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Phylogenetic and chemo-ecological aspects effecting the host-selection behaviour of the thistle tortoise beetle *Cassida rubiginosa*

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy

at
Lincoln University
by
Dilani Kasundara Hettiarachchi

Lincoln University
2018

To my father,

Who always believed in me,

Who taught me to dream bigger and aim higher.

- *D.K.*

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

Phylogenetic and chemo-ecological aspects affecting the host-selection behaviour of the thistle tortoise beetle *Cassida rubiginosa*

by

Dilani Kasundara Hettiarachchi

Most species of insects are selective feeders that not only choose the plant but also choose specific plant organs. Insect host-finding behaviour involves several cues such as olfactory, visual, tactile and gustatory. The responses to these cues depend on the context in which the signals are perceived and on the host-insect system. Knowing the process of host-finding behaviour of insects is important for management practices. The oligophagous, chrysomelid beetle *Cassida rubiginosa* was introduced to New Zealand in 2007 mainly to control *Cirsium arvense* (Californian thistle), one of the most noxious weeds found in the world. New Zealand does not have any native thistles and very few economically important ones. Therefore, there is potential for using this beetle to target secondary weeds and to better manage the beetle for weed biocontrol. Host selection behaviour of the beetle was investigated by conducting experiments in a phylogenetic context giving emphasis to chemical cues.

Host range was investigated for constitutive volatiles as well as induced volatiles. Host-range testing with constitutive volatiles was conducted by using 19 Asteraceae plants (16 Cardueae and 3 non-Cardueae) which were selected according to phylogeny. A series of single-choice and dual-choice olfactometer experiments and adult host-choice experiments were carried out investigating the beetles' choice of constitutive volatiles. Adult host-choice experiments were performed to test the feeding and oviposition preference of the beetle. Volatiles were induced by conspecific larval damage and 13 Cardueae plant species were used. Using induced volatiles, the differential attraction of *C. rubiginosa* in olfactometer experiments comparing undamaged leaves with conspecific larval damaged leaves was investigated. Volatiles were collected and analysed using GC-MS.

Compatibility of the beetle can change according to other biocontrol agents. Investigations were carried out with two fungal pathogens (*Puccinia punctiformis* and *Sclerotinia sclerotiorum*) infesting *C. arvense* by studying the olfactory behaviour of adult *C. rubiginosa* towards fungus-infected *C. arvense*. A series of olfactometer experiments were conducted to evaluate the host selection of the beetle; dual-choice experiments were performed to investigate its feeding preference. Adult beetles were given the choice between healthy thistle leaves and leaves infected by one of the pathogens. Volatiles were collected from healthy and fungus-infected plants and analysed using GC-MS.

The multi-targeting biocontrol potential of *C. rubiginosa* was tested with marsh thistle (*Cirsium palustre*), a species closely related to the primary host and shown to be equally preferred in previous experiments. To test if the beetle can reduce the fitness of marsh thistle a potted plant experiment was established with four treatments (0, 50, 100 and 200 larvae/plant). Plant growth (width, height and number of branches) and reproductive performance (number of flowers, seeds, seed weight and % germination) parameters were measured.

The results show that as phylogenetic distance from the primary host plant increases, the beetles' preference for alternative hosts decreases. This was the case for all three parameters: olfactory choice for constitutive volatiles, feeding and oviposition preference. Olfactory experiments showed similar host range to host-choice experiments across the Cardueae tribe. When presented with herbivore-induced volatiles (HIPVs) the beetle increased attraction towards five hosts that it was not attracted to when tested with constitutive volatiles. Chemical compounds such as cis-β-ocimene and β-caryophyllene were detected in the HIPVs blend in the five species of plants that showed beetle attraction, expanding the olfactory host range of the beetle. However, the beetles' attraction towards *C. arvense* was reduced when infected with the biotrophic rust fungus *P. punctiformis* while the necrotroph *S. sclerotiorum* had no effect. Both pathogens, however, reduced adult beetle leaf consumption. Volatile profiles showed a unique blend of compounds where the *P. punctiformis*-infected thistle leaves were characterised by the emission of benzenoids and indole, *S. sclerotiorum*-infected leaves by green leaf volatiles. Thus, having multiple biocontrol agents can reduce the efficacy of at least one of the involved agents and should be taken into consideration.

The multi-targeting potential of the beetle is predicted to follow the phylogenetic trend in host preference. As such, the greatest multi-targeting potential is likely for the equally preferred species, particularly the *Cirsium* and *Carduus* genera. Though the beetle was found to be equally attracted to *C. palustre* in all of the experiments, it did not show an effect in reducing *C. palustre* individual plant growth or reproductive performance under the imposed experimental conditions. If the beetle is to have an impact on other thistle weeds, it will likely need to attack early growth stages (seedlings), or smaller size classes of plants to prevent reproduction.

Keywords: Cardueae, host-range, conspecific cues, herbivore-induced volatiles, constitutive volatiles green thistle beetle, seed viability, alternative host, *Cirsium*, *Carduus*, *Centaurea*, *Cynara*, centrifugal phylogeny, oviposition, feeding, *Puccinia*, *Sclerotinia*, feeding, weed biological control, insect herbivore-fungal pathogen interaction.

Publications from this thesis

Full papers

Hettiarachchi, D.K., Cripps, M., Jackman, S., van Koten, C., Sullivan, J. & Rostás, M. (2018) Impact of the biocontrol beetle, *Cassida rubiginosa*, on the secondary weed target, marsh thistle (*Cirsium palustre*). *New Zealand Plant Protection,* 71.

Extended abstracts

Hettiarachi, D.K., Cripps, M.G., Cruikshank, R.H. & Rostás, M. (2017) A phylogenetic approach to assessing olfactory host selection in *Cassida rubiginosa* (Chrysomelidae). In Invertebrate Ecology of Australasian Grasslands. Proceedings of the Ninth ACGIE (ed S.N. Johnson), pp. 59-61. Western Sydney University, Hawkesbury, NSW, Australia. doi: https://doi.org/10.6084/m9.figshare.4910033.v1.

Conference proceedings

- Wake up and smell the thistles! Using plant volatiles to assess host suitability in an oligophagous biocontrol beetle. (2016). Ninth Australian Conference on Grassland Invertebrate Ecology (ACGIE), Western Sydney University, Sydney 4-7 April 2016 - Poster presentation
- Follow the smell! "Volatiles" for locating host plants. (2016). Lincoln University Postgraduate Conference, Lincoln University, Lincoln, New Zealand. 24 and 25August 2016 - Oral presentation
- Thistle phylogeny determines volatile-mediated host plant recognition in the weed biocontrol agent *Cassida rubiginosa*. (2017). The 2nd joint meeting of the 33rd annual meeting of the International Society of Chemical Ecology (ISCE), and the 9th meeting of the Asia-Pacific Association of Chemical Ecologists (APACE), Ryukoku University, Kyoto, Japan. 22-27 August 2017 – Poster presentation
- Tripartite interactions between *Cassida rubiginosa* and two fungal biocontrol agents on *Cirsium arvense*: plant volatile perspective. (2018). The 34th annual meeting of the International Society of Chemical Ecology (ISCE), Budapest, Hungary. 12-18 August 2017 – Poster presentation
- The role of thistle phylogeny on long-range and short-range host selection behaviour of the biocontrol agent *Cassida rubiginosa*. (2018). International Symposium on Biological Control of Weeds (ISBCW). Engelberg, Switzerland. 26-31 August 2018 – Oral presentation

Acknowledgements

This journey hasn't been easy and so far it has been the most difficult path I have walked in my life. If it wasn't for committed supervisors, an inspiring family, a fantastically supportive husband and kids, and patient friends I might not have completed this adventurous journey.

I extend my sincere thanks to my main academic supervisor Dr Michael Rostás for his guidance and support throughout, as well as his patience during my hardships. It has been a pleasure working together and exchanging ideas. Your passion for science, vast knowledge and logical way of thinking have been extremely helpful. It is due to your valuable guidance and friendly nature that I was able to complete my PhD research. I would also like to extend my gratitude to Dr Mike Cripps and Dr Jon Sullivan who are part of my supervisory team that has given me good suggestions and unrelenting support throughout this process. To Dr Robert Cruickshank, even though you have only been part of this journey for a short while, you have been supportive during many of my hardships. Your ideas and suggestions have made me think differently. Thank you very much for that. Sarah Jackman gave me immense support with my field and lab trials by finding and planting seeds as well as being a companion during our field visits, which is greatly appreciated. My heartfelt thanks goes to Chikako van Koten for helping me with the statistical analysis and providing useful feedback. To Dr Graeme Bourdôt, even though you are not part of my research team, exchanging ideas with you broadened my viewpoints. I will forever value the help you have given me. Thank you to Mark McNeill, AgResearch, Lincoln, for providing me with olfactometers, and to Rob McGregor, Glass Workshop, Canterbury University, for making me still-air olfactometers. I must also extend my gratitute to Jason Breitmeyer who kindly gave me technical support when analysing all the GC-MS samples. Josefina Narciso, Jenny Brookes, Myles Mackintosh, Brian Kwan and Fariba Nourozi, thank you for helping and encouraging me all the time. To Brent Richards and Leona Meachen from the Lincoln University Nursery who have taken care of my plants for the time I conducted experiments. Smith Bethanne and Laura Nixon helped me with some of my experiments, which is greatly appreciated. Thank you to IT Services and Library, Teaching and Learning and the Student health and support center staff at Lincoln University, for their support.

To all the staff at the Bio-Protection Research Centre, thank you for your help and encouragement. In particular, I would like to thank Prof. Travis Glare, Dr Andrew Holyoake, Prof. John Hampton, Assoc. Prof. Susan Worner, Janine Johnson, Dianne Fyfe, Sandy Wilson, Claire Tee and Susan Paterson for assisting and supporting me whenever needed, and in record time. I would like to extend my appreciation to my colleagues from the institution that I am associated with in Sri Lanka, Rajarata University of Sri Lanka, for the support they have given me throughout my course of study. To all my friends, your friendship means a lot to me and thank you for tolarating all my nagging. Each and every one of you is

precious and you have a special place in my life. The love, the tears and the smiles we share will always be cherished and valued. Thank you for letting me have a huge family in New Zealand.

I do not have the words to express my gratefulness towards my husband and my two loving kids. I know this journey has been a challenge to all three of you, especially because we were apart. To my daughter Senadee Dilansa Doranegama and my son Senuth Dinhas Doranegama, you are the most amazing kids any mom could ever dream of, and I am blessed to have you as mine. The understanding you showed with my studies and the encouraging words you have given me whenever I have been down have actually paved my way to achieving this goal. I was amazed by the way you two handled everything, while being so little and innocent. I love you loads and thank you for that. To my dearest husband, Sumedha Doranegama, you are my pillar right next to my dad. I will always lean on you because I know you will always be there to lift me up. Thank you for being my life partner and always encouraging me to aim higher. I wouldn't have done it without you. To my in-laws Upali Doranegama and Manel Doranegama, if it wasn't for your sacrifices I would not have stepped foot in New Zealand. You took me as your own daughter and encouraged me to pursue a PhD. You took care of my home and relieved me to do my studies. Not many in-laws would do that. Thank you very much mom and dad. I am blessed to have two moms and two dads in my lifetime. To my mom, Kusuma Hettiarachchi, dad (late) Jayampathi Hettiarachchi, if it wasn't for you I wouldn't dream high. I know how much you wanted me to do a PhD and it is a shame that you are not here today to witness it. Thus, this is for you dad. Mom, you have seen all my tears and never let me down. I feel like you are the only person that can understand me fully in the whole world. Thank you for everything. My sisters (Dirandi, Lahiri, Nimesha and Iresha) and my brothers (Tharinda, Anoj, Shashika and Buddhika) thank you for your craziness throughout this time. Thank you all for believing in me, it kept me going.

This PhD would not be possible if it wasn't for the New Zealand Commonwealth Scholarship granted by the Ministry of Foreign Affairs and Trade (MFAT), New Zealand. In addition, operational funding was provided by the New Zealand Ministry of Business, Innovation, and Employment through AgResearch core funding of pasture weed research. Both these funding bodies are greatly appreciated. Last but not least, I would like to mention all my colleagues and friends back in Sri Lanka who have given me moral support and encouragement throughout, and who are waiting for me to return to spoil me more. Thank you very much.

Table of Contents

Acknowledgements .... **vi**

Table of Contents .... **viii**

List of Tables .... **x**

List of Figures .... **x**

Chapter 1 General Introduction **1**

1.1 Reference 7

Chapter 2 The role of thistle phylogeny on long-range and short-range host-selection behaviour of the biocontrol agent *Cassida rubiginosa* **11**

2.1 Introduction 11

2.2 Material and Methods 13

- 2.2.1 Plants 13
- 2.2.2 Insect 15
- 2.2.3 Olfactometer experiments 16
- 2.2.4 Volatile extraction 18
- 2.2.5 Host-choice experiments 19
- 2.2.6 Statistical analysis 21

2.3 Results 22

- 2.3.1 Olfactometer experiments 22
- 2.3.2 Volatile extraction 25
- 2.3.3 Host-choice experiments 27

2.4 Discussion 31

2.5 Acknowledgement 35

2.6 References 35

Chapter 3 Conspecific larval feeding elicits differential olfactory attraction in *Cassida rubiginosa* towards thistles **39**

3.1 Introduction 39

3.2 Material and methods 41

- 3.2.1 Study system 41
- 3.2.2 Olfactometer experiments 43
- 3.2.3 Volatile analysis 43
- 3.2.4 Data Analysis 45

3.3 Results 45

- 3.3.1 Olfactometer experiments 45
- 3.3.2 Volatile analysis 46

3.4 Discussion 51

3.5 Acknowledgements 53

3.6 References 54

Chapter 4 Effect of two pathogenic fungi on *Cirsium arvense* volatiles and preference of *Cassida rubiginosa* **58**

4.1 Introduction 58

4.2 Material and methods 61

- 4.2.1 Olfactometer experiments 62
- 4.2.2 Volatile extraction 63

4.2.3 Dual-choice feeding performance tests 64
4.2.4 Data analysis 65
4.3 Results....................65
4.3.1 Olfactometer experiments 65
4.3.2 Volatile extraction....................66
4.3.3 Dual choice feeding performance tests 70
4.4 Discussion 70
4.5 Acknowledgement 74
4.6 References....................74

Chapter 5 Impact of the biocontrol beetle, *Cassida rubiginosa,* on the secondary weed target, marsh thistle *(Cirsium palustre)* 80
5.1 Introduction....................80
5.2 Materials and Methods....................82
5.2.1 Study system 82
5.2.2 Data Analysis 83
5.3 Results....................83
5.4 Discussion 85
5.5 Acknowledgments....................86
5.6 References....................86

Chapter 6 General Discussion 89
6.1 Key objectives and findings....................89
6.2 Direction of future research 92
6.1 Conclusion 94
6.2 References....................94
Appendices 96

List of Tables

Table 2.1 Summary of the olfactometer and adult host choice experiments with larval survival results from Cripps et al. (2016). CA – *Cirsium arvense*, CV - *Cirsium vulgare*, CP - *Cirsium palustre*, CT - *Carduus tenuiflorus*, CN - *Carduus nutans*, SM - *Silybum marianum*, AL - *Arctium lappa*, CL - *Carthamus lanatus*, CyS - *Cynara scolymus*, CyC - *Cynara cardunculus*, PA - *Ptilostemon afer*, CeC - *Centaurea cyanus*, OA - *Onopordum acanthium*, CeN - *Centaurea nigra*, ER - *Echinops ritro*, CM - *Centaurea macrocephala*. **Bold** - preferred equally or higher than that of *C. arvense*. * - preference is higher than that of *C. arvense* 34

Table 3.1 Comparison of constitutive volatiles vs herbivore-induced volatile emissions. P-values are given for the pairwise comparisons (t-tests) between the concentrations of each class of volatile with damaged and undamaged leaves. GLV –green leaf volatiles. Bold – statistically significant (P < 0.05). 49

Table 4.1 Volatile organic compounds emitted by *Puccinia punctiformis*-infected *Cirsium arvense* (PP), *Sclerotinia sclerotiorum*-infected *Cirsium arvense* (SS) and healthy *Cirsium arvense* (CA). S - sesquiterpenoid, M - monoterpenoid, B - benzenoid, F - furans, K - ketones and X - unknown. 69

Table 4.2 *Cassida rubiginosa* responses to feeding performance experiment, offering healthy *Cirsium arvense* leaves and pathogen infected *Cirsium arvense* leaves. N = 20..........70

Table 5.1 Mean (± standard error) reproductive and growth parameters of marsh thistle (*Cirsium palustre*) after treatment with 0, 50, 100, or 200 larvae per plant of the folivorous beetle, *Cassida rubiginosa*. Treatment means sharing a common letter do not differ at the 5% significance level. 84

List of Figures

Figure 2.1 Chronogram of the 16 Cardueae test plant species pruned from a comprehensive phylogeny of the tribe (Barres et al., 2013). Branch length depicts phylogenetic distance in millions of years (Cripps *et.al.*, 2016). *Cirsium arvense* was taken as the starting point when calculating the phylogenetic distance..15

Figure 2.2 Olfactometer set up a) no-choice experiment where the plant leaf is compared with a paper-mimic and cotton ball inside the olfactometer and b) choice experiment where *Cirsium arvense* was compared with a leaf from another test plant 18

Figure 2.3 Relationship between phylogenetic distances (millions of years) from *Cirsium arvense* and preference of *Cassida rubiginosa* towards leaf chamber over the control chamber of the olfactometer. PI = (Number of beetles that chose plant leaf chamber– Number of beetles that chose control chamber) / Total number. Index value is between –1 and +1 inclusive, with (+1) indicating perfect preference of test plant chamber over control chamber and (–1) perfect preference towards the control chamber over the test plant chamber. Preference index of plant = 1 – 2 / (1 + exp (– 0.0198 * Phylogenetic distance + 1.02146)) where a = 0.0198 ± 0.0046 (estimate ± standard error of estimate) and b = 1.02146 ± 0.2182. CA - *Cirsium arvense*, CV - *Cirsium vulgare,* CP - *Cirsium palustre,* CT - *Carduus tenuiflorus,* CN - *Carduus nutans,* SM - *Silybum marianum,* AL - *Arctium lappa,* CL - *Carthamus lanatus,* CyS - *Cynara scolymus,* CyC - *Cynara cardunculus,*

PA - *Ptilostemon afer,* CeC - *Centaurea cyanus,* OA - *Onopordum acanthium,* CeN - *Centaurea nigra,* ER - *Echinops ritro,* CM - *Centaurea macrocephala.* 23

Figure 2.4 Relationship between phylogenetic distances (millions of years) from *Cirsium arvense* and olfactory preference of *C. arvense* volatiles. PI = (Number of beetles that chose test plant – Number of beetles that chose *C. arvense*) / Total number. Index value is between −1 and +1 inclusive, with (+1) indicating preference of test plant over *C. arvense* and (−1) preference towards *C. arvense* over the test plant. Preference index value = 1 – 2 / (1 + exp (0.0182 * Phylogenetic distance − 0.0886)) where $a = 0.0182 \pm 0.0045$ and $b = 0.0886 \pm 0.1957$. CA - *Cirsium arvense*, CV - *Cirsium vulgare,* CP - *Cirsium palustre,* CT - *Carduus tenuiflorus,* CN - *Carduus nutans,* SM - *Silybum marianum,* AL - *Arctium lappa,* CL - *Carthamus lanatus,* CyS - *Cynara scolymus,* CyC - *Cynara cardunculus,* PA - *Ptilostemon afer,* CeC - *Centaurea cyanus,* OA - *Onopordum acanthium,* CeN - *Centaurea nigra,* ER - *Echinops ritro,* CM - *Centaurea macrocephala.* 24

Figure 2.5 Relative abundance of chemical compound detected in the 16 Cardueae plant species. 25

Figure 2.6 Hierarchical cluster dendrogram using nearest neighbour method and Bray-Curtis dissimilarity grouping plant species with similar constitutive volatiles of 16 Cardueae plant species...26

Figure 2.7 Relationship between phylogenetic distances (million years) from *Cirsium arvense* with a) feeding preference [Feeding hole number = exp (4.3275–0.0033*Phylogenetic distance)] and b) total egg mass number per female of *Cassida rubiginosa* [Total egg mass number = exp (1.9580–0.0057* Phylogenetic distance] towards each of the test plants in a no-choice cage experiment. CA - *Cirsium arvense*, CV - *Cirsium vulgare,* CP - *Cirsium palustre,* CT - *Carduus tenuiflorus,* CN - *Carduus nutans,* SM - *Silybum marianum,* AL - *Arctium lappa,* CL - *Carthamus lanatus,* CyS - *Cynara scolymus,* CyC - *Cynara cardunculus,* PA - *Ptilostemon afer,* CeC - *Centaurea cyanus,* OA - *Onopordum acanthium,* CeN - *Centaurea nigra,* ER - *Echinops ritro,* CM - *Centaurea macrocephala.* 28

Figure 2.8 Relationship between phylogenetic distances (million years) from *Cirsium arvense* and a) total feeding hole numbers per test plant [Feeding preference index = 1 – 2 / (1 + exp (0.0332 * Phylogenetic distance – 0.8780)) where $a = 0.0332 \pm 0.0092$ and $b = 0.8780 \pm 0.4050$] b) egg laying behaviour during dual choice cage experiment [Egg laying preference index = 1 – 2 / (1 + exp (0.0477 * Phylogenetic distance – 0.6073)) where $a = 0.0477 \pm 0.0094$ and $b = 0.6073 \pm 0.2856$]. The PI of CA over each test plant = (Number of the feeding holes/egg masses on test plant − Number of feeding holes/egg masses on CA) / Total number. Index value is between −1 and +1 inclusive, with (-1) indicating perfect preference of *CA* over the test plant and (+1) perfect preference of the test plant over CA. CA - *Cirsium arvense*, CV - *Cirsium vulgare,* CP - *Cirsium palustre,* CT - *Carduus tenuiflorus,* CN - *Carduus nutans,* SM - *Silybum marianum,* AL - *Arctium lappa,* CL - *Carthamus lanatus,* CyS - *Cynara scolymus,* CyC - *Cynara cardunculus,* PA - *Ptilostemon afer,* CeC - *Centaurea cyanus,* OA - *Onopordum acanthium,* CeN - *Centaurea nigra,* ER - *Echinops ritro,* CM - *Centaurea macrocephala.* 30

Figure 3.1 Mean number of adult female beetles *Cassida rubiginosa* choosing the constitutive volatiles (leaf chamber) and the control chambers (chamber with paper mimic and cotton ball) of the olfactometer during no-choice tests. * $P < 0.05$, ** $P < 0.001$. Plants were arranged according to phylogenetic distance from *Cirsium arvense.* N = 10......45

Figure 3.2 Mean number of adult female *Cassida rubiginosa* beetles choosing the constitutive volatiles (undamaged leaves) and herbivore induced volatiles (larval damaged leaves)

inside the olfactometer during olfactory preference tests. * P <0.05, ** P < 0.001. Plants were arranged according to phylogenetic distance from *C. arvense.* N = 20 46

Figure 3.3 Comparison of total emitted volatiles in larval damaged and undamaged plants according to their chemical classes (benzenoids, green leaf volatiles, monoterpenes and sesquiterpenes) in the five plant species attracted by *Cassida rubiginosa*. * P <0.05, ** P < 0.001 (*t*-test). 48

Figure 3.4 Hierarchical cluster dendrogram using nearest neighbour method and Bray-Curtis dissimilarity for the conspecific herbivore-induced volatiles. CA - *Cirsium arvense*, CV - *Cirsium vulgare,* CP - *Cirsium palustre,* CT - *Carduus tenuiflorus,* CN - *Carduus nutans,* SM - *Silybum marianum,* AL - *Arctium lappa,* CL - *Carthamus lanatus,* CyS - *Cynara scolymus,* CyC - *Cynara cardunculus,* PA - *Ptilostemon afer,* CeC - *Centaurea cyanus,* OA - *Onopordum acanthium,* CeN - *Centaurea nigra,* ER - *Echinops ritro,* CM - *Centaurea macrocephala.* 50

Figure 4.1 Mean ± SE number of *Cassida rubiginosa* that entered either the chamber containing pathogen infected or the healthy leaves of *C. arvense* (CA) during olfactometer experiments. Three adult females per replicate. Statistical comparisons are within groups, *Puccinia punctiformis* and *Sclerotinia sclerotiorum*. N = 20. *P < 0.05. PP: *Puccinia punctiformis* and SS: *Sclerotinia sclerotiorum*...65

Figure 4.2 Principle component analysis distinguished clusters of emitted volatiles according to the treatment/infection. a) Score plot of the different classes of volatile compounds according to the treatment. b) Loading plot with volatiles analyzed using GC-MS. Axis 1 eigenvalue 13.31 / explained variance 30.2; Axis 2: 8.98 / 20.4; Axis 3: 5.57 / 12.65. PP - *Puccinia punctiformis* infected *Cirsium arvense* plants; SS - *Sclerotinia sclerotiorum* infected *Cirsium arvense* plants; and CA – healthy *Cirsium arvense*. G – green leaf volatiles; B – benzenoids; O – C_8 compounds; M – monoterpenoid; S – mesquterpenoid; I – indole; F – furan; K – ketone and X - unknown 67

Figure 4.3 Plant volatiles emitted in accordance with the pathogen infections and the healthy *Cirsium arvense*. Mean amount of volatiles taken cumulatively according to the classes for each treatment. Mean ± SE shown. PP – *Puccinia punctiformis*-infected *Cirsium arvense* plants; SS – *Sclerotinia sclerotiorum*-infected *Cirsium arvense* plants; and CA – healthy *Cirsium arvense*. G – green leaf volatiles; B – benzenoids; O – C_8 compounds; M – monoterpenoid; S – sesquiterpenoid; I – indole and X – unknown 68

Chapter 1
General Introduction

Not all insects are attracted to all plants and the ones that are attracted to a particular plant have a certain location on the plant, such as leaf, stem, or roots, that it is attracted to. There is a meticulous selection taking place between plants and phytophagous insects. What drives an insect towards a plant? How does it select that particular plant part it feeds on? Why is it not attracted to another plant? How does an insect know where to find that special plant? These are some of the interesting questions scientists have been trying to answer. As insect behaviour towards plants is usually host-specific, these questions still prevail in different insect-plant systems and finding the answers is important, especially in fields such as weed biological control.

