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Lace Plant: a Novel System for Studying Developmental Programmed Cell Death

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ABSTRACT

The submerged aquatic plant, lace plant (*Aponogeton madagascariensis*, Aponogetonaceae) has a highly unusual leaf form, in which holes perforate the leaf blade in a regular lattice-like pattern. These perforations are formed by programmed cell death (PCD) shortly after leaf emergence from the apical region of the corm. Because PCD occurs in predictable locations in relation to the leaf vein pattern and at a known stage of leaf expansion, and because the thin, aquatic leaves are tractable to live-imaging microscopy, lace plant is an attractive model system for studying developmentally-regulated PCD in plants. One limitation of carrying out developmental and physiological analyses is the unknown effects of associated micro-organisms found in aquarium culture. Therefore, a primary objective of this study has been to establish and maintain axenic cultures of this species. We first characterized the formation of perforations through PCD in axenic cultures and found that the PCD process in culture did not differ significantly from that previously described. Since ethylene is an important component of PCD signaling pathways during many forms of plant PCD and also functions in growth regulation of other submerged aquatics, we also carried out experiments using 0.05 mM $AgNO_3$ as an inhibitor of ethylene perception and found a significant reduction in the number of perforations formed per leaf under treatment conditions compared to control plants. While preliminary, these experiments indicate that axenically cultured lace plant has considerable potential as a model system for the study of developmentally-regulated PCD in plants.

1. INTRODUCTION

Lace plant (*Aponogeton madagascariensis*, (Mirbel) van Bruggen, Aponogetonaceae) is native to Madagascar where it grows in river habitats as a submerged aquatic (Kasselmann 2003). Mature leaf blades are highly unusual in that they are perforated (fenestrated) with holes that extend through the blade, forming an open lattice pattern (**Figs. 1A, 1B**). Perforations are positioned equidistantly between the longitudinal and transverse veins, and are large and rectangular near the midvein, but smaller and rounder near the margin. Immature leaves are rolled longitudinally, but unfurl as they expand from the apical region of the corm-like tuber to form a flat leaf blade with a simple shape. The formation of perforations within the panels of epidermal and mesophyll tissue delimited by the grid-like vein pattern is a relatively late developmental process, occurring when the leaves have reached about 10% of full expansion, and secondarily converts the blade from a simple to a complex shape. The perforations expand as the leaf grows, and the gradient in perforation size from midvein to margin reflects the distribution of growth of the leaf blade. This unusual perforated leaf form is thought to function to reduce resistance to water flow and also to increase leaf surface area for the diffusion of CO_2 and mineral nutrients to photosynthetic tissues.

Programmed cell death (PCD) is a genetically encoded, active process that results in the death of individual cell, tissues, or whole organs. Plants employ PCD in a wide range of developmental processes, including the differentiation of individual cell types such as tracheary elements, the development of whole tissues such as aerenchyma and, less commonly, the morphogenesis of whole organs or organ assemblages (Barlow 1982, Morgan and Drew 2004). For instance, initially bisexual flowers become unisexual through the loss of gynoecium primordia to PCD (Caporali *et al.* 2003). The employment of PCD to form complex shapes during leaf morphogenesis is extremely rare. Lace plant and certain species of the genus *Monstera* and related genera of the Araceae are the only vascular plants known to use PCD as part of normal leaf morphogenesis (Kaplan 1984, Gunawardena *et al.* 2004 2005, Gunawardena and Dengler 2006). In lace plant, the first signs of PCD are an alteration of cytoplasmic streaming and a simultaneous loss of anthocyanin coloration which occur in a discrete subpopulation of epidermal and mesophyll cells located centrally within a perforation site. Nuclear DNA is degraded, but not fragmented into internucleosomal units, a common characteristic of PCD in animal apoptosis (Gunawardena *et al.* 2004). The cytoplasm contracts and collapses away from the cell wall and ultrastructural analyses show that, while the cytoplasm thins, organelles remain intact until late stages of PCD. PCD is initiated in these centrally located epidermal and mesophyll cells and then extends outward toward the veins, stopping about five cells distant from the vascular tissue.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; **AVG**, aminoethoxyvinylglycine; **HR**, hypersensitive cell death; **MS**, Murashige and Skoog medium; **2,5-NBD**, 2,5 norbomadiene; **PCD**, programmed cell death; **TUNEL assay**, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Mesophyll cells at the rim of the perforation differentiate as epidermal cells, thus maintaining the continuity of the epidermis in the mature leaf. Although some of the steps of cell death execution have been identified in this unique case of developmentally-regulated PCD in plants, nothing is known about the developmental cues, signaling pathways, or molecular regulation of the PCD process during perforation formation.

