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An Improved Tissue Culture System for Embryogenic Callus Production and Plant Regeneration in Switchgrass (*Panicum virgatum* L.)

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Abstract The increased emphasis on research of dedicated biomass and biofuel crops begs for biotechnology method improvements. For switchgrass (*Panicum virgatum* L.), one limitation is inefficient tissue culture and transformation systems. The objectives of this study were to investigate the utility of a new medium described here, LP9, for the production and maintenance of switchgrass callus and its regeneration, which also enables genetic transformation. LP9 medium is not based on Murashige and Skoog (MS) medium, the basal medium that all published switchgrass transformation has been performed. We demonstrate an efficient tissue culture system for switchgrass Alamo 2, which yields increased viability of callus and the ability to maintain callus for a duration of over 6 months. This longevity gives a greater useful callus lifetime than for published switchgrass MS-based media. This increased longevity enables greater potential efficiency and throughput for a transformation pipeline. Callus produced on LP9 is categorized as type II callus, which is more friable and easier to multiply, maintain and transfer than type I callus obtained from previously described tissue culture systems.

Keywords Biotechnology · Bioenergy feedstock · Biofuels · Callus · Media · Somatic embryogenesis · Tissue culture · Transformation

Abbreviations

BA	Benzyladenine
BAP	6-Benzylaminopurine
2,4-D	2,4-Dichlorophenoxyacetic acid
SEM	Scanning electron microscopy
TDZ	Thidiazuron

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Introduction

Increasing interest in the production of biofuels has warranted research in the production and genetic manipulation of high biomass crops such as switchgrass (*Panicum virgatum* L.), a warm-season perennial grass native to North America. With this increased interest, it is necessary to develop higher throughput transformation systems that are enabled by an efficient and reliable tissue culture system for target tissue production and plant regeneration. Stable transformation, in turn, enables the reverse genetics research for cell wall manipulation and plant growth improvement. Current switchgrass tissue culture and transformation systems are not very efficient and limited to derivatives of a single variety: Alamo. There are currently two described tissue culture systems in switchgrass: embryogenic callus [1–5] and seed-derived callus [6, 7]. Limitations of these systems include longevity of embryo viability (typically, less than 2 months) and high genetic and response variability in the seed-derived system. Despite the increased interest in switchgrass tissue culture, there has been little recent progress to enhance switchgrass tissue culture systems.

Callus in grasses can be classified as type I or type II, based upon color, texture, regeneration system, and the amount of time required for callus initiation. The morphology of callus has been reported and described in the important agronomic monocot crops such as maize [8–14], rice [15–17], sorghum [18], sugarcane [19], wheat [20], and various nonfood grasses [21–26]. Type I callus is the typical and most prevalent callus formed in monocot

species. It is characterized by compact form, slow-growth, white to light yellow in color, and highly organized [27]. This callus is composed almost entirely of cytoplasmic meristematic cells that lack large vacuoles. In maize, type I callus can only be maintained for only a few months and cannot be used in suspension cultures; whereas, type II callus can be maintained in culture for extended periods of time and is able to form cell suspensions [8, 27, 28]. Type II callus derived from maize has been described as soft, friable, rapidly growing, and exceedingly regenerative but is typically formed at lower frequencies than type I callus [8, 27–29]. Switchgrass callus described to date has all been of type I. In this study, we describe a novel non-Murashige and Skoog (MS)-based media and culture conditions that result in the production of type II callus in switchgrass that can be useful for the production of transgenic plants (Figs. 1 and 2).

Methods

Callus Induction, Maintenance, and Plant Regeneration

Switchgrass Alamo 2 tillers grown in the greenhouse were excised from plants at the E2 to E4 stage [30]. Inflorescences were cut 7 mm above the fourth node [1]. Pieces of inflorescence were then sterilized for 35 min in 75% commercial bleach containing 1% Tween 20. Inflorescences were subsequently washed with sterile water three times. Ends were then removed and discarded, and inflorescences were cut longitudinally in half, placed on MS + benzyladenine (BA), and incubated at 25°C in the dark as performed previously [1]. It is at this point in our protocol that we have diverged from previous tissue culture methodologies of embryogenic callus production [1] to produce friable type II callus.