Host specificity testings in the field of biological control primarily rely on the centrifugal phylogenetic method (Wapshere 1974), which consider the morphology and biochemical similarity of the plant groups as well as the distantly related economically important plants. The systematic and phylogenetic analysis of plants was traditionally based on morphological characters. The main question addressed in the centrifugal phylogenetic method is what are the plants that need to be safeguarded from the potential biological control agent?

Later Briese (1996), in their paper, discussed the usefulness of phylogenetic studies to biological control by using the two weevil genera *Larinus* and *Rhinocyllus* as examples. Briese (1996) highlights the use of quantitative methods in calculating the phylogenetic distance. The advancement of science has led to discovering the plant phylogenies by using molecular data that increased the accuracy levels of relatedness and give quantitative measurements regarding phylogenetic distances. The main question asked would be what are the plants likely to be attacked by the potential biological control agent? By using the modifications suggested by Briese (1996), the number of potential plant species that are needed to be used in host-specificity testing experiments can be reduced, and the threshold of prediction of likeliness of attack towards a particular plant species will increase.

Schoonhoven et al. (2005) stated that the insects in some way or another are able to identify the taxonomic relationship of plants which highlight the importance of plant phylogeny in host-specific testing. Most herbivores used in biological control are specialized and therefore, their interactions are restricted to either a single plant family or a lower taxonomic group (Bernays & Chapman 1994). More closely related plant species are more similar in morphology and chemistry, and more likely to possess the cues that will make a certain plant recognisable as a potential host by a phytophagous insect (Briese 2003, 2006). Taxonomic relationships are often synonymous with biochemical relatedness (Schoonhoven et al. 2005). Moreover, secondary metabolites have often been shown to be similar within members of a clade (Wink 2003), which can be taken to show plant relatedness. However, not many plant taxa have resolved volatile profiles. If a specialised insect shows a phylogenetically conserved pattern in host-plant selection across its potential host range, that would indicate the insect is tracking (through evolutionary time) traits that are shared by cosely related species. On the other hand, if an insect does not show a phylogenetically conserved pattern of host plant selection, this would suggest that it is tracking key traits that may have arisen through convergent evolution.

Insect host-plant finding behaviour involves the following cues; olfactory, visual, tactile and gustatory (Bernays & Chapman 1994; Schoonhoven et al. 2005). Long range host finding is mainly due to olfactory cues. An insect flying from a distance orient towards the host plants by using the plant smell/ plant volatiles. At proximity, both the visual and olfactory cues may play a role in plant identification. Insects may also use tactile cues, once they have landed on the prospective host. Some have to feed on the plant to identify it to be a suitable host. Short distance identification can involve all the above mentioned cues. Any one of these cues can dominate at any stage of the host finding process. To aid this process, insects have evolved a finely tuned sensory systems, for detection of host cues, and a nervous system, capable of processing the sensory inputs. And the responses to cues depend on the context in which the signals are perceived (Bruce 2015).

Large amounts of plant volatile organic compounds, either constitutive or induced, are released to the plant surface and with the air, they are transported away from their site of production (Visser 1986). An insect flying at a distance can detect these specific volatile blends (Bruce et al. 2005). Insects can either orient towards or away from the direction of the scent (Unsicker et al. 2009). For example, the pepper weevil, *Anthonomus eugenii*, has the ability to orient towards its host plants by using olfactory cues (Addesso & McAuslane 2009), whereas a non-

host plant, *Chrysanthemum morifolium* was reported to repel ovipositing females of the diamondback moth (*Plutella xylostella*) (Wang et al. 2008). Even though the orientation is due to olfaction in the above case some studies have demonstrated the use of other cues in host selection coupled with olfaction. For example, the host plant selection behaviour of adult *Lygus hesperus* depended on both olfactory and visual cues (Blackmer & Canas 2005). Further, the queen ant of *Pheidole minutula* uses volatiles in identifying its host plant *Maieta guianensis* over long distance and uses volatile and tactile cues to identify its host plant at short distance (Dattilo et al. 2009). The importance of these cues in finding their host can be used in pest management practices (Kerr et al. 2017). All of the above mentioned studies focused on constitutive volatiles, which are always emitted regardless of any biotic or abiotic stresses and are largely controlled by genetic and environmental conditions (Delphia et al. 2009; Niederbacher et al. 2015). In addition, volatiles are induced by both abiotic factors such as temperature, light and water (Yuan et al. 2009; Blande et al. 2014) and biotic factors such as pathogens and herbivores (Dicke & Baldwin 2010; Franco et al. 2017). The volatile situation changes when it is damaged by herbivory (Holopainen & Gershenzon 2010; Lucas-Barbosa et al. 2011)). Herbivore induced plant volatiles (HIPVs) can either attract or repel conspecifics and other herbivores or attract enemies of the herbivore, which is why HIPVs are considered as a plant's 'cry for help' (Takabayashi & Dicke 1996; Van Poecke & Dicke 2004; Mithöfer & Boland 2012; Dicke 2015; Shivaramu et al. 2017). For example Addesso et al. (2011) have shown the change in attraction of pepper weevil, *Anthonomus eugenii*, towards its host plant in the precence of HIPVs induced by conspecifics.

Over the past decades, there has been an increase in studies on plant-emitted volatiles in understanding plant-plant, plant-insect or plant-microbe interactions (Dicke & Loreto 2010; Beck et al. 2014). Plant-insect interactions are a major part of biological control. Some plant-insect interactions are mediated by chemicals. It is imporatnt to anlyse these chemical factors to exploit potentially co-evolved relationships which in turn may help explain the mechanisms underlying host-plant utilisation. Understanding mechanisms underlying host-plant utilisation is important in the case of biological control (Wheeler & Schaffner 2013). Currently chemoecological methods are considered for testing the suitability of biological control agents (Wheeler & Schaffner 2013; Park et al. 2018).

The thesis presented here used a phylogenetic approach in evaluating the responses of the beetle *Cassida rubiginosa* Mull. (Coleoptera: Chrysomelidae) towards constitutive and

induced volatiles of plants belonging to the tribe Cardueae. This is the first comprehensive study that follows the modifications to the centrifugal phylogenetic method proposed by Briese (2003). This was done in the context of olfaction, feeding and ovipositing of the beetle *C. rubiginosa*. I have used 16 Cardueae test species (*Cirsium arvense* (L) Scop., *Cirsium vulgare* (Savi) Ten., *Cirsium palustre* (L) Scop., *Ptilostemon afer* (Jacq.) Greuter., *Carduus nutans* L., *Silybum marianum* (L) Gaertn., *Carduus tenuiflorus* Curt., *Centaurea cyanus* L., *Centaurea macrocephala* Puschk., *Centaurea nigra* L., *Cynara cardunculus* L., *Cynara Scolymus* L., *Onopordum acanthium* L., *Carthamus lanatus* L., *Arctium lappa* L., *Echinops ritro* L.). These were pruned from a comprehensive phylogeny of the Cardueae tribe based on nuclear ribosomal DNA and chloroplast DNA markers (Barres et al. 2013; Cripps et al. 2016). Phylogenetic distance was calculated from the total branch lengths seperating each species from *C. arvense*. I have used the same distances used by Cripps et al. (2016) and the phylogeny will be used in Chapters 2 and 3 as the basis of plant selection. Three non-Cardueae species (*Taraxacum officinale* Weber. (Cichorieae), *Inula hookeri* C.B.Clarke (Inuleae), *Tragopogon porri* L. (Cichorieae)) that all belong to Asteraceae were used to see the preference of the beetles towards plants that do not belong to tribe Cardueae. These non-Cardueae plants were selected based on the literature from European countries where *Cassida* (not *C. rubiginosa*) feeding has been reported (Mike Cripps, personal communication).

Scientific studies of the beetle, *C. rubiginosa*, date back to 1917 (Kosior 1975). It is a medium sized (6 – 8 mm) green coloured beetle with yellowish elytral margins (Majka & Lesage 2008) and the life cycle consists of eggs, five larval stages, pupa and adult (Kosior 1975; Cripps et al. 2016). The eggs of *Cassida* are laid in batches on the foodplant. *Cassida rubiginosa* usually deposit eggs on the under surface of the leaves, more rarely on the upper surface and sporadically on the stem. Majority of egg masses (ootheca) will be found on the forepart of leaves, often near the main nerve or at the end of the blade (Kosior 1975) and the number of eggs in a batch ranged from 2 to 9 (Salem et al. 2017). Immediately after hatching larvae begin to feed on the foodplant. Activity of the larve increase with the instars, meaning that the fourth instar larvae is more active than the second instar larvae (Kosior 1975). The body of larva is dorsoventrally flattened and variably elongated. The head is ventrally curved and usually not visible from above. At the body sides 16 featherlike spines are set while the anal segment has a pair of long hairs called abdominal furca (Kosior 1975). Abdominal furca serve as base for the formation and carrying of the covering from exuviae and excrements. Larval development

depend on the climate factors such as air temperature, air humidity and rains (Kosior 1975; Ward & Pienkowski 1978). After the five larval stages they become pupa.

From pupa emerge the young adult which will be greenish yellow in colour and later it will turn out to be green. They will start scraping the leaf tissues where the leaf will have a webbed stucture. Unlike the larvae, adults are mobile even though it is unsure how far they can fly per day. Therefore, they have the opportunity to chose favourable food plants. Young will feed as much as possibe and overwinter under the debris (Cripps et al. 2015). Adults usually become active in early spring (Majka & Lesage 2008), i.e. around mid-September in New Zealand. After overwintering they tend to feed a lot and become sexually mature. Sexual maturity occurs almost at the same time in females and males and mating occurs. In *C. rubiginosa* the female lay eggs within 3 to 7 days after mating (Kosior 1975). Both the adult and the larvae feed on the same host plant and are foliage feeders (Ward & Pienkowski 1978). It is oligophagous on plants belonging to the family Asteraceae (Zwölfer & Eichhorn 1966; Kosior 1975) and mostly found in tribe Cardueae subtribe Carduinae (Cripps et al. 2016). However, the beetle shows marked preference for *Cirsium arvense* (L.) Scop. (Asteraceae, Cardueae) which is considered as its primary host plant (Zwölfer & Eichhorn 1966; Cripps et al. 2016).

The beetle was deliberately introduced to New Zealand in 2007 to control *Cirsium arvense* (Californian thistle or creeping thistle). *Cirsium arvense* is a dioecious perennial herb that is indigenous to Eurasia (Ang et al. 1995) but now found throughout the temperate regions of the world, is considered a noxious weed in agricultural systems (Cripps et al. 2011). The plant reproduces both vegetatively, which is the prominent form of reproduction, as well as from seeds (Ang et al. 1994). To control this thistle an integrated approach using spraying, mowing and biological control was used (Eerens et al. 2002; Burns et al. 2013). Biological control was especially important in areas where the terrain is not easily accessible to machinary and introducing biological control agents may offer the best potential for management of Californian thistle (Eerens et al. 2002). According to Cripps et al. (2011) seven agents were deliberately introduced to control *C. arvense*, namely *Altica carsuorum* Guer (Coleoptera, Chrysomelidae), *Cassida rubiginosa* Muller (Coleoptera, Chrysomelidae), *Ceratapion onopordi* (Kirby) (Coleoptera, Brentidae), *Hadroplontus litura* (F.) (Coleoptera, Curculionidae), *Lema cyanella* (L.) (Coleoptera, Chrysomelidae), *Rhinocyllus conicus* (Frolich) (Coleoptera, Curculionidae) and *Urophora cardui* (L.) (Diptera, Tephritidae). In contrast *Puccinia punctiformis* (Str.) Rohl. (Uredinales, Pucciniaceae) was an unintended

introduction that has shown to affect the plant (Cripps et al. 2011). From these introductions *C. rubiginosa*, *R. conicus* and *P. punctiformis* are estabilished in New Zealand (Cripps et al. 2011) and the best agent so far is the beetle *C. rubiginosa* (Cripps et al. 2015). This chrysomelid is oligophagous but the prospect of this agent to find and attack other thistle weeds in New Zealand had not been investigated. Therefore, investigations were carried out on the host-selection behaviour, giving special reference to olfaction, of *C. rubiginosa* when presented with Cardueae plant species. As mentioned above, 16 plant species (11 Carduinae plants, 4 Centaureinae plants and 1 Echinopsinae plant) belonging to tribe Cardueae were selected to better represent the thistles found in New Zealand. There are no native plants in the tribe Cardueae in New Zealand (Webb et al. 1988; Cripps et al. 2010) and a cost-benefit analysis concluded found that there is no potential damage to economic plants (e.g. Artichoke, safflower and cornflower)(Barratt et al. 2010). This gave an ample opportunity to experiment with the tribe Cardueae without many restrictions. Attempt was taken to combine the discipline of chemical ecology with biological control wherever possible.

In chapter 2, the question was raised whether the beetles' olfactory behaviour towards Cardueae plants is due to the blend of constitutive volatiles they emit and whether the response is phylogenetically conserved. Oviposition and feeding were evaluated in phylogenetically aligned host-choice experiments.

In chapter 3, the role of herbivore-induced volatiles in this system were assessed. This study also tested whether HIPVs change the olfactory preference of the beetle in comparision to constitutive volatiles (chapter 2). Insects may find the host plant using constitutive host-plant volatile cues but once the site has been colonised, feeding damage changes the quality and quantity of the released plant volatiles. This can potentially alter the behaviour of conspecific beetles, rendering the preferred plant less attractive or vice versa, thus expanding the beetle's realised host range.

In chapter 4, the preference of *C. rubiginosa* towards *C. arvense* plants when infected with the biotrophic rust fungus *Puccinia punctiformis* (Str.) Röhl (Basidiomycota, Pucciniaceae) or the generalist necrotroph *Sclerotinia sclerotiorum* (Lib) de Bary (Ascomycota, Sclerotiniaceae) was investigated. Both fungi and chrysomelid are important as biological control agents and require the same niche, i.e. thistle leaves. In weed biological control, it is common practice to introduce multiple agents for a single host plant. But considering the extensive amount of time

and costs required for biological control programmes, it is necessary to ensure the introduced agents do not interact negatively.

Finally, in chapter 5, incorporating the knowledge of the preference and performance of *C. rubiginosa*, its multi-targeting potential was investigated with *Cirsium palustre* (marsh thistle). This species was selected because it is closely related, and equally prefered, by the beetle. The impact of the biocontrol beetle was tested on this secondary weed target by measuring the reduction in seed germination due to foliar feeding of the beetle larvae.

1.1 Reference

Addesso KM, McAuslane HJ 2009. Pepper weevil attraction to volatiles from host and nonhost plants. Environmental entomology 38(1): 216-224.

Addesso KM, McAuslane HJ, Alborn HT 2011. Attraction of pepper weevil to volatiles from damaged pepper plants. Entomologia Experimentalis et Applicata 138(1): 1-11.

Ang BN, Kok LT, Holtzman GI, Wolf DD 1994. Competitive Growth of Canada Thistle, Tall Fescue, and Crownvetch in the Presence of a Thistle Defoliator, *Cassida rubiginosa* Müller (Coleoptera: Chrysomelidae). Biological Control 4(3): 277-284.

Ang BN, Kok LT, Holtzman GI, Wolf DD 1995. Canada Thistle [*Cirsium arvense* (L) Scop.] Response to Density of *Cassida rubiginosa* Müller (Coleoptera: Chrysomelidae) and Plant Competition. Biological Control 5(1): 31-38.

Barratt B, Howarth F, Withers T, Kean J, Ridley G 2010. Progress in risk assessment for classical biological control. Biological control 52(3): 245-254.

Barres L, Sanmartín I, Anderson CL, Susanna A, Buerki S, Galbany-Casals M, Vilatersana R 2013. Reconstructing the evolution and biogeographic history of tribe Cardueae (Compositae). American Journal of Botany 100(5): 867-882.

Beck JJ, Smith L, Baig N 2014. An overview of plant volatile metabolomics, sample treatment and reporting considerations with emphasis on mechanical damage and biological control of weeds. Phytochemical Analysis 25(4): 331-341.

Bernays EA, Chapman RF 1994. Host-Plant Selection by Phytophagous Insects. London, Chapman &Hall.

Blackmer J, Canas L 2005. Visual cues enhance the response of *Lygus hesperus* (Heteroptera: Miridae) to volatiles from host plants. Environmental entomology 34(6): 1524-1533.

Blande JD, Holopainen JK, Niinemets Ü 2014. Plant volatiles in polluted atmospheres: stress responses and signal degradation. Plant, Cell & Environment 37(8): 1892-1904.

Briese D 1996. Phylogeny: can it help us to understand host choice by biological weed control agents. Proceedings of the 9th International Symposium On Biological Control of Weeds. Pp. 19-26.

Briese D 2003. The centrifugal phytogenetic method used to select plants for host-specificity testing of weed biological control agents: Can and should it be modernised? Jacob HS, Briese DT ed. Glen Osmond, Crc for Australian Weed Management. 23-33 p.

Briese D 2006. Host specificity testing of weed biological control agents: initial attempts to modernize the centrifugal phylogenetic method. Proceedings of the fifth California conference on biological control. Pp. 25-27.

Bruce TJA 2015. Interplay between insects and plants: dynamic and complex interactions that have coevolved over millions of years but act in milliseconds. Journal of Experimental Botany 66(2): 455-465.

Bruce TJA, Wadhams LJ, Woodcock CM 2005. Insect host location: a volatile situation. Trends in Plant Science 10(6): 269-274.

Burns EE, Prischmann-Voldseth DA, Gramig GG 2013. Integrated Management of Canada Thistle (*Cirsium arvense*) with Insect Biological Control and Plant Competition under Variable Soil Nutrients. Invasive Plant Science and Management 6(4): 512-520.

Cripps M, Gassmann A, Fowler S, Bourdôt G, McClay A, Edwards G 2011. Classical biological control of *Cirsium arvense*: Lessons from the past. Biological Control 57(3): 165-174.

Cripps M, Jackman S, Roquet C, van Koten C, Rostás M, Bourdôt G, Susanna A 2016. Evolution of Specialization of *Cassida rubiginosa* on *Cirsium arvense* (Compositae, Cardueae). Frontiers in Plant Science 7: 1261.

Cripps MG, Jackman SD, van Koten C, Vink CJ, Dodd MB 2015. Overwintering habitat and the survival of the biocontrol beetle, *Cassida rubiginosa*.

Cripps MG, Edwards GR, Bourdôt GW, Saville DJ, Hinz HL, Fowler SV 2010. Enemy release does not increase performance of *Cirsium arvense* in New Zealand. Plant Ecology 209(1): 123-134.

Dattilo W, Izzo TJ, Inouye BD, Vasconcelos HL, Bruna EM 2009. Recognition of Host Plant Volatiles by *Pheidole minutula* Mayr (Myrmicinae), an Amazonian Ant-Plant Specialist. Biotropica 41(5): 642-646.

Delphia CM, Rohr JR, Stephenson AG, De Moraes CM, Mescher MC 2009. Effects of genetic variation and inbreeding on volatile production in a field population of horsenettle. International journal of plant sciences 170(1): 12-20.

Dicke M 2015. Herbivore-induced plant volatiles as a rich source of information for arthropod predators: Fundamental and applied aspects. Journal of the Indian Institute of Science 95(1): 35-42.

Dicke M, Baldwin IT 2010. The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. Trends in Plant Science 15(3): 167-175.

Dicke M, Loreto F 2010. Induced plant volatiles: from genes to climate change. Trends in plant science 15(3): 115-117.

Eerens J, Seefeldt S, Garry G, Armstrong M 2002. Controlling Californian thistle (*Cirsium arvense*) through pasture management. New Zealand Plant Protection: 111-115.

Franco FP, Moura DS, Vivanco JM, Silva-Filho MC 2017. Plant–insect–pathogen interactions: a naturally complex ménage à trois. Current Opinion in Microbiology 37: 54-60.

Holopainen JK, Gershenzon J 2010. Multiple stress factors and the emission of plant VOCs. Trends in Plant Science 15(3): 176-184.

Kerr JL, Kelly D, Bader MK-F, Brockerhoff EG 2017. Olfactory cues, visual cues, and semiochemical diversity interact during host location by invasive forest beetles. Journal of chemical ecology 43(1): 17-25.

Kosior A 1975. Biology, Ecology and Economic Importance of *Cassida* (Coleoptera, Chrysomelidae, Cassidinae) of the Ojcow National Park. Actazoologica 31(VIII): 252-388.

Lucas-Barbosa D, van Loon JJA, Dicke M 2011. The effects of herbivore-induced plant volatiles on interactions between plants and flower-visiting insects. Phytochemistry 72(13): 1647-1654.

Majka CG, Lesage L 2008. Introduced leaf beetles of the Maritime Provinces, 7: *Cassida rubiginosa* Muller and *Cassida flaveola* Thunberg (Coleoptera: Chrysomelidae). Zootaxa 1811(1): 37-56.

Mithöfer A, Boland W 2012. Plant defense against herbivores: chemical aspects. Annual review of plant biology 63: 431-450.

Niederbacher B, Winkler J, Schnitzler J 2015. Volatile organic compounds as non-invasive markers for plant phenotyping. Journal of experimental botany 66(18): 5403-5416.

Park I, Eigenbrode SD, Cook SP, Harmon BL, Hinz HL, Schaffner U, Schwarzländer M 2018. Examining olfactory and visual cues governing host-specificity of a weed biological control candidate species to refine pre-release risk assessment. BioControl: 1-13.

Salem H, Bauer E, Kirsch R, Berasategui A, Cripps M, Weiss B, Koga R, Fukumori K, Vogel H, Fukatsu T and others 2017. Drastic Genome Reduction in an Herbivore's Pectinolytic Symbiont. Cell 171(7): 1520-1531.e13.

Schoonhoven LM, Van Loon JJ, Dicke M 2005. Insect-plant biology, Oxford University Press on Demand.

Shivaramu S, Jayanthi PDK, Kempraj V, Anjinappa R, Nandagopal B, Chakravarty AK 2017. What signals do herbivore-induced plant volatiles provide conspecific herbivores? Arthropod-Plant Interactions 11(6): 815-823.

Takabayashi J, Dicke M 1996. Plant—carnivore mutualism through herbivore-induced carnivore attractants. Trends in Plant Science 1(4): 109-113.

Unsicker SB, Kunert G, Gershenzon J 2009. Protective perfumes: the role of vegetative volatiles in plant defense against herbivores. Current Opinion in Plant Biology 12(4): 479-485.

Van Poecke R, Dicke M 2004. Indirect defence of plants against herbivores: using Arabidopsis thaliana as a model plant. Plant Biology 6(04): 387-401.

Visser J 1986. Host odor perception in phytophagous insects. Annual review of entomology 31(1): 121-144.

