PROFILING PHENOLIC COMPOUNDS IN *APONOGETON MADAGASCARIENSIS* AND INVESTIGATING THEIR ROLE IN PROGRAMMED CELL DEATH DURING LEAF DEVELOPMENT

by

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Abstract

The lace plant (*Aponogeton madagascariensis*) is an aquatic monocot native to Madagascar that undergoes a form of programmed cell death (PCD) during early leaf development. This process results in a perforated leaf morphology that is extremely rare among plants. Young leaves emerge with a strong red pigmentation due to the presence of anthocyanins, a family of phenolic compounds. As lace plant leaves develop, they form a predictable pattern of perforations throughout the leaf lamina, starting in the centre of areoles located in between the lateral and longitudinal veins. The first sign of this PCD process is the disappearance of anthocyanin pigmentation in the young window stage leaf. Phenolic compounds are ubiquitous among plant life, and are known to be involved in a variety of plant stress responses and defense mechanisms. The production and regulation of phenolics are known to be heavily linked with jasmonates, a small family of phytohormones. Although the mechanisms of phenolic involvement in stress response are widely understood, their role in plant PCD remains to be elucidated.

In this study, a profiling of anthocyanin species and other phenolics was conducted in key developmental stages of lace plant leaf development via microscopy, spectrophotometry, and mass spectrometry. *In vivo* pharmacological experiments were conducted on whole plants by treatments with vanillin, phenidone, and methyl jasmonate to quantify their effects on leaf perforation formation.

Results indicate that anthocyanin and vanillin content were significantly higher in young leaves prior to and during PCD. In addition, anthocyanins are heavily localized in the apices of lace plant leaves. Under stresses such as high pH, light deprivation, and crowded growth, the apex of the leaf is the location of the formation of membrane-less anthocyanic vacuolar inclusions (AVIs). Treatment with inhibitors of endogenous phenolics through the jasmonate pathway, phenidone and vanillin, showed significant reduction of anthocyanin, total phenolics, DPPH radical scavenging capacity, and PCD in the lace plant. Treatment with methyl jasmonate (MeJA) caused a significant increase in anthocyanin production and DPPH radical scavenging, but did not affect the number of perforations**.** Analyses by Triple Quadrupole Mass Spectrometry analyses allowed for the identification of anthocyanin species present in the lace plant for the first time. Relative abundance analyses from TQ-MS revealed a substantial decrease in delphinidin species during leaf development after the PCD process is complete. Additionally, these results also revealed potentially novel compounds that have not been documented in any other species to date. The effects of plant phenolics on PCD mechanisms could shed light on future applications in regulating cell death for pharmacological and agricultural research.

List of Abbreviations and Symbols Used

Bad	Bcl2-associated agonist of cell death
Bak	Bcl2 antagonist/killer
Bax	Bcl2-associated x
Bcl-2	B-cell lymphoma 2
Bid	BH3-interacting domain death agonist
CE	catechin equivalents
Cy3R	cyanidin-3-rutinoside
Cy3Re	cyanidin-3-rutinoside equivalents
dH_2O	distilled water
DPPH	2,2-diphenyl-1-picrylhydrazyl
EI-MS	electrospray ionization-mass spectrometry
FA	formic acid
FADD	Fas-associated protein with death domain
Fas	Fas cell surface death receptor
HCl	hydrochloric acid
JA	jasmonic acid
JA-Ile	jasmonic acid-isoleucine
MeJA	methyl jasmonate
MeOH	methanol
NaOH	sodium hydroxide
PARP	poly(ADP-ribose)polymerase

PCD	programmed cell death
ROS	reactive oxygen species
SA	salicylic acid
Th	Thomson unit
TQ-MS	triple quadrupole-mass spectrometry
TRAIL	TNF related apoptosis inducing ligand

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CHAPTER 1

INTRODUCTION

1.1 Programmed Cell Death

Programmed cell death (PCD) is a highly regulated process of cell elimination and is present in both prokaryotic and eukaryotic life. The mechanisms of PCD were originally investigated in the development of the nematode *Caenorhabditis elegans*, which provided the foundation for continued investigation into cellular mechanisms behind PCD (Gumienny et al. 1999). PCD is critical in many developmental processes, and serves a variety of purposes depending on both species and environment. It is a complex energy-dependent process involving many biochemical modifications including protein cleavage, surface protein cross-linking, DNA degradation, phagocytic recognition, and activation of cysteine-aspartic proteases (caspases) and other death-linked proteins (Kroemer et al. 2005; Kroemer and Reed 2000). A summary of commonly documented cell death-related proteins and their known functions is presented in Table 1. Caspases are a family of proteases known to be highly involved in the PCD process, from initiation (caspase-2,-8,-9,-10), execution (caspase-3,-6,-7) to inflammation (caspase-1,-4,-5; Elmore, 2007). True caspases are documented only in animal PCD, and the mechanisms of animal PCD are much more widely understood than those in plants (Rantong and Gunawardena 2015).

Plant cell death is categorized into three types: apoptotic-like cell death, autophagic cell death, and necrosis (Galluzi et al. 2012). The former two types of cell death are more intricately controlled, due to the complex and highly regulated series of

cellular events that are involved in the process. This commonly includes an accumulation of reactive oxygen species, compromising of the mitochondrial membrane potential followed by the release of cytochrome c into the cytosol, activation of cell death proteases, DNA fragmentation, and vacuolar membrane (tonoplast) rupture (Obara et al. 2001).

PCD in plants is essential for a variety of critical developmental processes including xylem differentiation, aerenchyma formation, root cap cell shedding, hypersensitive response, leaf morphogenesis and senescence (Pennell and Lamb 1997). Xylem differentiation is the evolutionary adaptation for land plants to enable water transportation from roots to shoots, and involves cells within the centre of their body patterning to undergo PCD, leaving behind only a hollow tube of cell walls to deliver water throughout the organism via transpiration (Fukuda 2000). Aerenchyma formation is an adaptive trait for plants to survive in water-logged environments. It is a process in which cells within the root cortex undergo PCD to form oxygen-storing compartments (Drew et al. 2000). In the root cap during root growth and development, cells around the outermost edge undergo PCD as they are released from the root tip, secreting metabolites that both lubricate the surrounding substrate to promote ease of root penetration into the soil, as well as influencing which microbial species co-exist in the root microbiome in order to benefit the plant (Vacheron et al. 2013). Hypersensitive response is the defense mechanism in plants which identifies and prevents the spread of pathogens by triggering PCD in infected cells, and establishing chemical defenses in surrounding cells (Morel and Dangl 1997). Leaf senescence represents the terminal stage in plant leaf development, and functions as a process to recycle and redistribute nutrients back into other plant

organs (Noodén 2012). Perforation formation during leaf development only occurs in a few known plant species, including *Monstera obliqua* and *Monstera deliciosa* of the Araceae family, and *Aponogeton madagascariensis* of Aponogetonaceae (Gunawardena et al. 2004; Gunawardena et al. 2005). Although this process has now been well-documented, the purpose of perforation formation during leaf development remains widely unknown (Gunawardena et al. 2004). Recent advancements in plant PCD research have begun to show the involvement of defence and development-related phytohormones such as ethylene, ABA, and jasmonates in PCD pathways (Dauphinée et al. 2012; Adie et al. 2007; Overmyer et al. 2000). Furthermore, the relationship between reactive oxygen species (ROS) and antioxidant phenolic compounds and their connection to these phytohormones during plant PCD is also being elucidated (Overmyer et al. 2000; Van Breusegem and Dat 2006).

1.2 Diversity of Phenolic Compounds

Phenolic compounds are a broad class of organic phytochemicals found ubiquitously among plant life from green algae to angiosperms (Steinberg 1984). Phenolics are one of three phytochemical groups classified as secondary metabolites (Figure 1). Many of the perceived sensory characteristics of plants are associated with these compounds, which can regulate the taste, smell, and visual hue of the species. All phenolics contain a phenol group (-OH bonded to an aromatic hydrocarbon ring). Their chemical structure is similar to that of alcohols. Since thehydroxyl group is bound to an unsaturated carbon, it has higher acidity, and greater reactivity due to the weak bond between the oxygen and hydrogen of the hydroxyl group. This structural feature contributes to their use as antioxidants (Kähkönen et al. 1999). Phenolic compounds

range in size from the simple phenol (C_6H_6O) at 94.1 g/mol, to large complex tannins that can well exceed 1000 g/mol (Hagerman et al. 1998). Their roles in plants are as diverse as their chemical compositions. Many of these compounds have been shown to play important roles in a range of plant development, growth, and defense mechanisms (Coley et al. 1985). More recently, they are being studied for their effects in a number of human health conditions, including neurological, cardiovascular, and chronic disease research in relation to their role in PCD mechanisms (Sun et al. 2008; Bertelli and Das 2009; Frankel et al. 1993). Furthermore, their implication in plant cell death is an emerging object of intense research, which may shed additional light on cell signaling pathways and PCD among all forms of life (Beckman 2000; Tamagnone et al. 1998).

1.2.1 Phenolic Acids and Aldehydes

The addition of a carboxyl group on a simple phenol characterizes this group of small phenolics. Hydroxybenzoic acids and hydroxycinnamates are ubiquitous and diverse groups that include some important signaling molecules such as ferulic acid, *p*-coumaric acid, gallic acid, salicylic acid and vanillic acid. For example, these small phenolics are critical in a variety of abiotic stress responses such as insect attack, oxidative stress, and plant-microbe symbioses (Berendson et al. 2012; Schützendübel and Polle 2002; Mandal et al 2010). Many phenolic acids are necessary precursors in the biosynthesis of more complex phenolics (El-Basyouni et al 1963). Salicylic acid functions as a crucial plant hormone, contributing to development, photosynthesis, and cell signaling (Rivas-San Vicente and Plasencia 2011; Fariduddin et al 2003; Lu et al 2003).

1.2.1.1 Vanillin

In phenolics, aldehyde groups are derived from the reduction of carboxylic acids, forming fragrant phenolic aldehydes such as vanillin. Vanillin is a widespread aldehyde found in higher plants, but is well known from its abundance in the pods of *V. planifolia*, and has become one of the most common flavouring agents in consumables and scented products worldwide. Additionally, given its popularity in the food industry, vanillin has been under pharmacological investigation and has been shown to have antioxidant (Tai et al. 2011), antisickling (Zhang et al. 2004), and both pro- and anti-apoptotic properties (Lirdprapamongkol et al. 2010; Moossmann et al. 1997).

1.2.2 Flavonoids

With over 13,000 identified structural species to date, flavonoids represent the largest and most extensively studied group of phenolics. Flavonoids can be further sub-categorized into flavonols, flavones, flavanols, flavanones, anthocyanidins, and isoflavones. This family is mostly recognized for potent antioxidant compounds (Rice-Evans et al. 1995), but have also exhibited pro-apoptotic effects in cancer cell lines (Middleton et al. 2000), and anti-apoptotic effects as a potential therapeutic for Alzheimer disease prevention (Williams and Spencer 2012). These compounds are most widely studied from their presence in fruit and leaf tissues (Einbond et al. 2004; Xiaonan 2000).

1.2.2.1 Anthocyanins

Belonging to the vast flavonoid family of phenolic compounds, anthocyanins are a group of phytochemicals responsible for the pink-red-purple pigmentation found

extensively among higher plants, save for betacyanins among Order Caryophyllales (Stafford 1994). Anthocyanins are derived from their aglycons, anthocyanidins (Tanaka et al. 2008). Of the 19 known anthocyanidins, the 6 most commonly found in well-studied plant species include pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (Iacobucci and Sweeny 1983). A number of enzymes such as glycosyl-transferases bind different sugar moieties to anthocyanidin R groups, creating a vast diversity in anthocyanin species (Tanaka et al. 2008). The identification of these anthocyanin species through high performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) analyses has been an invaluable tool in anthocyanin-apoptosis research (Prior et al. 2001).

In plants, anthocyanins are known to serve a variety of functions including UV protection (Steyn et al. 2002), nitrogen fixation signaling (Bond 1951), auxin transport inhibition (Besseau et al. 2007), and attractants for pollination and seed dispersal (Saito and Harborne 1992). Under various environmental stresses, of which light exposure has been the most commonly studied, plant tissues produce membrane-less, proteinaceous matrices named anthocyanic vacuolar inclusions (AVIs; Markham et al. 2000). These inclusions are localized in the vacuole and act as reservoirs for anthocyanidin-3,5-diglucosides or acylated anthocyanins, and allows the cells to concentrate anthocyanins above normal levels in vacuolar solution (Markham et al. 2000; Conn et al. 2003).

Common anthocyanin species, particularly cyanidin and delphinidin derivatives, have been subject to investigation in apoptosis. In animal research, anthocyanins have been shown to have prominent effects on vision by preventing retinal inflammation (Miyake et al. 2012). Additionally, their antioxidant capacities have been used to combat

heart disease (Mazza 2007), and they have shown inhibitory and/or apoptotic roles with a variety of cancer cell types (Zhao et al. 2004; Lala et al. 2006; Malik et al. 2003; Reddivari et al. 2007; Hui et al. 2010). For example, cyanidin-3-rutinoside caused peroxide accumulation-induced apoptosis in HL-60 leukemic cells (Feng et al. 2007). The powerful antioxidant properties of these compounds have been attracting research in both medicine and food science (Einbond et al. 2004).

1.2.3 Coumarins

Coumarins are highly reactive bicyclic compounds derived from the C_6-C_3 skeleton common to any phenolics. In plants they are known for their involvement in pathogen and herbivory defense responses (Conrath et al. 2002). Their primary use in medicine has been as an anticoagulant agent in pharmaceuticals such as warfarin, which inhibits the vitamin K conversion cycle (Pauli et al. 1987). Other medicinal applications have been limited due to the hepatotoxic properties of coumarins in mammalian metabolism (Cohen 1979).

1.2.4 Stilbenes

Commonly found in the bark of trees and the exocarp of fruit, the endogenous value of stilbenes is linked to pathogen and herbivory resistance (Chong et al. 2009). Stilbenes have a C_6-C_2-C_6 structure, and can be hydroxylated to form stilbenoid derivatives, which can be produced by both plants and a single species of Gram-negative bacterium *Photorhabdus luminsescens* (Joyce et al. 2008). Resveratrol, the most widely studied stilbene derivative, is highly abundant in wine, and has been shown to promote cardiovascular health by decreasing the number of apoptotic cardiomyocytes (Tanabe et

al. 2005).

1.2.5 Tannins

Tannins are a highly abundant group of phenolics that are ubiquitous among both vegetative and woody plant tissues. Tannins can be classified into two groups: proanthocyanidins (condensed tannins) and hydrolysable tannins. These compounds are unique compared to other natural phenolics in their ability to precipitate proteins from solution, and have been used historically as a tanning agent of animal skins (Scalbert 1991), hence their name. Tannins are most widely known for their potent toxicity to animal predators and guard against microbial attack (Scalbert 1991). However, they have also been noted as powerful antioxidants (Hagerman et al 1998) and, more recently, have been investigated as potential treatments against cancer (Li et al. 2003). These anticancer properties of tannins, however, are one of the more controversial issues in phenolic pharmaceutics. Some studies, such as in esophageal cancer, have linked consumption of tannin-rich foods with a carcinogenic effect (Chung et al. 1998).

1.2.6 Suberin

Suberin is a cell wall-associated protective polymer comprised of phenolic and aliphatic domains, and in plants plays an important role in wound healing and in microbial defense (Bernards 2002; Lulai and Corsini 1998). It has been shown to have a role in a variety of cellular processes in plants, from the formation of aerenchyma, to cork differentiation (Enstone and Peterson 2005; Soler et al. 2007). Hydroxycinnamic acids and their derivatives are assembled into this suberin poly(phenolic) domain (SPPD) through localized peroxidase activity and polymerization (Bernards and Razem 2001). In

human research, suberin has been shown to combat colorectal cancer by resisting degradation in the colon and adsorbing hydrophobic carcinogens (Ferguson and Harris 1996).

1.3 Biosynthesis and Function

1.3.1 Phenolics

The ability for plants to synthesize phenolic compounds was an evolutionary advancement that has helped modern day plant lineages thrive. This adaptation allowed plants to produce compounds that could address specific needs for survival in their environment. Once plants moved onto land more than half a billion years ago, they evolved a way to counter the cleavage of chemical bonds caused by UV light, and did this by adapting a phenolic "UV light screen" (Boudet 2007). For example, an *Arabidopsis* mutant lacking these phenolic sunscreens exhibits excessive damage from UV-B and oxidative stress (Landry et al. 1995). Apart from UV stress, plants express phenolic production in response to a variety of stresses including microbial invasion (Schimel et al. 1996), heavy metal contamination (Michalak 2006), drought (André et al. 2009) and other oxidative related stress responses (Apel et al. 2004).

The biosynthetic pathways responsible for the production of plant phenolics are the Shikimate (phenylpropanoid) pathway which produces phenylpropanoids derived from either phenylalanine or tyrosine, and the polyketide (acetate/malonate) pathway which produces simple phenols (Quideay et al. 2011). Between these two pathways, plants are able to synthesize the vast diversity of polyphenols for their various physiological purposes. Although most of these are synthesized for structure in plant cell walls, many

are produced for the regulation of growth, development and stress response as previously mentioned. Chemical modifications within this pathway have enabled land plants to successfully adapt to their changing environments. Cytochrome P450 and other condensing enzymes form the phenolic parent skeletons, while methylation (O-methyltransferase), acylation (acyltransferase), and glycosylation (glycosyltransferase) represent some of these key fundamentals, which alter the phenolic structures' polarity, volatility, stability and ability to interact with other compounds (co-pigmentation). These enzymatic modifications are capable of producing thousands of molecular variants (Cheynier et al. 2013). Many phenolics contribute to a reduction of reactive oxygen species (ROS) in cells, and some have been shown to perform crucial roles in phytohormone transport and signal transduction pathways. One of the more well understood phenolics in signal transduction is a phenolic acid, salicylic acid (SA). SA plays an important role in a variety of physiological processes, such as the innate immune response during pathogen attack (Cheynier et al. 2013).

In regulating the induction of phenolics, interactions with various phytohormones are very important. Flavonoids are known regulators of auxin transport (Jacobs and Rubery 1988), and interactions between phenolics and abscisic acid (Jiang and Joyce 2003) and ethylene (Ke and Saltveit 1989) have also been documented. A variety of phenolics have been shown to antagonize the ABA induced inhibition of hypocotyl growth (Ray et al 1980), and reverse ABA-induced stomatal closure (Rai et al 1986). Ethylene is known to induce phenolic accumulation (Heredia and Cisneros-Zevallos 2009), and salicylic acid is a well-documented inhibitor of ethylene biosynthesis (Leslie and Romani 1988). However, the jasmonate family of phytohormones is possibly the

most important of the hormones in terms of their direct relationship with phenolic induction (Keinänen et al. 2001).

1.3.2 Jasmonates

Plants have a unique and complex ability to cope with both biotic and abiotic stresses, and given their adaptation to sedentary life, are able to utilize a variety of phytohormonal-dependent defense mechanisms for survival. Some of the primary hormones involved with plant stress responses are lipid-derived compounds known as jasmonates (Turner et al. 2002). This hormone family includes jasmonic acid (JA), methyl jasmonate (MeJA), jasmonic acid-isoleucine (JA-Ile), and is classified within a larger family known as oxylipins. Oxylipins are released from chloroplast membranes, and their biosynthesis is induced by dioxygenases, which (in plants) includes heme-dependent fatty acid oxygenases, and lipoxygenases (LOX). Due to their involvement in plant defense, lipoxygenases and the products of their activity have been the subject of cell death research in recent years (Wasternack 2007; Christensen et al. 2015). Some well known inhibitors of LOX include vanillin (Laughton et al. 1991) and phenidone (Cucurou et al. 1991), which were employed in this study.

The induction of jasmonate biosynthesis is most commonly documented as a response to herbivory, and accumulation of JA and MeJA and subsequent proteinase inhibitors was originally documented in response to insect attack (Farmer and Ryan, 1990). It has since been discovered that jasmonate accumulation is also associated with microbial and pathogen attack (Gundlach et al. 1992). The mechanism by which jasmonates act in these defense responses primarily involves the production of secondary

metabolites and other leaf volatiles that are cytotoxic against microbial and pathogen attack, and discourage herbivory (Wasternack 2007). The evolution of these lipid-derived phytohormones and their cell signaling mechanisms have been critical for the success of modern plant life.

1.4 Phenolics in Animal Cell death

The relationship between ROS and PCD is well elucidated, as ROS are highly reactive signalling molecules that interact heavily with and are produced primarily by mitochondria, in addition to other cellular components such as chloroplasts and the plasma membrane. Common ROS include singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals (Apel and Hirt 2004). While low concentrations of these molecules are critical for many signal transduction pathways, an excessive accumulation in cells unable to maintain homeostasis will result in cell death. The systematic release of cytochrome c from the mitochondria, activating caspases and the death cascade in animal PCD systems seems to be heavily linked with ROS accumulation (Simon et al. 2000). This accumulation of ROS can lead to age-related diseases in humans, such as Alzheimer's Disease, cardiovascular disease, and various forms of cancer (Castro and Freeman 2001). This gives an obvious value in disease prevention to phenolics given their strong antioxidant capacities (Al-Saikan et al. 1995). Aside from the general health benefits associated with antioxidants, phenolic compounds have been shown to be beneficial for applications in targeting specific apoptotic mechanisms such as death-related protein families, and the mitochondrial membrane potential. A compilation of recent advancements in phenolic research in apoptosis are summarized below in Table 2.

1.5 Phenolics in Plant Cell Death

The apoptotic mechanisms in animal cells through application of exogenous plant phenolics are being investigated extensively (Roy et al. 2002; Selassie et al. 2005; Yi et al. 2005), however the role of endogenous phenolics in plant cell death remains widely unknown. It has been proposed that plants have specialized cells at potential points of microbial entry where phenolics are heavily produced and stored in vacuoles for rapid oxidation, lignification, suberization, and cell death to seal off infection or injury (Beckman 2000). The direct relationship between phenolics and jasmonates has provided some interesting insight into potential roles of phenolics in plant PCD in recent years.

Regulation of cell death in root systems is critical for plant viability. Root cap cells begin to undergo PCD shortly after being produced by the proximal meristem before being systematically sloughed off during root growth, producing mucilages that aid in root tip lubrication and influence the surrounding rhizosphere (Rougier and Chaboud 1985). Gene transcription profiles of *Zea mays* root cap cells revealed an upregulation of jasmonate-induced response genes including lipoxygenase and 12-oxophytodienoate reductase (Jiang et al. 2006). This supports a previous study which suggested that JA activity is a key player in the lateral root cap cell shedding of *Arabidopsis thaliana* (Birnbaum et al. 2003). It has also been shown that during aerenchyma formation, JA increased expression of tissue remodeling gene *TaBWPR-1.2* in *Triticum aestivum* (Haque et al. 2014). Additionally, an *A. thaliana* mutant (*fad3-2 fad7-3 fad8*), which cannot accumulate jasmonates was unable to undergo hypersensitive response under pathogen attack compared to the wild-type. Application of MeJA recovered this PCD

process (Vijayan et al. 1998). Finally, application of MeJA to *A. thaliana* leaves induced senescence, and JA related gene expression was increased in senescing leaves (He et al. 2002). Given the relationship between jasmonates and the induction of a variety of plant PCD processes, it seems useful to further investigate the potential roles of phenolics in these systems. An objective of this study was to investigate the potential roles of MeJA in developmentally regulated PCD of the lace plant.

1.6 Phenolics in Lace Plant PCD

The lace plant (*Aponogeton madagascariensis*) is an aquatic monocot of the Aponogetonaceae family, native to Madagascar (Figure 2). It has a unique developmental leaf morphology, and is a member of one of only two known plant families to form holes in their leaves during development, the other being *Monstera spp*. in family Araceae. The lace plant is an excellent model organism for studying PCD due to the following: a) the predictability of perforation formation during leaf development; b) the establishment of axenic cultures for experimentation; and c) transparent leaves for microscopic observation. There are five specific stages of lace plant leaf development: (1) preperforation or furled, (2) window, (3) perforation formation, (4) perforation expansion, and (5) mature (Gunawardena et al. 2004). Three major stages - furled, window, and mature – are represented in Figure 3A. All leaves are connected to an underground stem known as a corm (Figure 2A). The first 3-4 juvenile leaves that emerge from the lace plant corm do not show a red pigmentation, and do not form perforations. Once these juvenile leaves have emerged, perforations begin to form in newly emerging leaves (window stage), eventually resulting in the lace-like appearance of the mature leaves (Gunawardena et al. 2004). This suberization is supported by evidence via Sudan

7B and Fluoral yellow 088 staining, in addition to autofluorescence detection by UV light (Gunawardena 2007). The earliest sign of PCD in the lace plant is the disappearance of anthocyanin pigmentation from the cells in the center of window stage leaf areoles. Prior to this change, areoles in the furled stage leaves have uniform pigmentation throughout (Figure 3B). In the window stage, the 4-5 cell layers surrounding the vasculature are considered non PCD cells (NPCD) because they do not undergo PCD, and retain their anthocyanin colouration (Figure 3C) until the cell death process is complete within each perforation, and the window stage leaf has matured (Figure 3D). The mesophyll cells at the perforation border transdifferentiate into epidermal cells, which deposit a layer of suberin, likely to protect the healthy cells from microbial invasion (Gunawardena 2007). However, the exact roles of these cell wall polymers in lace plant PCD remains to be elucidated. This leads to an important question regarding the role of phenolic compounds in early lace plant leaf development.

1.7 Objectives of This Study

The objectives of this study are: (1) to profile the phenolic compounds present in the lace plant throughout leaf development; (2) to investigate the effect of exogenous phenolics (vanillin) on developmental PCD in lace plant leaves; and (3) to investigate the effect of exogenous MeJA on developmental PCD in lace plant leaves. I hypothesize that phenolic compounds play a key role in the developmental pathway of lace plant PCD, and by altering endogenous phenolic production I can manipulate the natural PCD process. I also hypothesize that the lace plant contains novel anthocyanin species.

Table 1. Cell death proteins. Summary of key protein families investigated in recent phenolic cell death research, highlighting their known role(s) in apoptosis.

