New Zealand willows (*Salix* spp.), their metabolites and their plant-herbivore interactions

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Summary

New Zealand is a unique environment which affects how species behave, survive and interact with each other. Introduced species of willow (*Salix* spp) are used in New Zealand for various purposes, and several varieties (clones) have been developed (Gunawardana et al., 2014; McIvor, 2013). Various insect species attack willows in New Zealand. It is not yet known how the New Zealand environment would affect the secondary metabolites and species resistance to insect pests in willows, and host preferences of some pests have not yet been characterized. In my thesis I aimed to characterize chemistry (metabolites and volatile organic compounds) in several willow clones, as well as differences in clone preference in insect pests – giant willow aphid (GWA) *Tuberolachnus salignus* Gmelin, 1790 (Hemiptera: Aphididae) and red gall sawfly *Pontania proxima* Serville, 1823 (Hymenoptera: Tenthredinidae). The incidence and chemical aspects of galling by *P. proxima* on New Zealand willow clones has not yet been characterised. This information is vital for the selection of resistant cultivars and to understand potential indirect impacts on other insect species (e.g., natural enemies of competing herbivores).

Chapter 1 is a literature review on plant secondary metabolites and their role in plant defence against insect herbivores, as well as review of willow insect pests with emphasis on *P. proxima* and giant willow aphid. This chapter questions how much we know about willows in New Zealand, what differences they present from the willows from other parts of the world, and how the New Zealand environment affects the insects that feed on willows. Do insect pests preferers the same species/clones in New Zealand as in other parts of the world? What makes *P. proxima* and GWA prefer certain clones? What makes some clones resistant and others susceptible?

In Chapter 2, I explored the levels of damage caused by *P. proxima* to willow clones. Twelve willow clones (PN221, PN249, PN721, PN693, PN357, PN676, NZ1040, NZ1130, PN218, PN356, PN736 and PN742) used in New Zealand were selected and surveyed for *P. proxima* damage. Willows showed a range of resistance levels to *P. proxima*. These levels of resistance show as differences in *P. proxima* larval development (explored in Chapter 3), damage level and gall size. For example, clones PN221 and PN249 did not present galls, while clones PN736 and PN742 presented the highest level of damage in our field survey. Other clones had an intermediate level of damage, and some clones present with malformed galls. The survey also found that top sections of shoots had a significantly higher level of damage, while location and side of the plant had no effect, possibly because the experimental field was homogeneous in sun exposure and other abiotic factors such as soil fertility. Gall induction is still a mystery in the *Salix* spp - *P. proxima* system, mainly because the cecidogenic factor is not yet known.

The clones used in Chapter 2 were further investigated in Chapter 3 to link plant resistance to *P. proxima* development and growth. Larval development was investigated and measured, and the phenolic and nutrient content of willow leaves was quantified. The resistance of willow clones to *P. proxima* appears to be guided by a combination of physical and chemical attributes of the plants. Overall, *P. proxima* appears to prefer clones with a lower phenolic content and lower leaf pilosity. This preference, however, is in contrast with previous European studies in which *P. proxima* showed preference for a higher level of phenolics. Plant phenology seems also to play a role in preference, with *P. proxima* preferring to oviposit in clones which develop earlier in the season, even if those clones do not offer optimal conditions for larval development. Among the studied clones, PN 221 and PN 249 (*S. purpurea*) showed the highest resistance levels to *P. proxima* with no galls. Among the clones that developed galls, PN676 (*S. alba*, female) was the clone that produced the smallest larva and is therefore considered

more resistant. The fact that *P. proxima* has shifted its preference from willows with a high content of phenolic glycosides in its native range to willows with a low content of phenolic glycosides in New Zealand may be due to low predator pressure in New Zealand. Further studies are needed to investigate this shift and test this hypothesis.

In Chapter 4, I investigated the metabolomics profiles of six willow clones (PN220, PN249, PN386, NZ04-106-073, PN218 and NZ1040) and whether the differences in metabolites influence the preference of insect pests to the six clones. With the metabolomic profile we are expecting to see differences in chemistry between the clones and their influence on the plants' pest resistance. The most important compounds found were apigenin, isorhamnetin-3-O-glucoside, procyanidin B2, epicatechin, petunidin-3-O-β-glucopyranoside, kaempferide, kaempferol-3-glucuronide, quercetin-7-O-rhamnoside, unknown 1, isorhamnetin, peonidin-3-O-β-D-glucoside, luteolin-7-O-glucoside, procyanidin B1 and isorhamnetin-3-O-rutinoside. Due to the limited number of clones and limited number of replicates, I cannot draw definitive conclusions about the pattern of secondary metabolites in relation to resistance to the two insect herbivores – *P. proxima* and GWA. To our knowledge the direct effect of those metabolites on *P. proxima* and GWA was never tested. The resistance to *P. proxima* and GWA appears to be more correlated with phenological and morphological features of willow plants than with their chemistry.

Chapter 5 is an investigation of the volatile profile of willow clones studied in Chapter 4. With the metabolomic and volatile profile we hoped to elucidate whether the chemistry of the clone influences clone resistance to *P. proxima* and GWA. These volatile organic compounds (VOCs) included two green leaf volatiles (GLVs), (*Z*)-3-Hexenyl acetate and (*Z*)-3-Hexenyl-α-methylbutyrate; one monoterpenes, (*Z*)-β-Ocimene; and eight sesquiterpenes, β-Elemene α-Cubebene, Copaene, Germacrene D, (*Z*)-β-Caryophyllene, (*E*)-α-Bergamotene, (α)-Farnesene, δ-Cadinene. The results show that willow clones have highly species-specific VOC blends, a

conclusion backed up by other authors. Due to the limited number of clones and limited number of replicates, it was not possible to draw definitive conclusions about the pattern of volatile emissions in relation to resistance to the two insect herbivores – *P. proxima* and GWA. Resistance to *P. proxima* and GWA appears to be more correlated with phenological and morphological features of willow plants than with their VOC emissions.

Chapter 6 is the recapitulation of the conclusions of the experimental chapters and relating it with the existing literature. The twelve willows tested in chapters 2-5 showed a range of metabolites, leaf volatiles (VOCs), and resistance levels to *P. proxima* which manifested as differences in *P. proxima* larval development, damage level and gall size. Overall, *P. proxima* appears to prefer clones with a lower phenolic content and lower leaf pilosity and those that develop earlier in the season. The VOCs in willow clones appear to be species-specific and are not clearly linked to insect resistance. We suggest that the levels of phenolic compounds and pilosity together better explain the preference of oviposition of *P. proxima.* The highest amount of secondary metabolites was found in clones NZ04-106-073 (*S. lasiolepis* × *S. viminalis*, Female), PN676 (*S. alba* L., Female) and PN221 (*S. purpurea* L., Male). NZ04-106-073 also showed the highest emission of VOCs. The most susceptible clones to *P. proxima* were PN736 (*S. fragilis* L., Male) and PN742 (*S. fragilis* L., Male). Tree willows are preferred by *P. proxima* to shrubs. Among the studied clones, PN221 and PN249 (both *S. purpurea*) showed the highest resistance levels to *P. proxima* with no galls. Among the clones that developed galls, PN676 (*S. alba*, female) was the clone that produced the smallest larvae and is therefore considered more resistant.

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Dedication

In memoriam of my biggest cheerleader Ademir Nascimento da Mota also known as the best daddy ever.

Preface

The thesis is article-based and comprises three main sections – the General Introduction, four experimental chapters and the General Discussion. Each experimental chapter contains Introduction, Materials and Methods, Results and Discussion. In Chapter 2 I examined gall formation and damage caused by willow red bean-gall sawfly *Pontania proxima* in twelve different willow *Salix* spp clones. The same twelve clones used on Chapter 2 were further investigated for leaf chemistry and larval development of the red gall sawfly *Pontania proxima* (Hymenoptera: Tenthredinidae) in Chapter 3. The metabolomic profile of six willow (*Salix* spp.) clones of interest in New Zealand are elucidated in Chapter 4. The same six willows studied in Chapter 2 are used to investigate their volatile profiles Chapter 5. Chapter 6 is the General Discussion. References are listed in the end of each chapter.

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(MeanDecreaseAccuracy); the variables with higher MDA are more important in explaining observed patterns. Abbreviations: apig = apigenin; isorhamgl = isorhamnetin-3-O-glucoside; procyB2 = procyanidin B2; epcat = epicatechin;; petbgl = petunidin-3-O-β-glucopyranoside; kaempferide = kaempferide; kaempgl = kaempferol-3-glucuronide; querham = quercetin-7-O-rhamnoside; isorhamrut = isorhamnetin-3-O-rutinoside; unk1 = Unknown 1; quercetinacetbgl= quercetin 3-(6-O-acetyl-β-glucoside); kaempferolgl = kaempferol-3-O-glucoside; luteogl = luteolin-7-O-glucoside; delphinidinarhambgl = delphinidin-3-O-(6"-O-α-rhamnopyranosyl-β-glucopyranoside); eriodgl = eriodictyol-7-O-glucoside ; luteo = luteolin; myricetingalac = myricetin-3-galactoside; myricitrin = myricitrin; myrxyl = myricetin-3-xyloside; eriod = eriodictyol; isoquerc = isoquercitrin; quercgl = quercetin-3-glucuronide; rhoif = rhoifolin; quercetinbglarham = quercetin-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside; quercetinbglpyr = quercetin-3,4'-O-di-β-glucopyranoside; syringalac = syringetin-3-O-galactoside; neod= neodiosmin; flavmar = flavanomarein; querc = quercetin; procyB1 = procyanidin B1.....148

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Chapter 1 Introduction

1.1 Plant secondary metabolites and their role in plant-herbivore interactions

Secondary metabolites are compounds that do not participate in the plant's primary metabolism but have other biological and ecological functions, such as plant communication and defence. These compounds can be tissue constituents in inactive forms or can be induced by herbivory (Belete, 2018; Kariñho-Betancourt, 2018; Mitchell et al., 2016; War et al., 2012).

Plant phenolics are the most common defence secondary metabolites and play a major role in plant resistance, not just against herbivory but also against microorganisms, and are also involved in plant intra-specific competition (Dixon, 1999; Grandmaison et al., 1993; Khokhani et al., 2013; Shalaby & Horwitz, 2015; War et al., 2012). Different classes of phenolic compounds (e.g., lignin, quinones and salicylates) have different modes of action. Lignin is a phenolic polymer with a major role in plant structure and it has also been found to have a role in plant defence. The increase in toughness due to increased lignin content can block pathogen passage through plant tissues, decrease herbivore feeding and affect the plant's nutritional content (Clissold et al., 2009; Johnson et al., 2010; Kamili et al., 2020; Lattanzio et al., 2006; Siegrist et al., 1994; Smit & Dubery, 1997; War et al., 2012). The oxidation of phenols creates quinones that bind with leaf proteins and decrease their digestibility (Steffens et al., 1994; War et al., 2012). Quinones have also been reported to exhibit toxicity in insects (Bhonwong et al., 2009; Jang et al., 2021; Rigsby et al., 2015; Steffens et al., 1994; War et al., 2012). Salicylates are constituents of plant tissues from the family Salicaceae and have been reported to act as anti-feedants to non-specialist insect herbivores (Belete, 2018; Kariñho-Betancourt, 2018; Pasteels & Rowell-Rahier, 1992; Rowell-Rahier, 1984; Rowell-Rahier & Pasteels, 1990; War et al., 2012).

Flavonoids play multiples roles in plant defence, including responses to abiotic and biotic stressors such as UV radiation, pathogens, and pests (Bentivenha et al., 2018; Jiang et al., 2012; Koskimäki et al., 2009; Tabashnik, 1987; Treutter, 2006; van de Staaij et al., 2002; War et al., 2012). For insect pests, flavonoids and isoflavonoids influence their behaviour, growth and development (Kariñho-Betancourt, 2018; Piubelli et al., 2003; Simmonds, 2003; War et al., 2012; Yao et al., 2019).

Tannins are well known feeding deterrents for insect pests (Barbehenn & Peter Constabel, 2011; Kariñho-Betancourt, 2018; War et al., 2012). Tannins impact insect growth and development by reducing the efficiency of nutrient absorption and causing midgut lesions (Barbehenn & Peter Constabel, 2011; Bernays et al., 1980; Steinly & Berenbaum, 1985). The mode of action of tannins is by precipitating proteins, including herbivorous digestive enzymes, and by chelating metal ions therefore reducing their availability to herbivores (Barbehenn & Peter Constabel, 2011; War et al., 2012).

Plants also display a range of plant defence proteins (War et al., 2012). Lectins are glycoproteins able to remain intact in the intestines of herbivores, where they play a role as antinutritive agents (Hopkins & Harper, 2001; Vandenborre et al., 2011). They bind to the membrane's glycosyl groups, which form an inner layer in the insect's digestive tract (Murdock & Shade, 2002; Peumans & Van Damme, 1995; Vandenborre et al., 2011). This binding creates harmful systemic reactions that interfere with nutrient digestion and absorption (Hopkins & Harper, 2001; Vandenborre et al., 2011; Vasconcelos & Oliveira, 2004). Protease inhibitors play a role in plant resistance by inhibiting digestive enzymes in the guts of insects, thus decreasing protein digestion, which results in a shortage of amino acids causing slower development of the insect (Gatehouse et al., 1979; Johnston et al., 1993; Murdock & Shade, 2002; War et al., 2012). Enzymes e.g., peroxidases, polyphenol oxidases, lipoxygenases, ascorbate peroxidases also disrupt insect nutrition, but use different action mechanisms

(Bhonwong et al., 2009; Lawrence & Koundal, 2002; Taggar et al., 2012; War et al., 2012; Woldemariam et al., 2018).

Plants also emit volatile compounds that attract natural enemies (parasitoids or predators) of their herbivores and mediate herbivore host-selection (Binyameen et al., 2021; Effah et al., 2019; Tumlinson, 2014; Turlings & Wäckers, 2004). Natural enemies of herbivores can detect herbivore-induced volatiles and use those volatiles to differentiate plant species, plant cultivar and plant phenological stages (Clavijo McCormick et al., 2012; Jönsson et al., 2005; Kappers et al., 2011). Not just the presence of herbivore-induced volatiles is important, but also their qualitative changes. Natural enemies display behavioural changes according to the qualitative change in volatiles due to different types and growth stages of herbivores, as well as changes in host or prey density (Geervliet et al., 1998; Girling et al., 2011; Takabayashi et al., 1995). The detection of prey is based on olfactory cues and the behavioural changes help to enhance the natural enemies' ability to detect potential prey (Clavijo McCormick et al., 2012; Steidle & Van Loon, 2003).

Herbivore-induced volatiles are easier to detect than volatiles from insect herbivores because they are often systematically emitted which makes their concentration stronger and easier to detect (Clavijo McCormick et al., 2012; Vet & Dicke, 1992). By detecting these volatiles, herbivore enemies optimize their foraging capacity, therefore increasing their fitness. The predator-prey specificity seems to play a role on how predators of herbivores can interpret plant volatiles (Cortesero et al., 1997; Steidle & Van Loon, 2003). Evidence suggests that specialist parasitoids can distinguish herbivore-induced volatiles emitted by their host from those emitted by non-hosts (Cortesero et al., 1997; De Moraes et al., 1998). Specialist parasitoids are reported to have a higher ability to discriminate between developmental stages of their host and to detect the presence of previously parasitized hosts based on herbivore-

induced plant volatiles (Clavijo McCormick et al., 2012; Gouinguené et al., 2003; Mattiacci & Dicke, 1995; Takabayashi et al., 1995; Yoneya et al., 2009).

1.2 Secondary metabolites in the Salicaceae family and their ecological roles

The unique characteristic of the Salicaceae family (which includes willows and poplars) is the presence of salicylates (phenolic glycosides) in their tissues, and this is the defining characteristic of this plant group. The concentration of these compounds as well as the compound mixture can vary according to the plant tissue and species (Boeckler et al., 2013; Fabisch et al., 2019; Pasteels & Rowell-Rahier, 1992).

Salicylates are the most important metabolite in the Salicaceae family, strongly affecting the diversity of insect pests that feed on plants of this family and their specialization. Willows, having a higher concentration of salicylates, have a higher number of specialist insects, suggesting that a certain degree of specialization is required to overcome the toxicity of salicylates. Thus, salicylate specialists can attack a wider variety of willow species. The high energy costs of production and the inefficiency of these compounds against specialist insects has led to the loss of salicylates in some *Salix* species (Boeckler et al., 2011; Volf et al., 2015). Some specialist insects have been reported as taking advantage of salicylates by using them as a source of glucose or for production of defensive compounds (Boeckler et al., 2011; Pasteels & Rowell-Rahier, 1992). Pasteels et al. (1986) investigated the presence of defence compounds in eggs and larva of chrysomelid beetles. The authors found that *Chrysomela populi* L., 1758, *C. tremulae* L., 1758, *C. saliceti* Suffrian, 1849, *C. vigintipunctata* Scopoli, 1763 and *Phratora vitellinae* (L., 1758) present salicin in their eggs in high concentrations and that larvae use that compound for production of salicylaldehyde. Leong et al. (2022) tested the effect of β-diversity in Salicaceae family on leaf-chewing insects from orders Hymenoptera, Coleoptera and

Lepidoptera. The authors found that the degree of specialisation did not differ in Coleoptera feeding on Salicaceae and feeding on other plant species which suggests that beetles can overcome Salicaceae chemistry. Contrastingly, the level of specialization in sawflies (Hymenoptera) and in Lepidoptera caterpillars did change with quantitative differences in willow metabolites, however, the level of specialization did not change for salicylates or flavonoids.

Phenolic glycosides are important components of the chemical defences of Salicaceae. In poplars, for example, the volatile glycoside derivative 6-Hydroxycylohex-2-en-1-one (6-HCH) has been linked to autotoxicity since leaf biomass can be easily killed in contact with 6-HCH (Clausen et al., 2010). This compound is typically absent in summer, but its concentration increases after the first frost, possibly due to the post-frost enzymatic breakdown of salicortin to 6-HCH. This may be advantageous because it can intoxicate browsing herbivores, protecting the plant against them. It also makes plant tissues unpalatable for mammals; hares and moose were observed to prefer willows with lower concentrations of phenolic glycosides (Clausen et al., 2010).

Phenolics are the most important bioactive substances in short rotation coppice willows and their concentration is typically higher in early spring and lower during summer (Tyśkiewicz et al., 2019). Another study performed with poplars, tested different clones for their phenolic contents in relation with temperature and found that poplar plants that were grown under elevated temperatures had lower concentrations of phenolic compounds (Kosonen et al., 2012). Both specialist and generalist insects used phenolics to select potential hosts, with specialists preferring poplar plants grown at lower temperatures (higher in phenolics), while generalists preferred to feed on plants grown at higher temperatures (lower in phenolics). However, this study concluded that the production of phenolics did not change with

temperature, but their dilution increased, causing their concentration to fall (Kosonen et al., 2012).

Volatile nitrogen-containing compounds and green leaf volatiles (GLVs) also play a role in indirect defence in Salicaceae. For instance, black poplar (*Populus nigra* L.) was reported to change its volatile blend in response to feeding by the spongy moth (*Lymantria dispar* L., 1758*)* (Clavijo McCormick, Boeckler, et al., 2014). Evidence was found that the parasitoid *Glyptapanteles liparidis* (Bouché, 1834) can identify and is more attracted to herbivore-damaged leaves of black poplar (Clavijo McCormick, Irmisch, et al., 2014). Laboratory and field tests showed that parasitoids are attracted to minor nitrogen containing compounds (e.g., benzyl cyanide and aldoximes) and green leaf volatiles emitted by damaged foliage. Furthermore, nitrogen-containing compounds were also found to repel the herbivore, having a dual role in direct and indirect defence (Irmisch et al., 2014). A study by Yoneya et al. (2009) on willows (*Salix eriocarpa* Franch. & Sav.) also reported attraction of a predatory ladybird (*Aiolocaria hexaspilota* Hope, 1831) towards leaf beetle (*Plagiodera versicolora* Laicharting, 1781)-damaged foliage emitting nitrogen-containing compounds and GLVs.

In a study by Broberg et al. (2010), two susceptible and two resistant poplar clones were tested for herbivory resistance against poplar and willow borer *Cryptorhynchus lapathi* L., 1758 (Coleoptera: Curculionidae). The most susceptible clone showed the lowest level of primary metabolites, especially linoleic and linolenic acids, and proteins, but did not differ significantly from other clones in the salicylate or phenolic content. Therefore, the authors suggested resistance in the tested clones is due to a novel salicylate-independent mechanism (Broberg et al., 2010).

1.3 Willow (*Salix* spp.) and their economic and ecological relevance in New Zealand

Together with poplars, willows belong to the family Salicaceae and are plants with a very large diversity of growth forms, morphology and ploidy. Willows can be found as erect trees (e.g., *S. matsudana* Koidz, *S. nigra* Marshall, *S. pentandra* L., *S. fragilis* L.), weeping trees (e.g., *S. babylonica* L., *S. alba* L.) and shrubs (e.g., *S. schwerinii* E. Wolf, *S. purpurea* L., *S. viminalis* L.). Leaves are alternate and can be long and narrow or larger and rounder. All willows produce catkins that are wind- and insect-pollinated. Willow catkins can vary in shape and length, and they may appear before the emergence of leaves (precocious), coincide with leaf emergence (coetaneous) or form after leaves are fully formed. Plants are dioecious with ploidy varying from diploid to dodecaploid (Karp, 2014; Van Kraayenoord & Hathaway, 1987).

Willows are a naturalised (non-native) species in New Zealand, where they have multiple uses. The first introduced species was *Salix babylonica* (weeping willow) which was planted in Akaroa, Banks Peninsula in 1839 for landscape use. Other willow species, such as the crack willow (*S. fragilis*), the golden willow (*S. vitellina* L.), the common osier (*S. viminalis*) and the grey willow (*S. cinerea* L.) were introduced later; by 1860 these species were established in different regions of the country (Group, 2007a; Van Kraayenoord & Hathaway, 1987).

Poplars and willows are the first plant choice for soil protection due to their fast growth from vegetative material. Willows, however, have further advantages over poplars because they can grow amongst grass and can serve as livestock fodder in drought periods. Different willow clones flower throughout the year, providing pollen and nectar to honeybees from spring to autumn (Sopow et al., 2017). In New Zealand, tree willows are used for soil conservation, shade, shelter, fodder and stabilising riverbanks, while shrub willows are used

for windbreaks, stream bank stabilisation, pollen sources for bees and for slopes and roadside stabilization (Environment Southland, 2020; Group, 2007b; McIvor, 2013). Table 1.1 shows the indicated uses for a range of willow cultivars in New Zealand.

Table 1.1: A selection of willow cultivars in New Zealand and their indicated uses (Kuzovkina, 2015; McIvor, 2013; van Kraayenoord et al., 1995).

Cultivar	**Species/Hybrid**	**Growth form**	**Indication of use**
Tangoio	*S. matsudana* x *S. alba*	Tree	Exposed hill slopes and drier sites, tunnel gullies, eroding gullies and streambeds, fodder
Hiwinui	*S. matsudana* x *S. alba*	Tree	Exposed hill slopes and drier sites, tunnel gullies, riverbank/stream bank planting
Moutere	*S. matsudana* x *S. alba*	Tree	Tunnel gullies, riverbank/stream bank planting, eroding gullies and streambeds, shelter, and shade
Glenmark	*S. purpurea*	Shrub	Riverbank/stream bank planting, eroding gullies and streambeds
Golden willow	*S. alba* var. vitellina	Tree	Gravel riverbeds and wet areas
Aokautere	*S. matsudana* x *S. alba*	Tree	Shelter and shade
Makara	*S. matsudana* x *S. alba*	Tree	Shelter and shade
Matsudana	*S. matsudana*	Tree	Shelter and shade
Kinuyanagi	*S. viminalis*	Shrub	Fodder

1.4 Willow insect pests in New Zealand

Willows and poplars are attacked by a diverse range of insects worldwide. The most important willow insect pests worldwide are the woolly poplar aphid (*Phloeomyzus passerinii* Signoret, 1875), giant willow aphid (*Tuberolachnus salignus* Gmelin, 1790), Asian longhorned beetle (*Anoplophora glabripennis* Motschulsky, 1853), poplar leaf beetle (*Chrysomela tremulae* Fabricius, 1787 and *C. populi* L., 1758), poplar-and-willow borer (*Cryptorhynchus lapathi* L., 1758), pinhole borer (*Megaplatypus mutatus* Chapuis, 1865), large poplar borer (*Saperda carcharias* L., 1758), small poplar borer (*S. populnea* Linnaeus, 1758), large aspen tortrix (*Choristoneura conflictana* Walker, 1863), poplar defoliator (*Clostera cupreata* Butler, 1886 and *C. fulguriata* Walker, 1865), poplar twig borer (*Gypsonoma aceriana* Duponchel, 1843), poplar clearwing moth (*Paranthrene tabaniformis* Rottemburg, 1775), poplar bent-wing (*Phyllocnistis unipunctella* Stephens, 1834), hornet moth (*Sesia apiformis* Clerck, 1759), willow sawfly (*Nematus oligospilus* Forster, 1854), willow red-bean gall sawfly (*Pontania proxima* Lepeletier, 1823), tremex wasp (*Tremex fuscicornis* Fabricius, 1787) and gall midges (*Dasineura spp.*) (Richardson, 2014).

In New Zealand, common species attacking willows include the giant willow aphid (*T. salignus*), the willow sawfly (*N. oligospilus*) and the red gall sawfly (*P. proxima*), alongside generalist insects such as cicadas. In the next sections more detail will be provided for the first three species.

1.4.1 The giant willow aphid (*Tuberolachnus salignus*)

The giant willow aphid (hereafter GWA) is a large aphid (body length 5.0-5.8 mm) belonging to the family Aphididae (Hemiptera) (Figure 1.1). In this species the reproduction is

anholocyclic i.e., absence of males and exclusive parthenogenic reproduction (Blackman & Eastop, 1994; Dixon, 1985; McIvor, 2014). This pest is considered to be of Asian origin but is spread throughout the world where willows are found (Blackman & Eastop, 1994). In New Zealand it was first found in Auckland on *Salix* × *fragilis* L in December 2013 spreading through the country within a few months afterwards (Gunawardana et al., 2014; Sopow et al., 2014).

Figure 1.1: Giant willow aphid (GWA) *Tuberolachnus salignus* (Gmelin, 1790) colony in laboratory conditions. Both adults and nymphs pictured.

GWA colonies typically appear during summer on the bases of willow trees and spread upwards as population numbers increase. When the density of individuals becomes high, alate forms are produced and disperse to other plants. Colonies start to diminish in numbers in late autumn, when temperatures start to decrease, but in mild climates they can persist throughout the winter (Blackman & Eastop, 1994; Collins, 2001; McIvor, 2014; Sopow et al., 2017).

GWA can affect willow growth and fitness in several ways (Jones et al., 2021; Sopow et al., 2017). As a phloem feeder, GWA can substantially decrease the amount of photo-assimilate that arrives to the roots and stems. The negative effects of GWA on plant growth can be seen even after one growth season (Collins, 2001; Jones et al., 2021). The effects of attack can vary from reduction of plant growth to plant death (Jones et al., 2021). The high production of honeydew can also affect the soil biota and their ecology, which in turn may affect plant growth (Tun, Clavijo McCormick, Jones, Garbuz, et al., 2020). Honeydew deposition on the plant can also stimulate the growth of fungi on the plant surface which can decrease photosynthetic levels. The weakness induced by aphid attack can also leave the plant vulnerable to pathogens (Sopow et al., 2017).

GWA does not only affect the willow host but other species as well. For example, honeydew is reported to attract a variety of insect species in New Zealand, among which are wasps, honeybees, flies, ants and coccinellids. Honeybees suffer particularly from the effects of GWA honeydew. This honeydew contains fructose, glucose and a trisaccharide called melezitose (Mittler, 1958). Melezitose has a low solubility and during the honey production it crystalizes with moisture loss. This creates granular honey called “cement honey”, which is more difficult to extract from the honeycomb with commercial extraction methods. Although some beekeepers report that willow honeydew honey is a good overwintering food for honeybees, causing reduction of feeding costs, some also report that the honeybees avoid the crystalised honey and expend energy to remove the crystals from the hive, or report that melezitose can cause dysentery in honeybees (Sopow et al., 2017).

Knowledge about GWA natural enemies is limited due to the lack of reported natural enemies, restricting their use in biological control. Some options, however, are promising for the New Zealand environment. The parasitoid *Pauesia salignae* Watanabe, 1939 (Hymenoptera: Braconidae: Aphidiinae) is reported to parasitize GWA in India, Japan, Korea

Taiwan and USA, with GWA being reported as its only host (Marsh, 1979; Paik, 1975; Sopow et al., 2017; Watanabe, 1939).

In New Zealand, GWA biological control has been initiated by Scion in 2020 using *Pauesia nigrovaria* (Provancher, 1888) (Sopow & Jones, 2020). First the host specificity was tested to ensure the safety of this parasitoid in the New Zealand environment. The results showed that *P. nigrovaria* is host specific and their release in New Zealand was of low environmental risk (Sopow et al., 2021). The first release was made in February 2020 (Sopow & Jones, 2020). The biological control program was a success with a significant decrease in the GWA population. In two years, the proportion of aphid-free trees had increased from 30 % to 86 %. Although hyperparasitism has been reported, the parasitoid has proven to be thriving in the New Zealand environment (Sopow et al., 2022).

In Australia, the ladybird *Harmonia testudinaria* Mulsant 1850 is a reported GWA predator (Dransfield & Brightwell, 2021). This generalist predator has not been reported in New Zealand and its introduction could have potential negative effects on native species and food webs (Sopow et al., 2017). Tun, Clavijo McCormick, Jones and Minor (2020) investigated the potential of the harlequin ladybird beetle *Harmonia axyridis* (Pallas, 1773), a recent arrival in New Zealand, as a biological control agent of GWA. Their results showed that *H. axyridis* can consume a considerable number of GWA but may prefer other food sources which can compromise its potential as biological control agent.

1.4.2 Willow sawflies, *Nematus oligospilus* and *Pontania proxima*

Two introduced species of sawflies (Tenthredinidae: Hymenoptera) attack willows in New Zealand, the willow sawfly (*N. oligospilus*) and the red gall sawfly (*P. proxima*). Sawflies are typically host-specific and in New Zealand their host range is expected to continue to be

restricted to the genus *Salix* (Berry, 1997). Both sexes can be found in the native range but in invasive ranges, such as New Zealand, only females are present and reproduction occurs by parthenogenesis.

The willow sawfly *N. oligospilus*, native to central and northern Europe and Asia, was first reported in New Zealand in 1997 (Berry, 1997). Adult sawflies are 8-10 mm long, with a pale yellowish-brown thorax and a greenish abdomen. Each female can lay about 60 eggs in their lifetime under natural conditions. Their larva is caterpillar-like (eruciform), with a dark green body and a length of up to 17 mm (Berry, 1997; Carleton, 1939). Larvae feed on willow leaves and have five to seven instars. This species overwinters as cocoons, which are found in leaf litter or on vegetation (Caron, 2017).

The red gall sawfly *P. proxima* is broadly spread throughout the northern hemisphere. It was introduced accidently into New Zealand and was first reported in 1991 (Valentine & Walker, 1991). This pest causes bean-shaped galls on willow leaves. Females prefer to oviposit in leaf buds and 12-19 days later the eggs hatch. The gall formation is completed after 14 days. A well-developed gall is approximately 7 mm long, 7 mm wide, 5 mm thick, and bean-shaped. The colour of galls varies depending on the side of the leaf. On the upper surface the galls are pink to red and on the lower surface they are yellowish green. The galls change according to the stage of larval development. Galls are initially solid with a cavity for the larva. This cavity increases in size with the development of the larva, due to feeding, until the gall walls are thin. The larva takes a total of 18 days to complete its development, passing through five instars. The larva chews a hole in the gall wall to exit and pupates in the soil or on the willow bark. The duration of the pupal stage depends on the ambient temperature and can vary from days to months. Red gall sawfly overwinters as a prepupa (Carleton, 1939; Naumann et al., 2002).

With respect to *N. oligospilus*, the parasitoids *Dibrachys* sp. (Hymenoptera: Chalcidoidea: Pteromalidae) and *Pteromalus* sp. (Hymenoptera: Pteromalidae) and *Pediobius*

sp. (Hymenoptera: Systasidae) have been reported to attack willow sawflies (Urban & Eardley, 1995). The predator *Macrorhapis* sp. is also reported as a natural enemy. In New Zealand, *Dibrachys*, *Pteromalus* and *Pediobius* are genera with both introduced and established species (Berry, 1997; Ward et al., 2020). However, their effectiveness as biocontrol agents requires further investigation.

Less is known about potential natural enemies of the red gall sawfly (*P. proxima*). Many species have been recorded but not many have been agreed with certainty to be parasitoids of *P. proxima* (Carleton, 1939). The species with little doubt are reported to be *Angitia vestigialis* Ratz., *Pimpla vesicaria* Ratz., *Mesochorus* sp. (Hymenoptera:Ichneumonidae), *Scopimenus pygobarbus* Roman 1937, *Bracon discoideus* Wesmael 1838, *Bracon picticornis* Wesmael 1838, *Eulophus tischbeinii* Ratz., *Pteromalus capreae* L. 1761 (Carleton, 1939). To our knowledge, no natural enemies of *P proxima* have been reported in New Zealand.

1.5 Willow clones' resistance to insect pests as a sustainable pest control method and its relevance in willow production in New Zealand

Integrated pest management (IPM) is a strategy to combat insect pests using a diversity of control methods such as plant resistance, botanical diversity, biological control, bait crops, etc (Stenberg, 2017). These practices aim to keep insect pest populations below the economic damage limit with minimal applications of chemical pesticides (Stenberg, 2017). Common cultural methods in forestry include reducing the tree stand density and selection of resistant clones. The choice of clone is often based on the most common pest in the region, but other ecological aspects must be considered (Richardson, 2014).

Given the current state of knowledge and the challenges associated with the introduction of biocontrol agents, the selection of resistant clones could be a suitable solution

to reduce pest impact on willows. Plant resistance occurs when plant structural or chemical traits deter herbivore feeding and thus minimize the amount of herbivore damage experienced by the plant (Mitchell et al., 2016). In integrated pest management, the selection of resistant crop varieties can help to suppress or reduce insect pest damage in conjunction with other direct control tactics. In the case of the giant willow aphid, different willow clones have been shown to vary in their level of susceptibility or resistance to aphid attack (Jones et al., 2021; Tun, Minor, et al., 2020). For example, the Japanese willow Kinuyanagi (*Salix schwerinii*), alongside other shrub willows, has been shown to be resistant to *N. oligospilus* and could be considered for new plantings. However, shrub willows have a less extensive root system than tree willows and may not be useful for the same purposes (New Zealand Poplar & Willow Research Trust, 2014). No studies have documented the resistance of willows against *P. proxima* attack.

The research about defence mechanisms and resistance to insect herbivores in New Zealand willow clones is limited. Recent studies have focused on determining the resistance levels of clones to GWA due to the severity of attack and the economic damage caused in apiculture (Jones et al., 2021; Sopow et al., 2017; Tun et al., 2021).

1.6 Research aims

The introduction of invasive insect pests to New Zealand poses a threat to native and naturalised plant species, among them willows (*Salix* spp.). Given the importance of willows in New Zealand and our fragmented understanding of their interactions with introduced insect pests, the main goal of this study was to explore the metabolites produced by relevant willow clones in New Zealand and investigate their role in plant-herbivore interactions. In previous work Tun (2020) used the GWA/willow system as a study model to investigate the interaction

complexity between introduced plants and insect species. This work follows up on previous research to explore willow chemistry in the context of plant-herbivore interactions, with special attention to red-bean sawfly, *P. proxima*, for which scarce information is available regarding willow susceptibility/resistance in New Zealand.

Our hypothesis is that, since *P. proxima* is a specialized insect pest, clones more susceptible to it will have higher levels of phenol glycosides and a lower trichome density. In section 1.2 we discussed how different levels of resistance may also show differences in the types of volatile organic compounds (VOCs) – for example, black poplar (*Populus nigra* L.) was reported to change its volatile blend in response to feeding by the spongy moth (*Lymantria dispar* L., 1758) (Clavijo McCormick et al., 2014). Natural enemies of insects can also identify plants VOCs and are more attracted to herbivore-damaged leaves (Clavijo McCormick et al., 2014; Irmisch et al., 2014; Yoneya et al., 2009). Clones with different levels of resistance may also show differences in the types of volatile organic compounds (VOCs) emitted as well as the differences in total volatile emissions.

Specific aims, developed as four separate research chapters, included:

i. Analyse and report differences in gall incidence caused by *P. proxima* in twelve different willow clones (*Salix* spp.) to investigate resistance.
ii. Compare larval development in *P. proxima* in twelve different willow clones (*Salix* spp.) used in aim (i) and correlate leaf nutrient analysis to resistance levels to *P. proxima*.
iii. Characterize the metabolite profile of six selected willow clones (*Salix* spp.) relevant to New Zealand, including different sexes (male/ female), growth habits (shrub/ tree) and levels of resistance to chewing, galling and phloem-feeding insects.

iv. Characterize the volatile organic compounds (VOCs) from the six willow clones (*Salix* spp.) used in aim (iii) and discuss their potential role in plant-herbivore interactions.

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Data collection and analysis was conducted by Claryssa de Oliveira Mota with the assistance of Maria Minor. Writing was led by Claryssa de Oliveira Mota and supported by Maria Minor and Trevor Jones. Figures were developed by Claryssa de Oliveira Mota

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Chapter 2 Gall formation and damage caused by willow red bean-gall sawfly *Pontania proxima* in twelve different willow *Salix* spp clones.

2.1 Introduction

Willows are a naturalised species in New Zealand (NZ), where they have multiple uses, such as for soil protection against erosion, livestock fodder in drought periods, and source of pollen and nectar for apiculture from spring to autumn (Environment Southland, 2020; Group, 2007; McIvor, 2013; Sopow et al., 2017). The red gall sawfly *Pontania proxima* (Hymenoptera: Tenthredinidae) is a common pest of willows and is widespread throughout the Northern hemisphere. It has been introduced into New Zealand and was first reported here in 1929 (Kay, 1980). This insect causes bean-shaped red galls on willow leaves within which the larvae develop. Galls can cause a decrease in chlorophyll levels and chloroplasts number, therefore decreasing the rate of photosynthesis (Slepyan & Gabarayeva, 1981; Slepyan, 1962). Although *P. proxima* is not considered as a severe pest, large infestations can decrease willow plant vitality and plant production (Carleton, 1939; Naumann et al., 2002).

Galls of *P. proxima* require the presence of meristematic parenchyma, a tissue which allows the differentiation and rapid growth of cells – therefore, the sawfly prefers to oviposit in younger leaves (Higton, 1991; Slepyan, 1962; Slepyan & Gabarayeva, 1981). However, *P. proxima* can have several generations per year, when the spring generation oviposits in leaf buds, while the autumn generation, however, oviposits in mature leaves (Carleton, 1939). According to Higton (1991); Slepyan (1962); Slepyan and Gabarayeva (1981), *P. proxima* females prefer to oviposit close to the leaf midrib.

Approximately 24-36 hours after oviposition, the gall starts to develop (Higton, 1991). The *P. proxima* gall is basically a mass of hypertrophic parenchyma cells. The normal leaf mesophyll is 10 to 11 cells thick, but within the gall it is approximately 37 cells thick. Inside the gall there is a cavity where the larva develops, feeding on gall mesophyll. At the end of the larval development, only the peripheral layers of the gall parenchyma remain, and the gall is hollow (Higton, 1991; Slepyan & Gabarayeva, 1981). Slepyan and Gabarayeva (1981) highlight that although the cells pass through a significant pathological transformation, some morphological isolation of tissues is maintained, for example the conductor tissue and the leaf veins are not destroyed with gall formation.

The incidence of galling by *P. proxima* and chemical changes caused in NZ willow clones as a result of galling have not yet been characterised. No studies have documented the resistance of willow clones against *P. proxima* attack in NZ. This information is vital for the selection of resistant cultivars.

This study aimed to analyse and compare the degree of damage caused by *P. proxima* and galling differences in twelve different willow cultivars (clones) used in NZ. The study asked the following research questions: 1) Is there a difference in *P. proxima* damage and gall development in different clones? 2) Is there a difference in *P. proxima* oviposition preference regarding leaf/shoot/plant position?

2.2 Materials and methods

2.2.1 Clone selection

Twelve clones were selected from the Aokautere willow collection at Lavello Estate, near Palmerston North (40°21'49.9"S, 175°39'43.1"E). The site was chosen due to high

homogeneity in plant and shoot age, with one-year-old shoots growing on coppiced willow stools. Clones were selected based on the level of galling, sex and species. A survey was performed to classify clones by the level of galling due to *P. proxima* into five categories: from 1 (no damage) to 5 (extensive damage). Those categories were created based on the frequency and size of the galls on the leaves. Survey took place on the 4th and 6th February 2021. Table 2.1 shows the selected clones and detailed information on their attributes and level of galling.

Table 2.1: Selected willow *Salix* spp clones and survey information highlighting the different level of resistance to the red gall sawfly *Pontania proxima*, and the presence of the giant willow aphid (GWA) *Tuberolachnus salignus*. Clones were selected based on their genetic diversity, sex, and level of resistance to insect pests. Y – 'yes'. Galling levels: 1 – no galls, 5 – extensive galling. All clones showed chewing damage.

Clone code	**Species**	**Sex**	**Growth form**	**Galling level**	**Presence of GWA**
PN221	*S. purpurea* L.	M	Shrub	1	
PN249	*S. purpurea* L.	F	Shrub	1	
PN721	*S. matsudana* Koidz	M	Tree	2	
PN693	*S. matsudana* Koidz	F	Tree	2	
PN357	*S. alba L*	M	Tree	3	Y
PN676	*S. alba L.*	F	Tree	3	
NZ1040	*S. matsudana* Koidz *x alba* L.	F	Tree	3	
NZ1130	*S. matsudana* Koidz *x alba* L.	M	Tree	3	Y (heavy)
PN218	*S. fragilis* L.	F	Tree	4	
PN356	*S. alba* L.	M	Tree	4	Y
PN736	*S. fragilis* L.	M	Tree	5	
PN742	*S. fragilis* L.	M	Tree	5	

2.2.2 Leaf damage, image collection and image processing

Three plants per clone were used. Three shoots (branches) were randomly collected from each plant on 17th March 2021. In total nine shoots per clone were collected. Each willow plant was divided into three parts: base, mid-section and top. Shoots were collected from the top section of the trees, at random positions keeping the same distance from each other (see diagram in Figure 2.1a). The collected shoots were labelled, placed in buckets with water and kept in the refrigerator (3-5°C). The average shoot length was 2.97 metres, but shoot length varied between clones.

Figure 2.1: Shoot collection from one year-old coppice willow (*Salix* spp.) plants. (a) Diagram showing direction of shoot collection. (b) Willow plant clone PN218 from Aokautere willow collection as example of plants condition at the time of shoot collection

In the laboratory, the shoots were measured and divided into three equal sections: top, middle and base. Twenty leaves per section were numbered, displayed on the white board labelled with clone name and shoot section, and photographed for percentage of damage analysis. The camera model used for this process was Canon EOS40D equipped with Canon EF-S 18–55mm lens. Subsequently, images were processed with ImageJ software version 1.53e and GIMP 2.10.32. Using the software, the total leaf area and the total damaged area (all

galled areas combined) were calculated for each leaf. These data were used to calculate the total leaf damage percentage per leaf in each clone, as (total galls area / total leaf area) *100.

2.2.3 Gall structure – image collection

Three shoots (branches) were collected from each of three plants per clone, in total nine shoots per clone, on 9th December 2021. Whole branches were collected randomly from the top part of trees, from opposite positions from each other in the same manner as described in the previous section. The collected shoots were placed in buckets with water and kept in the refrigerator (3-5°C). Shoots were processed starting on the following day to avoid desiccation of leaves. One shoot was taken at a time and all leaves from the shoot were removed and put in a zip lock bag with a humid paper towel. The bag was immediately taken to the laboratory where galls were photographed, and larvae were extracted. Please see Chapter 3 for larvae analysis. Intact and open galls were photographed, and gall area was measured using image processing software CellSens Dimension version 1.6. The gall depth was measured with a digital calliper. Gall volume was calculated based on the area and the depth (volume = area*depth).

Figure 2.2 shows photographs of two selected willows clones (the top section of shoots in both) with galling damage, as an example of our method. Figure 2.3, Figure 2.4 and Figure 2.5 show details of galling in clones PN693, NZ1040, and PN676.

Figure 2.2: Willow *Salix* spp clones leaf photos highlighting the galling caused by red gall sawfly *Pontania proxima*, Clones with different levels of galling. Galling levels: 1 – no galls, 5 – extensive galling. Clone NZ1130 had galling level 3, clone PN742 had galling level 5.