The plant hormone ethylene is an important component of PCD signaling pathways during development and response to pathogens and abiotic stresses (Matoo and Handa 2004). For instance, hypoxia-induced aerenchyma formation in maize roots (Gunawardena *et al.* 2001), epidermal cell death at the site of adventitious root emergence in rice (Mergmann and Sauter 2000), senescence of pea carpels (Orzaez and Granell 1997), death of maize endosperm cells (Young and Gallie 2000), and camptothecin-induced death in tomato cell suspension cultures (de Jong *et al.* 2002) are well-studied examples where ethylene appears to function at early stages of the PCD process. Host defense in response to pathogens is expressed as the hypersensitive response (HR) which results in the death of infected cells and involves ethylene in at least some cases (Gilchrist 1998, Podile and Sripriya 2002, Matoo and Handa 2004). Cell death induced by ozone in tomato leaves is preceded by a rapid increase in ethylene biosynthesis (Matoo and Handa 2004), and transcript levels for ethylene biosynthesis enzymes and ethylene receptor genes are up-regulated in response to ozone treatment (Moeder *et al.* 2002). Ethylene diffuses more slowly in water than in air and is employed in the growth responses of certain aquatic plants, such as in stem and petiole elongation (Ridge 1987, Voesenek *et al.* 2003). Whether ethylene plays a role in the signaling pathways that lead to PCD during perforation formation in lace plant is completely unknown but, because ethylene functions as a developmental signal for some submerged aquatic plants and as a trigger of PCD in diverse systems, it is a promising candidate hormone for the experimental manipulation of perforation development process in lace plant.

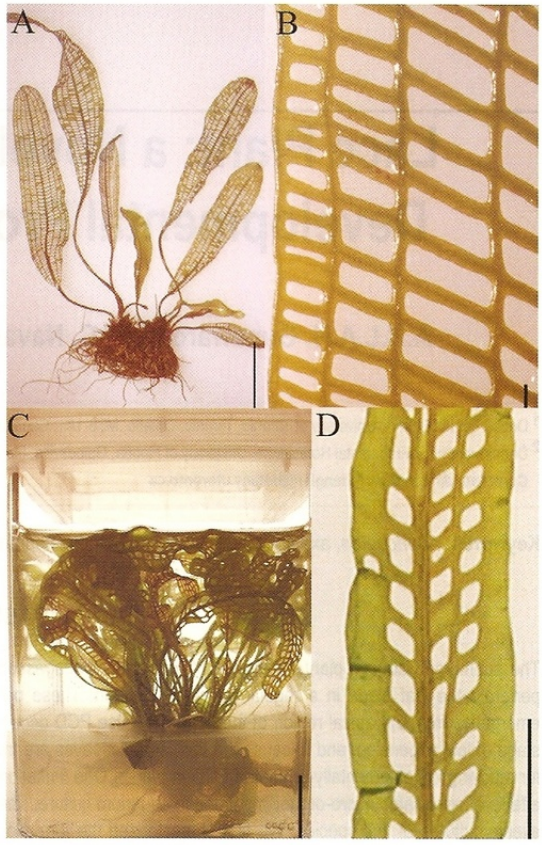


Fig. 1 Whole lace plant (*Aponogeton madagascariensis*, Aponogetonaceae) with perforated (fenestrated) leaves grown under aquarium conditions (**A, B**) and in axenic culture in Magenta GA7 vessels (**C, D**). Scale bars = A, 5 cm; B, 1 mm; C, 1.25 cm; D, 0.5 cm.

