Characterisation of volatile constituents of six native New Zealand ferns and changes in volatile emission in response to herbivore, mechanical wounding and phytohormone treatments.

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Abstract

Evolution has led to the development of countless defence strategies in terrestrial plants to deal with the threat of herbivory and disease. The production of specialised morphological structures, such as thorns and trichomes, is a prominent defence mechanism that directly deters potential herbivores. However, plants are capable of enlisting the aid of natural predators and parasites of attacking herbivores as a means of indirect defence, through the production and release of volatile organic compounds. This has prompted much research into the regulation and ecological roles of volatile organic compounds in many higher plant groups. However, similar studies are seldom in lower plants such as the Monilophytes thus we know very little concerning the ecological importance of plant emitted volatiles in this group. In this study, I investigated the volatile compounds released by six abundant native fern species using direct solvent extraction and headspace collections, and characterized the volatile emissions under natural herbivory, phytohormone treatment, and mechanically induced stress. Solvent extracts and headspace collections were analysed using gas chromatography coupled with mass spectrometry allowing the quantitative and qualitative description of the volatile profiles. These results were then used to relate volatile emission to the growth mode and other potential defence strategies of these species. A total of 15 volatile compounds were identified over the course of this thesis with links to fern physiology. Quantitative results revealed no differences in emissions under phytohormone treatment or artificially induced stress. The comparison of two methods, solvent extraction and headspace sampling, reveals the limitations the solvent extraction method has on elucidating fern-insect interactions. Research on fern volatiles could give insight into the evolution of anti-herbivore defence mechanisms in plants and the interactions between native ferns and arthropod communities. Potential applications of research in fern chemistry include pharmaceutical or perfumery uses and fern conservation, which should be incentives for further work. The results and conclusions made from this thesis does not only contribute to the limited pool of knowledge in this field of research but may also serve as a foundation for future studies.

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List of Abbreviations

VOC -	Volatile Organic Compounds
CT -	Control
JA -	Jasmonic Acid
PD -	Mechanical Damage
W –	Weta
PVH -	Passion Vine Hopper
GC-MS -	Gas Chromatography–Mass Spectrometry
AB -	*Asplenium bulbiferum*
AO -	*Asplenium oblongifolium*
CD -	*Cyathea dealbata*
CM -	*Cyathea medullaris*
DS -	*Dicksonia squarrosa*
MP -	*Microsorum pustulatum*

Chapter 1 : General Introduction and Literature Review

Plant Secondary Metabolites

Metabolites are the products and intermediates of metabolism, a process cells within all living organisms undergo, and is usually restricted to small molecules. Metabolites have various functions including the conversion of food into energy, stimulation/inhibition of enzymes and defence (Demain & Fang, 2000; Vining, 1990). They also play roles in interactions between other organisms such as their importance in the production of odours and pheromones (Hook et al., 1991; Stacey, 2003). Countless years of modern biochemistry have separated metabolites into two separate categories, primary and secondary, according to their involvement in the development of living cells. Primary metabolites are described as having roles in basic life functions such as growth, respiration, cell division and reproduction, whereas secondary metabolites are not directly involved in these processes but usually serve important ecological functions (Bourgaud et al., 2001).

The concept of secondary metabolites can be attributed to Stahl (1888) who proposed the name 'Schutzexkrete' for essential oils, alkaloids, tannins and other secondary metabolites. Kossel (1891) then defined these as separate to primary ones. It was not until research by Czapek (1921) who dedicated a volume of his 'plant biochemistry' series to what he called 'endproduckt', that secondary metabolites became widely recognized.

Unlike the main molecules found in plants, secondary metabolites were then characteristically defined by their low abundance - less than 1% of total carbon occurring in organs or dedicated cells (Rao & Ravishankar, 2002). Through the rapid improvement and development of analytical techniques, such as chromatography, more and more of these molecules could be recovered and as a result, phytochemistry established as a discipline during the 20th century.

Although paper chromatography initially identified some molecules to be pigments, other functions of these secondary compounds within plants remained unknown. However, with the continuous improvement of biochemical techniques and the birth of modern molecular biology, these secondary products have been shown to evidently play a role in how plants adjust and interact with their environment (Bourgaud et al., 2001). Secondary metabolites typically arise as offshoots from the pathways of primary metabolism (Geissman & Crout, 1969). They can be classified according to their composition (e.g. contain nitrogen), chemical structure (e.g. rings) but usually they are classified according to their biosynthetic pathways (Rosenthal & Berenbaum, 2012).

A few words of clarification must be said before we proceed, the term 'secondary metabolites' is rather misleading and may cause many misunderstandings amongst those unfamiliar with chemical ecology. This term is rather vague to begin with as it indiscriminately covers a very wide range of unrelated compounds and implies an unimportant, 'secondary' role for them. As we begin to increase our understanding and knowledge regarding secondary metabolites, the previously held belief that they were merely just functionless molecules or waste products resulting from 'mistakes' of primary metabolism has become a largely inaccurate and misguided view of these compounds. Rather many of these 'secondary' products are key components of active and potent defence mechanisms and are also significant components in signalling molecules (Robbins, 2000).

Although this way of thinking is relatively new and has only recently gained traction amongst the scientific community, many have come to the consensus that these products are part of a long-standing evolutionary arms race in a sort of 'chemical warfare' fought between plants and their pests and pathogens as proposed several years earlier by Bennett and Wallsgrove (1994).

Role and Importance

In higher plants, secondary metabolites contribute to the specific tastes, odours and colours observed in plants (Bennett & Wallsgrove, 1994), and are crucial to the human industry as they are unique sources for pharmaceutical products, food additives, cosmetics and multiple other products (Ravishankar & Rao, 2000). In the environment, these compounds are key to plant survival and many have several important ecological functions. These functions can be condensed into three distinct roles: 1) They serve as attractants for pollinators, seed-dispersing animals and herbivores (Cipollini & Levey, 1997; Kessler & Baldwin, 2007); 2) Protect plants against biotic and abiotic stressors (Bartwal et al., 2013; Edreva et al., 2008); and 3) Function as agents of plant-microbe symbiosis and mediate plant-plant communication (Bais et al., 2004; Reichling, 2010).

By nature, plants are immobile organisms vulnerable to their surrounding environment and must therefore adapt in order to survive. To compensate for their inability to flee from or fight against incoming threats, plants have evolved unique defence mechanisms to cope with herbivores and pathogens. These mechanisms are often divided into direct and indirect defences. Direct plant defences (direct resistance) refer to traits that act upon the attacker directly (Gols, 2014) and include the production of specialized morphological structures (e.g. thorns, spines, thicker leaves, trichomes etc.) and chemical substances (e.g. alkaloids,

phenolics, cyanogenic glucosides, etc.) (Chen, 2008) to discourage herbivores from causing physical damage or eliciting physiological reactions in the plant (War et al., 2012).

In contrast, indirect defences promote the efficiency of natural enemies to control plant antagonists (Heil, 2008) and include the release of volatile organic compounds (VOC's) from flowers, leaves and fruits into the immediate atmosphere (Maffei, 2010) as well as from the roots (Rasmann et al., 2005) into the soil to attract the natural enemies of their attackers (Dicke & Sabelis, 1987).

Classification

Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups: phenolics, terpenoids and nitrogen-containing compounds. Each group is chemically distinct from one another and are unique components of defence mechanisms in plants (Bennett & Wallsgrove, 1994). However, that is not to say that other minor chemical groups are less important to plant physiology and fitness but rather, the diversity and broadness of these classes would be of more use to analyse when there is much left yet to understand regarding fern physiology.

Terpenes (terpenoids), make up the largest class of secondary metabolites and are generally insoluble in water as they consist mostly of resins and essential oils (Singh & Sharma, 2015). Although the vast majority of terpenes are secondary compounds likely involved in plant defences, there are a select few compounds directly involved in plant growth or development and as such can be considered primary rather than secondary metabolites (e.g. gibberellins, brassinosteroids) (Campbell & Reece, 2002). Terpenes have been shown to be key factors in the resistance of several pathogens and insect pests (Harborne, 2014). For example, in conifers, monoterpenes accumulate in resin ducts found in the trunk, needles and twigs (Franceschi et al., 2005) and are toxic to the bark-beetle species that infest these plants. However, this is entirely dependent on the conifer and beetle species in question, observations of these monoterpenes across related species show that it can act as both a repellent and attractant of herbivore pests suggesting a co-evolutionary arms race between the beetles and plant.

Terpenes are also prime examples of how plants indirectly defend themselves against herbivores via enhancing the effectiveness of the natural enemies of the herbivores. Research on cotton plants by Rodriguez-Saona et al. (2003) demonstrate that, in the absence of

competing herbivores, natural enemies respond to both the volatile and non-volatile emissions of terpenoid compounds emitted by the beet armyworm through herbivory.

Phenolic compounds are appropriately defined by their characteristic phenol group: a hydroxyl functional group on an aromatic ring. Plant phenolics are a ubiquitous, chemically heterogeneous group with a majority of compounds expressing a diverse range of chemical properties (Hopkins & Huner, 2009).

Like terpenes, phenolics function both to attract and repel different organisms within the plants surroundings. Phenols are capable of acting as inhibitors, protective agents, pesticides and natural animal toxicants against attacking organisms (such as herbivores and bacterial/fungal pathogens) (Bhattacharya et al., 2010). Examples of phenolics acting as inhibitors of enzymes and feeding deterrents of herbivores are documented (Cheeke, 1989), however evidence in their role in resistance against bacteria and fungi pathogens are more pronounced (Bennett & Wallsgrove, 1994). For instance, the production of certain phytoalexin (anti-microbial) compounds (polyphenols) against the growth of fungal pathogens in two grapevine species (Favaron et al., 2009; Timperio et al., 2012) emphasizes the dynamic role which phenolic compounds possess in the deterrence of microbial organisms compared with that of plant-herbivore stress.

Phenolics are also recognised as allelochemicals for competitive plants and weeds (Xuan et al., 2005). Some phenolic metabolites (e.g. salicylic acid) are phytotoxic and can accumulate in the soil and subsequently inhibit seedling growth and seed germination of nearby plants (Baleroni et al., 2000).

Nitrogen-containing compounds are as common as the previous classes of metabolites. Alkaloids and cyanogenic glycosides in particular are of significant interest not only due to their effectiveness as anti-herbivore defences (Schardl & Chen, 2010) but also because of their medicinal properties and toxicity to humans. These compounds are mostly products of common amino acids (Lazar et al., 2003). It was initially assumed that alkaloids were nitrogenous wastes (i.e. urea and uric acid) however there was very little evidence in support of these hypotheses and ultimately refuted (Meyers, 1987). However, although some believe it to function as defence against herbivores due to their toxicity and general deterrence capability (Hopkins & Huner, 2009) many have yet to agree since there are areas concerning alkaloids left unclear (Aniszewski, 2015).

It has long been known that alkaloids have some effect on herbivorous insects, studies dating back almost four decades (Hesse, 2002) have demonstrated that alkaloids do play a role in plant-insect resistance. For example, increasing concentrations of alkaloids by *Solanum* (potato genus) were positively correlated with reduction in leaf hopper infestation, a common pest (Sanford et al., 1990). Similarly, in barley, concentrations of the alkaloid gramine decreased as the plant matured, this decrease in alkaloid concertation was directly correlated with increases in the rate of survival and feeding of aphid pests suggesting a deterrence function of gramine in barely seedlings (Zúñiga et al., 1986).

Plant Volatiles and their Ecological Roles

As discussed in the previous section, plants can produce a wide array of metabolic compounds some of which are volatile. There is substantial evidence showing that higher plants are capable of producing a large variety of volatile compounds (Knudsen et al., 1993). Volatile compounds generally consist of lipophilic substances with high vapour pressures capable of crossing membranes freely in the absence of a diffusion barrier (Pichersky et al., 2006). Plant volatiles are usually complex mixtures of the chemical compounds produced by the plant (Baldwin, 2010) and the composition and intensity of the volatile blends can carry vital information concerning the physiological status of the emitting plant and the biotic and abiotic stresses it is experiencing (Holopainen & Gershenzon, 2010; Vickers et al. 2009).

The availability of elements such as carbon, sulphur and nitrogen in addition to the energy provided through primary metabolism dictate the biosynthesis of volatile organic compounds (VOC's) within the plant (Vallarino et al. 2018). Depending on the chemical structure of these compounds, plant volatiles can be classed into alcohols, aldehydes, esters, ethers, hydrocarbons and ketones. However, finer classifications of these volatiles can also be done based on their biosynthetic origin and are generally divided into terpenoids, fatty acid derivatives, amino acid derivatives and benzenoids/phenylpropanoids.

Terpenoids (isoprenoids) and other steroids are products of three biosynthetic pathways; mevalonate, methylerythritol phosphate and Deoxyxylulose phosphate (Eisenreich et al., 2001), fatty acids and polyketides come from the acetate pathway, and aromatic amino acids and phenylpropanoids originate from the shikimate (shikimic acid) pathway (Dewick, 2002; Ribera & Zúñiga, 2012; Tzin & Galili, 2010).

Although these compounds are generally found in relatively small amounts when compared to the total weight of the plant, collectively they possess important ecological functions (Rosenkranz & Schnitzler, 2016). They are primarily associated with plant indirect and direct defences by either attracting the natural enemies of herbivores (Paré & Tumlinson, 1999) or by acting as feeding and/or oviposition deterrents (Arimura et al., 2009). For example, many volatile terpenes emitted by plants are non-specific toxins which are active against a range of organisms (e.g. bacteria, animals) (Rosenkranz & Schnitzler, 2016).

