Characterising Poplar and New Zealand Native Plant Resins and New Zealand Propolis using Volatile Organic Compounds

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Abstract

Recent trends show a growing understanding and acceptance of natural remedies in health care. One such remedy is propolis, a substance produced by honeybees and sold commercially. The natural mixture of beeswax and resin sourced from nearby trees has a role in maintaining the health of the colony. Several health benefits, including antimicrobial, anticarcinogenic and antiviral properties have been demonstrated internationally. These properties are not limited to bees, and their role in human health has been, and continues to be, investigated.

One of the challenges of dealing with a natural product involves natural variation. In the case of propolis, the most notable cause of variation is the botanical source of resin. Countries with differing flora produce different types of propolis. At a finer scale, propolis variation has also been identified within some countries, again often correlating with variation in available or preferred botanical sources. Diversity in the sources of resin collected during propolis production can lead to diversity in the chemical configuration of the final product. Such variation in the chemical make-up of propolis has also been demonstrated to correlate with variation in antimicrobial activity. Before propolis can be used as a health supplement for humans or bees, an understanding of the botanical sources used, and the chemical properties of the resulting propolis is required.

The resin produced by plants is a tacky, water insoluble substance with a range of functions. The complex chemistry of plant resins can be species specific but can also vary greatly both within and between families. One way to identify the compounds present in the resin is to investigate its volatile organic compounds (VOCs). VOCs are emitted by all plants and plant-products and mediate biotic and abiotic interactions, having biological activity against pests and diseases. Propolis, a glue-like substance produced by honeybees, is composed of beeswax mixed with plant resins. Propolis has biological activity that benefits both bees and humans. This biological activity has been shown to vary depending on the geographical and therefore botanical origin of propolis. While research has been moving toward a geographical based approach, this is yet to be done comprehensively in New Zealand. This study aimed to

investigate 1) the chemistry of the VOCs of poplar hybrids commonly planted in New Zealand, and that of species native to New Zealand, and 2) the VOCs of New Zealand propolis collected from nine different regions.

The volatile profiles of the resin of six poplar hybrids and 17 native species collected in autumn and/or spring were analysed using gas chromatography-mass spectrometry (GC-MS). A total of 111 compounds were tentatively identified. Principal component analysis (PCA) showed divergence between the volatile profiles of the poplar resin and the native resin, and the season during which samples were collected. PCA also indicated some variance amongst the poplar hybrids, and between clones of the same hybrid. Cluster dendrograms were used to visualise the divergence between groups and showed that the resin of some poplar clones was more similar to native species than to other clones of the same hybrid. This analysis provides a better understanding of the chemical profiles of the resin of several common plant species in New Zealand, and how they relate to each other.

74 propolis samples were collected from nine different regions across New Zealand, and 91 compounds were tentatively identified. Principal component analysis revealed that the region from which samples were collected was not sufficient to explain the variation amongst the volatile profiles, although some regions were separated and clustered together. A cluster dendrogram highlighted the variation between the propolis samples, and the seemingly random relation between different samples. Additionally, initial suggestions were made regarding the potential contributions of resin from both poplar and native botanical sources. This analysis furthers our knowledge of the chemical profile of New Zealand propolis, its sources of variation, and the potentially contributing botanical sources.

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Chapter 1

General Introduction

Chapter 1: General Introduction

Propolis, or bee glue, is a mixture made of resin collected from the leaves, bark and buds of certain plants. Only a small proportion of bees in the hive are tasked with collecting resin. After collection, worker bees chew the resin and mix it with salivary enzymes, beeswax, and some pollen to produce propolis (Alvarez-Suarez, 2017; Crane, 2009). Bees use propolis to seal unwanted spaces and maintain the structural integrity of the hive (Ghisalberti, 1979), but it is also thought to be an important component of social immunity (Borba et al., 2015; Evans & Spivak, 2010; Simone-Finstrom & Spivak, 2010; Wilson-Rich et al., 2009) having biological activity against pests and diseases (Antúnez et al., 2008; Bastos et al., 2008; Simone et al., 2009; Wilson et al., 2015). Correlation between propolis production, the viability of offspring, and the lifespan of adult workers has been demonstrated by Nicodemo et al. (2014).

A number of the chemical compounds present in propolis have been investigated for their activity against human illnesses. Propolis has been found to have antibacterial, antioxidative, antifungal, anti-inflammatory and anticarcinogenic properties, amongst other health benefits (Bankova et al., 1983; Cheng & Wong, 1996; Khayyal et al., 2003; Marcucci, 1995; Ozcul et al. 2005; Russo et al., 2004; da Silva Frozza et al., 2013; Xu et al., 2009). Consequently, its use in alimentary supplements and bio-cosmetics has been increasing rapidly. These potential benefits have driven an increased interest to improve the quality, consistency, and quantity of propolis produced by commercial colonies. Hence, this literature review aims to summarize some of the common features of propolis, including its botanical sources and volatile organic compounds, its role in honeybee health, and antimicrobial activity.

Botanical sources of propolis

Based on the plant from which resin is collected, there are thought to be around seven varieties of propolis (Catchpole et al., 2015), often classified by their geographic distribution. However, as analysis of propolis continues, the reality of the possible botanical sources used by honeybees is proving to be more varied and complex than once thought. The distinction between propolis of different locations is a result of their botanical sources, as well as the part of the plant used, or its stage of development (Roberto et al., 2016).

More often than not, New Zealand propolis is absent from lists of global propolis sources. When it is listed, it is classified as European/temperate/poplar type propolis (Bankova et al., 2000; Bloor et al., 2019). Still, on lists such as that constructed by Bankova et al. (2014), propolis from New Zealand is noticeably absent from an otherwise comprehensive review. In more recent lists, such as those constructed by Kasote et al. (2022), New Zealand is still included under the umbrella of temperate or poplar type propolis. However, even within this group, it is clear that very little is known about the chemical composition of New Zealand propolis. Kasote et al. (2022) constructed a heat map showing the chemical composition of studied propolis samples from different countries. According to this map, the only chemical groups found in propolis are aromatic acids and flavones and their esters.

While the resin produced by different plant species may be similar in their qualitative composition, their quantitative composition often differs (Bankova et al., 2002). Because most of the chemicals of bud exudates are included in propolis without chemical modification by bees (Greenaway et al., 1990; Salatino et al., 2011), and generally only one source is collected per resin-foraging trip (Wilson et al., 2013), the chemical composition of the propolis can often be used to identify the species from which the resin was collected (Bankova et al., 1998; Greenaway et al., 1990).

Resin from trees of the genus *Populus* appears to be the most common source for propolis production in temperate areas (Tomás-Barberán et al., 1993). Within the genus *Populus*, exudates from species (and hybrids) of the section *Aigeiros* (cottonwoods) are used in propolis production preferentially (Bankova et al., 2002; Greenaway et al., 1990). The section Aigeiros includes poplar species *P. deltoides* (Eastern Cottonwood), *P. nigra* (Black Poplar), and *P. fremontii* (Fremont Cottonwood), as well as many hybrids among the species (Greenaway et al., 1990). Exudates from other poplars, despite the species being widespread, do not appear to be as widely collected (Greenaway et al., 1990; Wilson et al., 2013).

As of 2014, more than 400 compounds had been identified in samples of poplar-type propolis internationally (Ristivojević et al., 2015). The biological activity of temperate propolis, the category into which New Zealand propolis is currently assigned, is generally attributed to flavonoids and phenolics. Caffeic acid phenethyl ester (CAPE), a major component of most poplar-type propolis, has been described as being responsible for a large portion of the biological activity ascribed to temperate propolis (Huang et al., 2014). Flavonoids typical of

poplar-type propolis include pinocembrin, pinobanksin, chrysin, galangin, kaempferol and quercetin (Huang et al., 2014). These compounds and others have been identified s as the likely cause of biological activity (Bankova, 2005; Vardar-Ünlü et al., 2008). Realistically, authors are probably unable to attribute biological activity to a single specific compound, as it is likely that the synergistic effect of numerous compounds occurring in different quantities are the cause of varying biological activity (Ristivojević et al., 2015). To that end, Ristivojević et al. (2015) recommend that poplar-type propolis should be characterized for pharmaceutical use by three criterion: total flavone and flavonols content, total flavanone and dihydroflavonol content, and total phenolic content.

Drescher et al. (2019) performed comparative chemical analysis of resin at the level of individual bee foragers in Germany. They also examined resin intake at the level of the colony to establish the effect of location, and therefore the composition and abundance of different tree species on variation in collected resin. When resin collected from foragers was compared to resin sampled from tree buds, the results showed that bees collected resin from several poplar species, as was expected. Somewhat unexpected was the finding that bees also collected resin from birch (*Betula alba*), horse chestnut (*Aesculus hippocastanum*) and conifers (*Picea abies* and *Pinus sylvestris*). Still, despite obvious compositional similarity, few close matches were found between bee-collected resins and those sampled from plants. Drescher et al. (2019) suggested this was likely due to the high variability found both within and among plant taxa; specifically, intra-specific variability in resin chemistry was relatively pronounced among individuals of birch and poplar. Both groups are known to contain a large number of hybrids that are difficult to distinguish morphologically, and often differ significantly in the chemical composition of their resin. Bees were shown to not necessarily collect resin from the closest source, but were instead highly selective, making choices for specific trees even among closely related and nearby species.

In 2016, Isidorov et al. conducted a comparison of propolis and resin samples from seven European countries – Latvia, Russia, Finland, Poland, Ukraine, Slovakia and France. Buds were collected from resin-producing trees occurring within the vicinity of hives sampled for propolis – downy birch (*Betula pubescens*), silver birch (*B. pendula*), common aspen (*Populus tremula*), black poplar (*P. nigra*), horse-chestnut (*Aesculus hippocastanum*), black alder (*Alnus glutinosa*) and Scots pine (*Pinus sylvestris*). Based on their chemical configuration, the propolis samples were separated into three groups, characterized as ‘Poplar-type’ (Finnish and Latvian

samples), 'Birch-type' (Russian and Polish samples), and 'Aspen-type' (Ukrainian, Slovakian and French samples). Based on their results, the authors drew two main conclusions.

First, when there are a variety of resin sources available, the chemical composition of propolis rarely matches exclusively to a single botanical source. Although all of the propolis samples were able to be matched relatively closely to a resin sample, nearly all of the samples contained compounds absent from their identified plant source, and vice versa. Secondly, Isidorov et al. (2016) highlight evidence for preferential selection by resin-collecting honeybees. None of the propolis samples contained any indication of the use of silver birch, horse-chestnut, black alder, or Scotch pine, all of which produce significant amounts of resin. Isidorov et al. (2016) suggest two hypotheses to explain their second conclusion. The first relates to potential variation in the levels of antimicrobial activity displayed by different resins. However, they report that the resins avoided by honeybees in their study showed relatively high biological activity against the bacteria assessed. Alternatively, avoided resins may possess deterrent or toxic compounds – although there is no suggestion currently as to the identity of these compounds.

Despite the seemingly vital contribution of resin by trees of Salicaceae family (poplars and willows), bees still produce propolis in areas lacking these species. Clearly, bees are able to, and actively will seek other botanical sources from which to retrieve resin when poplars or willows are not available (Greenaway et al., 1990).

The botanical sources of propolis in Brazil are of significant interest, as Brazil is the only tropical country that exports a considerable amount of propolis (Bankova et al., 2006). Propolis produced in southern Brazil is termed 'green' propolis. Such propolis is collected from the buds of *Baccharis dracunculifolia* (family Asteraceae) (Chan et al., 2013; Devequi-Nunes et al., 2018; Ferreira et al., 2017; Park et al., 2002). Conversely, northern Brazil is characterized by red propolis, from the plant *Dalbergia ecastaphyllum* (family Fabaceae) (Devequi-Nunes et al., 2018). There have also been reports of 'brown' propolis, thought to be sourced from *Copaifera sp.* (family Fabaceae) (Devequi-Nunes et al., 2018; Machado et al., 2016).

Ethanolic extracts of red, green and brown Brazilian propolis were analysed for differences in their chemical compounds by Devequi-Nunes et al. (2018). Red propolis extracts showed 23.89% more phenolic compounds, and 29.56% more flavonoid compounds than green propolis. Similarly, red propolis extracts showed 48% more phenolic compounds, and 84.13%

more flavonoid compounds than brown propolis. Red propolis was also reported to display the best antimicrobial activity, likely linked to the compounds reported above, and to have the highest levels of antioxidant compounds. These results are in accordance with similar comparative studies, in which samples with the lowest concentrations of phenolic compounds or flavonoids (brown propolis) demonstrate less antimicrobial activity than those with higher concentrations of the compounds (red propolis) (Machado et al., 2016). Still, it is noteworthy that all samples – red, green and brown – demonstrate some level of antimicrobial and antioxidant activity (Machado et al., 2016).

Samples of red propolis in Brazil were collected in areas dominated mainly by *D. ecastaphyllum* (Daugsch et al., 2008). Following chemical analysis, it was concluded that *D. ecastaphyllum* was in fact the botanical origin of the propolis. Samples of red propolis have also been collected in areas where *D. ecastaphyllum* is scarce. Such samples contained lower concentrations of the compounds found in *D. ecastaphyllum* resin (Daugsch et al., 2008). They also appeared to have lower levels of antimicrobial activity. This suggests that although bees are able to collect resin from many botanical sources, they choose those with the greatest biological activity (Daugsch et al., 2008). It is not completely clear why some trees are preferred for resin-collection. Suggestion has been made that honey bees are able to detect chemical cues on plant surfaces that indicate the presence of particular biologically active substances (Salatino et al., 2011).

In other parts of South America, the origin of some propolis has been identified as trees of the genus *Clusia* (family Clusiaceae). The resin from such trees is also used by meliponine and euglossine bees as a pollinator reward, and by stingless and orchid bees as nest building material (Bankova et al., 2006). The dioecious plants secrete resin from glandular tissues in both the male and female flowers. The phenolic compounds of propolis samples produced in Venezuela by *Apis mellifera* and five indigenous species of stingless bees were analysed by Tomás-Barberán et al. (1993). A majority of the propolis samples contained the polyprenylated benzophenones also exuded by the flowers of *Clusia major* and *C. minor*. Visual observations of *A. mellifera* visiting the flowers of *C. minor* were also recorded. *C. rosea* has been identified as a likely source of resin for propolis in Cuba. *C. rosea* is widely distributed across Cuba, and its floral resin is an abundant source of polyisoprenylated benzophenones. Work by Cuesta-Rubio et al. (2002) showed that the chemical nemorosone is

one of the major components of Cuban propolis, and that the same chemical has been isolated from *C. rosea*.

The importance of utilising a diverse range of available botanic resources by bees has been recognized by Drescher et al. (2014). The authors highlight three ways resource diversity can improve the well-being of any organism, in this case specifically the stingless bees of Australia (*Tetragonula carbonaria*). First, functional redundancy refers to the idea that the ability to exploit a variety of resources means resources are continuously available. Secondly, using a variety of resources may allow optimization of nutritional intake or other health benefits, coined 'functional balance' by Drescher et al. (2014). In this case, lower concentrations of a compound in one resource may be compensated by the presence of a higher concentration of the same compound in another. Finally, functional complementarity indicates a variety of resources can improve a specific function.

Scientists have repeatedly questioned why the number of resin sources visited by honeybees is seemingly so limited. Particularly in Brazil, home to more than 56,000 native species (Giulietti et al., 2005), it seems surprising that propolis chemotypes are usually attributed to just a single species. Saltinao and Salitino (2017) have shown that resin producing plants must meet two requirements to be used by honeybees as a resin source. First, plants must produce resin with significant biological activity. This requirement does not seem to eliminate many candidates, as a considerable number of resins have been demonstrated to possess antimicrobial activity. It is, however, the second requisite that Salatino and Salatino (2017) suggest is the most limiting factor. Plants must produce resin in a way that it is accessible to resin-collecting bees. Latex is likely too sticky for collection. Conversely, some resins are likely too solid. Resin-collecting honeybees are therefore limited to botanical sources that meet these two requirements

The identification of the botanical origins of propolis is important in determining its quality, and for product standardization (Bankova et al., 2000; Bankova et al., 2006; Ghisalberti, 1979). This knowledge would also be useful for beekeepers, to ensure the correct plants occur within the flight range of their hives (Alqarni et al., 2015; Bankova et al., 2000). The inability to collect propolis is known to negatively affect colonies, and bees are even reported to use "propolis substitutes" such as paints and asphalt (Alqarni et al., 2015; Bankova et al., 2000). In terms of standardization of propolis types, marker substances have been proposed (Salatino

et al., 2011). To be considered a marker for a particular propolis type, a substance must be of known botanical origin, and must be abundant and constant in the propolis type. Standardization of propolis is an important issue, as human demand for its medicinal properties increases.