One limitation of carrying out a developmental analysis and physiological experiments with a submerged aquatic such as lace plant is the unknown role of associated micro-organisms. The immediate environment of a developing lace plant leaf undergoing perforation formation is presumably rich in the products of nucleic acid, protein and carbohydrate degradation, and microscope analysis indicates that a substantial flora and fauna is present in aquarium culture. Therefore, one objective of our ongoing research has been to establish and maintain axenic cultures of lace plants. We also wished to compare the PCD process in plants maintained in axenic culture with those grown in aquaria. In this chapter we report on our success in establishing such cultures and on initial experiments using inhibitors of ethylene action to test the hypothesis that ethylene plays a role in the induction of PCD during perforation formation in this species.

2. AXENIC CULTURE OF LACE PLANT, INCLUDING PLANT GROWTH

2.1. Establishment of axenic cultures

Plants of *A. madagascariensis,* bearing mature fruits, were supplied by Mr. Brad McLane, Florida Aquatic Nurseries, Inc., Ft. Lauderdale, FL. Fruits were excised from the donor plants, surface sterilized in 0.6% sodium hypochlorite (10% v/v Clorox Ultra bleach) for 8 min and then rinsed in sterile distilled deionized (D&D) water for 5 min. Embryos, enclosed by thin seed coats, were aseptically excised from the fruit and surface sterilized in 1.2% sodium hypochlorite (20% v/v Clorox Ultra bleach) for 10 min and then rinsed for 5 min three times in sterile D&D water.

Embryos were separated from their seed coat and inoculated individually into separate 150 x 25 mm glass culture tubes containing 12 ml liquid medium consisting of liquid half-strength MS mineral salts (Murashige and Skoog 1962), 0.4 mg/L thiamine, 100 mg/L myo-inositol, 30 g/L sucrose, and 1 mg/L N_6-[2-isopentyl]adenine (2iP). Medium was adjusted to pH 5.7 with 0.1 N KOH prior to autoclaving at 1.2 kg cm^{-2} and 121°C for 20 min. Cultures were maintained in a Percival incubator at 25°C under 16h light/8h dark cycles provided by cool-white fluorescent lamps (General Electric F20WT12·CW) at 40 µmol m^{-2} s^{-1} as measured at culture level.

Of seventy two embryo cultures attempted, eight were visibly contaminated while six exhibited no growth or mortality by 28 days culture. During this culture period, responsive seedlings developed multiple basal shoots bearing partly perforated leaves. All visibly uncontaminated cultures were indexed for cultivable microbes using Liefert and Waites Sterility Test Medium as described by Kane (2000). Before subdividing shoots, each initial culture was assigned a genotype number. Clonal lines, derived from individual seedlings were maintained separately. Established plants were transferred to fresh medium at 28 day intervals.

2.2. Subculturing experimental plants

All transfers were carried out in a laminar flow hood. Autoclaved MS medium (2.15 g MS basal salts, 100 mg *myo*-inositol, 0.4 mg thiamine-HCl,

30 g sucrose and distilled water to make one liter) containing 1% agar was poured (100 ml) while still molten into autoclaved Magenta GA7 boxes and allowed to solidify for 45 min. Stock plants were removed from culture vessels and mature leaves and roots were dissected away from the corm, using sterile forceps and scissors. The corm was subdivided into 2-8 pieces, depending on size and each piece was placed in the center of the solidified MS medium layer. Sterile liquid MS (0% agar) was poured into the magenta box to fill approximately ¾ of the volume. The boxes were covered and sealed with parafilm. Cultures were maintained in a Conviron E15 growth chamber with 18h light/6h dark cycles at 24°C and 290 µmol m^{-2} s^{-1} from cool white fluorescent lights (Sylvania F72T12 CW/HVO).