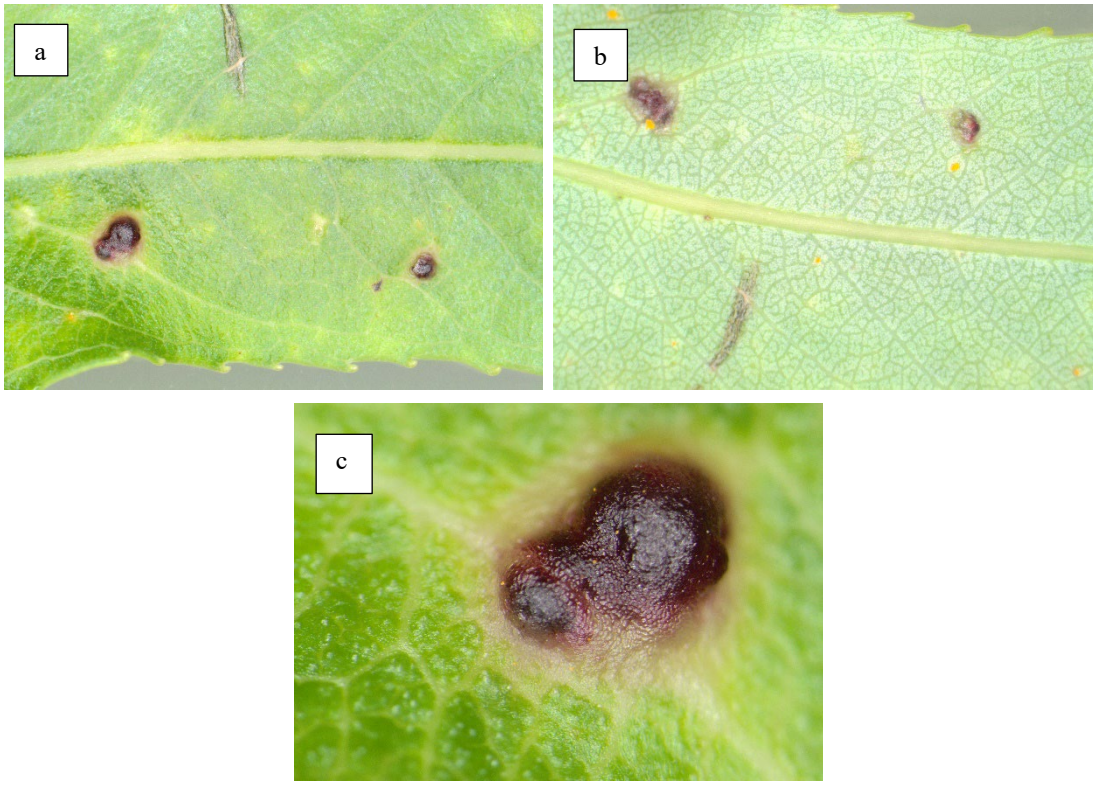

Figure 2.3: Malformed galls (galls that are small in size and do not support larval development) caused by red gall sawfly *Pontania proxima* in willow *Salix* spp clone PN693. (a) Adaxial surface of leaf. (b) Abaxial surface of leaf. (c) Close-up photo of gall.

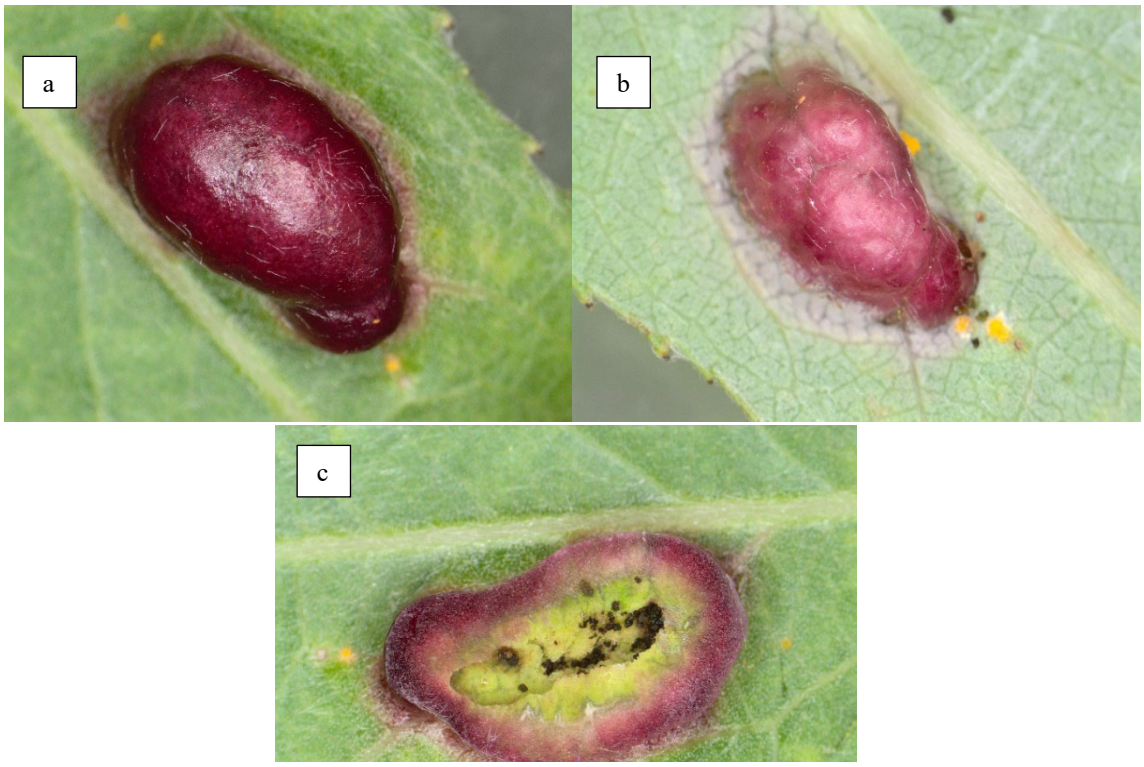

Figure 2.4: Gall caused by red gall sawfly *Pontania proxima* in willow *Salix* spp clone NZ1040. (a) Adaxial surface of leaf. (b) Abaxial surface of leaf. (c) Close-up photo of opened gall.

Figure 2.5: Gall caused by red gall sawfly *Pontania proxima* in willow *Salix* spp clone PN676. (a) Adaxial surface of leaf. (b) Abaxial surface of leaf. (c) Close-up photo of opened gall.

2.2.4 Data analyses

To compare the damage percentage between clones, first a normality test was performed to ensure data followed a normal distribution. A Shapiro-Wilk normality test was performed as well as construction of different density plots to visualize the data distribution in R (version 2023.04.21) using significance level of 0.05. It was verified that the data did not follow a normal distribution.

The generalized linear models (GLM) were used to test if the total level of damage per leaf area and mean gall volume were different between clones, plants, branches and sections of shoots. Branch was included to account for the effect of location on the tree, and shoot section

was included because different generations may prefer the top or the lower parts of shoots. Nested GLM models were run using normal, inverse and gamma distributions, and the most appropriate model was chosen based on the lowest Akaike information criterion (AIC). Where the GLM showed significant difference, a post hoc Tukey test was run with Bonferroni correction. A Pearson correlation test was run to check if gall size (volume) and *P. proxima* larval size (measured as maximum head capsule width, see Chapter 3 of this thesis) were correlated across all clones.

2.3 Results

The generalized linear model (GLM) showed a significant effect of willow clone and shoot section (top-middle-base) on the total leaf damage (%) caused by *P. proxima* (Table 2.2). Most of the leaf damage occurred in the top sections of the shoots. Clones that presented the highest level of damage were PN356 and PN357, and clones with the lowest level of damage were PN221 and PN249, with no damage. Figure 2.6 and Figure 2.7 show the results for individual clones and shoot sections. The post-hoc test results for individual clones are shown in Figure 2.6, and for shoot sections in Figure 2.7.

Table 2.2: Generalized linear model (GLM) comparing total leaf damage (%) caused by red gall sawfly *Pontania proxima* in different clones, plants and shoot sections of willow *Salix* spp. ** significant at alpha = 0.05.

Effect	LogLik	Model Df	Residual Df	Chisq	p-value
Clone	1182,6	11	13	294.13	0.000 **
Plant	1182.6	2	4	0.0867	0.958
Shoot section	1182.6	2	4	0.0927	0.012 **
Branch	1182.6	2	4	0.0927	0.761

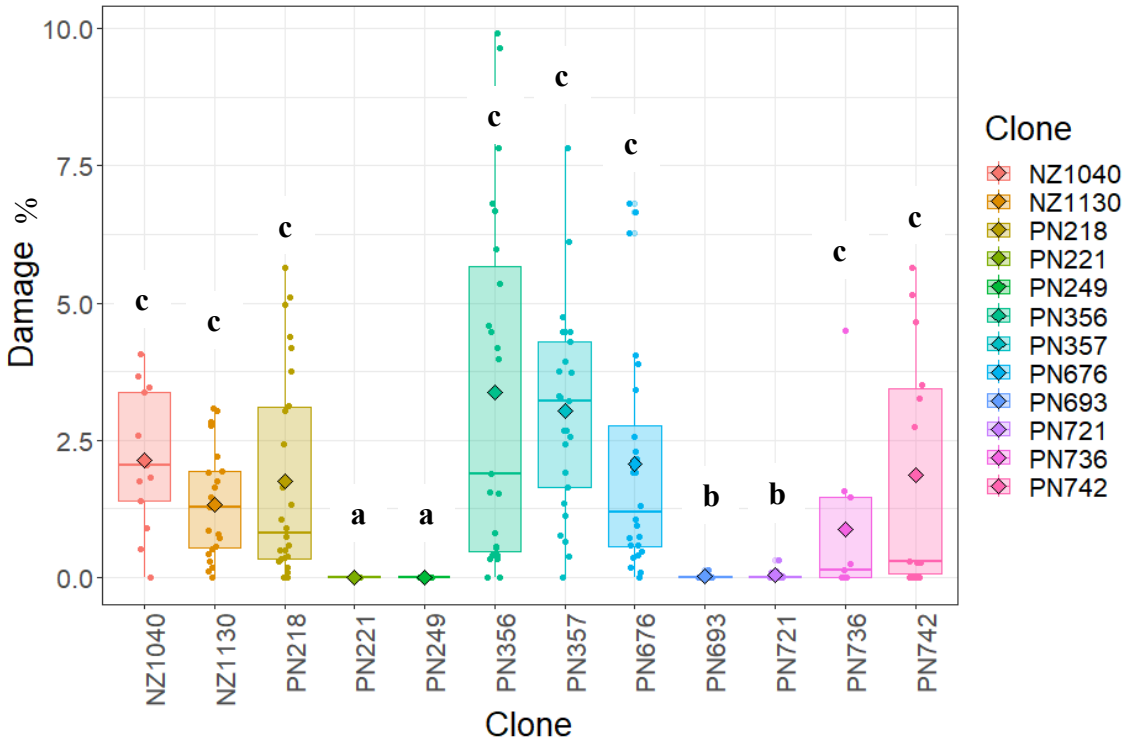

Figure 2.6: Total leaf damage (%) caused by red gall sawfly *Pontania proxima* in different willow *Salix* spp clones. The median is indicated by the line across the box. The mean is indicated by the diamond. Border of boxes represent 25-75% of the percentages. Letters indicate the results from Tukey test, means with the same letter are not significantly different at alpha=0.05. PN221 and PN249 showed no galls therefore no damage level.

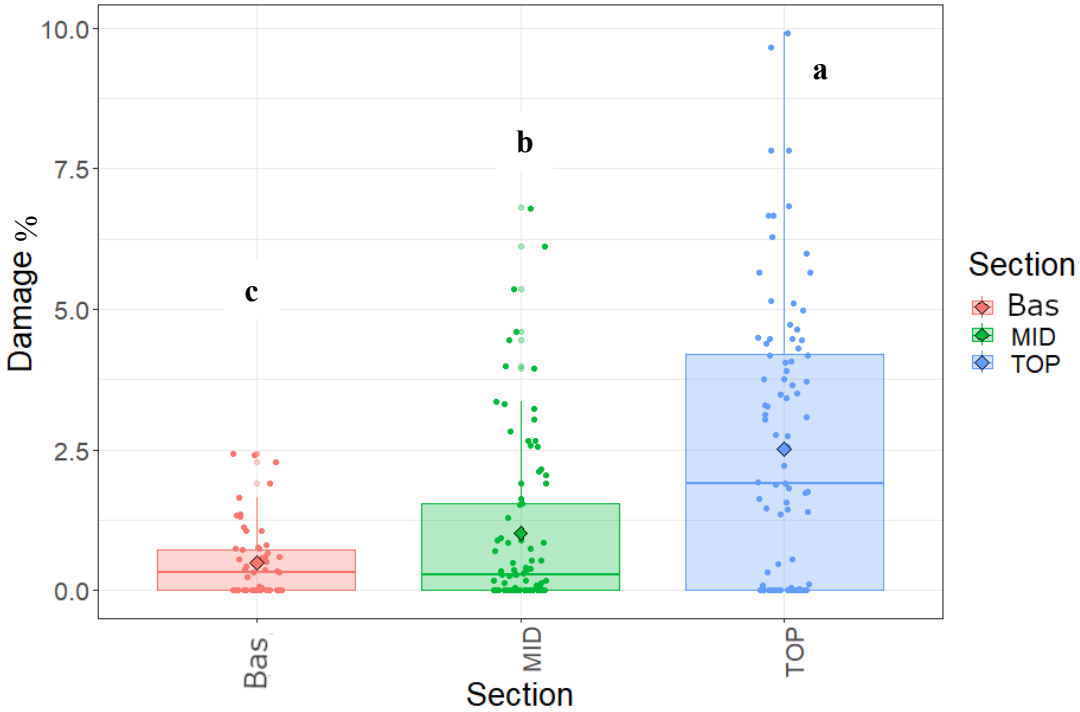

Figure 2.7: Total leaf damage (%) caused by red gall sawfly *Pontania proxima* in different sections of the willow *Salix* spp shoots (base, middle and top). The median is indicated by the line across the box. The mean is indicated by the diamond. Letters indicate the results from Tukey test, means with the same letter are not significantly different at alpha=0.05. Top vs Middle $p < 0.001$; Top vs Base $p < 0.001$; Middle vs Base $p = 0.018$.

The GLM showed a significant effect of clone on gall size (mm^3) caused by *P. proxima* ($p < 0.001$, df = 7, LogLik = -1888.7, Chisq = -180.25). Figure 2.8 shows the results for individual clones and their post-hoc test results. The biggest galls were found on clones PN218, PN736 and PN742. Smallest galls were found on PN676. Interestingly, the clones with the largest galls (Figure 2.7) were not the same ones which had the highest total leaf damage (Figure 2.6).

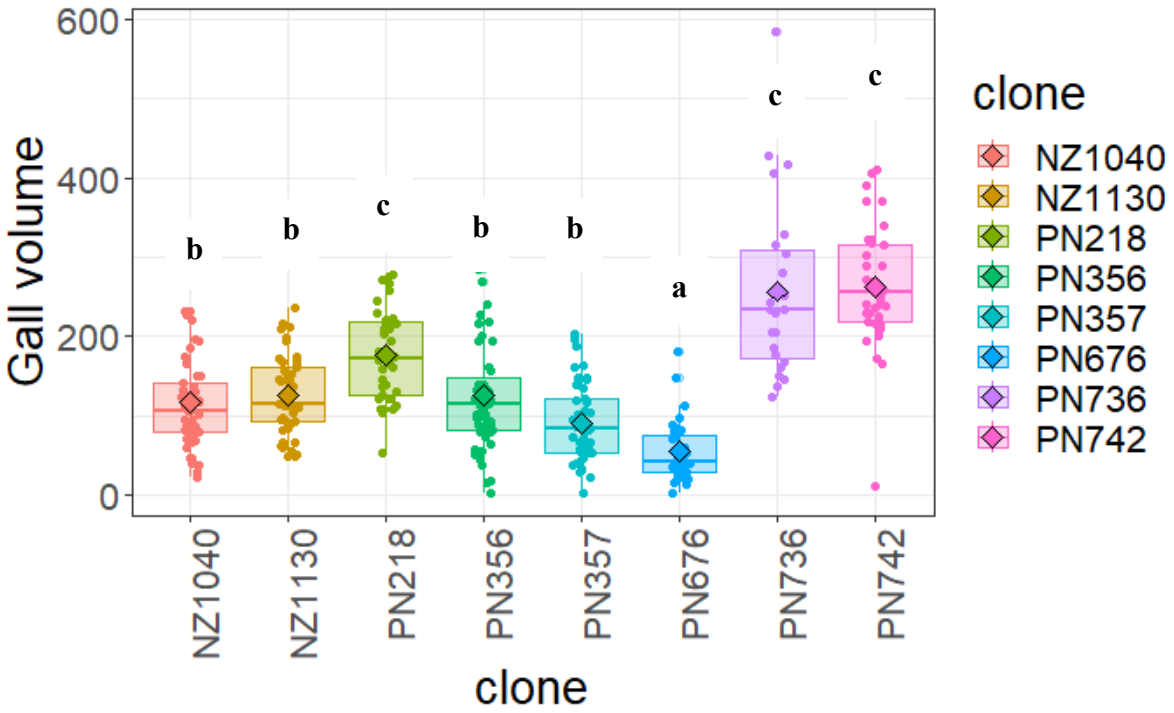

Figure 2.8: Mean size (mm^3) of galls caused by red gall sawfly *Pontania proxima* in different willow *Salix* spp clones. The median is indicated by the line across the box. The mean is indicated by the diamond. Letters indicate the results from Tukey test, means with the same letter are not significantly different at alpha=0.05.

2.4 Discussion

Willow clones used for planting in New Zealand show significant variability in species, sex, growth type, chemistry, morphology and anatomy. In addition to these differences, they

show different susceptibility to diseases and pests (Tun, 2020; Tun et al., 2021; Van Kraayenoord & Hathaway, 1986; Van Kraayenoord & Hathaway, 1987).

Our survey categorized clones in five different levels of galling: from 1 – no galls, to 5 – extensive galling, while analyses of damages in leaves and gall volume present three levels: no damage (clones represented by letter "a" on Figure 2.6), low damage (clones represented by letter "b" on Figure 2.6) and high damage (clones represented by letter "c" on Figure 2.6). All clones that presented galls showed the same level of damage in the leaf damage analyses, except clones PN721 and PN693 which presented malformed galls. Clones PN736, PN742 and PN218 presented the largest galls and PN676 presented the smallest galls.

The leaves for the gall damage and the gall size were from different collection events, so no correlation between total leaf damage and the average gall size (volume) could be made.

Weis and Abrahamson (1986) investigated the diameter of galls caused by *Eurosta solidaginis* (Diptera: Tephritidae) and concluded that gall size variation results from genetic variation among flies. In this insect species, small galls are more vulnerable to attack by parasitoid wasps, while larger galls are more vulnerable to attack by bird predators. Since *P. proxima* reproduce by parthenogenesis (Carleton, 1939), the genetic variability is not considered to be a factor. However, predator pressure may be important in the evolution of the species in its native range. In New Zealand, as parasitoids for *P. proxima* larvae have not yet been reported, it is not a factor that would influence gall size. In other countries, where there is parasitoid and predator pressure, this may play a role in determining gall size. Reported predators of *P. proxima* in the UK are: *Angitia vestigialis*, *Pimpla vesicaria*, *Mesochorus sp*., *Scopimenus pygobarbus*, *Bracon discoideus*, *Bracon picticornis*, *Eulophus tischbeinii*, *Pteromalus capreae* (Carleton, 1939). In Ireland the ectoparasitoid *Pnigalio nemati* is reported to attack *P. proxima* (Al-Saffar & Aldrich, 1998).

Our results show that *P. proxima* causes more damage in the top section of the shoots. This is in accordance with the previous study by Shanahan (1957), who showed higher numbers of galls in the top section of the shoots, in the second *P. proxima* generation. There are several possibilities for this preference. For example, females may have some distinctive mechanism to choose the upper part of the tree at the height of female flight, or differences in microclimates in different parts of the tree (Shanahan, 1957). However, in Shanahan (1957) the first oviposition by overwintered *P. proxima* seemed to be at random along the shoot. In our study, the insects were from the second-generation post-winter, which may be the cause of the oviposition location at the top of the shoot. Another reason for the selection of oviposition sites, may be the difference of predator or parasitoid attack. Females may change where to oviposit, depending on the severity of attack (Shanahan, 1957). This phenomenon happens in other species of insects, such as *Culex pipiens* (subspecies *quinquefasciatus*) (Chesson, 1984), *Leptinotarsa decemlineata* (Hermann & Thaler, 2018), and *Pyrrhosoma nymphula* (Rehfeldt, 1990). In New Zealand, however, there are no reported parasitoids for *P. proxima* larvae, although we can assume that generalist predators such as birds and spiders will attack the adults. During the dissection of larvae for Chapter 3, we have not found any evidence that would suggest the larvae are being parasitized, although there was evidence of gall herbivory. Another hypothesis suggested by Shanahan (1957) is that *P. proxima* mainly reproduce by parthenogenesis, and that sexual and parthenogenetic forms may develop in different parts of the tree. This hypothesis, however, still needs to be tested.

We know that some willow species develop galls by *P. proxima*, and others do not (Naumann et al., 2002; Van Kraayenoord & Hathaway, 1986). What makes some willow clones and some shoot sections more attractive to *P. proxima* than others? Can it be chemistry? Can it be their morphology and anatomy? Leaf toughness or leaf age? The author of this study observed that some willow clones have malformed galls. What causes these galls to be

malformed? What starts gall formation? There are many theories regarding gall induction, but what exactly causes the start of gall development (cecidogenesis) is not known for many host/pest systems (Higton, 1991). The cecidogenesis hypotheses include: 1) physical injury during oviposition, or the presence of egg/larva is the cecidogenic factor; 2) female insect injects a gall-forming substance during oviposition; 3) larva excretes cecidogenic substances while feeding. Higton (1991) highlights that galls caused by *P. proxima* start their development with oviposition, and do not require the presence of an egg or larva. Some authors were successful in producing callus on leaves, but to our knowledge, no one has been successful in producing formed galls. The cecidogenic agent is not known for the *P. proxima*/*Salix* system (Higton, 1991; Slepyan, 1962; E. I. Slepyan & N. Gabarayeva, 1981). Whatever triggers gall induction is not clear, and what keeps the development of galls going is also not clear. It is possible that secondary metabolites play a role in gall development, and that is determined by the clone or genotype.

Gall induction is still poorly understood in sawflies (Roininen et al., 2005). For the majority of species, the collaterial fluid injected during the oviposition seems to be enough for the initial growth, although in some species the feeding stimulus caused by the larvae is necessary for the gall to reach full size (McCalla et al., 1962; Roininen et al., 2005; Smith, 1970).

Although the cecidogenic agent is not known in *P. proxima*, it seems that plant hormones play a role in gall development (Raman, 2021). Yamaguchi et al. (2012) demonstrated that sawfly larvae (*Pontania* sp.), when analysed as a whole larva, contain high concentrations of auxin IAA and t-zeatin. The contents of the accessory glands of adult sawflies were investigated, and they contained a high concentration of t-zeatin riboside, an active form of the plant hormone cytokinin. The contents of the accessory glands are injected into the plant upon oviposition, which suggests that those hormones play a role in gall development. The role

of phytohormones in gall development is also supported in other species, such as *Leptocybe invasa* (Hymenoptera: Eulophidae), that causes galls on *Eucalyptus* trees and *Eurosta solidaginis* that causes galls on goldenrod (Mapes & Davies, 2001; Wang et al., 2022).

In general, not just the insect stimulus is necessary, but a stress response from the plant also has to take place for gall induction and development (Desnitskiy et al., 2023; Raman, 2012, 2021). A stimulating agent activates the cell division and hyperplasia (Harper et al., 2004; Raman, 2012). This stimulating agent can be proteins (Carango et al., 1988), bruchins (Doss et al., 2000) and mitogenic lipids (Farmer, 2000). After the first stimulus, stress responses are induced in the injured location in the plant and genes are expressed (Raman, 2011, 2012). These stress responses can, for example, activate peroxisomes (Corpas et al., 2004) and glyxysomes (Kim et al., 2004). Insect presence, however, may be necessary for the complete development of the gall (Raman, 2012).

For some organisms, however, the gall induction mechanism is better known. Carango et al. (1988) investigated gall induction in tall goldenrod (*Solidago altissima* L.) by larva of the tephritid fly *Eurosta solidaginis.* They concluded that the hyperinduction of a 58 kilodalton protein in the second and third week of gall growth suggests that a substance secreted by the larva may serve as a trans-acting gene regulator. Interestingly, not all organisms induce galls on oviposition. Guiguet et al. (2019) studied the comparison between two species of micromoths belonging to the genus *Caloptilia*, a gall-inducer *Caloptilia cecidophora* (Lep., *Gracillariidae*) and a non-gall-inducing *C. ryukyuensis*. Gall induction did not occur when the second instar leaf-mining larva were killed. This indicates that the cecidogenic compound is released from the third larval instar onwards.

Williams and Whitham (1986) investigated the premature leaf abscission in two species of cottonwoods as a response to herbivore attack by two *Pemphigus* gall aphid species. The authors proved that early leaf abscission is an effective way to encourage aphids to shift host,

and they hypothesized that it may be the reason why galling aphids are 3.5 times more likely to shift host than non-galling aphids. This may explain why we had differences in damage level when we analysed the damage percentage in leaves than when we surveyed the plants. The willow shoots with more galls may have lost more leaves than those with a lower number of galls, leaving us with less leaves to analyse in clones with heavier *P. proxima* attack.

There are some hypotheses regarding hybridisation and resistance to galling insects. Boecklen and Larson (1994) tested hybridisation hypotheses in eight species of gall-forming wasps (Hymenoptera: Cynipidae) on *Quercus grisea* x *Q. gambelii* oak hybrids. The null hypotheses were: H_0 no differences between the two host taxa, H_1 hybrid hosts support higher densities of herbivores than parental taxa, H_2 hybrid hosts support intermediate densities than parental taxa, and H_3 hybrid hosts support lower densities of herbivores than parental hosts. Evidence to support each hypothesis were found in different species. H_0 was supported by two species, H_1 were supported by two species, H_2 by three species and H_3 were supported by one species. Total herbivore population supported H_2. The authors concluded that there is no universal rule for density variation in Cynipidae.

It is known that the galls can cause a decrease in chlorophyll levels and chloroplasts number (Slepyan, 1962; E. I. Slepyan & N. Gabarayeva, 1981). Does *P. proxima* prefer to oviposit in leaves more exposed to sunlight to compensate for the decrease in photosynthetic activity? In our study we investigated if plants and shoots had an influence on galling by *P. proxima* oviposition. Our results did not show significant results for plant and shoot location. Our study site, however, was very well exposed to sunlight and very homogeneous since the plants were planted in the same year and coppiced at the same time, therefore shoots were uniform in height and position. In fields where the distribution of sunlight is irregular this may influence the oviposition site preference by *P. proxima*. This hypothesis, however, needs to be tested.

2.5 Conclusions

Willows show differences in resistance levels to *P. proxima*. These levels of resistance show as differences in larval development (explored in Chapter 5), leaf damage level and gall size.

The willow clones used in New Zealand show differences in leaf damage and gall size. Some clones present with malformed galls. PN221 and PN249 did not present galls, clones PN721 and PN693 presented malformed galls, clones PN357, PN676, NZ1040 and NZ1130 presented low intermediate damage level, PN218 and PN356 presented intermediate damage level and PN736 and PN742 presented the highest level of damage in our field survey. When we analysed percentage of damage as proportion of total leaf area, however, all clones that presented galls showed the same level of damage, except clones PN721 and PN693 that presented malformed galls.

The effect of plant and shoot on differences in leaf damage within the clones were not significant. This may be due to our experimental field being very homogenic in sun exposure, although aspect and sun levels were not measured directly. Shoot section showed significant differences in leaf damage, with the top section of the shoots having a higher level of leaf damage than the middle and basal section. Regarding gall volume, clone PN676 showed the lowest gall volume, clones NZ1040, NZ1130, PN357 and PN356 showed intermediate gall volume and clones PN218, PN736 and PN742 had the galls with largest volume.

Gall induction is still a mystery in the *Salix* spp - *P. proxima* system, mainly because the cecidogenic factor is not yet known.

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Chapter 3 Leaf chemistry and larval development of the red gall sawfly *Pontania proxima* (Hymenoptera: Tenthredinidae) in twelve different willow clones.

3.1 Introduction

Willows are a naturalised species in New Zealand, where they have multiple uses. Willows (*Salix* spp.) are widely used for soil conservation to stabilise pastoral hill country, and riverbanks, due to their fast growth from vegetative material (Wilkinson, 1999). Furthermore, willows can serve as shade for livestock and fodder in drought periods, and they provide an important source of spring pollen and nectar for the apiculture industry from spring to autumn (Sopow et al., 2017).

The red gall sawfly *Pontainia proxima* (Hymenoptera: Tenthredinidae) is widespread throughout the Northern hemisphere. It was first found in New Zealand in 1929 (Kay, 1980). This insect causes bean-shaped red galls on willow leaves, and it is a common pest of willows within its natural range. Although it is not considered as a severe pest, large infestations can decrease plant vitality and therefore plant production (Carleton, 1939; Naumann et al., 2002). Eggs are deposited during spring in the leaf-buds and the galls grow with the leaves (Carleton, 1939). In the autumnal brood, however, eggs may be laid on mature leaves (Carleton, 1939). Eggs hatch in around 12-19 days and the larval phase consists of five instars that take approximately 15 days to conclude (Carleton, 1939). The larva then leaves the gall and pupates in the soil around the plant or in bark cracks (Carleton, 1939). The incidence of galling by *P. proxima* and the plant chemical responses as a result of galling have not yet been characterised. Although the levels of resistance of willow clones against GWA have been categorized (Tun et al., 2020), until now (Chapter 2) there have been no studies that document the resistance of willow clones against *P. proxima* attack in New Zealand. This information is vital for the

selection of resistant cultivars and to understand the potential indirect impacts on other insect species (e.g., natural enemies of competing herbivores).

Insects have complex nutrient requirements for full development. Carbohydrates are needed as source of energy for metabolic activities (e.g., reproduction, synthesis of fat and glycogen production), nitrogen is used for the production of proteins, lipids play roles in reproduction as well as components of cellular membranes, vitamins and minerals are involved in other metabolic processes that are required for the full development and greater fitness of the insect (e.g., zinc and manganese play a role in the hardening of mandibles) (Bala et al., 2018; Behmer, 2008; Genç, 2006). Insect herbivores feed exclusively on plants and thus are relying on them for all the nutrients required for development and reproduction. Higher nitrogen content in plants is linked to a higher growth rate and fecundity of insect populations as well as increased life expectancy of insects (Bala et al., 2018; Jansson et al., 1991; McClure, 1980; Ren et al., 2013; Shah, 2017). Higher levels of phosphorus can also impact the insect population size; populations may increase or decrease depending on the insect species (Apple et al., 2009; Bala et al., 2018; Bishop et al., 2010; Jansson & Ekbom, 2002; Joern et al., 2012; Pitan et al., 2000; Schade et al., 2003; Shah, 2017). Insects that do not have their nutrient needs met, may increase food consumption in order to get the needed nutrients, which can affect rates of growth and weight (Morales-Ramos et al., 2011; Slansky, 1982). Larval development conditions have a great impact on adult fitness and reproduction. Larvae feeding on less nutritious diets can arrive to adult stage with a smaller storage of glycogen and lipids and will have lower reproductive rates than siblings that were fed more nutritious diets (Boggs & Freeman, 2005; Morales-Ramos et al., 2011; Morimoto et al., 2022; Rodrigues et al., 2015; Takken et al., 2013; Tigreros, 2013). Understanding the difference in nutrient content, and therefore feed quality, of different willow cultivars can provide a better insight into sawfly oviposition preference, fitness and reproduction rate, and this information can be correlated

with plant resistance and used in insect pest population management (Cárcamo et al., 2005; Rizvi & Raman, 2017).

Plant phenolics are the most common defence secondary metabolites and play a major role in plant resistance, not just against herbivory but also against microorganisms, and are also involved in plant competition (Harborne, 1994; Lattanzio et al., 2006; War et al., 2012). Different classes of phenolic compounds (e.g., lignin, quinones and salicylates) have different modes of action. Lignin is a phenolic polymer with a major role in plant structure and it has also been found to have a role in plant defence. The increase in toughness can block pathogen passage through plant tissues, decrease herbivore feeding and affect the plant's nutritional content (Lattanzio et al., 2006; War et al., 2012). The oxidation of phenols creates quinones that bind with leaf proteins and decrease their digestibility; quinones have also been reported to exhibit toxicity in insects. Salicylates are constituents of plant tissues from the family Salicaceae and have been reported to act as anti-feedants to non-specialist insect herbivores (Pasteels & Rowell-Rahier, 1992; War et al., 2012). The diversity of phenolic glycosides also plays a role on insect attraction in specialist insects. *Euura lasiolepis*, a specialist insect, has been reported to prefer certain kinds of phenolic glycosides over others. Roininen et al. (1999) investigated the oviposition preference of the gall-inducing sawfly *Euura lasiolepis* to different *Salix* species (*S. lasiolepis*, *Salix caprea*, *S. myrsinifolia*, *S. pentandra*, *S. phylicifolia, S. purpurea*, and *S. rosmarinifolia*), the poplar species *Populus tremula* and filter paper embedded with different phenolic glycosides (arbutin, salicin, and salicylalcohol, tremulacin and salicortin). The authors concluded that tremulacin was the key oviposition stimulant. This compound is the major phenolic glycoside found in *S. lasiolepis*, reported to be the host plant for *E. lasiolepis*. This study, however, tested the content of pure phenolic glycosides and did not take into consideration other factors such as additional volatiles, leaf pilosity and epicuticular waxes that can also influence the oviposition preference.

Willows (*Salix* spp.) are members of the Salicaceae family. Members of this family produce secondary metabolomic compounds called phenolic glycosides. Besides phenolic glycosides, willows also have other phenolic compounds such as flavonoids and tannins (Hegnauer, 1973; Piątczak et al., 2020). Different species may have different compound mixtures and different compound contents in different plant tissues (Boeckler et al., 2011; Hegnauer, 1973; Julkunen-Tiitto, 1986; Pasteels & Rowell-Rahier, 1992; Thieme, 1965; Torp et al., 2013). Hegnauer (1973) described and compared the chemistry of different species of the Salicaceae family and reported that leaves of *S. alba* L. do not contain salicin derivates (e.g., salicin, salicortin) but present a high content of leucoanthocyanins, while leaves of *S. fragilis* L. and *S. purpurea* L present salicin derivatives but do not contain leucoanthocyanins (Hegnauer, 1973). Julkunen-Tiitto (1986), however, reported that *S. alba* in Finland contained trace amounts of salicin derivates. Differences in phenolic content can also be observed between sexes, with males having lower amounts of phenolics (Price et al., 1989; Ruuhola et al., 2018). The presence of phenolic glycosides also affects the diversity of insect herbivores that feed on willows: plants that have phenolic glycosides in their leaves attract more specialized insect herbivores, while willows without these compounds attract more generalist herbivores (Boeckler et al., 2011; Braccini et al., 2013; Rowell-Rahier, 1984).

This study aimed to analyse the larval development of *P. proxima* on twelve different willow cultivars (clones) used in New Zealand and relate larval development to the nutrients and total amount of phenolic compounds in the leaves of the host plants. The study asked the following research questions: 1) Is there a difference in *P. proxima* larval growth in different clones? 2) Do clones have a significative difference in leaf nutrients and total phenolic content? 3) Is larval development influenced by nutrients and/or phenolic content? 4) Is there a difference in nutrient and/or phenolic content between galled leaves and healthy leaves?

3.2 Materials and methods

3.2.1 Clone selection

Twelve clones were selected from the willow collection at the Lavello Estate poplar and willow nursery, at Aokautere near Palmerston North (40°21'49.9"S 175°39'43.1"E). The site was chosen due to the high homogeneity in plant age, with one-year-old shoots on coppiced plants. Clones were selected based on the level of galling, sex and species. A survey was performed to classify clones by the level of galling due to *P. proxima* into 5 categories: from 1 (no damage) to 5 (extensive damage). Presence of damage due to chewing herbivores and piercing-sucking herbivores such as the giant willow aphid *Tuberolachnus salignus* were also recorded (Table 3.1). This survey took place on the 4th and 6th February 2021. Table 3.1 shows the selected clones and detailed information on their attributes and the level of galling observed in the field.

Table 3.1: Selected willow *Salix* spp clones and survey information highlighting the different levels of resistance of the clones to red gall sawfly *Pontania proxima* and giant willow aphid *Tuberolachnus salignus*. Clones were selected based on their species, sex, and level of resistance to insect pests. Y – 'yes', M – male, F – female. Galling levels: 1 – no galls, 2 – galls not fully developed, 3 to 5 – progressively more extensive galling. All clones showed damage from chewing insect herbivores. Details about the morphological characteristics from Glenny and Jones (2019) and Newstrom-Lloyd et al. (2015). Details about flowering time from Glenny and Jones (2019) and Newstrom-Lloyd et al. (2015).

Clone code	Species	Sex	Galling level	Presence of giant willow aphid	Flowering time	Leaf hairs	Hairs on last season's branchlets
PN 221	*S. purpurea* L.	M	1		25 Aug – 15 Sep	Absent on upper and lower surface lamina	Absent
PN 249	*S. purpurea* L.	F	1		7 Sept – 5 Oct	Absent on upper surface lamina; absent to moderate on lower lamina surface	Absent
NZ 1040	*S. matsudana* Koidz *x alba L.*	F	3		18 Sept – 8 Oct	Absent on upper surface lamina; sparse to moderate on lower surface lamina	Absent
NZ 1130	*S. matsudana* Koidz *x alba L.*	M	3	Y (heavy)	29 Aug – 27 Sept	Absent on upper surface lamina;	Absent

						sparse to moderately dense on lower surface lamina	
PN 218	*S. fragilis* L.	F	4		-	Absent on upper surface lamina; sparse to moderate on lower surface lamina	Can be present
PN 736	*S. fragilis* L.	M	5		-	Absent on upper surface lamina; sparse to moderately dense on lower surface lamina	Absent
PN 742	*S. fragilis* L.	M	5		-	Absent on upper surface lamina; sparse to moderately dense on lower surface lamina	Absent
PN 676	*S. alba* L.	F	3		28 Sept – 14 Oct	Sparse to moderately dense on lower and upper surface lamina	Present
PN 356	*S. alba* L.	M	4	Y	22 Sept – 27 Oct	Sparse to moderately dense on lower and upper surface lamina	Present

PN 357	*S. alba* L	M	3	Y	15 Sept – 13 Oct	Sparse to moderately dense on lower and upper surface lamina	Present
PN 693	*S. matsudana* Koidz	F	2		2 Sept -21 Sept	Absent on upper and lower surface lamina	Absent
PN 721	*S. matsudana* Koidz	M	2		-	Absent on upper and lower surface lamina	Absent

Twelve clones were selected for this study, but only eight clones were able to support *P. proxima* larval development (Table 3.1, galling level 3-5) and these eight clones were used for larvae collection and analysis described in the following sections.

3.2.2 Nutrient and total phenolics sampling and analysis

Leaf samples for this analysis were collected on the 18th of February 2021. The one-year-old shoots on the willow plants were divided into three parts: base, mid-section and top. Fully formed leaves were collected from the apical section of the shoots. For clones with large leaves, a sample of around 20 leaves were collected, and for clones with smaller leaves, a sample of around 30 leaves were collected. For each clone, samples of leaves with and without galls were collected. There were three plants per clone in the willow collection, and one sample of leaves with galls and one sample without galls per plant were collected. Thus, for each clone three samples with galls and three samples without galls were collected. The leaf samples were placed in a cooler with dry ice and then transported to the laboratory where they were freeze-dried and stored at -20 °C until further processing.

For nutrient and total phenolics analysis, the samples were manually ground. After grinding, individual samples from different plants were mixed into pooled samples for each clone (with galls and without galls, separately) and sent to the Nutrition Laboratory (Massey University, Palmerston North) for analysis. Pooling was done to meet sample weight requirements for the analysis.

Not all clones were chemically analysed (due to expense of this analysis). Clones were separated into groups based on sample weight, clone resistance, willow species, and galling level (i.e., the success of larval development); samples from a total of seven clones were sent for chemical analysis (Table 3.2).

Table 3.2: Selected willow clones *Salix* spp analysed for nutrients and total phenolics. Selection of clones was based on the weight of available samples, clone resistance, willow species and galling level. Galling levels: 1 – no galls, 2 – galls not fully developed, 3 to 5 – progressively more extensive galling. Clones were grouped according with galling level. Group 1: minimal galling (PN721 and PN221), Group 2: medium galling (NZ1130 and PN676), Group 3: heavy galling (PN742, PN218 and PN356).

Clone name	**Group**	**Galling level**	**Weight of sample (g)**	
			With galls	**Without galls**
PN221	1	1	-	11.3437
PN721	1	2	-	13.5430
PN676	2	3	11.8598	8.9670
NZ1130	2	3	9.7831	9.5569
PN218	3	4	17.8254	12.0229
PN356	3	4	13.8833	9.7622
PN742	3	5	16.2637	16.4284

Ash content was determined by AOAC Official Method 942.05, a method of laboratory analysis for animal feed with the furnace temperature set to 550°C (Thiex et al., 2019). Moisture was determined following AOAC 925.10 and 930.15, crude protein according to AOAC 968.06 (Dumas method) and crude fat following AOAC 2003.06 (Thiex et al., 2003). Total phenolic content was established according to Folin and Ciocalteu (1927). Carbohydrate content was obtained as the total carbohydrates by the difference method.

3.2.3 Larvae collection and measurement

The collection of galls for larval analysis was performed for the eight clones (NZ1040, NZ1130, PN218, PN736, PN742, PN676, PN356 and PN357) which supported larval development (galling level 2 to 5 in Table 3.1). Three shoots per plant were collected from three plants per clone on the 9$^{th\ of}$ March 2021. The method of shoot collection was as described

in Chapter 2. The collected shoots were placed in buckets with water and kept in the refrigerator (3-5°C) at Massey University, Palmerston North. The shoots started to be processed on the following day to avoid desiccation of the leaves. One shoot was taken at a time and leaves were removed and put in a zip lock bag with a humid paper towel. The bag was immediately taken to the laboratory where the galls were photographed, and larvae were extracted.

Galls were opened longitudinally with a scalpel and larvae extracted from the galls with fine tweezers and placed in 2 ml tubes containing 70% ethanol for preservation. Larvae were measured after all the larvae were extracted from the shoots. To measure larvae head capsule width, each larva was dissected, and the larval head capsule was placed on a watch glass with a small amount of 70% ethanol. Head capsule maximum width was measured (µm) using image processing software CellSens Dimension version 1.6 under a dissecting microscope (Figure 3.1).

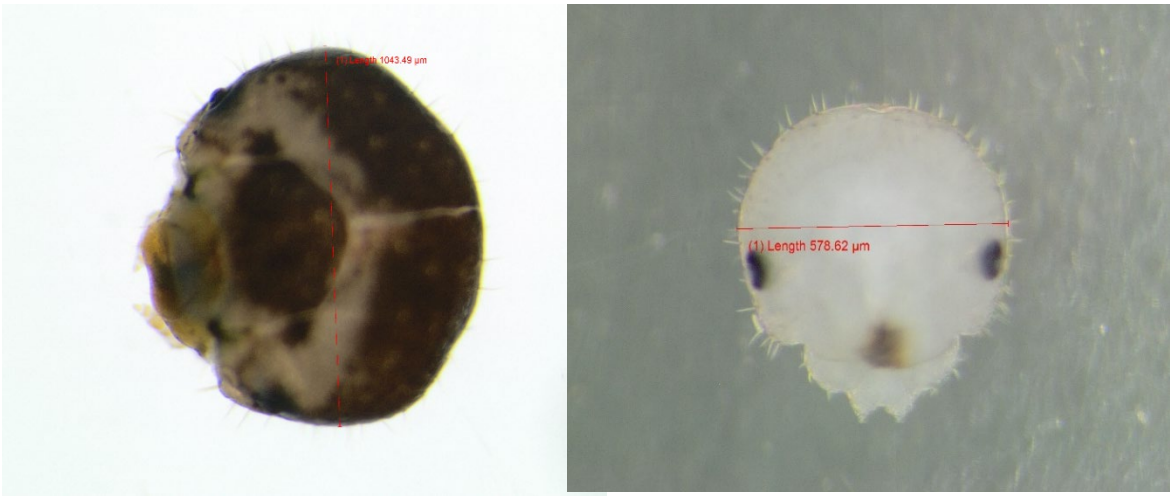

Figure 3.1: *Pontania proxima* larvae head capsules extracted from *Salix* spp clone PN742 (right and left images: larvae from two different instars). Head capsule maximum width measurement (µm) as shown in photo.

3.2.4 Statistical analyses

3.2.4.1 Data analysis for nutrients and total phenolics

All statistical analyses mentioned in this section were performed in RStudio (version 2022.07.1) using significance level of 0.05.

First, a Shapiro-Wilk normality test was performed as well as the construction of different density plots to visualize data distribution. Groups of clones (Table 3.2) and presence of galling were used as contrasts. A principal component analysis (PCA) using the "FactoMineR" and "factoextra" packages was performed. The PCA biplots and corresponding scores of variable contributions were then used to identify clusters and to visualise the overall differences in nutrients and phenolic content between the groups of clones based on galling levels, and in clones with and without galls. Generalized linear models (GLM) were run with normal, inverse and gamma distributions, and the most appropriate one was chosen based on the Akaike information criterion (AIC).

First, GLM was used to test for the effect of gall presence on nutrients and phenolics in leaves across all the groups of clones based on galling levels, and for groups 2 and 3 only (groups with galls present); this was followed by GLM testing for groups' significance. A linear regression was run for each of the nutrients and for total phenolics with larval head width as dependent variable, to determine the relationship between nutrients/phenolics and larval head size. Only instars four and five were included in the analyses, as they were the instars present in all the clones. A permutational multivariate analysis of variance (PERMANOVA) was run to test differences between groups two and three for nutrient content.

3.2.4.2 Instar classification and data analyses

To evaluate the relationship between nutrients/phenolics in leaves and *P. proxima* larval development (using larval head width), larvae were first classified by instar.

To separate different larval instars based on the head measurements, the kernel density estimation method was used, as described by Sukovata (2019). Data analyses were run in Rstudio software version 4.1.0 (2021-05-18). The packages used were "stats", "tidyverse", "mixtools" and "ggplot2". The significance level of 0.05 was used for all tests. The following workflow was carried out for each willow clone:

1. A frequency distribution graph of larval head capsule widths was plotted to visualize the data.
2. A kernel density estimate was constructed and the bandwith was optimized based on the method described by Shimazaki and Shinomoto (2010) (Figure 3.2). To avoid overfitting, the bandwith was adjusted manually when needed.