Wang H, Guo W-F, Zhang P-J, Wu Z-Y, Liu S-S 2008. Experience-induced habituation and preference towards non-host plant odors in ovipositing females of a moth. Journal of chemical ecology 34(3): 330-338.

Wapshere A 1974. A strategy for evaluating the safety of organisms for biological weed control. Annals of applied biology 77(2): 201-211.

Ward RH, Pienkowski RL 1978. Biology of *Cassida rubiginosa*, a thistle-feeding shield beetle. Annals of the Entomological Society of America 71(4): 585-591.

Webb C, Sykes W, Garnock-Jones P 1988. Flora of New Zealand Vol. 4. Naturalized Pteridophytes, gymnosperms, dicotyledons.

Wheeler GS, Schaffner U 2013. Improved understanding of weed biological control safety and impact with chemical ecology: a review. Invasive Plant Science and Management 6(1): 16-29.

Wink M 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64(1): 3-19.

Yuan JS, Himanen SJ, Holopainen JK, Chen F, Stewart Jr CN 2009. Smelling global climate change: mitigation of function for plant volatile organic compounds. Trends in Ecology & Evolution 24(6): 323-331.

Zwölfer H, Eichhorn O 1966. The host ranges of *Cassida* spp.(Col. Chrysomelidae) attacking *Cynareae* (Compositae) in Europe. Zeitschrift für angewandte Entomologie 58(1-4): 384-397.

Chapter 2
The role of thistle phylogeny on long-range and short-range host-selection behaviour of the biocontrol agent *Cassida rubiginosa*

Abstract

The oligophagous thistle tortoise beetle *Cassida rubiginosa* was introduced to New Zealand to control the noxious weed *Cirsium arvense*. A phylogenetic approach was used to study the importance of plant volatiles in the long-range and short-range host selection process of female *C. rubiginosa* beetles, and the effect of phylogeny on their feeding and oviposition behaviour was investigated.

A series of single-choice and dual-choice olfactometer and adult host-choice experiments were carried out. The target plant *C. arvense* was tested against 15 species of Cardueae and three non-Cardueae species. Adult host choice experiments using the same plant species were performed to test the feeding and oviposition preference of the beetle.

The results show that as phylogenetic distance from the primary host plant increases, the beetles' preference for alternative hosts decreases. This was the case for all three parameters: olfactory choice, feeding and oviposition preference. Furthermore, larval performance showed a narrower host-range compared with adult performance. Olfactory experiments showed similar host range to host choice experiments across the Cardueae tribe, suggesting that olfactory experiments may be sufficient to predict host range.

Keywords Centrifugal phylogeny, oviposition, feeding, Cardueae, host-range

2.1 Introduction

Degree of specialisation is a key point that determines the safety of an insect biocontrol agent used in weed biological control programme. The centrifugal phylogenetic method of Wapshere (1974) has been used to select test plants for candidate biological control agents. This method is based on taxonomic circumscription which uses a sequence of plants ranging from most closely related to more distantly related plants to the weed species, until the host range has been adequately circumscribed. However, this method involves testing an extensive number of plant species and is time-consuming and the basic aim is to ensure the minimal risk of non-target damage to economic and native species. Despite 99% of safety predictions when using this method, non-target attacks still occur from deliberately introduced biocontrol agents (Suckling & Sforza, 2014; Willis et al., 2003). Thus, there are now concerns among scientists to modernise the procedure of selecting test plant species. Briese (2003) proposed a model that makes use of recent advances in plant phylogeny based on the selection of test plant species on representative clades by using measurable phylogenetic separation from the target weed. This

put emphasis on plants that would rather be attacked by the agent (Briese, 2005), whereas in taxonomic circumscription the focus is towards the plants that need to be safeguarded from attacks. The new model will focus on the host-range rather than the individual plant assuming that more closely related plant species are more similar in aspects such as morphology and chemistry and more likely to possess cues that will make a certain plant recognisable as a potential host by a phytophagous insect (Briese, 2005). Specialised phytophagous insects often show a phylogenetically conserved pattern to host-plant utilisation allowing biological control practitioners to use phylogeny to reduce the list of test plant species.

Weed biocontrol practitioners traditionally relied on a series of no-choice and choice tests in the laboratory followed by garden experiments to assess the feeding, oviposition and development of the selected insect species during the pre-release host-specificity testing procedure (Briese, 2005). Often behavioural responses to olfactory cues are neglected (Park et al., 2018). Plant volatiles play an important role in locating and recognising a host plant (Bruce et al., 2005; Bernays and Chapman, 1994). Host recognition often involves identifying a particular blend of volatiles rather than a single key component. It is also thought that the ratios of volatile compounds involved in making the specific blend allow the insect to clearly discriminate between an appropriate and inappropriate host (Bruce and Pickett, 2011; de Bruyne and Baker, 2008; Bruce et al., 2005). Volatiles work as long- and short-range directional cues for insects in identifying plants. Thus, odour cues elicit certain behavioural responses in insects. Ehrlich and Raven (1964) have shown that many herbivores tend to seek plant food sources that share common characteristic classes of chemicals. Numerous studies have shown the involvement of plant chemistry in the process of host location, acceptance and determining the pattern of utilisation of plants by herbivorous insects (Bruce et al., 2005; Beck et al., 2014; Becerra, 1997; Ehrlich and Raven, 1964). Rapid evolutionary changes in plant chemistry have been shown to effect the efficacy of the biological control agents. For example a study using Chinese tallow (*Triadica sebifera*) populations from the introduced and native ranges to generalist (*Cnidocampa flavescens*) and specialist herbivores (*Gadirtha inexacta*) in the native range have shown that the specialists grew larger and consumed more mass on an invasive plant population, whereas generalist herbivores showed a similar performance between them. Further, the chemical analysis demonstrated that the invasive plants had lower tannin content, suggesting that a novel biotic environment may elicit evolutionary changes in the chemical composition of the plant species that affect the performances of the insects acting upon it (Huang et al., 2010). Therefore, ultimately, chemically mediated interactions have been

shown to influence the success (impact on target weed) or failure (impact on non-target plants) of classical biological control projects (Wheeler and Schaffner, 2013). Thus, it is also important to include testing on chemical cues during the pre and post release testing process of a candidate biological control agent (Park et al., 2018).

Previously, survival of the larvae of *Cassida rubiginosa* was assessed in relation to plant defensive traits (specific leaf area, leaf pubescence, flavonoid concentration, carbon and nitrogen content) in phylogenetically controlled experiments with 16 Cardueae plants (Cripps et al., 2016). There was a strong phylogenetic signal for *Cassida* survival where survival of *Cassida* larvae decreased with increasing phylogenetic distance. The larvae of *Cassida* have low mobility, therefore, most of the time larvae are forced to feed on the plant the adult female beetle has chosen to lay its eggs. The beetle is considered oligophagous and attacks on other thistles have been recorded (Hettiarachchi et al., 2018; Zwölfer and Eichhorn, 1966). But the level of preference towards those thistle plants that the beetle has chosen has not been documented. This is the first study to assess the plant host range with their preference level for each selected plant of *C. rubiginosa* within New Zealand, which will lead to exploring additional benefits in terms of population reduction of other thistles. Further, it is understood that this is the first phylogenetically controlled herbivore olfactory host-selection study. Here, behavioural bioassays of olfactory cues are employed followed by feeding and oviposition experiments conducted with plants belonging to tribe Cardueae to determine the host range of the thistle tortoise beetle, *C. rubiginosa* in New Zealand. Quantitative measures of phylogenetic distance were used rather than taxonomic circumscription.

This study hypothesised that 1) the host choice, feeding and oviposition of the beetle is phylogenetically conserved and the beetle would make host plant choices that are adaptive for the survival of their offspring, and 2) the preference of the beetle decreases according to phylogenetic distance and it is based on the decreased similarity of the volatile blend.

2.2 Material and Methods

2.2.1 Plants

The Cardueae is considered a monophyletic tribe that originated during the mid-Eocene and subtribal diversification originated throughout the Oligocene – Miocene period (Barres et al.,

2013). Tribe Cardueae is one of the largest tribes included in the family Asteraceae and has a nearly completely resolved phylogeny (Barres et al., 2013; Susanna et al., 2006;). Sixteen plant species within tribe Cardueae (*Cirsium arvense* (L) Scop., *Cirsium vulgare* (Savi) Ten., *Cirsium palustre* (L) Scop., *Ptilostemon afer* (Jacq.) Greuter., *Carduus nutans* L., *Silybum marianum* (L) Gaertn., *Carduus tenuiflorus* Curt., *Centaurea cyanus* L., *Centaurea macrocephala* Puschk., *Centaurea nigra* L., *Cynara cardunculus* L., *Cynara Scolymus* L., *Onopordum acanthium* L., *Carthamus lanatus* L., *Arctium lappa* L., *Echinops ritro* L.) were selected according to their phylogenetic relationships (Figure 2.1). The phylogenetic distances of the 16 Cardueae test species were reported previously by Cripps et al. (2016). Three non-Cardueae (Compositae); *Taraxacum officinale* Weber. (Cichorieae), *Inula hookeri* C.B.Clarke (Inuleae), *Tragopogon porri* L. (Cichorieae) were also included in the testing procedure. Plant species in which beetle feeding has been reported in other countries, which are present in New Zealand, and where there is a potential of being a target, were considered when selecting test plants that do not belongs to tribe Cardueae. All the test species used in this study were either deliberately or inadvertently introduced species to New Zealand (Cripps et al., 2013). Selected Cardueae plants for this study provide good representation of New Zealand thistles, as they include species from three (Carduinae, Echinopinae and Centaureinae) of the four subtribes and contain geographically widespread species-rich genera (e.g. *Cirsium*, *Centaurea*) as well as narrowly distributed species-poor genera (e.g. *Ptilostemon*, *Cynara*) (Cripps et al., 2016; Barres et al., 2013; Susanna et al., 2006).

All the plants were grown from seeds, either collected from the field or purchased from a commercial supplier (Kings Seeds NZ Ltd.). Seeds were sown from 15 June 2015 to 2 September 2015 for the first batch, from 9 August 2016 to 6 September 2016 for the second batch and 12 September 2017 for the third batch. The same conditions were provided for the plants during the process. Seedlings were grown in the glasshouse at AgResearch, Lincoln, and final potting size was 12 L. Standard potting mix (54% aged bark, 45% sand, 1% nutrients, by weight) containing added nutrients of Osmocote® 17-11-10 (N-P-K), lime, superphosphate, sulphate of potash and calcium nitrate were used to grow the plants. Plant identifications were verified by the expert Trevor James at AgResearch.

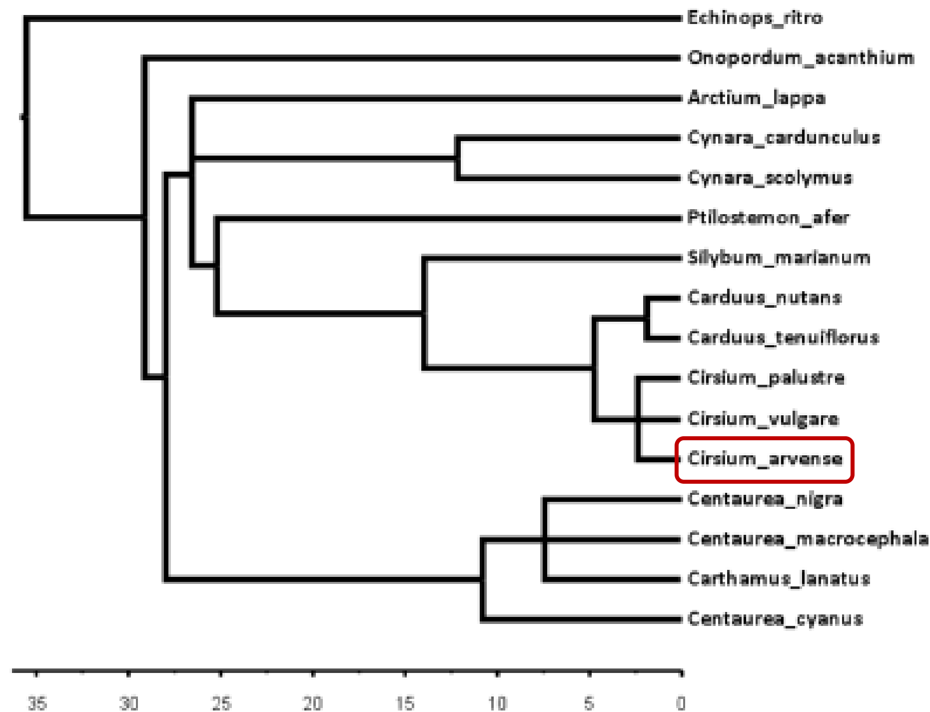

Figure 2.1 Chronogram of the 16 Cardueae test plant species pruned from a comprehensive phylogeny of the tribe (Barres et al., 2013). Branch length depicts phylogenetic distance in millions of years (Cripps et.al., 2016). *Cirsium arvense* was taken as the starting point when calculating the phylogenetic distance.

2.2.2 Insect

The tortoise beetle, *Cassida rubiginosa* Müller (Coleoptera: Chrysomelidae), is specialised to host plants belonging to tribe Cardueae. Its primary host *Cirsium arvense* is a noxious weed found worldwide (Zwölfer and Eichhorn, 1966). The beetle is native to the Palearctic region and considered an oligophagous feeder that feeds on a range of Cardueae plants (Zwölfer and Eichhorn, 1966; Webb et al., 1988). The beetle was deliberately introduced to New Zealand in 2007 (Cripps et al., 2011) and found to have potential to control *C. arvense* (Cripps et al., 2010; Bacher and Schwab, 2000; Ang et al., 1995). The beetle is univoltine and both the adult and larvae are leaf feeders. Adults overwinter under debris and hedges or forest margins and emerge in spring seeking host plants where they feed and deposit their egg masses. Egg masses or oothecae are mostly found on the underside of the leaves. Their life cycle consists of five larval stages and are mostly confined to the plant their mother has chosen for them until they become adults (Tipping, 1993).

The beetles were field collected from farms in Wairarapa area and kept in 2 L ventilated plastic boxes (the lid was modified by cutting an area of 105 mm x 155 mm and covering that area with a mesh of 0.5 mm) under constant temperature of 20 ºC, inside CT rooms (controlled-temperature room; 16h light: 8h dark period) at Lincoln University and fed with *C. arvense* clippings. Only adult female beetles (weight of a beetle ≤ 19 mg, taken as males; and beetles >19 mg, taken as females) were used and a beetle was used only once for an experiment. Randomly selected beetles were preserved using 70% alcohol and dissected later to confirm the sex.

2.2.3 Olfactometer experiments

Small still air olfactometers consisted of a glass Petri dish (140 mm diameter x 20 mm high) were used (Van Tol et al., 2002). Two holes, 12 mm diameter and 90 mm apart in the Petri dish lid were demarcated by small glass tubes of 25 mm high. Small glass cylinder (length 70 mm; diameter 55 mm) was placed on top of that glass tube such that the glass tube was positioned in centre. A glass cup (length 75mm; diameter 59 mm) with a rim was placed above the glass cylinder, interlocking them (Van Tol et al., 2002) and making a chamber (Figure 2.2). Olfactometers were washed with water, distilled water, acetone (100%) and hexane (99.5%) prior to each experiment in order to make sure that they were void of impurities and odour. Rigid, green colour nylon mesh (1 mm x 1 mm) was placed between the cylinder and the cup to prevent the beetles entering the glass cup where the plant material was kept. The green colour mesh was used to decrease the visibility of the plant material. Plant leaves were cut, and the cut part of the leaves was immediately wrapped in wet cotton balls and then with 2 inch wide laboratory film (Parafilm; PM-992) to prevent the drying of cotton wool. The experiments were started around 10:00-10:30 am where beetles were active inside the CT (23 ºC) rooms in Lincoln University during November to February 2015, 2016 and 2017. The humidity of the olfactometers were maintained between 55–60% and measured using a hygrometer (Martinez and Hardie, 2009). The beetles had two choices: to enter one of the chambers through the glass tubes of the olfactometer. The beetles that remained on the base of the olfactometer were not taken into consideration.

Preliminary experiments

It was essential to be certain that the beetles' choice was purely due to the volatiles. Therefore, two experiments were conducted. The first experiment was conducted by covering the leaf material and the control, which was the one used with no-choice experiments (described below), with gauze, ensuring that the material inside was not visible. The second experiment was conducted using a transparent glass plate between the glass tube and the glass cylinder so that the beetle could see the plant material inside the cylinder, but the volatiles were blocked. *Cirsium arvense* and *P. afer* were used in the chambers of the olfactometer. *Ptilostemon afer* is morphologically different to the primary host *C. arvense* and larvae did not survive on this plant during previous experiments (Cripps et al., 2016). For each plant species twenty replicates were conducted with 20 olfactometers. Three adult female beetles were used in each replicate.

No-choice tests

Plant leaves of similar age (2nd or 3rd leaves from the top) were cut from the petiole. Leaf petioles were covered with damp cotton wool to prevent wilting. A leaf of each plant species was then carefully placed inside one of the test chambers of the olfactometer. Inside the other chamber, damp cotton wool (2 ml of water was used to dampen it to maintain humidity) was placed with a green paper cut in the shape of a leaf; here after "paper mimic" (Figure 2.2 a). A paper mimic and damp cotton wool were used to minimise the effect of visual cues and standardise the experiment (Martinez and Hardie, 2009). Three adult female beetles were used in each of the olfactometer experiment and those beetles that were used were not used repeatedly. The beetles were given 6 hours to choose one arm and the arm that they chose was recorded. For each of the 19 plant species ten replicates were carried out. In total, 570 (19x3x10) adult female beetles were used for the experiment. Beetles were kept on *C. arvense* prior to the experiment.

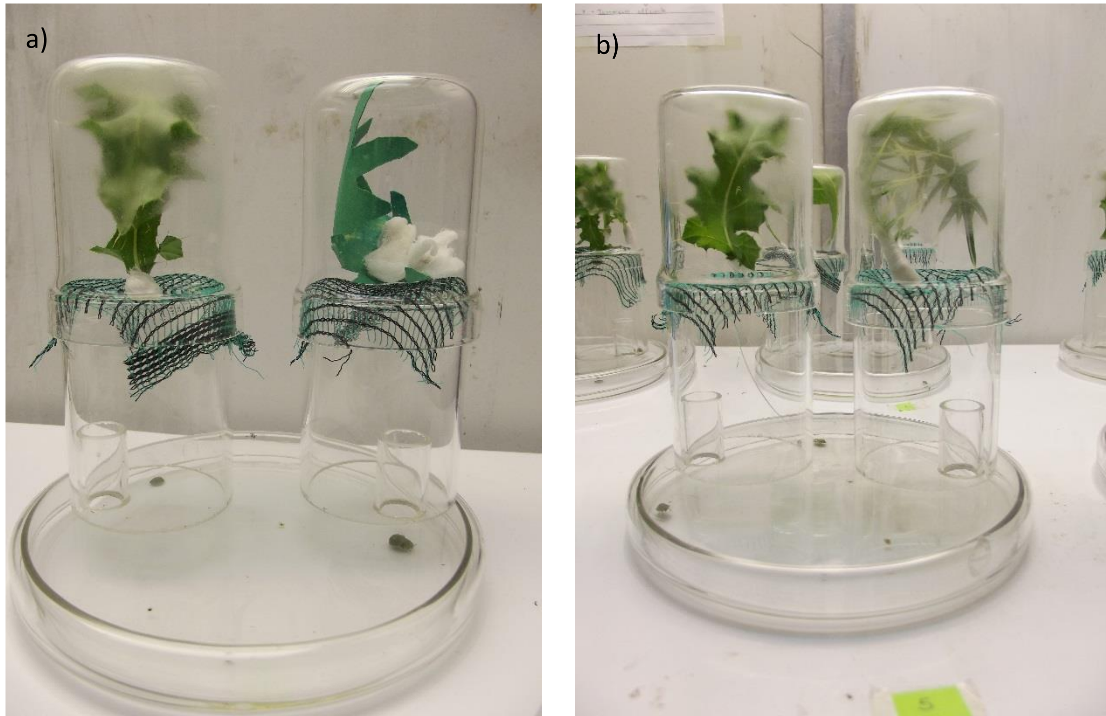

Figure 2.2 Olfactometer set up a) no-choice experiment where the plant leaf is compared with a paper-mimic and cotton ball inside the olfactometer and b) choice experiment where *Cirsium arvense* was compared with a leaf from another test plant

Choice test

Choice was given to the beetles by having *C. arvense* leaves (2nd or 3rd leaves from the top) in one chamber and test plant species leaves (2nd or 3rd leaves from the top) in the other chamber (Figure 2.2 b). The experiment was set up similar to the no-choice test with the same conditions. Three adult female beetles were used once in each of the olfactometer experiments. The beetles were given 6 hours to choose one arm and their choice was recorded. For each of the 18 plant species, 10 replicates were used with 540 (18x3x10) adult female beetles.

2.2.4 Volatile extraction

Volatile organic compounds (VOCs) were collected from undamaged plant leaves. In addition, empty odour-source vessels (control) were sampled to detect impurities in the system. Four leaves (from 2nd leaf till 5th leaf) were placed in one glass vessel (volume 520 ml). The cut end was immediately submerged in 30 ml of water to reduce the emission of volatiles and desiccation during volatile collection. At the beginning of each collection, odour source vessels were thoroughly washed with distilled water, acetone (100%) and hexane (95.5%) respectively,

to minimise impurities and odour. A push-pull system was used as described in Rostás and Eggert (2008). After setting up, the vessels were left for 48 hours to allow the volatiles to accumulate. Filtered air (activated charcoal filter, 400 ml; Alltech, Deerfield, IL, USA) originating from a compressed air cylinder was pushed into the vessels through one of the teflon tubes at a rate of 0.8 $lmin^{-1}$ and the air was pulled out through a trapping filter consisting of a glass tube (7 cm) containing 30 mg of 80–100 mesh SuperQ absorbent (Alltech, Deerfield, IL, USA), at the same rate by using a vacuum pump (Me2, Vacuubrand, Wertheim, Germany). Each collection lasted for 6 hours. The absorbed compounds were eluted from the trapping filter with 100 µl dichloromethane and 200 ng tetralin (Sigma-Aldrich, Australia) was added after the elution as an internal standard. All extracts were stored at -80 °C until analysed.

Samples were analysed for VOCs using an adaption of the method described by Rostás et al. (2015). A Shimadzu GCMS-QP2010 Ultra (Shimadzu, Japan) gas chromatography mass spectrometer (GC-MS) was fitted with a Restek Rtx-5ms fused silica capillary column (30.0 m x 0.25 mm internal diameter, 0.25 µm film thickness, Bellefonte, PA, USA). A CTC-Combi PAL auto sampler (PAL LHX-xt) was used to inject 1µL of sample into the gas chromatography (GC) injection port at 220 °C, operating in split-less mode at 241.0 kPa pressure for 39 seconds. After injection, the column oven was held at 35 °C for 3 minutes, then heated to 320 °C at 8 °C min^{-1} and held at this temperature for 8 minutes. Helium was used as the carrier gas with the constant linear velocity set at 44.0 cm/sec (1.5 ml min^{-1}) in split mode (30:1) after the high-pressure split-less injection. The mass spectrometer (MS) was operated in electron impact ionisation mode with 70eV and mass range of 33 to 550 m/z. The temperature of the capillary interface was 320 °C, with the source temperature set at 230 °C.

Tentative identification of detected peaks was made by matching their retention indices and mass spectra with the spectra of reference compounds found in the databases NIST EPA/NIH Mass Spectral Library database (National Institute of Standards and Technology, NIST11) and Wiley Registry of Mass Spectral Data 10th edition (John Wiley & Sons, Hoboken, New Jersey, USA).

2.2.5 Host-choice experiments

Adult host-choice experiments were conducted at AgResearch, Lincoln, during October 2015 and 2016 (detailed in section 2.2.1). In no-choice experiments, all 19 plant species were used,

whereas in choice experiments the 18 plant species mentioned above (detailed in section 2.2.1) were compared with *C. arvense*.

No-choice tests

Experiments were conducted during October 2015 at AgResearch, Lincoln, outside in an enclosed compound. Four replicates were conducted with the 19 plant species arranged in a randomised complete block design. Plants were individually covered with a cloth mesh sleeve, 125 cm long; 96 cm wide with 1 x 1 mm holes for ventilation, with two wire struts, that held the sleeve off the plant. Watering was done three times per day for 5 minutes via an irrigation system that delivered water directly to the soil of the pot via a Dutch spike. Four adult beetles, two females and two males, were released on to the soil of each potted plant species and left on the plants without disturbing. Plants were observed for the number of feeding holes and egg masses 5 days after the initial setup. At the end of the experiment, randomly selected beetles (71 beetles) were preserved, after weighing, in 70% alcohol and dissected to confirm their sex. Egg masses were collected and observed until all the larvae were deemed to have emerged from the egg masses. Total number of larvae emerged were counted for egg masses found on each plant species.