Protein(s)	Apoptotic Mechanism	Reference
Bax, Bak, Bad, Bid	Mediates mitochondrial membrane permeabilization	Chipuk et al. 2004; Scorrano et al. 2003; Wang et al. 1999; Li et al. 1998
Bcl-2	Blocks cytochrome c release from mitochondria	Yang et al. 1997; Kluck et al. 1997
Caspase-1,-4,-5	Inflammation	Martinon et al. 2002 ; Martinon et al. 2004
Caspase-2,-8,-9,-10	Initiation	Huang and Strasser 2000; Chen and Wang 2002
Caspase-3,-6,-7	Execution	Rhéaume et al. 1997; Hatai et al. 2000
FADD	Initiation binds Fas death receptors	Chinnaiyan et al. 1995; Zhang et al. 1998
PARP	ATP depletion	Ha and Snyder 1999; Lieberthal et al. 1998
TRAIL	Activates death receptors and caspases	Almasan and Ashkenazi 2003; Suliman et al. 2001

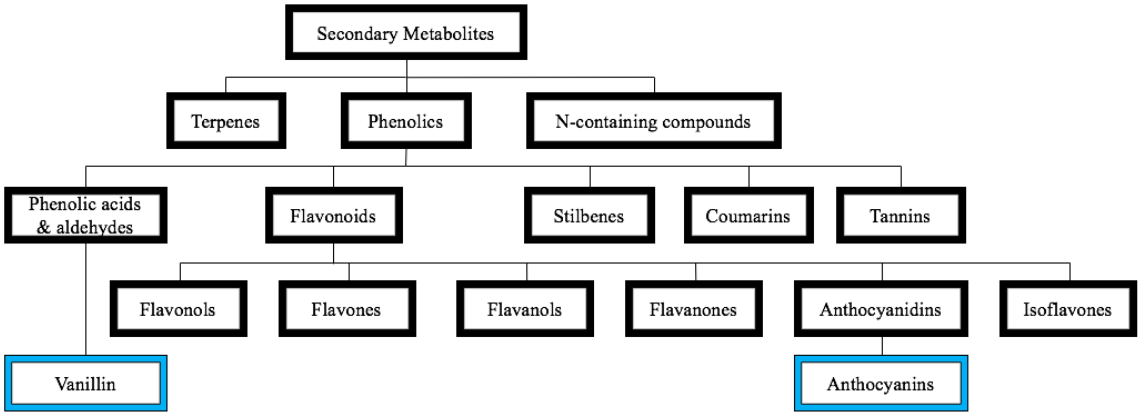

Figure 1. Classification of plant secondary metabolites. Phenolics represent one of three classes of secondary metabolites. The major families of phenolic compounds are represented in this flow chart. Flavonoids represent an extensive family of phenolics, containing over 13,000 identified compounds to date. Highlighted compounds represent central players in this study. Information gathered from Vermerris and Nicholson (2008), and Crozier et al. (2006).

Table 2. Recent applications in apoptosis. Notable phenolic compounds and their associated mechanisms in cell death research in recent years. Arrows indicate either an increase (↑) or a decrease (↓) in activity. Apoptotic mechanisms of proteins listed are outlined in Table 1.

Compound(s)	Family	Cellular Mechanism	Cancer Type	Reference
Resveratrol	Stilbenes	↑ Bax ↑cyt c release ↑ caspase-6	Colon	Mahyar-Roemer et al. 2001 Lee et al. 2009
Resveratrol	Stilbenes	↑ TRAILR1, TRAILR2 ↑ Bax, Bak, Bid, Bad ↑ caspase-9 ↑ caspase-3	Prostate	Shanker et al. 2007 Aziz et al. 2006 Benitez et al. 2007
Quercetin	Flavonols	↓ Bcl-2 ↑ caspase-6,-8,-9 ↑ Bax	Breast	Chou et al. 2010 Chien et al. 2009
Tannic acid	Phenolic acids	↑ Bak, FADD	Colon	Cosan et al. 2009
Cuphiin D1	Hydrolysable tannin	↓ Bcl-2	Leukemia	Wang et al. 2000
Ellagic acid and punicalagin	Phenolic acids/ Hydrolysable tannin	↑ cyt c release ↓ bcl-2 ↑ procaspase-3,-9	Colon	Larrosa et al. 2006
Delphinidin	Anthocyanidins	↓ PARP, Bax ↑ bcl-2	Skin	Afaq et al. 2007
Vanillin	Phenolic aldehydes	↑ TRAIL	Cervix	Lirdprapamongkol et al. 2010
Polyphenols (pomegranate)	-	↑ caspase-3	Breast	Dai et al. 2010

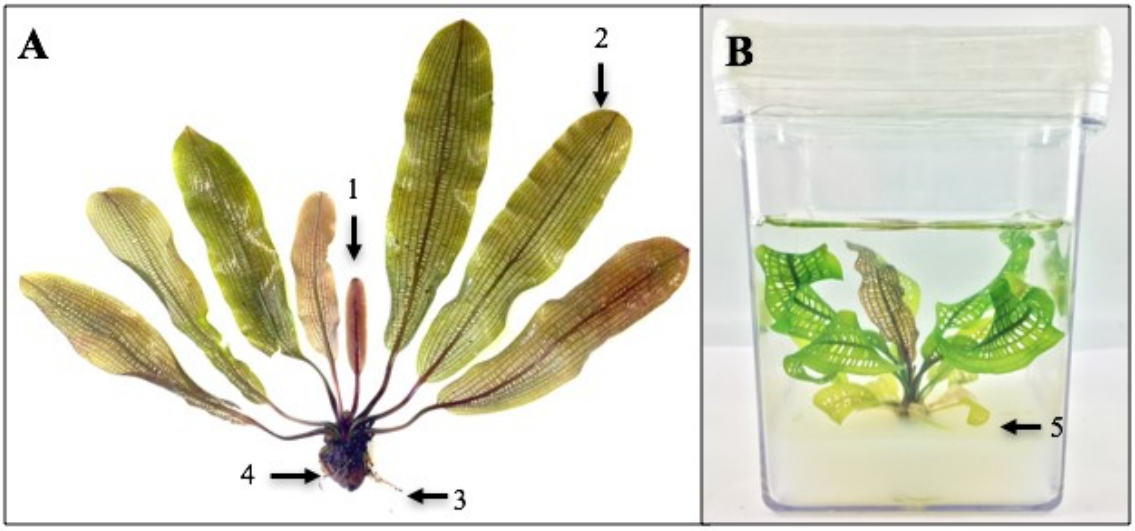

Figure 2. Lace plant (*Aponogeton madagascariensis*). (A) Aquarium-grown lace plant depicting young window stage leaf (1) with strong red pigmentation. Perforations form during this leaf stage, before losing red pigmentation once reaching maturity (2). Both leaves and roots (3) are produced from an underground corm (4). (B)A lace plant grown under aseptic conditions in a magenta box. During initial growth and emergence from the corm, three juvenile leaves (5) are produced. These juvenile leaves do not have red pigmentation, and do not form perforations.

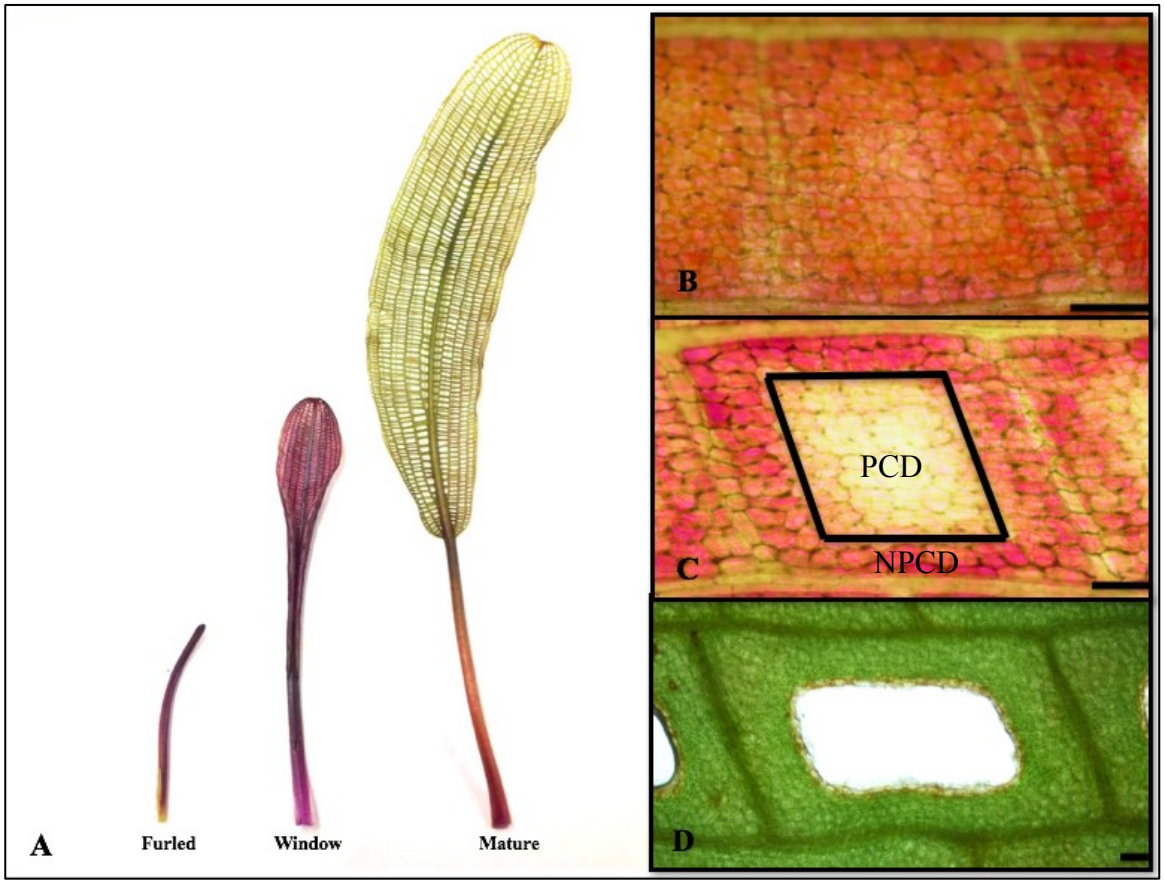

Figure 3. Lace plant leaf developmental stages. (A) Three successively older leaves at different stages of development corresponding to light microscopy micrographs of areoles (B, C, D) showing loss of anthocyanin pigmentation; the first sign of programmed cell death. (B) An areole from a pre-perforation stage leaf where anthocyanin pigmentation can be seen throughout cells. (C) An areole from a window stage leaf where a visible gradient delineating non programmed cell death (NPCD) cells from programmed cell death (PCD) cells, signified by loss of anthocyanin pigmentation. (D) An areole from a mature leaf where perforation has completely developed and a deposition of suberin (indicated by arrow) has taken place around the perforation border. Scale bar = 150 μm.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Materials and Chemicals

Lace plants were propagated as described in Gunawardena et al. (2006) in C1770 glass jars (PhytoTechnology Laboratories, KS, USA). In brief, plants were propagated from corms under sterile conditions in a laminar flow hood and grown in magenta boxes containing solid and liquid Murashige and Skoog (MS) medium (BioShop Canada Inc., Burlington, ON, Canada; Figure 2B). Once 3-4 perforated leaves had developed (approximately 4-6 weeks), individual plants were transferred into jars containing 40 mL solid MS medium with 1.5% agar (PhytoTechnology Laboratories, KS, USA) and 100 mL liquid MS medium. All plants were grown in 12h light/12h dark cycles (~125 μmol m-2 s-1) provided by fluorescent bulbs (Philips, Daylight Deluxe, F40T12/DX, Markham, ON, Canada) at 25ºC. Concentrations of chemical treatments of vanillin, phenidone, and MeJA were optimized and applied to liquid media in jars containing individually transplanted plants. Plants were then treated as described above and incubated for a 14 day period before harvesting for quantification of perforations and spectrophotometric assays. Experiments used both window and mature stage leaves (Figure 3A) unless otherwise stated.

Formic acid and HPLC-grade methanol used in tissue extractions were purchased from Fisher Scientific (Nepean, ON, Canada). Vanillin, catechin, cyanidin-3-rutinoside, phenidone, MeJA, DPPH (2,2-diphenyl-1-picrylhydrazyl), and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (Oakville, ON, Canada). All other chemicals were

purchased from PhytoTechnology Laboratories (KS, USA).

2.2 Microscopy

2.2.1 Localization of Anthocyanins

Leaf sections (n = 14 for each group) were excised from the apex, center, and base of lamina tissues from both window and mature lace plant leaves. Wet mounts were prepared from tissue pieces of approximately 1 cm^2. Coverslips were sealed onto glass slides with transparent nail polish and allowed to dry before inverting them and viewing under the microscope. Leaf tissues were observed on a Nikon Eclipse T*i* confocal microscope fitted with a digital camera (Nikon DS-Fi1). EZ-C1 3.80 imaging software (Nikon Canada, Mississauga, ON, Canada) was used for obtaining images at an excitation of 561 nm and emission of 595/50 nm for anthocyanin autofluorescence. Z-stack imaging was conducted to produce 3D images of tissues, scanning at 1 μm intervals. Using NIS Elements software (Nikon Canada, Mississauga, ON, Canada), maximum fluorescence projection images were selected from z-stacks and 25,000 μm^2 regions expressing the strongest fluorescence within each maximum projection were identified and used for quantification by recording software-projected fluorescence values (arbitrary units; Figure 4).

2.2.2 Anthocyanic Vacuolar Inclusion Quantification

Leaf apices ($n \geq 7$ per group) were excised from harvested window stage leaves grown under the following conditions: high pH (6.7), no light exposure (etiolated), high density (overgrown), low density (control), and aquarium-grown. Wet mounts were prepared from pieces of tissue of approximately 1 cm^2 from the apex, and observed on a

Nikon 90i light microscope (Nikon Canada, Mississauga, ON, Canada) using differential interference contrast (DIC) optics. Images were captured using a paired digital camera (DXM 1200c). Cell layers were scanned and anthocyanic vacuolar inclusions (AVIs) were counted using NIS Elements AR Version 3.0 software (Figure 5).

2.3 Extraction

2.3.1 Anthocyanin Extraction

Different anthocyanin extraction methods were used for both spectrophotometry and mass spectrometry work as described below.

2.3.1.1 Extraction for Spectrophotometric Analysis of Anthocyanins

Fresh leaf tissues (20 mg) were ground and macerated in a 5:95 formic acid (FA):MeOH solution for 50 minutes at room temperature. Samples were then centrifuged at 10,000 x g for 10 minutes, and the supernatant was collected. This protocol was modified from Li et al. (2010).

2.3.1.2 Mass Spectrometry

Tissue samples (500 mg) were taken from furled, window, mature, and senescent leaves, and inflorescences. They were ground and macerated in 5 mL of solution consisting of 85% acetone, 10% dH_2O and 5% FA and the resulting supernatant was filtered through glass wool. This was repeated two more times to obtain a total filtered extraction of approximately 15 mL. Polar compounds were then separated from other plant pigments and oils from the extract by adding a 1:1 ratio of chloroform:extract in a glass beaker. The top layer containing polar compounds was collected with a needle and syringe. The extract was then placed in a dark fume hood and allowed to evaporate for

two hours. The residue was then re-dissolved in 5 mL acidified dH_2O (5% FA) and run through a C18 Sep-Pak cartridge to adsorb the hydrophyllic phenolics. The cartridge was then washed with 5 mL acidified dH_2O and eluted with 5 mL acidified MeOH (5% FA). This solution was again placed into a dark fume hood to allow for evaporation. Anthocyanin residues were then re-dissolved in 1 mL of acidified MeOH (5% FA) before MS analysis. This protocol was modified from Garcia-Viguera et al. (1998).

2.3.2 Total Phenolic Extraction

For total phenolic extraction, 20 mg of tissue samples ($n \geq 9$ per group) from each window and mature stage leaf were ground and macerated in 20:80 dH_2O:Methanol and boiled for 30 minutes at 95^oC in a dry bath incubator. Samples were then incubated for an additional 24 hours in the dark at 4^oC, centrifuged for 10 minutes, and the supernatant collected for use in the Folin-Ciocalteu and DPPH assays to assess the extracts for total phenolic content, and free radical scavenging capacity, respectively. This protocol was modified from Menga et al. (2010).

2.3.3 Chlorophyll and Carotenoid Extraction

Tissue samples (200 mg; $n = 9$ per group) were taken from each window and mature stage leaf, placed in 2 mL DMSO in 1.5 mL micro-centrifuge tubes, wrapped in tinfoil and placed on a shaker at 60 rpm for 24 hours in room temperature. This protocol was modified from Chappelle and Kim (1992).

2.4 Spectrophotometry

All spectrophotometric analyses were performed in triplicate, where three individual readings were performed for each replicate and averaged. Blanks were used in

all spectrophotometric analyses, replacing tissue extract with corresponding solvent: either 5:95 formic acid:dH_2O (anthocyanin), 20:80 dH_2O:MeOH (phenolics), or DMSO (non-phenolic pigments).

2.4.1 Anthocyanin

Once final extracts were collected, they were read immediately in a Bio-Rad SmartSpec™ Plus spectrophotometer and their absorbance was recorded at a wavelength of 520 nm. A standard curve was generated using cyanidin-3-rutinoside as a standard to allow for the conversion of absorbance units (AU) into mg cyanidin-3-rutinoside equivalents per gram of tissue. The anthocyanin assay protocol was modified from Tsai et al. (2002).

2.4.2 Vanillin

Aliquots (1 mL) of the lace plant leaf extracts were transferred to 10 mL flasks and mixed with 2 mL of 5% sodium nitrite and 1 mL 10% HCl. Solutions were allowed to stand for 5 minutes at room temperature before adding 1 mL of a 10% NaOH solution and 5 mL dH_2O. Solutions were then measured at 385 nm against blank (replacing extract with 1 mL 20:80 dH2O:MeOH) corresponding to vanillin content (n = 6 per group). This protocol was modified from Backheet (1998).

2.4.3 Total Phenolic Content

Leaf extracts (100 μL) were added to 200 μL of 10% (vol/vol) Folin-Ciocalteu reagent and vortexed. 800 μL of a 700 mM solution of Na_2CO_3 was then added and tubes were incubated in the dark for two hours before measuring absorbances at 765 nm for

total phenolic content ($n \geq 9$ per group). A standard curve was generated using catechin as the standard, and results were expressed as catechin equivalents (CE). This protocol was modified from Agbor (2014).

2.4.4 DPPH Radical Scavenging

For the DPPH assay, 5 µL phenolic extract was added to 200 µL of a 0.3mM stock solution of DPPH in MeOH and briefly vortexed. Samples were then placed in the dark for 30 minutes at room temperature before measuring absorbance at 515 nm for radical scavenging capacity ($n \geq 9$ per group). DPPH has a strong purple pigmentation. Radical scavenging capacity of extracts corresponds with a reduction of purple colour into a colourless liquid, thus lowering absorbance at 515 nm. This protocol was modified from Saha et al. (2008)

2.4.5 Chlorophyll and Carotenoids

Absorbances of DMSO extracts were measured at 664 nm, 648 nm, and 470 nm for chlorophyll A, chlorophyll B, and carotenoids, respectively ($n = 9$ per group). This protocol was modified from Chappelle and Kim (1992).

2.5 Triple Quadrupole Mass Spectrometry

Anthocyanin extractions were prepared (2.3.1.2) from furled, window, and mature leaves, as well as inflorescence tissues.

Mass spectra were obtained on a 4000 Q TRAP LC-MS/MS triple quadrupole mass spectrometer (AB Sciex Instruments, Framingham, MA, USA) equipped with a Turbo Spray ESI source. Nitrogen was used as a desolvation gas at 400^{o}C. MS spectra within

the range of 50-1000 Th (unit of *m/z* ratio) at a scan rate of 1000 Th/s were performed in positive ion mode for anthocyanins.

Precursor-ion analyses were conducted using cyanidin-3-rutinoside as a standard. The most abundant m/z values among all extract samples were obtained for comparative analysis. For identity confirmations, product-ion and common-neutral-loss analyses were conducted on the most abundant mass/charge (m/z) detected. Product-ion analyses reveal the anthocyanidin structures contained within a given mass (Figure 6A). Common-neutral-loss analyses (Figure 6B) allows for detection of specific uncharged molecules released during fragmentation. Common-neutral-loss analyses were performed for three common sugar moieties: glucose (MW 162), rhamnose (MW 146), and xylose (MW 132).

Data were compiled and compared with existing anthocyanin database for identity confirmations (Buckingham and Munasinghe 2016).

2.6 Statistical Analyses

Statistical analyses were conducted using a general linear model (GLM) analysis of variance (ANOVA) with Dunnett test at 95% confidence on Minitab Express statistical software (Minitab Inc., State College, PA, USA) for all whole plant treatment experiments (perforation quantification, lamina width/length and spectrophotometric assays) to compare effect of treatments to control plants. A general linear model (GLM) analysis of variance (ANOVA) with Tukey test at 95% confidence was conducted for anthocyanin localization and anthocyanin/vanillin spectrophotometric assays for different developmental leaf stages to compare differences between all groups. Data are

represented as mean ± standard error. All data were normally distributed and had statistical power > 0.80 as determined by JMP 12 software (JMP, NC, USA).

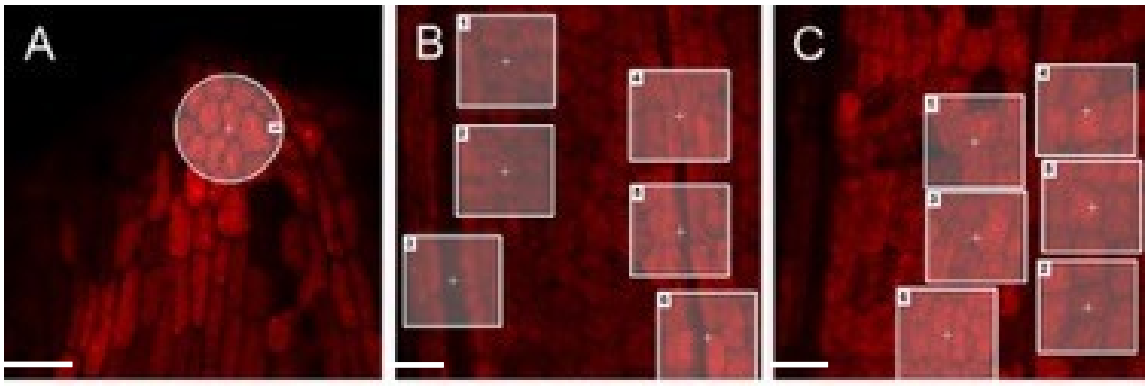

Figure 4. Anthocyanin localization. Maximum projection image of a window stage leaf generated by NIS Elements AR Version 3.0 software of anthocyanin autofluorescence obtained using a Nikon ti confocal microscope at an excitation/emission of 595/50 nm. The brightest 25,000 μm^2 regions (as indicated by boxes) were determined by the software, generating an arbitrary level of fluorescence for comparative analysis. Tissue pieces analyzed were either from the apex (A), mid portion (B), or base (C) of the lamina of the leaves. Scale bars = 150 μm.

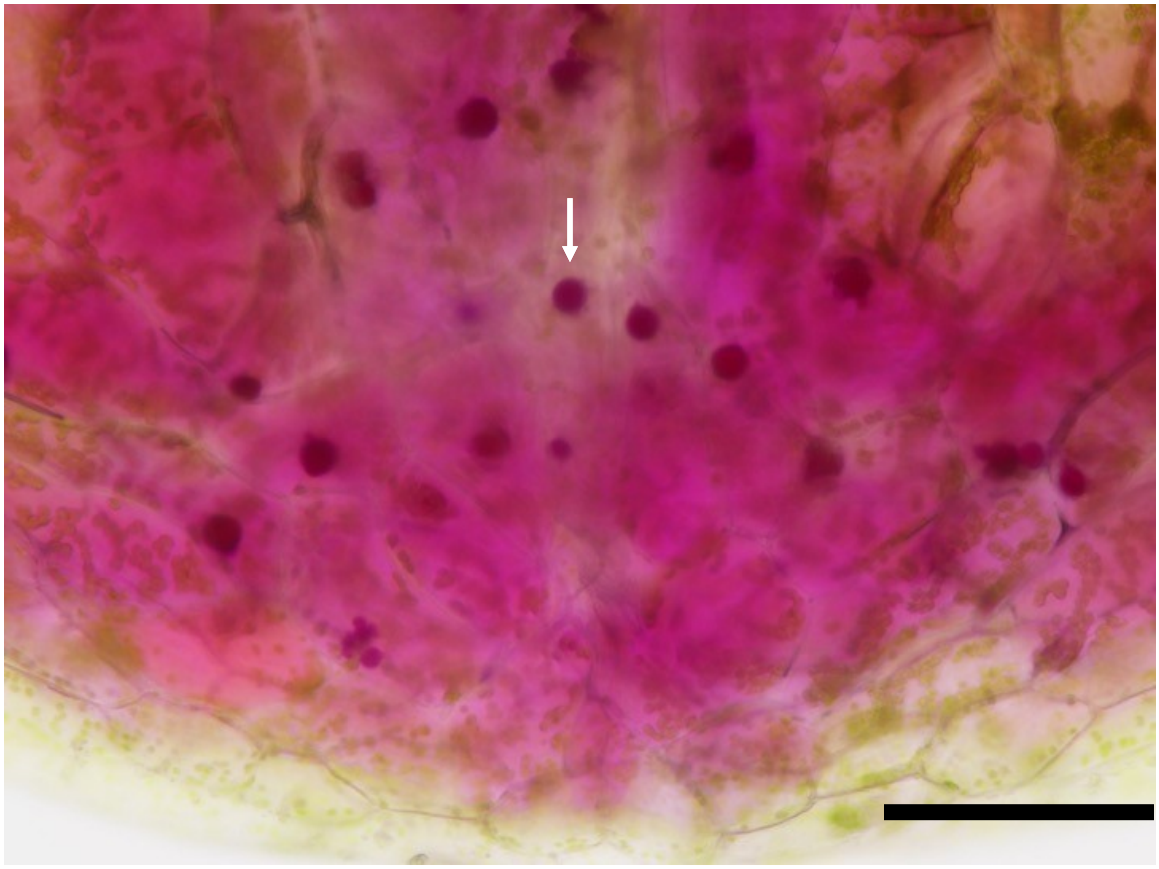

Figure 5. AVI quantification. Micrograph of leaf apex of a lace plant from a window stage leaf taken from an overgrown magenta box. Image was taken on a Nikon 90i light microscope fitted with a digital camera. Dark membrane-less AVIs indicated by an arrow can be observed in the cells. Scale bar = 100 μm.