Despite the significant variation in botanical origin of propolis based on geographic location, similar biological activity has been observed in samples from all over the world, despite differences in chemical composition. The active components of propolis are still in the process of being studied, and there are likely many unknown components occurring in small quantities that contribute to the anthropologically desired biological activity. Kujumgiev et al. (1999) found that although propolis from multiple different geographic locations consisted of significantly different chemical composition, they all displayed significant antifungal and antibacterial properties, with most also exhibiting antiviral activity. This seemingly surprising finding is, in retrospect, quite reasonable, as the natural use of propolis is in honeybee health.

Wollenweber and Buchmann (1997) investigated the botanical sources of propolis in the Sonoran Desert. In this area, vegetation is scattered, and is dominated by desert shrubs and cacti. Poplars are quite rare. Despite this, propolis samples were found to match resin from *P. fremontii*. It therefore appears that despite their scarcity, honey bees actively sought the resin of the *Populus* genus. Only one chemical was found that was not derived from *Populus* and was instead thought to have originated from the leaf exudate of an *Ambrosia* or *Baccharis* species. The question of preference or availability is provoked. Notably, are particular botanical sources for propolis preferred by different honeybee races, or is the source of resin solely determined by what is available in the flight range of the hive?

Volatile Organic Compounds

Plant compounds involved in primary metabolic pathways aid growth, development, and reproduction. Plants also produce other compounds known as “secondary” metabolites. Plant secondary metabolites serve ecological roles and are responsible for the medicinal and therapeutic properties of many plants (Gullo, 2013). Among these secondary metabolites are volatile organic compounds (VOCs). VOCs are released from almost all types of tissue as aromatic compounds, nitrogen-containing compounds and green leaf volatiles (Vivaldo et al., 2017). VOCs are characterized as lipophilic liquids with physical properties that allow them

to move across cellular membranes and be released into the environment readily (Pichersky et al., 2006). More than 1700 VOCs have been identified (Knudsen et al., 2006). These compounds are involved in the interactions of plants with their surrounding abiotic and biotic environment (Spinelli et al., 2011). Such interactions include communication between plants (Baldwin et al., 2006; Effah et al., 2019), deterrence or attraction of insect pests and pollinators respectively (Dudareva & Pichersky, 2000; Mumm et al., 2003; War et al., 2012), attraction of natural enemies of herbivores (Clavijo McCormick et al., 2012), and as an adaptation to environmental stresses (Holopainen & Gershenzon, 2010).

There is significant variation in the volatiles of different plant families, species, and even individuals (Bankova et al., 2014). Because honey bees incorporate plant resins to propolis without significant chemical alteration (Greenaway et al., 1990; Salatino et al., 2011), the volatiles present in propolis can be used in the characterisation and identification of the botanical sources of propolis samples. This method is commonly employed (e.g., Falcão et. al, 2016; de Oliveira Sartori et al., 2021; Pellati et al., 2013).

Although historic analyses of propolis reported up to 10% volatiles, recent studies converge on a much lower percentage, usually about 1%, and rarely up to 2-3% (Bankova et al., 2014). Despite the seemingly small quantitative contribution of volatiles to the chemical composition of propolis, this group of constituents represent an important part of the chemical signature of propolis. The volatiles found in propolis are responsible for its pleasant aroma (Drescher et al., 2017; Yang et al., 2010). Not only is this aroma attractive for human consumers, honeybees are also known to respond to odours in numerous behavioural situations (Krofczik et al., 2009). The volatiles present in propolis also contribute to the biological activity demonstrated against both human and honeybee diseases (Huang et al., 2014).

Biological activity

In recent years, particularly the past decade, there has been an influx of research investigating the biological activity of propolis and propolis extracts against common bacterial and fungal isolates. In particular, the potential role of propolis in human-medicine has been of increasing importance as scientists and consumers alike look toward natural remedies for ongoing illnesses; as numerous pathogens develop resistance to modern antibiotics, researchers are

looking toward natural products that may possess similar antimicrobial properties. Additionally, the role of propolis in protecting honeybee health has been highlighted as an important area of research. The chemical variation of propolis caused by the available botanical sources can also contribute to the biological activity of propolis.

Social insects are subject to higher rates of disease transmission, and subsequently, the increased demand on the immune system can be energetically costly. It was therefore surprising that when the bee genome was sequenced it appeared to have relatively limited diversity of immune system related genes (Simone et al., 2009; Wilson-Rich et al., 2009). Simone et al. (2009, 2012) suggests the use of propolis to prevent the spread of diseases could be considered self-medication, with the colony acting as an individual, or the 'self'. Because such a small portion of the colony acts as resin foragers (1 to 3%), the overall energy expenditure of the colony during resin foraging and propolis production is likely minimal, especially when compared with the energy involved in the up-regulation of complex individual immune systems. This down-regulation of the immune system may allow saved energy expenditure to be reallocated in honey production, brood rearing or overwintering survival (Borba et al., 2015). Thus, propolis appears to be a key substance mediating social immunity.

In countries of diverse climates and therefore botanical resin sources, regional variation in activity against honeybee diseases may be detected. This has been demonstrated in the United States by Wilson et al. (2015). Propolis was collected from 12 locations across 12 climatically diverse states. Samples from Nevada, Texas and Colorado were the most inhibitory against the growth of *Paenibacillus larvae*, the larval disease of honey bees known as American foulbrood that kills colonies worldwide, while propolis from Minnesota, Louisiana and New York did not show any inhibition of *P. larvae* over the concentrations tested. Samples were then collected from different sites in Nevada and Minnesota – the states with the highest and lowest inhibitory activity respectively. Interestingly, the new samples from Nevada showed similar results, while the new samples from Minnesota, collected within 65km of the original samples, showed increased activity, but still did not completely inhibit *P. larvae* growth. *Ascophora apis* (causative agent of chalkbrood) was found to be far more susceptible to propolis samples than *P. larvae.* All samples inhibited *A. apis* – samples from Nevada were again amongst the strongest inhibitors of bacterial growth, while those from Minnesota were the least inhibitory. The results of the resampling from Nevada and Minnesota were the same as they were for the inhibitory activity against *P. larvae*.

Propolis has also been demonstrated to have biological activity against common human diseases. Bosio et. al (2000) investigated the antibacterial activity of two propolis extracts collected in Italy against *Streptococcus pyogenes* strains. The sample that displayed the higher levels of antibacterial activity also contained higher concentrations of the flavonoids galangin and pinocembrin. The authors linked the higher inhibitory effect of this sample to the higher concentrations of these compounds, which was in turn associated with the presence of different botanical species in the locations of collection.

Bonvehí and Gutiérrez (2012) measured the antimicrobial activity of propolis extracts collected from Basque Country, Spain. The minimum inhibitory concentration (MIC) of propolis extracts against three Gram-positive bacteria (*Streptococcus mutans*, *Streptococcus pyogenes* and *Staphylococcus aureus*), three Gram-negative bacteria (*Helicobacter pylori*, *Salmonella enterica* and *Escherichia coli*), and two fungal strains (*Candida albicans* and *Saccharomyces cerevisae*) were recorded. All the Gram-positive bacteria, particularly *S. mutans* and *S. aureus*, were demonstrated to be highly sensitive to a lower concentration of propolis. *Escherichia coli* was insensitive to all of the concentrations tested and showed negligible inhibition. *Salmonella enterica* and *H. pylori* were shown to be sensitive to the propolis extracts, as were the fungal strains tested. The authors also noted a positive correlation between the content of flavonoids and phenolic compounds in samples, and the level of antimicrobial activity demonstrated.

Conclusion

Despite a recent trend of greater propolis research, a number of uncertainties remain, opening avenues for future research in the field of propolis. Importantly, there is limited information regarding New Zealand propolis and several lines of research are yet to be investigated. In particular, the resins collected by honey bees and whether there is any association with native plants.

Because of its natural use by honeybees as a form of self-medication, the biological activity of propolis is not surprising. Still, the level of antimicrobial activity has been shown to vary amongst propolis samples. Samples from different countries, and even samples from different regions or locations within countries have also varied in their ability to inhibit the growth of

common bacteria. Despite this proven variation, there is still a common trend to treat propolis collected from across an entire country as a single sample. The characterisation of propolis for use in food and cosmetic products will require a more detailed understanding of the variation between samples.

There is significant variation in the amount of research concerning propolis conducted in different countries. The review conducted by Bankova et al. (2014) provides a relatively comprehensive summary of the propolis research conducted globally. It also clearly depicts the differences in the quantity of research between countries. The various types of propolis found in Brazil have been studied extensively, and such studies feature heavily in Bankova's review. However, there are many countries with propolis-producing bees, and appropriate resin producing trees, absent from the list. Included in this group is New Zealand. Where New Zealand propolis is included in recent reviews such as that composed by Kasote et al. (2022), it is clear that the depth of knowledge about New Zealand propolis is still very low.

At the writing of the current review, there has been no regional survey of the propolis produced in New Zealand spanning the entire country. While the work of Markham et al. (1996) used samples based loosely on a regional structure, regions were still mixed, and an absence of samples collected in the South Island. As a country characterised by a very successful honey industry, there is potential to improve our propolis market by considering new avenues of differentiation and characterisation. The flora of New Zealand varies significantly across the country. Complementary variation in the chemical composition of propolis may therefore be expected.

Thesis aims and objectives

The central objective of this study is to identify potential regional variation in the propolis produced in New Zealand, specifically regional variation in the botanical sources used. Based on the information gathered from this study, my aim was to provide beekeepers and those involved in the commercialisation of propolis with guidance for tree planting and beehive locality that would support collection of high quality propolis. Primarily, I was interested in any variation between the resin of different species or clones, particularly commonly planted poplars and native trees, and whether this variation was detectable in the propolis produced regionally.

I carried out the majority of the planning, conducting, and analysing of the research presented in each chapter. My supervisors were Alastair Robertson and Andrea Clavijo-McCormick of Massey University, and Michelle Taylor from The New Zealand Institute for Plant and Food Research Limited.

Chapter 2: Characterisation of poplar and New Zealand native plant resin samples based on volatile organic compounds

In this chapter, I aimed to answer the question "is there variation in the volatile organic compounds of the resin of collected native species and commonly planted poplars in select regions throughout New Zealand?". I predicted that the two groups, poplar resin and native resin, would be significantly different in their chemical composition, and would share little overlap. I also predicted that there would be much more variation in chemical composition within the native group than within the closely related poplar hybrids, and that there would be very little variation between poplar clones.

Chapter 3: Characterisation of regionally collected New Zealand propolis using volatile organic compounds

In this chapter, I aimed to answer the question "is there variation in the volatile organic compounds of some New Zealand propolis, and can this variation be explained by region?" Additionally, I aimed to tentatively identify the potential contribution of native and poplar species in the collected propolis. I expected the propolis samples to somewhat overlap in their chemical composition, with some variation visible. I expected that such variation would be explained by the region from which samples were collected. Based on previous classification of New Zealand propolis as 'poplar-type', I expected to see poplars as the dominating contributor of resin to New Zealand propolis.

Chapter 4: Overall discussion, conclusions and recommendations

In the final chapter, I summarised the results of Chapter 2 and 3, and discussed the implications of my findings. The results of this study indicate that there may be more variation in New Zealand propolis than suggested in previous reviews. Some of this variation may be attributed to the native flora of New Zealand. While the region from which propolis was collected does not seem to completely explain the variation between samples, a more detailed analysis of

regionally collected samples would further support or refute this claim and allow an opportunity to characterise New Zealand propolis.

Chapter 2

Characterisation of poplar and New Zealand native plant resin samples based on volatile organic compounds

Chapter 2: Characterisation of poplar and New Zealand native plant resin samples based on volatile organic compounds

1. Introduction

Plant resin is a tacky, water insoluble substance secreted by plants to protect wounded tissue and leaf buds from herbivore and pathogen attack (Langenheim, 2003). The chemistry of plant resins is complex, and can comprise more than 300 compounds (Langenheim, 2003). The chemical composition of resin can be species specific. However, it can also vary greatly, both quantitatively and qualitatively, within and between plant families, and even between closely related individuals (Drescher et al., 2019; Langenheim, 2003).

Propolis is a resinous product collected by bees from the exudates of plants and mixed with beeswax (Agüero et al., 2011). Bees use propolis to seal cracks and crevices within the hive. Propolis is an incredibly varied product, with the chemical composition of propolis differing depending on the botanical source from which resin was collected. The presence of propolis has been linked to the low occurrence of bacteria and mould within the hive (Agüero et al., 2011). Propolis is a biologically active product with antimicrobial, anticarcinogenic, antifungal, antioxidant and antibacterial properties. The relative strength of each of these properties has been shown to differ depending on the chemical composition of the propolis, which is of interest to researchers and beekeepers alike.

The chemical composition of propolis is determined by its botanical source. The plants visited by resin-collecting bees affect the resulting chemical make-up of the propolis. The plants available to resin-foragers often differ according to the geographical location of collection due to variation in terrain, climate, water availability and land use (Park et al., 2002). Therefore, it is common to see variation in propolis samples that correlate with variation in the botanical sources at different geographical locations.

Volatile organic compounds (VOCs) are a useful way to characterise plant resins. The flora available to resin-collecting bees has been shown to determine the subsequent chemical composition of propolis, including the volatile component of its chemical signature (Bankova et al., 2014). It has been hypothesized that VOCs could play a role in providing odours to

resin-foraging bees that could allow preferential selection of plants (Bankova et al., 2014). Chemicals found in the VOCs of propolis can be linked to those found in the resin of local flora (Jerković and Mastelić, 2003).

New Zealand propolis is generally considered akin to European propolis that is collected from the resin of poplar trees. Much of the New Zealand landscape is characterised by farmland in which poplar trees are common and several poplar hybrids have been developed specifically to suit the New Zealand niche. Preferred poplar clones differ across the country due to differences in climate and environment (Wilkinson, 1999). However, New Zealand also has a unique native flora whose role in propolis is yet to be considered. To date, research into New Zealand propolis has not yet considered the role of native flora in its production, nor any potential variation in contributing poplar hybrids.

The aim of this chapter was to characterise the VOCs in resin collected from common native species and poplar hybrids. I expected to identify significant variation between the native and poplar groups. Within each group, I predicted that the native species would vary more than the closely related poplar hybrids with very little variation between the poplar hybrids, and even less variation between hybrid clones.

2. Methods

2.1 Autumn Resin Sample Collection

Poplar resin samples were collected in April 2021 from Plant and Food Research's poplar and willow orchard in Aokautere, New Zealand. Apical leaf buds were collected from six clones of commonly planted poplars, all from the same parent species *Populus deltoides x nigra*: Veronese, Pakaraka, Argyle, Selwyn, Fraser and Weraiti. There were three individuals of each clone. Buds were removed from each individual and stored on ice until extraction took place on the same day.

Resin samples from native New Zealand plants were collected from Massey University Manawatu campus in April 2021. Samples were collected in the same manner as the poplar samples.

2.2. Spring Resin Sample Collection

Poplar resin samples were collected again in November 2021 from Plant and Food Research's poplar and willow orchard in Aokautere, New Zealand. Apical leaf buds were collected from three individuals of the same six clones of poplars as the April collection. Buds were removed from each individual and stored at -4°C until extraction.

2.3. Labelling of Samples

Samples were labelled based on the season in which they were collected, the species from which they were collected, and the replicate number (Table 2.1). Samples collected in autumn were labelled AUT, and samples collected in spring were labelled SPR. Samples were given a single letter to indicate the species or hybrid from which they were sampled. The number at the end of each sample indicates the replicate.

