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BRIEF COMMUNICATION

Leaf bisection for the enzymatic isolation of mesophyll protoplasts from *Saintpaulia ionantha*

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Abstract

We describe a rapid and efficient procedure for isolating leaf mesophyll protoplasts from *Saintpaulia ionantha* (African violet). Because of the unusual surface properties of the leaf, efficient digestion required bisection of the lamina into abaxial and adaxial halves to expose the mesophyll. Enzymatic digestion occurred during a 3 h incubation with 2 % (m/v) driselase in 0.50 M mannitol, yielding 2 - 3 × 10^5 protoplasts g^{-1} (f.m.), with 70 % viability. Protoplasts ranged in size from 50 to 100 μm, and were derived from mostly non-photosynthetic mesophyll parenchyma.

Key words: African violet, driselase, mannitol, viability

Bilkey and Cocking (1982) described a technique for the physical, non-enzymatic isolation of protoplasts from callus of *Saintpaulia ionantha* H. Wendl. The procedure used cultured callus cells and required considerable time (3 - 10 months) for callus cultures to become suitable sources for protoplast isolation. Since the callus cultures were not able to be sub-cultured, fresh callus was necessary for each protoplast isolation. Winkelmann and Grunewaldt (1992) prepared *Saintpaulia* protoplasts from *in vitro* regenerated white shootlets and shoot primordia, and then regenerated plantlets from these protoplasts.

Despite the fact that African violet is an economically important horticultural crop which is widely used as a laboratory model for plant tissue culture, there exist no procedures for the enzymatic isolation of leaf mesophyll protoplasts from this species. The availability of a technique to routinely prepare mesophyll protoplasts

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from fresh plant tissue would make it possible to achieve significant progress in studying the molecular biology, genetics, and virus susceptibility of *Saintpaulia*.

S. ionantha plants, cvs. White Cascade and Startler, were obtained from Granger Gardens (Medina, OH), a gift from the research committee of the African Violet Society of America, Beaumont, Texas. Plants were grown in a medium of *Pro-Mix BX* (*Premier Brands*, Stamford, USA), and were fertilized monthly with a solution of *Peter's Fertilizer* (20-20-20). Plants were grown in a laboratory (temperature range 20 - 24 °C) 20 cm under 40 J s^{-1} *Sylvania Gro-Lux* lamps with an average irradiance of 65 μmol m^{-2} s^{-1} at the plant surface.

Following leaf excision, all manipulations were performed in a laminar flow chamber. Tissue of fully expanded leaves was surface sterilized 5 min in 70 % (v/v) ethanol containing 1 % (v/v) *BacDown* antimicrobial soap (*Deacon Laboratories*, Bryn Mawr, USA); followed by 5 min in 1.05 % (v/v) sodium hypochlorite solution containing 1 % *BacDown* antimicrobial soap; and a final 5 min rinse in sterile distilled water.

The lamina was bisected into abaxial and adaxial halves using a sterile razor blade. Excised tissue was floated, cut side down, on the driselase-mannitol solution for protoplast isolation (see below).

Many sources of enzymes (pectinases, cellulases, hemicellulases and driselase) were evaluated, both alone and in combination. The most efficient digestion occurred in 2.0 % (m/v) driselase (*Plenum Scientific*, Hackensack, USA) in 0.50 M mannitol. Before use, the solution was centrifuged for 10 min at 1100 *g* in an *IEC Model HN-SII* (Needham Heights, USA) clinical centrifuge. The supernatant was carefully recovered and placed in a 10 cm^3 syringe for filter sterilization through a 0.22 μm syringe filter (*Corning*, Corning, USA). The filtrate was placed in sterile polystyrene Petri dishes, 50 mm diameter. Abaxial and adaxial sections of tissue were floated on the filtrate for 3 h without shaking under the same laboratory conditions described above.

Protoplasts were gently filtered through 4 layers of sterile cheesecloth into a 15 cm^3 polypropylene centrifuge tube, were collected by pelleting at 200 *g* for 10 min, and were resuspended in 0.50 M mannitol and 10 mM $CaCl_2$. Protoplasts were counted with a hemacytometer and viability percentage was determined with two staining procedures: (*1*) with 0.1 % (m/v) *Evan's Blue* (*Sigma Chemical Co.*, St. Louis, USA) in 0.50 M mannitol; and (*2*) fluorescein diacetate (FDA) (*Sigma Chemical Co.*), 2.5 mg cm^{-3} (acetone), using the procedure of Power and Chapman (1985). Viability data for both procedures were in agreement.

