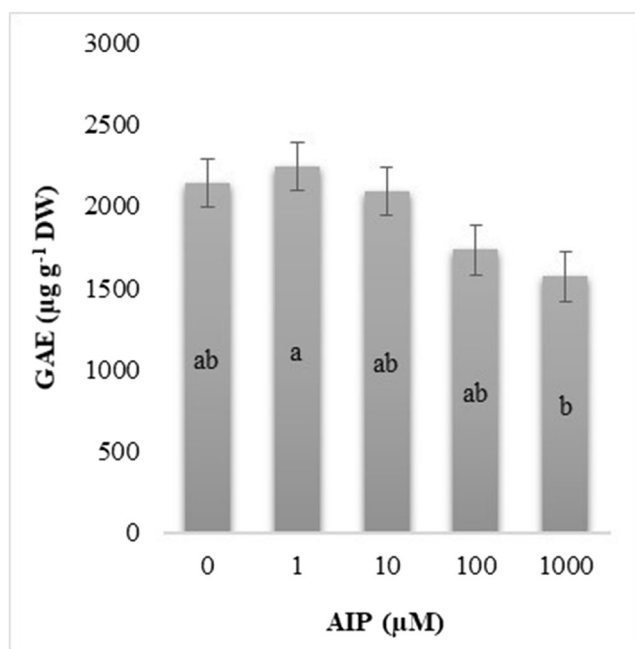


Figure 7 Regenerated *Miscanthus × giganteus* J.M.Greef, Deuter ex Hodk., Renvoize ‘M161’ calluses in rooting medium (a), liquid tillering medium (b), and after removal from tillering medium after approximately 28 d of incubation (c).

(87.3%). Similar side effects caused by drastic inhibition of the phenylpropanoid biosynthetic pathway have been documented previously and include increased incidence of hyperhydricity caused by reduced lignification and reduced lignin production for cell wall formation and development of shoots (Cvikrová *et al.* 2003). This could be of particular significance for grass species, as phenylpropanoids are major components of their cell walls and play important roles in cell growth/development and intercellular adhesion (Jones and Saxena 2013).

Another deleterious effect of high AIP levels in other systems is the hindrance of root formation and growth, as demonstrated in *Secale cereale* L. (Reuber *et al.* 1993). In contrast, average root development peaked in calluses incubated on 1000- μ M AIP in the fourth culture month (18.8). The frequency of calluses that exhibited root regeneration was more strongly represented in cultures maintained on media with higher auxin levels, which could be explained by the adventitious rooting promoting effects of 2,4-D (Viana and Mantell 1998). As with shoot regeneration, roots were induced for the extent of the culture period, although investigation of root culture with roots derived from callus tissue requires further examination in *M. x giganteus*.

Conclusions

The results presented here demonstrate that the regeneration capacity of *M. x giganteus* can be maintained for relatively long-term culture on semi-solid callus maintenance media, and that the percentage of calluses demonstrating regeneration can be enhanced with the addition of 10- and 100- μ M AIP. 2-Aminoindan-2-phosphonic acid functions to inhibit the phenylpropanoid biosynthetic pathway and thus can mediate phytohormone levels, cell wall development, and auxin metabolism (Hrubcová *et al.* 2000). Although soluble phenolic content decreased in a dose-dependent manner with AIP, a more targeted approach at specific phenolic content changes, including cell wall-bound phenols, and to account for potential interfering compounds could contribute to a better understanding of the regeneration capacity of this species. The findings of this study will help to develop options for alternative propagation methods through somatic embryogenesis and synthetic seeds and help to develop a foundation for *in vitro* breeding strategies.

