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Isolation of Leaf Protoplasts from the Submerged Aquatic Monocot *Aponogeton madagascariensis*

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ABSTRACT

Aponogeton madagascariensis is the only aquatic plant which forms perforations in its leaves through a process known as programmed cell death (PCD). Although PCD can be studied *in vivo*, isolated protoplasts maintain many of the same physiological properties of the intact plant and, due to their accessibility and reduced complexity, make ideal research tools to investigate plant processes including PCD. The conditions for the isolation of protoplasts from the lace plant were examined. Several factors including leaf age, carbohydrate source, and enzyme incubation time all significantly influenced protoplast yield and viability from lace plant tissue. Viable protoplasts were successfully isolated from leaf tissue approximately 1 month in age, using an enzyme mixture comprised of 2% w/v cellulase onozuka R10 and 0.5% pectolyase Y-23 dissolved in 0.005 M MES and 0.6 M sorbitol, pH 5.5. Approximately 60.35 ± 1.08 x 10^5 protoplasts/g fresh weight, with a viability of 92.75 ± 2.25%, were obtained from 4 hour isolations in the dark. Following successful isolation, these lace plant protoplasts can be used as an excellent model system for the study of environmentally induced PCD and can provide the first ever opportunity to compare this form of PCD with its developmentally regulated counterpart within one species of plant.

Keywords: anthocyanin, lace plant, programmed cell death (PCD), protoplast isolation
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **DIC**, differential interference contrast; **FITC**, fluorescein isothiocyanate; **MES**, 2-*N*-morpholino-ethanesulfonic acid; **MS**, Murashige and Skoog medium; **PCD**, programmed cell death; **TRITC**, tetramethyl rhodamine iso-thiocyanate

INTRODUCTION

Protoplasts

Protoplasts are essentially naked plant cells lacking a cell wall. They are spherical in shape with the cytoplasm contained within a plasma membrane. Protoplasts serve as a highly valuable and versatile cell based system and have been used to observe cellular processes and activities such as cell wall degradation, synthesis and communication, embryogenesis, cell division, photosynthetic activity and particle uptake, as well as stress and hormone responses in a variety of plant species (Pongchawee *et al.* 2006; Yoo *et al.* 2007).

The processes of protoplast isolation and regeneration have been of specific interest to the cereal industry given they represent some of the most economically important groups of plants for man. Protoplast isolation, culture and regeneration have been reported in the monocotyledonous cereals rice (Yamada *et al.* 1986), maize (Sheen 1993), pearl millet (Vasil and Vasil 1974), wheat (Harris *et al.* 1988) and sorghum (Battraw and Hall 1991). Protoplast manipulation has also been perfected in the model dicotyledonous species *Arabidopsis thaliana* (Yoo *et al.* 2007). To date, there are few reports of protoplast isolation and culture from freshwater aquatic plant species. The few species of freshwater plants which have been studied include the monocotyledonous angiosperm *Potamogeton lucens* L. (Staal *et al.* 1988), the dicotyledonous angiosperm *Ranunculus penicillatus* ssp. *Pseudofluitans* (Newman and Raven 1999), the monocotyledonous aquarium species *Anubias nana* Engler (Pongchawee *et al.* 2006) and *Cryptocoryne wendtii* De Wit (Pongchawee *et al.* 2007) and, as will be discussed here, the submerged monocotyledonous angiosperm *Aponogeton madagascariensis*.

The lace plant

The lace plant (*Aponogeton madagascariensis*) is an aquatic monocot native to the river systems of Madagascar. The lace plant is secured in gravel substrate by roots borne adventitiously on a spherical corm. Leaves are produced