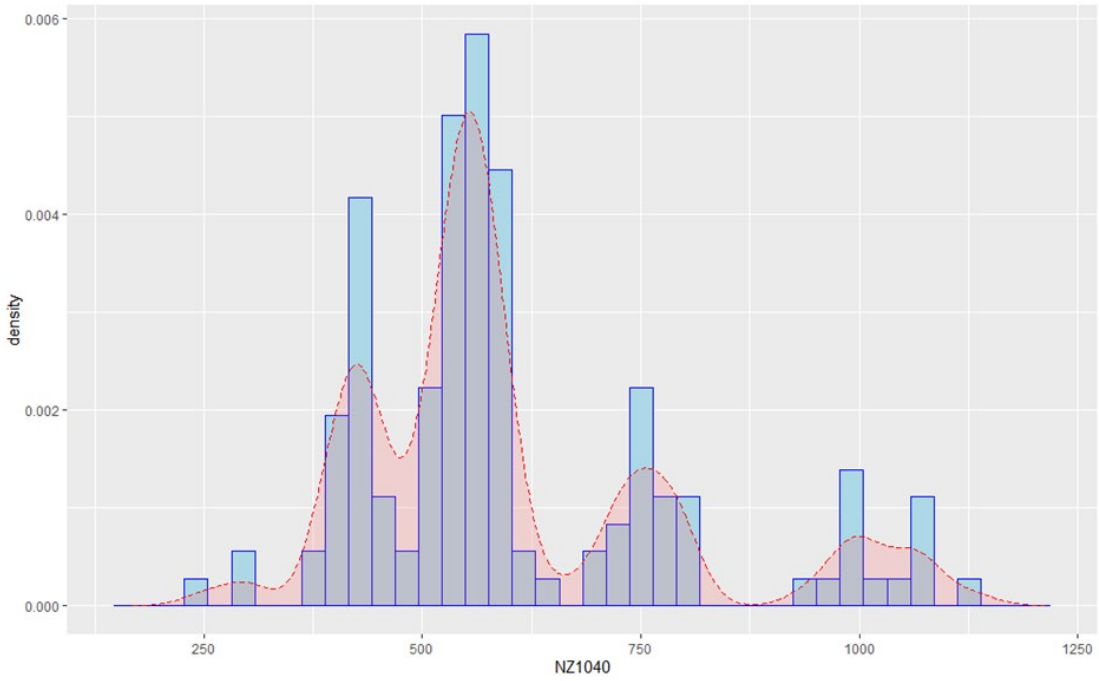

Figure 3.2: Data histogram and probability density function (y-axis) for larval head capsule measurements of red gall sawfly *Pontania proxima* (x-axis, micrometres), suggesting five larval instars; example for clone NZ1040. Similar graphs were constructed for all the susceptible clones.

3. Values of head sizes corresponding to peaks in the probability density function were calculated. These values were used as starting values in the next step.

4. To obtain the mean and the variance for head size of each instar, Gaussian curves were used to model larval instar. A Gaussian mixture model was used to fit Gaussian curves for each instar peak (this model fits a mixture of five gaussians to the data). Peak means from step 3 were used as the starting conditions for the model. Summaries of this step for clone PN742 are presented in Table 3.3 and Figure 3.3. Results for other clones are in Figure 3.7A-1 – Figure 3.7A-7.

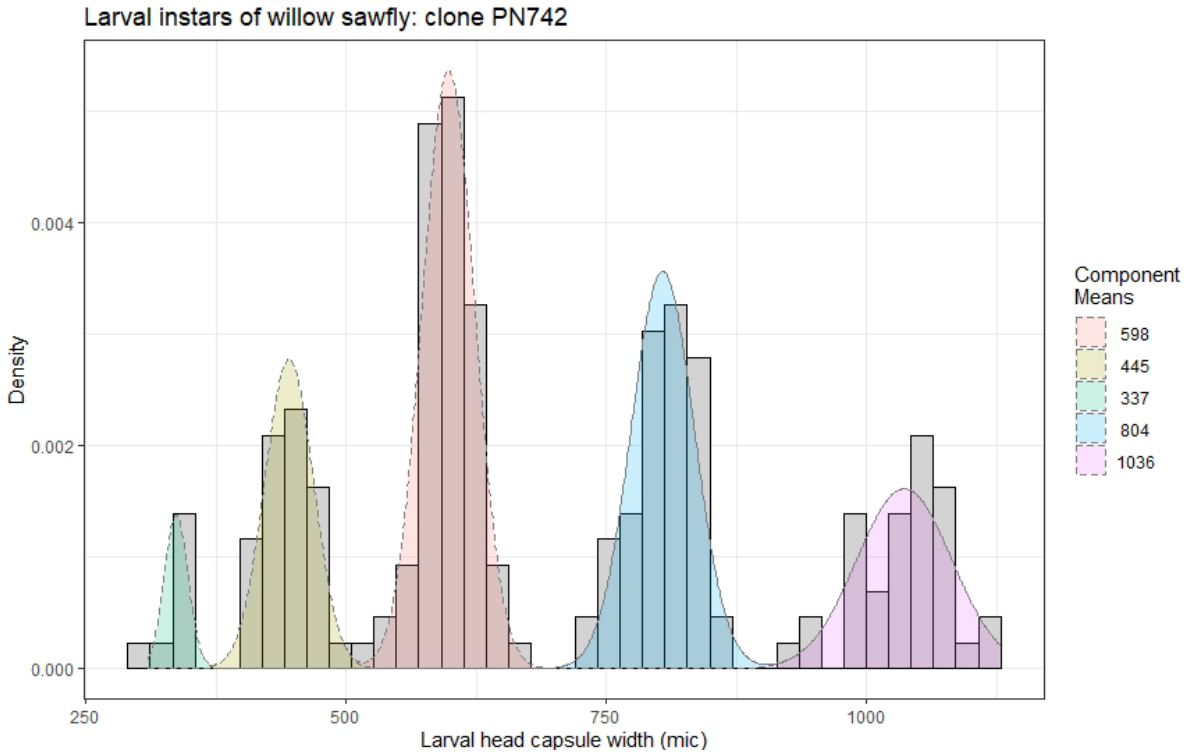

Figure 3.3: Predicted mean head capsule widths (Component Means) for five larval instars of red gall sawfly *Pontania proxima* developing in willow clones; example for willow clone PN742. The graph represents the probability density function based on mixed Gaussian model.

Table 3.3: Means and variances of head size for different instars of *Pontania proxima* larvae, example for clone PN742: summary of normalmixEM object (Gaussian mixture model). Comp 1-5 are the component Gaussian curves for five larval instars; mu is the mean, sigma is the variance, lambda is the scaling variable.

	comp 1	comp 2	comp 3	comp 4	comp 5
Lambda	0.0224	0.2143	0.4957	0.1631	0.1045
Mu	282.330	423.860	553.609	751.514	1027.204
Sigma	23.275	22.209	28.426	36.195	49.908

Graphs with kernel density estimate (Figure 3.2) were plotted for each of the eight clones. Figure 3.3 shows the Gaussian mixture model (probability density function) predicting instars and their mean head capsule widths for clone PN742. A similar graph was built for every one of the eight clones that developed larvae (Figure 3.7A-1 to Figure 3.7A-73.7 Appendix).

For clones NZ1130, PN356, PN357 and PN676 Gaussian models were not able to explain means for all five instars. To predict the missing instars, Dyar's law (linear regression) was performed using the obtained means from Gaussian models (Dyar, 1890). An OLS linear regression was performed in Microsoft® Excel® for Microsoft 365 MSO (Version 2209). Table 3.4 shows R^2 value for clones.

Table 3.4: Dyar's law R2 value for *Pontania proxima* instars extracted from willow *Salix* spp clones NZ1130, PN356, PN357 and PN676. OLS regression used to develop equation to predict the larval head capsule width.

Clone	R^2	Equation	P-value
NZ1130	0.9939	y=222.31x-112.95	0.04957
PN356	0.9986	y=238.51x-159.99	0.02413
PN357	0.9788	y=199.3x+1.96	0.0107
PN676	0.9923	y=173.35x+77.57	0.0560

To compare the larval development between clones, the mean and variance for instars IV and V obtained from the Gaussian mixture modelling were used in a one-way analysis of variance (ANOVA). When the overall F-test was significant, this was followed by a Tukey's one tail *post-hoc* test. Significance levels for the post-hoc tests were adjusted using the Benjamini–Hochberg procedure with the false discovery rate set at 0.025 and 0.055.

3.3 Results

3.3.1 Nutrients and total phenolics in the leaves of the willow clones

Data for leaf nutrients and leaf phenolic content in different clones can be found in Table 3.5 and

Figure 3.4. Healthy leaves of clone PN721 and PN742 showed the lowest phenolic content while healthy leaves of PN221 showed the highest (

Figure 3.4 and Table 3.5). Crude protein levels were highest in healthy leaves of clones PN742 and PN356, and smallest in healthy leaves of PN221. Carbohydrate levels were highest in healthy leaves of PN221 and lowest in healthy leaves in clone PN356. Fat levels were highest in healthy leaves of PN221 and PN356 while PN218 with galls showed the lowest levels. Clone PN721 (healthy leaves) showed the highest levels of ash and PN221 showed the lowest.

Table 3.5: Nutrient and total phenolics content in healthy and leaves galled by *Pontania proxima* for different clones of willows *Salix* spp. Total phenolics in milligrams of gallic acid equivalents per gram. All results are on a dry matter basis. Galling levels: 1 – no galls, 2 – galls not fully developed, 3 to 5 – progressively more extensive galling.

Clone	Galls on leaves	Galling level	Ash (%)	Crude Protein (%)	Fat (%)	Carbohydrates (%)	Total Phenolics (mg GAE/g)
PN221	No	1	4.82	13.81	5.25	76.23	188.33
PN721	No	2	8.54	15.55	4.27	71.63	78.31
PN676	Yes	3	7.44	16.19	3.39	72.98	135.45
PN676	No	3	7.99	15.43	3.28	73.30	139.93
NZ1130	Yes	3	6.22	17.69	3.49	72.60	102.29
NZ1130	No	3	6.15	19.32	4.06	70.36	92.21
PN218	Yes	4	6.89	15.08	1.86	76.17	118.69
PN218	No	4	8.11	17.20	4.93	69.88	108.00
PN356	Yes	4	6.60	18.48	4.29	70.52	119.36
PN356	No	4	7.35	20.20	5.60	66.85	111.31
PN742	Yes	5	6.01	16.17	3.83	74.10	102.51
PN742	No	5	7.47	20.56	4.44	67.53	83.55

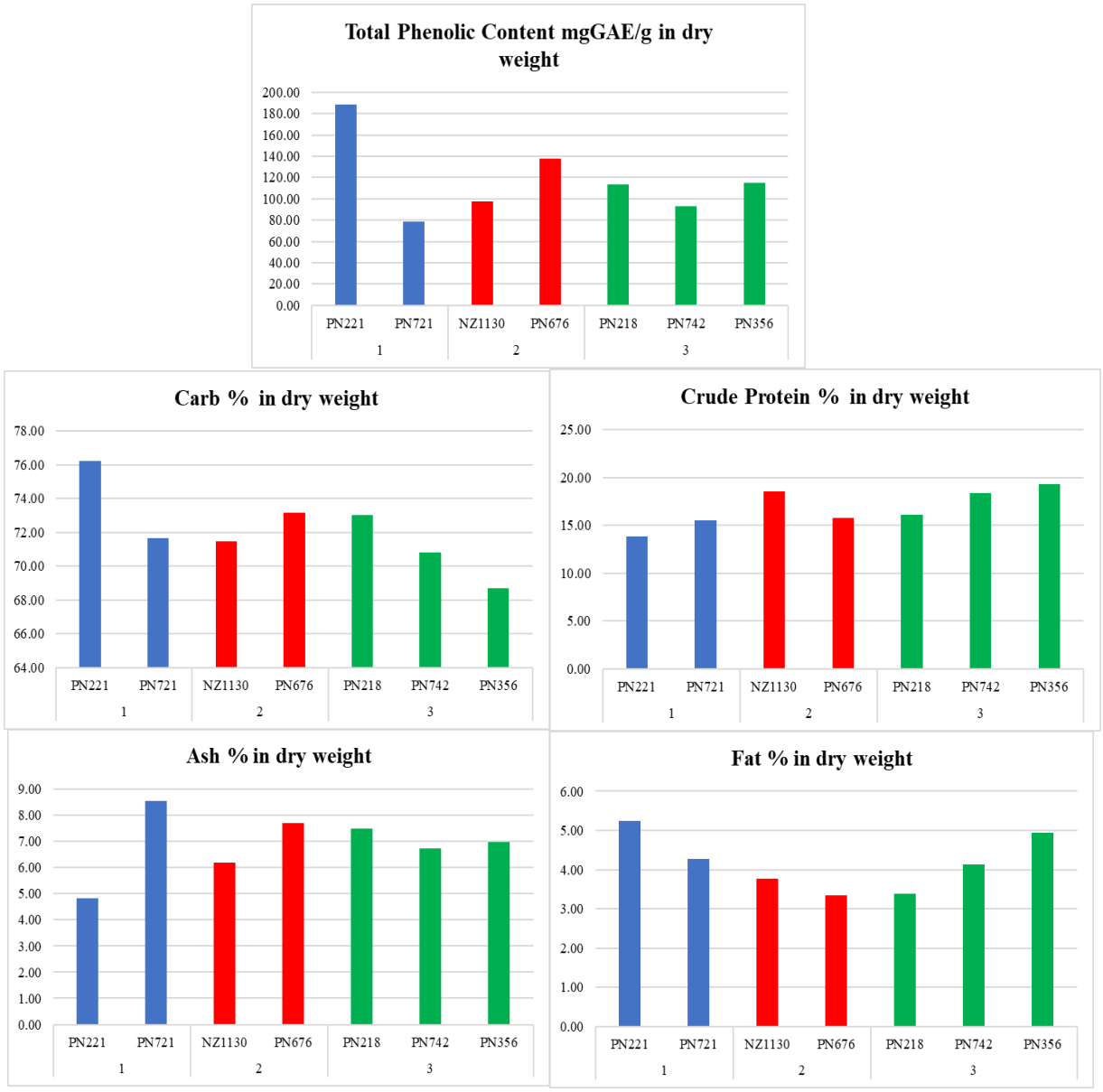

Figure 3.4: Crude protein, carbohydrates, fat, ash and total phenolics content in leaves of different willow *Salix* spp clones. Clones were grouped according to galling level; data are the average for galled and non-galled leaves. Group 1: minimal galling (PN721 and PN221), Group 2: medium galling (NZ1130 and PN676), Group 3: heavy galling (PN742, PN218 and PN356). Results for phenolic content are in milligrams of gallic acid equivalents per gram of dry matter. For nutrients, the results are in weight percentage of dry matter.

Figure 3.5 shows the principal component analysis (PCA) ordination for nutrients and total phenolics according to the galling group. PC1 and PC2 together explained 81.2% of the variation in leaf nutrients and total phenolics in the different groups. The main contributors of PC1 were protein, carbohydrates, and total phenolic content, while the main PC2 contributors were ash, fat and total phenolic contents. No distinct clusters were observed.

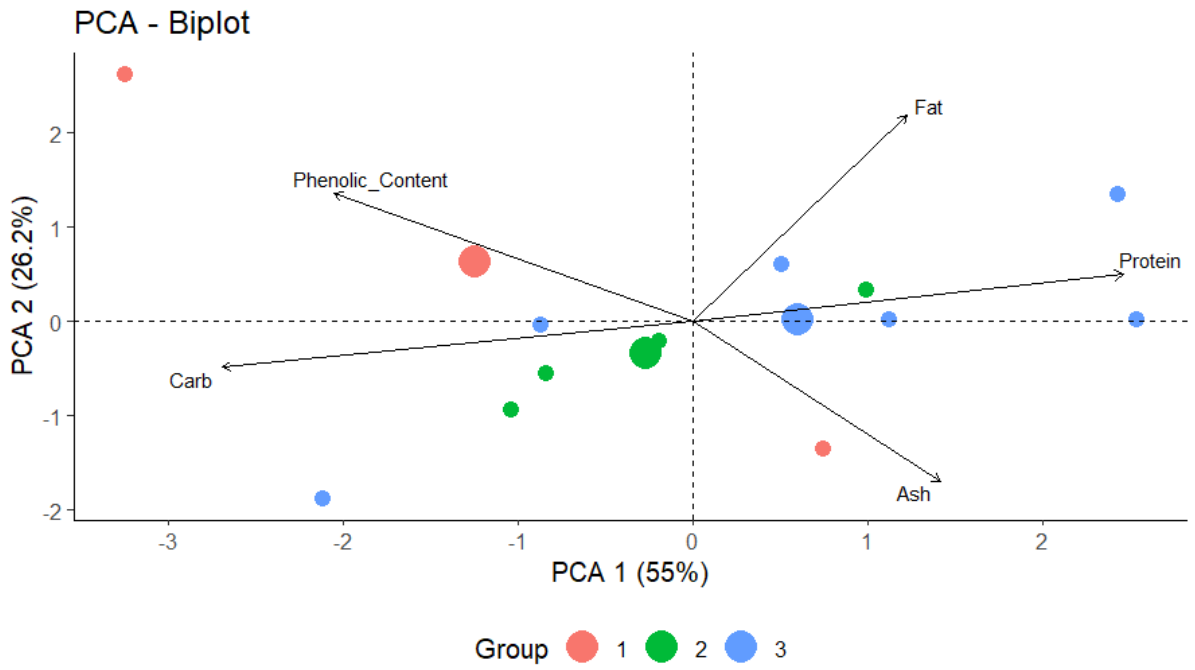

Figure 3.5: Principal component analysis (PCA) biplot showing differences in nutrients and phenolic content in different clones of willows *Salix* spp. Larger symbols are group centroids. Groups formed by differences in resistance to *Pontania proxima* galling. Group 1: minimal galling (PN721 and PN221), Group 2: medium galling (NZ1130 and PN676), Group 3: heavy galling (PN742, PN218 and PN356).

Figure 3.6 shows the PCA ordination for nutrients and total phenolics in leaves with and without galls, but only for groups that developed galls (groups 2 and 3). PC1 and PC2 together explained 89.9% of the variation in leaf nutrients. The main contributors of PC1 were protein, carbohydrates and fat, while the main contributors of PC2 were ash and total phenolic contents. Distinct clusters were observed. PCA ordination suggests that gall presence on leaves

is associated with a higher content of carbohydrates and phenolics, and lower content of fat and protein.

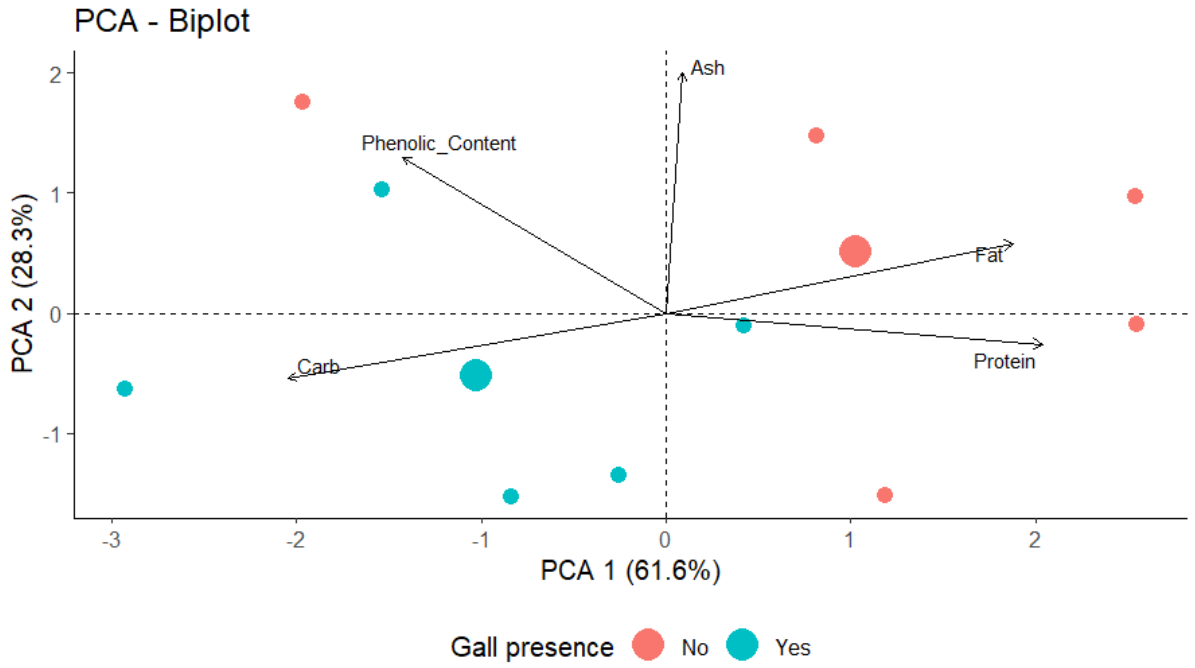

Figure 3.6: Principal component analysis (PCA) biplot showing nutrients and phenolic content in susceptible clones of willow *Salix* spp, in leaves with and without galls caused by red gall sawfly *Pontania proxima*. Larger symbols are group centroids. Resistant clones (PN221 and PN721, group 1 in Table 2) were excluded due to the lack of galls caused by *P. proxima*.

The galling groups did not show a significant difference for the nutrient or phenolic content (Table 3.6). Although the clones did show differences (Table 3.5), the statistical significance could not be tested due to low number of samples. Fat showed a significant difference between leaves with and without galls across all groups (Figure 3.7). Carbohydrates were significantly different between leaves with and without galls for groups 2 and 3 (Figure 3.7). None of the other nutrients showed significant effects of galling (Table 3.6). The permutational multivariate analysis of variance (PERMANOVA) did not find a significant difference in leaf nutrient content among clones (p-value = 0.459) (Table 3.7).

There was no significant relationship between the analysed nutrients and mean larval head width in the *P. proxima* IV and V instars (Table 3.8, Table 3.9).

Table 3.6: Generalized linear models (GLM) comparing the nutrient content in leaves of different clones of willow *Salix* spp. Clones were divided into groups based on the level of galling caused by red gall sawfly *Pontania proxima*. Group 1: minimal galling (PN721 and PN221), Group 2: medium galling (NZ1130 and PN676), Group 3: heavy galling (PN742, PN218 and PN356). ** significant model at $\alpha = 0.05$.

Model comparison	Nutrient	Df model	LogLik	Df residual	Chisq	p-value
By group, all groups	Ash	2	-17.196	4	0.2273	0.8926
	Protein	2	-25.515	4	5.2501	0.0724
	Fat	2	-16.538	4	2.4705	0.2908
	Carbohydrates	2	-29.654	4	2.0705	0.3551
	Phenolic content	2	-55.973	4	1.4705	0.4794
By gall presence, all groups	Ash	1	-17.196	3	0.9667	0.3255
	Protein	1	-25.515	3	0.3644	0.5461
	Fat	1	-16.538	3	5.4334	0.0198 ***
	Carbohydrates	1	-29.654	3	2.3465	0.1256
	Phenolic content	1	-55.973	3	0.0053	0.942
By gall presence, groups 2 and 3	Ash	1	-10.936	3	3.4425	0.0635
	Protein	1	-20.275	3	2.7679	0.0962
	Fat	1	-13.878	3	3.7995	0.0513
	Carbohydrates	1	-24.423	3	5.7919	0.0161 **
	Phenolic content	1	-42.351	3	06843	0.4081

Table 3.7: Permutational multivariate analysis of variance (PERMANOVA) comparing the nutrient content in leaves of different clones of willow *Salix* spp. Clones were divided into groups based on the level of galling caused by red gall sawfly *Pontania proxima*. Group 1: minimal galling (PN721 and PN221), Group 2: medium galling (NZ1130 and PN676), Group 3: heavy galling (PN742, PN218 and PN356). PERMANOVA comparing just group 2 and 3 between nutrients and phenolic content.

	df	**Sum of Squares**	**R^2**	**F**	**p value**
Gall presence	1	234.31	0.0794	0.6895	0.459
Residual	8	2718.54	0.9207		
Total	9	2952.85	1.000		

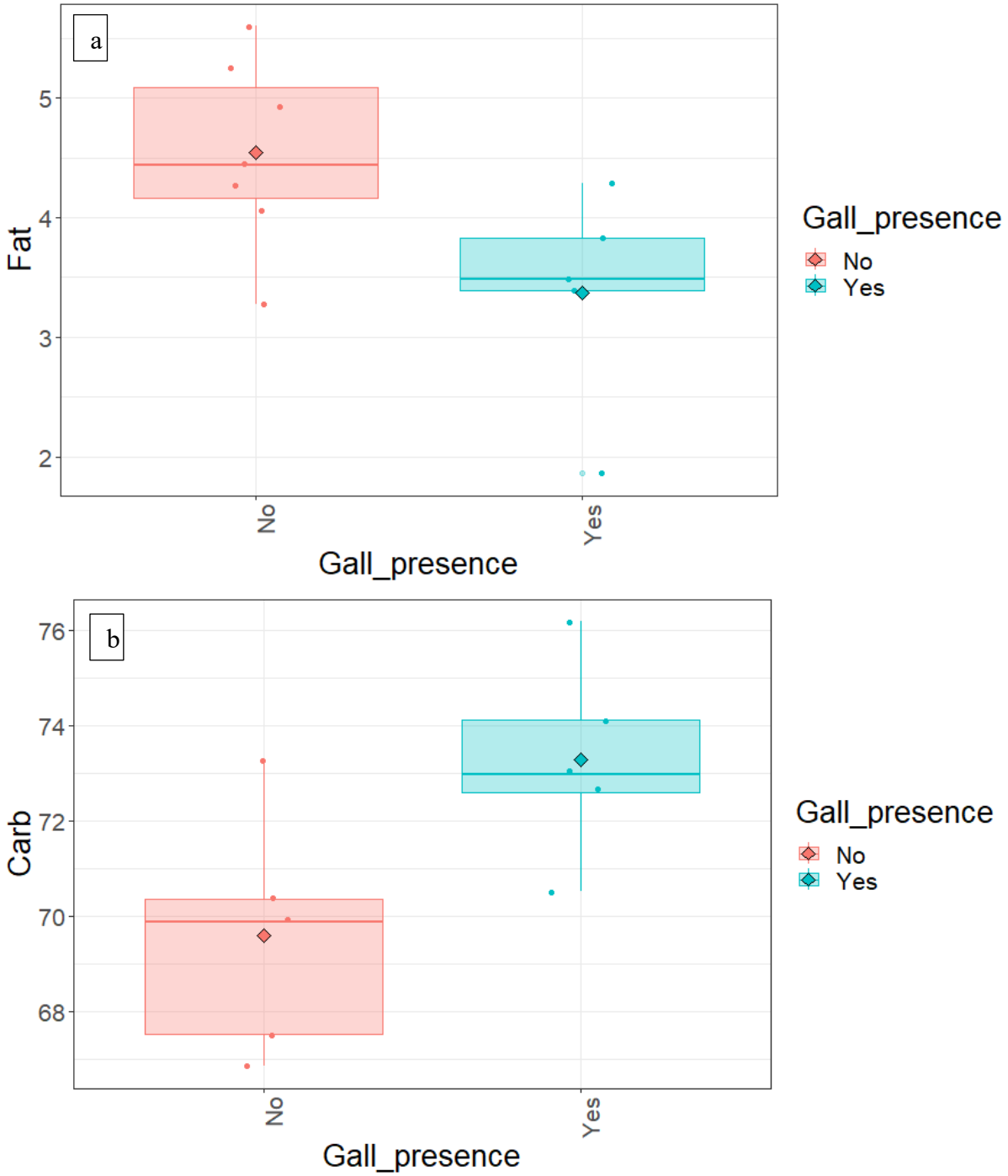

Figure 3.7: Box plots of the fat content (%, a) and carbohydrates content (%, b) in leaves with and without galls caused by the red gall sawfly *Pontania proxima*.from different willow *Salix* spp clones (Group1, 2 and 3) Clones were divided in groups based on the galling level. Group 1: minimal galling (PN721 and PN221), Group 2: medium galling (NZ1130 and PN676), Group 3: heavy galling (PN742, PN218 and PN356). The diamond symbols show the mean, the horizontal line the median, the vertical boxes the 25th and 75th percentiles, and the vertical lines extend to the minimum and maximum values.

Table 3.8: Linear regression describing the relationship between nutrients and total phenolics content in leaves of clones of willow *Salix* spp and mean larval head width of instar IV of red gall sawfly *Pontania proxima*.

Nutrient	R^2	standard error	p value
Ash	0.4315	22.51	0.2285
Crude protein	0.020	12.349	0.8192
Fat	0.0001	18.4195	0.973
Carbohydrate	0.0756	7.822	0.6545
Total Phenolic content	0.3302	0.9975	0.3109

Table 3.9: Linear regression describing the relationship between nutrients and total phenolics content in leaves of clones of willow *Salix* spp and mean larval head width of instar V of red gall sawfly *Pontania proxima*.

Nutrient	R^2	standard error	p value
Ash	0.644	17.72	0.102
Crude protein	0.0832	11.891	0.638
Fat	0.1192	17.21	0.5693
Carbohydrate	0.0141	8.041	0.8492
Total Phenolic content	0.4662	0.886	0.2039

3.3.2 Comparison of larval head capsule width between the willow clones

The range of the *P. proxima* larval head sizes (instars IV and V) in the willow clones are displayed in Figure 3.8. (See also Table 3.10 for the number of collected larvae, minimum and maximum larval head capsule size). Table 3.11 shows the estimated mean head capsule

widths, the variance and the *post-hoc* comparison results for different instars of *P. proxima* for the eight willow clones that developed larvae. Clone PN676 presented the smallest larvae and was different from all the other clones. Clones PN736 and PN742 did not show differences between each other but were different from the other clones and showed the largest larvae from all studied clones. Clones NZ1040, NZ1130, PN218, PN356 and PN357 did not show differences between each other and presented intermediate larvae size.

Table 3.10: Number of collected *Pontania proxima* larvae in galls from eight willow *Salix* spp clones, and minimum and maximum larval head capsule width (all instars).

Clone	Number of larvae	Min size (µm)	Max size (µm)
NZ1040	134	249.82	1131.3
NZ1130	181	329.09	1112.35
PN218	85	249.98	1092.51
PN356	162	225.29	1135.8
PN357	125	320.84	1135.5
PN676	86	239.74	994.43
PN736	100	250.46	1128.14
PN742	200	309.55	1128.33

Table 3.11: Estimated mean head capsule width (micrometres) of *Pontania proxima* larvae for different instars in eight willow *Salix* spp clones. Means obtained by mixed Gaussian models; underlined means obtained by Dyar's law as described in Methods. Variance in brackets. Graphs representing the HCW for each instar can be found in 3.7 Appendix.

Clone	Instar I	Instar II	Instar III	Instar IV	Instar V
NZ1040	291.80 [541.707]	426.42 [493.24]	554.93 [808.05]	756.85 [1310.10]	999.57 [2490.80]
NZ1130	109.36	331.67	564.00 [4446.30]	756.27 [1101.15]	1008.62 [4357.54]
PN218	320.13 [6481.55]	439.49 [127.12]	588.69[562.40]	787.62 [1533.74]	1012.41 [1915.84]
PN356	78.52	317.03	560.76[3459.88]	783.60 [1206.46]	1037.78 [2628.90]
PN357	201.26	434.62	562.99[8558.60]	770.65 [452.98]	1031.16 [3498.13]
PN676	259.76 [1150.66]	424.27	597.62	735.60 [2091.91]	970.85 [2636.57]
PN736	314.61 [1198.45]	462.38 [508.73]	603.81[439.61]	819.12 [1931.48]	1040.77 [2042.07]
PN742	339.64[645.49]	441.65 [554.34]	597.45[134.53]	810.75 [915.61]	1044.45 [2093.95]

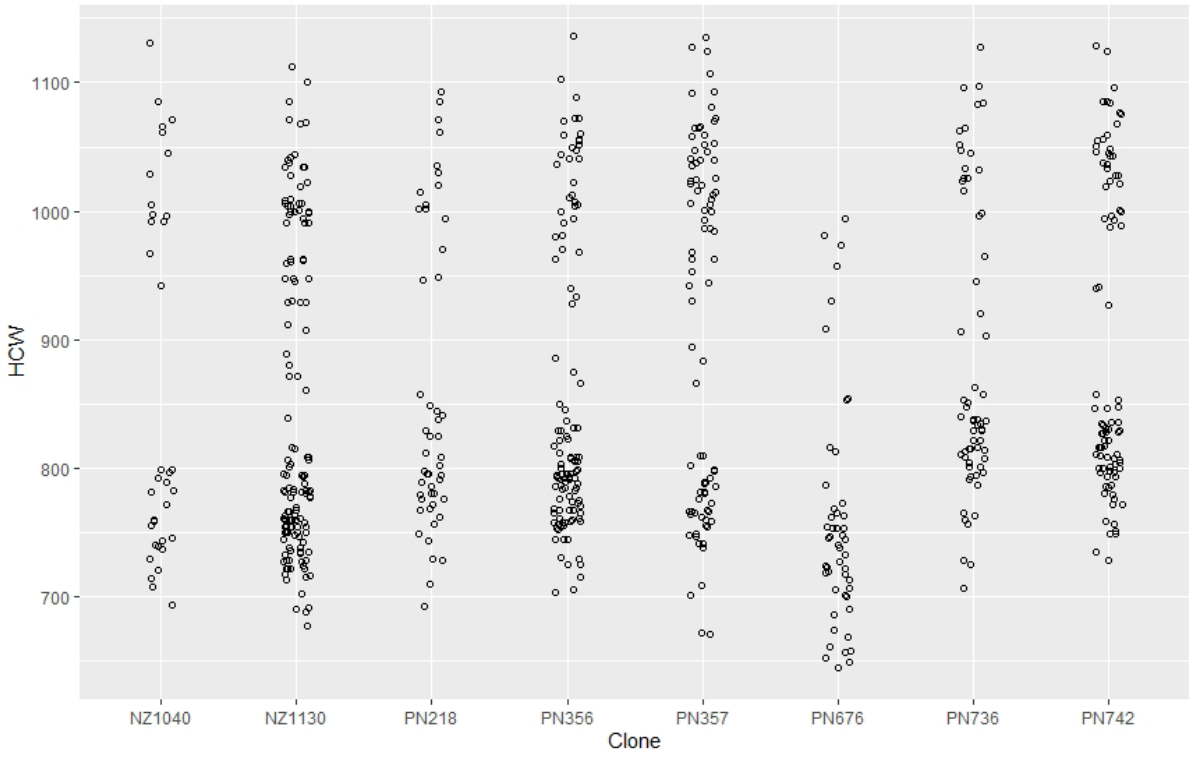

Figure 3.8: Head capsule widths (HCW, micrometres) of red gall sawfly *Pontania proxima* larval instars IV and V developing on different clones of willows *Salix* spp.

ANOVA results show that clone identity had a significant effect on development of *P. proxima* for both instars IV ($F = 80.277$ and $p < 0.001$) and V ($F = 31.738$ and $p < 0.001$). Full ANOVA results in

Table 3.7A-1 and Table 3.7A-2.

The post-hoc test results for individual clones are shown in Table 3.7A-3 andTable 3.7A-4. Some clone comparisons showed different results in different instars (Table 3.12). However, there were comparisons that showed significant and consistent results in both instars. For example, *P. proxima* larvae from clone PN676 were significantly smaller than larvae from clones PN356, PN736 and PN742. Clones PN736 and PN742 did not show difference in larval development between each other, and larvae from these two clones were significantly larger compared with clones NZ 1040, NZ1130, PN676 in both instars. Figure 3.9 shows the larval head capsule difference between group 2 and 3 for instars IV and V.

Table 3.12: Post-hoc Tukey test (α=0.05) comparing the larval development (head capsule widths for larval instar IV and V) of red gall sawfly *Pontania proxima* between different clones of willow *Salix* spp.

Clone	Mean instar IV	Post-hoc results	Clone	Means instar V	Post-hoc results
PN676	735.60	a	**PN676**	970.85	a
NZ1130	756.27	b	**NZ1040**	999.57	a
NZ1040	756.85	b	**NZ1130**	1008.62	a
PN357	770.65	b	**PN218**	1012.41	a
PN356	783.60	b	**PN357**	1031.16	b
PN218	787.62	b	**PN356**	1037.78	c
PN742	810.75	c	**PN736**	1040.77	c
PN736	819.12	c	**PN742**	1044.45	c

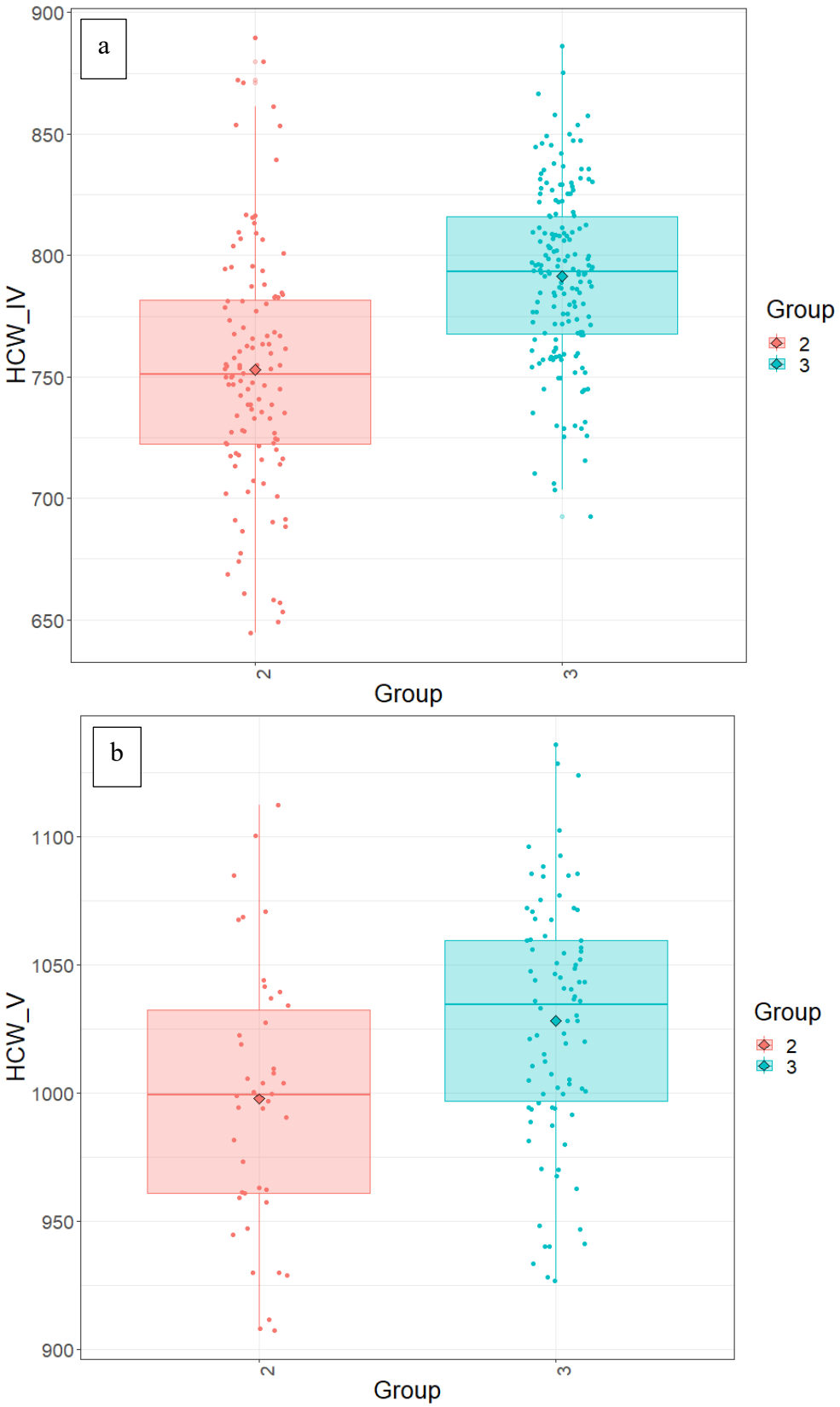

Figure 3.9: Box plots of the larval head capsule width (HCW) of red gall sawfly *Pontania proxima* larvae of instar IV (a) and instar V (b) extracted from willow leaves of different willow *Salix* spp clones (Group 2 and 3). Clones were divided into groups based on the galling level. Group 1: minimal galling (PN721 and PN221), Group 2: medium galling (NZ1130 and PN676), Group 3: heavy galling (PN742, PN218 and PN356). The median for HCW is indicated by the line across the box. The mean is indicated by the diamond.

3.4 Discussion

Willow clones used for planting in New Zealand show significant variability in species, sex, growth type, chemistry, morphology and anatomy. Given these differences, they show different susceptibility to diseases and pests (Tun et al., 2021; Tun et al., 2020; Van Kraayenoord & Hathaway, 1986; Van Kraayenoord & Hathaway, 1987).

Our results show that twelve *Salix* spp. clones considered in this Chapter, display differences in susceptibility to *P. proxima*, with PN221 and PN249 not developing galls, and PN693 and PN721 not developing fully formed galls. Since underdeveloped galls can be observed on clones PN721 and PN693, they are able to attract ovipositing *P. proxima* females, but the larva and therefore the gall, do not develop. Discussion about the causes of gall malformation can be found in Chapter 4. Clones that developed galls also presented different levels of larval development. Clones PN676, NZ1130 and NZ1040 supported significantly smaller *P. proxima* larvae than PN736 and PN742, which can be linked with secondary metabolites and plant nutrients.

Plants can utilise a wide range of mechanisms of defence against insect pests. Different species of plants can release different volatiles that could attract herbivores, or they may have different concentrations or different variety of secondary metabolites which could make them unpalatable to the insect pest. They can also have different morphological attributes such as waxy cuticles and/or the development of spines, setae and trichomes (Belete, 2018; Mitchell et al., 2016; War et al., 2012).

The relationship between willow flower volatiles and host location by *P. proxima* was investigated by Kehl et al. (2010). The authors proved that, within the tested *Salix* species, *S. fragilis* and *S.* x *rubens*, the percentage of plants that presented galls was higher for flowering plants than for non-flowering ones. The authors link the sensitivity of *P. proxima* to floral

volatiles, and nectar being the main source of energy to adults. The overlap between the first generation of sawflies and the flowering period of willows can be a cause for the attraction that *P. proxima* have toward clones PN693 and PN721 (*S. matusdana*) in our study, although these clones do not provide a suitable environment for larval development. These two clones belong to the species *S. matsudana* which flowers during the month of September (New Zealand Spring) with flowers and leaves appearing together (Van Kraayenoord & Hathaway, 1987). As *P. proxima* oviposit in leaf buds, the synchrony in development of flowers and new leaves provides an attractive site for oviposition.

P. proxima display preferences for clones that produce leaves earlier in the season, even if the clones do not provide optimum conditions for larval development (Kehl & Rambold, 2011). This may explain the reason why clones NZ 1040, NZ1130, PN356 and PN357 (belonging to species *S. matsudana* Koidz x *alba* L. and *S. alba* L.) had a high number of larvae, although the larval size was not the largest. Van Kraayenoord and Hathaway (1987) report the flowering period of NZ1130 and NZ1040 to be late August to early October and the flowering of *S. alba* males (in which category PN356 and PN357 belong) to be early September to late October. The *S. fragilis* species to which PN742 and PN736 belong, however, is reported to start flowering later in October (Tun et al., 2021). This suggests a later development of the *S. fragilis* in the season.

After insect attraction to a host plant, other factors (e.g., visual cues, pilosity, wax chemical profile) are important during the probing phase and we speculate that those differences are probably responsible for differences in galling severity between clones. The willows species and clones are known to differ in their chemical profiles (Tun et al., 2020). The volatiles and the cuticular plant compounds of different species of *Salix* spp. have been found to influence the oviposition of the willow sawfly *Nematus oligospilus* (Braccini et al., 2015), with *N. oligospilus* preferring to oviposit on leaves with a more diverse wax profile. Fernández et al.

(2019) further analysed weather phenolic glycosides (compounds that characterize the Salicaceae family) were present in the wax of different species of *Salix* and whether these compounds influenced oviposition of *N. oligospilus*. They found that *N. oligospilus* prefers to oviposit on plants with a higher content of phenolic glycosides in the wax than on plants that did not present these compounds. Some insects are "phenolic glycosides specialists", with these insects reported to be more sensitive to secondary metabolites, preferring plants with a higher phenolic glycosides content. They have a higher growth rate in the presence of phenolic glycoside than generalist insect herbivores; with some phenolic glycosides specialists even sequestering these chemicals for their own defence (Bernays et al., 2000; Boeckler et al., 2011; Kolehmainen et al., 1995; Matsuki & MacLean, 1994; Orians et al., 1997; Pasteels & Rowell-Rahier, 1992; Rank et al., 1998). As *N. oligospilus* is a specialist herbivore, this insect probably prefers host plant species with a higher phenolic glycosides content, although this alone cannot fully explain oviposition preference.

Following oviposition by the adult sawfly, the larvae must find a good environment to develop, which includes feeding on a diet with good nutrient content. Each nutrient will play a different role in insect development (e.g., carbohydrates are a source of energy for metabolic activities, nitrogen for the production of proteins, lipids play a role in reproduction as well as components of cellular membranes). Insects feeding on a carbohydrate-rich diet tend to be bigger, with sugars as well-known phagostimulants (Bernays & Simpson, 1982; Joern & Behmer, 1997). Larvae fed on high-nitrogen content diets grow bigger in comparison with larvae feeding in lower-nitrogen content diets (e.g., grasshopper *Ageneotettix deorum*, butterfly *Pieris rapae*, caterpillar *Manduca sexta*) (Joern & Behmer, 1997; Slansky Jr. & Feeny, 1977; Wilson et al., 2019). Matsuki and MacLean (1994) studied larval development of five insect herbivore species (a willow sawfly, *Nematus calais*; tiger swallowtail butterfly, *Papilio canadensis*; and three species of chrysomelid beetles, *Gonioctena occidentalis*, *Calligrapha*

verrucosa, and *Chrysomela falsa*) on ten species of willows (S. *brachycarpa*, *S. interior*, S. *lasiandra*, *S. novae-angliae*, *S. alaxensis*, *S, arbusculoides*, *S. monticola*, *S. bebbiana*, *S. glauca*, *S. planifolia*). The authors found that high nitrogen and water levels and low toughness increase larval size of early-season herbivores. *Nematus oligospilus* prefer to oviposit in more tender leaves with higher phenolic glycoside content (Braccini et al., 2013). We suggest that *P. proxima* may share the preference for tender tissues for oviposition. Further exploration is essential to substantiate this theory.

