Multitrophic Interactions Involving the Giant Willow Aphid, *Tuberolachnus salignus* (Gmelin)

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Summary

The giant willow aphid *Tuberolachnus salignus*, Gmelin, 1790 has become an important pest of willows, causing negative impact on host plant physiology and growth, and indirectly posing a unique problem in the apiculture industry. As it is a new invasive species to New Zealand, aphid-host interactions, extent of damage and ecological impacts of aphid infestation are not yet known. Aphid interactions with host plants, associated insect species and soil microbes were addressed in this thesis to fill knowledge gaps in formulating sustainable aphid management.

Chapter 1 presents a literature review on what is known about *T. salignus* in New Zealand and identifies multiple knowledge gaps, some of which are addressed in this thesis, including: the resistance or susceptibility of different willow cultivars to *T. salignus* attack; the plant emission of herbivore-induced volatile organic compounds in responses to aphid attack; the influence of the willow cultivar and age on honeydew composition (e.g., melezitose production); the ecological impacts of honeydew deposition on soil properties and biota; and the biocontrol potential of introduced natural ladybird predator *Harmonia axyridis*.

The main objective of Chapter 2 was to identify willow cultivars resistant or susceptible to *T. salignus*. *Tuberolachnus salignus* was found year-round and appears not to hibernate in NZ North Island conditions. Aphid population numbers and the extent of plant damage were cultivar-specific, with wide variations between resistant and susceptible cultivars. Two of the cultivars (*Salix eriocephala* and *S. lasiolepis* × *S. viminalis*) were identified as resistant, consistently showing low population levels of *T. salignus*. The remaining cultivars were classified as moderately resistant, susceptible, or highly susceptible, based on the aphid population levels. Aphid infestation delayed the flowering time, extended the duration of flowering, and decreased the catkin length in

susceptible cultivars. Interestingly, aphid infestation was found to increase the total floral output of some willow cultivars. Aphid infestation had no measurable effect on the number of shoots of willow cultivars, but reduced the survival, height, and shoot diameter of the plants by the end of the second growth season.

In Chapter 3, I explored VOC emissions by different cultivars and their changes in response to *T. salignus* infestation. The VOC emissions were cultivar-specific and varied with plant type (tree vs. shrub willows). The results also showed that resistant cultivars appear to emit more green leaf volatiles than other cultivars, suggesting that there can be a link between *T. salignus* resistance and VOC emission in willows, which deserves further exploration. However, most cultivars did not experience significant changes in their VOC emissions after aphid attack, while few have reduced emissions.

Due to the impact of melezitose in the apiculture industry, in Chapter 4 I investigated if the melezitose concentration in the honeydew varied depending on the plant cultivar upon which *T. salignus* was feeding. I showed that melezitose concentration in *T. salignus* honeydew did not vary with willow cultivar or plant age, but concentrations of other sugars (such as fructose) did. There was no obvious link between willow susceptibility to *T. salignus* and melezitose content, however, total honeydew sugar concentration was lower while fructose content was higher in highly susceptible cultivars identified in Chapter 1.

Tuberolachnus salignus honeydew deposition has multiple ecological impacts. Copious amounts of honeydew fall on the understory vegetation or directly on the soil surface, resulting in irregular occurrence of black sooty mould areas under aphid-infested plants (**Figure 5.1**). This carbon-rich energy source is utilized by soil microorganisms (fungi, bacteria and yeasts), in turn increasing the abundance of fungivores and their predators in honeydew-receiving soil. In Chapter 5, I found confirmation of this

honeydew-mediated cascading effect, which was directly linked with honeydew availability and the level of input, with strongest effect in black sooty mould spots.

Due to severe impacts of *T. salignus,* finding sustainable control strategies, such as the use of natural enemies, is crucial. However, as an invasive species *T. salignus* lacks natural enemies in New Zealand. Chapter 6 explores the potential of the ladybird predator *Harmonia axyridis* (Coleoptera: Coccinelidae) as biocontrol agent against *T. salignus.* The results show that although this predator can feed on *T. salignus,* the aphid is not its preferred prey. *H. axyridis* that fed on immature *T. salignus* developed slower than on alternative prey, and preferentially selected other aphid prey species in dual choice tests, rejecting *T. salignus.* This lack of preference, coupled with the huge appetite, wide prey spectrum, and rapid population build-up of *H. axyridis,* presents a possibility of it outcompeting native natural enemies and native prey insect species, causing a potential risk of biodiversity loss in New Zealand. The results suggested that *H. axyridis* should not be promoted as a biocontrol agent for *T. salignus.*

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Dedication

To my wife Cho Htwe, daughter Thiri Htet and son Naing Min Khant

For their love, support and encouragement that inspired me to complete this study

Preface

The thesis is article-based and comprises three main sections – the General Introduction, five experimental chapters and the General Discussion. Aphid population fluctuations and their infestation effect on flowering and growth of different willow cultivars are investigated in Chapter 2. Emissions of volatile organic compounds (VOCs) from willow cultivars in response to aphid infestation are studied in Chapter 3. In Chapter 4 I examined the effect of willow cultivar and plant age on melezitose sugar concentration in aphid honeydew. The effects of aphid honeydew deposition on soil biota and soil biochemical indicators were investigated in Chapter 5. Chapter 6 examines the predation potential of the harlequin ladybird beetle to control the giant willow aphid. Each experimental chapter contains Abstract, Introduction, Materials and Methods, Results and Discussion. As VOC sampling and honeydew collection were done in the same willow field trial (mentioned in Chapter 2), duplication of cultivar information and experimental design could not be avoided in Chapter 2, 3 and 4. Supplementary information and author's statement of contribution to publications/manuscripts (DRC-16) are presented in the Appendix of each experimental chapters. All references are listed after Chapter 7 General Discussion.

Table of contents

Summary iii

Acknowledgements vi

Dedication viii

Preface ix

Table of contents x

List of Tables xvi

List of Figures xvii

Chapter 1 1

General Introduction 1

1.1 Overview 2

1.2 Willows in New Zealand: a valued plant or a weed? 2

1.3 Host range and biology of *Tuberolachnus salignus* 4

1.4 *Tuberolachnus salignus* in multitrophic interactions 7

1.4.1 Direct impact on the host plant 7

1.4.2 VOC emission and response to *T. salignus* infestation 8

1.4.3 Host-mediated effect on sugar composition of aphid honeydew 10

1.4.4 Effect of aphid honeydew on below-ground soil processes 11

1.4.5 Selecting natural enemies for controlling *T. salignus* 12

1.5 Research questions 15

Chapter 2 17

2.1 Abstract 19

2.2 Introduction 20
2.3 Materials and Methods 23
2.3.1 Study site and soil preparation 23
2.3.2 Cultivar selection and experimental design 23
2.3.3 Aphid inoculation and population monitoring 24
2.3.4 Flowering phenology and catkin measurements 25
2.3.5 Sapling survival and growth measurement 26
2.3.6 Statistical analysis 28
2.4 Results 28
2.4.1 Aphid population monitoring 28
2.4.2 Flowering parameters 31
2.4.3 Sapling survival and growth parameters 35
2.5 Discussion 36
2.6 Conclusions 45
2.7 Appendix 47
Chapter 3 57
Volatile profiling of fifteen willow cultivars and their responses to giant willow aphid infestation 57
3.1 Abstract 59
3.2 Introduction 60
3.3 Materials and Methods 62
3.3.1 Study site and plant material 62

3.3.2 Aphid inoculation 63
3.3.3 VOCs sampling 65
3.3.4 Gas chromatography–mass spectrometry (GC-MS) analysis 66
3.3.5 Statistical analysis 67
3.4 Results 69
3.4.1 Characterisation of willow VOCs 69
3.4.2 VOC response of willow cultivars to *T. salignus* infestation 74
3.4.3 Correlation between aphid infestation and VOC emission 75
3.5 Discussion 75
3.6 Conclusions 81
3.7 Appendix 82
Chapter 4 89
Effect of willow cultivar and plant age on the melezitose content of giant willow aphid (*Tuberolachnus salignus*) honeydew 89
4.1 Abstract 91
4.2 Introduction 92
4.3 Materials and Methods 95
4.3.1 Willow field trial 95
4.3.2 Aphids 96
4.3.3 Honeydew collection 96
4.3.4 Honeydew sugar analysis 97
4.3.5 Statistical analysis 98

4.4 Results 99

4.5 Discussion 101

4.6 Appendix 108

Chapter 5 117

Honeydew deposition by the giant willow aphid (*Tuberolachnus salignus*) affects soil biota and soil biochemical properties 117

5.1 Abstract 119

5.2 Introduction 120

5.3 Materials and Methods 123

5.3.1 Willow field trial 123

5.3.2 Aphids 124

5.3.3 Soil sampling 124

5.3.4 Soil chemical properties 125

5.3.5 Soil fauna 125

5.3.6 Soil enzymes 126

5.3.7 Yeasts 127

5.3.8 Microbial properties 128

5.3.9 Data analysis 129

5.3.10 Structural equation modelling 130

5.4 Results 132

5.4.1 Soil chemical properties 132

5.4.2 Soil meso-fauna 134

5.4.3 Soil enzymes 134

5.4.4 Soil microbial properties and yeast CFU 136

5.4.5 Principal Component Analysis-Linear Discriminant Analysis 138

(PCA-LDA) 138

5.4.6 Structural Equation Modelling (SEM) 140

5.5 Discussion 142

5.6 Conclusions 145

5.7 Appendix 146

Chapter 6 151

The potential of harlequin ladybird beetle *Harmonia axyridis* as a predator of the giant willow aphid *Tuberolachnus salignus*: voracity, life history and prey preference 151

6.1 Abstract 153

6.2 Introduction 154

6.3 Materials and Methods 156

6.3.1 Rearing prey insects 156

6.3.2 Rearing harlequin ladybird beetles 157

6.3.3 Voracity of different stages of *H. axyridis* 158

6.3.4 Growth, development and larval survival of *H. axyridis* on different diets 159

6.3.5 Prey preference of *H. axyridis* 159

6.3.6 Statistical analysis 160

6.4 Results 162

6.4.1 Voracity 162

6.4.2 Development, growth and survival of immature *H. axyridis* on different diets 162

6.4.3 Prey preference between *M. persicae* and *T. salignus* 165

6.5 Discussion 167

6.6 Appendix 170

Chapter 7 171

General discussion 171

7.1 Synthesis 172

7.2. The impact of invasive herbivores on multitrophic interactions and biological control strategies 177

7.2.1. Bottom-up biocontrol mechanisms 178

7.2.2 Top-down biocontrol mechanisms 180

7.3 Knowledge contribution to controlling *T. salignus* and reducing honeydew-related problems 181

7.4 Knowledge gaps for future research 183

References 186

List of Tables

Table 2.1. List of willow cultivars used in the field trial. 27

Table 3.1. Willow cultivars (*Salix* spp.) used in characterising and identifying the willow VOC response to *T. salignus* infestation. 64

Table 3.2. GC-MS analysis of VOCs released from the foliage of fifteen willow cultivars. 71

Table 4.1. List of willow cultivars used in the experiment. 95

Table 4.2. Concentration of different sugars (g L^{-1}) in honeydew from willows and effect of cultivar, plant age and their interaction on individual and pooled sugar production after fitting linear model. The values are mean ± SE. *Salix matsudana* × *S. alba* (1) and (2) represent clones NZ 1040 and NZ 1184, respectively. 102

Table 5.1. Soil chemical properties before aphid inoculation (baseline) and after aphid inoculation (control, aphid-infested and black sooty mould spots), during the first and second year of the experiment. Values are the means ± SE. Different letters in each column indicate significant differences between the treatments at each sampling time (Tukey's HSD test, $\alpha = 0.05$). 132

Table 6.1. Developmental time and weight at moulting (mean ± SE) for the different life stages of *H. axyridis*, fed with either first instar *T. salignus*, or frozen *E. kuehniella* eggs. 164

List of Figures

Figure 1.1. *Tuberolachnus salignus* feeding on willow stem, April 2019, the National Willow Collection in Palmerston North, New Zealand. 6

Figure 1.2. Multitrophic interactions of *T. salignus* investigated in this thesis. 15

Figure 2.1. Population levels of *T. salignus* on susceptible willow cultivars, from the first inoculation to the end of experimental period; data represent the average for 13 willow cultivars. Two resistant cultivars (*S. lasiolepis* × *S. viminalis* and *S. eriocephala*) on which aphid numbers never reached high level (>4) were excluded. See **Figure S2.4** for the data on all the individual cultivars. 30

Figure 2.2. Total number of weeks with high *T. salignus* abundance (greater than 4 on the proxy-log scale) for the willow cultivars; combined data from 2018 and 2019. The values represent mean ± SE. Different letters indicate significant differences at $\alpha = 0.05$, Tukey's HSD test. 31

Figure 2.3. The effect of *T. salignus* infestation on the flowering phenology, catkin number and catkin size of the willow cultivars. Values represent mean ± SE; clear bars are the control plants, black bars are the aphid-infested plants. Catkin length was not measured for *S. lasiolepis*. The F-tests are for the cultivar × aphid infestation interaction effect. The significant effect of aphid infestation in the individual willow cultivars is shown with an asterisk (*), Tukey's HSD test, $\alpha = 0.05$. 33

Figure 2.4. The total floral output of the willow plants as affected by willow cultivar and aphid infestation. Values represent the mean ± SE; clear bars are the control plants, black bars are the aphid-infested plants. The F-test showed the significant interaction effect of willow cultivar and aphid infestation on the total floral output. 34

Figure 2.5. Survival of the willow plants inoculated with *T. salignus*. The survival days were counted from the date of the first successful aphid inoculation to the end of the two-

year field trial. “Others” represents the twelve cultivars, other than *S. candida, S. eriocephala* and *S. viminalis*. The Chi-square value represents the result of the log rank test, comparing the survival distribution of the fifteen willow cultivars. 35

Figure 2.6. The effect of *T. salignus* infestation on the maximum plant height of the willow cultivars, measured at the end of each growth season. The F-tests are for the interaction effect of cultivar and aphid infestation on the plant height. Asterisks indicate a significant aphid effect within the cultivars, Tukey’s HSD test, $\alpha = 0.05$. 37

Figure 2.7. The effect of *T. salignus* infestation on the mean number of shoots in the willow cultivars. The F-tests are for the interaction effect of aphid infestation and cultivar. The asterisk indicates a significant aphid effect within the cultivar, Tukey’s HSD test, $\alpha = 0.05$. 38

Figure 2.8. Mean shoot diameter of the willow cultivars, as affected by *T. salignus* infestation. The F-tests are for the interaction effect of aphid infestation and cultivar. Asterisks indicate a significant aphid effect within the cultivars, Tukey’s HSD test, $\alpha = 0.05$..... 39

Figure 3.1. VOC sampling from willow foliage using a portable volatile assay system. Suitable branches were enclosed in plastic bags, into which clean air was circulated by a pump..... 66

Figure 3.2. Non-metric Multi-Dimensional Scaling plot of similarities in VOC profiles released by the fifteen willow cultivars. Bray-Curtis dissimilarities were calculated on the square-root transformed VOC profiles, containing 19 compounds. Each point represents a headspace sample of each species or hybrids (n=6/cultivar). Most samples at the left side of the dotted line correspond to shrub willows, whereas most samples at the right of the dotted line are tree willows. 72

Figure 3.3. Relative proportions of each major VOC class emitted by the fifteen willow cultivars. The values and bars indicate mean ± SE. Different letters represent statistically significant differences at 0.05% level by multiple comparison using Tukey's HSD test. 73

Figure 3.4. Total volatile emissions released by the fifteen willow cultivars for the control and aphid-infested treatments. The values and bars indicate mean ± SE. Asterisks indicate significant differences between the treatments for each cultivars by Tukey's HSD test. 74

Figure 4.1. Melezitose, glucose, fructose and glucose concentration on *T. salignus* honeydew from thirteen willow cultivars in year one (clear bars) and year two (black bars) of the experiment. Values represent mean ± SE. Different letters indicate significant differences among all possible treatment combinations (cultivar*age) after a Tukey HSD test ($\alpha = 0.05$). *Salix matsudana* × *S. alba* (1) and (2) represent clones NZ 1040 and NZ 1184, respectively. 103

Figure 4.2. Pearson correlation (R) between honeydew melezitose concentration (g L^{-1}) and that of other sugars. 104

Figure 5.1. The black sooty mould spots under the canopy of the willow plants. 121

Figure 5.2. The effect of *T. salignus* honeydew deposition on the abundance of soil meso-fauna: Collembola (**a**), Gamasida (**b**), Astigmata (**c**), Oribatida (**d**) and others (**e**), prior to aphid inoculation (before infestation), and after aphid inoculation (control, aphid-infested, and black sooty mould spots) in the first and second year. The values are the means ± SE. Different letters indicate statistically significant differences between treatments within each sampling time (Tukey's HSD test, $\alpha = 0.05$). 133

Figure 5.3. The activity of the soil enzymes: dehydrogenase (**a**), urease (**b**), amidase (**c**), β-amylase (**d**), invertase (**e**) and α-glucosidase (**f**) under the canopies of willow plants,

prior to aphid inoculation (before infestation), and after aphid inoculation (control, aphid-infested, and black sooty mould spots) in the first and second year. The values are the means ± SE. Different letters indicate significant differences between the treatments at each sampling time (Tukey's HSD test, α = 0.05). 135

Figure 5.4. The effect of *T. salignus* honeydew deposition on the geometric mean of soil enzyme activities, prior to aphid inoculation (before infestation), and after aphid inoculation (control, aphid-infested, and black sooty mould spots) in the first and second year. The values are the mean ± SE. Different letters represent statistically significant differences between the treatments at each sampling time (Tukey's HSD test, α =0.05). 136

Figure 5.5. The effect of *T. salignus* honeydew deposition on (a) soil microbial biomass (μg C g^{-1} soil), (b) basal respiration (μg CO_2–C g^{-1} soil h^{-1}), and (c) metabolic quotient over time (μg CO_2–C μg^{-1} Cmic h^{-1}), prior to aphid inoculation (before infestation), and after aphid inoculation (control, aphid-infested, and black sooty mould spots) in the first and second year. The values represent means ± SE. Different letters indicate statistically significant differences at each sampling time (Tukey HSD test,α=0.05). 137

Figure 5.6. The yeast colony forming units (CFU) per gram of dry soil, collected under the canopies of willow plants prior to aphid inoculation (before infestation), and after aphid inoculation (control, aphid-infested, and black sooty mould spots) in the first and second year. The values are the means ± SE. Different letters indicate statistically significant differences at each sampling time (Tukey HSD test,α=0.05). 138

Figure 5.7. PCA-LDA bi-plots of the soil biochemical variables, classified (a) by treatment, and (b) by sampling time. The PCA was run to reduce the dimensions, followed by LDA to separate the treatments and sampling times. The variables were scaled and

centred prior to the analysis. The shaded ellipses represent the 95% confidence areas. 139

Figure 5.8. Paths diagrams for the effects of *T. salignus* honeydew deposition on (a) soil biochemical properties, and (b) meso-fauna communities. The circles above the rectangles indicate the error terms. The solid and dotted arrows represent significant and non-significant associations, respectively. The path coefficients are the standard regression weights, with asterisks showing different levels of significance (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The squared multiple correlations (r^2) are expressed above the top right corner of each rectangle.....141

Figure 6.1. Voracity of third instar and adult males and females of *H. axyridis* fed first instar *T. salignus* in laboratory experiment (n=14-16). The values represent means ± SE; different letters indicate statistically significant differences between means (LS means with Tukey-Kramer adjustment, α=0.05).....162

Figure 6.2. Survival density functions of immature *H. axyridis* fed first instar *T. salignus* vs. frozen *E. kuehniella* eggs, with the starvation treatment as control. 165

Figure 6.3. Selection of first prey by different stages and sexes of *H. axyridis* when offered a choice between *M. persicae* and *T. salignus* aphids, n = 45. Different letters within each bar indicate significant differences (α=0.05).....166

Figure 6.4. Preference index (varies from 0 to 1, with 1 indicating 100% prey preference) for *M. persicae* and *T. salignus* prey in different stages and sexes of *H. axyridis*. The error bars are the SE of mean preference index. Different letters within and across age groups indicate significant differences (LS means with Tukey-Kramer adjustment, α=0.05). 166

Chapter 1

General Introduction

1.1 Overview

The giant willow aphid, *Tuberolachnus salignus* Gmelin, 1790 (Hemiptera: Aphididae), was first discovered on *Salix* × *fragilis* in the North Island of New Zealand (Auckland) in late 2013, and only few months after the first detection its presence was reported throughout the country, including the South Island (Gunawardana *et al.*, 2014). This aphid is regarded as a pest of economic importance due to its direct negative effect on the survival and biomass production of host plants (willows), as well as aphid honeydew-related problems in apiculture. When feeding, *T. salignus* produce copious amounts of honeydew, which is rich in melezitose sugar and attracts foraging honeybees. This honeydew is not nutritionally suitable for bees, causing bee dysentery and reducing survival of overwintering bees (Seeburger *et al.*, 2020). Melezitose-containing honey crystalizes in the comb ('cement honey'), making extraction difficult and reducing honey yield and quality (Sopow *et al.*, 2017). Since *T. salignus* is a new species in New Zealand, not much is known about the control measures and ecological relationships of this aphid with other organisms already present in willow agroecosystems. Thus, comprehensive studies are indispensable for gaining greater understanding of the ecological role of *T. salignus* in the willow systems and for investigating short-term risk mitigation options and long-term sustainable management (Sopow, 2016).

1.2 Willows in New Zealand: a valued plant or a weed?

Willows (*Salix* spp., family Salicaceae) are deciduous dioecious plants, encompassing about 450 species with growth forms ranging from low shrubs to tall trees (Argus, 1999; McIvor, 2013; Phillips & Daly, 2008). Willows were introduced to New Zealand with early European settlers around the 1840's as ornamental plants in their gardens (Thompson & Reeves, 1993). Willows are now distributed throughout New Zealand (Phillips & Daly, 2008) because of their ability to survive under various

environmental conditions, to produce rapid growth rates, and to reproduce both sexually and vegetatively (Webb *et al.*, 1988). Fifty-nine taxa (consisting of 38 species and 21 hybrids) are currently grown in New Zealand (Newstrom-Lloyd *et al.*, 2015), and different cultivars have been selected for different purposes. The willows in New Zealand fall in three groups: tree willows that can grow up to 20 m tall and produce a single stem with 90cm in diameter; osier willows (basket willows) that are medium-sized shrubs and produce multiple stems; shrub willows (sallow) that have multiple stems with stout branching patterns (McIvor, 2013). New willow hybrids are being developed to improve growth form, growth rate and the resistance to pest and disease infestation.

Willows have a long history of various uses in New Zealand, and are extensively grown for soil conservation, river bank stabilization, horticultural shelterbelts, shelters and amenity trees (Karp *et al.*, 2011; McIvor, 2013; Wilkinson, 1999). They are also valued as livestock fodder (McIvor, 2013), providing the foliage biomass of 36,000 tonnes during a summer drought (Moore *et al.*, 2003). Willows flower from late July to December in New Zealand, providing pollen in early Spring – an important protein source for maintaining population growth and brood feeding of honey bees (Newstrom-Lloyd *et al.*, 2015). Willows are woven to make baskets, living walls, wicker cones for climbing vegetables and cloches for protecting plants from direct sunlight (Lilian, 2016). Ability to establish rapidly and to tolerate high level of pollutants make some willow species well suited to use for alleviating elevated soil cadmium (Cd) concentrations in New Zealand, resulting from repeated superphosphate fertiliser application (Bramley, 1990). Deep root system and high evapotranspiration rate of willows enable their use to reduce nitrate leaching associated with intensive dairy farming (Franklin *et al.*, 2016; Ministry for the Environment, 2007). Additionally, *Salix* spp. render other environmental and ecological benefits, e.g., providing cover for eels, trout and native plants, and habitats for birds

(Green *et al.*, 1989; Phillips & Daly, 2008), increasing water quality (Styles *et al.*, 2016) and soil carbon storage (Cunniff *et al.*, 2015), and balancing greenhouse gas emissions (Volk *et al.*, 2016).

Although effective for multipurpose uses, willows are viewed as an invading weed in New Zealand's wetlands and native vegetation (West, 1994). Dense willow plantings and cracked stems obstruct waterways, causing localized flooding and erosion (West, 1994) and causing regular maintenances needs, especially in low energy rivers/streams (Phillips & Daly, 2008). Dense canopy of invasive *Salix cinereal* is known to outcompete low stature native plant communities, altering the structure and functioning of a wetland (Phillips & Daly, 2008). Furthermore, increased water take-up due to willow invasion can decrease the carrying capacity of streams and lakes, impacting native wildlife communities and decreasing in-stream arthropod abundance (Denyer, 2015). Non-cracking and sterile (male) erosion control cultivars have been developed to minimize their invasive spread to wetlands and waterways (Stace, 2017).

Whether willows are considered as valued species or weeds, depends on which species or hybrids are used for what purpose, and where the willows are planted/grown. In the current study I assume that willows are a valued plant, and that control measures are needed to protect these multipurpose species.

1.3 Host range and biology of *Tuberolachnus salignus*

As the name implies, the giant willow aphid is one of the largest species (5.8 mm in abdominal length) among 120 willow-feeding Homopteran (Hill *et al.*, 2020). This species is normally found in clusters on willow stems (**Figure 1.1**). *Tuberolachnus salignus* is believed to have originated from Asia (Charles *et al.*, 2014), but has become a cosmopolitan species invading many parts of the world where host willow species are

grown (Collins & Leather, 2001). The aphid is autoecious, completing its life cycle on a single host (Dixon, 1985). It feeds mainly on willows (*Salix* sp.), but can also be found feeding on poplar, apple, and pear trees, especially near willow shelter belts (Martin, 2017). By 2014, ten willow clones and one poplar species were identified as the potential hosts of *T. salignus* in New Zealand (Gunawardana *et al.*, 2014). The host range of *T. salignus* has expanded in 2017, when fifty willow clones and one poplar species were confirmed to be host trees in New Zealand (Sopow *et al.*, 2017). An even wider host range of *T. salignus* can be expected in New Zealand in the future.

The giant willow aphid is suggested to be anholocyclic, i.e., males are totally absent (Blackman & Spence, 1996) and the females reproduce parthenogenetically (Williams & Dixon, 2007). *Tuberolachnus salignus* exhibits low clonal diversity, with only 16 different genotypes recorded from 27 populations of this aphid from five countries (Aradottir *et al.*, 2012).

Winged and wingless morphs have different patterns of survivorship, and winged adults exhibit higher mortality compared to wingless adults in the lab (Collins & Leather, 2001). Winged morphs also produce fewer offspring than wingless morphs (Collins & Leather, 2001). An adult female of *T. salignus* can produce 35-71 nymphs during its lifetime (Collins & Leather, 2001). Several overlapping generations are found in a colony containing a large number of individuals (Martin, 2017). There are four nymphal instars and the duration of each instar is temperature-dependent (Collins & Leather, 2001; Özder *et al.*, 2007). Optimum temperature for development, fecundity and survival is 25°C (Özder & Sağlam, 2008). The total nymphal duration is four times shorter at 25°C (10.1 days) than at 10°C (40.6 days) (Collins & Leather, 2001). The life cycle of *T. salignus* takes 2-3 weeks depending on the temperature (Collins & Leather, 2001). Apterous *T. salignus* require 196 degree days with 5.5°C threshold from birth to final ecdysis (Collins,

2001). Survival rate of nymphal instars also increases with temperature (Özder *et al.*, 2007), but decreases at a constant temperature of 27.5°C (Özder & Sağlam, 2008). The mortality rate of immature *T. salignus* depends on the host plant species; for example, higher mortality was found on *S. babylonica* than on *S. matsudana* (Özder *et al.*, 2007).

Figure 1.1. *Tuberolachnus salignus* feeding on willow stem, April 2019, the National Willow Collection in Palmerston North, New Zealand.

The giant willow aphid exhibits a peculiar pattern of phenology: in the UK, the aphid disappears from willow trees in spring, when host trees are thought to be nutritionally superior (Collins, 2001) due to the higher nutrient translocation at the rapid growth stage of host plants (Dixon, 1985). Instead, aphid populations start to build up in late summer on less nutritious willow trees and then continue to increase on leafless willows up to late autumn (Blackman & Eastop, 1994). In New Zealand, this aphid appears to be less active in winter and more active in the warmer seasons (Sopow *et al.*, 2017), with highest population densities occurring in February and March (Sopow *et al.*,

2017); the aphid is not found on willows from late May to August (Gunawardana *et al.*, 2014). An increased seasonal abundance of *T. salignus* has been observed in Spring, Summer and Autumn (2013-2014, 2014-2015, 2015-2016) since its initial invasion of New Zealand (Jones & McIvor, 2016). In this study, the aphid was found on New Zealand host willows year-round in 2018, without apparent hibernation.

1.4 *Tuberolachnus salignus* in multitrophic interactions

The giant willow aphid exists in a multitrophic system. Like many other aphid species, population densities of *T. salignus* in tritrophic communities are hypothesized to be controlled by bottom-up (e.g., plant genotype) and top-down (e.g., presence/absence of mutualistic partners and predators) forces (Johnson, 2008).

1.4.1 Direct impact on the host plant

Nutritional limitation of resistant plant genotypes can affect the population fluctuation of insect herbivores which feed on these host plants (Price *et al.*, 2005). While some herbivorous insects can discriminate the susceptible hosts from unpalatable resistant ones, others cannot. Whether *T. salignus* can discriminate between hosts or not remains unclear. Collins (2001) reported no difference in attractiveness of six willow clones to *T. salignus* in a greenhouse trial. On the other hand, Aradottir *et al.* (2009) suggested that *T. salignus* preferred specific willow clones to others in laboratory olfactometry tests. Further studies are required to explore host-plant selection by this aphid.

Overall, host-plant quality for an insect herbivore is determined by specific traits that are mainly driven by the plant's genotype (Underwood & Rausher, 2000). The difference in susceptibility of willow clones to other insects (Kendall *et al.*, 1996; Nordman *et al.*, 2005) and pathogens (Pei *et al.*, 1996) can depend on genotypes (both within and among populations) and species (Collins, 2001). The genus *Salix* possesses

high genetic diversity, and new hybrids and cultivars are being developed with specific attributes to meet human needs. In New Zealand, Plant & Food Research Ltd. is working to identify resistant willow genotypes and clones, to substitute the existing clones susceptible to *T. salignus* (Sopow, 2016). *Tuberolachnus salignus* infestation is known to reduce survival and biomass production of some willow cultivars (Collins, 2001), but the extent of damage to resistant and susceptible willow cultivars requires further investigation. Studying the association of *T. salignus* with different willow cultivars will offer a unique opportunity to explore the role of genetic variation in the resistance of willows to this herbivore.

Seasonal weather patterns and host nutritional quality (host genotype and age) are known to influence aphid population densities (Day *et al.*, 2010; Kindlmann & Dixon, 1996). Aphid infestation can change the flowering phenology of the host (Buntin & Raymer, 1994) and cause changes in the population patterns of pollinators (Rafferty & Ives, 2011). Understanding and identifying the drivers governing *T. salignus* population fluctuations and their ecological impacts may help us to formulate a sustainable management strategy for controlling this aphid.

1.4.2 VOC emission and response to *T. salignus* infestation

Plants emit a large quantity of volatile organic compounds (VOCs) into the neighbouring environment (Staudt & Lhoutellier, 2007). This VOC emission plays various ecological and physiological roles (Jaeger *et al.*, 2016), shaping the assemblage of herbivorous insects within plant communities (Inui *et al.*, 2003; Poelman *et al.*, 2008). The VOC blends are highly diverse and mainly contain terpenoids, benzenoids, phenylpropanoids, and fatty acid and amino acid derivatives (Effmert *et al.*, 2012). Both quality and quantity of VOC assemblages are known to depend on biotic and abiotic factors (Holopainen & Gershenzon, 2010).

Willow plants are also known to release various VOCs (Copeland *et al.*, 2012; Hakola *et al.*, 1998) and these emissions are cultivar-specific (Peacock *et al.*, 2001). Host-specific VOC emission is related to both direct and indirect plant defences (Goggin, 2007) and plays an important role in host selection by aphids (Ahmed *et al.*, 2019). Therefore, characterizing the VOCs released from different willow cultivars is an important step in understanding plant-herbivore interactions.

Sometimes, a unique array of VOCs is released following insect feeding on a host plant (Kigathi *et al.*, 2009). This herbivore-induced VOC emission has been found important in studying interaction between plants, herbivores, and their natural enemies (Boeve *et al.*, 1996; Dicke *et al.*, 1990; Knudsen *et al.*, 1993) in a community. Different plant species release different odours after being infested by the same herbivore species (De Moraes *et al.*, 1998), and different herbivores can lead to different VOC emissions on the same plant (McCormick *et al.*, 2014a).

Some studies have been conducted to explore the VOC responses of willow to insects. For example, multiple compounds are emitted by *Salix eriocarpa* infested by the leaf beetle *Plagiodera versicolora* Laicharting, 1781, which inform a natural enemy, the predatory ladybird *Aiolocaria hexaspilota* Hope, 1831 of the suitable state of their prey (Yoneya & Takabayashi, 2013), showing a role of VOCs in indirect defence. Also, a negative relationship between the emission of green leaf volatiles (fatty acid derivatives) in infested plant parts and resistance to two leaf beetles (*Galerucella lineola* Fabricius, 1781 and *Phratora vulgatissima* Linnaeus, 1758) was observed in ten willow species (Peacock & Herrick, 2000), suggesting the VOC involvement in direct defence.

To our knowledge, no previous work has been done in exploring the volatile communication between willow cultivars and *T. salignus*. Studying VOC response of

different willow cultivars to *T. salignus* infestation may explain why aphid populations prefer some cultivars over others, and why some willow cultivars are more resistant than others.

1.4.3 Host-mediated effect on sugar composition of aphid honeydew

The giant willow aphids assimilate required nutrients (carbohydrates, amino acids and lipids) from the phloem sap of a willow host (Sopow *et al.*, 2017). As phloem sap contains an unbalanced nutrient composition, with high carbohydrate (mainly sucrose) content and low amino acid content, aphids have to ingest large quantities of plant sap (Douglas, 2009). An apterous *T. salignus* adult can ingest a photosynthetic assimilate of 5-20 cm^2 of leaf day^{-1} (Mittler, 1958a). Unassimilated phloem sap is excreted from the aphid's anus as droplets of sugar-rich honeydew (Sharma *et al.*, 1995). Mittler (1957) estimated that an apterous *T. salignus* adult could excrete 1.71-2.08 mm^3 h^{-1} of honeydew, and early instar honeydew production ranged from 0.45 to 1.43 mm^3 h^{-1}.

Aphid honeydew is mainly dominated by carbohydrates (both plant-derived phloem sugars and aphid-synthesized sugars) and amino acids (Hogervorst *et al.*, 2007; Woodring *et al.*, 2004). Both sugar and amino acid composition of honeydew corresponds more or less to the composition of phloem sap (Sabri *et al.*, 2013), which differs between and within host plants (Schillewaert *et al.*, 2017). Sugar profiles of honeydew correspond to the chemistry of the host plant (Fischer *et al.*, 2005), with oligosaccharides in the honeydew resulting from the transformation of simple sugars present in the phloem sap of host plants (Karley *et al.*, 2005). Melezitose is a dominant sugar in most aphid honeydews (Fischer & Shingleton, 2001; Fischer *et al.*, 2005; Fischer *et al.*, 2002). The identity of host plant species can influence the quantity of melezitose and concentration of other carbohydrates in the excreted honeydew (Fischer & Shingleton, 2001). Moreover, phloem sap quality can vary with plant age, which in turn determines the sugar

concentration of scale insect honeydew (Beggs *et al.*, 2005). Melezitose from *T. salignus* honeydew is known to cause problems in New Zealand apiculture industry, reducing yield and quality of honey (Sopow *et al.*, 2017). In addition, the *T. salignus* honeydew in New Zealand is foraged by the *Vespula* and *Polistes* wasps (Hymenoptera: Vespidae), leading to localized increase of these pests (Gunawardana *et al.*, 2014). Wasps become a major factor in population reduction of honey bees as they compete for available resources, attack and kill bees, and rob the hives (Harris, 1991; Lester *et al.*, 2013). Exploring whether (and how) plant factors such as willow cultivar and age may influence melezitose content of *T. salignus* honeydew can provide basic information to help lessen honeydew-related problems in NZ apiculture industry.

1.4.4 Effect of aphid honeydew on below-ground soil processes

Herbivorous insects have a dominant role in the functioning of most ecosystems (Schowalter, 2016). Their negative effects on host plants are well known and diverse. Less is understood about their positive contribution to the cycling of carbon and nitrogen, decomposition processes, nutrient mineralization in the soil, and plant productivity (Hartley & Jones, 2008). The input of carbon- and nitrogen-rich insect excrements and cadavers into the soil links the above- and below-ground processes in the food web (Hunter, 2001). These supplementary energy inputs accelerate soil decomposition processes (Pastor & Cohen, 1997). The accelerated breakdown of organic matter, in turn, can improve soil nutritional status and plant productivity (Hartley & Jones, 2008). Insect populations, especially at high population densities, can exhibit an influential effect on nutrient cycling in perennial systems (Fogal & Slansky, 1985). Lightfoot and Whitford (1990) also suggested that herbivorous insects can have an influence on nitrogen cycling and can promote high rates of nitrogen turnover, especially from highly nutritious hosts.

A large proportion of aphid-produced honeydew is directly deposited onto the soil surface (Milcu *et al.*, 2015). If it does not fall directly onto the soil surfaces, this excretion coats the vegetation understory and anything under the infested host trees (Martin, 2017). Finally, the honeydew runs off onto the soil surface as the rain washes it out from the coated surfaces. The effect of aphid honeydew deposition on soil nutrient cycling and availability has been of interest since 1970s (Owen, 1978, 1980; Owen & Wiegert, 1976; Petelle, 1980). Honeydew provides a readily available source of labile carbon, inducing an increase in soil microbial populations (Jílková *et al.*, 2018), which in turn could lead to an increase in abundance of soil mesofauna (springtails and mites) (Eisenhauer *et al.*, 2007; Men'ko *et al.*, 2006) and produce positive effects on host plants (Stadler & Müller, 1996). Honeydew deposition has also been shown to increase soil nitrogen fixation by non-symbiotic bacteria, leading to an increase in plant productivity (Owen & Wiegert, 1976), However, studies are still needed to explore the short- and long-term effects of honeydew deposition on soil biological and chemical properties in order to better understand the effects of aphid herbivory on soil biotic community, nutrient cycling, and performance of host plants.

1.4.5 Selecting natural enemies for controlling *T. salignus*

Aphids are considered to be one of the most destructive pests in agriculture and forestry (Kumar, 2019). Aphids are normally confronted by a wide range of natural enemies, including microbes, predators and parasitoids (Rabasse & van Steenis, 1999; Yano, 2006). These organisms not only increase aphid mortality, but also cause avoidance behaviours, reducing aphid reproductive potential and host feeding (Goggin, 2007). Successful examples of aphid biological control include the use of fungus (*Zoophthora radicans* Batko, 1964) against yellow clover aphid/spotted alfalfa aphid (*Therioaphis trifolii* Monell, 1882) (Milner *et al.*, 1982), and the use of parasitoids (*Aphidius* spp. and

Aphelinus spp.) and predator (*Aphidoletes aphidimyza* Rondani, 1847) against green peach aphid (*Myzus persicae* Sulzer, 1776), cotton aphid (*Aphis gossypii* Glover, 1877) and potato aphid (*Macrosiphum euphorbiae* Thomas, 1878) (van Lenteren, 2000). Biological control of aphids using natural enemies had been extensively practised for multiple reasons: the urge to reduce chemical pesticide use, the development of insecticide resistance, and the increased use of biocontrol agents against other pests, necessitating a comparable control measure for managing aphids (Furk & Hines, 1993; Rabasse & van Steenis, 1999).

As *T. salignus* is a new addition to New Zealand fauna (Martin, 2016), the major problem in establishing a biocontrol program was the lack of known natural enemies (Sopow *et al.*, 2017). To solve this problem, potential natural enemies have been investigated, and the parasitoid *Pauesia salignae* Watanabe, 1939 (Hymenoptera: Braconidae) and harlequin ladybird beetle *Harmonia axyridis* Pallas, 1773 (Coleoptera: Coccinellidae) were ranked as two most promising biocontrol agents against *T. salignus* (Sopow *et al.*, 2017). *Pauesia salignae* was imported to New Zealand in 2017 to test its potential and host range in containment facilities (Foster, 2017). High host specificity in the trials make this parasitoid a promising candidate for controlling *T. salignus* (Sopow, 2018). *Harmonia axyridis* was first detected in the North Island of New Zealand (Auckland) in 2016 and was often found to be associated with *T. salignus* (Martin, 2016). At first, the biocontrol effect of *H. axyridis* was not appreciable, but one year after its arrival, *H. axyridis* was able to decrease the population densities of *T. salignus* considerably (Martin, 2017). Currently, no further information on how *H. axyridis* interacts with *T. salignus* in the New Zealand ecosystems is available.

Harmonia axyridis is a polyphagous predator (Osawa, 1992), native to eastern and central Asia (Roy *et al.*, 2016). This species was introduced to many countries as a

classical biocontrol agent of many aphid species, but also had many other regions outside the range of intentional release (Brown *et al.*, 2008). Ladybird beetles (Coleoptera: Coccinellidae) are generalist predators and important biocontrol agents of aphids and other phytophagous pests (Pervez & Omkar, 2006; Powell & Pell, 2007). The beetle possesses a eurytopic nature (i.e., is able to tolerate a wide range of habitats or ecological conditions) with a wide prey spectrum, high phenotypic plasticity and high population build-up (Roy & Brown, 2015). They can easily switch to alternative prey when their target prey species are absent/rare (Omkar & Pervez, 2003). Their ability to survive at low prey densities and high fecundity when offered high prey densities contribute to their rapid establishment in non-native ranges (Agarwala & Bhowmik, 2011).

The polyphagous behaviour can make the ladybird species useful in Integrated Pest Management (IPM) programs, because of the ease of mass rearing and augmentation on alternative or artificial food sources (Guedes & Almeida, 2013). On the other hand, the wide prey spectrum and high competitive abilities make *H. axyridis* a dominant predator species that could have a strong negative impact on biodiversity, reducing the number of non-target insects and driving out native ladybirds and other predator species (Koch & Galvan, 2008). In New Zealand, the beetle was found to feed on pip fruit, blemishing the surface, and to aggregate within grape bunches, damaging the quality of juice and wine products (Ministry for Primary Industries, 2016). Because of their negative effect on biodiversity and fruit crop production (Koch & Galvan, 2008), *H. axyridis* has a possibility of becoming a pest that needs to be managed (Kenis *et al.*, 2008; Sopow *et al.*, 2017).

To avoid potential risks and maximize biocontrol efficacy of *H. axyridis*, their biocontrol potential needs to be tested in New Zealand environment to define the

relationship between *H. axyridis* and the target prey species. The results will help to establish whether *H. axyridis* is a suitable biocontrol agent for *T. salignus*.

1.5 Research questions

Multidisciplinary research can help to understand the complex associations of *T. salignus* with its host plant, environment, and predators, which in turn, can elucidate the ecological role of aphids in a multitrophic context (**Figure 1.2**).

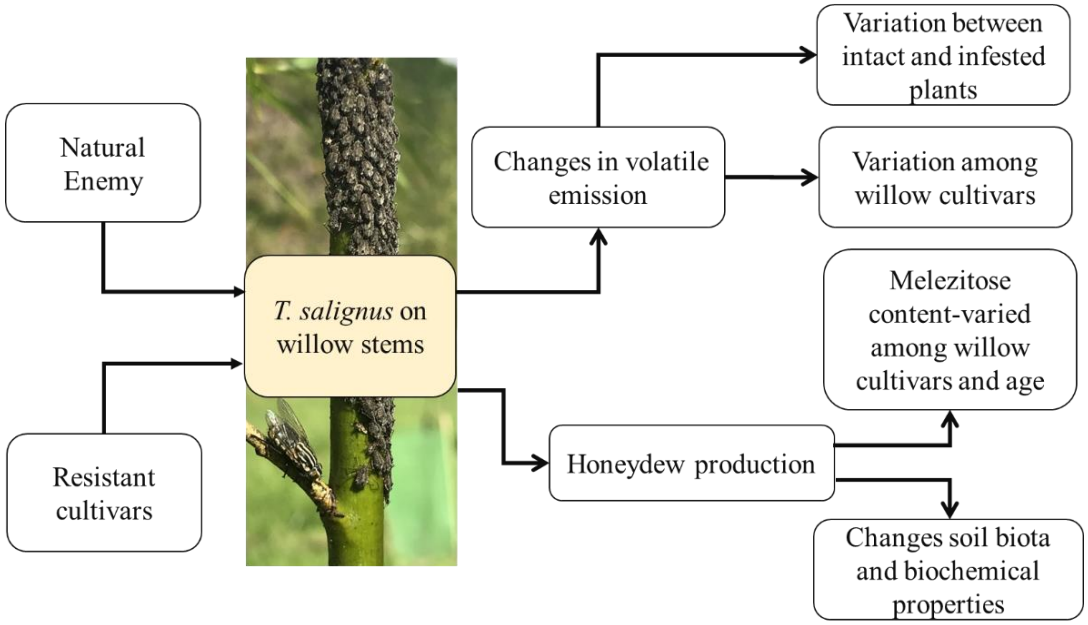

Figure 1.2. Multitrophic interactions of *T. salignus* investigated in this thesis.

The present study investigated interactions of *T. salignus* with the host plants, the environment, and a predator. The study focused on the following research questions:

(i) Are different willow cultivars equally susceptible to *T. salignus*, and how are flowering and growth influenced by the *T. salignus* infestation in these cultivars?

(ii) How does *T. salignus* infestation affect the VOC emission from willows, and are there differences among willow cultivars?

(iii) How do the willow cultivar identity and plant age influence the melezitose concentration of aphid honeydew?

(iv) How does aphid honeydew deposition affect soil biota and soil biochemical properties?