After 10 days on MS + BA, inflorescences were removed from the media and placed into a sterile Petri dish, further cut into 1-cm-long segments and placed onto LP9, a new callus induction media modified from the callus induction medium by Lu et al. [31]. This media by Lu et al. [31] was altered by adding the auxin 2,4-dichlorophenoxy-acetic acid (2,4-D; 5 mg l^{-1}) in place of dicamba, removing BA and myo-inositol from the media, and decreasing the amount of proline from 500 to 100 mg l^{-1}. These determinations were made after informal experimentation with switchgrass (data not shown). After 14 days on LP9, callus developed (Fig. 3b). Callus was then excised from the explants and cultured further on fresh LP9.

Antibiotic Selection

Callus production under hygromycin selection was performed to determine optimal selection rates that can be used for genetic transformation. Four concentrations of hygromycin B (0, 50, 60, and 70 mg l^{-1}; Calbiochem, Gibbstown, NJ, USA) in LP9 (5 mg l^{-1} 2,4-D, 100 mg proline, N_6 macroelements, B_5 microelements, Fe-EDTA, supple-

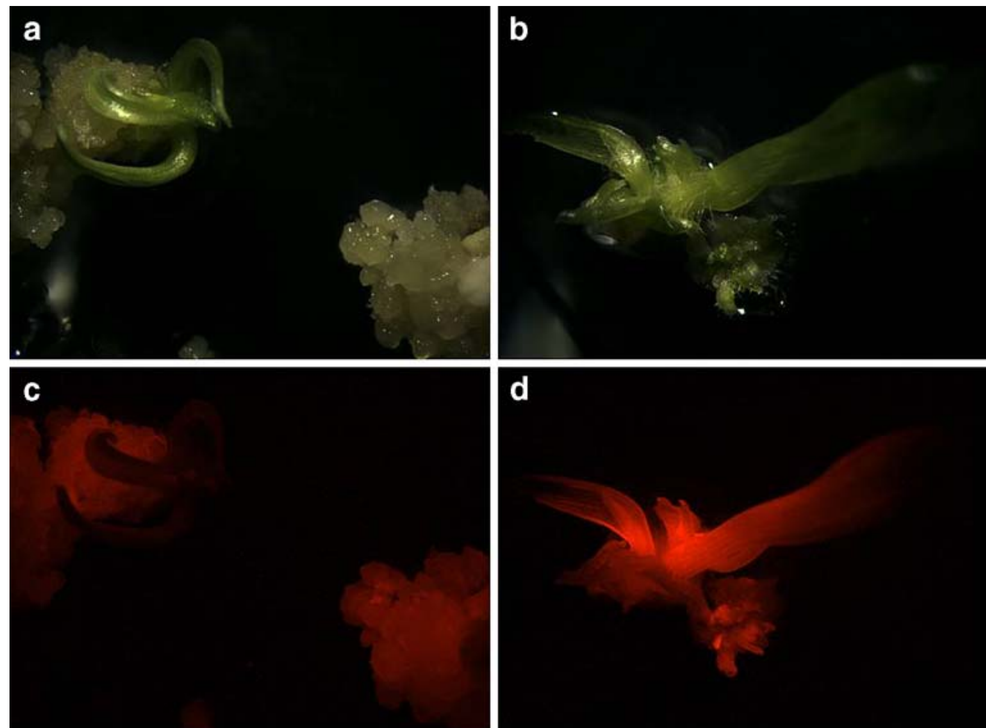

Fig. 1 Nontransgenic and transgenic switchgrass. **a** Nontransgenic and **b** switchgrass transformed with pporRFP under 3 ms white light exposure. **c** Nontransgenic and **d** switchgrass transformed with pporRFP excited with 535/30 nm light and emissions filter 600/50 nm exposed for 30 s