Plant-emitted volatiles mediate communication between plants and other organisms. Seed dispersers and pollinators are attracted by volatile compounds thus increasing the reproductive advantage of the plant (Mumm & Dicke, 2010; Pichersky & Gershenzon, 2002). Ironically, as a product of coevolution, certain phytophagous insects are also capable of using volatiles to locate suitable host plants and mates (Landolt & Philips, 1997; Reddy & Guerrero, 2004) yet, in most cases, plant volatiles primarily function as an indirect defence mechanism against them.

Changes in the emission of VOC's as a result of herbivore damage attract natural enemies (predators and parasites) of feeding herbivores to the plant (Turlings et al., 1995). Depending on the type of stress and the plant species involved, quantitative and qualitative differences between volatile bouquets help herbivore enemies discriminate between status of host plants (Olson et al., 2009) and herbivore species (De Boer et al., 2004; De Moraes et al., 1998; McCormick et al., 2012). These tritrophic interactions involved in plant defence and the intricate mechanisms (Mercke et al., 2004) behind them illustrate the complex role plant secondary metabolites serve in plant-insect relations.

Plant volatiles have a variety of other functions in their environment. Plant volatiles do not only reduce the amount of attacking herbivores (Kessler & Baldwin, 2001) through typical plant-parasite/predator interactions but can also alert neighbouring plants (both conspecifics and heterospecifics) of any immediate herbivore or pathogen danger (Shulaev et al., 1997). Volatiles can induce the expression of defence genes in neighbouring plants (Arimura et al., 2000) or prepare these plants to respond more efficiently to attacks in the future (Kessler et al., 2006). Certain plant-emitted volatiles are also capable of adhering to neighbouring heterospecific plants and subsequently affect their interactions with their environment (Himanen et al., 2010).

In addition to the examples and literature mentioned previously, there have been countless others which have demonstrated the use of plant-emitted volatiles as an indirect mechanism where plants make use of the natural enemies of herbivores to serve as 'bodyguards' (Takabayashi et al., 1991; Van Poecke & Dicke, 2004). Although the effect herbivore-induced plant volatiles have on attacking herbivores remains more or less unchallenged in the current literature, while some agree plant fitness is hugely affected by the ecological functions of their secondary metabolites (Hopkins & Huner, 2009; Lazar et al., 2003); others remain unclear whether the release of these volatiles have any net fitness gain for the plant. Therefore, more studies into the evolutionary ecology of herbivore induced volatiles are required in order to fully grasp these mechanisms (Dicke & Baldwin, 2010).

Secondary Compounds in Ferns

This review touches briefly upon a small number of studies that provide evidence of secondary metabolites used as successful defence strategies in higher plant groups. Unfortunately, I have yet to come across publications that directly investigate the effect secondary metabolites have on the defensive capabilities in the fern taxa after extensively probing the literature. The most recent review on the potential biological functions of secondary metabolites in ferns by Vetter (2018) provides a comprehensive summary on the chemical and biochemical aspects and uses but only briefly addresses their role in plant defence. The production of cyanogenic glycosides to work against biological attackers and the effectiveness of tannin molecules as deterrence to phytophagous insects were reported in this review.

Other reviews have investigated fern phytochemicals such as that of Cao et al. (2017) in which they provide an extensive summary of chemicals found in an array of fern species throughout the world. Though they note evidence suggesting potent antimicrobial activity in multiple studies and the toxicity of bracken ferns from sesquiterpene glycosides, their conclusions and discussions concerning fern chemicals revolve around the potential medicinal and pharmaceutical applications. However, Cao et al. (2017) also briefly report a case were methanol fern extracts were successful as an insecticide but do not elaborate further.

Similar studies have shown evidence which suggests that secondary metabolites of fern species *Lygodium venustum* having antibiotic uses in chemotherapy though nothing has been investigated into their effectiveness against plant pathogens (Morais-Braga et al., 2012). Although not directly attributed to secondary compounds, nectar secretion of fern species *Pleopeltis crassinervata* were associated with lower levels of herbivore damage (Koptur et al., 2013). It is obvious from gleaning through the literature that recent research regarding fern

phytochemicals are led by medical and pharmaceutical journals mainly as novel treatments or approaches to microbial studies (Khan & Ullah, 2018; Nath et al., 2018; Ullah et al., 2018). Older research such as that by Soeder (1985) only provides a comprehensive survey of fern constituents and does not give any insight as to their ecological importance.

Compared to non-volatile secondary compounds, there is noticeably more literature directly concerning fern-emitted volatile organic compounds. Although there are some which do not investigate the ecological importance of these volatiles (Froissard et al., 2011), there are a handful which investigate the relationship between these volatiles and herbivory. Surprisingly, there is evidence (Imbiscuso et al., 2009; Kessler et al., 2015) that certain fern species are able to release herbivore-induced volatiles, however, both studies did not directly measure the effect these volatiles had on the herbivores and herbivore activity.

Imbiscuso et al. (2009) observed an 'oxidative burst' in the fern species *Pteris vittata* and the subsequent emission of volatile terpenoids following herbivore damage which is a trend many other higher plants express upon herbivory. Similarly, Kessler et al. (2015) in their investigation of six related species of fern; *Melpomene firma, Melpomene wolfii, Alansmia laxa, Cochlidium serrulatum, Lellingeria subsessilis,* and *Mycopteris taxifolia*, reported compounds that were well known among angiosperms as herbivore deterrents, particularly the mono- and sesquiterpenes.

This is not to say there is no evidence against the use of volatiles as defence mechanisms in ferns. Radhika et al. (2012) tested the volatile emission following herbivory of two different species (one generalist, one specialist) on the bracken fern *Pteridium aquilinum* and concluded that the jasmonic acid levels in both species were not sufficient enough to elicit volatile emissions from the fern. However, it is important to know that the current amount literature concerning this topic is insufficient and inadequate to make solid conclusions about the defence strategies in ferns and thus further research into the ecology of fern volatiles is needed.

Fern Insect Interactions

Most major groups of vascular plants, to some degree, are used by phytophagous insects as a source of food and ferns are no exception. However, there is relatively little information in the literature on fern-insect interactions. The most recently relevant studies broadly discussing this topic were written over three decades ago (Auerbach & Hendrix, 1980; Balick et al., 1978; Cooper-Driver, 1978; Hendrix, 1980). Since these publications there has been a paucity of studies building onto their work and only very few publications on specific fern-arthropod interactions over this time.

It is a widespread belief that ferns have few insect attackers. Schneider (1892) was the first to describe the relatively poor number of insects feeding on ferns compared to flowering plant and conifers. The phenomenon was reiterated again by Brues (1920) and challenged soon after by Swezey (1922) through his observations in the Hawaiian Islands though his conclusions were later called into caution (Hendrix, 1980). While over the years other authors repeatedly reported poor herbivory on ferns (Hoo & Fraenkel, 1964; Kaplanis et al., 1967), these reports were generally based on anecdotal evidence. Balick et al. (1978) was one of the first to systematically survey the literature to solve the long-held observation as well as the earliest to suggest a possible coevolution between arthropod and ferns prior to and after the radiation of angiosperms. Hendrix (1980) conducted a similar survey of the literature and additionally compared this to Balick et al. (1978) and concluded that the total number of insects that utilized ferns as a food source was significantly fewer than expected but suggested that the amount of insects recorded to have fed on ferns were not a representative sample of phytophagous insects as a whole thus emphasising the need for more studies. Hendrix (1980) was also one of the first to highlight the potential importance of chemical defences and ecological characteristics in fern-insect interactions.

Flaws regarding the age-old generalization that only few arthropods feed on ferns were pointed out by Auerbach and Hendrix (1980) suggesting comparisons to angiosperms were invalid due to biases involving plant growth form and unequal sampling efforts and reiterated again by Hendrix and Marquis (1983) and by Mehltreter and Tolome (2003) in observations on herbivore damage in tropical ferns, which are more diverse than those in the temperate regions of the Northern hemisphere, where the majority of reports originated.

Studies have shown that ferns have evolved adaptations in response to predators, such as the release of nectar from the fronds to recruit ants (Tempel, 1983), the relation between fern-feeding moths and fern phylogenies (Weintraub et al., 1995), and changes in the sporing season to avoid predation by spore feeders (Sawamura et al., 2009).

There is also ample fossil evidence suggesting a long history of interactions between insects and ferns. Examples include the use of insect vectors for seed fern pollen dispersal in the Upper Carboniferous (Scott & Taylor, 1983), pteridophyte spore predation all throughout the Palaeozoic (Scott et al., 1985), and direct defence mechanisms against herbivory by a seeded fern in the Late Carboniferous (Krings et al., 2003). Together, this evidence supports a standing relation between insects and ferns and prompts for further investigation.

Methods for Volatile Collection and Analysis

Recently, interest into the ecology and biochemistry of plant volatile organic compounds has increased, leading to the development of a variety of techniques and systems for the collection and analysis of volatiles (Linskens & Jackson, 1997). Through the design of sensitive and relatively inexpensive bench-top instruments for gas chromatography-mass spectrometry (GC-MS), volatile analysis over the last couple decades has improved quite significantly. Traditionally, methods such as steam distillation or solvent extractions were used (Veith & Kiwus, 1977) to acquire the volatile compositions of living plants, however, with the rise in developed headspace analysis techniques providing more representative volatile profiles, the former methods of analyses are, although still practiced (Ormeno et al., 2011), less used in modern volatile analyses.

The methods and techniques discussed in this section will primarily overview the practical approaches to volatile analyses published by Tholl et al. (2006). This is because it is currently the latest review regarding the practices involved with plant volatile analyses. Thus, if further detail is required, it is worthwhile reading this publication as each technique is described in more detail there.

There is no single, widely accepted method for acquiring plant volatiles but rather, the most suitable system and techniques that are required are usually dependent on the plant material/organ and biological question being investigated (Tholl et al., 2006). For example, whether analyses of volatiles are done on plants under laboratory or field conditions will result in completely different sampling techniques and use of instrumentation, each having their respective advantages and disadvantages. Field systems will therefore be more simplified and

have been modified for portable volatile collections, whereas laboratory systems allow for more precision and manipulation using intricate set-ups and additional devices to control for other variables. Whether the experiment requires a 'snapshot' of the volatile profile released or whether developmental or stress related changes need to be measured must also be considered during experimental design (Tholl et al., 2006).

Solvent extraction is one of the oldest, more destructive methods of volatile sampling (Harborne, 1998) but recently, the investigation into the airspace (headspace) surrounding above-ground plant material, is being adopted by many studies (Bicchi et al., 2008; Fan & Almirall, 2014; Ha et al., 2014) due to their non-destructive and more realistic volatile profiles. There are two main headspace sampling techniques commonly used throughout the literature: static and dynamic headspace sampling, each with their own strengths and limitations (Tholl et al., 2006).

Analysis of the static headspace involves enclosing the entire plant (or parts of it) into a container and subsequently extract volatiles using an adsorbent material. This technique does not circulate air within the chamber, thus static, disallowing impurities from a continuous airstream mixing in. The recent development of solid phase microextraction in this sort of system allows for the sampling of volatiles at the 'parts per billion' level to become practical and efficient (Tu et al., 2014). In contrast, dynamic headspace sampling uses a continuous air stream and is the most frequently used technique in volatile analysis. There are two systems associated with this sampling technique: closed-loop stripping and 'push'/'pull' systems (Tholl et al., 2006). Closed-loop systems circulate the air in closed chambers whereas 'push'/'pull' systems use a constant flow of air taken up from the outside and leaves the system with the volatiles in tow.

These sampling methods trap plant volatile organic compounds on adsorbent material and are then routinely analysed by the standard technique of Gas-Chromatography (GC). For this review, it is not practical to discuss the standard protocols used in GC analysis but there are publications within the literature (Dewulf et al., 2002) who have. For GC analysis, plant volatiles are either injected into the heated injector as solvent extracts or desorbed from the adsorbent via a thermal desorption tube (Tholl et al., 2006). Mass spectrometry (MS) detectors are the most widely used detectors in GC analysis of plant volatiles and are crucial in obtaining ion chromatograms. Once compound profiles have been thoroughly analysed using the GC-MS, identification of these compounds are deduced by comparing the results to the mass spectral data from popular databases (e.g. NIST MS). Correct identification of these compounds can

simply be done using retention indices or similarity comparisons with library mass spectral data (Tholl et al., 2006).

Research Objectives

Much of what we know regarding the regulation and ecological function of volatile organic compounds comes from studies of higher plants, however, very little is known about their function in lower plant groups such as ferns. This research will help elucidate ecological interactions involving native ferns and their associated arthropod communities as well as give insight into the evolution of anti-herbivore defence mechanisms in plants.

The purpose of this research is to characterise the volatile emissions of six New Zealand ferns and to investigate whether these ferns emit volatile organic compounds as a response to damage and whether this emission is related to other forms of direct defence (i.e. phenolics). I further elaborate on whether they can differentiate between herbivore damage, phytohormone treatment and mechanical wounding. Overall, this study aims to increase the information on the subject and serve as a foundation for future studies in this topic.