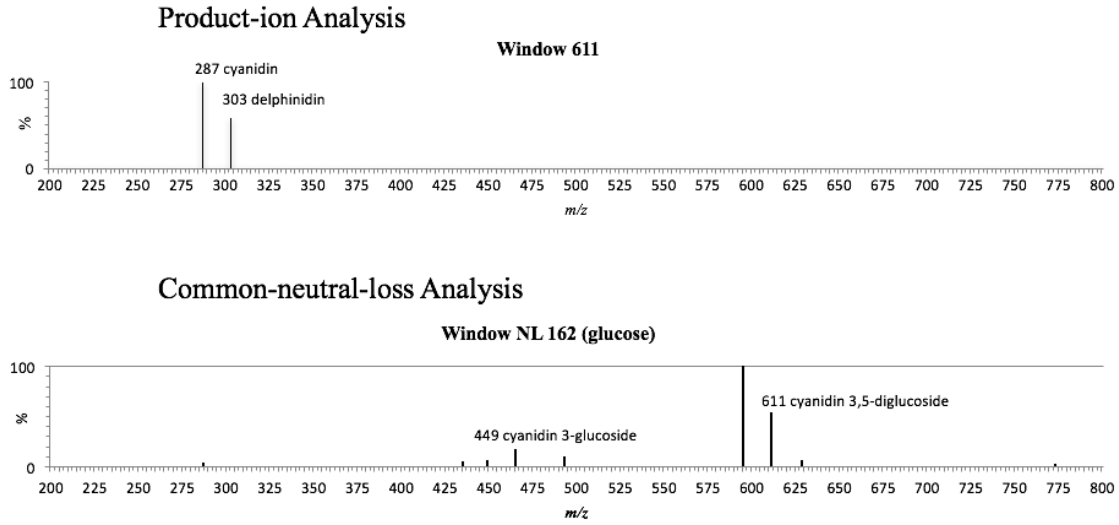

Figure 6. Product-ion and common-neutral-loss analyses. Sample MS spectra from a window stage leaf extract for product-ion 611 *m/z*. Analysis reveals both cyanidin and delphinidin precursors. Common-neutral-loss selecting for MW 162 (glucose) reveals *m/z* products of 611 and 449, corresponding to cyanidin 3,5-diglucoside, and cyanidin-3-glucoside, respectively.

CHAPTER 3

RESULTS

3.1 Anthocyanin Localization

Confocal autofluorescence analysis of anthocyanin localization across leaf lamina in window and mature stage leaves is shown in Figure 7. Anthocyanins in window stage leaves were most abundant in the apex of the leaf, compared to the middle and basal lamina ($P < 0.0001$). No significant difference was observed between the middle and basal laminar tissues ($P = 0.8798$). Similarly, in mature leaves anthocyanin levels were substantially higher in the apex of the leaf when compared to the middle and base of the lamina ($P < 0.0001$). No difference was observed between the middle and basal laminar tissues ($P = 1.0000$). There were significantly higher levels of anthocyanins detected in all three regions of the leaf in window stage leaves versus mature leaves ($P < 0.0001$). Raw data and results of statistical analyses are presented in Appendix 1.

3.2 Anthocyanic Vacuolar Inclusions

The number of anthocyanic vacuolar inclusions formed in the apex of window stage leaves was significantly lower in (control) lace plants grown in magenta boxes compared to all other stress-related growth conditions (Figure 8). Increased pH ($P = 0.0332$), etiolated ($P = 0.0052$), and overgrown ($P = 0.0027$) conditions all resulted in a greater number of AVIs. Although aquarium-grown plants had more AVIs than those grown in magenta boxes, the difference was not statistically significant ($P = 0.0530$;

Figure 8). Raw data and statistical analyses are presented in Appendix 2.

3.3 Anthocyanin and Vanillin Throughout Lace Plant Leaf Development

Both anthocyanin and vanillin content were significantly highest in window stage leaves compared to all other stages (Figure 9). Furled leaves had higher vanillin and anthocyanin content than mature and senescent leaves, but lower levels than window stage leaves. There was no significant difference observed between mature and senescent leaves for either vanillin or anthocyanin ($P = 1.000$).

3.4 Whole Plant Experiments

The effect of select chemical applications (vanillin, phenidone, and methyl jasmonate) on lace plant PCD was evaluated by quantifying the inhibition of perforations after a 14-day incubation period. Samples of leaves at different developmental stages from optimal treatment applications are shown in Figure 10. A strong inhibition of perforations can be observed in both vanillin and phenidone treated plants (Figure 10B&C), especially in the first couple of leaves post-treatment (leaves 1 and 2). In later leaves, perforation recovery is observed (leaves 3 and 4). In the MeJA treated plants, perforations form normally, but colouration of the leaf is darker with tones of red from anthocyanin, and leaves appear to have stunted growth (Figure 10D).

To quantify leaf morphology, the length and width of the lamina were measured for each treatment. Vanillin and phenidone treatments both significantly affected lamina length and width only at the highest concentration applied (Figure 11A and B, D and E; Appendix 4-5). All concentrations of methyl jasmonate applied had a significant effect on both lamina length and width in relation to control plants (Figure 11C,F; Appendix 5).

Both sequential leaf perforation recovery (Figure 12A,C,E) and total mean perforations pre- and post-treatment (Figure 12B,D,F) are shown for comparative trends. For vanillin-treated plants, concentrations of 2 mM or greater ($p = <0.0001$) caused a significant inhibition of perforation formation (Figure 12B; Appendix 3). In phenidone-treated plants, concentrations of 100 μM ($P = 0.0168$) and 200 μM ($P < 0.0001$) inhibited perforation formation (Figure 11D; Appendix 4). When comparing individual leaf perforation recovery trends in vanillin and phenidone treated plants, the strongest PCD inhibition was observed in the first leaf that developed after application of the treatment. The number of perforations/cm^2 returned to normal levels in subsequent leaves as the inhibitory effect of the treatment wore off over the 14 day incubation (Figure 12A,C,E). There was no significant change in perforation number among plants treated with methyl jasmonate (Figure 12F; Appendix 5).

3.5 Spectrophotometry Assays

For the anthocyanin assay, a standard curve was established using cyanidin-3-rutinoside (Appendix 6) to express results as mg cy3Re/g. Anthocyanin concentration was significantly lower in window stage leaves in both 2 mM vanillin ($P = 0.0002$) and 100 μM phenidone ($P = 0.0003$) treated plants compared to control (Figure 13A; Appendix 6). Anthocyanin concentration was significantly higher in plants treated with 2.5 μM MeJA in both window ($P = 0.0098$) and mature ($P < 0.0001$) stage leaves (Figure 13A; Appendix 6). In the total phenolic content assay, the only change in endogenous phenolics was observed in the vanillin treatment, which was lower in window stage leaves in relation to the control ($P = 0.0011$; Figure 13B; Appendix 6). TPC is expressed as catechin equivalents (CE) generated by standard curve (Appendix 6). In the DPPH

radical scavenging assay, effects on radical scavenging capacity were observed in all treatment groups (Figure 13). Vanillin treated plants had a lower radical scavenging capacity in both window ($P = 0.031$) and mature ($P = 0.002$) leaves (Appendix 6). Phenidone also reduced radical scavenging capacity in window stage leaves ($P = 0.0403$) but did not affect radical scavenging capacity of mature leaves ($p = 0.5956$). Treatment with MeJA significantly increased radical scavenging capacity in mature stage leaves ($P = 0.0195$), but did not have any effect on window stage leaves in relation to the control ($P = 1.000$). After conducting comparative analyses of non-phenolic pigments (chlorophyll A, chlorophyll B, and carotenoids) from leaf extracts of treated lace plants, it was observed that both vanillin and phenidone-treated plants had higher chlorophyll A ($P = 0.0427, 0.0008$, respectively) and carotenoid content ($P = 0.0123, 0.0016$, respectively). Chlorophyll B content was unaffected by either treatment, and MeJA had no significant effect on any of these pigments (Figure 13; Appendix 6).

3.6 Triple Quadrupole Mass Spectrometry

The most abundant mass/charge (*m/z*) species from the precursor-ion analyses using cyanidin-3-rutinoside were collected from MS spectra and compiled for relative abundance between tissue types (Figure 14). The most abundant masses were 433 *m/z*, 449 *m/z*, 595 *m/z*, 611 *m/z*, 623 *m/z*, 727 *m/z*, 741 *m/z*, 757 *m/z*, 773 *m/z*. The *m/z* of 595 was substantially higher than all other *m/z* detected, and was used as the relative abundance maximum on the y-axis of Figure 14. The second-most abundant *m/z* (611) fell between 5-30% of 595, depending on tissue type. The masses of particular interest were those that showed a substantial decrease in abundance throughout leaf development

(highest during PCD phases), and thus *m/z* 595, 611, and 773 were selected for product-ion and common-neutral-loss analyses.

Upon product-ion analyses, the precursor aglycone delphinidin (MW 303) was discovered in all three masses of interest. Relative abundances of cyanidin (287) and delphinidin (303) precursors were plotted, revealing a decrease in delphinidin species throughout lace plant leaf development (Figure 15).

Common-neutral-loss analyses were conducted with three common sugar moieties: glucose (MW 162), rhamnose (MW 146), and xylose (MW 132) to identify neutral molecule precursors in select masses for identification. Of the cyanidin or delphinidin-containing compounds, cyanidin-3-rutinoside, cyanidin-3,5-diglucoside, cyanidin-3-sophoroside-5-glucoside, delphinidin-3-rutinoside, and delphinidin-3-rutinoside-5-glucoside IDs were proposed (Figure 15).

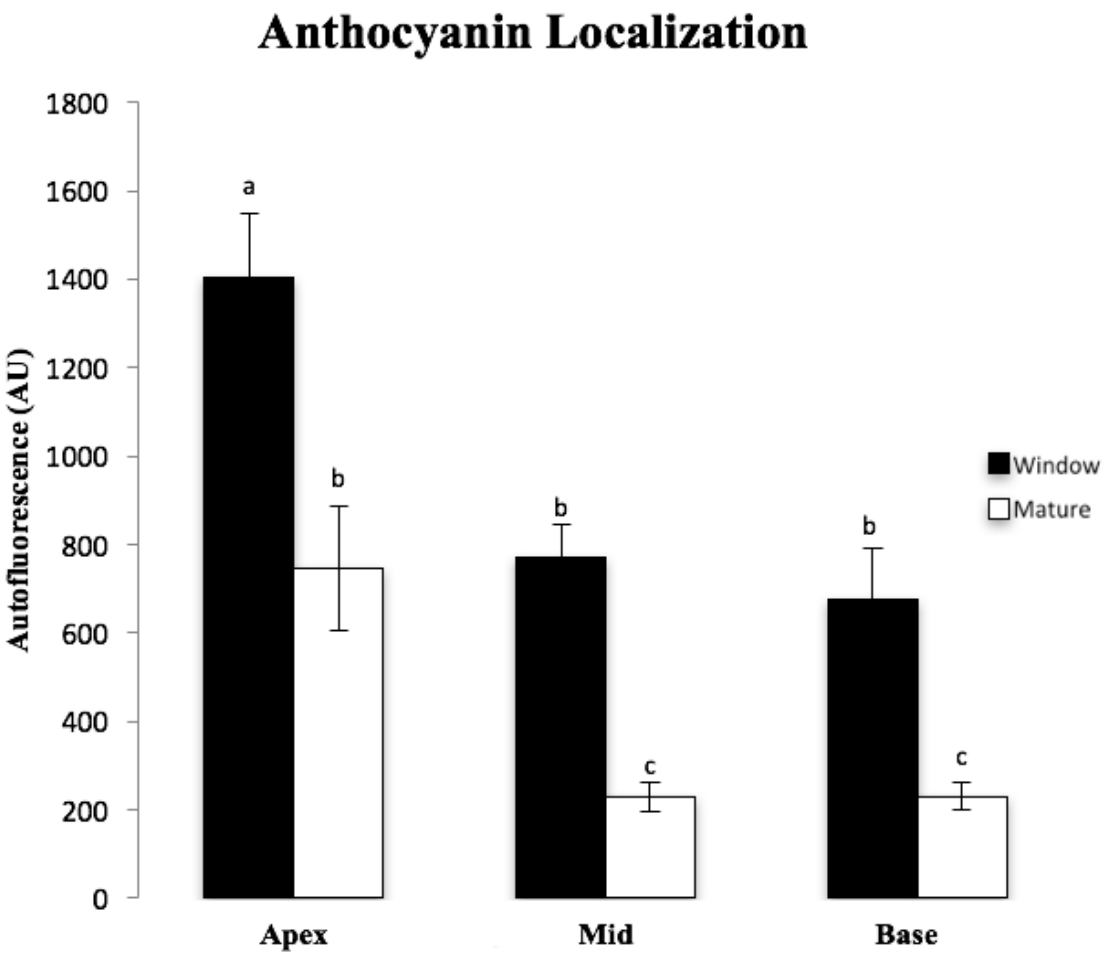

Figure 7. Localization of anthocyanin in leaf laminas. Anthocyanin autofluorescence quantified by confocal microscopy using NIS-Elements software. Apex, middle, and basal laminar tissues were sampled from both window and mature stage leaves. Autofluorescence values are expressed as arbitrary units (AU). Data are expressed as mean ± standard error. (n = 14). Data represented by different letters are significantly different ($P \leq 0.05$). Tukey test was conducted at 95% confidence to compare all groups.

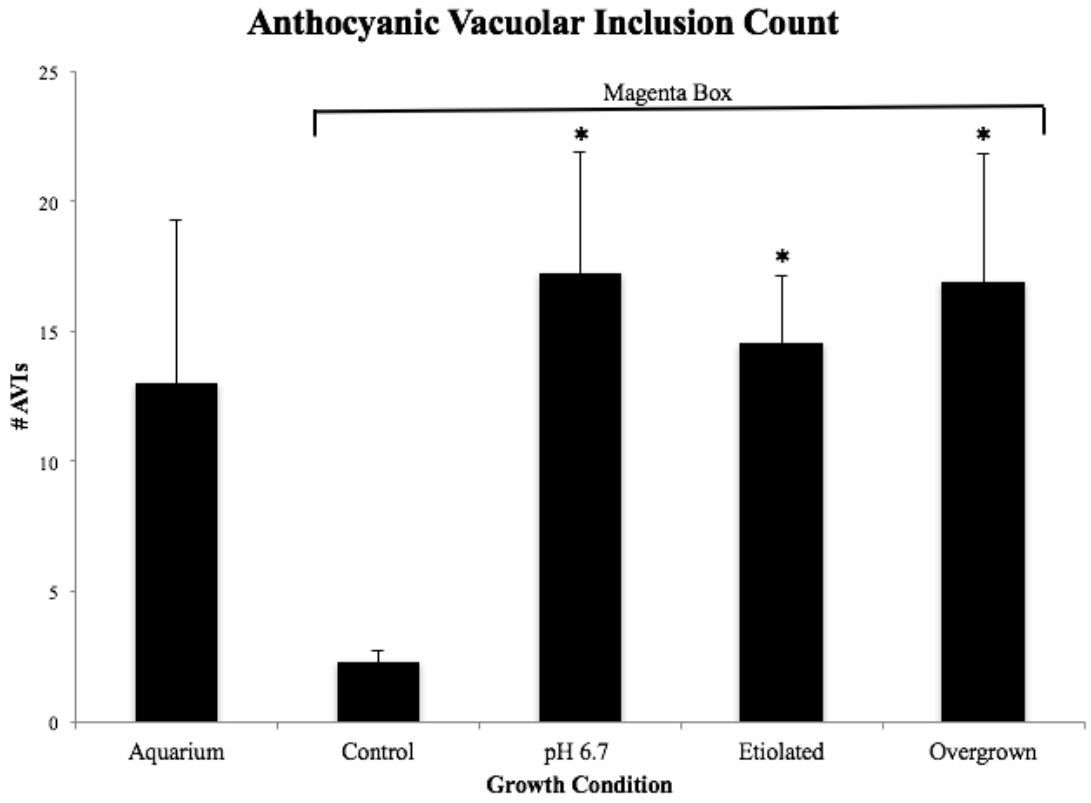

Figure 8. Anthocyanin Vacuolar Inclusion (AVI) quantification in window stage leaf apices under various growth conditions. Using light microscopy and NIS-elements software, AVIs present in lace plant window stage leaf apices were counted. Growth conditions marked with * are significantly different from control (standard culture growth conditions). The three stress-related growth conditions (pH 6.7, etiolated, and overgrown) all resulted in higher AVI counts. Data are expressed as mean ± standard error. ($n \geq 7$). Data represented with an asterisk are significantly different from control ($P \leq 0.05$). Dunnett test at 95% confidence was conducted to compare individual means to control.

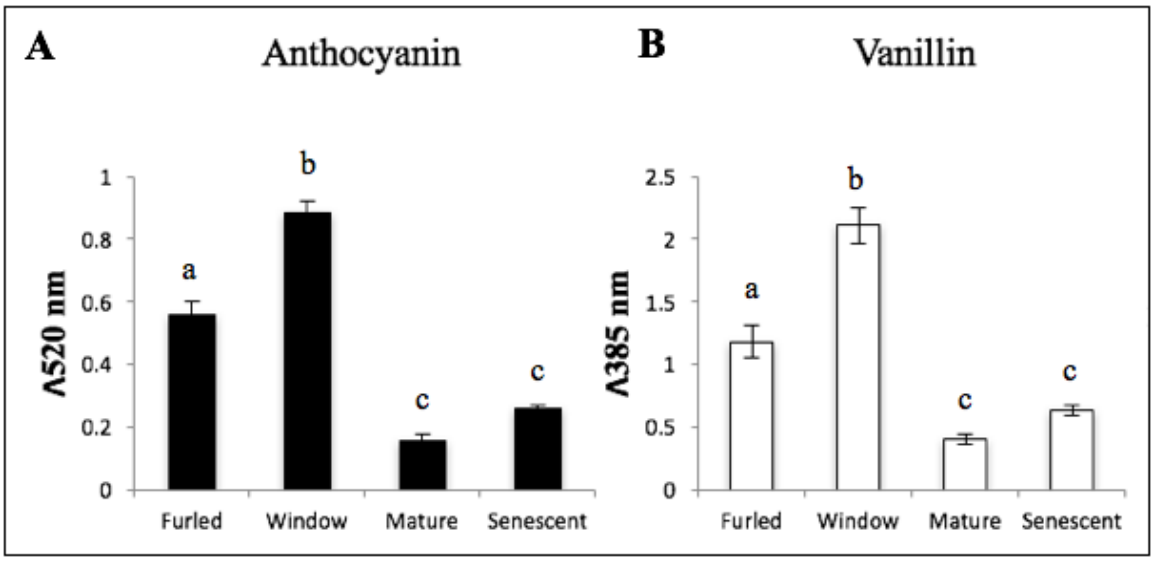

Figure 9. Anthocyanin and vanillin content throughout lace plant leaf development. Comparative absorbances for (A) anthocyanin (520 nm) and (B) vanillin (385 nm) were taken from leaves at different stages of development. Data are expressed as mean ± standard error. ($n \geq 6$). Data represented with different letters are significantly different from each other ($P \leq 0.05$). Tukey test was conducted at 95% confidence to compare all groups.

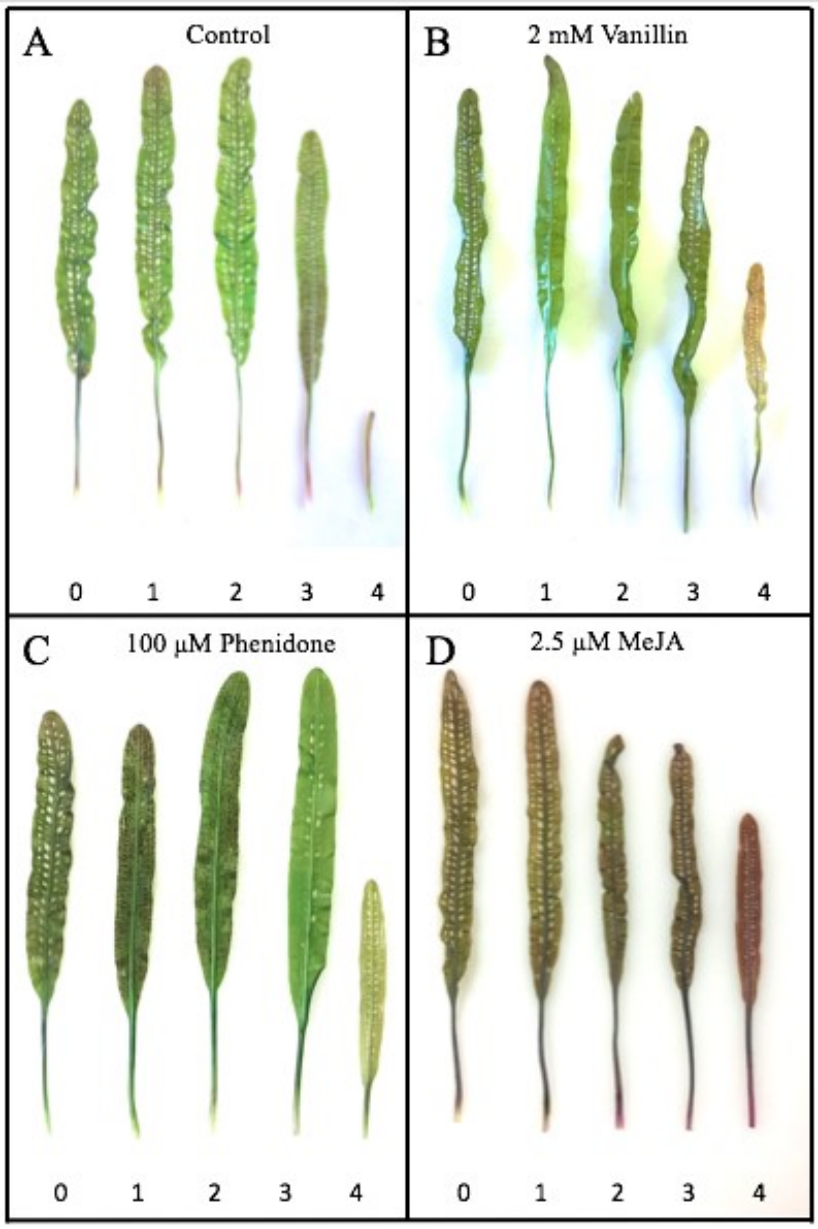

Figure 10. Sample leaves from optimal treatment concentrations. Images of leaves showing the effect of each treatment on leaf morphology. Leaf 0 for each grouping acts as internal control because it had already reached window stage of development upon treatment. Leaves 1-4 developed during the incubation period of the treatment. Normal perforation development in (A) control, compared to perforation inhibition and recovery is observed in (B) vanillin and (C) phenidone treated plants. (D) MeJA did not affect perforation formation, but stunted the growth of leaves and caused a strong red pigmentation to occur.

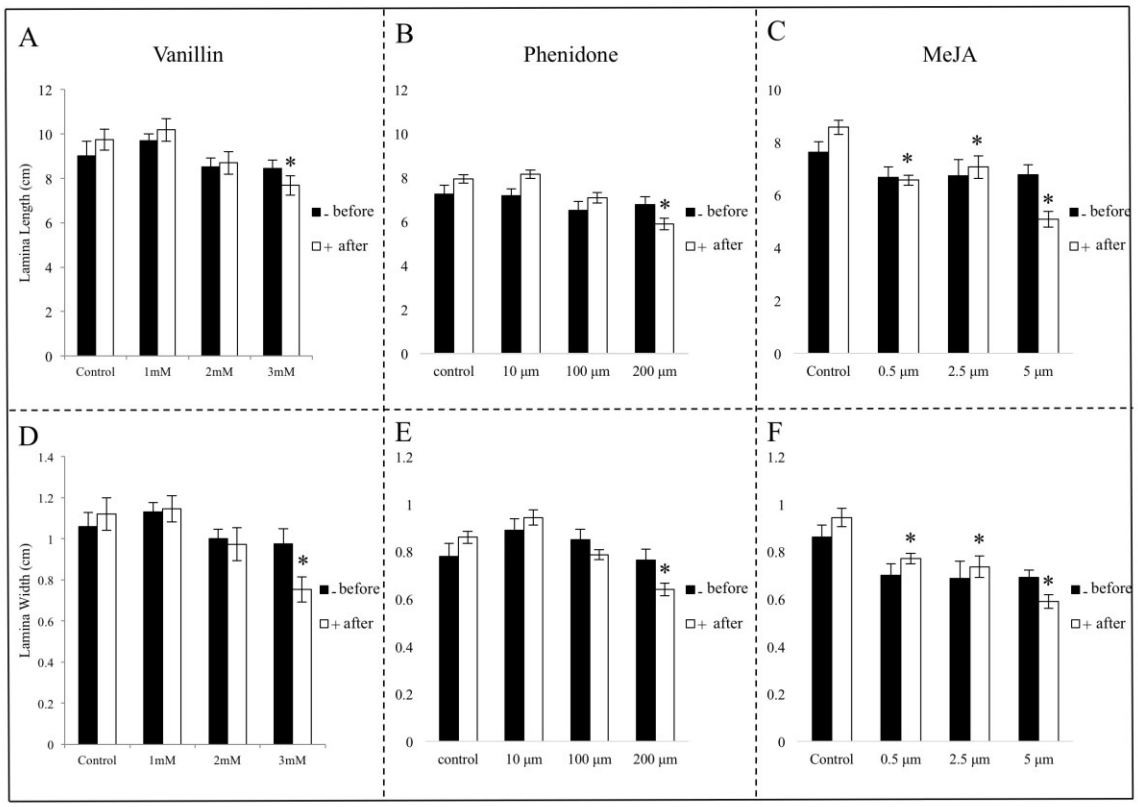

Figure 11. Effect of Vanillin, Phenidone and Methyl Jasmonate on the growth of the lamina of lace plants. Leaves of treated plants were harvested and measured for length and width. Both leaves produced before (-) and after (+) incubation in treatments are presented. Vanillin and phenidone treatment only had an effect on lamina width and length at the highest concentration used (A and B, D and E). MeJA treated plants were smaller in length and width at all concentrations compared to controls. Data are expressed as mean ± standard error. ($n \geq 9$). Data represented with an asterisk are significantly different from control ($P \leq 0.05$). Dunnett test at 95% confidence was conducted to compare individual means to control.