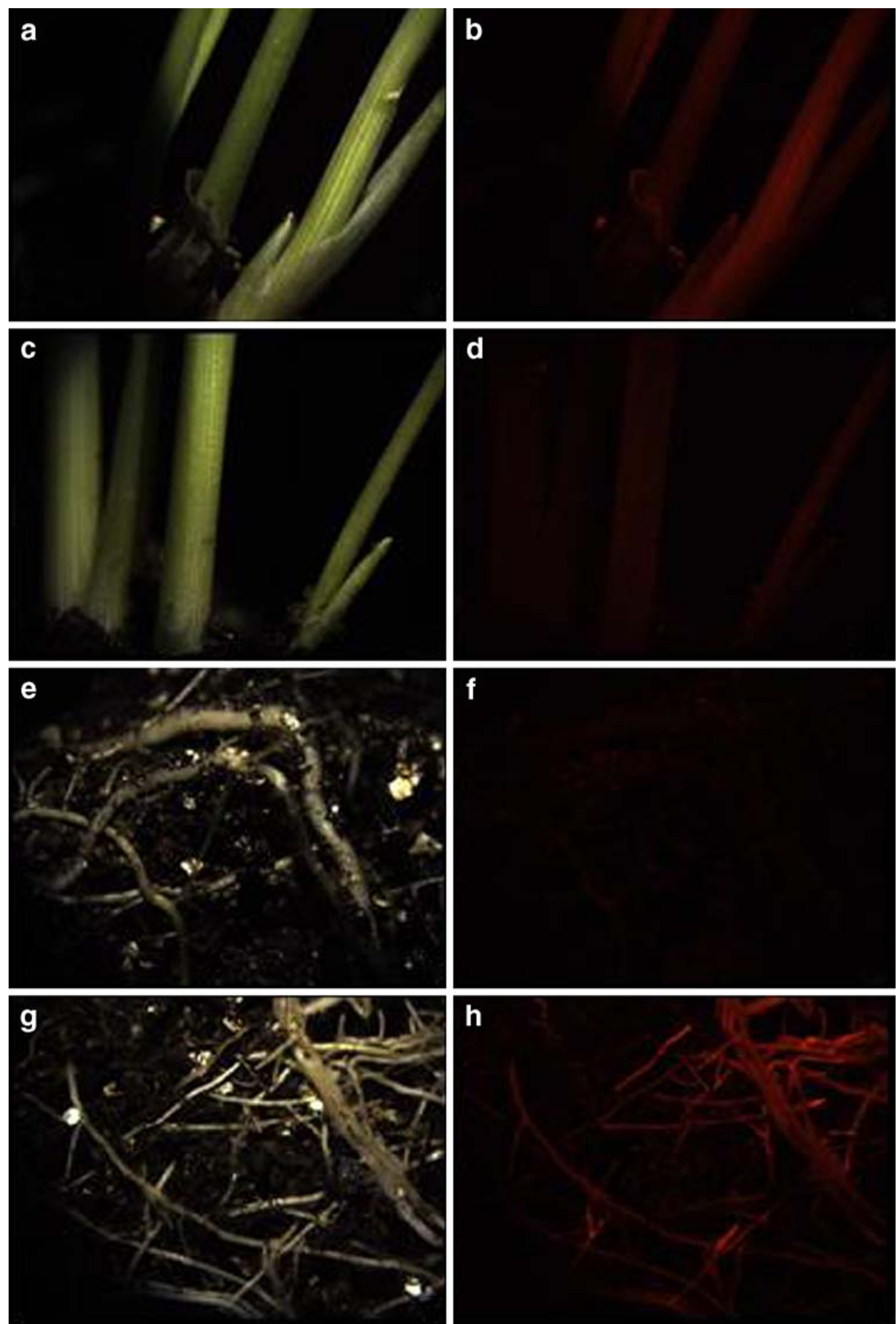

Fig. 2 Nontransgenic and transgenic switchgrass transplanted into bark media in pots. The dissecting micrographs were produced using the lowest power of an epifluorescence dissecting microscope. **a** Switchgrass transformed with pporRFP and **c** nontransgenic under 3 ms white light exposure. **b** Switchgrass transformed with pporRFP and **d** nontransgenic excited with 535/30 nm light and emission filter 600/50 nm exposed for 15 s. **e** Nontransgenic roots of switchgrass and **g** switchgrass transformed with pporRFP under white light and **f** nontransgenic excited and **h** switchgrass transformed with pporRFP excited with 535/30 nm light and emissions filter 600/50 nm exposed for 15 s

mented with Gamborg's vitamins, 500 mg l^{-1} casein hydrolysates, 500 mg l^{-1} glutamine, 30 g l^{-1} sucrose, 3 g l^{-1} Gelzan) [31] were used to determine kill curves for six replicated plates for each treatment containing 20 explants per plate. Growth was assessed by weighing fresh callus pieces at days 0 and 60. Subculturing was performed at 3-week intervals. Data were analyzed by analysis of variance (ANOVA) using the general linear model (SAS 9.2, Cary, NC, USA). Duncan's multiple range test was used to compare treatment mean values when significant differences (at the 0.05 probability level) were found.

Vector Construction

The binary vector contained a red fluorescent protein (*pporRFP* gene from the coral *Porites porites*) gene under