Dual-choice tests

The above mentioned 18 plant species were compared to test the host preference of the beetle with its primary host *C. arvense* by using ten replicates for each plant species. Insect rearing tents (L60 x W60 x H60 cm, Bugdorm, Meview Science Ltd, Taiwan) were used for this experiment. The first experiment, with 18 test plant species, started on 18 November 2016 and others followed every three consecutive days, where with the last one set-up on 21 December 2016. By the time the experiments were set up, the plants were about 3 months old and the rosettes had more than five leaves on them. Two plants (*C. arvense* and the test plant) were placed 15 cm apart from each other inside the cage and the beetles, two females and two males, were placed in the centre of the cage between the two pots (7.5 cm from each potted plant). The plants were watered (15 ml) on the second day regardless of the weather and left undisturbed for 3 days letting the beetles choose a preferred plant. The number of feeding holes and egg masses on each plant species were recorded. A beetle was used only once, and new plants were used for each replicate during the experiment. At the end of the experiment, randomly selected beetles (100 beetles) were preserved, after weighing, in 70% alcohol and dissected to determine their sex. Egg masses were collected and observed inside Petri dishes

under 20 °C, until all the larvae were deemed to have emerged from the egg masses. Total number of larvae emerged were counted for egg masses found on each replicate of each plant species.

2.2.6 Statistical analysis

Data analysis of the olfactometer experiments was conducted using generalised linear model analysis (GLM) assuming Poisson distribution through log link function. Only the beetles that had made a choice were included in the analysis. Preference index (PI) was calculated following the below equation for olfactometer no-choice tests:

$$\frac{(\#\ \textit{Cassida}\ \text{beetles preferring test leaf} - \#\ \textit{Cassida}\ \text{beetles preferring the control chamber})}{\text{Total number}}$$

For olfactometer choice tests:

$$\frac{(\text{Number of}\ \textit{Cassida}\ \text{beetles preferring test plant} - \text{Number of}\ \textit{Cassida}\ \text{beetles preferring CA})}{\text{Total number}}$$

The analysis for feeding and oviposition behaviour of the beetle during the adult host-choice experiment, no choice condition, were conducted using a generalised linear model (GLM) assuming plant-species-specific negative binomial distributions through log link function. This GLM consisted of only a single factor plant, and was applied only to data from plant groups having non-zero means. The comparison of each of these plant groups with non-zero means to groups with zero-means was made with the Fisher's exact test (also known as two-sample binomial exact test), by comparing the probability of having at least one feeding hole/ one egg mass between them. The relationship of the number of feeding holes to phylogenetic distance was investigated using another negative binomial GLM with log link function, which consisted of only phylogenetic distance covariate. Comparisons of each of the feeding and oviposition with that of the *C. arvense* (CA) during choice tests was made on each choice experiment separately from the others using GLM consisting of a single factor plant with two levels: CA or test plant. The observed larval numbers were $\log_e(x+1)$-transformed. Then, the comparison between CA and test plant was made on each choice experiment separately from the others, using analysis of variance (ANOVA). Each ANOVA consisted of a single factor plant. For experiments in which no larvae were observed on test plant in all replicates, the mean value of the $\log_e(x+1)$-transformed larval numbers on CA was compared against zero value, using one-sample *t* test. Minitab 17.0 and SAS version 9.3 was used for the analysis. PI was calculated for choice experiments by using the following equation:

$$\frac{\text{(Number of } \textit{Cassida} \text{ beetles preferring test plant - Number of } \textit{Cassida} \text{ beetles preferring the CA)}}{\text{Total number}}$$

In each case PI was compared with phylogenetic distance of the test plants from *C. arvense*. The index value lies between (-1) and (+1) inclusive, with (+1) indicating perfect preference towards the test plant and (-1) indicating perfect preference towards the CA. Because of this limited range of possible index values, a nonlinear relationship described by the following logistic curve was assumed, and this relationship - i.e. two parameters *a* and *b*, were estimated with nonlinear regression analysis by using Minitab version 16:

$$\text{Preference index value} = 1 - 2 / (1 + \exp(a * \text{Phylogenetic distance} - b))$$

Cirsium arvense leaves were not tested against *C. arvense* leaves which would have resulted in a near zero value for the PI and would not accommodate the possibility for the beetle to show greater preference towards other test plant species.

The volatile data were analysed for phylogenetic relationship using Bray-Curtis similarity measures and hierarchical cluster analysis.

2.3 Results

2.3.1 Olfactometer experiments

Preliminary experiments

The beetle showed significant preference (Chi-Sq. = 7.56, df = 1, P = 0.006) towards the volatiles emitted by *C. arvense* leaves covered by gauze over the cotton ball plus paper mimic covered by gauze when tested in the same set up. But it did not show a significant preference (Chi-Sq. = 0.78, df = 1, P = 0.376) towards any chamber when tested with *P. afer* and *C. arvense* with a glass plate separating the cylinder from the tube of the olfactometer that blocked volatiles. Plant volatiles play a role in host finding behaviour of adult *C. rubiginosa*.

No-Choice tests

Female *C. rubiginosa*, showed a significant preference towards the olfactometer chamber containing *C. arvense* (Chi Sq. = 13.72, df = 1, P < 0.0001, PI = 0.69), *C. vulgare* (Chi Sq. = 10.82, df = 1, P = 0.01, PI = 0.70), *C. palustre* (Chi Sq. = 4.61, df = 1, P = 0.032, PI = 0.41), and *C. tenuiflorus* (Chi Sq. = 5.48, df = 1, P = 0.019, PI = 0.48) compared to the control

chamber. Preference indices were used in measuring the relationship with the phylogenetic distance. The beetles' preference towards *C. vulgare* (PI = 0.70) was close to its' primary host (PI = 0.69). The beetles' preference towards test plants decreased significantly (nonlinear regression, P = 0.001) as the phylogenetic distance of the plant increased from the main host *C. arvense* (Figure 2.3). Non-Cardueae species did not show any significant attraction during the experiment (Appendix II).

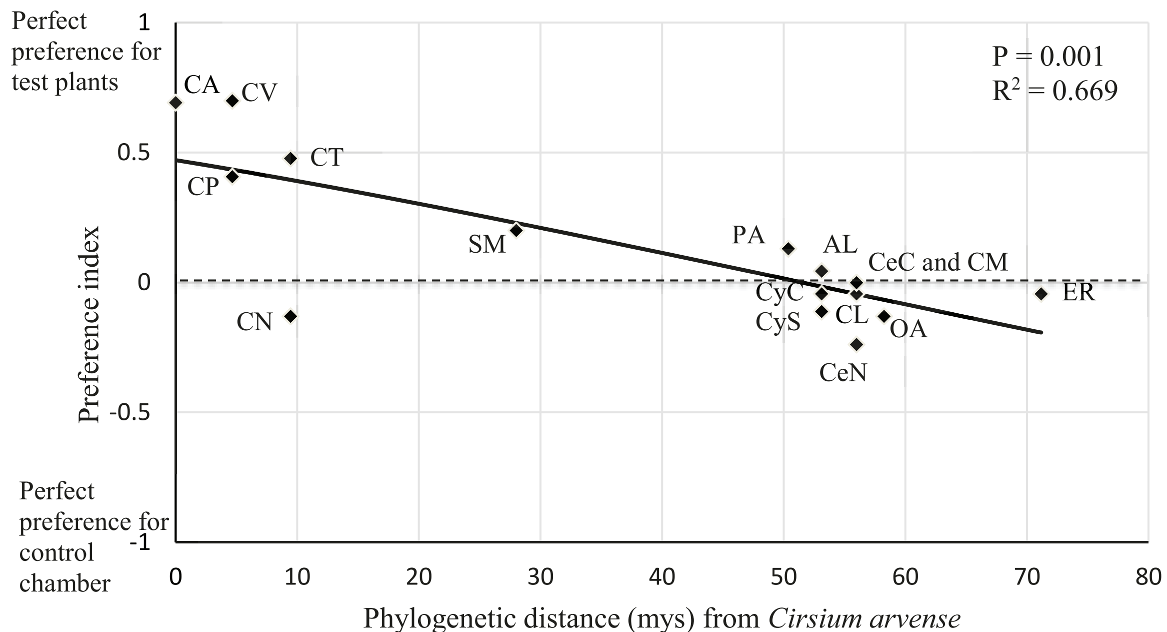

Figure 2.3 Relationship between phylogenetic distances (millions of years) from *Cirsium arvense* and preference of *Cassida rubiginosa* towards leaf chamber over the control chamber of the olfactometer. PI = (Number of beetles that chose plant leaf chamber– Number of beetles that chose control chamber) / Total number. Index value is between –1 and +1 inclusive, with (+1) indicating perfect preference of test plant chamber over control chamber and (–1) perfect preference towards the control chamber over the test plant chamber. Preference index of plant = 1 – 2 / (1 + exp (– 0.0198 * Phylogenetic distance + 1.02146)) where a = 0.0198 ± 0.0046 (estimate ± standard error of estimate) and b = 1.02146 ± 0.2182. CA - *Cirsium arvense*, CV - *Cirsium vulgare,* CP - *Cirsium palustre,* CT - *Carduus tenuiflorus,* CN - *Carduus nutans,* SM - *Silybum marianum,* AL - *Arctium lappa,* CL - *Carthamus lanatus,* CyS - *Cynara scolymus,* CyC - *Cynara cardunculus,* PA - *Ptilostemon afer,* CeC - *Centaurea cyanus,* OA - *Onopordum acanthium,* CeN - *Centaurea nigra,* ER - *Echinops ritro,* CM - *Centaurea macrocephala.*

Choice test

When beetles were given a choice between *C. arvense* leaves and test plant leaves no significant preference was shown for *C. vulgare* (Chi Sq. = 0.04, df = 1,P = 0.847, PI = - 0.04), *C. palustre* (Chi Sq. = 0.17, df = 1, P = 0.683, PI = - 0.08), *C. tenuiflorus* (Chi Sq. =1.40, df = 1, P = 0.237,

PI = 0.23), *C. nutans* (Chi Sq. = 0.36, df = 1, P = 0.548, P = - 0.12), *C. scolymus* (Chi Sq. =3.06, df = 1, P = 0.080, PI = - 0.33), *A. lappa* (Chi Sq. =1.84, df = 1, P = 0.175, PI = - 0.26), *C. macrocephala* (Chi Sq. = 0.57, df = 1, P = 0.449, PI = - 0.14) and *C. lanatus* (Chi Sq. = 1.52, df = 1, P = 0.218, PI = 0.25). Based on volatile cues these species were equally preferred by the beetle compared with *C. arvense*. *Carduus tenuiflorus* (PI = 0.23) was preferred by the beetle over the *C. arvense*, its primary host. None of the non-Cardueae species showed significant attraction when compared with *C. arvense* (Appendix III). Dual-choice olfactometer tests showed that the beetle's relative preference for alternative host plant volatiles decreased with increasing phylogenetic distance from *C. arvense* (nonlinear regression, P = 0.001, Figure 2.4).

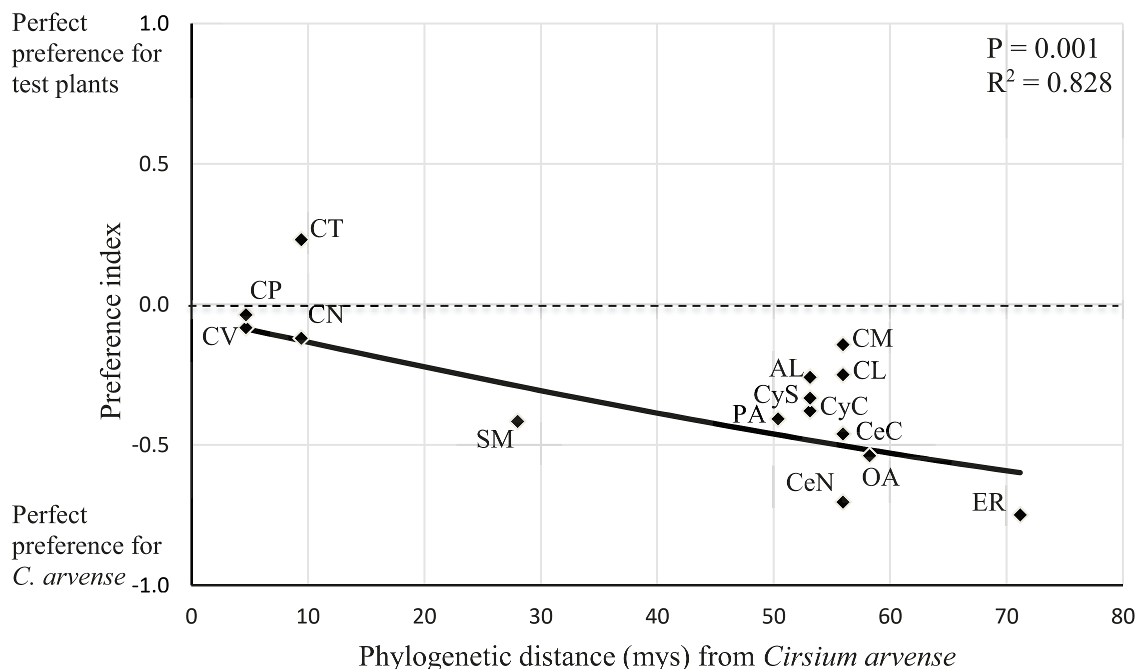

Figure 2.4 Relationship between phylogenetic distances (millions of years) from *Cirsium arvense* and olfactory preference of *C. arvense* volatiles. PI = (Number of beetles that chose test plant – Number of beetles that chose *C. arvense*) / Total number. Index value is between –1 and +1 inclusive, with (+1) indicating preference of test plant over *C. arvense* and (–1) preference towards *C. arvense* over the test plant. Preference index value = 1 – 2 / (1 + exp (0.0182 * Phylogenetic distance – 0.0886)) where $a = 0.0182 \pm 0.0045$ and $b = 0.0886 \pm 0.1957$. CA - *Cirsium arvense*, CV - *Cirsium vulgare,* CP - *Cirsium palustre,* CT - *Carduus tenuiflorus,* CN - *Carduus nutans,* SM - *Silybum marianum,* AL - *Arctium lappa,* CL - *Carthamus lanatus,* CyS - *Cynara scolymus,* CyC - *Cynara cardunculus,* PA - *Ptilostemon afer,* CeC - *Centaurea cyanus,* OA - *Onopordum acanthium,* CeN - *Centaurea nigra,* ER - *Echinops ritro,* CM - *Centaurea macrocephala.*

2.3.2 Volatile extraction

A total of 104 constitutive volatile compounds were found in the 16 Cardueae test plant species. This included six benzenoids, four green leaf volatiles, 20 monoterpenes, 48 sesquiterpenes, ten other volatiles and 16 unidentified volatiles. Commonly appearing volatiles were sesquiterpenoids and monoterpenes (Figure 2.5). Sesquiterpenoids such as: β-caryophyllene (% plant species), germacrene D, α-murolene, δ-cadinene, cyclosativene, α-copaene and monoterpenes such as: α-pinene, sabinene, β-myrcene, β-ocimene were found in most of the plant species (Appendix I). However, no combination of volatiles was found to be specific to the plants that were attractive to the beetle in the olfactometer experiments. There were some unknown sesquiterpenes that were found unique to certain plant species (Appendix I). Chemical compounds such as β-cis-ocimene, β-caryophyllene, α-copaene and cyclosativene were found in all *Cirsium* and *Carduus* species. Certain compounds were only absent in one plant species from the tested group of *Carduus* and *Cirsium* plants: α- humulene was absent only in *C. tenuiflorus*, germacrene D was absent only in *C. vulgare*, δ-cadinene was absent only in *C. palustre*, and caryophyllene oxide was absent only in *C. nutans.* There was no phylogenetic relationship between the collected volatiles (Bray-Curtis dissimilarity measure, P = 0.434). Hierarchical cluster dendrogram (Figure 2.6) have shown neighbouring relationships between *C. palustre* and *C. tenuiflorus* (both branched from *C. arvense* at 0.54).

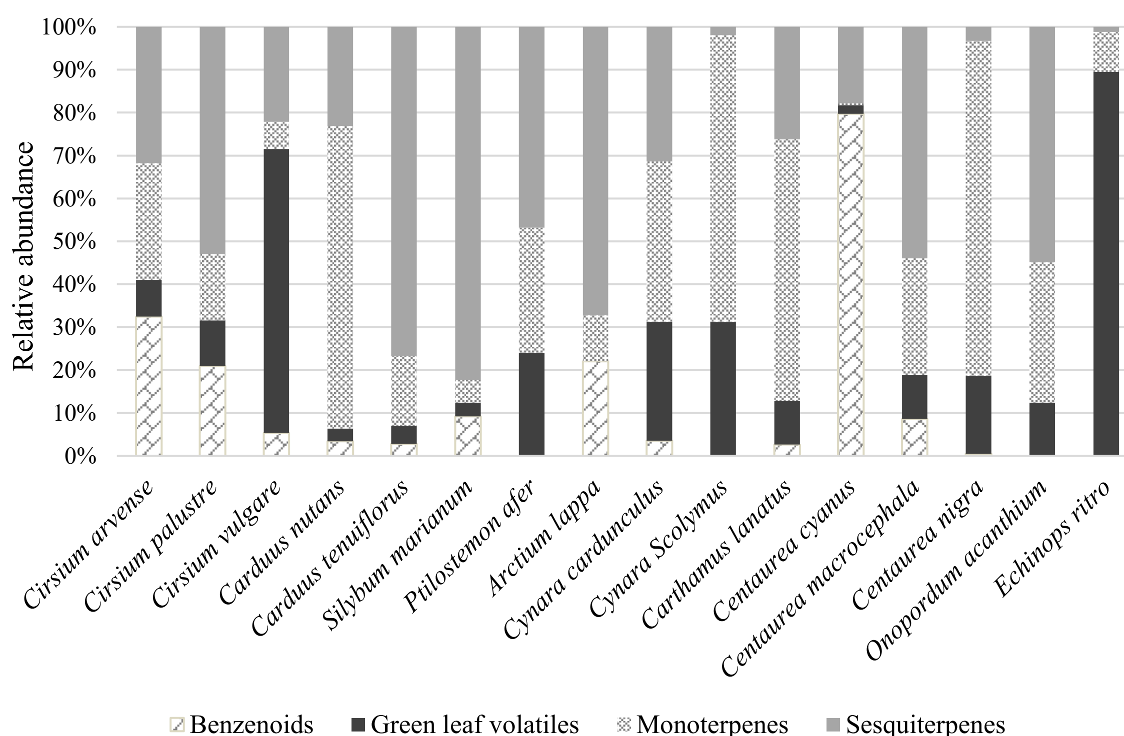

Figure 2.5 Relative abundance of chemical compound detected in the 16 Cardueae plant species.

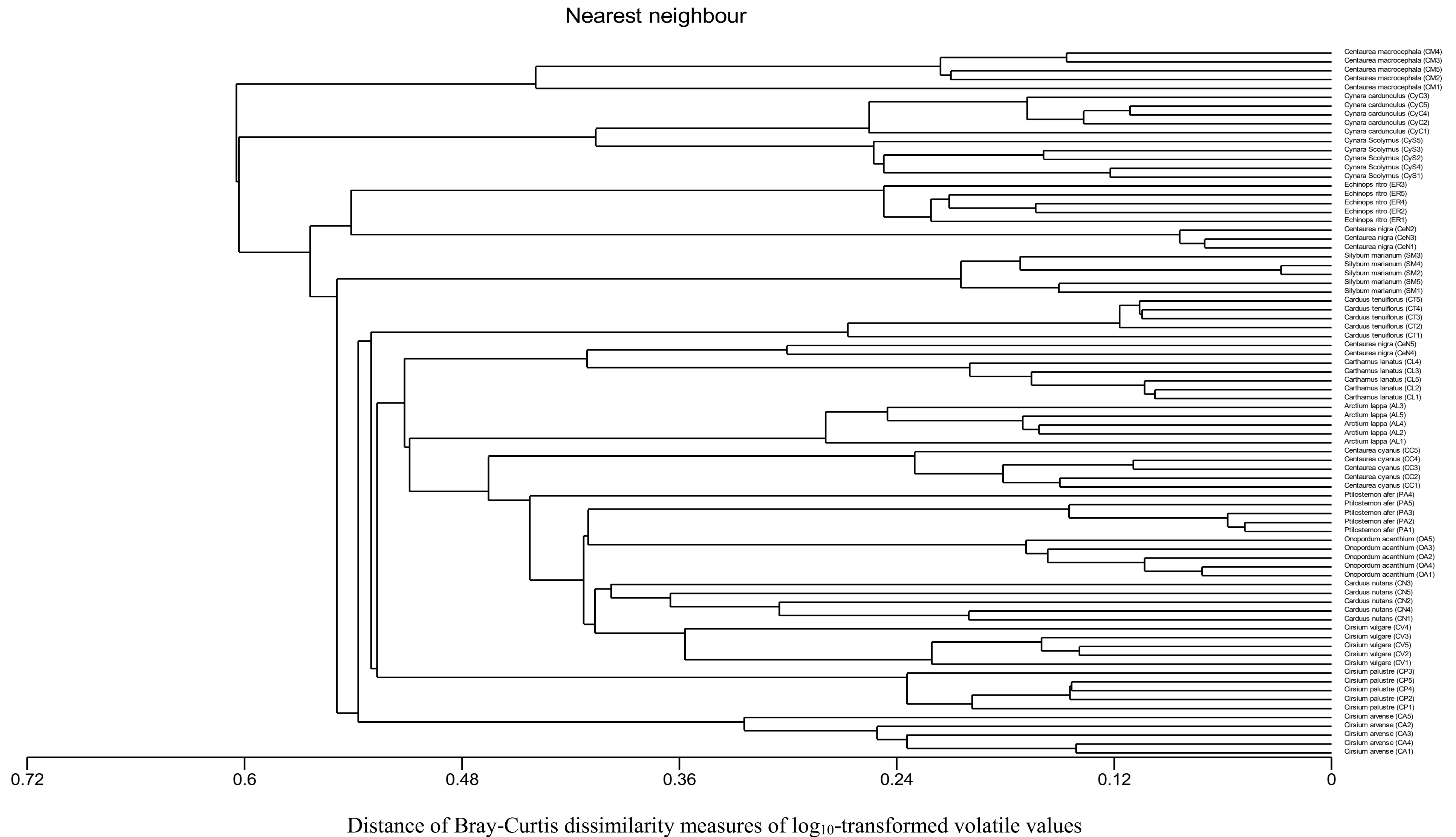

Figure 2.6 Hierarchical cluster dendrogram using nearest neighbour method and Bray-Curtis dissimilarity grouping plant species with similar constitutive volatiles of 16 Cardueae plant species.

2.3.3 Host-choice experiments

No-choice experiment

The number of feeding holes showed no statistically significant phylogenetic relationship (P = 0.523, Figure 2.7a). Total egg mass number per female showed a statistically significantly decrease (P < 0.001) with the phylogenetic distance from *C. arvense* (Figure 2.7b). The greatest number of feeding holes and egg masses were found on *S. marianum* and *C. palustre* (Appendix II) respectively. The greatest number of larvae emerged from *A. lappa* but *C. arvense* was shown to have the greatest number of larvae emerged per egg mass (Appendix II). From all the parameters measured beetles' performance is higher in genus *Cirsium* and *Carduus* (subtribe Carduinae) (Appendix II). The number of larvae emerged from egg masses deposited by the beetle in a particular test plant species when compared with *C. arvense* was not statistically significant (nonlinear regression, P = 0.140). Feeding or oviposition was not observed in the tested non-Cardueae species except *T. porri* where one egg mass was found in which no larvae emerged.