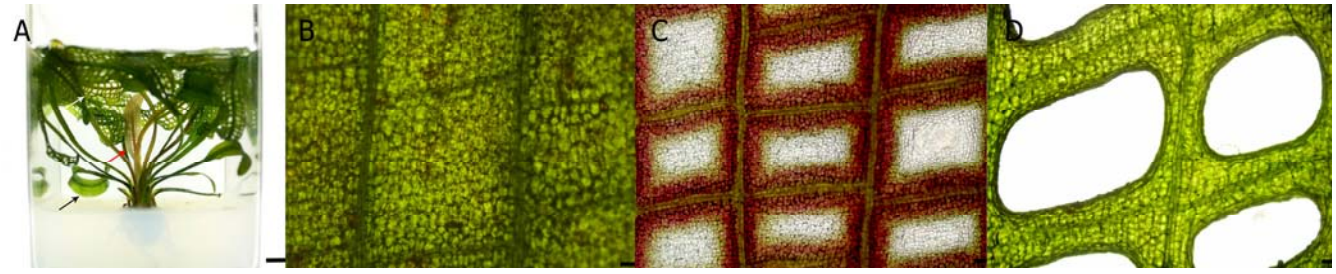

Fig. 1 Sterile *A. madagascariensis* in magenta box, white arrow indicating mature leaf, red arrow indicating window stage leaf, and black arrow indicating heteroblast leaf (**A**). Scale bar = 1 cm. Micrograph of heteroblast leaf (**B**) window stage leaf (**C**) and mature leaf (**D**). Scale bar (**B-D**) = 100 µm.

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from a shoot apical meristem (SAM) at the apex of the corm. The leaves of the lace plant form in a heteroblastic series; the first 1-3 leaves produced by the SAM are the juvenile leaves in the heteroblastic series and do not form perforations (**Fig. 1A, 1B**); the following leaves produced by the SAM are adult leaves, which do form perforations. There are only two families in the vascular plant kingdom which produce perforations in their leaves via programmed cell death (PCD): the Araceae and Aponogetonaceae. The lace plant is one of forty species in the monogeneric family Aponogetonaceae and is the only species that produces perforations in its leaves through the PCD process (Gunawardena and Dengler 2006).

PCD and the lace plant

Programmed cell death is the regulated death of cells within an organism. PCD is omnipresent throughout the eukaryotes, occurring in both unicellular and multicellular organisms. PCD in plants can be either environmentally induced or developmentally regulated (Gunawardena 2008). Environmentally induced PCD is an outcome of external biotic and abiotic factors and can be induced by such factors as heat shock (McCabe *et al.* 1997; McCabe and Leaver 2000), UV radiation (Danon and Gallois 1998), pathogens (Mittler and Lam 1997), low oxygen (Gunawardena *et al.* 2001), and density and salinity changes (McCabe *et al.* 1997). In contrast, developmentally regulated PCD occurs as part of normal, unperturbed development and presumably is a response to internal signals. Examples of developmentally regulated PCD include elimination of transitory organs and tissues (Browder and Iten 1998), xylem differentiation (Fukuda *et al.* 1998), and leaf morphogenesis (Gunawardena *et al.* 2004, 2005) as is seen in the lace plant. In the lace plant, each leaf reaches a length of approximately two cm before PCD is initiated. Through the PCD process, a simple, tongue-shaped leaf is converted to a more complex structure characterized by open perforations located between longitudinal and transverse veins. The perforations radiate outward before they stop growth four to five cells from the perimeter veins, creating a lattice like pattern over the entire leaf surface (Gunawardena *et al.* 2004, 2006, 2007; Gunawardena 2008; Wright *et al.* 2009).

The developmental morphology of the adult perforated lace plant leaf has been divided into five stages. Initially, stage 1 (tissue pattern) involves longitudinally rolled pink leaves where no perforations are present. This pink coloration is due to the pigment anthocyanin, which is found in the vacuole of the mesophyll cells; interestingly this pigment is not found in the epidermal cells. Stage 2 (window) is characterized by distinct transparent regions forming in the center of the vascular tissue due to the loss of pigments such as chlorophyll and anthocyanin (**Fig. 1C**); stage 3 (perforation formation) involves the degradation of the cytoplasm and the cell wall, resulting in the loss of transparent cells in the center of the window; stage 4 (perforation expansion) is characterized by the expansion of the perforation within the areole and lastly, stage 5 (mature) results in a completed perforation (**Fig. 1D**; Gunawardena *et al.* 2004). Despite being a fully submerged macrophyte a cuticle is present on the leaves, commencing as very thin in the window stage and thickening moderately into the mature stage.

The lace plant is an excellent model system for the study of developmentally regulated PCD in plants (Gunawardena *et al.* 2004, 2006, 2007; Gunawardena 2008; Wright *et al.* 2009; Elliott and Gunawardena 2010). Perforation formation within the leaf is predictable, with perforations forming at very specific stages of leaf development and only between longitudinal and transverse veins. The leaf tissue is also very thin, approximately four to five cell layers thick, making examination via microscopy possible. This thin structure also makes the leaf nearly transparent, allowing for live cell imaging within the plant. There is also a well developed protocol for the sterile culture of the lace plant (**Fig. 1A**), allowing for generation of a large amount of experimental plant material with no microbial contamination (Gunawardena *et al.* 2006).