(v) Can *H. axyridis* be used as a potential biocontrol agent for *T. salignus*, based on the voracity, prey suitability and prey preference tests?

Chapter 2

Population fluctuations of the giant willow aphid *Tuberolachnus salignus* Gmelin (Hemiptera: Aphididae) and the effect on the flowering and growth of willow cultivars

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2.1 Abstract

The giant willow aphid *Tuberolachnus salignus* is a large phloem-feeding insect which colonizes the stems of infested willow trees. This aphid is a relatively new invasive species in New Zealand, and the inter- and intra-annual population patterns, as well as the damage it can cause to willow cultivars are not known. Therefore, a two-year field trial (2017-2019) was established to investigate the aphid population dynamics, and the effect of this aphid on flowering, growth and survival of fifteen willow cultivars.

Aphid population levels on the willow cultivars were found to fluctuate in the two study seasons, with considerable variation among the cultivars. Two of the cultivars (*Salix eriocephala* and *S. lasiolepis* × *S. viminalis*) were identified as resistant, consistently showing low population levels of *T. salignus*. The remaining cultivars were classified as moderately resistant, susceptible, or highly susceptible, based on the aphid population levels. Aphid numbers were lower and the timing of the peak population was delayed in 2019, possibly reflecting weather patterns, differences in host plant quality (first- vs. second-year plants), or a combination of both. Aphid infestation delayed the flowering time, extended the duration of flowering, and decreased the catkin length in susceptible cultivars. Interestingly, aphid infestation was found to increase the total floral output of some willow cultivars. Aphid infestation had no measurable effect on the number of shoots of willow cultivars, but reduced the survival, height, and shoot diameter of the plants by the end of second growth season. It can be concluded that aphid population numbers and the extent of plant damage are cultivar-specific, with wide variations between resistant and susceptible cultivars.

2.2 Introduction

Willows are perennial deciduous plants in the genus *Salix* (family Salicaceae). The genus has a wide genetic diversity, encompassing about 450 species worldwide (Argus, 1997). The rapid growth rate and the ability to easily propagate vegetatively and to rapidly re-sprout after multiple cutbacks make some willow species suitable for biomass production in short rotation coppice systems (Sennerby-Forsse & Zsuffa, 1995; Timothy *et al.*, 2004). Additionally, modern willow growing systems are used as multi-purpose systems for biomass production, bioremediation, phytoremediation, nutrient management, and stream bank stabilization (Smart *et al.*, 2005). In New Zealand (NZ) willows are also used for erosion control, as windbreaks, for basket making, as a source of pollen for honeybees, and as livestock shelter and fodder (McIvor, 2013; Newstrom-Lloyd *et al.*, 2015; Wilkinson, 1999). Many willow cultivars with desirable traits have been developed through hybridization to meet specific needs linked to their uses. In NZ, there are currently over 50 planted willow cultivars consisting of many species and hybrids (Sopow *et al.*, 2017).

Among the 450 arthropod species (including 120 species of aphids) that feed on willows (Kennedy & Southwood, 1984), the giant willow aphid, *Tuberolachnus salignus* Gmelin (Hemiptera: Aphididae) has been identified as an economically important willow pest (Collins, 2001; Sopow *et al.*, 2017). This is one of the largest aphid species (5.8 mm in length) and can occupy more than half of the stem surface of young infested trees (Collins, 2001; Gunawardana *et al.*, 2014). It was first detected in Auckland in the North Island of New Zealand in 2013 and is now found throughout the country, where willows are grown (Martin, 2017). Fifty willow cultivars and one poplar species were confirmed as hosts of *T. salignus* in NZ (Sopow *et al.*, 2017), but a wider host range can be expected. The inconvenience of using conventional control measures such as chemical spray, and

the lack of natural enemies in NZ (Charles *et al.*, 2014) have brought to attention the need to test willow cultivars for resistance against *T. salignus*.

In the UK, Aradottir *et al.* (2009) suggested that *T. salignus* exhibited significant preferences for specific willow cultivars, as supported by different infestation levels in both field trials and laboratory bioassays. Therefore, exploring the aphid population densities and their fluctuation in different willow cultivars under natural or semi-natural conditions may be useful to identify resistant cultivars.

Besides monitoring aphid population levels and their fluctuations over time, measuring different plant fitness-related parameters can help to elucidate the impacts of the aphid on its host plants. In general, aphids manipulate the resource allocation within the plant, having the ability to negatively affect plant growth, survival, and reproduction (Goggin, 2007; Snow & Stanton, 1988). Severe aphid infestation has been shown to have long-lasting effects on plant growth in other tree species. For example, a single infestation event by the green spruce aphid *Elatobium abietinum* was found to reduce the vertical growth rate of *Picea sitchensis* for at least 3 years (Carter, 1977). Devastating effects on host survival have also been reported when an invasive aphid encounters a susceptible host, as occurred in Iceland, where the introduction of the pine woolly aphid *Pineus pini* in the late 1930's caused elevated mortality of Scots pine (*Pinus sylvestris*), leading to the ending of commercial plantings of this tree (Heiðarsson *et al.*, 2020). The impacts of aphid infestation on tree reproduction have been less studied, but there is evidence that aphids can affect the fertility and fecundity of annual species (Snow & Stanton, 1988).

Willows have a seasonal flowering phenology (Frankie *et al.*, 1974), known as 'mass flowering', producing all the catkins within a few weeks (Bawa, 1983; Newstrom-Lloyd *et al.*, 2015). The onset and duration of flowering determines the plants reproductive success (Bucher & Roemermann, 2020). Many abiotic (temperature and

photoperiod) and biotic factors (genotype, pollinators, seed dispersers and floral pathogens) influence the flowering phenology of plant species (Elzinga *et al.*, 2007; Nagahama & Yahara, 2019). The changes in flowering phenology due to insect herbivory have been rarely investigated, but decreased numbers of catkins per plant and delays in reproduction have been observed when different levels of vole (*Microtus agrestis* (L.) and *Clethrionomys glareolus* Schreb) bark damage were simulated in boreal willow, *Salix myrsinifolia-phylicifolia* (Elmqvist *et al.*, 1987). In *Silene alba* (Carophyllaceae), the plants susceptible to the fungus *Ustilago violacea* flowered earlier than resistant plants, and fungal infestation could delay flowering in susceptible plants (Biere & Antonovics, 1996).

Willow pollen is a good source of protein for honey bees during spring brood feeding, and for maintaining bee colonies after the winter rest (Newstrom-Lloyd *et al.*, 2015). In NZ, willow flowering in early spring is important for honey bees, due to a lack of alternative sources of pollen. Both the flowering phenology and the number of catkins produced are important parameters in willow-honey bee interactions. Changes in these two parameters can affect the availability of pollen (Juenger & Bergelson, 1997), and the plants reproductive success (Galloway & Burgess, 2009). To our knowledge, to what extent *T. salignus* infestation affects the flowering phenology and catkin production of willow cultivars has not been evaluated previously.

In this study, we monitored the *T. salignus* populations on fifteen willow cultivars and quantified the effect of *T. salignus* infestation on the flowering phenology, flower production, and biomass growth of young willow plants. The study asked the following research questions: 1) Do the fifteen willow cultivars have the same or different populations of *T. salignus*? 2) How do aphid populations change on first- and second-year plants? 3) How does *T. salignus* infestation influence the flowering phenology and

catkin production of willow cultivars? 4) To what extent does aphid infestation affect the survival and growth of willow cultivars?

2.3 Materials and Methods

2.3.1 Study site and soil preparation

This present study was conducted in a 4,000 m^2 (50 × 80 m) paddock at the Plant Growth Unit, Massey University, New Zealand (40°22'41.70"S, 175°36'30.67"E). All four sides of the experimental area (with farm road at the north side) were enclosed by shelterbelts with pines and poplar/willow trees. On May 16, 2017, the field was assessed to make sure that the experimental area was well positioned to avoid potential differences in shading from shelterbelts (5-17 m away from the fences). Six 75 m long rows were marked out with 4 m spacing between rows. The herbicide glyphosate was sprayed to kill the weeds at the rate of 10 ml L^{-1} along the rows with the width of 1 m. The areas of the rows were cultivated (rotary hoe) prior to planting the willow cuttings on June 7, 2017. The pH of the soil was slightly acidic (6.1 ± 0.05) and mean nitrogen content in air-dried soil samples was 0.25 ± 0.01%.

2.3.2 Cultivar selection and experimental design

Stem cuttings of fourteen willow cultivars were obtained from the willow collection at the RST Environmental Solutions nursery at Aokautere, and the Hawke's Bay Regional Council Allen Road nursery, while commercial willow growers in Otago provided cuttings of one other willow cultivar (*S. viminalis*, PN 220). The detailed information on the selected willow cultivars can be seen in **Table 2.1.** Seventy-two cuttings of each willow cultivar, measuring 20 cm in length and 13 ± 2.6 mm in diameter were collected, soaked in water, and stored in a temperature-controlled room (4°C) before

planting in the field. On June 16, 2017, the willow cuttings were manually planted by gently tapping them with a hammer to the depth of 15 cm, in six rows with 0.4 m spacing between cuttings in the rows. Twelve cuttings of each willow cultivar were planted together in a row plot in each row, with each row containing 180 cuttings of 15 different willow cultivars.

The experiment was laid out in three replicated blocks. Each block contained two rows, with each row randomly allocated to one of the two treatments: *T. salignus* (aphids added by inoculation) and control (no aphids). The layout represents a split-plot design, with aphid treatment as a whole-plot factor and willow cultivars as a sub-plot factor (see **Figure S2.1** for details of the experimental layout).

After planting the willow cuttings, plastic tree guards (Poly Logic Plastics Ltd) were installed to protect the young plants from damage by rabbits and hares. The young plants were irrigated in November and December 2017, during a period of dry weather. Weeds in a 1 m width cultivation zone in the rows were controlled by spraying with the herbicide Buster® (glufosinate-ammonium), and weeds inside the tree guards were removed manually.

Daily weather data (maximum and minimum temperature, precipitation, and relative humidity) were downloaded from the nearest weather station (Palmerston North Ews, Agent Number-21963) (NIWA, 2020), located 680 m away from the field trial site. The daily measurements were averaged to obtain weekly weather readings during the experimental period (**Figure S2.2**).

2.3.3 Aphid inoculation and population monitoring

Inoculation with five *T. salignus* adults per tree was done on the willow cultivars in the aphid treatment rows on January 25-27, 2018 and on December 6-7, 2019 (the onset

of the population build-up in both years). Because of unsuccessful aphid establishment on some plants, additional inoculations were done with 10 adult aphids on February 13-14, 2018 and January 30, 2019. In the control rows that were kept free of aphids, any dispersing aphids were manually removed from the plants on a weekly basis. Whenever manual control was not feasible, the insecticide Mavrik® (Tau-fluvalinate) was applied at the rate of 10 ml L^{-1} to control the aphid populations in the control rows.

The *T. salignus* population density was monitored on all the plants of the fifteen willow cultivars in the aphid-infested rows, on a weekly basis for two years, in order to evaluate the resistance levels of willow cultivars used in this experiment. The aphid population density was difficult to quantify, because of their dense patchy colonies. Therefore, proxy-log abundance classes, modified from Collins (2001), were used: 0=<5 aphids, 1=5-20 aphids, 2=20-50 aphids, 3=50-100 aphids, 4=100-300 aphids, 5=300-600 and 6=>600 aphids per plant. Time series plots were generated to visualize the aphid population fluctuation on the willow cultivars over time. The number of weeks with high aphid abundance (greater than 4 on the proxy-log scale) was calculated to compare the peak aphid populations on the willow cultivars in 2018 and 2019.

2.3.4 Flowering phenology and catkin measurements

The effect of *T. salignus* infestation on the flowering phenology of the willow cultivars was investigated from August to October 2018 (Southern hemisphere spring). At every weekly sampling visit, the fully opened catkins were counted on each plant, and then removed from the plants in both the aphid-infested and control rows. The number of days to first flowering was recorded from the day of planting the willow cuttings to the day when a plant produced its first fully opened catkin. The flowering duration (days) was calculated from the first to the last day of flowering. The weekly numbers of removed catkins were summed up to get the total catkin number per plant. Ten catkins were

collected from each plant at the peak flowering time of each willow cultivar, and put into labelled paper bags. The catkin lengths (cm) were measured in the lab. Total floral output was calculated by multiplying the mean catkin length with the catkin number per plant. The first and last plants in the row plots (plants no. 1 & 12) of each willow cultivar were excluded from the flower measurement to avoid edge effects. Abnormal and deformed catkins were removed from the plants, but excluded from the measurements.

2.3.5 Sapling survival and growth measurement

The plants in the aphid infested rows were monitored weekly to determine the effect of *T. salignus* on the survival of the willow cultivars. Maximum plant height (m) was measured on the tallest shoot of each plant from the aphid-infested and control rows, with a telescopic height pole (Senshin 8 m SK-88, Osaka, Japan) on August 8, 2018, and June 15, 2019, at the end of the first and second growth seasons. The number of shoots and shoot basal diameters (cm) of each plant were measured on August 9, 2018 and June 3, 2019. Mean shoot basal diameter was calculated by dividing the sum of the diameters of all shoots produced by each plant, by the total number of shoots.

Table 2.1. List of willow cultivars used in the field trial.

Symbol	Species	Code	Common name	Type	Sex
A	*S. candida*	PN 385	Furry Ness	Shrub	Male
B	*S. eriocephala*	PN 376	Americana	Shrub	Male
C	*S. lasiolepis*	PN 751	-	Shrub	Male/female
D	*S. lasiolepis* × *S. viminalis*	NZ 04-106-073	-	Shrub	Male
E	*S. purpurea*	PN 249	Booth	Shrub	Female
F	*S. schwerinii*	PN 386	Kinuyanagi	Shrub	Male
G	*S. viminalis*	PN 220	Gigantea	Tree/Shrub	Male/female
H	*S.* × *reichardtii*	PN 714	-	Shrub	Male
J	*S. alba*	PN 357	-	Tree	Male
K	*S. lasiandra*	PN 747	-	Tree	Male
L	*S. matsudana*	PN 227	Kew	Tree	Female
M	*S. matsudana* × *S. alba* (1)*	NZ 1040	Tangoio	Tree	Female
N	*S. matsudana* × *S. alba* (2)*	NZ 1184	Moutere	Tree	Male/female
P	*S. matsudana* × *S. lasiandra*	NZ 03-003-073	-	Tree	Male
Q	*S.* × *fragilis*	PN 218	Russelliana	Tree	Female

***** Referred to as *S. matsudana* × *S. alba* (NZ 1040) and *S. matsudana* × *S. alba* (NZ 1184) in the text.

2.3.6 Statistical analysis

A linear mixed model (LMM) was used to compare the number of weeks with high aphid numbers (proxy-log scale >4) among the willow cultivars in the *lme4* package. In the model, the willow cultivar and monitoring year were used as fixed factors, and row number as a random factor. Repeated measure LMM was fitted to investigate the effect of willow cultivars on the aphid population level, using the weekly monitoring date as a repeated factor. The LMMs with row number as a random factor were fitted to compare the flowering (days to first flowering, duration of flowering, catkin number per plant, catkin length and total floral output) and plant growth (maximum plant height, shoot number and mean shoot basal diameter) measurements of the willow cultivars between treatments (aphid-infested and control). The flowering and plant growth data were square-root transformed if the assumption of normality was not met. Whenever a significant effect was detected in the model output, a *post-hoc* means comparison was conducted using Tukey's HSD test in the *multcomp* and *lsmean* packages.

The Kaplan-Meier method was used to estimate the survival functions of willow cultivars from the aphid-infested rows, using the *suvminor* and *survival* packages (Therneau, 2015). I then ran the log-rank test to compare the survival curves. All the analyses and graphical displays were done in R version 4.0.0 (R Development Core Team, 2019).

2.4 Results

2.4.1 Aphid population monitoring

Aphid populations increased rapidly after the first successful inoculation (February 14, 2018), reaching the peak in April 2018, slightly reducing in May 2018 and then rapidly diminishing in July 2018 (**Figure 2.1**). Aphid numbers remained low from

early August until early October 2018, and the population started to build up again at the end of October 2018, followed by a gradual increase in December 2018 to January 2019. The aphid population increased gradually after the second inoculation (January 30, 2019), reaching the peak density in May 2019, and then decreased sharply during July-August 2019 (**Figure 2.1**). During the second year (2019), the peak aphid population occurred earlier, lasted longer, and had higher sustained abundance than in 2018.

When comparing different willow cultivars, I found significant differences in the number of weeks with high aphid abundance (>4 on the proxy log-scale) among the willow cultivars ($F_{14,828}$=82.37, P<0.001) (**Figure 2.2**). Aphid abundance never reached >4 on two of the cultivars (*S. lasiolepis* × *S. viminalis* and *S. eriocephala*). At the other end, *S. viminalis* had the greatest number of weeks with high aphid abundance (**Figure 2.2**). Based on the number of weeks with high aphid abundance, the willow cultivars were classified into four categories: resistant – zero weeks with high aphid infestation (*S. lasiolepis* × *S. viminalis* and *S. eriocephala*), moderately resistant – one to four weeks (*S. lasiandra*, *S. purpurea, S.* × *reichardtii*), susceptible – five to eight weeks (*S. matsudana* × *S. lasiandra, S. matsudana, S.* × *fragilis, S. lasiolepis, S. alba, S. matsudana* × *S. alba* (2), *S. schwerinii* and *S. matsudana* × *S. alba* (1)), and highly susceptible – over nine weeks (*S. candida* and *S. viminalis*).

The number of weeks with high aphid abundance decreased significantly in 2019, compared to 2018 ($F_{1,828}$=919.24, P<0.001) (**Figure S2.3**). The willow cultivar*year interaction effect was also significant ($F_{14,828}$=19.90, P<0.001). With the exception of *S. viminalis,* all the willow cultivars had lower aphid populations in 2019 compared to 2018 (**Figure S2.4**).

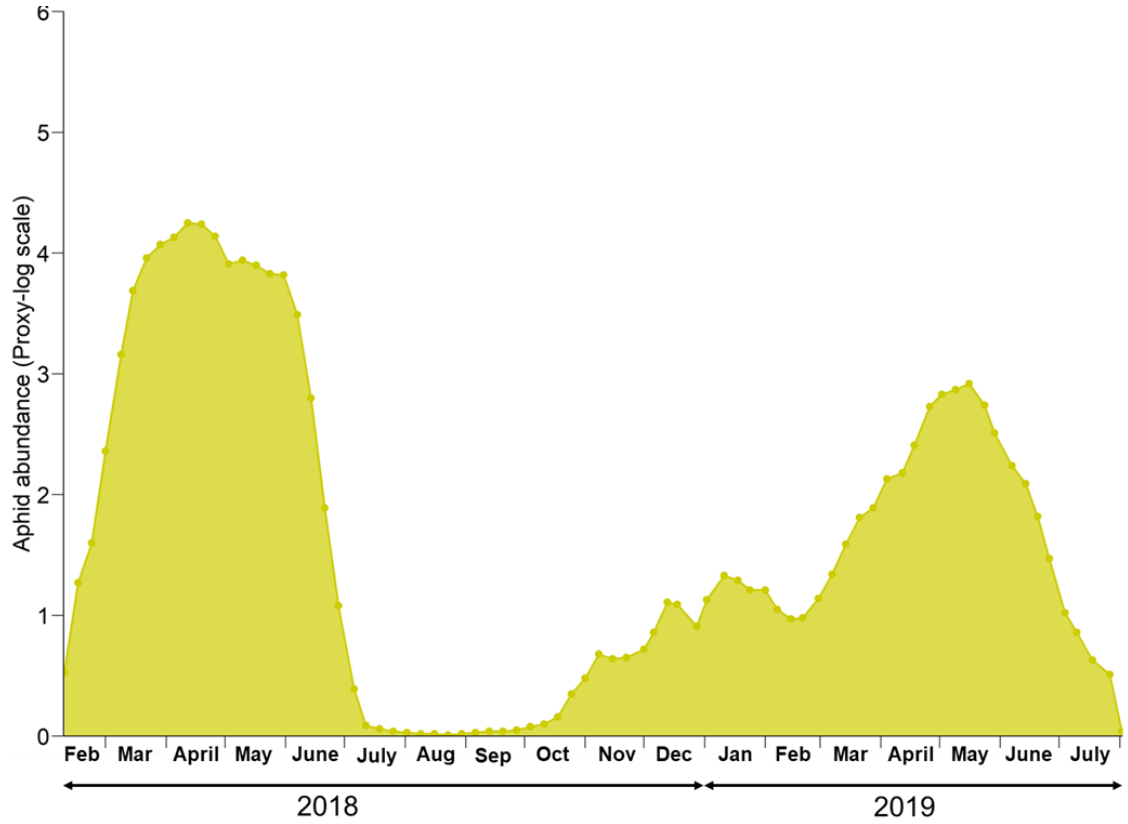

Figure 2.1. Population levels of *T. salignus* on susceptible willow cultivars, from the first inoculation to the end of experimental period; data represent the average for 13 willow cultivars. Two resistant cultivars (*S. lasiolepis* × *S. viminalis* and *S. eriocephala*) on which aphid numbers never reached high level (>4) were excluded. See **Figure S2.4** for the data on all the individual cultivars.

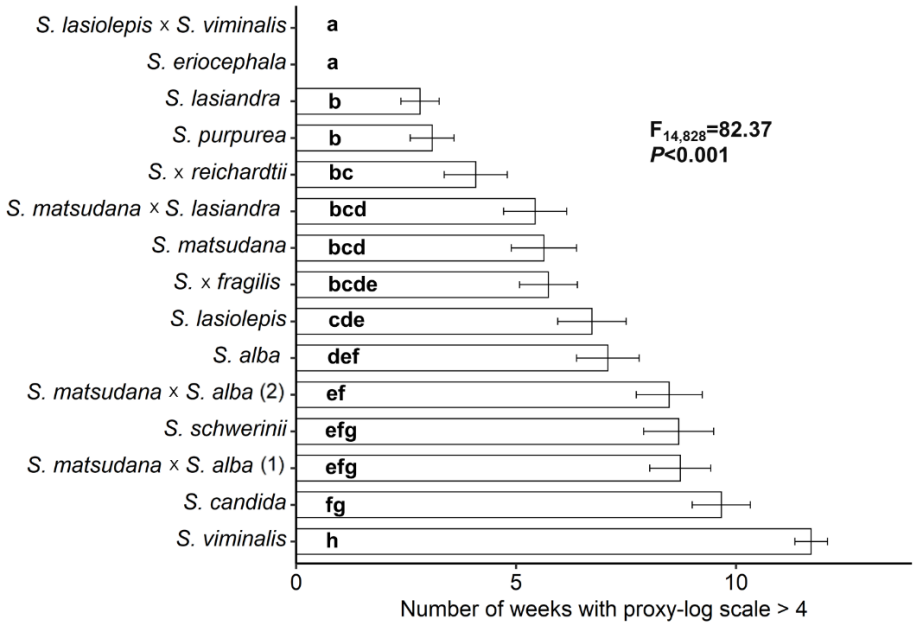

Figure 2.2. Total number of weeks with high *T. salignus* abundance (greater than 4 on the proxy-log scale) for the willow cultivars; combined data from 2018 and 2019. The values represent mean ± SE. Different letters indicate significant differences at α = 0.05, Tukey's HSD test.

2.4.2 Flowering parameters

The number of days to first flowering differed significantly among the willow cultivars ($F_{14,768}$=757.71, P<0.001). *Salix lasiolepis*, *S. matsudana* × *S. lasiandra* and *S. lasiolepis* × *S. viminalis* were early flowering cultivars (late July to late August), while *S.* × *fragilis*, *S. candida*, *S. purpurea*, *S. lasiandra* and *S. viminalis* flowered later, starting from early October (**Figure S2.5).** The number of days to first flowering was not affected by *T. salignus* infestation ($F_{1,768}$=1.47, P=0.224, **Table S2.1**), but the cultivar*aphid treatment interaction was significant ($F_{14,768}$=11.30, P<0.001). Infestation by *T. salignus* delayed the first flowering of *S. candida* by 17 days and *S.* × *reichardtii* by 7 days (**Figure S2.6**), while *S. eriocephala* in the aphid-infested rows bloomed 9 days earlier than in the control rows (**Figure 2.3a**).

The duration of flowering differed among the willow cultivars ($F_{14,768}$=82.80, P<0.001) (**Figure 2.3b & Figure S2.5**). *Salix eriocephala* had the longest duration of flowering (40 days), followed by *S. viminalis* (38 days), *S. schwerinii* (37 days), *S. candida* (37 days) and *S. matsudana* × *S. lasiandra* (36 days). Among the remaining cultivars, *S. purpurea*, *S. matsudana* × *S. alba* (NZ 1184), *S. lasiolepis*, *S. lasiandra*, *S.* × *reichardtii*, *S. alba* and *S. lasiolepis* × *S. viminalis* flowered for a shorter period, with the duration ranging from 5 to 17 days (**Table S2.1**). *Tuberolachnus salignus* infestation significantly extended the flowering duration of the willow cultivars ($F_{1,768}$=82.26, P<0.001). The effect of aphid infestation was most pronounced in *S. candida*, *S. matsudana*, *S. viminalis* and *S.* × *reichardtii*, with a 7 to 19 day increase in the flowering duration in the aphid-infested plants, compared to the control plants (**Figure 2.3b**).

The number of catkins per plant differed among the willow cultivars ($F_{14,812}$=190.71, P<0.001), with significantly higher catkin production in *S. schwerinii* (627 ± 34), *S. matsudana* (286 ± 19) and *S. eriocephala* (246 ± 18). The lowest number of catkins were produced by *S. purpurea*, *S. lasiolepis*, and *S. matsudana* × *S. alba* (NZ 1184) (**Table S2.1**). The number of catkins per plant increased significantly in the aphid-infested plants of *S. schwerinii* ($F_{1,812}$=190.71, P<0.001) (**Figure 2.3c**).

The mean catkin length was significantly different among the willow cultivars ($F_{13,716}$=1378.85, P<0.001), with considerably longer catkin length in *S. lasiandra*, *S. alba*, *S.* × *fragilis* and *S. lasiolepis* × *S. viminalis*, than in the other cultivars (**Table S2.1**). Infestation by *T. salignus* significantly reduced the catkin length of the willow cultivars ($F_{1,716}$=64.71, P<0.001). The interaction effect of willow cultivar and infestation treatment was significant, with catkin length significantly reduced in the aphid-infested *S. candida*, *S. alba*, *S. viminalis* and *S.* × *reichardtii* (**Figure 2.3d & Figure S2.7).**

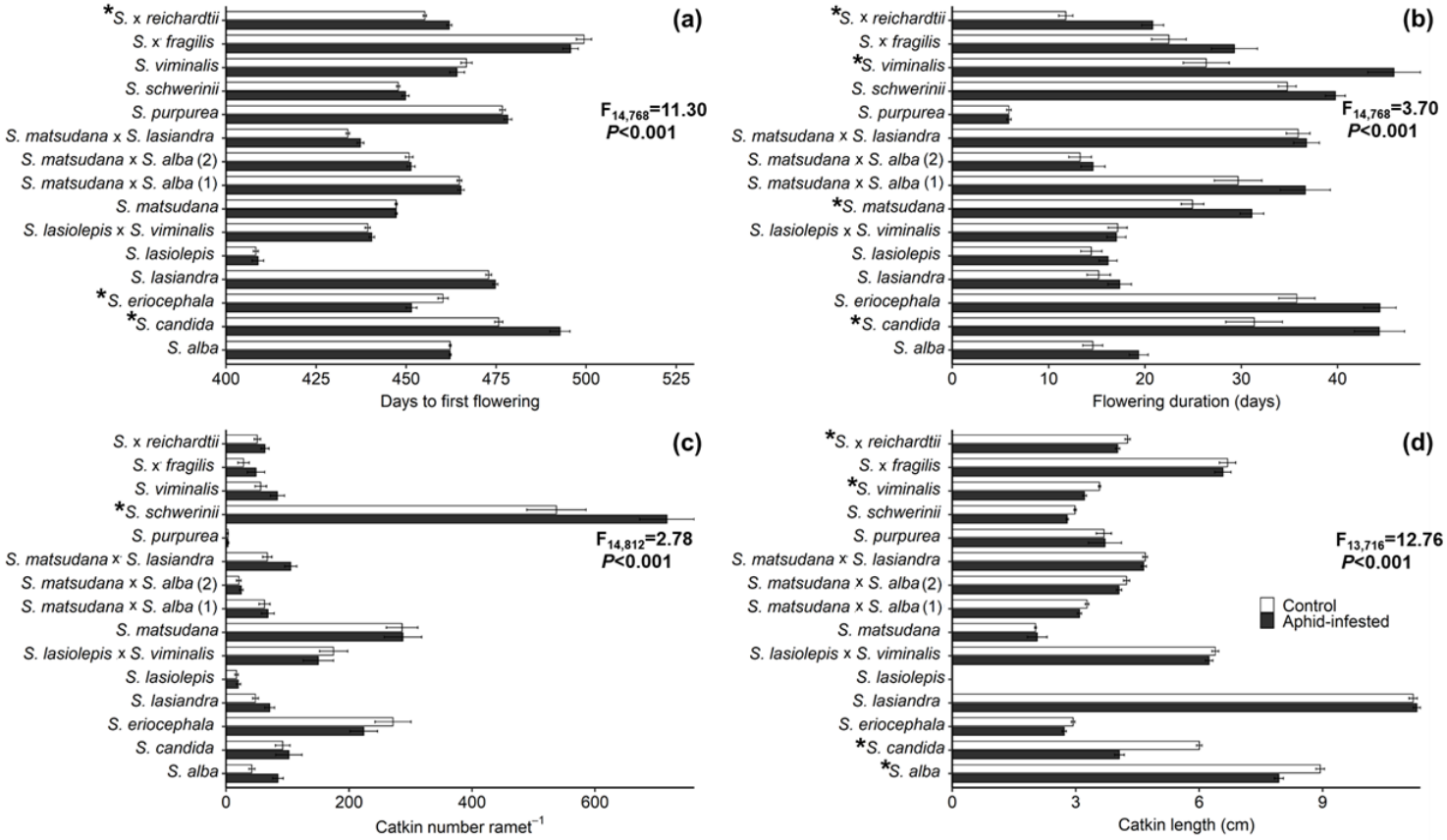

Figure 2.3. The effect of *T. salignus* infestation on the flowering phenology, catkin number and catkin size of the willow cultivars. Values represent mean ± SE; clear bars are the control plants, black bars are the aphid-infested plants. Catkin length was not measured for *S. lasiolepis*. The F-tests are for the cultivar × aphid infestation interaction effect. The significant effect of aphid infestation in the individual willow cultivars is shown with an asterisk (*), Tukey's HSD test, $\alpha = 0.05$.

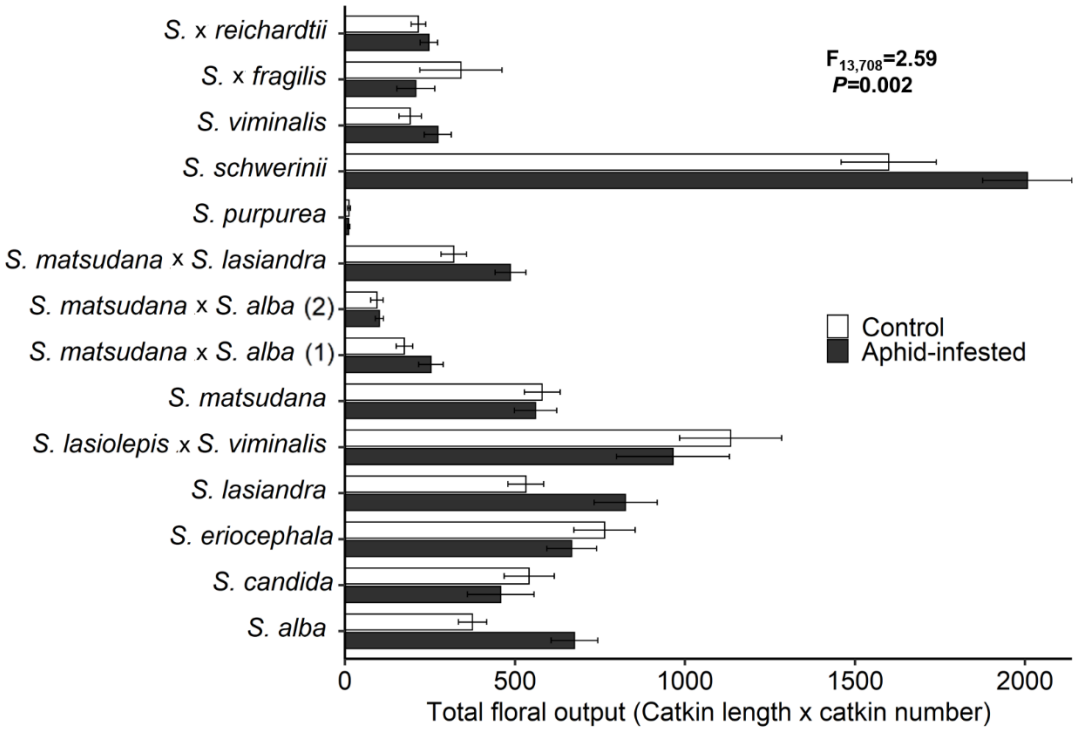

Figure 2.4. The total floral output of the willow plants as affected by willow cultivar and aphid infestation. Values represent the mean ± SE; clear bars are the control plants, black bars are the aphid-infested plants. The F-test showed the significant interaction effect of willow cultivar and aphid infestation on the total floral output.

Overall, the aphid-infested willow plants produced more numerous but smaller catkins than the control plants (**Table S2.1**). The total floral output differed significantly among the willow cultivars ($F_{13,705}$=79.73, P<0.001) (**Figure 2.4**). *Salix schwerinii* and *S. lasiolepis* × *S. viminalis* produced the highest floral output, while the lowest was observed in *S. purpurea* and *S. matsudana* × *S. alba* (NZ 1184) (**Table S2.1**). *Tuberolachnus salignus* infestation significantly increased the total floral output of the willow cultivars ($F_{1,705}$=6.55, P=0.011). The interaction effect was significant, but the floral output did not differ within each cultivar in the control and aphid-infested treatments (**Figure 2.4**).

2.4.3 Sapling survival and growth parameters

Aphid infestation significantly influenced the survival of the willow cultivars (χ^2_{14}=134, P<0.001). While all the plants of 12 of the cultivars were alive at the end of the trial, the survival of the *S. viminalis*, *S. eriocephala* and *S. candida* plants was reduced to 86.7%, 78.6% and 62.1%, respectively (**Figure 2.5**).

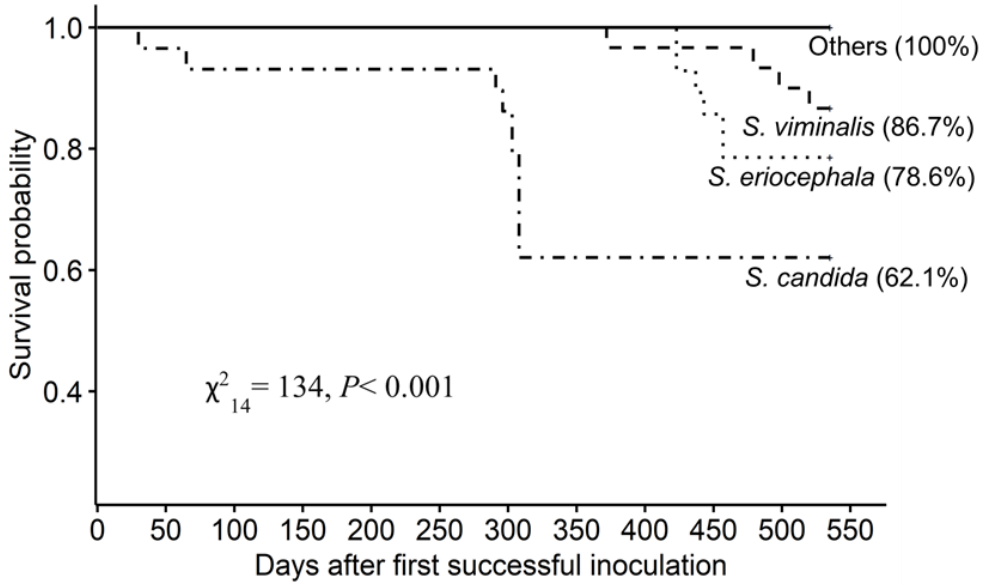

Figure 2.5. Survival of the willow plants inoculated with *T. salignus*. The survival days were counted from the date of the first successful aphid inoculation to the end of the two-year field trial. "Others" represents the twelve cultivars, other than *S. candida*, *S. eriocephala* and *S. viminalis*. The Chi-square value represents the result of the log rank test, comparing the survival distribution of the fifteen willow cultivars.

Aphid infestation did not affect the maximum plant height at the end of the first growth season in 2018 ($F_{1,841}$=0.11, P=0.76), but reduced the plant height at the end of the second growth season in 2019 ($F_{1,818}$=168.02, P=0.03, **Table S2.2**). The reduction in height was significant in *S. candida* and in *S. matsudana* × *S. lasiandra* (**Figure 2.6**).

Maximum plant height, shoot number, and mean shoot diameter differed significantly among the willow cultivars (**Table S2.2**). The effects of aphid infestation

and the interaction between infestation and willow cultivar were quite variable (**Table S2.2 & Figs. 2.6-8**).

Across all the willow cultivars, *T. salignus* infestation did not affect the shoot number at the end of first ($F_{1,841}$=0.13, *P*=0.73) and second ($F_{1,818}$=1.19, *P*=0.34) growth seasons (**Table S2.2**). However, the cultivar*infestation interaction effects were significant, and in the second growth season (2019) the infestation by *T. salignus* significantly reduced the shoot number of *S. viminalis* (**Figure 2.7**).

Similarly, there was no effect of *T. salignus* infestation on the mean shoot diameter at the end of the first growth season in 2018 (**Table S2.2** & **Figure 2.8**). At the end of the second growth season (2019), the aphid infestation considerably reduced the mean shoot diameter of the cultivars ($F_{1,818}$=28.48, *P*=0.006). The interaction effect of infestation*cultivar was significant, and compared to the control plants, the shoot diameter was greatly decreased in the aphid-infested *S. alba* and *S. matsudana* × *S. lasiandra* (**Figure 2.8**).

2.5 Discussion

In the current study, the aphid population level was found to fluctuate on the willow cultivars in the two consecutive monitoring years, with considerably higher numbers on the susceptible cultivars in both growth seasons. Two of the cultivars (*S. eriocephala* and *S. lasiolepis* × *S. viminalis*) were identified as resistant, consistently showing low population levels of *T. salignus*, whereas *S. candida* and *S. viminalis* were highly susceptible. Aphid numbers were lower, and the peak population time was delayed in 2019, which may reflect the different weather patterns and/or the host plant quality in the first and second growth seasons.

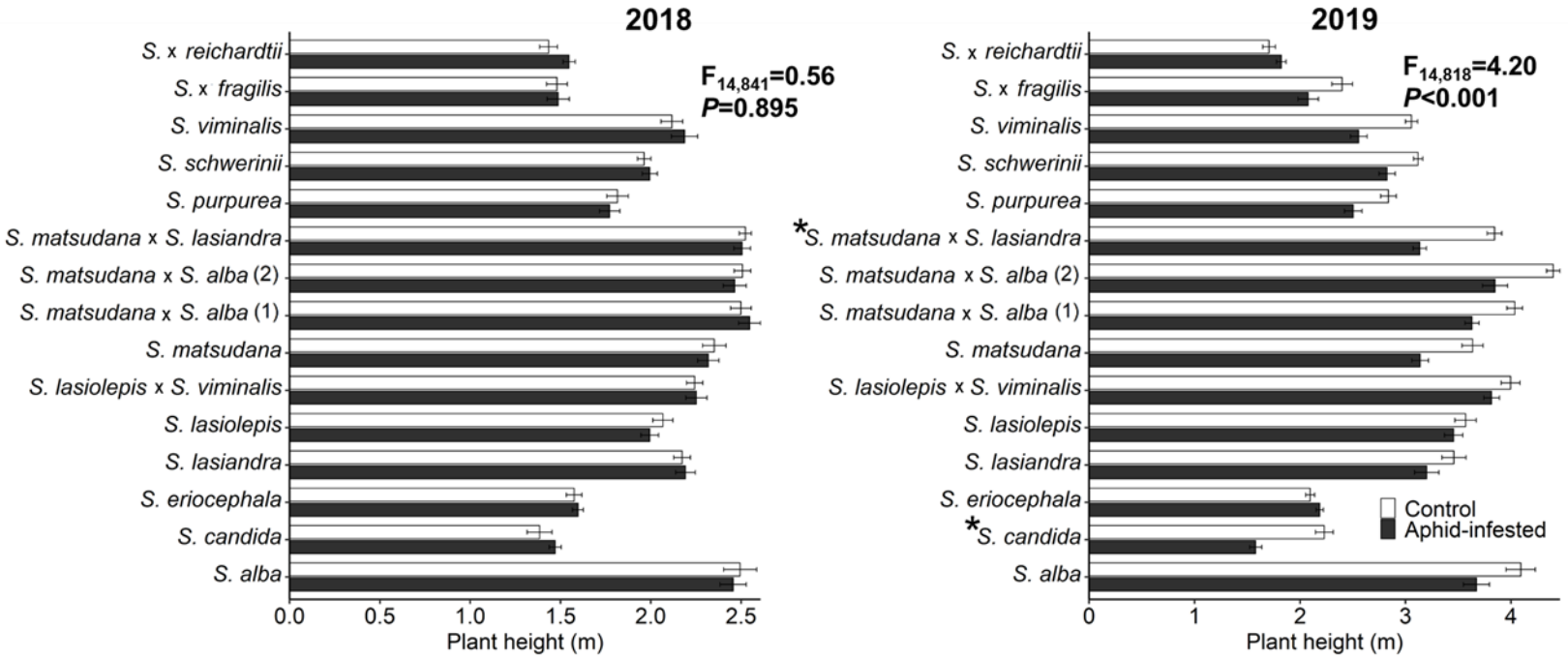

Figure 2.6. The effect of *T. salignus* infestation on the maximum plant height of the willow cultivars, measured at the end of each growth season. The F-tests are for the interaction effect of cultivar and aphid infestation on the plant height. Asterisks indicate a significant aphid effect within the cultivars, Tukey's HSD test, $\alpha = 0.05$.

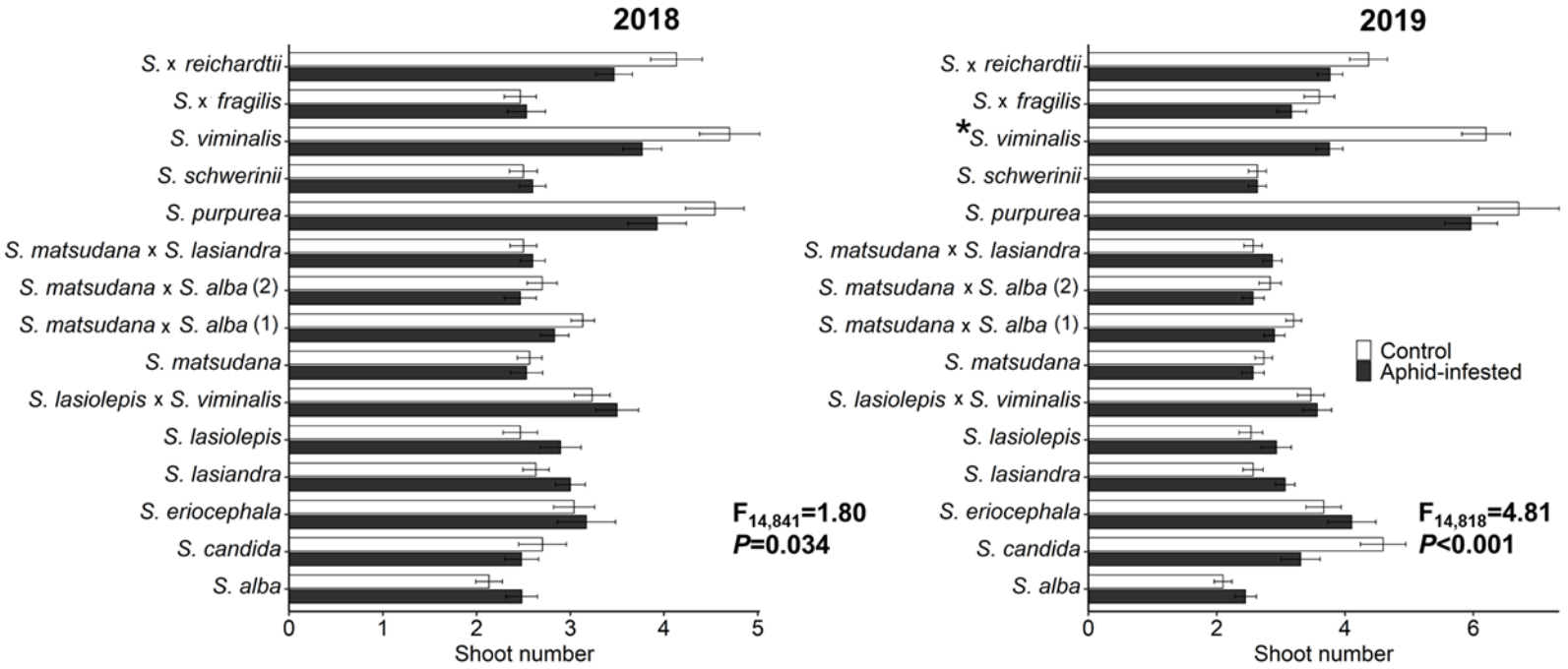

Figure 2.7. The effect of *T. salignus* infestation on the mean number of shoots in the willow cultivars. The F-tests are for the interaction effect of aphid infestation and cultivar. The asterisk indicates a significant aphid effect within the cultivar, Tukey's HSD test, $\alpha = 0.05$.