In our study, the three groups analysed based on *P. proxima* galling level did not show significant differences in nutrients or total phenolics. When the clones were analysed by gall presence (across all groups), fat percentage was significantly higher in healthy leaves than in galled leaves. When just clones that produce galls (groups 2 and 3) were analysed, carbohydrate percentage was higher in galled leaves. Individual clones showed a diverse range of values in nutrient content, although statistical comparison of individual clones could not be performed due to the low number of replicates. Clone PN742 showed the highest content of crude protein, while clones PN221 and PN676 had the lowest protein levels. This is well in accordance with the *P. proxima* larval development data, where we found that clone PN742 had the largest larvae, while PN676 had the smallest larvae. This suggests that clone PN742 provides more nutrition and therefore the larvae can develop better.

Phenolics can also influence the growth and fitness of insect herbivores (Harborne, 1994; Lattanzio et al., 2006; Mason et al., 2021; War et al., 2012). The Salicaceae family are characterized by the presence of phenolic glycosides. Some *Salix* species, however, may not present phenolic glycosides (Julkunen-Tiitto, 1986; Pasteels & Rowell-Rahier, 1992; Rowell-Rahier, 1984). Matsuki and MacLean (1994) tested the growth rate of five insect herbivores (*Nematus calais*, *Papilio canadensis*, *Chrysomela falsa*, *Calligrapha verrucosa* and *Gonioctena occidentalis*), and found that the type and the concentration of phenolic glycosides

influenced the development of insect herbivores. The authors demonstrated that the presence of phenolic glycosides had a significant effect on the growth of four out of the five examined species, with growth of "phenolic glycosides specialists" not affected or even enhanced by the presence of glycosides, while non-specialists showed a lower growth rate in the presence of glycosides.

The difference in the performance of generalist and specialist insect herbivores in the presence of phenolic glycosides is well documented in the literature. Specialists are reported to be more sensitive to secondary metabolites, and prefer plants with a higher phenolic glycosides content and have a higher growth rate in the presence of phenolic glycoside than generalist insect herbivores, with some specialists even sequestering these chemicals for their own defence (Bernays et al., 2000; Boeckler et al., 2011; Kolehmainen et al., 1995; Matsuki & MacLean, 1994; Orians et al., 1997; Pasteels & Rowell-Rahier, 1992; Pasteels et al., 1983; Prudic et al., 2007; Rank et al., 1998; Rowell-Rahier, 1984; Rowell-Rahier & Pasteels, 1990).

Our results showed that clone PN221, which did not present galls, and clone PN676 with level 3 galling and poor *P. proxima* larval development, showed the highest levels of phenolics, while clone PN742 with level 5 galling and the best larval development, had the lowest phenolic content. These results are in contrast with Hjältén et al. (2007) and Soetens et al. (1991) who found that the galling caused by the sawflies *Pontania triandrae* and *P. proxima*, respectively, were higher in species of willows with a higher phenolic content. These studies, however, did not investigate larval development. The Hjältén et al. (2007) and Soetens et al. (1991) studies were carried out in Europe (Belgium and Sweden, respectively), where *P. proxima* and *P. triandrae* have natural enemies.

In New Zealand *P. proxima* does not have any reported natural enemies (Kay, 1980). Since *P. proxima* is a specialist herbivore, it is resistant to the damage phenolics can cause, although the detoxication, in most cases, is believed be energetically costly (Boeckler et al., 2011;

Lindroth, 1988; Lindroth et al., 1986; Pasteels & Rowell-Rahier, 1992; Rowell-Rahier, 1984). Predators of specialists, however, are reported to be sensitive to phenolic glycosides (Boeckler et al., 2011; Kearsley & Whitham, 1992; Pasteels & Rowell-Rahier, 1992; Rowell-Rahier & Pasteels, 1990). We can speculate, that predators of *P. proxima* may be sensitive to phenolics, which forces *P. proxima* to choose higher phenolic content hosts in its native range. With the lack of predators in New Zealand, *P. proxima* does not have the predator pressure to choose a willow with higher level of phenolics, and therefore may choose willows with lower phenolic content to save energy that would be used for detoxication. We suggest that the lack of predators influenced *P. proxima* in New Zealand to choose host plants with a lower level of phenolic compounds. To our knowledge, there is no literature that reports this preference shift in *P. proxima* and our hypothesis needs to be tested.

Results presented in this chapter show that there was no difference in nutrients and phenolic content between galled and healthy willow leaves, except for carbohydrate content where galled leaves showed a higher level of carbohydrates than non-galled leaves. Hartley (1998) analysed young and mature leaves of *S. alba* for nitrogen, carbohydrates and total phenolics, and found that leaves galled by *P. proxima* had significantly higher levels of phenolics and carbohydrates than healthy leaves, while nitrogen levels did not show a difference between healthy and galled leaves. These results suggest that *P. proxima* does manipulate the carbohydrate content in leaf tissues.

Besides the mentioned chemical defences, plants protect themselves against herbivory with morphological features such as waxy cuticles and/or the development of spines, setae and trichomes (Belete, 2018; Mitchell et al., 2016; War et al., 2012). Epicuticular lipids can influence oviposition, movement and feeding of insect herbivores on plants (Alfaro-Tapia et al., 2007; Bernaola et al., 2021; Brennan & Weinbaum, 2001; Eigenbrode et al., 1995; Rutledge & Eigenbrode, 2003; White & Eigenbrode, 2000). Plant surface wax can also influence

population numbers of their parasitoids and predators (Eigenbrode & Espelie, 1995; Eigenbrode & Jetter, 2002; Eigenbrode et al., 1995). Plant waxes are reported to affect the performance of insect herbivores, influencing the ability to adhere to the plant, as well as the performance of predators (Alfaro-Tapia et al., 2007; Brennan & Weinbaum, 2001; Eigenbrode & Jetter, 2002; Eigenbrode et al., 1995; Rutledge & Eigenbrode, 2003; White & Eigenbrode, 2000). Higher levels of epicuticular waxes are reported to decrease feeding in sugar cane leaf hopper *Deltocephalus menoni* (Hemiptera: Cicadellidae) (Chanchala et al., 2020).

Salix species present variable leaf cuticular morphology. Tomaszewski (2004) studied the leaf cuticular morphology of four *Salix* species: *Salix alba*, *S. fragilis*, *S. triandra* and *S. pentandra*, and found that *S. pentandra* has an amorphous film, while the other species present cuticular structures referred to as "conicoids and splinters". The species with larger conicoids was *S. alba,* which gives the leaf cuticle a very rough surface look. Although our study did not aim to characterise and differentiate the cuticle of different willow clones, we suggest that the levels of epicuticular wax, morphology and their chemical differences may influence the preference and performance of *P. proxima* on willow clones.

In our study, when we compared all groups by gall presence (Group 1: minimal galling (PN721 and PN221), Group 2: medium galling (NZ1130 and PN676), Group 3: heavy galling (PN742, PN218 and PN356)), the percentage of fat was significantly higher in healthy leaves than in galled leaves. When the analyses were carried out with just the clones that produced galls (groups 2 and 3), fat did not show a higher percentage. This led us to believe that those in group 1 have a higher fat percentage in the leaf tissues than in groups 2 and 3. This higher fat percentage in group 1 can be linked to a thicker leaf cuticle, which was observed by the author during sample grinding, with clone PN693 visually showing a much thicker cuticle than the other clones. Bernaola et al. (2021) found in their study that rice water weevils *Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae) prefer to oviposit in rice mutants with lower

levels of wax, and that the fall armyworm *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) larvae gained more weight on the mutant low-wax plants than on the wild types with normal wax levels. We suggest that *P. proxima* may also prefer to oviposit in plants with lower levels of epicuticular waxes. Our hypothesis, however, has not yet been tested.

Besides epicuticular waxes, trichomes can also serve as a physical defence against insect herbivores, and this has been reported expensively in the literature (Belete, 2018; Handley et al., 2005; Mitchell et al., 2016; War et al., 2012). *Pontania proxima* has been reported to prefer hosts with lower trichome density. Soetens et al. (1991) correlated the level of pilosity in willow leaves with the number of galls caused by *P. proxima* and found more galls in *Salix* species with higher levels of phenol glucosides and lower trichome density. Another study (Kehl & Rambold, 2011) correlating the *P. proxima* population abundance with phenological and morphological characteristics, also confirmed that *P. proxima* prefers clones with a lower trichome density.

We used in this study the willow species *S. purpurea* (clones PN221 and PN249), *S. matsudana* x *alba* (clones NZ1040 and NZ1130), *S. fragilis* (clones PN218, PN736 and PN742), *S. alba* (clones PN676, PN356 and PN357) and *S. matsudana* (clones PN693 and PN721). Among these, the *S. alba* species shows the highest levels of leaf pilosity, and the species *S. matsudana*, *S. purpurea* and *S. fragilis* have the lowest levels (Glenny & Jones, 2019; Van Kraayenoord & Hathaway, 1987). In our study, *P. proxima* showed better development in clones PN742 and PN736 which belong to the species *S. fragilis*. The clone that showed the poorest larval development was clone PN676, which belongs to the species *S. alba*. Our results are in accordance with previous findings showing that *P. proxima* prefers willows with a lower density of trichomes, although the pilosity alone cannot explain preference and was not quantified in our study.

3.5 Conclusions

Willow clones showed differing resistance to *P. proxima*. Overall, *P. proxima* appears to prefer clones with a lower phenolic content and lower pilosity. This preference, however, is in contrast with previous studies in which *P. proxima* shows preference for a higher level of phenolics. More studies reporting the response of *P. proxima* to willow volatiles need to be undertaken, as well as studies testing *P. proxima* preference to different classes of phenolic glycosides.

Among the studied clones, PN 221 and PN 249 (*S. purpurea*) showed the highest resistance levels to *P. proxima* with no galls. Among the clones that developed galls, PN676 (*S. alba*, female) was the clone that produced the smallest larva and is therefore considered the most resistant.

Our study revealed that, on our experimental field in the year when the experiment was conducted, *P. proxima* has shifted its preference from willows with a high content of phenolic glycosides, to willows with a low content of phenolic glycosides. We hypothesize that may have occurred due to low predator pressure. Further studies are needed to investigate this shift and test this hypothesis.

3.6 References

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3.7 Appendix

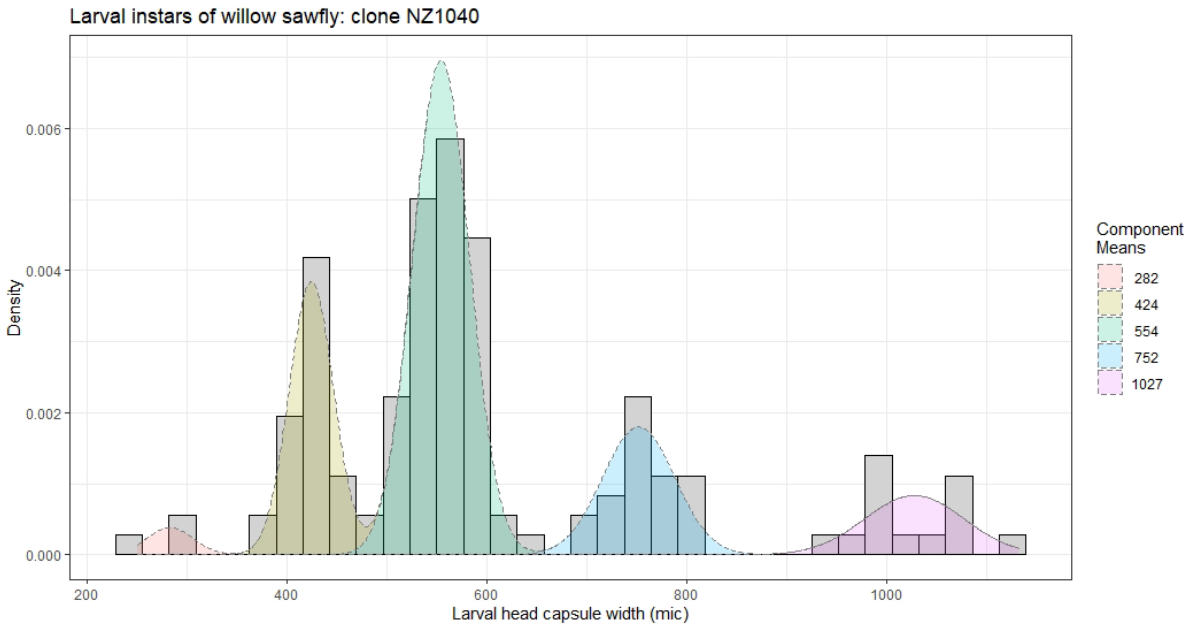

Figure 3.7A-1: Predicted mean head capsule widths (Component Means) for five larval instars of red gall sawfly *Pontania proxima* developing in willow clones; example for willow clone NZ1040. The graph represents the probability density function based on mixed Gaussian model.

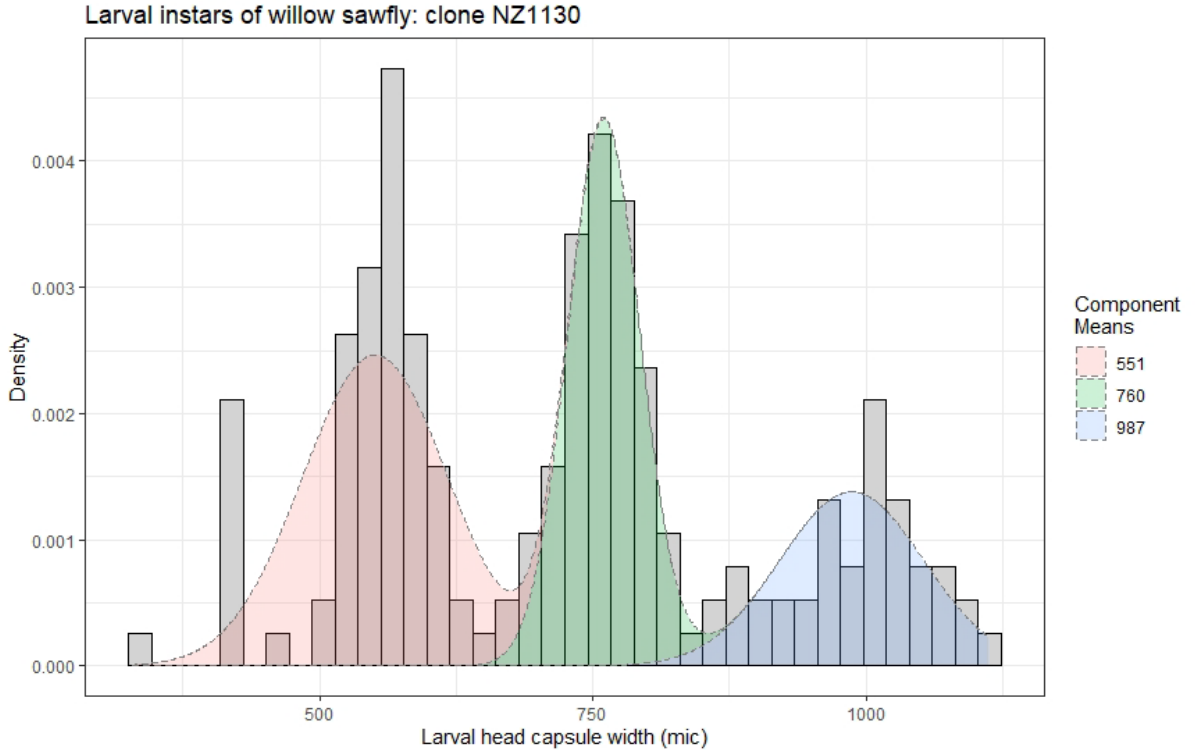

Figure 3.7A-2: Predicted mean head capsule widths (Component Means) for five larval instars of red gall sawfly *Pontania proxima* developing in willow clones; example for willow clone NZ1130. The graph represents the probability density function based on mixed Gaussian model.

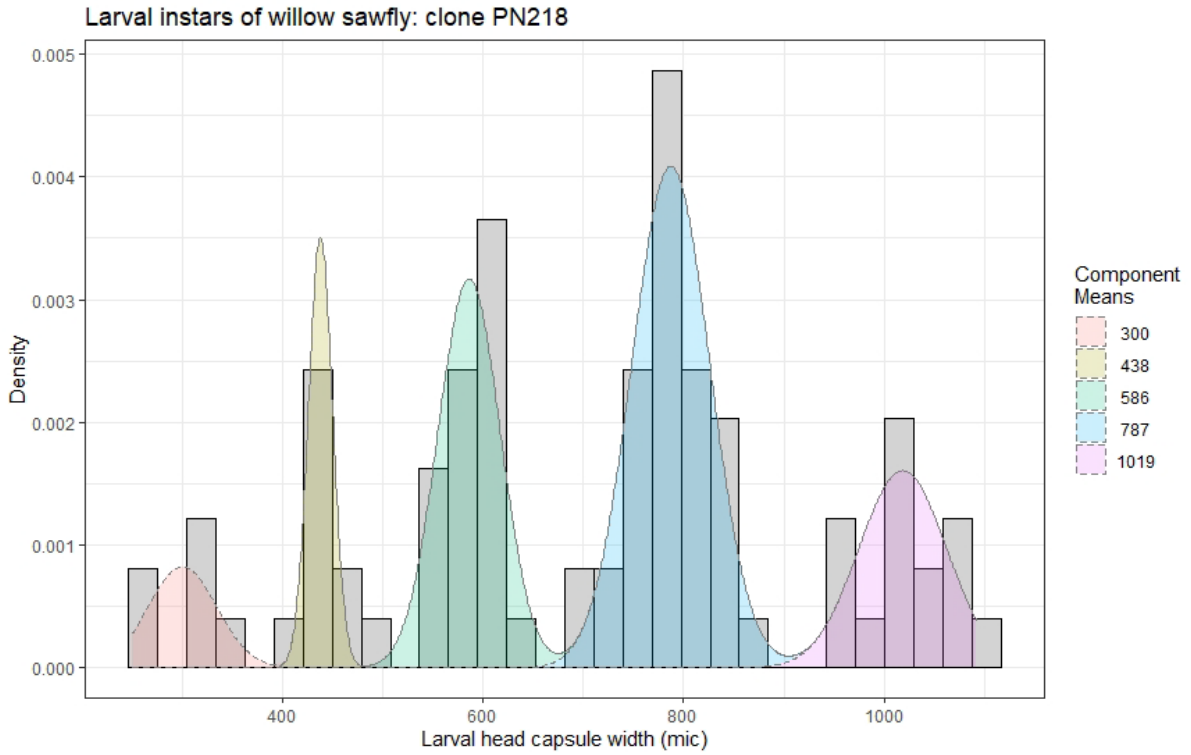

Figure 3.7A-3: Predicted mean head capsule widths (Component Means) for five larval instars of red gall sawfly *Pontania proxima* developing in willow clones; example for willow clone PN218. The graph represents the probability density function based on mixed Gaussian model.

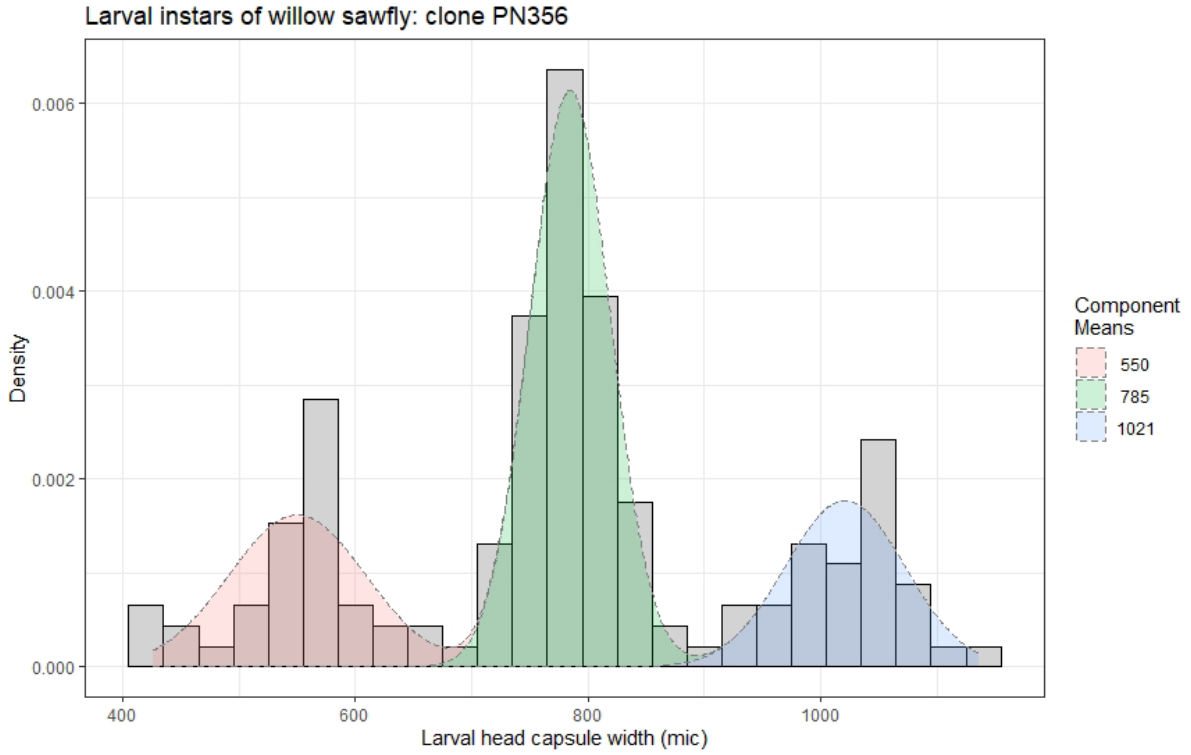

Figure 3.7A-4: Predicted mean head capsule widths (Component Means) for five larval instars of red gall sawfly *Pontania proxima* developing in willow clones; example for willow clone PN356. The graph represents the probability density function based on mixed Gaussian model.

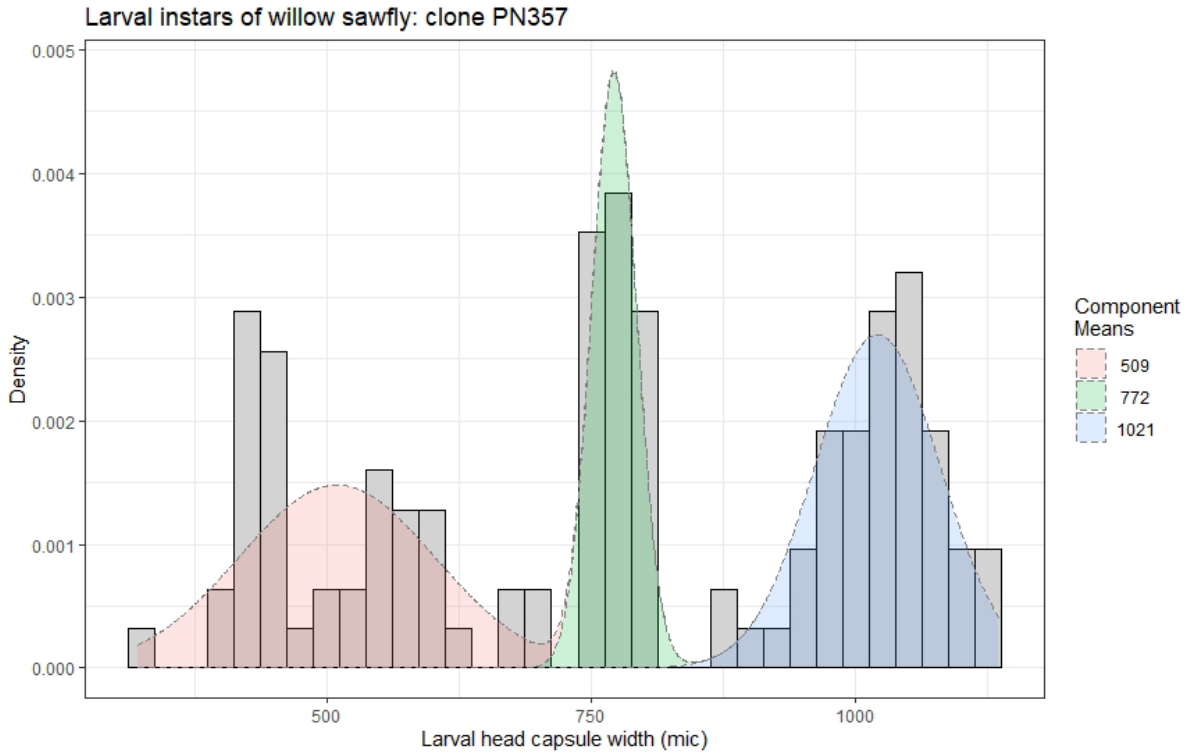

Figure 3.7A-5: Predicted mean head capsule widths (Component Means) for five larval instars of red gall sawfly *Pontania proxima* developing in willow clones; example for willow clone PN357. The graph represents the probability density function based on mixed Gaussian model.

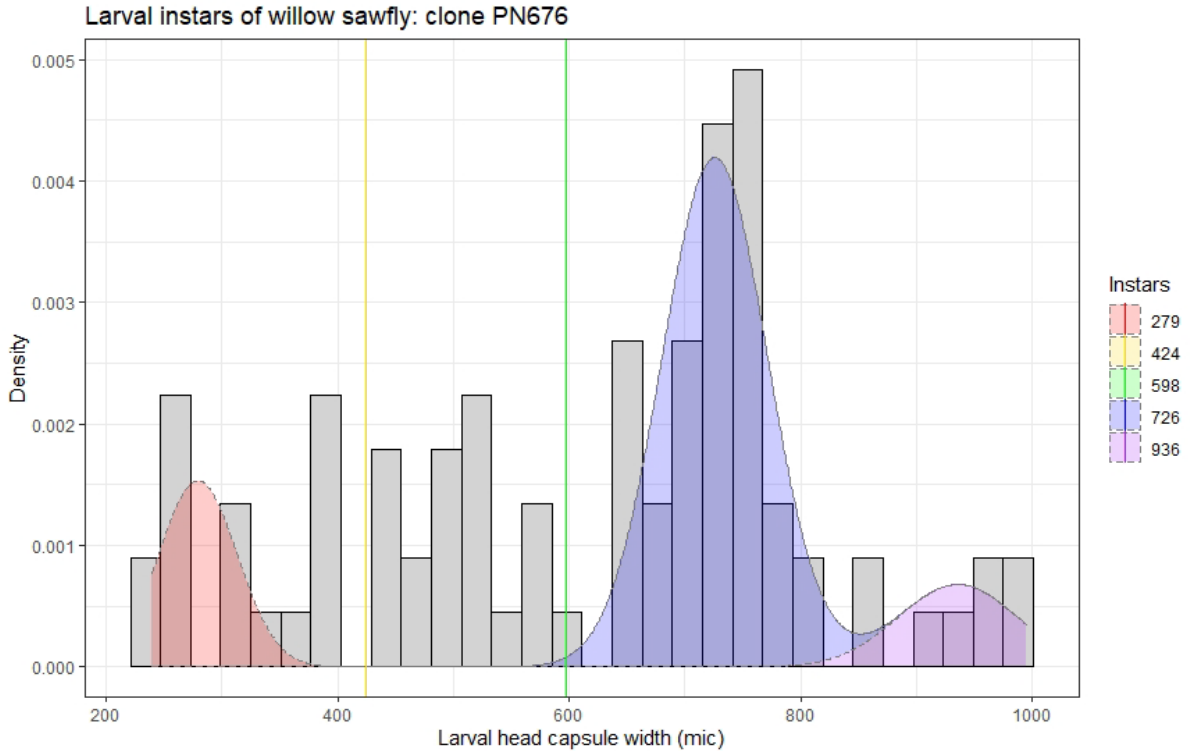

Figure 3.7A-6: Predicted mean head capsule widths (Component Means) for five larval instars of red gall sawfly *Pontania proxima* developing in willow clones; example for willow clone PN676. The graph represents the probability density function based on mixed Gaussian model.

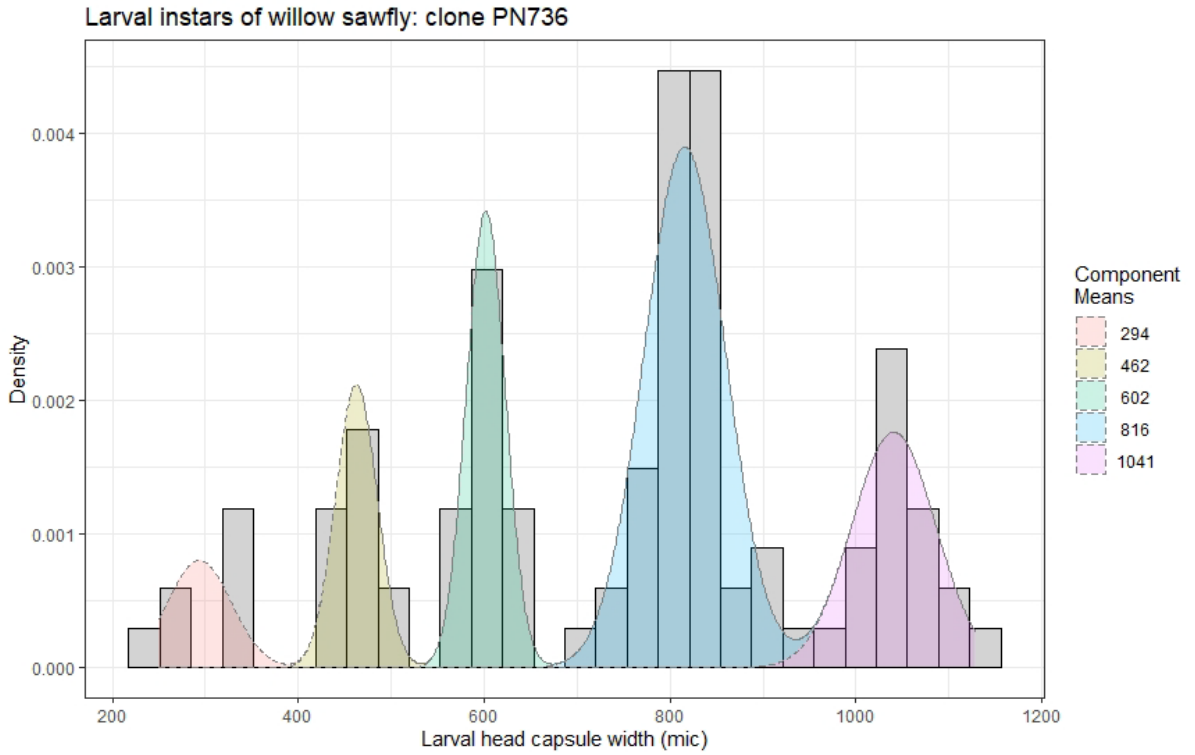

Figure 3.7A-7: Predicted mean head capsule widths (Component Means) for five larval instars of red gall sawfly *Pontania proxima* developing in willow clones; example for willow clone PN736. The graph represents the probability density function based on mixed Gaussian model.

Table 3.7A-1: Analysis of variance (one-way ANOVA) comparing the larval development (head capsule widths for instar IV) of red gall sawfly *Pontania proxima* between eight clones of willow *Salix* spp.

Source of Variation	Sum of Squares	d.f.	Mean Squares (MS)	F	p
Clone	245074.789	7	106381.412	80.277	<0.001
Error	499595.096	377	1325.186		
Total	744669.885	384			

Table 3.7A-2: Analysis of variance (one-way ANOVA) comparing the larval development (head capsule widths for instar V) of red gall sawfly *Pontania proxima* between eight clones of willow *Salix* spp.

Source of Variation	Sum of Squares	d.f.	Variance	F	p
Clone	70455.248	7	100893.117	31.738	<0.001
Error	635796.569	200	3178.983		
Total	706251.817	207			

Table 3.7A-3: Post-hoc Tukey test (α=0.05) comparing the larval development (head capsule widths for larval instar IV) of red gall sawfly *Pontania proxima* between different clones of willow *Salix* spp. 'B-H critical value' –Benjamini–Hochberg procedure value (B-H) at a set false discovery rate (FDR) and the significance of each comparison. 'ns' – not significant. *** - significant.

Comparison	Raw p-value	B-H critical value at FDR=0.025	B-H significance at 0.025 FDR	B-H critical value at FDR=0.055	B-H significance at 0.055 FDR
NZ1040 vs NZ1130	0.4734	0.0170	ns	0.0373	ns
NZ1040 vs PN218	0.0038	0.0089	***	0.0196	***
NZ1040 vs PN356	0.0032	0.0080	***	0.0177	***
NZ1040 vs PN357	0.0622	0.0143	ns	0.0314	ns
NZ1040 vs PN676	0.0287	0.0134	ns	0.0295	***
NZ1040 vs PN736	0	0.0009	***	0.0020	***
NZ1040 vs PN742	0.0001	0.0018	***	0.0039	***
NZ1130 vs PN218	0.001	0.0045	***	0.0098	***
NZ1130 vs PN356	0	0.0009	***	0.0020	***
NZ1130 vs PN357	0.0028	0.0071	***	0.0157	***
NZ1130 vs PN676	0.0057	0.0107	***	0.0236	***
NZ1130 vs PN736	0	0.0009	***	0.0020	***
NZ1130 vs PN742	0	0.0009	***	0.0020	***
PN218 vs PN356	0.3035	0.0161	ns	0.0354	ns
PN218 vs PN357	0.0155	0.0125	ns	0.0275	***
PN218 vs PN676	0	0.0009	***	0.0020	***
PN218 vs PN736	0.0011	0.0063	***	0.0138	***
PN218 vs PN742	0.0057	0.0098	***	0.0216	***
PN356 vs PN357	0.0080	0.0116	***	0.0255	***
PN356 vs PN676	0	0.0009	***	0.0020	***
PN356 vs PN736	0.0001	0.0027	***	0.0059	***
PN356 vs PN742	0.0001	0.0054	***	0.0118	***
PN357 vs PN676	0.0001	0.0036	***	0.0079	***
PN357 vs PN736	0	0.0009	***	0.0020	***
PN357 vs PN742	0	0.0009	***	0.0020	***
PN676 vs PN736	0	0.0009	***	0.0020	***
PN676 vs PN742	0	0.0009	***	0.0020	***
PN736 vs PN742	0.1666	0.0152	ns	0.0334	ns

Table 3.7A-4: Post-hoc Tukey test (α=0.05) comparing the larval development (head capsule widths for larval instar V) of red gall sawfly *Pontania proxima* between different clones of willow *Salix* spp. 'B-H critical value' –Benjamini–Hochberg procedure value (B-H) at a set false discovery rate (FDR) and the significance of each comparison. 'ns' – not significant. *** - significant at specified FDR.

Comparison	Raw p-value	B-H critical value at FDR = 0.025	B-H significance at 0.025 FDR	B-H critical value at FDR=0.055	B-H significance at 0.055 FDR
NZ1040 vs NZ1130	0.2946	0.0205	ns	0.0452	ns
NZ1040 vs PN218	0.2379	0.0188	ns	0.0413	ns
NZ1040 vs PN356	0.0163	0.0071	ns	0.0157	***
NZ1040 vs PN357	0.0341	0.0107	ns	0.0236	ns
NZ1040 vs PN676	0.1499	0.0170	ns	0.0373	ns
NZ1040 vs PN736	0.0149	0.0036	ns	0.0079	***
NZ1040 vs PN742	0.0082	0.0018	ns	0.0039	***
NZ1130 vs PN218	0.4003	0.0241	ns	0.0530	ns
NZ1130 vs PN356	0.0152	0.0045	ns	0.0098	***
NZ1130 vs PN357	0.0422	0.0116	ns	0.0255	ns
NZ1130 vs PN676	0.0806	0.0152	ns	0.0334	ns
NZ1130 vs PN736	0.0170	0.0080	ns	0.0177	***
NZ1130 vs PN742	0.0064	0.0009	ns	0.0020	***
PN218 vs PN356	0.0491	0.0134	ns	0.0295	ns
PN218 vs PN357	0.1042	0.0161	ns	0.0354	ns
PN218 vs PN676	0.0707	0.0143	ns	0.0314	ns
PN218 vs PN736	0.0428	0.0125	ns	0.0275	ns
PN218 vs PN742	0.0232	0.0098	ns	0.0216	ns
PN356 vs PN357	0.2973	0.0214	ns	0.0471	ns
PN356 vs PN676	0.0161	0.0063	ns	0.0138	***
PN356 vs PN736	0.4142	0.025	ns	0.055	ns
PN356 vs PN742	0.3040	0.0223	ns	0.0491	ns
PN357 vs PN676	0.0224	0.0089	ns	0.0196	ns
PN357 vs PN736	0.2427	0.0196	ns	0.0432	ns
PN357 vs PN742	0.1534	0.0179	ns	0.0393	ns
PN676 vs PN736	0.0152	0.0054	ns	0.0118	***
PN676 vs PN742	0.0119	0.0027	ns	0.0059	***
PN736 vs PN742	0.3974	0.0232	ns	0.0511	ns

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In which chapter is the manuscript/published work?		Chapter 4	
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Chapter 4 Metabolomic profile of six willow (*Salix* spp.) clones of interest in New Zealand.

4.1 Introduction

Secondary metabolites are compounds that do not participate in the plant's primary metabolism but have other biological and ecological functions, such as plant communication and defence. These compounds can be tissue constituents in inactive forms or can be induced by herbivory (Belete, 2018; Kariñho-Betancourt, 2018; Mitchell et al., 2016; War et al., 2012). Plant phenolics are the most common defence secondary metabolites and play a major role in plant resistance, not just against herbivory but also against microorganisms, and are also involved in plant intra-specific competition (Dixon, 1999; Grandmaison et al., 1993; Khokhani et al., 2013; Li et al., 2010; Shalaby & Horwitz, 2015; War et al., 2012). Different classes of phenolic compounds (e.g., lignin, quinones and salicylates) have different modes of action.

Willows (*Salix* spp) are naturalised in New Zealand, where they have multiple uses including soil protection against erosion, providing source of livestock fodder in drought periods, and a source of pollen and nectar for apiculture (McIvor, 2013; Oppong et al., 2001; Sopow et al., 2017). The unique characteristic of the Salicaceae family (which includes willows and poplars) is the presence of salicylates (phenolic glycosides) in their tissues, and this is the defining characteristic of this plant group. The concentration of these compounds as well as the compound mixture may vary depending on some factors. These factors may be gender, species, season of the year, growing conditions (e.g., fertilizer, shade, temperature), shoot length, different plant tissues and species (Boeckler et al., 2013; Fabisch et al., 2019; Pasteels & Rowell-Rahier, 1992; Price et al., 1989; Thieme, 1965).

Salicylates strongly affect the diversity of insect pests that feed on plants of this family and their specialization. Willows, that have high concentration of salicylates, have a higher number of specialist insect herbivores, suggesting that a certain degree of specialization is required to overcome the toxicity of salicylates. Thus, salicylate specialists can attack a wider variety of willow species. The high energy costs of production and the inefficiency of these compounds against specialist insects has led to the loss of salicylates in some *Salix* species (Boeckler et al., 2011; Volf et al., 2015). Some specialist insects have been reported as taking advantage of salicylates by using them as a source of glucose or for production of their own defensive compounds (Boeckler et al., 2011; Pasteels & Rowell-Rahier, 1992).

The giant willow aphid (GWA) *Tuberolachnus salignus* is a large aphid belonging to the family Aphididae (Hemiptera) (Blackman & Eastop, 1994; Dixon, 1985; McIvor, 2013). This pest in New Zealand and his aphid was first found in December 2013, spreading through the country in time afterwards (Gunawardana et al., 2014; Sopow et al., 2014). GWA can substantially decrease the amount of photo-assimilate that arrives to the roots and stems of the willow plant, the impact can range from reduction of plant growth to plant death; the negative effects on plant growth can be seen even after one growth season with GWA infestation (Collins, 2001; Jones et al., 2021; Sopow et al., 2017). Aphid honeydew deposition can stimulate the growth of fungi on the plant surface which can decrease photosynthetic levels, as well as affect other species including honeybees and soil biota (Sopow et al., 2017; Tun, 2020). The direct effects of willow secondary metabolites on GWA are yet to be reported.

The red gall sawfly *Pontania proxima* Lepeletier, 1823 (Tenthredinidae: Hymenoptera) was introduced into New Zealand accidently and was first reported in 1991 (Valentine & Walker, 1991). This insect causes multiple bean-shaped galls on willow leaves and heavy infestations can decrease plant vitality and therefore plant production (Carleton, 1939; Naumann et al., 2002). The resistance of New Zealand willows clones to *P. proxima* has not

been characterized yet, nor the correlation between secondary metabolite compounds in NZ willow clones and their resistance to *P. proxima*.

This study aimed to analyse the secondary metabolite compounds in leaves of six different willow cultivars (clones) used in New Zealand and to correlate differences in secondary metabolite compounds to different characteristics of the plants, such as growth form and resistance to insect herbivores. The study asked the following research questions: 1) How do the secondary metabolite profiles of these six clones compare? 2) If selected clones have a significant difference in secondary metabolite profiles, can we correlate these differences to resistance to herbivores (GWA, *P. proxima* and others), growth form, or induction by a herbivore (GWA)? This information is vital for the selection of resistant cultivars and to understand the potential indirect impacts on other insect species (e.g., natural enemies of competing herbivores).

4.2 Materials and Methods

4.2.1 Clone selection and sample collection

Willow leaf samples were collected in April 2019 (NZ Autumn) from two-years old willow plants belonging to different clones planted at the Plant Growth Unit (PGU) at Massey University, Palmerston North, New Zealand (40°22’46” S, 175°36’29” E), as part of a previous study to explore the impact of the giant willow aphid on different host plants (Tun et al., 2020). The soil type is Manawatu fine sandy loam (LRIS Portal, 2021), classified as fluvial recent soil (Hewitt, 2010). Medium annual rainfall is 900-1000 mm with median annual average temperature of 13-14°C (Chappell, 2015).

The previous study (Tun et al., 2020) consisted of fifteen clones and seventy-two plants per clone, half of which were treated with a contact insecticide at the beginning of spring and a subsequent manual control of insect pests was administered throughout the season. The other half of plants remained untreated to allow insect attack. The plants were distributed in six rows consisting of twelve plants per clone per row (see study design details in (Tun et al., 2020)). Six tissue samples of the clones were collected in February 2019 (28th January to February 1st) corresponding to two plants per clone per row. The sample consisted of twelve apical leaves of a randomly selected branch for each plant, from both treatments (induced – plants attacked by GWA, control – plants protected from GWA herbivory). Samples were immediately frozen in liquid nitrogen after collection. Afterwards, samples were transferred to laboratory where they were stored at -80 °C. The leaves were stored whole wrapped in foil and stored in zip lock bags.

From the tissue bank of fifteen clones, six clones were selected for this metabolomic study. In total, 12 leaf samples were analysed per clone, six of those from control plants and six induced by GWA. The selection was made to include both male and female plants, different growth forms (trees and shrubs), and varying degrees of susceptibility to insect pests (e.g., GWA, willow red gall sawfly *Pontania proxima* Lepeletier, 1823 (Tenthredinidae: Hymenoptera) and leaf chewing insects (eg. *Nematus oligospilus* Forster, 1854 (Tenthredinidae: Hymenoptera) as well as their frequency of use in New Zealand and genetic distinction between selected clones (Table 4.1). Resistance to GWA was based on Tun et al. (2020) data; resistance to *P. proxima* was from field data in this study.

Table 4.1: Selected willow *Salix* spp clones and survey information highlighting the different level of resistance of the clones to red gall sawfly *Pontania proxima* and giant willow aphid (GWA) *Tuberolachnus salignus*. Clones were selected based on their genetic diversity between themselves, sex, and level of resistance to insect pests. Details about the morphological characteristics from Glenny and Jones (2019); resistance to GWA from Tun et al. (2020); resistence to *P. proxima*: this study.

Species/Hybrid	**Code**	**Usage**	**Growth form**	**Sex**	**Susceptibility to insects**	**Leaf hairs**	**Hairs on last season's branchlets**
S. viminalis	PN220	Commonly planted	Shrub	Male	Very susceptible to GWA Resistant to *P. proxima*	Absent on upper surface lamina Sparse to moderate on lower lamina surface	Present
S. purpurea	PN249	Commonly used for riverbank stabilization	Shrub	Female (polyploid)	Moderately susceptible to GWA Resistant to *P. proxima*	Absent on upper surface lamina Absent to moderate on lower lamina surface	Absent
S. schwerinii	PN386	Commercial clone, commonly used for riverbank stabilization	Shrub	Male	Moderately susceptible to GWA Resistant to *P. proxima*	Sparse to moderately dense on upper surface lamina Very dense on lower surface lamina	Absent
S. lasiolepis × ***S. viminalis***	NZ04-106-073	Recently released for planting to city councils	Shrub	Male	Resistant to GWA Moderately resistant to *P. proxima*	Absent on upper and lower surface lamina	Absent
S. fragilis	PN218	Most common naturalised willow in NZ	Tree	Female	Moderately susceptible to GWA Very susceptible to *P. proxima*	Absent on upper surface lamina Sparse to moderate on lower surface lamina	Can be present
S. matsudana × ***S. alba***	NZ1040	Most planted willow in NZ	Tree	Female	Moderately susceptible to GWA Very susceptible *P. proxima*	Absent on upper surface lamina Sparse to moderate on lower surface lamina	Absent

4.2.2 Sample processing

Leaves of our six selected clones that were stored in 2019 in -80°C, were freeze-dried, ground and subsequently stored at -20 °C in September 2020. Samples not used for this study were left in storage. For each sample, 50 mg of ground leaf tissue was weighed, placed into 2 ml microcentrifuge tube, and stored at -20 °C. Sample leftovers were kept in storage in-20 °C. Then, 1500 µl of chilled MeOH: H2O 80: 20 v/v were added to the tube, homogenised with one bead for 5 minutes and kept for 1 hour at -20°C. After the extraction period, samples were centrifuged at 11000 rpm at 4°C for 10 mins. Following that, 500 µl of the supernatant was transferred into a tapered 1500 µl microcentrifuge tube (sample tube) and 500 µl supernatant was placed into a chilled vial for quality control (QC tube). Samples were centrifuged again under the same conditions as the first centrifugation. Then, 200 µl of the supernatant was transferred into a 2 ml microcentrifuge tube and dried under nitrogen gas (flow 3 min^{-1}) at 30°C for 35 min. All QC tubes were stored at -20°C for subsequent analyses.