The present study aimed to optimize the isolation of viable, high density protoplasts from the freshwater monocot *Aponogeton madagascariensis*.

MATERIALS AND METHODS

Subculture and plant materials

Lace plants used for all experimental purposes were grown in axenic culture in magenta boxes (**Fig. 1A**) as described by Gunawardena *et al.* (2006). Plants were maintained at 23.5°C with 12 h light/ 12 h dark cycles provided by daylight simulating fluorescent bulbs (Philips, Daylight Deluxe, F40T12/DX, Markham, Ontario) at approximately 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$. All experimental plant materials were propagated via subculture according to the protocol of Gunawardena *et al.* (2006). All chemicals were purchased from Bio Shop (Burlington, Ontario, Canada) unless otherwise stated.

Protoplast isolation

Protoplast isolation was modified from Staal *et al.* (1988), Pongchawee *et al.* (2006) and Pongchawee *et al.* (2007). Leaf samples from boxed plants between 1-2 months of age were excised and rinsed with demineralized water. The central vein from each leaf was removed and the remaining two halves were cut longitudinally into strips approximately 1 cm in width. The strips were placed in 100 ml protoplast buffer solution (0.005 M 2-*N*-morpholino-ethanesulfonic acid, MES; Sigma-Aldrich, Oakville, Ontario, Canada), 0.6 M sorbitol, pH 5.5 (adjusted with dilute potassium hydroxide) for 15-20 min (Staal *et al.* 1988). All strips totaling one gram in wet fresh weight were transferred to a 100 × 20 mm Petri dish containing enzyme solution. Enzyme solution consisted of 2% w/v Cellulase R10, and 0.5% Pectolyase Y-23 (Yakult Pharmaceutical Industries, Tokyo, Japan) dissolved in 10 ml protoplast buffer solution (Pongchawee *et al.* 2006, 2007). Leaf strips were incubated for 4 hrs at 27°C in the dark; the Petri dish was shaken at 50 rpm for the final 30 min of the enzyme incubation. The suspension was then pipetted through a wet 70 µm mesh into a 50-ml centrifuge tube leaving the leaf debris in the Petri dish. This leaf debris was then rinsed with 10 ml of clean protoplast buffer and swirled for an additional 10 min at 50 rpm to release the remaining protoplasts. This suspension was then collected, pipetted through mesh and collected in the same 50-ml centrifuge tube. The combined protoplast suspension was then centrifuged at 100 × *g* for 20 min with no brake at room temperature. The pellet was rinsed with 15 ml of clean buffer solution, re-centrifuged, and resuspended in approximately 1 ml of clean protoplast buffer.

Density and viability

Protoplast density was determined via a hemocytometer technique in which protoplasts were counted in small areas under square coverslips and the data were extrapolated to the entire sample. Viability was determined through fluorescein diacetate (FDA) staining (Sigma-Aldrich, Oakville, Ontario, Canada).

Microscopy

Light microscopic observations were performed using glass coverslips and slides on a Nikon eclipse 90i compound light microscope (Nikon, Mississauga, Ontario, Canada) fitted with a digital camera (Nikon DXM 1200c) and using NIS Elements AR 3.0 imaging software and I Control. Light microscope observations were performed using differential interference contrast (DIC) optics with complimentary fluorescent images taken via a fluorescent B2A filter (excitation 440-520 nm and emission 485-585 nm).

Laser confocal scanning observations were performed using a droplet cell culture chamber on a Nikon Eclipse T*i* confocal microscope (Nikon, Mississauga, Ontario, Canada) fitted with a digital camera (Nikon DS-Fi1) and using EZ-C1 3.80 imaging software and Ti Control. Confocal microscope observations were performed using DIC optics with complimentary fluorescent images