Table 2.1: Resin samples labelling key

SAMPLE ID	COMMON NAME	SCIENTIFIC NAME
AUTA1	ARGYLE	*Populus euramericana 'Argyle'*
AUTA2	ARGYLE	*Populus euramericana 'Argyle'*
AUTA3	ARGYLE	*Populus euramericana 'Argyle'*
AUTF1	FRASER	*Populus euramericana 'Fraser'*
AUTF2	FRASER	*Populus euramericana 'Fraser'*
AUTF3	FRASER	*Populus euramericana 'Fraser'*
AUTP1	PAKARAKA	*Populus euramericana 'Pakaraka'*
AUTP2	PAKARAKA	*Populus euramericana 'Pakaraka'*
AUTP3	PAKARAKA	*Populus euramericana 'Pakaraka'*
AUTS1	SELWYN	*Populus euramericana 'Selwyn'*
AUTS2	SELWYN	*Populus euramericana 'Selwyn'*
AUTS3	SELWYN	*Populus euramericana 'Selwyn'*
AUTV1	VERONESE	*Populus euramericana 'Veronese'*
AUTV2	VERONESE	*Populus euramericana 'Veronese'*
AUTV3	VERONESE	*Populus euramericana 'Veronese'*
AUTW1	WERAITI	*Populus euramericana 'Weraiti'*
AUTW2	WERAITI	*Populus euramericana 'Weraiti'*
AUTW3	WERAITI	*Populus euramericana 'Weraiti'*
AUTC1	PŪRIRI	*Vitex lucens*
AUTG1	KARAKA	*Corynocarpus laevigatus*
AUTH1	WHAU	*Entelea arborescens*
AUTH2	WHAU	*Entelea arborescens*

AUTI1	PUKATEA	*Laurelia novae-zelandie*
AUTK1	KAWAKAWA	*Piper excelsum*
AUTK2	KAWAKAWA	*Piper excelsum*
AUTL1	LEMONWOOD	*Pittosporum eugenioides*
AUTN1	KĀNUKA	*Kunzea ericoides*
AUTO1	KŌHŪHŪ	*Pittosporum tenuifolium*
AUTQ1	KAURI	*Agathais australis*
AUTR1	NGAIO	*Myoporum laetum*
AUTT1	MĀPOU	*Myrsine australis*
SPRA1	ARGYLE	*Populus euramericana 'Argyle'*
SPRA2	ARGYLE	*Populus euramericana 'Argyle'*
SPRA3	ARGYLE	*Populus euramericana 'Argyle'*
SPRF1	FRASER	*Populus euramericana 'Fraser'*
SPRF2	FRASER	*Populus euramericana 'Fraser'*
SPRF3	FRASER	*Populus euramericana 'Fraser'*
SPRP1	PAKARAKA	*Populus euramericana 'Pakaraka'*
SPRP2	PAKARAKA	*Populus euramericana 'Pakaraka'*
SPRP3	PAKARAKA	*Populus euramericana 'Pakaraka'*
SPRS1	SELWYN	*Populus euramericana 'Selwyn'*
SPRS2	SELWYN	*Populus euramericana 'Selwyn'*
SPRS3	SELWYN	*Populus euramericana 'Selwyn'*
SPRV1	VERONESE	*Populus euramericana 'Veronese'*
SPRV2	VERONESE	*Populus euramericana 'Veronese'*
SPRV3	VERONESE	*Populus euramericana 'Veronese'*
SPRW1	WERAITI	*Populus euramericana 'Weraiti'*
SPRW2	WERAITI	*Populus euramericana 'Weraiti'*
SPRW3	WERAITI	*Populus euramericana 'Weraiti'*
SPRE1	TŌTARA	*Podocarpus totara*
SPRD1	REWAREWA	*Knightia excelsa*
SPRH1	WHAU	*Entelea arborescens*
SPRK1	KAWAKAWA	*Piper excelsum*
SPRM1	MĀHOE	*Melicytus ramiflorus*
SPRI1	PUKATEA	*Laurelia novae-zelandie*
SPRJ1	MĀNUKA	*Leptospermum scoparium*
SPRN1	KĀNUKA	*Kunzea ericoides*
SPRO1	KŌHŪHŪ	*Pittosporum tenuifolium*
SPRQ1	KAURI	*Agathais australis*
SPRR1	NGAIO	*Myoporum laetum*
SPRT1	MAPOU	*Myrsine australis*
SPRU1	CABBAGE TREE	*Cordyline australis*
SPRX1	HARAKEKE	*Phormium tenax*

SPRC1	PŪRIRI	*Vitex lucens*
SPRZ1	KŌWHAI	*Sophora microphylla*

2.4. Resin extraction procedure

One gram (1g) of leaf buds were weighed and transferred into a conical flask. 10ml of 95% purity hexane and an internal standard (10nanograms per microliter of nonyl acetate) were added to each flask, which were then sealed with cling film. The samples were stored for five hours at 16°C. After this time, leaf buds were removed, and the flask resealed and stored at -80°C overnight. Each sample was passed through a 0.2µl mesh filter and 200µl of the prepared liquid was transferred to a small vial and stored at -80°C until chemical analysis. This process was used for all resin samples in both autumn and spring.

2.5. GC-MS analysis

The samples were analysed using gas chromatography-mass spectrometry (GC-MS). Compounds were separated using a 30m x 250um x 0.25um TG-5MS capillary column. Helium was used as the carrier gas, and was supplied at 53.5kPa pressure, linear velocity 36.6 cm/s, total flow 14.0 mL/min, and purge flow 3.0mL/min. The initial oven temperature was 50°C held for 3 minutes, then raised 9°C/min until 200°C, which was maintained for 3 minutes. Compounds were tentatively identified by comparing target spectra to the mass spectra library from the National Institute of Standards and Technology (NIST) using the GC-MS postrun analysis software supplied by Shimadzu Corporation. Peaks were quantified relative to the internal standard, then divided by the dry weight of the leaf buds and sampling time (in hours) to estimate the emissions per dry weight per hour. A blank sample of just hexane and the internal standard was run, and any compounds found in this sample were excluded from the sample runs.

2.6. Qualitative and quantitative analysis

Statistical analysis was performed using RStudio (RStudio Team (2022). RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/). Compounds identified in only one sample were excluded from analysis. Principal component analysis was performed using the "prcomp" package and the "FactoMiner" package. Random Forest analysis was conducted using the "RandomForest" package. Tentatively identified compounds were given abbreviated names to make them

compatible with RStudio. Table 2.2 contains a full list of compound abbreviations. Both (*Z*)-α-Bergamotene and Spathulenol were identified twice in the same sample and were not able to be separated into stereoisomers. They have therefore been listed twice in Table 2.2 and separated by numbering them. An outlier, SPRA1, was identified due to its containing exceptionally high levels of α and β pinene when the poplar resin samples were analysed on their own and was removed from analysis (Appendix 2).

Table 2.2: Compound abbreviation key

IUPAC NAME	COMPOUND	ABBRIEVIATION
3-methylbut-2-enyl acetate	Prenyl acetate	PryAce
2,6,6-trimethylbicyclo[3.1.1]hept-2-ene	α-Pinene	aPin
6,6-dimethyl-2-methylidenebicyclo[3.1.1]heptane	β-Pinene	bPin
1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane	Eucalyptol	Euc
2-phenylethanol	Phenylethyl Alcohol	PEAlc
2-[(1S)-4-methylcyclohex-3-en-1-yl]propan-2-ol	L-α-Terpineol	LaTerp
(1R,4E,9S)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene	Caryophyllene	Cary
(1S,4S,7R)-1,4-dimethyl-7-prop-1-en-2-yl-1,2,3,4,5,6,7,8-octahydroazulene	α-Guaiene	aGua
2,6,6,9-tetramethylcycloundeca-1,4,8-triene	α-Caryophyllene	aCary
(3R,5S)-3,8-dimethyl-5-prop-1-en-2-yl-1,2,3,3a,4,5,6,7-octahydroazulene	δ-Guaiene	dGua
2-[(1R,3S,4S)-4-ethenyl-4-methyl-3-prop-1-en-2-ylcyclohexyl]propan-2-ol	Elemol	Ele
(1R,4R,6R,10S)-4,12,12-trimethyl-9-methylidene-5-oxatricyclo[8.2.0.04,6]dodecane	Caryophyllene oxide	CaryOx
2-[(3S,5R,8S)-3,8-dimethyl-1,2,3,4,5,6,7,8-octahydroazulen-5-yl]propan-2-ol	Guaiol	Gua
1,1,7-trimethyl-4-methylidene-2,3,4a,5,6,7,7a,7b-octahydro-1aH-cyclopropa[e]azulene	Alloaromadendrene	Aden
2,3-ditert-butylphenol	Di-tert-butylphenol	Diter
1,3,4-trimethylcyclohex-3-ene-1-carbaldehyde	3-Cyclohexene-1-carboxaldehyde, 1,3,4-trimethyl-	3Cyc1CA
(1S,5S,6R)-2,6-dimethyl-6-(4-methylpent-3-enyl)bicyclo[3.1.1]hept-2-ene	(*Z*)-α-Bergamotene	TaB1
6-methyl-2-(4-methylcyclohex-3-en-1-yl)hept-5-en-2-ol	α-Bisabolol	aBis
6,6-dimethyl-2-(3-oxobutyl)bicyclo[3.1.1]heptan-3-one	6,6-Dimethyl-2-(3-oxobutyl)bicyclo[3.1.1]heptan-3-one	DiBi
4-methyl-1-propan-2-ylcyclohex-3-en-1-ol	4-Terpineol	4Terp
4-ethenyl-1,2-dimethoxybenzene	3,4-Dimethoxystyrene	DiSty
(1S,5S,6R)-2,6-dimethyl-6-(4-methylpent-3-enyl)bicyclo[3.1.1]hept-2-ene	(*Z*)-α--Bergamotene	TaB2
2,6,6,9-tetramethyltricyclo[5.4.0.02,8]undec-9-ene	Tricyclo[5.4.0.0(2,8)]undec-9-ene, 2,6,6,9-tetramethyl-	TriUnd
3,4-bis(ethenyl)-3-methylcyclohexene	Cyclohexene, 3,4-diethenyl-3-methyl-	CycHex
(1R,2S,6S,7S,8S)-1,3-dimethyl-8-propan-2-yltricyclo[4.4.0.02,7]dec-3-ene	Copaene	Cop

(1S,4aS,8aR)-4,7-dimethyl-1-propan-2-yl-1,2,4a,5,6,8a-hexahydronaphthalene	α-Muurolene	aMuur
7-methyl-4-methylidene-1-propan-2-yl-2,3,4a,5,6,8a-hexahydro-1H-naphthalene	γ-Cadinene	gCad1
(1S,8aR)-4,7-dimethyl-1-propan-2-yl-1,2,3,5,6,8a-hexahydronaphthalene	δ-Cadinene	dCad
1,1,4,7-tetramethyl-1a,2,3,4,4a,5,6,7b-octahydrocyclopropa[e]azulene	α-Gurjunene	aGurj
(1S,4aR,8aS)-4,7-dimethyl-1-propan-2-yl-1,2,4a,5,6,8a-hexahydronaphthalene	α-Amorphene	aAmor
2-(6,8-dimethyl-3-tricyclo[4.4.0.02,7]dec-8-enyl)propan-2-ol	α-Copaen-11-ol	aCop11
(1aR,4R,4aR,7aR,7bR)-1,1,4-trimethyl-7-methylidene-2,3,4,4a,5,6,7a,7b-octahydro-1aH-cyclopropa[e]azulene	β-Gurjunene	bGurj
(3E,6E)-3,7,11-trimethyldodeca-1,3,6,10-tetraene	α-Farnesene	aFarn
1,1,4,7-tetramethyl-1a,2,3,4,5,6,7,7b-octahydrocyclopropa[e]azulene	Isoledene	Isol
(6E)-3,7,11-trimethyldodeca-1,6,10-trien-3-ol	Nerolidol	Nero
(1R,5S,6R,7S,10R)-10-methyl-4-methylidene-7-propan-2-yltricyclo[4.4.0.01,5]decane	β-Cubebene	1HCyc
7-methyl-4-methylidene-1-propan-2-yl-2,3,4a,5,6,8a-hexahydro-1H-naphthalene	γ-Cadinene	gCad2
(1aR,4aR,7S,7aR,7bR)-1,1,7-trimethyl-4-methylidene-1a,2,3,4a,5,6,7a,7b-octahydrocyclopropa[h]azulen-7-ol	Spathulenol	Spath1
(6Z)-3,7,11-trimethyldodeca-1,6,10-trien-3-ol	D-Nerolidol	Dnero
methyl 2-hydroxybenzoate	Salicylic acid, methyl ester	SalAMe
oct-1-en-3-ol	Amyl vinyl carbinol	AVC
(4R)-1-methyl-4-prop-1-en-2-ylcyclohexene	D-Limonene	dLim
3,7-dimethylocta-1,6-dien-3-ol	Linalool	Lin
(1,7,7-trimethyl-2-bicyclo[2.2.1]heptanyl) acetate	2-Camphanol acetate	2CampA
(1E,6E,8S)-1-methyl-5-methylidene-8-propan-2-ylcyclodeca-1,6-diene	Germacrene D	GermD
cyclopentanol	Cyclopentanol	CycPen
cyclopentanone	Dumasin	Dum
hexan-3-ol	Ethylpropylcarbinol	EPCar
cyclopent-2-en-1-one	2-Cyclopenten-1-one	2Cyc1
[(Z)-hex-3-enyl] acetate	(*Z*)-3-Hexen-1-ol, acetate	ZHexAc
2,2-dimethyl-3-methylidenebicyclo[2.2.1]heptane	Camphene	Camp
7-methyl-3-methylideneocta-1,6-diene	β-Myrcene	bMyrc
nonal	Nonanal	Non
(1S,4S)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one	L-camphor	lCamp
dodecan-2-yl acetate	2-Acetoxydodecane	2Aceto
2-(4-methylcyclohex-3-en-1-yl)propan-2-yl acetate	α-Terpenyl acetate	aTerpA
(1R,5S,6R,7S,10R)-4,10-dimethyl-7-propan-2-yltricyclo[4.4.0.01,5]dec-3-ene	α-Cubebene	aCub
hexyl octyl sulfite	Sulfurous acid, hexyl octyl ester	Sahex
(6E)-7,11-dimethyl-3-methylidenedodeca-1,6,10-triene	(*Z*)-β-Farnesene	ZbFarn