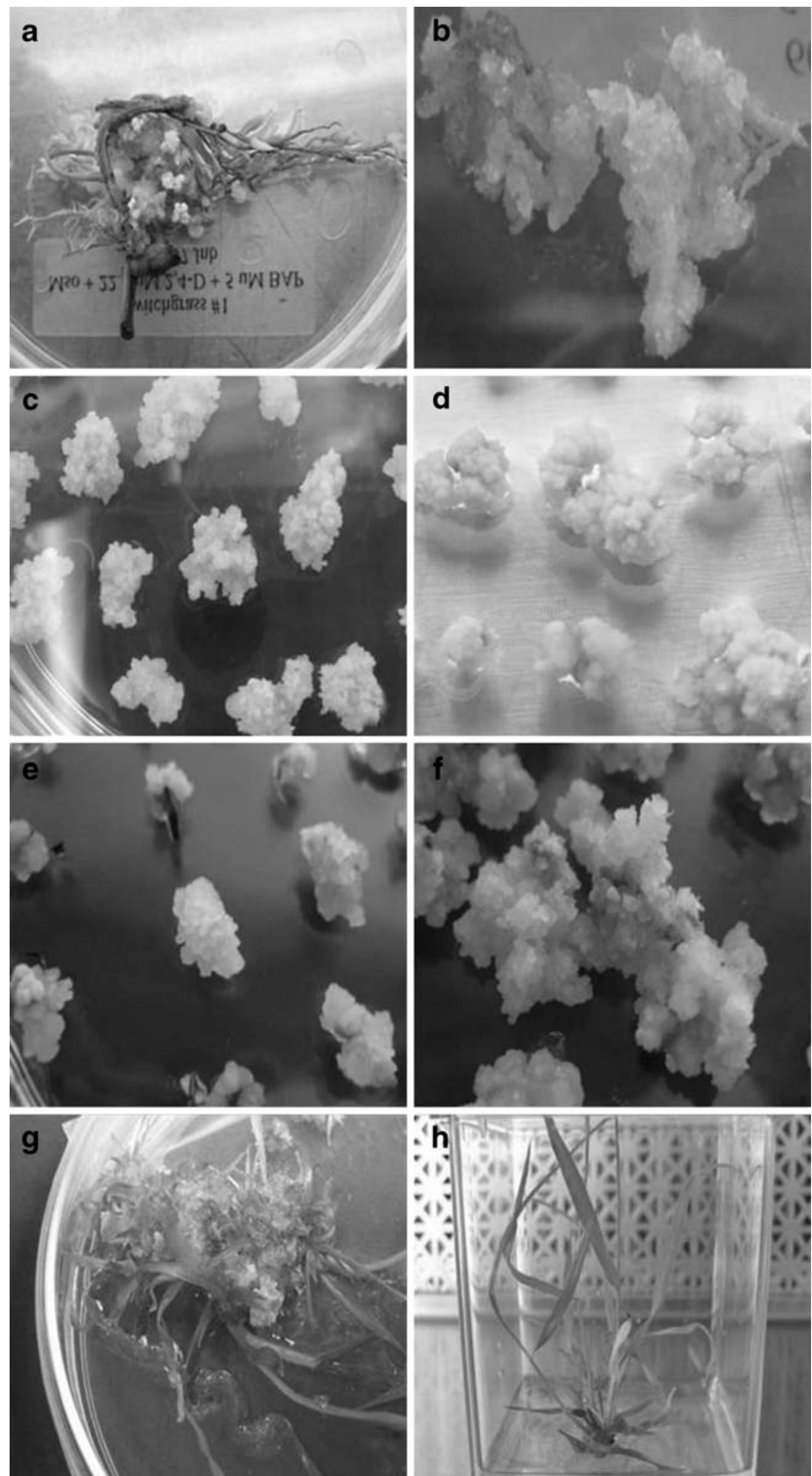

Fig. 3 Two tissue culture media were used to produce callus from Alamo 2 inflorescence. **a** Callus derived from the methods of [1] and **b** callus derived from LP9 media. Type II callus was observed on **c** LP9, and type I callus was observed on **d** MSO + 2,4-D + BAP media [1] in switchgrass. Alamo 2 callus grown on LP9 for **e** 1 and **f** 6 months. Formation of **g** Alamo 2 shoots at 2 weeks and **h** rooted Alamo 2 shoot

the control of the maize ubiquitin (*ZmUbi*1) promoter and the selectable marker gene encoding hygromycin phosphotransferase (*hph*) that was under the control of the rice actin 1 (*OsAct*1) promoter. The ZmUbi1 promoter from the pAHC25 plasmid [32] was polymerase chain reaction (PCR) amplified and cloned into pCR4B-TOPO (Invitrogen, Carlsbad, CA, USA). The pEarleyGate 304 plasmid contains the Gateway compatible cassette *attR1*-Cm^{R}-*ccdB*-*attR2* coupled with an AcV5 epitope and the *OCS* terminator [33]. This cassette was PCR amplified and cloned directly

downstream of the *ZmUbi*1 promoter in pCR4B-TOPO. To confer resistance to hygromycin, a cassette containing the *OsAct*1 promoter and *hph* gene was cloned upstream of the *ZmUbi*1- *attR1*-Cm^R-*ccdB*-*attR2* cassette in pCR4B-TOPO. The *OsAct*1-*hph* and *ZmUbi*1- *attR1*-Cm^R-*ccdB*-*attR2* cassettes were excised with *Sbf*I and *Asc*I and gel purified with QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The pPZP201BK binary backbone [34] was digested with *Pst*I and *Asc*I, purified with the QIAquick PCR Purification Kit (Qiagen), and ligated with the *OsAct*1-*hph* and *ZmUbi*1-*attR1*-Cm^R-*ccdB*-*attR2* cassettes. The *pporRFP* gene [35] was kindly provided by Dr. Mikhail Matz and was cloned into pCR8/GW/TOPO and recombined into the expression vector using Gateway® LR Clonase® II enzyme mix (Invitrogen). All amplified regions and resulting plasmids were sequence verified at the University of Tennessee Molecular Biology Resource Facility.