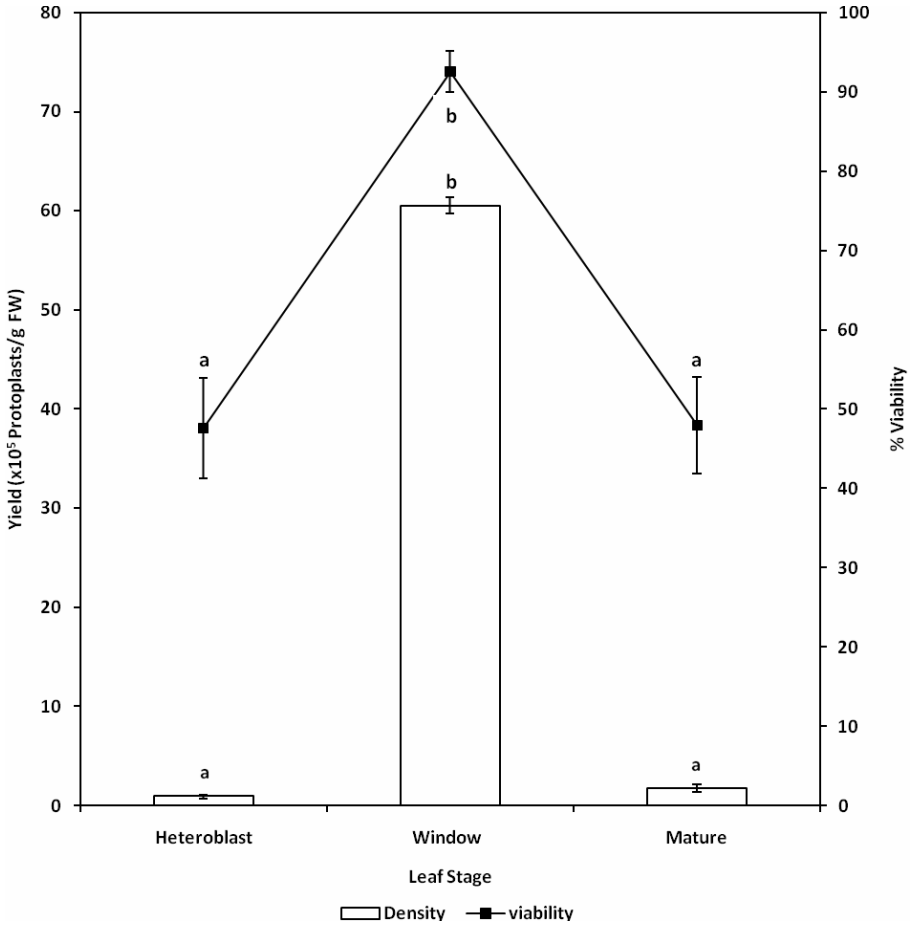

Fig. 2 Effect of leaf stage on protoplast isolation from *A. madagascariensis*. Bar graph represents average protoplast yield from each stage while the line graph represents average viability. All samples were isolated with enzyme combination 2. Data represents mean ± standard error of four replicates.

taken via a fluorescent fluorescein isothiocyanate (FITC; excitation 460-500, nm emission 510-560 nm) or tetramethyl rhodamine iso-thiocyanate (TRITC; excitation 527-552 nm, emission 577-632 nm) laser. All composite plates were assembled using Adobe Photoshop Elements version 6.0.

Leaf stage, carbohydrate source, and enzyme incubation time

Following preliminary trials in which the proper enzyme combination for lace plant protoplast isolation was determined (data not shown), the effects of varied leaf stage (juvenile, window and mature), carbohydrate source in buffer (0.6 M sorbitol, mannitol, sucrose or glucose) and enzyme incubation time (2, 4 or 6 hrs in the dark) were examined. Protoplasts were isolated and collected as previously described. Protoplast density and viability were recorded in order to determine the effect of these variables on protoplast isolation.

Statistical analysis

All data were assessed by a general linear model of variance (GLM ANOVA) and the means were compared by the Tukey test at 95% confidence intervals ($P < 0.05$). All statistical analyses were carried out using Minitab 15 Statistical Software- English (Minitab Inc., State College, PA, USA).

RESULTS

Protoplast isolation

Leaf stage was found to significantly affect the yield and viability of protoplast isolates ($P = 0.000$). The window stage of leaf development resulted in the highest yield ($60.50 \pm 0.80 \times 10^5$ protoplasts/ g fresh weight) and viability (92.50 ± 2.59%) of protoplasts when compared to the heteroblast and mature leaf samples (**Fig. 2**) as determined by heamcytometer and FDA techniques respectively. The window stage cells were consistently spherical in shape and contained many chloroplasts and mitochondria (**Fig. 3C**). Protoplast density decreased significantly when using heteroblast leaf ($0.80 \pm 0.24 \times 10^5$/g fresh weight) and mature stage leaf samples ($1.99 \pm 0.44 \times 10^5$/g fresh weight), as compared to window stage leaf material at $P < 0.05$. Protoplast viability also decreased significantly when using heteroblast leaf (53.25 ± 3.71%) and mature stage leaf samples (52.60 ± 5.03%), as compared to window stage leaf material at $P < 0.05$.

