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Isolation of Protoplasts from *Nepenthes* - A Plant Carnivore

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Abstract

Protoplasts were isolated from the lamina of greenhouse grown *Nepenthes ampullaria* and the hybrid *N*. 'Rokko' Exotica in order to develop a protocol for protoplast isolation suitable for wild species of *Nepenthes*. Various molarities utilizing mannitol or sorbitol and different enzyme mixtures and concentrations as well as incubation times were evaluated to maximize protoplast yield and viability. The most effective treatment, a 4 hrs incubation at 40 rpm and 25°C in a solution consisting of 0.5 M sorbitol, 5% cellulase 'Onozuka' R-10, 0.5% macerozyme R-10, and 0.3% pectolyase Y-23, generated 4.35×10^6 protoplasts/ gfw of which 62.1% were viable. Culture was attempted in respect of regeneration of the cell wall, however, no cell division was observed.

Introduction

The genus *Nepenthes* is represented by approximately 135 species with greatest diversity in Southeast Asia. These plants are easily recognized by modification of their leaves into photosynthetic lamina and fluid-filled pitchers. These pitchers primarily act to attract, capture and digest arthropods.

All individuals are dioecious, the plants developing as a single, fixed sex. Males are more common in wild populations, possibly to ensure effective cross pollination (Adam et al. 2011). The high rate of endemism and restriction to specific habitats make these plants vulnerable to extinction. The majority of species are confined to small ranges with undersized populations. For example, 13 of the 39 Bornean species are found only on a single mountain or mountain complex (McPherson and Robinson 2012).

An increasing number of species are threatened or endangered due to habitat destruction, collection of species for plant trade, and exploitation by hobbyists. Habitat destruction due to deforestation (both legal and illegal) with subsequent

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drainage has impacted a large number of taxa, while poaching has threatened several with extinction. Tissue culture is the most common commercially used method for plant propagation and helps to reduce collection pressure among wild populations (Sukamto et al. 2011). While this method is effective, it comes with a cost to plant survival. Large amounts of plant tissue may be used as is the case with meristem, shoot tip, or node culture (Latha and Seeni 1994) and in some cases the whole plant may be sacrificed. *Nepenthes* populations consisting of a few individuals of one sex can therefore be more damaged than protected.

Plantlet regeneration from *Nepenthes* calli produced from isolated protoplasts offers the opportunity to mass propagate these enigmatic plants, thus reducing pressure on wild populations. In this report, we describe a simple, routine procedure to isolate *Nepenthes* protoplasts from leaf lamina of the greenhouse raised plants, which were used to simulate explants from wild individuals.

Materials and Methods

Apical leaf tissue was collected from *N. ampullaria* or *N*. 'Rokko' Exotica grown under greenhouse conditions. Laminae were washed 1 hr under running water with dish soap, thereafter soaked in 10% (v:v) commercial chlorine bleach with three drops of Tween 20/100 ml for 10 min. Following surface sterilization, the lamina was rinsed three times with sterilized distilled water. Each lamina was cut into 1 - 2 mm wide slivers approximately 4 cm long and 0.5 g transferred to 5 ml of a preplasmolysis solution or directly to 55 mm Petri dishes containing 5 ml of 0.22 μ filter sterilized enzyme solution of variable osmotic and enzyme concentrations.

The preplasmolysis solution consisted of 0.7 M mannitol or sorbitol and 5 mM MES in cell and protoplast washing solution (Frearson et al. 1973) adjusted to pH 6.0 and autoclaved. Slivers were incubated for 1 hr at 40 rpm and 25°C. Different permutations of protoplast isolation involving preplasmolysis with or without vacuum were performed.

Ideal osmolality was determined by manipulation of the isolation solution molarity by the use of mannitol or sorbitol. Test concentrations included ten-fold dilutions ranging from 1.0 - 0.1 M. Other parameters were: 1, 2 or 3% cellulase for 5 hrs at 27°C and 40 rpm. Protoplasts were evaluated subjectively for overall appearance and stained with fluorescein diacetate (FDA) to determine the extent of viability of the cells. For comparison, the osmolality of the cells was directly measured on an osmometer (Advanced Instruments, Inc., Norwood, MA) and used to calculate the appropriate molarity of the enzyme solution (Sarhan and Cesar 1988).