4.2.3 Sample analyses

The analyses of samples were conducted at AgResearch Ltd, Lincoln, NZ. The samples arrived dried and cryogenically packaged; the first step of analyses was reconstitution. Samples were dissolved in 200uL acetonitrile: water (1:9 v/v) and used after four times dilution of the original sample volume. Samples were then analysed using a Nexera X2 high-performance liquid chromatography (UHPLC) system (Shimadzu, Japan) consisting of a SIL-30AC autosampler coupled to a LCMS-9030 quadrupole time-of-flight (Q-TOF) mass spectrometer (Shimadzu, Japan) equipped with an electrospray ionization source. Two µl of sample was injected into a reverse-phase Ascentis® Express C18 UHPLC column (2.1 x 100 mm, 2 µm particle size; Sigma, USA) and eluted at 30°C over a 20 min gradient with a flow rate of 400

μL/min. The mobile phase solvent A was a mixture of Milli-Q water and 0.1% formic acid (v/v), and solvent B consisted of acetonitrile with 0.1% formic acid. The solvent gradient program started at 5% solvent B from 0 to 0.5 min, increased to 99% B within 12.5 min, held at 99% B for 2 min, decreased to 5% B within 1 min and stored at 5% B until the end of the elution run.

Mass spectrum results were obtained at *m/z* 55-1100 in both positive and negative ionization modes with a spray voltage of 4.0 kV and -3.0 kV, respectively, at a scan rate of 10 spectra/s. Ion source was operated under an optimal condition: nebulizing gas flow 3.0 L/min; heating gas flow 10.0 L/min; interface temperature 300°C; drying gas flow 10.0 L/min; desolvation line temperature 250°C and heat block temperature 400°C. Data independent acquisition (DIA) MS/MS scan for precursors *m/z* 100 to *m/z* 900 with 20 Da *m/z* width was used to confirm the fragmentation data of precursors. In each ionisation mode, the sequence comprised blanks followed by samples interspersed with QC samples once in every ten samples. The intensity of internal standards (MSK-QC Kit, Cambridge Isotope Laboratories Inc., MA, USA), retention times and mass accuracy were monitored using LabSolutions Insight software version 3.50SP2 (Shimadzu, Japan).

4.2.4 Data processing

The MS-DIAL platform was used for data pre-processing, peak picking, sample alignment and compound identification (Tsugawa et al., 2015). The normalisation method used was Lowess. Both ion modes are centroid. Retention time for both modes started at 3 min to 15 min. Mass range begin at 55 and ended in 1100 and MS2 mass range begin at 90 and ending in 910. Centroid parameters were: MS1 tolerance 0.01 MS2 tolerance 0.025. Peak detection parameters were smoothing method LinearWeightedMovingAverage, smoothing level 3,

minimum peak width 5 and Minimum peak height 10000. Peak spotting parameters were mass slice width 0.1, exclusion mass list (mass & tolerance). Sigma window value was 0.5, MS2 Dec amplitude cut off 0. Library used for identification was the massBank which was downloaded directly from the MSdial website (Tsugawa et al.). Retention time tolerance was 100, Accurate mass tolerance (MS1) 0.01, Accurate mass tolerance (MS2) was 0.05, identification score cut off was 80% using retention time for scoring False and using retention time for filtering False. Adduct ion setting for positive ion mode was [M+H]+. Adduct ion setting for negative mode was [M-H]-. Alignment parameters setting reference file used was the QC file with retention time tolerance 0.05, MS1 tolerance 0.015, retention time factor 0.5, MS1 factor 0.5, peak count filter 0. Ion mobility data was not used.

4.2.5 Statistical Analysis

All statistical analyses in this chapter were done in RStudio (2023.06.1 Build 524 © 2009-2023 Posit Software, PBC) using R version 4.3.1. Secondary metabolites were ranked by importance using randomForest function according to mean decrease accuracy. Random Forest variable selection was chosen based on its ability to decrease the out-of-the-bag (OOB) error compared with other statistics methods (Breiman, 2001; Ranganathan & Borges, 2010). The mean decrease accuracy (MDA) was chosen over Gini index in Random Forest analysis because we wanted to know the importance of the compound for the prediction of the outcome and the stability of this predictor (Nicodemus, 2011).

A principal component analysis (PCA) using “FactoMineR” and “factoextra” packages was performed. The PCA biplots and corresponding scores of variable contributions were then used to identify clusters and to visualise overall secondary metabolite differences between clones and groups of clones with similar resistance to GWA (susceptible, resistant, and

moderately resistant), similar resistance to *P. proxima* (susceptible, resistant, and moderately resistant), and growth form (tree or shrub). Differences in sex were not tested as sex was confounded with growth form. A permutational multivariate analysis of variance (PERMANOVA) using the Adonis function with Euclidean distance matrix and 999 permutations with Bonferroni p-value correction was performed to compare clones and groups of clones. Additionally, a general linear model (GLM) was done to compare total metabolite concentration across clones and groups. A normal, inverse and gamma GLM was performed and the type with the lowest Akaike information criterion (AIC) score was chosen.

4.3. Results

Tentative identification of compounds is showed on Table 4.2 and Table 4.3. The reference column shows literature where the compound was found in willows or in the Salicaceae family.

Table 4.2: Summary of compounds in negative ion mode identified by MS Dial using massBank database matching. Reference that supports the presence of the compound on *Salix* genus or Salicaceae family.

Average	**Average**	**Tentative identification**				**Reference**
RT	**Mz**	Name	Reference m/z	Formula	Confidence level (%)	
3.113	179.0349	Caffeic acid	179.03499	C9H8O4	94.3	Pobłocka-Olech et al. (2010)
4.717	269.04544	Apigenin	269.04553	C15H10O5	95.1	Aboul-Soud et al. (2020)
5.768	269.0455	Isomer of Apigenin	269.04553	C15H10O5	94.5	Aboul-Soud et al. (2020)
3.985	285.04028	Isomer of Luteolin	285.04047	C15H10O6	87.7	Tegelberg et al. (2003)
5.213	285.04083	Luteolin	285.04047	C15H10O6	92.8	Tegelberg et al. (2003)
3.922	287.05594	Isomer of Eriodictyol	287.05612	C15H12O6	95.9	Noleto-Dias et al. (2020)
5.141	287.05615	Eriodictyol	287.05612	C15H12O6	96.5	Noleto-Dias et al. (2020)
3.296	289.07187	Epicatechin	289.07175	C15H14O6	95.5	Wiesneth et al. (2018)
5.901	299.05609	Isomer of Kaempferide	299.05612	C16H12O6	85.2	Zhou et al. (2022)
4.445	299.05615	Isomer of Kaempferide	299.05612	C16H12O6	88.1	Zhou et al. (2022)
4.78	299.05627	Kaempferide	299.05612	C16H12O6	86.4	Zhou et al. (2022)
3.963	301.03488	Isomer of Quercetin	301.03537	C15H10O7	80.2	Zhou et al. (2022)
4.657	301.03537	Quercetin	301.03537	C15H10O7	85.1	Zhou et al. (2022)
4.375	445.07779	Unknown 1	445.07764	C21H18O11	90.4	
4.495	447.09332	Isomer of Luteolin-7-O-glucoside	447.09329	C21H20O11	81.7	González-Alamilla et al. (2019)
4.258	447.09406	Quercetin-7-O-rhamnoside	447.09329	C21H20O11	96.5	Argus and McJannet (1992)

3.985	447.09421	Luteolin-7-O-glucoside	447.09329	C21H20O11	93.8	González-Alamilla et al. (2019)
3.864	449.07285	Myricetin-3-Xyloside	449.07254	C20H18O12	92.2	No ref [1]
4.432	449.10886	Flavanomarein	449.10895	C21H22O11	89.3	No ref
3.928	449.11023	Eriodictyol-7-O-glucoside	449.10895	C21H22O11	92.5	Förster et al. (2021)
4.012	461.07303	Kaempferol-3-Glucuronide	461.07254	C21H18O12	91.5	Randriamanana et al. (2014)
3.577	463.0878	Isoquercitrin	463.0882	C21H20O12	91.6	He et al. (2023)
3.946	463.08957	Myricitrin	463.0882	C21H20O12	95	Muklada et al. (2020)
3.973	477.06812	Quercetin-3-Glucuronide	477.06747	C21H18O13	94.1	Nissinen et al. (2017)
4.98	477.1041	Petunidin-3-O-β-glucopyranoside	477.1033	C22H23O12	89.6	No ref
4.298	477.10464	Isorhamnetin-3-O-glucoside	477.10385	C22H22O12	95	Piątczak et al. (2020)
3.619	479.08371	Myricetin-3-Galactoside	479.0831	C21H20O13	93.8	Stolter et al. (2013)
4.134	505.09943	Quercetin 3-(6-O-acetyl-β-glucoside)	505.09875	C23H22O13	93	No ref
4.332	505.09979	Isomer of Quercetin 3-(6-O-acetyl-β-glucoside)	505.09875	C23H22O13	92	No ref
4.276	507.11447	Syringetin-3-O-galactoside	507.11441	C23H24O13	90.5	No ref
3.513	577.13507	Isomer of Procyanidin B2	577.13513	C30H26O12	90.1	Zaiter et al. (2016)
3.315	577.13507	Procyanidin B1	577.13513	C30H26O12	88.1	Zaiter et al. (2016)
3.089	577.13556	Procyanidin B2	577.13513	C30H26O12	91.8	Zaiter et al. (2016)

[1] References for compounds marked as “no ref” were not found for Salicaceae family.

3.891	577.13574	Isomer of Procyanidin B2	577.13513	C30H26O12	85.8	Zaiter et al. (2016)
4.14	577.15582	Rhoifolin	577.15625	C27H30O14	88.8	No ref in *Salix*
4.211	607.1676	Neodiosmin	607.16687	C28H32O15	84.4	No ref
3.113	609.1463	Isomer of Quercetin-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside	609.14612	C27H30O16	82.9	Mosaddik et al. (2006)
3.638	609.1463	Luteolin-3',7-di-O-glucoside	609.14612	C27H30O16	81.9	No ref
3.43	609.14661	Quercetin-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside	609.14612	C27H30O16	88.3	No ref
3.822	609.14697	Delphinidin-3-O-(6"-O-α-rhamnopyranosyl-β-glucopyranoside)	609.14557	C27H31O16	94.3	No ref
4.145	623.1618	Isorhamnetin-3-O-rutinoside	623.16174	C28H32O16	90.9	Piątczak et al. (2020)
3.473	625.14093	Quercetin-3,4'-O-di-β-glucopyranoside	625.14105	C27H30O17	80.3	No ref
3.975	895.19489	Kaempferol-3-O-glucoside	895.19385	C21H20O11	82	Keefover-Ring et al. (2022)

Table 4.3: Summary of compounds in positive ion mode identified by MS Dial using massBank database matching.

Average RT	Average Mz	Identification				Reference
		Name	Reference m/z	Formula	Confidence level (%)	
4.361	287.05405	Isomer of Luteolin	287.05502	C15H10O6	92.1	Tegelberg et al. (2003)
4.459	287.05405	Isomer of Kaempferol	287.05502	C15H10O6	89.9	Budny et al. (2021)
4.243	287.0542	Kaempferol	287.05502	C15H10O6	91	Budny et al. (2021)
5.215	287.05432	Isomer of Luteolin	287.05502	C15H10O6	93.2	Tegelberg et al. (2003)
3.983	287.05435	Luteolin	287.05502	C15H10O6	92.9	Tegelberg et al. (2003)
3.93	289.06995	Eriodictyol	289.07068	C15H12O6	98	Noleto-Dias et al. (2020)
5.144	289.0701	Isomer of Eriodictyol	289.07068	C15H12O6	98	Noleto-Dias et al. (2020)
4.436	301.06979	Isomer of Kaempferide	301.07068	C16H12O6	90.6	Zhou et al. (2022)
5.898	301.06989	Kaempferide	301.07068	C16H12O6	89.9	Zhou et al. (2022)
3.623	303.04898	Isomer of Quercetin	303.04993	C15H10O7	87.2	Zhou et al. (2022)
4.135	303.04901	Isomer of Quercetin	303.04993	C15H10O7	92.5	Zhou et al. (2022)
3.467	303.04904	Isomer of Quercetin	303.04993	C15H10O7	92.9	Zhou et al. (2022)
4.332	303.0491	Isomer of Quercetin	303.04993	C15H10O7	91.8	Zhou et al. (2022)
3.827	303.04922	Isomer of Quercetin	303.04993	C15H10O7	92.7	Zhou et al. (2022)
3.955	303.04922	Quercetin	303.04993	C15H10O7	92.7	Zhou et al. (2022)
4.27	303.04938	Isomer of Quercetin	303.04993	C15H10O7	93.5	Zhou et al. (2022)

4.985	317.06461	Rhamnetin	317.06558	C16H12O7	87.7	Lavola et al. (2018)
4.757	317.06476	Isomer of Isorhamnetin	317.06558	C16H12O7	93.7	Pobłocka-Olech et al. (2021)
4.535	317.06488	Isomer of Isorhamnetin	317.06558	C16H12O7	94.4	Pobłocka-Olech et al. (2021)
3.729	317.06494	Isomer of Isorhamnetin	317.06558	C16H12O7	93.3	Pobłocka-Olech et al. (2021)
4.148	317.06494	Isomer of Isorhamnetin	317.06558	C16H12O7	89.4	Pobłocka-Olech et al. (2021)
4.299	317.06525	Isorhamnetin	317.06558	C16H12O7	93.5	Pobłocka-Olech et al. (2021)
3.888	319.04443	Myricetin	319.04483	C15H10O8	96.5	Mizuno et al. (1989)
3.616	319.04446	Isomer of myricetin	319.04483	C15H10O8	95	Mizuno et al. (1989)
4.378	447.09158	Unknown 1		C21H18O11		
4.261	449.10724	Quercitrin	449.10785	C21H20O11	93.4	Kompantsev and Glyzin (1973)
3.708	449.10727	Isomer of quercitrin	449.10785	C21H20O11	87.1	Kompantsev and Glyzin (1973)
3.982	449.10785	Luteolin-7-O-glucoside	449.10785	C21H20O11	87.4	Pobłocka-Olech et al. (2021)
3.864	451.08664	Myricetin-3-Xyloside	451.0871	C20H18O12	83.6	No ref[2]
4.406	463.12326	Peonidin-3-O-β-D-glucoside	463.12292	C22H23O11	90.1	No ref
3.96	465.10175	Isoquercitrin	465.10275	C21H20O12	93	He et al. (2023)
3.571	465.10178	Isomer of Hyperoside (quercetin-3-O-galactoside)	465.10275	C21H20O12	90.3	Piątczak et al. (2020)
3.923	465.10199	Isomer of isoquercitrin	465.10275	C21H20O12	93.5	He et al. (2023)

[2] References for compounds marked as “no ref” were not found for Salicaceae family.

4.981	479.11731	Petunidin-3-O-β-glucopyranoside	479.11786	C22H23O12	81.8	No ref
4.297	479.11819	Isorhamnetin-3-O-glucoside	479.11841	C22H22O12	85.1	Piątczak et al. (2020)
3.618	481.09732	Unknown 2		C21H20O13		
3.958	487.08441	Hyperoside (syn. quercetin-3-O-galactoside)	487.08469	C21H20O12	85.4	Piątczak et al. (2020)
4.332	507.11246	Isomer of quercetin 3-(6-O-acetyl-β-glucoside)	507.11331	C23H22O13	83.8	No ref
4.332	529.09454	Quercetin 3-(6-O-acetyl-β-glucoside)	529.09528	C23H22O13	84.7	No ref
3.092	579.14935	Procyanidin B1	579.14972	C30H26O12	81	Zaiter et al. (2016)
4.144	579.16943	Rhoifolin	579.17084	C27H30O14	85	No ref
3.679	617.14734	Kaempferol-3-O-rutinoside	617.14771	C27H30O15	82.6	No ref
4.148	625.17584	Isorhamnetin-3-O-rutinoside	625.17633	C28H32O16	82.5	Piątczak et al. (2020)
4.147	647.15753	Isomer of isorhamnetin-3-O-rutinoside	647.15826	C28H32O16	90.2	Piątczak et al. (2020)
3.729	647.15778	Isomer of isorhamnetin-3-O-rutinoside	647.15826	C28H32O16	84.5	Piątczak et al. (2020)

After contaminants were excluded, 32 compounds were identified in negative ionization mode and 23 compounds were identified in positive ionization mode. From those, eight compounds showed separation from the others based on their mean decrease accuracy (MDA) in RandomForest function (MDA values over 160) in positive mode and nine compounds showed separation in negative mode (MDA>140), suggesting that they are good predictors of the differences between willow clones, with an OOB estimate of error rate of 1.39% for negative and 2.78% for positive mode (Figure 4.1 and Figure 4.2). For negative mode, compounds selected for further analyses were apigenin, isorhamnetin-3-O-glucoside, procyanidin B2, epicatechin, petunidin-3-O-β-glucopyranoside, kaempferide, kaempferol-3-glucuronide, quercetin-7-O-rhamnoside and isorhamnetin-3-O-rutinoside (Table 4.4). For positive mode, compounds selected for further analyses were an unidentified compound we called unknown 1, isorhamnetin, isorhamnetin-3-O-glucoside, peonidin-3-O-β-D-glucoside, kaempferide, luteolin-7-O-glucoside, procyanidin B1 and isorhamnetin-3-O-rutinoside (Table 4.5). Most clones had all mentioned metabolites in positive or negative mode. Clone that did not have all metabolites was PN386 (*S. schwerinii*) with seven metabolites in negative mode (Table 4.4).

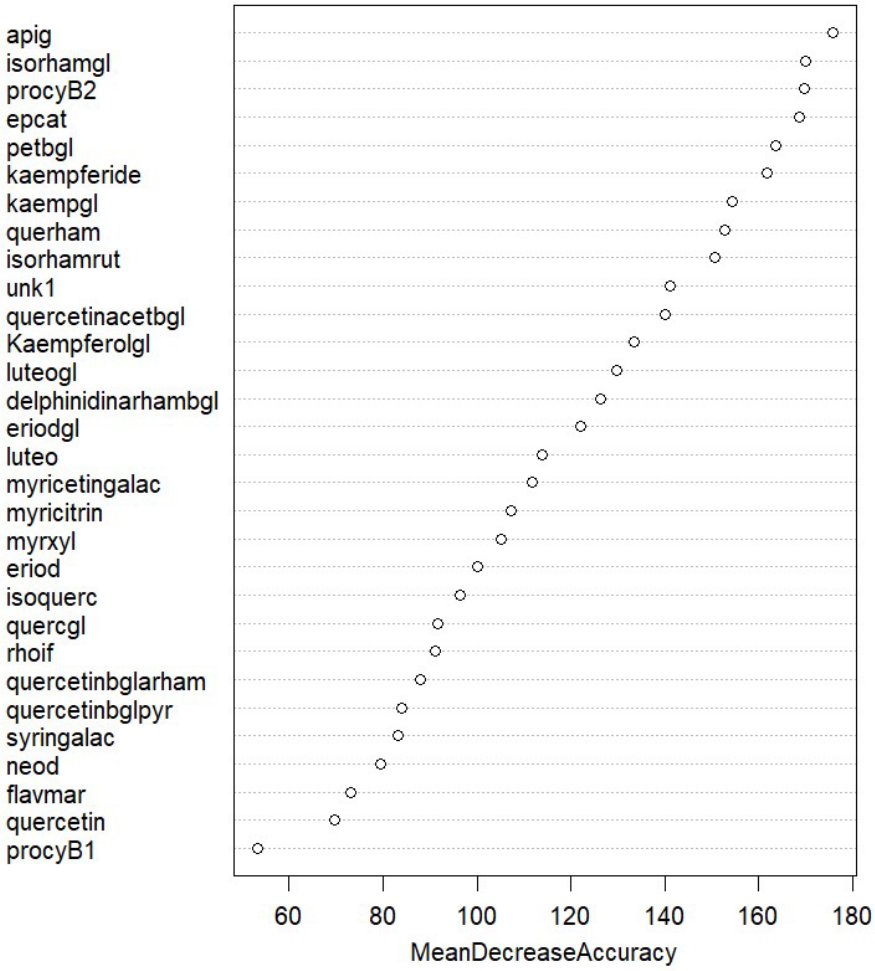

Figure 4.1: Importance scores obtained by RandomForest analysis of metabolites data (high-performance liquid chromatography, negative ionization mode) collected from six *Salix* clones commonly used in New Zealand. Plotted are variable importance scores (MeanDecreaseAccuracy); the variables with higher MDA are more important in explaining observed patterns. Abbreviations: apig = apigenin; isorhamgl = isorhamnetin-3-O-glucoside; procyB2 = procyanidin B2; epcat = epicatechin;; petbgl = petunidin-3-O-β-glucopyranoside; kaempferide = kaempferide; kaempgl = kaempferol-3-glucuronide; querham = quercetin-7-O-rhamnoside; isorhamrut = isorhamnetin-3-O-rutinoside; unk1 = Unknown 1; quercetinacetbgl= quercetin 3-(6-O-acetyl-β-glucoside); kaempferolgl = kaempferol-3-O-glucoside; luteogl = luteolin-7-O-glucoside; delphinidinarhambgl = delphinidin-3-O-(6"-O-α-rhamnopyranosyl-β-glucopyranoside); eriodgl = eriodictyol-7-O-glucoside ; luteo = luteolin; myricetingalac = myricetin-3-galactoside; myricitrin = myricitrin; myrxyl = myricetin-3-xyloside; eriod = eriodictyol; isoquerc = isoquercitrin; quercgl = quercetin-3-glucuronide; rhoif = rhoifolin; quercetinbglarham = quercetin-3-O-β-glucopyranosyl-7-O-α-rhamnopyranoside; quercetinbglpyr = quercetin-3,4'-O-di-β-glucopyranoside; syringalac = syringetin-3-O-galactoside; neod= neodiosmin; flavmar = flavanomarein; querc = quercetin; procyB1 = procyanidin B1.

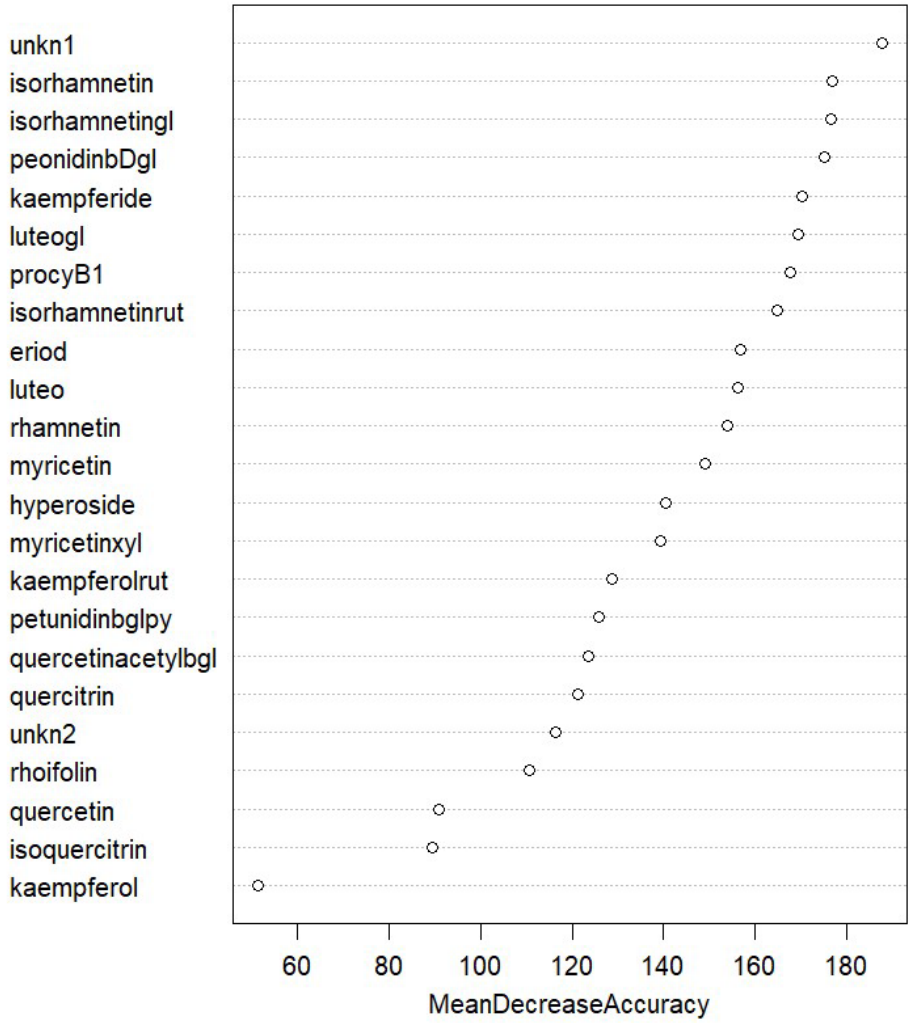

Figure 4.2: Importance scores (MeanDecreaseAccuracy) obtained by RandomForest analysis of metabolites data (high-performance liquid chromatography, positive ionization mode) collected from six *Salix* clones commonly used in New Zealand. The variables with higher MDA are more important in explaining observed patterns. Abbreviations: unkn1 = Unknown 1; isorhamnetin = isorhamnetin; isorhamnetingl = isorhamnetin-3-O-glucoside; peonidinbDgl = peonidin-3-O-β-D-glucoside; Kaempferide = kaempferide; luteogl = luteolin-7-O-glucoside; procyB1 = procyanidin B1; isorhamnetinrut = isorhamnetin-3-O-rutinoside, eriod = eriodictyol; luteo = luteolin; rhamnetin = rhamnetin; myricetin = myricetin; hyperoside = hyperoside; myricetinxyl = myricetin-3-xyloside; kaempferolrut = kaempferol-3-O-rutinoside; petunidinbglpy = Petunidin-3-O-β-glucopyranoside; quercetinacetylbgl = quercetin 3-(6-O-acetyl-β-glucoside); quercetrin = quercetrin; unkn2= Unknown 2; rhoifolin = rhoifolin ; quercetin = quercetin; isoquercitrin = isoquercitrin; kaempferide = kaempferide.

Table 4.4: LC-MS analysis of metabolites in negative ion mode from six *Salix* clones commonly used in New Zealand. Metabolites are classified by chemical group.

		Flavanol		**Flavone**	**Proanthocyanin**	**Phenol glycoside**					
Clone	**Species**	Epicatechin	Kaempferide	Apigenin	Procyanidin B2	Isorhamnetin-3-O-glucoside	Petunidin-3-O-β-glucopyranoside	Quercetin-7-O-rhamnoside	Isorhamnetin-3-O-rutinoside	Kaempferol-3-O-glucoside	**Number of metabolites**
PN220	*S. viminalis*	+	+	+	+	+	+	+	+	+	9
PN249	*S. purpurea*	+	+	+	+	+	+	+	+	+	9
PN386	*S. schwerinii*	+	+	+	+	+	+	+	0	0	7
NZ04-106-073	*S. lasiolepis* × *S. viminalis*	+	+	+	+	+	+	+	+	+	9
PN218	*S. fragilis*	+	+	+	+	+	+	+	+	+	9
NZ1040	*S. matsudana* ×*S. alba*	+	+	+	+	+	+	+	+	+	9
	Frequency in six clones	6	6	6	6	6	6	6	5	5	

Table 4.5: LC-MS analysis of metabolites in positive ion mode from six *Salix* clones commonly used in New Zealand. Metabolites are classified by chemical group.

		Flavanol		**Flavanone**	**Proanthocyanin**	**Anthocyanin**	**Phenol glycoside**			
Clone	**Species**	Isorhamnetin	Kaempferide	Unknown 1	Procyanidin B1	Peonidin-3-O-β-D-glucoside	Luteolin-7-O-glucoside	Isorhamnetin-3-O-glucoside	Isorhamnetin-3-O-rutinoside	**Number of metabolites**
PN220	*S. viminalis*	+	+	+	+	+	+	+	+	8
PN249	*S. purpurea*	+	+	+	+	+	+	+	+	8
PN386	*S. schwerinii*	+	+	+	+	+	+	+	+	8
NZ04-106-073	*S. lasiolepis* × *S. viminalis*	+	+	+	+	+	+	+	+	8
PN218	*S. fragilis*	+	+	+	+	+	+	+	+	8
NZ1040	*S. matsudana* × *S. alba*	+	+	+	+	+	+	+	+	8
	Frequency in six clones	6	6	6	6	6	6	6	6	

4.3.1 Results for negative ion mode

Figure 4.3 shows a PCA ordination for the nine most important metabolites identified in Table 4.4. PC1 and PC2 together explained 68.9% of the variation in metabolite profile in six willow clones. In 4.7 Appendix, Table 4.7A-1 shows the values for the dimensions of the PCA. The main contributors of PC1 were kaempferide, apigenin, kaempferol-3-glucuronide and Procyanidin B2, while PC2 main contributors were epicatechin, isorhamnetin-3-O-rutinoside, quercetin-7-O-rhamnoside and petunidin-3-O-β-glucopyranoside. Distinct clusters were observed. PCA ordination suggests that clone NZ1040 had higher concentration of isorhamnetin-3-O-rutinoside and clone NZ 04-106-073 had higher concentration of apigenin and kaempferide. The differences between compound concentrations in clones are shown in Figure 4.4.

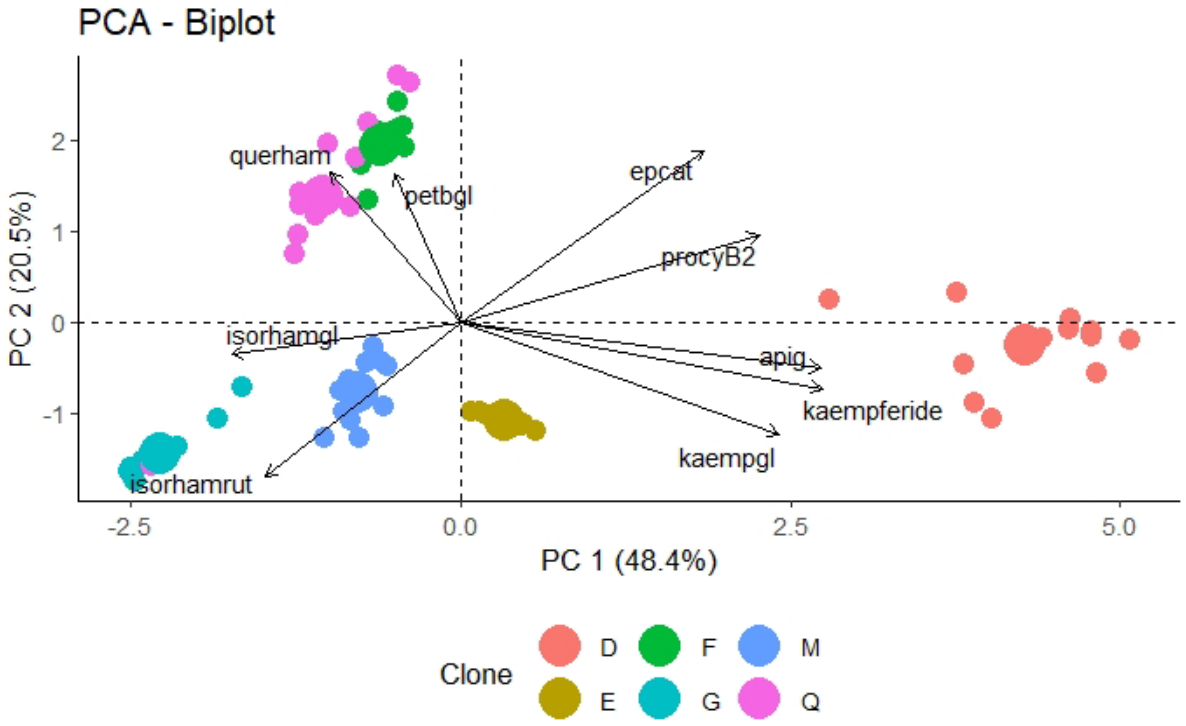

Figure 4.3: Principal component analysis (PCA) biplot showing differences in metabolites in different willow *Salix* spp clones. Larger symbols are group centroids. Clones: G = PN220, E= PN249, F = PN386, D = NZ 04-106-073, Q = PN218, M = NZ1040. Abbreviations: epcat = epicatechin; procyB2 = procyanidin B2; apig = apigenin; kaempferide = kaempferide; kaempgl = kaempferol-3-glucuronide; isorhamrut = isorhamnetin-3-O-rutinoside; isorhamgl = isorhamnetin-3-O-glucoside; querham = quercetin-7-O-rhamnoside; petbgl = petunidin-3-O-β-glucopyranoside.

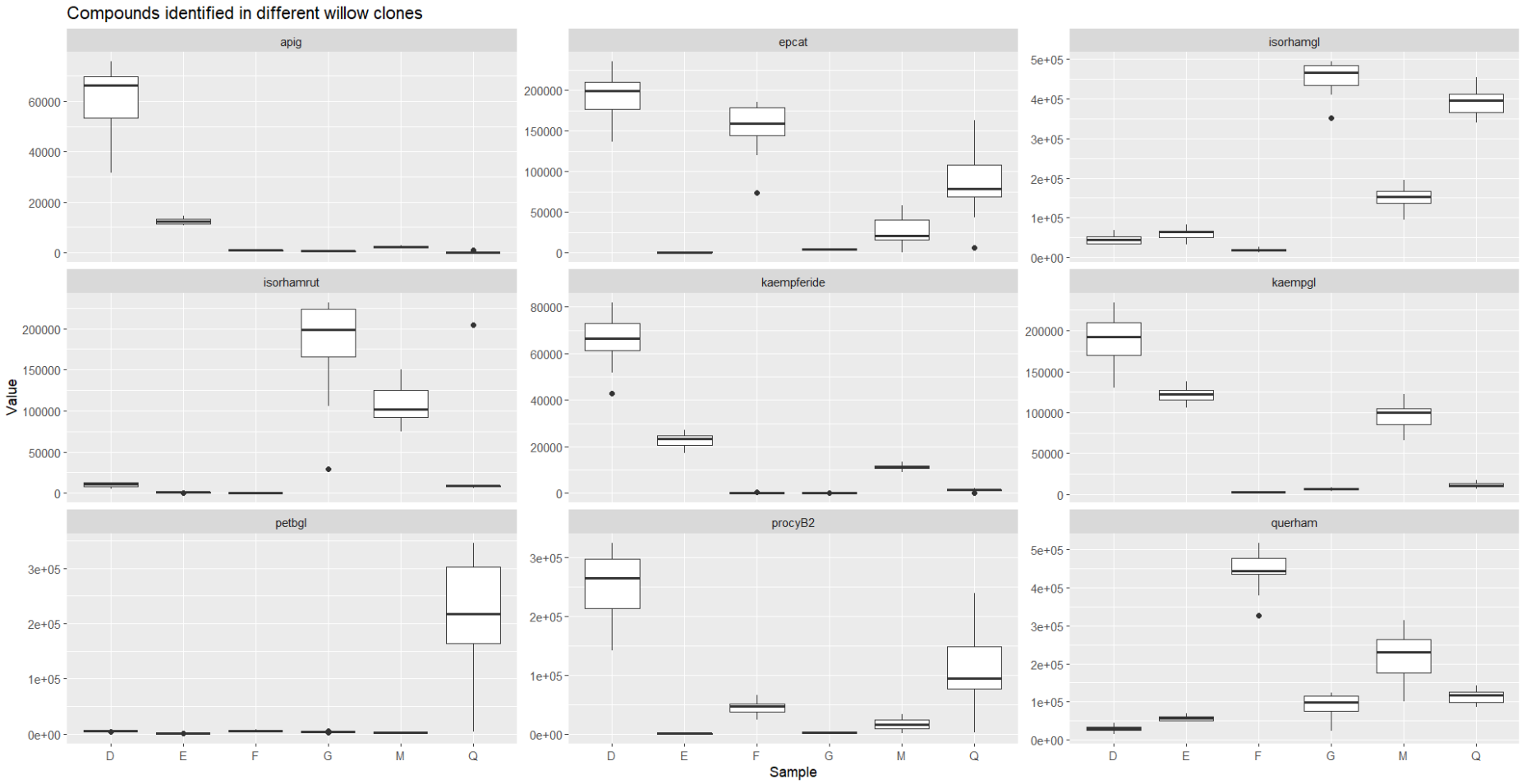

Figure 4.4: Different compounds concentrations in different willow clones in negative ion mode. Abbreviations: see Figure 4.3.

PERMANOVA showed significant differences in metabolites among clones (Pseudo-F=156.02; $p<0.001$). Pairwise comparisons between clones showed significative differences between all clones.

Figure 4.5 shows PCA ordination for the nine most important metabolites identified in Table 4.4, with clones grouped according to resistance to GWA. The main contributors of PC1 and PC2 same as above (Figure 4.3: Principal component analysis (PCA) biplot showing differences in metabolites in different willow *Salix* spp clones. Larger symbols are group centroids. Clones: G = PN220, E= PN249, F = PN386, D = NZ 04-106-073, Q = PN218, M = NZ1040. Abbreviations: epcat = epicatechin; procyB2 = procyanidin B2; apig = apigenin; kaempferide = kaempferide; kaempgl = kaempferol-3-glucuronide; isorhamrut = isorhamnetin-3-O-rutinoside; isorhamgl = isorhamnetin-3-O-glucoside; querham = quercetin-7-O-rhamnoside; petbgl = petunidin-3-O-β-glucopyranoside.Figure 4.3). Distinct clusters were observed. PCA ordination suggests that moderately susceptible clones had higher concentration of quercetin-7-O-rhamnoside and petunidin-3-O-β-glucopyranoside and resistant clones had higher concentration of apigenin and kaempferide.

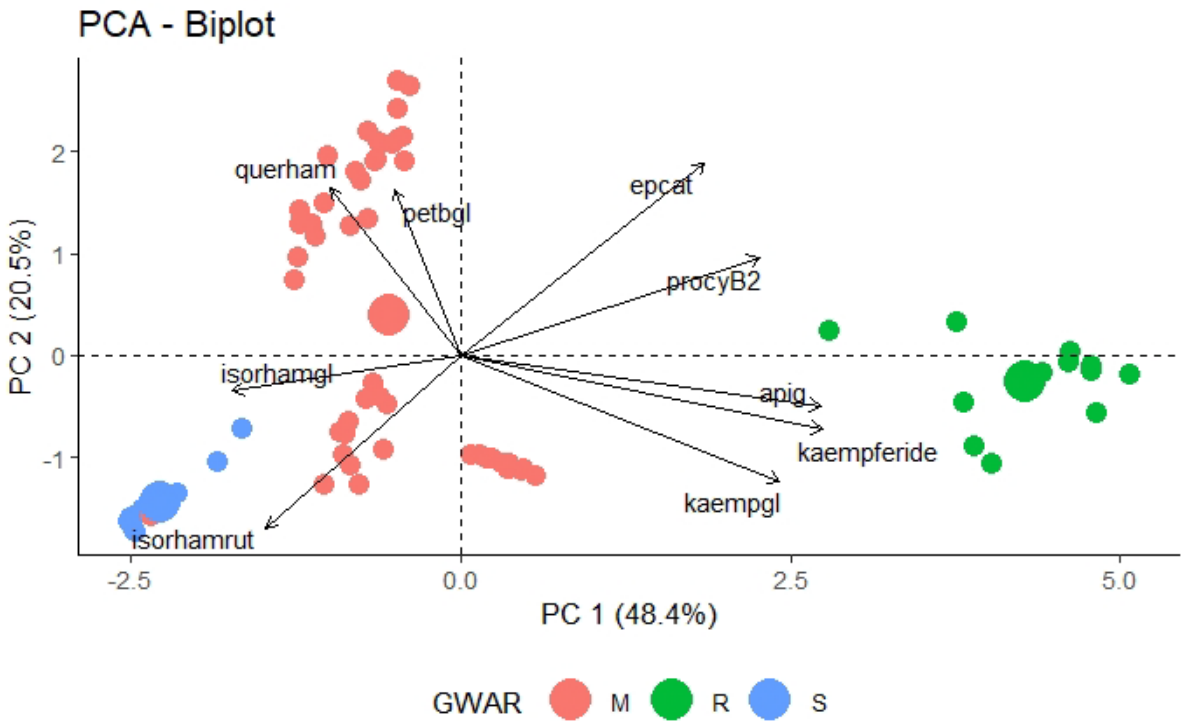

Figure 4.5: Principal component analysis (PCA) biplot showing differences in metabolites in different willow *Salix* spp clones grouped according to resistance to the giant willow aphid (GWA). Larger symbols are group centroids. 'GWAR' indicates the clones' level of resistance to GWA – susceptible (S), resistant (R), and moderately susceptible (M). See Figure 4.3 for metabolites abbreviations.

PERMANOVA showed a significant difference in metabolites among clones which were susceptible (S), moderately susceptible (M) and resistant (R) to GWA (Pseudo-F = 25.838; $p < 0.001$). All levels of resistance showed significant differences in metabolite profiles (S x R, Pseudo-F = 307.34, p = 0.001; S x M, Pseudo-F = 19.721, $p < 0.001$; R x M, Pseudo-F = 21.096, $p < 0.001$).

Figure 4.6 shows a PCA ordination for the nine most important metabolites identified in Table 4.4 with clones grouped according to resistance to *P. proxima* sawfly. The main contributors of PC1 and PC2 are the same as above (Figure 4.3). This PCA ordination suggests that moderately susceptible clones had higher concentration of apigenin and kaempferide.

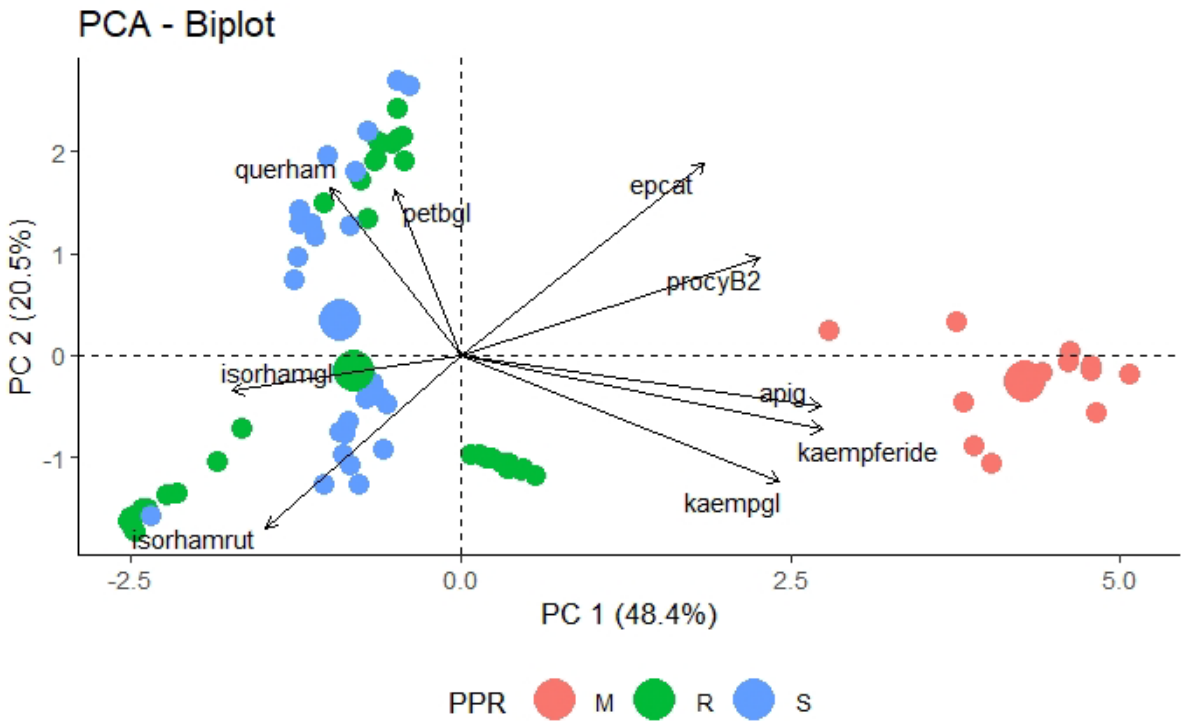

Figure 4.6: PCA biplot showing differences in metabolite compounds in different willow *Salix* spp cultivars grouped according to the level of resistance to *Pontania proxima*. Larger symbols are group centroids. 'PPR' indicates the clones susceptible (S), resistant (R), and (M) moderately resistant to *P. proxima*. See Figure 4.3 for metabolites abbreviations.

PERMANOVA showed significant difference in metabolites among clones which were susceptible (S), moderately resistant (M) and resistant (R) to *P. proxima* (Pseudo-F = 15.081; $p < 0.001$). All the three groups showed significant difference (S x R, Pseudo-F = 5.6586, p = 0.003; S x M, Pseudo-F = 36.276, p = 0.001; R x M, Pseudo-F = 19.111, p = 0.001).

Figure 4.7 shows a PCA ordination for nine most important metabolites identified in Table 4.4 with clones grouped by induction by GWA. The PCA ordination showed no distinctive clusters. PERMANOVA showed no significant difference in metabolites between induced and control plants (control x induced, Pseudo-F = 0.0515; p = 0.995).