Fig. 3 depicts both DIC and corresponding FDA fluorescent images from all three leaf samples, illustrating both protoplast density and viability respectively. Density and viability in the heteroblast leaf isolations (**Fig. 3A, 3B**) are drastically reduced compared to window stage samples (**Fig. 3C, 3D**), and cells also appear to be less spherical in shape. Density and viability are also reduced in the mature stage samples (**Fig. 3E, 3F**) when compared to window stage isolates. These mature cells appear to maintain a more spherical shape then the heteroblast cells but display less chloroplasts when compared to window stage isolates. Note the presence of anthocyanin in the vacuole of some cells (**Fig. 3C, 3E**), suggesting the presence of an intact tonoplast membrane.

Window stage protoplast isolations exhibited high yields of cells (**Fig. 4A**), with organelles clearly present within the majority of isolates (**Fig. 4B, 4C**). Chloroplasts (**Fig. 4B** red arrow) and the nucleus (**Fig. 4B** black arrow) can also be visualized within the cells, suggesting viable isolations. The plasma membrane can also be seen on all cell isolates (**Fig. 4C** red arrow). Lastly, note the presence of anthocyanin in the vacuole (**Fig. 4C**), suggesting the presence of an intact

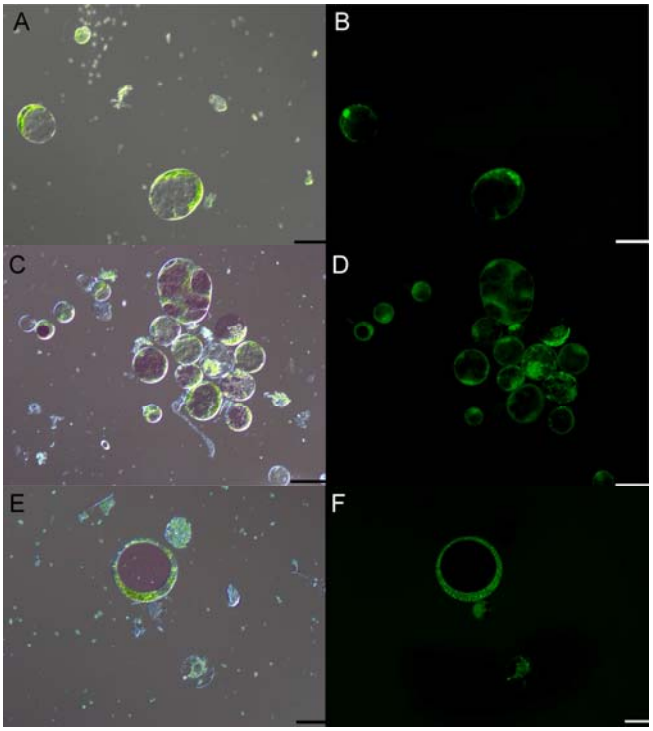

Fig. 3 Light micrographs of isolated protoplasts from *A. madagascariensis* heteroblast leaves (**A, B**), window stage leaves (**C, D**) and mature leaves (**E, F**). DIC images (**A, C, E**) and corresponding fluorescent images depicting FDA staining of living cells (**B, D, F**). The pink coloration within the cells is the pigment anthocyanin; the presence of this pigment within the cells indicates an intact tonoplast membrane, and hence viable cells. These pink cells are isolated from the mesophyll of the leaf, while the clear cells are isolated from the epidermis. All scale bars = 100 µm.