The thesis addresses the following objectives:

1) To characterize the volatile emission of six native fern species

2) To investigate changes in volatile emission of six native fern species under three treatments: herbivore damage, phytohormone treatment and mechanical wounding

3) To compare the volatile emissions between treatments and establish if qualitative or quantitative differences occur

4) To establish volatile emission correlates with mode of growth (tree, epiphytic or shrub) or other potential defence traits such as phenolics

Chapter 2 : Materials and Methods

Study Site and Fern Species

The ferns used in this study were found naturally growing in two vegetative areas within the Manawatu Massey University campus (see **Fig.2-1**) during the last two months of 2016 and first two months of 2017. The following six fern species were used for volatile analyses: silver tree-fern (*Cyathea dealbata*); black tree-fern (*Cyathea medullaris*); rough tree-fern (*Dicksonia squarrosa*); hound's tongue-fern (*Microsorum pustulatum*); hen and chicken fern (*Asplenium bulbiferum*); and the shining spleenwort (*Asplenium oblongifolium*) (see **Fig.2-2**).

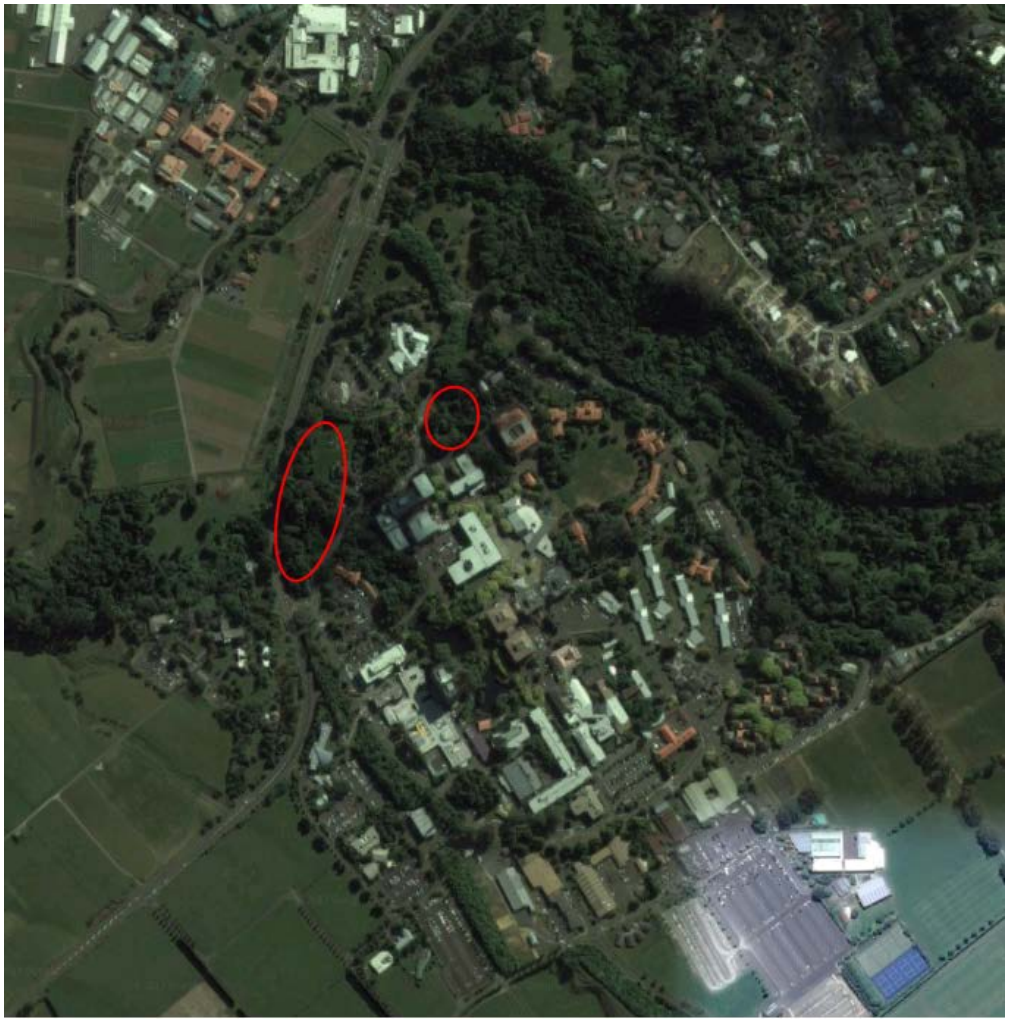


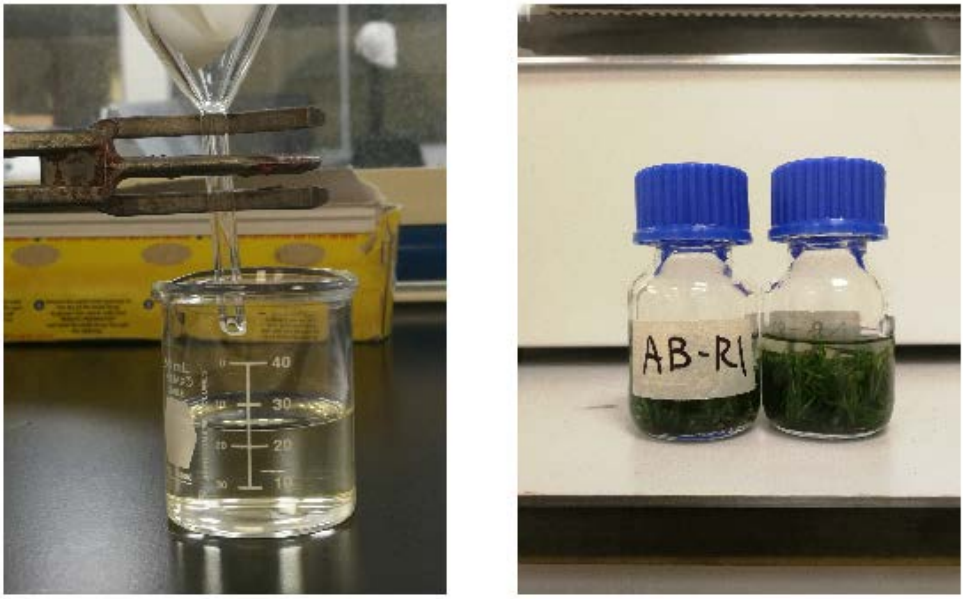
Figure 2-1. The location of vegetative areas (red circles) within Massey University (Google Earth).

Only individuals with relatively small damage to their fronds were selected for treatments, minor lacerations and sunburn to fronds were ignored. Ferns in positions where high environmental stress may potentially occur, such as high exposure to sunlight or wind, were avoided. Vegetative material used for volatile collections was undamaged and free of invertebrate activity.

Figure 2-2. Native fern species used for volatile analysis; (a) *Dicksonia squarrosa*, (b) *Microsorum pustulatum*, (c) *Asplenium bulbiferum*, (d) *Asplenium oblongifolium*, (e) *Cyathea dealbata*, (f) *Cyathea medullaris.*

Solvent Extraction

To characterise the volatile compounds of the six species, a solvent extraction method was initially used. For this purpose, fresh aerial parts of ferns were collected from the same vegetative areas of Massey University mentioned previously. Parts from the ferns species: *C. dealbata*; *C. medullaris*; *D. squarrosa*; *M. pustulatum*; *A. bulbiferum*; and *A. oblongifolium* were used for this analysis. Fresh plant material from over five individuals of each species were cut into small pieces and accurately weighed to 3 grams. The samples were extracted using a 30 mL solution of high grade hexane (CAS 110-5-3, ≥99%, Sigma Aldrich, St Louis, MO, USA) and a known concentration of nonyl acetate ($C_{11}H_{22}O_2$) (10ng/mL) in a sealed container (**Fig.2-3**) for 72 hours in a climate-controlled room (22°C). During this extraction process the samples were systematically shaken every 24 hours to increase the efficiency of extraction. Extracts were then filtered through 70mm glass microfiber filters and again using 0.2µm syringe filters before GC-MS analysis. The experiment was conducted in triplicate.



a) b)

Figure 2-3. Examples of solvent extraction process: (a) initial filtering process using 70mm glass microfiber filter, (b) sealed containers containing fern material submerged in solvent/internal standard solution.

Phytohormone and Mechanical Damage

To test the effect of different treatments of volatile emission, a dynamic headspace volatile collection was carried out on site, using the same species described above. This method was selected since it was non-destructive allowing to investigate changes over time. I submitted each fern species to the following treatments 1) mechanical wounding; 2) mechanical wounding plus phytohormone (jasmonic acid), and 3) control (no applied damage). Jasmonic acid was used since it is the main phytohormone involved in plant anti-herbivore responses and it is known to elicit herbivore-induced volatiles (Hopke et al., 1994).

Each treatment consisted of 5 replicates per fern species and had a total volatile collection time of 3 hours during the same time of day (10:30-13:30) for each measurement. Mechanical wounding of the plant material was conducted via standardised (30cm) linear tissue scarring with a razor blade on the adaxial surface of the fronds. Application of the phytohormone jasmonic acid was done using a spray bottle containing a 10mg/L solution of the compound diluted in MiliQ H_2O. Proceeding the tissue scarring of the plant material, 1mL of the solution was sprayed evenly across the artificially damaged portions of the fronds prior to headspace collection.

For VOC collection, the fronds were bagged using unused commercial oven bags enclosing roughly 20-40 cm of the fronds (see. **Fig.2-4**). Collection of volatiles were conducted in the field using a portable volatile assay system (PVAS22, Volatile Assay Systems, Rensselaer, NY, USA) coupled with Teflon and silicon tubing for dynamic (push-pull airflow) headspace collections. A 90 'push' and 80 'pull' airflow scheme was set to create positive pressure within the system. Volatile organic compounds were collected using an adsorbent (HayeSep Q, Volatile Assay Systems, Rensselaer, NY, USA) filter at the 'pull' portion of the system. All collections were conducted on dry, sunny weather conditions to minimise environmental influence on volatile release. Sampled plant material subsequently had their fresh weight measured immediately following volatile collection and dry weight after a minimum of 72 hours of drying in an 80°C oven.

Figure 2-4. Example of a field experimental setup of the dynamic volatile headspace collection system using a PVAS22 unit on *Dicksonia squarrosa*.

The HayeSep Q filters were eluted using 200µL of high grade pentane (CAS, 109-66-0, ≥99%, Thermofisher Acros Organics, Geel, Belgium) and a 1ng/mL nonyl acetate internal standard solution. A 10ng/mL concentration of the internal standard was used for 1 replicate in the control treatment for optimization purposes. Volatile solutions were then stored in -80°C temperatures and briefly thawed prior to GC-MS analysis.

Polyphenol & Chlorophyll Collection

Analysis of polyphenol and chlorophyll content were conducted using a Dualex Scientific+™ (Force-A, Paris, France) sensor. A total of 10 measurements were randomly collected from undamaged leaf material across each replicate of fern species. The embedded datalogger was used to acquire the flavonol, anthocyanin, chlorophyll and nitrogen balance measurements.

Herbivore Damage

Assessment of the effect herbivore damage have on fern volatile emissions were tested on the four fern species: *C. dealbata*; *D. squarrosa*; *A. bulbiferum*; and *A. oblongifolium* using the Wellington tree weta (*Hemideina crassidens*) and passion vine hopper (*Scolypopa australis*) as herbivores. These species were selected based on field observations, where they were found to feed on ferns. Preliminary herbivore damage tests were conducted to estimate the damage extent and overall effectiveness of the *H. crassidens* as a fern herbivore (**Fig.2-5**). These trials consisted of using undamaged fresh frond cuttings from a variety of ferns and leaving them in ice-cream containers with a single weta over the course of 48 hours. Observations from these trials assisted with the conception of the primary experiment.

Figure 2-5. Example of herbivore damage by *H. crassidens* on frond cuttings of *C. medullaris* in preliminary trials.

Due to the difficulty and limitations associated with conducting herbivory experiments in the field, these trials were held using potted fern species acquired from Fronds NZ Ltd (Cambridge, New Zealand). It was unclear as to the origin of each fern individual though Fronds NZ Ltd state that they source the majority of their ferns from pine forest areas around the country and are subsequently replanted at their nursery's and grown in ideal conditions. A total of 12 individuals for each of the target fern species were used for the experiment. During the course of this study these plants were kept in a nursery alongside other non-fern New Zealand native plants and watered regularly. The herbivores used to elicit volatile responses were sourced locally. The Wellington tree wetas (*H. crassidens*) were gathered from a nearby patch of native bush (Bledisloe Park) while the passion vine hopper's (*S. australis*) was collected from vegetation all around the campus grounds.

H. crassidens were gathered six weeks prior to volatile collections and were fed a non-fern diet consisting of leaves from the native tree mahoe (*Melicytus ramiflorus*) and cubes of carrot. They were kept in a climate-controlled room (16°C) with a fixed light regime (07:00-19:00 light) and released back into the native bush at the conclusion of the experiment. In contrast, *S. australis* were collected prior to volatile measurements and released the following day in a completely different area from the place of collection. A new batch of *S. australis* were collected for each volatile measurement.

Figure 2-6. Example of a laboratory experimental setup of the dynamic volatile headspace collection system using a PVAS22 unit on *D. squarrosa*.