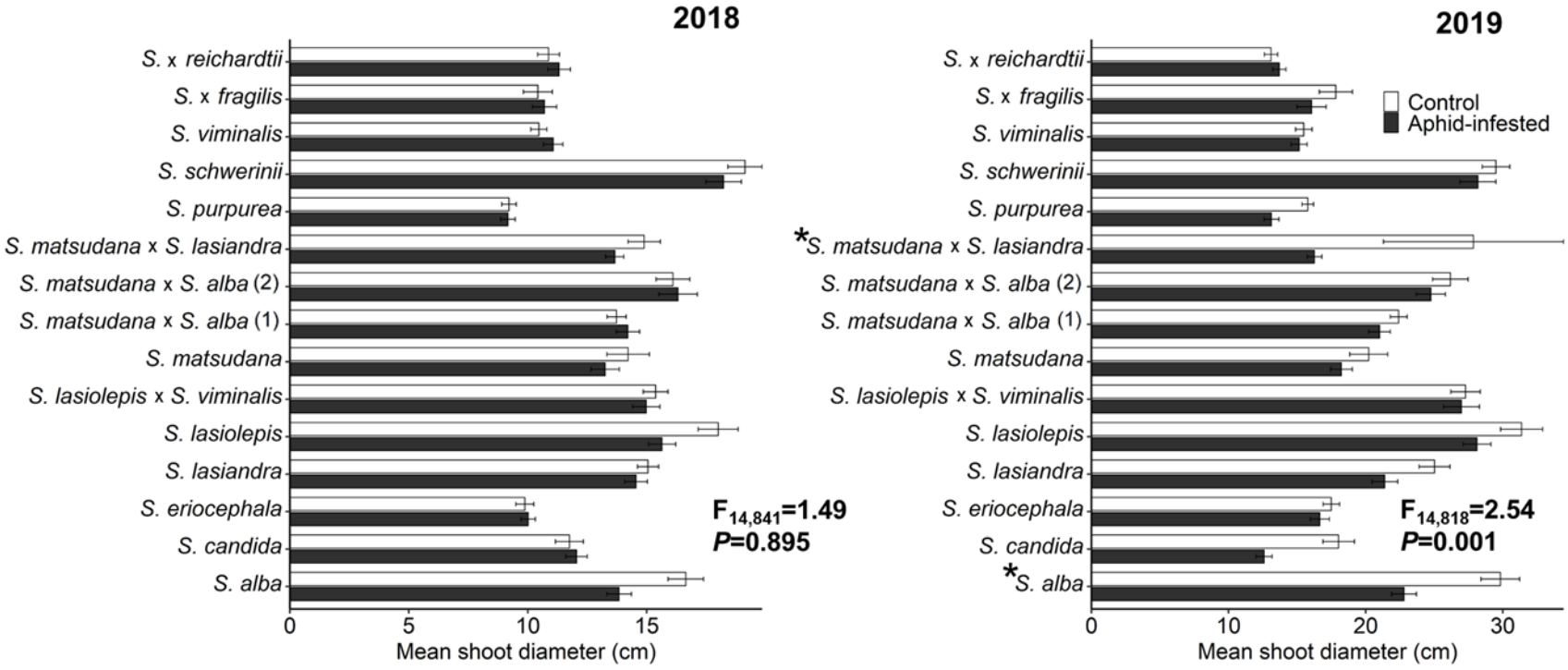

Figure 2.8. Mean shoot diameter of the willow cultivars, as affected by *T. salignus* infestation. The F-tests are for the interaction effect of aphid infestation and cultivar. Asterisks indicate a significant aphid effect within the cultivars, Tukey's HSD test, $\alpha = 0.05$.

Seasonality determines population fluctuations in tree-dwelling aphids (Dixon *et al.*, 1996). Seasonal changes in tree nutritional quality, such as the phloem sap quality (Sequeira & Dixon, 1996) and the timing of sap flow, are known to affect the physiology of adult aphids. For example, smaller body size and reduced reproduction of aphids were associated with lower concentrations of plant amino-nitrogen (amine, amides, amino acids and proteins) at the end of growth season (Kidd, 1985; Kindlmann & Dixon, 1996). In our study, the population densities of *T. salignus* fluctuated widely within each year, probably reflecting the effects of seasonal weather patterns (Day *et al.*, 2010), and seasonal changes in the host plant quality (Kindlmann & Dixon, 1996), on the aphid's physiology and survival. Seasonal changes in weather patterns and host plant quality are closely related to each other (Kindlmann & Dixon, 1996) in shaping the population patterns of *T. salignus* on the willow cultivars. The delayed time of the peak population abundance in 2019 might be related to the hotter weather and prolonged drought following the aphid inoculation in 2019, which made it hard for *T. salignus* to successfully establish on willow plants. No aphids were present on *S. matsudana*, *S. purpurea*, *S. viminalis*, *S.* × *fragilis and S.* × *reichardtii* from early June to early October 2018, which probably indicated the lack of assimilate transport. The giant willow aphid does not seem to hibernate in New Zealand, with small numbers of aphids occurring in winter on *S. lasiandra* and *S. schwerinii*. Further study should focus on how the aphid overwinters on these cultivars in New Zealand.

Both tree age and tree size can affect the aphid population densities (Straw *et al.*, 2020). When the plant ages, the stem structure changes, with the bark increasing in thickness and density (Sonmez *et al.*, 2007). A maturing tree could also change the local micro-climate (Donaldson *et al.*, 2006), thus changing the insect habitat. Our study compared aphid population numbers on first- and second-year willow plants; although

we observed aphid populations to decline between the first and the second years of monitoring, a longer observation period, and a different study design will be needed to examine the effect of tree age on *T. salignus* populations.

The "see-saw effect" in insect population dynamics is the negative relationship between initial and final population densities across different seasons (spring, summer and autumn in the current study), and years (Kindlmann & Dixon, 2010; Sequeira & Dixon, 1997). Aphid physiology, host plant quality at the end of the growth season, and their interaction effects, cumulatively influence the occurrence of the see-saw effect in aphid population dynamics (Kindlmann & Dixon, 1996). Our experimental results show the evidence of a see-saw effect in *T. salignus* population dynamics. Early and high aphid population build-up uses up available host resources and reduces host plant quality later in the growth season (Bumroongsook & Harris, 1991; Denno *et al.*, 2000), as observed in the *S. viminalis* cultivar (**Figure S2.4**). Heavy infestation in the first-year plants may have reduced the host quality of some willow cultivars (susceptible shrub willow cultivars), decreasing the aphid population numbers and delaying the population peaks in the second year. Further investigation should focus on changes in aphid body size, fecundity and population fluctuations across seasons and over a number of years, to investigate the see-saw population dynamics of *T. salignus* on different willow cultivars, and to separate it from weather effects.

Plant genotypic variation in resistance is an important feature explaining population dynamics of associated insect species (Faticov *et al.*, 2020; Tomescu & Nef, 2007). Differences in the susceptibility of willow cultivars to insect pests can reflect their genotypes, both within and among willow populations and species (Kendall *et al.*, 1996; Nordman *et al.*, 2005). Plant genotypes can vary in the composition of sugars (mainly sucrose) and amino acids in their phloem sap that determines aphid fecundity (Febvay *et*

al., 1988) and abundance (Honěk, 1987). Aphid performance is directly correlated with the amino acid composition of different plant cultivars (Sandström *et al.*, 2000). However, host nutritional quality alone cannot explain plant resistance to insect herbivory (Febvay *et al.*, 1988). The allelochemical content of phloem sap can affect host plant quality (Baldwin *et al.*, 2001) and vary with host developmental stages (Parker *et al.*, 2000) and genotypes (Killiny, 2017). These differences can influence aphid performance and abundance on different willow cultivars (Karley *et al.*, 2002; Parker *et al.*, 2000). Further research is advised to link *T. salignus* abundance and host quality, to better understand why aphid abundance is higher on some cultivars than others, and why some cultivars are more resistant than others.

In the current study, aphid infestation had no measurable effect on the flowering and growth parameters of resistant and moderately resistant cultivars. In susceptible cultivars, aphid infestation decreased plant growth but extended flowering time and caused infected plants to produce more catkins than control plants (**Table S2.1**). The results are in agreement with (Collins, 2001) who reported that *T. salignus* infestation negatively affected shoot and root production of *S. viminalis,* although their study did not consider flowering.

Insect herbivory can change the plant ontogeny (a resource allocation process, Dayrell *et al.*, 2018) that leads to changes in floral traits, flowering time and production (Hoffmeister *et al.*, 2016; Rusman *et al.*, 2020). The extent to which herbivory affects flowering phenology appears to relate to the time and ability of the host plant to compensate for the damage (Freeman *et al.*, 2003; Oesterheld & McNaughton, 1991). For example, the shrub willows showed more pronounced changes in flowering parameters than the tree willows. Susceptible cultivars in the aphid-infested rows seemed to take a longer time to restore the resources required for flowering (Nagahama & Yahara, 2019),

and produced more catkins less synchronously over a longer flowering duration, while those in the control rows flowered simultaneously within a shorter period of time. The amount of flower production is directly related to the flowering duration in some trees (Otárola *et al.*, 2013). The date of the first and last flowering determines flowering duration, that in turn affects the number of flowers that the plants produce (Dorji *et al.*, 2020).

The willow cultivars used in the current study appear to have different ontogenetic shifts between growth and reproduction. The shorter catkin length in the aphid-infested *S. candida, S. × reichardtii, S. viminalis, and S. alba* cultivars suggests resource limitation due to aphid herbivory (Barber *et al.*, 2015). These cultivars seem to invest less resources than are needed for producing normal-sized catkins. Plants exposed to herbivorous insects early in the developmental stages are known to produce smaller flowers compared to the control plants (Hoffmeister *et al.*, 2016). Some aphid-infested cultivars (e.g., *S. schwerinii*) produced more catkins than the control plants, which is likely to be an over-compensatory response (Agrawal, 2000) to *T. salignus* infestation. There seems to have been a close relationship between the decrease in catkin size and increase in flowering duration of *S. candida, S. × reichardtii* and *S. viminalis.*

As willows and pollinators are mutualistic partners (Tumminello, 2016), a shift in the flowering phenology and duration can have ecological consequences in willow agro-ecosystems (Hovenden *et al.*, 2008). First, changes in the phenology can force plants to pollinate assortatively: early flowering plants can only be pollinated by other plants that flower early, and the same is true for late flowering plants (Ismail & Kokko, 2020). Second, phenological shifts can influence the flower display, growth form, and reproductive potential of the forthcoming plant generation (Galloway & Burgess, 2009). Third, phenological delays and changes in flowering duration can lead to a mismatch

between peak flowering and peak pollinator visitation, causing a negative impact on the plant-pollinator interaction (Rafferty & Ives, 2011), and on the ecosystem service that pollinators provide (Kearns *et al.*, 1998). Finally, the shift in flowering time and total floral output can cause pollen limitation (Juenger & Bergelson, 1997) or surplus (pollen unused by pollinators), changing the community structure of pollinators and associated antagonists (Elzinga *et al.*, 2007).

Infestation by *T. salignus* is known to decrease plant survival (Kumar *et al.*, 2003; Sopow *et al.*, 2017). In this study, it was found that aphid infestation reduced the survival of *S. viminalis* and *S. candida*. In *S. candida*, aphid infestation severely affected the plant health, and decreased plant survival was pronounced in the spring of the second growth season. Dead *S. viminalis* plants were observed only in the second growth season. Similarly, high infestation of Scots pine (*Pinus sylvestris*) by the pine woolly aphid (*Pineus pini*) caused significant mortality in the second year post-infestation (Heiðarsson *et al.*, 2020). The elevated mortality of the resistant cultivar *S. eriocephala* in 2019 was probably not related to aphid infestation. This cultivar is drought-susceptible, and the decreased plant survival coincided with a prolonged summer drought in the 2019 growth season.

Aphid infestation affects the photosynthesis rate and growth of host plants (Patankar *et al.*, 2011). Woody plants respond to herbivore damage by increasing the photosynthesis rate to compensate for the losses of plant growth and carbohydrates in the plant tissues (Nykänen & Koricheva, 2004). Collins et al. (2001) found that compared to the control plants, shoot biomass decreased when *S. viminalis* ‘Jorr’ saplings were inoculated with *T. salignus*. Infestation decreased the water content and amount of woody tissues in the willow shoots, while increasing the photosynthetic rate and leaf nitrogen content (Collins *et al.*, 2001b), making the host plants more palatable for other herbivores

(Charles *et al.*, 2014), and more susceptible to fungal (*Botryosphaeria parva*) attack (Sopow *et al.*, 2017).

In the current study, the impact of *T. salignus* infestation on willow growth varied greatly. The magnitude and intensity depended on the aphid population densities, host genotypes, and host ability to compensate for aphid damage (Dedryver *et al.*, 2010; Freeman *et al.*, 2003). Susceptible cultivars (*S. viminalis* and *S. candida*) hosted high aphid population loads in both years and showed a decrease in the maximum plant height and shoot number. Mean shoot diameter did not decrease in these shrub willow cultivars, but decreased in the susceptible tree willows (*S. alba* and *S. matsudana* × *S. lasiandra*), suggesting that the effects of aphid infestation may be cultivar-specific. Cultivars that hosted large aphid populations (such as *S. lasiolepis* and *S. schwerinii*) showed little or no reduction in growth due to aphid infestation, which might be attributed to their higher ability to compensate the losses caused by *T. salignus* (Ney *et al.*, 2013). Generally, willows possess a large nutrient stock and biomass in the underground parts (Cunniff *et al.*, 2015), and so the cultivars with deep-rooted systems (tree willows in the current study) appear to be able to rapidly regenerate phloem sap lost to *T. salignus*. Further research should focus on the effect of aphid infestation on the below- and above-ground biomass production of resistant and susceptible cultivars.

2.6 Conclusions

The feeding behaviour of major herbivores affects the productivity of their host plants. In the case of willow plants, our results demonstrate that the impact of *T. salignus* infestation differs between willow cultivars. Overall, the infestation by this aphid affected flowering and decreased plant survival and growth of some cultivars, while increasing floral output in other cultivars. The observed differences in the willow responses to *T. salignus* indicate variable resistance and compensatory ability among the

cultivars, with *S. viminalis and S. candida* being highly susceptible to aphid infestation, and *S. eriocephala* and *S. lasiolepis* × *S. viminalis* being resistant. Further studies are needed to investigate the impact of seasonality and host plant biochemistry on aphid populations, and to explore the effect of aphid infestation on the above- and below-ground biomass production in different willow cultivars.

2.7 Appendix

Table S2.1. Effect of cultivar and *T. salignus* aphid infestation on flowering phenology, catkin production, catkin size and total floral output of willows. Values are mean ± SE. Different letters in each column indicate significant difference at α=0.05, Tukey's HSD test.

Individual effect	Flowering parameters				
	Days to first flowering	Flowering duration (days)	Catkin number	Catkin length (cm)	Total floral output
Cultivar					
S. alba	462.24±0.17 gh	16.97±0.77 b	62.07±5.62 c	8.46±0.05 j	525.26±44.40 ef
S. candida	482.51±1.79 j	36.53±2.25 ef	95.74±10.85 c	5.30±0.05 g	508.81±58.58 e
S. eriocephala	455.62±1.18 f	40.32±1.38 f	246.42±18.18 e	2.82±0.016 b	712.39±57.25 fg
S. lasiandra	473.83±0.52 i	16.28±0.86 b	59.43±4.89 c	11.25±0.04 k	668.98±54.66 f
S. lasiolepis	408.56±0.88 a	15.32±0.72 b	17.92±2.14 b	n/a	n/a
S. lasiolepis × *S. viminalis*	439.90±0.50 c	17.10±0.69 b	162.47±16.72 d	6.31±0.03 h	1049.53±111.53 g
S. matsudana	447.27±0.19 d	28.02±0.93 cd	286.92±19.73 e	1.92±0.01 a	570.32±40.32 ef
S. matsudana × *S. alba* (1)	465.02±0.55 h	33.15±1.83 de	65.32±6.77 c	3.19±0.02 c	214.70±22.35 bc
S. matsudana × *S. alba* (2)	451.12±0.73 e	13.93±0.85 b	22.78±2.40 b	4.17±0.03 e	97.82±10.73 ab
S. matsudana × *S. lasiandra*	435.57±0.56 b	36.33±0.91 ef	86.13±6.59 c	4.67±0.02 f	403.51±31.22 de
S. purpurea	477.50±0.67 ij	5.88±0.16 a	1.14±0.36 a	3.61±0.11 d	11.27±2.80 a
S. schwerinii	448.82±0.53 de	37.27±0.76 ef	627.20±34.54 f	2.89±0.01 b	1802.98±98.64 h
S. viminalis	465.28±1.34 h	37.45±2.30 ef	71.78±7.81 c	3.37±0.02 d	238.99±27.30 cd
S. × *fragilis*	497.61±0.51 k	25.73±1.55 c	27.17±6.25 b	6.68±0.07 i	263.95±60.09 cd
S. × *reichardtii*	458.72±0.61 fg	16.45±0.91 b	56.32±4.23 c	4.12±0.02 e	231.63±16.79 cd
F value	$F_{14,768}$=757.71	$F_{14,768}$=82.80	$F_{14,812}$=190.71	$F_{13,716}$=1378.85	$F_{13,705}$=79.72
P value	<0.001	<0.001	<0.001	<0.001	<0.001
Treatment					
Aphid-infested	456.01±1.06	28.57±0.72	140.72±9.89	4.42±0.05	598.72±33.94
Control (aphid-free)	455.51±1.04	22.92±0.61	117.59±8.35	4.76±0.05	525.78±30.24
F value	$F_{1,768}$=1.47	$F_{1,768}$=82.26	$F_{1,812}$=190.71	$F_{1,716}$=64.71	$F_{1,705}$=6.55
P value	0.224	<0.001	<0.001	<0.001	0.011

Table S2.2. Effect of willow cultivar and *T. salignus* infestation on maximum tree height, stem number and mean stem diameter in different willow cultivars at the end of first (2018) and second (2019) growth season. Values are mean±SE. Different letters in each column indicate significant difference at α=0.05, Tukey's HSD test.

Individual effect	Tree height (m)		Shoot number		Shoot basal diameter (cm)	
	2018	2019	2018	2019	2018	2019
Cultivar						
S. alba	2.48±0.06 f	3.89±0.10 g	2.31±0.11 a	2.27±0.11 a	15.27±0.49 efg	26.37±0.96 hi
S. candida	1.43±0.04 a	1.97±0.07 ab	2.59±0.16 ab	3.96±0.25 de	11.90±0.37 cd	15.36±0.75 ab
S. eriocephala	1.59±0.03 a	2.14±0.03 bc	3.11±0.19 bcd	3.90±0.24 de	9.96±0.24 ab	17.05±0.46 bcd
S. lasiandra	2.18±0.04 de	3.33±0.08 f	2.82±0.11 abc	2.82±0.11 abc	14.81±0.32 efg	23.21±0.76 fgh
S. lasiolepis	2.03±0.04 cd	3.51±0.07 f	2.68±0.14 ab	2.72±0.15 ab	16.85±0.53 gh	29.78±0.94 i
S. lasiolepis × *S. viminalis*	2.25±0.04 e	3.91±0.06 g	3.37±0.15 cd	3.52±0.15 cde	15.18±0.39 efg	27.13±0.84 hi
S. matsudana	2.34±0.04 ef	3.39±0.07 f	2.55±0.11 ab	2.65±0.11 ab	13.74±0.54 de	19.23±0.80 cde
S. matsudana × *S. alba* (1)	2.52±0.04 f	3.83±0.06 g	2.98±0.10 bc	3.05±0.10 bcd	13.98±0.32 e	21.71±0.51 efg
S. matsudana × *S. alba* (2)	2.49±0.04 f	4.12±0.08 g	2.58±0.12 ab	2.70±0.12 ab	16.22±0.53 fg	25.46±0.84 ghi
S. matsudana × *S. lasiandra*	2.52±0.03 f	3.49±0.07 f	2.55±0.10 ab	2.72±0.10 ab	14.28±0.40 ef	22.06±3.36 def
S. purpurea	1.79±0.04 b	2.66±0.06 d	4.22±0.22 e	6.31±0.37 g	9.19±0.22 a	14.38±0.39 ab
S. schwerinii	1.98±0.03 c	2.97±0.05 de	2.55±0.10 ab	2.63±0.10 ab	18.69±0.52 h	28.84±0.82 i
S. viminalis	2.15±0.05 cde	2.83±0.06 e	4.23±0.20 e	5.00±0.27 f	10.77±0.27 abc	15.32±0.42 ab
S. × *fragilis*	1.48±0.04 a	2.24±0.07 c	2.50±0.13 ab	3.38±0.17 bcde	10.57±0.39 abc	16.94±0.81 abc
S. × *reichardtii*	1.49±0.03 a	1.77±0.04 a	3.80±0.17 de	4.07±0.18 ef	11.10±0.33 bc	13.40±0.34 a
F value	$F_{14,841}$=103.97	$F_{14,818}$=168.01	$F_{14,841}$=18.82	$F_{14,818}$=36.34	$F_{14,841}$=50.35	$F_{14,818}$=49.32
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Treatment						
Aphid-infested	2.06±0.02	2.95±0.04	2.95±0.06	3.29±0.07	13.32±0.18	19.76±0.34
Control (aphid-free)	2.06±0.024	3.27±0.04	3.01±0.06	3.53±0.09	13.84±0.21	22.67±0.59
F value	$F_{1,841}$=0.11	$F_{1,818}$=10.81	$F_{1,841}$=0.13	$F_{1,818}$=1.19	$F_{1,841}$=0.98	$F_{1,818}$=28.48
P value	0.758	0.030	0.733	0.336	0.377	0.006

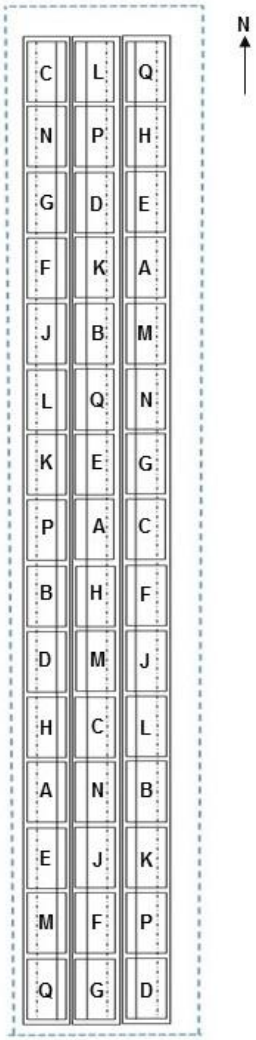

Figure S2.1. Experimental layout for the field trial to test the resistance of willow cultivars to *T. salignus*. The outermost rectangle represents the fence encircling the experimental area. The three long and narrow rectangles indicate the blocks. The smallest rectangles and different letters (see in **Table 2.1**) indicate the randomization of the willow cultivar row plots within each block, and the vertical lines indicate the control (dotted lines) and aphid-infested (solid lines) treatments.

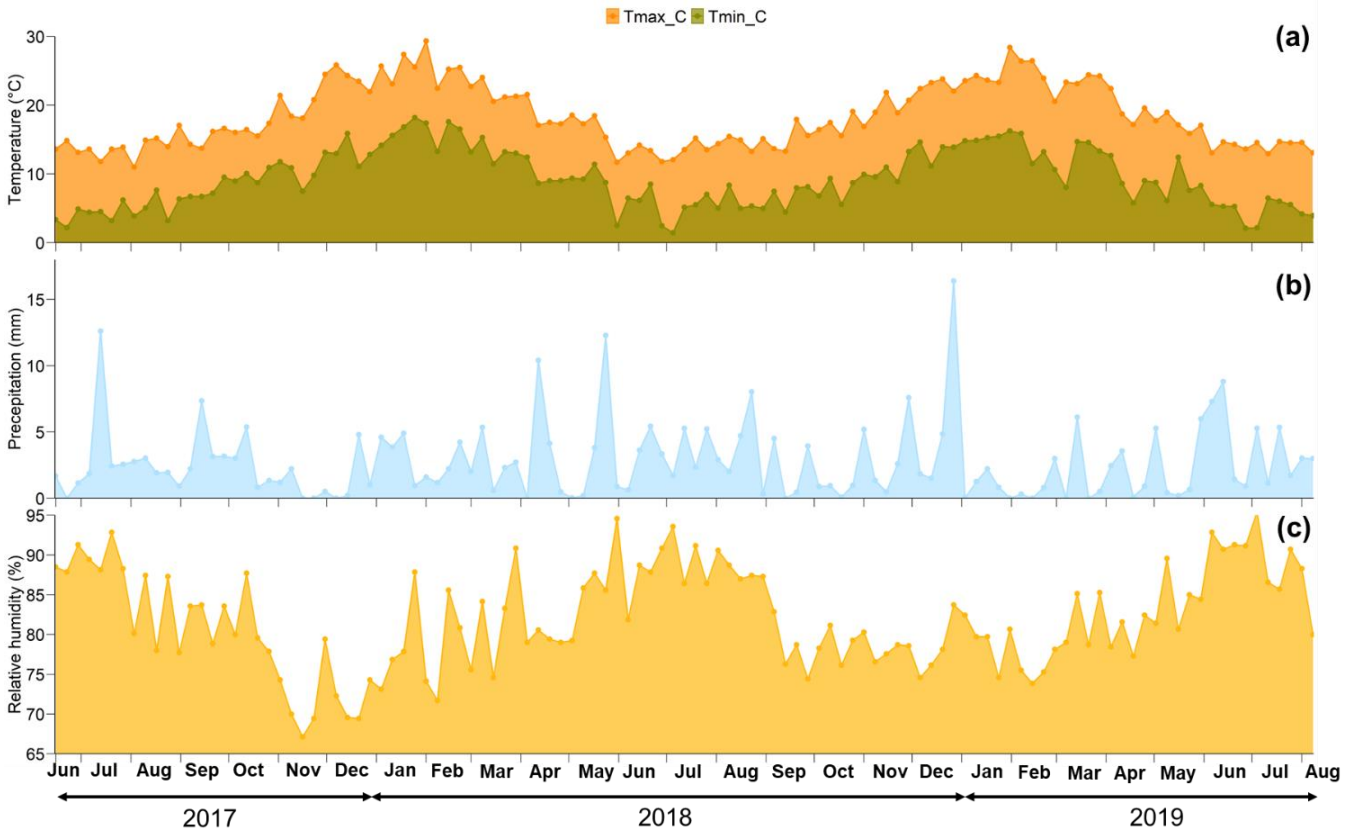

Figure S2.2. Weather conditions during the experimental period from June 15, 2017 to August 8, 2019. (**a**) Maximum and minimum temperature, (**b**) precipitation and (**c**) relative humidity.

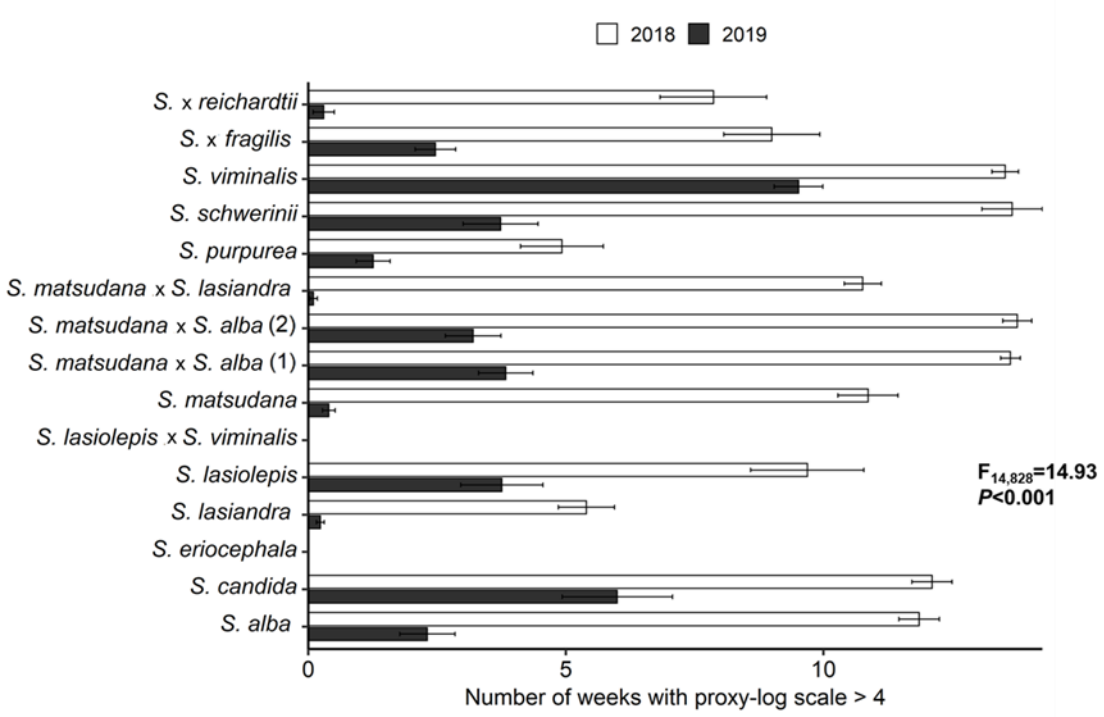

Figure S2.3. The number of weeks in 2018 and 2019 with high *T. salignus* abundance (greater than 4 on the proxy-log scale, or >300 individuals per plant) for the willow cultivars, and the F-test for the cultivar*year interaction effect. The values are the mean ± SE.

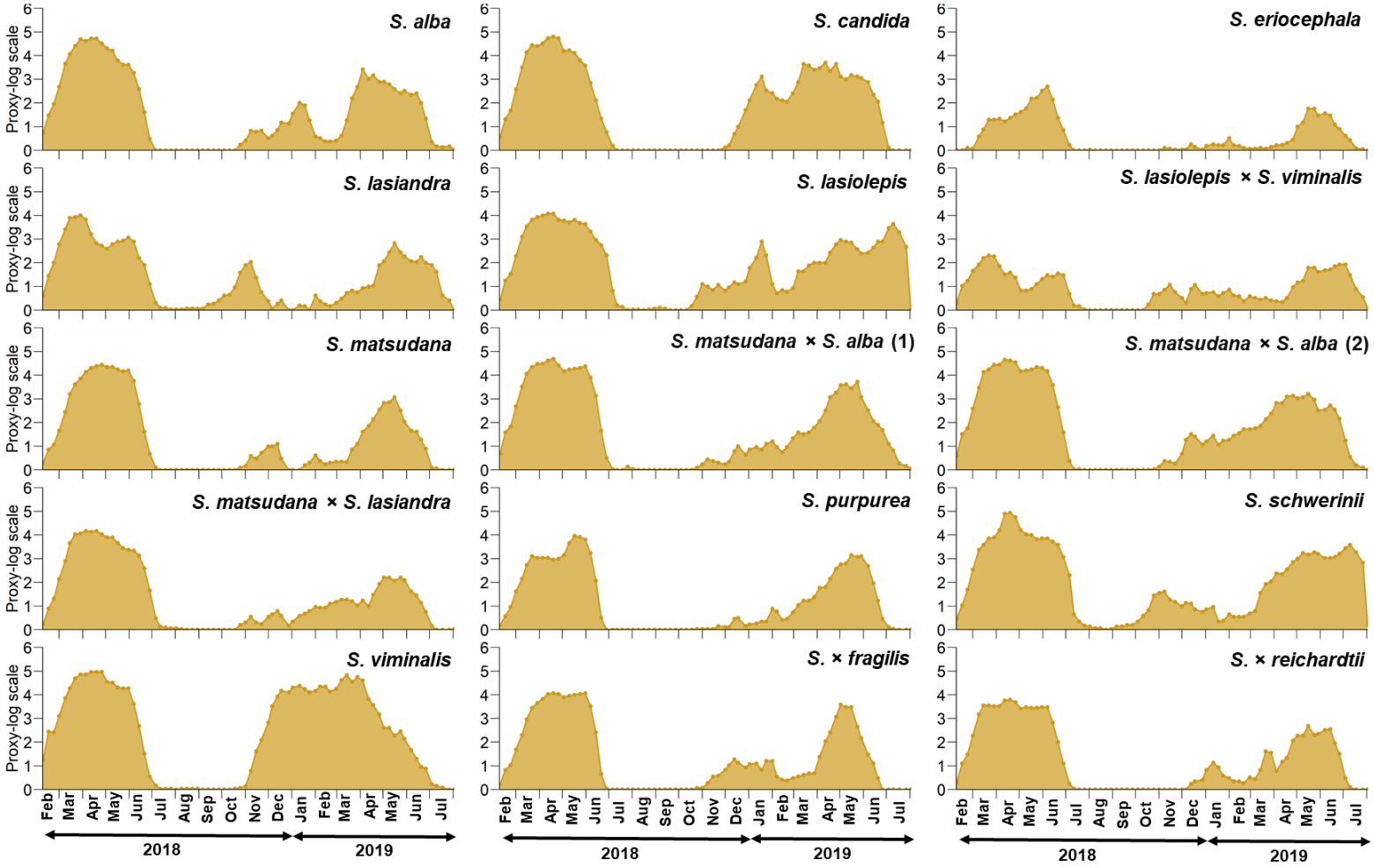

Figure S2.4. Aphid population patterns on the willow cultivars from the first inoculation until the end of experimental period.

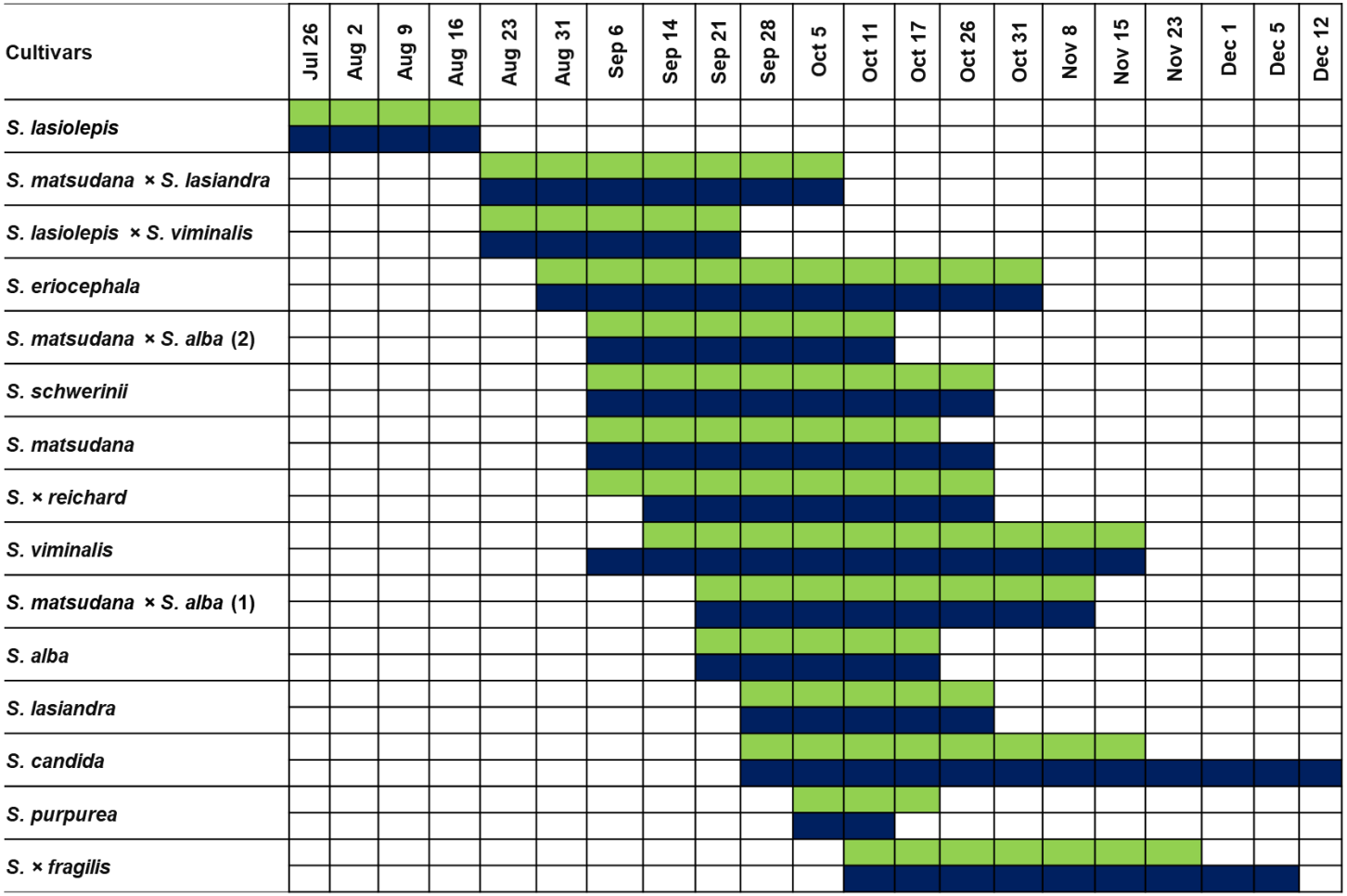

Figure S2.5. Flowering duration of the willow cultivars in 2018; green – control (aphid-free) plants, dark blue – aphid-infested plants. The fully opened catkins were removed from plants on a weekly basis, to estimate the flowering duration. Abnormal catkins were excluded.

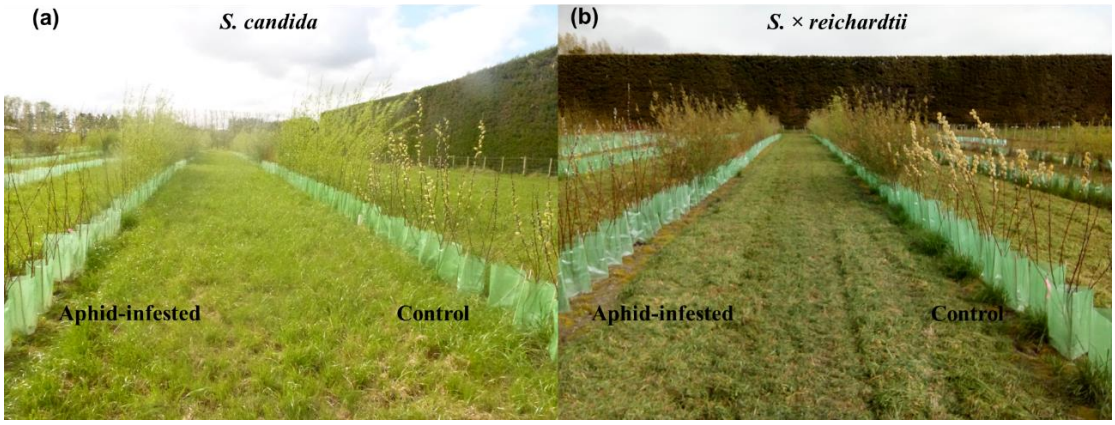

Figure S2.6. Delayed flowering of (**a**) *S. candida* and (**b**) *S.* × *reichardtii*, in the aphid-infested and control plants in the paired-row plantings.

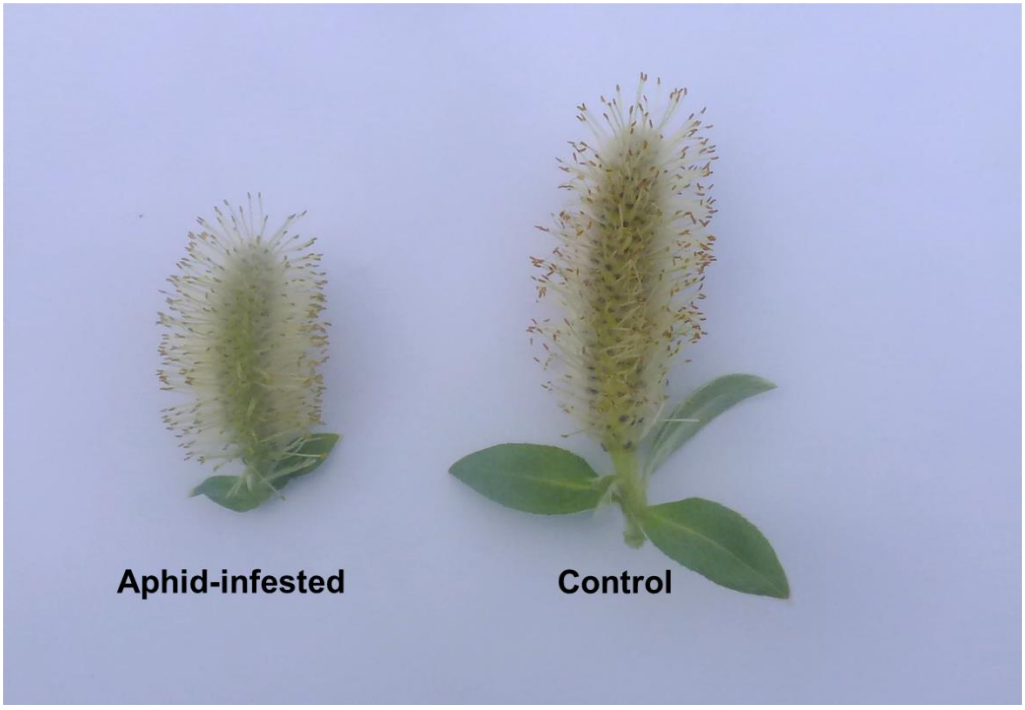

Figure S2.7. The effect of aphid infestation on the catkin length in *S. candida*.

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Chapter 3

Volatile profiling of fifteen willow cultivars and their responses to giant willow aphid infestation

This chapter has been published as:

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Reproduced here with some minor modifications in style and formatting.

3.1 Abstract

The giant willow aphid (*Tuberolachnus salignus*) is a large stem-feeding insect which forms dense colonies on infested plants. Since *T. salignus* is a new invasive species in New Zealand, we have a poor understanding of the plant chemical responses to aphid infestation. This study aimed to characterize the volatile organic compounds (VOCs) emissions of fifteen different willow cultivars growing in New Zealand, and to evaluate changes in response to *T. salignus* attack in a field trial. Volatiles were collected using a headspace sampling technique and analysed using gas chromatography-mass spectrometry (GC-MS). We found high variability in the volatile profiles of different cultivars, with (*Z*)-3-hexenyl acetate and (*E*)-β-ocimene being the only common components to all blends. Taxonomically related plants showed an overlapping pattern of VOC emission, and there seemed to be a clear separation between shrub and tree willows. Responses to aphid infestation were variable, with only four cultivars showing changes in their total VOC emission, or that of at least one class of VOCs. A weak positive correlation between aphid population estimates and VOC emissions suggests that responses are cultivar-specific and not infestation-dependent. These results reveal useful information about the interaction between *T. salignus* and its potential host plants for biological control and pest management purposes.

3.2 Introduction

Plants naturally release a wide array of volatile organic compounds (VOCs) into the environment to perform various ecological and physiological processes (Jaeger *et al.*, 2016). Chemically, VOCs are low molecular weight lipophilic molecules, consisting of terpenoids, benzenoids, green leaf volatiles (GLVs), fatty acid and amino acid derivatives (Dudareva & Pichersky, 2008). The production and release of these VOCs are highly responsive to biotic and abiotic factors, making them an excellent source of information for surrounding organisms (McCormick, 2016; Vivaldo *et al.*, 2017). VOCs mediate multiple ecological interactions: they can repel herbivores (De Moraes *et al.*, 2001; Irmisch *et al.*, 2014), attract the natural enemies of herbivores (predators and parasitoids), and lure pollinators and seed dispersers, which are key elements to the plant's defense and reproduction (Amo *et al.*, 2013; Dudareva & Pichersky, 2000; McCormick *et al.*, 2014b). However, VOC can also enhance the attractiveness of plants to some herbivores, harming the emitting plants (Baldwin *et al.*, 2002). Plants also release VOCs to protect themselves from environmental stress, such as heat or UV-radiation (Holopainen & Gershenzon, 2010; Owen & Peñuelas, 2005).

A variety of factors are known to influence both the quality and quantity of VOCs emitted by plants (McCormick *et al.*, 2012). Among them, the plant species is a determining factor, since some biosynthetic pathways are taxon-specific (Arneth *et al.*, 2007; Winters *et al.*, 2009). As VOC emission quantitively and qualitatively differs from one species to another, volatile blends (scents) are good indicators of a plant's identity and of evolutionary relationships among plant groups (Vivaldo *et al.*, 2017). Some VOCs are released only from specific plant species, or plant groups, while others are ubiquitous to all plant species (Kesselmeier & Staudt, 1999). Within the same plant species, VOCs emission can vary within species, and with the phenological state, age and sex of the plant

(Ashman *et al.*, 2005; Masante-Roca *et al.*, 2007; Michereff *et al.*, 2011; Shiojiri & Karban, 2006).

Although plants constitutively emit VOCs, both quantitative and qualitative changes in VOCs blends are detected when plants are attacked by insect herbivores (Dudareva *et al.*, 2013). These herbivore-induced plant volatiles (HIPVs) mainly consist of GLVs, aromatics and terpenoids (Giacomuzzi *et al.*, 2016). The HIPV emission is a dynamic process between the host plant and herbivorous insects (McClung, 2006), where the abundance and identity of the attacker influences the responses to herbivore attack (McCormick *et al.*, 2014a). The feeding mode of the herbivore appears to play a key role in regulating the plant's responses, activating different signaling pathways (Walling, 2000). Previous studies suggest that phloem feeders induce less pronounced changes in the volatile emission of their host plants than chewing insects (Joó *et al.*, 2010; Rodriguez-Saona *et al.*, 2003).

Willow plants are known to release various VOCs, consisting of acetaldehydes, acetones, acetic acids, isoprenes, methanols, methyl ethyl ketones, methyl vinyl ketones and monoterpenes (Copeland *et al.*, 2012; Hakola *et al.*, 1998). These emissions are species-specific (Peacock *et al.*, 2001), have been related to both direct and indirect plant defenses (Goggin, 2007), and play an important role in host selection by aphids (Ahmed *et al.*, 2019) and other insects (Inui *et al.*, 2003). As willows possess different growth forms (Argus, 1999), VOC blends and response to herbivore attack can differ between tree and shrub types.