Agrobacterium-Mediated Transformation

Transformation was conducted using *Agrobacterium tumefaciens* strain EHA105 containing the vector described above. *Agrobacterium* was grown in YEP supplemented with 50 mg l^{-1} kanamycin at 27°C for 2 days. Cultures were centrifuged at 4,000 rpm for 30 min. Supernatant was removed, and *Agrobacterium* was resuspended in 25 ml of liquid LP9, 100 μM of acetosyringone, and 10 μl of Silwet. *Agrobacterium* solution was then shaken at 150 rpm at room temperature for 30 min. Callus pieces, 0.5 cm, were placed in the *Agrobacterium* solution and incubated at room temperature under 0.53 atm vacuum for 30 min. Callus pieces were then coincubated on LP9 for 3 days. Callus pieces were placed in liquid LP9 + 400 mg l^{-1} timetin and vortexed to remove excess *Agrobacterium* growth. Callus pieces were placed on LP9 + 60 mg l^{-1} hygromycin + 400 mg l^{-1} timetin. Calli were transferred to new LP9 media + antibiotics every 2 weeks until transgenic callus formed and grew to 0.5 cm. Calli were then placed on MSO + 5 μM BAP + 60 mg l^{-1} hygromycin + 400 mg l^{-1} timetin. Shoots were formed after 14 days and were placed onto MS as described above to regenerate plants.

Histology

Scanning electron microscopy (SEM) was performed whereby tissues (5 weeks) were fixed in 3% (*v*/*v*) glutaraldehyde and 0.1 M cacodylate buffer. Samples were then rinsed three times (10 min each) in cacodylate buffer and subsequently postfixed in cacodylate-buffered 2% osmium tetroxide for 90 min and subsequently dehydrated in acetone in series (25%, 50%, 75%, 90%, 100%, and dry 100%). Following acetone dehydration, the samples were critical point dried with liquid carbon dioxide (Ladd Research Industries Critical Point Dryer). Dried samples were stored under vacuum until viewing. Five calli were affixed to two-sided carbon tape on a stub and sputtered with gold (SPI Sputter coater) prior to viewing with SEM. Samples were observed with a LEO (Zeiss) 1525 FE-SEM.

Results

Callus Induction, Maintenance, and Plant Regeneration

LP9 medium produced stable type II friable callus (Fig. 3b) for switchgrass Alamo 2 that is morphologically and functionally unique compared with that from previous research (Fig. 3a, d) [1]. Callus produced from this media is brittle and white (Fig. 3b, c), which allowed for easy multiplication and transfer, and less “artistic judgment” to select callus that will subsequently proliferate. This morphology is consistent with descriptions of type II callus previously shown in maize [8, 10, 13]. Callus forms quickly, after 2 weeks, on LP9 media from inflorescent explants on 100% of inflorescent explants, which is similar to that achieved from the published methods [1]. Callus can easily be removed from the cut explants and placed onto fresh media, forming type II callus, which proliferated at an efficiency of 33%; i.e., approximately 67% of the callus is type I. Once formed, type II callus was transferred every 3 weeks until it was used for transformation experiments or plant regeneration. Callus induced from LP9 media has demonstrated longevity in our laboratory, thus far, for over 6 months compared with embryogenic callus derived from MS + 2,4-D + 6-benzylaminopurine (BAP) that tends to become unresponsive after 3–4 months (Fig. 3e, f) ([1]; data not shown). In practice, since callus can be divided and utilized for longer than 6 months, a genetic transformation pipeline is potentially much more efficient and faster since new explants are not needed as frequently (Fig. 3f). After 1 month on LP9, callus can be selected and used for *A. tumefaciens*-mediated transformation or particle bombardment, and plants begin to be regenerated within 3 weeks of callus initiation.

Shoots were readily produced in 2 weeks after placement onto MS medium containing 5 μM BAP [1] (Fig. 3g). Once shoots were produced, individual shoots were separated from the clump and placed into MS in Magenta GA7 boxes, and rooting occurred in about 2 weeks (Fig. 3h).