2.3. Growth analysis

Growth was monitored in a subset of cultures by recording the number and lengths of all leaves present. Digital images were taken of these plants every 2 days (**Fig. 2**). Subcultured corms produced leaves at a rate of one every 2-3 days. The first-formed leaves were small, entire and lacked perforations (**Figs. 2A-C**). Typically the fourth or fifth leaf formed on a sub-cultured corm was the first to show evidence of perforations as it emerged from the corm apex (**Figs. 2D-F**); these perforations were located near the leaf midrib and were discontinuous in that only some of the panels of interveinal tissue formed a perforation, while most remained intact (**Fig. 2F**). As new leaves were formed, a greater proportion of perforation sites underwent PCD, so that by the sixth leaf formed after sub-culturing, each interveinal panel formed a perforation (**Figs. 2G-I**, arrow). Thus after about four weeks in culture, plants consistently formed leaves with perforations at predictable sites. Mature, perforate leaves were much smaller than those of plants maintained in aquaria, however, and perforations tended to be restricted to the tissue adjacent to the midvein and major longitudinal veins, but to be absent near the leaf margin (**Figs. 1C, 1D**).

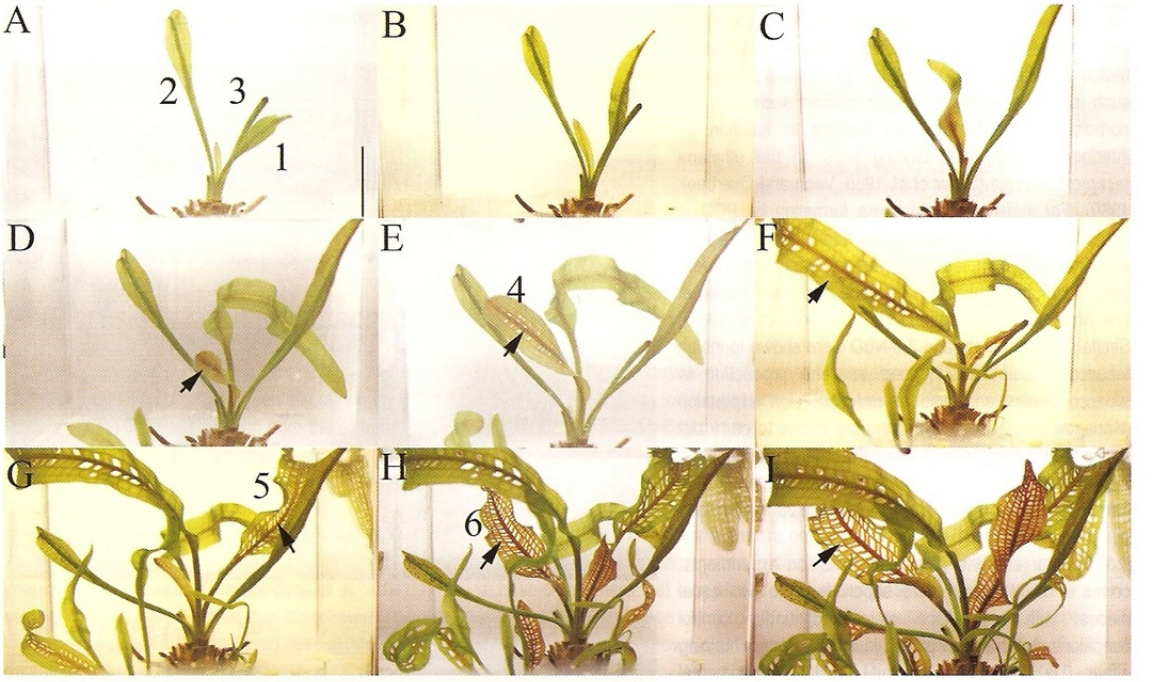


Fig. 2 Development of a subcultured lace plant corm on MS medium. (**A-D**), The first three leaves are small and lack perforations. (**E-F**) The fourth leaf is larger and forms a small number of perforations between the midrib and first longitudinal vein (arrows). (**G**) The fifth leaf forms perforations more consistently between the midrib and the first longitudinal vein and between the first and second veins (arrow). (**H-I**) The sixth leaf produced after subculture produces perforations at each perforation site (except for the marginal area, arrows). Leaves are numbered in order of appearance. Additional leaves arise from adventitious shoot meristems on the corm. Scale bar = 2.5 cm.

3. PROGRAMMED CELL DEATH IN AXENIC CULTURE

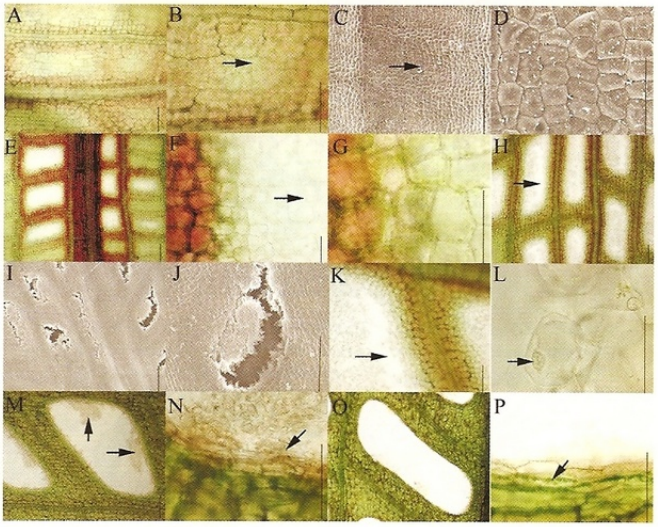