Willows (*Salix* spp.) are known for their high genetic diversity encompassing more than 400 wild and cultivated species and hybrids all over the world (Argus, 2007). New Zealand (NZ) currently has 59 species and hybrids (Glenny & Jones, 2019). Some of them are widely planted in NZ for biomass production, soil conservation on pastoral hill

country and river banks, and as sources of spring pollen and nectar for honey bees (Isebrands *et al.*, 2014; Newstrom-Lloyd *et al.*, 2015; Wilkinson, 1999). Willows are now being infested by the giant willow aphid, *Tuberolachnus salignus* Gmelin (Hemiptera: Aphididae), a stem-feeding insect that was first reported in NZ in December 2013 (Martin, 2017). Aphid infestation reduces the amount of photosynthetic storage in willow roots and stems, leading to changes in plant performance and growth (Sopow *et al.*, 2017). Long-term sustainable management solutions are needed to reduce the impact of aphid infestation, such as the selection of resistant willow species or hybrids for planting (Sopow, 2016). A key aspect for selection is a better understanding of the aphid interaction with its host plant, including the plant's production and emission of volatile organic compounds (VOCs) in response to herbivore attack.

Some studies have explored the role of willow VOCs in host selection by herbivores, such as the willow sawfly (*Nematus oligospilu*s) and willow leaf beetles (*Phratora* spp. and *Plagiodera* spp.) (Braccini *et al.*, 2015; Fernandez *et al.*, 2007; Peacock *et al.*, 2001; Yoneya *et al.*, 2010) However, there is scarce information about willow responses to attacks by phloem-feeding herbivores (Aradottir *et al.*, 2009). Therefore, in this study, we explored the intra-genus (*Salix*) variation in VOCs from fifteen cultivars, and changes in VOCs emissions in response to infestation by *T. salignus*. We expect these results will shed some light on the aphid–plant interaction, and inform pest management decisions for successful willow growing.

3.3 Materials and Methods

3.3.1 Study site and plant material

This study was conducted in a willow field trial at the Orchard Block, Plant Growth Unit, Massey University, NZ (40°22'41.70" S, 175°36'30.67" E). The field trial was set up to investigate the interactions of the giant willow aphid with its host plants and

the environment. Fifteen willow cultivars, from different geographical origins (Glenny & Jones, 2019), were grown in three blocks of paired rows, with the position of each species being random within each paired row. Twelve ramets (an individual of plant species, vegetatively reproduced from a single parent plant) of each species or hybrid were planted within each row. Within each block, one row was randomly selected as a control (aphid exclusion), while the adjacent row was aphid-infested. Information on the willow cultivars, and the field trial layout are provided in in **Table 3.1** and **Table S3.1.**

The willow field trial was planted using stem cuttings in June 2017, with 0.4 m spacing between cuttings within the rows, and 4 m spacing between rows. The willow plants in the control rows were inspected for colonising aphids on a weekly basis, and any aphids found were removed manually. Mavrik® insecticide (Nelson, NZ) was applied on 28 February 2018 and 17 January 2019, when manual control was impractical due to high population densities of *T. salignus*.

3.3.2 Aphid inoculation

Willow plants in the aphid-infested rows were inoculated with five adult aphids per plant on January 25-27, 2018 and December 6-7, 2019. Additional inoculations with ten adult aphids per plant were done on February 13-14, 2018 and January 30, 2019. The aphid infestation per species or hybrids was quantified immediately before VOC collection using a visual scale from zero to six, with 0 = less than five aphids, 1 = 2 to 20 aphids, 2 = 20 to 50 aphids, 3 = 50 to 100 aphids, 4 = 100 to 300 aphids, 5 = 300 to 600 aphids, and 6 = 600 aphids or more per plant.

Table 3.1. Willow cultivars (*Salix* spp.) used in characterising and identifying the willow VOC response to *T. salignus* infestation.

Species/hybrids	Code	Type	Sex	Geographical origin
S. candida	PN 385	Shrub	Male	North America
S. eriocephala	PN 376	Shrub	Male	North America
S. lasiolepis	PN 751	Shrub	Male	North America
S. lasiolepis × *S. viminalis*	NZ 04-106-073	Shrub	Male	Hybridized in New Zealand
S. purpurea	PN 249	Shrub	Female	Europe, North Africa
S. schwerinii	PN 386	Shrub	Male	Eastern Asia
S. viminalis	PN 220	Shrub	Male	Europe, Western Asia
S. × *reichardtii*	PN 714	Shrub	Male	Europe
S. alba	PN 357	Tree	Male	Europe, Western and Central Asia
S. lasiandra	PN 747	Tree	Male	North America
S. matsudana	PN 227	Tree	Female	Eastern Asia
S. matsudana × *S. alba* (1)	NZ 1040	Tree	Female	Hybridized in New Zealand
S. matsudana × *S. alba* (2)	NZ 1184	Tree	Male/female	Hybridized in New Zealand
S. matsudana × *S. lasiandra*	NZ 03-003-073	Tree	Male	Hybridized in New Zealand
S. × *fragilis*	PN 218	Tree	Female	Europe and Western Asia

Numbers (1) and (2) represent *S. matsudana* × *S. alba* (NZ 1040) and (NZ 1184), and will be used in subsequent tables and figures.

3.3.3 VOCs sampling

VOCs from willow branches were collected using the push-pull headspace sampling method as described in Effah *et al.* (2020). Among the twelve plants in the row plots of each species or hybrid, one of the middle ramets (plants 5, 6 or 7) per plot was chosen to ensure that the VOCs collected were released from that treatment, without receiving VOCs from different neighboring plants, for a total of six plants per species. Willow branches of a suitable size, without visible sign of damage by insects and pathogens, were selected and enclosed in oven cooking bags (Glad®, Melbourne, Australia). One inlet and one outlet tube were fastened with cable binders at each end of the bag. The portable volatile assay system (PVAS22 pump, VAS Rensselaer NY) was used to circulate carbon-filtered air through the bag (**Figure 3.1**). Incoming air was pumped at 1.70 L/min and outgoing air was pulled at 1.20 L/min creating a slight overpressure to avoid contaminants from entering the bag. The VOCs emitted from the willow foliage were trapped in Haysep-Q filters attached to the outlet (pull) tubes. The pump ran for two hours, and then the filters were removed and individually wrapped with labelled pieces of aluminum foil, and stored in a cooler box to prevent contamination and evaporation of the collected volatiles. The willow branches were cut just below the bags, and the oven-dried weight of the branches was measured after drying at 60 °C for 72 h; therefore, volatiles measured are presented in nanograms per dry weight (g) per hour ($ng \cdot g \cdot DW^{-1} \cdot h^{-1}$). Negative controls were also included by taking air samples from empty bags to exclude potential contaminants.

The first VOC sampling was performed on 17–23 January 2018, to characterize the willow VOCs (n = 6 branches/cultivar, 90 in total) before giant willow aphid inoculation. To estimate the effect of *T. salignus* on the VOC emissions of the willow

plants, second VOC sampling was done shortly after aphid inoculation from both the control and aphid-infested plants on 15–17 March 2019.

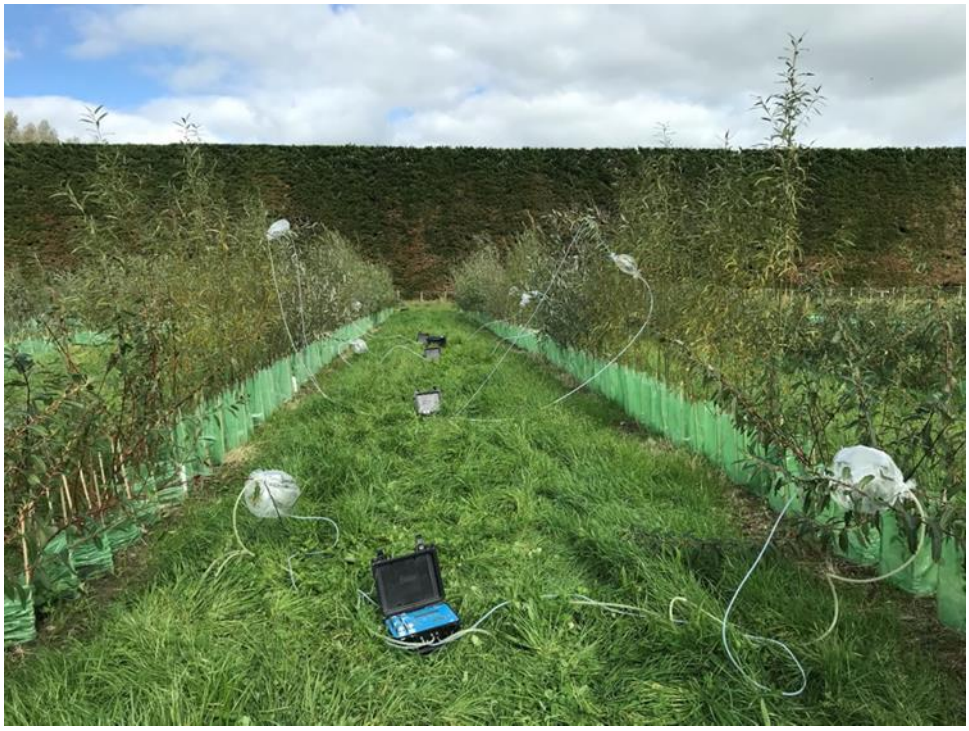

Figure 3.1. VOC sampling from willow foliage using a portable volatile assay system. Suitable branches were enclosed in plastic bags, into which clean air was circulated by a pump.

3.3.4 Gas chromatography–mass spectrometry (GC-MS) analysis

The volatile compounds in the filters were eluted using a solvent solution with an internal standard (200 µL hexane with 10 ng/mL of nonyl acetate) into gas chromatography-mass spectrometry (GC-MS) vials and then stored in a −80 °C freezer before analysis. The willow volatiles were separated and identified 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 µm × 0.25 µm). Helium (He) was used as a carrier gas with the flow rate of

0.5 mL/min into split mode (10:1). The injector port and detector were set up at 250 and 230 °C, respectively. The oven temperature was initially held at 50 °C for 3 min, then increased by 5°C/min to 95 °C, and then ramped to 240 °C at 15°C/min, where it was maintained for three minutes. VOCs were tentatively identified using the NIST (National Institute of Standards and Technology) Mass Spectral Library and confirmed by comparing their retention times with those of commercial standards whenever available. Post-run analyses were carried out using the Shimadzu Lab Solutions software (version 2.50). VOCs were quantified by dividing their peak area by that of the internal standard and expressed as nanogram per microliter per gram of dry weight of foliage per hour ($ng \cdot g^{-1} \cdot h^{-1}$). Contaminants (toluene, p-xylene, o-xylene, diethyl phthalate, etc.) that were consistently identified in negative controls (empty oven cooking bags) were excluded from further analyses.

3.3.5 Statistical analysis

The R statistical software (Version 3.6.1) (Version 3.6.1, R Development Core Team, 2019) was used for all analyses. For the VOC profiling of the fifteen willow cultivars, 19 VOCs were chosen, based on their consistent occurrence in the samples of at least one species or hybrid. One replicate of *S.* × *fragilis* that did not emit the selected compounds was dropped from analyses. The VOC data were square-root transformed to achieve normality (Zhang *et al.*, 2018), and to allow rare VOCs to have equal weight by reducing the overestimation of highly-occurring VOCs in the headspace samples (Sheehan *et al.*, 2014). We performed a permutational multivariate analysis of variance (PERMANOVA) with Bonferroni adjustment to differentiate the VOC blends of the fifteen willow cultivars. The analysis was done using the Adonis function with Bray–Curtis distance matrix and 999 permutations (Arbizu, 2019). Non-metric Multi-Dimensional Scaling (NMDS) was performed to depict differences in the VOC profiles

of the willow cultivars. The PERMANOVA and NMDS were performed using the *vegan* (Oksanen *et al.*, 2010) package.

The relative proportion of each major VOC class (aldehydes, GLVs, monoterpenes and sesquiterpenes) was calculated by summing up the specific VOC concentrations for each group, and then dividing by the whole blend as described by Digilio *et al.* (2010). We constructed linear mixed model (LMM) ANOVAs on the square root transformed relative VOC proportions using the *lme4* package, to further differentiate the emission of VOC class within each species or hybrid. The fifteen willow cultivars were treated as a fixed factor, while the row number of the VOC sampling was considered as a random variable. The linear mixed model was fitted on a proxy-log scale and a multiple comparison was then performed using Tukey's HSD test in *multcomp* and *lsmeans* packages.

To test the response of the willows to aphid infestation, a Tweedie generalized linear model with gamma distribution and log-link function was used to compare the total VOC emissions, and that of each of the four major VOC groups (aldehydes, GLVs, monoterpenes and sesquiterpenes). The concentrations of specific VOCs were summed up to become the total concentrations of the major VOC classes. A one-way ANOVA, followed by Tukey's HSD test was used to compare aphid population levels on willow species, monitored just before VOC sampling. Finally, a Spearman's rank correlation was performed to correlate the aphid population level (proxy-log scale) and the total VOC emissions for the aphid-infested willows; the relationship was visualized using the *ggpubr* package.

3.4 Results

3.4.1 Characterisation of willow VOCs

The VOCs in the headspace samples from the willow plants, before inoculation with aphids, included: one aldehyde (nonanal), four GLVs ((*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl benzoate and (*Z*)-3-hexenyl-α-methylbutyrate), four monoterpenes ((*E*)-β-ocimene, (*Z*)-β-ocimene, α-ocimene and β-myrcene) and ten sesquiterpenes (α-cubebene, (*E*,*E*)-α-farnesene, (*E*)-β-famesene, germacrene D, *δ*-cadinene, (*E*)-α-bergamotene, copaene, (*Z*,*E*)-α-farnesene, β-caryophyllene and cedrene) (**Table 3.2**). *Salix candida* and *S. schwerinii* released the largest number of VOCs (15), whereas *S. matsudana*, *S. matsudana* × *S. alba* (NZ 1040), *S. purpurea*, *S. lasiandra*, *S. lasiolepis* × *S. viminalis* and *S. matsudana* × *S. alba* (NZ 1184) emitted less than six out of the 19 selected VOCs. The remaining cultivars produced 7 to 11 VOCs in their headspace samples. The two VOCs released by all willows in this study were (*Z*)-3-hexenyl acetate and (*E*)-β-ocimene. More than 50% of the cultivars released α-farnesene, (*Z*,*E*)-α-farnesene and α-ocimene. The VOCs (*E*)-β-famesene, germacrene D, *δ*-cadinene, β-myrcene, cedrene, (*Z*)-3-hexenyl-α-methylbutyrate, α-cubebene, copaene and (*E*)-α-bergamotene were released from 25% of the willow cultivars. *Salix candida* was the only species that emitted (*Z*)-3-hexenyl benzoate (**Table 3.2**).

The VOC profiles of the tested willow cultivars differed significantly (PERMANOVA; Pseudo-$F_{14,88}$ = 5.83, $P < 0.001$). Due to the high overlap of the VOC profiles, the NMDS algorithm yielded a low stress value (badness-of-fit; 0.20). However, some of the willow cultivars were distinguishable from others. For instance, there was clear differentiation between *S. lasiandra* and *S. lasiolepis*, *S. schwerinii*, *S. matsudana* and *S. matsudana* × *S. alba* (NZ 1040). *Salix schwerinii* had no overlap with *S. eriocephala*, *S.* × *reichardtii*, *S. lasiolepis* × S. *viminalis*, *S. lasiandra* and *S. matsudana*

× *S. alba* (NZ 1040) (**Figure 3.2**). As expected, the closely related cultivars had more similar VOC profiles, as observed for *S. matsudana* and its hybrids. Tree willows were clustered separately from the shrub willows (**Figure 3.2**).

The fifteen willow cultivars emitted different proportions of the four classes of VOCs. The relative proportions of long-chain aldehydes in the whole blends differed significantly among the willow cultivars ($F_{14,88} = 3.78$, $P < 0.001$) (**Figure 3.3a**). The (LMM) ANOVA results also showed a significant fixed effect in GLVs proportion ($F_{14,88} = 8.49$, $P < 0.001$). Three species (*S. lasiolepis* × *S. viminalis*, *S.* × *reichardtii* and *S. eriocephala*) released more GLVs than five other species or hybrids (*S. schwerinii*, *S. matsudana*, *S. matsudana* × *S. lasiandra*, S. *viminalis* and *S.* × *fragilis*) (**Figure 3.3b**). Monoterpene production varied greatly among the willow cultivars ($F_{14,88} = 9.24$, $P < 0.001$). *Salix matsudana*, *S. matsudana* × *S. alba* (NZ 1184), *S. purpurea*, *S. viminalis* and *S. matsudana* × *S. alba* (NZ 1040) had the largest monoterpene emissions, while *S. lasiandra* and *S. lasiolepis* × *S. viminalis* released the lowest amount (**Figure 3.3c**). There were significant differences in the relative proportion of sesquiterpenes too ($F_{14,88} = 12.03$, $P < 0.001$). *Salix schwerinii*, *S. lasiolepis*, *S. lasiandra*, *S. matsudana* × *S. lasiandra*, *S. candida*, and *S. alba* were all high sesquiterpene-emitters, while *S. matsudana* × *S. alba* (NZ 1040) and *S. matsudana* had zero emissions (Figure **3.3d**).

Table 3.2. GC-MS analysis of VOCs released from the foliage of fifteen willow cultivars.

Cultivar	Major VOC groups																		
	Aldehyde	**GLVs**				**Monoterpenes**				**Sesquiterpenes**									
	Nonanal	(Z)-3-Hexenol	(Z)-3-Hexenyl acetate	(Z)-3-Hexenyl benzoate	(Z)-3-Hexenyl-α-methylbutyrate	(Z)-β-Ocimene	(*E*)-β-Ocimene	α-Ocimene	β-Myrcene	α-Cubebene	α-Farnesene	(Z,E)-α-Farnesene	(*E*)-β-Famesene	Germacrene D	β-Caryophyllene	Cedrene	Copaene	δ-Cadinene	(*E*)-α-Bergamotene	Number of VOC emitted
S. alba	o	+	+	o	+	o	+	o	o	o	+	+	o	o	+	o	o	o	+	8
S. candida	+	+	+	+	+	o	+	+	+	+	+	+	o	+	+	+	+	o	o	15
S. eriocephala	o	+	+	o	+	o	+	o	o	+	+	+	o	o	+	o	+	o	o	9
S. lasiandra	+	o	+	o	o	o	+	o	o	o	+	+	o	o	o	o	o	o	o	5
S. lasiolepis	o	o	+	o	o	o	+	+	o	+	+	+	o	+	+	+	+	+	o	11
S. lasiolepis × *S. viminalis*	+	+	+	o	o	o	+	o	o	o	+	+	o	o	o	o	o	o	o	6
S. matsudana	o	o	+	o	o	+	+	+	o	o	o	o	o	o	o	o	o	o	o	4
S. matsudana × *S. alba* (1)	o	+	+	o	o	+	+	+	o	o	o	o	o	o	o	o	o	o	o	5
S. matsudana × *S. alba* (2)	+	o	+	o	o	+	+	+	o	o	+	o	o	o	o	o	o	o	o	6
S. matsudana × *S. lasiandra*	+	o	+	o	o	+	+	+	o	o	+	+	o	o	o	o	o	o	+	8
S. purpurea	o	+	+	o	o	o	+	+	o	o	+	o	o	o	o	o	o	o	o	5
S. schwerinii	+	o	+	o	+	+	+	+	+	+	+	+	o	o	+	+	+	+	+	15
S. viminalis	o	+	+	o	o	+	+	+	+	o	+	+	+	o	o	o	o	o	+	10
S. × *fragilis*	+	+	+	o	o	o	+	+	o	o	+	o	+	o	o	o	o	o	o	7
S. × *reichardtii*	+	+	+	o	o	o	+	o	o	o	+	+	o	o	+	o	o	o	o	7
Number of cultivars	8	9	15	1	4	6	15	10	3	4	13	10	2	2	6	3	4	2	4	

*The "+" and "o" symbols indicate the presence and absence of the compounds in the headspace samples, respectively.

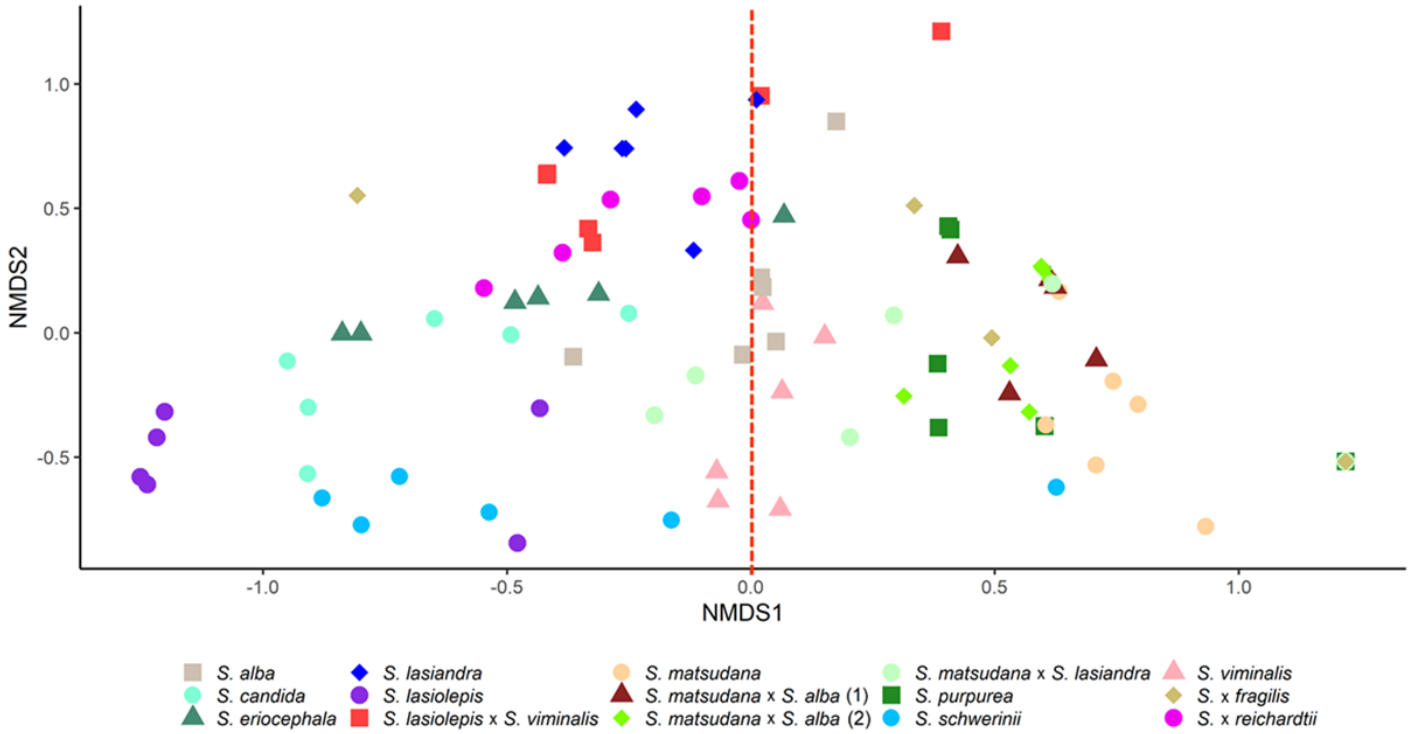

Figure 3.2. Non-metric Multi-Dimensional Scaling plot of similarities in VOC profiles released by the fifteen willow cultivars. Bray-Curtis dissimilarities were calculated on the square-root transformed VOC profiles, containing 19 compounds. Each point represents a headspace sample of each species or hybrids (n=6/cultivar). Most samples at the left side of the dotted line correspond to shrub willows, whereas most samples at the right of the dotted line are tree willows.

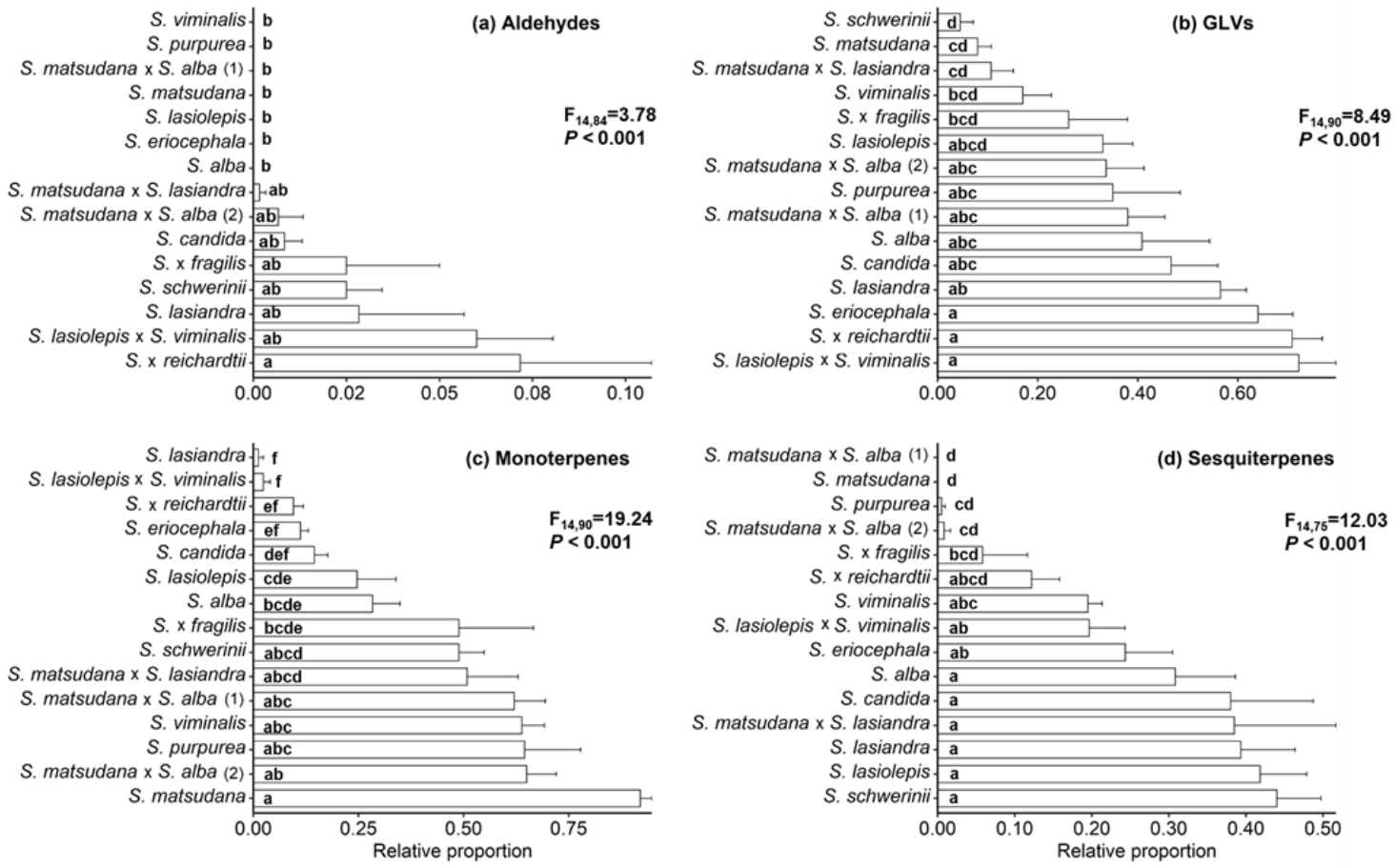

Figure 3.3. Relative proportions of each major VOC class emitted by the fifteen willow cultivars. The values and bars indicate mean ± SE. Different letters represent statistically significant differences at 0.05% level by multiple comparison using Tukey's HSD test.

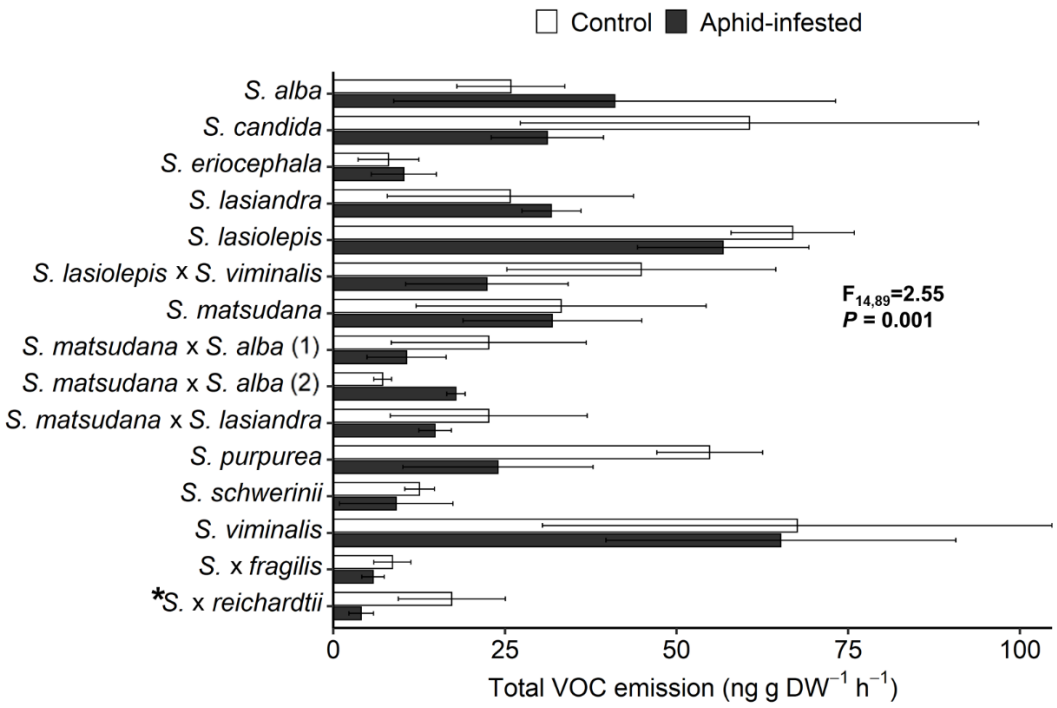

Figure 3.4. Total volatile emissions released by the fifteen willow cultivars for the control and aphid-infested treatments. The values and bars indicate mean ± SE. Asterisks indicate significant differences between the treatments for each cultivars by Tukey's HSD test.

3.4.2 VOC response of willow cultivars to *T. salignus* infestation

Eighteen VOCs were identified in the headspace samples from aphid-infested plants (**Table S3.2**) and different willow species responded differently to *T. salignus* infestation. In most cases, the VOC profiles of aphid-infested willow plants did not differ significantly from those of control plants (**Figure 3.4**). However, upon closer inspection, aphid infestation was found to significantly decrease total VOC emission in *S.* × *reichardtii* (**Figure 3.4**), GLV emission in *S. matsudana* × *S. lasiandra*, monoterpene emission in *S.* × *reichardtii* and *S. candida*, and sesquiterpene emission in *S.* × *reichardtii*, *S. matsudana* × *S. alba* (NZ 1184) and *S. candida* (**Figure 3.5**).

3.4.3 Correlation between aphid infestation and VOC emission

Our observation of aphid populations before VOC collection revealed different degrees of infestation among willow cultivars (**Figure 3.6**), with *S. eriocephala*, *S. matsudana*, and *S. lasiolepis* × *S. viminalis* having very low infestation rates (most plants having 20 aphids or less) and *S. viminalis* having the highest infestation rates (most plants having 300 aphids or more), followed by *S. candida* (most plants having over a 100 aphids). Correlation analysis revealed only a weak relationship between aphid infestation and VOC emissions ($n = 45$, $\rho = 0.34$, $P = 0.02$).

3.5 Discussion

Our results showed variation in the VOC profiles of the different willow cultivars, attributed to the diversity of *Salix* spp. used in current study (**Table 3.1**). The VOC emissions were found to be more similar between closely related plants, as shown by close clustering between *S. matsudana* and its hybrids (*S. matsudana* × *S. alba* (NZ 1040), *S. matsudana* × *S. alba* (NZ 1184) and *S. matsudana* × *S. lasiandra*). VOC composition was distinct between shrub and tree willows, supporting the genetic clustering between these two growth forms as recently reported by Ngantcha (2010).

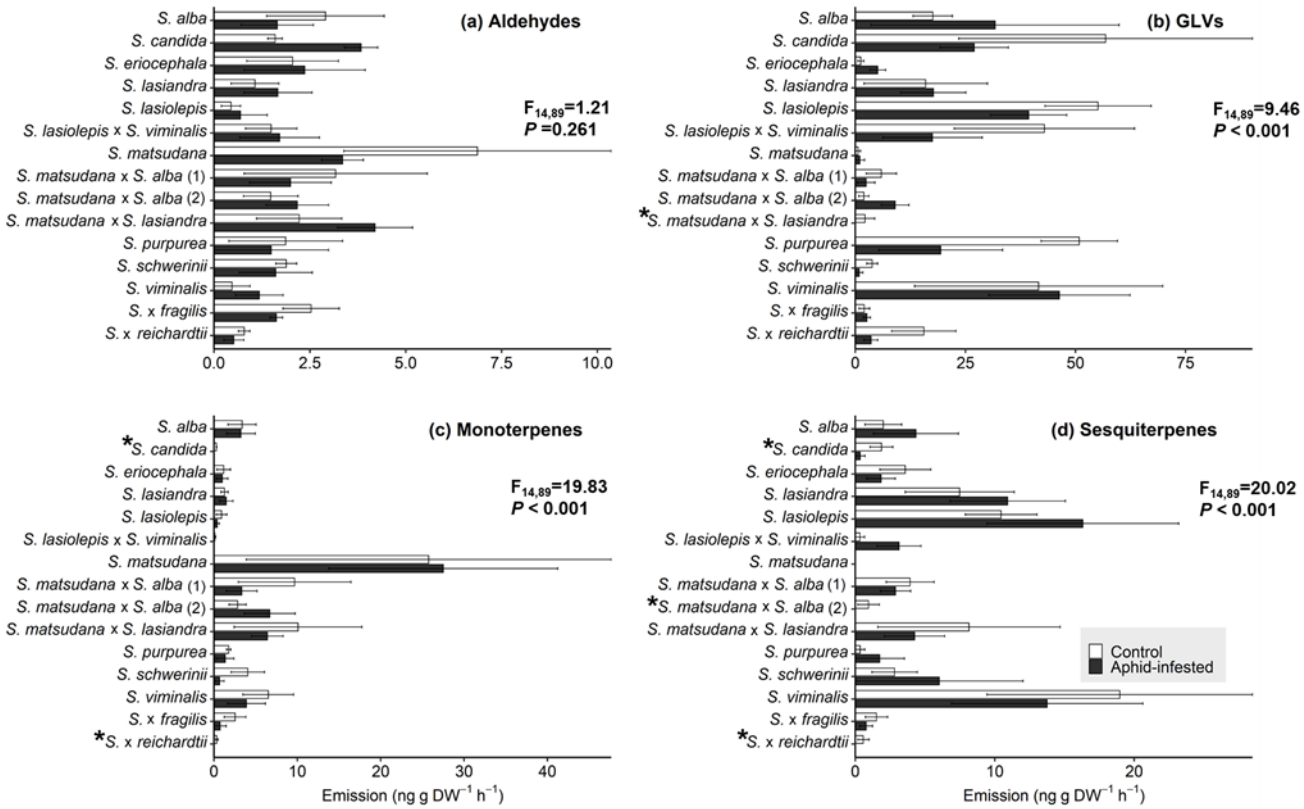

Figure 3.5. Total emissions of the four major VOC classes by the fifteen willow cultivars for the control and aphid-infested treatments. Values and bars indicate mean ± SE. Asterisks indicate significant differences between the treatments for each cultivars by Tukey's HSD test. Detailed multiple comparisons can be seen in **Table S3.3**.

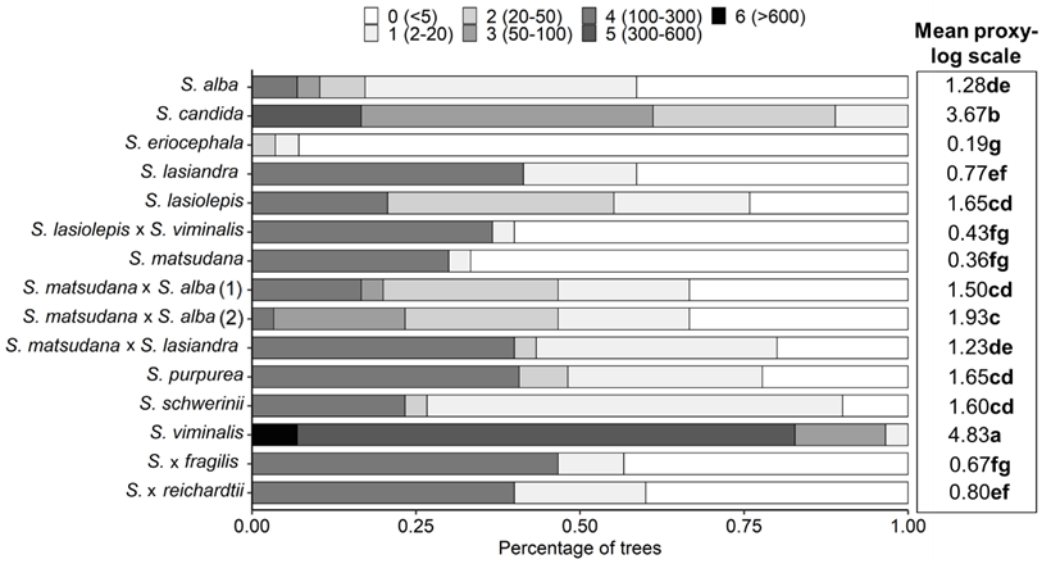

Figure 3.6. Proportion of willow plants hosting different population levels of *T. salignus* as monitored on March 15, 2019. The numbers on left side of each bar represent mean proxy-log scales. Different symbols behind the values indicate statistically significant differences by Tukey's HSD test (α=0.05). Numbers (1) and (2) represent *S. matsudana* × *S. alba* (NZ 1040) and (NZ 1184).

Although cultivars varied in the number of compounds emitted, two compounds were common to all blends: GLVs (*Z*)-3-hexenyl acetate and monoterpene (*E*)-β-ocimene. Both compounds have been reported for *S. eriocarpa* (Yoneya *et al.*, 2010), *S. viminalis* (Fernandez *et al.*, 2007) and in related poplar trees (*Populus nigra* and *P. trichocarpa*) (Danner *et al.*, 2011; McCormick *et al.*, 2014b), suggesting that they are ubiquitous to plants species in the family Salicaceae. Studies suggest that these two compounds play important ecological roles. For example, (*Z*)-3-hexenyl acetate has been identified as a key compound associated with herbivore damage in different willow varieties and in poplar species, and is known to attract natural enemies (Frost *et al.*, 2008; McCormick *et al.*, 2014b; Peacock *et al.*, 2001). In other willow and poplar species (*E*)-

β-ocimene is involved in within-plant communication as a signal emitted by damaged plant parts to alert nearby undamaged parts of potential attack (Frost *et al.*, 2007). In our study, some cultivars reduced their emissions of these two compounds in response to *T. salignus* herbivory (**Figure S3.1**, **Table S3.4**). The reasons behind this reduction are not yet known and deserve further investigation.

Few studies have explored willow VOC responses to herbivore attack. HIPV emission by willows can be highly specific, varying with the life stage of their attacker (larvae vs. adult), and informing natural enemies about the suitable stage of their prey (Yoneya *et al.*, 2009). The predatory ladybird (*Aiolocaria hexaspilota*) was more attracted to VOC blends induced by willow beetle (*Plagiodera versicolora*) larvae, containing higher amounts of the GLVs (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate, the monoterpenes (*E*)-β-ocimene, (*Z*)-β-ocimene, allo-ocimene and linalool, the sesquiterpene (*E*)-α-farnesene, and two oximes (nitrogenous compounds) (Yoneya *et al.*, 2009). These results show that HIPVs play a role in host selection by herbivores, and in indirect defense in willows.

In the present study, we observed that not all willow cultivars reacted to aphid damage in the same way. While some willows (*S.* × *reichardtii*, *S. matsudana* × *S. lasiandra*, *S. matsudana* × *S. alba*, and *S. candida*) responded by decreasing their VOC emissions, the majority of cultivars did not show a significant change. Other studies on phloem feeders show contrasting results, with some reporting increases in VOC emissions after attack (Blande *et al.*, 2010; Giorgi *et al.*, 2012; Joó *et al.*, 2010; Schwartzberg *et al.*, 2011; Ye *et al.*, 2019). For instance, infestation by spiral gall aphid *Pemphigus spyrothecae* (Hemiptera: Aphididae) on leaf tissue can alter leaf's photosynthetic activity that in turn triggers jasmonate transportation from petiole to lamina and finally modifies VOC emission in poplar (*Populus* × *petrovskiana*) (Ye *et al.*,

2019). However, there are also reports showing reductions in VOC emissions or no response at all (Rodriguez-Saona *et al.*, 2003; Schwartzberg *et al.*, 2011; Turlings *et al.*, 1998). Furthermore, studies comparing chewers and phloem feeders typically indicate that the latter have a less pronounced effect on VOC emissions than chewing herbivores (Rodriguez-Saona *et al.*, 2003; Turlings *et al.*, 1998).

The lack of response in most cultivars may be due to the fact that the giant willow aphid does not directly damage the photosynthetically active tissue (leaves), nor causes severe mechanical damage (as chewing herbivores do), and thus may not trigger strong changes in VOC emissions (Turlings *et al.*, 1998). However, it has been suggested that aphids actively suppress plant responses to escape their natural enemies (Schwartzberg *et al.*, 2011). This manipulation of plant responses is possibly mediated by microbial endosymbionts of aphids in order to protect their hosts (Frago *et al.*, 2017). Further studies are required to clarify the mechanism behind the observed responses (or lack of them).

The VOC emission reduction observed in some cultivars may also be due to a trade-off between indirect and direct defense (i.e., production of VOCs vs. non-volatile secondary metabolites) (Ballhorn *et al.*, 2008; Koricheva *et al.*, 2004; Rudgers *et al.*, 2004; Wei *et al.*, 2011). Both direct and indirect defenses have a metabolic cost to the plant, and plants typically favor one type of defense over another. For instance, a study investigating wild and cultivated accessions of lima bean (*Phaseolus lunatus*) found that plants producing high levels of cyanogenic compounds (direct defenses) released low amounts of VOCs (indirect defenses) and vice versa (Ballhorn *et al.*, 2008). In this study, some cultivars hosted lower aphid populations and appear to be more naturally resistant to aphid attack than others (**Figure 3.6**). This resistance is possibly associated with the

presence of physical defense mechanisms (e.g., rough bark of resistant species), suggesting that defense trade-offs could exist in different types of willows.

Plant volatiles are known to play a role in deterring herbivores and attracting natural enemies in related tree species (Irmisch *et al.*, 2014; McCormick *et al.*, 2014b; Yoneya *et al.*, 2009), but considering the costs involved in VOC production and emission (Niinemets, 2004; Robert *et al.*, 2013), it would be disadvantageous for a plant to increase its emissions if there was an elevated fitness cost (e.g., higher appetency to generalist herbivores) with no net benefit (e.g., no attraction of natural enemies); such as in our case, where the invasive aphid lacks specialist natural enemies.

The degree of infestation (**Figure 3.6**) could also have contributed to different outcomes. The emission of HIPVs can qualitatively and quantitively vary depending on the population density of the insect feeding on host plants (De Backer *et al.*, 2015; Miresmailli *et al.*, 2012), with studies typically reporting a positive correlation between herbivore population density and VOC emission (De Boer *et al.*, 2004; Horiuchi *et al.*, 2003; Rioja *et al.*, 2018). However, we only found a weak correlation between aphid infestation and VOC emission, and responding cultivars (except *S. candida*) were not heavily infested. This shows that responses are host-specific and less dependent on the degree of infestation, although within the responding cultivars, changes in aphid density may affect VOC emissions. Further studies are required to test this hypothesis.

A study by Aradottir *et al.* (2009) found that *T. salignus* was significantly attracted to certain willow varieties but not to others in laboratory olfactometry tests. Although the compounds involved were not identified, this evidence shows that the giant willow aphid uses plant volatiles to choose their host plants. Therefore, future research should explore the role of VOCs in *T. salignus* host selection and colonization, and the behavioral responses of potential natural enemies of *T. salignus*, such as the harlequin ladybird

Harmonia axyridis (Tun *et al.*, 2020a) to willow VOCs. Our results suggest that some naturally resistant cultivars (*S. lasiolepis* × *S. viminalis* and *S. eriocephala*) are rich GLV emitters, which are known to repel herbivores and attract natural enemies in other systems (Scala *et al.*, 2013). Representative compounds from this group, such as (*Z*)-3-hexenyl acetate, are good candidates for further testing.

In this study, plants belonging to the same species or hybrid had the same sex, so we did not explore the influence of plant sex on VOC emissions or responses to herbivory; this is an aspect that requires further investigation. Being an exploratory study, our results were limited to a low number of replicates, and therefore, we encourage additional studies with higher replication to confirm these findings. Aphid infestation was unequal between plants, as we wanted to explore aphid behavior in nature, and in doing so, were able to identify some cultivars which appear to be naturally more resistant than others to aphid attack. We are following this lead towards the selection of resistant cultivars for sustainable willow growing.

3.6 Conclusions

To summarize, there was a high variation in VOC emissions by different willow cultivars, with clear clustering between tree and shrub species. Most cultivars did not show significant changes in their VOC emissions in response to *T. salignus* infestation, but in those that did, this response was typically a reduction in VOC emissions. Whether this occurs due to the lack of response by the plant, trade-offs between direct and indirect defenses, or the active suppression of plant defenses by the aphid, requires further testing. Our study provides the foundation to further explore the role of willow VOCs in host selection by *T. salignus*. This information will contribute to the selection of willow cultivars for future planting, to reduce the ecological and economic impacts of this emerging pest.

3.7 Appendix

Table S3.1. Split-plot experimental layout of the willow field trial.

