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## Switchgrass (*Panicum virgatum* L.) cell suspension cultures: Establishment, characterization, and application

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### ABSTRACT

Switchgrass (*Panicum virgatum* L.) is a warm-season perennial grass that has received considerable attention as a potential dedicated biofuel and bioproduct feedstock. Genetic improvement of switchgrass is needed for better cellulosic ethanol production, especially to improve cellulose-to-lignin ratios. Cell suspension cultures offer an *in vitro* system for mutant selection, mass propagation, gene transfer, and cell biology. Toward this end, switchgrass cell suspension cultures were initiated from embryogenic callus obtained from genotype Alamo 2. They have been established and characterized with different cell type morphologies: sandy, fine milky, and ultrafine cultures. Characterization includes histological analysis using scanning electron microscopy, and utility using protoplast isolation. A high protoplast isolation rate of up to 10<sup>6</sup> protoplasts/1.0 g of cells was achieved for the fine milky culture, whereas only a few protoplasts were isolated for the sandy and ultrafine cultures. These results indicate that switchgrass cell suspension type sizably impacts the efficiency of protoplast isolation, suggesting its significance in other applications. The establishment of different switchgrass suspension culture cell types provides the opportunity to gain insights into the versatility of the system that would further augment switchgrass biology research.

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### 1. Introduction

Switchgrass (*Panicum virgatum* L.) is a warm-season perennial grass that is native to the prairies of North America and grown in monoculture for hay, grazing, and erosion control. It has received considerable interest for its potential as a bioenergy crop owing to its high biomass production and wide adaptation [1–4]. Genetic manipulation of switchgrass is needed for the development of improved switchgrass for better cellulosic ethanol production [5–8]. Recently, *in vitro* techniques have been developed and advanced as of great potential for assaying the target genes in switchgrass [9–15]. Yet, stable transformation is time-consuming because of low efficiency inherent in the current system; switchgrass is considered to be recalcitrant toward genetic transformation.

Cell suspension cultures offer an *in vitro* system that can be used as a tool for various studies in switchgrass. They can be used in

experiments involving mutant selection, mass propagation, protoplast isolation, gene transfer, and cell biology to study cell wall traits. It is now accepted that plants and cultured cells metabolize foreign compounds in qualitatively similar ways [16,17], but with qualifications.

Here, we report the development of three novel switchgrass cell suspension cultures, each with distinct morphological features. Our initial evaluation indicated the significance of the cell type in effectiveness of protoplast isolation. The different cell types among the switchgrass suspension cultures provide an opportunity to gain further insights into their potential applications in cell wall biology and biotechnology.

### 2. Materials and methods

#### 2.1. Production of switchgrass cell suspension cultures

##### 2.1.1. Initiation

Initial cell suspension culture was generated essentially as described [18] with some variations. Embryogenic callus was formed from *in vitro*-developed inflorescences of nodal segments of genotype Alamo 2 [19]. Approximately 1.0 g fresh weight of callus was transferred to 125-ml flasks containing 30 ml Murashige and Skoog [20] medium supplemented with 9  $\mu$ M 2,4-

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Fig. 1. Switchgrass cell suspension types sandy, fine milky, and ultrafine in the exponential growth phase, 5 days after subculture.

dichlorophenoxyacetic acid (2,4-D), 4.4  $\mu\text{M}$  6-benzylaminopurine (BAP) (both from Sigma, St. Louis, MO, USA), and 3% maltose (Fisher, Fair Lawn, NJ, USA). Flasks were incubated on a rotary shaker at 120 rpm and maintained at 22–24 °C in the dark. Feeding of the cultures with fresh medium was done at 10–14 days intervals, at which the suspensions were allowed to settle and 6–8 ml of supernatant were removed and replaced by 12–14 ml of fresh medium. This procedure was repeated for about 7 weeks. The supernatant was then filtered through a 210- $\mu\text{m}$  mesh (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The resultant suspension derived from the filtrate was used as primary source for the subsequent establishment of different types of cell suspension cultures.