The primary experiment consisted of 2 treatments, damage by *H. crassidens* and *S. australis*, coupled with a control for each of the four fern species. A total of four replicates were used for each treatment with volatile measurements taking place before the application of the herbivore (15:00-17:00), during herbivore application (17:00-19:00), and 14 hours following the removal of the herbivore from the plant to test for delayed volatile release (09:00-11:00). Collection times were determined to accommodate activity times of *H. crassidens*. Volatile measurements were acquired similarly to that of the phytohormone experiment (see **Fig. 2-6**) in a climate-controlled room (17°C) with a fixed light regime (08:00-20:00 light). Control measurements consisted of volatile collections in the absence of herbivores, weta treatments involved placing both a single male and female weta on the fronds, and passion vine hopper treatments involved 6 individuals freshly gathered individuals placed on the fronds (**Fig.2-7**).

Figure 2-7. Examples of insects interacting with ferns: (a*) S. australis* resting on D. squarrosa, (b) *H. crassidens* consuming pinna leaves of *D. squarrosa*.

Following volatile collections of herbivore damage, insects were immediately removed from the plant and kept in containers overnight and separately measured the following day to identify non-plant emitted volatiles from the samples. This was repeated for each replicate in each of the two treatments. Prior to each herbivore damage experiment *H. crassidens* were starved for roughly 50 hours each time to ensure feeding of fern material and reduce the likelihood of frass occurring.

Volatile samples contained in HayeSep Q filters were eluted using 200µL of high grade pentane (CAS, 109-66-0, ≥99%, Thermofisher Acros Organics, Geel, Belgium) and analysed through a GC-MS. Volatile solutions were then stored in -80°C temperatures and briefly thawed prior to GC-MS analysis.

Gas Chromatography-Mass Spectre Analysis

Analyses for fond extracts, phytohormone treatment, mechanical damage, and herbivory experiments were performed using a Shimadzu benchtop gas chromatography – mass spectrometer system (GCMS-QP2010, Shimadzu Corp., Kyoto, Japan) equipped with a TG-5MS capillary column (30 m x 0.25 mm x 0.25 µm, Thermo Fisher Scientific, Waltham, MA, USA). The injection volume of each sample was 1 µL. Helium (99.999%) was used as the carrier gas at a column flow-rate of 1 mL/min. Carrier gas conditions include pressure control mode of 53.5 kPa, total flow (14.0 mL/min), linear velocity (36.3 cm/sec), purge flow (3.0 mL/min and a split ratio of 10.0. Injection port temperature was 230 °C with the following column temperature program: 50 °C for 3 min, followed by an increase to 95 °C at a rate of 5 °C/min, an increase to 145 °C at a rate of 15 °C/min, an increase to 200 °C at a rate of 10 °C/min and maintenance at 270°C for 3 min. The MS settings are as follows: ion source temperature (200°C), interface temperature (200°C), solvent cut time (2 min) with a total program time of 23.83 minutes.

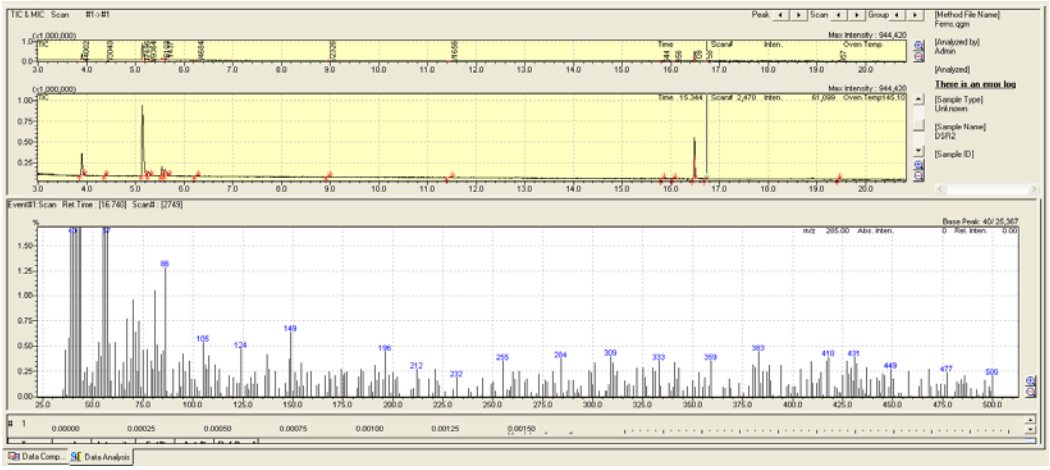


Figure 2-8. Example of manual peak integration using ion chromatography.

Peaks in chromatograms of volatile and solvent profiles were manually integrated and analysed (see **Fig.2-8**). The NIST05 MS library (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used for mass spectral analyses and compound identification according to reference compounds in the database (see **Fig.2-9**). Compounds were identified based off known retention times of external standards. Only compounds with high similarity values (≥80) were reported, headspace and internal standard contamination (i.e. noise) were excluded from the analysis. In cases where compounds showed low similarity values (<80) but retention times corresponded with previous reports, the compound was considered for analysis.

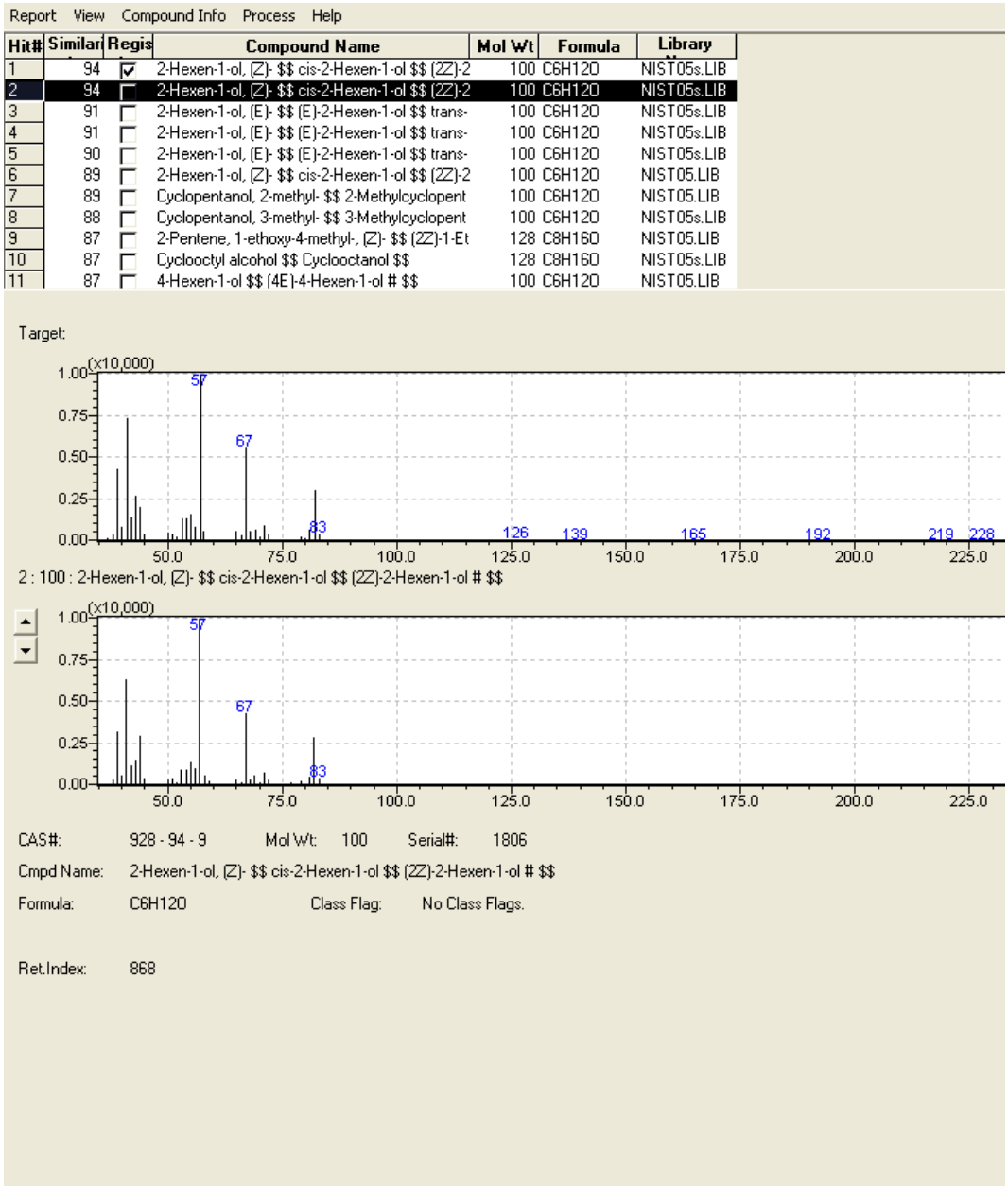


Figure 2-9. Example of compound identification comparing mass spectrum data from samples to target reference values within the NIST05 MS library.

Statistical Analyses

Extract constituents were quantified by comparing unknown peak areas with the internal standard (nonyl acetate). The constituents for both the phytohormone experiment and the solvent extracts were quantified in the same manner. Analysis of volatile constituents acquired from the herbivore experiments were done on the basis of presence/absence due to the scarcity of data. Ion chromatogram characteristics such as peak area, peak height, and area/height ratio were transferred to Microsoft Excel spreadsheets from all datasets. Means and standard errors for the emission rate of each compound in the phytohormone and solvent extract experiments were calculated and tabulated for qualitative analysis.

Values concerning the phytohormone treatment are expressed in ng g^{-1} DW h^{-1} (nanograms per gram of dry weight per hour). Content concerning the solvent extract experiment are expressed as the ng g^{-1} FW (nanograms per gram of fresh weight). Calculation of average total emission rates per treatment (C, JA, or PD) were completed using the collective volatile emission rates for each of the five replicates within the corresponding treatment. Emission rates between fern species and growth mode (whether tree, shrub or epiphyte) were calculated using the total sum of volatiles released per species per replicate. Comparisons between species and growth type solvent extract analyses were conducted using the average of the total sum of concentrations per replicate of species and plotted with 95% confidence intervals.

One-way analyses of variances (ANOVA) as well as Tukey Honest Significant Differences (HSD) post-hoc tests were used for treatment comparisons. All statistical analyses were conducted using the R statistical computing software (Version 3.4.2; R Core Team, 2013). Bar plots were constructed using Minitab 17 Statistical Software (2010).

Chapter 3 : Results

Solvent Extraction

A total of 7 compounds were detected and identified from six fern species using the solvent extraction method (**Table. 3-1**). The concentrations of constituents were lowest in *M. pustulatum* and its compound list (3 total) was also much lower than the other fern species investigated. By contrast, *D. squarrosa* contained the highest concentrations of compounds and released the highest number of compounds along with *A. oblongifolium* (7 total).

Comparison of the total concentration for constituents extracted based on species indicated a difference in values ($F=477.4$, $p<0.01$). Post-hoc analyses reveals significant differences between all individuals with the exception of CD-AB and CM-AO (**Fig.3-1a**). Comparison of concentrations according to growth method determined differences in groups ($F=3.73$, $p=0.04$) although further post-hoc analyses indicate no statistically significant differences between any of the groups (**Fig.3-1b**).

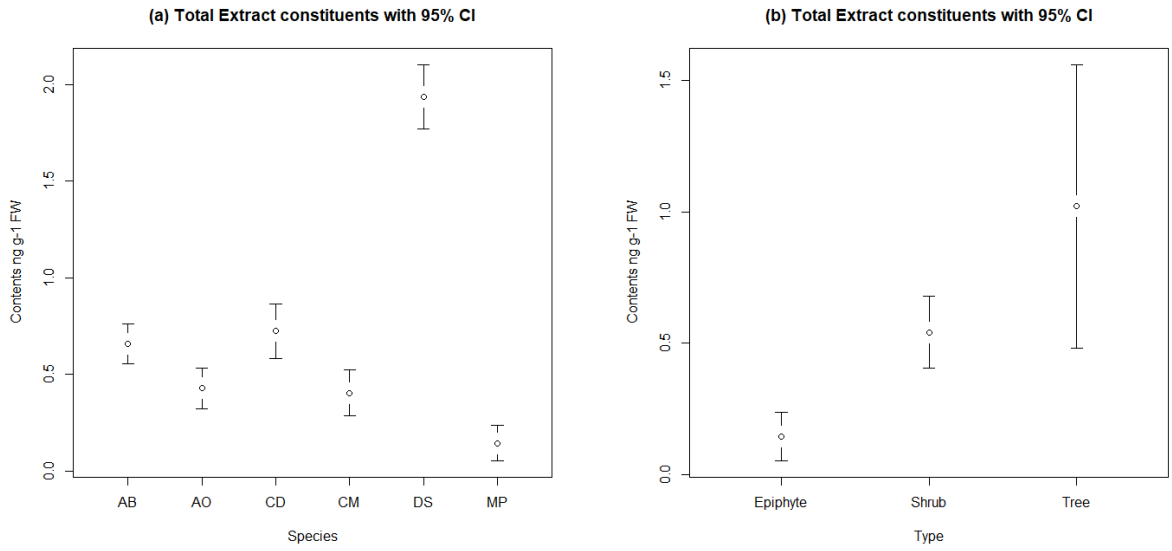


Figure 3-1. Comparisons of confidence intervals (95%) and mean values of the concentrations of fern extracts according to species (a) and method of growth (b).