Row 1	Row 2	Row 3	Row 4	Row 5	Row 6
Control	**Infested**	**Infested**	**Control**	**Infested**	**Control**
S. lasiolepis	*S. lasiolepis*	*S. matsudana*	*S. matsudana*	*S. × fragilis*	*S. × fragilis*
S. matsudana × S. alba (2)	*S. matsudana × S. alba* (2)	*S. matsudana × S. lasiandra*	*S. matsudana × S. lasiandra*	*S. × reichardtii*	*S. × reichardtii*
S. viminalis	*S. viminalis*	*S. lasiolepis × S. viminalis*	*S. lasiolepis × S. viminalis*	*S. purpurea*	*S. purpurea*
S. schwerinii	*S. schwerinii*	*S. lasiandra*	*S. lasiandra*	*S. candida*	*S. candida*
S. alba	*S. alba*	*S. eriocephala*	*S. eriocephala*	*S. matsudana × S. alba* (1)	*S. matsudana × S. alba* (1)
S. matsudana	*S. matsudana*	*S. × fragilis*	*S. × fragilis*	*S. matsudana × S. alba* (2)	*S. matsudana × S. alba* (2)
S. lasiandra	*S. lasiandra*	*S. purpurea*	*S. purpurea*	*S. viminalis*	*S. viminalis*
S. matsudana × S. lasiandra	*S. matsudana × S. lasiandra*	*S. candida*	*S. candida*	*S. lasiolepis*	*S. lasiolepis*
S. eriocephala	*S. eriocephala*	*S. × reichardtii*	*S. × reichardtii*	*S. schwerinii*	*S. schwerinii*
S. lasiolepis × S. viminalis	*S. lasiolepis × S. viminalis*	*S. matsudana × S. alba* (1)	*S. matsudana × S. alba* (1)	*S. alba*	*S. alba*
S. × reichardtii	*S. × reichardtii*	*S. lasiolepis*	*S. lasiolepis*	*S. matsudana*	*S. matsudana*
S. candida	*S. candida*	*S. matsudana × S. alba* (2)	*S. matsudana × S. alba* (2)	*S. eriocephala*	*S. eriocephala*
S. purpurea	*S. purpurea*	*S. alba*	*S. alba*	*S. lasiandra*	*S. lasiandra*
S. matsudana × S. alba (1)	*S. matsudana × S. alba* (1)	*S. schwerinii*	*S. schwerinii*	*S. matsudana × S. lasiandra*	*S. matsudana × S. lasiandra*
S. × fragilis	*S. × fragilis*	*S. viminalis*	*S. viminalis*	*S. lasiolepis × S. viminalis*	*S. lasiolepis × S. viminalis*

Table S3.2. GC-MS analysis of VOCs released from the foliage of 15 willow cultivars, for the control and aphid-infested treatments.

Cultivars	Treatment	Aldehyde	GLVs						Monoterpenes				Sesquiterpene						
		Nonanal	1-Hexanol	2-He+enol acetate	(*E*)-2-Hexenal	(*Z*)-3-Hexenol	(*Z*)-3-Hexenol acetate	(*Z*)-3-Hexenyl-α-methylbutyrate	α-Pinene	α-Ocimene	(*E*)-β-Ocimene	Linalool	(*E*)-α-Bergamotene	(E) -β-caryophyllene	Copaene	δ-Cadinene	α-Cubebene	α-Farnesene	(*E*, *Z*)-α-Farnesene
S. candida	Control	+	o	o	+	+	+	+	o	o	+	o	o	o	+	o	+	+	o
	Infested	+	o	o	+	+	+	o	o	o	o	o	o	o	+	o	o	o	+
S. eriocephala	Control	+	+	o	o	+	+	o	o	o	+	o	+	+	+	o	o	+	o
	Infested	+	o	o	o	+	+	o	o	o	+	+	o	o	o	o	o	+	+
S. lasiolepis	Control	+	o	+	+	+	+	+	o	+	+	o	+	+	+	+	+	+	o
	Infested	+	+	+	+	+	+	+	o	o	+	o	o	+	+	+	+	+	+
S. lasiolepis × *S. viminalis*	Control	+	o	+	+	+	+	+	o	o	+	o	o	o	o	o	o	+	o
	Infested	+	o	o	o	+	+	o	o	o	+	o	o	o	+	o	o	+	+
S. purpurea	Control	+	o	o	+	+	+	+	o	o	+	o	o	o	o	o	o	+	o
	Infested	+	o	o	o	+	+	+	o	o	+	o	o	o	o	o	o	+	+
S. schwerinii	Control	+	o	o	+	+	o	+	o	+	+	o	+	o	o	o	o	+	o
	Infested	+	o	o	o	+	o	+	o	o	+	o	+	o	+	o	o	+	+
S. viminalis	Control	+	o	o	o	+	+	+	+	o	+	o	+	o	o	o	o	+	+
	Infested	+	o	o	o	+	+	+	+	+	+	o	+	o	o	o	o	+	o
S. × *reichardtii*	Control	+	o	o	o	+	+	o	o	o	+	o	o	+	o	o	o	o	+
	Infested	+	o	o	o	+	+	+	o	o	o	o	o	o	o	o	o	o	+
S. alba	Control	+	o	o	o	+	+	o	o	o	+	+	o	+	o	o	o	+	+
	Infested	+	o	o	o	+	+	o	o	o	+	+	o	+	o	o	o	+	+
S. lasiandra	Control	+	o	o	o	+	+	+	o	o	+	o	+	+	o	o	o	+	+
	Infested	+	o	o	o	+	+	+	o	o	+	o	+	+	o	o	o	+	+
S. matsudana	Control	+	o	o	o	o	+	o	o	+	+	+	o	o	o	o	o	o	+
	Infested	+	o	o	o	o	+	o	o	+	+	o	o	o	o	o	o	o	+
S. matsudana × *S. alba* (1)	Control	+	o	o	o	+	+	o	o	+	+	o	o	+	+	+	o	o	+
	Infested	+	o	o	o	+	+	o	o	o	+	o	o	o	+	+	o	o	+
S. matsudana × *S. alba* (2)	Control	+	o	o	o	+	+	o	+	+	+	o	o	+	o	+	o	+	+
	Infested	+	o	o	o	+	+	o	+	+	+	+	o	o	o	o	o	o	+
S. matsudana × *S. lasiandra*	Control	+	o	+	o	+	+	o	+	+	+	+	+	o	o	o	o	+	+
	Infested	+	o	o	o	o	o	o	o	+	+	o	+	o	o	o	o	+	+
S. × *fragilis*	Control	+	o	+	o	+	+	o	+	+	+	o	+	+	o	o	o	+	+
	Infested	+	o	o	o	+	+	o	o	o	+	o	o	+	o	o	o	o	+

*The “o” and “+” represent absence and presence of each VOC in the headspace sample of willow cultivars, respectively.

Table S3.3. Mean emissions of total and major VOC classes as influenced by willow cultivars and aphid infestation.

Cultivars	Infestation	VOC concentration (ng g DW^{-1} h^{-1})				
		Total	**Aldehyde**	**GLV**	**Monoterpene**	**Sesquiterpene**
S. alba	Infested	40.98±32.19ab	1.65±0.94	31.75±28.15abc	3.23±1.72abcd	4.35±3.04ab
	Control	25.86±7.87ab	2.91±1.53	17.59±4.48abcde	3.36±1.69abcd	2.01±1.31ab
S. candida	Infested	31.18±8.19ab	3.84±0.44	27.00±7.78abcd	0.00±0.00f	0.34±0.34b
	Control	60.61±33.38a	1.59±0.19	56.85±33.38a	0.30±0.04de	1.87±0.81ab
S. eriocephala	Infested	10.28± 4.74ab	2.37±1.58	5.08±1.77bcdef	1.00±0.68abcde	1.85±1.02ab
	Control	8.03±4.43ab	2.05±1.20	1.23±0.68cdef	1.16±0.82abcde	3.58±1.84ab
S. lasiandra	Infested	31.77±4.32ab	1.67±0.89	17.71±7.41abcde	1.46±0.81abcde	10.93±4.13ab
	Control	25.78±17.93ab	1.07±0.62	15.97±14.02abcde	1.26±0.43abcde	7.49±3.92ab
S. lasiolepis	Infested	56.78±12.49a	0.69±0.69	39.38±8.65ab	0.39±0.26bcde	16.32±6.88a
	Control	66.90±8.98a	0.44±0.25	55.11±12.05a	0.90±0.65bcde	10.45±2.57ab
S. lasiolepis × *S. viminalis*	Infested	22.37±11.85ab	1.71±1.04	17.53±11.28abcde	0.00±0.00f	3.14±1.58ab
	Control	44.85±19.59ab	1.49±0.67	42.94±20.48ab	0.10±0.10e	0.33±0.33b
S. matsudana	Infested	31.90±13.03ab	3.35±0.54	1.05±1.05def	27.49±13.73a	0.00±0.00c
	Control	33.20±21.12ab	6.87±3.49	0.58±0.58f	25.74±21.89a	0.00±0.00c
S. matsudana × *S. alba* (1)	Infested	10.66±5.77ab	2.00±1.06	2.47±1.98bcdef	3.32±1.84abcd	2.88±1.08ab
	Control	22.65±14.20ab	3.17±2.39	5.88±3.44bcdef	9.66±6.75abc	3.93±1.72ab
S. matsudana × *S. alba* (2)	Infested	17.87±1.34ab	2.17±0.81	9.02±3.13bcdef	6.68±3.06abcd	0.00±0.00c
	Control	7.19±1.28abc	1.48±0.71	1.94±1.15bcdef	2.83±1.02abcd	0.94±0.78ab
S. matsudana × *S. lasiandra*	Infested	14.85±2.37ab	4.20±0.98	0.00±0.00g	6.39±1.90abcd	4.25±2.16ab
	Control	22.63±14.35ab	2.22±1.11	2.19±2.19bcdef	10.07±7.67ab	8.15±6.54ab
S. purpurea	Infested	23.97±13.84ab	1.49±1.49	19.39±14.06abcde	1.33±1.07abcde	1.76±1.76ab
	Control	54.82±7.71a	1.87±1.48	50.86±8.68ab	1.76±0.25abcde	0.34±0.34b
S. schwerinii	Infested	9.14±8.27ab	1.61±0.95	0.86±0.86ef	0.66±0.55bcde	6.02±6.02ab
	Control	12.57±2.15ab	1.88±0.27	3.82±1.24bcdef	4.05±2.01abcd	2.81±1.63ab
S. viminalis	Infested	65.17±25.48a	1.18±0.62	46.36±16.05ab	3.88±2.29abcd	13.76±6.88a
	Control	67.58±37.12a	0.47±0.47	41.63±28.20ab	6.51±3.05abcd	18.97±9.53a
S. × *fragilis*	Infested	5.78±1.61abc	1.63±0.16	2.65±0.79bcdef	0.73±0.73	0.78±0.46ab
	Control	8.59±2.72ab	2.53±0.74	2.03±1.20bcdef	2.53±1.30abcde	1.51±0.80ab
S. × *reichardtii*	Infested	4.06±1.80c	0.52±0.26	3.54±1.56bcdef	0.00±0.00f	0.00±0.00c
	Control	17.25±7.78	0.79±0.15	15.55±7.30abcde	0.35±0.15cde	0.57±0.41ab
$F_{14,89}$ value		2.55	1.21	9.46	19.83	20.02
P value		0.001	0.261	<0.001	<0.001	<0.001

Table S3.4. Mean emissions of selected VOCs as influenced by willow cultivars and aphid infestation.

Cultivars	Infestation	VOC concentration (ng g DW^{-1} h^{-1})			
		α-farnesene	**(*E*)-β-ocimene**	**(*Z*)-3-Hexenol**	**(*Z*)-3-Hexenol acetate**
S. alba	Infested	2.30±2.30ab	8.62±4.91abcd	4.13±4.13abcd	27.62±24.02ab
	Control	1.49±1.49ab	9.56±4.78abcd	2.25±1.39^{abcd}	15.34±4.29abc
S. candida	Infested	0.00±0.00c	0.00±0.00d	8.55±1.66abc	17.28±6.61ab
	Control	0.80±0.80ab	1.21±0.17cd	25.82±19.80a	27.06±10.91ab
S. eriocephala	Infested	1.85±1.02ab	2.12±1.09bcd	2.80±1.18abcd	2.27±0.79abcd
	Control	2.53±1.82ab	2.28±1.86bcd	0.98±0.70abcd	0.25±0.25d
S. lasiandra	Infested	5.83±2.12a	5.86±3.22abcd	5.57±3.94abc	11.26±4.63abc
	Control	4.27±2.16ab	5.03±1.70abcd	2.51±1.09abcd	13.07±12.60abc
S. lasiolepis	Infested	3.78±1.83ab	1.55±1.02bcd	2.68±1.23abcd	25.25±3.82ab
	Control	2.97±1.30ab	3.28±2.28bcd	2.97±1.21abcd	37.82±6.63a
S. lasiolepis × *S. viminalis*	Infested	2.70±1.37ab	0.00±0.00d	0.80±0.44abcd	16.72±10.92ab
	Control	0.33±0.33ab	0.40±0.40c	3.05±1.63abcd	37.41±17.31a
S. matsudana	Infested	0.00±0.00c	101.03±49.36a	0.00±0.00e	1.05±1.05bcd
	Control	0.00±0.00c	96.10±82.24a	0.00±0.00e	0.58±0.58cd
S. matsudana × *S.alba* (1)	Infested	0.00±0.00c	13.27±7.35abc	0.55±0.55bcd	1.91±1.44abcd
	Control	0.00±0.00c	33.62± 22.88ab	0.11±0.11d	5.77±3.45abcd
S. matsudana × *S.alba* (2)	Infested	0.00±0.00c	20.78±9.66abc	0.44±0.44bcd	8.58±3.56abc
	Control	0.11±0.11b	10.08±3.96abcd	0.23±0.23cd	1.7±1.18abcd
S. matsudana × *S.lasiandra*	Infested	2.87±0.95ab	23.28±6.79abc	0.00±0.00e	0.00±0.00e
	Control	5.18±3.63ab	36.34±26.74ab	1.13±1.13abcd	1.06±1.06bcd
S. purpurea	Infested	1.76±1.76ab	5.32±4.26abcd	8.78±8.06abc	9.78±5.25abc
	Control	0.34±0.34ab	7.04±0.98abcd	27.54±4.31a	16.63±3.51ab
S. schwerinii	Infested	4.11±4.11ab	2.63±2.20bcd	0.52±0.52bcd	0.00±0.00e
	Control	2.33±1.23ab	15.26±7.55abc	1.15±0.83abcd	0.00±0.00e
S. viminalis	Infested	6.04±3.06a	13.43±7.36abc	12.86±3.04ab	28.91±10.73ab
	Control	8.78±5.20a	25.00±12.47abc	11.98±6.42ab	26.31±19.63ab
S. × *fragilis*	Infested	0.00±0.00c	2.94±2.94bcd	0.87±0.44abcd	1.78±0.37abcd
	Control	1.07±0.55ab	9.59±4.85abcd	0.64±0.64abcd	1.39±0.70abcd
S. × *reichardtii*	Infested	0.00±0.00c	0.00±0.00d	0.93±0.37abcd	2.61±1.22abcd
	Control	0.00±0.00c	1.39±0.59bcd	3.20±1.40abcd	12.35±5.90abc
$F_{14,89}$ value		33.55	19.840	14.98	18.79
P value		<0.001	<0.001	<0.001	<0.001

Different letters in each row indicate statistically significant differences using Tukey' HSD test at $P<0.05$. *Salix matsudana* × *S. alba* (1) and (2) represent clones NZ 1040 and NZ 1184, respectively.

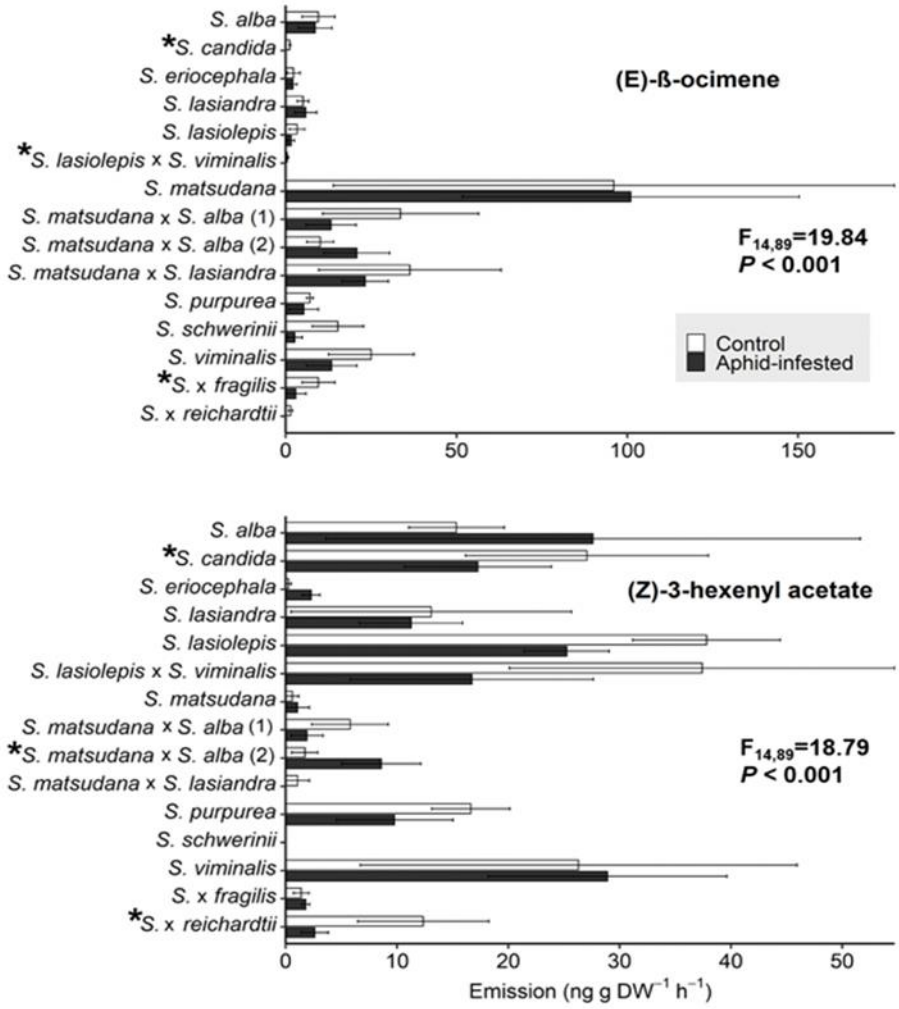

Figure S3.1. Total emissions of the terpenoid (*E*)-β-ocimene and the GLV (*Z*)-3-hexenyl acetate. Asterisks indicate significant differences between the treatments within each cultivar, Tukey's HSD test, α=0.05. Details can be seen in **Table S3.4**.

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Chapter 4

Effect of willow cultivar and plant age on the melezitose content of giant willow aphid (*Tuberolachnus salignus*) honeydew

This chapter was accepted for publication in the *Agricultural and Forest Entomology* as:

Tun, K. M., Minor, M., Jones, T., and McCormick, A. C. (In Press). Effect of willow cultivar and age on the melezitose content of giant willow aphid (*Tuberolachnus salignus*) honeydew.

Reproduced here with some minor modifications in style and formatting.

4.1 Abstract

- The giant willow aphid *Tuberolachnus salignus* is an invasive pest in New Zealand attacking over fifty cultivars of willow. The aphids produce copious amounts of honeydew, which is used by other insects as a food source.

- When foraged by honeybees, *T. salignus* honeydew causes honey to crystallize in the comb and affects bee health; these effects are associated with the elevated melezitose content in the honeydew. The impact of host-plant related factors on *T. salignus* honeydew melezitose content remains unknown.

- This study investigated the effect of willow cultivar and plant age on the melezitose content (and that of other sugars) of *T. salignus* honeydew. To do so, we conducted high performance liquid chromatography analyses of honeydew samples from thirteen willow clones collected in the same season (autumn) from one- and two-year old plants under field conditions.

- Melezitose was the most abundant of the measured sugars in most samples, but its content did not vary significantly with willow cultivar or plant age. In contrast, sucrose was significantly affected by both factors. Fructose and glucose were significantly impacted by willow plant age and cultivar, respectively. A significant cultivar*age interaction was observed for all sugars.

4.2 Introduction

The giant willow aphid, *Tuberolachnus salignus* Gmelin (Hemiptera: Aphididae) is a phloem feeding insect which forms dense colonies on the stems of infested willow (*Salix* spp.) plants (Collins, 2001). This is the largest among 120 aphid species that are known to feed on willows (Hill *et al.*, 2020). *Tuberolachnus salignus* is now spread world-wide, and found anywhere willows are grown (Charles *et al.*, 2014). Originally from Asia, this species was first reported in New Zealand (NZ) in 2013 and has spread rapidly throughout the country, attacking over 50 species and hybrids of willow (Gunawardana *et al.*, 2014). This aphid reproduces parthenogenically (Blackman & Eastop, 1994) and has several overlapping generations during the year (Sharma & Thakur, 1993). In NZ the aphid can be found on willows year-round, with peak population numbers during late summer and autumn (February to April) (Sopow *et al.*, 2017).

Like other aphids, *T. salignus* takes up the required nutrients (carbohydrates, amino acids, and lipids) from the phloem sap of plants, in this case willows (Howse, 2017). The phloem sap of the host plant normally contains high concentrations of sugars with a small fraction of amino acids (Douglas, 2009). Because of this imbalance in the nutrient composition (Jerković *et al.*, 2010), the aphids have to take up enough phloem sap to obtain the amino acids they need for survival and reproduction (Sabri *et al.*, 2013). As a result, the excess carbohydrates are excreted as a sugary honeydew (Mercer, 2020). *Tuberolachnus salignus* honeydew contains melezitose [O-α-D-Glucopyranosyl-(1→3)-β-D-fructofuranosyl-α-D-glucopyranoside] (Mittler, 1958a), which is a trisaccharide synthesized in the aphid gut from two units of glucose and one unit of fructose (break up products of the disaccharide sucrose). Due to its lower solubility in water, melezitose helps aphids to reduce the osmotic stress caused by elevated sugar ingestion (Ashford *et al.*, 2000; Bacon & Dickinson, 1957; Rhodes *et al.*, 1997).

Aphid honeydew provides an energy-rich food source for other insects, especially ants, bees and wasps (Fischer & Shingleton, 2001). Under natural conditions, honeydew deposition can have positive ecological impacts, such as attracting natural enemies (predators and parasitoids) which help to regulate aphid populations (Fischer & Shingleton, 2001; Monticelli *et al.*, 2020). However, in areas where aphids are invasive and lack natural enemies, such as in NZ, excessive honeydew production can have negative economic and ecological impacts (Seeburger *et al.*, 2020). Willows are used in NZ for the spring production of pollen and nectar for honeybees (*Apis mellifera*), and for soil conservation to stabilise stream banks and pastoral hill country. During autumn and winter, when floral resources are low, honeybees are attracted to and forage on the giant willow aphid honeydew (Sopow *et al.*, 2017). However, the melezitose contained in this honeydew may result in honey crystallizing in the comb ('cement honey') (Imdorf *et al.*, 1985; Sopow *et al.*, 2017), making it difficult to extract and thereby reducing the honey yield and quality (Côté, 2007). Melezitose is also considered to be a poor quality food for honeybees, and has been linked to dysentery, abdominal swelling, reduced mobility and low overwintering survival of bees (Seeburger *et al.*, 2020). Additionally, *Vespula* wasps are also found feeding on *T. salignus* honeydew in NZ and Canada (Isebrands *et al.*, 2014; Sopow *et al.*, 2017), and increased wasp populations can pose a problem for honey bees, as wasps compete for available food sources, and attack and kill the bees (Harris, 1991; Lester *et al.*, 2013; Moller & Tilley, 1989). Therefore, quantifying the sugar content of *T. salignus* honeydew is urgently needed to provide basic information to solve honeydew-related problems in the apiculture industry.

Some aphid species produce honeydew with an elevated melezitose content, while others produce very little. For example, *Thieroaphis riehmi* (Hemiptera: Aphididae) feeding on *Quercus* spp. produces honeydew with 49% of melezitose in its total sugar

content, while *Macrosiphum euphorbiae* (Hemiptera: Aphididae) feeding on tomato produces honeydew with only 0.7% melezitose (Hendrix *et al.*, 1992). The honeydew melezitose content, and in general its sugar composition, depend not only on the insect species but also on the plant species the insects are feeding on (Hendrix *et al.*, 1992). As phloem sap is the only available food source for sap-sucking insects, the chemical composition of honeydew reflects the plant sap composition, which can vary depending on the plant species, variety or cultivar, and age (Karley *et al.*, 2002). For example, the black bean aphid *Aphis fabae* (Hemiptera: Aphididae) was observed to produce differing amounts of melezitose when feeding on different plant species (broad bean, goosefoot, beetroot, and poppy) (Schillewaert *et al.*, 2017). This variation can be observed even in closely related plant species. Fischer and Shingleton (2001) reported that *Chaitophorus populeti* and *C. populialbae* (Hemiptera: Aphididae) produced more melezitose when feeding on European aspen (*Populus tremula*) than on the closely related white poplar (*Populus alba*).

For *T. salignus*, the effect of host-plant related factors on the content of melezitose and other sugars in the honeydew remains unknown. This information could be useful for selecting suitable willow cultivars for future planting, in order to reduce the melezitose problem in the NZ apiculture industry. With this in mind, this study aimed to explore the host-mediated variations in the *T. salignus* honeydew sugar content. Specifically, we investigated the effects of two plant-related factors (willow cultivar and plant age) on the *T. salignus* honeydew melezitose content and that of its precursor sugars (glucose, fructose and sucrose), under field conditions.

4.3 Materials and Methods

4.3.1 Willow field trial

Tuberolachnus salignus honeydew was collected from willow plants grown in a field trial at the Plant Growth Unit, Massey University, New Zealand. The field trial was located on flat alluvial land, with a Manawatu fine sandy loam soil, at latitude 40°22'41.70"S, longitude 175°36'30.67"E, and elevation 35 m a.s.l. The mean annual rainfall is 980 mm, and the mean annual temperature is 13.3°C (NIWA, 2020).

Table 4.1. List of willow cultivars used in the experiment.

Species	PFR Code	Common name	Type	Sex
S. candida	PN 385	Furry Ness	Shrub	Male
*S. eriocephala**	PN 376	Americana	Shrub	Male
S. lasiolepis	PN 751		Shrub	Male/female
S. lasiolepis × *S. viminalis**	NZ 04-106-073		Shrub	Male
S. purpurea	PN 249	Booth	Shrub	Female
S. schwerinii	PN 386	Kinuyanagi	Shrub	Male
S. viminalis	PN 220	Gigantea	Tree/Shrub	Male/female
S. × *reichardtii*	PN 714		Shrub	Male
S. alba	PN 357		Tree	Male
S. lasiandra	PN 747		Tree	Male
S. matsudana	PN 227	Kew	Tree	Female
S. matsudana × *S. alba* (1)	NZ 1040	Tangoio	Tree	Female
S. matsudana × *S. alba* (2)	NZ 1184	Moutere	Tree	Male/female
S. matsudana × *S. lasiandra*	NZ 03-003-073		Tree	Male
S. × *fragilis*	PN 218	Russelliana	Tree	Female

No honeydew samples were collected from clones marked with asterisk (*), reflecting the resistance of these willow clones to giant willow aphid infestation. PFR code is the Plant & Food Willow Collection code.

The willow plants in the field trial comprised fifteen willow cultivars (**Table 4.1**), including tree and shrub willow cultivars. The willow cultivars were grown from stem cuttings (20 cm length, 1 - 2 cm diameter), which were planted in the field in June 2017. The cuttings were planted in rows, with 0.4 m spacing within rows, and 4.0 m between rows. The experiment was laid out in a split plot design with three replicated blocks, each

block containing two rows. Within each block, the rows were randomly allocated to either the control or aphid infestation treatment. This trial was port of a broader project investigating the ecological impacts of the giant willow aphid. For the purposes of this study, the honeydew sampling was done only on the plants in the aphid-infested rows.

The soil was prepared by rotary hoeing prior to planting, and the weeds were controlled by manual weeding and spraying with the herbicide (Glufosinate-ammonium, Bayer NZ Ltd). Within each row, the fifteen cultivars were planted in a random order, with each cultivar planted as a row plot of 12 ramets. Rows No. 2, 3 & 5 (aphid infestation treatment) were used in this study (**Table S4.1**), using 2 to 3 plants per cultivar per row.

4.3.2 Aphids

The willow plants in the aphid infestation rows were inoculated with five adult aphids per plant on January 25-27, 2018 and on December 6-7, 2019. Additional inoculations with ten adult aphids per plant were done on February 13-14, 2018, and January 30, 2019, to ensure successful aphid establishment. *T. salignus* populations on the willows increased rapidly, peaking in April, and then declined naturally in May and June in response to colder weather. Two of the cultivars (*S. eriocephala* and *S. lasiolepis* × *S. viminalis*) were excluded from the study because the aphids did not produce harvestable amounts of honeydew for analysis.

4.3.3 Honeydew collection

Tuberolachnus salignus, feeding on the willow stems, deposits honeydew droplets of various sizes at irregular intervals (**Figure S4.1a**). The honeydew droplets were collected using disposable plastic cups (7.5 cm diameter, 10 cm height), covered with 2 mm × 2 mm poly mesh to prevent the removal of honeydew by foraging honeybees, flies and wasps. The cups and mesh were fastened with wire and attached to the willow stems below the aphid colonies (**Figure S4.1b**).

Honeydew sampling was done in autumn (from March to May) 2018 and 2019. The honeydew was collected over nine days in each year (**Figure S4.2**). Collection was conducted from 9:00 am to 12:00 pm (noon) to minimize the impact of changing environmental conditions. Samples were collected on clear sunny days to prevent potential dilution by rain and dew (Murphy & Kelly, 2003), and to avoid warm afternoon weather that would increase the viscosity of the collected honeydew (Kelly et al., 1992). This time frame is also ecologically relevant, as honeybee foraging typically occurs during this period. Ambient temperature during the sampling period was about 20°C with relative humidity around 75%, so we assume low sample evaporation under these conditions (Fukatani et al., 2016). The weather data for the sampling periods, obtained from a nearby weather station, are shown in **Figure S4.2** and **Table S4.2**.

After sampling from 9:00 am to 12:00 pm, the droplets in each collection cup were diluted with a known volume of water (1-1.5 ml), recovered using a 20-200 µl micropipette, put in a labelled 1.5 ml Eppendorf tube, and immediately placed in a portable ice box to be transported to the lab, where the samples were stored in a -20°C freezer until they were analysed. The number of samples ranged from 4 to 6 per cultivar in 2018, and 6 to 7 per cultivar in 2019 (in some cases, not enough honeydew for analysis was recovered). On each sampling day, different plants were randomly selected to avoid duplicate samples from the same plant.

4.3.4 Honeydew sugar analysis

For analysis, the honeydew samples were defrosted at room temperature for 3 to 6 hours. Sample preparation was done according to Fischer and Shingleton (2001). In short, 0.1-0.5 µl of sample was diluted in 50 µl of Milli-Q-Water in Eppendorf tubes. The suspension was taken up into a glass syringe (Sigma-Aldrich, Z314552-1EA) and passed

through the Minisart® Syringe Filter (Sartorius, 0.2 µm). Then, the samples were put into 2 ml clear screw top vials.

The separation and quantification of the sugars in the honeydew samples was done by high-performance liquid chromatography (HPLC), using a Shodex HPLC with an ASI 100 automated sample injector. A 10 µl aliquot was injected into a Sugar Pak I column (Waters, 6.5 by 300 mm) that was kept at 75°C. A 75% acetonitrile: 25% water solution was used as the mobile phase, with a flow rate of 1 ml min^{-1}. The peak areas of the sugars in the samples were evaluated using a RI detector (Shodex RI-101), to calculate the micrograms of each sugar in the 10 µl aliquot.

We used a standard solution containing the four sugars: melezitose (99% purity, Sigma-Aldrich), fructose (99% purity, Thermo Fisher Scientific), sucrose (99.5% purity, Sigma-Aldrich) and glucose (99% purity, Thermo Fisher Scientific). Although our main interest was the detection and quantification of the melezitose in the samples, we included its precursors glucose, fructose and sucrose in the analysis as a reference. These four sugars are known to occur in *T. salignus* honeydew (Mittler, 1958a). A calibration curve was made by plotting the peak area of the external standards with predetermined concentrations. The concentrations of the four sugars were determined from the integrated peak areas, and the sugar concentration was expressed in g L^{-1}.

4.3.5 Statistical analysis

We used R statistical software version 3.6.1 (R Development Core Team, 2019) to perform all the data analyses. Normality was checked using Shapiro-Wilk test. A square-root transformation was done whenever necessary to meet the assumption of normality. The linear model was fitted to examine the effect of the willow cultivar and plant age on the concentration of melezitose, glucose, fructose and sucrose, and their combined total. Whenever the main effects were not significant, but the interaction effect

was significant, the simple effects - the cultivar effect within a single plant age, and the plant age effect within a cultivar, were analyzed using ANOVA and t tests, respectively. The post-hoc means comparison by Tukey's HSD was conducted using the *multcomp* and *lsmean* packages, whenever a global F-test was significant. Pearson correlation was used to establish the relationships between the content of melezitose and the other sugars.

4.4 Results

Weather conditions during the honeydew collection in 2018 and 2019 were different: the 2019 autumn was drier than the 2018 autumn, with higher maximum temperature and lower precipitation (**Figure S4.2**).

The HPLC analysis of the honeydew showed that melezitose was the dominant sugar, followed by sucrose and fructose (**Table 4.2, Figure 4.1**). Glucose was the least abundant sugar, detected in only 41% of the honeydew samples.

Despite some variation, the melezitose content in the *T. salignus* honeydew was not significantly influenced by willow cultivar and plant age, but there was a significant cultivar*age interaction (**Table 4.2, Figure 4.1**). The willow cultivar had no significant effect on the melezitose concentration in year one (**Table S4.3**). In year two the cultivar effect was significant, with considerably higher melezitose content in honeydew from *S.* × *fragilis* and *S. lasiolepis* than from *S. lasiandra* (**Table S4.4**). Apart from *S.* × *fragilis* (t_{11}=-3.09, P=0.010), the plant age did not affect the melezitose content of honeydew from the willow cultivars (**Figure 4.1**).

The sucrose content of the honeydew was significantly influenced by both willow cultivar and plant age; the interaction effect was also significant (**Table 4.2**). Honeydew samples contained significantly more sucrose in year one, overall. In year one, honeydew from *S. lasiandra*, *S. lasiolepis*, *S. matsudana* and *S. schwerinii* had significantly higher sucrose levels than honeydew from *S. candida*. In year two, honeydew from *Salix*

lasiolepis had significantly higher sucrose levels than that from *S. candida, S.matsudana, S. matsudana* × *S. alba* (NZ 1040), *S. matsudana* × *S. lasiandra, S. purpurea, S. viminali*s and *S.* × *fragilis* (**Figure 4.1**).

The fructose content of the honeydew was significantly influenced by plant age. Similar to sucrose, aphids feeding on year one plants produced honeydew with a higher fructose content (**Table 4.2, Figure 4.1**). The cultivar*plant age interaction was significant (**Figure 4.1**). The significant effect of the cultivar was only found in year two, with higher fructose content in honeydew from *S.* × *fragilis* than from *S. schwerinii* and *S. viminalis* (**Table S4.4**).

The glucose content of the honeydew was highly variable. It was low (< 5 g L^{-1}) in most of the samples and was not detected in the *S. lasiolepis* and *S. schwerinii* year two samples (**Figure 4.1**). The glucose concentration in honeydew was significantly influenced by the willow cultivar in both years. *S.* × *reichardtii* in the year one (compared to *S. lasiolepis*, *S. matsudana* × *S. alba* (NZ 1040), *S. schwerinii* and *S.* × *fragilis*) and *S.* × *fragilis* in year two samples (compared to *S. alba*, *S. lasiandra*, *S. lasiolepis*, *S. matsudana*, *S. matsudana* × *S. alba* (NZ 1184) and *S. schwerinii*) contained a significantly higher content of this sugar (**Table S4.3 & S4.4**). The cultivar*plant age interaction was significant (**Table 4.2**); honeydew from *S.* × *reichardtii* in the year one and from *S.* × *fragilis* in year two had a higher glucose content than most of the honeydew samples (**Figure 4.1**). Plant age had a significant effect on the glucose content of honeydew from *S. alba* (t_{11}=2.33, *P*=0.040) and *S.* × *fragilis* (t_{11}=-6.81, *P*<0.001).

When the measured sugars were pooled together, there was a significant effect of the plant age, with a lower total sugar concentration in the honeydew from the year two plants. There was no significant effect of cultivar or the cultivar*plant age interaction (**Table 4.2**).

The melezitose content had a weak but significant positive correlation with sucrose and glucose (Pearson R < 0.3), and a significant positive correlation with fructose (0.73) (**Figure 4.2**).

4.5 Discussion

In our study, willow cultivar and plant age had little influence on the melezitose concentration of the *T. salignus* honeydew, but did significantly influence the glucose, fructose, and sucrose concentration in the honeydew. I assume that this can be attributed to differences in the sucrose content of the phloem sap (Fischer & Shingleton, 2001), with sucrose being the predominant sugar in the phloem sap of willows (Mittler, 1958a). The sucrose is hydrolyzed into glucose and fructose in the aphid's digestive tract, and transformed into melezitose for osmoregulation of the ingested phloem sap (Shaaban *et al.*, 2020). Our findings support those of Baqui and Kershaw (1993) and Beggs *et al.* (2005) indicating that plant age is an important determinant of honeydew composition, and those of Fischer and Shingleton (2001), showing that even closely related plants can cause noticeable differences in the honeydew composition.

Table 4.2. Concentration of different sugars (g L^{-1}) in honeydew from willows and effect of cultivar, plant age and their interaction on individual and pooled sugar production after fitting linear model. The values are mean ± SE. *Salix matsudana* × *S. alba* (1) and (2) represent clones NZ 1040 and NZ 1184, respectively.

		Sugar concentration (g L^{-1})				
	N	**Melezitose**	**Sucrose**	**Fructose**	**Glucose**	**Total**
Cultivar						
S. alba	13	68.30±5.15	27.61±3.10	17.09±1.66	0.49±0.24	113.49±9.34
S. candida	13	61.78±4.33	13.53±1.88	18.74±1.98	3.13±1.33	97.18±7.08
S. lasiandra	11	56.72±8.97	26.12±4.58	16.38±2.94	1.02±0.60	100.24±15.99
S. lasiolepis	12	84.12±6.20	46.40±4.25	18.39±1.71	0.08±0.08	148.99±11.08
S. matsudana	13	68.85±4.92	21.46±4.42	14.87±1.21	0.38±0.14	105.57±7.78
S. matsudana × *S. lasiandra*	13	71.22±6.72	15.20±3.72	17.72±1.76	1.91±0.60	106.05±10.73
S. matsudana × *S. alba* (1)	13	70.51±5.14	26.75±3.25	17.47±1.58	0.44±0.19	115.16±8.69
S. matsudana × *S. alba* (2)	13	69.15±4.43	11.99±3.27	17.18±1.10	2.56±0.81	100.89±7.06
S. purpurea	9	74.64±10.62	16.28±4.77	16.84±2.12	2.02±0.53	109.78±12.25
S. schwerinii	13	61.77±5.71	32.54±3.56	14.20±1.26	0.13±0.1	108.64±9.92
S. viminalis	13	71.77±5.98	15.15±3.26	16.94±2.25	1.21±0.40	105.06±9.61
S. × *fragilis*	13	80.30±7.54	15.69±3.25	19.25±1.61	2.94±0.70	118.18±9.50
S. × *reichardtii*	13	74.19±6.33	19.87±4.28	19.46±1.70	4.31±1.65	117.83±9.27
$F_{12,136}$		1.48	11.49	1.11	6.18	1.77
P value		0.139	<0.001	0.359	<0.001	0.058
Plant age						
Year one	71	69.86±2.39	28.26±1.64	20.49±0.72	1.33±0.35	119.94±3.96
Year two	91	70.51±2.54	17.38±1.59	14.79±0.54	1.79±0.29	104.47±3.80
$F_{1,136}$		0.001	61.1	46.76	2.01	9.54
P value		0.978	<0.001	<0.001	0.158	0.003
Interaction cultivar*age						
$F_{12,136}$		2.09	4.87	2.42	3.69	1.54
P value		0.02	<0.001	0.007	<0.001	0.118

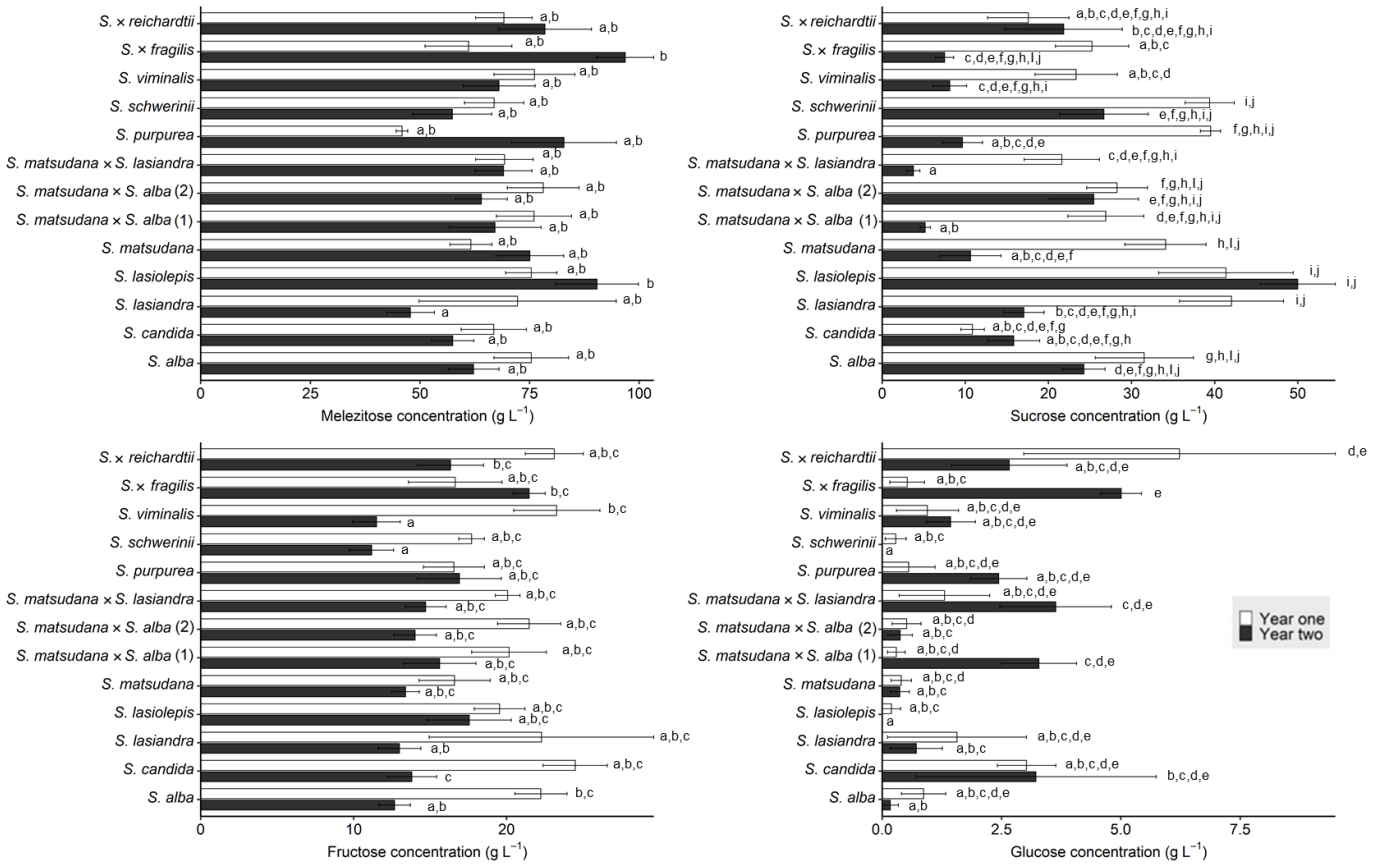

Figure 4.1. Melezitose, glucose, fructose and glucose concentration on *T. salignus* honeydew from thirteen willow cultivars in year one (clear bars) and year two (black bars) of the experiment. Values represent mean ± SE. Different letters indicate significant differences among all possible treatment combinations (cultivar*age) after a Tukey HSD test (α = 0.05). *Salix matsudana* × *S. alba* (1) and (2) represent clones NZ 1040 and NZ 1184, respectively.