(5R)-2-methyl-5-[(2S)-6-methylhept-5-en-2-yl]cyclohexa-1,3-diene	Zingiberene	Zing
(4S)-1-methyl-4-(6-methylhepta-1,5-dien-2-yl)cyclohexene	β-Bisabolene	bBis
4-methoxy-6-prop-2-enyl-1,3-benzodioxole	Myristicin	Myris
3-methylidene-6-propan-2-ylcyclohexene	β-Phellandrene	bPhel
(3E)-3,7-dimethylocta-1,3,6-triene	(*E*)-β-Ocimene	EbOci
(3Z)-3,7-dimethylocta-1,3,6-triene	(Z)-β-Ocimene	ZbOci
decan-1-ol	Capric alcohol	CapAl
(1S,2S,4R)-1-ethenyl-1-methyl-2,4-bis(prop-1-en-2-yl)cyclohexane	β-Elemene	bElem
(6E)-7,11-dimethyl-3-methylidenedodeca-1,6,10-triene	(*E*)- β-farnesene	EbFarn
4-methyl-1-propan-2-ylbicyclo[3.1.0]hex-2-ene	Thujene	Thuje
4-ethenyl-4-methyl-1-propan-2-yl-3-prop-1-en-2-ylcyclohexene	δ-EIemene	dEle
[(2Z)-3,7-dimethylocta-2,6-dienyl] acetate	Neryl acetate	Nace
(1aR,7R,7aS,7bR)-1,1,4,7-tetramethyl-1a,2,3,5,6,7,7a,7b-octahydrocyclopropa[e]azulene	Ledene	Led
(3S)-3-[(2R)-6-methylhept-5-en-2-yl]-6-methylidenecyclohexene	β-Sesquiphellandrene	bSes
(1S,2S)-1-ethenyl-1-methyl-4-propan-2-ylidene-2-prop-1-en-2-ylcyclohexane	γ-Elemene	yEle
1,1,4,7-tetramethyl-2,3,4,5,6,7,7a,7b-octahydro-1aH-cyclopropa[h]azulen-4a-ol	Palustrol	Palus
(1aR,4R,4aS,7R,7aS,7bS)-1,1,4,7-tetramethyl-2,3,4a,5,6,7,7a,7b-octahydro-1aH-cyclopropa[e]azulen-4-ol	Ledol	Ledo
1,7,7-trimethyltricyclo[2.2.1.02,6]heptane	Tricyclene	TriCyc
4-methylidene-1-propan-2-ylbicyclo[3.1.0]hexane	Sabinene	Sab
1-methyl-5-prop-1-en-2-ylcyclohexene	Cyclohexene, 1-methyl-5-(1-methylethenyl)-	Cyc1M
(1R,4Z,9S)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene	Isocaryophyllene	ICary
(4S)-1-methyl-4-[(2E)-6-methylhepta-2,5-dien-2-yl]cyclohexene	(*Z*)-α- Bisabolene	ZaBis
(1R,4S,4aR,8aS)-1,6-dimethyl-4-propan-2-yl-3,4,4a,7,8,8a-hexahydro-2H-naphthalen-1-ol	δ-Cadinol	dCad
1-methyl-4-prop-1-en-2-ylcyclohexan-1-ol	β-Terpineol	dTerp
2-(4-methylcyclohex-3-en-1-yl)propan-2-ol	Terpineol	Terp
3-methylbut-3-en-1-ol	Methallyl carbinol	Mcarb
hexanal	Hexanal	Hex
5,6,7,7a-tetrahydro-4aH-cyclopenta[d][1,3]dioxin-4-one	Tetrahydrocyclopenta[1,3]dioxin-4-one	Tetdi
1-methyl-4-propan-2-ylbenzene	p-Cimene	pCim
2-hydroxybenzaldehyde	Salicylal	Sali
2-methoxy-3-prop-2-enylphenol	3-Allylguaiacol	3Ally
(1S,4S)-1,6-dimethyl-4-propan-2-yl-1,2,3,4-tetrahydronaphthalene	Calamenene	Cala
(1aR,4aR,7S,7aR,7bR)-1,1,7-trimethyl-4-methylidene-1a,2,3,4a,5,6,7a,7b-octahydrocyclopropa[h]azulen-7-ol	Spathulenol	Spath2

(1aS,1bS,2R,4aS,5S,7aR)-1,1,2,5-tetramethyl-decahydro-1H-cyclopropa[e]azulen-5-ol	Veridiflorol	VeFlor
(*E*)-hex-2-enal	Leaf aldehyde	LAld
(*E*)-pent-3-en-2-ol	Methyl propenyl carbinol	MPC
(2Z,7Z)-1,7-dimethyl-4-propan-2-ylcyclodeca-2,7-dien-1-ol	1-Hydroxy-1,7-dimethyl-4-isopropyl-2,7-cyclodecadiene	HDIC
2-[(1S,3Z,7Z)-4,8-dimethylcyclodeca-3,7-dien-1-yl]propan-2-ol	Hedycaryol	Hedy
hexan-1-ol	Amylcarbinol	AmylC
heptan-2-ol	Amyl methyl carbinol	AMC
(*Z*)-hex-3-en-1-ol	Leaf alcohol	LAlc
2-methyl-5-propan-2-ylcyclohexa-1,3-diene	α-Phellandrene	aPhel
1-methyl-4-propan-2-ylcyclohexa-1,4-diene	Crithmene	Crith
1-methyl-4-propan-2-ylidenecyclohexene	Terpinolene	Terpin
1,7,7-trimethylbicyclo[2.2.1]heptan-2-one	Camphor	Camph
(6E)-2,6-dimethyl-10-methylidenedodeca-2,6-diene	β-Farnesene	bFarn
(1S,2R,5S)-2,6,6,8-tetramethyltricyclo[5.3.1.01,5]undec-8-ene	α-Cedrene	aCed
2-methylbut-2-en-1-ol	2-Buten-1-ol, 2-methyl-	2But
3-methylbut-2-enyl benzoate	Prenyl benzoate	PB
2,6,6-trimethylcyclohexene-1-carbaldehyde	β-Cyclocitral	bCyc
(4aR)-3,5,5,9-tetramethyl-1,2,4a,6,7,8-hexahydrobenzo[7]annulene	β-Himachalene	bHim
hexan-2-ol	2-Hexanol	2Hex
(*Z*)-hex-3-enal	(*Z*)-3-Hexenal	Z3Hex

3. Results

I analysed 64 resin samples, 30 collected in autumn and 34 collected in spring. Six commonly planted poplar hybrids were sampled, and 20 native species. 111 compounds were tentatively identified. None of the compounds were identified in all the samples. None were identified in all of the autumn samples, or in all of the spring samples. Additionally, none of the compounds were identified in all of the native samples. One compound, Methallyl carbinol, was identified in all of the poplar samples. Two additional compounds, salicylal and 3-Allyguaiacol, were identified in all of the spring poplar samples. 31 compounds that were identified in native samples, were absent in poplar samples. 17 compounds that were identified in poplar samples, were absent in native samples. The full dataset can be found here.

Resin samples from poplars and from native trees grouped by the season during which they were collected were classified using principal component analysis (PCA) based on the compounds identified. The chemical profiles from the four groups overlapped. The spring poplar samples were tightly clustered (Fig. 2.2). The first principal component (PC1) explained 7.8% of the variability. PC1 was characterised by high scores for Dumasin, α-

Cubebene and β-pinene. Principal component two (PC2) accounted for 7.2% of the total variance. Compounds such as Eucalyptol, (*Z*)-α-Bergamotene and Camphene contributed more to this dimension (Supplementary table 1, Fig. 2.1). Principal components 1-22 accounted for about 91% of the total variance in the data, and compounds with higher contributions to these components (Supplementary table 1) were considered in subsequent analysis.

The most important compound for separating the four combinations of resin type (native spring and autumn, and poplar spring and autumn) identified by the random forest model was Methallyl carbinol (Fig. 2.3) which was most prevalent in the autumn poplar resins. 3-Allylguiacol and Salicylal also separated the sample types reasonably well and were most abundant in spring poplar resins. Other compounds such as Phenylethyl alcohol, Caryophyllene, and Tetrahydrocyclopenta[1,3]dioxin-4-one
also had higher discrimination among resin types. The out of bag (OOB) score for this model is 21.88%, indicating that a high number of samples can be successfully allocated to their respective group, either autumn or spring and native or poplar, using this method.

The resin samples did not clearly separate into native or poplar groups when presented as a cluster dendrogram (Figure 2.4). Some species and hybrids clustered closely together, such as the spring Veronese samples, both the spring and autumn Kawakawa samples, and the spring Weraiti samples. Other poplar hybrids were intermixed with native samples. While there was some overlap between autumn and spring samples, there was also a clear group of samples only collected in autumn. This group contained only poplar samples and a single Lemonwood sample. There also appeared to be a smaller group of poplar samples collected in the spring, further separating the poplar and native samples.

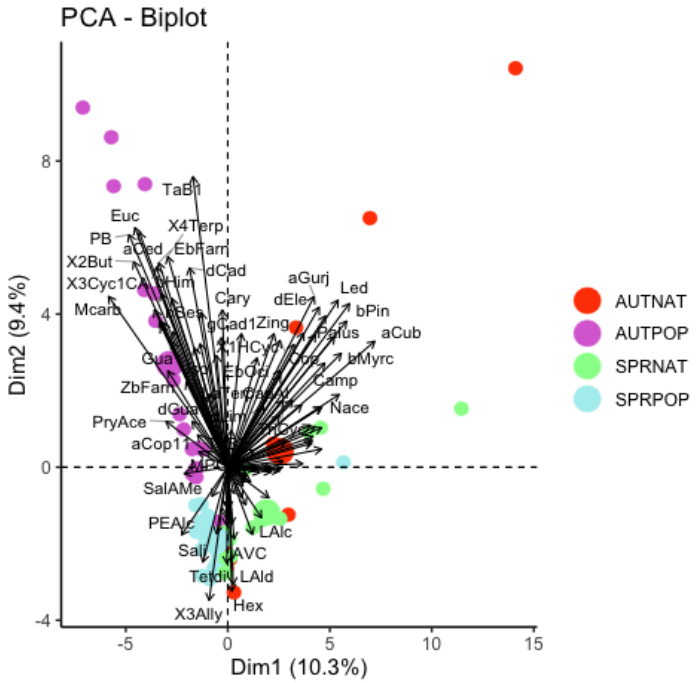

Figure 2.1: Biplot of resin samples and identified compounds grouped by native or poplar, and spring or autumn collection. Each small symbol represents an individual sample. The larger symbols are the centre of each group of samples.

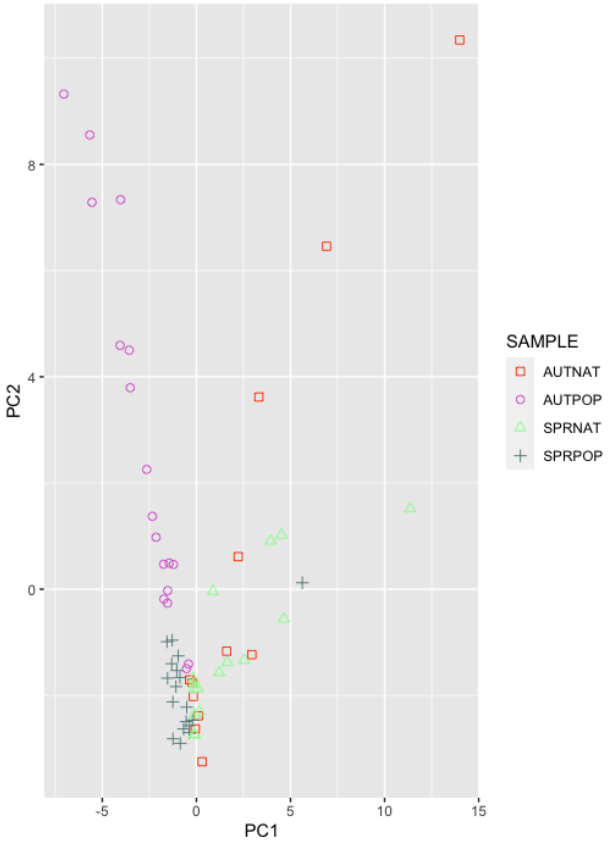

Figure 2.2: PCA plot of resin samples grouped by native or poplar, and spring or autumn collection.

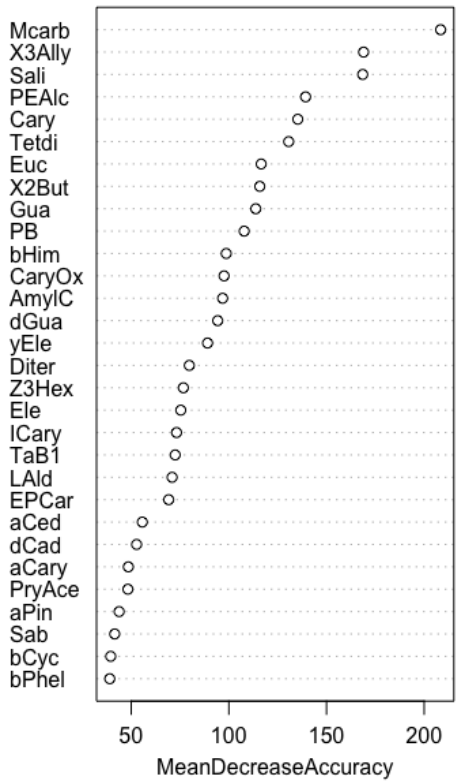

Figure 2.3: Random Forest plot of resin samples grouped by native or poplar, and spring or autumn collection.

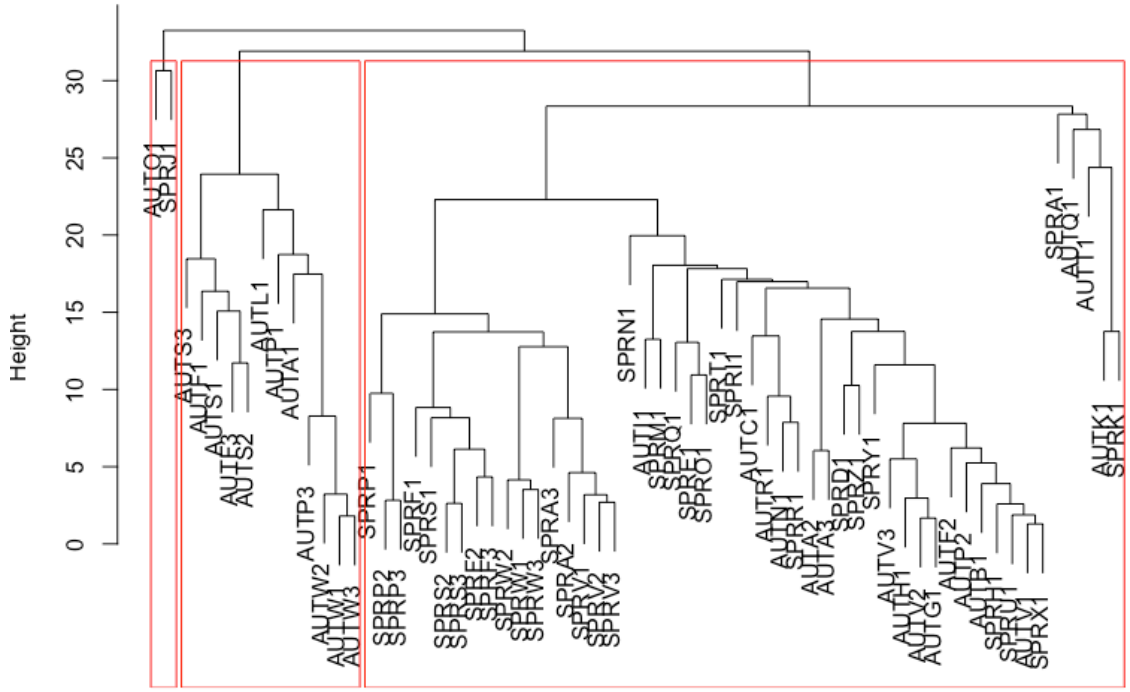

Figure 2.4: Cluster dendrogram of resin samples grouped by native or poplar, and spring or autumn collection.

Resin samples from poplars were classified again on their own with a new principal component analysis (PCA) based on the compounds identified. The chemical profiles from the four poplar groups overlapped, although some samples did appear to cluster together (Fig. 2.6). SPRW, SPRV, SPRS, AUTW, SPRP each formed their own tight cluster (Fig. 2.6). The first principal component (PC1) explained 27% of the variability. PC1 was characterised largely by Eucalyptol, α-Pinene and prenyl benzoate. Principal component two (PC2) accounted for 12.2% of the total variance. Compounds such as Caryophyllene, α-Guaiene and δ-Guaiene contributed more to this dimension (Supplementary table 2, Fig. 2.5). Principal components 1-22 accounted for about 98% of the total variance in the data, and compounds with higher contributions to these components (Supplementary table 2) were considered in subsequent analysis.

Looking just at the spring and autumn poplar resins, the most important distinguishing compound identified by the model was Eucalyptol (Fig. 2.7) which was most abundant in autumn. Other compounds such as phenylethyl alcohol, α-caryophyllene, and 6,6-Dimethyl-2-(3-oxobutyl)bicyclo[3.1.1]heptan-3-one also had higher contributions. The OOB score for this model is 8.57%, indicating that a very high number of the samples can be successfully allocated to their respective poplar hybrid using this method.

When presented as a cluster dendrogram, the poplar resin samples separated clearly into the season from which they were collected (Figure 2.8). One spring group is identified, and two autumn groups. One of the autumn groups is more closely related to the spring group than it is to the other autumn group. While many of the clones from each poplar hybrid appear closely related, for example the spring and autumn Weraiti clones, some are more divergent, for example the autumn Pakaraka clones.