The development of perforations in leaves of sub-cultured plants does not differ significantly from that previously described for aquarium-grown plants (Gunawardena *et al.* 2004). During Stage 1, leaves extend from the shoot apex region and begin to unfurl, revealing the grid-like pattern of longitudinal and transverse veins (**Fig. 3A**). At this stage, epidermal and mesophyll cells located between the veins appear to be normal, with no cytological indication of perforation formation (**Figs. 3B-D**). Subcultured plants differ from aquarium-grown plants in that anthocyanin is only weakly expressed (**Figs. 3A, 3B**). During Stage 2 (the "window" stage), distinct transparent regions are observed at the sites of perforation, resulting from the loss of chlorophyll and anthocyanin (if present) (**Figs. 3E-G**). These regions contrast sharply with cells outside the perforation site, which retain chlorophyll and vacuolar anthocyanin (**Figs. 3F, 3G**). At Stage 3 (perforation formation), epidermal and mesophyll cells undergoing PCD appear to separate from adjacent cells, allowing rupture of the blade at these locations (**Figs. 3H-L**). The cytoplasm of cells at the site of rupture is shrunken and pulled away from the cell wall, although nuclei remain intact (**Fig. 3L**). During Stage 4, perforations enlarge as the leaf expands (**Figs. 3M, 3N**). One notable difference between sub-cultured and aquarium-grown plants is that cell wall degradation during PCD is reduced in axenic culture. Large patches of dead cells adhere to the rim of the perforation at one or two locations (**Fig. 3M**, arrows) and dead cells are retained along the whole periphery of the perforation (**Fig. 3N**). In mature leaves (Stage 5), the perforations have reached full size and the living cells at the rim of the perforation differentiate as epidermal cells, with an elongated shape, few chloroplasts, and cell walls

impregnated with brownish phenolic substances (**Figs. 3O, 3P**). Although more detailed analyses employing the TUNEL assay for nuclear DNA degradation, video-photography of cytoplasmic streaming and collapse, and electron microscopy of cytoplasmic changes (see Gunawardena *et al.* 2004) were not carried out for these axenically-cultured plants, these features observed by light and scanning electron microscopy indicate that the process of PCD is similar under both growth conditions.