Table 3-1. Average concentration of fern extract compounds from fronds of six species. Values indicated with a '*' represent data from a single replicate. Values are presented in ng g-1 FW. Note: n.d – no data available.

Compounds	*Asplenium oblongifolium*	*Asplenium bulbiferum*	*Cyathea dealbata*	*Cyathea medullaris*	*Dicksonia squarrosa*	*Microsorum pustulatum*
Hexanal	0.13 ± 0.02	0.50 ± 0.05	0.47 ± 0.02	0.27 ± 0.03	2.03 ± 0.06	0.07*
2-Hexenal	0.26 ± 0.03	0.69 ± 0.03	2.00 ± 0.16	0.59 ± 0.12	6.83 ± 0.12	n.d
3-Hexen-1-ol, (Z)-	0.47 ± 0.01	n.d	0.80 ± 0.01	0.48 ± 0.06	0.54 ± 0.07	n.d
2-Hexen-1-ol, (Z)-	0.65 ± 0.06	0.58 ± 0.06	n.d	n.d	1.06 ± 0.12	n.d
2-Octen-1-ol, (E)-	n.d	n.d	0.06 ± 0.01	0.57*	0.15 ± 0.03	0.14 ± 0.03
1-Hexanol	0.82 ± 0.03	1.37 ± 0.08	n.d	n.d	1.01 ± 0.23	n.d
2-Nonen-1-ol	0.13 ± 0.03	0.14 ± 0.01	0.30 ± 0.02	n.d	n.d	n.d
1,6-Octadien-3-ol, 3,7-dimethyl-, acetate	n.d	n.d	n.d	n.d	n.d	0.18 ± 0.03

Phytohormone and Mechanical Damage

According to GC-MS analysis of headspace samples: 9 volatile organic compounds were detected within the control (**Table. 3-2**), 10 volatile compounds from the phytohormone treatment (**Table. 3-3**), and 11 compounds from the mechanical damage treatment (**Table. 3-4**). The species of fern found to emit the fewest number of volatile compounds was *A. bulbiferum* (8 total), followed by *C. dealbata* (9 total). *D. squarrosa* was found to emit the highest number of compounds (12 total). The compound *2-Octen-1-ol, (E)* - was consistently detected throughout all fern species across both treatments and control. It was also the most prominently released volatile compound in the control and phytohormone treatments. The following compounds; *1,6-Octadien-3-ol, 3,7-dimethyl-*, and *3-Hexen-1-yl, acetate, (Z)-* were detected only once during the control treatments. *1S-.alpha.-Pinene* was found in several fern species but never recurred in non-control treatments. Volatile compounds *3-Hexen-1-ol*, *3-Hexenal, (Z)-*, *2-Hexenal, (E)-*, and *1-Octene, 3,7-dimethyl-* were present only in non-control treatments.

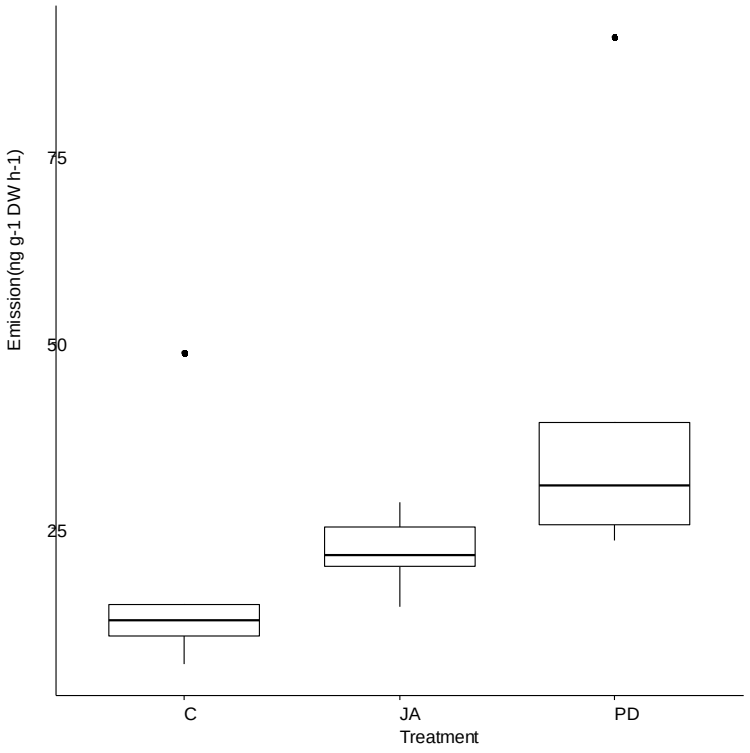


Figure 3-2. Comparison of total volatile emission rates for each treatment; control - no induced damage (C) and two treatments - mechanical damage (PD), and phytohormone treatment (JA).

Table 3-2. Average emission and SE of volatile organic compounds from fronds of six fern species within the control treatment. Values indicated with a '*' represent data from a single replicate. Values are presented in ng g^{-1} DW h^{-1}. Note: n.d – no data available.

Compounds	*Asplenium oblongifolium*	*Asplenium bulbiferum*	*Cyathea dealbata*	*Cyathea medullaris*	*Dicksonia squarrosa*	*Microsorum pustulatum*
(Z,Z)-.alpha.-Farnesene	n.d	n.d	n.d	n.d	n.d	24.23 ± 20.45
1,6-Octadien-3-ol, 3,7-dimethyl-	n.d	n.d	n.d	1.82*	n.d	n.d
1S-.alpha.-Pinene	n.d	n.d	n.d	8.57*	1.26*	24.58*
2-Octen-1-ol, (E)-	19.49 ± 6.23	31.64 ± 13.39	7.79 ± 3.49	4.49 ± 1.12	7.62 ± 0.39	102.35 ± 66.26
2-Pentanone, 4-hydroxy-4-methyl-	3.64*	n.d	1.67*	n.d	2.33*	18.05*
3-Hexen-1-yl, acetate, (Z)-	n.d	n.d	n.d	n.d	6.39*	n.d
3-Octanol	6.63 ± 4.83	15.27 ± 6.02	n.d	0.97*	1.09*	n.d
3-Octanone	7.06 ± 1.88	7.59 ± 2.58	1.97*	2.99*	n.d	16.61*
Copaene	5.65 ± 2.44	6.40 ± 1.27	3.34	n.d	n.d	5.23*

Table 3-3. Average emission and SE of volatile organic compounds from fronds of six fern species within the phytohormone (jasmonic acid) treatment. Values indicated with a '*' represent data from a single replicate. Values are presented in ng g^{-1} DW h^{-1}. Note: n.d – no data available.

Compounds	*Asplenium oblongifolium*	*Asplenium bulbiferum*	*Cyathea dealbata*	*Cyathea medullaris*	*Dicksonia squarrosa*	*Microsorum pustulatum*
(Z,Z)-.alpha.-Farnesene	n.d	6.86*	n.d	n.d	1.27*	29.26 ± 26.60
1-Hexanol	2.60*	6.61 ± 0.81	n.d	n.d	13.00*	n.d
1-Octene, 3,7-dimethyl-	3.61 ± 0.09	2.88 ± 0.49	3.77 ± 0.68	4.82 ± 1.46	4.79 ± 0.45	9.74 ± 2.84
2-Hexenal, (E)-	4.73 ± 0.2	12.50 ± 2.42	n.d	6.46 ± 0.1	12.41 ± 7.61	10.78 ± 7.83
2-Octen-1-ol, (E)-	24.23 ± 7.39	60.90 ± 12.92	32.12 ± 5.81	13.89 ± 4.81	20.40 ± 7.56	222.15 ± 51.49
2-Pentanone, 4-hydroxy-4-methyl-	n.d	n.d	n.d	n.d	n.d	13.23*
3-Hexen-1-ol	2.09 ± 0.43	n.d	11.92 ± 4.30	3.70 ± 0.94	15.63 ± 7.02	n.d
3-Octanol	4.41 ± 1.61	22.47 ± 6.87	9.36*	n.d	17.63 ± 12.9	n.d
3-Octanone	3.91 ± 0.36	15.94 ± 4.59	4.41 ± 0.96	11.41*	2.32*	11.20 ± 4.06
Copaene	5.83 ± 1.51	13.93 ± 6.94	n.d	n.d	n.d	10.82 ± 3.32

Table 3-4. Average emission and SE of volatile organic compounds from fronds of six fern species within the mechanical damage only treatment. Values indicated with a '*' represent data from a single replicate. Values are presented in ng g^{-1} DW h^{-1}. Note: n.d – no data available.

Compounds	*Asplenium oblongifolium*	*Asplenium bulbiferum*	*Cyathea dealbata*	*Cyathea medullaris*	*Dicksonia squarrosa*	*Microsorum pustulatum*
(Z,Z)-.alpha.-Farnesene	4.57*	2.40*	n.d	n.d	n.d	38.24*
1-Hexanol	5.36 ± 1.22	5.87 ± 0.67	n.d	3.97*	11.20 ± 2.97	21.32*
1-Octene, 3,7-dimethyl-	3.25 ± 0.98	1.73*	4.70 ± 0.415	6.02 ± 1.71	5.48*	6.62*
2-Hexenal, (E)-	3.02*	8.03 ± 1.09	7.35 ± 1.55	n.d	60.27 ± 20.10	56.34 ± 44.95
2-Octen-1-ol, (E)-	50.15 ± 18.89	64.85 ± 10.53	53.87 ± 18.24	23.15 ± 3.88	75.50 ± 45.44	322.99 ± 146.91
2-Pentanone, 4-hydroxy-4-methyl-	n.d	n.d	n.d	8.66*	n.d	n.d
3-Hexen-1-ol	7.24 ± 3.20	n.d	33.05 ± 16.30	7.86 ± 2.23	39.04 ± 23.16	20.64 ± 2.68
3-Hexenal, (Z)-	59.82 ± 22.99	30.07 ± 5.92	72.99 ± 37.09	31.81 ± 5.97	156.12 ± 57.84	172.79 ± 88.19
3-Octanol	13.81 ± 5.59	28.98 ± 5.70	n.d	2.23*	15.76 ± 6.76	n.d
3-Octanone	11.45 ± 2.37	13.43 ± 1.99	8.21 ± 2.06	5.77 ± 0.57	n.d	12.64 ± 1.41
Copaene	11.35 ± 3.87	14.16 ± 3.75	n.d	n.d	n.d	20.02 ± 7.12

Comparison of total emission according to treatment yielded no significant differences in means (F=2.16, p=0.158, **Fig.3-2**). Across all fern species investigated the control treatment averaged 18.72, 95% CI [5.42, 32] ng g^{-1} DW h^{-1}, jasmonic acid application 21.94, 95% CI [17.8, 26.1] ng g^{-1} DW h^{-1}, and mechanical damage 41.98, 95% CI [20, 64] ng g^{-1} DW h^{-1}.

Comparison of total emissions between species showed no significant differences for the mechanically damaged treatments (F=2.096, p=0.101) and control treatments (F=1.952, p=0.123) (see.**Fig.3-3**). Differences only within the phytohormone treatment were detected (F=9.156, p<0.01) with TukeyHSD tests indicating differences between species; *M. pustulatum-A. bulbiferum* (p=0.04), *M. pustulatum-A. oblongifolium* (p<0.01), *M. pustulatum-C. dealbata* (p<0.01), *M. pustulatum-C. medullaris* (p<0.01), and *M. pustulatum-D. squarrosa* (p<0.01).

Post-hoc analysis of emissions via treatments within species showed differences in; AB (F=4.98, p=0.03) between PD-CT (p=0.03), CD (F=4.37, p=0.04) between PD-CT (p=0.03), CM (F=13.66, p<0.01) between PD-CT (p<0.01) and PD-JA (p=0.02), and DS (F=5.03, p=0.03) between PD-CT (p=0.03). There were no significant differences between treatments in AO (F=3.42, p=0.07) and MP (F=1.82, p=0.2).