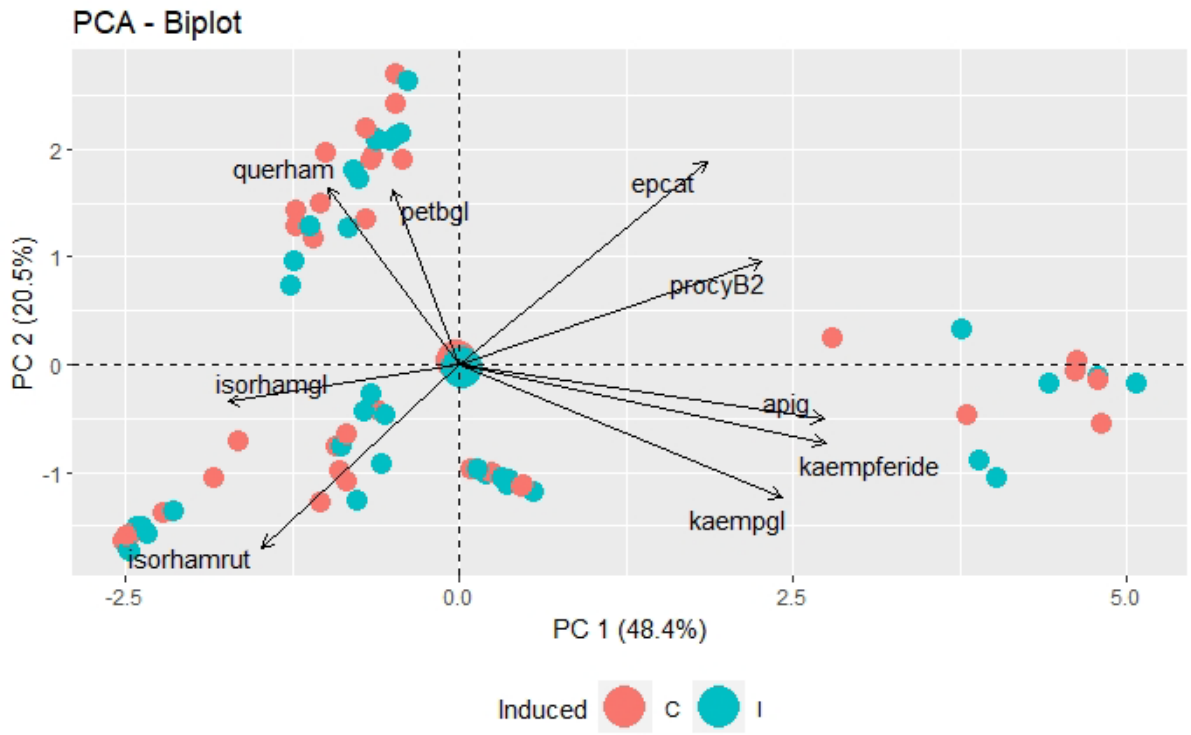

Figure 4.7: PCA biplot showing differences in metabolite compounds in willow *Salix* spp clones grouped as control plants and plants induced by GWA. Larger symbols are group centroids. C – control, I – induced. See Figure 4.3 for metabolites abbreviations.

Figure 4.8 shows a PCA ordination for the nine most important metabolites identified in Table 4.4 with clones grouped by plant growth form. The PCA showed no distinct clusters. PERMANOVA showed a significant difference in metabolites between two plant growth forms (tree vs. shrub, Pseudo-F = 7.0892; $p < 0.001$).

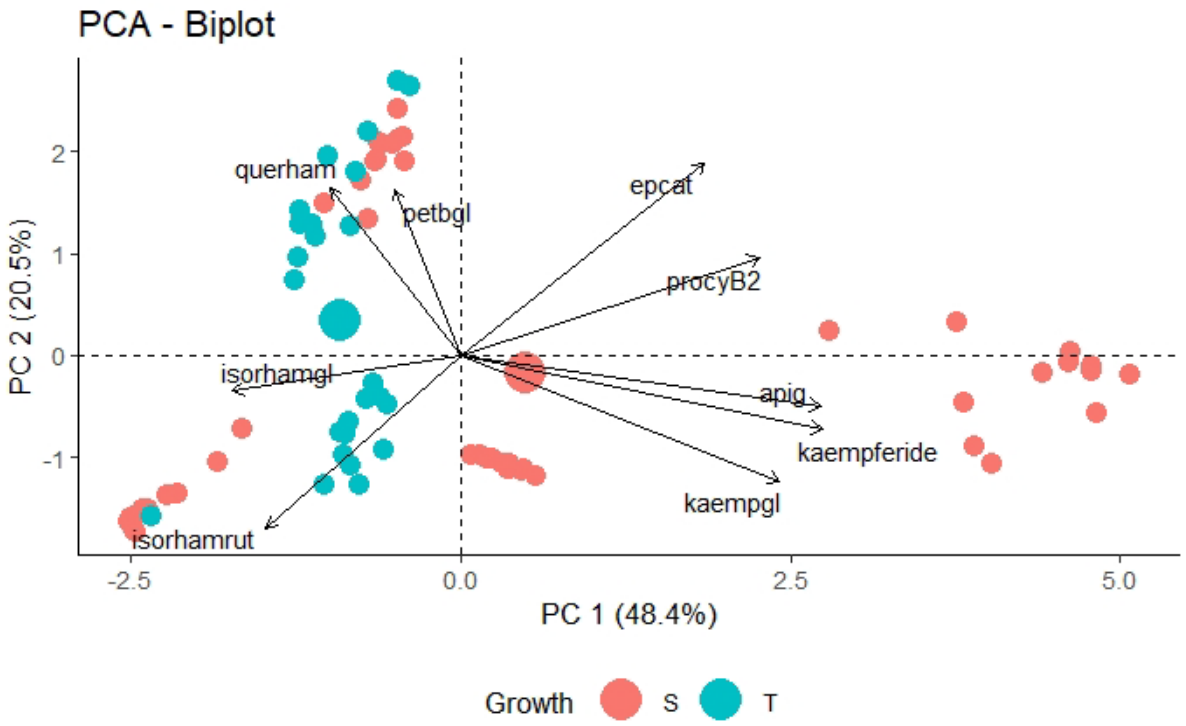

Figure 4.8: PCA biplot showing differences in metabolite compounds in different willow *Salix* spp clones grouped by growth form. Larger symbols are group centroids. S – shrub, T – tree. See Figure 4.3 for metabolites abbreviations.

4.3.2 Results for positive ion mode

Figure 4.9 shows a PCA ordination for the eight most important metabolites identified in Table 4.5. PC1 and PC2 together explained 75.7% of the variation in metabolite profiles in six willow clones. In 4.7 Appendix, Table 4.7A-2 shows the values for each dimension of the PCA. The main contributors of PC1 were luteolin-7-O-glucoside, kaempferide, peonidin-3-O-β-D-glucoside, isorhamnetin and isorhamnetin-3-O-glucoside, while PC2 main contributors were isorhamnetin-3-O-rutinoside, procyanidin B1, kaempferide and peonidin-3-O-beta-D-glucoside. Distinct clusters were observed. PCA ordination suggests that clone PN249 had higher concentration of luteolin-7-O-glucoside, clone NZ 04-106-073 had higher emission of procyanidin B1 as well as Kaempferide, unknown and peonidin.

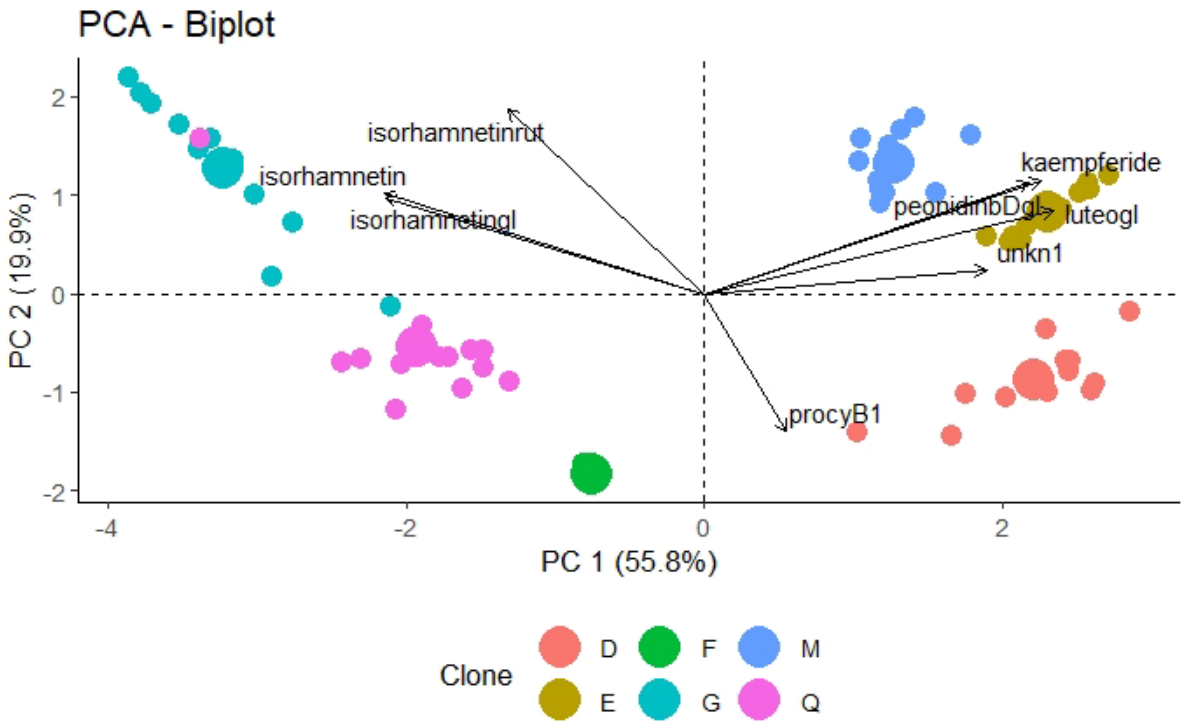

Figure 4.9: Principal component analysis (PCA) biplot showing differences in metabolites in different willow *Salix* spp clones. Larger symbols are group centroids. Clones: G = PN220, E= PN249, F = PN386, D = NZ 04-106-073, Q = PN 218, M = NZ1040. Abbreviations: peonidinbDgl = peonidin-3-O-beta-D-glucoside; kaempferide = kaempferide; luteogl = luteolin-7-O-glucoside; unkn1 = unknown 1; procyB1 = procyanidin B1; isorhamnetingl = isorhamnetin-3-O-glucoside; isorhamnetin = isorhamnetin; isorhamnetinrut = isorhamnetin-3-O-rutinoside.

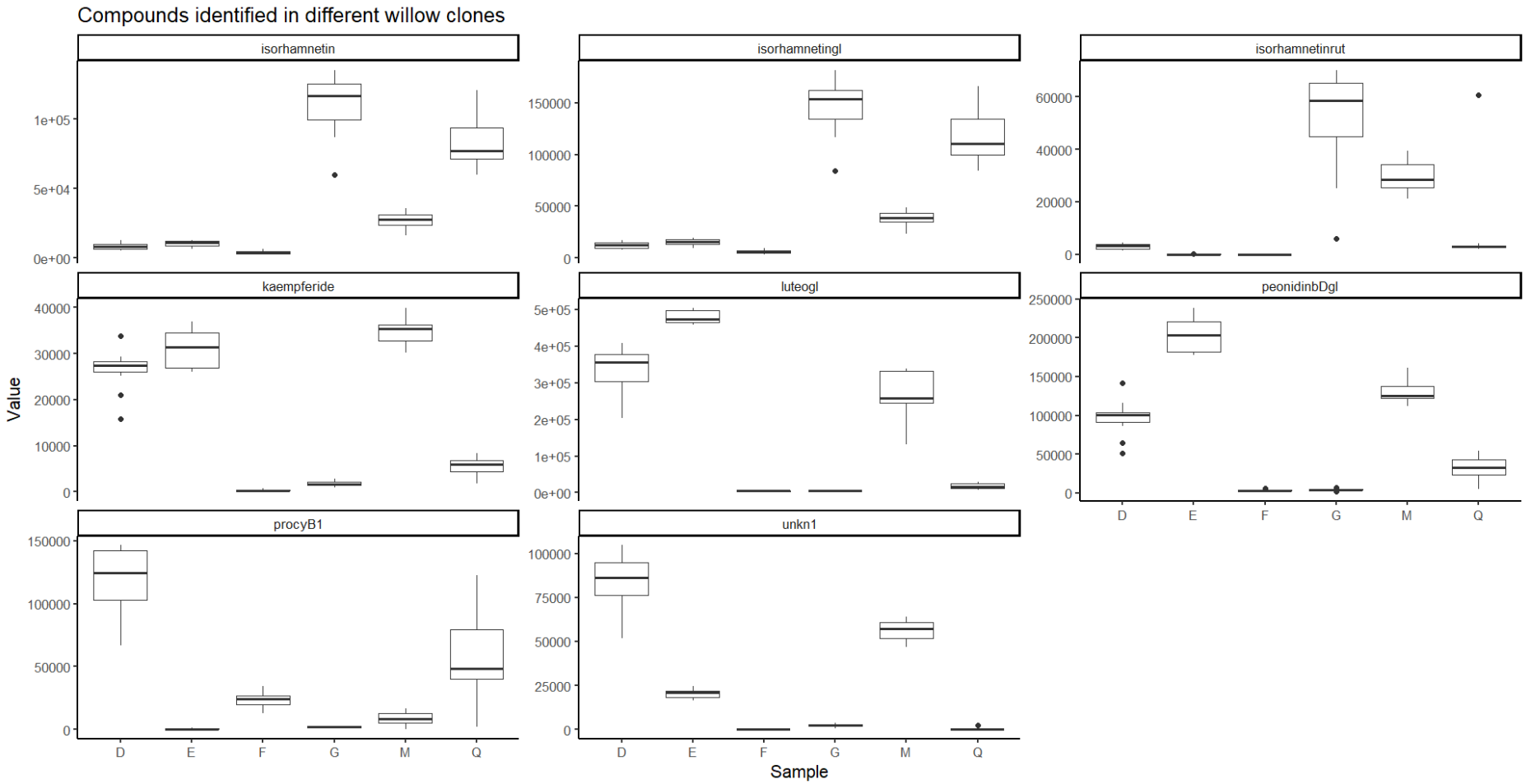

Figure 4.10: Different compound concentrations in different willow clones in positive ion mode. Abbreviations: on Figure 4.9.

PERMANOVA showed significant differences in metabolites among clones (Pseudo-F = 274.89; $p < 0.001$). Pairwise comparisons between clones showed significative differences between all clones.

Figure 4.11 shows a PCA ordination for the eight most important metabolites identified in Table 4.5 with clones grouped according to their resistance to GWA. PERMANOVA showed significant differences in metabolites among clones which were susceptible (S), moderately susceptible (M) and resistant (R) to GWA (Pseudo-F =14.124; $p < 0.001$). All level of resistance showed significant differences in metabolite profiles (S x R, Pseudo-F = 269.79, $p < 0.001$; S x M, Pseudo-F = 12.377, $p<0.001$; R x M, Pseudo-F = 8.1078, $p < 0.002$). In PCA no distinct pattern is observed in relation to susceptibility, suggesting that the ordination and PERMANOVA results reflect clone-level differences was observed in relation to susceptibility.

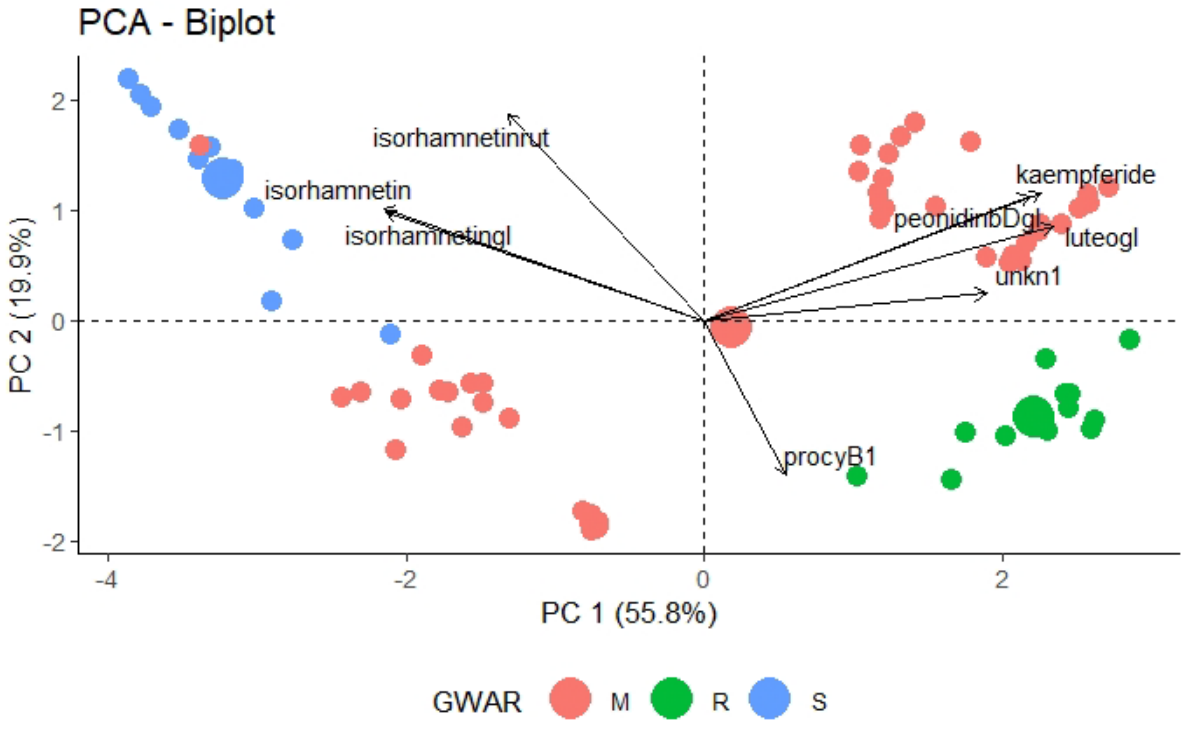

Figure 4.11: Principal component analysis (PCA) biplot showing differences in metabolites in different willow *Salix* spp clones grouped according to resistance to the giant willow aphid (GWA). Larger symbols are group centroids. 'GWAR' indicates the clones' level of resistance to GWA – susceptible (S), resistant (R), and moderately susceptible (M). See Figure 4.9 for metabolites abbreviations.

Figure 4.12 shows a PCA ordination for the eight most important metabolites identified in Table 4.5 with clones grouped according to resistance to *P. proxima*. PERMANOVA showed significant differences in metabolites among clones which were susceptible (S), moderately resistant (M) and resistant (R) to *P. proxima* (Pseudo-F = 6.361; p = 0.003). Susceptible and resistant clones did not show significant different from each other (S x R, Pseudo-F = 0.9234, p = 0.359) while both susceptible and resistant clones were significantly different from moderately susceptible clones (S x M, Pseudo-F = 23.065, p < 0.001; R x M, Pseudo-F = 8.2291, p = 0.006). No distinct clusters are observed in PCA related to susceptibility, suggesting that the ordination and PERMANOVA results reflect clone-level differences relating to susceptibility.

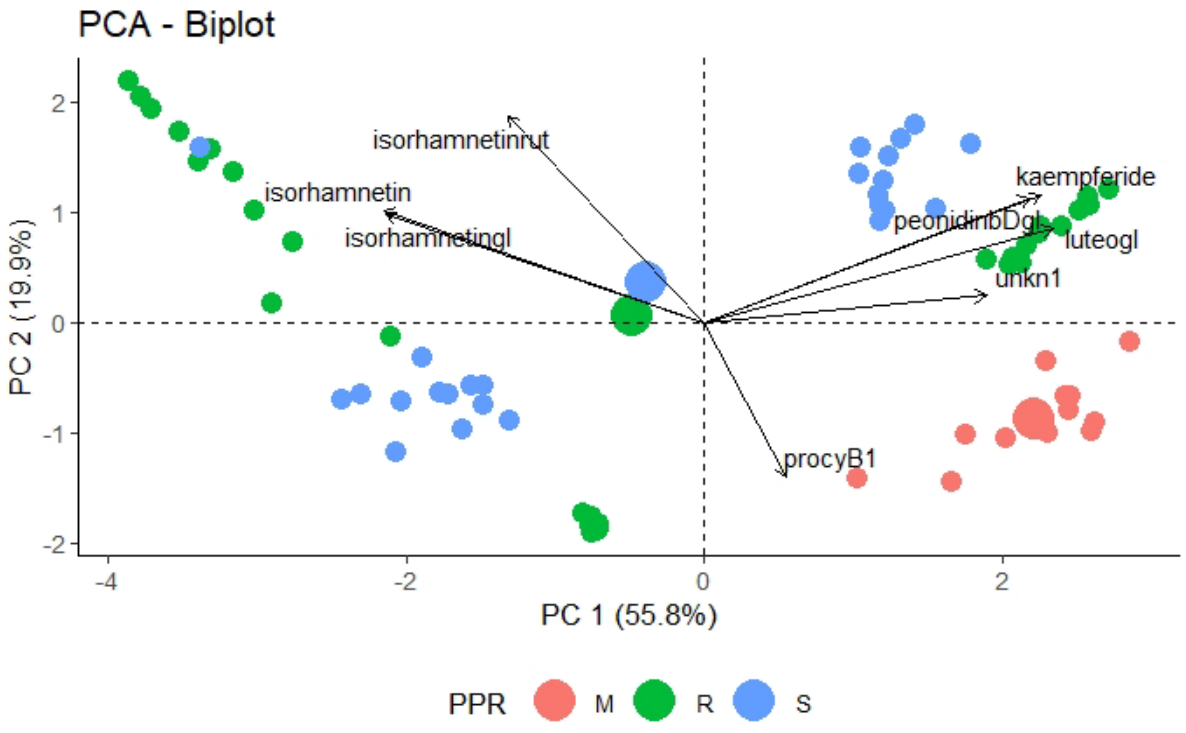

Figure 4.12: PCA biplot showing differences in metabolites compounds in different willow *Salix* spp cultivars grouped according to the level of resistance to *Pontania proxima*. Larger symbols are group centroids. 'PPR' indicates the clones susceptible (S), resistant (R), and (M) moderately resistant to *P. proxima*. See Figure 4.9 for metabolites abbreviations.

Figure 4.13 shows a PCA ordination for the eight most important metabolites identified in Table 4.5 with clones grouped by induction by GWA. PERMANOVA showed no significant differences in metabolites between control and induced plants (control vs. induced, Pseudo-F = 0.0244; p = 0.996). No distinct clusters were observed.

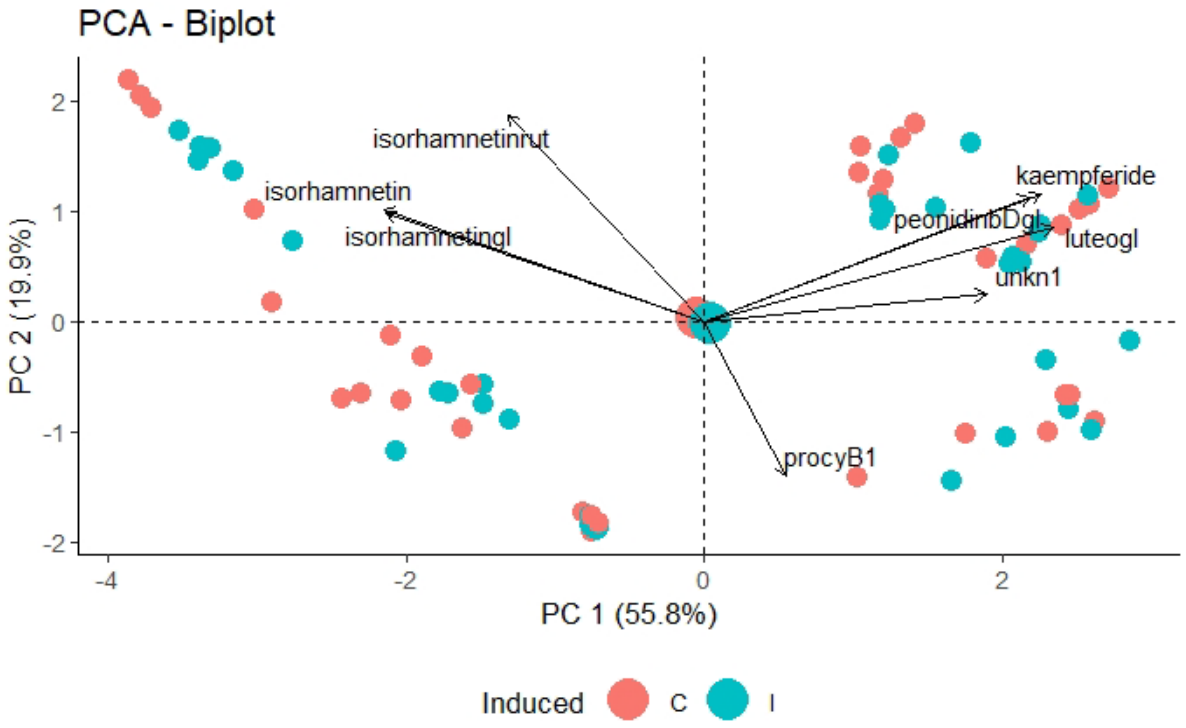

Figure 4.13: PCA biplot showing differences in metabolite compounds in willow *Salix* spp clones grouped as control plants and plants induced by GWA. Larger symbols are group centroids. C – control, I – induced. See Figure 4.9 for metabolites abbreviations.

Figure 4.14 shows a PCA ordination for the eight most important metabolites identified in Table 4.5 with clones grouped by growth form. PERMANOVA showed no significant difference in metabolites between two plant growth mode (tree vs. shrub, Pseudo-F = 2.414; p = 0.106). No distinct clusters were observed, suggesting that the ordination and PERMANOVA results reflect clone-level differences.

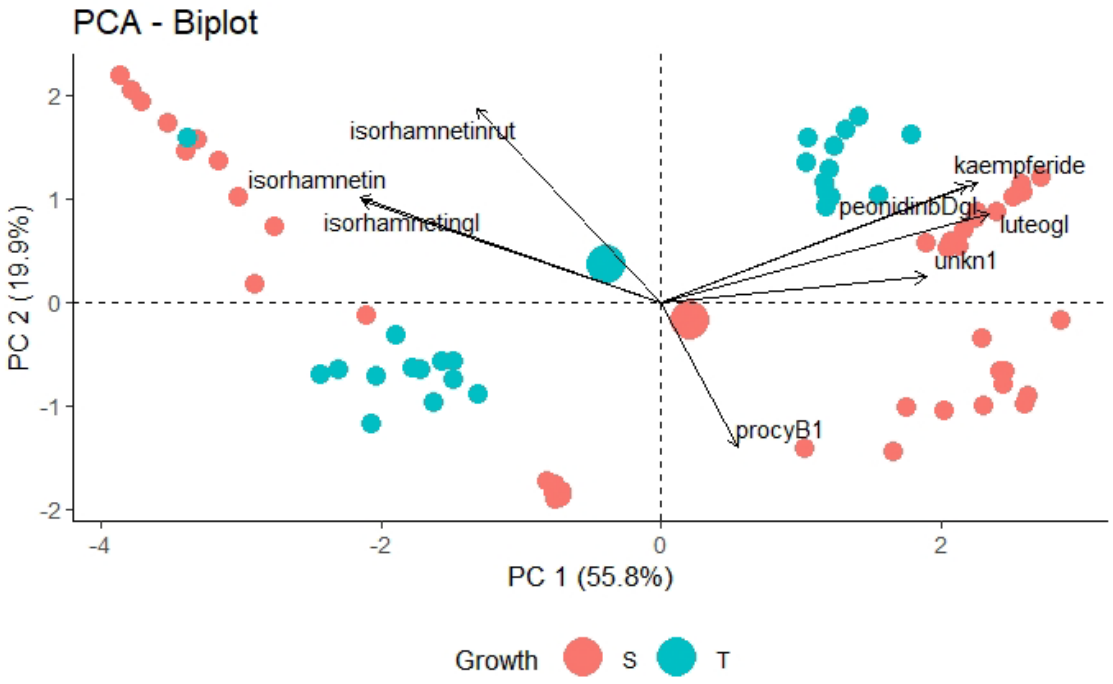

Figure 4.14: PCA biplot showing differences in metabolite compounds in different willow *Salix* spp clones grouped by growth form. Larger symbols are group centroids. S – shrub, T – tree. See Figure 4.9 for metabolites abbreviations.

4.3.3 Total concentration of the analysed secondary metabolites

The general linear models (GLM) showed significant effect of clone, GWA resistance and *P. proxima* resistance, but not plant growth form, on the total concentration of the analysed secondary metabolites (Table 4.6, Figure 4.15, Figure 4.16 and Figure 4.17). The post-hoc Tukey test with Bonferroni correction for clones showed significant differences among all clones except for clones PN220 vs PN249 (std error = 0.0000, z value = 2.379, p = 0.2606), NZ1040 vs PN249 (std error = 0.0000, z value= -2.238, p = 0.3781), PN 218 vs PN249 (std error = 0.0000, z value = 0.717, p = 1) and PN 218 vs PN220 (std error = 0.0000, z value = -1.730, p = 1). For the full Tukey test results please see Table 4.7A-3 in 4.7 Appendix. Regarding GWA resistance, post-hoc test showed significant difference in total metabolites concentration between resistant vs. moderately susceptible clones and resistant and susceptible

(R x M, p = 0.000; S x M, p= 0.646; S x R, p= 0.0000; Figure 4.16). For *P. proxima* resistance, all groups showed significant differences in total metabolite concentration (R x M, p = 0.0000; S x M, p = 0.000; S x R, p= 0.000; Figure 4.17).

Table 4.6: Generalized linear model (GLM) comparing total concentration of the analysed secondary metabolites (Totalmet) (arbitrary units) in different clones of willow *Salix* spp grouped by different factors. GWA= giant willow aphid. ** significant at alpha = 0.05.

Model comparison	Df residual	LogLik	Df model	Chisq	p-value
Clone	7	-1002.24	5	99.731	0.0000**
GWA resistance	4	-1005.56	2	59.849	0.0000**
***P. proxima* resistance**	4	-1005.56	2	73.943	0.0000**
Growth form	3	-1001.6	1	0.0931	0.7603

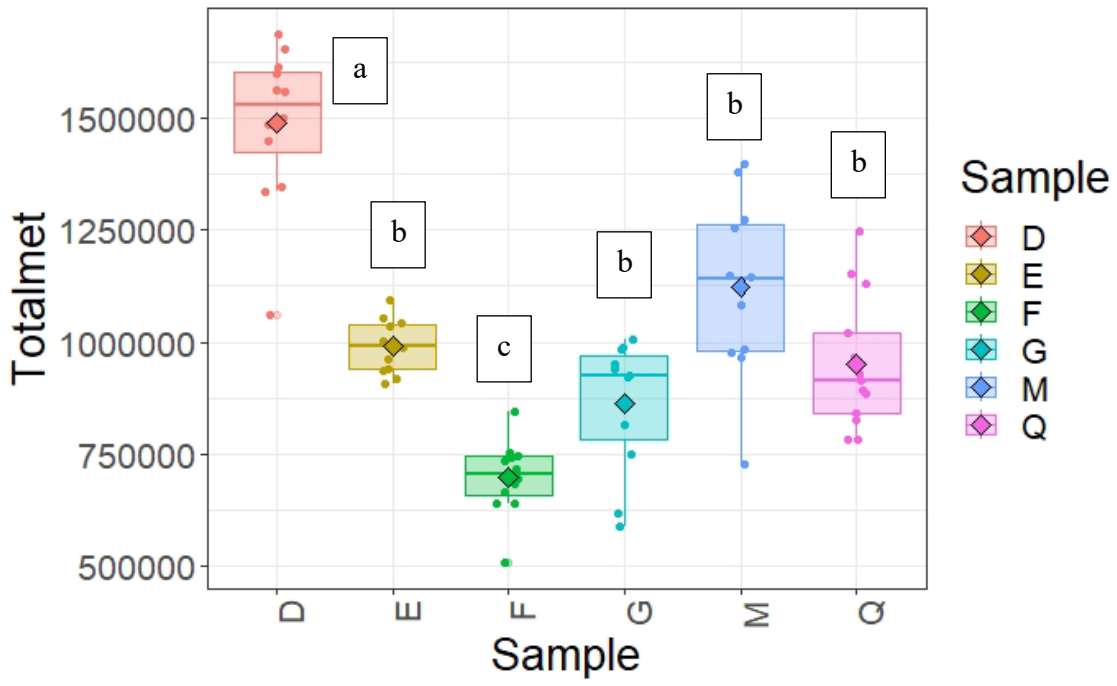

Figure 4.15: Total concentration of the analysed secondary metabolites (Totalmet) (arbitrary units) in different willow *Salix* spp clones. The median is indicated by the line across the box. The mean is indicated by the diamond. Clones: G = PN220, E= PN249, F = PN386, D = NZ 04-106-073, Q = PN 218, M = NZ1040. Letters indicate the results of the Tukey post-hoc test.

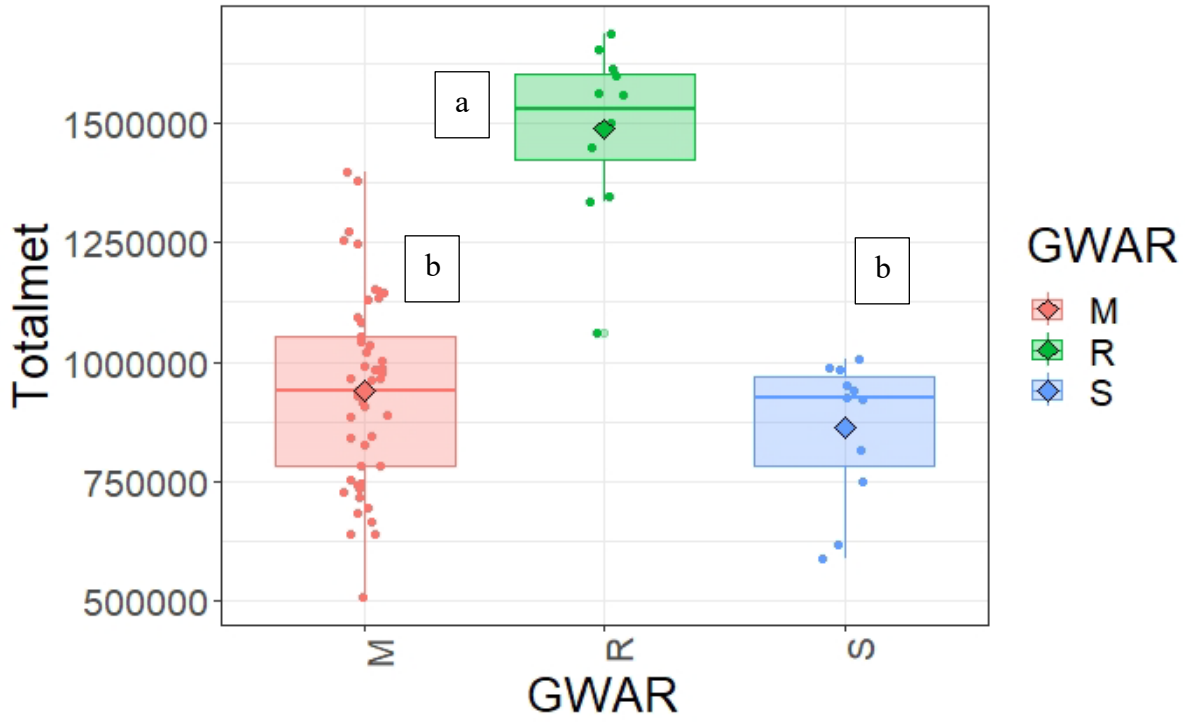

Figure 4.16: Total concentration of the analysed secondary metabolites (Totalmet) (arbitrary units) in different willow *Salix* spp clones grouped based on resistance to giant willow aphid. The median is indicated by the line across the box. The mean is indicated by the diamond. GWAR= giant willow aphid resistance: susceptible (S), moderately susceptible (M) and resistant (R). Letters indicate the results of the Tukey post-hoc test.

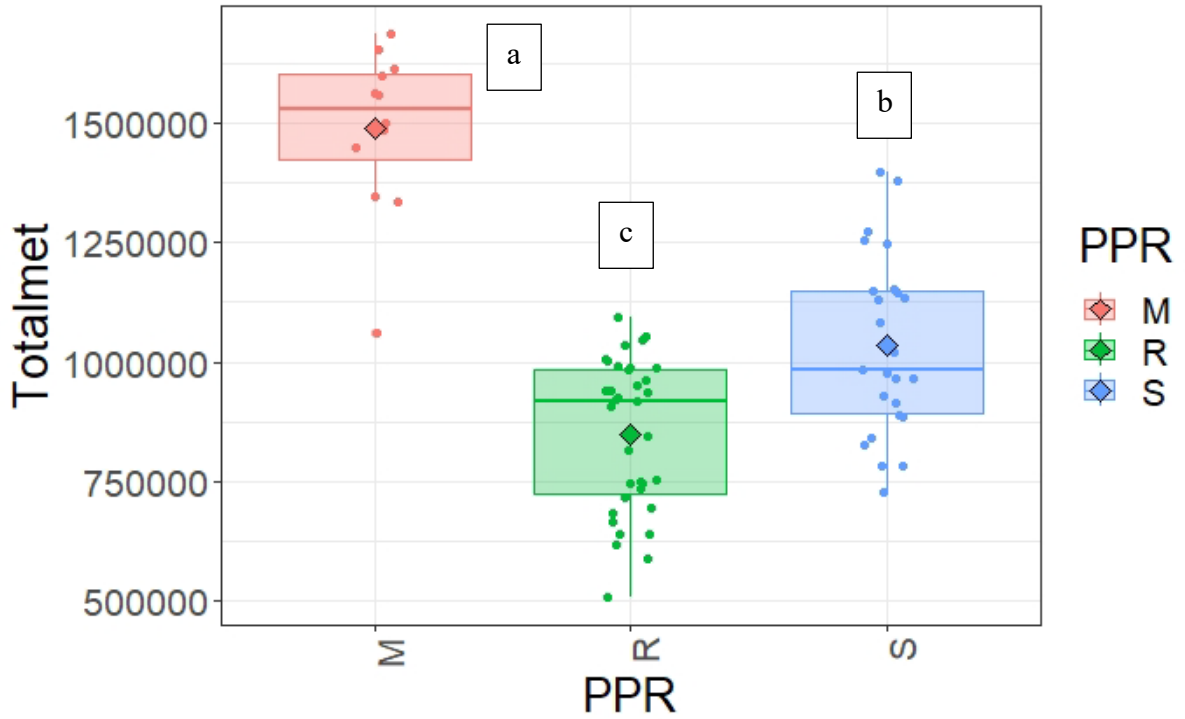

Figure 4.17: Total concentration of the analysed secondary metabolites (Totalmet) (arbitrary units) in different willow *Salix* spp clones grouped based on resistance to *Pontania proxima*. The median is indicated by the line across the box. The mean is indicated by the diamond. PPR= *Pontania proxima* resistance. Susceptible (S), moderately resistant (M) and resistant (R). Letters indicate the results of the Tukey post-hot test.

4.4 Discussion

Willow clones used for planting in New Zealand show significant variability in species, sex, growth type, chemistry, morphology and anatomy. Due to all these differences, they show different susceptibility to diseases and pests (Tun et al., 2021; Tun et al., 2020; Van Kraayenoord & Hathaway, 1986; Van Kraayenoord & Hathaway, 1987).

All clones showed significant differences in their metabolomic profiles. Clone PN220 showed highest concentration of isorhamnetin-3-O-rutinoside, isorhamnetin-3-O-glucoside, isorhamnetin. Clone NZ 04-106-073 showed higher concentration of apigenin, epicatechin, kaempferide, procyanidin B2, kaempferol-3-glucuronide, kaempferide, procyanidin B1, luteolin-7-O-glucoside and the unknown compound 1. Clone PN218 petunidin-3-O-β-glucopyranoside, isorhamnetin-3-O-glucoside, isorhamnetin and procyanidin B1. Clone PN386 showed higher concentration of epicatechin and quercetin-7-O-rhamnoside. Clone PN249 showed higher concentration of kaempferide, luteolin-7-O-glucoside and peonidin-3-O-beta-D-glucoside. Clone NZ1040 showed higher concentrations of isorhamnetin-3-O-rutinoside, kaempferide, luteolin-7-O-glucoside, peonidin-3-O-beta-D-glucoside and the unknown compound 1. Most clones contained all the analysed compounds except for PN386 (*S. schwerinii*). The compounds that this clone did not possess were isorhamnetin-3-O-rutinoside and kaempferol-3-glucuronide. Regarding the total concentration of metabolites, clone PN249 (*S. purpurea*) did not show significant differences with clones PN220 (*S. viminalis*), NZ1040 (*S. matsudana* × *S. alba*) and PN218 (*S. fragilis*) and PN220 (*S. viminalis*) did not show difference with clone PN218 (*S. fragilis*). Clone PN386 (*S. schwerinii*) showed the lowest total metabolites concentration and clone NZ04-106-073 (*S. lasiolepis* × *S. viminalis*) presented the highest.

Plant phenolics are the most common defence secondary metabolites and play a major role in plant resistance (Dixon, 1999; Grandmaison et al., 1993; Khokhani et al., 2013; Li et al., 2010; Shalaby & Horwitz, 2015; War et al., 2012). Different classes of phenolic compounds (e.g., lignin, quinones and salicylates) have different modes of action. Among the compounds we found, some classes of compounds have their effect on insects described in the literature. Quercetin and isorhamnetin are reported to inhibit aphid (*Aphis fabae* Scopoli) reproduction on cowpea *Vigna* spp. (Lattanzio et al., 2000). Onyilagha et al. (2004) reports isorhamnetin-3-sophoroside7-glucoside and kaempferol-3,7-diglucoside as effective deterrents to bertha armyworm (*Mamestra configurata* Walker) and 7,4'-dihydroxyflavone and dihydroquercetin as phagostimulants. Apigenin and kaempferol are reported to decrease the intensity of plant sap ingestion in pea aphid *Acyrthosiphon pisum* (Harris) (Stec et al., 2021). Kim and Mullin (2007) report isorhamnetin 3-O-neohesperidoside as phagostimulant for adult beetles of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) that feed on corn (*Zea mays* L.) pollen. Epicatechin is reported to decrease larval growth on *Ectropis grisescens* (Geometridae, Lepidoptera) (Li et al., 2022). The effect of some phenolic compounds, however, can be modified by glycosylation or hydroxylation with some increasing their detrimental effects and others increasing their detrimental effect (Elliger et al., 1980; Jones & Klocke, 1987; Laks & Pruner, 1989).

The production of secondary metabolites is intrinsic to genotype and species and is genetically determined. An interesting study about the effects of hybridization on production of secondary metabolites was conducted by Orians et al. (2000). The authors measured the concentrations of phenolic glycosides and condensed tannins in parental linages of *S. sericea* and *S. eriocephala* and in their F1 hybrid to investigate chemical variation among hybrids. These two species have contrasting chemical characteristics; *S. sericea* produces phenolic glycosides and low concentrations of condensed tannin while *S. eriocephala* does not produce

phenolic glycosides but has high concentrations of condensed tannins. The authors found that the concentration of phenolic glycosides in the hybrid was lower than the parental midpoint which indicates directional dominance. For condensed tannins, however, F1 hybrids were intermediate between parental lines which indicates predominantly additive inheritance or balanced ambidirectional dominance. The authors concluded that the production of phenolic glycosides is controlled by one or more recessive alleles.

Regarding the level of resistance to GWA, clones with all levels of resistance showed differences in their metabolomic profiles. Due to our low number of clones, our conclusions about which compounds can cause resistance to GWA are limited. Our susceptible clone showed higher concentration of isorhamnetin and its isomers. Interestingly, our resistant clone, NZ 04-106-073, is a hybrid of *S. viminalis*, which is the species of PN220, our susceptible clone. Those clones showed differences in their metabolomic profile and in the resistance to GWA. NZ 04-106-073 showed a higher concentration of secondary metabolites than PN220. To our knowledge there have been no previous studies conducted with GWA and their preference for lower phenol glycoside concentration. However, the resistance of *Populus angustifolia* to aphid *Pemphigus betae*, a galling aphid, was correlated with the presence of high phenol concentrations (Zucker, 1982). The authors demonstrated that the trees with the higher aphid numbers per leaf have lower phenol levels in their leaves. Chlorogenic acid and 3,5-dicaffeoyl quinic acid were identified as anti-feedants for Leaf Beetle *Plagiodera versicolora* and 1,2-di[(9Z,12Z,15Z)-octadeca-9,12,15-trienoyl]-3- β -D-galactopyranosyl-sn-glycerol as a feeding stimulant (Jassbi, 2003).

For resistance to *P. proxima*, we faced the same issue due to the low number of clones in the study. The pattern of resistance to *P. proxima* was more difficult to draw conclusions on than for GWA resistance. The susceptible clones showed diverse chemical profiles and did not have a particular class of compounds in higher or in lower concentration. The same was

observed with the resistant clones. *P. proxima* is reported to prefer higher concentration of phenol glycosides (Hjältén et al., 2007; Soetens et al., 1991), however, in our study we did not find this pattern. Causes for this were discussed in Chapter 3, where we discussed in more detail damage caused by *P. proxima*, and larval development.