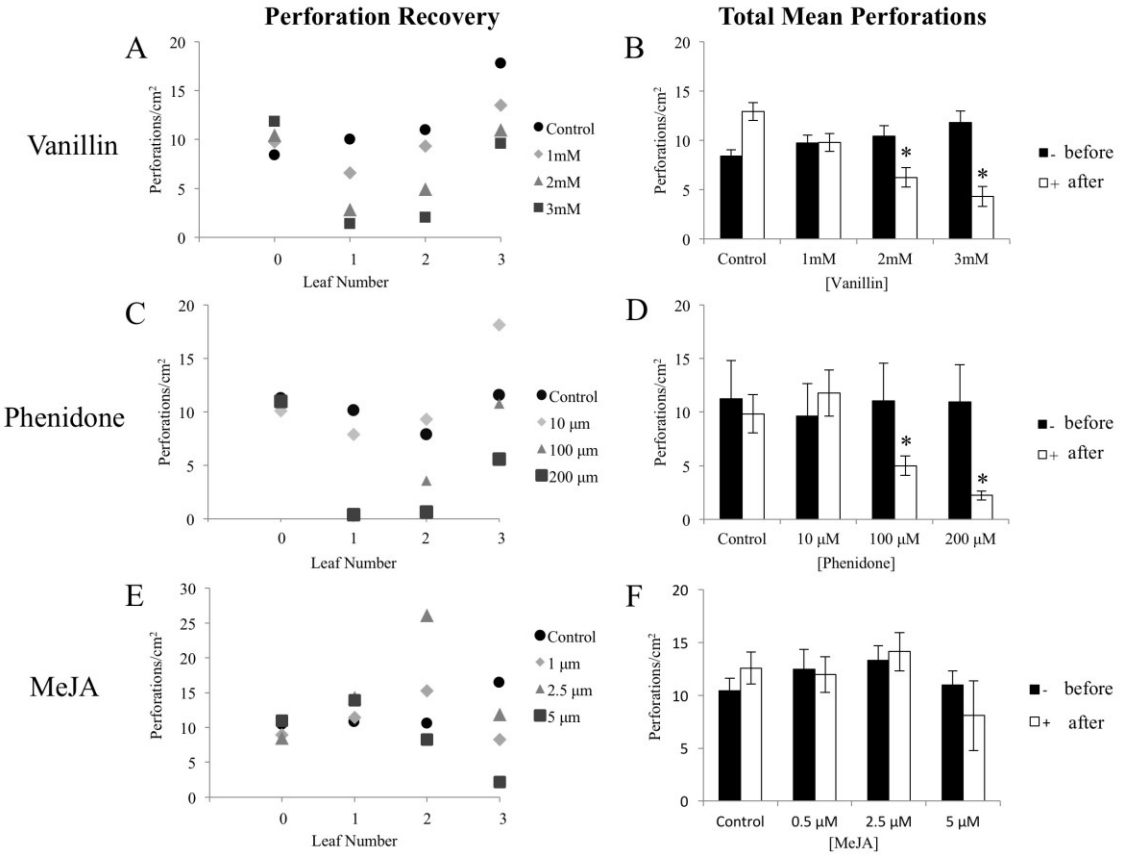

Figure 12. Effect of Vanillin, Phenidone and Methyl Jasmonate on lace plant perforation development. Treated plants were harvested and quantified for effect on perforation formation. Both leaves produced before (-) and after (+) incubation in treatments are presented. Individual leaf perforation recovery (A,C,E) and combined means pre- and post-treatment (B,D,F) are presented. Leaf 0 represents the youngest perforating leaf upon chemical application. Leaves 1-3 are produced during incubation in the treatment. The formation of perforations was inhibited by vanillin (>2 mM) and phenidone (>100 uM), Perforation formation was unaffected by MeJA treatment. Data are expressed as mean ± standard error. ($n \geq 9$). Data represented with an asterisk are significantly different from control ($P \leq 0.05$). Dunnett test at 95% confidence was conducted to compare individual means to control.

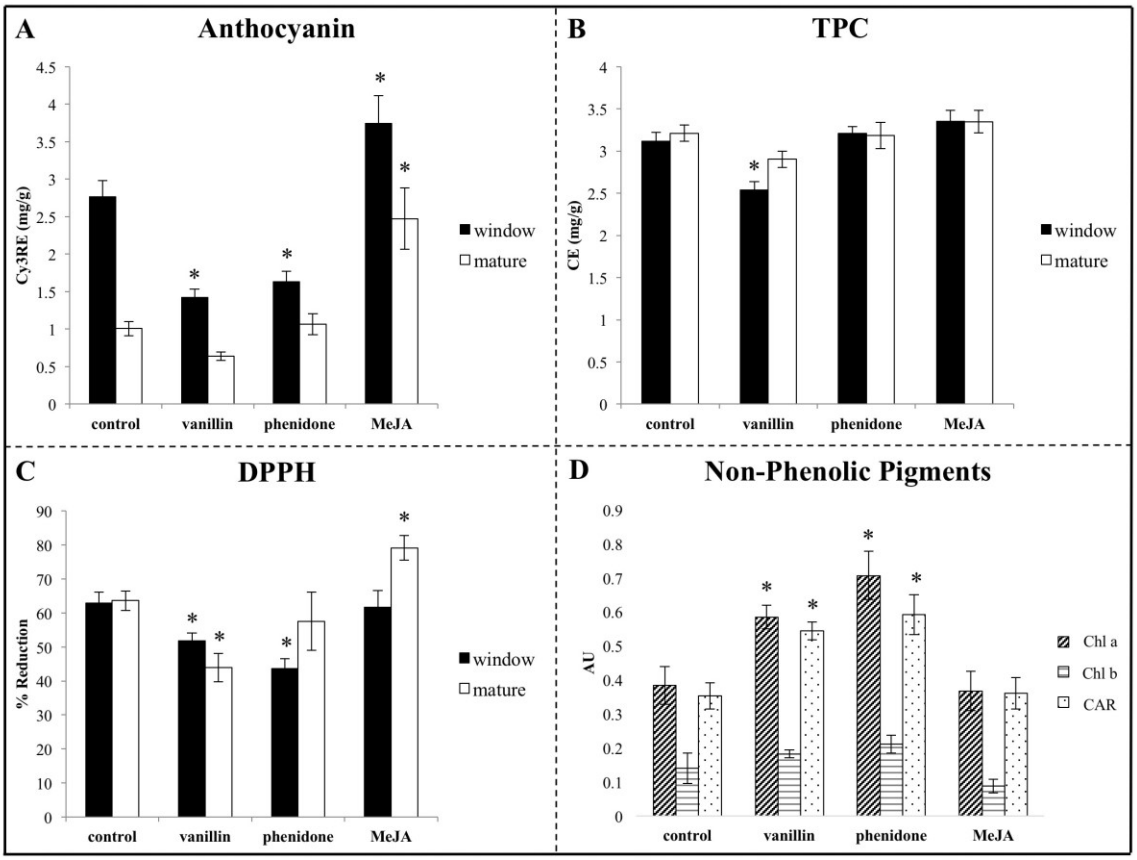

Figure 13. Spectrophotometric assays of lace plant leaf extracts. (A) Anthocyanin content, (B) total phenolic content (TPC), (C) free radical scavenging capacity (DPPH), and (D) non-phenolic pigments including chlorophyll A (664 nm), chlorophyll B (648 nm), and carotenoid contents (470 nm) were spectrophotometrically determined from prepared extracts from lace plant leaves treated with 2 mM vanillin, 100 μM phenidone, and 2.5 μM MeJA. Anthocyanin content is expressed as cyanidin-3-rutinoside equivalents (mg/g), TPC as catechin equivalents (mg/g), DPPH as percent reduction of radical, and non-phenolic pigments expressed as absorbance units (AU). Data are expressed as mean ± standard error. ($n \geq 9$). Data represented with an asterisk are significantly different from corresponding control ($P \leq 0.05$). Dunnett test at 95% confidence was conducted to compare individual means to control.

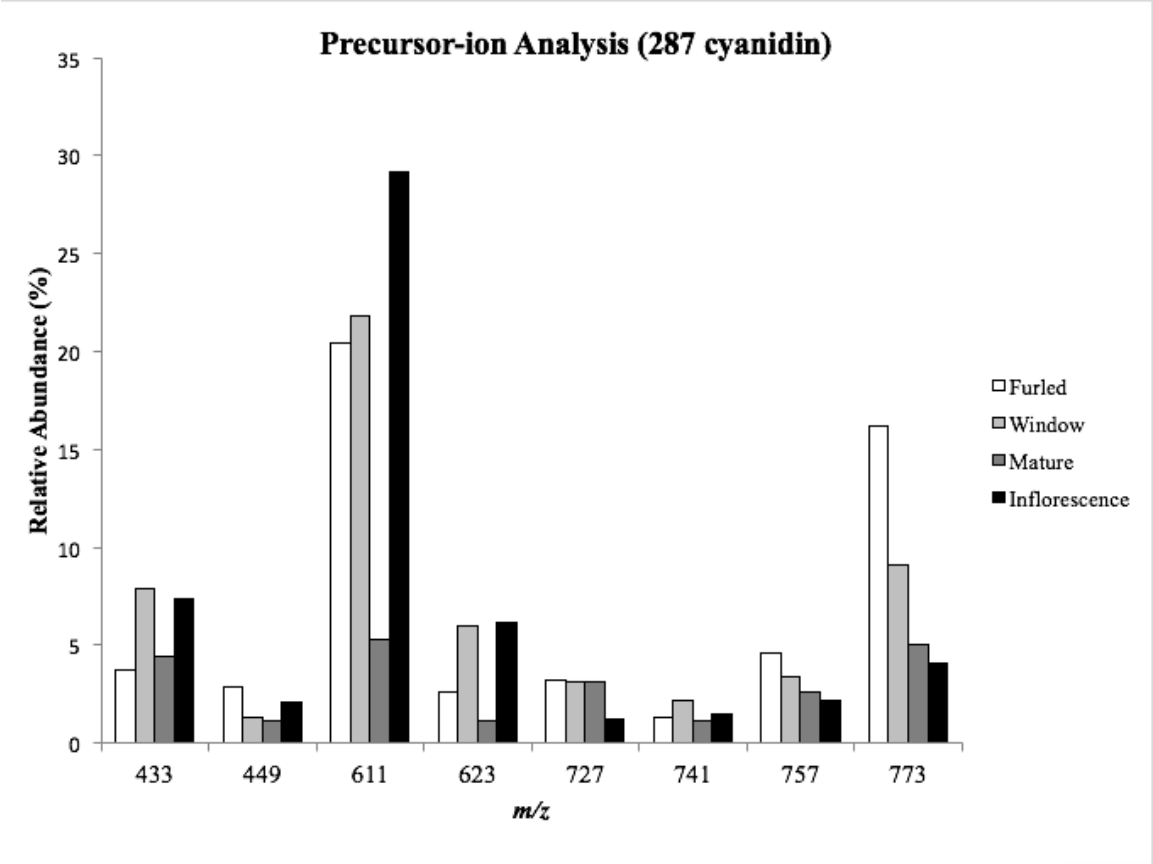

Figure 14. Relative abundance of product ions with cyanidin precursor. The most abundant anthocyanin products containing cyanidin (MW 287) in extracts from furled, window, and mature leaves, as well as inflorescence tissue. All masses are compared to the 595 *m/z* maxima (y-axis). The most abundant *m/z*(595, 611, 773) were selected based on these results for additional investigation in product-ion and common-neutral-loss analyses.

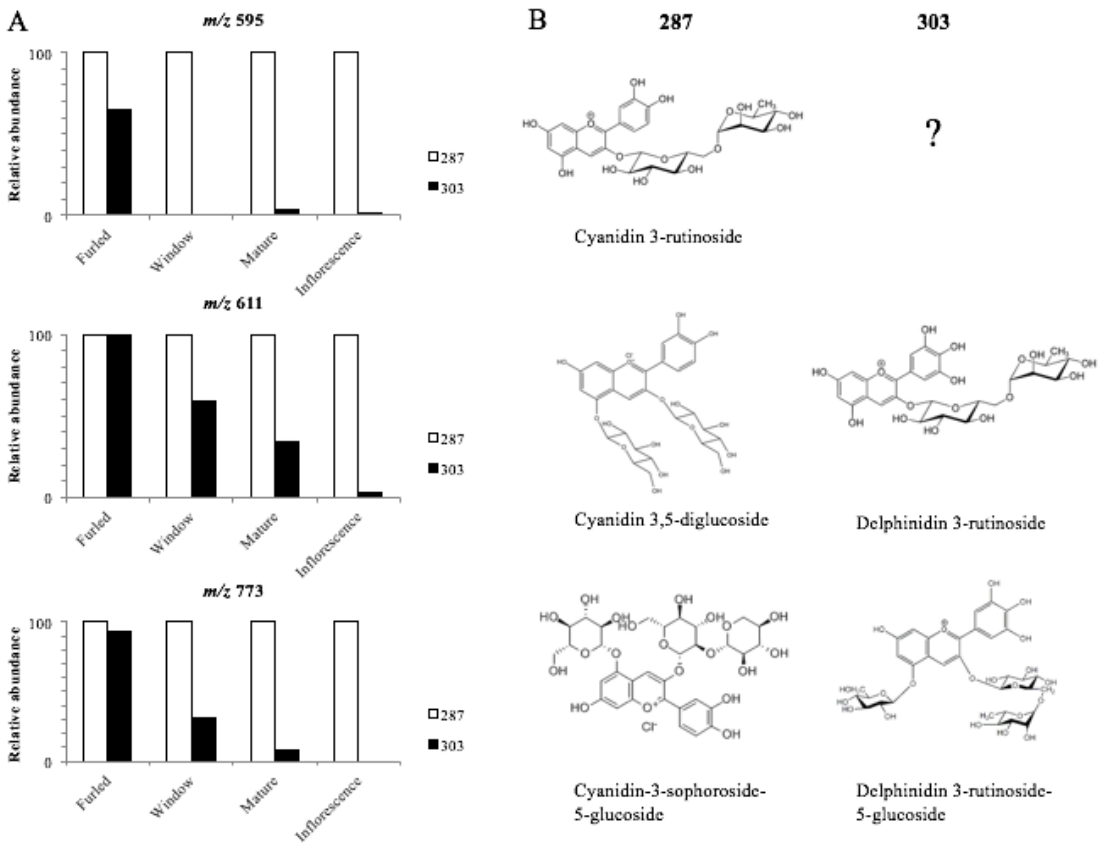

Figure 15. Relative abundances of cyanidin and delphinidin anthocyanins and their proposed IDs. Relative abundances of cyanidin and delphinidin-based anthocyanin species for (A) the three most represented masses (left column) in the lace plant. Proposed ID's for cyanidin (287) and delphinidin (303) species of select masses are presented (B).

Table 3. Proposed IDs of anthocyanins in the lace plant as determined by triple quadrupole mass spectrometry. Proposed IDs of anthocyanins and closely related species obtained from mass spectra. Mass/charge, precursor ions, and neutral loss data are paired with proposed IDs. Results were compared with flavonoid database to infer IDs (Buckingham and Munasinghe 2015).

m/z	Precursor Ion	Neutral Loss	Proposed ID
433	287	146	kaempferol rhamnoside
449	287	162	cyanidin 3-glucoside
579	287	-	procyanidin
595	287	146	cyanidin 3-rutinoside
595	303	-	delphinidin sp. (?)
611	287	162	cyanidin 3,5-diglucoside
611	303	146	delphinidin 3-rutinoside
727	287	132, 162	cyanidin 3-xylosylrutinoside
727	303	-	delphinidin sp. (?)
757	287	146, 162	cyanidin 3-rutinoside-5-glucoside
757	303	-	delphinidin sp. (?)
773	287	162	cyanidin 3-sophoroside-5-glucoside
773	301	-	peonidin sp. (?)
773	303	146, 162	delphinidin 3-rutinoside-5-glucoside

CHAPTER 4

DISCUSSION

4.1 Lace plant as a model for programmed cell death research

The formation of perforations during leaf development is an extremely rare occurrence in nature, and has only been documented in two members of Araceae, and a single species in Aponogetonaceae (Gunawardena et al. 2004; Gunawardena et al. 2005). The lace plant of Aponogetonaceae is a very effective model for the field of plant PCD research, due to the predictability of the formation of perforations throughout its leaf lamina. Additionally, the transparency of its leaves provides a very suitable system to observe cellular events of PCD via microscopy (Gunawardena et al. 2004). Many of the cellular events that occur during lace plant PCD have been documented, including the disappearance of anthocyanin pigmentation, the increased activity of transvacuolar strands, perinuclear localization of chloroplasts, loss of chlorophyll pigmentation, formation of vacuolar aggregates, and retraction and collapse of the tonoplast (Wertman et al. 2012). Previous molecular investigations have revealed the following: (1) PCD and actin degradation are prevented by treatment with a caspase-1 inhibitor (Ac-YVAD-CMK; Lord et al. 2013); (2) treatment with an ethylene biosynthesis inhibitor (aminoethoxyvinylglycine) reduces the formation of perforations; and (3) ethylene receptors (AmERS1a and AmERS1c) are regulators of ethylene-induced PCD in the lace plant (Rantong et al. 2015). In animal PCD, our knowledge of apoptotic mechanisms is very broad and comprehensive in relation to plant PCD. The role of caspases, as well as other death-related protein families as key executers of cell death are well understood

(Kumar 2007; Zhou and Doctor 2003). In plants, there are a number of "caspase-like" proteins such as saspases, metacaspases, phytaspases, and vacuolar processing enzymes which are known to catalyze very similar cell death mechanisms when compared to their animal counterparts (Bosch and Franklin-Tong 2007). Gaining a better understanding of the role of these enzymes in lace plant PCD can reveal potentially conserved mechanisms in cell death between plants and animals.

Although the active phases of lace plant leaf PCD mechanisms have been documented, the induction phase remains to be elucidated. RNA sequencing of the lace plant would contribute substantially to our understanding of this early induction, as genes that are highly expressed during the early phases of PCD could be identified and targeted for future experimentation. Additionally, establishing an effective protocol for lace plant transformation will also aid tremendously in the investigation of additional molecular hallmarks of developmental PCD. Futhermore, the presence of anthocyanins throughout the lace plant PCD process invites additional investigation into the roles of phenolic compounds in plant PCD.

4.2 A new role for anthocyanins?

Anthocyanins represent the most widely documented family of secondary metabolites produced in plants in terms of their involvement in responses to stressors such as UV radiation (Steyn et al. 2002). These compounds are also often visible in deciduous leaves prior to seasonal senescence (Hoch et al. 2001). A much less frequent occurrence is the presence of anthocyanins in young developing leaves. Tropical woody tree species such as *Pometia pinnata* J. R. Forst and G. Forst (Sapindaceae)*, Mesua*

ferrea Linn. (Cluciaseae), *Grewia tomentosa* Juss. (Malvaceae), *Ficus viridicarpa* Bl. (Moraceae), *Quercus coccifera* L. (Fagaceae) and *Atta columbica* Guérin-Méneville (Formicidae) contain high levels of anthocyanins in their young developing leaves (Lee and Lowry 1980; Karageorgou and Manetas 2006; Coley and Aide 1989). Proposed functions for anthocyanins in these species include protection against herbivory, fungal infections, and UV radiation damage. However, there are certain cases where the presence of anthocyanin in juvenile leaves remains unknown. For example, in the juvenile leaves of *Syzygium spp.* R.Br. (Myrtaceae) it was shown that anthocyanins were not contributing to protection against UV-A or UV-B radiation, though their purpose is not yet understood (Woodall and Stewart 1998). One of the noteworthy aspects of anthocyanin research in plants, is that it is predominantly conducted on terrestrial species, which typically produce anthocyanins exclusively in their epidermal cells, although there are some known exceptions (Kytridis et al. 2006). For example, in species which produce anthocyanins in the mesophyll, there is a stronger resistance to oxidative damage (Kytridis et al. 2006). The lace plant, being an aquatic species, likely does not acquire its anthocyanins for the purpose of UV protection. Additionally, lace plant anthocyanins are produced only in mesophyll cell layers and not in the epidermis (Lord and Gunawardena 2010).

An intriguing process during lace plant PCD is the early disappearance of visible anthocyanin pigmentation in cells destined for death, and the retention of the pigmentation in cell layers that will resist PCD (Gunawardena et al. 2004). This study investigated anthocyanins during the PCD process, and identified the most abundant anthocyanin species throughout leaf development. Spectrophotometric analysis of

anthocyanins present throughout lace plant leaf development indicated that anthocyanin levels were highest in younger leaves (furled and window stages) compared to leaves in the mature or senescent leaves (Figure 9). Furthermore, this indicates a strong relationship between the presence of anthocyanins and the manifestation of PCD, as chemical treatments that inhibited perforations (i.e. vanillin and phenidone) also reduced anthocyanin content in leaves (Figure 12; Figure 13A). It is also evident that lace plant anthocyanins have a strong antioxidant capacity, which was observed when comparing the anthocyanin levels to the DPPH radical scavenging assay (Figure 13A,C). Where PCD inhibitor treatments reduced anthocyanin content in leaves, their radical scavenging capacity also significantly decreased. MeJA treatments also yielded a positive correlation between anthocyanin content and DPPH scavenging capacity (Figure 13A,C). These results support the powerful antioxidant capacity of anthocyanin-rich cells in the mesophyll (Kytridis et al. 2006), and also points to an important relationship between anthocyanins and ROS during normal lace plant leaf development.

There are hundreds of identified anthocyanin species in plants (Buckingham and Munasinghe 2015). In order to gain a better understanding of the role of anthocyanins in lace plant PCD, it is important to determine the profile of anthocyanin species that are produced by the lace plant, specifically during the early stages prior to and during PCD events. With TQ-MS we were able to compile the first profile of the most abundant anthocyanin species in the lace plant at various stages of development. This profile was dominated by cyanidin and delphinidin-derived anthocyanin species at all stages of leaf development, as well as the inflorescence (Figure 15). Interestingly, the delphinidin-derived species were shown to dramatically decrease in abundance compared to the

cyanidin derivatives at progressively later stages of leaf development (Figure 15), indicating that these delphinidin derivatives may be contributing an important function during PCD. Of the compounds revealed in this work, based on precursor-ion, product-ion, and common-neutral-loss analyses, there are some species that warrant further elucidation specifically delphinidins. A deeper investigation into these unknown compounds through NMR analysis may unveil novel anthocyanin species that could lead to future research in animal systems for potential pharmacological application.

Membrane-less bodies termed AVIs are known to form in plant tissues when grown in a variety of stressors, and are known to concentrate 3,5-diglucosides and acylated anthocyanins within the vacuole, but segregated from the harsh vacuolar solution (Markham et al. 2000). In the lace plant, we found that AVI production significantly increases in the following conditions compared to control: a) high pH (6.7), b) light deprivation (etiolated), and c) overgrown (crowded) window stage leaves (Figure 8). The number of AVIs in aquarium-grown plants increased from control, but not significantly. Additional replicates are needed to determine potential significance here. Furthermore, the MS analyses revealed a high abundance of cyanidin 3,5-diglucoside throughout the lifespan of the lace plant leaf. This particular cyanidin species is known for its endogenous role in protecting plants against oxidative stress (Youdim et al. 2000). Cyanidin 3,5-diglucoside may be a primary compound sequestered by AVIs in the lace plant, and heavily produced in response to environmental stresses. However, the conspicuous decrease in delphinidin species throughout lace plant leaf development when compared to the cyanidin derivatives, make delphinidins an intriguing target for future investigation in plant PCD research.

4.3 Delphinidins

Based on our results from TQ-MS abundance analyses of anthocyanin species, delphinidin-derived anthocyanin species seem to be the most relevant anthocyanins in lace plant PCD. A number of delphinidin species have been used in a variety of pharmacological research programs including cancer and neurological studies. Typically anthocyanins have attracted attention of researchers because of their antioxidant capacities and ability to prevent cell death by removing harmful ROS from the cell (Zafra-Stone et al. 2007). However, certain species have been shown to display pro-apoptotic effects (Katsube et al. 2003; Lazzè et al. 2004). A common delphinidin derivative, delphinidin 3-sambubioside, has been shown to induce apoptosis in human leukemia cells through the ROS-mediated mitochondrial pathway (Hou et al. 2005). Interestingly, delphinidin itself has been shown to induce apoptosis in human prostate cancer cells, both in vitro and in vivo through activation of caspases (Hafeez et al. 2008). Furthermore, a study that tested the pro-apoptotic effects of a variety of anthocyanins determined that delphinidin was the most potent compound involved in apoptosis of promyelocytic leukemia cells, through caspase-3 activation and triggering cells to generate intracellular hydrogen peroxide, a potent ROS (Hou et al. 2003). It is possible that the abundance of delphinidin species in leaf stages prior to and during developmental PCD may be directly involved as an activator in the signaling pathway in relation to caspase-like enzymes during the induction and/or execution phases of PCD.

Recent studies have shown that animal caspase inhibitors have the potential to inhibit developmental PCD in the lace plant, suggesting a strong similarity in enzymatic activity (Lord et al. 2013). These findings could be investigated through additional

studies detailing the effects of lace plant delphinidins in an animal system. Moreover, delphinidins have shown additional pharmacological value, including protection from ischemia-induced heart injury (Scarabelli et al. 2009), antiangiogenesis models (Favot et al. 2003), and neurotoxicity prevention (Hyo-Shin et al. 2009) through antioxidant-related activity.

Of the delphinidin species we profiled in this work (Table 3), there are some notable results from previous research groups. One of the primary challenges in anthocyanin research for medicine and agriculture is the low bioavailability of the compounds due to their low stability (Manach et al. 2005; McGhie and Walton 2007). Delphindin-3-rutinoside, which was identified in the lace plant, is an exception to this, and has been shown to remain intact through the bloodstream in both rat and human systems (Matsumoto et al. 2001; Matsumoto et al. 2006). Additionally, this compound has been shown to relax the bovine ciliary smooth muscle by stimulating the production of nitric oxide, inhibiting muscle contraction (Matsumoto et al. 2005). Little pharmacological work has been done on the delphinidin species identified in the lace plant, but based on the strong evidence for pro-apoptotic properties of delphinidin, these compounds are intriguing candidates for future investigation.