For most plant species, a standard technique for protoplast isolation begins with the physical removal of the epidermis by peeling. Epidermal peeling was attempted with *S. ionantha*, but was unsuccessful because *Saintpaulia* leaves are covered with a thick layer of epicuticular wax.

Besides peeling, several other methods were attempted to expose the *Saintpaulia* mesophyll for digestion: brushing, with or without added abrasives; "feathering" leaf tissue into 1 mm sections; and vacuum infiltration of enzyme and mannitol into sliced tissue pieces (Table 1). None of these procedures achieved satisfactory protoplast isolation without considerable cytological damage.

Table 1. A comparison of protoplast isolation techniques, showing yield (protoplasts per g fresh mass) and viability (percentage of viable protoplasts) of protoplasts for African violet cvs. White Cascade and Startler. Numbers represent mean values obtained from at least five separate replicates of each technique.

Procedure	Cultivar	Yield	Viability
Leaf bisection	White Cascade	2.9×10^5	65
	Startler	2.2×10^5	76
Epidermal peeling	White Cascade	3.3×10^3	60
	Startler	5.7×10^3	57
Epidermal Brushing	White Cascade	4.0×10^4	34
	Startler	3.1×10^4	21
Feathering leaf tissue (1 mm sections)	White Cascade	5.9×10^4	63
	Startler	4.8×10^4	48
Vacuum infiltration of enzyme	White Cascade	8.4×10^3	35
	'Startler'	9.1×10^3	41

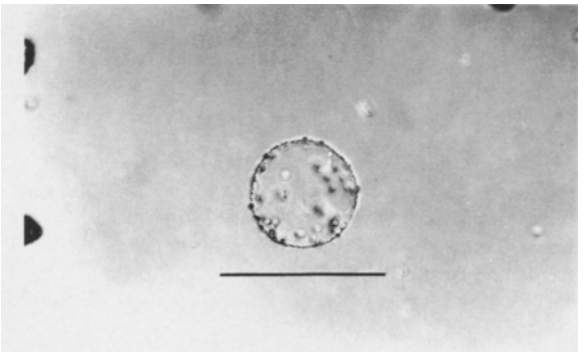

Fig. 1. Photomicrograph of leaf mesophyll protoplast of *Saintpaulia ionantha* cv. White Cascade. Bar = 100 μm.

After evaluating these techniques for their ability to expose mesophyll tissue, the only highly efficient technique involved bisection of the lamina into upper and lower halves. Following enzymatic digestion with 2.0 % driselase, 2 - 3 $\times$ 10^5 protoplasts per g fresh mass were produced (Table 1). The viability averaged 70 %, and ranged from 65 - 80 % from experiment to experiment. The population of protoplasts was derived from mostly non-photosynthetic mesophyll parenchyma cells (Fig. 1). Approximately 5 % of the population of protoplasts were smaller (30 - 50 μm) and were derived from photosynthetic chlorenchyma. Most of these chlorenchymous cells remained attached to tissue sections or appeared as partially digested separated cells after up to 16 h digestion.

Tissue bisection to expose abaxial and adaxial halves is an unusual procedure, and to our knowledge, has not been previously exploited for mesophyll protoplast

isolation. This bisection could only be accomplished because *Saintpaulia* has a very thick lamina, and the procedure proved to be a gentle technique which effectively exposed mesophyll cells without significant cell damage. We found in fact, that without leaf bisection, *Saintpaulia* protoplast isolation is not practicable.

Winkelmann and Grunewaldt (1992) have already shown that non-photosynthetic *S. ionantha* protoplasts derived from *in vitro* cultured cells can be regenerated into plantlets. It is likely that *S. ionantha* non-photosynthetic mesophyll protoplasts can be cultivated in the same way.

The rapid generation of mesophyll protoplasts from fresh tissue, without the need for *in vitro* cultured sources, will facilitate further studies. For example, Sulzinski *et al.* (1994) demonstrated that *S. ionantha* will support limited virus infection following tobacco mosaic virus inoculation. The nature of the virus susceptibility or resistance can be further determined at the cellular level by inoculating mesophyll protoplasts with virus or viral RNA. Moreover, the ability to produce mesophyll protoplasts will provide a means to engineer transgenic *Saintpaulia* plants to exhibit desirable horticultural characteristics in this economically important ornamental plant species.

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