### 2.1.2. Establishment

Different cell types were established using the original source suspension culture. Subsequent filtrations of the suspension through a 210- $\mu\text{m}$  mesh at 2-week intervals maintained the suspension in a dilute stage, which resulted in an ultrafine type culture. Whereas feeding the suspension, as described above, for about 2 months transformed the suspension into a sandy type culture. Occasionally, over time, the sandy type cultures tended to aggregate into cell clusters. Eventually, these aggregated pieces began to release fine cells into the medium giving the supernatant a milky appearance, which we termed fine milky type culture.

### 2.2. Histology

Cells were collected by centrifugation and the supernatant was replaced with 3% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA, USA). After 45 min, the glutaraldehyde was removed and the cells were washed with cacodylate buffer three times for 10 min each. The cells were post-fixed in 2% osmium tetroxide (Electron Microscopy Sciences) for 45 min and subsequently dehydrated in an acetone series (25%, 50%, 75%, 90%, 100%, and dry 100%). Following acetone dehydration, the cells were critical point dried with liquid carbon dioxide. The samples were sprinkled onto two-sided carbon tape on a stub and sputtered with gold (SPI sputter coater) prior to viewing with a LEO (Zeiss) 1525 FE-scanning electron microscope (SEM).

### 2.3. Protoplast isolation

Details of protoplasts isolation were essentially the same as described in our previous work [21] with several modifications. Protoplasts were isolated from suspension cultures in the exponential growth phase, 4–5 days after subculture. Approximately 1.0 g fresh weight suspension cells were used per 7 ml enzyme solution (0.6 M mannitol, 10 mM MES [pH 5.7], 6% cellulase (Onozuka RS) (Serva/Crescent Chemicals, Islandia, NY, USA), 1% macerozyme R-10 (PlantMedia, Dublin, OH, USA), 1% driselase (Sigma), 0.5% pectolyase Y-23 (MP Biomedicals LLC, OH, USA), 0.1% BSA, 70 mM  $\text{CaCl}_2$ ). Prior to addition of the cells, the enzyme solution was

dissolved by incubating at 55 °C for 10 min, cooled to room temperature, and filtered through a 0.45  $\mu\text{m}$  microfilter (Millipore, Billerica, MA, USA). The digestion was carried out in the dark under gentle shaking at 50 rpm for 3–4 h at room temperature. After incubation, the solution was filtered sequentially through 70 and 40  $\mu\text{m}$  Falcon cell strainer (BD Biosciences, Bedford, MA, USA) placed in Petri-dish. One volume of W5 washing solution (154 mM NaCl, 125 mM  $\text{CaCl}_2$ , 5 mM KCl, 2 mM MES) was added and protoplasts were collected by centrifugation at 1300 rpm for 7 min and re-suspended in 500  $\mu\text{l}$  of Mmg solution (0.6 M mannitol, 15 mM  $\text{MgCl}_2$ , 4 mM MES [pH 5.7]). Protoplasts were observed under bright field light using an Olympus BX51 microscope and quantified by a hemocytometer. Viability of the protoplasts was examined by Evans blue staining assay as described in our previous work [21].

### 3. Results and discussion

Switchgrass cell suspension cultures were initiated from embryogenic callus derived from inflorescences of nodal segments. Different types of cell suspension cultures were developed from the primary source culture. The sandy cell type culture was dense in texture and precipitated rapidly, whereas the fine milky and ultrafine types had a tendency to remain suspended in the media (Fig. 1). The growth color and texture of the sandy type culture to a certain extent depended on duration of feeding the cells. They became thick and pale yellow in color when fed more than 2 weeks. The sandy type culture grew at much higher rate than others. After approximately 5–6 weeks, culling of sandy type cells was required during feeding to keep them from becoming overpopulated. Large quantities of homogenous cells could be obtained after 3 months feeding (Fig. 2). On the contrary, the fine milky type cells grew slowly, yet despite their low cell density, viscosity of the culture increased within one week of establishment. It was visually apparent that the fine milky type culture became markedly viscous after two weeks of feeding the cells. Viscosity of cell suspension culture of some certain monocots has shown to be related to secreted polysaccharide pectinaceous substances from cells [22]. Interestingly, in the present study, only the fine milky type culture resulted in viscosity, which differentiated this cell type from other switchgrass cultures.