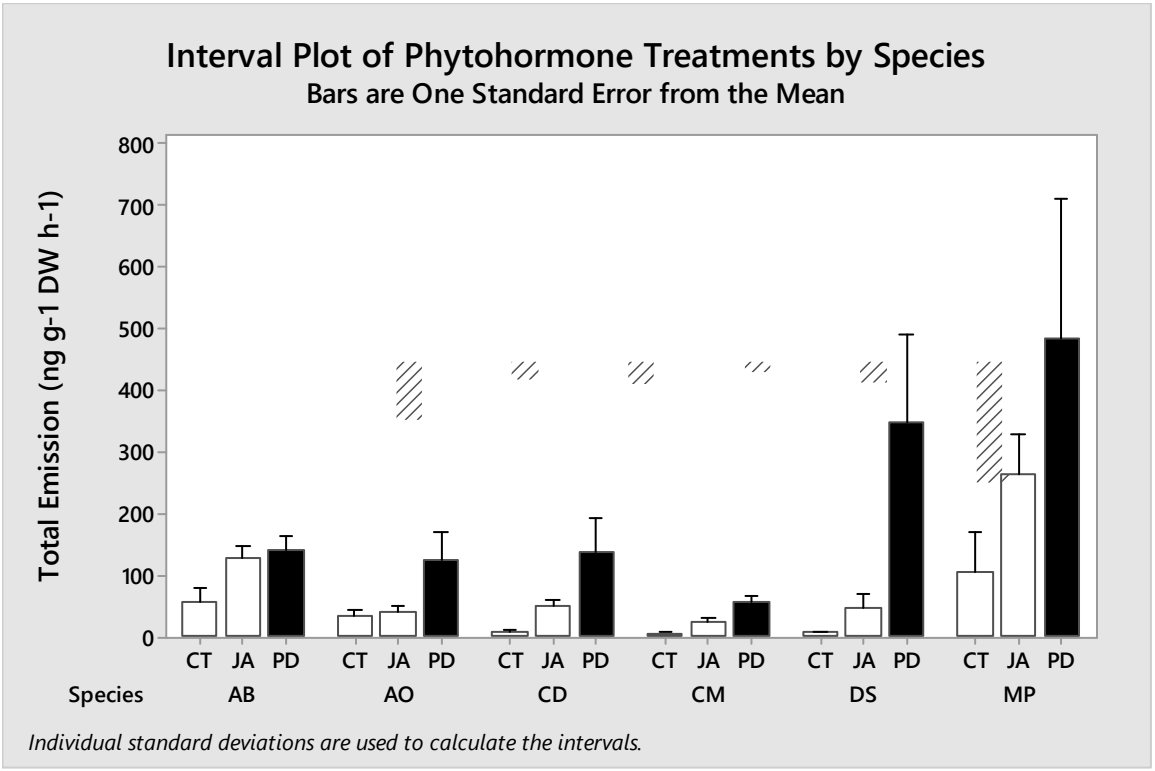


Figure 3-3. Comparison of total volatile emission rates for each treatment according to fern species. Control (CT) with two treatments - mechanical damage (PD), and phytohormone treatment (JA).

Comparison of total emissions according to the growth method showed statistically significant differences within control treatments (F=5.214, p=0.012), phytohormone treatments (F=19.22, p<0.01), and mechanical damage treatments (F=3.42, p=0.048) (see **Fig.3-4**). Subsequent post-hoc tests indicate differences with epiphyte-tree species in the control (p=0.01), with epiphyte-tree (p<0.01) and epiphyte-shrub (p<0.01) species in the phytohormone treatment, and with epiphyte-shrub (p=0.047) species in the mechanical damage treatment.

Post-hoc analysis of total emissions via treatments within growth modes showed differences in; tree species (F=7.60, p<0.01) between PD-CT (p<0.01) and PD-JA (p=-0.01), and shrub species (F=5.75, p<0.01) between PD-CT (p=0.01). There were no significant differences within treatments for the epiphyte species (F=1.82, p=0.2).

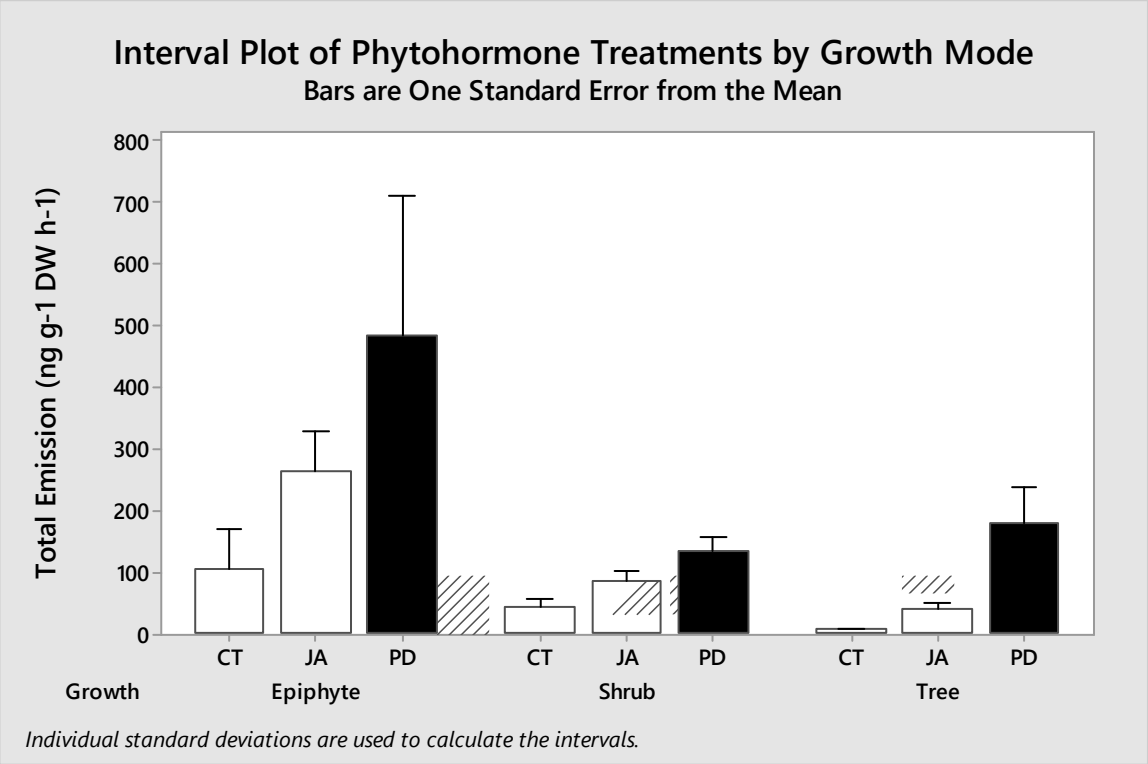


Figure 3-4. Comparison of total volatile emission rates for each treatment according to fern growth mode. Control (CT) with two treatments - mechanical damage (PD), and phytohormone treatment (JA). (Note: Tree = CD, CM and DS; Epiphyte = MP; Shrub = AB and AO).

Polyphenol and Chlorophyll Analysis

Comparison of chlorophyll contents between species of ferns indicated a significant difference in means (F=70.42, p<0.001, **Fig.3-5a**), post-hoc analysis shows difference between all species with the exception of CD-DS and CM-MP. Subsequent analyses on flavonol content (F=73.41, p<0.001, **Fig.3-5b**), anthocyanin content (F=6.91, p<0.001, **Fig.3-5c**), and the nitrogen balance index (NBI) (F=34.71, p<0.001, **Fig.3-5d**) similarly indicated a significant difference of species means regarding each phenolic. Post-hoc tests showed no differences in means of AB-MP, CD-AO, CM-AO for flavonol and CM-AB, DS-AB, MP-AO, DS-CM for the NBI. Post hoc of anthocyanin shows differences in means of only between MP-DS, DS-CM, DS-AB, and AO-AB while other comparisons had no differences.

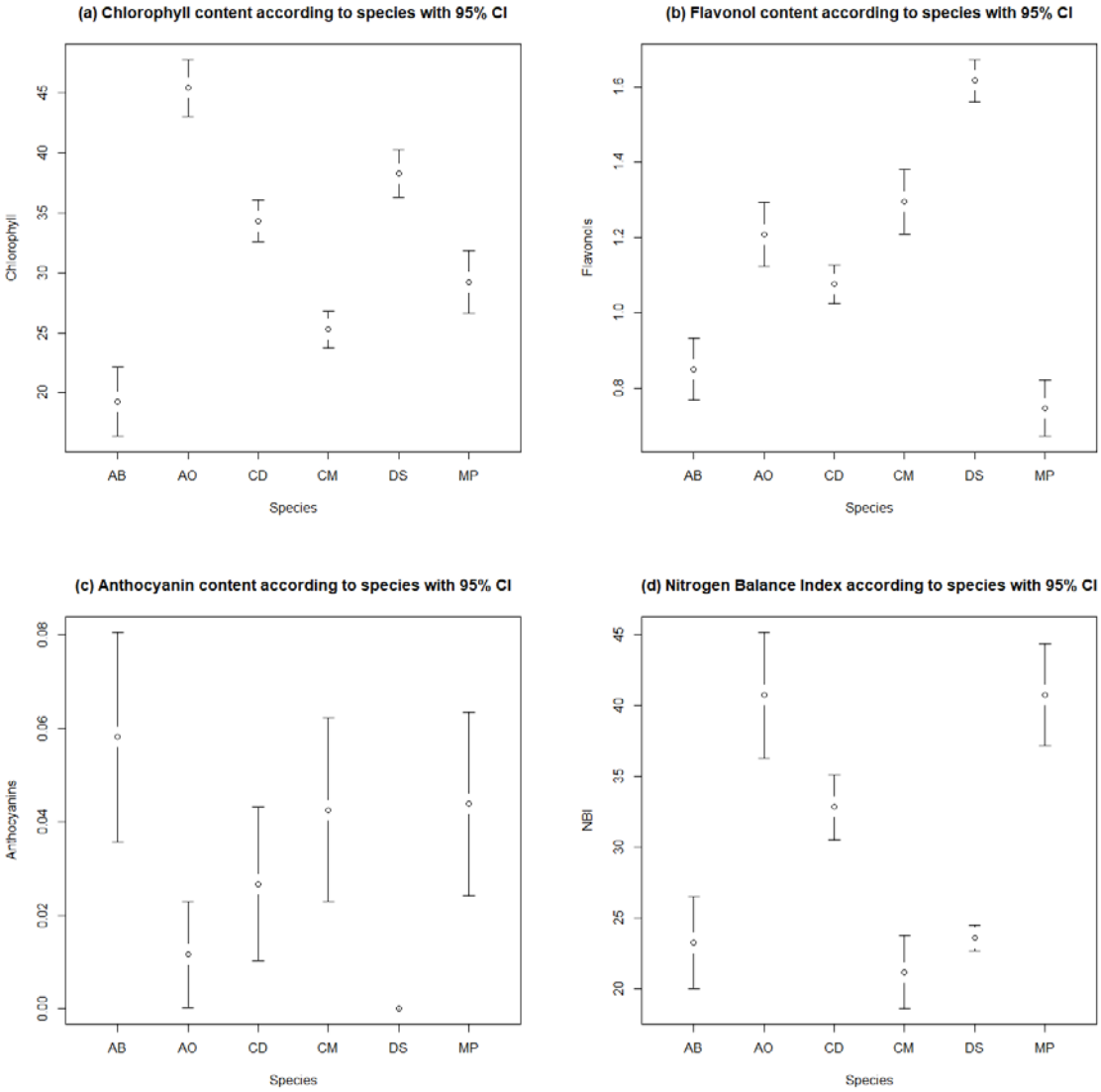


Figure 3-5. Comparisons of confidence intervals (95%) and mean values of chlorophyll content (a); flavonols (b); anthocyanins (c); and nitrogen balance index (d) of the six native fern species.

Analysis of other metabolic constituents according to method of growth resulted in no significant differences in chlorophyll content (F=1.70, p=0.184, **Fig.3-6a**) and anthocyanins (F=2.54, p=0.081, **Fig.3-6c**) but significant differences of values in all growth modes for flavonols (F=69.56, p<0.001, **Fig.3-6b**) and the nitrogen balance index (F=28.44, p<0.001, **Fig.3-6d**).

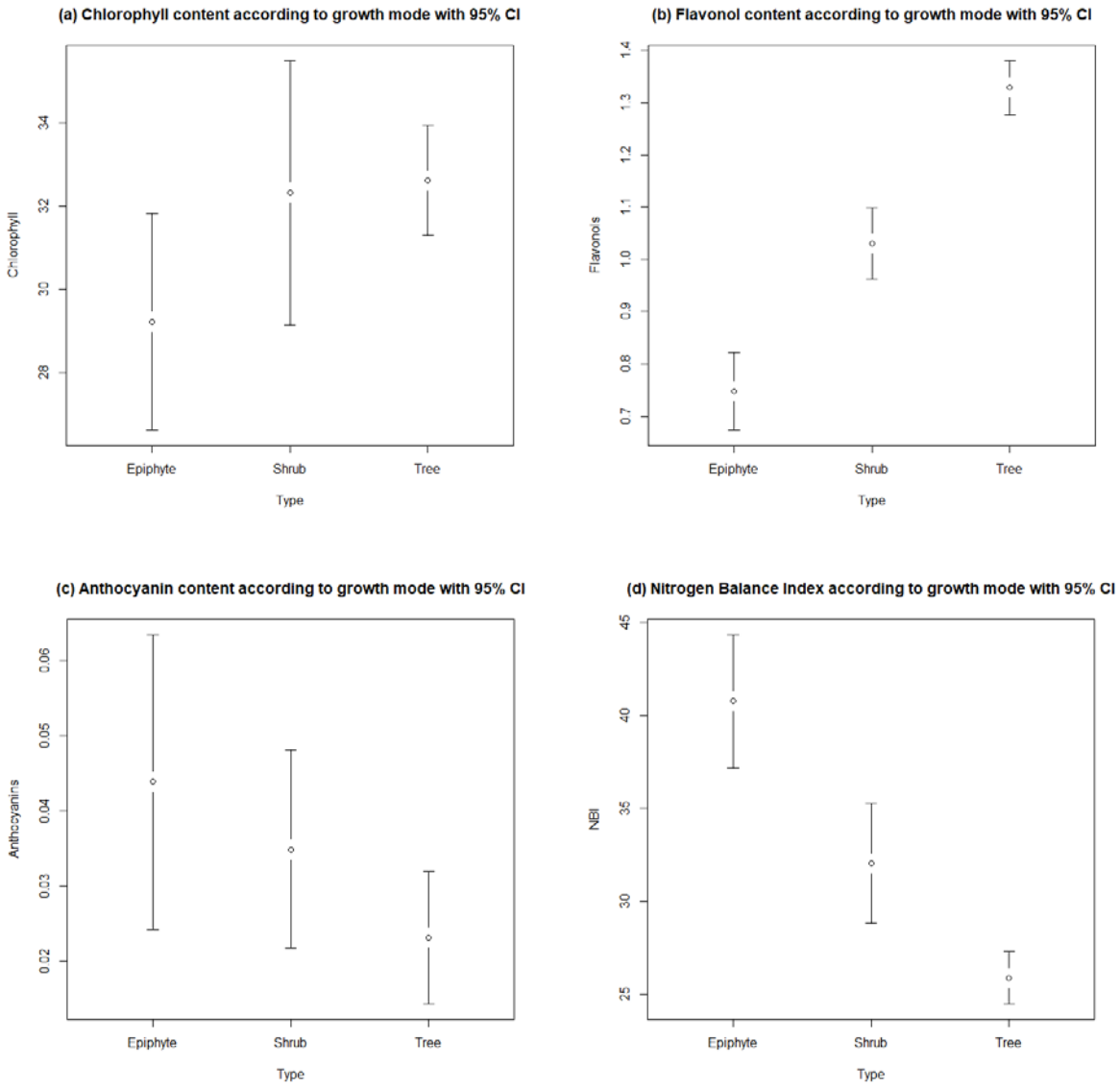


Figure 3-6. Comparisons of confidence intervals (95%) and mean values of chlorophyll content (a); flavonols (b); anthocyanins (c); and nitrogen balance index (d) of the three growth modes. (Note: Tree = CD, CM and DS; Epiphyte = MP; Shrub = AB and AO).