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References

- Březinová A, Holík J, Zažimalová E, Vlasáková V, Malá J (1996) Somatic embryogenesis in oak (*Quercus robur* L.). Plant Physiol Biochem (spec.issue) Proc.10th FESPP Congress, Florence, Italy, Abstract No. S03-18, p 31
- Cvikrová M, Binarová P, Eder J, Vágner M, Hrubcová M, Zoň J, Macháčková I (1999) Effect of inhibition of phenylalanine ammonia-lyase activity on growth of alfalfa cell suspension culture: alterations in mitotic index, ethylene production, and contents of phenolics, cytokinins and polyamines. Plant Physiol 107:329–337
- Cvikrová M, Malá J, Eder J, Hrubcová M, Vágner M (1998) Abscisic acid, polyamines and phenolic acids in sessile oak somatic embryos in relation to their conversion potential. Plant Physiol Biochem 36: 247–255
- Cvikrová M, Malá J, Hrubcová M, Eder J, Zoň J, Macháčková I (2003) Effect of inhibition of biosynthesis of phenylpropanoids on sessile oak somatic embryogenesis. Plant Physiol Biochem 41:251–259
- Engbers H, Deen B (2013) Field-scale agricultural biomass research and development project final report. Agriculture and Agri-Food, Canada https://www.ontariosoilcrop.org/wp-content/uploads/2015/11/biomass_final_report-january2014-h.engbers_b.deen.pdf. Accessed 3 Feb 2018
- Fitch MMM, Moore PH (1990) Comparison of 2,4-D and picloram for selection of long-term totipotent green callus cultures of sugarcane. Plant Cell Tissue Organ Cult 20:157–163
- Folin O, Ciocalteu V (1927) On tyrosine and tryptophane determinations in proteins. J Biol Chem 73:627–650
- Gairi A, Rashid A (2004) TDZ-induced somatic embryogenesis in non-responsive caryopses of rice using a short treatment with 2,4-D. Plant Cell Tissue Organ Cult 76:29–33
- Głowacka K, Balachandran SM, Kaczmarek Z (2010a) Impact of colchicine application during callus induction and shoot regeneration on micropropagation and polyploidisation rates in two *Miscanthus* species. In Vitro Cell Dev Biol-Plant 46:161–171
- Głowacka K, Jezowski S, Kaczmarek Z (2010b) 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
- Gubišová M, Gubiš J, Žofajová A, Mihálik D, Kraic J (2013) Enhanced *in vitro* propagation of *Miscanthus x 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*. Glob Chang Biol 14: 2000–2014
- Heaton EA, Long SP, Voigt TB, Jones MB, Clifton-Brown J (2004) *Miscanthus* for renewable energy generation: European Union experience and projections for Illinois. Mitig Adapt Start GI 9:433–451
- 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
- Hrubcová M, Cvikrová M, Eder J, Zoň J, Macháčková I (2000) Effect of inhibition of phenylpropanoid biosynthesis on peroxidase and IAA-oxidase activities and auxin content in alfalfa suspension cultures. Plant Physiol Biochem 38:949–956
- Jones AMP, Chattopadhyay A, Shukla M, Zoň J, Saxena PK (2012) Inhibition of phenylpropanoid biosynthesis increases cell wall digestibility, protoplast isolation, and facilitates sustained cell division in American elm (*Ulmus americana*). BMC Plant Biol 12:75
- Jones AMP, Saxena PK (2013) Inhibition of phenylpropanoid biosynthesis in *Artemisia annua* L.: a novel approach to reduce oxidative browning in plant tissue culture. PLoS One 8:e76802