4.4 Vanillin as an inhibitor of plant PCD

Vanillin has been a popular subject for pharmacological investigation due to its economic importance and its relevance in the human diet. In apoptosis research, vanillin has been investigated as a pro-apoptotic agent against a variety of cancer cell types. In human colorectal cancer cell line HT-29, apoptosis was induced by G0/G1 cell cycle

arrest (Ho et al. 2009). In HeLa cells, vanillin caused TRAIL-induced apoptosis by inhibiting activation of NF-κB; a protein complex that controls DNA transcription and cell survival (Lirdprapamongkol et al. 2010). Vanillin has also been shown to have antiapoptotic effects in other animal-based experimentation. This phenolic aldehyde caused a significant neuroprotective effect in the hippocampus by blocking oxidative damage in gerbils subjected to transient ischemia (Kim et al. 2007).

In the lace plant model, endogenous vanillin was first assessed by using a spectrophotometric assay. Results indicated that, similar to anthocyanins, vanillin content was significantly higher in young leaves during the process of PCD, compared to the older mature and senescent leaves (Figure 9). Given the level of specificity of this spectrophotometric assay, there may have been structurally similar compounds influencing the result. However, this result suggests that there may be an important function for vanillin in the early stages of leaf development during the PCD process. The next phase of this research was to apply exogenous vanillin to lace plants in order to observe the effect of vanillin on perforation formation. Vanillin suppressed production of anthocyanins and total phenolics, significantly reduced DPPH radical scavenging capacity, and led to an increase in chlorophyll A and carotenoid content (Figure 13). These results suggest that the application of exogenous vanillin is causing a number biosynthetic alterations within the plant leading to PCD inhibition. The resulting high abundance of vanillin in lace plant tissues in stages during and prior to PCD indicate vanillin may have an important anti-apoptotic function within the cells during development. Additionally, it seems that the lace plant substitutes chlorophyll A and carotenoid pigments in response to a reduction of phenolic pigments. This could be an

adaptive mechanism for the plant to attempt to cope with a loss in photoprotection. Interestingly, even though vanillin and phenidone showed similar experimental results in perforation quantification and spectrophotometry experiments, vanillin was the only compound to cause a significant change in the total phenolic content of the lace plant (Figure 9B). This result suggests that vanillin is affecting an additional PCD mechanism within the MeJA pathway. However, it is likely that both vanillin and phenidone are inhibiting the MeJA pathway, because a similar decrease in anthocyanin production was observed. MeJA is introduced into cells by lipoxygenase activity on membrane-derived fatty acids.

Vanillin and phenidone are both well documented inhibitors of LOX (Laughton et al. 1991; Stern et al. 1993), which may explain the observed results in the suppressed phenolic production, and inhibition of perforations in the lace plant system. LOXs are a family of iron dioxygenases which catalyze the conjugation of polyunsaturated fatty acids into hydroperoxides, and form oxylipins such as the jasmonates. These organic conjugates include both lipid (LOOH) and cholesterol (ChOOH) hydroperoxides, and act as signaling intermediates during lipid peroxidation: a degenerative process affecting cell membranes and other lipid-containing bodies experiencing oxidative stress (Girotti 1998). LOXs are ubiquitous among plants and animals and have important roles in cell signaling and metabolism. Some well-known roles for plant LOXs include activation of early events in germination, defense response to abiotic stressors, and in the initiation of leaf senescence (Lynch and Thompson 1984). LOXs can be subdivided into 5-, 12- or 15-lipoxygenases based on the addition of a hydroperoxy group at the corresponding carbons. Arachidonic, linoleic, and linolenic acids are known substrates for LOXs, due to

the selective oxidation that only targets fatty acids containing a 1-cis,4-cis pantadiene system. Oxygen can be added to either end of this structure, and in the case of linoleic and linolenic acids, leads to two possible products: 9- and 13-hydroperoxy fatty acids. These products then lead to a series of biosynthetic pathways responsible for producing a variety of signaling molecules and green leaf volatiles (Royo et al. 1996). LOX activity is known to induce a cascade of defense mechanisms in plants, from the introduction of reactive oxygen species into cells via lipid degradation, and the generation of oxylipins such as jasmonates (Wasternack 2007). More specifically, the action of 13-LOX (the 13-hydroperoxy fatty acid pathway) on linolenic acid allows for the production of 12-oxo-phytodienoic acid (12-OPDA) and jasmonates as downstream products. The 9-LOX (9-hydroperoxy fatty acid pathway) produces oxylipin 10-oxo-11,15-phytodienoic acid (10-OPDA) and 10-oxo-11-phytoenoic acid (10-OPEA), which are structurally similar to jasmonates and have similar physiological roles (Christensen et al. 2015). To date, it is generally understood that the 13-LOX pathway is involved in stress response and survival, while the 9-LOX pathway is more closely linked with cell death (Montillet et al. 2005; Christensen et al. 2015). The involvement of LOX in plant PCD systems remains widely unknown, however, current studies are investigating LOX in plant PCD to provide useful insight into the importance of lipid signaling in these processes. Plants have the potential to provide an insightful basis for LOX research, due to the presence of both cytosolic and chloroplastic LOX (Bell et al. 1995). Additional investigation into the abundance and activity of LOX or LOX related genes throughout lace plant leaf development could shed important light on some of the initial signals involved in the

PCD cascade.

4.5 Reactive oxygen species and phenolics

Although we did not directly quantify amounts of endogenous ROS in this study, it is still clear through our experimental results that the balance between ROS and phenolics is critical for the normal development of lace plant leaves. Upon inhibiting the formation of perforations, we observed a significant decrease in anthocyanin content, which was matched with a decrease in radical scavenging capacity as per our DPPH assay results (Figure 13). ROS are known to promote apoptosis in both plant and animal systems (Van Breusegem and Dat 2006; Simon et al. 2000). Pairing that with the well-elucidated antioxidant properties of phenolic compounds provides evidence that suggests there is a balance between ROS and phenolics occurring during the formation of perforations in the lace plant. ROS are not only apoptosis-inducing compounds, but are also involved in many cell signaling pathways (Thannickal et al. 2000). Although our results suggest that there is an important relationship between ROS and phenolics in the lace plant during development, the exact signaling mechanisms that are affected by these antagonists remain to be elucidated.

4.6 Proposed model for role of phenolics in the formation of perforations in the lace plant

Many unknown mechanisms remain in the signaling cascade of PCD in lace plant leaf development. In regards to the role of phenolics, this study has shown that MeJA treatment leads to an increase in anthocyanin content. Additionally, higher phenolic content in leaves results in a higher free radical scavenging capacity (Figure 13). The suggested model (Figure 16) shows that upstream of the jasmonates, LOX may be very

important players in this cascade. Although the initial signal is unknown, however, in other species it is understood that LOX activity induces ROS (Lopez et al. 2011; Sun and Chen 2011). 13-LOX is responsible for inducing the production of jasmonates in plant cells, while 9-LOX produce "death acids", which are structurally very similar to jasmonates, but are primarily involved in cell death activity, rather than in response to stress (Christensen et al. 2015). In my proposed model, the 13-LOX derived jasmonates induce the accumulation of anthocyanins in the leaf cells, as supported by our experimental observations in a greater anthocyanin accumulation upon treatment with MeJA. 9-LOX activity could be a potential step in the process leading to an accumulation of death acids and ROS in the centre of the areole, where there is a lower concentration of antioxidant phenolics. This accumulation would then lead to subsequent death of the innermost cells of the areole, but stop where the gradient of phenolics around the veins is high enough to counteract the accumulation of ROS.

In terms of the involvement of the PCD inhibition treatments in this model, it is suggested that the perforation inhibitors vanillin and phenidone, are inhibiting LOX activity, and subsequently reducing the production of jasmonates, phenolics, and ROS released into the cell (Figure 17). This inhibition could account for the observed reduction in anthocyanin content (and TPC content in terms of vanillin-treated window stage leaves). Additionally, to account for the reduction in antioxidants, the ROS are likely consequently low in order to maintain oxidative homeostasis within the cell. If ROS were allowed to accumulate in the cells without sufficient antioxidants present, one would expect an increase in cell damage and death events. Due to the lack of PCD gradient in the vanillin and phenidone treatments, it is suggested that there are lower

concentrations of ROS than available phenolics, thus all cells within the areoles remain viable throughout the life cycle of the leaf.

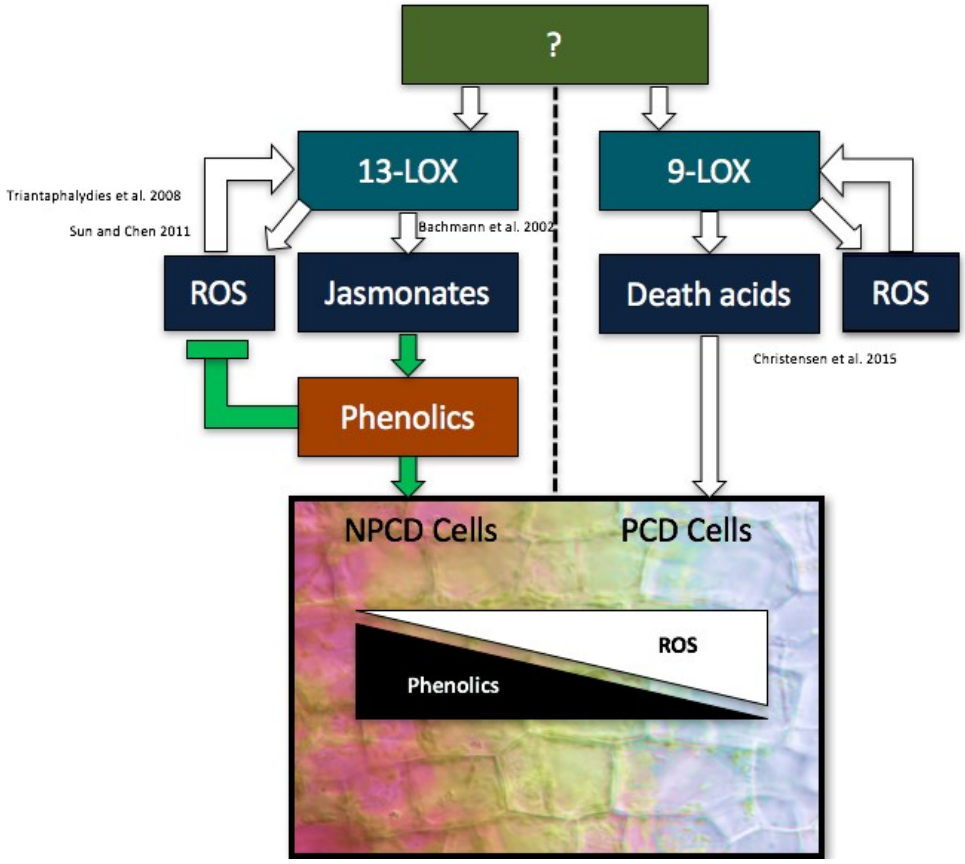

Figure 16. Proposed model for role of phenolics in the formation of perforations in the lace plant. Flowchart representing the proposed model for the involvement of phenolic compounds in the lace plant PCD pathway. The initiating signal remains unknown (question mark). 13-LOX leads to the production of jasmonates, phytohormones largely involved in stress responses and the production of secondary metabolites. These jasmonates induce the synthesis of phenolics in the vascular tissues, resulting in a gradient towards the centre of each areole, thereby delineating NPCD and PCD cell zones. On the programmed cell death (PCD) cell side of the model, there is an accumulation of ROS and death acids following the accumulation of 9-LOX. This imbalance causes a predominance of ROS which will subsequently lead PCD and the formation of perforations. Green arrows denote evidence supported by this study.

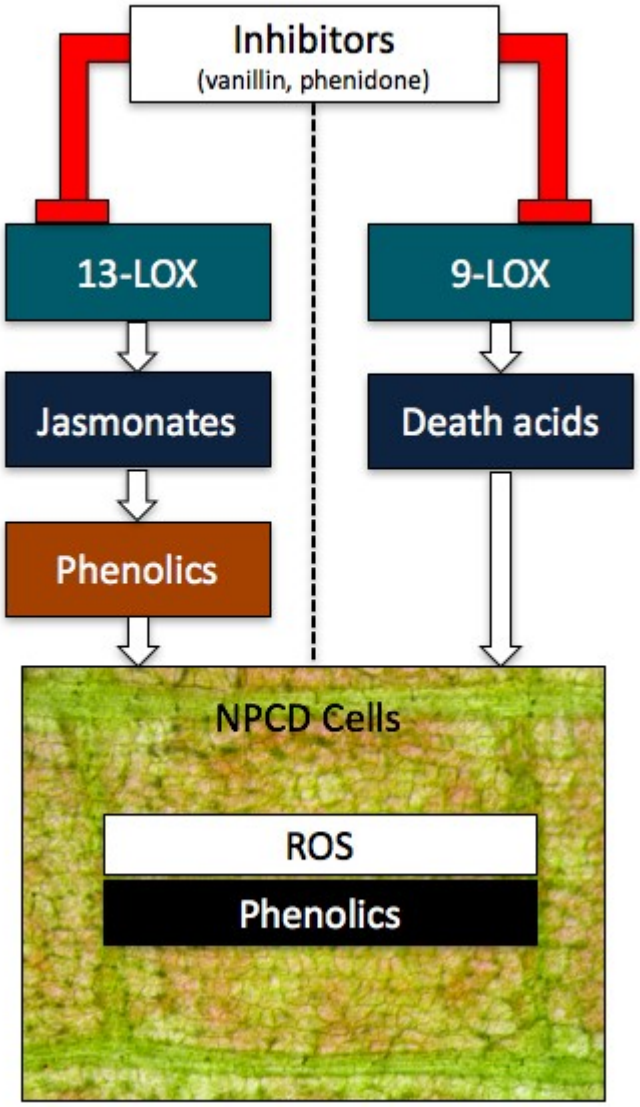

Figure 17. Proposed model for role of inhibitors in formation of perforations in the lace plant. Flowchart representing the proposed model for the involvement of exogenous vanillin and phenidone treatments in the lace plant PCD pathway. There is LOX inhibition, which subsequently reduces production of jasmonates, secondary metabolites, and ROS. There is no death gradient in the areoles, as all cells remain viable. In order for this cell viability to occur, the ROS levels are unable to accumulate to the point of PCD.

4.7 Conclusions

Little is known about the molecular mechanisms involved in various forms of plant PCD, specifically those involved in PCD during leaf development. Compared to animal PCD, more work is needed to gain a better understanding of key compounds involved in plant cell death including caspase-like enzymes, phytohormones, ROS, and phenolics. A previous study has shown an important relationship between antioxidants and ROS during lace plant PCD (Dauphinée et al. 2017). In this study, we gained insight into the potential roles of phenolics in developmental PCD of the lace plant. We found that both anthocyanin and phenolic aldehyde vanillin are significantly higher in younger leaf tissues when the PCD process is taking place, suggesting a close relationship with the cell death process. In addition, a variety of growth conditions were investigated in order to assess the production of membrane-less anthocyanin-containing vesicles, AVIs. Environmental stresses such as an increased pH, light deprivation, and overcrowding all led to a significant increase in AVI production.

We also assessed endogenous phenolics throughout normal lace plant development, including the profiling of specific anthocyanin species for the first time. Cyanidin and delphinidin derivatives are the most dominant species present throughout lace plant development. Of particular interest is the resulting high abundance of delphinidin species in the younger leaf tissues when PCD is occurring.

Plants treated with LOX inhibitors vanillin and phenidone developed significantly fewer perforations, and completely inhibited perforation development in the first leaf to develop after applying treatment. Additionally, treatment with these inhibitors significantly reduced anthocyanin production and free radical scavenging capacity. MeJA

treatment led to an increase in anthocyanin content in leaves, and subsequently an increase in free radical scavenging capacity. Furthermore, in compensation of reduced anthocyanin pigmentation, chlorophyll A and carotenoid content increased which is likely related to photoprotection.

Taken together these results indicate that there is an important relationship between ROS and phenolics during normal lace plant leaf development, and that the manipulation of this pathway via the application of exogenous phenolics alters cell fate by blocking PCD. By treating lace plants with endogenous lace plant phenolic extracts, we may gain additional insight into the effects of these compounds on plant PCD. Vanillin, phenidone, and delphinidins have shown promising results in animal system apoptosis research, and our study has begun to establish important links between the cell death mechanisms effected by these compounds in our plant model. Future work should further investigate potentially novel phenolic compounds in the lace plant with separation through chromatography, and analysis by NMR. Compound isolation from lace plant leaves may prove to be very beneficial in testing their effects on PCD in a variety of cell systems such as cancer. We can learn a lot from studying plant PCD, especially when considering the advancements made in animal cell death research due to a vast array of phytochemicals. Phenolic compounds provide endless opportunities for investigating PCD provided their vast diversity, and the range of applications that have already been investigated in pharmacology and agriculture alike.

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APPENDIX

1. Anthocyanin Localization Raw Data and Statistical Analyses

	Window				Mature		
	apex	mid	base		apex	mid	base
Exp 1	1448	685.64	494.26	Exp 1	797.99	282.88	264.87
	1493.2	865.63	987.16		1351.58	239.02	230.14
Exp 2	1095.91	599.77	531.39	Exp 2	1284.92	263.1	363.88
	1309.12	791.77	114.19		855.12	271.84	179.72
Exp 3	865.07	713.24	832.19	Exp 3	481.25	252.31	271.68
	1684.9	598.65	487.03		723.87	224.28	206.39
Exp 4	1047.69	658.78	743.93	Exp 4	450.96	87.93	199.5
	1091.67	1015.17	816.73		663.36	156.44	135.31
Exp 5	1373.79	600.12	594.11	Exp 5	212.43	105.33	124.74
	1476.19	765.43	520.26		570.74	220.35	240.39
Exp 6	1643.29	1010.31	1185.98	Exp 6	1033.34	342.38	346.58
	1948.1	1110.47	804.84		778.61	305.53	262.62
Exp 7	1233.88	710.19	707.67	Exp 7	561.72	267.67	193.95
	1947.78	663.9	646.32		660.15	201.08	197.67
Mean	1404.185	770.64786	676.14714	**Mean**	744.71714	230.01	229.81714
SD	327.1952	168.28365	257.2698	**SD**	313.36625	72.36363	69.085184
SE	146.32614	75.258735	115.05455	**SE**	140.14165	32.361999	30.895833

Grouping Information Using the Tukey Method and 95% Confidence

Region	N	Mean	Grouping		
window apex	14	1404.19	A		
window mid	14	770.65		B	
mature apex	14	744.72		B	
window base	14	676.15		B	
mature mid	14	230.01			C
mature base	14	229.82			C

Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
mature base-mature apex	-514.90	85.88	(-765.70, -264.10)	-6.00	<0.0001
mature mid-mature apex	-514.71	85.88	(-765.51, -263.91)	-5.99	<0.0001
window apex-mature apex	659.47	85.88	(408.67, 910.27)	7.68	<0.0001
window base-mature apex	-68.57	85.88	(-319.37, 182.23)	-0.80	0.9670
window mid-mature apex	25.93	85.88	(-224.87, 276.73)	0.30	0.9997
mature mid-mature base	0.19	85.88	(-250.61, 250.99)	0.00	1.0000
window apex-mature base	1174.37	85.88	(923.57, 1425.17)	13.67	<0.0001
window base-mature base	446.33	85.88	(195.53, 697.13)	5.20	<0.0001
window mid-mature base	540.83	85.88	(290.03, 791.63)	6.30	<0.0001
window apex-mature mid	1174.18	85.88	(923.37, 1424.98)	13.67	<0.0001
window base-mature mid	446.14	85.88	(195.34, 696.94)	5.19	<0.0001
window mid-mature mid	540.64	85.88	(289.84, 791.44)	6.30	<0.0001
window base-window apex	-728.04	85.88	(-978.84, -477.24)	-8.48	<0.0001
window mid-window apex	-633.54	85.88	(-884.34, -382.74)	-7.38	<0.0001
window mid-window base	94.50	85.88	(-156.30, 345.30)	1.10	0.8798

Individual confidence level = 99.54%

2. Anthocyanic Vacuolar Inclusion Quantification and Statistical Analyses

	AVI Quantification				
Rep #	**Aquarium**	**Etiolated**	**pH 6.7**	**Control**	**Overgrown**
1	3	12	1	1	10
2	21	27	19	0	7
3	5	9	14	2	0
4	12	9	33	1	13
5	47	3	4	2	42
6	0	0	16	5	17
7	3	16		3	33
8		19		0	13
9		20		0	
10		24		1	
11		21		0	
12				2	
13				7	
14				5	
15				1	
16				4	
17				1	
18				1	
19				0	
20				6	
21				5	
	Aquarium	**Etiolated**	**pH 6.7**	**Control**	**Overgrown**
Mean	14.6666667	14.8	17.2	2.3	17.8571429
SD	16.6032125	8.66445193	11.4673449	2.21144211	13.8918013
SE	7.42518238	2.61243055	4.68152397	0.48257623	4.91149344

Grouping Information Using the Dunnett Method and 95% Confidence

Condition	N	Mean	Grouping
Control (control)	21	2.2381	A
Overgrown	8	16.875	
Etiolated	11	14.545	
pH 6.7	6	14.500	
Aquarium	7	13.000	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
Aquarium-Control	10.762	4.231	(-0.102, 21.625)	2.54	0.0530
Etiolated-Control	12.307	3.608	(3.043, 21.572)	3.41	0.0052
Overgrown-Control	14.637	4.028	(4.295, 24.979)	3.63	0.0027
pH 6.7-Control	12.262	4.488	(0.739, 23.784)	2.73	0.0332

Individual confidence level = 98.66%

3. Vanillin Whole Plant Harvest Data and Statistical Analyses

Vanillin Harvest Data						
[Vanillin]	Leaf	Petiole (cm)	Lamina (cm)	Width (cm)	# Perfs	Perfs/cm^2
Control-1	0	17.9	10.9	1.2	148	11.31498471
	1	18	10.8	1.4	204	13.49206349
	2	17.5	10.4	1.1	116	10.13986014
	3	13.8	8.6	1	196	22.79069767
	4	4	2.7	0.3	-	
Control-2	0	18.4	10.6	1	76	7.169811321
	1	19.4	10.9	1.2	130	9.93883792
	2	19.4	11.3	1.6	158	8.738938053
	3	14.3	7.6	1	187	24.60526316
	4	6.1	3.4	0.4	-	
Control-3	0	19.6	11.8	1.5	116	6.553672316
	1	22	13	1.5	160	8.205128205
	2	18.3	8.8	0.9	184	23.23232323
	3	16.3	7.8	0.9	254	36.18233618
	4	4.8	2.7	0.3	-	
Control-4	0	12.1	9.3	1	75	8.064516129
	1	15.2	10.4	1.1	88	7.692307692
	2	15.8	11.3	1.2	90	6.637168142
	3	16.3	11.9	1.4	162	9.723889556
	4	4.3	3.1	0.4	-	
Control-5	0	12.6	9	1.4	69	5.476190476
	1	16	10.7	1.5	108	6.728971963
	2	18.5	10.8	1.5	130	8.024691358
	3	18.2	13.2	1.8	189	7.954545455
	4	7	4.5	0.6	-	
Control-6	0	9.8	6.8	0.8	58	10.66176471
	1	11.3	7.6	0.9	76	11.11111111
	2	12.1	8.4	1	77	9.166666667
	3	13	9.4	0.9	77	9.101654846
	4	6.8	4.7	0.6	-	
Control-7	0	19.9	12.4	1.3	144	8.933002481
	1	21.8	13	1.5	178	9.128205128
	2	23.2	13.9	1.4	262	13.4635149
	3	17.2	9.3	1	290	31.1827957
	4	13.2	8.1	1	-	

Control-8	0	18.5	11.3	1.1	110	8.849557522
	1	17.1	11.6	1.3	146	9.681697613
	2	18.8	12.6	1.5	168	8.888888889
	3	19	12.7	1.3	172	10.41792853
	4	7.7	4.9	0.7	-	
Control-9	0	13.5	9.3	1	118	12.68817204
	1	15.9	10.9	1.1	112	9.341117598
	2	19.5	12.4	1.2	208	13.97849462
	3	14.2	8.7	1	244	28.04597701
	4	3.6	2.8	0.2	-	
Control-10	0	15.1	8.9	1.1	67	6.84371808
	1	17.9	11	1.2	145	10.98484848
	2	19.5	11.4	1.3	176	11.87584345
	3	18.9	10.4	1.3	233	17.23372781
	4	5.7	3.3	0.4	-	
Control-11	0		5	0.7	50	14.28571429
	1		6.5	0.8	89	17.11538462
	2		7	0.8	75	13.39285714
	3		7.2	0.9	77	11.88271605
	4		4.2	0.6	-	
Control-12	0		8.3	0.9	37	4.953145917
	1		8.5	0.9	63	8.235294118
	2		9.3	0.9	82	9.796893668
	3		6.6	0.8	84	15.90909091
	4		2.1	0.3	-	
Control-13	0		9.1	1.1	96	9.59040959
	1		9.3	1	134	14.40860215
	2		8.9	1	122	13.70786517
	3		8.1	0.9	120	16.46090535
	4		4.3	0.6	-	
Control-14	0		6.6	0.8	26	4.924242424
	1		8	0.8	58	9.0625
	2		8.1	0.8	43	6.635802469
	3		8.5	0.9	85	11.11111111
	4		4.9	0.6	-	
Control-15	0		9.2	1.2	106	9.601449275
	1		10.2	1.2	114	9.31372549
	2		11.1	1.3	154	10.67221067
	3		9.4	1.2	196	17.37588652

	4		5	0.7	-	
Control-16	0		6	0.7	23	5.476190476
	1		6.7	0.9	64	10.61359867
	2		7.5	0.9	94	13.92592593
	3		8.3	1	100	12.04819277
	4		5.4	0.8	-	
Control-17	0		6.1	0.8	22	4.508196721
	1		7	0.8	25	4.464285714
	2		6.9	0.9	58	9.339774557
	3		6.3	0.9	86	15.1675485
	4		3.2	0.4	-	
Control-18	0		8.8	1.1	83	8.574380165
	1		10.1	1.1	126	11.34113411
	2		9.9	1.2	108	9.090909091
	3		9.1	1	156	17.14285714
	4		5.5	0.7	-	
1 mM-1	0	13.7	9.6	1	60	6.25
	1	13.9	9.1	1	19	2.087912088
	2	16.1	11.3	1.2	32	2.359882006
	3	13.5	9.8	1.2	58	4.931972789
	4	7.2	5	0.6	-	
1 mM-2	0	11	7.7	1	98	12.72727273
	1	12.1	8.1	0.9	72	9.87654321
	2	12.5	8.3	1	96	11.56626506
	3	11.7	8.7	1	110	12.64367816
	4	5	3.9	0.6	-	
1 mM-3	0	14.7	9.3	1	134	14.40860215
	1	16.6	10.8	1	142	13.14814815
	2	18.4	11.3	1.8	151	7.423795477
	3	15.2	9.7	1.5	193	13.26460481
	4	7.7	4.4	0.6	-	
1 mM-4	0	15	10.9	1.2	85	6.498470948
	1	15.6	11.1	1.2	66	4.954954955
	2	17.1	11.5	1.5	113	6.550724638
	3	17.5	11.3	1.1	108	8.688656476
	4	3.4	3	0.2	-	
1 mM-5	0	15.4	10.4	1.2	104	8.333333333
	1	18.5	12.1	1.2	96	6.611570248
	2	16	8.7	0.9	130	16.60280971