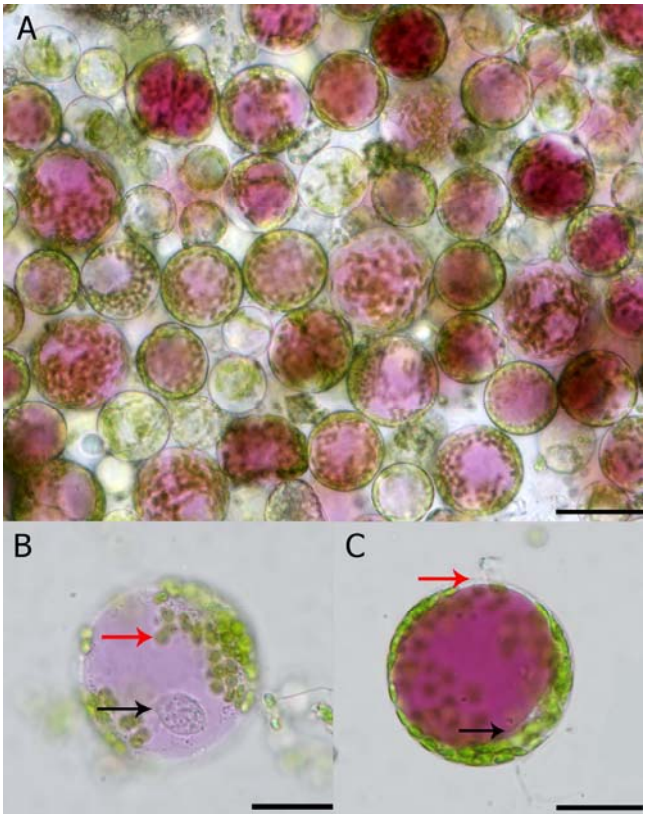

Fig. 4 Confocal micrographs of isolated protoplasts from a window stage leaf sample. Freshly isolated protoplasts released from window stage lace plant leaf. The pink cells are isolated from the mesophyll of the leaf, while the clear cells are isolated from the epidermis (**A**). Scale bar = 100 µm. Individual isolated protoplast, red arrow indicates chloroplasts, black arrow indicates nucleus (**B**). Individual isolated protoplast, red arrow indicates plasma membrane, black arrow indicates tonoplast membrane (**C**). Scale bar (**B, C**) = 50 µm

tonoplast membrane (**Fig. 4C** black arrow).

Trials experimenting with four different carbohydrate sources, each at 0.6 M, in protoplast buffer and enzyme mixture depicted that sorbitol significantly influenced protoplast yield and viability ($P = 0.000$; **Fig. 5**). Sorbitol at 0.6 M resulted in the highest yield ($60.42 \pm 0.89 \times 10^5$ protoplasts/ g fresh weight) and viability ($92.75 \pm 2.95\%$) when compared to other carbohydrate sources, as determined by heamcytometer and FDA techniques respectively. Using mannitol, protoplast yield ($7.85 \pm 0.53 \times 10^5$/g fresh weight) and viability ($71.25 \pm 2.04\%$) were significantly decreased when compared to sorbitol samples at $P < 0.05$ (**Fig. 5**). Using glucose, protoplast yield ($4.17 \pm 0.43 \times 10^5$/g fresh weight) and viability ($52.22 \pm 3.53\%$) were also significantly decreased when compared to sorbitol samples at $P < 0.05$ (**Fig. 5**). Lastly, when using sucrose, protoplast yield ($9.98 \pm 0.51 \times 10^5$/g fresh weight) and viability ($74.87 \pm 1.84\%$) were significantly decreased when compared to sorbitol samples at $P < 0.05$ (**Fig. 5**).

The amount of time protoplasts were allowed to digest in enzyme solution (2, 4 and 6 hrs) was found to significantly influence protoplast yield and viability ($P = 0.000$; **Fig. 6**). Protoplast digestion for 4 hrs resulted in the highest yield ($60.35 \pm 1.08 \times 10^5$ protoplasts/g fresh weight) and viability ($92.75 \pm 2.25\%$) when compared to 2 and 6 hour digestion times, as determined by hemocytometer and FDA techniques respectively. Digestion for 2 hrs resulted in significantly lower yield ($2.02 \pm 0.27 \times 10^5$) and viability ($61.37 \pm 1.61\%$) at $P < 0.05$ when compared to 4 hour digestions (**Fig. 6**). Digestion for 6 hrs also resulted in significantly lower yield ($1.92 \pm 0.34 \times 10^5$) and viability ($51.55 \pm 2.94\%$) at $P < 0.05$ when compared to 4-hr digestion (**Fig. 6**). There was no significant difference in protoplast yield between 2- and 6-hr digestions at $P > 0.05$, although significant differences in viability were noted between the same two trials at $P < 0.05$ (**Fig. 6**).

DISCUSSION

Factors effecting protoplast isolation

Several factors, including leaf age, carbohydrate source in buffer and enzyme solution, and enzyme incubation time all significantly influenced protoplast yield and viability from lace plant tissue.