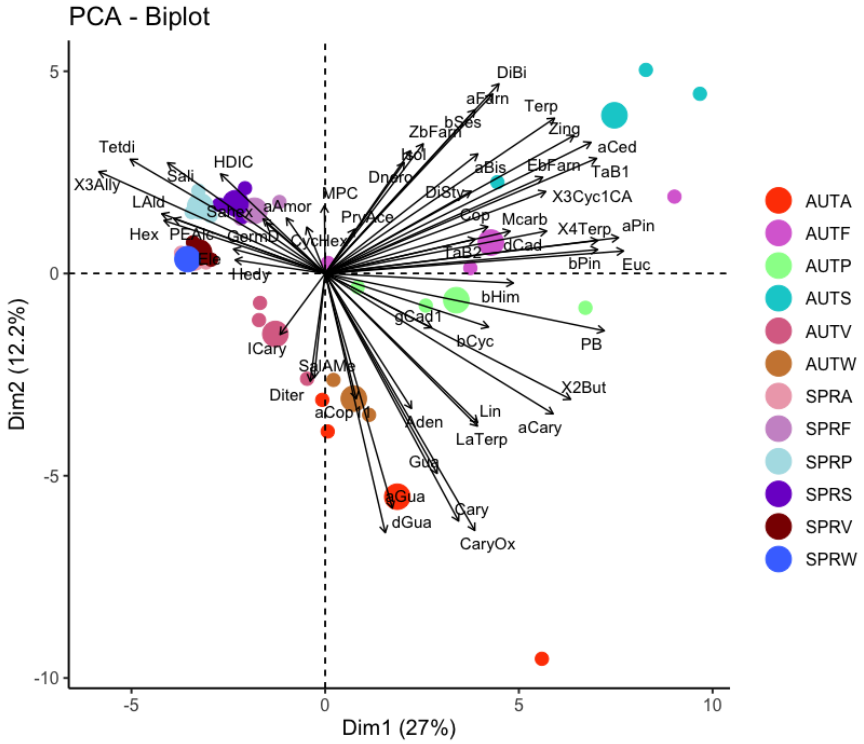

Figure 2.5: Biplot of poplar resin samples and identified compounds. Each small symbol represents an individual sample. The larger symbols are the centre of each group of samples.

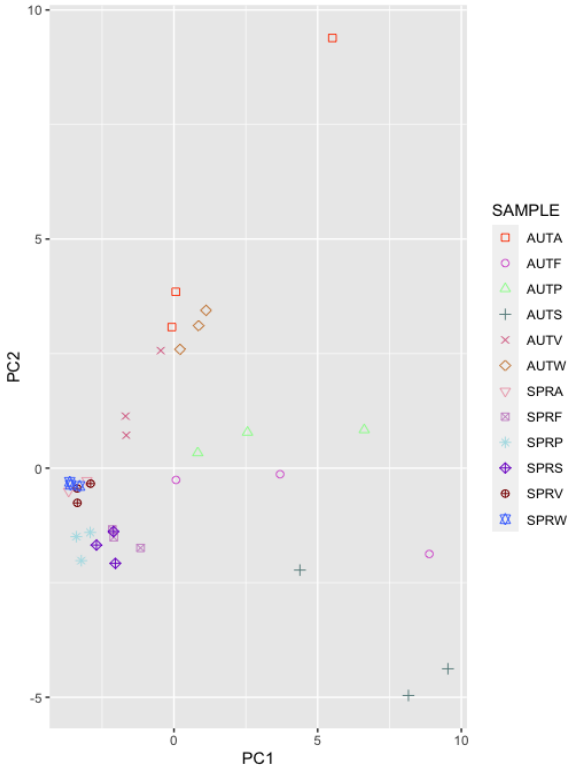

Figure 2.6: PCA plot of poplar resin samples.

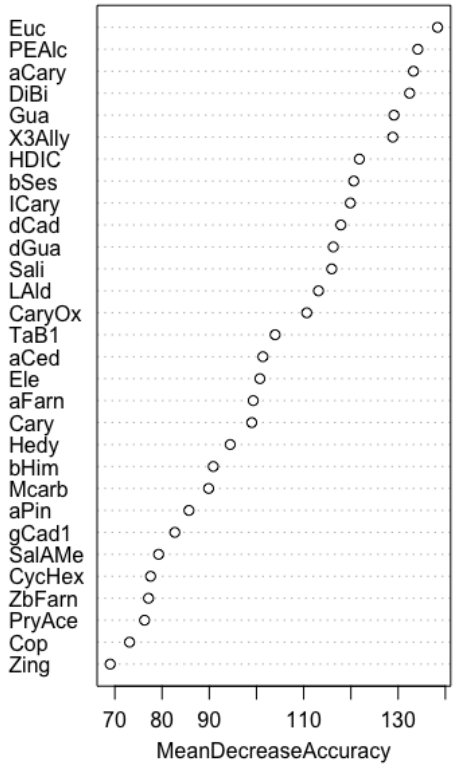

Figure 2.7: Random Forest plot of poplar resin samples.

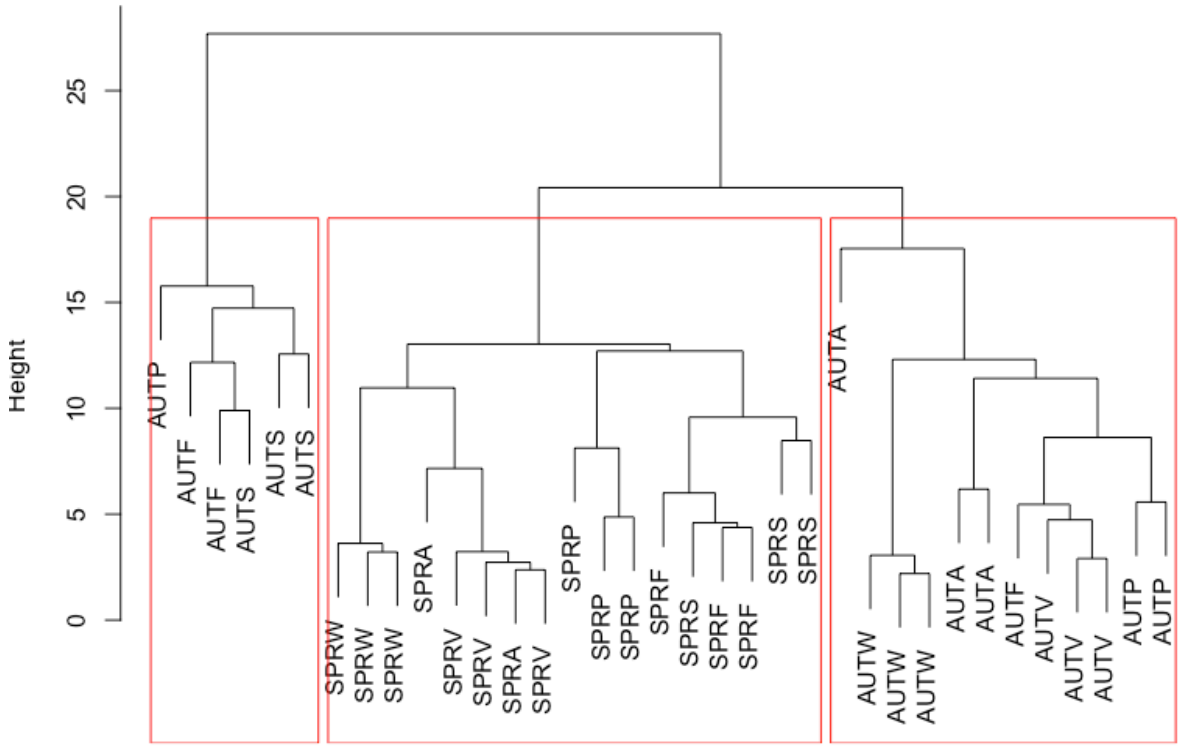

Figure 2.8: Cluster dendrogram of poplar resin samples.

4. Discussion

The aim of this chapter was to characterise the VOCs in resin collected from common native species and poplar hybrids. I had predicted that there would be significant divergence between the poplar and native groups. Figures 2.1 and 2.2 showed clear divergence between the two groups collected in autumn and in spring. Figure 2.4 depicted a similar separation. While there was some overlap between poplar and native samples, there was reasonably clear separation, as well as separation between the two seasons.

I also expected that the native group would be far more varied than the poplar group. However, Figure 2.5 suggests similar levels of variance between the two groups. This is due to the unexpected level of variance within the poplar group. Some hybrids, such as the Weraiti clones, were more closely related (Figure 2.5), while some were more varied.

Variation was identified amongst the collected resin samples, as seen in figure 2.2. While a large number of the samples collected formed a dense cluster, several samples separated from this large cluster. Further analysis began to identify some more specific variation.

When comparing poplar resin and native species resin based on the season in which they were collected, despite some overlap, four reasonably identifiable clusters occurred (Figure 2.6). Apart from one outlier, which was removed, the poplar samples collected in spring formed a very dense cluster. The poplar samples collected in autumn overlapped slightly with the other three groups, but formed a distinct, more spread cluster. The variation in both autumn groups was displayed more by PC2, and these groups displayed a more significant spread.

Methallyl carbinol was identified by the random forest analysis as being the most important compound for differentiating samples into their respective groups (Figure 2.3). It was identified in various quantities in all the poplar samples but was not identified in any of the native samples.

(*Z*)-α-Bergamotene was identified in 12 of the 20 Autumn poplar samples (it was absent in all replicates of Argyle and Veronese samples), but was only identified in two native autumn samples, five spring poplar samples, and no spring native samples.

Phenylethyl alcohol was identified by the random forest analysis as being the 4th most important compound for differentiating samples into their respective groups (Figure 2.3). Phenylethyl Alcohol was detected in high levels in all spring poplar samples but was not detected in any native samples collected in either season.

Eucalyptol was identified in all, but three poplar samples collected in autumn (the three Weraiti samples) but was only identified in one native species (both autumn and spring Kawakawa samples) and in all spring Argyle and Pakaraka samples, and one spring Selwyn sample. Eucalyptol was identified as being the 7th most important compound for differentiating samples into their respective groups by the random forest analysis (Figure 2.3).

Therefore, while there was some overlap and numerous compounds identified at similar levels in both poplar and native samples, the chemical composition of these two groups, and the two seasons in which they were collected, was distinctly different (Figure 2.1 and 2.4).

When considering only poplar resin samples, most of the clones clustered together closely in their triplicates (Figure 2.5 and 2.6). The most obvious exception to this is a single Argyle poplar sample collected in spring, which was removed as an outlier (Appendix 2). Still, another Argyle poplar clone collected in autumn was significantly separated from the main cluster. The resin collected from this tree was characterized by exceptionally high levels of both α and β pinene. A-Pinene was detected only in one other spring poplar sample, and β pinene was not identified in any other spring poplar sample. The sample collected from the same tree in autumn contained several compounds not identified in the other two argyle samples, including β pinene, L-α-Terpineol, Caryophyllene, 4-Terpineol, Linalool, Prenyl benzoate and β-Cyclocitral.

Eucalyptol was identified by the random forest analysis as the most important compound when differentiating poplar samples into their hybrid groups (Figure 2.7). Eucalyptol was identified in all the autumn poplar samples except the three Weraiti samples. In the spring poplar samples, it was only identified in the Argyle and Pakaraka clones, and one Selwyn clone. Within the samples in which Eucalyptol was identified, there were large variations in quantities, with Eucalyptol being the most abundant compound in some samples.

Phenylethyl alcohol was identified by the random forest analysis as being the 2nd most important compound for differentiating samples into their respective groups (Figure 2.7). It was identified in all the spring poplar samples. However, it was only detected in the three autumn Argyle and Weraiti samples, and in two autumn Selwyn samples.

Therefore, initial conclusions can be drawn regarding possible variation within and between poplar hybrids. Variation was identified between poplar clones, and this variation can be attributed to the presence, absence, or quantity of different compounds. Similarly, while most clones of each hybrid cluster closely together, some variation is still visible. This is most significant within the Argyle hybrid.

Similar variation between poplar species was identified by Dresher et al. (2019). In this study, the authors chemically compared the resin being collected by resin-foraging honeybees from 25 colonies for use in propolis production. When comparing poplar, birch, horse chestnut, and coniferous resins, poplars were identified as having the highest levels of intraspecific variability. *P. canadensis* resin varied between individuals from different sites, and even between neighbouring individuals. Three different chemotypes were characterised from *P. canadensis* resin. Additionally, when considering the role of resin in propolis production, several of the propolis samples did not match unambiguously to a single resin source. Instead, they contained numerous compounds found exclusively in poplar resin. The authors concluded that while poplar resin likely contributed to these samples, that other resin sources must have been visited.

VOCs have been used to link propolis with its botanical origin. For example, Agüero et al. (2011) compared samples of Andean Argentinian propolis with exudates of the native *Larrea nitida* and found them to be a close match. They concluded there was substantial evidence that the propolis samples studied were sourced from *L. nitida*. Still, several compounds were only detected in propolis samples, suggesting that additional botanical sources may have contributed resin.

Conclusions and future outlook

Not all my initial predictions were confirmed by the results. There was significant variation detected between the poplar and native groups, as I had predicted. There was also variation detected between seasons. While variation was detected within the native group, there was also more variation in the poplar group than anticipated. Some poplar hybrids displayed more intraspecific variation than others. The next steps in this area should involve sampling more species available to resin-collecting honeybees in New Zealand. Additionally, sampling the loads carried by returning resin-foragers and the resin from all the available species within forging-range of the hive would allow more detailed conclusions to be drawn (Drescher et al., 2019). The current study provides interesting and somewhat unexpected findings regarding the variation of resin in New Zealand and will allow further hypotheses to be created based on these results.

Chapter 3

Characterisation of regionally collected New Zealand propolis using volatile organic compounds

Chapter 3: Characterisation of regionally collected New Zealand propolis using volatile organic compounds

1. Introduction

Propolis is a product produced by most bee species composed mainly of beeswax and resin collected from local flora (Ghisalberti, 1979). The word propolis is derived from the Greek *pro*-, meaning in defence, and *polis*-, meaning the city (Ghisalberti, 1979), therefore, defence of the city, or, in the case of bees, the hive. Propolis is used to fill cracks and crevices within the hive to control airflow, and to embalm pests that die within the hive. Evidence for propolis as a form of 'self-medication' by bees has also been established (Borba et al., 2015; Evans & Spivak, 2010; Simone-Finstrom & Spivak, 2010; Wilson-Rich et al., 2009).

While wild bees often produce copious amounts of propolis to create an encompassing propolis envelope (Bankova et al., 2014; Seeley and Morse, 1976), honeybees bred for commercial practices tend to produce lower quantities of propolis due to the uniform nature of commercial hives (Hodges et al., 2019), and genetic selection against propolis production due to its inconvenience when harvesting honey (Delaplane, 2007). However, with increasing commercial interest in propolis, some beekeepers are looking to improve the quantity and quality of propolis production. Commercial beekeepers can use plastic propolis mats placed at the top of the hive to stimulate increased propolis deposition. Propolis mats can be frozen, and the propolis removed and used as a commercial product.

Propolis has been used as a natural remedy since ancient times (Castaldo and Capasso, 2002), and its popularity has been retained. Internationally, there is growing interest in propolis products in the biocosmetic and natural health industries (Sforcin and Bankova, 2011). This increased interest from consumers has created heightened engagement from researchers. The investment of research globally has not been evenly conducted, with some products receiving significantly more attention (Bankova et al., 2014).

Brazil currently dominates the international propolis market. This domination correlates with the prevalence of research into Brazilian propolis. There have been three main types of propolis identified in Brazil. Some authors have further divided these three types into 12

using finer analytical processes (Park et al., 2002). Their geographic location, botanical source and biological activity have been investigated in great detail (Park et al., 2002; Salatino et al., 2005). This detailed understanding of Brazilian propolis has made it an international preference when sourcing propolis and has helped shape a new focus on collecting and studying propolis not only based on the country of origin, but on the geographic location of collection.

The regional variation detected in international propolis samples can be of importance. Variation in the botanical sources of resin used have been shown to alter the biological activity of propolis. Such variation is critical when considering the application and standardisation of propolis as a commercial product. Therefore, it is becoming more common to analyse propolis samples by the region or area from which they were collected, rather than lumping all samples from a single country together.

New Zealand propolis remains largely understudied. New Zealand propolis is broadly considered 'European', and to be collected from poplar species (Kasote et al., 2022). However, to categorise New Zealand propolis so broadly does not account for any variation in botanical source geographically, nor does it allow for the potential contribution of distinct native species.