	3	14.7	8.4	1	122	14.52380952
	4	10.4	6.3	0.7	-	
1 mM-6	0	15.2	9.3	1.4	198	15.20737327
	1	16.6	11	1.6	214	12.15909091
	2	18.4	12.1	1.6	220	11.36363636
	3	17.8	9.4	0.9	178	21.04018913
	4	8.5	4.1	0.6	-	
1 mM-7	0	15.7	11.2	1.3	80	5.494505495
	1	17.1	11.3	1.2	82	6.04719764
	2	17.8	11.8	1.1	120	9.244992296
	3	18.7	11.2	1.1	114	9.253246753
	4	12.3	7.3	0.9	-	
1 mM-8	0	13	9.6	1.1	120	11.36363636
	1	14.8	10.8	1.3	132	9.401709402
	2	15.7	11.7	1.1	156	12.12121212
	3	11	7.7	1.1	181	21.36953955
	4	3.9	3.1	0.3	-	
1 mM-9	0	13.7	9.2	1.1	91	8.992094862
	1	15	10.3	1	13	1.262135922
	2	14.7	8.8	1	72	8.181818182
	3	13.2	8.8	0.9	154	19.44444444
	4	1.8	1.4	0.2	-	
1 mM-10	0	13.4	9.7	1	81	8.350515464
	1	15.3	10.1	1.1	4	0.360036004
	2	13.6	9.2	0.9	65	7.850241546
	3	16.2	11.3	1	110	9.734513274
	4	6.5	4.1	0.5	-	
2 mM-1	0	10.8	8	1	28	3.5
	1	12	8.1	0.9	0	0
	2	14	9.9	1.1	29	2.662993572
	3	11.6	8	1	42	5.25
	4	5.7	4	0.4	-	
2 mM-2	0	12.9	8.8	1	86	9.772727273
	1	13	8.7	1	9	1.034482759
	2	14.1	10.2	1.1	28	2.495543672
	3	15.7	10.9	1.2	32	2.44648318
	4	7.3	4.8	0.5	-	
2 mM-3	0	12.3	8.6	1	88	10.23255814
	1	12.5	8.3	0.9	39	5.220883534

	2	11.7	8.5	1	78	9.176470588
	3	11.7	8.5	1	92	10.82352941
	4	2.9	2.6	0.2	-	
2 mM-4	0	18.1	11.9	1.3	122	7.886231416
	1	18.2	10.7	1.1	52	4.418011895
	2	21	11.8	1.1	76	5.855161787
	3	17.6	10	1.3	146	11.23076923
	4	13.5	6.8	0.7	-	
2 mM-5	0	11.1	7.9	0.9	96	13.5021097
	1	14	9.5	1	7	0.736842105
	2	16.6	11.4	1	96	8.421052632
	3	18.8	11	1.2	128	9.696969697
	4	9.3	6.4	0.6	-	
2 mM-6	0	12.2	8.8	1.2	39	3.693181818
	1	12.3	9.2	1.1	0	0
	2	18	10.4	1.3	72	5.325443787
	3	22	13.2	1.5	148	7.474747475
	4	10.2	6.9	0.9	-	
2 mM-7	0	12.9	8.8	1.1	51	5.268595041
	1	14.8	8.6	1	8	0.930232558
	2	19.9	11.7	1	15	1.282051282
	3	17.3	10.6	1.1	185	15.86620926
	4	3.7	2.6	0.2	-	
2 mM-8	0	14	10	1	70	7
	1	16.7	10.4	1	3	0.288461538
	2	16.3	10	0.7	18	2.571428571
	3	9.7	5.6	0.4	24	10.71428571
	4	2.7	1.8	0.2	-	
2 mM-9	0	10.5	7.3	0.7	119	23.28767123
	1	10.6	6.3	0.5	0	0
	2	9.5	5.3	0.6	0	0
	3	6.7	4.8	0.5	0	0
	4	1.3	0.8	0.1	-	
2 mM-10	0	11.5	8.3	0.9	86	11.51271754
	1	12.1	7.5	0.7	0	0
	2	12.2	8.3	0.8	14	2.108433735
	3	12	7.8	0.9	138	19.65811966
	4	2.5	2	0.2	-	
2 mM-11	0		8.5	1	72	8.470588235

	1		8.8	0.9	15	1.893939394
	2		8	0.9	2	0.277777778
	3		8	0.9	59	8.194444444
	4		5.4	0.6	-	
2 mM-12	0		9	1.1	118	11.91919192
	1		8.4	0.9	62	8.201058201
	2		10.2	2	67	3.284313725
	3		8	0.9	174	24.16666667
	4		5.6	0.6	-	
2 mM-13	0		8.3	0.8	92	13.85542169
	1		6.6	0.7	5	1.082251082
	2		6.3	0.8	0	0
	3		6.1	0.7	74	17.33021077
	4		2.9	0.4	-	
2 mM-14	0		7.4	0.9	108	16.21621622
	1		9.2	1.1	98	9.683794466
	2		10.8	1.1	142	11.95286195
	3		7.5	0.8	124	20.66666667
	4		5.3	0.6	-	
2 mM-15	0		6.6	1	56	8.484848485
	1		6.3	0.9	32	5.643738977
	2		7.5	0.9	24	3.555555556
	3		7.5	0.9	60	8.888888889
	4		3.1	0.4	-	
2 mM-16	0		9.2	1.2	128	11.5942029
	1		11.4	1.1	38	3.03030303
	2		10.6	1	134	12.64150943
	3		10	1	102	10.2
	4		8.2	0.8	-	
2 mM-17	0		7.8	1	100	12.82051282
	1		7.8	0.9	13	1.851851852
	2		6.8	0.8	62	11.39705882
	3		8.4	1	70	8.333333333
	4		6.5	0.7	-	
2 mM-18	0		10.1	1	83	8.217821782
	1		10.1	1	60	5.940594059
	2		9.2	1	32	3.47826087
	3		9	1	48	5.333333333
	4		3.7	0.4	-	

2 mM-19	0		6.4	0.8	46	8.984375
	1		6.4	0.9	20	3.472222222
	2		6.4	0.8	7	1.3671875
	3		7.2	1	88	12.22222222
	4		3.8	0.5	-	
2 mM-20	0		10.1	1.1	118	10.62106211
	1		11.4	1.2	52	3.801169591
	2		12	1.2	152	10.55555556
	3		9.7	1.1	124	11.62136832
	4		5.6	0.6	-	
3 mM-1	0	15	9.3	1.1	97	9.481915934
	1	18.4	8.9	0.9	0	0
	2	18.4	9.3	0.9	3	0.358422939
	3	15	8	0.7	15	2.678571429
	4	11	5.9	0.6	-	
3 mM-2	0	11.7	6.2	0.8	122	24.59677419
	1	15	8.8	0.8	0	0
	2	16.7	11.5	1	20	1.739130435
	3	10.2	6.2	0.5	32	10.32258065
	4	0.9	0.6	0.1	-	
3 mM-3	0	12.8	6.6	0.6	89	22.47474747
	1	12.2	6.2	0.5	0	0
	2	12.3	5.8	0.6	0	0
	3	13.5	6.5	0.5	0	0
	4	8.5	4.4	0.4	-	
3 mM-4	0	12	9	0.9	78	9.62962963
	1	12.1	5.9	0.5	1	0.338983051
	2	11.4	6.9	0.7	0	0
	3	8.8	5.5	0.5	0	0
	4	3.4	2.6	0.3	-	
3 mM-5	0	13.3	9.3	1.2	100	8.960573477
	1	12.3	7.3	0.9	48	7.305936073
	2	14.7	9.5	0.9	6	0.701754386
	3	14.5	7.7	0.7	0	0
	4	8.5	5.9	0.6	-	
3 mM-6	0	14.8	9.8	1.2	172	14.62585034
	1	15	9	0.9	0	0
	2	14.7	9.3	0.9	5	0.597371565
	3	12.8	7.9	0.8	13	2.056962025

	4	3.3	2.9	0.2	-	
3 mM-7	0	13.1	9.2	1	50	5.434782609
	1	13	6.9	0.8	26	4.710144928
	2	14.2	7	0.8	0	0
	3	15.8	10.8	0.9	29	2.983539095
	4	0.9	4.6	0.5	-	
3 mM-8	0	11.5	7.7	1	88	11.42857143
	1	15.1	7.6	0.8	0	0
	2	16	9.9	1	142	14.34343434
	3	12.5	7.3	0.7	144	28.18003914
	4	4.1	2.8	0.3	-	
3 mM-9	0	14	9.2	1.2	130	11.77536232
	1	17	9.5	1.1	5	0.4784689
	2	15.8	7.3	0.9	16	2.435312024
	3	12.2	6.2	0.6	104	27.95698925
	4	3.7	2.5	0.3	-	
3 mM-10	0	8.8	6.7	0.7	49	10.44776119
	1	9	6	0.6	4	1.111111111
	2	7.8	5.6	0.6	0	0
	3	8.3	6.2	0.6	80	21.50537634
	4	1	0.7	0.1	-	

Grouping Information Using the Dunnett Method and 95% Confidence

VANtreatment	N	Mean	Grouping
Control (+) (control)	54	12.8871	A
3 mM (-)	10	12.886	A
2 mM (-)	20	10.342	A
1 mM (+)	30	9.8023	A
1 mM (-)	10	9.763	A
Control (-)	18	8.2483	
2 mM (+)	60	6.2626	
3 mM (+)	30	4.327	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
1 mM (-)-Control (+)	-3.125	2.018	(-8.541, 2.292)	-1.55	0.5491
1 mM (+)-Control (+)	-3.085	1.335	(-6.667, 0.498)	-2.31	0.1299
2 mM (-)-Control (+)	-2.545	1.535	(-6.663, 1.573)	-1.66	0.4681
2 mM (+)-Control (+)	-6.625	1.100	(-9.576, -3.674)	-6.02	<0.0001
3 mM (-)-Control (+)	-0.002	2.018	(-5.418, 5.415)	0.00	1.0000
3 mM (+)-Control (+)	-8.560	1.335	(-12.143, -4.978)	-6.41	<0.0001
Control (-)-Control (+)	-4.639	1.596	(-8.921, -0.357)	-2.91	0.0263

Individual confidence level = 99.22%

Grouping Information Using the Dunnett Method and 95% Confidence

VANtreatment	N	Mean	Grouping
Control (-) (control)	18	8.2483	A
Control (+)	54	12.8871	
3 mM (-)	10	12.886	A
2 mM (-)	20	10.342	A
1 mM (+)	30	9.8023	A
1 mM (-)	10	9.763	A
2 mM (+)	60	6.2626	A
3 mM (+)	30	4.327	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
1 mM (-)-Control (-)	1.514	2.312	(-4.506, 7.534)	0.65	0.9730
1 mM (+)-Control (-)	1.554	1.748	(-2.997, 6.105)	0.89	0.8896
2 mM (-)-Control (-)	2.094	1.905	(-2.865, 7.053)	1.10	0.7620
2 mM (+)-Control (-)	-1.986	1.576	(-6.088, 2.116)	-1.26	0.6459
3 mM (-)-Control (-)	4.637	2.312	(-1.383, 10.657)	2.01	0.1973
3 mM (+)-Control (-)	-3.921	1.748	(-8.472, 0.629)	-2.24	0.1193
Control (+)-Control (-)	4.639	1.596	(0.485, 8.793)	2.91	0.0218

Individual confidence level = 99.02%

Grouping Information Using the Dunnett Method and 95% Confidence

VANtreatment	N	Mean	Grouping
Control (+) (control)	54	9.6167	A
1 mM (+)	30	10.1900	A
1 mM (-)	10	9.6900	A
Control (-)	18	8.8556	A
2 mM (+)	60	8.8467	A
2 mM (-)	20	8.5900	A
3 mM (-)	10	8.3000	A
3 mM (+)	30	7.6833	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
1 mM (-)-Control (+)	0.0733	0.5997	(-1.5361, 1.6827)	0.12	1.0000
1 mM (+)-Control (+)	0.5733	0.3967	(-0.4912, 1.6379)	1.45	0.6263
2 mM (-)-Control (+)	-1.0267	0.4560	(-2.2504, 0.1970)	-2.25	0.1492
2 mM (+)-Control (+)	-0.7700	0.3268	(-1.6469, 0.1069)	-2.36	0.1165
3 mM (-)-Control (+)	-1.3167	0.5997	(-2.9261, 0.2927)	-2.20	0.1694
3 mM (+)-Control (+)	-1.9333	0.3967	(-2.9979, -0.8688)	-4.87	<0.0001
Control (-)-Control (+)	-0.7611	0.4741	(-2.0335, 0.5112)	-1.61	0.5067

Individual confidence level = 99.22%

Grouping Information Using the Dunnett Method and 95% Confidence

VANtreatment	N	Mean	Grouping
Control (-) (control)	18	8.8556	A
1 mM (+)	30	10.1900	A
1 mM (-)	10	9.6900	A
Control (+)	54	9.6167	A
2 mM (+)	60	8.8467	A
2 mM (-)	20	8.5900	A
3 mM (-)	10	8.3000	A
3 mM (+)	30	7.6833	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
1 mM (-)-Control (-)	0.8344	0.6871	(-0.9544, 2.6233)	1.21	0.6796
1 mM (+)-Control (-)	1.3344	0.5194	(-0.0178, 2.6867)	2.57	0.0547
2 mM (-)-Control (-)	-0.2656	0.5660	(-1.7391, 1.2080)	-0.47	0.9958
2 mM (+)-Control (-)	-0.0089	0.4682	(-1.2278, 1.2100)	-0.02	1.0000
3 mM (-)-Control (-)	-0.5556	0.6871	(-2.3444, 1.2333)	-0.81	0.9261
3 mM (+)-Control (-)	-1.1722	0.5194	(-2.5244, 0.1800)	-2.26	0.1158
Control (+)-Control (-)	0.7611	0.4741	(-0.4733, 1.9955)	1.61	0.4034

Individual confidence level = 99.02%

Means

VANtreatment	N	Mean	StDev	95% CI
1 mM (-)	10	1.13000	0.14181	(0.99205, 1.26795)
1 mM (+)	30	1.14667	0.23596	(1.06702, 1.22631)
2 mM (-)	20	1.00000	0.14510	(0.90246, 1.09754)
2 mM (+)	60	0.97333	0.23855	(0.91702, 1.02965)
3 mM (-)	10	0.97000	0.21628	(0.83205, 1.10795)
3 mM (+)	30	0.75333	0.17367	(0.67369, 0.83298)
Control (-)	18	1.03889	0.23044	(0.93607, 1.14171)
Control (+)	54	1.10926	0.24669	(1.04990, 1.16862)

Pooled StDev = 0.221368

Grouping Information Using the Dunnett Method and 95% Confidence

VANtreatment	N	Mean	Grouping
Control (+) (control)	54	1.10926	A
1 mM (+)	30	1.14667	A
1 mM (-)	10	1.13000	A
Control (-)	18	1.03889	A
2 mM (-)	20	1.00000	A
2 mM (+)	60	0.97333	
3 mM (-)	10	0.97000	A
3 mM (+)	30	0.75333	

Means not labeled with the letter A are significantly different from the control level mean.

Grouping Information Using the Dunnett Method and 95% Confidence

VANtreatment	N	Mean	Grouping
Control (-) (control)	18	1.03889	A
1 mM (+)	30	1.14667	A
1 mM (-)	10	1.13000	A
Control (+)	54	1.10926	A
2 mM (-)	20	1.00000	A
2 mM (+)	60	0.97333	A
3 mM (-)	10	0.97000	A
3 mM (+)	30	0.75333	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
1 mM (-)-Control (-)	0.09111	0.08731	(-0.13619, 0.31842)	1.04	0.7994
1 mM (+)-Control (-)	0.10778	0.06600	(-0.06405, 0.27960)	1.63	0.3860
2 mM (-)-Control (-)	-0.03889	0.07192	(-0.22613, 0.14835)	-0.54	0.9904
2 mM (+)-Control (-)	-0.06556	0.05949	(-0.22044, 0.08933)	-1.10	0.7601
3 mM (-)-Control (-)	-0.06889	0.08731	(-0.29619, 0.15842)	-0.79	0.9337
3 mM (+)-Control (-)	-0.28556	0.06600	(-0.45738, -0.11373)	-4.33	0.0002
Control (+)-Control (-)	0.07037	0.06025	(-0.08648, 0.22723)	1.17	0.7134

Individual confidence level = 99.02%

5. Phenidone Whole Plant Harvest Data and Statistical Analyses

Phenidone Harvest Data						
Treatment	**Leaf**	**Petiole (cm)**	**Lamina (cm)**	**Width (cm)**	**Perf #**	**perfs/cm^2**
Control-1	0	10.4	7.1	0.9	16	2.503912363
	1	9.8	7.1	0.9	30	4.694835681
	2	13.5	9.2	0.9	80	9.661835749
	3	11.5	7.8	0.8	136	21.79487179
	4	5.3	4.1	0.6	-	
Control-2	0	10.4	7	0.8	60	10.71428571
	1	10.6	7.3	0.9	114	17.35159817
	2	12.5	9.5	1	72	7.578947368
	3	11.9	9.1	1	78	8.571428571
	4	9.1	6.8	0.8	-	
Control-3	0	11.6	7.8	0.9	52	7.407407407
	1	11.4	7.8	0.9	74	10.54131054
	2	10.1	7.1	0.8	78	13.73239437
	3	9.8	6.6	0.9	76	12.79461279
	4	7.5	5.7	0.7	-	
Control-4	0	11.2	7.7	1	72	9.350649351
	1	10.9	7.6	1	62	8.157894737
	2	12.2	8.6	1.1	25	2.642706131
	3	13.5	9.5	1	18	1.894736842
	4	9.5	6.5	0.8	-	
Control-5	0	9.7	7.3	0.9	86	13.08980213
	1	10.6	7.9	1	28	3.544303797
	2	9.5	7.1	0.8	22	3.873239437
	3	7.1	5.9	0.7	74	17.91767554
	4	2	1.7	0.1	-	
Control-6	0	7.2	5	0.8	49	12.25
	1	8.9	6.4	0.8	55	10.7421875
	2	10.2	7.7	1	71	9.220779221
	3	10.4	7.7	0.9	90	12.98701299
	4	6.9	5.5	0.7	-	
Control-7	0	11.4	8.2	0.7	58	10.10452962
	1	12.4	8.6	0.7	56	9.302325581
	2	12.5	8.7	0.9	44	5.619412516

	3	11.1	8.3	0.9	50	6.693440428
	4	5.1	3.9	0.4	-	
Control-8	0	8.2	5.6	0.5	70	25
	1	9.1	6.6	0.5	53	16.06060606
	2	10	7.3	0.6	29	6.621004566
	3	9.1	6.7	0.7	42	8.955223881
	4	6	4.8	0.6	-	
Control-9	0	13.1	9.5	0.8	76	10
	1	13.6	10	0.8	88	11
	2	14.1	9.6	1	109	11.35416667
	3	12.5	8.7	1	110	12.64367816
	4	5.7	4.3	0.5	-	
Control-10	0	10.3	7.3	0.5	44	12.05479452
	1	11.4	8.3	0.7	58	9.982788296
	2	10.7	7.8	0.8	53	8.493589744
	3	10	7.8	0.8	69	11.05769231
	4	3.3	2.3	0.3	-	
10 um-1	0	12	8.3	1	104	12.53012048
	1	11.6	8.4	0.9	55	7.275132275
	2	12.6	9.5	1.1	78	7.464114833
	3	10	7.2	0.8	196	34.02777778
	4	3.5	2.5	0.3	-	
10 um-2	0	11.5	7.6	0.7	68	12.78195489
	1	9.3	6.4	0.7	43	9.598214286
	2	9.4	7.4	0.8	68	11.48648649
	3	8.6	6.5	0.8	116	22.30769231
	4	2.2	1.8	0.2	-	
10 um-3	0	12.5	8.9	0.9	72	8.988764045
	1	11.3	8.6	0.9	56	7.235142119
	2	11.3	8.8	0.9	82	10.35353535
	3	10.5	8.2	1	100	12.19512195
	4	5.4	4.2	0.6	-	
10 um-4	0	9.1	6.9	0.9	61	9.822866345
	1	10.8	8.2	1	66	8.048780488
	2	11.8	9.1	1.1	60	5.994005994
	3	10.4	7.8	0.8	102	16.34615385
	4	4.4	3.4	0.5	-	
10 um-5	0	10.6	7.1	1	74	10.42253521
	1	12.1	8.4	1	40	4.761904762

	2	12.2	8.7	1	64	7.356321839
	3	11.1	7.6	0.9	7	1.023391813
	4	3.1	2.4	0.3	-	
10 um-6	0	10.2	6	1.1	43	6.515151515
	1	11.6	7.5	1.4	58	5.523809524
	2	14	8.5	1.2	63	6.176470588
	3	16.7	9.5	1.1	97	9.282296651
	4	8	5.5	0.7	-	
10 um-7	0	10	6.7	1	36	5.373134328
	1	14	9.4	1.2	44	3.90070922
	2	15.2	10.3	1.1	105	9.267431598
	3	13.3	9.1	1.1	125	12.48751249
	4	8	5.5	0.6	-	
10 um-8	0	8.8	6	0.6	47	13.05555556
	1	10.2	7.1	0.7	53	10.6639839
	2	9.8	7.2	0.7	77	15.27777778
	3	7.9	6	0.6	103	28.61111111
	4	2.1	1.9	0.2	-	
10 um-9	0	11.3	7.6	0.9	48	7.01754386
	1	12.4	8.8	0.9	92	11.61616162
	2	14.1	9.4	1	126	13.40425532
	3	10.6	6.9	0.9	204	32.85024155
	4	3.1	2.3	0.3	-	
10 um-10	0	9.2	6.7	0.8	79	14.73880597
	1	10.7	8.1	1	83	10.24691358
	2	10.9	8.3	0.9	48	6.425702811
	3	9.8	7.5	0.8	74	12.33333333
	4	3.1	2.6	0.3	-	
100 um-1	0	13.3	9.1	1	42	4.615384615
	1	11.6	8.8	0.8	3	0.426136364
	2	13.8	9.6	1	112	11.66666667
	3	12.7	8.4	0.8	220	32.73809524
	4	9.3	5.2	0.6	-	
100 um-2	0	9.7	6.4	0.8	94	18.359375
	1	9.8	6.8	0.8	0	0
	2	10.8	7.5	0.8	4	0.666666667
	3	11.8	8.6	0.8	76	11.04651163
	4	7.9	5.5	0.7	-	
100 um-3	0	12.2	8.2	1	80	9.756097561

	1	11.5	8.2	0.9	0	0
	2	11.3	8.5	0.8	64	10.66666667
	3	13.3	9.8	0.9	76	8.616780045
	4	9.2	7.1	0.8	-	
100 um-4	0	8	5.5	0.8	75	17.04545455
	1	7.8	5.5	0.8	4	0.909090909
	2	8.8	6	0.8	0	0
	3	8.7	6.7	0.9	22	3.648424544
	4	5	4	0.5	-	
100 um-5	0	10	6.5	0.9	91	15.55555556
	1	9.6	6.9	0.8	0	0
	2	10.3	7	0.8	2	0.357142857
	3	12.8	8.8	0.9	50	6.313131313
	4	6	4.7	0.6	-	
100 um-6	0	9.9	6	1	56	9.333333333
	1	10.9	6.9	0.9	0	0
	2	11.6	7.3	1	1	0.136986301
	3	10.3	6.9	0.6	70	16.90821256
	4	2.3	1.9	0.2	-	
100 um-7	0	10.3	6.4	0.9	38	6.597222222
	1	12.4	6.9	0.9	28	4.508856683
	2	14.2	8.2	0.9	8	1.08401084
	3	11.5	7	0.6	70	16.66666667
	4	7.5	5.5	0.5	-	
100 um-8	0	7.9	5.1	0.6	25	8.169934641
	1	8.6	5.6	0.6	0	0
	2	8.7	5.7	0.6	0	0
	3	9.4	6.4	0.7	6	1.339285714
	4	4.5	3.1	0.4	-	
100 um-9	0	9.3	6.6	0.8	18	3.409090909
	1	9.9	6.5	0.7	0	0
	2	10.9	7.4	0.8	3	0.506756757
	3	9.3	6.9	0.8	35	6.34057971
	4	2.9	2.2	0.3	-	
100 um-10	0	6.9	5.2	0.7	65	17.85714286
	1	7	4.8	0.6	0	0
	2	7.5	5.3	0.6	35	11.00628931
	3	7	5.3	0.7	16	4.312668464
	4	5.2	4.2	0.5	-	