- Kim HS, Zhang G, Juvik JA, Widholm JM (2010) *Miscanthus × giganteus* plant regeneration: effect of callus types, ages and culture methods on regeneration competence. *Glob Change Biol 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
- Le Ngoc Huyen T, Rémond C, Dheilly RM, Chabbert B (2010) Effect of harvesting date on the composition and saccharification of *Miscanthus × giganteus*. *Bioresour Technol* 101:8224–8231
- Lewandowski I (1997) Micropropagation of *Miscanthus × giganteus*. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry*, vol 39, High-tech and micropropagation Springer, New York, 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
- Liu G, Gilding EK, Godwin ID (2015) A robust tissue culture system for sorghum [*Sorghum bicolor* (L.) Moench]. *S Afr J Bot* 98:157–160
- Lozovaya V, Gorshkova T, Yablokova E, Zabolotina O, Ageeva M, Rummyantseva N, Kolesnichenko E, Waranyuwat A, Widholm J (1996) Callus cell wall phenolics and plant regeneration ability. *J Plant Physiol* 148:711–717
- MacDonald MJ, D’Cunha GBD (2007) A modern view of phenylalanine ammonia lyase. *Biochem Cell Biol* 85:273–282
- Malá J, Cvikrová H, Březinová A, Hrubcová M, Eder J, Vágner M, Cvikrová M (2000) Endogenous contents of phytohormones and phenylpropanoids in sessile oak somatic embryos in relation to their conversion potential. *J Forest Sci* 46:197–204
- Mehrotra S, Goel MK, Kukreja AK, Mishra BN (2007) Efficiency of liquid culture systems over conventional micropropagation: a progress towards commercialization. *Afr J Biotechnol* 6:1484–1492
- Morrish F, Vasil V, Vasil IK (1987) Developmental morphogenesis and genetic manipulation in tissue and cell cultures of the Gramineae. *Adv Genet* 24:431–499
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Owaga Y (2015) Long-term maintenance of high regeneration ability of switchgrass embryogenic callus. *Plant Biotech* 32:1–4
- 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
- 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
- Reuber S, Leitsch J, Krause GH, Weissenböck G (1993) Metabolic reduction of phenylpropanoid compounds in primary leaves of rye (*Secale cereale* L.) leads to increased UV-B sensitivity of photosynthesis. *Z Naturforsch* 48:749–756
- Singleton VL, Orthofer R, Lamuela-Raventós RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol* 299:152–178
- Singleton VL, Rossi JA (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* 16:144–158
- Ślusarkiewicz-Jarzina A, Ponitka A, Ceraży-Waliszewska J, Wojciechowicz MK, Sobańska K, Jeżowski S, Pniewski T (2017) Effective and simple *in vitro* regeneration system of *Miscanthus sinensis*, *M. × giganteus* and *M. sacchariflorus* for planting and biotechnology purposes. *Biomass Bioenergy* 107:219–226
- Taşkın H, Baktemur G, Kurul M, Büyükalaca S (2013) Use of tissue culture techniques for producing virus-free plant in garlic and their identification through real-time PCR. *Sci World J* 2013:1–5
- Tejera MD, Heaton EA (2017) Description and codification of *Miscanthus × giganteus* growth stages for phenological assessment. *Front Plant Sci* 8:1–12
- Vasil IK, Vasil V (1994) *In vitro* culture of cereals and grasses. In: Vasil IK, Thrope TA (eds) *Plant cell and tissue culture*. Kluwer Academic Publishers, Dordrecht, Netherlands, pp 293–312
- Viana AM, Mantell SH (1998) Comparative uptake and metabolism of 2-[¹⁴C]-2,4-dichlorophenoxyacetic acid in callus cultures of monocot (*Dioscorea* spp.) and dicot (*Nicotiana tabacum* L.) plants. *Braz J Bot* 21:89–99
- Vreugdenhil D, Boogaard Y, Visser RGF, de Bruijn SM (1998) Comparison of tuber and shoot formation from *in vitro* cultured potato explants. *Plant Cell Tissue Organ Cult* 53:197–204
- Withers KK (2015) Morphological adaptations and membrane stabilizing mechanisms of overwintering *Miscanthus* (Poaceae). Unpublished thesis. University of Guelph, Guelph, ON, Canada <http://atrium.lib.uoguelph.ca/xmlui/handle/10214/8708>. Accessed 4 Oct 2017
- Xue S, Kalinina O, Lewandowski I (2015) Present and future options for *Miscanthus* propagation and establishment. *Renew Sust Energ Rev* 49:1233–1246
- Zaid A (1987) *In vitro* browning of tissues and media with special emphasis to date palm cultures—a review. *Acta Hort* 212:561–566
- Zawadzki M, Ragauskas A (1999) Quantitative determination of quinone chromophores in isolated lignins. IPST Technical Paper Series Number 815:1–28