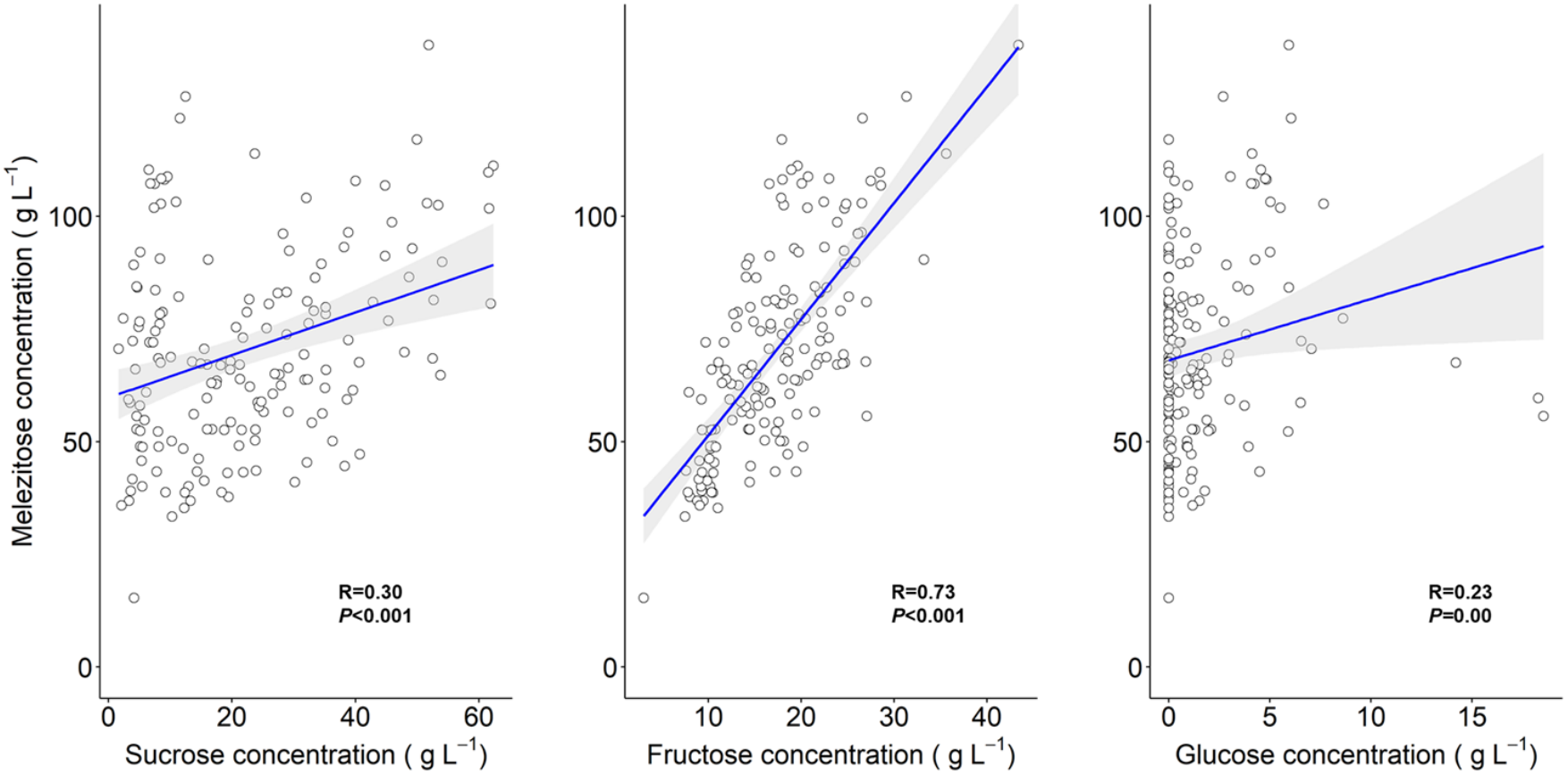

Figure 4.2. Pearson correlation (R) between honeydew melezitose concentration (g L^{-1}) and that of other sugars.

Other factors such as day/night cycles, seasonality, aphid developmental stages and aggregation density are also known to influence the honeydew production and composition (Hargreaves & Llewellyn, 1978; Llewellyn *et al.*, 1974). Differences in weather conditions in honeydew collection years in our study could have had a direct influence on the sugar concentration of aphid honeydew. The willow plants were under drought stress during the summer in year two, which can affect the aphid establishment, delay the peak population time, and lower the aggregation density of aphids on the willow cultivars. These population differences can determine the amount of honeydew production (**Figure S4.3 & 4.4**) and indirectly the sugar concentration of the honeydew. However, we tried to minimize these impacts by collecting honeydew samples at the same time of the day, during the same season, under the same atmospheric conditions, and by inoculating the same number of aphids of the same developmental stage per plant. Other factors such as plant phenology, secondary metabolites, and the location of the aphids on the plant can have an effect on the *T. salignus* honeydew production and sugar composition (Jakobs *et al.*, 2019; Lundborg *et al.*, 2016; Savage, 2020), and require further testing.

The sugar composition of the *T. salignus* honeydew in this study is typical of a homopteran honeydew, consisting of higher proportion of sucrose, fructose, and oligosaccharides (such as melezitose), with a small fraction of glucose (Shaaban *et al.*, 2020). Melezitose was the most abundant sugar, followed by fructose and sucrose, with only trace amounts of glucose. Glucose was also the least abundant sugar in the honeydew of *Coccus hesperidum* (Hemiptera: Coccoidea) (Golan & Najda, 2011). However, the honeydew of *Bemisia argentzjblii* (Homoptera: Aleyrodidae), another phloem feeder, had an opposing pattern with less sucrose than glucose and fructose (Golan & Najda, 2011).

These differences may be attributed to variation in the metabolism of these insect groups, probably in relation to their gut microbiota (Dillon & Dillon, 2004).

The elevated melezitose content in our samples suggest that *T. salignus* is able to efficiently convert the ingested sucrose into melezitose (an oligosaccharide) to reduce osmotic pressure (Ashford *et al.*, 2000; Bacon & Dickinson, 1957). The high correlation between the fructose and melezitose content suggests that fructose is a good predictor of the presence of melezitose in *T. salignus* honeydew.

The melezitose content plays an important role in the attraction of hymenopteran species to honeydew. For example, ants respond most intensively to honeydew containing high amounts of melezitose (Kiss, 1981; Schmidt, 1938; Völkl *et al.*, 1999), and hymenopteran parasitoids are also attracted to melezitose-containing honeydew (Bouchard & Cloutier, 1985; Hatano *et al.*, 2008). Melezitose elicits feeding responses in the braconid parasitoid *Cotesia glomerata*, and its consumption increases parasitoid longevity (Hausmann *et al.*, 2005; Wäckers, 2001). Therefore, the attraction of honeybees to *T. salignus* honeydew is not surprising, although its consumption (especially in high quantities) may be non-adaptive. Honeybees are unable to digest melezitose, which accumulates in the hindgut, altering the gut microbial composition and causing malnutrition and high mortality in overwintering bees (Seeburger *et al.*, 2020). Further studies are needed to investigate the factors influencing the attraction of honeybees towards *T. salignus* honeydew and its consumption, and to determine the ingestion rates and the health impacts on the bees.

Our results show that although host plant-related factors influence the sugar composition of the *T. salignus* honeydew, the melezitose concentration is high and does not vary significantly among willow cultivars. Therefore, to lessen the impact of melezitose-related problems in the apiculture industry, we recommend the selection of

willow cultivars that are resistant to *T. salignus*, such as *S. eriocephala* and *S. lasiolepis* × *S. viminalis* (unpublished data), and further investigation of potential biocontrol agents against the giant willow aphid (Tun *et al.*, 2020a). Further research is needed to evaluate the variation in the sugar composition of honeydew in *T. salignus* feeding on mature willow trees and alternative host plants, and to explore the impact of other environmental, aphid- and plant-related factors on honeydew production and composition.

4.6 Appendix

Table S4.1. Split-plot experimental layout of the field trial where VOC sampling was carried out.

Row 1	Row 2	Row 3	Row 4	Row 5	Row 6
Control	**Infested**	**Infested**	**Control**	**Infested**	**Control**
S. lasiolepis	*S. lasiolepis*	*S. matsudana*	*S. matsudana*	*S. × fragilis*	*S. × fragilis*
S. matsudana × S. alba (2)	*S. matsudana × S. alba* (2)	*S. matsudana × S. lasiandra*	*S. matsudana × S. lasiandra*	*S. × reichardtii*	*S. × reichardtii*
S. viminalis	*S. viminalis*	*S. lasiolepis × S. viminalis*	*S. lasiolepis × S. viminalis*	*S. purpurea*	*S. purpurea*
S. schwerinii	*S. schwerinii*	*S. lasiandra*	*S. lasiandra*	*S. candida*	*S. candida*
S. alba	*S. alba*	*S. eriocephala*	*S. eriocephala*	*S. matsudana × S. alba* (1)	*S. matsudana × S. alba* (1)
S. matsudana	*S. matsudana*	*S. × fragilis*	*S. × fragilis*	*S. matsudana × S. alba* (2)	*S. matsudana × S. alba* (2)
S. lasiandra	*S. lasiandra*	*S. purpurea*	*S. purpurea*	*S. viminalis*	*S. viminalis*
S. matsudana × S. lasiandra	*S. matsudana × S. lasiandra*	*S. candida*	*S. candida*	*S. lasiolepis*	*S. lasiolepis*
S. eriocephala	*S. eriocephala*	*S. × reichardtii*	*S. × reichardtii*	*S. schwerinii*	*S. schwerinii*
S. lasiolepis × S. viminalis	*S. lasiolepis × S. viminalis*	*S. matsudana × S. alba* (1)	*S. matsudana × S. alba* (1)	*S. alba*	*S. alba*
S. × reichardtii	*S. × reichardtii*	*S. lasiolepis*	*S. lasiolepis*	*S. matsudana*	*S. matsudana*
S. candida	*S. candida*	*S. matsudana × S. alba* (2)	*S. matsudana × S. alba* (2)	*S. eriocephala*	*S. eriocephala*
S. purpurea	*S. purpurea*	*S. alba*	*S. alba*	*S. lasiandra*	*S. lasiandra*
S. matsudana × S. alba (1)	*S. matsudana × S. alba* (1)	*S. schwerinii*	*S. schwerinii*	*S. matsudana × S. lasiandra*	*S. matsudana × S. lasiandra*
S. × fragilis	*S. × fragilis*	*S. viminalis*	*S. viminalis*	*S. lasiolepis × S. viminalis*	*S. lasiolepis × S. viminalis*

Table S4.2. Weather conditions recorded during sampling of honeydew deposition by *T. salignus* feeding on year one willow saplings, May 17, 2018. Weather station: Palmerston North Ews, Agent Number-21963, located 680 m away from the field trial site.

Time, am	Temperature (°C)			Relative humidity (%)	Rain (mm)	Wind speed (m/s)
	Maximum	Minimum	Average			
9:00	13.2	12.2	12.7	71	0	4.7
10:00	14.3	12.9	13.6	70	0	4.4
11:00	14.5	12.9	13.9	67	0	5.0
12:00	14.9	12.8	14	66	0	6.2

Table S4.3. The effect of willow cultivars on the concentration of four sugars in year one honeydew samples.

Cultivar	N	Sugar concentration (g L^{-1})			
		Melezitose	**Sucrose**	**Fructose**	**Glucose**
S. alba	6	75.38±8.50	31.53±5.92abc	22.25±1.71	0.87±0.46ab
S. candida	6	66.83±7.45	10.87±1.41a	24.49±2.10	3.02±0.61ab
S. lasiandra	4	72.28±22.50	42.01±6.27bc	22.29±7.34	1.57±1.46ab
S. lasiolepis	5	75.37±5.85	41.35±8.09bc	19.55±1.65	0.19±0.19a
S. matsudana	6	61.62±4.78	34.09±4.88bc	16.60±2.33	0.40±0.21ab
S. matsudana × *S. alba* (1)	6	75.99±8.56	26.89±4.55abc	20.16±2.45	0.30±0.19a
S. matsudana × *S. alba* (2)	6	78.09±8.18	28.25±3.66abc	21.48±2.06	0.51±0.30ab
S. matsudana × *S. lasiandra*	6	69.26±6.58	21.61±4.53abc	20.07±0.80	1.31±0.95ab
S. purpurea	2	45.91±1.32	39.49±1.21abc	16.56±1.99	0.55±0.55ab
S. schwerinii	6	66.92±6.76	39.39±2.95abc	17.72±0.83	0.28±0.22a
S. viminalis	6	76.11±9.25	23.33±4.96abc	23.28±2.82	0.95±0.65ab
S. × *fragilis*	6	61.06±9.90	25.24±4.40abc	16.65±3.07	0.52±0.36a
S. × *reichardtii*	6	69.14±6.43	17.58±4.89ab	23.12±1.90	6.23±3.27b
$F_{12,58}$ value		0.65	3.94	1.18	2.73
P value		0.789	<0.001	0.317	0.005

Table S4.4. The effect of willow cultivars on the concentration of four sugars in year two honeydew samples.

Cultivar	N	Sugar concentration (g L^{-1})			
		Melezitose	**Sucrose**	**Fructose**	**Glucose**
S. alba	7	62.22±5.81ab	24.25±2.58de	12.67±1.03ab	0.17±0.17a
S. candida	7	57.45±4.81ab	15.82±3.12bcde	13.81±1.62ab	3.22±2.52abcde
S. lasiandra	7	47.82±5.44a	17.05±2.42bcde	12.99±1.40ab	0.71±0.55abcd
S. lasiolepis	7	90.37±9.43b	50.00±4.52f	17.56±2.75ab	0.00±0.00a
S. matsudana	7	75.05±7.72ab	10.64±3.64abcd	13.40±0.90ab	0.37±0.20abc
S. matsudana × *S. alba (1)*	7	67.13±10.46ab	5.18±0.63ab	15.63±2.37ab	3.29±0.79de
S. matsudana × *S. alba (2)*	7	64.01±5.92ab	25.46±5.38de	14.02±1.38ab	0.38±0.26ab
S. matsudana × *S. lasiandra*	7	69.06±6.47ab	3.75±0.78a	14.71±1.34ab	3.64±1.17cde
S. purpurea	7	82.85±11.94ab	9.65±2.42abcd	16.92±2.74ab	2.44±0.58bcde
S. schwerinii	7	57.36±9.03ab	26.67±5.33e	11.18±1.44a	0.00±0.00a
S. viminalis	7	68.04±8.18ab	8.13±2.02abc	11.50±1.55a	1.44±0.52abcde
S. × *fragilis*	7	96.80±6.55b	7.51±1.10abc	21.49±1.04b	5.01±0.43e
S. × *reichardtii*	7	78.52±10.61ab	21.83±7.04cde	16.33±2.16ab	2.66±1.21abcde
$F_{12,78}$ value		2.79	12.91	2.44	7.39
P value		0.003	<0.001	0.010	<0.001

Figure S4.1. Honeydew deposition by *T. salignus* (a), and plastic cups with mesh cover (b) for honeydew collection.

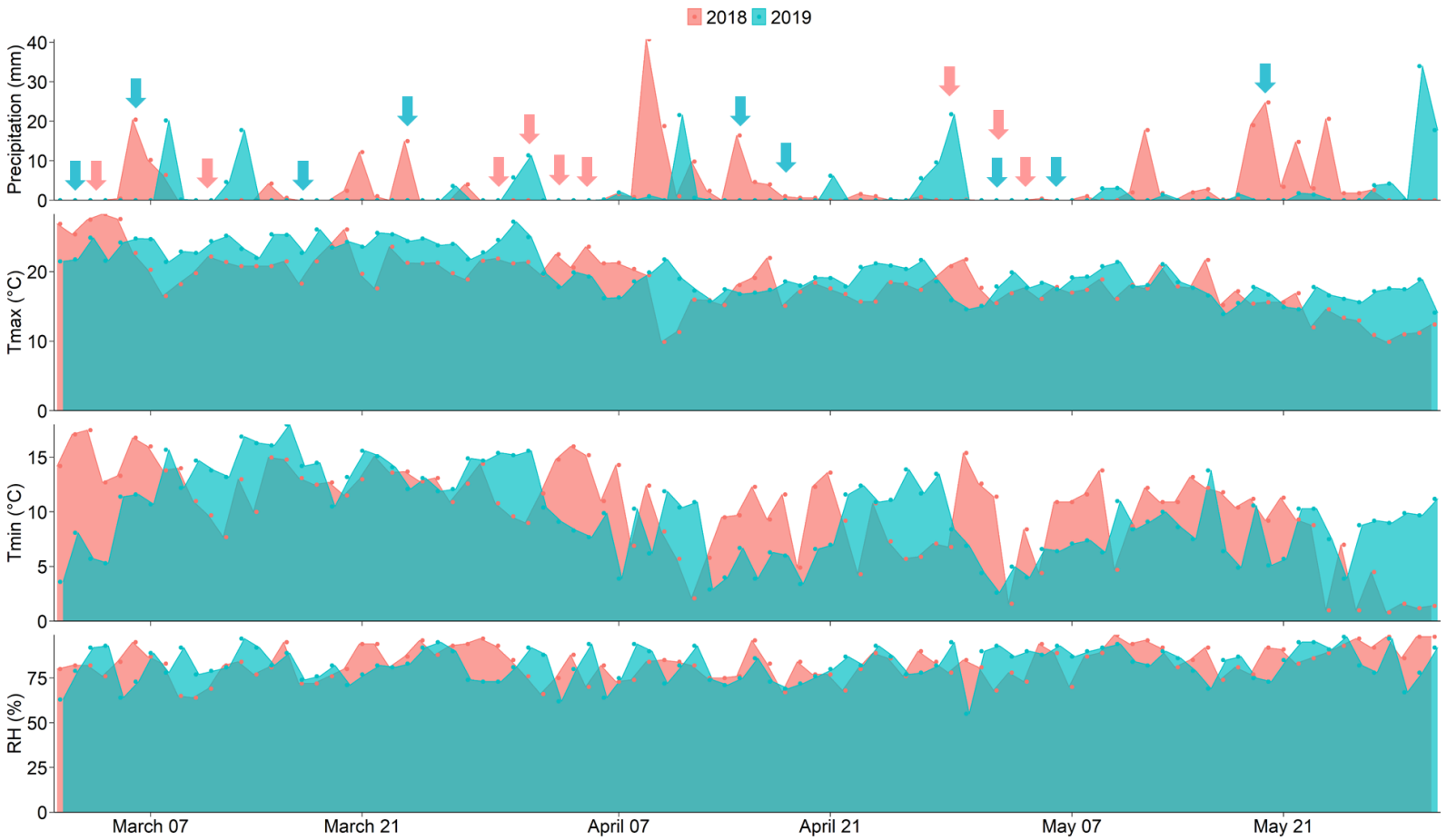

Figure S4.2. Weather condition (precipitation, maximum temperature (Tmax), minimum temperature (Tmin) and relative humidity (RH)) during honeydew collection period in 2018 and 2019. Different coloured arrows and area represent honeydew collection date in different year, as described in the legend.

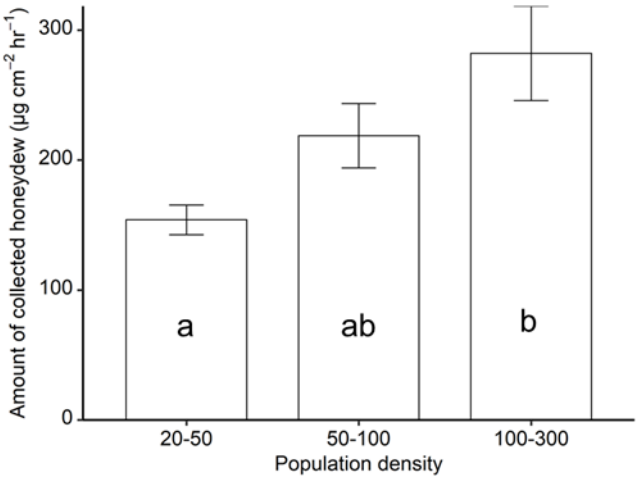

Figure S4.3. Effect of *T. salignus* population density on the amount of honeydew collected on first-year willow plants. The amount of honeydew collected is expressed in µg cm^{-2} hr^{-1} (amount of honeydew per surface area of the cup per collection time). Aphid population density was estimated visually just before the cups were attached to the stems and assigned to an infestation level as described by Collins (2001) (<5, 5-20, 20-50, 50-100, 100-300 and >300 individuals per cluster).

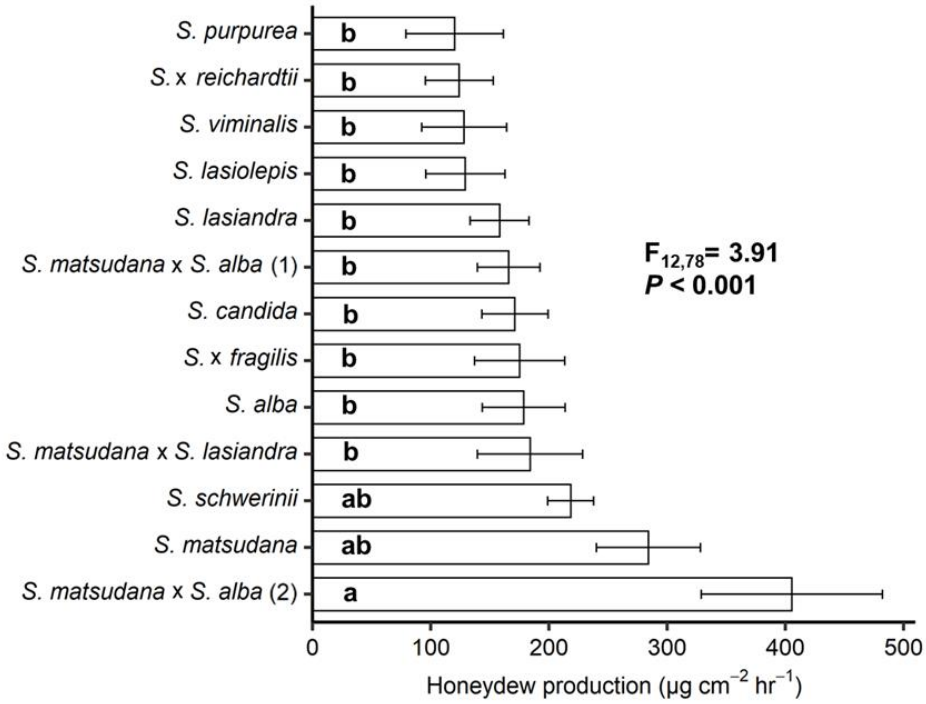

Figure S4.4. The honeydew deposition rate of *T. salignus* feeding on first year plants of the willow cultivars over three hours sampling period, May 7, 2018. Bars show the means ± SE. The different letters represent statistically significant differences (Tukey's HSD test, $P<0.05$). *S. matsudana* × *S. alba* (1) and (2) represent clones NZ 1040 and NZ 1184, respectively. Population level had no significant effect on honeydew deposition when run together with willow cultivars in a linear mixed model with row numbers as a random factor.

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Chapter 5

Honeydew deposition by the giant willow aphid (*Tuberolachnus salignus*) affects soil biota and soil biochemical properties

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Reproduced here with minor modifications in style, numbering of Figures and Tables, and reference format.

5.1 Abstract

Infestation of willow plants by the giant willow aphid *Tuberolachnus salignus* (Hemiptera: Aphididae) is associated with copious deposition of sugar-rich honeydew under the plant canopy. We explored the effect of aphid honeydew on the soil biota and biochemical indicators in a two-year field trial. Soil samples from under aphid-infested and control willow trees, as well as samples from black sooty mould spots under the aphid-infested willows were compared; soil samples before aphid inoculation were used as a baseline. The honeydew deposition had a positive effect on the total soil carbon (C), but not on the total soil nitrogen content or soil pH. Microbial biomass C, basal respiration, number of yeast colony forming units, and the geometric mean of activities for six enzymes were significantly higher in honeydew-affected soils than in the control treatment on both years. The honeydew deposition also increased soil meso-fauna abundance, especially in the black sooty mould spots. The soil biochemical properties, which differed before and after aphid infestation, showed considerable overlap between the first and second year post-infestation. The results highlight the cascading effects of *T. salignus* on soil biological activity and the importance of using a multitrophic approach to explore similar scenarios.

5.2 Introduction

The giant willow aphid, *Tuberolachnus salignus* (Gmelin) (Hemiptera: Aphididae), is an invasive stem-feeding pest of willow trees, which has recently arrived in New Zealand (Sopow *et al.*, 2017). Willows (*Salix* spp.) are important multi-purpose farm trees used for biomass production, bioremediation, erosion control, and soil nutrient management (McIvor, 2013; Smart *et al.*, 2005). As *T. salignus* is a new species in New Zealand, not much is known about the ecological consequences associated with its presence in willow growing systems, such as its effects on the soil biota.

One of the prominent features of infested willow plantings is the deposition of copious amounts of honeydew by aphid colonies, and the growth of black sooty mould on the leaves, stems, and on the soil surface ((Sopow *et al.*, 2017); see also **Figure 5.1**). A single adult *T. salignus* can exude 1.71–2.08 mm^3 of honeydew per hour (Mittler, 1957, 1958b). Chemically, the honeydew is a mixture of water, carbohydrates (90–95% dry weight), amino acids (< 5%), lipids and other nutrients (Byrne & Miller, 1990; Dhami *et al.*, 2011). When this energy-rich liquid is deposited on the leaves and understory plants, it is splashed onto the soil surface by rainfall (Beggs & Wardle, 2006). It can be hypothesized that deposition of *T. salignus* honeydew on the soil surface will initiate a cascade of changes in soil processes, causing modifications in soil chemical properties, microbial activities and in the abundance of soil microbivores. Previous studies have linked the labile carbon (C) input from aboveground aphid herbivory to changes in belowground biochemical properties (Hunter, 2001; Jílková *et al.*, 2018; Jílková *et al.*, 2020; Reynolds & Hunter, 2001). These effects are linked to aphid population density (Michalzik *et al.*, 1999) and the identity of the aphid species (Milcu *et al.*, 2015).

Figure 5.1. The black sooty mould spots under the canopy of the willow plants.

Aphid honeydew deposition on the soil surface is expected to increase nutrient availability, fuel the growth of microbial communities in belowground systems (Domisch *et al.*, 2009; Jílková *et al.*, 2018), and influence the soil decomposition processes (Rousk *et al.*, 2009; Stadler *et al.*, 2006). Microorganisms (bacteria, fungi, and other taxa) contribute to the functioning of soil ecosystems (Nannipieri *et al.*, 2017), regulating the processes of organic matter decomposition and nutrient cycling (Cregger *et al.*, 2012). Soil microorganisms are assumed to be energy-limited, and as a result, mostly remain dormant when C resources are scarce (Blagodatskaya & Kuzyakov, 2013). The daily addition of sugar to the soil can cause a 2.5-fold increase in bacterial diversity, compared to control treatments, as sugar supplementation encourages the soil microbes to become active (Shi *et al.*, 2011). Aphid honeydew is a suitable growing medium for various saprophytic microbes (Stadler & Müller, 1996) and has been shown to increase the activities of soil microorganisms (Jílková *et al.*, 2018; Stadler *et al.*, 2006). Among soil

microbes, yeasts are important degraders and saprotrophs (Connell *et al.*, 2008; Mašínová *et al.*, 2018), utilizing various C and nitrogen (N) sources (Yurkov, 2018). Soil yeasts are ubiquitously present in many agroecosystems (Elena & Renata, 2003) and in nutrient-rich forest environments (Mašínová *et al.*, 2018), and exhibit a quick response to changes in soil nutrient content (Birkhofer *et al.*, 2012).

Changes in soil microbial activity can also be reflected in the activities of soil enzymes (García-Ruiz *et al.*, 2008). Soil enzymes activity is a commonly used soil bioindicator (Nosrati *et al.*, 2011), because of their quick response to subtle changes in available resources such as organic C input (Torres *et al.*, 2015; Wei *et al.*, 2006), and the ease of enzyme quantification (Rao *et al.*, 2014). So far, soil enzyme activities have not been used to explore honeydew-mediated changes in soil quality. Measuring the changes in the activities of soil enzymes following aphid infestation can provide a good tool to quantify the soil microbial responses to honeydew deposition (Cregger *et al.*, 2012; Yu *et al.*, 2017).

An increase in microbial biomass could have potential consequences for soil meso-fauna, as their abundance is likely to be affected through food web interactions (Milcu *et al.*, 2015; Sinka *et al.*, 2009). Soil meso-fauna (Collembola and Acari) live in top soil layers and play different functional roles in soil processes and nutrient cycling (Coleman *et al.*, 2017). Collembola, Astigmata, and Oribatida are dominant soil microbivores (Hopkin, 1997; Hoy, 2008; Men'ko *et al.*, 2006; Mylonakis *et al.*, 2002; Schon *et al.*, 2012; Whalen & Sampedro, 2010), while Gamasida are mobile predators of meso-fauna (Walter *et al.*, 1988). Although some studies have been conducted to explore the effect of honeydew deposition on soil meso-fauna abundance (Milcu *et al.*, 2015; Sinka *et al.*, 2009), the results are inconclusive and different taxa respond in different ways to the sugar addition – some increase in abundance, while other decrease.

The aim of this study was to investigate the cascading effects of honeydew deposition by *T. salignus* on soil chemical properties (pH, C and N), soil microbial biomass and basal respiration, soil yeasts, abundance of soil meso-fauna (Acari and Collembola), and soil enzyme activity. Six enzymes were selected based on their sensitivities and importance in the electron transport system (dehydrogenase), cycling of C (glucosidase, invertase and amylase), and N (amidase and urease) in the soil. We analysed and compared soil biochemical properties and biota under control plants, under aphid-infested plants and in black sooty mould spots under aphid-infested plants.

5.3 Materials and Methods

5.3.1 Willow field trial

A willow field trial with an area of 4000 m^2 (50 m × 80 m) was established at the Orchard Block, Plant Growth Unit, Massey University, Palmerston North, New Zealand (40°22′41.70″ S, 175°36′30.67″ E, 30 m a.s.l). Average annual rainfall at the study site is 980 mm, ranging from 64 mm in the driest month (February) to 99 mm in the wettest month (July). Average annual temperature is 13.3°C, fluctuating from 8.6 (July) to 18.1 °C (February) (NIWA, 2020). The soil type in the experimental area is a Manawatu fine sandy loam (Weathered Fluvial Recent Soil; Hewitt, 1998). Prior to planting willows, weeds were killed with glyphosate herbicide on 16 May 2017 and on 25 May 2017 the soil was rotary hoed in six rows. Each row was 1 m wide and 75 m long, with 4.0 m spacing between rows. The field trial was arranged in a split-plot layout, with three replicated blocks. Each block included two rows of willows; each row contained row plots of 12 ramets of fifteen willow cultivars. Willow cuttings (20 cm in length and 13 ± 2.6 mm in diameter) were planted on 16 June 2017, with 0.4 m spacing between cuttings within rows. After planting, the weeds were controlled by manual weeding and by spraying with Buster® herbicide (Glufosinate-ammonium, Bayer NZ Ltd). The two

treatments, presence of *T. salignus* and aphid-free control, were randomly allocated to the two rows within each block.

5.3.2 Aphids

Willow plants in the aphid-infested rows were inoculated with five adult aphids per plant on 25–27 January 2018 and 6–7 December 2019. Additional inoculations with ten adult aphids per plant were performed on 13–14 February 2018 and 30 January 2019. The willow plants in the control rows were inspected for colonizing aphids on a weekly basis, and any aphids found were removed manually. The plants in control rows were sprayed with Mavrik® insecticide on 28 February 2018 and on 17 January 2019, when manual control was impractical due to high population densities of *T. salignus*.

5.3.3 Soil sampling

Soil samples were collected on the willow field trial site on 16 May 2017 prior to willow planting to assess spatial heterogeneity of the site. Following willow planting, samples were collected under the canopy of willow plants, in the 1.0 m wide cultivation zone, before aphid inoculation on 24 January 2018, after aphid inoculation on 22 June 2018, and on 2 July 2019. Three sampling points that were 20 m equidistant from each other, were marked along each of the six rows of the field trial (**Figure S5.1**).

Eighteen samples (one per sampling point), consisting of nine replicates from the aphid-infested and control rows, were collected during each sampling visit. Although all the plants in infested rows were inoculated with aphids, the honeydew was unevenly deposited, reflecting the distribution pattern of the aphid colonies. Therefore, additional soil samples were taken from black sooty mould spots (**Figure 5.1**) on the soil surface in the aphid-infested rows. Three samples (one per row) were collected on 22 June 2018, and six samples (two per row) were collected on 2 July 2019. Soil moisture content was measured three times at each sampling point using a TDR 300 Soil Moisture Probe

(Spectrum Technologies Inc., Aurora, IL, USA), and the average of the three readings was then recorded. Soil temperature at 5 cm depth was measured with a QM7216 Digital Stem Thermometer. At each sampling point, two samples were collected, one for soil fauna extraction and another for analysing the soil chemical properties, microbial respiration and enzyme activities. The samples were put into labelled plastic bags, placed in an ice chest and immediately brought to the laboratory.

The samples used to quantify the soil meso-fauna (Collembola and Acari) were taken using a 25 cm^2 soil corer to 5 cm depth. The sample (300–500 g) for soil chemical properties, microorganisms and enzymes was collected using a spade to 5 cm depth from five spots within a 1.0 m diameter circle around each sampling point, and then mixed thoroughly in a plastic tray to get a homogenous sample. Earthworms, plant roots, moss, stones, and other debris were removed before sieving the soil through a 2 mm drum sieve. The sieved soil was then divided into two subsamples. The subsample for analysing the soil chemical properties and enzyme activities was air-dried at room temperature, ground and sieved through a 1 mm mesh drum sieve. The subsample for determining the yeast colony forming unit (CFU), microbial respiration and biomass was frozen at −20°C.

5.3.4 Soil chemical properties

The soil pH was measured in a slurry containing 5 g of soil and 12.5 mL of distilled water, using an Orion Star™ A214 pH/ISE Benchtop Meter (Thermo Scientific, Waltham, MA, USA). The total C and N content were determined by Vario Macro Cube (Elementar Analysensysteme GmbH, Langenselbold, Germany) from the mixture of soil (75–100 mg) and tungsten oxide powder (25–50 mg).

5.3.5 Soil fauna

The soil samples for meso-fauna extraction were processed within 24 h. The fauna were extracted from the soil cores using a modified Berlese-Tullgren apparatus, as

described by Oliver and Beattie (1996). Extraction was performed under 15 W light bulbs (Sylvania, OH, USA, 240–250 W) in a 17 to 30°C temperature gradient in a temperature-controlled room for 7 days. The animals were collected into 70% ethanol and examined using an Olympus SZX12 stereomicroscope (Spach Optics Inc., Rochester, NY, USA). The Collembola were identified to an order level. The soil Acari were assigned to three suborders: Oribatida, Astigmata and Gamasida. The other meso- and macro-fauna, including small insects, spiders, centipedes, Isopoda, Diplura, Symphyla, annelid worms and Pauropoda, were grouped as "others". The densities of the fauna were expressed as the number of individuals per m^2.

5.3.6 Soil enzymes

The urease (EC 3.5.1.5), invertase (EC 3.2.1.26), β-amylase (EC 3.2.1.2), α-glucosidase (EC 3.2.1.20), dehydrogenase (EC 1.1.1.1) and amidase (EC 3.5.1.4) activities were assessed according to the protocols developed by Shcherbakova (1983), Frankenberger and Johanson (1983), Ross (1983), Mfombep and Senwo (2012), Serra-Wittling *et al.* (1995), Alef and Nannipieri (1995) and Frankenberger (1980), with slight modifications. Moist soil (1 g dry weight equivalent) was treated with 1.6 mL of triphenyltetrazolium chloride (TTC) before incubating at 30°C for 24 h; 5 mL of acetone was added followed by incubation in the dark for 2 h to measure the dehydrogenase activity (Alef & Nannipieri, 1995). After incubating 0.25 g of soil with 2 mL of urea in phosphate buffer, and 20 µL of toluene at 37°C for 4 h, the urease activity was assayed using a Genova Nano Micro-Spectrophotometer (Jenway, Stone, Staffordshire, UK) as the amount of nitrate released from urea (Shcherbakova, 1983). The amidase activity was assessed using formamide substrate, and the amount of ammonia released during hydrolysis of the enzyme was measured at a wavelength of 400 nm in the above-mentioned spectrophotometer (Frankenberger, 1980). The invertase activity was

estimated by measuring the amount of glucose and fructose released from sucrose, after incubating soil samples (0.3 g) with toluene and modified universal buffer at pH 5 (Frankenberger & Johanson, 1983). The amylase activity was measured using starch solution as a substrate, according to Wainwright *et al.* (1982). Determination of the α-glucosidase consisted of incubating soil samples (1 g) with toluene (0.2 mL), 67 mM sodium acetate buffer (4.3 mL, pH 5.0) and 50 mM maltose (0.5 mL) in plastic tubes at 37°C for 1 h; the activity of this enzyme was assayed after placing the tubes in a boiling water bath for 5 min (Mfombep & Senwo, 2012). The activity of each enzyme was expressed based on 1 g of dry soil. One gram of each fresh soil sample was used to estimate the dry-weight equivalent conversion factor. The samples were oven-dried at 80°C for 72 h until constant weight was achieved, and the dry weight measured.

The geometric mean of the enzyme activities (GMea) is regarded as a sensitive indicator for soil quality and soil health assessment (García-Ruiz *et al.*, 2009; Hinojosa *et al.*, 2004; Puglisi *et al.*, 2006). The GMea calculation is based on the activities of all the assayed enzymes (García-Ruiz *et al.*, 2009), and it is a more reliable index than any of the specific enzyme activities alone (Paz-Ferreiro *et al.*, 2014). In this study, the GMea for six enzyme activities in the aphid-infested and control rows, and the black sooty mould soil spots was calculated according to Paz-Ferreiro *et al.* (2014) as follows:

$$GMea = \sqrt[6]{Ure \times Inv \times Amy \times Glu \times Dehy \times Ami} \quad (1)$$

where *Ure*, *Inv*, *Amy*, *Glu*, *Dehy* and *Ami* represent urease, invertase, β-amylase, α-glucosidase, dehydrogenease and amidase, respectively.

5.3.7 Yeasts

The frozen soil samples were incubated at 25°C for 24 h. Fresh soil was divided into three plastic tubes (1 g dry soil equivalent each), and suspended in Milli Q water to

obtain three dilutions (*v*/*w*, 1:5, 1:10 and 1:20) according to Yurkov *et al.* (2012). After shaking the suspensions on an orbital shaker for 1 h, 0.1 mL aliquots were plated in triplicate on casein-peptone glucose yeast extract agar, supplemented with chloramphenicol (0.1 g L^{-1}). Lactic acid was added to acidify the medium to pH 4.5. The plates were incubated at 25°C for 2 days and then transferred to a chiller (5°C) to prevent mould development. Visible colonies were counted weekly for three consecutive weeks. The yeast counts were expressed as the colony forming units (CFU) per 1 g of dry soil, multiplied by the dilution factor (5, 10 and 20).

5.3.8 Microbial properties

The microbial biomass C (Cmic) and basal respiration (BR) were determined by the substrate-induced respiration (SIR) method (Ananyeva *et al.*, 2011). The frozen soil samples were incubated for 24 h at 25°C. The samples (1 g dry weight equivalent) were weighed and placed into 22 mL glass vials. After dropwise addition of 0.1 mL glucose solution (8 mg g^{-1} soil), the vials were closed with airtight lids containing a septum in the centre. The vials were incubated at 22°C for 3–5 h. Air samples were collected using a syringe, and then injected into the CO_2 analyser (HP 3396 Series II Integrator, Hewlett Packard, Palo Alto, CA, USA). The Cmic (μg C g^{-1} soil) was calculated as: Cmic = SIR (μL CO_2 g^{-1} soil h^{-1}) × 40.04 + 0.37, according to Anderson and Domsch (1978). A similar procedure was used for determining the BR, but only 0.1 mL of water was added to the vials prior to the 24 h incubation period at 22°C. The BR of the soil samples was measured as μg CO_2-C g^{-1} dry soil h^{-1}. The microbial metabolic quotient (qCO_2) was calculated by dividing the BR by the Cmic, and expressed in μg CO_2-C mg^{-1} Cmic h^{-1} (Ananyeva *et al.*, 2016).

5.3.9 Data analysis

All analyses were performed in R version 3.5.1 (R Development Core Team, 2019). A Shapiro–Wilk test was used to check whether the data distributions met the assumption of normality. Generalized linear models (GLMs) were used for analysis of the treatment effects on the soil chemical and microbial properties, and enzyme activities. The gamma distribution with log-link function was used for non-normally distributed variables, while the normally distributed data were fitted using the gaussian distribution with identity link. Count data (meso-fauna and yeast CFU counts) were analysed in the GLM using the Poisson and quasi-poisson error distributions. The GLMs were fitted separately for the two sampling times, after the willow plants were inoculated with aphids. The pre-treatment sampling on 24 January 2018, prior to the aphid inoculation, was used as the baseline measurement, but was not included in the analysis of the aphid infestation treatment vs. control. The library "*multcomp*" was used for multiple comparisons using Tukey's HSD test, whenever the GLM results showed global significant differences of the means. Full results of all GLM tests are provided in the Supplemental **Table S5.1**.

The principal component analysis-linear discriminant analysis (PCA-LDA) was used to visualise the data and maximize the treatment segregation (Patel *et al.*, 2014; Walsh *et al.*, 2007). All variables were square-root transformed, scaled, and centred (divided by their respective standard errors) to assure equal variance, before conducting the PCA-LDA (Stenberg *et al.*, 1998). First, the PCA was performed to produce the principal components (PCs) in the "*FactoMineR*" and "*factoextra*" packages. The first eight PCs together explained more than 80% of the variance in the data and were used in the linear discriminant analysis (LDA). The "*caret*", "*MASS*" and "*tidyverse*" packages (Patel *et al.*, 2014) were used to perform LDA.

In the PCA-LDA evaluating the honeydew-related changes in the soil biochemical properties among the control, aphid-infested treatments, and black sooty mould spots, the baseline measurements (collected before aphid inoculation) were excluded, as the aphid absence on the plants meant no honeydew was deposited on the soil surface. In the PCA-LDA, which compared the selected soil indicators over time, the baseline measurements as well as data from first and second year after aphid inoculation were included. Soil temperature and moisture measurements were excluded from the time analysis to remove bias due to weather.

In the current study, the effect of the willow cultivars was excluded from consideration. Previous research had showed the total sugar content in the honeydew of *T. salignus* was not statistically different among the willow cultivars (Tun *et al.*, 2020b). The soil samples were taken from under the canopies of the same willow cultivars, in the aphid-infested and control rows.

5.3.10 Structural equation modelling

Structural equation modelling (SEM) is a more reliable approach than univariate correlations and regressions, because it provides path coefficients to examine the multiple associations in a multi-layered system (Grace, 2006). SEM was constructed to explore how honeydew deposition could influence soil biochemical processes and functions, and to quantify the relative contribution of the different variables, which form a network of causal relationships (Hatcher, 1996).

The *a priori* hypothetical model was first constructed to describe the causal relationships for the effects of aphid honeydew on the soil environment, linking *T. salignus* honeydew deposition to Cmic, specific enzyme activities, meso-fauna abundance, and the geometric mean of the enzyme activities (GMea). This was based on a modification of the path model constructed by Milcu *et al.* (2015). Variables for the

model were selected based on the PC scores (**Figure S5.2**) and previous literature. We then used SEM to calculate the coefficients associated with each path in SPSS Amos 25 (IBM, Armonk, NY, USA) (Arbuckle, 2014).

The data on the soil analysis from the two sampling dates after aphid inoculation were pooled for this analysis; the pre-treatment data, prior to aphid inoculation, were excluded. The honeydew input was coded as a categorical (ordinal) variable, with 0 for control, 1 for aphid-infested, and 2 for black sooty mould spots. The Cmic, total C and N contents were selected to evaluate the direct effect of honeydew deposition. The Cmic was chosen to estimate the indirect effect of honeydew deposition on the Gmea and meso-fauna abundance, as Cmic has been shown to associate with soil fauna (Milcu *et al.*, 2015), soil chemical properties (total C, total N) and enzyme activities (Cheng *et al.*, 2013).

The critical ratio of the multivariate kurtosis and squared Mahalanobis distance were checked for multivariate normality and the presence of outliers (Sutton-Grier *et al.*, 2010). The pooled dataset, containing the selected variables over the two sampling times, was square-root transformed to meet the assumption of multivariate normality. The maximum likelihood estimation was used to test the path coefficients in the SEM models. Standardized coefficients were calculated for all the variables in the paths diagram (Grace & Bollen, 2005; Kwan & Chan, 2011). The Chi-square test, probability value of the likelihood ratio test, comparative fit index (CFI), root mean square error of approximation (RMSEA), and Akaike's information criterion (AIC) were used to evaluate the model fit.

5.4 Results

5.4.1 Soil chemical properties

Before the willows were planted, the soil of the field trial site had a mean pH value of 6.1 ± 0.05, 2.4 ± 0.08% total C and 0.25 ± 0.01% total N. None of these parameters exhibited any significant spatial differences prior to willow planting. The baseline measurements for soil chemical properties after planting prior to aphid infestation are included in **Table 5.1**.

Table 5.1. Soil chemical properties before aphid inoculation (baseline) and after aphid inoculation (control, aphid-infested and black sooty mould spots), during the first and second year of the experiment. Values are the means ± SE. Different letters in each column indicate significant differences between the treatments at each sampling time (Tukey's HSD test, $\alpha = 0.05$).

Parameter	Treatment	Sampling Time		
		Before aphid infestation	First year	Second year
pH	Baseline	5.75 ± 0.03		
	Control		6.102 ± 0.032a	6.073 0.029a
	Aphid-infested		6.177 ± 0.052a	6.058 ± 0.065a
	Black sooty mould spots		6.163 ± 0.091a	6.067 ± 0.040a
Total C (%)	Baseline	2.40 ± 0.06		
	Control		2.146 ± 0.076b	2.278 ± 0.110b
	Aphid-infested		2.260 ± 0.061b	2.297 ± 0.084b
	Black sooty mould spots		2.490 ± 0.023a	2.547 ± 0.070a
Total N (%)	Baseline	0.27 ± 0.01		
	Control		0.238 ± 0.006a	0.247 ± 0.011a
	Aphid-infested		0.249 ±0.005a	0.242 ±0.008a
	Black sooty mould spots		0.237 ± 0.009a	0.247 ± 0.006a
C : N	Baseline	8.79 ± 0.13		
	Control		9.008 ± 0.136b	9.207 ± 0.178b
	Aphid-infested		9.076 ± 0.107b	9.483 ± 0.216b
	Black sooty mould spots		10.547 ± 0.306a	10.302 ± 0.140a

Honeydew deposition resulted in higher total C content in the black sooty mould spots compared to the control and aphid infestation treatments in both first and second year after aphid infestation (**Table 5.1**). There was no effect of the treatments on the soil pH values or total N content in both years. The C:N ratio was significantly higher in the black sooty mould spots in both years (**Table 5.1**).