200 um-1	0	7.1	4.6	0.7	38	11.80124224
	1	6.5	3.4	0.5	0	0
	2	7.4	3.4	0.5	0	0
	3	7.6	3.9	0.5	0	0
	4	5.3	3.7	0.5	-	
200 um-2	0	9.6	6.3	0.8	45	8.928571429
	1	8.6	4.8	0.6	0	0
	2	10.2	5.4	0.6	0	0
	3	11.2	6.6	0.7	0	0
	4	9.3	6.4	0.7	-	
200 um-3	0	13.4	7.3	0.8	11	1.883561644
	1	15.2	7.4	0.8	0	0
	2	12.4	6.4	0.6	0	0
	3	11.5	6.3	0.6	2	0.529100529
	4	2.7	1.8	0.2	-	
200 um-4	0	11.8	7.5	1	107	14.26666667
	1	12.6	7	0.9	26	4.126984127
	2	13.9	7.6	0.7	0	0
	3	14.5	9.1	0.8	141	19.36813187
	4	6.3	4.5	0.4	-	
200 um-5	0	10	6.9	0.9	22	3.542673108
	1	10.2	6.8	0.9	0	0
	2	9.3	6.1	0.8	35	7.172131148
	3	8.8	6	0.8	39	8.125
	4	5.2	3.7	0.5	-	
200 um-6	0	12	7.7	0.8	61	9.902597403
	1	11.1	7.3	0.8	0	0
	2	10.5	6.7	0.6	0	0
	3	10	7	0.8	118	21.07142857
	4	3.1	2.3	0.3	-	
200 um-7	0	7.4	5.6	0.7	74	18.87755102
	1	6.7	4.2	0.5	0	0
	2	7.4	5	0.6	0	0
	3	7.3	5.3	0.6	0	0
	4	3.1	2.5	0.4	-	
200 um-8	0	11.3	8.1	0.9	80	10.9739369
	1	12.1	8.2	0.9	3	0.406504065
	2	12.7	8.2	0.9	0	0
	3	9.2	6.5	0.7	26	5.714285714

	4	3.6	2.6	0.2	-	
200 um-9	0	11.2	7.6	0.5	46	12.10526316
	1	10.3	6.4	0.5	0	0
	2	9.2	6	0.5	0	0
	3	6.2	4.7	0.5	0	0
	4	4	2.5	0.2	-	
200 um-10	0	10.2	7.6	0.7	48	9.022556391
	1	9.5	6.3	0.7	0	0
	2	9.3	6.6	0.6	0	0
	3	7.3	5.7	0.7	28	7.01754386
	4	2.1	1.4	0.1	-	
200 um-11	0	7.7	5.2	0.6	60	19.23076923
	1	7.3	4.1	0.4	0	0
	2	7.6	4.4	0.5	0	0
	3	5.4	3.9	0.5	0	0
	4	1	0.8	0.1	-	

Grouping Information Using the Dunnett Method and 95% Confidence

PHENtreatment	N	Mean	Grouping
Control (+) (control)	30	9.8495	A
10 µM (+)	30	11.785	A
Control (-)	10	11.248	A
100 µM (-)	10	11.070	A
200 µM (-)	11	10.958	A
10 µM (-)	10	10.1246	A
100 µM (+)	30	4.996	
200 µM (+)	33	2.2282	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
10 µM (-)-Control (+)	0.275	2.243	(-5.738, 6.288)	0.12	1.0000
10 µM (+)-Control (+)	1.935	1.586	(-2.316, 6.187)	1.22	0.7669
100 µM (-)-Control (+)	1.220	2.243	(-4.792, 7.233)	0.54	0.9958
100 µM (+)-Control (+)	-4.854	1.586	(-9.106, -0.602)	-3.06	0.0168
200 µM (-)-Control (+)	1.108	2.166	(-4.696, 6.912)	0.51	0.9971
200 µM (+)-Control (+)	-7.621	1.550	(-11.775, -3.467)	-4.92	<0.0001
Control (-)-Control (+)	1.398	2.243	(-4.615, 7.411)	0.62	0.9906

Individual confidence level = 99.19%

Grouping Information Using the Dunnett Method and 95% Confidence

PHENtreatment	N	Mean	Grouping
Control (-) (control)	10	11.248	A
10 µM (+)	30	11.785	A
100 µM (-)	10	11.070	A
200 µM (-)	11	10.958	A
10 µM (-)	10	10.1246	A
Control (+)	30	9.8495	A
100 µM (+)	30	4.996	
200 µM (+)	33	2.2282	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
10 µM (-)-Control (-)	-1.123	2.748	(-8.201, 5.955)	-0.41	0.9971
10 µM (+)-Control (-)	0.537	2.243	(-5.242, 6.316)	0.24	0.9999
100 µM (-)-Control (-)	-0.178	2.748	(-7.256, 6.900)	-0.06	1.0000
100 µM (+)-Control (-)	-6.252	2.243	(-12.031, -0.473)	-2.79	0.0289
200 µM (-)-Control (-)	-0.290	2.684	(-7.205, 6.625)	-0.11	1.0000
200 µM (+)-Control (-)	-9.019	2.218	(-14.732, -3.306)	-4.07	0.0005
Control (+)-Control (-)	-1.398	2.243	(-7.177, 4.381)	-0.62	0.9699

Individual confidence level = 98.91%

Grouping Information Using the Dunnett Method and 95% Confidence

PHENtreatment	N	Mean	Grouping
Control (+) (control)	30	7.9433	A
10 µM (+)	30	8.1467	A
Control (-)	10	7.2500	A
10 µM (-)	10	7.1800	A
100 µM (+)	30	7.1400	A
200 µM (-)	11	6.7636	
100 µM (-)	10	6.5000	
200 µM (+)	33	5.9606	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
10 µM (-)-Control (+)	-0.7633	0.4432	(-1.9511, 0.4244)	-1.72	0.4059
10 µM (+)-Control (+)	0.2033	0.3134	(-0.6365, 1.0432)	0.65	0.9882
100 µM (-)-Control (+)	-1.4433	0.4432	(-2.6311, -0.2556)	-3.26	0.0091
100 µM (+)-Control (+)	-0.8033	0.3134	(-1.6432, 0.0365)	-2.56	0.0680
200 µM (-)-Control (+)	-1.1797	0.4278	(-2.3262, -0.0331)	-2.76	0.0405
200 µM (+)-Control (+)	-1.9827	0.3062	(-2.8033, -1.1622)	-6.48	<0.0001
Control (-)-Control (+)	-0.6933	0.4432	(-1.8811, 0.4944)	-1.56	0.5162

Individual confidence level = 99.19%

Grouping Information Using the Dunnett Method and 95% Confidence

PHENtreatment	N	Mean	Grouping
Control (-) (control)	10	7.2500	A
10 µM (+)	30	8.1467	A
Control (+)	30	7.9433	A
10 µM (-)	10	7.1800	A
100 µM (+)	30	7.1400	A
200 µM (-)	11	6.7636	A
100 µM (-)	10	6.5000	A
200 µM (+)	33	5.9606	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
10 µM (-)-Control (-)	-0.0700	0.5428	(-1.4682, 1.3282)	-0.13	1.0000
10 µM (+)-Control (-)	0.8967	0.4432	(-0.2450, 2.0383)	2.02	0.1739
100 µM (-)-Control (-)	-0.7500	0.5428	(-2.1482, 0.6482)	-1.38	0.5131
100 µM (+)-Control (-)	-0.1100	0.4432	(-1.2516, 1.0316)	-0.25	0.9999
200 µM (-)-Control (-)	-0.4864	0.5303	(-1.8524, 0.8797)	-0.92	0.8420
200 µM (+)-Control (-)	-1.2894	0.4381	(-2.4180, -0.1608)	-2.94	0.0187
Control (+)-Control (-)	0.6933	0.4432	(-0.4483, 1.8350)	1.56	0.3931

Individual confidence level = 98.91%

Grouping Information Using the Dunnett Method and 95% Confidence

PHENtreatment	N	Mean	Grouping
Control (+) (control)	30	0.86000	A
10 µM (+)	30	0.94333	A
10 µM (-)	10	0.89000	A
100 µM (-)	10	0.85000	A
100 µM (+)	30	0.78667	A
Control (-)	10	0.78000	A
200 µM (-)	11	0.76364	A
200 µM (+)	33	0.65455	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
10 µM (-)-Control (+)	0.03000	0.05373	(-0.11400, 0.17400)	0.56	0.9951
10 µM (+)-Control (+)	0.08333	0.03799	(-0.01849, 0.18516)	2.19	0.1644
100 µM (-)-Control (+)	-0.01000	0.05373	(-0.15400, 0.13400)	-0.19	1.0000
100 µM (+)-Control (+)	-0.07333	0.03799	(-0.17516, 0.02849)	-1.93	0.2815
200 µM (-)-Control (+)	-0.09636	0.05187	(-0.23537, 0.04264)	-1.86	0.3217
200 µM (+)-Control (+)	-0.20545	0.03712	(-0.30494, -0.10597)	-5.54	<0.0001
Control (-)-Control (+)	-0.08000	0.05373	(-0.22400, 0.06400)	-1.49	0.5719

Individual confidence level = 99.19%

Grouping Information Using the Dunnett Method and 95% Confidence

PHENtreatment	N	Mean	Grouping
Control (-) (control)	10	0.78000	A
10 µM (+)	30	0.94333	
10 µM (-)	10	0.89000	A
Control (+)	30	0.86000	A
100 µM (-)	10	0.85000	A
100 µM (+)	30	0.78667	A
200 µM (-)	11	0.76364	A
200 µM (+)	33	0.65455	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
10 µM (-)-Control (-)	0.11000	0.06580	(-0.05952, 0.27952)	1.67	0.3310
10 µM (+)-Control (-)	0.16333	0.05373	(0.02492, 0.30174)	3.04	0.0142
100 µM (-)-Control (-)	0.07000	0.06580	(-0.09952, 0.23952)	1.06	0.7441
100 µM (+)-Control (-)	0.00667	0.05373	(-0.13174, 0.14508)	0.12	1.0000
200 µM (-)-Control (-)	-0.01636	0.06429	(-0.18198, 0.14925)	-0.25	0.9999
200 µM (+)-Control (-)	-0.12545	0.05312	(-0.26228, 0.01137)	-2.36	0.0838
Control (+)-Control (-)	0.08000	0.05373	(-0.05841, 0.21841)	1.49	0.4408

Individual confidence level = 98.91%

5. Methyl Jasmonate Whole Plant Harvest Data and Statistical Analyses

MeJA Harvest Data						
Treatment	**Leaf**	**Petiole (cm)**	**Lamina (cm)**	**Width (cm)**	**Perf #**	perfs/cm^2
Control-1	0	13	8.5	1	104	12.23529412
	1	18.3	10.7	1.4	152	10.14686248
	2	15.1	8.5	1.2	87	8.529411765
	3	11.9	6.5	1	99	15.23076923
	4	6.9	3.8	0.5	-	
Control-2	0	11.9	5.5	1	28	5.090909091
	1	18.4	11.6	1.1	83	6.504702194
	2	18.2	11.5	1.3	169	11.30434783
	3	12.6	7.7	1	231	30
	4	2.2	1.3	0.1	-	
Control-3	0	10.5	7.2	0.7	90	17.85714286
	1	10.5	6.8	0.6	52	12.74509804
	2	13.4	8.4	0.7	38	6.462585034
	3	14.1	9.3	0.8	34	4.569892473
	4	9.1	4.9	0.5	-	
Control-4	0	10.5	7.2	0.7	42	8.333333333
	1	11.1	7.8	0.8	103	16.50641026
	2	12.3	9.1	0.9	103	12.57631258
	3	12.6	8.8	1	116	13.18181818
	4	7.6	5.7	0.6	-	
Control-5	0	11.6	8.6	0.9	116	14.9870801
	1	13.2	9.5	1	115	12.10526316
	2	12.8	9.7	1.3	162	12.84694687
	3	8.6	6.5	0.8	226	43.46153846
	4	3.9	3.1	0.4	-	
Control-6	0	12.5	8.9	1	101	11.34831461
	1	14.3	9.8	1	172	17.55102041
	2	14.4	10.2	1.3	276	20.81447964
	3	12.2	8.7	1.3	156	13.79310345
	4	6.1	4.4	0.6	-	
Control-7	0	10.4	7.8	1	69	8.846153846
	1	11.1	7.9	1	77	9.746835443
	2	12	9.3	1	49	5.268817204
	3	9.8	7.7	0.8	8	1.298701299

	4	2.8	2.3	0.3	-	
Control-8	0	8.4	6	0.7	42	10
	1	9.5	7	0.8	57	10.17857143
	2	9.7	7.2	0.8	72	12.5
	3	9.6	7.3	0.7	76	14.87279843
	4	5.6	4.4	0.5	-	
Control-9	0	11.9	7.9	0.7	37	6.690777577
	1	13.7	9.2	1	57	6.195652174
	2	13.4	8.4	1	91	10.83333333
	3	12.9	7.9	0.7	107	19.34900542
	4	7.4	4.8	0.6	-	
Control-10	0	12.4	9.1	1	81	8.901098901
	1	13.2	10.2	1.1	66	5.882352941
	2	13.3	10.1	1	48	4.752475248
	3	10.5	7.9	0.8	56	8.860759494
	4	5.4	3.7	0.5	-	
0.5 um-1	0	8	6.1	0.6	66	18.03278689
	1	8.1	6.1	0.7	60	14.05152225
	2	9	6.8	0.8	70	12.86764706
	3	9.8	6.8	0.8	124	22.79411765
	4	4.6	3.6	0.5	-	
0.5 um-2	0	11.4	9	1	98	10.88888889
	1	10.2	8	1	116	14.5
	2	9.9	7.4	0.9	82	12.31231231
	3	8	5.7	0.7	77	19.29824561
	4	4	3	0.3	-	
0.5 um-3	0	7.3	5.2	0.6	70	22.43589744
	1	7.3	5.4	0.7	68	17.98941799
	2	8.4	6.4	0.7	48	10.71428571
	3	8.3	6	0.7	78	18.57142857
	4	2.9	2.3	0.3	-	
0.5 um-4	0	10.2	7.6	0.8	82	13.48684211
	1	12.4	9.1	1	48	5.274725275
	2	12.2	8.7	1	33	3.793103448
	3	8.2	5.9	0.7	108	26.15012107
	4	2.5	2.2	0.2	-	
0.5 um-5	0	10.4	8.1	0.8	76	11.72839506
	1	8.8	6.7	0.8	96	17.91044776
	2	10.8	8.3	0.9	101	13.52074967

	3	7.5	5.3	0.8	112	26.41509434
	4	2.6	2.3	0.2	0	
0.5 um-6	0	8.5	6.2	0.7	41	9.447004608
	1	8.3	6	0.6	68	18.88888889
	2	8.9	7	0.7	51	10.40816327
	3	9.4	7.1	0.7	67	13.48088531
	4	3.2	2.8	0.3	-	
0.5 um-7	0	8.7	6.4	0.6	71	18.48958333
	1	8.8	6.4	0.7	35	7.8125
	2	10	6.7	0.8	24	4.47761194
	3	9.4	6.4	0.7	31	6.919642857
	4	7.3	5	0.6	-	
0.5 um-8	0	7.3	5.2	0.5	29	11.15384615
	1	8.1	5.6	0.8	46	10.26785714
	2	9.2	6.3	0.8	32	6.349206349
	3	9	6.5	0.9	18	3.076923077
	4	5.4	4.3	0.5	-	
0.5 um-9	0	8	6	0.6	27	7.5
	1	7.5	5.4	0.5	41	15.18518519
	2	7	4.9	0.6	30	10.20408163
	3	8.1	6	0.7	30	7.142857143
	4	3.9	2.8	0.4	-	
0.5 um-10	0	9.9	6.9	0.8	10	1.811594203
	1	9.1	5.9	0.8	21	4.449152542
	2	9.9	6.1	0.7	9	2.107728337
	3	13	8.2	0.9	15	2.032520325
	4	7.8	4.9	0.7	-	
2.5 um-1	0	6.4	3.6	0.4	15	10.41666667
	1	7.2	4.2	0.4	20	11.9047619
	2	7	4.7	0.4	53	28.19148936
	3	5	3.2	0.4	2	1.5625
	4	3.3	2.1	0.4	-	
2.5 um-2	0	14	9.8	0.7	50	7.288629738
	1	12.1	8.6	0.6	44	8.527131783
	2	7.5	4.8	0.5	40	16.66666667
	3	4.5	3.1	0.5	14	9.032258065
	4	5.5	2.7	0.4	-	
2.5 um-3	0	10.1	6.5	0.7	62	13.62637363
	1	10.2	6.6	0.5	48	14.54545455

	2	6.6	4.5	0.5	53	23.55555556
	3	5.8	3.8	0.4	15	9.868421053
	4	6	3.8	0.4	-	
2.5 um-4	0	7.6	4.5	0.5	12	5.333333333
	1	9.7	6.5	0.7	70	15.38461538
	2	8.5	6.1	0.5	126	41.31147541
	3	8.1	5.4	0.7	73	19.31216931
	4	3.2	2.3	0.4	-	
2.5 um-5	0	8.2	5.1	0.5	23	9.019607843
	1	8	5.2	0.7	87	23.9010989
	2	9.7	6.2	0.8	90	18.14516129
	3	9.8	6.8	0.9	48	7.843137255
	4	8.1	5.1	0.7	-	
2.5 um-6	0	10.6	6.4	0.4	13	5.078125
	1	12.5	6.9	0.8	64	11.5942029
	2	11.5	6.5	0.6	112	28.71794872
	3	12.4	6.7	0.6	95	23.6318408
	4	10.1	4.2	0.5	-	
2.5 um-7	0	7.9	5.3	0.5	94	35.47169811
	1	9.2	6.7	0.7	96	20.46908316
	2	10.9	7.2	0.7	54	10.71428571
	3	11.2	7	0.9	108	17.14285714
	4	4.3	2.9	0.5	-	
2.5 um-8	0	9.7	6.5	0.6	64	16.41025641
	1	10.1	7	0.8	128	22.85714286
	2	14.2	9.5	1	61	6.421052632
	3	15.5	9.3	1	168	18.06451613
	4	10	5.5	0.7	-	
2.5 um-9	0	14.7	10.2	1.1	144	12.8342246
	1	15.7	9.3	1.1	92	8.99315738
	2	23.1	13.9	1.6	244	10.97122302
	3	17.7	10.4	1.2	252	20.19230769
	4	6.7	3.5	0.4	-	
2.5 um-10	0	8	5.5	0.7	28	7.272727273
	1	8.7	5.5	0.6	20	6.060606061
	2	7.5	5.2	0.6	58	18.58974359
	3	8.6	5	0.6	18	6
	4	7.2	4.4	0.5	-	
2.5 um-11	0	12.5	9.7	1	182	18.7628866

	1	11.8	9.2	1.1	192	18.97233202
	2	17	12.3	1	92	7.479674797
	3	18.1	13.1	1	70	5.34351145
	4	8.6	5.1	0.6	-	
2.5 um-12	0	10.2	7.5	0.8	120	20
	1	10.2	0.2	0.8	110	687.5
	2	8.9	5.8	0.7	26	6.403940887
	3	8.6	5.6	0.5	88	31.42857143
	4	7	4.4	0.5	-	
5.0 um-1	0	10.7	7.5	0.6	23	5.111111111
	1	11	7.5	0.7	70	13.33333333
	2	11.6	7.3	0.7	21	4.109589041
	3	11.9	7.7	0.8	55	8.928571429
	4	4.7	2.7	0.3	-	
5.0 um-2	0	10.8	7.3	0.8	25	4.280821918
	1	9.6	6.2	0.8	30	6.048387097
	2	8.9	5.8	0.5	42	14.48275862
	3	3.4	5.8	0.5	8	2.75862069
	4	4.3	2.7	0.4	-	
5.0 um-3	0	9.4	6.3	0.5	43	13.65079365
	1	5.8	3.8	0.5	42	22.10526316
	2	6.5	4	0.4	5	3.125
	3	6.7	3.7	0.5	0	0
	4	2.9	1.8	0.3	-	
5.0 um-4	0	13.1	9.6	0.8	134	17.44791667
	1	13.1	9.1	0.9	108	13.18681319
	2	9.5	6.8	0.9	120	19.60784314
	3	6	3.5	0.5	9	5.142857143
	4	3.3	2.2	0.3	-	
5.0 um-5	0	8.1	6.1	0.7	47	11.00702576
	1	8.3	6.3	0.9	69	12.16931217
	2	6.5	5.1	0.7	46	12.88515406
	3	6.3	4.5	0.7	10	3.174603175
	4	6.7	4.5	0.6	-	
5.0 um-6	0	8.7	5.9	0.7	27	6.537530266
	1	7.6	5.3	0.7	37	9.973045822
	2	8.4	3.9	0.5	0	0
	3	5.4	3.6	0.5	0	0
	4	2.6	2.1	0.4	-	

5.0 um-7	0	7.5	5.7	0.6	30	8.771929825
	1	7.3	5.3	0.6	52	16.35220126
	2	6	3.5	0.5	18	10.28571429
	3	5.6	3.9	0.5	0	0
	4	5.3	3.7	0.4	-	
5.0 um-8	0	8	5.9	0.8	58	12.28813559
	1	6	4.5	0.5	49	21.77777778
	2	5.6	3.9	0.4	0	0
	3	5.6	3.9	0.4	0	0
	4	2.2	1.8	0.3	-	
5.0 um-9	0	9	6.5	0.7	39	8.571428571
	1	5.7	4.8	0.4	3	1.5625
	2	5.5	4	0.5	36	18
	3	5.3	3.3	0.4	0	0
	4	3.4	2.4	0.5	-	
5.0 um-10	0	10	7	0.7	108	22.04081633
	1	8.1	5.8	0.6	78	22.4137931
	2	7.8	5.1	0.6	0	0
	3	7.1	4.5	0.6	4	1.481481481
	4	2.2	1.7	0.3	-	

Grouping Information Using the Dunnett Method and 95% Confidence

MeJAtreatment	N	Mean	Grouping
Control (+) (control)	30	12.602	A
2.5 µM (+)	36	34.37	A
2.5 µM (-)	12	13.460	A
0.5 µM (-)	10	12.497	A
0.5 µM (+)	30	11.966	A
5 µM (-)	10	10.971	A
Control (-)	10	10.429	A
5 µM (+)	30	8.097	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.5 µM (-)-Control (+)	-0.10	19.32	(-51.85, 51.64)	-0.01	1.0000
0.5 µM (+)-Control (+)	-0.64	13.66	(-37.23, 35.95)	-0.05	1.0000
2.5 µM (-)-Control (+)	0.86	18.07	(-47.55, 49.26)	0.05	1.0000
2.5 µM (+)-Control (+)	21.77	13.08	(-13.26, 56.80)	1.66	0.4436
5 µM (-)-Control (+)	-1.63	19.32	(-53.38, 50.11)	-0.08	1.0000
5 µM (+)-Control (+)	-4.51	13.66	(-41.10, 32.08)	-0.33	0.9998
Control (-)-Control (+)	-2.17	19.32	(-53.92, 49.57)	-0.11	1.0000

Individual confidence level = 99.18%

Grouping Information Using the Dunnett Method and 95% Confidence

MeJAtreatment	N	Mean	Grouping
Control (-) (control)	10	10.429	A
2.5 µM (+)	36	34.37	A
2.5 µM (-)	12	13.460	A
Control (+)	30	12.602	A
0.5 µM (-)	10	12.497	A
0.5 µM (+)	30	11.966	A
5 µM (-)	10	10.971	A
5 µM (+)	30	8.097	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.5 µM (-)-Control (-)	2.07	23.66	(-58.81, 62.94)	0.09	1.0000
0.5 µM (+)-Control (-)	1.54	19.32	(-48.17, 51.24)	0.08	1.0000
2.5 µM (-)-Control (-)	3.03	22.66	(-55.25, 61.31)	0.13	1.0000
2.5 µM (+)-Control (-)	23.94	18.91	(-24.72, 72.60)	1.27	0.5929
5 µM (-)-Control (-)	0.54	23.66	(-60.33, 61.42)	0.02	1.0000
5 µM (+)-Control (-)	-2.33	19.32	(-52.04, 47.37)	-0.12	1.0000
Control (+)-Control (-)	2.17	19.32	(-47.53, 51.88)	0.11	1.0000

Individual confidence level = 98.90%

Grouping Information Using the Dunnett Method and 95% Confidence

MeJAtreatment	N	Mean	Grouping
Control (+) (control)	30	8.7067	A
Control (-)	10	7.6700	A
5 µM (-)	10	6.7800	
2.5 µM (+)	36	6.7222	
2.5 µM (-)	12	6.7167	
0.5 µM (-)	10	6.6700	
0.5 µM (+)	30	6.5700	
5 µM (+)	30	5.0800	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.5 µM (-)-Control (+)	-2.0367	0.6582	(-3.7995, -0.2738)	-3.09	0.0151
0.5 µM (+)-Control (+)	-2.1367	0.4654	(-3.3832, -0.8902)	-4.59	<0.0001
2.5 µM (-)-Control (+)	-1.9900	0.6157	(-3.6390, -0.3410)	-3.23	0.0098
2.5 µM (+)-Control (+)	-1.9844	0.4456	(-3.1779, -0.7910)	-4.45	0.0001
5 µM (-)-Control (+)	-1.9267	0.6582	(-3.6895, -0.1638)	-2.93	0.0249
5 µM (+)-Control (+)	-3.6267	0.4654	(-4.8732, -2.3802)	-7.79	<0.0001
Control (-)-Control (+)	-1.0367	0.6582	(-2.7995, 0.7262)	-1.57	0.5066

Individual confidence level = 99.18%

Grouping Information Using the Dunnett Method and 95% Confidence

MeJAtreatment	N	Mean	Grouping
Control (-) (control)	10	7.6700	A
Control (+)	30	8.7067	A
5 µM (-)	10	6.7800	A
2.5 µM (+)	36	6.7222	A
2.5 µM (-)	12	6.7167	A
0.5 µM (-)	10	6.6700	A
0.5 µM (+)	30	6.5700	A
5 µM (+)	30	5.0800	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.5 µM (-)-Control (-)	-1.0000	0.8062	(-3.0738, 1.0738)	-1.24	0.6114
0.5 µM (+)-Control (-)	-1.1000	0.6582	(-2.7933, 0.5933)	-1.67	0.3288
2.5 µM (-)-Control (-)	-0.9533	0.7718	(-2.9389, 1.0322)	-1.24	0.6153
2.5 µM (+)-Control (-)	-0.9478	0.6444	(-2.6054, 0.7098)	-1.47	0.4496
5 µM (-)-Control (-)	-0.8900	0.8062	(-2.9638, 1.1838)	-1.10	0.7117
5 µM (+)-Control (-)	-2.5900	0.6582	(-4.2833, -0.8967)	-3.93	0.0007
Control (+)-Control (-)	1.0367	0.6582	(-0.6566, 2.7299)	1.57	0.3840