Present authors compared the effects of multiple cellulase concentrations and cellulase preparations in various combinations with macerozyme R-10 (0 - 2% w/v) (PhytoTechnology Laboratories, Shawnee Mission, KS), pectolyase Y-23 (0 - 0.3% w/v) (PhytoTechnology Laboratories, Shawnee Mission, KS), funcelase (0-1%) (PhytoTechnology Laboratories, Shawnee Mission, KS), and pectinase (0.07%) (Sigma Chemical Company, St. Louis, MO). The cellulases utilized were Onozuka R-10, Onozuka F A and Onozuka RS (PhytoTechnology Laboratories, Shawnee Mission, KS) in concentrations ranging from 0 - 5%. All enzymes were dissolved in osmotically adjusted ultra pure water containing 1 mM $CaCl_2$, 5 mM MES, ampicillin (135 μM), antioxidants (0.568 μM ascorbic acid, 0.781 μM citric acid) and in some instances beta(β)-mercaptoethanol (10 mM), and the pH adjusted to 5.8. Lamina slivers were digested at 40 rpm, 25°C, in the dark for 4 - 16 hrs.

The digestion media containing protoplasts were extracted from Petri dishes using a wide bore pipette with gentle suction and then strained through a sterile 100 μm mesh filter into 50 ml centrifuge tubes to remove debris. The filtered protoplasts were centrifuged four times in a swinging bucket rotor at 100 × g for 3 min with osmotic solution (0.5 M sorbitol, 1 mM $CaCl_2$, 5 mM MES, pH 6.0) to remove all traces of enzymes. Purified protoplasts were resuspended in various modifications of MS supplemented with different auxins and cytokinins. The yield was determined by counting the number of protoplasts per gram of lamina using a hemocytometer.

Isolated protoplasts were assessed for viability in side-by-side comparisons of their ability to exclude Evans blue (Nagata and Takebe 1970) or to accumulate fluorescein (Widholm 1972). Calcoflur White staining to detect the presence of any cellulostic wall material was performed as described by Nagata and Takabe (1970).

For each treatment, a minimum of 2 Petri dishes were used, each containing 5 ml of digestion enzyme solution. All experiments were repeated a minimum of three times. Analysis of variance (GraphPad Software, Inc.) was used for comparison between treatments and the comparison of viability methods. Two-by-two differences were tested by applying the Bonferroni post hoc test (GraphPad Software, Inc.).

Results and Discussion

Nepenthes leaves are known to have a high lignin, wax/cutins, and suberin content, all associated with leaf sclerotization (Osunkoya et al. 2008). A thick cuticle covers the upper epidermis which is formed by small cells while a thinner cuticle covers the lower epidermis. Aquiferous tissue, formed by three - four

layers of isodiametrical cells, separates the 2 - 3 cell thick palisade parenchyma from the epidermis. A thick spongy parenchyma is formed by rounded cells containing small auriferous spaces between the cells. Within the mesophyll are sclerids and calcium oxalate druses. A sclerenchyma sheath surrounds the vascular bundles (Toma et al. 2002). The leaf anatomy led us to experiment with a variety of cellulases, in combination with other maceration enzymes, in order to determine the most appropriate choice for digestion of *Nepenthes* lamina, which can differ between the many species of this genus. *N. ampullaria* has a much thicker and hirsute lamina compared to *N*. 'Rokko' Exotica which is less "leathery" and glabrous. Lamina from apices were used because of their softer texture, compared with older leaves that have a thick and leathery lamina in both plants (Pavlovič et al. 2007).

Preplasmolysis was utilized in a number of experiments to determine if it had any effect on the yield of protoplasts. Preplasmolysis with or without vacuum infiltration had no effect on protoplast isolation (data not shown). Sorbitol rather than mannitol appeared to impart the least amount of stress to the protoplasts at 0.5 M as indicated by the spherical shape of the cells and observation of cytoplasmic streaming. Subsequent determination of optimal molarity by measuring the osmolality of the expressed sap of the leaves, confirmed the use of a 0.5 M osmoticum, although salts and other medium components were not taken into account and could have a significant effect on the protoplasts (Russell 1993). Osmolality is a variation of molality (moles/kg) and was used as a direct inference of molarity (moles/l) since water was the solvent and makes both values comparable. Briefly, lamina was frozen in liquid nitrogen and ground in a mortar. The homogenate was then centrifuged at 25,000 × g for 20 min at 4°C and the osmolality of the supernatant determined by freezing point depression, with optimum osmolality (molarity) of the isolation medium equivalent to 1.8 times that measured in the lamina (Sarhan and Cesar 1988).