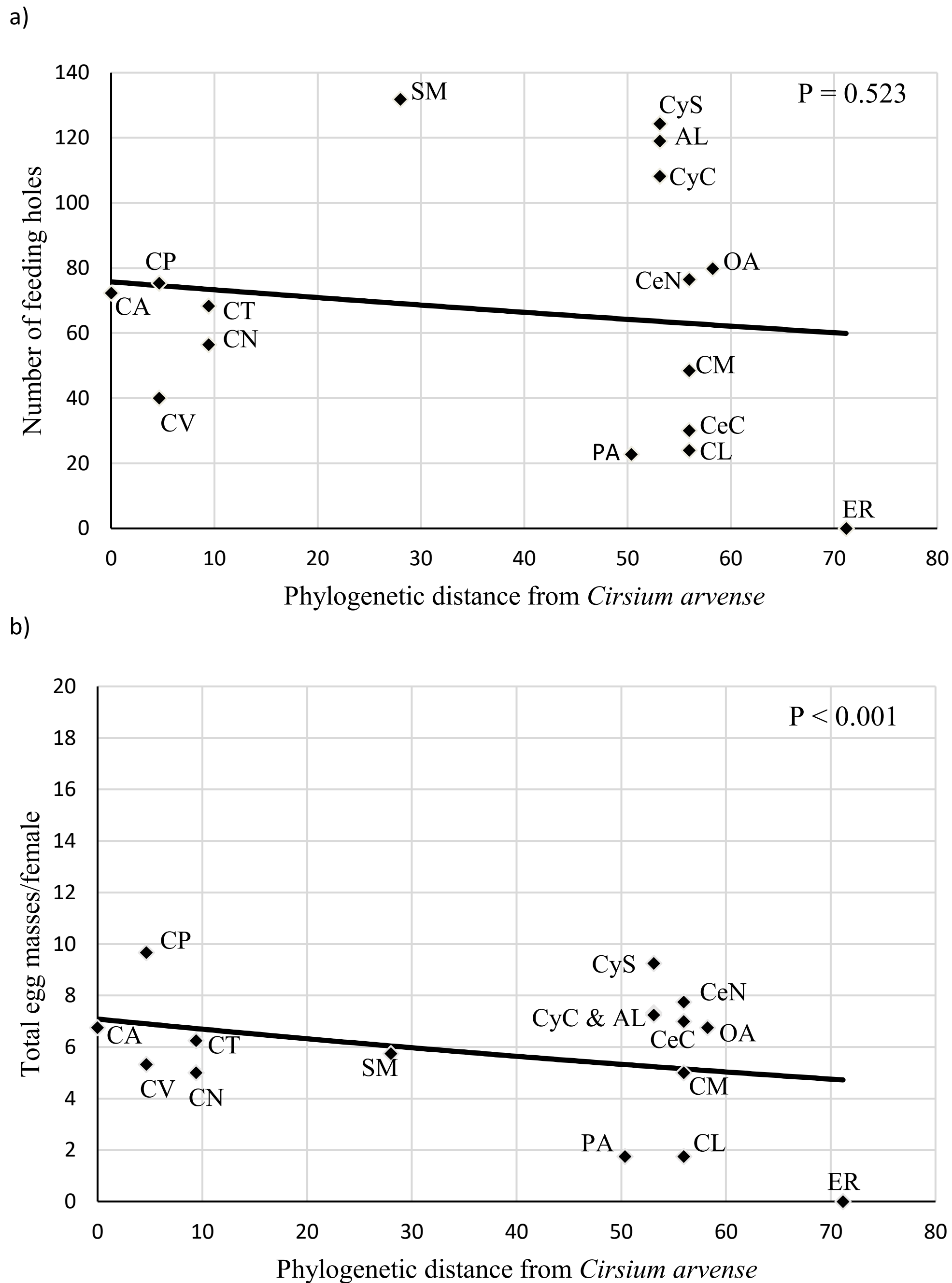

Figure 2.7 Relationship between phylogenetic distances (million years) from *Cirsium arvense* with a) feeding preference [Feeding hole number = exp (4.3275–0.0033*Phylogenetic distance)] and b) total egg mass number per female of *Cassida rubiginosa* [Total egg mass number = exp (1.9580–0.0057* Phylogenetic distance] towards each of the test plants in a no-choice cage experiment. CA - *Cirsium arvense*, CV - *Cirsium vulgare,* CP - *Cirsium palustre,* CT - *Carduus tenuiflorus,* CN - *Carduus nutans,* SM - *Silybum marianum,* AL - *Arctium lappa,* CL - *Carthamus lanatus,* CyS - *Cynara scolymus,* CyC - *Cynara cardunculus,* PA - *Ptilostemon afer,* CeC - *Centaurea cyanus,* OA - *Onopordum acanthium,* CeN - *Centaurea nigra,* ER - *Echinops ritro,* CM - *Centaurea macrocephala.*

Dual-choice experiment

Mean feeding hole numbers when compared with *C. arvense* was highest in *C. vulgare* whereas non-Cardueae plants were not selected by the beetle (Appendix III). Number of feeding holes were statistically significantly higher in *C. vulgare* (PI = 0.72) and *C. tenuiflorus* (PI = 0.29) than the primary host *C. arvense* (Appendix III). Seven plant species: *S. marianum* (PI = 0.14), *C. palustre* (PI = 0.10), *A. lappa* (PI = - 0.01), *C. scolymus* (PI = - 0.05), *C. nutans* (PI = - 0.06), *C. cardunculus* (PI = - 0.08) and *C. nigra* (PI = - 0.11) did not show any significant difference when compared with the primary host (Appendix III). There is statistical evidence (nonlinear regression, P = 0.003) that feeding preference (Figure 2.8a) and egg laying behaviour (nonlinear regression, P < 0.001, Figure 2.8b) decreases with the increasing phylogenetic distance from *C. arvense* to the test plant species. Oviposition was found highest in *C. vulgare* (Appendix III). *Cirsium vulgare* (PI = - 0.42), *C. palustre* (PI = - 0.24), *C. tenuiflorus* (PI = 0.12), *C. scolymus* (PI = - 0.07), *C. nutans* (PI = - 0.22), *S. marianum* (PI = - 0.46), *A. lappa* (PI = - 0.66) and *C. nigra* (PI = - 0.71) have not showed any statistical significant difference with oviposition when compared with the primary host (Appendix III). The number of larvae emerged from egg masses deposited by the beetle in a particular test plant species statistically significantly (nonlinear regression, P < 0.001) decreased with the phylogenetic distance from *C. arvense*. The beetle did not show any feeding or oviposition on tested non-cardueae species.

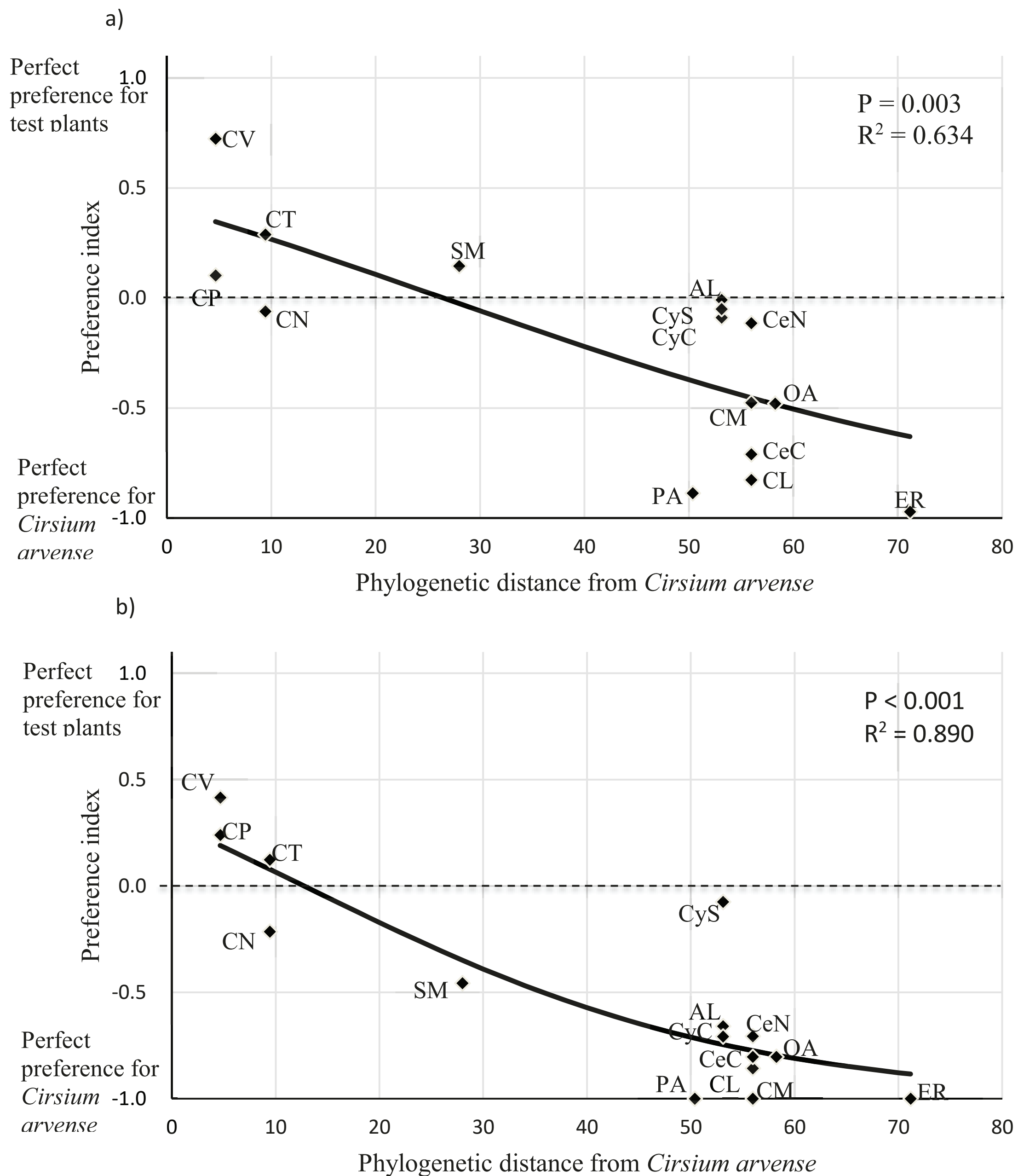

Figure 2.8 Relationship between phylogenetic distances (million years) from *Cirsium arvense* and a) total feeding hole numbers per test plant [Feeding preference index = 1 – 2 / (1 + exp (0.0332 * Phylogenetic distance – 0.8780)) where a = 0.0332 ± 0.0092 and b = 0.8780 ± 0.4050] b) egg laying behaviour during dual choice cage experiment [Egg laying preference index = 1 – 2 / (1 + exp (0.0477 * Phylogenetic distance – 0.6073)) where a = 0.0477 ± 0.0094 and b = 0.6073 ± 0.2856]. The PI of CA over each test plant = (Number of the feeding holes/egg masses on test plant – Number of feeding holes/egg masses on CA) / Total number. Index value is between –1 and +1 inclusive, with (-1) indicating perfect preference of *CA* over the test plant and (+1) perfect preference of the test plant over CA. CA - *Cirsium arvense*, CV - *Cirsium vulgare,* CP - *Cirsium palustre,* CT - *Carduus tenuiflorus,* CN - *Carduus nutans,* SM - *Silybum marianum,* AL - *Arctium lappa,* CL - *Carthamus lanatus,* CyS - *Cynara scolymus,* CyC - *Cynara cardunculus,* PA - *Ptilostemon afer,* CeC - *Centaurea cyanus,* OA - *Onopordum acanthium,* CeN - *Centaurea nigra,* ER - *Echinops ritro,* CM - *Centaurea macrocephala.*

2.4 Discussion

The results of this study demonstrated that chrysomelid beetle *Cassida rubiginosa* shows a phylogenetically conserved pattern during long-range and short-range host-finding behaviour, as well as feeding and oviposition. Beetles were specifically attracted to the scent of *Cirsium* and *Carduus* species, close relatives of their primary host, during the no-choice olfactometer experiments, whereas the host range was broader during olfactometer choice experiments. This may be due to the change of blend inside the olfactometers. When test plants were placed inside the olfactometer during choice experiments, the interior volatile situation become complex and there is an effect on the choice of the beetle from cumulative volatiles from both plant species. It is possible that the blended volatiles have distracted the beetle towards a less preferred/ sub-optimal host. This depends on how well the insects' sensory system has been fine-tuned to identify the volatiles as well as the degree that they rely upon the olfactory cues when finding the most suitable host. *Cassida rubiginosa* has been identified on other Cardueae species (Zwölfer and Eichhorn, 1966). Moreover, chemical similarity of different plants attract similar insects regardless of the phylogeny (Becerra, 1997). The presence of similar volatiles to both plant species (*C. arvense* and the test plant species) inside the olfactometer might have distracted the beetle towards sub-optimal hosts, in which beetle can still survive. This has led to a broader host range resulting from the choice olfactometer experiment compared to the no-choice experiment. Beetles have not shown any significant attraction towards volatiles of any of the non-Cardueae species we tested which means they are well capable of identifying the food-plant range by using olfaction.

Even though closely related plants tend to have similar chemistry (Wang et al., 2018; Wink et al., 2010) our study did not find any similarity in volatiles between closely related plants, except in *C. palustre* and *C. tenuiflorus*. Although the behaviour of the beetle towards olfaction is phylogenetically conserved, our results did not show a phylogenetic pattern in the emitted volatiles. The lack of a phylogenetic pattern might be due to two reasons: 1) method sensitivity - the method adopted was not sensitive enough to identify the lower concentration of compounds present in the volatile bouquet of test plant species. The beetle is highly sensitive at detecting the chemicals that are not experimentally detectable that led it towards the plants according to the plant phylogeny, 2) identification by different ratios – the beetle is attracted to a blend of the volatiles that were detected as common in Cardueae plants, which are mostly terpenes, but in different ratios (Bruce et al., 2005a) and composition for individual plant

species. A more sensitive method is suggested hereafter to increase the accuracy with Cardueae plants. Either, thermo-desorption of VOCs using GC-MS where the sample mixture is heated to promote desorption into the headspace of the tube before putting it onto GCMS (Hatipoglu et al., 2016) or silicone tubing method described by Kallenbach et al. (2015) could be more sensitive than the method used here. Another approach could be to focus only on terpenes by extracting them from the plant tissue where it is possible to have higher amounts of compounds (Becerra, 1997; Mooney and Emboden, 1968). It should be noted that there are some studies that do not show volatile phylogenetic signals. A study conducted by Wei et al. (2007) has shown a partly phylogenetically conserved pattern of volatiles emitted by healthy plants belonging to several families. Becerra (1997) reconstructed the molecular phylogenies of the *Blepharida* (Coleoptera) and *Bursera* (Burseraceae) using terpene extractions and suggested that chemical similarity is partially independent of plant phylogeny. Peakall et al. (2010) mentioned polyphyly of the active floral compounds (six compounds of 2,5-dialkylcyclohexan-1,3-diones called chiloglottones) from the phylogeny of the *Chiloglottis* orchids. Further, Courtois et al. (2009) studied volatiles of 55 species of tropical tree, by mechanically damaging the leaves, did not find any pattern of chemical similarities within genera. It is less clear to what degree plant volatiles are reliable in a particular species identification; however, in broad taxonomic scale many plant groups have unique volatile profiles (Pearse et al., 2013; Courtois et al., 2009) that lead insects towards their host plants. The lack of chemical equivalence with phylogeny of tested Cardueae plants could be due to growth forms of the plants. Schrader et al. (2017) have shown by using 13 Brassicaceae species representing all four evolutionary lineages that plant growth form is more important than phylogenetic relatedness when it comes to composition of constitutive and induced volatiles.

According to ‘appropriate/inappropriate landing’ theory plant selection involves three linked chains of events involving 1) olfactory cues, 2) visual cues and 3) non-volatile plant chemical cues (Finch and Collier, 2000). For insects that use odour conditioned movements, decisions have to be made at distance and later at proximity of the plant or at contact. Adult host-choice experiments were conducted giving beetles’ freedom to use any of the cues. During the no-choice condition the beetles feeding was not found to be phylogenetically conserved, whereas oviposition adhered to phylogeny. When the beetle was not given a choice of food, significant amount of feeding on many sub-optimal and less-preferred Cardueae plant species was observed. I expect that this is mainly for the survival of the beetle. Increased number of feeding holes may be due to the plant being sub-optimal in nutrition or it can be due to repeated nibbling

by the beetle as it searches of better food (Schoonhoven et al. 2005). However, *Cassida* have not shown significant feeding or oviposition on non-Cardueae species (Appendix III). Oviposition depend on individual fitness and age among many more other factors (Jaenike, 1978) which was not given attention during the study. Further, it is not clear whether the female *C. rubiginosa* beetle can dissolve the eggs without laying them when forced with unsuitable host. In the study by Cripps et al. (2016), larval survival showed a phylogenetically conserved pattern and the survival rates of *C. rubiginosa* larvae on *C. tenuiflorus*, *C. palustre*, *C. vulgare* and *A. lappa* were statistically equivalent to *C. arvense*. Adult choice experiments showed a broader host range than the larval host choice, although it is more concise when compared with adult no-choice tests (Appendix III). During adult host-choice experiments the beetles' choice was not restricted to *Cirsium* and *Carduus* species. Zwölfer and Eichhorn (1966) have documented *C. rubiginosa* feeding on 21 plant species which included *Arctium*, *Carduus*, *Cirsium*, *Silybum*, *Cynara* and *Onopordum* genus. With limited time, some individuals might make good decisions after taking more time whereas some might make rapid decisions and move with less quality plant species (Bernays, 2001). There is a trade-off between having quality food for both the adults and the larvae or merely surviving the adult time. As the beetle is oligophagous on tribe Cardueae it is not crucial to find the primary host *C. arvense*. However, in both olfactory and adult host-choice experiments, beetles did not miss choosing *Cirsium* and *Carduus* species (Table 2.1). Therefore, the potential for multi-targeting is higher with *Cirsium* and *Carduus* species, whereas in the non-Cardueae species tested, it was of no interest to the beetle. When considering the overall results (Table 2.1) the host range of *C. rubiginosa* is similar for both the olfactory and adult host-choice experiments.

This comprehensive study has shown that the choice of this biological control agent is always phylogenetically conserved, except in no-choice feeding which is an exceptional case that will be rare in natural environments. Therefore, quantitative phylogenetic measures can be used to predict the plant species likely to be chosen by the beetle. Also, we can give a threshold of phylogenetic distance where *Cassida* performance will significantly decrease. Therefore, incorporating the suggestions by Briese (2003), as in the case of this study, will be advantageous for biological control practitioners, as it will reduce the amount of energy, money and time put into doing experiments identifying host-range of a potential biological control agent. Further, olfactometer experiments identified all of the plants that were likely to be attacked by the beetles and all of the plants that were optimal for the larval survival (Table 2.1). Olfaction can therefore be considered as one of the most important factors when it comes to

biological control host range testing. Olfactory experiments are also much quicker than feeding and oviposition experiments at revealing the possible plants that are likely to be attacked. Recently there are suggestions to include olfactory experiments when testing with a potential biological control agent (Park et al. 2018; Wheeler and Schaffner, 2013). This study is a positive example which shows that olfactory studies can be used in biological control experiments. More similar olfactory studies will need to be conducted using different biological control agents before these methods are routinely included in biological control best practise.

Table 2.1 Summary of the olfactometer and adult host choice experiments with larval survival results from Cripps et al. (2016). CA – *Cirsium arvense*, CV - *Cirsium vulgare*, CP - *Cirsium palustre*, CT - *Carduus tenuiflorus*, CN - *Carduus nutans*, SM - *Silybum marianum*, AL - *Arctium lappa*, CL - *Carthamus lanatus*, CyS - *Cynara scolymus*, CyC - *Cynara cardunculus*, PA - *Ptilostemon afer*, CeC - *Centaurea cyanus*, OA - *Onopordum acanthium*, CeN - *Centaurea nigra*, ER - *Echinops ritro*, CM - *Centaurea macrocephala*. **Bold** - preferred equally or higher than that of *C. arvense*. * - preference is higher than that of *C. arvense*

Olfactometer	**Adult host choice tests Single-choice**		**Adult host choice tests Dual-choice**		**Larval survival**
Preference	**Feeding**	**Oviposition**	**Feeding**	**Oviposition**	
CP	**CP**	**CP**	**CP**	**CP**	**CP**
CV	**CV**	**CV**	**CV***	**CV**	**CV**
CN	**CN**	**CN**	**CN**	**CN**	CN
CT*	**CT**	**CT**	**CT***	**CT**	**CT**
SM	**SM**	**SM**	**SM**	**SM**	SM
PA	PA	PA	PA	PA	PA
AL	**AL**	**AL**	**AL**	**AL**	**AL**
CyC	**CyC**	**CyC**	**CyC**	CyC	CyC
CyS	**CyS**	**CyS**	**CyS**	**CyS**	CyS
CL	CL	CL	CL	CL	CL
CeC	CeC	**CeC**	CeC	CeC	CeC
CM	**CM**	**CM**	CM	CM	CM
CeN	**CeN**	**CeN**	**CeN**	**CeN**	CeN
OA	**OA**	**OA**	OA	OA	OA
ER	ER	ER	ER	ER	ER

This is likely to be the first study incorporating both olfactometer testing and adult host-choice testing experiments to evaluate the host range of an insect biological control agent according

to the plant phylogeny. The study investigated the plant chemistry and the biological control agents' response to that chemistry in predicting the host range. The importance of including olfactory studies when testing for a potential biocontrol agent is emphasised. Further, incorporating chemical studies can identify the evolutionary changes that may occur in plant secondary chemistry of invasive weeds when the plant is exposed to novel biotic and abiotic factors that might lead to host shift. These studies can also identify the secondary compounds that might influence the performance of some biological control agents (Wheeler and Schaffner, 2013; Ehrlich and Raven, 1964) and greatly aid in the success of the biological control programme. Current resolved phylogenies with measurable values act as a good predictor when selecting plants to be tested with a potential biocontrol candidate.

2.5 Acknowledgement

I thank Jason Breitmeyer for his technical assistance with GC-MS analysis. This study was funded by the New Zealand Ministry of Business, Innovation, and Employment through AgResearch core funding of pasture weed research. I would also like to acknowledge the Commonwealth scholarship offered by the Ministry of Foreign affairs and Trade for their financial assistant to the principle researcher.

2.6 References

Ang, B., Kok, L., Holtzman, G., Wolf, D., 1995. Canada Thistle [*Cirsium arvense* (L) Scop.] Response to Density of *Cassida rubiginosa* Müller (Coleoptera: Chrysomelidae) and Plant Competition. Biological Control. 5**,** 31-38.

Bacher, S., Schwab, F., 2000. Effect of Herbivore Density, Timing of Attack and Plant Community on Performance of Creeping Thistle *Cirsium arvense* (L.) Scop. (Asteraceae). Biocontrol Science and Technology. 10**,** 343-352.

Barres, L., Sanmartín, I., Anderson, C. L., Susanna, A., Buerki, S., Galbany-Casals, M., Vilatersana, R., 2013. Reconstructing the evolution and biogeographic history of tribe Cardueae (Compositae). American Journal of Botany. 100**,** 867-882.

Becerra, J. X., 1997. Insects on plants: macroevolutionary chemical trends in host use. Science. 276**,** 253-256.

Beck, J. J., Smith, L., Baig, N., 2014. An overview of plant volatile metabolomics, sample treatment and reporting considerations with emphasis on mechanical damage and biological control of weeds. Phytochemical Analysis. 25**,** 331-341.

Bernays, E., 2001. Neural limitations in phytophagous insects: implications for diet breadth and evolution of host affiliation. Annual Review of Entomology. 46**,** 703-727.

Bernays, E. A., Chapman, R. F., 1994. Host-plant selection by phytophagous insects. Springer Science & Business Media.

Briese, D., 2003. The centrifugal phytogenetic method used to select plants for host-specificity testing of weed biological control agents: Can and should it be modernised? Crc for Australian Weed Management, Glen Osmond.

Briese, D., 2005. Translating host-specificity test results into the real world: the need to harmonize the yin and yang of current testing procedures. Biological Control. 35**,** 208-214.

Bruce, T. J., Wadhams, L. J., Woodcock, C. M., 2005. Insect host location: a volatile situation. Trends in Plant Science. 10**,** 269-274.

Bruce, T. J. A., Pickett, J. A., 2011. Perception of plant volatile blends by herbivorous insects – Finding the right mix. Phytochemistry. 72**,** 1605-1611.

Courtois, E. A., Paine, C. T., Blandinieres, P.-A., Stien, D., Bessiere, J.-M., Houel, E., Baraloto, C., Chave, J., 2009. Diversity of the volatile organic compounds emitted by 55 species of tropical trees: a survey in French Guiana. Journal of Chemical Ecology. 35**,** 1349.

Cripps, M., Gassmann, A., Fowler, S., Bourdôt, G., McClay, A., Edwards, G., 2011. Classical biological control of *Cirsium arvense*: Lessons from the past. Biological Control. 57**,** 165-174.