Herbivore Damage

According to dynamic headspace GC-MS analysis; 7 volatile organic compounds (**Table.3-5**) were detected over the course of this experiment. *A. bulbiferum* was found to have the most number of volatile compounds emitted (7 total) in contrast to the rest which was observed to emit a total of 4 compounds. GC-MS data concerning measurements acquired from the *S. australis* - passion vine hopper (PVH) treatments were virtually non-existent with only *A. oblongifolium* emitting enough to be detected on the GC-MS. Compounds *(Z,Z)-.alpha.-Farnesene, 3-Octanol,* and *Copaene* were observed only on *A. bulbiferum*. Compounds *2-Penten-1-ol, (E)-* and *2-Hexene, (E)-* were only detected during the application of wetas to fern material. *2-Hexene, (E)-* was detected in one instance 14 hours after herbivore removal.

In samples with volatile data spanning across the three time periods measured, peak area during the herbivore application period were relatively smaller compared to that of measurements acquired prior to and 14 hours after their application (see **Fig.3-7**)

Table 3-5. List of compounds detected from the herbivory experiments and corresponding treatments they were observed within. Control (CT), Weta (W) - *H. crassidens*, Passion Vine Hopper (PVH) – *S. australis*. n.d – no data available.

Compounds	*Asplenium oblongifolium*	*Asplenium bulbiferum*	*Cyathea dealbata*	*Dicksonia squarrosa*
(Z,Z)-.alpha.-Farnesene	n.d	CT only	n.d	n.d
2-Hexene, (E)-	W only	W only	W only	W only
2-Octen-1-ol, (E)-	CT/PVH/W	CT/W	CT/W	CT/W
2-Penten-1-ol, (E)-	W only	W only	W only	W only
3-Octanol	n.d	CT	n.d	n.d
3-Octanone	CT/PVH/W	CT/W	W only	W only
Copaene	n.d	CT only	n.d	n.d

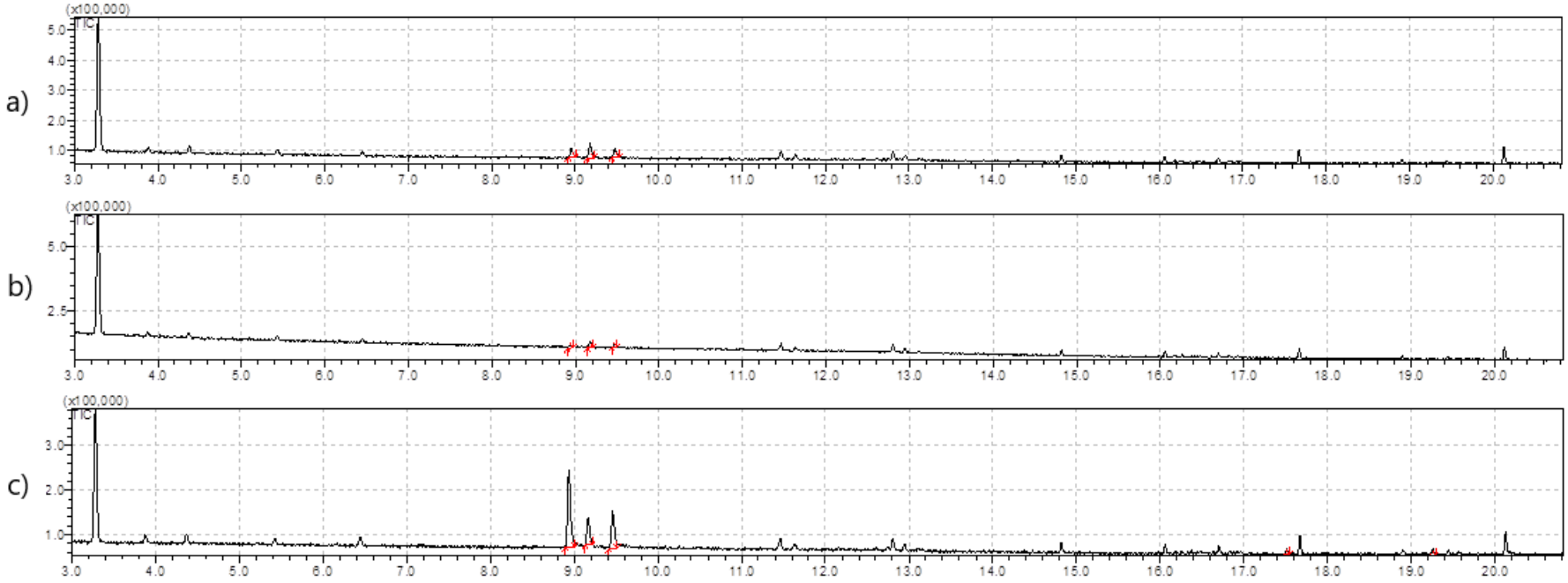


Figure 3-7*.* Example of three ion chromatograms of a control replicate for *A. bulbiferum*. (a) Chromatogram of volatile measurements prior to herbivore application period, (b) Chromatogram of volatile measurements during the herbivore application period, (c) Chromatogram of volatile measurements 14 hours after herbivore application period. Note: Red lines indicate target volatile compounds.

Chapter 4 : General Discussion

Solvent Extraction vs. Headspace Collection

There have only been two studies published to date which have used solvent extraction to analyse the metabolic constituents of ferns (Fons et al., 2010; Froissard et al., 2011). Another study (Halarewicz & Szumny, 2010) analysed volatile oils extracted from the bracken fern (*Pteridium aquilinum* sub. *Aquilinum*) using a similar method. Solvent extraction was also used to investigate the VOC's of six horsetails (Equisetum), a subclass of plants within the fern lineage (Pryer et al., 2001), in a study which reported a sizable seventy-five VOC's from these plants (Fons et al., 2013). Although, much of my methods used for solvent extraction were derived from Froissard et al. (2011), we obtained differing results. In their publication, they reported roughly 30 compounds for each fern species analysed containing a range of aromatic compounds, polyketides, monoterpenes and isoprenoid derivatives. In contrast, the highest number of compounds detected in my samples was 7.

Froissard et al. (2011) investigated 6 ferns (*Asplenium trichomanes*, *Dryopteris dilatate*, *Polystichum setiferum*, *Gymnocarpium dryopteris*, *Pteridium aquilinum*, and *Phegopteris connectilis*). Upon comparison of constituent profiles between the two data sets, I found no recurring compounds. Comparison with Fons et al. (2010) and their investigation of French ferns (*Adiantum capillus-veneris*, *Athyrium filix-femina*, *Blechnum spicant*, *Dryopteris filix-mas*, *Oreopteris limbosperma*) yielded equivalent results. I hypothesize that this may either be the result of the physiological and/or environmental differences between species or the differences in extraction methods. Froissard et al. (2011) had macerated and extracted fern constituents for a period of one week whereas I had only extracted for a period of 72 hours with scheduled shaking events. It is possible that due to the nature of ferns, longer extraction periods and the crushing of plant cells may be necessary to obtain a higher number of compounds, yet, crushing the leaves will damage the cell walls, releasing other compounds, which are not normally found in the fern scent in nature.

Other studies have investigated the volatiles of ferns using headspace sampling techniques, which are less invasive and do not require the destruction of the sample and are therefore more similar to natural emissions (Imbiscuso et al. 2009; Kessler et al. 2015; Radhika et al. 2012). Comparison of data sets indicate recurring compounds (3-hexen-1-ol) with Imbiscuso et al. (2012) and (a-pinene) with Kessler et al. (2015). A major reason for this difference in volatile profiles is likely due to the fact that none of these studies measured the headspace directly in the field but rather used dried (Kessler et al., 2015) samples or greenhouse propagated plants (Imbiscuso et al., 2009; Radhika et al., 2012). As these conditions may not be representative of

natural stresses, volatile profiles were expected to differ. Despite being lab-based collections, the number of compounds detected were similar to the ones reported in this study (Imbiscuso et al., 2009; Radhika et al., 2012) with the exception of Kessler et al. (2015) identifying over 20 compounds from dried samples. As methodologies were different, it is very difficult to make direct comparisons. Fern species used by Imbiscuso et al. (2009); Kessler et al. (2015); and Radhika et al. (2012) are described in Chapter 1 of this thesis.

A direct comparison of both methods in this study revealed that headspace collections allowed to recover a higher amount of compounds, although some compounds can be consistently recovered by both methods. In terms of answering important ecological questions, in-field headspace volatile analysis of plants would be more appropriate and provide a better output of ecologically relevant compounds for further testing (i.e. in herbivory experiments). In contrast, solvent extraction may be ideal for breaking down plant material in order to identify key compounds in fern metabolism, but these may not necessarily be involved in fern defence. As suggested by Froissard et al. (2011) solvent extraction can also be useful for describing likely scents in plants for perfumery applications or even act as a compound check for GC-MS analysis.

Phytohormone Treatment and Mechanical Damage

Jasmonic acid (JA) is a naturally occurring organic compound found in several plant species with the function of regulating plant responses to biotic and abiotic stresses in addition to plant growth and development (Delker et al.2006). Like with a similar study by Radhika et al. (2012) I had expected the jasmonic acid to stimulate anti-pest defences within all ferns species and thus increase its production and release of its volatile organic compounds, ultimately however, these expectations were not met. Collectively, the fern species investigated did not change their emission rates regardless of whether plant-defences were induced or not, but as individual species, plants with epiphytic growths modes (*M. pustulatum*) appeared to produce more volatile compounds compared to their more larger counterparts when exposed to JA. As my results indicate, *M. pustulatum* has significantly higher concentrations of volatile emissions compared to the other 5 fern species when damaged and stimulated by jasmonic acid. In contrast, it was also identified as the species with the lowest concentration of volatile constituents extracted. Though post-hoc analyses showed no significant differences between growth modes, the uncertainty in this area may be clarified if the experiment were to be repeated with larger samples sizes and a more representative array of species for each mode of growth.

Studies investigating volatile emissions of epiphytic plants have yet to be published thus currently, it is difficult to draw conclusions and support or deny any existing theories for this observation. Based on my analysis, I suggest this explanation; epiphytes are under lower habitat pressures save their need for a host for physical support, therefore the energy investment to structures such as roots or bark are redirected into production of volatile compounds for plant defences thus explaining why they may significantly release higher concentrations. Alternatively, epiphytes are ideal habitats for animals, fungi and bacteria (Everhart et al., 2009) thus it wouldn't be uncommon for these plants to attract symbionts or herbivorous predators towards the plant with their high emission concentrations. This may also explain the fact that low concentrations of volatile constituents were extracted from this species. It is possible that chemical reactions needed for synthesis of volatile compounds only take place when cells on the surface are damaged. My conclusions concerning jasmonic acid on epiphytic ferns are partly consistent with those of Radhika et al. (2012), indicating that exogenously applied JA can trigger indirect defence responses (changes in VOC emission) in some fern species.

Comparisons of volatile profiles between treatments however did yield interesting information, *1,6-Octadien-3-ol, 3,7-dimethyl-*, *(Z)-3-Hexen-1-yl, acetate*, and *1S-alpha-Pinene* were only ever detected in control treatments but never when mechanical damage was applied. This suggests one of two scenarios; the first being that these compounds are passively released by ferns but at incredibly low concentrations very difficult to detect and mechanical damage dilutes these compounds even further on the GC-MS, whereas the second scenario being that these compounds are released as a result of different stresses but due to artificial wounding of plant organs, the plant subsequently prioritizes the new, more prominent stressor, thus ceases or reduces the synthesis of 'minor' volatile compounds to more suitable compounds that may effectively deal with the current threat. This hypothesis has yet to be directly investigated within the literature however, studies by Geervliet et al. (1997) and Van Den Boom et al. (2004) have expressed in their data similar observations where a small handful of volatile compounds, non-identical to those in this study, were only observed in undamaged treatments. Though not robust evidence for this hypothesis, it does warrant investigation in future studies as it could be a potential discovery in plant defence research.