Because the composition of carbohydrates in the leaves of *Nepenthes* is poorly known, we experimented with different concentrations of enzymes to optimize cell wall digestion. Cellulase acts together with other enzymes to degrade cell walls by modifying celluloses, lichenin, and cereal β-D-glucans. It modifies cellulose by hydrolyzing 1,4-β-D-glucosidic linkages. Cellulase activity is inhibited by glucose and cellobiose. Moreover, it is completely inhibited by Hg^{2+} and slightly inhibited by Mn^{+}, Ag^{2+}, Zn^{2+} and Cu^{2+}. Cellulase Onozuka R-10, derived from *Trichoderma viride*, contains high cellulase activity. It is a multi-enzymatic system consisting of cellulase, α-amylase, hemicellulase, pectinase, and protease activity. Other enzymes such as α-amylase break down 1,4-α-D-

glucosidic linkages in polysaccharides, while 1,4-α-D-galastosiduronic linkages in galacturans are randomly cleaved by pectinase.

Onozuka RS is another multi-component enzyme system with high cellulose activity obtained from a mutant *T. viride* derived from the parent strain for cellulase Onozuka R-10. It also consists of cellulase, α-amylase, hemicellulase, pectinase, and protease activity. Onozuka RS contains xylanase activity that is three times higher than cellulase Onozuka R-10.

Onozuka F A is yet another cellulase derived from *T. viride* with activities similar to cellulase, protopectinase, hemicellulase, and others. It is typically used to measure the rate of digestion of dried feeds.

Funcelase is also isolated from *T. viride* and is a multi-system enzyme mixture consisting of ß-1,3-glucanase, chitinase, cellulase, and xylanase. More frequently used in protoplast isolation from fungi, it is also effective in the preparation of protoplasts from woody plants and true grasses when used in combination with other enzymes such as macerozyme.

Macerozyme R-10 is a multi-enzyme mixture derived from *Rhizopus* sp. that possesses high pectinase and hemicellulase and low cellulase activity. Pectinase is a general term for enzymes such as pectolyase Y-23, derived from *Aspergillus japonicas*. Pectolyase Y-23 is a purified maceration enzyme that contains two types of pectinase: endo-polygalacturonase and endo-pectinlyase and has optimum activity at pH 5.5.

ANOVA shows a significance difference ($p \leq 0.001$) between enzyme selections for protoplast release. Bonferroni post hoc testing indicates that 5% cellulase Onozuka R-10 protoplast yields in combination with 0.5% macerozyme R-10 and 0.3% pectolyase Y-23 are significantly greater ($p \leq 0.01$) than the cellulases Onozuka F A and Onozuka RS as well as funcelase, also in combination with macerozyme and pectolyase, and that there was no significant difference between the Onozuka F A, Onozuka RS, and funcelase. In all experimental conditions, regardless of enzyme mixture, temperature, and duration of exposure, leaf tissue never completely broke down and required mechanical assistance for maximum protoplast release (Fig. 1).

Protoplasts settled to the bottom of the tube following washing. These varied in size and were transparent in color with the green chloroplasts regularly arranged around a central vacuole (Fig. 2). No cell wall remained when the protoplasts were stained with calcofluor white. Viability of isolated protoplasts was determined by fluorescein diacetate hydrolysis assay or by exclusion of Evan's blue. A side-by-side comparison of viability determination for three different cellulases was performed. ANOVA revealed no statistical difference between the techniques ($p = 0.69$); so either method is equally effective at

evaluating viability (Fig. 3). Five percent Onozuka R-10 in combination with macerozyme (0.5%) and pectolyase Y-23 (0.03%) released protoplasts with 62.1% viability.