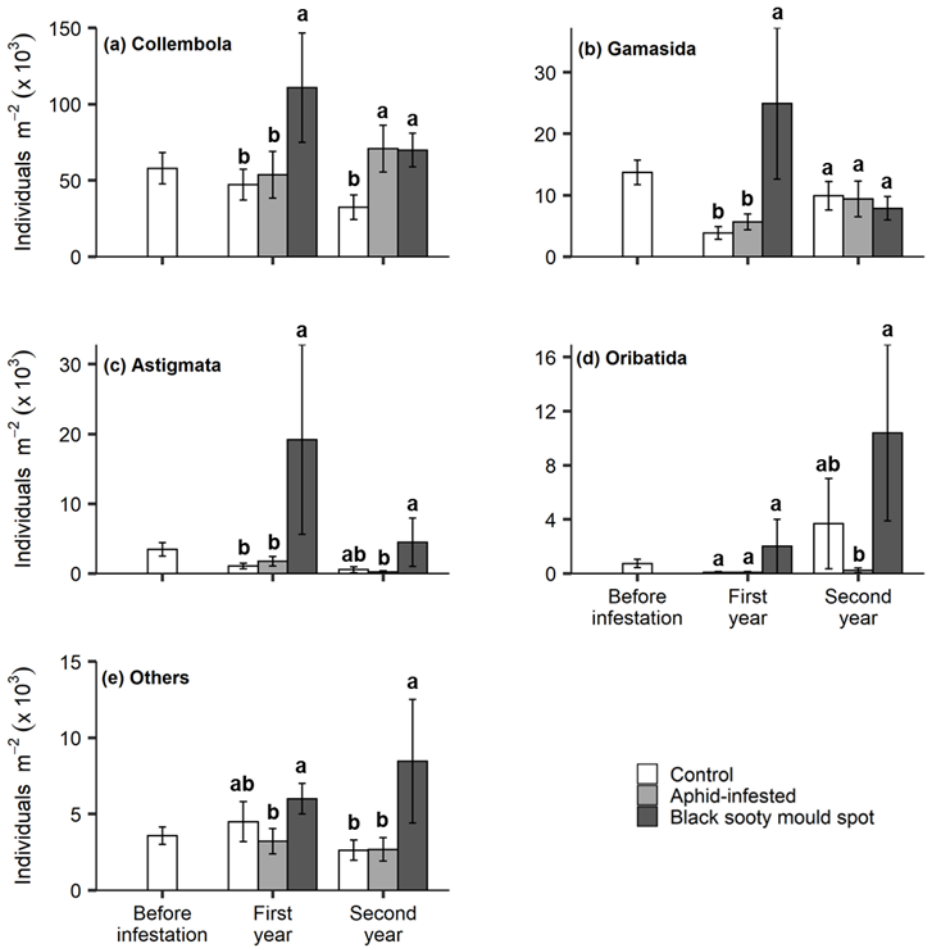

Figure 5.2. The effect of *T. salignus* honeydew deposition on the abundance of soil meso-fauna: Collembola (**a**), Gamasida (**b**), Astigmata (**c**), Oribatida (**d**) and others (**e**), prior to aphid inoculation (before infestation), and after aphid inoculation (control, aphid-infested, and black sooty mould spots) in the first and second year. The values are the means ± SE. Different letters indicate statistically significant differences between treatments within each sampling time (Tukey's HSD test, $\alpha = 0.05$).

5.4.2 Soil meso-fauna

Of the 4.80×10^6 soil meso-fauna collected in samples, Collembola was the most dominant taxon, comprising 75.5% of the total. Significantly higher Collembola densities were observed in the black sooty mould spots in the first year, and in both the aphid-infested rows and black sooty mould spots in the second year, compared to the control treatments (**Figure 5.2a**).

The soil Acari (0.92×10^6 individuals) accounted for 19.3% of the soil meso-fauna. Gamasida was the most abundant mite taxon, comprising 67.9% of the Acari. The aphid infestation had a significant effect on gamasid mites only in the first year, with higher densities in the black sooty mould spots (**Figure 5.2b**). Astigmata accounted for 19.3% of the total soil mites. Their densities were higher in the black sooty mould spots than the control and aphid infestation treatments in first year (**Figure 5.2c**). Oribatida was the least abundant group (12.8%) of soil mites. In the second year, significantly higher densities of Oribatida were recorded in the black sooty mould spots than in aphid infestation treatment but not in the control treatment (**Figure 5.2d**). The black sooty mould spots also had higher population densities of the 'other' fauna in the second year (**Figure 5.2e**).

5.4.3 Soil enzymes

In general, honeydew deposition affected the soil enzyme activities, which tended to be higher in black sooty mould spots than in aphid-infested and control treatments (**Figure 5.3**). The dehydrogenase and β-amylase had significantly higher activities in the black sooty mould spots in both years; in the second year the activity of these enzymes was also higher in the aphid infestation treatment than in the control (**Figure 5.3a,d**). The soil urease activity was consistent across the two years and showed a significant response to honeydew deposition in the order: black sooty mould spots > aphid-infested > control

(**Figure 5.3b**). Both the amidase and invertase had significantly higher activities in the black sooty mould spots in the first year; in the second year the trend remained, but the differences were not significant (**Figure 5.3c,e**). The activity of soil α-glucosidase was significantly higher in black sooty mould spots than in the control treatment in both years, but there was no difference between the control and aphid-infested treatments (**Figure 5.3f**). Similar to the specific enzyme activities, the Gmea for the six enzymes was significantly influenced by the honeydew deposition; the Gmea in the first and second year was in the order: black sooty mould spots > aphid-infested > control (**Figure 5.4**).

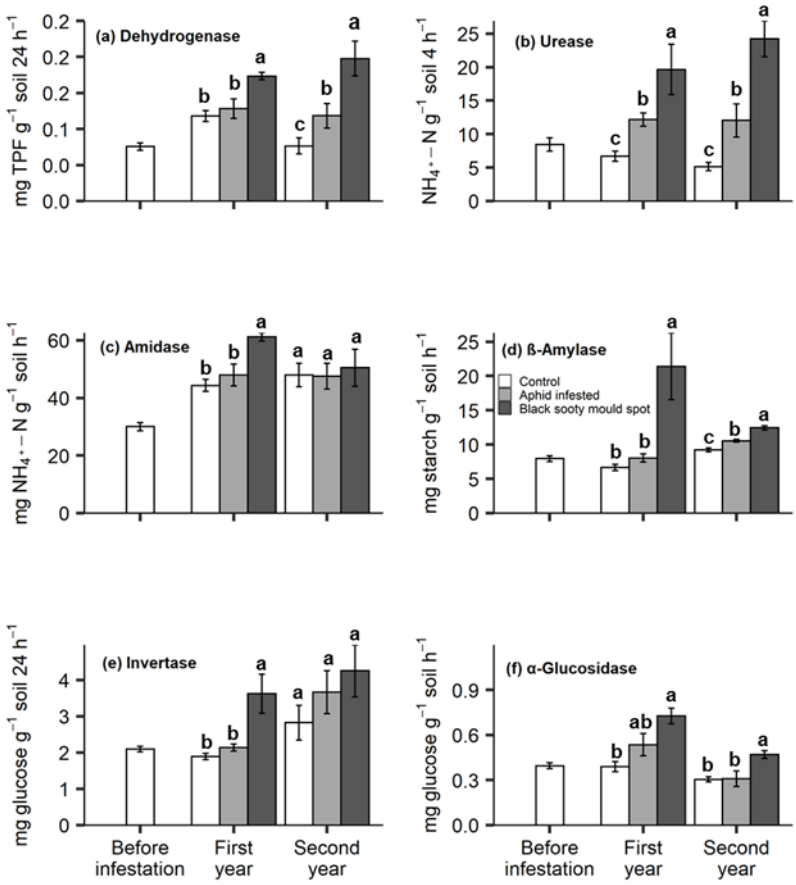

Figure 5.3. The activity of the soil enzymes: dehydrogenase (**a**), urease (**b**), amidase (**c**), β-amylase (**d**), invertase (**e**) and α-glucosidase (**f**) under the canopies of willow plants, prior to aphid inoculation (before infestation), and after aphid inoculation (control, aphid-infested, and black sooty mould spots) in the first and second year. The values are the

means ± SE. Different letters indicate significant differences between the treatments at each sampling time (Tukey's HSD test, α = 0.05).

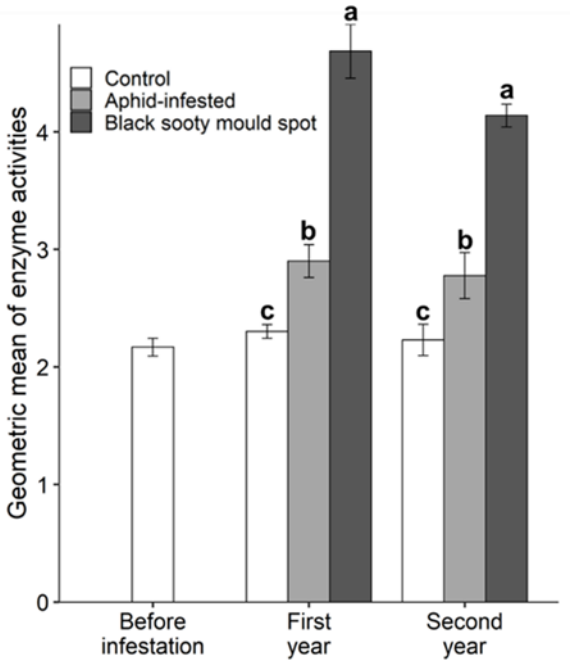

Figure 5.4. The effect of *T. salignus* honeydew deposition on the geometric mean of soil enzyme activities, prior to aphid inoculation (before infestation), and after aphid inoculation (control, aphid-infested, and black sooty mould spots) in the first and second year. The values are the mean ± SE. Different letters represent statistically significant differences between the treatments at each sampling time (Tukey's HSD test, α =0.05).

5.4.4 Soil microbial properties and yeast CFU

In both years, soil Cmic (**Figure 5.5a**), basal respiration (**Figure 5.5b**) and yeast CFU (**Figure 5.6**) increased in the order: control > aphid-infested > sooty mould spots, with all differences being significant. The microbial quotient (qCO_2) was significantly higher in the black sooty mould spots than in the aphid-infested and control treatments only in the second year (**Figure 5.5c**).

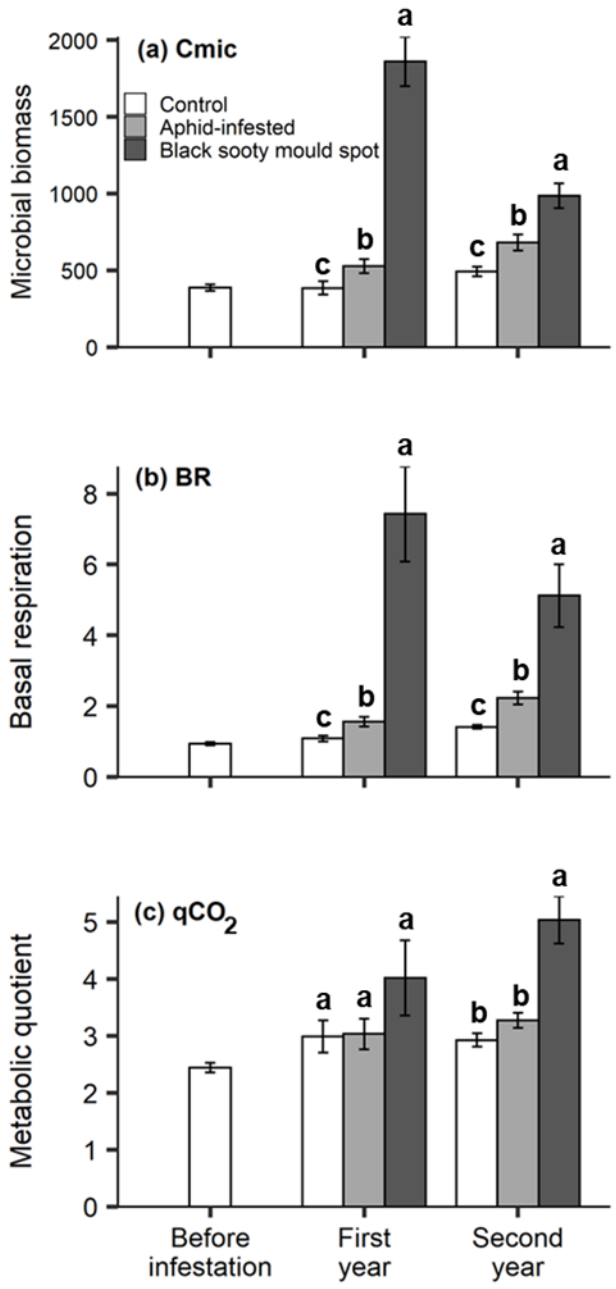

Figure 5.5. The effect of *T. salignus* honeydew deposition on (a) soil microbial biomass (µg C g^{-1} soil), (b) basal respiration (µg CO_2–C g^{-1} soil h^{-1}), and (c) metabolic quotient over time (µg CO_2–C $µg^{-1}$ Cmic h^{-1}), prior to aphid inoculation (before infestation), and after aphid inoculation (control, aphid-infested, and black sooty mould spots) in the first and second year. The values represent means ± SE. Different letters indicate statistically significant differences at each sampling time (Tukey HSD test,α=0.05).

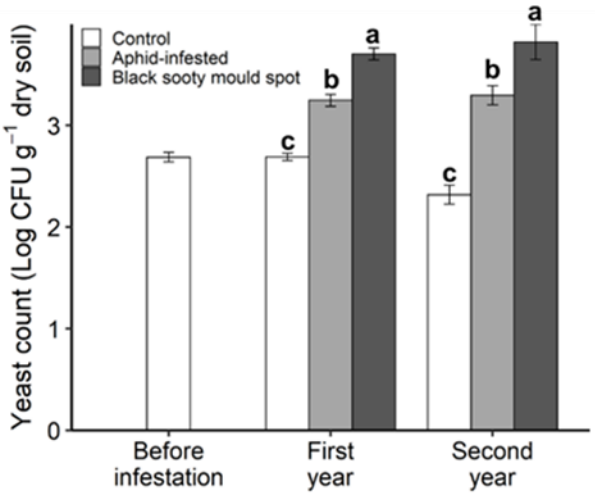

Figure 5.6. The yeast colony forming units (CFU) per gram of dry soil, collected under the canopies of willow plants prior to aphid inoculation (before infestation), and after aphid inoculation (control, aphid-infested, and black sooty mould spots) in the first and second year. The values are the means ± SE. Different letters indicate statistically significant differences at each sampling time (Tukey HSD test,α=0.05).

5.4.5 Principal Component Analysis-Linear Discriminant Analysis (PCA-LDA)

The PCA-LDA showed the localized effect of the honeydew deposition in the black sooty mould spots, and the change after aphid infestation (**Figure 5.7**).

The first PCA-LDA clearly separated the black sooty mould spots from the other two treatments, but there was some overlap between the control and the aphid-infested treatment (**Figure 5.7a**). For the three treatments, the first (LD1) and second (LD2) discriminant functions explained 95.8 and 4.2% of the total variability, respectively. The GMea, BR, Cmic, C:N ratio, urease, β-amylase, qCO_2, and yeast CFU were the variables that contributed the most to the separations along the PC1 (**Figure S5.2a**), which had the highest discriminant coefficient in the first linear discriminant (LD1).

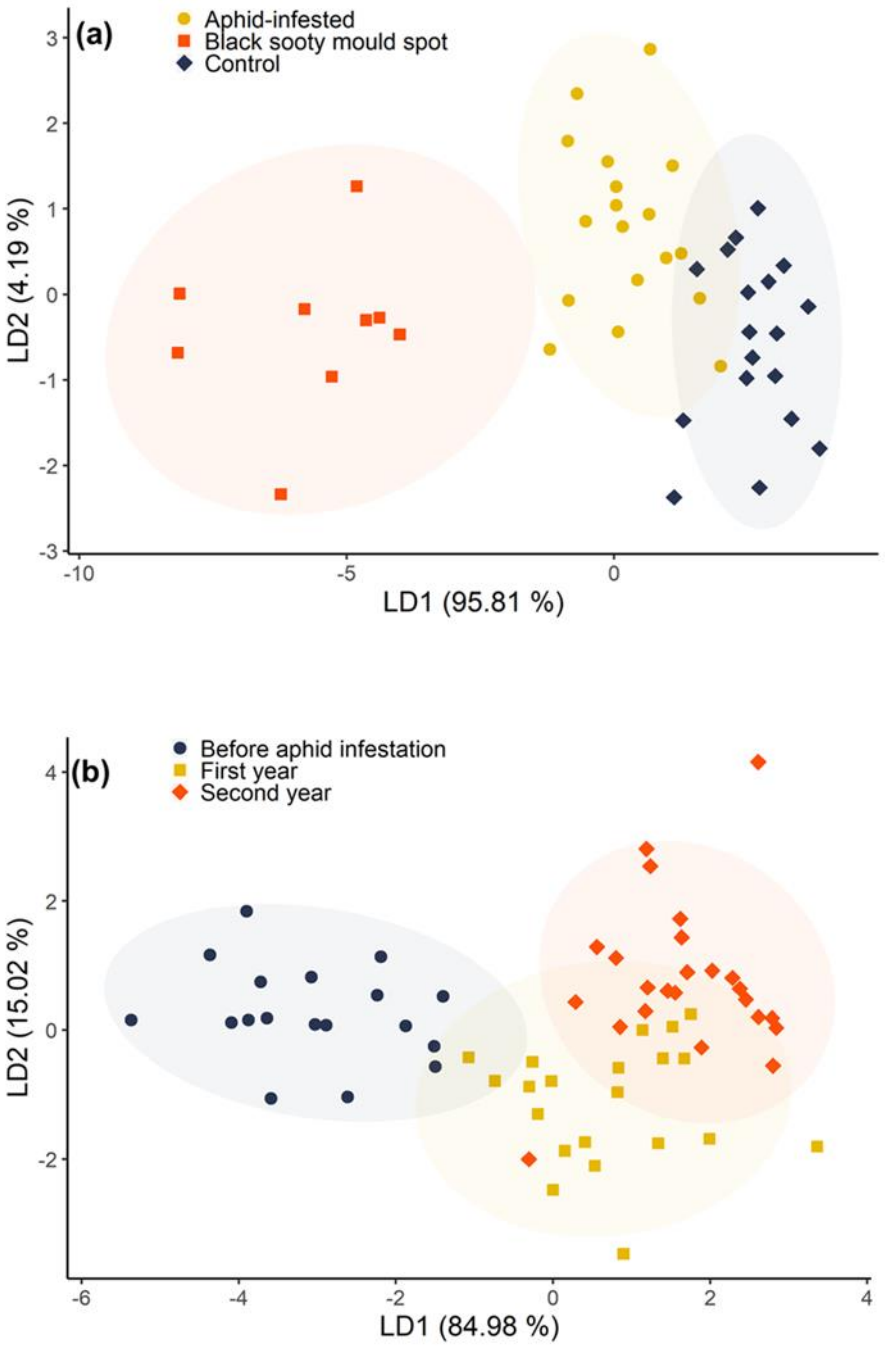

Figure 5.7. PCA-LDA bi-plots of the soil biochemical variables, classified (a) by treatment, and (b) by sampling time. The PCA was run to reduce the dimensions, followed by LDA to separate the treatments and sampling times. The variables were scaled and centred prior to the analysis. The shaded ellipses represent the 95% confidence areas.

The second PCA-LDA clearly separated the sampling times prior to aphid inoculation (before aphid infestation), and after aphid inoculation in the first and second years, but there was considerable overlap between the first and second year post-infestation (**Figure 5.7b**). The GMea, BR, Cmic, C:N ratio, urease, β-amylase, qCO_2, and yeast CFU were also the variables that contributed the most to the separation along PC1 (**Figure S5.2b**) which had highest weight in separating sampling times in LD1 (84.9% of the total variability) (**Figure 5.7b**).

5.4.6 Structural Equation Modelling (SEM)

The SEM for the soil enzymatic response revealed a fairly good fit to the data (χ^2 = 125.7, $df_{(66–28)}$ = 38, $P < 0.001$, RMSEA = 0.00, CFI = 1, AIC = 181.7). The honeydew deposition had a positive direct effect on the total soil C and microbial biomass C (Cmic), but not on the total N (**Figure 5.8a**). Total soil C also increased the Cmic, while the total N had significant negative effect on Cmic. The Cmic increased the activities of all six assayed enzymes (**Figure 5.8a**), but the degree of influence was largest for β-amylase, urease, and dehydrogenase. The increased activities of urease, α-glucosidase and invertase contributed most to the geometric mean of the enzyme activities (GMea).

The SEM for the soil meso-fauna abundance had a better fit to the data (χ^2 = 87.3, $df_{(36–19)}$ = 17, $P < 0.001$, RMSEA = 0.00, CFI = 1, AIC = 72.0). The honeydew deposition increased the Cmic, which increased the abundance of the Collembola and Astigmata, but no significant effect was found for Oribatida mites (**Figure 5.8b**). The abundance of Gamasida was positively correlated with the abundance of their prey-Collembola, Astigmata and Oribatida.

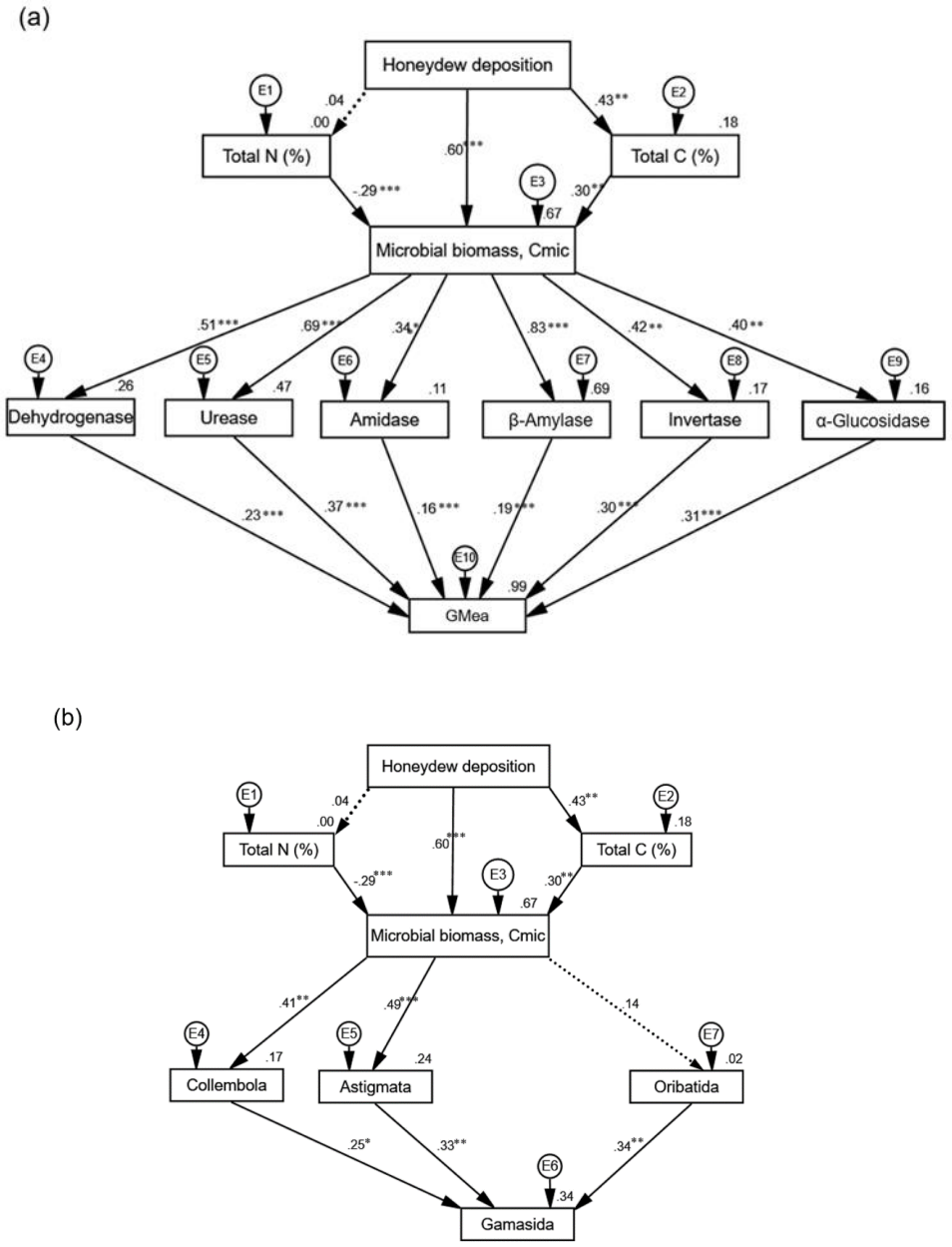

Figure 5.8. Paths diagrams for the effects of *T. salignus* honeydew deposition on (a) soil biochemical properties, and (b) meso-fauna communities. The circles above the rectangles indicate the error terms. The solid and dotted arrows represent significant and non-significant associations, respectively. The path coefficients are the standard regression weights, with asterisks showing different levels of significance (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The squared multiple correlations (r^2) are expressed above the top right corner of each rectangle.

5.5 Discussion

In the willow field trial, the development of black sooty mould spots on the soil surface (**Figure 5.1**) is an indication of a high population density of *T. salignus*. As expected, we found that the copious deposition of honeydew on the soil surface affected the soil biological and biochemical properties, especially in the spots marked by the black sooty mould. Overall, the soil biological indicators (microbial properties, enzyme activities and meso-fauna abundance) were found to be more sensitive to aphid honeydew deposition than the soil chemical properties.

In our study, *T. salignus* honeydew deposition increased the soil total C content but did not change soil total N content. Stadler *et al.* (2006) found that honeydew input increased the dissolved organic C in litter, but reduced inorganic N content, and suggested that net N immobilization had occurred. Aphid honeydew is a C-rich but N-poor resource [5], inducing the population of soil microorganisms to increase and then compete for the limited N, which can increase the N immobilization rate and result in the depletion of inorganic N (Milcu *et al.*, 2011; Stadler *et al.*, 2006). Thus, the honeydew deposition could indirectly decrease the soil N content through enhanced microbial activity (Domisch *et al.*, 2009; Stadler *et al.*, 2008), where microorganisms could emerge as potential competitors of willow plants for nitrogen resources (Kaye & Hart, 1997). However, there is some evidence that N limitations can be compensated through increased non-symbiotic N_2-fixation by soil microorganisms (Petelle, 1984).

The results of our study showed that sugar supplementation in the honeydew increased the yeast CFU count, microbial biomass C and microbial respiration. Soil microorganisms are mostly energy limited and remain dormant in the absence of a suitable substrate (Blagodatskaya & Kuzyakov, 2013), so honeydew addition could increase their population numbers and respiration rate by promoting favourable growth

conditions (Joergensen & Stefan, 1999). Soil yeasts prefer nutrient-rich environments and are known to utilize low molecular weight sugars (Mašínová *et al.*, 2018) that are the major components of aphid honeydew. The increase in microbial biomass C (Cmic) and basal respiration are in accordance with the study of Milcu *et al.* (2015), who found that Cmic and basal respiration increased by 330% and 58.4%, respectively, in honeydew treatments. However, care should be taken in interpreting the microbial response to honeydew addition, as the sugar component of honeydew can shut down the metabolism of some microbes (Islam & Wright, 2004). Further studies using molecular techniques are advised to determine the changes in the soil microbial community structure following honeydew deposition.

The activity of soil enzymes is regarded as a direct measurement of the metabolic response of the soil microbial communities to nutrient availability (García-Ruiz *et al.*, 2008). Our results show a significant effect of nutrient supplementation from aphid honeydew on the soil enzyme activities. Although honeydew contains an unbalanced ratio of C to N, we found that both C-hydrolysing (β-amylase, invertase, α-glucosidase), and N-hydrolysing (urease and amidase) enzymes positively responded to the honeydew deposition. Dehydrogenase was also found to be a sensitive indicator of increased microbial activity (García-Ruiz *et al.*, 2012; Ruzhen *et al.*, 2014) as a result of the supplementary C input from honeydew. The interpretation of enzyme activity results should be treated cautiously as enzyme assays generate the highest potential estimates, under optimum substrate, pH and temperature conditions, rather than the actual values (Alef & Nannipieri, 1995). However, both the specific enzyme activities and the GMea were suitable indicators discriminating the black sooty mould spots from the control, with GMea being the best predictor.

Soil meso-fauna (Collembola and Acari) normally live in the topsoil layers, and play different functional roles in the soil processes and nutrient cycling (Coleman *et al.*, 2017). Collembola counts in the honeydew-affected soil were higher than in control. Sinka *et al.* (2009) found no significant influence of honeydew deposition on Collembola abundance, while Milcu *et al.* (2015) reported the decline in Collembola and mite numbers in soil treated with synthetic honeydew. Seeger and Filser (2008) noted that the effect varied for different collembolan taxa. The mite groups Astigmata and Oribatida are fungivores and saprophages (Behan-Pelletier, 1999), while the Gamasida are predators of other soil mites and Collembola (Jung *et al.*, 2010). In our study, the abundance of Oribatida and Gamasida varied over time, while that of Astigmata was fairly consistent. All three mite groups are known to respond to external resources (Bedano *et al.*, 2006; Cao *et al.*, 2011), with the degrees of response to the aphid honeydew reflecting their different life histories. The Astigmata are known for their rapid response to the changing environment, due to their faster metabolism, shorter generation time, and higher fecundity than the Oribatida (Behan-Pelletier, 1999). On the other hand, the higher population density of Gamasida reflects the presence of the prey on which they feed (Walter *et al.*, 2013).

The SEMs were a useful tool to assess the multiple linkages between the honeydew and the soil biological and biochemical indicators, linking the aboveground herbivory to the below-ground soil processes. The path diagrams show that honeydew deposition by *T. salignus* has a multitrophic cascading effect on the soil biota, similarly as in Michalzik *et al.* (1999), Milcu *et al.* (2015) and Stadler *et al.* (1998). In the current study, the Cmic was positively correlated with the soil total C content, but not with the total N. Our results are in line with those of Cheng *et al.* (2013) and Johnson *et al.* (2005),

who suggested that the Cmic was mainly dependent on the soil C source, and additional N input could decrease the Cmic.

5.6 Conclusions

The deposition of *T. salignus* honeydew affects the various soil biotic and abiotic properties through a multitrophic cascade. The aphid honeydew provides an energy-rich source for the soil microbes, causing an increase in the Cmic, that leads to increased soil enzyme activities. These processes affect the abundance of soil meso-fauna microbivores and their predators. This example illustrates the importance of multitrophic interactions, and the cascading effects of an aphid herbivore on soil chemical properties and soil biological communities.

5.7 Appendix

Table S5.1. Summary of treatment effects of *T. salignus* honeydew deposition on soil biochemical properties, soil enzymes, and soil meso-fauna abundance in first and second year of the experiment (Generalized Linear Models, α=0.05).

Parameter	Unit	First year			Second year		
		df1, df2	F	*P*	df1, df2	F	*P*
pH	-	2,20	0.75	0.485	2,23	0.03	0.973
Total C	%	2,20	3.55	**0.043**	2,23	3.62	**0.044**
Total N	%	2,20	3.26	0.061	2,23	0.1	0.904
C : N	-	2,20	18.1	**0.001**	2,23	7.12	**0.004**
Collembola	ind. m^{-2}	2,20	3.27	**0.032**	2,23	3.79	**0.039**
Gamasida	ind. m^{-2}	2,20	11.45	**0.001**	2,23	0.15	0.858
Astigmata	ind. m^{-2}	2,20	13.49	**0.001**	2,23	4.14	**0.03**
Oribatida	ind. m^{-2}	2,20	0.82	0.311	2,23	3.97	**0.043**
Other mesofauna	ind. m^{-2}	2,20	3.01	**0.045**	2,23	5.97	**0.011**
Dehydrogenase	mg TPF g^{-1} soil 24 h^{-1}	2,20	3.64	**0.042**	2,23	10.25	**0.001**
Urease	NH_4^+-N g^{-1} soil 4 h^{-1}	2,20	17.29	**0.001**	2,23	20.7	**0.001**
Amidase	mg NH_4^+-N g^{-1} soil h^{-1}	2,20	3.62	**0.047**	2,23	0.09	0.915
β-amylase	mg starch g^{-1} soil 24 h^{-1}	2,20	34.14	**0.001**	2,23	27.03	**0.001**
Invertase	mg glucose g^{-1} soil 24 h^{-1}	2,20	21.86	**0.001**	2,23	0.26	0.821
α-glucosidase	mg glucose g^{-1} soil h^{-1}	2,20	4.87	**0.02**	2,23	3.9	**0.036**
Gmea	-	2,20	50.37	**0.001**	2,23	23.99	**0.001**
Cmic	µg C g^{-1} soil	2,20	44.14	**0.001**	2,23	19.9	**0.001**
BR	µg CO_2–C g^{-1} soil h^{-1}	2,20	89.54	**0.001**	2,23	45.53	**0.001**
qCO_2	µg CO_2–C $µg^{-1}$ Cmic h^{-1}	2,20	1.51	0.247	2,23	28.34	**0.001**
Yeast CFU	log CFU g^{-1} soil	2,62	70.99	**0.001**	2,71	44.952	**0.001**

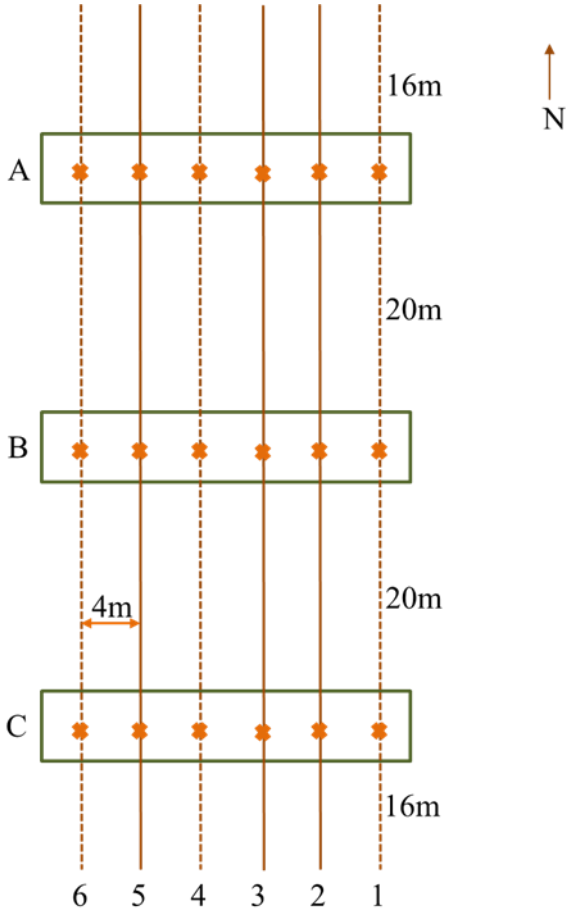

Figure S5.1. Sampling layout for the soil samples. The numbers (1-6) represent the rows of willow plants, and the letters (A-C) indicate the sampling points along the rows. The willow plants in rows 1, 4 and 6 were kept free of aphids, by weekly manual removal or insecticide spraying, while plants in rows 2, 3 and 5 were inoculated with aphids.

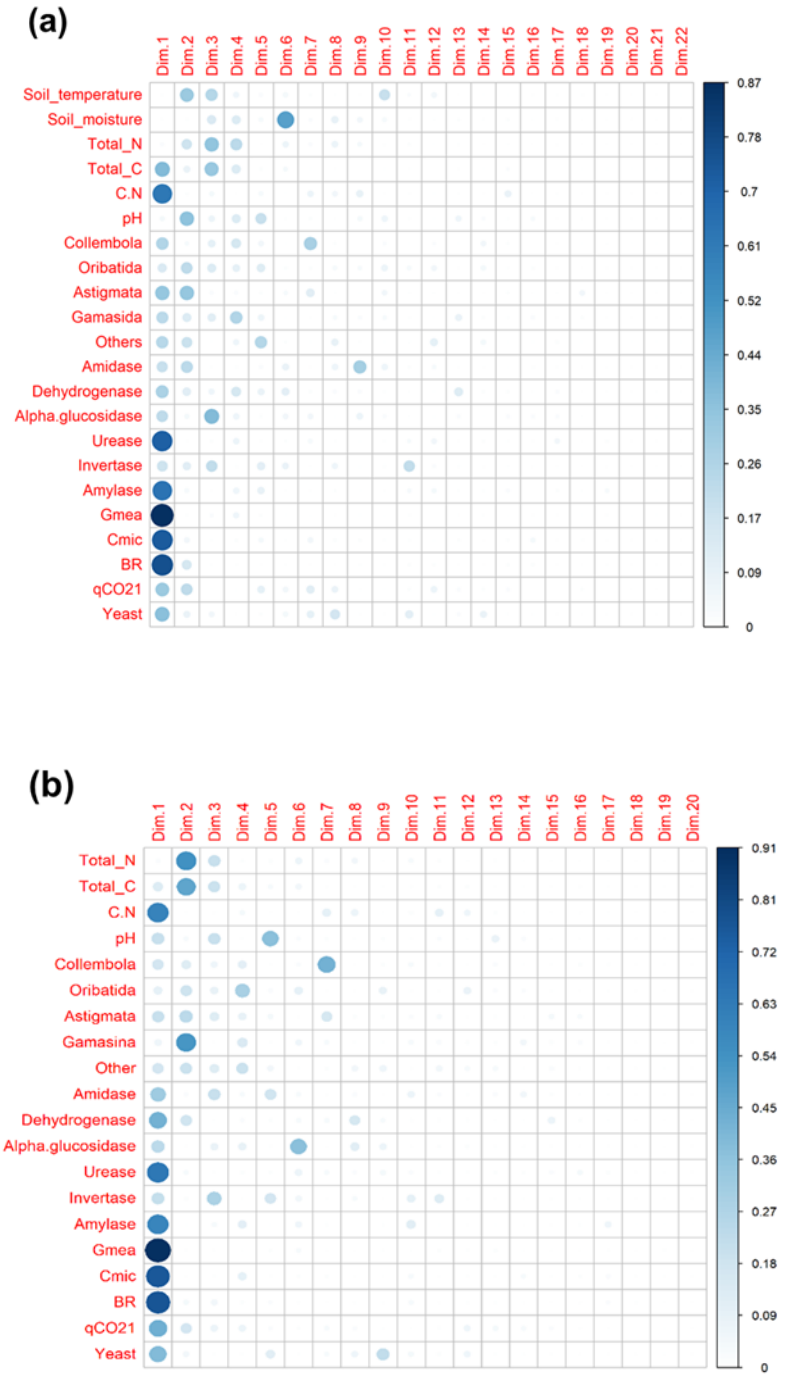

Figure S5.2. Correlation plots for contributing variables to each dimension in the PCA for (a) treatments, and (b) sampling times. The variables that contributed the most to the first few dimensions were selected for SEM.

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Name of candidate:	Kyaw Min Tun
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In which chapter is the manuscript /published work:	Chapter 5

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Chapter 6

The potential of harlequin ladybird beetle *Harmonia axyridis* as a predator of the giant willow aphid *Tuberolachnus salignus*: voracity, life history and prey preference

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6.1 Abstract

The giant willow aphid, *Tuberolachnus salignus* is an invasive insect in New Zealand for which control measures are being sought due to its detrimental effects on willow cultivation and apiculture. We evaluated the biocontrol potential of the harlequin ladybird beetle, *Harmonia axyridis* by measuring voracity and feeding preference of larvae and adults in laboratory feeding trials. Results show that *H. axyridis* consumes *T. salignus*, with females being more voracious than males and larvae. However, *H. axyridis* fed *T. salignus* took longer to develop, gained less weight and had lower survival comparing with those fed eggs of the Mediterranean flour moth, *Ephestia kuehniella*. In a choice test, larval and adult *H. axyridis* preferred the green peach aphid, *Myzus persicae* as the prey item, rejecting *T. salignus*. We suggest that *H. axyridis* is likely to use *T. salignus* only as a facultative prey, and so cannot be prioritised as a potential biocontrol agent.

6.2 Introduction

The giant willow aphid, *Tuberolachnus salignus* Gmelin (Hemiptera: Aphididae), is an invasive willow sap feeder that establishes in dense colonies covering a large portion of stem surfaces of 1-3-year-old willow saplings (Collins, 2001). The infestation can negatively affect above- and below-ground biomass and reduce the survival of young trees; therefore this aphid has been identified as an important pest of willows (Collins *et al.*, 2001a). In addition, *T. salignus* excretes copious amounts of honeydew containing the aphid-synthesized sugar melezitose, which is foraged by bees and creates a unique problem for the bee-keeping industry in New Zealand (Sopow *et al.*, 2017). Although most of the honeydew components are beneficial to the bees, indigestible sugars (such as melezitose) can cause dysentery (Huang, 2012). Melezitose crystallizes easily, clogging the filters during honey extraction, causing up to 31% loss in honey yields (Sopow *et al.*, 2017). Moreover, *T. salignus* honeydew attracts common wasps, *Vespula vulgaris* Linnaeus (Hymenoptera: Vespidae), one of the most destructive pests of the apiculture industry (Gunawardana *et al.*, 2014). Therefore, considerable attention has been given to controlling this recently arrived aphid species in New Zealand.

Tuberolachnus salignus is thought to originate from Asia but is now found in most parts of the world where willows are grown (Blackman & Spence, 1996). The aphid was first reported in New Zealand in 2013 (Gunawardana *et al.*, 2014) and in Australia in 2014 (State Government Victoria Department of Environment and Primary Industries, 2015). In New Zealand, a sustainable management strategy is being developed to lessen the impact of *T. salignus* on multiple industries where willows are used (Sopow, 2016), with biological control regarded as one of the options for long-term sustainable management (Sopow, 2016). Sopow *et al.* (2017) listed the potential natural enemies of *T. salignus* worldwide, including two parasitoids, four insect predators and an

entomopathogenic fugus, and then ranked the harlequin ladybird, *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) and the braconid wasp, *Pauesia salignae* Watanabe (Hymenoptera: Braconidae) as the most promising biocontrol agents.

*Harmonia axyridi*s is native to eastern Asia but is now extensively distributed all over the world (Tedders & Schaefer, 1994). It was first detected in Auckland in 2016 and has spread rapidly throughout the North Island of New Zealand (Martin, 2016). No overlapping distribution of *T. salignus* and *H. axyridis* has been reported in their native range. However, *H. axyridis* was found to be associated with *T. salignus* populations in Europe (Dransfield & Brightwell, 2015; Edkins, 2002) and New Zealand (Martin, 2017), where it is one of the four ladybird species closely associated with *T. salignus* populations (Martin, 2016). Although *H. axyridis* could potentially contribute to controlling the population of *T. salignus* (Martin, 2017), no scientific investigation has been yet undertaken to explore their relationship.

An understanding of the basic biological parameters and diet breadth of a predator species is needed to evaluate its potential for reducing pest populations (Furlong & Zalucki, 2010). Similarly, estimating the voracity of a predator is a pre-requisite to establish its biocontrol potential against a specific pest species (Lucas *et al.*, 1997; Meyling *et al.*, 2003). Investigating the relationship of *H. axyridis* and its prospective prey species is also crucial due to the extensive invasion and broadly polyphagous nature of this ladybird (de Castro-Guedes *et al.*, 2016), and the negative impacts it has on non-target species in its introduced range (Koch, 2003).

The present investigation was designed to explore the voracity, life history and prey preference of the predatory ladybird *H. axyridis* using *T. salignus* and alternative prey species under laboratory conditions. The first objective was to compare the voracities of larval and adult *H. axyridis* on *T. salignus* to estimate the stage-specific prey

suppression potential. Second, prey suitability of *T. salignus* was assessed by comparing growth, development and survival of *H. axyridis* fed on *T. salignus* and on the reference diet (eggs of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae)). Finally, the prey preference of *H. axyridis* was evaluated when given a choice between *T. salignus* and an alternative aphid prey, the green peach aphid (*Myzus persicae* Sulzer (Hemiptera: Aphididae)).

6.3 Materials and Methods

6.3.1 Rearing prey insects

The starter population of *T. salignus* was collected from the willow biomass field trial and the National Willow Collection of the New Zealand Poplar & Willow Research Trust, at the Plant Growth Unit of Massey University (Palmerston North, New Zealand) in summer 2019. The aphid was maintained in the lab on hydroponically grown saplings of *Salix viminalis* in a controlled environment at 25 ± 1°C, 75% relative humidity and a photoperiod of 16L: 8D. Willow cuttings (1 cm diameter, 25 cm height) were grown in plastic containers (8.5 cm diameter, 10 cm height), with screw lid having 1.5 cm hole in the centre to accommodate the cutting. Two-thirds of the container was filled with 25% strength Hoagland's hydroponic solution that was replenished as required and completely changed on a fortnightly basis. Twenty adult *T. salignus* from lab-maintained populations were allowed to reproduce for a day and then removed from each sapling to get age-matched cohorts for all trials.

Laboratory colonies of the green peach aphid, *Myzus persicae*, were obtained from the Entomology and IPM Laboratory at Massey University in May 2019 and maintained on cabbage plants (Ranfurly Mini F.1 Hybrid). The cabbage seeds were sown in pots (10.5 cm diameter, 8.5 cm height) using Shrub and Tub Mix (Oderings Garden

Centres) in a glasshouse at the Seed Technology Laboratory, Massey University. One-month-old plants were brought to the temperature-controlled room and then inoculated with five adult *M. persicae* per plant. The pots were watered every second day. Aphid mummies and whiteflies were regularly removed to maintain a clean *M. persicae* population until the preference testing.

The initial population of the Mediterranean flour moth was obtained from the Entomology and IPM Laboratory at Massey University in March 2019. The moth populations were kept in plastic containers (8.5 cm diameter, 10 cm height) with ventilated lids. The larvae were fed on a standard diet containing 43.5% wholemeal wheat flour, 43.5% maize meal, 3.0% yeast and 10% glycerine (Lima *et al.*, 2001). All emerging adults were reared in cages (19 cm × 12 cm × 20 cm). The eggs were harvested every four days and stored in a -20°C freezer before being used for trials.