4. A ROLE FOR ETHYLENE IN PROGRAMMED CELL DEATH IN LACE PLANT?

Ethylene has been hypothesized to have a role in PCD and certain other aspects of plant growth, based on studies of mutants deficient in ethylene biosynthesis, perception or signaling or on pharmacological experiments using inhibitor treatments that interfere with either ethylene biosynthesis or perception in a wide variety of plants (Wang *et al.* 2002, Matoo and Handa 2004). Inhibitors of ethylene biosynthesis such as aminoethoxyvinylglycine (AVG) competitively interact with the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, the enzyme that is thought to be the important rate controlling step near the terminus of this major route of ethylene synthesis (Wang *et al.* 2002, Matoo and Handa 2004). Inhibitors of ethylene perception such as silver salts or cyclic olefins such as 2,5-norbornadiene (2,5-NBD) are thought to function by interfering directly with binding sites on the ethylene receptor molecule (Sisler *et al.* 1986, Veen and Overbeek 1989). For instance, aerenchyma formation by PCD in maize roots is promoted by the addition of 0.1 µl L^{-1} ethylene, but completely prevented when roots were treated simultaneously with ethylene and silver nitrate at low, non-toxic concentrations (0.6 µM; Drew *et al.* 1981). Similarly, silver nitrate and 2,5-NBD were shown to inhibit tobacco mosaic virus-induced ethylene production in tobacco leaves and also to reduce lesion expansion (Knoester *et al.* 2001). We therefore chose to carry out preliminary experiments using silver nitrate to test the hypothesis that ethylene perception has a role in the formation of perforations during leaf development in lace plant.

Fig. 3 Perforation development in the sixth or later leaves produced on lace plant corms. (**A-D**), Stage 1 (pre-perforation). Note that cells in the perforation site that will undergo PCD (arrows) resemble those near the veins that will not in light microscope (**A, B**) and scanning electron microscope (**C, D**) views. (**E-G**) Stage 2 ("window" formation). Note that cells in the perforation site lose chlorophyll pigmentation and become transparent (arrow), while anthocyanin accumulates in cells near the veins. (**H-L**) Stage 3 (perforation formation). Scanning electron micrographs show small elongate or oval ruptures at the center of perforation sites with presumably dead cells remaining attached along the line of rupture (**I, J**). Light micrographs show the rupture (arrow) and a broad zone of dying cells within each perforation site (**H, K**). Dying cells have a collapsed cytoplasm, but intact nuclei at this stage (arrow, **L**). (**M, N**) Stage 4 (leaf expansion). The perforation enlarges as the leaf expands, retaining large patches of dead cells (**M**, arrows) or a narrow rim of dead cells (**N**, arrow) at the margin. (**O, P**) Stage 5 (mature leaf). Dead cells may be present, but living cells at the margin differentiate as epidermal cells (**P**, arrow). Scale bars = A, B, F, K, N, P, 100 µm; D, G, L, 50 µm; C, E, H, I, J, M, O, 300 µm.