Individual confidence level = 98.90%

Grouping Information Using the Dunnett Method and 95% Confidence

MeJAtreatment	N	Mean	Grouping
Control (+) (control)	30	0.97333	A
Control (-)	10	0.87000	A
0.5 µM (+)	30	0.77000	
2.5 µM (+)	36	0.73333	
0.5 µM (-)	10	0.70000	
5 µM (-)	10	0.69000	
2.5 µM (-)	12	0.65833	
5 µM (+)	30	0.59000	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.5 µM (-)-Control (+)	-0.27333	0.07028	(-0.46156, -0.08510)	-3.89	0.0010
0.5 µM (+)-Control (+)	-0.20333	0.04970	(-0.33643, -0.07024)	-4.09	0.0005
2.5 µM (-)-Control (+)	-0.31500	0.06574	(-0.49107, -0.13893)	-4.79	<0.0001
2.5 µM (+)-Control (+)	-0.24000	0.04758	(-0.36743, -0.11257)	-5.04	<0.0001
5 µM (-)-Control (+)	-0.28333	0.07028	(-0.47156, -0.09510)	-4.03	0.0006
5 µM (+)-Control (+)	-0.38333	0.04970	(-0.51643, -0.25024)	-7.71	<0.0001
Control (-)-Control (+)	-0.10333	0.07028	(-0.29156, 0.08490)	-1.47	0.5837

Individual confidence level = 99.18%

Grouping Information Using the Dunnett Method and 95% Confidence

MeJAtreatment	N	Mean	Grouping
Control (-) (control)	10	0.87000	A
Control (+)	30	0.97333	A
0.5 µM (+)	30	0.77000	A
2.5 µM (+)	36	0.73333	A
0.5 µM (-)	10	0.70000	A
5 µM (-)	10	0.69000	A
2.5 µM (-)	12	0.65833	A
5 µM (+)	30	0.59000	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
0.5 µM (-)-Control (-)	-0.17000	0.08608	(-0.39144, 0.05144)	-1.97	0.1899
0.5 µM (+)-Control (-)	-0.10000	0.07028	(-0.28080, 0.08080)	-1.42	0.4817
2.5 µM (-)-Control (-)	-0.21167	0.08242	(-0.42368, 0.00034)	-2.57	0.0505
2.5 µM (+)-Control (-)	-0.13667	0.06880	(-0.31366, 0.04033)	-1.99	0.1857
5 µM (-)-Control (-)	-0.18000	0.08608	(-0.40144, 0.04144)	-2.09	0.1504
5 µM (+)-Control (-)	-0.28000	0.07028	(-0.46080, -0.09920)	-3.98	0.0006
Control (+)-Control (-)	0.10333	0.07028	(-0.07747, 0.28414)	1.47	0.4500

Individual confidence level = 98.90%

6. Spectrophotometry Data and Statistical Analyses

Anthocyanin	Cy3RE (mg/g)	leafTPC	ce (mg/g)	leafDPPH	% Reduction
controlW-1	2.402033467	controlW-1	3.617504052	controlW-1	57.00440529
controlW-2	2.548188943	controlW-2	2.756482982	controlW-2	70.30837004
controlW-3	1.160771023	controlW-3	3.317666126	controlW-3	55.28541226
controlW-4	1.508155052	controlW-4	3.459481361	controlW-4	46.24735729
controlW-5	1.126879898	controlW-5	2.989465154	controlW-5	70.87737844
controlW-6	1.421309045	controlW-6	3.194084279	controlW-6	74.10147992
controlW-7	2.878627409	controlW-7	2.703808752	controlW-7	58.31889081
controlW-8	1.976276213	controlW-8	2.930713128	controlW-8	64.47140381
controlW-9	1.287862741	controlW-9	2.855753647	controlW-9	67.63431542
controlW-10	3.393348867	controlW-10	3.903160454	controlW-10	80.25164114
controlW-11	2.124549883	controlW-11	2.693679092	controlW-11	48.63238512
controlW-12	0.99978818	controlW-12	3.923419773	controlW-12	82.60393873
controlW-13	1.27938996	controlW-13	2.981361426	controlW-13	55.11163337
controlW-14	3.029019276	controlW-14	3.262965964	controlW-14	79.55346651
controlW-15	3.0629104	controlW-15	3.068476499	controlW-15	73.88379205
controlW-16	2.185977547	controlW-16	2.442463533	controlW-16	91.42066421
controlW-17	1.470027537	controlW-17	2.76458671	controlW-17	92.38929889
controlW-18	3.660241474	controlW-18	3.025931929	controlW-18	61.58405172
controlW-19	0.773141284	controlW-19	2.957050243	controlW-19	69.12715517
controlW-20	0.921414954	controlW-20	3.457455429	controlW-20	77.26293103
controlW-21	1.400127092	controlM-1	2.580226904	controlW-21	53.87713998
controlW-22	1.120525312	controlM-2	2.748379254	controlW-22	60.775428
controlW-23	2.452870155	controlM-3	3.102917342	controlW-23	36.3796576
controlW-24	0.385511544	controlM-4	3.327795786	controlW-24	49.17142857
controlW-25	0.470239356	controlM-5	2.726094003	controlW-25	26.85714286
controlM-1	1.084515992	controlM-6	3.291329011	controlW-26	30.82857143
controlM-2	0.576149121	controlM-7	3.309562399	controlM-1	40.88105727
controlM-3	0.275365389	controlM-8	3.396677472	controlM-2	71.76211454
controlM-4	1.253971616	controlM-9	2.541734198	controlM-3	41.80761099
controlM-5	0.665113323	controlM-10	3.60534846	controlM-4	40.80338266
controlM-6	0.843041728	controlM-11	3.348055105	controlM-5	55.28541226
controlM-7	1.417072654	controlM-12	3.591166937	controlM-6	74.36575053
controlM-8	0.728659182	controlM-13	3.238654781	controlM-7	62.78162912
controlM-9	0.626985808	controlM-14	2.961102107	controlM-8	65.3812825
controlM-10	1.033679305	controlM-15	3.248784441	controlM-9	90.29462738
controlM-11	1.306926499	controlM-16	2.803079417	controlM-10	76.03938731

controlM-12	0.699004448	controlM-17	2.98541329	controlM-11	65.9190372
controlM-13	1.406481678	controlM-18	4.026742301	controlM-12	74.78118162
controlM-14	3.005719127	controlM-19	4.111831442	controlM-13	34.31257344
controlM-15	1.328108452	vanillinW-1	1.984602917	controlM-14	60.28202115
controlM-16	2.768481254	vanillinW-2	2.86993517	controlM-15	60.06116208
controlM-17	4.266045329	vanillinW-3	2.193273906	controlM-16	77.72140221
controlM-18	1.408599873	vanillinW-4	2.474878444	controlM-17	74.35424354
controlM-19	1.686083457	vanillinW-5	2.572123177	controlM-18	58.40517241
controlM-20	0.243592459	vanillinW-6	2.300648298	controlM-19	54.63362069
controlM-21	4.369836899	vanillinW-7	2.104132901	controlM-20	70.68965517
controlM-22	3.766151239	vanillinW-8	3.023905997	controlM-21	39.90433031
vanillinW-1	0.872696463	vanillinW-9	2.499189627	controlM-22	52.71903323
vanillinW-2	1.520864224	vanillinW-10	2.630875203	controlM-23	76.13293051
vanillinW-3	0.440584622	vanillinW-11	3.135332253	controlM-24	89
vanillinW-4	1.842829909	vanillinW-12	2.641004862	controlM-25	76.97142857
vanillinW-5	0.796441432	vanillinM-1	2.831442464	controlM-26	69.05714286
vanillinW-6	0.660876933	vanillinM-2	2.584278768	vanillinW-1	38.98678414
vanillinW-7	1.565346325	vanillinM-3	2.448541329	vanillinW-2	45.33039648
vanillinW-8	1.20737132	vanillinM-4	2.882090762	vanillinW-3	44.84581498
vanillinW-9	1.910612158	vanillinM-5	2.636952998	vanillinW-4	49.5243129
vanillinM-1	0.588858293	vanillinM-6	2.768638574	vanillinW-5	48.3615222
vanillinM-2	2.548188943	vanillinM-7	2.811183144	vanillinW-6	45.91966173
vanillinM-3	1.811056979	vanillinM-8	3.046191248	vanillinW-7	47.79029463
vanillinM-4	0.669349714	vanillinM-9	3.672204214	vanillinW-8	51.25649913
vanillinM-5	3.359457742	vanillinM-10	2.936790924	vanillinW-9	53.37954939
vanillinM-6	2.776954035	vanillinM-11	3.376418152	vanillinW-10	40.70021882
vanillinM-7	0.781614065	vanillinM-12	2.819286872	vanillinW-11	53.78555799
vanillinM-8	3.084092353	phenidoneW-1	3.147487844	vanillinW-12	52.07877462
vanillinM-9	2.056767634	phenidoneW-2	3.418962723	vanillinM-1	39.73568282
phenidoneW-1	1.402245287	phenidoneW-3	3.530388979	vanillinM-2	30.74889868
phenidoneW-2	2.200804914	phenidoneW-4	3.117098865	vanillinM-3	30.30837004
phenidoneW-3	1.438254607	phenidoneW-5	3.145461912	vanillinM-4	69.34460888
phenidoneW-4	1.425545435	phenidoneW-6	2.916531605	vanillinM-5	31.71247357
phenidoneW-5	1.080279602	phenidoneW-7	3.040113452	vanillinM-6	23.78435518
phenidoneW-6	1.770811269	phenidoneW-8	2.948946515	vanillinM-7	62.26169844
phenidoneW-7	6.22537598	phenidoneW-9	3.017828201	vanillinM-8	61.6117851
phenidoneW-8	1.086634188	phenidoneW-10	3.329821718	vanillinM-9	44.8440208
phenidoneW-9	1.059097649	phenidoneW-11	3.698541329	vanillinM-10	46.6083151
phenidoneW-10	2.546070748	phenidoneM-1	2.450567261	vanillinM-11	42.77899344

phenidoneW-11	2.118195298	phenidoneM-2	3.418962723	vanillinM-12	42.99781182
phenidoneW-12	1.237026054	phenidoneM-3	2.732171799	phenidoneW-1	68.44036697
phenidoneW-13	0.978606227	phenidoneM-4	3.04821718	phenidoneW-2	63.42507645
phenidoneM-1	0.652404152	phenidoneM-5	3.082658023	phenidoneW-3	39.37562941
phenidoneM-2	0.847278119	phenidoneM-6	3.10089141	phenidoneW-4	57.88016113
phenidoneM-3	0.796441432	phenidoneM-7	3.1636953	phenidoneW-5	47.98590131
phenidoneM-4	1.811056979	phenidoneM-8	3.248784441	phenidoneW-6	40.2
phenidoneM-5	1.27938996	phenidoneM-9	3.21636953	phenidoneW-7	44.85714286
phenidoneM-6	2.34696039	phenidoneM-10	3.978119935	phenidoneW-8	39.74285714
phenidoneM-7	2.709171786	phenidoneM-11	4.223257699	phenidoneW-9	34.14285714
phenidoneM-8	1.347823	mejaW-1	3.291329011	phenidoneW-10	33.08571429
phenidoneM-9	1.129648	mejaW-2	3.242706645	phenidoneW-11	55.68571429
mejaW-1	3.283202711	mejaW-3	2.651134522	phenidoneM-1	54.98470948
mejaW-2	5.214996823	mejaW-4	3.169773096	phenidoneM	38.53211009
mejaW-3	5.475534844	mejaW-5	3.15356564	phenidoneM	28.82678751
mejaW-4	3.805973311	mejaW-6	3.224473258	phenidoneM	22.88519637
mejaW-5	1.24761703	mejaW-7	3.382495948	phenidoneM	26.23363545
mejaW-6	2.107604321	mejaW-8	3.04821718	phenidoneM	69.37142857
mejaW-7	2.736708325	mejaW-9	3.378444084	phenidoneM	61.17142857
mejaW-8	1.368354162	mejaW-10	4.109805511	phenidoneM	72.11428571
mejaW-9	2.937936878	mejaW-11	4.196920583	phenidoneM	60.82857143
mejaM-1	1.520864224	mejaM-1	2.843598055	phenidoneM	87.51428571
mejaM-2	4.251217962	mejaM-2	2.910453809	phenidoneM	89.08571429
mejaM-3	4.48210125	mejaM-3	3.149513776	mejaW-1	41.18831823
mejaM-4	2.721880957	mejaM-4	3.242706645	mejaW-2	76.38469285
mejaM-5	3.077737767	mejaM-5	3.252836305	mejaW-3	39.90433031
mejaM-6	1.446727388	mejaM-6	3.315640194	mejaW-4	60.28571429
mejaM-7	1.60347384	mejaM-7	3.179902755	mejaW-5	70.11428571
mejaM-8	2.937936878	mejaM-8	3.240680713	mejaW-6	81.02857143
mejaM-9	1.60347384	mejaM-9	3.281199352	mejaW-7	73.02857143
		mejaM-10	4.19286872	mejaW-8	52.8
		mejaM-11	4.196920583	mejaW-9	60.05714286
				mejaM-1	75.0755287
				mejaM-2	75.74018127
				mejaM-3	77.59315206
				mejaM-4	83.11428571
				mejaM-5	91.34285714
				mejaM-6	78.85714286
				mejaM-7	87.25714286

				mejaM-8	74.45714286
				mejaM-9	89.05714286

Grouping Information Using the Dunnett Method and 95% Confidence

leaftpc	N	Mean	Grouping
controlW (control)	20	3.11528	A
mejaW	11	3.3499	A
mejaM	11	3.3460	A
phenidoneM	11	3.2422	A
phenidoneW	11	3.21011	A
controlM	20	3.20948	A
vanillinM	12	2.90117	A
vanillinW	12	2.5358	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
controlM-controlW	0.0942	0.1275	(-0.2494, 0.4378)	0.74	0.9745
mejaM-controlW	0.2308	0.1514	(-0.1771, 0.6386)	1.52	0.5401
mejaW-controlW	0.2346	0.1514	(-0.1732, 0.6425)	1.55	0.5215
phenidoneM-controlW	0.1269	0.1514	(-0.2810, 0.5347)	0.84	0.9512
phenidoneW-controlW	0.0948	0.1514	(-0.3130, 0.5027)	0.63	0.9898
vanillinM-controlW	-0.2141	0.1473	(-0.6108, 0.1826)	-1.45	0.5919
vanillinW-controlW	-0.5795	0.1473	(-0.9762, -0.1827)	-3.93	0.0011

Individual confidence level = 99.17%

Grouping Information Using the Dunnett Method and 95% Confidence

leaftpc	N	Mean	Grouping
controlM (control)	20	3.20948	A
mejaW	11	3.3499	A
mejaM	11	3.3460	A
phenidoneM	11	3.2422	A
phenidoneW	11	3.21011	A
controlW	20	3.11528	A
vanillinM	12	2.90117	A
vanillinW	12	2.5358	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
controlW-controlM	-0.0942	0.1275	(-0.4378, 0.2494)	-0.74	0.9745
mejaM-controlM	0.1365	0.1514	(-0.2713, 0.5444)	0.90	0.9304
mejaW-controlM	0.1404	0.1514	(-0.2674, 0.5483)	0.93	0.9208
phenidoneM-controlM	0.0327	0.1514	(-0.3752, 0.4405)	0.22	1.0000
phenidoneW-controlM	0.0006	0.1514	(-0.4072, 0.4085)	0.00	1.0000
vanillinM-controlM	-0.3083	0.1473	(-0.7051, 0.0884)	-2.09	0.2037
vanillinW-controlM	-0.6737	0.1473	(-1.0704, -0.2769)	-4.57	0.0001

Individual confidence level = 99.17%

Grouping Information Using the Dunnett Method and 95% Confidence

leafdpph	N	Mean	Grouping
controlW (control)	26	62.845	A
mejaM	9	81.388	
controlM	26	63.629	A
mejaW	9	61.644	A
phenidoneM	11	55.595	A
phenidoneW	11	47.711	
vanillinW	12	47.663	
vanillinM	12	43.895	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
controlM-controlW	0.784	4.193	(-10.551, 12.119)	0.19	1.0000
mejaM-controlW	18.544	5.847	(2.738, 34.350)	3.17	0.0131
mejaW-controlW	-1.201	5.847	(-17.007, 14.605)	-0.21	1.0000
phenidoneM-controlW	-7.249	5.438	(-21.949, 7.451)	-1.33	0.7017
phenidoneW-controlW	-15.134	5.438	(-29.834, -0.434)	-2.78	0.0403
vanillinM-controlW	-18.950	5.276	(-33.213, -4.687)	-3.59	0.0034
vanillinW-controlW	-15.181	5.276	(-29.444, -0.918)	-2.88	0.0310

Individual confidence level = 99.20%

Grouping Information Using the Dunnett Method and 95% Confidence

leafdpph	N	Mean	Grouping
controlM (control)	26	63.629	A
mejaM	9	81.388	
controlW	26	62.845	A
mejaW	9	61.644	A
phenidoneM	11	55.595	A
phenidoneW	11	47.711	
vanillinW	12	47.663	
vanillinM	12	43.895	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
controlW-controlM	-0.784	4.193	(-12.119, 10.551)	-0.19	1.0000
mejaM-controlM	17.760	5.847	(1.953, 33.566)	3.04	0.0195
mejaW-controlM	-1.985	5.847	(-17.791, 13.821)	-0.34	0.9998
phenidoneM-controlM	-8.033	5.438	(-22.733, 6.667)	-1.48	0.5956
phenidoneW-controlM	-15.918	5.438	(-30.618, -1.218)	-2.93	0.0269
vanillinM-controlM	-19.734	5.276	(-33.997, -5.471)	-3.74	0.0020
vanillinW-controlM	-15.965	5.276	(-30.229, -1.702)	-3.03	0.0202

Individual confidence level = 99.20%

Grouping Information Using the Dunnett Method and 95% Confidence

leafanthocyanin	N	Mean	Grouping
controlW (control)	24	2.7633	A
mejaW	9	3.7454	
mejaM	9	2.4712	A
phenidoneW	13	1.6294	
vanillinW	9	1.4194	
phenidoneM	9	1.1035	
controlM	21	1.00725	
vanillinM	9	0.63946	

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
controlM-controlW	-1.7560	0.2292	(-2.3776, -1.1345)	-7.66	<0.0001
mejaM-controlW	-0.2920	0.2999	(-1.1051, 0.5210)	-0.97	0.9136
mejaW-controlW	0.9821	0.2999	(0.1691, 1.7952)	3.28	0.0098
phenidoneM-controlW	-1.6598	0.2999	(-2.4728, -0.8467)	-5.54	<0.0001
phenidoneW-controlW	-1.1339	0.2642	(-1.8502, -0.4176)	-4.29	0.0003
vanillinM-controlW	-2.1238	0.2999	(-2.9369, -1.3108)	-7.08	<0.0001
vanillinW-controlW	-1.3438	0.2999	(-2.1569, -0.5308)	-4.48	0.0002

Individual confidence level = 99.20%

Grouping Information Using the Dunnett Method and 95% Confidence

leafanthocyanin	N	Mean	Grouping
controlM (control)	21	1.00725	A
mejaW	9	3.7454	
controlW	24	2.7633	
mejaM	9	2.4712	
phenidoneW	13	1.6294	A
vanillinW	9	1.4194	A
phenidoneM	9	1.1035	A
vanillinM	9	0.63946	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
controlW-controlM	1.7560	0.2292	(1.1360, 2.3760)	7.66	<0.0001
mejaM-controlM	1.4640	0.3057	(0.6373, 2.2907)	4.79	<0.0001
mejaW-controlM	2.7381	0.3057	(1.9114, 3.5648)	8.96	<0.0001
phenidoneM-controlM	0.0962	0.3057	(-0.7305, 0.9229)	0.31	0.9999
phenidoneW-controlM	0.6221	0.2707	(-0.1102, 1.3544)	2.30	0.1353
vanillinM-controlM	-0.3678	0.3057	(-1.1945, 0.4589)	-1.20	0.7832
vanillinW-controlM	0.4122	0.3057	(-0.4145, 1.2389)	1.35	0.6826

Individual confidence level = 99.19%

leafpigments	664	648	470
Control-1	0.734	0.409	0.625
Control-2	0.549	0.179	0.395
Control-3	0.316	0.076	0.331
Control-4	0.437	0.314	0.389
Control-5	0.334	0.069	0.24
Control-6	0.194	0.021	0.236
Control-7	0.282	0.069	0.31
Control-8	0.308	0.069	0.332
Control-9	0.296	0.054	0.319
Vanillin-1	0.49	0.15	0.488
Vanillin-2	0.476	0.115	0.436
Vanillin-3	0.698	0.212	0.621
Vanillin-4	0.686	0.208	0.613
Vanillin-5	0.461	0.186	0.44
Vanillin-6	0.576	0.196	0.563
Vanillin-7	0.501	0.155	0.501
Vanillin-8	0.692	0.212	0.62
Vanillin-9	0.683	0.207	0.609
Phenidone-1	0.909	0.31	0.784
Phenidone-2	0.76	0.258	0.649
Phenidone-3	0.674	0.21	0.585
Phenidone-4	0.597	0.151	0.492
Phenidone-5	0.84	0.241	0.738
Phenidone-6	0.93	0.276	0.732
Phenidone-7	0.348	0.083	0.303
Phenidone-8	0.424	0.108	0.341
Phenidone-9	0.877	0.257	0.697
MeJA-1	0.51	0.128	0.5
MeJA-2	0.375	0.117	0.396
MeJA-3	0.412	0.121	0.411
MeJA-4	0.411	0.101	0.408
MeJA-5	0.443	0.099	0.403
MeJA-6	0.654	0.177	0.547
MeJA-7	0.118	0	0.129
MeJA-8	0.215	0.03	0.254
MeJA-9	0.163	0.013	0.195

Grouping Information Using the Dunnett Method and 95% Confidence

Treatment	N	Mean	Grouping
Control (control)	9	0.38333	A
Phenidone	9	0.70656	
Vanillin	9	0.58478	
MeJA	9	0.36678	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
MeJA-Control	-0.01656	0.07943	(-0.21241, 0.17930)	-0.21	0.9936
Phenidone-Control	0.32322	0.07943	(0.12737, 0.51907)	4.07	0.0008
Vanillin-Control	0.20144	0.07943	(0.00559, 0.39730)	2.54	0.0427

Individual confidence level = 98.08%

Grouping Information Using the Dunnett Method and 95% Confidence

Treatment	N	Mean	Grouping
Control (control)	9	0.14000	A
Phenidone	9	0.21044	A
Vanillin	9	0.18233	A
MeJA	9	0.08733	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
MeJA-Control	-0.05267	0.04021	(-0.15181, 0.04647)	-1.31	0.4271
Phenidone-Control	0.07044	0.04021	(-0.02869, 0.16958)	1.75	0.2106
Vanillin-Control	0.04233	0.04021	(-0.05681, 0.14147)	1.05	0.5936

Individual confidence level = 98.08%

Grouping Information Using the Dunnett Method and 95% Confidence

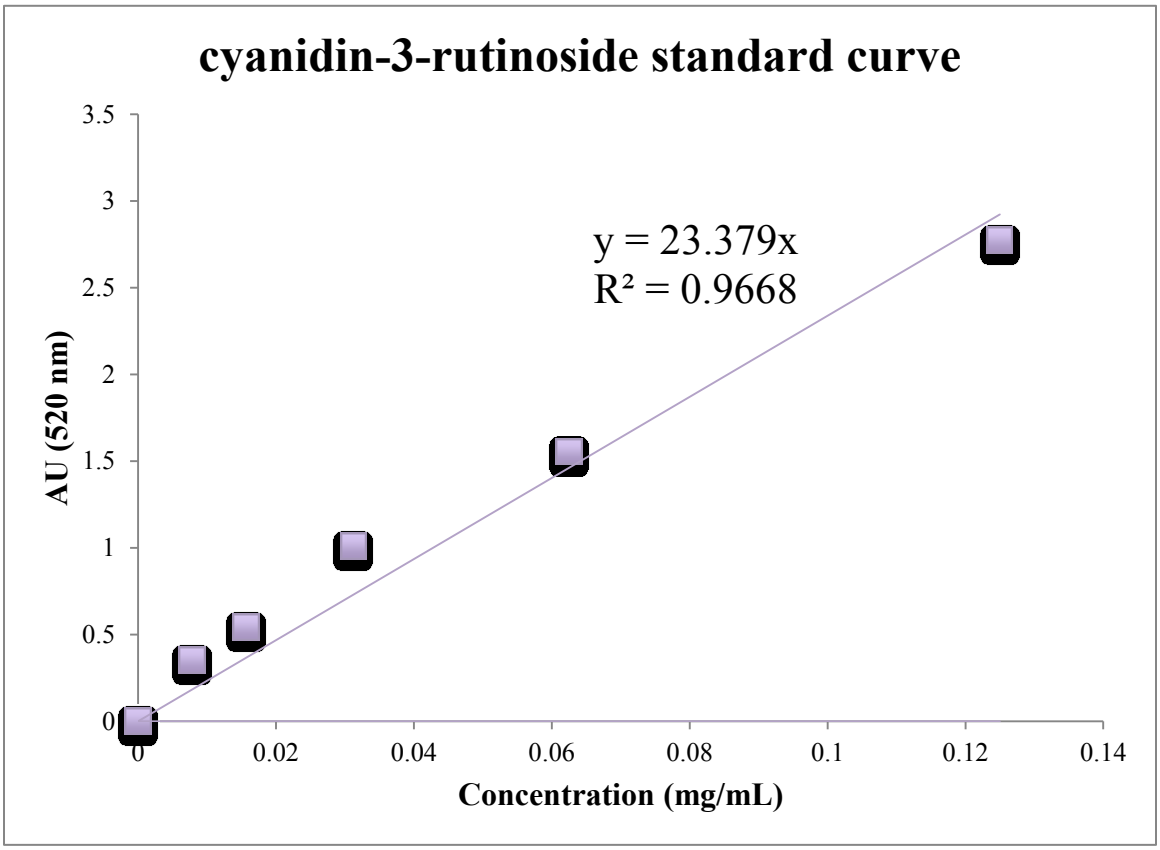
Treatment	N	Mean	Grouping
Control (control)	9	0.35300	A
Phenidone	9	0.59122	
Vanillin	9	0.54344	
MeJA	9	0.36033	A

Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
MeJA-Control	0.00733	0.06231	(-0.14629, 0.16096)	0.12	0.9988
Phenidone-Control	0.23822	0.06231	(0.08460, 0.39185)	3.82	0.0016
Vanillin-Control	0.19044	0.06231	(0.03682, 0.34407)	3.06	0.0123

Individual confidence level = 98.08%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R² = 0.9668
AU (520 nm)
Concentration (mg/mL)
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3
2.5
2
1.5
1
0.5
0
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14