Window stage leaves from plants approximately 1 month of age resulted in the highest yield and viability of protoplast isolates when compared to juvenile heteroblast and mature leaf samples. This difference in yield and viability based on leaf age has been seen in other species including *Anubias nana* (Pongchawee *et al*. 2006), *Cryptocoryne wendtii* (Pongchawee *et al.* 2007) and *Sesbania bispinosa* (Zhao *et al.* 1995). Similar trends to *Anubias nana* and *Cryptocoryne wendtii* were seen in the lace plant in that the youngest and oldest leaves gave lower protoplast yield and viability when compared to middle aged samples. There are several reasons for this occurrence, foremost among them being that the youngest leaves often do not contain thick deposits of cellulose or pectin, resulting in the enzyme mixture degrading the leaf instead of isolating protoplasts from it. Secondly, older leaves tend to have a thicker cuticle and more lignin in their cell walls then younger leaves, making them difficult to digest. Middle stage samples such as the window stage have a very thin cuticle and generally contain low amounts of lignin, resulting in healthy, round, organelle rich protoplasts from isolation.

Using mannitol, sucrose, or glucose in protoplast buffer and enzyme mixture resulted in a significantly lower number of protoplasts isolated when compared to sorbitol. It is possible that mannitol, sucrose and glucose all resulted in hypo or hypertonic solutions, causing the burst or collapse

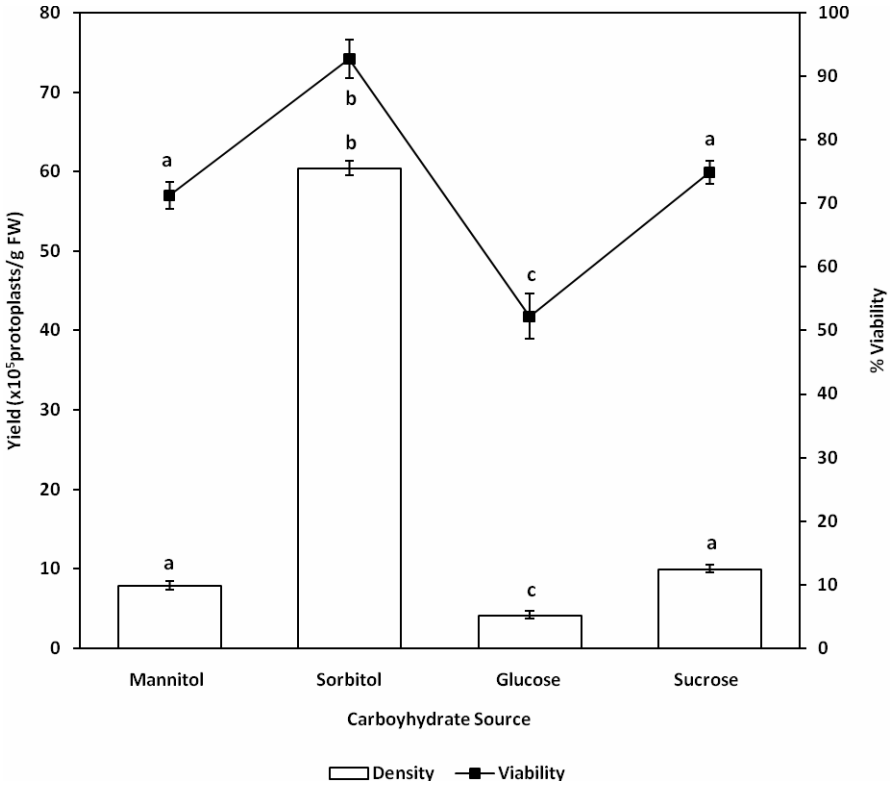

Fig. 5 Effect of various carbohydrate sources on protoplast isolation from *A. madagascariensis* window stage leaves. Bar graph represents average protoplast yield from each stage while the line graph represents average viability. Data represents mean ± standard error of four replicates.