For our ethylene perception inhibition experiments, corms of stock plants were subdivided into two equal pieces, with one piece maintained under control conditions and the other placed in MS medium containing either 0.005 mM or 0.05 mM $AgNO_3$. Experimental treatment with 0.005 $AgNO_3$ did not appear to affect leaf form, so experiments with the higher concentration 0.05 $AgNO_3$ only were continued (n = 11/treatment). After a six week experimental period, treatment plants were generally smaller, and fully expanded leaves appeared smaller than in control plants (**Figs. 4A, 4B**). Treatment plants also appeared to retain anthocyanin in mature leaves, giving them a brownish color (**Fig. 4B**). At the end of the experiment, leaves were removed from the control and treatment corms and the number of leaves, leaf length, and number of perforations per leaf recorded (**Fig. 4C**). The data were variable, but there was no significant difference in the total number of leaves produced during the experimental period or in the proportion of those leaves that developed perforations between the experimental and control plants (**Fig. 5A**). Silver nitrate treatment significantly reduced the length of later-formed leaves that developed perforations, but not the length of the early-formed imperforate leaves (**Fig. 5B**). Silver nitrate also reduced the number of perforations per leaf (**Fig. 5C**), suggesting that inhibition of ethylene perception suppresses PCD at potential perforation sites, resulting in a smaller number of perforations per mature leaf. These experiments are not conclusive, however, since there is a positive correlation between leaf length and perforation number for both control and treatment plants, with no significant difference between slopes of the regression lines (**Fig. 5D**).

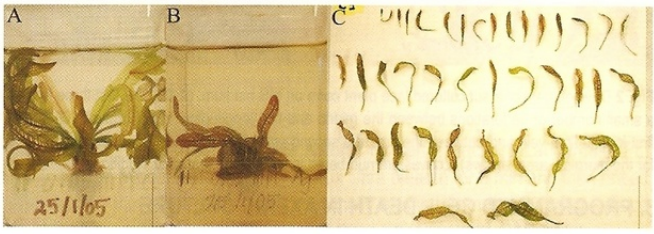


Fig. 4 Ethylene perception inhibition experiments using 0.05 mM $AgNO_3$. (**A**) Control plant after 45 days of subculture. (**B**) Treatment plant derived from the same parent corm after 45 days. (**C**) Leaves produced on one control plant after 45 days. Scale bars = A, B, 2 cm C, 4 cm.