Transformation

Five independent transgenic events originating from separate callus pieces were obtained using this method from 113 callus clusters exposed to *Agrobacterium*, i.e., 4.4% transformation efficiency. However, these 113 callus clus-

ters consisted of type I and type II (approximately half of each), but only type II callus yielded transgenic plants; therefore, this transformation efficiency can likely be increased by selecting only type II callus for transformation experiments. These results are comparable to published efficiency from prior methods. Somleva et al. [36] confirmed that 27 transgenic events were recovered from 794 callus clusters exposed to *Agrobacterium*, i.e., 3.4% efficiency. Entire plant expression of pporRFP was visualized using the lowest power of an epifluorescence dissecting microscope excited with 535/30 nm light and emissions filter 600/50 nm exposed for 15 s (Figs. 1 and 2). PporRFP has an excitation maximum of 578 nm and an excitation maximum of 595 nm [35] and gives bright fluorescence in transgenic plants (Figs. 1 and 2).

Antibiotic Selection

After 2 months on selection, fresh weights were found to be significantly different in hygromycin treatments ($P<0.05$; Fig. 4). Fresh weights obtained from calli grown on selection with 60 mg l^{-1} hygromycin was not significantly different from those grown on 70 mg l^{-1} hygromycin selection ($P<0.05$), but these were much less than those grown on 50 mg l^{-1} hygromycin (Fig. 4). Thus, the apparent optimal concentration for subsequent selection of transgenic calli was determined to be 60 mg l^{-1} hygromycin.

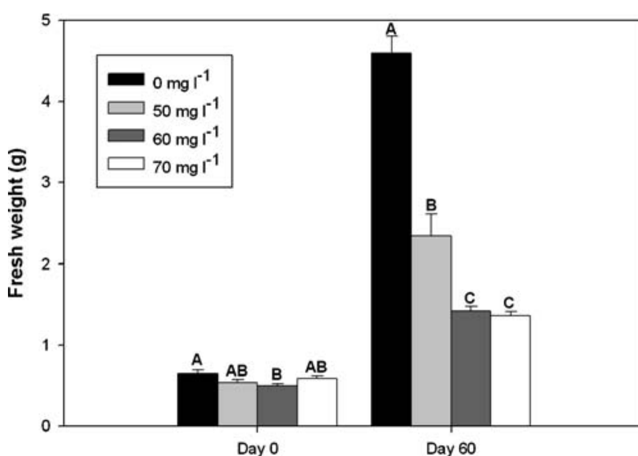

Fig. 4 Fresh weights (grams) of callus grown under hygromycin selection. Six plates of callus were grown in LP9 with each of four concentrations of hygromycin (0, 50, 60, or 70 mg l^{-1}). Fresh weights were recorded on day 0 and 60. Data were analyzed by ANOVA using the general linear model (SAS 9.2, Cary, NC, USA). Duncan's multiple range test was used to compare treatment mean values within time points for significant differences (at the 0.05 probability level). *Error bars* indicate standard error. Different *letters* at each time point indicates significant differences

Histology

A scanning electron micrograph of callus produced on LP9 (Fig. 5) shows clusters of somatic embryos that are found as protrusions along the leftmost surface. Various morphologies were observed in this type II callus produced on LP9 (Fig. 5). These morphologies include irregular surfaces with globular, unorganized tissue types. Tissues of this type have demonstrated to have rapid growth and be highly friable.

Discussion

Recent progress to improve switchgrass tissue culture systems and transformation has been limited. Previous tissue culture systems for switchgrass have manipulated various auxins and cytokinins in an MS-based system [3–6]. Plant regeneration by both somatic embryogenesis and organogenesis occurred from both mature caryopses and young leaf segments of switchgrass Alamo at 45 and 5 μM BAP, respectively, in combination with 22.5 μM 2,4-D [3]. Denchev and Conger [4] examined the influence of type and concentration of two auxins, 2,4-D and picloram, in combination with the cytokinin, BA, on callus induction and regeneration in switchgrass Alamo mature caryopses and determined that 11.3–45.0 μM 2,4-D in combination with 15.0 or 45.0 μM BA produced optimal results. Various combinations of 2,4-D and another cytokinin, thidiazuron (TDZ), have also been researched for their ability to produce a highly regenerative tissue culture system in switchgrass cv. Alamo [6]. The best