Histological analysis using scanning electron microscopy (SEM) demonstrated distinct morphological features between the types of the cell cultures—the sandy type consisted of large and often elongated cells; the fine milky type contained small, generally rounded cells; and the ultrafine type included various intermediate stages of the enlarged and small rounded cells (Fig. 3). The cells were generally present in compact small groups and only occasionally as single cells. The proportion of the two intermediate stages of cells in the ultrafine type culture depended on duration of filtration. Usually in durations longer than 2-week filtration intervals, the number of elongated cells in the suspension culture increased. Extracellular matrix (ECM)-like layer was observed on the surface of the fine milky cells (Fig. 3). Presence of ECM has been linked to

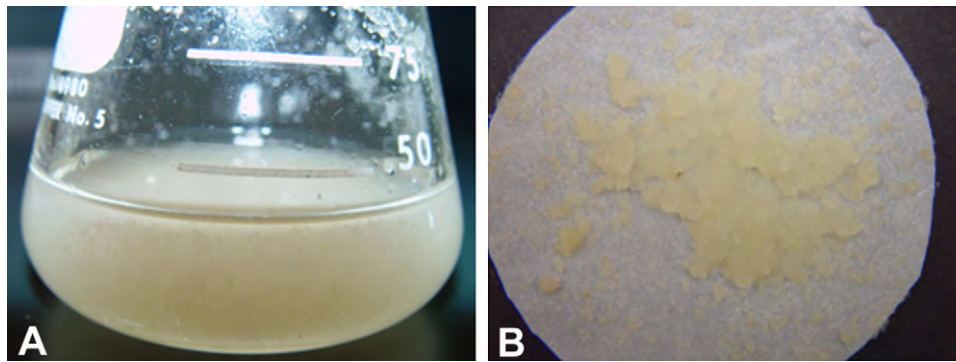


Fig. 2. Switchgrass cell suspension sandy type after 3 months feeding. (A) Dense suspension cells grown in flask and (B) cells filtrated through Whatman filter paper.

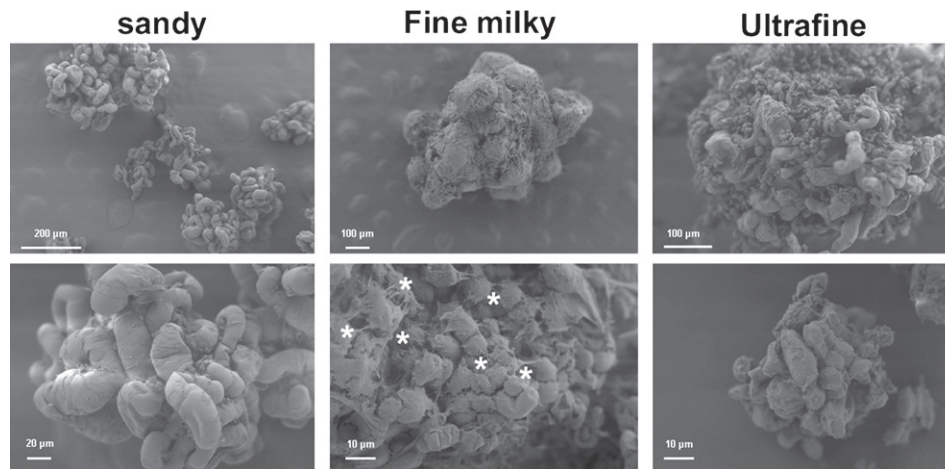


Fig. 3. Scanning electron micrograph of switchgrass cell suspension types sandy, fine milky, and ultrafine. Asterisks indicate extracellular matrix-like layer on the surface of the fine milky cells.

the formation of pro-embryogenic cells or the shift of embryogenic competence [23–25].

To evaluate the usefulness of the cell cultures in one application, isolation of protoplasts was performed on each type of culture. A high protoplast isolation rate, up to  $10^6$  protoplasts/1.0 g of cells, was obtained for the fine milky type culture (Fig. 4). The viability of these protoplasts was up to 90%. These results are consistent with our previous work on leaf and root-derived protoplasts where an efficient protoplast isolation and transient gene expression was demonstrated [21]. However, only a few protoplasts were isolated for the sandy and ultrafine type cultures, which were associated with a considerable amount of the undigested cells (data not shown). Increasing the enzymatic incubation period time over a

time course up to 28 h did not improve disassociation of the undigested cells (Fig. 5). Although the majority of the cells appeared to be plasmolyzed, protoplasts in these cells often failed to be released because of unbroken cell walls (Fig. 5). These observations suggest that the difference between these cell type cultures is likely related to cell wall properties, which suggests an obvious application in cell biology for cell wall research.