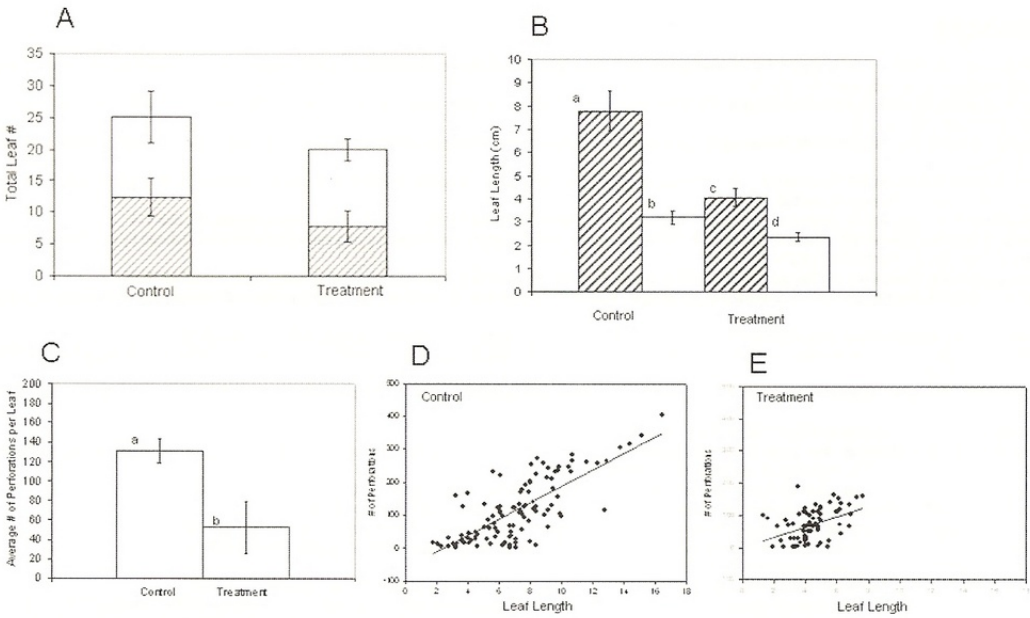


Fig. 5 Ethylene perception inhibition experiments using 0.05 mM $AgNO_3$. (A) Total number of leaves produced after 45 days by control and treatment plants. Hatched portion of bar indicates proportion of total leaves having perforations. (**B**) Mean lengths of leaves produced on control and treatment plants. Hatched bars indicate lengths of perforated leaves and open bars lengths of imperforate leaves. (**C**) Total number of perforations per leaf in perforated leaves of control and treatment plants. (**D**) Linear regression analysis of perforation number (dependent variable) and leaf length in control and treatment plants. The same letter associated with histogram bars indicates that mean values are not significantly different at $P < 0.05$, $n = 11$ plants / treatment.

5. CONCLUSIONS AND FUTURE PROPECTS

The development of complex leaf shape through perforation formation is a highly unusual morphogenetic mechanism, yet one that holds promise for the investigation of developmentally regulated PCD in plants. First, perforations form in predictable locations and at a known developmental stage in young leaves expanding from the shoot apex of lace plant corms. PCD is initiated in a population of cells at the center of the perforation site, and then a wave of PCD is propagated outward through epidermal and mesophyll cells until it reaches tissue that is about 5 cells from the veins. This spatial pattern makes it possible to predict which cells are going to die and to identify adjacent cells of the same age that will not undergo the PCD process. Thus, although study of PCD in lace plant is at an early stage, this system may eventually lend itself to the investigation of positional regulators of developmental PCD. Second, leaf blades of lace plant are only four cell layers thick and are relatively transparent, facilitating whole-mount experimental procedures such as the TUNEL assay and *in situ* hybridization and time-lapse and live-cell imaging of living leaves (Gunawardena *et al.* 2004). Third, we have shown here that lace plant can be grown and readily propagated in axenic culture, providing large amounts of clonal material for experimental study that is free of micro-organisms. This preliminary report indicates that the cell biology of PCD in cultured leaves does not differ from that in aquarium grown plants, except perhaps for a reduced level of cell wall degradation during perforation formation.

The nature of the developmental signals that trigger the PCD process at a predictable time and place is unknown. The placement of perforations at a consistent distance from leaf veins suggests that a vascular-derived signal might be involved in the induction of PCD (Nelson and Dengler 1997). Equally, the veins might be a source of an inhibitory molecule that blocks the PCD process or prevents perception of the inductive signal, and cells might respond at a threshold level of one or more diffusible molecules. Identification of such putative signaling molecules is extremely challenging, although it is possible that elements of a signaling system could be identified through microarray or differential display methodologies (Matz and Lukyanov 1998). An alternative approach is to test candidate molecules that have been associated with PCD in other systems for physiological activity in lace plant. We have chosen to test the efficacy of our axenic culture system by such an approach. As ethylene has been shown to promote PCD in a number of systems and inhibitors of ethylene biosynthesis or perception to suppress PCD, we have carried out a series of pharmacological experiments using $AgNO_3$, an inhibitor of ethylene perception. We have shown that 0.05 mM $AgNO_3$ is sufficient to suppress perforation formation in treated lace plants. Suppression is not complete, but leaves form fewer perforations, suggesting that leaf tissue responds in an all-or-nothing threshold manner. Under the concentrations of $AgNO_3$ used, leaf expansion was also affected, so that it will be necessary to experiment with a greater range of concentrations, as well as other inhibitors and sources of ethylene production such as Ethephon, in future experiments. Nevertheless, these initial experiments indicate that lace plants maintained in axenic culture will be tractable material for future experimentation. We are currently working on micro-propagation and transformation methodologies for lace plant that will contribute to our understanding of the execution of cell death, as well as its induction and signaling pathways, in this unique example of developmentally regulated PCD.

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