In relation to this theory, another interesting piece of information was revealed during qualitative analysis, which was that the following compounds *3-Hexen-1-ol*, *(Z)-3-Hexenal*, *(E)-2-Hexenal*, and *1-Octene, 3,7-dimethyl-* were only ever present in treated samples and absent in the controls, usually, at relatively high concentrations compared to other compounds.

Likewise, with the studies mentioned above (Geervliet et al., 1997; Van Den Boom et al., 2004), several compounds have also been reported to be present in only damaged samples. This may support the second scenario I proposed but due to the paucity of research concerning this, I cannot make any solid conclusions.

A brief look into the recent literature demonstrates that some of these compounds have important roles in plant ecology; *3-Hexen-1-ol* having shown to induce arthropod resistance (Losvik et al, 2018), *3-Hexenal* as a precursor to other important green-leaf volatiles (such as *(E)-2-Hexenal*) (Kunishima et al., 2016), which has been shown to attract parasites (Vieira et al., 2014). All these compounds are green leaf volatiles, often reported to mediate plant-arthropod interactions in flowering plants (Light et al., 1993; Shiojiri et al., 2006; Whitman & Eller, 1990). *1-Octene, 3,7-dimethyl-* is an identified monoterpene but no direct plant-insect function has been so far reported in the literature. However, other compounds belonging to this group have been shown to play important roles in responses against abiotic stress and natural enemy recruitment (Loreto et al., 1998; Wang et al., 2008; Yamasaki et al., 2007).

For the majority of fern species analysed, total volatile emissions differed significantly between mechanical damage and control treatments. Similarly, when categorised according to method of growth, these two treatments were also found to differ. Though this may not provide much insight into the ecological aspect of fern volatiles it does provide evidence which shows, for some fern species, that there are quantitative differences in the volatile emissions which indicates that ferns respond to damage to cells, at least to some degree. These observations are consistent with those of Van Den Boom et al. (2004) who also observed higher volatile emissions in mechanical damage treatments compared to those collected from undamaged leaves and other treatments for some compounds amongst a few higher plants. A possible explanation as to why we observe this may be that because the plant responses are not regulated by compounds such as JA, they may overcompensate for the immediate stress by producing more than normal amounts of volatile compounds to “play it safe”, whereas when responses are regulated, they would prioritise efficiency for energy spent thus producing the fewer compounds for maximum efficiency.

Polyphenol and Chlorophyll Analyses

As depicted by **Fig.3-5**, there does not seem to be any pattern or trend in chlorophyll and polyphenolic content amongst the fern genus. The similarities and differences in the means of each species do not give us any indication as to the evolutionary development of these compounds. Consultation of fern phylogenies (Pryer et al. 2004; Schneider et al., 2004) suggests relatedness of *Dicksonia* and *Cyathea* genus and a more distantly related *Asplenium* and subsequent correlation of these phylogenies to the differences inferred from data suggests high variability in the production of these compounds or an environmentally driven physiology. What is also noteworthy is the fact that there is no consistency with phenolic and chlorophyll content between species of the same genus for instance, with the exception of anthocyanins; chlorophyll, NBI and flavonols contents were significantly different amongst species of the same genus (*Asplenium* and *Cyathea*). Although this isn't sufficient evidence to support any claim, it does raise the possibility that biosynthesis of volatile compounds in plants could potentially be governed by habitat features rather than their genetic make-up.

Remarkably, we see a pattern in polyphenol production based on plant growth mode (**Fig.3-6**). Leaf flavonols, in general, function to protect plants cells from UV, accumulates phenolics as well as gives an indication as to the leaf light environment (Williams & Harborne, 1977). Likewise, the nitrogen balance index (NBI) is an index used to estimate crop nitrogen nutrition (Schröder et al. 2003). Given that tree fern species are more exposed to the sunlight compared to epiphytes and shrub species it is understandable they invest more into the production of leaf flavonols for extra UV protection. In contrast, epiphytes do not need to invest in such compounds as they live mostly in the shade of their hosts. In addition to this, some epiphytes serve as important nitrogen fixing plants in their habitat (Han et al. 2010) thus may explain the relatively high nitrogen content in their leaves. In relation to the use of these phenolics as a potential for defence against herbivory, there could be a relation between the high nitrogen content and the high concentration of volatile compounds emitted in fern epiphytes however these inferences also warrant future studies into this topic.

Herbivore Damage

Compared to volatile measurements acquired from other experiments in this thesis, GC-MS analysis of volatiles from the herbivory experiment, were rather poor. The majority of replicates across the four fern species investigated didn't register any volatile compounds. There are several likely explanations for this occurrence: the fact that measurements were conducted in controlled conditions may have affected volatile release. These conditions may not be reflective of the plants natural habitat ergo the plant had no reason to synthesize volatiles. Likewise, greenhouse-grown plants may not respond equally to those growing in the wild. Another explanation may be the fact that only 2 hours were used for volatile collection, this time period may not have been sufficient to adsorb enough compounds to register in the GC-MS. The environmental settings in the climate-controlled room (temperature, humidity, photoperiod, etc.), may have not been optimal for VOC emissions (Gouinguené & Turlings, 2002). Comparison of volatile emissions under both field and laboratory conditions by Kigathi et al. (2009) suggested that volatile release is likely to be influenced by additional biotic and abiotic factors.

Interestingly, in control replicates where volatiles were registered in all or two of the three volatile measurements, peak area of target volatile compounds appeared to have dropped during the period of herbivore application only to increase again in the next measurement (**Fig.3-7**). Although there isn't any statistical evidence to support this explanation, it is possibly due to the time of day used for measurements. First measurement occurred in the late afternoon, followed by another in the early evening with the last in the following morning. Volatile concentrations would seem to drop during the early evening and increase again the following morning. This may indicate a temporal factor in volatile emissions in ferns however, a dedicated study investigating this is required to confirm these observations. Other studies investigating volatile emission dynamics have also found changes in emission during the course of herbivory and with changes in photoperiod (McCormick et al., 2014).

Data regarding measurements associated with the passion vine hopper *S. australis* were effectively none. Initially, this insect was chosen as a potential herbivore due to observations of eggs and nymphs located on the underside of several fern species. In addition, this insect has piercing and sucking mouth parts which serve as a contrast to the ripping and chewing feeding method *of H. crassidens*. My results suggest-three possible scenarios; a) that the damage done by them was insufficient to elicit a large response from the plant, b) that the insects were simply not feeding during the volatile measurements either due to saturation of

food prior to collection or their peak activity times were inverse to the collection period, or c) that they may not feed at all in some of the tested species. Either way, it is difficult to narrow down the likely causes for these observations.

Lastly, the most important observation in this experiment is the emission of 2 compounds: *(E)-2-Penten-1-ol*, and *(E)-2-Hexene* in weta herbivory treatments. The emission of these volatiles corresponds with observations of fern material consumption by the weta and were detected only during measurements when herbivores were present in the headspace. As no volatile compounds were detected when insects were separately analysed or in any of the controls these two compounds can be considered to have been herbivore induced. *(E)-2-Penten-1-ol* has been recorded to be found only in herbivore treatments in the literature (Geervliet et al., 1997; Van Den Boom et al., 2004) however, I have yet to find a study documenting its effectiveness in plant-insect interactions. *(E)-2-hexene* is a green leaf volatile, green leaf volatiles have been reported to mediate several plant arthropod interactions (Light et al., 1993; Shiojiri et al., 2006; Whitman & Eller, 1990) but I have found no studies which report *(E)-2-hexene* to be associated with herbivory. Further comparison with real standards is required to validate the identity of these compounds and potentially use them in bioassays with natural enemies of the herbivores.

Final Remarks

This is the first study, as far as I am aware, to investigate the volatile constituents of New Zealand ferns under both field and laboratory conditions. As far as exploratory research goes, I believe my work and the discussion above establishes a clear foundation on which future studies can build upon. Through my experimentations, I have set a baseline on what to expect when working with field-based trials with similar methodologies. Choosing the study species for this thesis was partly due to their relative abundance in the natural environment and partly due to their genetic and biological traits. I aimed to cover a range of aspects with what little resources and time I was given to test the waters and, hopefully, establish certain priorities for this topic. In addition, the two treatments I have carried out in the field (phytohormone, mechanical), are common place in this line of work and the subsequent analyses, both qualitative and quantitative analyses were somewhat successful in characterising the fern volatiles I had initially intended to describe. Though I had expected to observe a larger variety and higher quantity of volatiles when I first started, the results I found and conclusions I ended up with leave me satisfied with the potential my research has on this issue.

Further observations into plant phenolics and fern growth mode provide a fresh perspective on the evolution and perhaps underlying mechanisms behind the emission of fern volatiles and even possibly fern-insect interactions. Though I failed to elucidate differences in volatile emissions according to herbivore feeding methods due to a number of possible reasons (lack of feeding by herbivores, greenhouse grown fern material, not optimal controlled conditions, etc.), my observations can provide important information for future research designs. The comparison between two collection methods was useful to determine the advantages and limitations of each method, although there is surely much room for improvement in this area.

Chapter 5 : Conclusions and Recommendations

Conclusions

The primary aim of this thesis was to characterise the volatile emissions and volatile constituents of six native fern species, to test their responses to herbivory, and investigate whether the release of these compounds was related to the method of growth. I believe, with my work through these sets of experiments, I have managed to fulfil the objectives I had set out to investigate.

Analysis of volatile collections suggests that total volatile emissions did not differ significantly between phytohormone induced and artificially induced damage however, there were differences in the emissions between species within each treatment and between different modes of growth. Polyphenol and chlorophyll content had no evident pattern between species but suggests a trend based on the ferns method of growth. Despite, the relative paucity of volatile data concerning my herbivory experiments, 2 compounds were correlated with direct herbivory. Chemical compositions of fern material extracts were underwhelmingly informative. Results from both extract and in-field phytohormone treatments show that solvent extraction did not provide a representative chemical profile of the ferns. Although, solvent extraction may serve as a potential check for particular compounds, it is not suitable for investigating fern-insect interactions. This re-enforces the idea that in field measurements and collections are crucial in determining fern-environment interactions. Lastly, qualitative analysis of volatile organic compounds has identified 15 organic compounds which have been consistent throughout my measurements and have been accurately identified with high similarity ratings, additionally these compounds have been linked to plant physiology in the literature.

As my review of the literature demonstrates, there is an overwhelming gap in our knowledge concerning the use of volatiles as defence mechanisms in ferns and other primitive plant groups. Most, if not all the data acquired from these experiments are new and unchallenged. As such, data from exploratory studies like mine may give insight into the evolution of anti-herbivore defence mechanisms in plants by describing volatile constituents of one of the oldest extant plant groups. Aside from the phylogenetic implications this research may have, this also opens potential applications in the conservation of threatened fern species through pest management using environmentally safe synthetic chemicals for biocontrol as well as the novel idea for these volatile bouquets to be used in perfumery. The work from this study will serve as a foundation for future projects in this growing field of research.

Recommendations for Future Work

Given that this field of research is relatively new, there are multiple areas for improvement based on exploratory studies such as this and of those before. Particularly, the use of the latest gas chromatography- mass spectrometry techniques and software are ideal for optimal efficiency and accuracy in the quantitative and qualitative analysis this research entails. Data processing can be overwhelming if investigating copious quantities without the use of advanced software and technology. The equipment I was using was relatively outdated which may have caused an overall decrease in the accuracy of my data - particularly the decade old software and NIST library used for peak integration and compound identification. More recent versions of these may have better resolutions or additional functions which may assist with several functions relating to compound integration.

This research, despite the time and methodological constrains, opens the door for future studies on fern chemical ecology. Future studies should include further comparisons between diverse extraction methods to optimise the outputs, as well as the identification of other potential herbivores, and new herbivory trials under natural conditions to elucidate the responses of ferns. Multiple behavioural tests are also required to understand plant-herbivore and plant-natural enemy interactions mediated by these volatiles. Additionally, a deeper look into the fern´s genome and metabolome should reveal the signalling pathways involved in the regulation of volatile emission and their evolutionary relevance.

Based on the results of my measurements concerning herbivory, it would be interesting to investigate the temporal aspects of fern volatiles. As particular compounds, both plant and artificial, have the potential to adhere to other plants, similar studies should be focused in areas where such 'noise' is minimised or at least controlled to some degree. It would also be crucial to dedicate entire studies solely on volatile profiles from different types of insect herbivory as it would undoubtedly serve as an indispensable reference and guide for the chemical bouquets we would expect from certain interactions and future research. Another aspect in this topic worth investigating would be to study the responses of ferns to salicylic acid, a plant phenolic known to induce flowering, cause allelopathy, and is essential to plant disease resistance (Hopke et al., 1994; Raskin, 1992). On a final note, I would recommend and welcome any interest into fern chemical ecology as it is an impoverished field of research, so any contribution is more than appreciated. I would like to end with a quote from Cooper-Driver (1978) which summarises the context of this thesis "is the apparent lack of feeding by insects on ferns a fact or is it really due to lack of observations or even interest?"

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