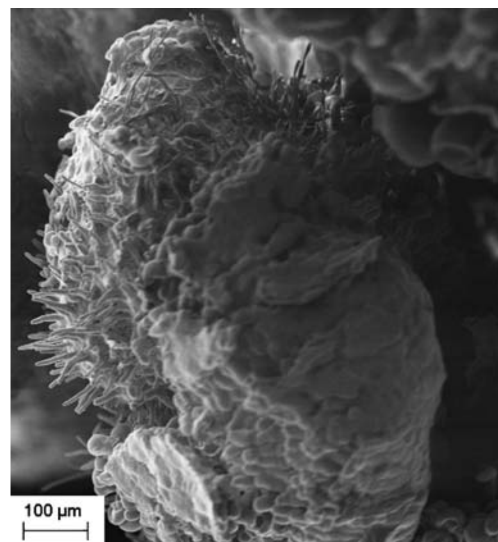

Fig. 5 Embryogenic culture of Alamo 2 switchgrass at 5 weeks. Scanning electron micrograph of surface features demonstrating clusters of somatic embryos (*leftmost*) and friable, type II callus features including globular, unorganized and smooth structures

combination was determined to be 4.5 μM 2,4-D and 18.2 μM TDZ [6]. Dutta Gupta and Conger [5] were able to establish embryogenic cell suspension cultures of switchgrass when MS media were supplemented with 9.0 μM 2,4-D and 4.4 μM BAP. However, tissue culture systems using MS in combination with 2,4-D and various cytokinins produced a callus that was hard, white, and compact at the coleptilar or scutellar stage of embryogenesis and, therefore, is classified as type I callus [3, 5, 37].

LP9 is not an MS-based tissue culture medium, but rather combines N_6 macroelements and B_5 microelements with the auxin, 2,4-D. It does not include any cytokinin. This system is novel and enables callus production and plant regeneration in switchgrass. Lu et al. [31] utilized media with similar components for bermuda grass tissue culture, but their protocol did not result in friable callus with type II characteristics. However, we have demonstrated that LP9 produces type II switchgrass callus that is highly friable and has increased longevity compared with prior media. Most maize tissue culture systems that produce type II callus demonstrating friability and increased longevity utilize N_6 macroelements as their media base [8, 10, 13]. However, we are the first to examine the callus induction and plant regeneration using N_6 macroelements combined with B_5 microelements in switchgrass tissue culture.

Armstrong and Green [8] found that the addition of L-proline to N_6 media produced a friable, type II callus from immature maize embryos and further concluded that proline might function to protect the cultures from various stresses. Interestingly, the addition of L-proline to MS media did not enhance formation of somatic embryos in maize [8].

LP9 supplemented with hygromycin (60 mg l^{-1}) can be used to select for transformants similar to those selected using bialaphos (10 mg l^{-1}) in an MS-based system [38, 39].

Here, we have demonstrated an improved tissue culture system for switchgrass Alamo 2 that features a friable, fast-growing callus derived from inflorescences that can be classified as type II callus using our new media, LP9. Callus produced on LP9 demonstrates increased viability and can be maintained for longer periods of time (greater than 6 months), enabling use in transformation experiments without having to regenerate callus from new tillers. This enhanced maintenance and regeneration reduces the time needed to produce whole transgenic plants by at least 1 month. In addition, type II callus is easily recognized by its friable and dry appearance and lighter color, which can be chosen without the aid of a microscope for subsequent proliferation. Further work needs to be done to compare transformation efficiencies of switchgrass callus produced on LP9 to those produced on media in the current literature as well as examine genotype specificity.

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