Moreover, only fine milky cell type culture led to efficient protoplast isolation. This may be because of the unique viscous texture of this cell type culture that facilitates the digestion of the cells. Protoplasts can be used in several studies, such as somatic hybridization, transient gene expression, and genetic transformation and subsequent regeneration of transgenic plants. A protoplast-to-plant

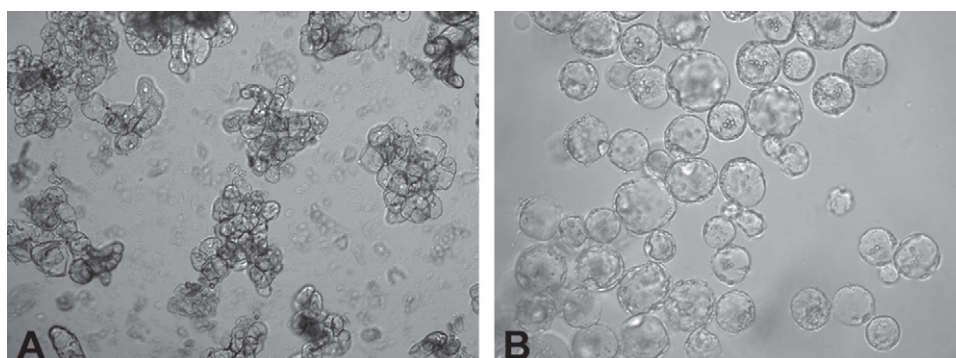
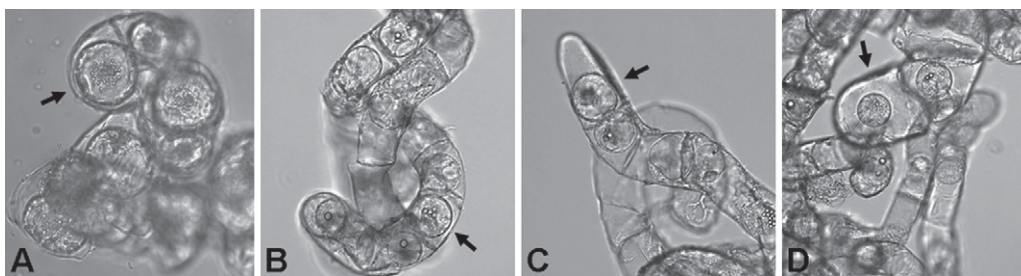


Fig. 4. Protoplasts isolated from switchgrass cell suspension fine milky type culture. (A) Suspension culture showing groups of tightly packed cells before the digestion (10 $\times$ ) and (B) protoplasts isolated after 4 h digestion (20 $\times$ ).





**Fig. 5.** Switchgrass cell suspension sandy type culture incubated in the enzyme solution throughout a time course. After 4 h (A), 7 h (B), 17 h (C), and 28 h (D) incubation (20 $\times$ ). Arrows indicate plasmolyzed cells containing protoplasts which failed to get released due to the unbroken cell walls.

regeneration system would facilitate genetic manipulation techniques. It is known that protoplasts from leaf or other plant tissues of grass species do not generally divide and protoplasts only regenerate into plants when they have been isolated from embryogenic cell suspensions [26,27]. Consistently, cell suspension-derived protoplasts have been shown to be competent for plant regeneration in a number of gramineous monocots [28,29]. Given our efficient protoplast isolation from the fine milky culture, it may be a potential value of this cell type for such application. The implication of such system would be an alternative route for the recalcitrant genetic transformation and regeneration of genetically modified switchgrass.

Additionally, it is of interest that whether these type cultures differ in the frequency of plant regeneration. Plant regeneration has been reported from switchgrass embryogenic ultrafine suspension cultures [18], given that our developed cell type cultures were initiated from embryogenic callus, they may have potential that can be used for such application.

In summary, the present study demonstrates the development of three different types of switchgrass cell suspension cultures with distinct morphological features that could be useful to the research community for various downstream studies, such as plant regeneration, mutant selection, mass propagation, gene transfer, and cell biology.

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