Markham et al. (1996) were the first to broadly investigate the chemical composition of New Zealand propolis and its potential regional variation, yet the samples analysed were largely mixtures from differing regions. Since then, work has focused mainly on the anti-carcinogenic properties of propolis. Still, there is a lack of understanding regarding any variation in the chemical composition of New Zealand propolis, and the potential contribution of region to such variation. Additionally, there has been little investigation into the botanical sources contributing to New Zealand propolis.

Volatile organic compounds (VOCs) have been suggested as a useful way to characterise propolis samples. VOCs compose a small but significant component of propolis, with some authors reporting between 5-10% (Castaldo and Capasso, 2002), while others report between 1-3% (Bankova et al., 2014). VOCs contribute to the pleasant aroma of propolis, as well as to its biological activity (Bankova et al., 2014). Additionally, the compounds identified in the

VOCs of propolis can be compared to the VOCs of the resin available to resin-collecting bees (Jerković and Mastelić, 2003).

The aim of this chapter was to characterise the VOCs in New Zealand propolis collected from different regions. I expected the chemical composition of many of the propolis samples to overlap, with other samples being notably varied. I predicted that this variation would be largely explained by the region from which samples were collected. I expected to detect the contribution of poplar resin due to the previous classification of New Zealand propolis as European or poplar-type. However, I also anticipated some contribution by native species.

2. Methods

2.1. Autumn Propolis Samples

Propolis mats were provided by Arataki Honey Ltd in April 2021. Mats were grouped together according to the apiary from which they were collected, and wrapped in plastic and burlap sacks, before being courier to Massey University. The propolis was removed from the mats and stored at -4°C until analysis.

2.2. Spring Propolis Samples

Beetek propolis mat (Ecrotek, New Zealand) were delivered to beekeepers to be placed on hives in late September. Mats remained in hives for between five to nine weeks. Mats from each site were wrapped in tinfoil and placed in a sealed plastic bag. The mats were couriered to Massey University, where the propolis was removed from the mats and stored at -4°C until analysis. Beekeepers were asked to provide information on the flora within 3km of the hive. The results of this request were vague and not compatible, so were excluded from further analysis.

2.3. Labelling

Propolis samples were labelled based on the season in which they were collected, and the region from which they originated. Samples collected in autumn were labelled AUT, and samples collected in spring were labelled SPR. When analysing the propolis samples irrespective of region, the samples collected in each season were numbered. Two propolis samples collected in autumn, AUT8 and AUT12, were excluded for analysis due to the

degradation of the samples. When considering region, each region was given a three-letter code as seen in table 3.1. Two samples, SPR9 and SPR15, were excluded from the regional analysis, as only one sample was provided from each region.

Table 3.1: Propolis samples labelling key

SAMPLE ID	REGION	REGIONAL + SEASONAL LABEL
AUT1	Waikato	AUTWAI
AUT2	Waikato	AUTWAI
AUT3	Waikato	AUTWAI
AUT4	Waikato	AUTWAI
AUT5	Waikato	AUTWAI
AUT6	Waikato	AUTWAI
AUT7	Waikato	AUTWAI
AUT9	Waikato	AUTWAI
AUT10	Waikato	AUTWAI
AUT11	Waikato	AUTWAI
AUT13	Waikato	AUTWAI
AUT14	Waikato	AUTWAI
AUT15	Waikato	AUTWAI
AUT16	Southland	AUTSOU
AUT17	Southland	AUTSOU
AUT18	Southland	AUTSOU
SPR1	Manawatu	SPRMAN
SPR2	Manawatu	SPRMAN
SPR3	Northland	SPRNOR
SPR4	Gisborne	SPRGIS
SPR5	Gisborne	SPRGIS
SPR6	Gisborne	SPRGIS
SPR7	Gisborne	SPRGIS
SPR9	Blenheim	
SPR10	Manawatu	SPRMAN
SPR11	Canterbury	SPRCAN
SPR12	Canterbury	SPRCAN
SPR13	Canterbury	SPRCAN
SPR14	Canterbury	SPRCAN
SPR15	Taranaki	
SPR16	Nelson	SPRNEL
SPR17	Nelson	SPRNEL
SPR18	Nelson	SPRNEL
SPR19	South Canterbury	SPRSCAN

SPR20	South Canterbury	SPRSCAN
SPR21	South Canterbury	SPRSCAN
SPR22	West Coast	SPRWC
SPR23	West Coast	SPRWC
SPR24	West Coast	SPRWC
SPR25	West Coast	SPRWC
SPR26	West Coast	SPRWC
SPR27	North Canterbury	SPRNCAN
SPR28	North Canterbury	SPRNCAN
SPR29	North Canterbury	SPRNCAN
SPR30	North Canterbury	SPRNCAN
SPR31	Hawkes Bay	SPRHB
SPR32	Hawkes Bay	SPRHB
SPR33	Hawkes Bay	SPRHB
SPR34	Hawkes Bay	SPRHB
SPR35	Hawkes Bay	SPRHB
SPR36	Hawkes Bay	SPRHB
SPR37	Hawkes Bay	SPRHB
SPR38	Hawkes Bay	SPRHB
SPR39	Hawkes Bay	SPRHB
SPR40	Hawkes Bay	SPRHB
SPR41	Hawkes Bay	SPRHB
SPR42	Hawkes Bay	SPRHB
SPR43	Hawkes Bay	SPRHB
SPR44	Hawkes Bay	SPRHB
SPR45	Waikato	SPRWAI
SPR46	Waikato	SPRWAI
SPR47	Waikato	SPRWAI
SPR48	Southland	SPRSOU
SPR49	Northland	SPRNOR
SPR50	Northland	SPRNOR
SPR51	Northland	SPRNOR
SPR52	Manawatu	SPRMAN
SPR53	Southland	SPRSOU
SPR54	Southland	SPRSOU
SPR55	Northland	SPRNOR
SPR56	Northland	SPRNOR
SPR57	Northland	SPRNOR
SPR58	Northland	SPRNOR
SPR59	Northland	SPRNOR

2.4. HS-SPME procedure

The methods used were modified from those reported by Pellati et al. (2013), as described. Extraction was performed using a manual holder and a 100µm polydimethylsiloxane (PDMS) fibre (Supelco, Bellefonte, PA, USA). Prior to undertaking GC-MS analysis, the fibres were conditioned in the injector of the GC, in accordance with the instructions provided by the manufacturer. Two grams (2g) of propolis was placed in a 10ml round-bottom headspace vial and sealed with a magnetic screw cap with a Silicone/PTFE septa. The sample was heated in a thermostatic bath for 30 minutes at 75°C. The SPME device was inserted into the vial, and the fibre was exposed to the headspace for 15 minutes. After samples had been analysed using the GC-MS, the SPME fibre was reconditioned for 5 minutes in the GC injector port at 250°C for reuse.

2.5. GC-MS analysis

The samples were analysed using gas chromatography-mass spectrometry (GC-MS). Compounds were separated using a 30m x 250um x 0.25um TG-5MS capillary column. Helium was used as the carrier gas, and was supplied at 53.5kPa pressure, linear velocity 36.6 cm/s, total flow 14.0 mL/min, and purge flow 3.0mL/min. The initial oven temperature was 50°C held for 3 minutes, then raised 9°C/min until 200°C, which was maintained for 3 minutes. Compounds were tentatively identified by comparing target spectra to the mass spectra library from the National Institute of Standards and Technology (NIST) using the GC-MS post-run analysis software supplied by Shimadzu Corporation.

2.6. Qualitative and quantitative analysis

Statistical analysis was performed using R Studio (RStudio Team (2022). RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/). Compounds identified in only one sample were excluded from analysis. The analysis was based on the relative abundance of compounds. Tentatively identified compounds were given abbreviated names to make them compatible with RStudio. Table 3.2 contains a full list of compound abbreviations. Both (*Z*)-α-Bergamotene and α-Muurolene were identified twice in the same sample and were not able to be separated into stereoisomers. They have therefore been listed twice in Table 3.2 and separated by numbering them. Principal component analysis was performed using the “prcomp” package and the “FactoMiner” package.

Table 3.2: Compound abbreviation key

IUPAC NAME	COMMON NAME	ABBRIEVIATION
3-methylbut-2-en-1-ol	Prenal acetate	PreAce
3-methylbut-2-enal	Prenal	Pre
2-methylbutanoic acid	α-Methylbutyric acid	aMBA
pent-4-enyl acetate	4-Pentenyl acetate	4PenAce
2,6,6-trimethylbicyclo[3.1.1]hept-2-ene	α-Pinene	aPin
3-methylbut-2-enyl acetate	Prenyl acetate	PryAce
(*E*)-2-methylbut-2-enoic acid	Cevadic acid	CevA
Benzaldehyde	Benzaldehyde	BA
Phenylmethanol	Benzyl Alcohol	Balc
3,7-dimethylocta-1,6-dien-3-ol	Linalool	Lin
2-phenylethanol	Phenylethyl Alcohol	PEAlc
buta-1,3-dien-2-ylbenzene	Phenoprene	Phen
Benzyl acetate	Benzyl acetate	BAce
2,6,6-trimethylcyclohexene-1-carbaldehyde	β-Cyclocitral	bCyc
4-phenylbutan-2-one	Benzylacetone	ByAce
2,3,4,5-tetramethyltricyclo[3.2.1.02,7]oct-3-ene	Tricyclo[3.2.1.02,7]oct-3-ene, 2,3,4,5-tetramethyl-	TriOct
(1S,5S,6R)-2,6-dimethyl-6-(4-methylpent-3-enyl)bicyclo[3.1.1]hept-2-ene	(*Z*)-α-Bergamotene	TaB1
(4aR)-3,5,5,9-tetramethyl-1,2,4a,6,7,8-hexahydrobenzo[7]annulene	β-Himachalene	bHim
1-methyl-4-(6-methylhept-5-en-2-yl)benzene	α-Curcumene	aCurc
2-(6,8-dimethyl-3-tricyclo[4.4.0.02,7]dec-8-enyl)propan-2-ol	α-Copaen-11-ol	aCop11
2,2-dimethyl-3-methylidenebicyclo[2.2.1]heptane	Camphene	Camph
6,6-dimethyl-2-methylidenebicyclo[3.1.1]heptane	β-Pinene	bPin
1-methyl-4-prop-1-en-2-ylcyclohexene	Limonene	Lim
(6E)-7,11-dimethyl-3-methylidenedodeca-1,6,10-triene	β-Farnesene	bFarn
2,6-dimethyl-6-(4-methylpent-3-enyl)bicyclo[3.1.1]hept-2-ene	(*Z*)-α-Bergamotene	TaB2
(1S,2R,5S)-2,6,6,8-tetramethyltricyclo[5.3.1.01,5]undec-8-ene	α-Cedrene	aCed
1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane	Eucalyptol	Euc
methyl 2-hydroxybenzoate	Salicylic acid, methyl ester	SAME
2-methylpropyl benzoate	Isobutyl benzoate	IBBen
4,10-dimethyl-7-propan-2-yltricyclo[4.4.0.01,5]dec-3-ene	α-Cubebene	aCub
3-methylbutyl benzoate	Isopentyl benzoate	IPBen
3-methylbut-2-enyl benzoate	Prenyl benzoate	PB
4,7-dimethyl-1-propan-2-yl-1,2,4a,5,6,8a-hexahydronaphthalene	α-Muurolene	aMuur1
(1Z,6Z)-1-methyl-5-methylidene-8-propan-2-ylcyclodeca-1,6-diene	Germacrene D	GermD
(1S,8aR)-4,7-dimethyl-1-propan-2-yl-1,2,3,5,6,8a-hexahydronaphthalene	δ-Cadinene	dCad
2-[(3S,5R,8S)-3,8-dimethyl-1,2,3,4,5,6,7,8-octahydroazulen-5-yl]propan-2-ol	Guaiol	Gua
6-methylhept-5-en-2-one	Sulcatone	Sulc
(4R)-1-methyl-4-prop-1-en-2-ylcyclohexene	D-Limonene	dLim

1,1,7-trimethyl-4-methylidene-2,3,4a,5,6,7,7a,7b-octahydro-1aH-cyclopropa[e]azulene	Alloaromadendrene	Aden
(1R,2S,6S,7S,8S)-1,3-dimethyl-8-propan-2-yltricyclo[4.4.0.02,7]dec-3-ene	Copaene	Cop
decanal	Decanal	Dec
1,3,4-trimethylcyclohex-3-ene-1-carbaldehyde	3-Cyclohexene-1-carboxaldehyde, 1,3,4-trimethyl-	3Cyc1CA
(3R,4aS,5R)-4a,5-dimethyl-3-prop-1-en-2-yl-2,3,4,5,6,7-hexahydro-1H-naphthalene	Valencene	Val
(1R,4aR,8aS)-7-methyl-4-methylidene-1-propan-2-yl-2,3,4a,5,6,8a-hexahydro-1H-naphthalene	γ-Cadinene	yCad
1-phenylethanone	Acetophenone	Acep
(6Z)-7,11-dimethyl-3-methylidenedodeca-1,6,10-triene	(*Z*)-β-Farnesene	ZbFarn
(6Z)-3,7,11-trimethyldodeca-1,6,10-trien-3-ol	D-Nerolidol	dNero
butyl prop-2-enoate	Acrylic acid butyl ester	AABE
3,7,11-trimethyldodeca-1,6,10-trien-3-ol	Nerolidol	Nero
1-(2-methoxyphenyl)ethanone	ortho-Methoxyacetophenone	oMAP
(3E,6E)-3,7,11-trimethyldodeca-1,3,6,10-tetraene	α-Farnesene	aFarn
6,6-Dimethyl-2-methylene-bicyclo[2.2.1]heptan-3-one	Bicyclo[2.2.1]heptan-3-one, 6,6-dimethyl-2-methylene-	bCych
1,1a,6,6a-tetrahydrocyclopropa[a]indene	Cycloprop[a]indene, 1,1a,6,6a-tetrahydro-	CycTet
2-methyl-5-propan-2-ylphenol	Carvacrol	Carv
cyclopropylidenemethylbenzene	Benzylidenecyclopropane	Bcyc
5-methyl-2-propan-2-ylphenol	Thymol	Thy
4-ethenyl-1,2-dimethoxybenzene	3,4-Dimethoxystyrene	DiSty
(6E)-7,11-dimethyl-3-methylidenedodeca-1,6,10-triene	(*E*)-β-Farnesene	EbFarn
(3Z,6E)-3,7,11-trimethyldodeca-1,3,6,10-tetraene	(*Z,E*)-α-Farnesene	ZEaFarn
nonanal	Nonanal	Non
(5-methyl-2-phenylpyrazol-3-yl) benzoate	Benzoic acid 5-methyl-2-phenyl-2H-pyrazol-3-yl ester	BAPy
(6E)-3,7,11-trimethyldodeca-1,6,10-trien-3-ol	(*E*)-Nerolidol	Enero
hexanal	Hexanal	Hex
6,6-dimethyl-2-(3-oxobutyl)bicyclo[3.1.1]heptan-3-one	6,6-Dimethyl-2-(3-oxobutyl)bicyclo[3.1.1]heptan-3-one	DiBi
2-methyl-5-(6-methylhept-5-en-2-yl)cyclohexa-1,3-diene	Zingiberene	Zing
(2R)-6-methyl-2-[(1R)-4-methylcyclohex-3-en-1-yl]hept-5-en-2-ol	α-Bisabolol	aBis
3-(6-methylhept-5-en-2-yl)-6-methylidenecyclohexene	β-Sesquiphellandrene	bSes
(1E,5E)-1,5-dimethyl-8-propan-2-ylidenecyclodeca-1,5-diene	Germacrene B	GermB
(4S)-1-methyl-4-(6-methylhepta-1,5-dien-2-yl)cyclohexene	β-Bisabolene	bBis
(3Z)-3,7-dimethylocta-1,3,6-triene	(*Z*)-β-Ocimene	ZbOci
3-methylbut-3-enyl benzoate	3-Methyl-3-butenyl benzoate	3M3BB
(1S)-2,2-dimethyl-3-methylidenebicyclo[2.2.1]heptane	Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1S)-	bCychS
4-methylidene-1-propan-2-ylbicyclo[3.1.0]hexane	Sabinene	Sab
(3E)-3,7-dimethylocta-1,3,6-triene	(*E*)-β-Ocimene	EbOci