Cripps, M., Jackman, S., Roquet, C., van Koten, C., Rostás, M., Bourdôt, G., Susanna, A., 2016. Evolution of Specialization of *Cassida rubiginosa* on *Cirsium arvense* (Compositae, Cardueae). Frontiers in Plant Science. 7**,** 1261.

Cripps, M. G., Bourdôt, G. W., Fowler, S. V., 2013. Sleeper thistles in New Zealand: status and biocontrol potential. New Zealand Plant Protection. 66**,** 99-104.

Cripps, M. G., Edwards, G. R., Bourdôt, G. W., Saville, D. J., Hinz, H. L., Fowler, S. V., 2010. Effects of pasture competition and specialist herbivory on the performance of *Cirsium arvense*. Biocontrol Science and Technology. 20**,** 641-656.

de Bruyne, M., Baker, T. C., 2008. Odor Detection in Insects: Volatile Codes. Journal of Chemical Ecology. 34**,** 882-897.

Ehrlich, P. R., Raven, P. H., 1964. Butterflies and plants: a study in coevolution. Evolution. 18**,** 586-608.

Finch, S., Collier, R., 2000. Host-plant selection by insects–a theory based on ppropriate/inappropriate landings' by pest insects of cruciferous plants. Entomologia Experimentalis et Applicata. 96**,** 91-102.

Hatipoglu, S. D., Zorlu, N., Dirmenci, T., Goren, A. C., Ozturk, T., Topcu, G., 2016. Determination of Volatile Organic Compounds in Fourty Five Salvia Species by Thermal Desorption-GC-MS Technique. Records of Natural Products. 10.

Hettiarachchi, D. K., Cripps, M., Jackman, S., van Koten, C., Sullivan, J., Rostás, M., 2018. Impact of the biocontrol beetle, *Cassida rubiginosa,* on the secondary weed target, marsh thistle (*Cirsium palustre*). New Zealand Plant Protection. 71.

Huang, W., Siemann, E., Wheeler, G.S., Zou, J., Carrillo, J. & Ding, J. (2010) Resource allocation to defence and growth are driven by different responses to generalist and specialist herbivory in an invasive plant. Journal of Ecology*,* **98,** 1157-1167.

Jaenike, J., 1978. On optimal oviposition behavior in phytophagous insects. Theoretical Population Biology. 14, 350-356.

Kallenbach, M., Veit, D., Eilers, E. J., Schuman, M. C., 2015. Application of silicone tubing for robust, simple, high-throughput, and time-resolved analysis of plant volatiles in field experiments. Bio-Protocol. 5.

Martinez, A. S., Hardie, J., 2009. Hygroreception in olfactometer studies. Physiological Entomology. 34, 211-216.

Mooney, H., Emboden, W., 1968. The relationship of terpene composition, morphology, and distribution of populations of *Bursera microphylla* (Burseraceae). Brittonia. 20, 44-51.

Park, I., Eigenbrode, S. D., Cook, S. P., Harmon, B. L., Hinz, H. L., Schaffner, U., Schwarzländer, M., 2018. Examining olfactory and visual cues governing host-specificity of a weed biological control candidate species to refine pre-release risk assessment. BioControl. 1-13.

Peakall, R., Ebert, D., Poldy, J., Barrow, R. A., Francke, W., Bower, C. C., Schiestl, F. P., 2010. Pollinator specificity, floral odour chemistry and the phylogeny of Australian sexually deceptive *Chiloglottis* orchids: implications for pollinator-driven speciation. New Phytologist. 188, 437-450.

Pearse, I. S., Gee, W. S., Beck, J. J., 2013. Headspace Volatiles from 52 oak Species Advertise Induction, Species Identity, and Evolution, but not Defense. Journal of Chemical Ecology. 39, 90-100.

Rostás, M., Cripps, M. G., Silcock, P., 2015. Aboveground endophyte affects root volatile emission and host plant selection of a belowground insect. Oecologia. 177, 487-497.

Rostás, M., Eggert, K., 2008. Ontogenetic and spatio-temporal patterns of induced volatiles in *Glycine max* in the light of the optimal defence hypothesis. Chemoecology. 18, 29-38.

Schoonhoven LM, Van Loon JJ, Dicke M 2005. Insect-plant biology, Oxford University Press on Demand.

Schrader, J., Unsicker, S. B., Bhattacharya, S., Mummenhoff, K., 2017. Growth form rather than phylogenetic relationship predicts broad volatile emission patterns in the Brassicaceae. Plant Systematics and Evolution. 303, 653-662.

Suckling, M. D., & Sforza, R. F. H. , 2014. What magnitude are observed non-target impacts from weed biocontrol? . PLoS One. 9.

Susanna, A., Garcia-Jacas, N., Hidalgo, O., Vilatersana, R., Garnatje, T., 2006. The Cardueae (Compositae) revisited: insights from ITS, trnL-trnF, and matK nuclear and chloroplast DNA analysis. Annals of the Missouri Botanical Garden. 150-171.

Tipping, P. W., 1993. Field studies with *Cassida rubiginosa* (Coleoptera: Chrysomelidae) in Canada thistle. Environmental Entomology. 22, 1402-1407.

Van Tol, R. W. H. M., Visser, J. H., Sabelis, M. W., 2002. Olfactory responses of the vine weevil, *Otiorhynchus sulcatus*, to tree odours. Physiological Entomology. 27, 213-222.

Wang, Y., Li, X., Jiang, Q., Sun, H., Jiang, J., Chen, S., Guan, Z., Fang, W., Chen, F., 2018. GC-MS Analysis of the Volatile Constituents in the Leaves of 14 Compositae Plants. Molecules. 23, 166.

Wapshere, A., 1974. A strategy for evaluating the safety of organisms for biological weed control. Annals of Applied Biology. 77, 201-211.

Webb, C. J., Sykes, W. R., Garnock-Jones, P. J., 1988. Flora of New Zealand DSIR, Botany Division, Christchurch.

Wei, J., Wang, L., Zhu, J., Zhang, S., Nandi, O. I., Kang, L., 2007. Plants Attract Parasitic Wasps to Defend Themselves against Insect Pests by Releasing Hexenol PLoS One. 2, e852.

Wheeler, G. S., Schaffner, U., 2013. Improved understanding of weed biological control safety and impact with chemical ecology: a review. Invasive Plant Science and Management. 6, 16-29.

Willis, A. J., Kilby, M., McMaster, K., Cullen, J., Groves, R., 2003. Predictability and acceptability: potential for damage to non-target native plant species by biological control agents for weeds. Improving the selection, testing and evaluation of weed biological control agents. Glen Osmond: South Australia CRC for Australian Weed Management. 35-49.

Wink, M., Botschen, F., Gosmann, C., Schäfer, H., Waterman, P. G., 2010. Chemotaxonomy seen from a phylogenetic perspective and evolution of secondary metabolism. Annual Plant Reviews. 40, 364-433.

Zwölfer, H., Eichhorn, O., 1966. The host ranges of *Cassida* spp.(Col. Chrysomelidae) attacking *Cynareae* (Compositae) in Europe. Zeitschrift für Angewandte Entomologie. 58, 384-397.

Chapter 3
Conspecific larval feeding elicits differential olfactory attraction in *Cassida rubiginosa* towards thistles

Abstract
Conspecific cues have often been omitted from host range testing. But conspecific damage induced volatiles can be transferring certain information to conspecifics as well as other herbivores. This study investigated the differential attraction of the biocontrol agent *Cassida rubiginosa* in olfactometer experiments comparing undamaged leaves with conspecific larval damaged leaves by using 13 Cardueae species selected according to their phylogeny. The beetles' attraction was towards tested *Cirsium* species and *Carduus tenuiflorus* with the constitutive volatiles. But with herbivore-induced plant volatiles (HIPVs) the beetle displayed increased attraction towards five hosts that it was not attracted to when tested with constitutive volatiles. Chemical compounds such as cis-β-ocimene and β-caryophyllene were detected in the blend of HIPVs from the five species of plants that showed beetle attraction. The induced plant volatiles did not show a strong phylogenetic pattern. In conclusion, HIPVs had a role in increasing the olfactory host range of *Cassida rubiginosa*. Thus, this study highlights the importance of investigating conspecific cues to identify the behaviour towards host plants.

Keywords: Cardueae, host-range, conspecific cues, herbivore-induced volatiles, phylogeny, larval damage

3.1 Introduction

Plants constitutively emit a plethora of volatile organic compounds, which can act as kairomones, that enable herbivorous insects to identify their host (Bernays and Chapman, 1994). These volatile organic compounds can be categorised as constitutive volatiles, which are expressed regardless of any abiotic and biotic stresses, and induced volatiles which are emitted due to abiotic (temperature, light, water, etc.) and biotic (herbivores, pathogens, etc.) stresses (Niederbacher et al., 2015; Dicke and Baldwin, 2010; Delphia et al., 2009). The perception of plant-derived odours and the oriented movement towards their source is an early step in the insect's host-selection process (Bruce, 2015; Szendrei and Rodriguez-Saona, 2010). For example, both males and females of the melon fly, *Zeugodacus cucurbitate*, a tephritid fruit fly that has a wide host range but preferring cucurbitaceous plants, responded to odours of cucumber and tomato plants confirming the importance of constitutive volatiles in locating its host plants (Njuguna et al., 2018). Also, as mentioned in Chapter 2, *Cassida rubiginosa* orient towards *Cirsium arvense* using odour cues. The situation changes when the plant is colonised by herbivores due to the altered quantity and quality of volatiles.

Herbivory induces a different set of volatiles mainly comprised of terpenoids, fatty acid derivatives, phenyl propanoids and benzenoids (Dudareva et al., 2004). They can be emitted either at the site of damage or systemically from undamaged parts of the plant (Heil and Ton, 2008). Those herbivore-induced plant volatiles (HIPVs) can play different roles in creating complex interactions among conspecific and heterospecific insects. For example, a study conducted by Shivaramu et al. (2017) with *Capsicum annum* (bell pepper) and its pest *Scirtothrips dorsalis* (chilli thrips), as a model system, showed that thrips were significantly attracted to HIPVs induced by conspecific damage and fruiting stage volatiles of the plants but the attraction was higher towards HIPVs. The omnivorous mirid bug, *Nesidiocoris tenuis*, showed gender-specific attractions to the HIPVs emitted due to conspecifics feeding on eggplants and sesame plants (Rim et al., 2018). Frati et al. (2008), on the other hand, found *Lugus rugulipennis* (mirid bug) attraction towards its host plant *Vicia faba* are less responsive to volatiles emitted by plants that have conspecific feeding damage than plants without any damage from conspecific bugs.

Not only conspecifics but also enemies of herbivores can respond to HIPVs. Reviews by Dicke (2015), Clavijo McCormick et al. (2012) and Takabayashi and Dicke (1996) have discussed many cases where the HIPVs play a role in attracting enemies of herbivores. In some cases, HIPVs can attract both the conspecifics and the predator as in the case of the pandemis leafroller moth, *Pandemis pyrusana*, larvae induced volatiles which attracted both male and female conspecific moths and the general predator the common green lacewing, *Chrysoperla plorabunda* to apple trees (El-Sayed et al., 2018). It is evident that these HIPVs can transmit herbivore-specific information that is detectable by the predators, especially for foraging parasitoids. For example, *Heliothis virescens* and *Helicoverpa zea* feeding on tobacco, cotton and maize produce distinct volatile blends. The specialist parasitic wasp *Cardiochiles nigriceps* was able to discriminate infestation by its host, *H. virescens*, from that by *H. zea* and the systematically produced plant volatiles (De Moraes et al., 1998). Thus, herbivore-specific plant volatile responses occur based on the plant family and the proportion. The variety of released compounds give different signals to herbivores that aid in behavioural plasticity of a certain insect species.

Even though there are studies investigating a particular plant-herbivore system, fewer studies address the behavioural changes due to conspecific cues in evaluating the host range of an insect herbivore in a phylogenetic context (Sutton et al., 2017). The tortoise beetle, *Cassida rubiginosa* Müller (Coleoptera: Chrysomelidae), is used as a biological control agent against *Cirsium arvense*, a noxious weed of temperate regions of the world. The beetle is oligophagous and feeds on variety of plants belonging to tribe Cardueae. Previous study (Chapter 2) investigated the beetles' responses to constitutive volatiles and showed that it exhibits an olfactory preference towards species of *Cirsium* and *Carduus*. This study investigated the olfactory preference of *Cassida rubiginosa* to phylogenetically selected thistles in the presence of conspecific larval feeding (HIPVs) without removing the larvae. It was hypothesised that conspecific HIPVs increase the attractiveness of the beetle towards thistles. Specifically, it was predicted that this would be evident for both (1) plant genera (*Cirsium* and *Carduus*) that are preferred with constitutive volatiles, and (2) other Cardueae genera that do not show preference towards constitutive volatiles, thus increasing the preference of thistle species that the beetle is attracted to when exposed to conspecific HIPVs. It should be noted that larvae plus the damaged leaf volatiles were included together as this is the near natural condition I wanted to assess. Therefore, whenever I say "damaged volatiles" this potentially includes the volatiles emitted by the larvae as well.

3.2 Material and methods

3.2.1 Study system

Cardueae is a monophyletic tribe which belongs to family Compositae containing more than 2500 species (Susanna et al., 2006). The native distribution of Cardueae plants are almost exclusively in the Northern hemisphere (Webb et al., 1988). The plants used in this study are native to Eurasia and were introduced inadvertently or deliberately as agricultural and ornamental plants (Cripps et al., 2013). When selecting the plants, previous records on larval feeding was taken into account (Chapter 2). We used a phylogenetic approach in selecting the 13 plant species: *Cirsium arvense*, *Cirsium palustre, Cirsium vulgare, Carduus nutans, Carduus tenuiflorus, Silybum marianum, Arctium lappa, Cynara scolymus, Cynara cardunculus, Centaurea cyanus, Centaurea macrocephala, Centaurea nigra, Onopordum acanthium,*. The phylogenetic distances of the 13 Cardueae test species were reported

previously by Cripps et al. (2016). Due to the constraint of finding enough beetles the experiments were conducted in two consecutive summers.

All plants were grown from seeds, either collected from the field or purchased from a commercial supplier (Kings Seeds NZ Ltd.). Seeds were sown from 9 August 2016 to 6 September 2016 for the first group and on 12 September 2017 for the second. Similar conditions were provided during the process. Seedlings were grown in a glasshouse at AgResearch, Lincoln, and subsequently transplanted into 12 L plant pots containing a standard potting mix (54% aged bark, 45% sand, 1% nutrients, by weight) with added nutrients of Osmocote® 17-11-10 (N-P-K), lime, superphosphate, sulphate of potash and calcium nitrate. Plants were watered once a day.

The thistle tortoise beetle, *Cassida rubiginosa* (Coleoptera: Chrysomelidae), is native to the Palearctic region and deliberately introduced to New Zealand in 2007 to control *C. arvense* (Cripps et al., 2013; Zwölfer and Eichhorn, 1966). Even though the primary host is *C. arvense* the beetle is oligophagous on other plants in the tribe Cardueae (Zwölfer and Eichhorn, 1966). Both the adult and larvae are leaf feeders. Adults overwinter under debris and hedges or forest margins and emerge in spring seeking host plants where they feed and deposit their egg masses. Egg masses or oothecae are mostly found on the underside of the leaves. Their life cycle consists of five larval stages and are mostly confined to the plant their mother has chosen for them until they become adults (Tipping, 1993). The larvae of *C. rubiginosa* collects their exuviae together with their faeces on two spines at their abdominal tip (Bacher and Luder, 2005) and use as a shield when disturbed by orienting it in the direction of predators (Bacher and Luder, 2005; Müller, 2002; Eisner et al., 1967).

For the experiments, beetles were field collected (from Wairarapa, approximately 1000 beetles) and kept in 2 L ventilated plastic boxes (the lid was modified by cutting an area of 105 mm x 155 mm and covering that area with a mesh of 0.5 mm) under constant temperature of 20 ºC, inside temperature-controlled rooms (CT room; 16h light: 8h dark period) at Lincoln University and fed with *C. arvense* clippings. Only adult female beetles (weight of a beetle ≤19 mg, taken as males; and beetles >19 mg, taken as females) were used and a beetle exposed to an experiment was not used repeatedly.

3.2.2 Olfactometer experiments

Small still air olfactometers (140 mm diameter x 20 mm high) were used to investigate the long-range host-selection behaviour of the beetle (see Chapter 2). The experiment was always started around 10:00 am–10:30 am inside the CT room with a constant temperature of 23°C and 55–60% humidity. Olfactometers were washed with water, distilled water, acetone (100%) and hexane (99.5%), respectively in order to make sure that they were void of impurities and odour. Plant leaves were cut from the petiole. The selected leaves were approximately the same age and size (2nd and 3rd leaf from the top). Leaf petioles were covered with a damp cotton wool to prevent wilting. The leaves of each plant species were then placed inside the arm chambers of the olfactometer without mechanically damaging the leaf material. The beetle had two choices; to enter the chamber with undamaged plant leaves or to the other chamber with damaged leaves. The beetles that did not make a choice and remained on the olfactometer base, were not counted. Twenty replicates were conducted for each test plant species during two consecutive summers (ten replicates in 2016 and ten replicates in 2017).

No-choice olfactory preference tests

The same experiment described in Chapter 2 was used as the no-choice olfactometer experiment which showed attraction towards constitutive volatiles.

Choice olfactometer preference tests

The experiment was conducted to compare attraction towards constitutive volatiles and induced volatiles of each of the selected test plant species. Undamaged leaves of tests plants were put in one chamber of the olfactometer. Twenty larvae (2nd to 3rd instar) of *C. rubiginosa* were introduced to the leaves in the other chamber. After 48 hours three adult female beetles were introduced to the base of the olfactometer. After 6 hours, the olfactory chamber of their choice was recorded.

3.2.3 Volatile analysis

Volatile organic compounds (VOCs) were collected from undamaged plant leaves and plant leaves with introduced twenty 2nd to 3rd instar *C. rubiginosa* larvae. In addition, empty odour-source vessels (control) were used to detect impurities in the system. Four leaves (from 2nd leaf till 5th leaf) were placed in one glass vessel (volume 520 ml). The cut end was immediately

submerged in 30 ml of water to reduce the emission of volatiles from the cut end and desiccation during volatile collection. At the beginning of each collection odour source vessels were thoroughly washed with distilled water, acetone (100%) and hexane (95.5%), respectively, to minimise impurities and odour. A push-pull system was used as described in Rostás and Eggert (2008). After setting-up, the vessels were left for 48 hours to allow the volatiles to accumulate. Filtered air (activated charcoal filter, 400 ml; Alltech, Deerfield, IL, USA) originating from a compressed air cylinder was pushed into the vessels through one of the teflon tubes at a rate of 0.8 $lmin^{-1}$ and the air was pulled out, through a trapping filter consisting of a glass tube (7 cm) containing 30 mg of 80–100 mesh SuperQ absorbent (Alltech, Deerfield, IL, USA) at the same rate by using a vacuum pump (Me2, Vacuubrand, Wertheim, Germany). Each collection lasted for 6 hours. The absorbed compounds were eluted from the trapping filter with 100 µl dichloromethane and 200 ng tetralin (Sigma-Aldrich, Australia) was added after the elution as an internal standard. All extracts were stored at -80 °C until analysed.

Samples were analysed for VOCs using an adaption of the method described by Rostás et al. (2015). A Shimadzu GCMS-QP2010 Ultra (Shimadzu, Japan) gas chromatography mass spectrometer was fitted with a Restek Rtx-5ms fused silica capillary column (30.0 m x 0.25 mm internal diameter, 0.25 µm film thickness, Bellefonte, PA, USA). A CTC-Combi PAL auto sampler (PAL LHX-xt) was used to inject 1µL of sample into the gas chromatography (GC) injection port at 220 °C, operating in split less mode at 241.0 kPa pressure for 39 seconds. After injection, the column oven was held at 35 °C for 3 minutes, then heated to 320°C at 8 °C min^{-1} and held at this temperature for 8 minutes. Helium was used as the carrier gas with the constant linear velocity set at 44.0 cm/sec (1.5 ml min^{-1}) in split mode (30:1) after the high-pressure split-less injection. The mass spectrometer (MS) was operated in electron impact ionization mode with 70eV and mass range of 33 to 550 m/z. The temperature of the capillary interface was 320 °C, with the source temperature set at 230 °C.

Initial identification of detected peaks was made by matching their retention indexes and mass spectra with the spectra of reference compounds found in the databases NIST EPA/NIH Mass Spectral Library database (National Institute of Standards and Technology, NIST11) and Wiley Registry of Mass Spectral Data 10th edition (John Wiley & Sons, Hoboken, New Jersey, USA).

3.2.4 Data Analysis

Olfactometer data were analysed using Generalized Linear Model (GLM) assuming Poisson distribution through log-link function. The difference in the concentration of volatile classes between damaged and undamaged leaves of each test species were analysed by *t*-tests using Minitab version 15 and the volatile data were analysed using Bray-Curtis similarity measure and Hierarchical cluster analysis.

3.3 Results

3.3.1 Olfactometer experiments

No-choice olfactory preference tests

Following from Chapter 2, adult female beetles are attracted to the constitutive volatiles produced by *C. arvense* (Chi Sq. = 13.72, df = 1, P < 0.0001, PI = 0.69), *C. vulgare* (Chi Sq. = 10.82, df = 1, P = 0.01, PI = 0.70), *C. palustre* (Chi Sq. = 4.61, df = 1, P = 0.032, PI = 0.41), and *C. tenuiflorus* (Chi Sq. = 5.48, df = 1, P = 0.019, PI = 0.48) species (Figure 3.1).

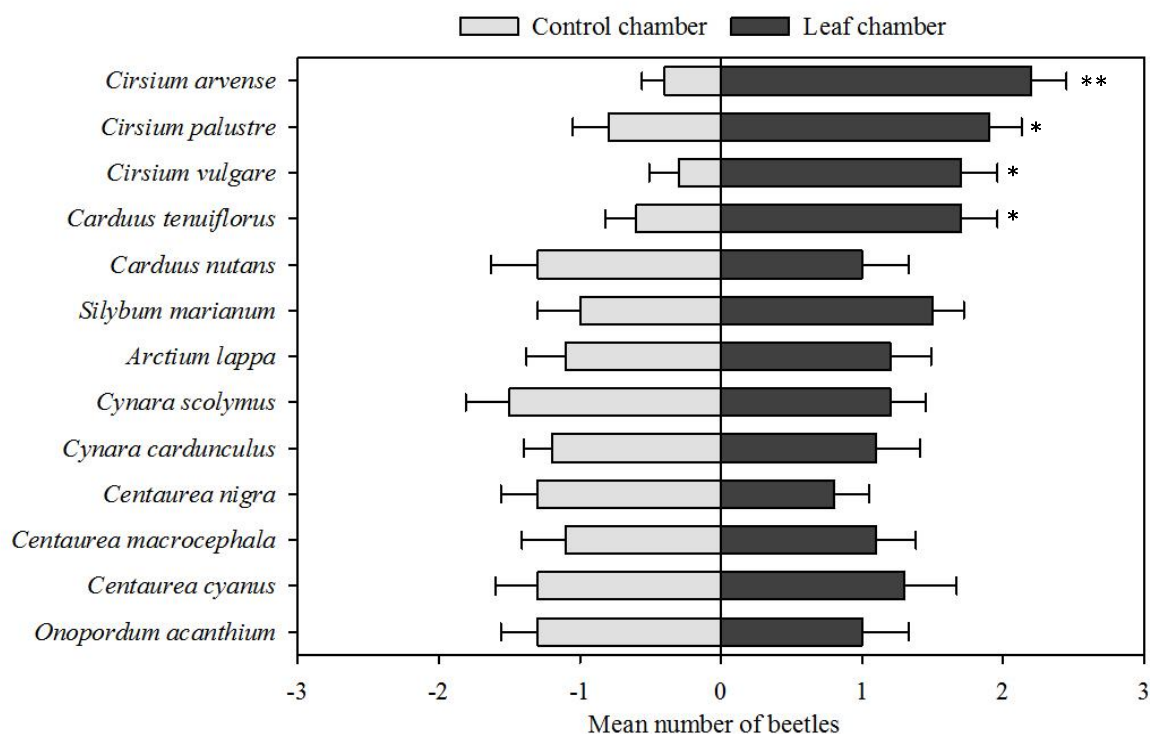

Figure 3.1 Mean number of adult female beetles *Cassida rubiginosa* choosing the constitutive volatiles (leaf chamber) and the control chambers (chamber with paper mimic and cotton ball) of the olfactometer during no-choice tests. * P <0.05, ** P < 0.001. Plants were arranged according to phylogenetic distance from *Cirsium arvense.* N = 10.