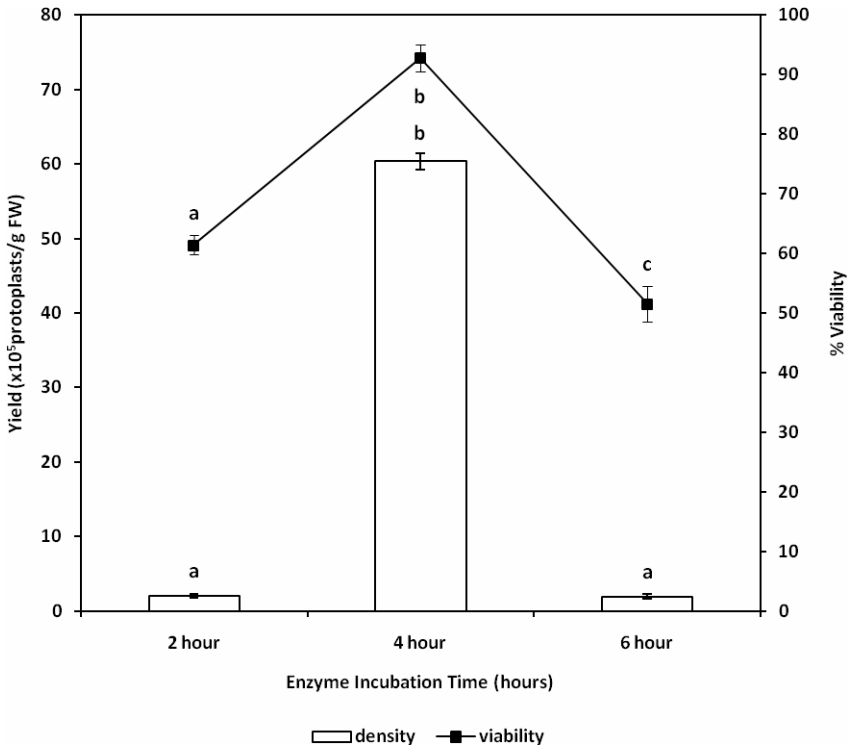

Fig. 6 Effect of various enzyme incubation periods on protoplast isolation from *A. madagascariensis* window stage leaves. Bar graph represents average protoplast yield from each stage while the line graph represents average viability. Data represents mean ± standard error of four replicates.

of protoplasts, respectively. Sorbitol as a carbohydrate seemed to provide the most suitable osmotic pressure for *A. madagascariensis* protoplasts, allowing for cells to remain intact; however the factors contributing to this finding should be investigated further (Pongchawee *et al.* 2006, 2007).

During enzyme incubation trials the optimal time to digest lace plant leaves was determined to be 4 hr. Incubation for a shorter period of time (2 hr) led to a decrease in protoplast yield, due to the tissue not being thoroughly digested. Large pieces of tissue still remained intact following digestion at 2 hr and contained many intact protoplasts. Conversely, the 6 hr incubation resulted in excessive digestion of the leaf material. This prolonged incubation of leaves could potentially have led to the dysfunction and breaking of cells. To allow for shorter incubation times, the concentration of the enzyme solution could have been raised and to allow for longer incubation times, the con-

centration of the enzyme solution could have been lowered (Pongchawee *et al.* 2006, 2007).

CONCLUSIONS AND FURTHER WORK

Initially, several protocols for the isolation of protoplasts from land plants such as *A. thaliana* (Yoo *et al.* 2007), maize (Sheen 1993) and wheat (Harris *et al.* 1988) were attempted with the lace plant, and resulted in low yield, non viable protoplasts from the plant. Here, the first efficient protocol for the isolation of high yield, viable protoplasts from the aquatic monocot *A. madagascariensis* was developed. Protoplasts were successfully isolated from window stage leaf tissue using an enzyme mixture comprised of 2% w/v cellulase R10 and 0.5% pectolyase Y-23 dissolved in 0.005 M MES, 0.6 M sorbitol, pH 5.5. Approximately 60.35 ± 1.08 × 10^5 protoplasts/g fresh weight, with a viability of 92.75 ± 2.25% were obtained from 4 hr isolations in the dark. Leaf age, carbohydrate source, and enzyme incubation time all significantly influenced both protoplast yield and viability.

At present time the isolated protoplasts will remain viable in culture medium consisting of 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 1.0 mg/L thidiazuron for up to 10 days with aggregates forming following 20 days of culture. Further research needs to be completed in order to determine precise hormone concentrations for the formation of callus and whole plant regeneration from the protoplasts. Whole plant regeneration from protoplasts would create an accelerated, cost effective method for producing sterile lace plants in large quantities.

Protoplasts from perforation expansion leaves have recently shown their suitability for environmentally induced PCD using heat treatment at 55°C for 20 min (Lord and Gunawardena 2010). This environmentally induced cell death has allowed for the opportunity to compare environmentally induced PCD with its developmentally regulated counterpart within one plant. In the future these protoplasts can be treated with other PCD inducers to further investigate changes in organelles, including the mitochondria, cytoskeleton, transvacuolar strands, vacuole and nucleus during PCD.

Lastly, these protoplasts can be utilized as a system for *in vivo* transient gene expression; by transforming the protoplasts with genes tagged with fluorescent markers and targeted to specific organelles, a more precise understanding of the order of events occurring within a cell undergoing PCD can be determined. This tagging would also permit for characterization of organelle origin, movement and fate.

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