2-[(1S)-4-methylcyclohex-3-en-1-yl]propan-2-ol	L-α-Terpineol	LaTerp
6,6-dimethyl-2-methylidenebicyclo[3.1.1]heptan-3-ol	L-(*Z*)-Pinocarveol	LtrPino
(6,6-dimethyl-2-bicyclo[3.1.1]hept-2-enyl)methanol	Myrtenol	Myrta
benzyl pentanoate	Valeric acid	VA
icosan-3-ylcyclohexane	3-Cyclohexyleicosane	3CIco
1,2,3-trimethylbenzene	Hemimellitene	Hemim
1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene	(*Z*)-Decalin	trDec
(1aR,4aR,7S,7aR,7bR)-1,1,7-trimethyl-4-methylidene-1a,2,3,4a,5,6,7a,7b-octahydrocyclopropa[h]azulen-7-ol	Spathulenol	Spath
butylcyclohexane	Butylcyclohexane	BCycB
2-methoxy-4-[(E)-prop-1-enyl]phenol	Isoeugenol	IsoE
(1S,3R,5S)-4-methylidene-1-propan-2-ylbicyclo[3.1.0]hexan-3-ol	Sabinol	Sabi
(1E,4E,8E)-2,6,6,9-tetramethylcycloundeca-1,4,8-triene	α-Caryophyllene	aCary
[2,2,4-trimethyl-3-(2-methylpropanoyloxy)pentyl] 2-methylpropanoate	Kodaflex txib	Koda
butyl 2-methylpropanoate	n-Butyl isobutyrate	nBIso
1-O-(2-methylpropyl) 4-O-propan-2-yl 2,2-dimethyl-3-propan-2-ylbutanedioate	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	PACI
(1R,4E,9S)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene	Caryophyllene	Cary
1,1,7-trimethyl-4-methylidene-2,3,4a,5,6,7,7a,7b-octahydro-1aH-cyclopropa[e]azulene	Aromadendrene	Aroma
(1S,4S)-1,6-dimethyl-4-propan-2-yl-1,2,3,4-tetrahydronaphthalene	Calamenene	Cala
dodecanoic acid	Lauric acid	Laur

3. Results

I collected 74 propolis samples. 16 samples were collected in autumn, and 58 were collected in spring. Nine of the 16 regions were sampled. Canterbury was further broken up into North Canterbury, South Canterbury, and mid-Canterbury (Table 3.1). 91 compounds were tentatively identified. None of the compounds were identified in all of the propolis samples. 11 compounds were identified in all of the autumn samples, and 40 compounds were identified in all of the spring samples. The full dataset can be found here.

Propolis samples grouped by the region from which they were collected were classified using principal component analysis (PCA) based on the compounds identified. The chemical profiles from the 13 regions overlapped, although the autumn Waikato samples were slightly clustered (Fig. 3.2). The three spring Southland samples also clustered separately from the other samples. The first principal component (PC1) explained 7.8% of the variability. PC1

was characterised largely by (*Z*)-α-Bergamotene, α-Curcumene and Zingiberene. Principal component two (PC2) accounted for 7.2% of the total variance. Compounds such as Copaene, δ-cadinene and β-pinene contributed more to this dimension (Supplementary table 4, Fig. 3.1). Principal components 1-22 accounted for about 75% of the total variance in the data, and compounds with higher contributions to these components (Supplementary table 4) were considered in subsequent analysis.

Because the region from which samples were collected did not account for the variation seen in propolis samples, a dendrogram was constructed to identify the relation between samples (Figure 3.3). SPR48, collected from Southland, is the most varied when compared to the rest of the group. Absent in this sample are the compounds Prenal acetate, Benzyl alcohol, both of which were identified in a large number of other samples. It has high levels of (Z)-β-Farnesene, which was only detected at low levels in a handful of other samples. Germacrene B, only in one other sample, high levels of Sabinene only detected at very low levels in two other samples, SPR54 and SPR55, collected from Southland and Northland respectively, are most closely related to each other, and also closely related to SPR48. Also in this cluster are samples SPR17, collected from Nelson, SPR24, collected from the West Coast and SPR43, collected from Hawkes Bay. The samples do not separate into region, nor do they separate into season from which they were collected.

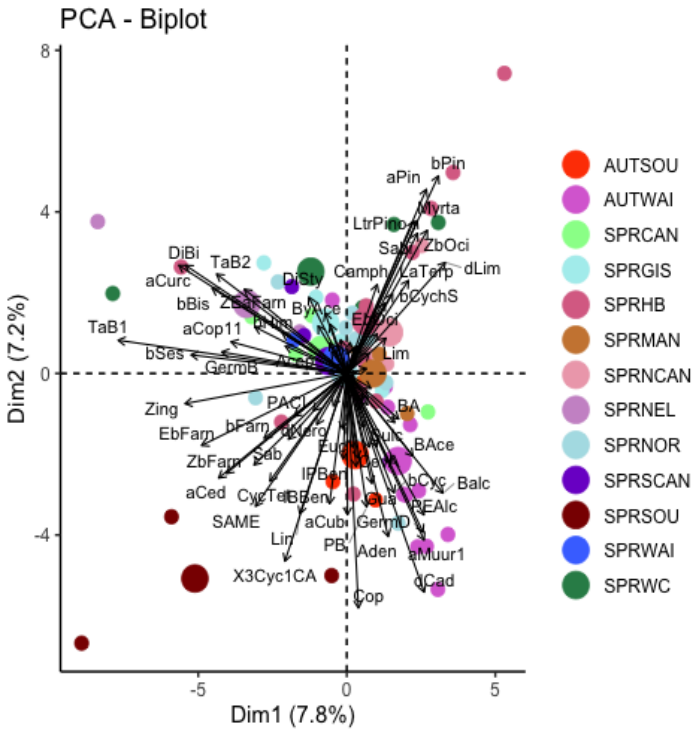

Figure 3.1: Biplot of propolis samples grouped by region and identified compounds. Each small symbol represents an individual sample. The larger symbols are the centre of each group of samples.

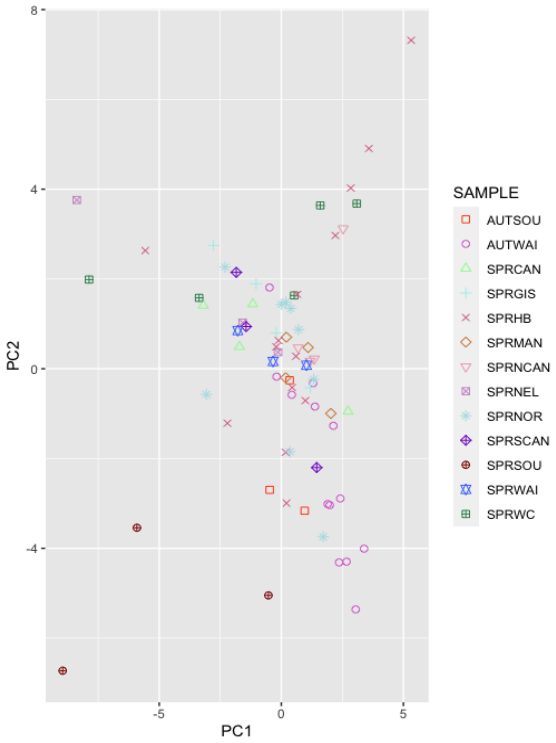

Figure 3.2: PCA plot of propolis samples grouped by region.

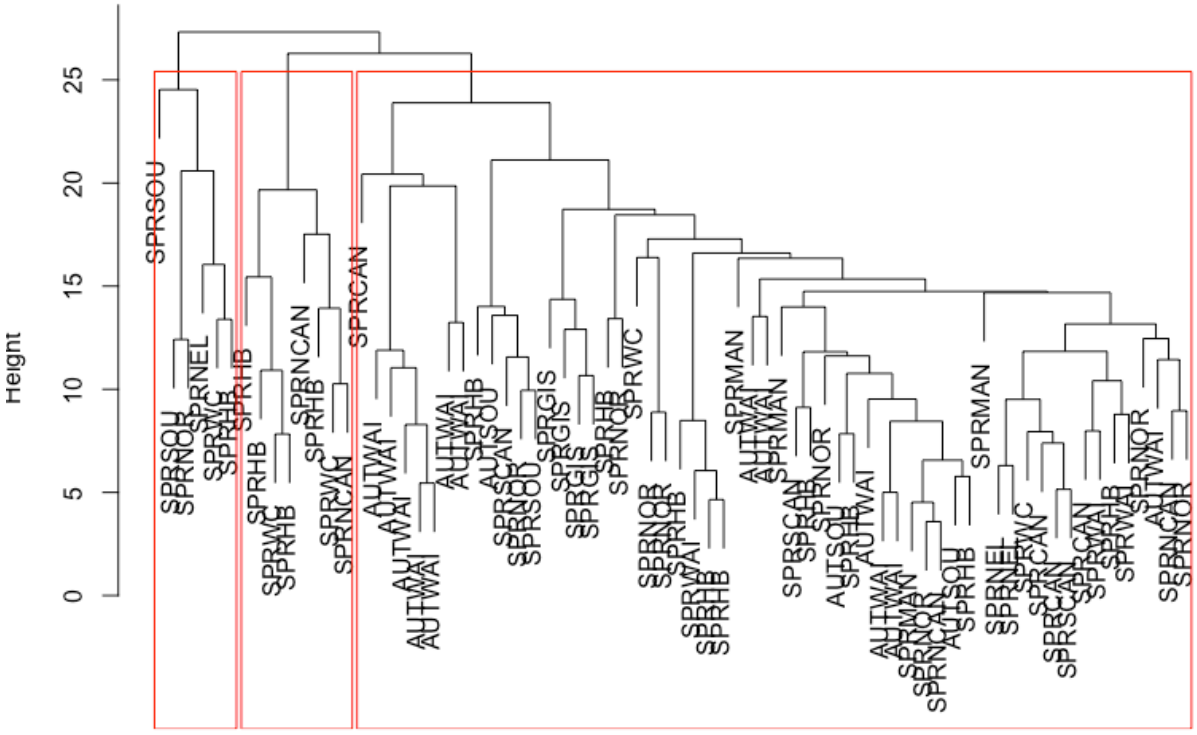

Figure 3.3: Dendrogram of propolis samples

4. Discussion

There was variation amongst the chemical profile of the collected propolis samples, as visualised by Figure 3.1. This variation could not be explained entirely by region, nor season, as displayed by Figure 3.3. However, while many of the regional samples overlapped, some regions appeared to form distinct clusters. This included samples from Southland collected in spring, and samples from Waikato collected in autumn.

The cluster formed by the three Southland spring samples was characterised by α-cedrene, linalool, and 3-Cyclohexen-1-carboxaldehyde, 1,3,4-trimethyl-, which were only identified in a small handful of other samples (Figure 3.1). Even within this cluster, variation occurred, as several compounds, including Prenal acetate, α-Curcumene, β-Farnesene, δ-Cadinene and Guaiol, were detected in only two of the samples. This divergence can be seen in Figure 3.3, as one Spring Southland sample was separated significantly from the other two.

Figure 3.3 also displays a close relationship between seven of the Waikato samples collected in Autumn. These samples were characterised by the compounds Prenal acetate, Benzyl alcohol, Phenylethyl alcohol and α-Copaen-11-ol. Even within this group, no compounds were identified in all the Autumn Waikato samples, and the compounds that were identified were recorded in varying quantities.

The two samples most separated from the main cluster are a Spring sample collected from the Hawkes Bay, and a Spring sample collected from Southland (Figure 3.1). The Hawkes Bay sample is characterised by the absence of Prenal acetate, Benzyl alcohol and (*Z*)-α-Bergamotene. These compounds were identified in 51, 36, and 52 of the 74 samples respectively. This sample was also characterized by the presence of δ-Limonene, Myrtenol, and Sabinol, which were only detected in 5, 2, and 1 other sample respectively. The Southland sample was also characterised by an absence of Prenal acetate and Benzyl alcohol, as well as the absence of α-Curcumene and β-pinene, which were identified in 39 and 29 of the 74 samples respectively. It was also characterised by the presence of Germacrene B and Sabinene, identified in only 2 other samples each. All these compounds are of botanical origin and have been previously identified in propolis.

Therefore, while the region from which samples were collected could not conclusively account for all of the variation between samples, it appears to have contributed in some cases, and should not be disregarded entirely. Similar findings were reported by Cheng et al. (2013), where samples were collected from four different regions of China. Of note, nine volatile compounds were identified in all four samples, and an additional ten compounds were detected in three of the four samples. While this aspect indicates there is some similarity among samples collected from different regions, several compounds were identified in only one sample, highlighting a divergence between regions. When PCA was applied to this dataset, the samples collected from each of the regions separated into four distinct clusters, indicating that geographical origin contributed largely to the variation.

Brazil has been a world leader in being able to separate propolis into chemotypes based on the botanical source. Researchers have been able to separate propolis types conclusively into red, green and brown, based on the geographical, and therefore botanical, origin of collection. For example, Machado et al. (2016) were able to compare the flavonoid and phenolic content

of samples under each category from different regions. Further, scientists have been able to identify the likely botanical sources of each propolis type - red propolis is sourced from *Dalbergia ecastaphyllum* (Neto et al., 2017), green propolis from *Baccharis dracunculifolia*, and brown propolis from species from the genus *Copaifera*.

In some cases, grouping of propolis types by region has not been possible. This is the case in research conducted by Falcão et al. (2016) where volatile oils isolated from propolis samples collected across Portugal did not allow the samples to be grouped based on their geographical origin. However, links could be made between the chemical composition of propolis samples, and that of the resin samples collected from nearby plants.

Some of the main volatile compounds identified in the New Zealand propolis samples collected included (*Z*)-α-bergamotene, α-curcumene, α-pinene and β-pinene, all of which are common plant volatiles and have been identified by other researchers in propolis samples (Nalbantsoy et al., 2022; de Oliveira et al., 2021; Ribeiro et al., 2021; Shin and Lee, 2013).

A and β pinene are monoterpenes and are both known to have several therapeutic properties. Salehi et al. (2019) summarise many of the known therapeutic properties of these compounds. Both compounds are known to be applied as fungicidal, antimicrobial, antibacterial, and antiviral agents. Additionally, both compounds have been found to have inhibitory effects on leukaemia and breast cancer.

A-curcumene has been identified as an important active compound with significant bioactivity. Widiakongko et al. (2021) identified α-curcumene, as well as other active components of ginger also identified in some of our propolis samples (α-farnesene, β-sesquiphelladrene, and zingebirin) as having the potential to inhibit the activity of the COVID-19 virus in the human body. α-curcumene has also been demonstrated to possess inhibitory activity against human ovarian cancer cells (Shin and Lee, 2013).

Four compounds identified in propolis samples were only detected in native resin samples. Nonal was identified in spring propolis samples from Gisborne, Canterbury, West Coast, Southland, and Northland, and in the autumn Kawakawa and spring Rewarewa sample. A-Cubebene was identified in autumn propolis samples from both Waikato and Southland, and

spring propolis samples from Northland and Southland. A-Cubebene was also identified in autumn samples of Kawakawa, Kōhūhū, and Kauri, and spring samples of Tōtara, Mānuka, Kānuka and Kauri. B-Bisbolene was identified in spring propolis samples from Nelson, West Coast, Hawkes Bay and Northland, and in autumn samples of Kawakawa, Kōhūhū, and Ngaio, and spring samples of Kawakawa, and Ngaio. E- β-Ocimene was identified in spring propolis samples from West Coast and Hawkes Bay, and in autumn samples of Lemonwood and Kauri. These compounds were not identified in any of the poplar clones sampled. This suggests that there is a potential contribution to New Zealand propolis by some native species.

Ten compounds identified in propolis samples were only detected in poplar resin samples. For example, α-Copaen-11-ol was identified in 40 of the 72 propolis samples collected, and in one autumn Pakaraka sample and all three autumn Weraiti samples. Phenylethyl Alcohol was identified in 33 of the 72 propolis samples, and in all autumn Argyle, and Weraiti samples, as well as in two autumn Selwyn samples, and in all spring poplar samples. B-Himachalene was identified in autumn Waikato samples, and spring samples collected from Gisborne, Canterbury, West Coast, Hawkes Bay, and Northland, and in all autumn Fraser, Pakaraka, Selwyn and Weraiti samples. As these compounds were not identified in any of the native species sampled, it can be hypothesised that resin from poplar species does contribute significantly to New Zealand propolis. This is in keeping with previous research that groups New Zealand propolis as ‘poplar-type’.