Choice olfactometer preference tests

Statistically significant preference towards herbivore-induced volatiles compared to constitutive volatiles was found in *Carduus nutans* (Chi Sq. = 3.99, df = 1, P = 0.046), *Silybum marianum* (Chi Sq. = 4.81, df = 1, P = 0.028), *Centaurea nigra* (Chi Sq. = 15.25, df = 1, P < 0.001), *Cynara carduncul*us (Chi Sq. = 5.15, df = 1, P = 0.023) and *Cynara scolymus* (Chi Sq. = 5.89, df = 1, P = 0.015) (Figure 3.2). There was no preference towards HIPVs in *Cirsium* and *Carduus* species where the beetles showed attraction to constitutive volatiles during no-choice test.

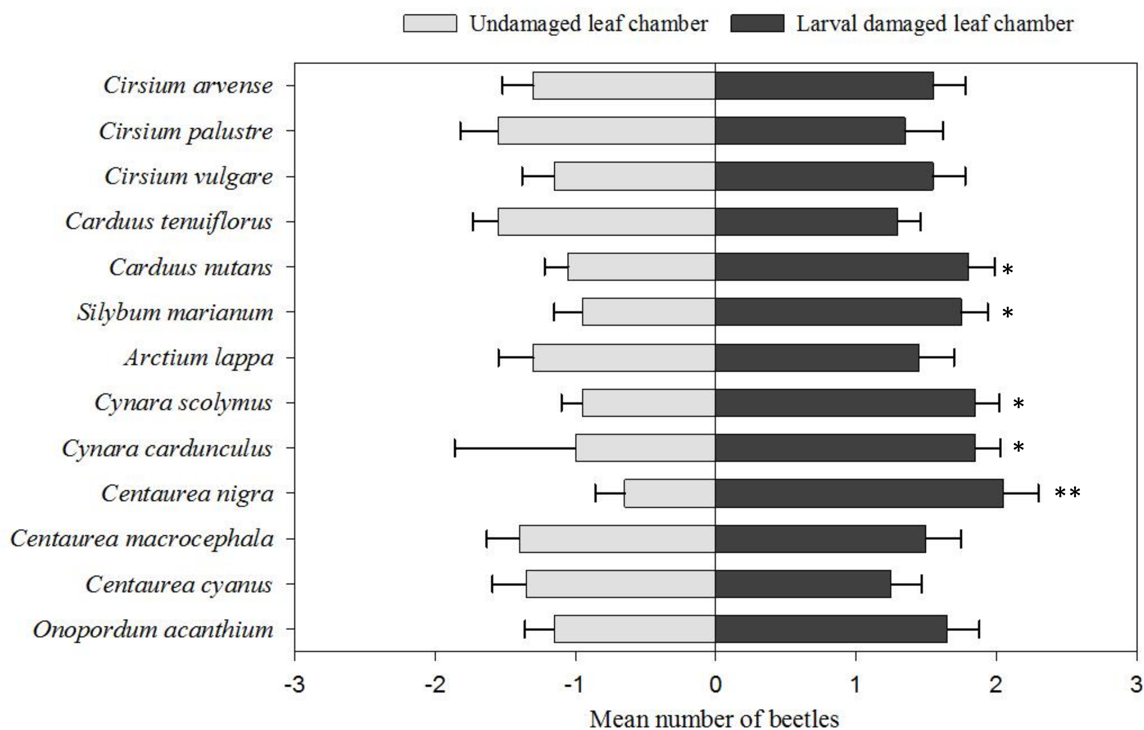

Figure 3.2 Mean number of adult female *Cassida rubiginosa* beetles choosing the constitutive volatiles (undamaged leaves) and herbivore induced volatiles (larval damaged leaves) inside the olfactometer during olfactory preference tests. * P <0.05, ** P < 0.001. Plants were arranged according to phylogenetic distance from *C. arvense.* N = 20

3.3.2 Volatile analysis

A total of 56 volatiles were collected from the five significantly attractive tests plants (*Carduus nutans*, *Silybum marianum*, *Cynara carduncul*us, *Cynara scolymus* and *Centaurea nigra*) with larval damage and 40 volatiles from the five conspecific undamaged test plants. Tentatively identified volatiles in the larval-damaged plants included two benzenoids, five green leaf

volatiles, two C_8 compounds, four monoterpenes and 36 sesquiterpenes, whereas the counter undamaged plants included one benzenoid, one green leaf volatile, 13 monoterpenes and 20 sesquiterpenes. Therefore, the herbivore-induced volatile bouquet of the plants that are attracted contained a higher variety of sesquiterpenes, whereas the undamaged plant volatile bouquet contained a higher variety of monoterpenes. There was a significant difference in the quantities of sesquiterpenes in *C. nutans* ($P < 0.0001$), *S. marianum* ($P = 0.0475$) and *C. nigra* ($P = 0.0021$); monoterpenes in *C. nigra* ($P = 0.019$) when comparing with undamaged plant leaves and the emission of green leaf volatiles was increased in *C. nigra* ($P = 0.023$), *C. cardunculus* ($P = 0.0064$) and *S. marianum* ($P = 0.006$) (Figure 3.3). Chemical compounds such as cis-β-ocimene and β-caryophyllene were detected in all five species whereas some compounds were present in four plant species: D-Limonene (was absent in *C. scolymus*), 2-ethyl-1-hexanol, (absent in *C. nutans*), α-humulene (absent in *C. scolymus*) and δ-cadinene (absent in *C. scolymus*). Some unidentified compounds were found uniquely in certain plant species (Appendix IV). Constitutive volatles were the same mentioned in Chapter 2 (Appendix I).

A total of 97 volatiles were collected from the 13 Cardueae plants with larval damage on them (Appendix IV). The volatile blend emitted in response to larval feeding was not phylogenetically conserved (Figure 3.4, Bray-Curtis similarity measure, P = 0.487). According to the hierarchical dendrogram, there is no relationship with HIPVs in phylogenetically close genera (Figure 3.4). Chemical compounds such as m-ethylacetophenone, α-copaene, β-caryophyllene and germacrene D were detected in most plant species. In most plant species there was a statistically significant difference when comparing the quantity of sesquiterpenes in damaged and undamaged plants (Table 3.1).

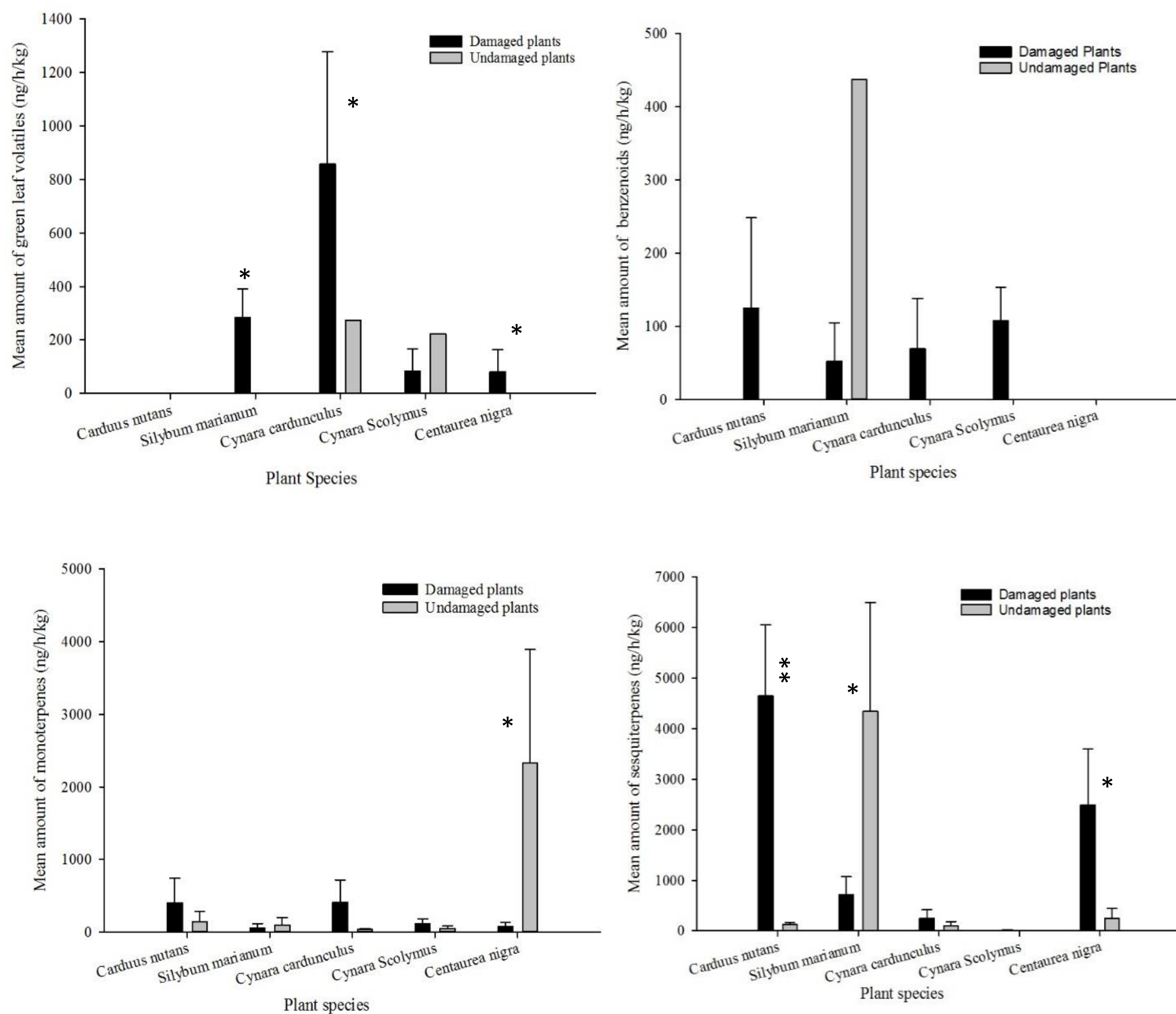

Figure 3.3 Comparison of total emitted volatiles in larval damaged and undamaged plants according to their chemical classes (benzenoids, green leaf volatiles, monoterpenes and sesquiterpenes) in the five plant species attracted by *Cassida rubiginosa*. * P <0.05, ** P < 0.001 (*t*-test).

Table 3.1 Comparison of constitutive volatiles vs herbivore-induced volatile emissions. P-values are given for the pairwise comparisons (t-tests) between the concentrations of each class of volatile with damaged and undamaged leaves. GLV –green leaf volatiles. Bold – statistically significant (P < 0.05).

Plant species	GLVs	Benzenoids	Monoterpenes	Sesquiterpenes
Cirsium arvense	0.9689	**0.0160**	0.4271	0.0742
Cirsium palustre	**0.0020**	**0.0010**	0.0648	**0.0012**
Cirsium vulgare	0.8643	0.1730	0.0527	**0.0001**
Carduus nutans	---------	0.0770	0.7651	**0.0001**
Carduus tenuiflorus	0.1350	---------	**0.0329**	**0.0005**
Silybum marianum	**0.0060**	0.1774	0.2544	**0.0475**
Arctium lappa	0.0030	---------	**0.0429**	**0.0084**
Cynara cardunculus	**0.0064**	0.1430	0.1043	0.9274
Cynara scolymus	0.4346	0.0540	0.4200	0.0530
Centaurea nigra	**0.0230**	---------	**0.0190**	**0.0021**
Centaurea macrocephala	**0.0055**	0.0514	0.0518	**0.0000**
Centaurea cyanus	**0.0330**	0.1810	**0.0122**	0.3510
Onopordum acanthium	**0.0202**	**0.0060**	0.6078	**0.0002**

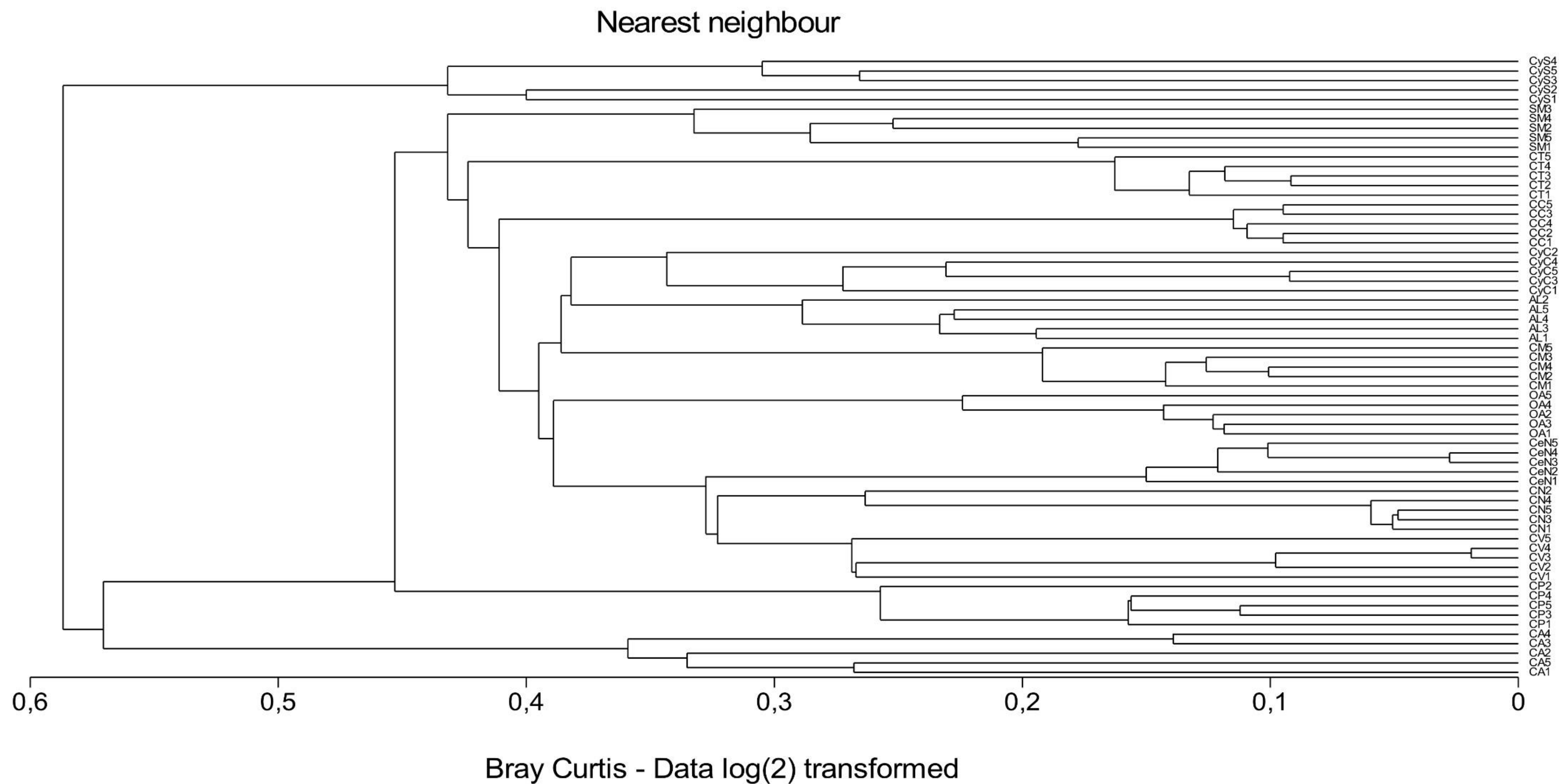

Figure 3.4 Hierarchical cluster dendrogram using nearest neighbour method and Bray-Curtis dissimilarity for the conspecific herbivore-induced volatiles. CA - *Cirsium arvense*, CV - *Cirsium vulgare,* CP - *Cirsium palustre,* CT - *Carduus tenuiflorus,* CN - *Carduus nutans,* SM - *Silybum marianum,* AL - *Arctium lappa,* CL - *Carthamus lanatus,* CyS - *Cynara scolymus,* CyC - *Cynara cardunculus,* PA - *Ptilostemon afer,* CeC - *Centaurea cyanus,* OA - *Onopordum acanthium,* CeN - *Centaurea nigra,* ER - *Echinops ritro,* CM - *Centaurea macrocephala.*

3.4 Discussion

Constitutive volatiles of *C. arvense, C. palustre, C. vulgare* and *C. tenuiflorus* species had previously been found to attract adult female *C. rubiginosa* beetles (see Chapter 2). Herbivory increased the attractiveness in some of the plants that did not show attraction to constitutive volatiles: such as *Carduus nutans*, *Silybum marianum*, *Centaurea nigra, Cynara scolymus* and *Cynara cardunculus,* due to the volatiles induced by conspecific larval feeding. Adult females attracted to a plant with conspecific larvae could lead to resource competition with regards to feeding and oviposition (Barnes and Murphy, 2018; Aukema and Raffa, 2002; Lamberti et al., 1987) and it could increase the risk of encountering host defences induced by the larvae (Shivaramu et al., 2017). Even though this study mainly focused on the olfactory orientation and individual feeding preference, in the field it is expected to have intraspecific competition for the resources, especially in the introduced range where there are less or no natural enemies (Cripps, 2009). Due to such potential negative effects, the question is, why would the females choose certain plant species that had been colonised by conspecific larvae? As the attractiveness of the preferred *Cirsium* and *Carduus* species did not change significantly with larval feeding, it is hypothesised that the attraction showed here might be due to the similar blend of volatiles to *Cirsium* and *Carduus* species in the HIPVs. Thus, when comparing the constitutive volatiles of *C. arvense* with HIPVs of the five attractive plant species, β-cis-ocimene and β-caryophyllene were found common to all. Another possible reason is the biology of the herbivore. Chrysomelid species tend to live in large aggregations (Pasteels et al., 1988). This aggregation can be for collective security (Vulinec, 1990; Treherne and Foster, 1980). Therefore, the attraction towards larval-damaged sub-optimal hosts instead of undamaged ones supported the aggregating behaviour of *Cassida* beetle. But surely volatiles have played a role in the attraction as not all the plants tested showed this aggregation. Aggregation of *Altica carduorum* (Coleoptera: Chrysomelidae) on *C. arvense* was reported more on leaves that have feeding damage, mechanical damage and larval faeces (Wan and Harris, 1996). Further investigations are needed to evaluate the effect of the volatiles emitted by larval exuviae and faeces in eliciting adult behaviour (Fernandez and Hilker, 2007).

Few studies have been conducted to investigate the behaviour of herbivores towards plant volatiles induced by conspecifics in host specificity testing (Sutton et al., 2017). Studies of such cues have been reported but mostly on insects and their preferred host plant, whereas in this study the effect of conspecifically induced volatiles from a range of phylogenetically related hosts was investigated. It has been shown that some herbivores are attracted to or repelled by

the volatiles emitted by plants due to damage from conspecifics (Bolter et al., 1997b). Examples of conspecific interactions include the Colorado potato beetles (*Leptinotarsa decemlineata*), where the beetle was more attracted to small potato plants when they were attacked with Colorado potato beetle larvae (Landolt et al., 1999; Bolter et al., 1997a) . Also, neonate fall armyworms, *Spodoptera frugiperda*, were more attracted to leaves that were fed by first instar conspecifics than undamaged plants (Carroll et al., 2008). In contrast, *Heliothis virescens* larvae induce volatiles during the night which repel the adult moth from *Nicotiana tabacum* plants (De Moraes et al., 2001). Kalberer et al. (2001) reported attraction of the leaf beetle *Oreina cacaliae* to its primary host plant *Adenostyles alliariae* was more attractive shortly after an attack by the conspecifics. Some studies showed differences in the attraction towards plants in the presence of different genders of conspecifics (Sutton et al., 2017; Tansey et al., 2005).

In this study, 3-hexenal, 3-hexene-1-ol, cis-β-ocimene, m-ethylacetophenone, cyclosativene, α-copaene, β-caryophyllene, germacrene D and caryophylene oxide were identified in all five plant species that were attractive to herbivores. Some of these chemicals, when individually tested, were found to be attractive to herbivores. For example, a study conducted with the Asian corn borer, *Ostrinia furnacalis*, using gas chromatographic-electroantennographic detection (GC-EAD) revealed that females respond to 3-hexen-1-ol and deposit fewer eggs on a wax paper treated with 3-hexen-1-ol, whereas neonate larvae are repelled (Huang et al., 2009). Germacrene D was reported as a compound responsible for the attraction of leaf beetle *Oreina cacaliae* towards the conspecific damaged *Adenostyles alliariae* plants (Kalberer et al., 2001). Volatiles emitted by tea plants infested by *Ectropis obliqua* larvae attracted more adult *E. obliqua* than uninfested plants and 3-hexenal were found to attract mated females (Sun et al., 2014). It was also detected that the quantity of green leaf volatiles and sesquiterpenes increased with herbivory. Some studies have showed high level of terpenoids and green leaf volatiles in HIPVs compare to constitutive (Aljbory and Chen, 2018; Mumm and Dicke, 2010; Dicke, 1994).

Considering all 13 Cardueae test plant species, m-ethylacetophenone, α-copaene, β-caryophyllene and germacrene D were found common to most of the plant species. For consistency only a volatile present in more than two samples were taken for analysis. Volatile detection can be vary even for the same plant species (Bruce et al., 2011). Thus, any plant volatiles that were not found in more than two samples were not taken into consideration. For example, germacrene D was found in only two of the five samples in *C. arvense* damaged

volatile blend and was therefore not included in calculations whereas it was found in all five samples in the *C. arvense* constitutive volatile blend and so was considered. Herbivore-induced volatiles did not show a phylogenetically conserved pattern. Evolution of the HIPVs as a defensive trait has not been studied much. Heil et al. (2004) have used the extra-foliar nectar secretion of Central American *Acasia* species to investigate the differential expression of resistance trait. A study conducted with terpenes of 202 Amazonian tree species, spanning the angiosperm diversity has shown strong phylogenetic signals and some compounds have displayed significant correlated evolution with at least one other compound (Courtois et al., 2016). During this study an increased amount of terpenes with herbivory was observed and it would be interesting to further investigate the terpene production with herbivory on thistles as an evolutionary trait of plant defence.

In summary, these results suggest that larval feeding by *C. rubiginosa* changes the plant volatile blend in several Cardueae species. This can affect the behaviour of the adult beetles, as the plants become more attractive. The presence of 3-hexen-1-ol, 3-hexenal and germacrene D could have possibly played a role in beetle attraction. Further research should be carried out to investigate the differential attraction towards the compounds identified, by using electrophysiological methods to determine which volatiles the herbivores perceive. It would be interesting to investigate the behavioural changes of adult *C. rubiginosa* with volatiles emitted in different larval density, growth stages and in the presence of different sexes of beetle as these behaviours are not well studied with *C. rubiginosa* (Fernandez and Hilker, 2007). Cues induced by conspecific are not usually considered in host-specificity testing procedures but a better understanding of their role in host-plant selection could improve the reliability of host specificity testing procedure.

3.5 Acknowledgements

I thank Jason Breitmeyer for his technical assistance with GC-MS analysis. This study was funded by the New Zealand Ministry of Business, Innovation, and Employment through AgResearch core funding of pasture weed research. And I would also like to acknowledge the Commonwealth scholarship offered by the Ministry of Foreign Affairs and Trade for their financial assistant to the principal researcher.

3.6 References

Aljbory, Z., Chen, M. S., 2018. Indirect plant defense against insect herbivores: a review. Insect Science. 25, 2-23.

Aukema, B. H., Raffa, K. F., 2002. Relative effects of exophytic predation, endophytic predation, and intraspecific competition on a subcortical herbivore: consequences to the reproduction of *Ips pini* and *Thanasimus dubius*. Oecologia. 133, 483-491.

Bacher, S., Luder, S., 2005. Picky predators and the function of the faecal shield of a cassidine larva. Functional Ecology. 19, 263-272.

Barnes, E. E., Murphy, S. M., 2018. Time-lagged intraspecific competition in temporally separated cohorts of a generalist insect. Oecologia. 186, 711-718.

Bernays, E. A., Chapman, R. F., 1994. Host-plant selection by phytophagous insects. Springer Science & Business Media.

Bolter, C. J., Dicke, M., Van Loon, J. J., Visser, J., Posthumus, M. A., 1997a. Attraction of Colorado potato beetle to herbivore-damaged plants during herbivory and after its termination. Journal of Chemical Ecology. 23, 1003-1023.

Bolter, C. J., Dicke, M., Van Loon, J. J. A., Visser, J. H., Posthumus, M. A., 1997b. Attraction of Colorado Potato Beetle to Herbivore-Damaged Plants During Herbivory and After Its Termination. Journal of Chemical Ecology. 23, 1003-1023.