Plants infected with GWA did not show significant differences in metabolites from control plants. This result is in accordance with Tun et al. (2020) who demonstrated that GWA does not cause differences in emissions of VOCs. The authors attributed the lack of response to GWA attack to the fact that these insects do not cause direct damage to the leaves or cause severe mechanical injury to the plant since they feed on sap. The authors also brought up the possibility that GWA may manipulate the plant defences by suppressing their response to attack as happens in pea aphids, *Acyrthosiphon pisum* (Schwartzberg et al., 2011). Schwartzberg et al. (2011), however, did not investigated how pea aphids suppress plant response. Turlings et al. (1998) investigated induction of volatile emissions in maize by three insect herbivores with different feeding habits: a caterpillar (*Spodoptera littoralis*), a stemborer (*Ostrinia nubilalis*), and an aphid (*Rhopalosiphum maidis*). The authors suggest that the aphids, due to their feeding habit, do not cause enough cell damage to trigger plant response. Will and van Bel (2008) developed a theory on how aphids may suppress plant response to their attack. The authors suggest that aphid's saliva injected during feeding contains proteins that bind with calcium and prevent calcium-induced sieve element occlusion which explains local suppression of plant defence. We suggest that GWA may also be able to suppress plant defences which explains the lack of differences in secondary metabolites in leaves from induced and control willow plants. Further investigation is necessary to prove those theories.

Regarding secondary metabolites production induction by aphids, Hodge et al. (2019) investigated leaf chemistry changes in *Arabidopis thaliana* caused by three species of aphid: *Myzus persicae*, a generalist with wide host range and *Brevicoryne brassicae* and *Lipaphis*

pseudobrassicae, two brassica specialists. These authors found that most glucosinolates were reduced in concentration by aphid feeding, except when generalist aphid *Myzus persicae* fed which caused increase in levels of 4-methoxy-indolyl-glucosinolate, jasmonic acid and salicyclic acid. Those compounds correlated positively with aphid density and feeding time. Similar results were found by Gianoli and Niemeyer (1997). The authors investigated hydroxamic acids accumulation in wheat *Triticum aestivum* infested by cereal aphid *Rhopalosiphum padi*. It was found that low cereal aphid population, below 25 aphids, do not cause changes in hydroxamic acids concentration. Time of infestation also affected the concentration of hydroxamic acids with 48 hours being the minimum time to trigger the induction of hydroxamic acid production. These authors also found that different concentrations of nitrogen fertilization had no effect on the aphid induction of hydroxamic acids. Similar results were also found pea *Pisum sativum* L.cv. Cysterski after infestation by the pea aphid *Acyrthosiphon pisum* (Morkunas et al., 2016).

It is advantageous to aphids if plants do not increase the concentration of secondary metabolites in their tissues. Although GWA is a specialized herbivore of willows, and therefore tolerant to phenol glycosides, these compounds may negatively affect GWA development and their fitness. To our knowledge there are no studies on the role of phenol glycosides in GWA specifically, but in general phenol glycosides are anti-feedants for insect herbivores (Lattanzio et al., 2006; Pasteels & Rowell-Rahier, 1992; Rowell-Rahier & Pasteels, 1990). In aspen (*Populus tremula*) the effects of *Chaitophorus* aphid infestation and of mechanical damage on condensed tannins and total phenolic were investigated (Gaur et al., 2022). The authors found that aphid fecundity was negatively correlated with condensed tannins concentrations and total phenolics. The authors also found that total phenolics concentration increased in response to damage while condensed tannins showed no increase or even a decrease below constitutive

levels in genotypes rich in condensed tannins. The authors concluded that total phenolics are more relevant to resistance than condensed tannins.

Another relevant point about our study is that we used untargeted metabolomics analysis by Q-TOF-MS therefore the number of features we captured was very high. Settled identification score cut off was 80%. This caused a high number of unidentified features. We may have lost some key metabolites that may shed a light on insect-pest resistance.

4.5 Conclusions

The willow clones we analysed appear to have highly species-specific (clone-specific) metabolomic profiles. Due to our limited number of clones and limited number of replicates, the untargeted metabolomics analysis by Q-TOF-MS method we used. We cannot draw definitive conclusions about the pattern of secondary metabolites in relation to resistance to the two insect herbivores – *P. proxima* and GWA. GWA feeding did not cause significative changes in the metabolomic profile of the analysed willow clones. The resistance to *P. proxima* and GWA may be more correlated with phenological and morphological features (such as leaf pilosity, bark thickness, flowering time) of willow plants than with metabolomic profile.

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4.7 Appendix

Table 4.7A-1: Principal component analysis (PCA)results table showing differences in metabolites in different willow *Salix* spp clones for negative ion mode. Abbreviations: epcat = epicatechin; procyB2 = procyanidin B2; apig = apigenin; kaempferide = kaempferide; kaempgl = kaempferol-3-glucuronide; isorhamrut = isorhamnetin-3-O-rutinoside; isorhamgl = isorhamnetin-3-O-glucoside; querham = quercetin-7-O-rhamnoside; petbgl = petunidin-3-O-β-glucopyranoside; Dim = dimension.

Feature	Dim 1	Dm 2	Dim 3	Dim 4	Dim 5
apig	20.7904	1.6555	0.6529	3.2410	1.7610
epcat	9.5622	23.4889	0.0017	17.3840	2.8413
kaempferide	21.0278	3.5090	0.2031	0.2555	0.2354
querham	2.7311	17.8280	24.1720	10.6725	21.3334
kaempgl	16.3268	10.0111	0.3488	3.8363	38.0798
petbgl	0.7133	17.5181	30.2182	9.5269	23.4438
isorhamgl	8.3725	0.8011	31.8891	5.2340	0.7863
procyB2	14.3313	6.0943	10.9102	7.1298	0.1803
isorhamrut	6.1447	19.0940	1.6040	42.7200	11.3387

Table 4.7A-2: Principal component analysis (PCA)results table showing differences in metabolites in different willow Salix spp clones for positive ion mode. Abbreviations: peonidinbDgl = peonidin-3-O-beta-D-glucoside; kaempferide = kaempferide; luteogl = luteolin-7-O-glucoside; unkn1 = unknown 1; procyB1 = procyanidin B1; isorhamnetingl = isorhamnetin-3-O-glucoside; isorhamnetin = isorhamnetin; isorhamnetinrut = isorhamnetin-3-O-rutinoside; Dim = dimension.

Feature	Dim 1	Dm 2	Dim 3	Dim 4	Dim 5
kaempferide	16.9076	12.2732	13.4466	0.08692	29.7013
isorhamnetin	15.3165	9.5614	7.2918	12.5237	0.4471
unkn1	11.8620	0.5531	28.6319	16.1266	4.8464
luteogl	18.1950	6.6517	0.0000	6.6686	41.9807
peonidinbDgl	15.7706	11.7198	1.5069	14.8048	0.0000
isorhamnetingl	15.1795	8.7751	7.4890	15.1096	2.6105
procyB1	1.0052	18.1351	50.6375	4.6375	6.1794
isorhamnetinrut	5.7635	32.3306	3.0983	30.0422	14.2347

Table 4.7A-3: Post-hoc Tukey test comparing the total concentration of analysed secondary metabolites in different clones of willow *Salix* spp. *** - significantly different at α=0.05.

Comparison	Z value	Pr(>|z|)
NZ04-106-073 vs PN249	7.004	0.000***
NZ04-106-073 vs PN386	12.086	0.000***
NZ04-106-073 vs PN220	8.844	0.000***
NZ04-106-073 vs NZ1040	4.917	0.000***
NZ04-106-073 vs PN218	7.850	0.000***
PN249 vs PN386	6.067	0.000***
PN249 vs PN220	2.379	0.2606
PN249 vs NZ1040	-2.238	0.3781
PN249 vs PN218	0.717	1
PN386 vs PN220	-3.671	0.0036***
PN386 vs NZ1040	-8.091	0.000***
PN386 vs PN218	-5.484	0.000***
PN220 vs NZ1040	-4.501	0.0001 ***
PN220 vs PN218	-1.730	1
NZ1040 vs PN218	3.000	0.0406***

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Data collection and analysis was conducted by Claryssa de Oliveira Mota with the assistance of Andrea Clavijo-McCormick and Evans Effah. Writing was led by Claryssa de Oliveira Mota and supported by Maria Minor and Andrea Clavijo-McCormick. Figures were developed by Claryssa de Oliveira Mota.

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Chapter 5 Volatile profiles of six willow (*Salix* spp.) clones of interest in New Zealand.

5.1 Introduction

Willows (*Salix* spp) are a naturalised species in New Zealand, where they have multiple uses due to their fast growth from vegetative material (Wilkinson, 1999). The most common use of willows in New Zealand is soil protection against erosion (Environment Southland, 2020; Group, 2007; McIvor, 2013). Willows can also serve as livestock fodder in drought periods (Oppong et al., 2001; Sopow et al., 2017). Different hybrids flower throughout the year, providing pollen and nectar for apiculture from spring to autumn (Sopow et al., 2017).

All plants emit volatile organic compounds (VOCs) that have multiple ecological functions, such as attracting natural enemies (parasitoids or predators) of their herbivores and mediating herbivore host-selection (Binyameen et al., 2021; Effah et al., 2019; Tumlinson, 2014; Turlings & Wäckers, 2004). VOCs can belong to multiple chemical classes including terpenoids, fatty acid-derived molecules – the category into which green leaf volatiles (GLVs) fall, and minor classes such as nitriles (Bouwmeester et al., 2019; Irmisch et al., 2014; Zhou & Jander, 2021). The differences in VOC emissions can vary depending on multiple factors such as plant genotype, environmental conditions (e.g., nutrient availability, precipitation, temperature) and presence of attacking herbivore (Chacón-Fuentes et al., 2023; Effah et al., 2020; Meihls et al., 2012; Otieno et al., 2023; Snoeren et al., 2010).

Different plant species will have different responses to changes in environment and herbivory. Effah et al. (2020) reported that the main factor influencing VOC emission from heather (*Calluna vulgaris*) was soil nutrients. Herbivory and temperature had little influence

and soil moisture had no impact on VOC emission. Swanson et al. (2021) studied the effects of temperature increase and infestation by gall-forming eriophyoid mites (*Aculus tetanothrix*) on VOC emission in willow *Salix myrsinites*. The authors found that raising temperature increases emissions of isoprene, while infestation by *A. tetanothrix* mites induces emissions of natural enemy attractants such as (E)-4,8-dimethyl-1,3,7-nonatriene, sesquiterpenes and GLVs. Another interesting study investigating willow volatiles was Tun et al. (2020). These authors studied the volatile profiles of 15 willow clones and the differences in VOCs emission following the giant willow aphid (GWA) *Tuberolachnus salignus* infestation. Tun et al. (2020) study showed that the differences in VOC emissions were more related to plant genotype than to herbivory response.

The giant willow aphid (GWA) *T. salignus* is a large aphid belonging to the family Aphididae (Hemiptera) (Blackman & Eastop, 1994; Dixon, 1985; McIvor, 2013). This pest is of Asian origin but is spread throughout the world where willows are found (Blackman & Eastop, 1994). In New Zealand this aphid was first found in December 2013, spreading through the country thereafter (Gunawardana et al., 2014; Sopow et al., 2014). GWA can substantially decrease the amount of photo-assimilate that arrives to the roots and stems of the willow plant, impacts that can range from reduction of plant growth to plant death; the negative effects on plant growth can be seen even after just one growth season with GWA infestation (Collins, 2001; Jones et al., 2021; Sopow et al., 2017). Aphid honeydew deposition can stimulate the growth of fungi on the plant surface which can decrease photosynthetic levels, as well as affect other species including honeybees and soil biota (Sopow et al., 2017; Tun, 2020).

Aradottir et al. (2009) showed that giant willow aphid responds positively to olfactory cues from different willow clones but did not correlate it with willow resistance to this insect pest. Tun et al. (2020) characterized volatiles released by willow clones to evaluate changes in

response to GWA infestation. Their results suggested that volatile profiles and responses to GWA are willow species-specific and not infestation-dependent (Tun et al., 2020).

The bean gall sawfly *Pontania proxima* Lepeletier, 1823 (Tenthredinidae: Hymenoptera) was introduced into New Zealand accidently and was first reported in 1929 (Kay, 1980). This insect causes multiple bean-shaped galls on willow leaves and heavy infestations may possibly decrease plant vitality and therefore plant production (Carleton, 1939; Naumann et al., 2002). The resistance of New Zealand willow clones to *P. proxima* has not been characterized yet, nor the correlation between volatile profile and *P. proxima* resistance. Kehl et al. (2010) tested antennae response of *P. proxima* to floral scent of willows. The authors proved that the sawfly's antennae respond positively to willow floral scents and that flowering willows showed a higher gall load than non-flowering willows.

This study aimed to analyse the volatile organic compounds (VOCs) emitted by six different willow cultivars (clones) used in New Zealand and to correlate differences in VOCs to different characteristics of the plants, such as resistance to insect herbivores, growth form and sex. The study asked the following research questions: 1) How do the VOC profiles of these six clones compare? 2) If selected clones have a significant difference in VOC emissions, can we correlate these differences to resistance to herbivores (GWA, *P. proxima* and others), plant growth form or plant sex? This information is vital for the selection of resistant cultivars and to understand the potential indirect impacts on other insect species (e.g., natural enemies of competing herbivores).

5.2 Materials and Methods

5.2.1 Clone selection and samples collection

Six willow clones were selected for this study. The selection was made to include both male and female plants, different growth forms (trees and shrubs) and varying degrees of susceptibility to insect pests such as GWA and willow red gall sawfly, as well as the clone frequency of use in New Zealand, and genetic distinction between selected clones (Table 5.1).

Willow cuttings were planted on 21st October 2020 (NZ Spring) at the Plant Growth Unit at Massey University, Palmerston North, New Zealand (40°22’46” S, 175°36’29” E). The soil type is Manawatu fine sandy loam (LRIS Portal, 2021), classified as fluvial recent soil (Hewitt, 2010). Medium annual rainfall in the area is 900-1000 mm, with annual average temperature of 13-14°C (Chappell, 2015).

Table 5.1: Willow *Salix* spp clones selected for volatile collection and analysis. The selection was made to include both male and female plants, different growth forms (trees and shrubs), varying degrees of susceptibility to insect pests (e.g., giant willow aphid (GWA) *Tuberolachnus salignus* and willow red gall sawfly *Pontania proxima*, as well as their frequency of use in New Zealand and genetic distinction between selected clones. Details about the morphological characteristics from Glenny and Jones (2019). Details about flowering time from Glenny and Jones (2019) and Newstrom-Lloyd et al. (2015).

Species/ Hybrid	**Code**	**Usage**	**Growth form**	**Sex**	**Susceptibility to insects**	**Flowering time**	**Leaf hairs**	**Hairs on last season's branchlets**
S. viminalis	PN220	Commonly planted for riverbank stabilisation	Shrub	Male	Very susceptible to GWA Resistant to *P. proxima*	27 Aug- 6 Oct	Absent on upper surface lamina Sparse to moderate on lower lamina surface	Present
S. purpurea	PN249	Commonly planted for riverbank stabilisation	Shrub	Female (triploid)	Moderately susceptible to GWA Resistant to *P. proxima*	7 Sept – 5 Oct	Absent on upper surface lamina Absent to moderate on lower lamina surface	Absent
S. schwerinii	PN386	Commonly planted for riverbank stabilisation	Shrub	Male	Moderately susceptible to GWA Resistant to *P. proxima*	-	Sparse to moderately dense on upper surface lamina Very dense on lower surface lamina	Absent
S. lasiolepis × ***S. viminalis***	NZ04-106-073	Recently released for riverbank stabilisation	Shrub	Male	Resistant to GWA Moderately resistant to *P. proxima*	-	Absent on upper and lower surface lamina	Absent

S. fragilis	PN218	Naturalised willow species in NZ	Tree	Female	Moderately susceptible to GWA Very susceptible to *P. proxima*	-	Absent on upper surface lamina Sparse to moderate on lower surface lamina	Can be present
S. matsudana* × *S. alba	NZ1040	Commonly planted for soil conservation	Tree	Female	Moderately susceptible to GWA Very susceptible *P. proxima*	18 Sept – 8 Oct	Absent on upper surface lamina Sparse to moderate on lower surface lamina	Absent

5.2.2 VOCs sampling

The collection of VOCs was performed from the 2nd to 4th of February 2022 using the push-pull headspace sampling method as described in Effah et al. (2020). Each willow clone had 6 plants as replicates summing to a total of 36 samples. All samples were collected during daytime hours on sunny and dry days with average temperature of 20.9°C (National Institute of Water and Atmospheric Research, 2023). The collection was performed in a randomized manner (the order of clones and clone replicates was randomised). The branches were not flowering. Healthy branches were selected and enclosed in oven-cooking bags (Glad®, Melbourne, Australia). Bags were sealed with cable binders with one inlet and one outlet at each end of the bag. We used a portable volatile assay system (PVAS22 pump, VAS Rensselaer NY) with carbon-filter to circulate air through the bag. To avoid contaminants being carried into the bags, an overpressure was created by pumping air at 1.70 L/min and pulling at 1.20 L/min. Filters with Haysep-Q polymer were attached to the outlet tubes to retain the volatiles. Negative controls were created with the same methodology by taking samples from empty bags to exclude potential contaminants in further analyses. The pump ran for two hours then the filters were removed, individually wrapped in pieces of aluminium foil and labelled. Packaged and labelled filters were then stored in a cooler box to prevent contamination and evaporation. The cooler box was immediately taken to a laboratory and filters eluted. Solvent solution used in the elution was internal standard consisting of 200 µL hexane with 10 ng/mL of nonyl acetate. Eluted samples were then stored in a -80 °C freezer until analyses.

After collection, the branches were cut just below the bag that enclosed them, and oven-dried at 60°C for 72 h. The weight of the branches was measured after drying. The volatiles measured are presented in nanograms per dry weight (g) per hour (ng/g/h).

5.2.3 Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

VOCs were analysed using the GC-2010 Plus Gas Chromatograph (Shimadzu, Japan) coupled to the AOC-20 I auto-injector, QP2010 SE- gas chromatograph-mass spectrophotometer and TG-5MS column (30 m 250 m 0.25 m) with Helium (He) used as carrier gas in flow rate of 0.5 mL/min into split mode (10:1). The injector port temperature was 250 °C and detector temperature were set up at 230 °C. The oven temperature was held at 50 °C for 3 minutes increasing by 5 °C/min until it reached 95 °C. Oven temperature was then boosted 15 °C/min until 240 °C and maintained for three minutes.

Post-run analyses were done using the Shimadzu Lab Solutions software (version 2.70) and tentative compound identification was done using the NIST (National Institute of Standards and Technology) Mass Spectral Library. Identification confirmation was achieved by comparing VOC retention times and mass spectrum with those of commercial standards whenever available. Kovats Index was calculated and compared (Kováts, 1958). Contaminants consistently identified in negative controls were excluded from further analyses.

5.2.4 Statistical Analysis

All statistical analyses in this chapter were done in RStudio (2023.06.1 Build 524 © 2009-2023 Posit Software, PBC) using R version 4.3.1. VOCs were ranked by importance using the RandomForest function according to mean decrease accuracy. Random Forest variable selection was chosen based on its ability to decrease the out-of-the-bag error compared with other statistics methods (Breiman, 2001; Ranganathan & Borges, 2010). The mean decrease accuracy (MDA) was chosen over Gini index in Random Forest analysis because we

wanted to know the importance of the volatile on the prediction of the outcome and the stability of this predictor (Nicodemus, 2011).

A principal component analysis (PCA) using "FactoMineR" and "factoextra" packages was performed. The PCA biplots and corresponding scores of variable contributions were then used to identify clusters and to visualise overall VOC differences between clones and groups of clones with similar resistance to GWA (susceptible, resistant, and moderately resistant), similar resistance to *Pontania proxima* (susceptible, resistant, and moderately resistant), and same growth form (tree or shrub). Analyses based on plant sex were not performed because most *Salix* species and hybrids used in this experiment did not have clones of both sexes. A permutational multivariate analysis of variance (PERMANOVA) using the Adonis function with Euclidean distance matrix and 999 permutations with Bonferroni p-value correction was performed to compare clones and groups of clones. Additionally, a general linear model (GLM) was done to compare total volatile emission across clones and groups. A normal, inverse and gamma GLM was performed and the type with the lowest Akaike information criterion (AIC) score was chosen.

5.3 Results

After contaminants were excluded, 37 VOCs were identified. From these 37, eleven compounds showed separation from the others based on their mean decrease accuracy (MDA) in RandomForest function with MDA values over 50, suggesting that they are good predictors of the differences between willow clones, with an OOB estimate of error rate of 25% (Figure 5.1). These VOCs included two GLVs, (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenyl-α-methylbutyrate; two monoterpenes, (*Z*)-β-ocimene and β-elemene; and seven sesquiterpenes,

α-cubebene, copaene, germacrene D, (*Z*)-β-Caryophyllene, (*E*)-α-bergamotene, (α)-farnesene, δ-cadinene (Table 5.2). These eleven compounds were used for further analyses.

The willow clone that released the highest number of volatiles was PN386 (*S. schwerinii*) which released nine different volatiles, followed by NZ04-106-073 (*S. lasiolepis* × *S. viminalis*), PN218 (S. fragilis), NZ1040 (*S. matsudana* × *S. alba*), PN249 (*S. purpurea*) and PN220 (*S. viminalis*) releasing 7, 7, 6, 5 and 4 different volatiles, respectively (Table 5.2). All six clones released (Z)-β-Ocimene. From the most important VOCs, clone PN386 did not release (*Z*)-3-hexenyl acetate and δ-cadinene. Half of the clones released (*Z*)-3-hexenyl-α-methylbutyrate, germacrene D, (Z)-β-caryophyllene and δ-cadinene. Four out of six clones released (*Z*)-3-hexenyl acetate, copaene, (*E*)-α-bergamotene and (α)-farnesene. Just two clones released β-elemene and α-cubebene (Table 5.2).

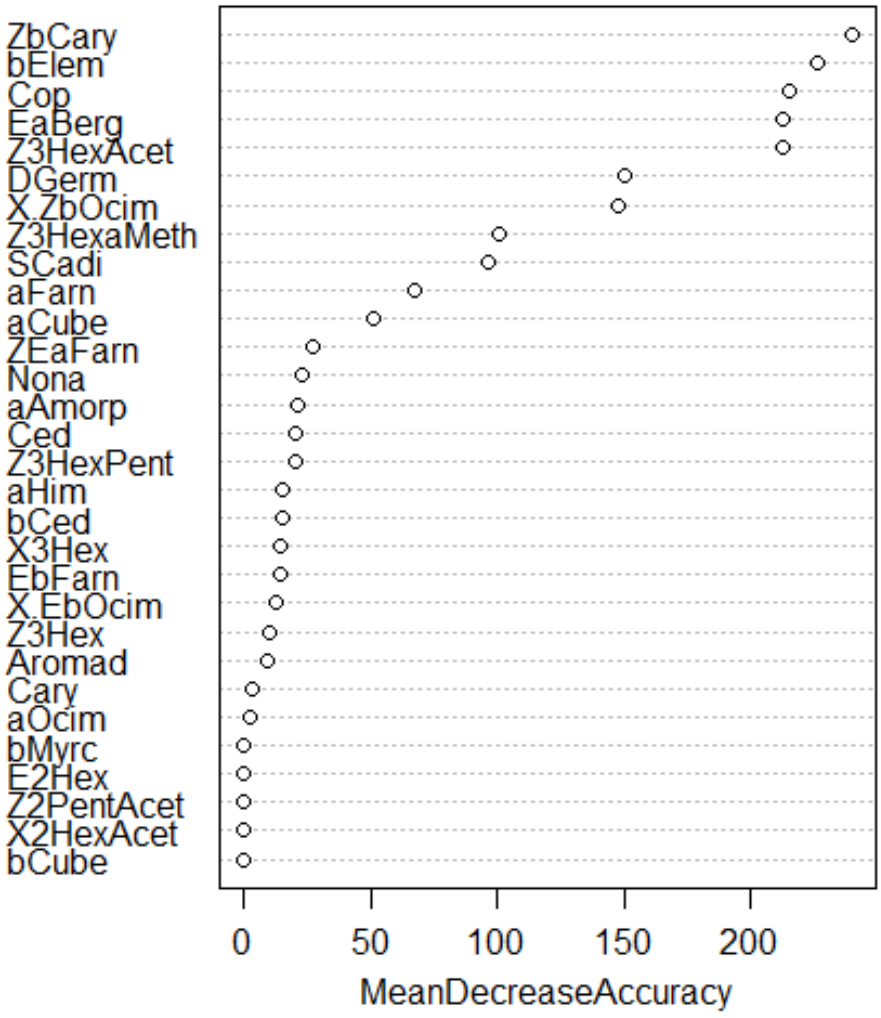

Figure 5.1: VOCs importance scores obtained by RandomForest analysis of data collected from six willow clones commonly used in New Zealand. Plotted are variable importance scores (MeanDecreaseAccuracy); the variables with higher MDA are more important in explaining observed patterns. Abbreviations: ZbCary = (Z)-β-caryophyllene; bElem = β-elemene; Cop = copaene; EaBerg = E-α-bergamotene; Z3HexAcet= (Z)-3-hexenyl acetate; DGerm = D-germacrene; ZbOcim = (Z)-β-ocimene; Z3HexaMeth = Z-3-hexenyl-α-methylbutyrate; SCadi = δ-cadinene; aFarn = α-farnesene; aCube = α-cubebene; ZEaFarn= (Z,E)-α-farnesene; Nona = nonanal; aAmorp= α-amorphene; Ced = cedrene; Z3HexPent = (Z)-3-hexenyl pentanoate; aHim = α-himachalene; bCed = β-cedrene; X3Hex= 3-hexen-1-ol; EbFarn = E-β-farnesene; EbOcim = (E)-β-ocimene; Z3Hex = (Z)-3-Hexen-1-ol; aromad = aromadendrene; Cary = caryophyllene; aOcim = α-ocimene; bMyrc = β-myrcene; E2Hex = (E)- 2-hexen-1-ol; Z2PentAcet = (Z)-2-pentenyl acetate; 2HexAcet = 2-hexen-1-ol, acetate; bCube = β-cubebene.

Table 5.2: GC-MS analysis of VOCs released from six willow clones commonly used in New Zealand. VOCs are classified by chemical group.

		Major VOC groups											
		GLVs		Monoterpenes	Sesquiterpenes								
Clone	**Species**	(*Z*)-3-hexenyl acetate	(*Z*)-3-hexenyl-α-methylbutyrate	(*Z*)-β-ocimene	β-elemene	α-cubebene	copaene	germacrene D	(*Z*)-β-caryophyllene	(*E*)-α-bergamotene	(α)-farnesene	δ-cadinene	Number of emitted VOC
PN 220	*S. viminalis*	+	0	+		0	0	0	0	+	+	0	4
PN 249	*S. purpurea*	+	+	+		0	+	0	0	0	0	+	5
PN 386	*S. schwerinii*	0	+	+	+	+	+	+	+	+	+	0	9
NZ 04-106-073	*S. lasiolepis* × *S. viminalis*	+	+	+		0	+	+	0	+	+	0	7
PN 218	*S. fragilis*	0	0	+	+	+	0	0	+	+	+	+	7
NZ 1040	*S. matsudana* × *S. alba*	+	0	+		0	+	+	+	0	0	+	6
	Frequency in six clones	4	3	6	2	2	4	3	3	4	4	3	

Figure 5.2 shows PCA ordination for eleven most important VOCs identified in Table 5.2. PC1 and PC2 together explained 40.9% of the variation in VOCs profile in willow clones. In 5.6 Appendix, Table 5.6A-1 shows the values for the dimensions of the PCA. The main contributors of PC1 were (E)-α-bergamotene, (Z)-3-hexenyl acetate and (α)-farnesene, while PC2 main contributors were copaene, germacrene D and total δ-cadinene. Distinct clusters were observed. PCA ordination suggests that clone NZ1040 had higher emission of δ-cadinene, clone PN249 had higher emission of (Z)-3-hexenyl-α-methylbutyrate and clone PN218 had higher emission of β-elemene .

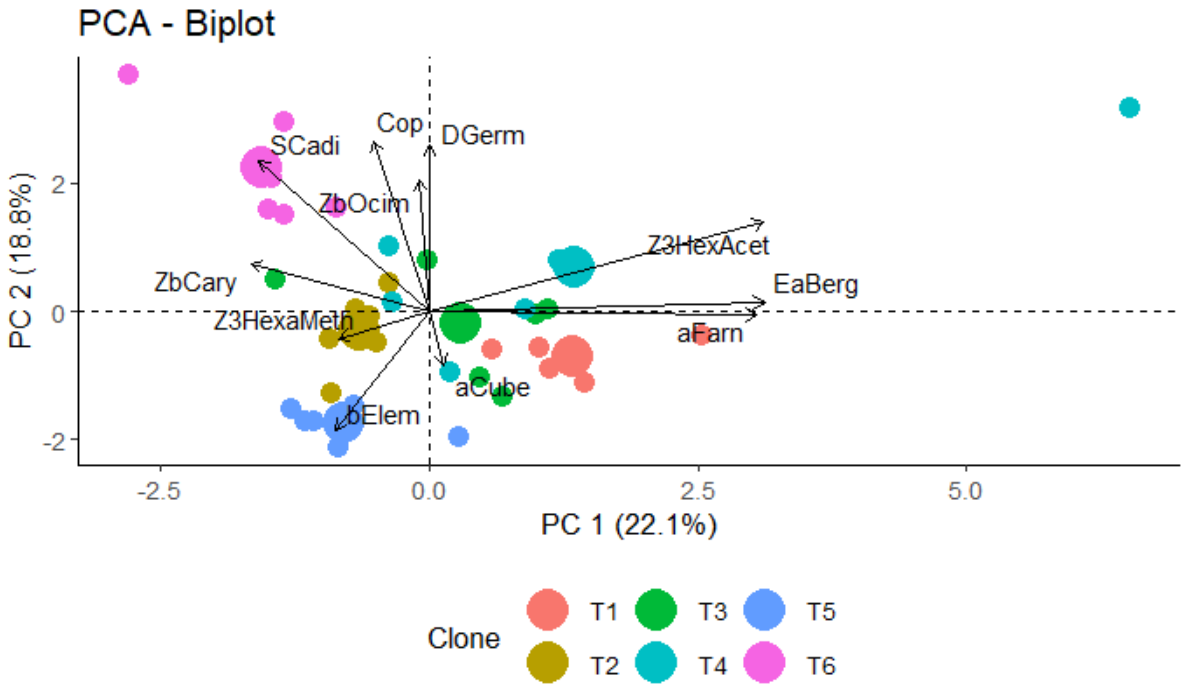

Figure 5.2: Principal component analysis (PCA) biplot showing differences in volatile compounds in different willow *Salix* spp clones. Clones: T1 = PN220, T2 = PN249, T3 = PN386, T4 = NZ 04-106-073, T5 = PN 218, T6 = NZ1040. VOCs: ZbCary = (Z)-β-caryophyllene; bElem = β-elemene; Cop = copaene; EaBerg = E-α-bergamotene; Z3HexAcet= (Z)-3-hexenyl acetate; DGerm = D-germacrene; ZbOcim = (Z)-β-ocimene; Z3HexaMeth = Z-3-hexenyl-α-methylbutyrate; SCadi = δ-cadinene; aFarn = α-farnesene; aCube = α-cubebene. Large circles are group centroids, small circles are data points.

PERMANOVA showed significant differences in VOCs among the clones (Pseudo-F=3.050; $p<0.001$). Pairwise comparisons between clones showed significant differences between all clones except within the pair PN220 (*S. viminalis*) and NZ04-106-073 (*S. lasiolepis* × *S. viminalis*), and the pair PN386 (*S. schwerinii*) and PN218 (*S. fragilis*).

Figure 5.3 shows PCA ordination for the same eleven VOCs with clones grouped according to resistance level to GWA. The percentage variation explained by PC1 and PC2, and the VOCs contributing to separation on PC1 and PC2 are the same as for Figure 5.2. No distinct clusters were observed, although the PCA ordination suggests that clones susceptible to GWA have higher emission of (*E*)-α-bergamotene and (α)-farnesene, and lower emission of germacrene D.

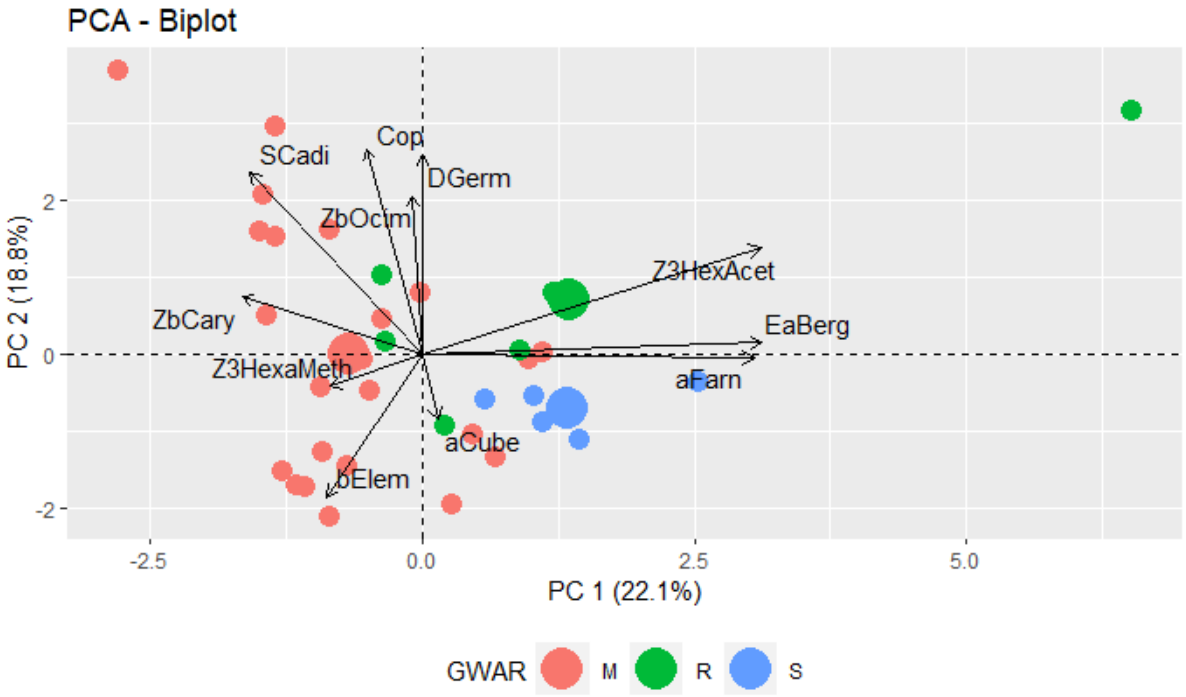

Figure 5.3: PCA biplot showing differences in volatile compounds in different willow *Salix* spp clones grouped according to resistance to the giant willow aphid (GWA). 'GWAR' indicates the clones' level of resistance to GWA – susceptible (S), resistant (R), and moderately susceptible (M). See Figure 5.2 for VOCs abbreviations.

PERMANOVA showed significant difference in VOCs among clones which were susceptible (S), moderately susceptible (M) and resistant (R) to GWA (Pseudo-F=5.816; p<0.005). However, susceptible and resistance clones did not show significant differences in VOC profiles while moderately susceptible clones showed significant difference to both susceptible and resistant clones (S x R, Pseudo-F=1.063, p=0.363; S x M, Pseudo-F=4.888, p=0.002; R x M, Pseudo-F=10.273, p<0.001).

Figure 5.4 shows PCA ordination for the same eleven VOCs with willow clones grouped according to their resistance level to *P. proxima*. The percentage variation explained by PC1 and PC2, and the VOCs contributing to separation on PC1 and PC2 are the same as for Figure 5.2. No distinct clusters were observed.

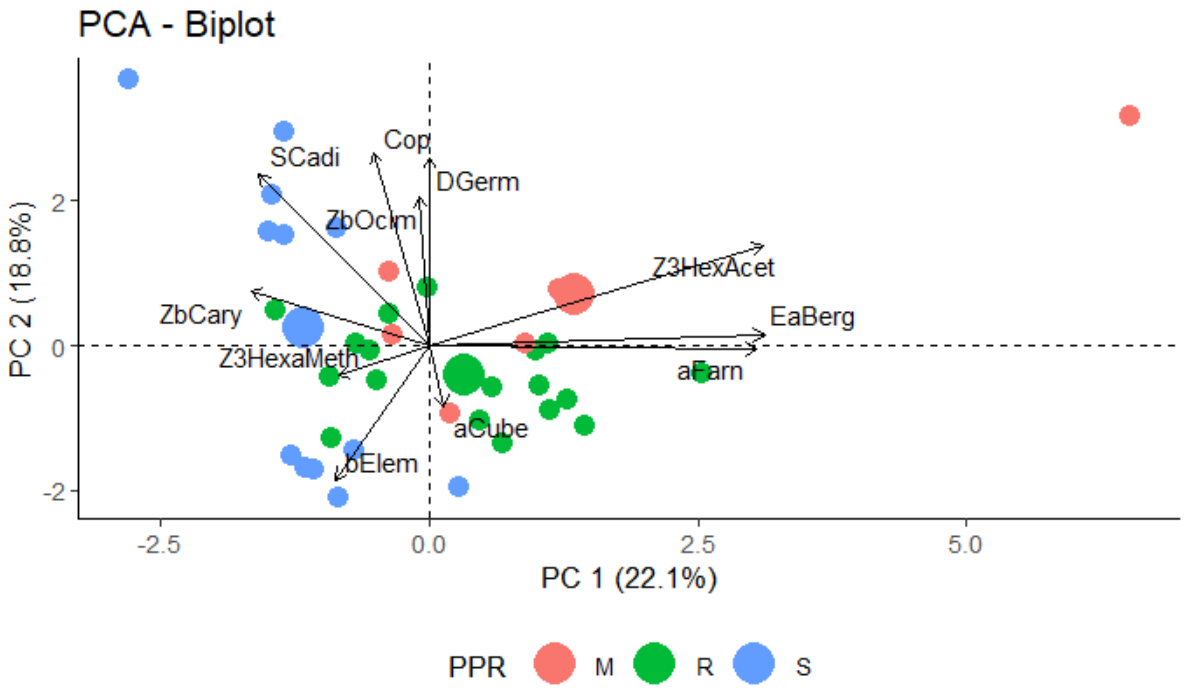

Figure 5.4: PCA biplot showing differences in volatile compounds in different willow *Salix* spp cultivars grouped according to the level of resistance to *Pontania proxima*. Larger symbols are group centroids. 'PPR' indicates the clones susceptible (S), resistant (R), and (M) moderately resistant to *P. proxima*. See Figure 5.2 for VOCs abbreviations.

PERMANOVA showed significant differences in VOCs among clones which were susceptible (S), moderately resistant (M) and resistant (R) to *P. proxima* (Pseudo-F=5.446; p=0.004). All the three groups showed significant difference (S x R, Pseudo-F=3.152, p=0.012; S x M, Pseudo-F=5.799, p=0.001; R x M, Pseudo-F=5.794, p=0.014).

Figure 5.5 shows PCA ordination for VOCs in different willow clones grouped according to the growth form (trees vs. shrubs). The % variation explained by PC1 and PC2, and the VOCs contributing to separation on PC1 and PC2 are the same as for Figure 5.2. Distinct clusters were not observed. PERMANOVA showed significant difference in VOCs between the two growth forms (shrub vs. tree, Pseudo-F=2.772; p=0.048).

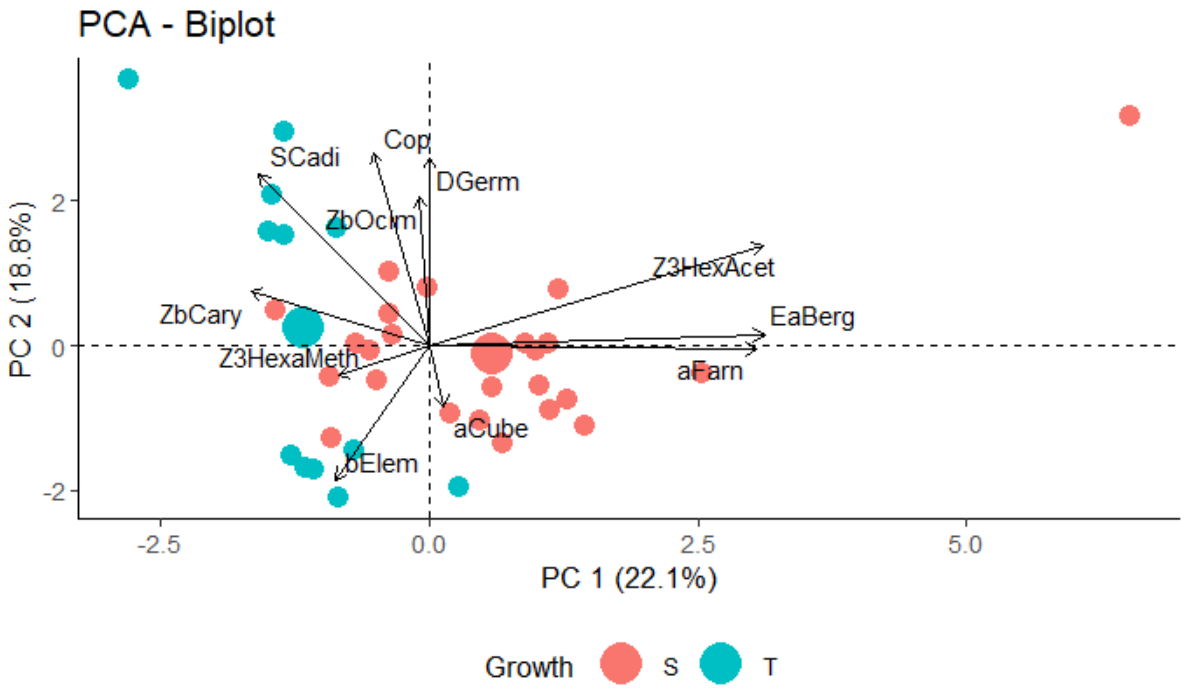

Figure 5.5: PCA biplot showing differences in volatile compounds in different willow *Salix* spp clones grouped by growth form. Larger symbols are group centroids. S – shrub, T – tree.

The general linear models (GLM) showed significant effect of clone, GWA resistance and *P. proxima* resistance, but not plant growth form, on the total VOC emission (Table 5.3, Figure 5.6, Figure 5.7 and Figure 5.8). The post-hoc Tukey test with Bonferroni correction for clones did not show significant differences among clones. Regarding GWA resistance, post-hoc test showed significant difference in total VOC emission between resistant vs. moderately susceptible clones (R x M, p=0.009; S x M, p=1.00; S x R, p=0.538; Figure 5.6). For *P. proxima* resistance, only resistant vs. moderately resistant willow clones showed significant differences in total VOC emission (R x M, p=0.034; S x M, p=0.090; S x R, p=1.00; Figure 5.8).

Table 5.3: Generalized linear model (GLM) comparing total volatile emission (ng/g/h) in different clones of willow *Salix* spp grouped by different factors. GWA= giant willow aphid. ** significant at alpha = 0.05.

Model comparison	**Df residual**	**LogLik**	**Df model**	**Chisq**	**p-value**
Clone	7	-1.3801	5	15.709	0.008**
GWA resistance	4	-1.3801	2	8.896	0.012**
***P. proxima* resistance**	4	-1.3801	2	8.1309	0.017**
Growth form	3	0.31100	1	0.7075	0.400

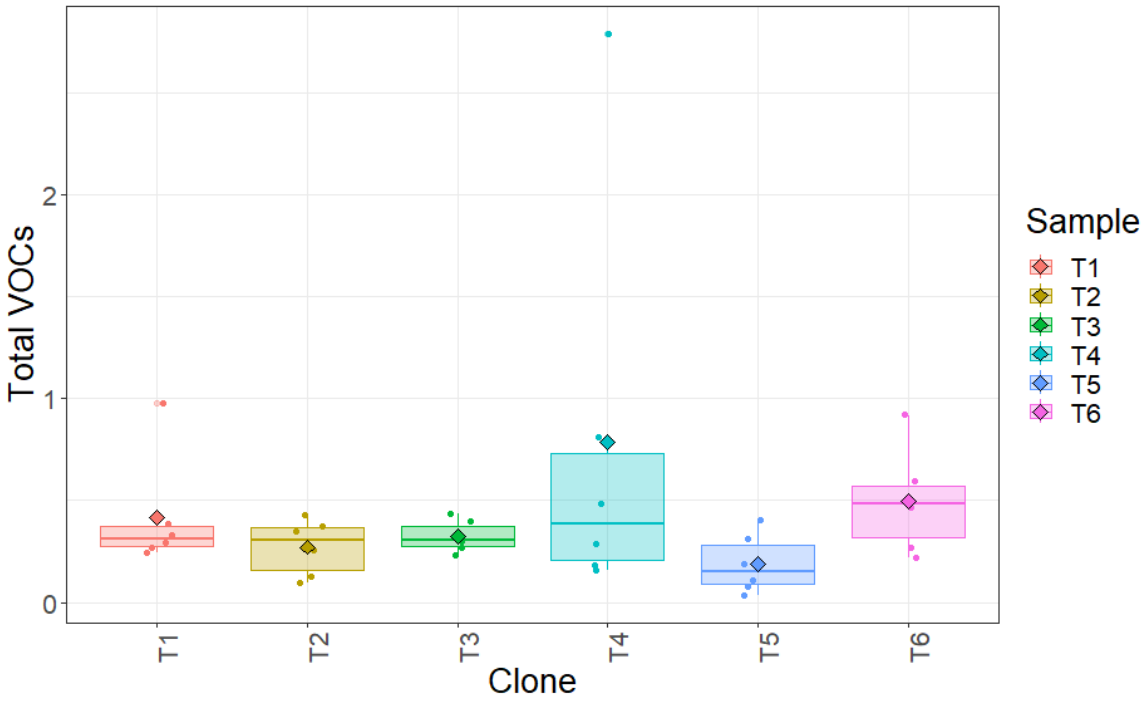

Figure 5.6: Total volatile emissions (Total VOCs) (ng/g/h) in different willow *Salix* spp clones. The median is indicated by the line across the box. The mean is indicated by the diamond. Clones: T1= PN220, T2= PN249, T3= PN386, T4= NZ04-106-073, T5= PN218, T6= NZ1040.