Additionally, more than 60 compounds were identified in the propolis samples that were not identified in any of the resin samples. This could indicate that 1) there are additional botanical species contributing to New Zealand propolis, 2) there may be chemical transformation of the original source compounds, or 3) these compounds may be unrelated to the botanical source of the propolis. There is some evidence to support the contribution of other botanical sources to New Zealand propolis. For example, α-curcumene, known to be of botanical origin and previously identified in propolis (Shin and Lee et al., 2013), was detected in more than half of the propolis samples, but was not identified in any of the resin samples. Additionally, limonene was identified in eight of the propolis samples, but was not identified in any of our resin samples, despite also being of botanical origin and having been

previously identified in propolis (Sarıkahya et al., 2021). These possible explanations require further investigation.

Conclusions and future outlook

In conclusion, many VOCs were detected in the collected propolis samples. There was significant variation amongst these samples, with some emerging trends able to be identified. While the region from which propolis samples were collected could not unambiguously account for the variation between samples, some regions appeared to cluster separately. There were four compounds identified in the propolis samples that were exclusively detected in native resin samples, and ten detected exclusively in poplar samples. This suggests that native species may also be contributing to New Zealand propolis, and that there may be more species not sampled in the current study also contributing to New Zealand propolis. The next step in this field would be to conduct more detailed metabolomics analysis to further build on the conclusions drawn in the present paper. The results from this study provide a strong basis on which to further develop an understanding of geographic variation in New Zealand propolis, and the contribution of different botanical sources. The potential contribution of native species and further unidentified botanical sources provides an exciting new avenue for researchers to explore.

Chapter 4

Final Discussion, Conclusions, and Recommendations

Chapter 4: Final Discussion, Conclusions and Recommendations

The intent of this study was to investigate potential chemical and antimicrobial variation present in New Zealand propolis and to investigate the chemical variation present in the resin of common poplar hybrids and native species. As demand for natural human health products and biocosmetics grows, so too does the need to adequately understand the range of natural products available. Similarly, as the beekeeping industry continues to explore ways in which to improve the health of their hives, so too is it vital that we have a better understanding of a very diverse product. Internationally a growing trend is emerging whereby propolis samples collected in the same country, and sometimes in very close proximity, are displaying different chemical and biological properties that correlate with and perhaps driven by several factors including geography, available flora and season. The first attempts to look at New Zealand propolis from a regional perspective were undertaken by Markham et al. (1996), in which samples were grouped together to form batches predominantly from one region, or a mixture of several regions. Therefore, the current study is the first attempt at a comprehensive overview of the geographic variation of propolis in New Zealand.

Chapter three of this study explored the potential variation in VOCs of propolis samples from 11 of the 16 regions of New Zealand, with Canterbury split further into North Canterbury, South Canterbury, and Canterbury. The aim of this section was to identify any variation in the propolis samples, and to investigate whether any identifiable variation correlated with the geographic origin of the propolis. From this information, I was able to obtain an understanding of the specific chemicals that contributed most to this variation. I observed that while the region from which propolis was collected did not completely account for the displayed variation, it did contribute somewhat, and more so for some regions, for example Southland and Waikato, than others. Still, most samples collected from all over the country displayed similar chemical profiles.

Additionally, this research was the first to investigate the chemical variation of trees potentially contributing resin to propolis production. The assumption that poplars are the main botanical source of New Zealand propolis, was supported by the results of the current study. However, there was evidence that some of our native species could also be

contributing. When the poplar hybrids were examined, there appeared to be some hybrids that varied more than others.

In addition to the two studies presented here, a pilot study was conducted to test methods for microbial work. Extraction methods were adapted from Devequi-Nunes et al. (2018). Different concentrations of propolis samples were inoculated with *E. coli* and *Phytophthora*. Bacterial colonies were measured each hour using a spectrophotometer. However, the extracted samples were too cloudy for bacterial colonies to be counted. I centrifuged the samples to remove some of the cloudiness, however the samples were still too cloudy for bacterial colonies to be counted. I instead attempted to inoculate agar plates with the propolis extractions. To achieve concentrations comparable to those tested by Devequi-Nunes required too much liquid meaning the plates did not set.

If the experiment using the spectrometer was to be repeated, I would recommend trialling another extraction method that uses a lower quantity of propolis, or involves more straining to reduce the cloudiness. If the agar plate experiment was to be repeated, I would recommend using an extraction method that produced a significantly more concentrated propolis extraction as this would require less liquid to be added to the agar solution.

I would also recommend focusing on propolis from Southland, Hawkes Bay and Waikato, as these samples were the most chemically diverged (Figure 3.1). A range of microbes should be studied, with a particular focus on common honey bee pathogens, such as *Paenibacillus larvae* and *Ascophora apis*.

Regional Variation and the role of resin

While there was significant overlap in the chemical composition of collected propolis samples, there was also visible spread. Several samples were separated from the main cluster. When considering the region from which propolis was collected, some regions clustered more closely than others. Samples collected during Spring from Southland and during autumn from Waikato both clustered separately from the main group.

Although the region from which propolis was collected did not completely account for the variation seen between samples, it did appear to contribute to some clusters, for example

those collected in Southland and Waikato. While propolis collected in larger countries such as Brazil and China can be distinctly separated into geographical and botanical chemotypes, this is not yet able to be done with New Zealand propolis. This is potentially not surprising, as the size of New Zealand limits the likely variation in botanical sources available to resin collecting bees. Still, there is variation seen in the local flora available in different regions. For example, Southland being the coldest part of the country may contribute to the difference in chemical composition of these samples when compared to the rest of the samples. Poplars are also rare in other parts of the country, and other plants may be used as shelter belts instead.

Investigation into the chemical profiles of common poplar hybrids and native species revealed some overlap, but also significant divergence. Specifically, both the poplar and native samples collected in autumn were more distinct than those collected in spring. There appeared to be far more divergence between poplars and natives in autumn than in spring.

Despite the poplars sampled being closely related hybrids, and the replicates of each hybrid being clones, there was still some variation in the chemical profile of the poplar resin samples. Interestingly, despite the same trees being sampled in both seasons, the hybrids did not necessarily cluster together. Some hybrids were more varied than others. One of the Argyle samples collected in spring was considered an outlier due to its divergence from the other samples. However, when removed from the analysis, a second Argyle sample collected in spring diverged significantly from the main cluster.

Due to the closely related nature of the poplar hybrids, and the closer relation of the hybrid clones, the variation in chemical composition seen is unexpected. This is of particular interest when considering the number of poplar hybrids available to resin-collecting bees across the country and has implications when considering the regional and therefore botanical source of propolis.

While the results from this study do not allow conclusive statements to be made regarding the contribution of specific poplar hybrids or native species to New Zealand propolis, some interesting implications are detectable. The fact that there were chemicals detected in the propolis samples only identified in poplar resin, and conversely compounds only identified in native resin, suggests that both groups contribute to New Zealand propolis. However, there

were further compounds detected in propolis samples not identified in either resin source. These compounds may be a result of other botanical sources, chemical transformation, or a factor other than resin. However, they more likely indicate the contribution of other unsampled botanical sources. Given that honeybees are known to collect resin from several different botanical sources, this prospect provides an exciting future avenue to explore.

Limitations

Delays and lockdowns imposed by Covid-19 affected the ability to collect onsite samples of resin. The initial plan involved visiting each apiary in the spring and collecting resin directly from nearby trees. However, the collection period coincided with the August-September lockdown, preventing travel. Post lockdown, the uncertainty of nationwide travel interfered with this happening later in the year. While the sampling of potential resin contributors without requiring long distance travelling was a suitable alternative, it did reduce the ability to link resin directly to propolis samples.

Future Research

While VOCs are a useful marker when considering variation in chemical composition, more comprehensive metabolomic analysis would provide further evidence when investigating the variation between samples, and the correlation between resin and propolis samples. Metabolomics involves the analytical profiling of small molecules, commonly referred to as metabolites, within the cell (Alseekh et al., 2021). In comparison to the methods used in the current study that focus on the identification of VOCs, a metabolomics analysis would identify biochemical markers such as sugars, simple amino acids, lipids and nucleotides. Metabolomics is increasingly being used to further understand the full complexity of biological systems and their response to changes and external influence. Such analysis was not undertaken in the current study as the extensive cost, skill and time required to do so was outside the scope of the study.

Using VOCs, I was able to draw initial conclusions regarding the relation between propolis samples and the region from which they were collected, and between propolis and resin samples. A full metabolomic analysis of propolis and resin samples collected across New Zealand would allow for more specific comparisons to be made, and correlations to be further confirmed or altered.

Similarly, due to the differing methods employed while collecting and extracting propolis and resin samples, direct comparisons could not be made, and therefore conclusive contributions to propolis are not identified. A smaller experiment conducted in a controlled environment where honeybee colonies were provided with a known number of potential resin sources would allow direct identification of honeybee preference and describe how these resins map to the compounds in the resulting propolis. To identify such a preference right down to the species or hybrid level would allow preferential selection of hive location for beekeepers looking to improve propolis quality or quantity or looking to plant new trees. Similarly, more native species and poplar hybrids should be sampled to further understand which botanical sources contribute most significantly to New Zealand propolis, and whether this differs with locality.

While the survey of New Zealand propolis is by far the most conclusive conducted in New Zealand so far, there is still room to conduct a larger scale collection. A handful of regions were not sampled, and some regions were sampled more heavily than others. For example, the three propolis samples collected from Southland appeared to cluster separately from the rest of the propolis samples, and it would be interesting to see if additional samples collected from Southland would cluster in the same way.

Conclusion

The results of this study suggest that while the chemical profiles of many of the propolis samples collected across New Zealand overlap, there is still visible variation. While this cannot yet be attributed solely to the region from which samples were collected, the contribution of geography did appear stronger in some regions than others. Therefore, regional variation cannot be ruled out entirely, and further research using metabolomics should aim to collect samples evenly from all regions of New Zealand to further answer this question. Similarly, while there was some overlap in the chemical composition of the resin samples collected, there was also some variation detected. More potential resin contributors should be sampled if this research was to be repeated. Initial conclusions regarding the contribution of different resin sources to the propolis collected will begin to help the New Zealand honeybee industry understand what makes New Zealand propolis unique. It will provide factors to be considered regarding propolis production, such as the botanical sources

of resin in the vicinity of hives, and the trees to be planted in the future. This study provides a basis on which to continue to build our understanding of New Zealand propolis and will allow beekeepers to find a starting point when considering the value of New Zealand propolis.

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Appendix 1

Variable Contributions of Compounds to Principal Component Analyses

Appendix 1: Variable contributions of compounds to principal component analyses

S1: Variable contribution of resin data grouped by poplar or native (aligns with Fig 2.1 and 2.2)

S2: Variable contribution of resin data poplar only (aligns with Fig 2.5 and 2.6)

S3: Variable contribution of resin data poplar only with outlier included (aligns with Fig A2.1 and Fig A2.2)

S4: Variable contribution of propolis data grouped by region (aligns with Fig 3.1 and 3.2)

Appendix 2

Resin Outlier

Appendix 2: Resin outliers

Resin samples from poplars were classified using principal component analysis (PCA) based on the compounds identified. The chemical profiles from the four groups overlapped, although some samples did appear to cluster together (Fig. A2.2). Spring collections of Weraiti, Veronese, Selwyn, Pakaraka, and autumn collections of Weraiti each formed their own tight cluster (Fig. A2.2). The first principal component (PC1) explained 22.9% of the variability. PC1 was characterised largely by Eucalyptol, (*Z*)-α-Bergamotene and prenyl benzoate. Principal component two (PC2) accounted for 11.2% of the total variance. Compounds such as α-pinene, β-pinene and Camphene contributed more to this dimension (Supplementary table 3, Fig. A2.1). Principal components 1-22 accounted for about 91% of the total variance in the data, and compounds with higher contributions to these components (Supplementary table 3) were considered in subsequent analysis.

The most important compound identified by the model was Eucalyptol (Fig. A2.3). Other compounds such as Guaiol, 6,6-Dimethyl-2-(3-oxobutyl)bicyclo[3.1.1]heptan-3-one, and phenylethyl alcohol also had higher contributions. The OOB score for this model is 8.33%, indicating that a very high number of samples can be successfully allocated to their respective poplar hybrid using this method.

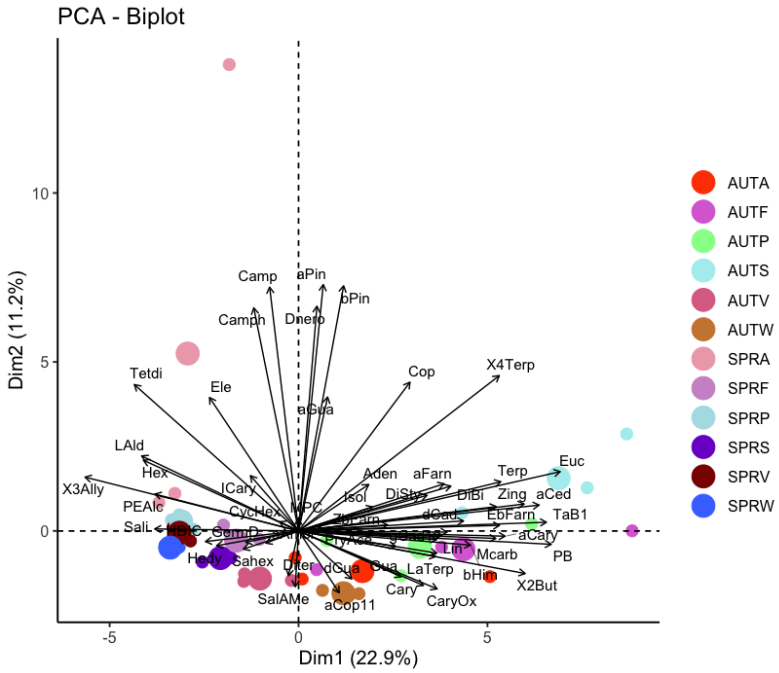

Figure A2.1: Biplot of poplar resin samples and identified compounds.

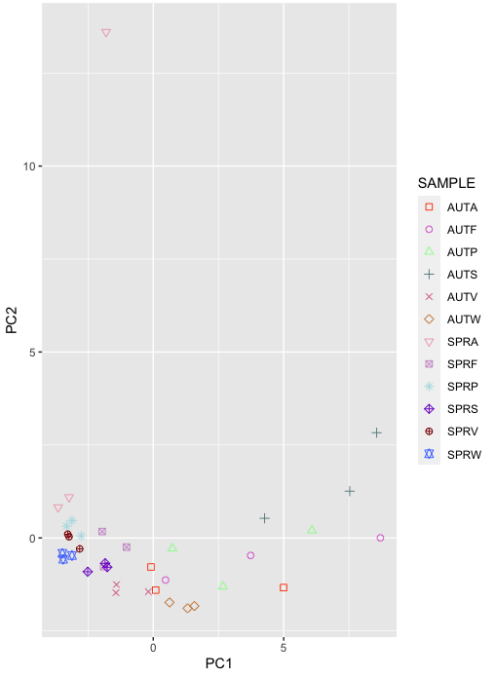

Figure A2.2: PCA plot of poplar resin samples

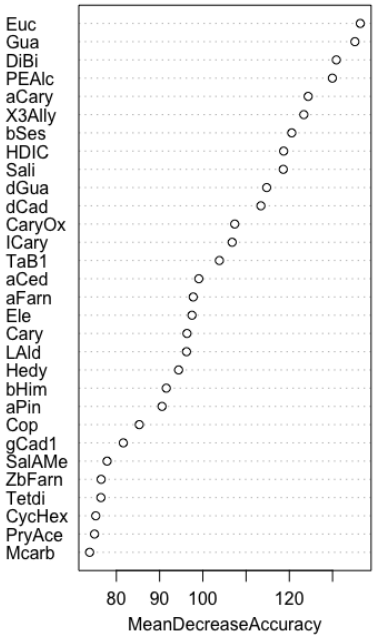

Figure A2.3: Figure 3: Random Forest plot of poplar resin samples