6.3.2 Rearing harlequin ladybird beetles

In summer 2019, adults and immature stages of *H. axyridis* were collected from the willow field trial and the National Willow Collection at the Plant Growth Unit of Massey University (Palmerston North, New Zealand). Adults were sexed as described by McCornack *et al.* (2007). Adult males and females were kept separately in plastic containers (8.5 cm diameter, 10 cm height) in the laboratory at 25 ± 1°C, 75% relative humidity and a photoperiod of 16L: 8D. The immature stages were reared to adults as described below. Adult *H. axyridis* were provided an *ad libitum* supply of *T. salignus*, artificial food, honey and water. The artificial food was prepared as described by Majerus *et al.* (1989). Honey and water were provided via a soaked cotton swab.

For *H. axyridis* breeding, pairs (one male and one female) were allowed to mate in plastic Petri dishes (5.5 cm diameter, 1.5 cm height) for 24 hrs. After removing the males, females were kept individually and checked daily for oviposition. The eggs were

transferred to Petri dishes (9 cm diameter, 1.5 cm height) lined with filter paper and supplied with a cotton swab soaked with 30% honey solution (raw Mānuka honey, DownUnder Honey, Ltd). Upon hatching, the larvae were individually confined into small Petri dishes to prevent sibling cannibalism (Agarwala & Dixon, 1992) and reared to the desired stages by offering the diet described above. For prey choice experiments, the newly emerged larvae of *H. axyridis* were reared on the Mediterranean flour moth's eggs, artificial food, honey and water to obtain predators with no previous experience of feeding on aphids.

6.3.3 Voracity of different stages of *H. axyridis*

The voracity of larval and adult *H. axyridis* on juvenile *T. salignus* was measured in no-choice tests. The experimental arena consisted of a hydroponic willow sapling, grown in a plastic container (8.5 cm diameter, 10 cm height) inside a 30 cm × 30 cm × 30 cm cage, as described in the rearing of *T. salignus*. Each experimental run (24 hrs period) consisted of four treatments of *H. axyridis*: (1) one third instar with 60 aphids, (2) one adult male with 60 aphids, (3) one adult female with 60 aphids, and (4) a control (60 aphids with no predator). This number of aphids was selected based on prior observations to exceed the maximum number of aphids consumed by *H. axyridis* in 24 hrs so that the predators were not prey-limited.

The adult ladybird beetles used in the trial were 7-9-day-old. All aphids offered to *H. axyridis* were 1-day-old (first instar). The predators were starved for 24 hrs before placing them into the experimental arenas. The control treatment with no predators was used to estimate the natural death rate of *T. salignus* during the experimental period (24 hrs). Each treatment was replicated 16 times. The number of *T. salignus* consumed by the different stages and sexes of *H. axyridis* was compared, and the voracity (V_0) was calculated based on the formula in Soares *et al.* (2003),

$$V_0 = (A\text{-}a_{24})\ ra_{24}$$

where A= number of *T. salignus* available, a_{24}= number of *T. salignus* alive after 24 hrs in the predator treatment, and ra_{24}= proportion of *T. salignus* found alive after 24 hrs in the control treatment.

6.3.4 Growth, development and larval survival of *H. axyridis* on different diets

The neonates of *H. axyridis* were divided into three groups, and individually housed in small Petri dishes (9 cm diameter, 1.5 cm height). They were provided with three diet regimes: (1) frozen *E. kuehniella* eggs (<1 mm) (2) first instars (1-2-day-old) of *T. salignus* (2.1 ± 0.1 mm) (3) starvation treatment (no food or water). The food was supplied *ad libitum*, and exuviae and leftovers of prey were removed daily. Petri dishes were changed weekly to prevent contamination. Immature survival was monitored twice a day until adult eclosion. Weight of first instars was measured soon after hatching from eggs. Weight and duration of life stages were recorded after the respective moults. Duration of prepupa was recorded from when the abdominal tip of fourth instar *H. axyridis* became attached to the surface of Petri dish. Weights of prepupae and pupae were measured with the Petri dish; the Petri dish was weighed after adult emergence and its weight subtracted. Each diet treatment consisted of 20 replicates.

6.3.5 Prey preference of *H. axyridis*

Preliminary studies were conducted to estimate the size and weight of the prey species, number of prey the third instar and adults of *H. axyridis* can consume during 2 hrs (experimental period), and to test whether different prey ratios affect predator preference. First instar *T. salignus* (body length 2.1 ± 0.1 mm) and newly emerged adult *M. persicae* (body length 1.9 ± 0.1 mm) were used to standardize prey size in prey selection behaviour. Petri dishes (9 cm diameter) containing 15 *T. salignus* and 15 *M.*

persicae were used as experimental arenas. An equal number of the two prey species was used to test predator preference, as our preliminary studies revealed that *H. axyridis* exhibited a constant strong preference towards certain prey, and consumed the same proportions of each prey even when provided with different ratios of *T. salignus* and *M. persicae*. Third instars and 1-week-old male and female adults of naïve *H. axyridis* were starved for 24 hrs prior to the dual-choice preference test. A single predator was introduced into each experimental arena and the identity of the first attacked prey species was recorded, usually a few minutes after releasing the predator. After 2 hrs, the number of prey species eaten was recorded. Partly consumed aphids were counted as 0.25 aphid (Finlayson *et al.*, 2010). These tests were replicated 15 times both for third instar and adult predators. A control treatment was set up with the same number of aphids to check whether there was natural death or escape of prey species during the trial period, but without releasing predators into the arenas.

Prey preference of ladybird beetles for *T. salignus* (α_1) was quantified using the equation from Chesson (1978) as follows:

$$\alpha_1=(\frac{r_1}{n_1}) / [\left(\frac{r_1}{n_1}\right)+\left(\frac{r_2}{n_2}\right)]$$

where r_1 is the number of attacked *T. salignus*, n_1 is the number of total *T. salignus*, r_2 is the number of attacked *M. persicae*, n_2 is the number of total *M. persicae*. Preference for *M. persicae*, (α_2) could then be calculated by subtracting the *T. salignus* preference from 1.

6.3.6 Statistical analysis

A generalized linear model (GLM, proc *glimmix* in SAS 9.4, SAS Institute) with the gamma distribution and log link function was used to compare the consumption of *T. salignus* by third instars and adults (females and males) of *H. axyridis*. Two larvae (out

of 16 tested) that moulted to the subsequent instar during the 24 hrs voracity test were excluded from this analysis. Development and weight data were analyzed by GLM with inverse-Gaussian de Castro-Guedes *et al.* (2016) or gamma distribution and a log link function using diet treatment as a predictor variable. The insects that did not complete development were excluded from the development and weight analysis. Least square means with Tukey-Kramer adjustment were used for multiple means comparisons. The Kaplan-Meier estimator for survival function was generated to evaluate the effect of diets (starvation, *T. salignus* and *E. kuehniella* egg) on the immature survival of *H. axyridis*, and then differences between diet treatments were determined using the log-rank test in *survival* (Therneau, 2015; Therneau & Grambsch, 2000) and *survminer* packages (Kassambara & Kosinski, 2019) in R version 3.6.0 (R Development Core Team, 2019). A logistic regression with a logit link function (proc *logistic*, SAS 9.4) was used to analyse the predators' choice data, to compare whether different stages and sexes of *H. axyridis* select *T. salignus* or *M. persicae* first. The preference index for two prey species was compared using a GLM with gamma distribution and a log link function. Life stages of predators and prey species were used as predictor variables.

6.4 Results

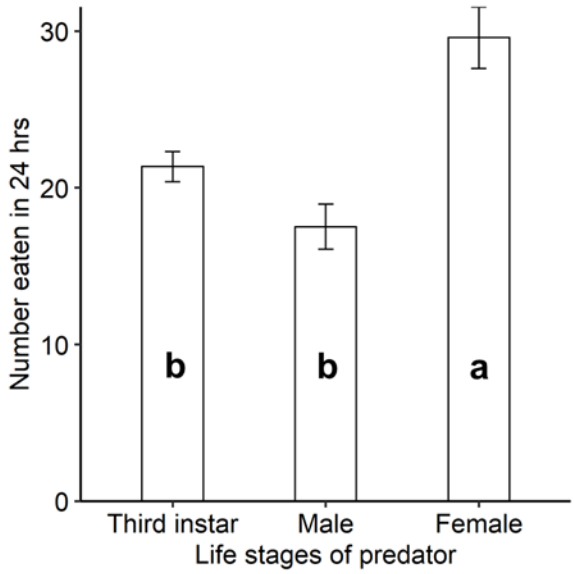

Figure 6.1. Voracity of third instar and adult males and females of *H. axyridis* fed first instar *T. salignus* in laboratory experiment (n=14-16). The values represent means ± SE; different letters indicate statistically significant differences between means (LS means with Tukey-Kramer adjustment, α=0.05).

6.4.1 Voracity

Voracity of third instar and adult (male and female) predators differed significantly ($F_{2,43}$=17.41, P<0.001). Third instar *H. axyridis* consumed slightly more prey than adult males, but significantly less than adult females (**Figure 6.1**). Adult females were the most voracious consuming on average, in 24h, 29.58 first instar *T. salignus,* while males and third instars consumed 17.52 and 21.35 aphid nymphs, respectively.

6.4.2 Development, growth and survival of immature *H. axyridis* on different diets

First instar *H. axyridis* fed on the *T. salignus* diet developed slower than those fed with eggs of *E. kuehniella* ($F_{1,30}$=20.01, P<0.001). The total developmental time was also

significantly longer for larvae receiving the *T. salignus* diet (16 days) vs. those fed with frozen eggs of *E. kuehniella* (15.11 days) ($F_{1,30}$= 17.36, P<0.001; **Table 6.1**).

Weights attained by first, second, third and fourth instars of *H. axyridis* did not differ significantly between the two diet treatments, although larvae fed on *T. salignus* tended to be lighter (**Table 6.1**). However, we observed significant differences in weight gain for prepupae ($F_{1,30}$= 20.36, P<0.001), pupae ($F_{1,30}$= 62.05, P<0.001) and newly eclosed adults ($F_{1,30}$= 30.85, P<0.001). Prepupae and pupae reared on *T. salignus* diet were 10.0 mg and 8.47 mg lighter respectively, than those reared *E. kuehniella* eggs (**Table 6.1**). The mean weight of newly emerged adults was also significantly lower for *H. axyridis* reared on *T. salignus* (**Table 6.1**). Because 7 out of 20 *H. axyridis* on the *T. salignus* diet died without completing development, we did not have enough replicates to consider development and growth of male and female *H. axyridis* separately.

Survival of *H. axyridis* was quantified as the number of days to death, from neonates to newly emerged adults. All individuals from the starvation treatment did not moult and died within the first three days of the trial. Diet treatment significantly affected the survival of immature *H. axyridis*, with greater survivorship detected in immature *H. axyridis* fed with *E. kuehniella* eggs than in those fed *T. salignus* nymphs (Log-rank test, χ_2^2=42.8, P<0.001). As shown in **Figure 6.2**, survival of *H. axyridis* was initially similar between the starvation and *T. salignus* diets because of difficulties of first instar *H. axyridis*, to handle similar-sized prey, but diverged over time, showing lower survival on aphid diet. Overall, *H. axyridis* consuming moth's eggs had longer survival (14.85 days) and reached adulthood in greater numbers (95%) than *H. axyridis* reared on *T. salignus* nymphs (11.8 days and 65% survival).

Table 6.1. Developmental time and weight at moulting (mean ± SE) for the different life stages of *H. axyridis*, fed with either first instar *T. salignus*, or frozen *E. kuehniella* eggs.

	Development period (days)						
Prey species	First instar	Second instar	Third instar	Fourth instar	Prepupa	Pupa	Total
T. salignus (n=13)	2.61±0.24	1.84±0.09	1.92±0.14	3.69±0.13	1.23±0.12	4.69±0.13	16.00±0.20
E. kuehniella (n=19)	2.00±0.00	1.84±0.10	1.74±0.10	3.74±0.10	1.10±0.07	4.68±0.11	15.11±0.13
$F_{1,30}$ value	20.01	0.00	0.88	0.07	1.43	0.05	17.36
P value	<0.01	0.98	0.36	0.80	0.24	0.96	<0.01
	Weight at moulting (mg)						
	First instar	Second instar	Third instar	Fourth instar	Prepupa	Pupa	Adult
T. salignus (n=13)	0.18±0.02	1.66±0.16	4.98±0.35	12.01±0.85	28.99±1.46	28.48±0.64	24.03±0.67
E. kuehniella (n=19)	0.19±0.03	1.71±0.10	5.62±0.19	12.70±0.77	38.65±0.85	36.68±0.82	29.35±0.75
$F_{1,30}$ value	0.17	0.31	2.42	2.00	20.36	62.05	30.85
P value	0.68	0.58	0.13	0.17	<0.01	<0.01	<0.01

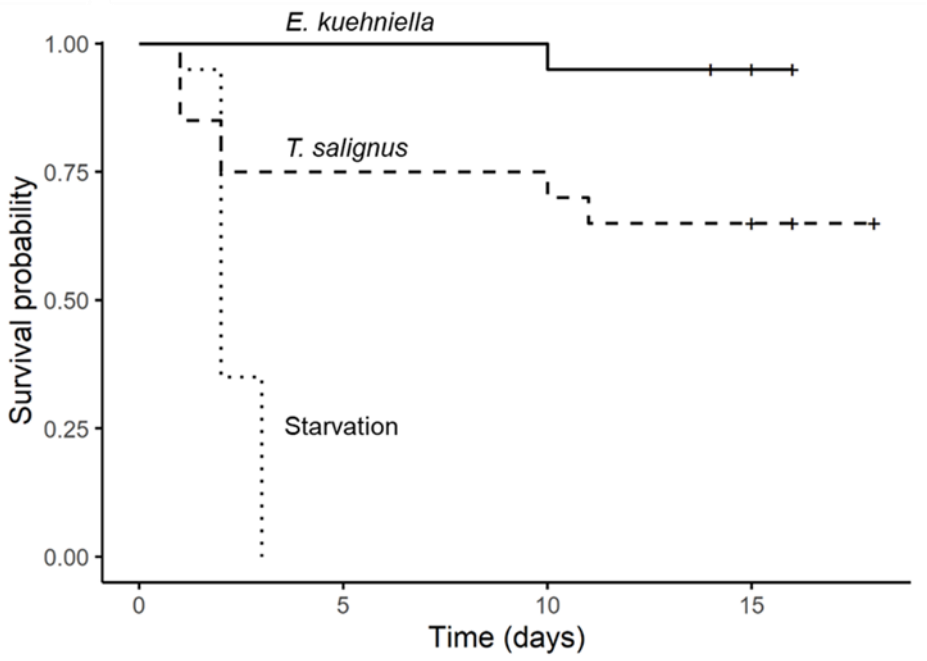

Figure 6.2. Survival density functions of immature *H. axyridis* fed first instar *T. salignus* vs. frozen *E. kuehniella* eggs, with the starvation treatment as control.

6.4.3 Prey preference between *M. persicae* and *T. salignus*

In 87% of cases (39 out of 45) *H. axyridis* attacked *M. persicae* first (χ^2_1= 18.22, P<0.001) (**Figure 6.3**). The choice of *M. persicae* as the first prey did not differ among third instars, males, and female *H. axyridis* (χ^2_2=1.08, P= 0.584). Female *H. axyridis* selected *T. salignus* in 3 out of 15 replicates (20%) while third instar and male predators choose this aphid once (6.67%) and twice (13.33%) only. Once selected as prey, none of the aphids were rejected, but in general *H. axyridis* appeared to avoid encounters with *T. salignus* after being introduced into the experimental arena.

The preference index for *M. persicae* (0.69 ± 0.02) was significantly higher than that for *T. salignus* (0.31 ± 0.02), indicating that *H. axyridis* expressed a clear preference for the former ($F_{1,84}$=52.51, P<0.001). This preference was consistent for all predators (third instars, males, and females) (**Figure 6.4**). No interaction effect was observed between the life stage of the predator and prey preference.

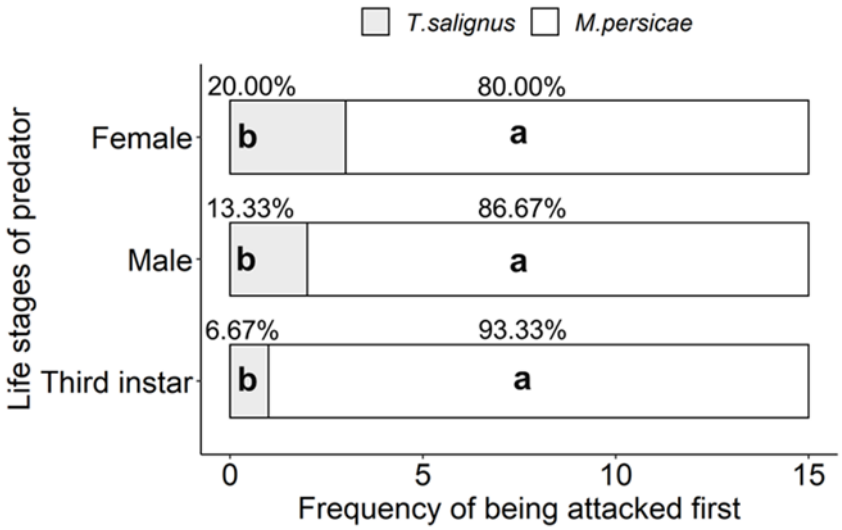

Figure 6.3. Selection of first prey by different stages and sexes of *H. axyridis* when offered a choice between *M. persicae* and *T. salignus* aphids, n = 45. Different letters within each bar indicate significant differences (α=0.05).

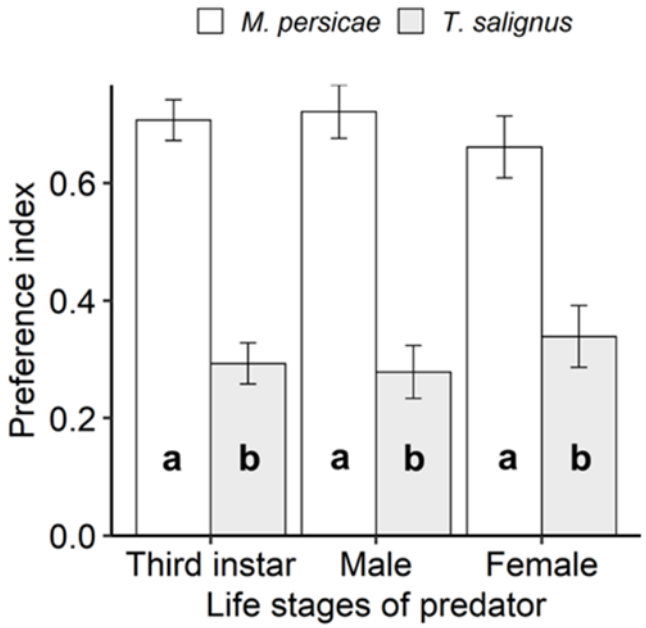

Figure 6.4. Preference index (varies from 0 to 1, with 1 indicating 100% prey preference) for *M. persicae* and *T. salignus* prey in different stages and sexes of *H. axyridis*. The error bars are the SE of mean preference index. Different letters within and across age groups indicate significant differences (LS means with Tukey-Kramer adjustment, α=0.05).

6.5 Discussion

The high voracity rates of third instar and adult *H. axyridis* make it a potential candidate for controlling early instars of *T. salignus*. Third instars had a slightly higher consumption rate than males, but neither was as voracious as the females, disproving the generalization that adult ladybirds consume more prey than larvae. Adult female *H. axyridis* consumed more *T. salignus* nymphs than males, as shown in other ladybird species (Omkar & James, 2004). Increased voracity of adult female ladybirds was explained by their bigger size and higher energy intake for metabolic requirement (Evans & Gunther, 2005; Pervez & Omkar, 2006). However, Cabral *et al.* (2009) reported that adult male *C. undecimpunctata* were as voracious as the females. In our study, even virgin females attacked more *T. salignus* than the males, showing a clear sex-bias in voracity.

The results also show that *H. axyridis* can reach adulthood feeding on first instar *T. salignus* only, suggesting nutritional suitability of young *T. salignus* compared to reference diet (*E. kuehniella*). However, this study also evidences that feeding on *T. salignus* alone will result in longer development times, reduced weight and higher pre-adult mortality. The longer development period of first instar *H. axyridis* fed on *T. salignus* nymphs can be explained by the small predator-prey size ratio (Reavey, 1993), making foraging on a large prey more strenuous (de Castro-Guedes *et al.*, 2016; Jalali *et al.*, 2009). The data suggests that neonate *H. axyridis* can survive and develop in the willow ecosystem, where *T. salignus* may be the only available prey, especially during New Zealand summer. Sibling cannibalism (Agarwala & Dixon, 1992) could be a risk for young *H. axyridis* under low *T. salignus* densities or when they are having trouble finding suitably sized prey.

Weights of the first instars to adult *H. axyridis* reared on *E. kuehniella* eggs in our experiment were similar to those reported in earlier studies conducted by de Castro-

Guedes *et al.* (2016) and Specty *et al.* (2003), who reared this ladybird species on fresh and frozen eggs of *E. kuehniella.* Thus, our results are reproducible and were not affected by external factors influencing *E. kuehniella* egg quality.

Pre-adult survival of *H. axyridis* differed significantly among the diet treatments in the current study. Mortality of *H. axyridis* was the highest (25%) in the first larval stage, likely due to the difficulty in feeding on similar-sized first instar of *T. salignus*. Two out of fifteen *H. axyridis* pupae from the *T. salignus* treatment did not emerge to the adult stage. Our results conform with the findings of Kindlmann *et al.* (2000), who suggested that the first and fourth larval stages of *H. axyridis* were the most vulnerable periods, responsible for their population decrease. We also observed a longer duration of the fourth instar *H. axyridis* fed on *T. salignus*, which is possibly related to the need to store more energy reserves to achieve the critical weight required for pupation, as shown in other ladybird species (Omkar & James, 2004) and other insects (Chambers & Klowden, 1990; Davidowitz *et al.*, 2003; Ribeiro & Von Zuben, 2010). Thus, *T. salignus* cannot be considered an optimal prey for *H. axyridis*. Frozen eggs of *E. kuehniella* were shown to be more nutritious than pea aphids because of the amino acid and fatty acid constituents (Specty *et al.*, 2003). Analysis of nutrient composition of *T. salignus* would provide more information about the quality and suitability of this aphid for *H. axyridis*.

Harmonia axyridis preferred *M. persicae* over *T. salignus* as their first prey item when offered a choice between the two aphid species. *Harmonia axyridis* exhibited a strong non-preference of *T. salignus* – the naïve predators refused to attack *T. salignus* in most first encounters with that prey, whereas *M. persicae* was immediately captured and consumed. This behaviour was not due to differences in prey size, as the prey used in the experiments were of similar size, and no prey defence response to predator was observed. Other generalist predators display prey switching in response to different prey ratios

(Enkegaard *et al.*, 2001; Jaworski *et al.*, 2013), but we observed no prey switching in *H. axyridis* in preliminary studies, so only equal proportion of prey species was used to evaluate the predator's preference. Our results suggest that, when faced with multiple prey options under natural conditions, *H. axyridis* may prefer other food sources to *T. salignus*, which would limit its potential as a biocontrol agent for managing this aphid. Further research should explore whether *H. axyridis* is able to handle larger instars and adult *T. salignus*, and also to evaluate the diet breadth and prey preference of *H. axyridis* between *T. salignus* and other co-existing prey species, especially native aphids (Koch, 2003).

This study is the first investigation exploring the voracity and prey preference of *H. axyridis* and its potential as a biocontrol agent to manage *T. salignus* in willow systems. Our results show that *H. axyridis* can consume considerable numbers of *T. salignus* and survive on this aphid, but takes longer to complete its development, has a lower weight and higher preadult mortality in comparison to a standard diet. Also, when faced with prey choice, *H. axyridis* preferred another aphid species over *T. salignus.* Altogether, our results suggest that *H. axyridis* could contribute to population reduction of early instar *T. salignus*, but strong preference for other prey sources may limit its potential as a candidate for augmentative biological control against *T. salignus*.

6.6 Appendix

DRC 16

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Kyaw Min Tun
Name/title of Primary Supervisor:	Dr. Maria Minor
In which chapter is the manuscript /published work:	Chapter 6

Please select one of the following three options:

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- Please provide the full reference of the Research Output:

Tun, K. M., Clavijo McCormick, A., Jones, T., & Minor, M. (2020). The potential of harlequin ladybird beetle Harmonia axyridis as a predator of the giant willow aphid Tuberolachnus salignus: voracity, life history and prey preference. BioControl, 65(3), 313-321. 10.1007/s10526-020-10010-5

( ) The manuscript is currently under review for publication – please indicate:

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Candidate's Signature:	Digitally signed by Kyaw Min Tun Date: 2020.06.30 11:14:24 +12'00'
Date:	30-Jun-2020
Primary Supervisor's Signature:	Maria Minor Digitally signed by Maria Minor Date: 2020.08.04 11:14:00 +12'00'
Date:	4-Aug-2020

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GRS Version 5 – 13 December 2019
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Chapter 7

General discussion

7.1 Synthesis

Willows in New Zealand are valued for multiple purposes (Gunawardana *et al.*, 2014; McIvor, 2013) and are now threatened by *T. salignus*. The aphid has a range of impacts: directly on host plant's physiology and growth, and indirectly on the New Zealand apiculture industry (Sopow *et al.*, 2017). Losses in multiple industries were estimated at $300 per year due to *T. salignus* (Sopow *et al.*, 2019). As it has only recently invaded New Zealand, the ecological relationships of *T. salignus* with host plants and organisms in other trophic levels are not yet known. This thesis was designed to explore multitrophic interaction of *T. salignus*, to better understand its ecological impact and to inform management decisions about its control.

Chapter 1 presents a literature review on what is known about *T. salignus* in New Zealand and other ranges. This chapter identifies multiple knowledge gaps, some of which are addressed in this thesis, including: the resistance or susceptibility of different willow cultivars to *T. salignus* attack, the plant's defence responses to aphid attack (e.g., emission of herbivore-induced volatile organic compounds to attract natural enemies), the influence of the host plant on honeydew composition (e.g., melezitose production), the ecological impacts of honeydew deposition (e.g., on soil properties and biota), and the biocontrol potential of introduced natural enemies (e.g., *Harmonia axyridis*).

The main objective of Chapter 2 was to identify willow cultivars resistant or susceptible to *T. salignus*. Aphid infestation levels varied among willow cultivars, and cultivar resistance was assessed based on the number of weeks with high aphid population densities (and other parameters investigated). *Tuberolachnus salignus* was found year-round and appears not to hibernate in NZ North Island conditions. Not all susceptible cultivars responded negatively to aphid infestation, which can be attributed by the differences in their compensatory ability. Death of young plants (apart from *S.*

eriocephala) and decreased shoot number could be linked with willow susceptibility to aphid herbivory.

In Chapter 3, I explored VOC emissions by different cultivars and their changes in response to *T. salignus* infestation. The results suggest that VOC emission is cultivar-specific, and varies with plant type (tree vs. shrub willows). The data also revealed that resistant cultivars appear to emit more green leaf volatiles (GLVs) than other cultivars, suggesting that there can be a link between *T. salignus* resistance and VOC emission in willows, which deserves further exploration. The GLV emissions increase after mechanical damage, and resistant willow cultivars have been found to emit higher concentration of (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenol than the cultivars susceptible to damage by willow leaf beetles *Phratora vulgatissima* Linnaeus, *P. vitellinae* Linnaeus and *Galerucella lineola* Linnaeus (all Coleoptera: Chrysomelidae) (Peacock *et al.*, 2001). However, the results in Chapter 3 show that most cultivars do not experience significant changes in their VOC emissions after aphid attack, while few have reduced emissions. This can be attributed to low levels of infestation, the fact that aphids do not damage photosynthetic tissue, or to active suppression of the plant responses by the aphid. These hypotheses remain to be tested.

Due to the impact of melezitose in the apiculture industry, in Chapter 4 I investigated if the melezitose concentration in the honeydew varied depending on the plant cultivar upon which *T. salignus* was feeding. I showed that melezitose concentration in *T. salignus* honeydew did not vary with willow cultivar or plant age, but concentrations of other sugars did. Melezitose is the dominant sugar in aphid honeydew, and the honeydew composition changes with host plants on which aphid feeds (Fischer & Shingleton, 2001; Pringle *et al.*, 2014), although Shaaban *et al.* (2020) suggested that host plant is not responsible for the melezitose content of aphid honeydew. There was no obvious link

between willow susceptibility to *T. salignus* and melezitose content, however, total honeydew sugar concentration was lower while fructose content was higher in highly susceptible cultivars identified in Chapter 1. The information on the connection between plant resistance and aphid sugar production is still limited, as no honeydew could be harvested from *T. salignus* on resistant cultivars. My data for honeydew collection show that willow cultivar had a stronger influence on the total amount of honeydew production than the size of aphid colony on the willow stem (**Figure S4.3 & S4.4)**.

Plant sex and plant type (tree vs. shrub) can influence both VOC emission (Chapter 3) and aphid honeydew production (Chapter 4). *Salix* spp. are known for their phenolic glycosides content in plant tissues, which are implicated as deterrents to insect and mammalian herbivory (Boeckler *et al.*, 2011; Fields & Orians, 2006), as well as toxins affecting feeding behaviour and fecundity of herbivores (Pasteels & Rowell-Rahier, 1992). Concentration of these phenolic glycosides can differ between male and female willows (Julkunen-Tiitto, 1986), and these differences can result in different herbivore loads, in turn affecting VOC emission and honeydew production from sexes of willow plants. In my project, the VOC blends of willow cultivars can be separated by plant type (Ch. 3), and honeydew collection data show higher honeydew production (**Figure S4.4)** from tree willows than from shrub willows, although plant type had no significant influence on sugar concentration of *T. salignus* honeydew.

Tuberolachnus salignus honeydew deposition has multiple ecological impacts. Copious amounts of honeydew fall on the understory vegetation or directly on the soil surface, resulting in irregular occurrence of black sooty mould areas under aphid-infested plants (**Figure 5.1**). This carbon-rich energy source is utilized by soil microorganisms (fungi, bacteria and yeasts) (Jílková *et al.*, 2020; Milcu *et al.*, 2015; Stadler *et al.*, 1998), in turn increasing the abundance of fungivores and their predators in honeydew-receiving

soil. In Chapter 5, I found confirmation of this honeydew-mediated cascading effect, which was directly linked with honeydew availability and the level of input, with strongest effect in black sooty mould spots (**Figure 5.7a**). My experimental results in Chapter 5 only consider honeydew-mediated effects on soil biota and biochemical properties. Other studies such as Milcu *et al.* (2015) can extend our understanding of the cascading effect of aphid honeydew to the host plant. For example, honeydew deposition under the tree canopy was shown to alter plant architecture (shoot: root ratio and primary: secondary branches ratio) and flowering phenology (flower number per branch) of a female willow *S. dasyclados* (Milcu *et al.*, 2015). Increased shoot: root biomass ratio can be attributed to the increased nutrient availability for the host plant (Poorter *et al.*, 2012), resulting from increased activities of nitrifying bacteria in honeydew-affected soils (Jílková *et al.*, 2020). Increased branching seems to be linked with an unknown root-related process (Collins, 2001) that deserves a closer look in future research. Honeydew addition can induce willow flowering through increased growth, or through plant-like hormone production by mycorrhizal fungi (Strzelczyk & Pokojska-Burdziej, 1984). The fungi regulate the hormone synthesis of diazotrophic bacteria (Barea *et al.*, 2002) and the increase in fungal growth can determine symbiotic association between mycorrhizal fungus and diazotrophic bacteria (Collins, 2001).

Due to severe impacts of *T. salignus*, finding sustainable control strategies, such as the use of natural enemies, is crucial. However, as an invasive species *T. salignus* lacks natural enemies in New Zealand. The introduced predatory ladybird *Harmonia axyridis* (Coleoptera: Coccinelidae) is known for its wide prey spectrum, consisting of over 77 prey species that feed on 85 host plant species (de Castro-Guedes *et al.*, 2016). The beetle mainly consumes aphids, psyllids, coccids, weevils, and spider mites (Hodek, 1993; Lucas *et al.*, 1997; McClure, 1986; Michaud, 2001; Stuart *et al.*, 2002) but can also feed

on pollen, nectar and fruits (Kovach, 2004; LaMana & Miller, 1996). In New Zealand, the beetle consumes *T. salignus* and tree aphids such as the Oak aphid *Tuberculatus annulatus* Hartig, 1841 and the Chinese elm aphid *Tinocallis ulmiparvifoliae* Matsumura, 1919 (Martin, 2016).

In Chapter 6, I explored the biocontrol potential of *H. axyridis.* The results show that although this predator can feed on *T. salignus,* this aphid is not its preferred prey. *H. axyridis* that fed on immature *T. salignus* developed slower than on alternative prey. Larvae and adults of both sexes preferentially selected other aphid prey species in dual choice test, rejecting *T. salignus.* This lack of preference, coupled with the huge appetite and rapid population build-up of *H. axyridis,* presents a possibility of it outcompeting native natural enemies and native prey insect species, causing a potential risk of biodiversity loss in New Zealand (Ministry for Primary Industries, 2017). Thirteen out of around 120 aphid species in New Zealand are indigenous and are now threatened by predation from introduced natural enemies (Teulon *et al.*, 2003). Further studies are needed to delve into the prey spectrum of *H. axyridis* and their effects on non-target pests and native ladybird species in the New Zealand situation. Sopow *et al.* (2017) suggested that *H. axyridis* should not be promoted as a biocontrol agent for *T. salignus,* and my results fully support their suggestion. In the field situation, the high voracity of *H. axyridis* can cause a localised population reduction of *T. salignus,* but the predator can quickly switch to more nutritious prey species. Other natural enemies, such as the parasitoid *Pauesia salignae* Watanabe, 1939 (Hymenoptera: Braconidae) require further consideration as biocontrol agents against *T. salignus.*

7.2. The impact of invasive herbivores on multitrophic interactions and biological control strategies

Multiple organisms in every ecosystem are ecologically linked to each other (Singh, 2003). These interactions generate complex food web structures, consisting of interrelated food chains (Cohen *et al.*, 2009; Singh, 2003). As primary producers, plants sustain these food webs, but must also have mechanisms to defend themselves from excessive herbivore loads (Turlings *et al.*, 1995; Zhu & Park, 2005). Plant defences include physical barriers, chemicals or semiochemicals (infochemicals), and can be classified as direct (having a direct impact on the herbivore) or indirect (affecting the herbivore through increased recruitment of natural enemies) defence (Dicke & Sabelis, 1987; Price, 1997; Price *et al.*, 1980).

Under natural situations, the interactions between plants, herbivores and other trophic levels are maintained in balance through natural selection, however, invasive herbivores like *T. salignus* impose great stress to their invaded systems, as they have not co-evolved with their new hosts which may lack adequate defence mechanisms, and typically lack natural enemies in their introduced range (Van Driesche & Hoddle, 2009). If invaders succeed in exploiting the new resources, they rapidly increase their population densities and become pests. The changes in herbivore community composition will inevitably impact the structure of trophic webs, and other non-trophic interactions affecting the whole community (David *et al.*, 2017). Aphids have an additional impact associated with the honeydew deposition, which provides a food source for some animals and promotes microorganism growth (Sopow *et al.*, 2017), extending their effects well beyond the plant-insect interaction.

This study evidences the direct effects of *T. salignus* on the growth, reproduction and survival of willow plants, but also their (potential) indirect effects on other community members through honeydew deposition (e.g., on soil properties and biota). Due to its multiple ecological impacts, it is important to establish a suitable biocontrol strategy that involves the identification of bottom-up (plant-derived) and top-down (natural enemy related) mechanisms to eliminate, reduce, or maintain low population densities of this pest insect.

7.2.1. Bottom-up biocontrol mechanisms

In this study I found that willow plant cultivar, type (tree or shrub) and age, influenced giant willow aphid attack and population density, suggesting that some cultivars are naturally more resistant than others. The reasons behind this resistance remain to be investigated; possible reasons include morphological barriers such as differences in bark structure in resistant vs. susceptible willow cultivars or chemical barriers. The differences in VOC emission suggest that tree and shrub willows behave chemically differently, and that resistant cultivars emit more green leaf volatiles that could act as deterrents. Further insight is needed into other direct chemical defences. Willows are known to possess salicinoids and phenolic glucosides that make the leaves of host plants unpalatable for generalist herbivores and can prolong their development and foraging time, and increase their movement, leading to increased exposure to natural enemies (Bernays, 1997; de Siqueira Neves *et al.*, 2011; Price *et al.*, 1980).

Plant sex was not investigated here, but can also act as a bottom-up force in determining population densities of herbivorous insects, predators, and their interactions (Kabir *et al.*, 2014). Female plants invest more in reproduction and defensive traits, and less in vegetative growth, than their male counterparts (Lloyd & Webb, 1977; Obeso, 2002). These intersexual differences can also impact host quality, pollen availability and

nectar composition (Dötterl *et al.*, 2014; Kabir *et al.*, 2014; Petry *et al.*, 2013), which in turn determine predator populations (Åhman, 1997). Insect herbivory on willow plants is assumed to be sex-biased (Åhman, 1997; Stenberg *et al.*, 2011b), resulting from higher quality of female plants than the males (Hunter & Price, 1992; Julkunen-Tiitto, 1986). Consequently, predator abundance on female plants increases, reflecting higher prey availability on more nutritious host plants (Stenberg *et al.*, 2011a). Some studies suggest that biocontrol efficacy is lower in Short Rotation Willow Coppices (SRC) plantations grown as a single clone/cultivar of same sex (mainly female) than in natural vegetation (such as in NZ) (Kabir *et al.*, 2014). Therefore, plant intersexual differences should be incorporated in future studies.

Changes in nutrient concentration of host plants can also exert a bottom-up cascading effect on herbivorous insects and their natural enemies (Teder & Tammaru, 2002). Feeding on more nutritious hosts can increase prey density and quality, which leads to increased performance of individual predators (Mayntz & Toft, 2001). Herbivorous insects consume more and spend more time in feeding than resting when feeding on a less nutritious host, and this increases their visibility and exposure to natural enemies (Fajer *et al.*, 1989; Moran & Hamilton, 1980; Price *et al.*, 1980). In my thesis, the nutrient content of willow tissues in response to *T. salignus* infestation was not investigated. However, other studies show that infestation by *T. salignus* and the willow leaf beetle *Plagiodera versicolora* Laicharting, 1781 (Coleoptera: Chrysomelidae) increased willow leaf nitrogen content (Collins *et al.*, 2001a; Kagata & Ohgushi, 2007). The relative growth rate of the ladybird beetle *Aiolocaria hexaspilota* Hope, 1831 (Coleoptera: Coccinellidae) also increased, which was attributed to increased quality of prey (*P. versicolora*) that fed on more nutritious plants (Kagata & Ohgushi, 2007).

Changes in nutrient availability and other bottom-up effects require additional exploration.

7.2.2 Top-down biocontrol mechanisms

In this study, we found no evidence supporting changes in VOC emissions upon herbivore attack, indicating that attraction of natural enemies through this means is unlikely to occur. This suggests that the willow cultivars investigated are investing more resources into direct than indirect defences, probably due to the fact that the insect is invasive and lacks natural enemies in its invasive range. However, a question remains open regarding the ability of the aphid to suppress plant defences, which requires future investigation.

Some evidence suggests that polyphagous predators could act as top-down biocontrol agents (Albajes & Alomar, 1999; Symondson *et al.*, 2002). However, caution is advised, as a new predator with broad prey range can have devastating consequences for natural ecosystems. Prey or host specificity is a preferred trait for natural enemies in biocontrol programmes. Arguments for this preference include: 1) specialists are more effective at maintaining pest populations at low densities in a stable way; 2) the use of generalist predators may increase predation of non-target species or interference with other natural enemies; and 3) some generalist predators can facultatively feed on plants, and may themselves become a pest (Albajes & Alomar, 1999). Our study supports the second argument, indicating that when offered a choice, the polyphagous predator *H. axiridis* always preferred other prey over *T. salignus.* Thus, the use of this species as biocontrol agent is not advised.

Other (specialist) natural enemies like *P. salignae* or mechanisms of top-down control remain to be investigated. Plants have multiple ways of luring predators of their herbivores, with VOC emission being just one mechanism. Plants can also provide

additional food in the form of floral or extra-floral nectar and refuge for natural enemies (such as domatia) (Kessler & Heil, 2011). Given the abundance of predatory arthropods interacting with willows in agroecosystems, future studies should explore their biocontrol potential and other forms of indirect defence.

7.3 Knowledge contribution to controlling *T. salignus* and reducing honeydew-related problems

This multitrophic study provides insights into top-down (natural enemies) and bottom-up (willow cultivars) control of *T. salignus*. My results will help the practitioners in making management decisions on *T. salignus* in New Zealand and elsewhere around the world.

Cultivar selection plays an important part in reducing the impact of aphid infestation and in lessening honeydew-related problems in apiculture industries. Through my studies, two resistant cultivars (*S. eriocephala* and *S. lasiolepis* × *S. viminalis*) were identified, whereas *S. viminalis* and *S. candida* were classed as most susceptible cultivars. Two-year weekly aphid monitoring can fill the lack of information on aphid surveillance in New Zealand. In the winter 2018, small aphid clusters were found on *S. lasiandra* and *S. schwerinii,* suggesting that the aphids could survive the North Island of New Zealand winter. This also leads to my speculation that eradicating the aphids on those cultivars in winter could decrease their population numbers in the forthcoming season.

I have reported for the first time that aphid infestation can increase floral output and extend the period of pollen availability to honeybees (Chapter 2). This is the good news for New Zealand beekeepers who are making use of willows for providing spring pollen for bee's brood feeding. Cultivar selection for aphid resistance vs. bee nutrition may need

to be compromised in designing aphid management program (for example, the susceptible *S. candida* is known for its good quality pollen), and needs further investigation. I discovered in Chapter 3 that aphid population levels have a negative relationship with GLV emission of willow cultivars and four VOCs have been identified for future investigation of *T. salignus*-willow interaction. The results of Chapter 2 and 3 contribute to understanding how *T. salignus* populations differ among willow cultivars, and why some willow cultivars are more resistant to aphid infestation than others, providing important information in cultivar selection for willow plantations and for future breeding programs.

Melezitose concentration did not vary with willow cultivar and plant age (Chapter 4). This finding could not support the selection of willow cultivars that contribute least to melezitose concentration of *T. salignus* honeydew. However, to lessen the impact of honeydew-related problems in apiculture industries, I would recommend using resistant cultivars (*S. eriocephala* and *S. lasiolepis* × *S. viminalis*) which had no honeydew production in our study in place of susceptible varieties, or if not feasible, to use lower melezitose-containing cultivars such as *S. lasiandra*, *S. lasiolepis* and *S. schwerinii*.

I acknowledged from predatory trials (Chapter 6) that harlequin ladybird beetle *H. axyridis* can reduce the aphid population to certain extent, but it should not be considered as an augmentative biocontrol agent for controlling *T. salignus*. *Harmonia axyridis* is known to have negative impacts on non-target arthropods and fruit production, as well as becoming a nuisance as household invader in North America (Koch & Galvan, 2008). Studies on other biocontrol agents (preferably specialists) are encouraged.

7.4 Knowledge gaps for future research

My thesis results provide insight into the aphid's ecological relationships and into controlling invasive species in New Zealand and elsewhere around the world. Given the lack of ecological studies in *T. salignus*, the current study covered some of the knowledge gaps, but also identified additional gaps to fill in. I provide some ideas for further research to be addressed by future ecologists/entomologists, as listed below:

- Evaluating the aphid infestation effect on willow resource allocation (growth vs. reproduction, and above- vs. below-ground biomass production). I have only shown that infestation can decrease above-ground willow growth, but it will be interesting to explore what happens below-ground too, as willows are used for erosion and flood control in New Zealand. This information will help river engineers in selecting cultivars suitable for specific purposes.
- Changes in quantity and quality of willow pollen, and willow seed production in response to aphid infestation merit further exploration. I only report that the aphid infestation changed flowering phenology and pollen output of willow cultivars (Chapter 2). Aphid-induced changes in willow flowering can alter the abundance and community composition of pollinators (honeybees and flies) and possibly predators (I observed the *H. axyridis* beetles feeding on willow pollen in Massey field trial).
- VOC-mediated host plant selection by aphids should be investigated, with special emphasis on candidate compounds highlighted in Chapter 3. Since there seems to be little evidence of herbivore-induced VOC emission, other forms of indirect defence (natural enemy recruitment) should be investigated.
- The characteristics that determine resistance or susceptibility in willow cultivars should be investigated, these could be either morphological or

chemical. I observed in the willow field trial that two resistant cultivars had harder bark, suggesting that aphid resistance may be related to bark characteristics and the rapid development of rough bark. Difference in salicinoids or phenolic glucosides could also explain the different levels of resistance/susceptibility.

- Chemical analyses of the phloem sap of willow cultivars must be conducted to establish its connection with carbohydrate and protein composition of aphid honeydew. Establishing honeydew production at different aphid population levels and over different seasons and years is also important, as honeydew availability can influence the abundance and community composition of honeydew foragers (honeybees, wasps, flies, ants, ladybird, and birds) and soil microorganisms (Chapter 2 and 5).
- A closer observation of the honey bee's honeydew foraging behaviour and the impacts of honeydew consumption on honey bee health is also needed. Melezitose is known to negatively impact honey bee health and survival and most cultivars produce it in high levels (Chapter 4). Honeydews from aphids feeding on different willow cultivars are thought to differ in nutrient composition, and these differences could be reflected in physical and behavioural traits, such as the size of acini (small oval bodies in hypopharyngeal gland) in nurse bees and frequency of visits by foraging bees.
- Honeydew deposition by *T. salignus* affects soil biota and soil biochemical properties (Chapter 5). It is already known that aphid honeydew increases the abundance of soil microbes, but which species/group of species are responding to honeydew addition is not yet known. DNA analysis will help to explore the diversity of microorganisms in honeydew-affected soils. The long-

term effects of the honeydew deposition on the soil health remain unknown, and require further investigation. In particular, how total soil C, soil microbial activity and soil mesofauna increases in honeydew-affected soil influence the growth and health of willow plants.

- Finally, testing efficacy of other candidate biocontrol agents and their compatibility with resistant willow cultivars will help in formulating an IPM package to lessen the impact of *T. salignus* infestation in the willow planting and apiculture industries.

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