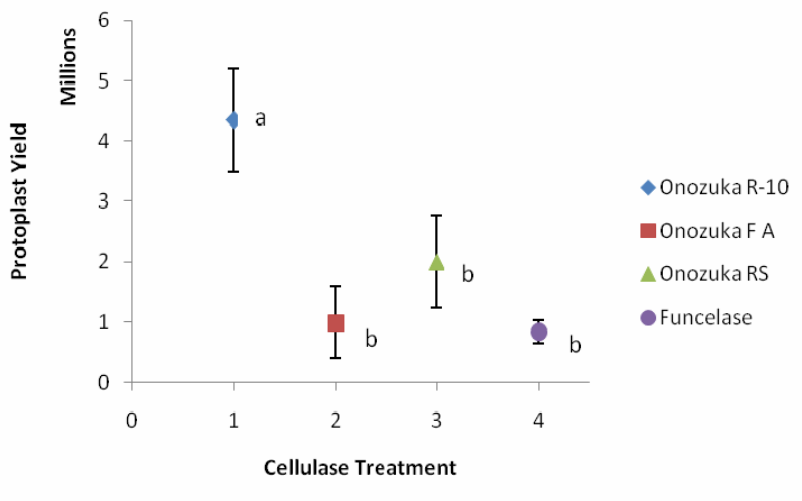

Fig. 1. The ability of different cellulases to digest *Nepenthes* lamina. Comparison of four different cellulases (5% w/v) in 0.5 M sorbitol solution containing 0.5% macerozyme R-10 and 0.03% pectolyase Y-23. Different letters indicate values statistically different at $p \leq 0.01$.

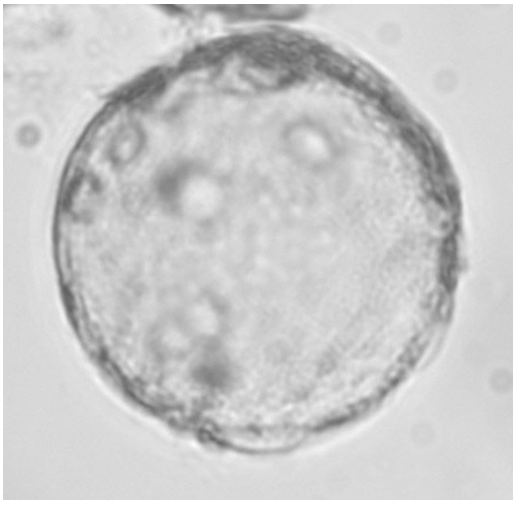

Fig. 2. A viable protoplast of *Nepenthes* 'Rokko' Exotica (400×).

The protoplasts were cultured at a number of densities (0.2 – 1 × 10^6 viable protoplasts/ml) in a variety of media of different compositions and by a variety of methods (hanging drop culture, embedded in low melting point agarose, alginate film, liquid culture, etc.) in the dark at 25°C in an attempt to stimulate sustained divisions and colony formation. Cultures were observed for changes in the protoplasts with respect to shape, size, division and the regeneration of the cell wall. Calcofluor white staining 5 - 7 days after cultures were initiated revealed the presence of regenerated cell walls. Viability testing at 5 - 7 days also indicated a large percentage of live cells (data not shown) although in no instance was cellular division observed.

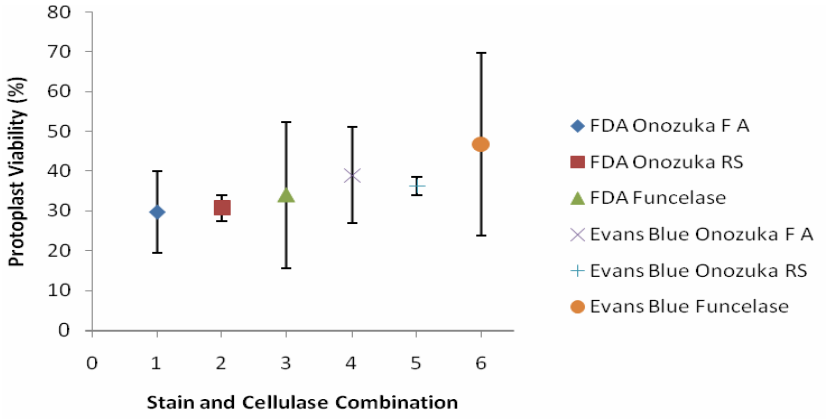

Fig. 3. Viability of *Nepenthes* protoplasts. Comparison of fluorescein diacetate hydrolysis or exclusion of Evan's Blue to determine viability of protoplasts isolated from *Nepenthes* by use of 5% Onozuka RS, Onozuka F A or funcelase. No significant difference exists among or between treatments (p= 0.69).

The most effective treatment for protoplast isolation, a 4 hr incubation at 40 rpm and 25°C in a solution consisting of 0.5 M sorbitol, 5% cellulase 'onozuka' R-10, 0.5% macerozyme R-10, and 0.3% pectolyase Y-23, generated 4.35 × 10^6 protoplasts/g fresh weight of which 62.1% were viable. On-going work will allow the authors to determine the best technique for cellular divisions and callus formation, ultimately leading to plant regeneration.

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