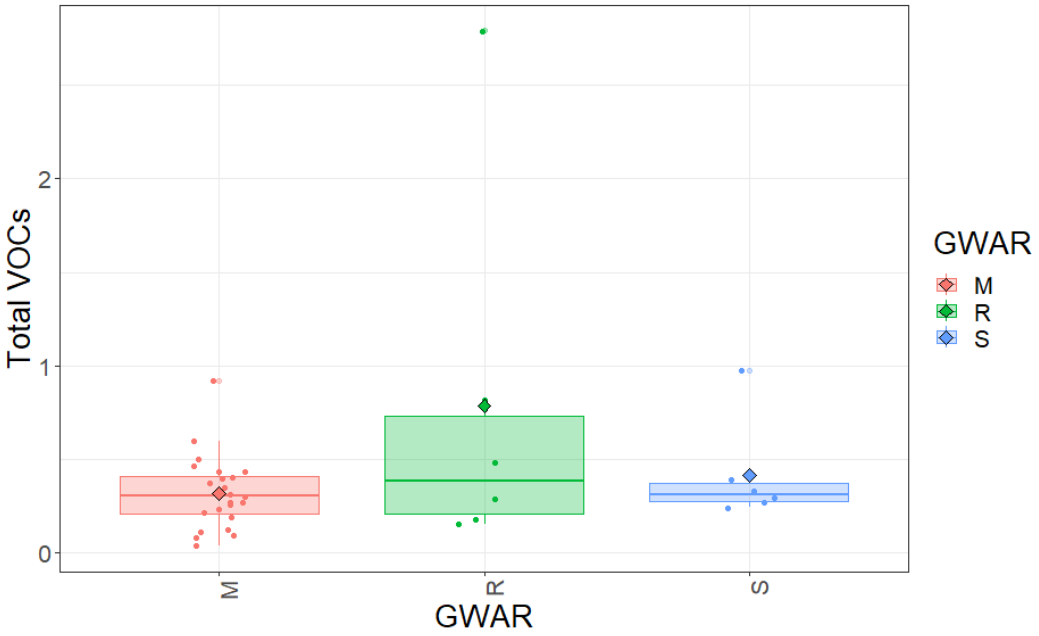

Figure 5.7: Total volatile emissions (Total VOCs) (ng/g/h) in willow *Salix* spp clones grouped based on resistance to giant willow aphid. The median is indicated by the line across the box. The mean is indicated by the diamond. GWAR= giant willow aphid resistance: susceptible (S), moderately susceptible (M) and resistant (R).

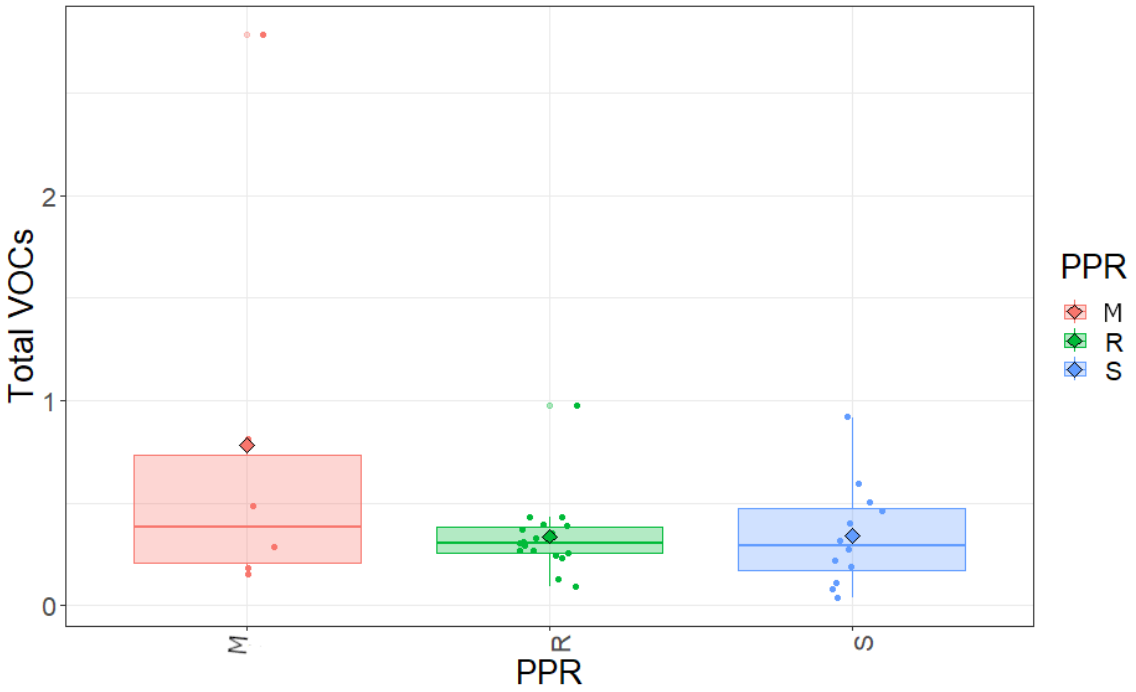

Figure 5.8: Total volatile emissions (Total VOCs) (ng/g/h) in different willow *Salix* spp clones grouped based on resistance to *Pontania proxima*. The median is indicated by the line across the box. The mean is indicated by the diamond. PPR= *Pontania proxima* resistance. Susceptible (S), moderately resistant (M) and resistant (R).

5.4 Discussion

Willow clones used for planting in New Zealand show significant variability in species, sex, growth type, chemistry, morphology and anatomy. In addition to these differences, they show different susceptibility to diseases and pests (Tun et al., 2021; Tun et al., 2020; Van Kraayenoord & Hathaway, 1986; Van Kraayenoord & Hathaway, 1987).

Keeping in mind how diverse willows are, it is not surprising that they would show differences in VOCs emissions. Our results show that the six *Salix* species considered in this chapter were significantly different in VOCs profiles, except the clone pair PN220 (*S. viminalis*) and NZ04-106-073 (*S. lasiolepis* × *S. viminalis*) and pair PN386 (*S. schwerinii*) and PN218 (*S. fragilis*). The genetic proximity of NZ04-106-073 and PN220 may explain why they

were not different in their VOCs profiles. PN386 and PN218 are not related and do not share similar features and their similarity in VOC profiles is unexpected. For total VOCs emission, the clones did not show significant differences.

Clone PN386 (*S. schwerinii*) showed the highest number of emitted VOCs among the studied clones and (*Z*)-β-Ocimene was the only VOC present in all clones. Our results are in accordance with previous studies in this region of New Zealand, where β-ocimene was also present in all clones (Tun et al., 2020). β-ocimene is an acyclic monoterpene reported to be present in a vast range of plant species (Ding et al., 2020; Fäldt et al., 2003; Huang et al., 2022; Jian et al., 2021; Navia-Giné et al., 2009). This compound has been reported as a generalist insect attractant, attracting pollinators and natural enemies of herbivores (Farré-Armengol et al., 2017; Jürgens et al., 2003; Kang et al., 2018; Krug et al., 2018; Mohammed et al., 2020; Pecetti et al., 2002). For herbivores, β-ocimene showed repellent effect in species such as *Spodoptera litura* (Lepidoptera: Noctuidae), *Myzus persicae* (Hemiptera: Aphididae), and *Ectropis obliqua* (Lepidoptera: Geometridae) (Jian et al., 2021; Jing et al., 2021; Kang et al., 2018). The effects of β-ocimene on our case study herbivores, GWA and *P. proxima*, have not been yet reported.

PCA ordination suggested that clone NZ1040 had higher emission of δ-cadinene, clone PN249 had higher emission of (Z)-3-hexenyl-α-methylbutyrate and clone PN218 had higher emission of β-elemene. Both δ-cadinene and β-elemene are sesquiterpenes and the European elm bark beetle *Scolytus multistriatus* is reported to positively respond to δ-cadinene and β-elemene (Millar et al., 1986). Literature about insect response to these specific VOCs, however, is scarce.

Growth forms (tree willows and shrub willows) showed significant differences in volatile profiles, but distinct clusters were not observed in PCA ordination; there was no difference in total VOC emissions. Tun et al. (2020) showed similar results, with shrubs and

tree showing different VOC profiles. We did not test for difference in VOCs between the two plant sexes, as sex was confounded with clone and growth form. However, Füssel et al. (2007) who found that in three out of the eight *Salix* species analysed, intraspecific differences in floral scent composition could be explained by sex. Keefover-Ring et al. (2022) analysed the floral VOCs of *S. purpurea* and found that males produced greater amounts of terpenoids in their floral volatiles, whereas females emitted more benzenoids.

We had a limited number of clones in this study; therefore, our results cannot be used to generalize for the whole *Salix* genus. Our conclusions related to the groups must be taken with caution, considering our limited number of clones – for example, resistant and susceptible 'groups' had just one clone each, while the moderately resistant group included four clones; in comparing levels of resistance, we may be just observing the clone-level differences. Similarly, plant sex, growth form and clone identity were confounded; for example, we did not have both sexes of the same species, so differences are likely to represent the clone effect. More clones would have to be studied to separate these effects.

GWA does show preference according to chemical cues, as was demonstrated by Aradottir et al. (2009). In their study, GWA attraction to willow varieties Bowles Hybrid (*S. viminalis* L), Discovery (*S. schwerinii* x (*S. viminalis* L. x *S. schwerinii* Wolf 'Bjorn')), Tordis (*S. viminalis* L. x *S. schwerinii* Wolf), Stott 10 (*S. burjatica* Nasarov x *S. viminalis* L.), Baldwin (*S. triandra* L), *S. gilgiana* Seemen, were tested in an olfactometer and the non-host poplar Unal (*Populus trichocarpa* L. x *P. deltoides* Bartr. ex Marsh) was used as control. The authors showed that GWA prefers *S. viminalis* both in the olfactometer and in the field. The insects for their study, however, were reared on the willow variety Resolution ((*S. viminalis* L. x *S. viminalis* 'Jorrun') x (*S. viminalis* x *S. schwerinii* Wolf 'Bjorn')) which is also a hybrid of *S. viminalis*. This may have influenced GWA choice, since insect herbivores often show preference for plant species on which they were reared (Barron & Corbet, 2000; Sandoz et al.,

2000; Tapia et al., 2015). Aradottir et al. (2009) did not analyse the VOC profiles of the studied clones, therefore conclusions about which VOC may attract GWA were not made.

Regarding *P. proxima* resistance, willow clones showed significant differences among each other, but no distinct clusters were observed on PCA ordination. For total VOC emission, resistant and moderately susceptible clones showed significant differences between each other. However, we failed to find a pattern that would suggest attraction or repellence to *P. proxima* in total VOC emission or in a specific VOC. The clone most susceptible to *P. proxima* in our study was PN218. This clone presented the lowest total VOC emission. The clone NZ1040 was also highly susceptible to the sawfly and presented the second highest total VOC emission. Both clones, however, are trees. This may suggest that *P. proxima* has preference for tree clones rather than shrub clones. Shanahan (1957) has raised the possibility that there may be a correlation between the adult sawfly flight height and the number of galls in higher shoots. To our knowledge, there have been no studies testing the sensitivity of *P. proxima* to specific volatile compounds, therefore we are unable to say which compounds the sawflies could be attracted to.

The relationship between willow flower volatiles (*S. fragilis* and *S.* x *rubens*) and host location by *P. proxima* was investigated by Kehl et al. (2010). The authors showed that the proportion of plants that presented galls was higher for flowering plants than for non-flowering ones. The authors linked the sensitivity of *P. proxima* to floral volatiles to the fact that nectar is the main source of energy to adult sawflies. Shrub willows flower before the leaves, but in tree willows such as *S. alba* and *S. fragilis*, the leaves and flowers open at the same time. *P. proxima* appears adapted to this behaviour, with a preference for flowering tree willows that will have newly opened young leaves. There were no catkins present on willows during the time of VOCs collection in our study, so we are unable to check if the clones more susceptible

to *P. proxima* attack had a higher floral VOC emission. This hypothesis, however, needs to be tested.

When we think about the biology of our studied insects, *P. proxima* and GWA, it is not farfetched to speculate that female *P. proxima* may rely more on VOCs emission for host location as well as being more motivated to find a new host than GWA. GWA can appear in wingless or winged form and wingless aphids live in large colonies (Blackman & Eastop, 1994). They will not disperse from tree to tree unless necessary, therefore locating a new host is not their main priority. Meanwhile, a female *P. proxima* sawfly is constantly looking for oviposition sites as well as nectar, which adults use as a food source, and VOCs may be an important component of host location. Braccini et al. (2015) reports that the willow sawfly *Nematus oligospilus* uses volatiles for distance host location and seek chemical contact clues for oviposition. The *P. proxima* antennae are reported to respond to green leaf volatiles and floral volatiles (Kehl et al., 2010). These examples support that *P. proxima* may rely more on volatiles cues than GWA. This theory, however, needs to be tested.

Plants can utilise a wide range of mechanisms of defence against insect pests. Different species of plants can release different volatiles that could attract herbivores, or they may have different concentrations or a different variety of secondary metabolites which could make them unpalatable to the insect pest (Belete, 2018; Mitchell et al., 2016; Pasteels & Rowell-Rahier, 1992; War et al., 2012). They can also have different morphological attributes such as waxy cuticles and/or the development of spines, setae and trichomes (Belete, 2018; Mitchell et al., 2016; War et al., 2012). Due to the complexity of factors that influence plant resistance to insect herbivores, multiple factors must be considered. The VOC profile alone is not enough to elucidate plant attraction or repellence to herbivores and their natural enemies.

5.5 Conclusions

Willow clones appear to have highly species-specific VOC blends. The growth form, however, can also explain some of the differences we found. Due to our limited number of clones and limited number of replicates, we could not draw definitive conclusions about the pattern of volatile emissions in relation to resistance to the two insect herbivores – *P. proxima* and GWA.

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5.6 Appendix

Table 5.6A-4: Principal component analysis (PCA) results table showing differences in organic volatile compounds (VOC) in different willow *Salix* spp clones. Abbreviations: ZbCary = (Z)-β-caryophyllene; bElem = β-elemene; Cop = copaene; EaBerg = E-α-bergamotene; Z3HexAcet= (Z)-3-hexenyl acetate; DGerm = D-germacrene; ZbOcim = (Z)-β-ocimene; Z3HexaMeth = Z-3-hexenyl-α-methylbutyrate; SCadi = δ-cadinene; aFarn = α-farnesene; aCube = α-cubebene.

VOC	Dim 1	Dm 2	Dim 3	Dim 4	Dim 5
Z3HexAcet	27.07364	6.3253	0.3597	2.5493	0.0871
ZbOcim	0.02173	13.8562	11.7126	8.5625	21.2981
Z3HexaMeth	2.0079	0.5737	26.1473	2.8277	7.8238
Cop	0.74958	23.2485	4.29	14.6013	0.0319
aCube	0.05633	2.346	8.4483	32.0253	30.1272
bElem	2.1411	11.3593	12.8705	15.4312	0.2924
DGerm	0.00003	22.0702	1.9857	2.774	20.3410
ZbCary	7.6856	1.8298	25.6807	7.782	2.8066
EaBerg	27.2675	0.07738	0.6504	6.5788	2.4615
aFarn	25.9967	0.0066	7.2047	1.3423	4.4676
SCadi	6.9999	18.3070	0.6501	5.5257	10.2628

Chapter 6 General discussion

In Chapter 2, we investigated the levels of damage in different willow clones, aiming to explore variations in *P. proxima* damage and gall development in different clones. Addicionally, we examined differences in *P. proxima* oviposition preference based on factors such as plant position or shoot position. We also discussed the lack of evidence for a cecidogenic factor for *P. proxima*.

In Chapter 3, our objective was to investigate differences in *P. proxima* larval growth among willow clones and determine whether variations in leaf nutrient content and total phenolic content influenced larval development.

Chapters 4 and 5 focused on secondary metabolites and volatile emissions, aiming to elucidate their role in insect pest resistance. In Chapter 4, we analysed the metabolomic profiles of six willow clones to assess how these profiles differed and whether these differences correlated with resistance to herbivores (GWA, *P. proxima*, and others), growth form, or induction by herbivory (GWA). Chapter 5 built upon this by examining the volatile profiles of the same six willow clones, analysing and comparing their emissions to determine potential correlations with herbivore resistance and plant growth form.

Table 6.1 provides summary of the characteristics for each clone, including their resistance to insect herbivores (GWA and *P. proxima*), their leaf morphology, as well as their significant volatiles and metabolites.

Regarding the amount of total metabolites, clone NZ04-106-073 (*S. lasiolepis* × *S. viminalis*) showed the highest amount followed by NZ1040, PN249, PN218, PN220 and PN386. For total amount of volatiles, the highest amount was emitted by NZ04-106-073 followed by NZ1040, PN386, PN249, PN220 and PN218.

Clone NZ04-106-073 was the clone that emitted the highest amount of volatiles and had the highest concentration of metabolites. Interestingly, this clone is resistant to GWA and is moderately resistant to *P. proxima* - the sawfly oviposits but the gall does not develop. Secondary metabolites are an effective way for plants to defend against insect herbivores (Belete, 2018; Kariñho-Betancourt, 2018; War et al., 2012). However, the same metabolites can be used by specialist insects for their own defence, and so they may prefer higher concentrations of secondary metabolites (Boeckler et al., 2011; Pasteels et al., 1986; Pasteels & Rowell-Rahier, 1992). Having a higher concentration of metabolites may not be advantageous to the plants depending on the herbivorous community around them, and some willows have been reported to lose their salicylates (Boeckler et al., 2011; Volf et al., 2015). Volatiles attract both herbivores and their natural enemies (Binyameen et al., 2021; Effah et al., 2019; Tumlinson, 2014; Turlings & Wäckers, 2004). Therefore, having a high emission of volatiles may not necessarily mean that the plant is more resistant to a specific insect. The blend of volatiles may play a more important role in determining resistance.

Table 6.1: Selected willow *Salix* spp clones used in Chapters 2-5 and the survey information, highlighting different levels of resistance of the clones to red gall sawfly *Pontania proxima* and giant willow aphid (GWA) *Tuberolachnus salignus*. Clones were selected based on their genetic diversity between themselves, sex, and level of resistance to insect pests. Galling levels: 1 – no galls, 5 – extensive galling. All clones showed chewing damage. Details about the morphological characteristics from Glenny and Jones (2019).

Species/Hybrid	**Code**	**Growth form**	**Sex**	**Susceptibility to insects**	**Highest emitted volatile**	**Highest compound concentration**	**Leaf hairs**	**Hairs on last season's branchlets**
***S. viminalis* L.**	PN220	Shrub	Male	Very susceptible to GWA Resistant to *P. proxima*	(*E*)-α-Bergamotene	isorhamnetin, isorhamnetin-3-O-glucoside and isorhamnetin-3-O-rutinoside	Absent on upper surface lamina Sparse to moderate in lower lamina surface	Present
***S. lasiolepis* Benth.** × ***S. viminalis* L.**	NZ04-106-073	Shrub	Male	Resistant to GWA Moderately resistant to *P. proxima*	(*Z*)-3-Hexenyl acetate	unknown compound 1, apigenin, epicatechin, procyanidin B1 and B2, kaempferide and kaempferol-3-glucuronide	Absent on upper and lower surface lamina	Absent
***S. purpurea* L.**	PN249	Shrub	Female (polyploid)	Moderately susceptible to GWA. Resistant to *P. proxima* (galling level 1)	(*Z*)-3-Hexenyl-α-methylbutyrate and (*Z*)-β-Ocimene	peonidin-3-O-beta-D-glucoside, and luteolin-7-O-glucoside	Absent and lower surface lamina Absent to moderate in lower lamina surface	Absent
***S. purpurea* L.**	PN221	Shrub	Male	Galling level 1, no presence of GWA in the field	no data	no data	Absent on upper and lower surface lamina	Absent
***S. schwerinii* E. Wolf**	PN386	Shrub	Male	Moderately susceptible to GWA.	α-Cubebene and α-Farnesene	quercetin-7-O-rhamnoside	Hairs sparse to moderately dense on upper surface lamina	Absent

				Resistant to *P. proxima*			Very dense on lower surface lamina	
***S. fragilis* L.**	PN218	Tree	Female	Moderately susceptible to GWA. Very susceptible to *P. proxima* Galling level 4, no presence of GWA in the field			Absent on upper surface lamina Sparse to moderate on lower surface lamina	Can be present
***S. fragilis* L.**	PN736	Tree	Male	Galling level 5, presence of GWA in the field	no data	no data	Absent on upper surface lamina Sparse to moderately dense on lower surface lamina	Absent
***S. fragilis* L.**	PN742	Tree	Male	Galling level 5, presence of GWA in the field	no data	no data	Absent on upper surface lamina Sparse to moderately dense on lower surface lamina	Absent
***S. matsudana* Koidz.**	PN721	Tree	Male	Galling level 2, no presence of GWA in the field	no data	no data	Absent on upper and lower surface lamina	Absent
***S. matsudana* Koidz.**	PN693	Tree	Female	Galling level 2, no presence of GWA in the field	no data	no data	Absent on upper and lower surface lamina	Absent

***S. alba* L.**	PN357	Tree	Male	Galling level 3, presence of GWA in the field	no data	no data	Sparse to moderately dense on lower and upper surface lamina	Present
***S. alba* L.**	PN676	Tree	Female	Galling level 3, no presence of GWA in the field	no data	no data	Sparse to moderately dense on lower and upper surface lamina	Present
***S. alba* L.**	PN356	Tree	Male	Galling level 4, presence of GWA in the field	no data	no data	Sparse to moderately dense on lower and upper surface lamina	Present
***S. matsudana* Koidz. × *S. alba* L.**	NZ1040	Tree	Female	Moderately susceptible to GWA. Very susceptible *P. proxima* Galling level 3, no presence of GWA in the field	D-Germacrene and (*Z*)-β-Caryophyllene		Absent on upper surface lamina Sparse to moderate on lower surface lamina	Absent
***S. matsudana* Koidz.*x S. alba* L.**	NZ1130	Tree	Male	Galling level 3, heavy presence of GWA in the field	no data	no data	Absent on upper surface lamina Sparse to moderately dense on lower surface lamina	Absent

Secondary metabolites alone cannot fully explain the preference/resistance of *P. proxima* or GWA but our study did show evidence that they play an important role in insect pest development and/or attraction to willows. Both GWA and *P. proxima* are specific pests of willows. The behaviour of phenol glycoside specialist insects and generalists may be different, and specialists may prefer higher concentrations of phenol glycosides and even take advantage of the presence of these compounds (Boeckler et al., 2011; Kosonen et al., 2012; Pasteels et al., 1986; Pasteels & Rowell-Rahier, 1992). As discussed in Chapter 3, *P. proxima* in New Zealand seem to prefer plants with lower phenolic content in contrast with other populations of *P. proxima* in other parts of the world. Due to this association, predicting the exact role that secondary metabolites play in the resistance becomes more challenging. Furthermore, the effects of specific secondary metabolites have not being tested in *P. proxima* or GWA.

Similar to *P. proxima,* GWA also seem to prefer for willows with lower total secondary metabolites. The most susceptible clone to GWA, PN220 (*S. viminalis*), showed the second lowest concentration of phenolics and second lowest total emission of volatiles. On the contrary, clones NZ04-106-073 and NZ1040 which had the highest total emission of VOCs and highest total concentration of metabolites were clones which were resistant and moderately resistant to GWA.

In our study, *P. proxima* showed better development in clones PN742 and PN736 which belong to the species *S. fragilis*. The clone that showed the poorest *P. proxima* larval development was clone PN676, which belongs to the species *S. alba*.

Importantly, *P. proxima* display preferences for clones that produce leaves earlier in the season, even if these clones do not provide optimum conditions for larval development (Kehl & Rambold, 2011).

6.1 Resistance of willows to willow red-bean sawfly *Pontania proxima*

Our results show that twelve *Salix* spp. clones (Chapter 2 and Chapter 3 of this thesis) display differences in susceptibility to *P. proxima*, with some clones (PN221 and PN249) not developing galls and other clones (PN693 and PN721) not developing fully formed galls, suggesting that these clones do attract ovipositing *P. proxima* females, but the larva, and therefore the gall, do not develop. Clones that developed fully formed galls presented different levels of larval development. Clones PN676, NZ1130 and NZ1040 supported significantly smaller *P. proxima* larvae than PN732 and PN742, which can be potentially linked to secondary metabolites and plant nutrients. For example, clone PN742 showed the highest content of crude protein in leaf tissue, while clones PN221 and PN676 had the lowest protein levels. This is well in accordance with the *P. proxima* larval development data, where we found that clone PN742 had the largest larvae, while PN676 had the smallest larvae. This suggests that clone PN742 provides more nutrition and therefore the larvae can develop better. The Pearson correlation coefficient was computed to assess the linear relationship between gall size and larvae size (measured as head capsule width, please see Chapter 3). There was no significant correlation between the two variables ($r(204) = 0.118$, $p = 0.091$). This fact may be evidence that the development of the gall depends on plant responses which are intrinsic to species. The larval development relates to the nutrition that the plant offers, and larval development may not affect gall development. This theory, however, needs to be investigated. No clear correlation between total leaf damage and the average gall size was observed, reasons are discussed in Chapter 2.

6.2 Role of hybridization

What is interesting to address, is the species role in the resistance to damage, not just the clone. *P. proxima* seem to prefer tree willows to shrub willows, with tree willows having the highest level of damage. The effect of hybridization is also visible, with *S. matsudana* clones (PN721 and PN693) not developing fully formed galls, but their hybrids with *S. alba* (NZ1040 and NZ1130) developing galls and able to support *P. proxima* larval development.

There are some hypotheses regarding plant hybridisation and resistance to galling insects. Boecklen and Larson (1994) tested hybridisation hypotheses in eight species of gall-forming wasps (Hymenoptera: Cynipidae) in *Quercus grisea* x *Q. gambelii* oak hybrids. The null hypotheses were: H_0 no differences between the two host taxa, H_1 hybrid hosts support higher densities of herbivores than parental taxa, H_2 hybrid hosts support intermediate densities than parental taxa, and H_3 hybrid hosts support lower densities of herbivores than parental hosts. Evidence to support each hypothesis was found in different species. H_0 was supported by two species, H_1 were supported by two species, H_2 by three species and H_3 was supported by one species. Total herbivore population supported H_2. The authors concluded that there is no universal rule for density variation in Cynipidae.

Hallgren (2003) studied the effects of hybridization on performance, development and survival rates of *Phratora vitellinae* (L. 1758, Coleoptera: Chrysomelidae) in *S. repens* L., *S. caprea* L. and their Fl hybrids. Hybrids between *Salix caprea* and *S. repens* are intermediate between the two parental species in phenolic glucoside concentration. Authors found that *P. vitellinae* has a higher development rate on *S. repens,* a species with high concentrations of phenolic glucosides, and equally low development rate on *S. caprea* and the Fl hybrid. They concluded that would be beneficial for the insect to have an ovipositional preference for *S.*

repens instead of *S. caprea* and intermediate preference for Fl hybrids. In our study, the hybrids seem to support the same density level of herbivores as one of the parental lines.

Orians et al. (1997) investigated the preference and performance of five leaf-feeding beetle species (*Calligrapha multipunctata bigsbyana* (Kirby), *Plagiodera versicolora* (Laicharting), *Chrysomela knabi* Brown, *Chrysomela scripta* Fabricius and *Popillia japonica* Newman) on two species of willows (*Salix sericea* and *S. eriocephala*) and their hybrids. The preference of the beetles for willow hybrids or the parental lineages was different for different species but experiments with different concentrations of purified salicortin demonstrated that this compound may play a role in insect preference. The beetles' performance also varied according to species and their preference did not match with their best performance. The authors concluded that the tolerance of insects for phenol glycosides plays a role in attraction and performance on willow plants, whether the insect is a specialist or not. Torp et al. (2013) investigated the willow hybrids resistance to the beetle *Phratora vulgatissima.* The authors used F2 hybrids of *Salix viminalis* and *Salix dasyclados* and found different patterns of resistance. Beetle survival on the hybrids was similar to that on one of the parents, while oviposition was intermediate. The most interesting part of this study was the consequences of hybridization on secondary metabolites, with novel compounds appearing in hybrids besides differences in concentrations. The authors concluded that genomic alterations led to changes in biosynthetic pathways.

6.3 Role of plant sex

Willows (*Salix* spp.) can vary in concentration of phenol glycosides and other phenolic compounds such as flavonoids and tannins depending on diverse factors such as species and depending on the plant tissue analysed (Boeckler et al., 2011; Hegnauer, 1973; Julkunen-Tiitto,

1986; Pasteels & Rowell-Rahier, 1992; Thieme, 1965; Torp et al., 2013). Differences in phenolic content can also be observed between sexes, with males having lower amounts of phenolics (Price et al., 1989; Ruuhola et al., 2018).

Füssel et al. (2007) studied the floral composition of eight *Salix* species and concluded that intraspecific differences in floral scent composition could be explained by sex. Keefover-Ring et al. (2022) analysed the floral VOCs of *S. purpurea* and found that males produced greater amounts of terpenoids in their floral volatiles, whereas females emitted more benzenoids. Tollsten and Knudsen (1992) tested the differences in floral scent in three different willow species: *Salix caprea*, *S. cinerea* L., and *S. repens*, and found that *Salix caprea* and *S. cinerea* showed very similar composition in scent between sexes while *S. repens* showed a lower resemblance. The authors concluded that species where the floral scents are more similar are more adapted to insect pollinators.

Plant sex may also play a role in our study, since PN676, a female clone of *S. alba*, presented a higher content of phenolic compounds and was not as advantageous for larval development as the male clones of *S. alba* (PN357 and PN356). In Chapter 4 and 5, the analysed clones were too few in number to analyse for effect of plant sex. To draw definitive conclusions on the role of plant sex, we would have to increase our number of replicates, to have a sufficient number of clones from the same species but with different sexes.

6.4 Host choice

The question that remains is: why would the sawfly females choose a host that does not offer the optimum conditions for larval development, like clone PN676? Many theories about oviposition preference had been developed, e.g., patch dynamics hypothesis, the time

hypothesis, and the parasite/grazer hypothesis (Thompson, 1988). Here we are going to discuss the cases more applicable to our study case of *P. proxima*.

Experiments by Wasserman and Futuyma (1981) revealed that oviposition preference of *Callosobruchus maculatus* Fabricius (Coleoptera: Bruchidae) for pigeon peas was negatively correlated with the number of eggs laid in a container. The authors attributed this preference to the avoidance by the beetle of beans marked with the oviposition pheromone. We hypothesise that this may be happening with *P. proxima*. When the most suitable clones achieve a high number of oviposition sites, the sawfly females may switch to using less suitable clones to ensure the survival of the offspring. This may not benefit a specific individual, but at a population level may assure a higher total number of individuals for the next generation.

An interesting study developed by Cha et al. (2009) investigated if exotic predators can change preferences of herbivores for certain hosts. They compared the efficiency of defence in a native beetle species *Chrysomela knabi* after feeding on two species of native willows, one high in phenolic glycosides (*Salix sericea*) and another low in phenol glycosides (*Salix eriocephala*). *C. knabi* showed preference and better performance on phenol glycoside-rich *S. sericea*. The insect also produced a higher salicylaldehyde content, consequently showing reduced predation by generalist exotic predators, ladybird *Harmonia axyridis* larvae and juvenile praying mantis *Tenodera aridifolia*. The authors concluded that the preference of the herbivore for *S. sericea* was due to better development and better larval survival. It's important to note that *C. knabi* is a specialized herbivore. Specialists are able to tolerate and even take advantage of phenol glycosides presence (Boeckler et al., 2011; Kosonen et al., 2012; Pasteels et al., 1986; Pasteels & Rowell-Rahier, 1992). The same behaviour may not appear in generalist herbivores. This may be another possibility why *P. proxima* chooses clone PN676 as a host although that clone does not give optimal larval survival conditions with high level of phenolics as demonstrated in Chapter 3. Denno et al. (1990) also found similar results when analysing

the oviposition patterns, larval performances and susceptibility to predators in two insects – *Phratora vitellinae*, a beetle Salicaceae specialist, and *Galerucella lineola,* a generalist beetle – in two *Salix* species rich in phenol glycosides, *Salix fragilis* and *S. dasyclados*, and one species poor on those compounds, *S. viminalis*. Although insect larvae developed well on *S. viminalis* in a predator-free environment, *P. vitellina* adults avoided ovipositing on it which authors concluded was due to the lower defences of larvae against predators when larvae fed in phenol glycosides-poor diet.

6.5 Effects of galls on plant chemistry and fitness

Our plants were in the field in natural conditions when volatiles were collected. GWA was not present and chewing damage was not identified. The presence of galls, however, was identified.

Our results showed that clone PN221, which did not present galls, and clone PN676 with low level of galling and poor *P. proxima* larval development, showed the highest levels of phenolics, while clone PN742 with highest level of galling and the best larval development had the lowest phenolic content. These results are in contrast with Hjältén et al. (2007) and Soetens et al. (1991) who found that the galling caused by the sawflies *Pontania triandrae* and *P. proxima*, respectively, were higher in species of willows with a higher phenolic content. Our study, however, analysed whole leaves with galls and whole leaves without galls, and the level of phenolic content within the gall itself may be different. An interesting study correlating the gall morphology with gall chemistry was performed by Nyman and Julkunen-Tiitto (2000). This study examined the concentration of 36 phenolic compounds in galls caused by six different species of monophagous sawflies belonging to the genus *Pontania*. The authors compared the concentration of phenolic compounds in gall cortex, gall interior, galled and

ungalled leaves, and found that gall interiors had fewer and different low molecular weight phenolics than leaves, with concentration of non-tannin compounds markedly reduced in the gall interior compared to leaves. On the other hand, concentration of condensed tannins was commonly higher in gall interior than in the leaves, although high concentration could also be found in gall exterior.

When we look into the literature for general gall chemistry, an interesting meta-analysis conducted by Hall et al. (2017) comes up. The authors analysed articles from 1982 to 2013 to access the available data on the chemical relationship of galling insects and host plant chemistry. Based on the available literature, the authors concluded that the ability to manipulate plant chemistry is broadly distributed among galling insects, independently of the insect taxa, host plants and habitats. Phenolics and tannins in the entire host plants showed an increase while VOCs showed no changes. The authors concluded that an increase in the concentration of these compounds can be a strategy to reduce competition with other herbivores, while a lack of change in VOCs can be a strategy for protection against natural enemies that can use olfactory cues for prey/host recognition.

Gall insects have been reported to suppress plants defence responses (Borges, 2018; Hall et al., 2017; Tooker & De Moraes, 2008; Tooker et al., 2008). Consistently, infested plants showed a higher emission of farnesene, β-bourbonene and eucalyptol. Alterations on the volatile blend was also found for gall midge *Dasineura oleae* in olive trees (Angeli et al., 2022).

The effect of galling parasites in plants can also be positive for the plant. Rocha et al. (2013) reported that *Eucalyptus camaldulensis* Dehnh plants infected with galls induced by *Leptocybe invasa* Fisher & LaSalle (Hymenoptera; Eulophidae) were less susceptible to cold injury than neighbouring plants without galls. The authors attributed the cold resistance difference to physiological changes induced by *L. invasa* which might have a positive indirect effect by inducing frost resistance. Tooker et al. (2008) investigated the indirect defensive responses of

goldenrod (*Solidago altissima*) induced by two gall-inducing species, the tephritid fly *Eurosta solidaginis* and the gelechiid moth *Gnorimoschema gallaesolidaginis* and two non-galling insects the meadow spittlebug, *Philaenus spumarius*, and *Heliothis virescens*, a generalist caterpillar. The authors found that the generalist caterpillar induced strong indirect defences while the gall-formers and the spittlebugs did not. However, infestation by *E. solidaginis* suppressed subsequent attacks by the generalist caterpillar. Klimm et al. (2020), however, obtained contrasting results when examining VOC emission of oak tree in response to infestation by gall wasp (*Neuroterus quercusbaccarum*).

6.6 Role of leaf morphology

Plants can utilise a wide range of mechanisms of defence against insect pests. Different species of plants can release different volatiles that could attract enemies of herbivores, or they may have different concentrations or different variety of secondary metabolites which could make them unpalatable to the insect pest (Barbehenn & Peter Constabel, 2011; Belete, 2018; Pasteels & Rowell-Rahier, 1992; War et al., 2012). Plants can also have different morphological attributes such as waxy cuticles and/or the development of spines, setae and trichomes. Epicuticular lipids can influence oviposition, movement and feeding of insect herbivores (Alfaro-Tapia et al., 2007; Bernaola et al., 2021; Brennan & Weinbaum, 2001; Eigenbrode & Espelie, 1995; Rutledge & Eigenbrode, 2003; White & Eigenbrode, 2000). Plant surface wax can also influence population numbers of parasitoids and predators (Eigenbrode & Espelie, 1995; Eigenbrode & Jetter, 2002; Eigenbrode et al., 1995).

As *P. proxima* oviposits in willow leaves, the most important morphological characteristics may be leaf tissue tenderness, chemical differences in foliar wax, thickness of cuticle wax and trichome density. In Chapter 3 we discuss the preference of *P. proxima* for

willow clones with glabrous (without trichomes on upper surface) leaves. Among clones considered in this thesis (Table 6.1), the *S. alba* species and *S. schwerinii* show the highest levels of leaf pilosity, and the species *S. matsudana*, *S. purpurea* and *S. fragilis* have the glabrous leaves (Glenny & Jones, 2019; Van Kraayenoord & Hathaway, 1987). *Salix schwerinii* (clone PN386) was resistant to *P. proxima* in our study. On the other hand, *Salix purpurea* (clone PN249) was also resistant to *P. proxima* but does not have trichomes on upper leaf surface (Glenny & Jones, 2019; Van Kraayenoord & Hathaway, 1987).

Existing literature suggest that trichome density does play a role in *P. proxima* resistance. Soetens et al. (1991) correlated the level of pilosity in willow leaves with the number of galls caused by *P. proxima*. The authors found more galls in *Salix* species with higher levels of phenol glucosides and lower trichome density. Kehl and Rambold (2011) correlated *P. proxima* population abundance with phenological and morphological characteristics of *Salix*, and also confirmed that *P. proxima* prefers clones with a lower trichome density. Our results are in accordance with findings showing that *P. proxima* prefers willows with a lower density of trichomes – however, due to our lower number of resistant clones, we cannot conclude that this is the main reason for resistance to *P. proxima;* the leaf pilosity alone cannot explain preference and was not quantified in our study.

For GWA, which is a phloem feeder that usually feeds on the branches and shoots of willows, leaf characteristics may not be as important as the bark thickness and/or trichomes on the young bark. Regarding branchlets trichome density, three of our clones have trichomes on the bark of branchlets: PN218 (*S. fragilis*), PN386 (*S. schwerinii*) and PN220 (*S. viminalis*) (Glenny & Jones, 2019; Van Kraayenoord & Hathaway, 1987). Among these clones, PN220 is susceptible to GWA and the other two are moderately susceptible. To our knowledge there are no studies correlating GWA resistance and trichome density in willows. In other species of aphids, however, more information can be found suggesting negative correlation with trichome

density on the host plant (Amin et al., 2017; Hao et al., 2020; Singh et al., 2021; Triplett et al., 2023).

The number of aphids *Aphis gossypii* Glover in ash gourd was found to be negatively correlated with trichome density (Khan et al., 2000). Varieties of sorghum resistant to aphid *Melanaphis sorghi* (Theobald) are also reported to have a higher trichome density (Triplett et al., 2023). Not just the density of trichomes is important but also the type of trichomes. Cho et al. (2017) analysed trichome morphology and aphid resistance and found that honeydew spots from the aphid *Macrosiphum euphorbiae* were negatively associated with glandular trichome density in wild species of potatoes *S. tuberosum*. The glandular exudate of type IV glandular trichomes (glandular trichome type IV are elongated with a glandular cell in the tip, trichome base is unicellular and flat (Glas et al., 2012)), is proven to cause delay in the time to first probe, reduction in the number of probes and a decrease in the feeding time of *M. euphorbiae* in wild tomato (Goffreda et al., 1988). Blanco-Sánchez et al. (2021) concluded that exudate from type IV glandular trichomes of tomato caused an overexpression of detoxication markers in *M. euphorbiae* demonstrating their detrimental effects on the aphid. Similarly, the aphid *Myzus persicae* (Sulzer) is reported to be significantly affected by the trichome density of IV type glandular trichome in tomato plants (Simmons et al., 2003). The literature for aphids seems to suggest that aphids are more sensitive to glandular trichomes than non-glandular. As GWA is a very big aphid, it may be less sensitive to trichome density than smaller aphid species.

Willows seem to enhance their trichome density following herbivory events (Björkman et al., 2008; Dalin & Björkman, 2003; Dalin et al., 2004). Depending on the willow species, the reaction to herbivores may be different. *Salix cinerea* increased trichome density after herbivory by adults of beetle *Phratora vulgatissima* L., and larval feeding decreases after that change (Dalin & Björkman, 2003). In *S. viminalis*, however, there was an overall decrease in

trichome density within the plant although leaves of beetle-defoliated plants had a higher trichome density compared to control plants (Dalin et al., 2004). The subsequent larval growth and feeding was not altered by changes in trichome density. The larvae appeared to be removing trichomes while feeding (Dalin et al., 2004). Soetens et al. (1991) investigated the influence of trichome density and phenol glucoside content on the distribution of *Phratora vitellinae* (L.), *Plagiodera versicolora* Baly (Coleoptera: Chrysomelidae) and *P. proxima* (Lepeletier 1823) (Hymenoptera: Tenthredinidae) in 76 willow hybrids (*S. alba* x *fragili*s). The abundance of *Ph. vitellinae* larvae, adults of *Pl. versicolora* and galls of *P. proxima* were correlated positively with a high phenol glycoside content and a low pilosity of the leaves. The distribution of adults of *Ph. vitellinae* and larvae of *Pl. versicolora* were not influenced by phenol glycosides nor by leaf pilosity. The influence of trichomes on insect predators were also investigated. Björkman and Ahrné (2005) studied the influence of trichome density of willow *S. cinerea* leaves on two natural enemies of *P. vulgatissima* – *Anthocoris nemorum* L. (Heteroptera: Anthocoridae) and *Ortothylus marginalis* L. (Heteroptera: Miridae). The authors discovered that the efficiency of those predators was not influenced by trichomes density in the greenhouse or in the field.

6.6 Conclusions

Our results help to elucidate *P. proxima* relationship with its host *Salix* spp., the differences that relationship may have in a unique environment such as New Zealand, and the consequences for herbivores-host relationship. We hope that our results will help to fill up the gaps in the knowledge and help to elucidate the possible differences in the NZ willow-insect herbivores system and the systems in other parts of the world. These advances will be important for plant breeding and sustainable pest management in NZ.

Similar to what was found by Tun et al (2020), the VOCs in willow clones appear to be species-specific and are not clearly linked to insect resistance. We suggest that the levels of phenolic compounds and pilosity together better explain the preference of oviposition of *P. proxima.* The highest amount of secondary metabolites was found in clones NZ04-106-073 (*S. lasiolepis* × *S. viminalis*, Female), PN676 (*S. alba* L., Female) and PN221 (*S. purpurea* L., Male). NZ04-106-073 also showed the highest emission of VOCs. Clone sex is an important factor for concentration of secondary metabolites. In clones in the same species that present different sex, females show a higher amount of secondary metabolites and higher resistance to *P. proxima*. The most susceptible clones to *P. proxima* were PN736 (*S. fragilis* L., Male) and PN742 (*S. fragilis* L., Male). Tree willows are preferred by *P. proxima* to shrubs. It was not yet investigated if New Zealand willow cultivars have different defence strategies compared with cultivars used in other parts of the world.

Our results help to elucidate the metabolomic profile of New Zealand willow clones and also help to build a bridge between the knowledge we have about types of resistance (e.g., antibiosis - adverse effects plants may have on herbivores such as reduction of growth, survival, and/or fecundity - or antixenosis - plant characteristics that affect herbivores reducing the preference or acceptance for that plant (Stout, 2014)) willows have against their insect pests in New Zealand. This improves our knowledge about the strategies willows have against their pests and what may have changed in herbivore- host relationship in the New Zealand environment. Our study is also important to further knowledge about willow clones developed in New Zealand and to help with the development of new varieties. We hope our contribution will help to improve the knowledge in willow resistance to insect herbivores and help select desirable characteristics for plant breeding.

6.7 Knowledge gaps for future research

The thesis results provide further insight into willow resistance to insect pests. Given the lack of studies about *P. proxima* and GWA, the current study covered some knowledge gaps, but also brought to light additional gaps. These gaps are:

- Since New Zealand is such a unique environment and invasive species often behave differently, is there any differences between species of willows in New Zealand than in other parts of the world? Do willows in New Zealand have more secondary metabolites? Perhaps less? What about morphology? Is it the same? I believe a deeper investigation of impacts that the New Zealand environment may have on willows is worth to be investigated.
- I explored the chemistry of willows and their impact on insect pests but there are insufficient data on willow anatomy and morphology (e,g, bark thickness, leaf cuticle thickness, trichome density) and how these impact willow resistance to GWA or *P. proxima*.
- I have explored the metabolomics profiles of willow clones, but the effects of the individual metabolites on GWA and *P. proxima* need to be studied. Would some of them have an attractive effect? Would some of them help the insect development? Is GWA able to metabolize some compounds and produce glucose like other pests of willows?
- My study of volatiles in willows was restricted to a small set of clones. A larger number of clones should be tested, including male and female clones from the same species, and possibly phenological changes in volatiles.
- To our knowledge there are no studies testing antennal or behavioural response of *P. proxima* or GWA to individual willow volatiles or blends. This information is essential to determine which volatiles attract these pests.

- I studied the metabolomic profile of six willow clones in New Zealand. This number is very small, and a higher number of clones should be studied since New Zealand provide such a unique environment which can impact the biology of invasive species.
- To our knowledge there are no studies on how *P. proxima* can manipulate the chemistry of willows, although we know in other systems, the gall-inducing insect is able to do so. Does *P. proxima* also have that ability? Does galling by *P. proxima* affect resistance of willows to diseases and other insect pests?
- There is evidence of gall predation in New Zealand. The natural enemies, however, need to be identified.

6.8 References

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