Bruce, T. J. A., Pickett, J. A., 2011. Perception of plant volatile blends by herbivorous insects – Finding the right mix. Phytochemistry. 72, 1605-1611.

Bruce, T. J. A., 2015. Interplay between insects and plants: dynamic and complex interactions that have coevolved over millions of years but act in milliseconds. Journal of Experimental Botany. 66, 455-465.

Carroll, M. J., Schmelz, E. A., Teal, P. E., 2008. The attraction of *Spodoptera frugiperda* neonates to cowpea seedlings is mediated by volatiles induced by conspecific herbivory and the elicitor inceptin. Journal of Chemical Ecology. 34, 291-300.

Clavijo McCormick, A., Unsicker, S. B., Gershenzon, J., 2012. The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. Trends in Plant Science. 17, 303-310.

Courtois, E. A., Dexter, K. G., Paine, C. E. T., Stien, D., Engel, J., Baraloto, C., Chave, J., 2016. Evolutionary patterns of volatile terpene emissions across 202 tropical tree species. Ecology and Evolution. 6, 2854-2864.

Cripps, M., Jackman, S., Roquet, C., van Koten, C., Rostás, M., Bourdôt, G., Susanna, A., 2016. Evolution of Specialization of *Cassida rubiginosa* on *Cirsium arvense* (Compositae, Cardueae). Frontiers in Plant Science. 7, 1261.

Cripps, M. G., Influence of natural enemies on Cirsium arvense—a biogeographic perspective. Lincoln University, 2009.

Cripps, M. G., Bourdôt, G. W., Fowler, S. V., 2013. Sleeper thistles in New Zealand: status and biocontrol potential. New Zealand Plant Protection. 66, 99-104.

De Moraes, C. M., Lewis, W., Pare, P., Alborn, H., Tumlinson, J., 1998. Herbivore-infested plants selectively attract parasitoids. Nature. 393, 570.

De Moraes, C. M., Mescher, M. C., Tumlinson, J. H., 2001. Caterpillar-induced nocturnal plant volatiles repel conspecific females. Nature. 410, 577.

Delphia, C. M., Rohr, J. R., Stephenson, A. G., De Moraes, C. M., Mescher, M. C., 2009. Effects of genetic variation and inbreeding on volatile production in a field population of horsenettle. International Journal of Plant Sciences. 170, 12-20.

Dicke, M., 1994. Local and systemic production of volatile herbivore-induced terpenoids: their role in plant-carnivore mutualism. Journal of Plant Physiology. 143, 465-472.

Dicke, M., 2015. Herbivore-induced plant volatiles as a rich source of information for arthropod predators: Fundamental and applied aspects. Journal of the Indian Institute of Science. 95, 35-42.

Dicke, M., Baldwin, I. T., 2010. The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. Trends in Plant Science. 15, 167-175.

Dudareva, N., Pichersky, E., Gershenzon, J., 2004. Biochemistry of Plant Volatiles. Plant Physiology. 135, 1893-1902.

Eisner, T., van Tassell, E., Carrel, J. E., 1967. Defensive Use of a "Fecal Shield" by a Beetle Larva. Science. 158, 1471-1473.

El-Sayed, A., Knight, A., Basoalto, E., Suckling, D., 2018. Caterpillar-induced plant volatiles attract conspecific herbivores and a generalist predator. Journal of Applied Entomology. 142, 495-503.

Fernandez, P., Hilker, M., 2007. Host plant location by Chrysomelidae. Basic and Applied Ecology. 8, 97-116.

Frati, F., Salerno, G., Conti, E., Bin, F., 2008. Role of the plant–conspecific complex in host location and intra-specific communication of *Lygus rugulipennis*. Physiological Entomology. 33, 129-137.

Heil, M., Greiner, S., Meimberg, H., Krüger, R., Noyer, J.-L., Heubl, G., Linsenmair, K. E., Boland, W., 2004. Evolutionary change from induced to constitutive expression of an indirect plant resistance. Nature. 430, 205.

Heil, M., Ton, J., 2008. Long-distance signalling in plant defence. Trends in Plant Science. 13, 264-272.

Huang, C. H., Yan, F. M., Byers, J. A., Wang, R. J., Xu, C. R., 2009. Volatiles induced by the larvae of the Asian corn borer (*Ostrinia furnacalis*) in maize plants affect behavior of conspecific larvae and female adults. Insect Science. 16, 311-320.

Kalberer, N. M., Turlings, T. C., Rahier, M., 2001. Attraction of a leaf beetle (*Oreina cacaliae*) to damaged host plants. Journal of Chemical Ecology. 27, 647-661.

Lamberti, G., Feminella, J., Resh, V., 1987. Herbivory and intraspecific competition in a stream caddisfly population. Oecologia. 73, 75-81.

Landolt, P. J., Tumlinson, J., Alborn, D., 1999. Attraction of Colorado potato beetle (Coleoptera: Chrysomelidae) to damaged and chemically induced potato plants. Environmental Entomology. 28, 973-978.

Müller, C., 2002. Variation in the effectiveness of abdominal shields of cassidine larvae against predators. Entomologia Experimentalis et Applicata. 102, 191-198.

Mumm, R., Dicke, M., 2010. Variation in natural plant products and the attraction of bodyguards involved in indirect plant defense. Canadian Journal of Zoology. 88, 628-667.

Niederbacher, B., Winkler, J., Schnitzler, J., 2015. Volatile organic compounds as non-invasive markers for plant phenotyping. Journal of experimental botany. 66, 5403-5416.

Njuguna, P. K., Murungi, L. K., Fombong, A., Teal, P. E., Beck, J. J., Torto, B., 2018. Cucumber and Tomato Volatiles: Influence on Attraction in the Melon Fly *Zeugodacus cucurbitate* (Diptera: Tephritidae). Journal of agricultural and food chemistry. 66, 8504-8513.

Pasteels, J., Rowell-Rahier, M., Raupp, M., 1988. Plant-derived defense in chrysomelid beetles. Novel aspects of insect-plant interactions. Wiley, New York. 235-271.

Rim, H., Uefune, M., Ozawa, R., Takabayashi, J., 2018. An omnivorous arthropod, *Nesidiocoris tenuis*, induces gender-specific plant volatiles to which conspecific males and females respond differently. Arthropod-Plant Interactions. 1-9.

Rostás, M., Cripps, M. G., Silcock, P., 2015. Aboveground endophyte affects root volatile emission and host plant selection of a belowground insect. Oecologia. 177, 487-497.

Rostás, M., Eggert, K., 2008. Ontogenetic and spatio-temporal patterns of induced volatiles in *Glycine max* in the light of the optimal defence hypothesis. Chemoecology. 18, 29-38.

Shivaramu, S., Jayanthi, P. D. K., Kempraj, V., Anjinappa, R., Nandagopal, B., Chakravarty, A. K., 2017. What signals do herbivore-induced plant volatiles provide conspecific herbivores? Arthropod-Plant Interactions. 11, 815-823.

Sun, X.-L., Wang, G.-C., Gao, Y., Zhang, X.-Z., Xin, Z.-J., Chen, Z.-M., 2014. Volatiles emitted from tea plants infested by *Ectropis obliqua* larvae are attractive to conspecific moths. Journal of Chemical Ecology. 40, 1080-1089.

Susanna, A., Garcia-Jacas, N., Hidalgo, O., Vilatersana, R., Garnatje, T., 2006. The Cardueae (Compositae) revisited: insights from ITS, trnL-trnF, and matK nuclear and chloroplast DNA analysis. Annals of the Missouri Botanical Garden. 150-171.

Sutton, G., Paterson, I., Compton, S., Paynter, Q., 2017. Predicting the risk of non-target damage to a close relative of a target weed using sequential no-choice tests, paired-choice tests and olfactory discrimination experiments. Biocontrol Science and Technology. 27, 364-377.

Szendrei, Z., Rodriguez-Saona, C., 2010. A meta-analysis of insect pest behavioral manipulation with plant volatiles. Entomologia Experimentalis et Applicata. 134, 201-210.

Takabayashi, J., Dicke, M., 1996. Plant—carnivore mutualism through herbivore-induced carnivore attractants. Trends in Plant Science. 1, 109-113.

Tansey, J., McClay, A., Cole, D., Keddie, B., 2005. Evidence for the influence of conspecific chemical cues on *Aphthona nigriscutis* (Coleoptera: Chrysomelidae) behaviour and distribution. Bio Control. 50, 343.

Tipping, P. W., 1993. Field studies with *Cassida rubiginosa* (Coleoptera: Chrysomelidae) in Canada thistle. Environmental Entomology. 22, 1402-1407.

Treherne, J., Foster, W., 1980. The effects of group size on predator avoidance in a marine insect. Animal Behaviour. 28, 1119-1122.

Vulinec, K., 1990. Collective security: aggregation by insects as a defense. Insect Defenses. 251-288.

Wan, F. H., Harris, P., 1996. Host finding and recognition by *Altica carduorum*, a defoliator of *Cirsium arvense*. Entomologia Experimentalis et Applicata. 80**,** 491-496.

Webb, C., Sykes, W., Garnock-Jones, P., 1988. Flora of New Zealand Vol. 4. Naturalized Pteridophytes, gymnosperms, dicotyledons.

Zwölfer, H., Eichhorn, O., 1966. The host ranges of *Cassida* spp.(Coleoptera: Chrysomelidae) attacking *Cynareae* (Compositae) in Europe. Zeitschrift für Angewandte Entomologie. 58**,** 384-397.

Chapter 4

Effect of two pathogenic fungi on *Cirsium arvense* volatiles and preference of *Cassida rubiginosa*

Abstract
In biological control, several herbivore and pathogen species are often released against the same target without knowing how these biological control agents interact. In New Zealand, two fungal pathogens (*Puccinia punctiformis* and *Sclerotinia sclerotiorum*) and the chrysomelid beetle *Cassida rubiginosa* are used to control the thistle *Cirsium arvense*. This study investigated the compatibility of these biocontrol agents by studying the olfactory behaviour of adult *C. rubiginosa* towards fungus-infected thistle leaves. A series of olfactometer experiments were conducted to evaluate the host selection of the beetle; dual choice experiments were performed to investigate its feeding preference. Adult beetles were given the choice between healthy thistle leaves and leaves infected by one of the pathogens. Volatiles were collected from healthy and fungus-infected plants and analysed using GC-MS. The beetles' attraction towards *C. arvense* was reduced when infected with the biotrophic rust fungus *P. punctiformis*, while the necrotroph *S. sclerotiorum* had no effect. Both pathogens, however, reduced adult beetle leaf consumption. Feeding on the infected leaves was confined to infection-free areas. Volatile profiles showed a unique blend of compounds where the *P. punctiformis*-infected thistles were characterised by the emission of benzenoids and indole, *S. sclerotiorum* infected plants by green leaf volatiles. This study shows that the adult beetle recognises rust infection by its typical scent and consequently avoids such low-quality plants. It is speculated that the beetle was deterred by benzenoids and indole as these were restricted to *P. punctiformis*, while *S. sclerotiorum* did not alter olfactory behaviour. The study highlights the complexity of the interactions between biocontrol agents. Knowing the underlying mechanisms may help to improve the success of weed biocontrol.

Keywords: *Puccinia*, *Sclerotinia*, feeding, weed biological control, insect herbivore-fungal pathogen interaction

4.1 Introduction

Weeds are concurrently attacked by natural enemies, both herbivores and fungal pathogens. Simultaneous attacks of herbivores and pathogens can have a detrimental effect on a weed but might also interfere with the efficacy or performance of biocontrol agents occupying the same niche in a specific host-plant species. Combining agents that use different niches and co-exist together are more important in biological control rather than an individual agent (Janisiewicz, 1996). In some instances, interspecific competition can cause failure of some insect biological control agents as in the case of the seed head weevil, *Larinus minutus*, and a gall-inducing fly, *Urophora affinis*, two biocontrol agents released against spotted knapweed in North America (Crowe and Bourchier, 2006). *Larinus minutus* attack rates were significantly lower in the presence of *U. affinis* compared to release treatments where *L. minutus* was attacking alone and as a consequence of the antagonistic interaction the overall impact on the plant was not as high as it could have been when the beetle was attacking on its own. Thus, using multiple

agents may actually reduce the success of the involved agents through competitive exclusion (Ehler and Hall, 1984), especially when the agents are acting on the same niche competing for the same resources (Crowe and Bourchier, 2006). Therefore, it is important to investigate the change in preference and performance if both agents are acting on the same host plant. This is an important consideration as the process of introducing a weed biocontrol agent is an often tedious, involving extensive amounts of money and time (McFadyen, 1998).

Plant-phytopathogen-herbivore interactions are complex as the physiology of the plant changes in response to pathogen infection, which in return can change the conditions (nutrient quality, palatability, etc.) for the insect herbivore (Tack and Dicke, 2013; Awmack and Leather, 2002). Pathogen infections can thus influence the choice and the magnitude of damage caused by the insect herbivore (Rostás et al., 2003; Awmack and Leather, 2002; Hatcher, 1995; Hatcher et al., 1994). The consequence of these interactions for an insect herbivore can be beneficial, detrimental or neutral, depending on the plant species, pathogen and the insect herbivore (Barbosa, 1991; Rostás et al., 2003). Considering the effort and cost put into identifying the best biological control agent for a particular weed, it is important to ensure the optimum performance of the agent in the introduced environment, especially when multiple introductions take place for a particular host. Therefore, it is vital to identify the antagonistic, additive or synergistic interactions among the potential agents for a target weed.

Both pathogens and insect herbivores induce plant defence responses. Pathogen damage to plants can lead to the changes in plant volatile and non-volatile secondary compounds, primary metabolites and also visual cues (Franco et al., 2017; Dicke, 2015; Rostás et al., 2006; Hatcher, 1995; Hatcher et al., 1994). Certain pathogenic fungi emit their own volatile organic compounds (VOCs) and/or induce plants to produce VOCs; in both cases this can change the behaviour of insect herbivores (Drakulic et al., 2017; Guo et al., 2014; Tack and Dicke, 2013; Morath et al., 2012; Tasin et al., 2009; Evans et al., 2008; Cardoza et al., 2003; Rostás et al., 2003). Some microorganisms use mimicry as a strategy to attract or deter certain herbivores (Ngugi and Scherm, 2006). For example, the sexual stages of rust fungi in the genera *Puccinia* and *Uromyces* produce a flowery smell to attract pollinators to amplify the transfer of gametes (Naef et al., 2002; Pfunder and Roy, 2000; Raguso and Roy, 1998). In addition, the type of microorganism might be influential on the emitted VOCs such that behaviour of insects differs depending on the pathogen (reviews by Franco et al., 2017, Raman and Suryanarayanan, 2017). But overall these VOCs are perceived and used by insects to predict the status of the plant to

make the decision either to consume or reject it (Rizvi et al., 2015; Tasin et al., 2012; Dötterl et al., 2009; Rostas and Hilker, 2002; Simon and Hilker, 2005; Janzen, 1977).

This study focused on the preference of *Cassida rubiginosa* Müller (Coleoptera, Chrysomelidae), when its host plant, *Cirsium arvense* (L.) Scop (Asteraceae, Cardueae), is infected with either *Puccinia punctiformis* (Str.) Röhl (Basidiomycota, Pucciniaceae) or *Sclerotinia sclerotiorum* (Lib) de Bary (Ascomycota, Sclerotiniaceae). Californian thistle (*C. arvense*), is a well-known noxious weed, indigenous to Eurasia, that is found abundantly in pasture lands in New Zealand resulting in huge economic losses (e.g. loss in pastoral farm gross revenue of $685 million during 2011-2012) to the country (Bourdôt et al., 2016; Kaye-Blake et al., 2010). It is a dioecious perennial that can reproduce by seeds as well as by vegetative means using root buds. Creeping roots make it difficult to control this weed and there is demand for less labour-intensive, more economically friendly and repeated control methods (Ang et al., 1995). Therefore, over time, seven biocontrol agents (Cripps et al., 2011b) were released deliberately to control Californian thistle. So far, the most successful biocontrol agent introduced to control *C. arvense* in New Zealand is the leaf-feeding tortoise beetle *Cassida rubiginosa* which was introduced in 2007. It is an oligophagous univoltine beetle that completes its life cycle on the host plant; both larvae and adults are foliage feeders. The beetle can feed on other thistles, but its primary host is *C. arvense* (Cripps, 2013; Zwölfer and Eichhorn, 1966). Apart from *C. rubiginosa*, the highly specialised biotrophic rust fungus *Puccinia punctiformis* and the generalist necrotrophic *Sclerotinia sclerotiorum* have also been reported attacking *C. arvense* (Cripps et al., 2011a; Brosten and Sands, 1986). Both pathogens are non-indigenous species now wild in New Zealand and both have gained attention as potential biocontrol agents due to their high virulence towards Californian thistle (Bourdôt et al., 2006; Bourdôt and Harvey, 1996). Necrotrophic fungi kill the plant tissue before extracting nutrients and biotrophic fungi extract nutrients without killing the plant until their life cycle is complete (Raman and Suryanarayanan, 2017).

Puccinia punctiformis is an obligate parasite that infects Californian thistle systemically and forms necrosis of the leaves and stem (Frantzen, 1994; Thomas et al., 1994). Orange coloured spermagonia/pycniospores and a strong smell, mimicking the flower, are the early signs of the *P. punctiformis* infection (Demers et al., 2006; Thomas et al., 1994). It affects both the shoots and the flowers (Demers et al., 2006; Thomas et al., 1994) and can reduce the Californian thistle populations (Cripps et al., 2014; Berner et al., 2013). Incidence of natural infections of *P.*

punctiformis is less frequent in the field and artificial inoculation is also difficult (Cripps et al., 2009; Demers et al., 2006).

In contrast to biotrophic rust, the necrotroph *S. sclerotiorum* is fairly easy to culture, inoculate and infect the shoots and roots of the Californian thistle (Bourdôt and Harvey, 1996; Brosten and Sands, 1986). In the early stages of infection, small dark coloured lesions develop and once the fungus progresses into the main stem, wilting typically occurs and often the infected leaves shred (Bolton et al., 2006). All three species, fungi and herbivore, are considered important in different biological control aspects: *C. rubiginosa* in classical biological control (Cripps et al., 2011b), *P. punctiformis* in augmentative biological control (Waipara et al., 2009) and *S. sclerotiorum* as a bio-herbicide (Bourdôt et al., 2006).

Several studies have investigated *C. arvense*-*C. rubiginosa*-fungal pathogen interactions but none have studied the effect of fungal infection on volatile emissions and herbivore host-finding behaviour together. There have been separate studies focusing on *C. rubiginosa*-*C. arvense* (Cripps et al., 2015; Asadi et al., 2013; Cripps, 2013; Koji et al., 2012; Cripps et al., 2010; Reed et al., 2006; Bacher and Schwab, 2000; Spring and Kok, 1997; Ang et al., 1995; Ang et al., 1994), studies on fungal pathogen-*C. arvense* (Bourdôt et al., 2006; Kluth et al., 2005; Kluth et al., 2003; Bourdôt and Harvey, 1996; Bourdôot et al., 1995; Thomas et al., 1994; Brosten and Sands, 1986), and a few focusing on *C. arvense*-fungal pathogen-*C. rubiginosa* (Kluth et al., 2002; Kluth et al., 2001). No studies have explored the adult *C. rubiginosa* choice and feeding performance when the plant leaf is infected with *P. punctiformis* or *S. sclerotiorum*, or the change of volatile blend and the long-range host selection. The host-finding behaviour was investigated with regards to olfactory cues and feeding behaviour of *C. rubiginosa* when *C. arvense* is infected either with *P. punctiformis* or with *S. sclerotiorum.* Furthermore, this study fills the gap between the two disciplines, chemical ecology and biological control of weeds, which provide opportunities to investigate both fundamental and applied chemical mechanisms behind insect-plant interactions where much remains to be studied (review Wheeler and Schaffner (2013)).

4.2 Material and methods

Puccinia punctiformis infected plants were field collected from two farms located at Lincoln (43°38'17.37"S: 172°27'6.13"E and 43°40'2.65"S: 172°22'32.43"E) on the 7 and 14 December

2017. Infected wild plants were collected. By the time of the experiment, infected leaves were covered with uredinial spores on the abaxial side. Experiments were conducted on the day material was collected.

Isolates (S36) of *S. sclerotiorum* were taken from the cultures belong to the Pathology group at Lincoln University. Used cultures were verified by the expert (Dr. Seona Casonato, Pathology group, Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln) and were sub-cultured. Green house grown plants were used in inoculation. The plant leaves were inoculated with the *S. sclerotiorum* by placing small blocks of potato-dextrose agar containing the pathogen. The leaves were covered with polythene bags after placing the agar block, in order to increase the moisture levels surrounding the plant leaves to increase the rate of inoculation and the bag was removed after 2-3 days. After 4-5 days the leaves that were found to be infected with the pathogen were used in the experiments.

Field-collected beetles (from several farms in Wairarapa) were kept in 2 L ventilated plastic boxes under constant temperature of 20 ºC, inside temperature-controlled (CT) rooms (16h light: 8h dark period) at Lincoln University and were fed with *C. arvense* clippings. Only adult female beetles (weight of a beetle ≤19 mg, taken as males; and beetles >19 mg, taken as females) were used.

4.2.1 Olfactometer experiments

Small still-air olfactometers consisting of a glass Petri dish (140 mm diameter x 20 mm high) were used to investigate the long-range, host-selection behaviour of the beetle (see Chapter 2). The experiment was conducted in a CT room with a constant temperature of 23 ºC and 61-65% humidity. Prior to each bioassay, olfactometers were washed with tap water, distilled water, acetone (100%) and hexane (99.5%), respectively, to ensure they were void of impurities. Both pathogen-infected and uninfected *C. arvense* leaves were cut and the cut parts of the leaves were immediately wrapped in wet cotton balls and then with 2-inch-wide laboratory film (Parafilm; PM-992) to prevent drying. Wrapped leaves were placed inside the chamber. Rigid, green colour nylon mesh (1 mm x 1 mm) was placed between the cylinder and the cup to prevent the beetles entering the glass cup where the plant material was kept. Three female adult beetles were introduced to the base of the olfactometer and allowed a 6-hour period to choose the

desired chamber. All experiments were started around 10:00-10:30 a.m. when beetles were active. The beetles had three choices: to enter the chamber with rust-infected plant leaves or to enter the chamber with healthy, uninfected plant leaves, in each case passing through the glass tubes on the petri dish lids or to remain on the base of the olfactometer. Beetles that remained on the base of the olfactometers were not considered. Twenty replicates were conducted for each pathogen species using 20 olfactometers.

To test whether the *C. arvense* flower scent attracts *C. rubiginosa* beetles, olfactometer experiments were conducted using healthy *C. arvense* leaves in one chamber and healthy *C. arvense* leaves with flowers in the other chamber. Twenty replicates were conducted following the same procedure.

4.2.2 Volatile extraction

Volatile organic compounds (VOCs) were collected from healthy, undamaged and pathogen-infected plant leaves. Four leaves (2nd to 5th leaf) were placed in one glass vessel (volume 520 ml). The cut end was immediately submerged in 30 ml of water to reduce the emission of volatiles from the cut end and desiccation during volatile collection. In addition, empty odour-source vessels (control) were used to detect background impurities. At the beginning of each collection, odour source vessels were thoroughly washed with distilled water, acetone (100%) and hexane (95.5%), respectively, to minimise collection of contaminants. A push-pull system was used as described in Rostás and Eggert (2008). After setting-up, the vessels were left for 48 hours to allow the volatiles to accumulate inside. Filtered air (activated charcoal filter, 400 ml; Alltech, Deerfield, IL, USA) originating from a compressed air cylinder was pushed into the vessels through one of the teflon tubes at a rate of 0.8 $lmin^{-1}$. The air was pulled out through a trapping filter consisting of glass tube (7 cm) containing 30 mg of 80 – 100 mesh SuperQ absorbent (Alltech, Deerfield, IL, USA) at the same rate by using a vacuum pump (Me2, Vacuubrand, Wertheim, Germany). Each collection lasted for 6 hours. The absorbed compounds were eluted from the trapping filter with 100 µl dichloromethane and 200 ng tetralin (Sigma-Aldrich, Australia) added after the elution as an internal standard. All extracts were stored at -80 °C until analysis.

Samples were analysed for VOCs using an adaption of the method described by Rostás et al. (2015). A Shimadzu GCMS-QP2010 Ultra (Shimadzu, Japan) gas chromatography mass spectrometer was fitted with a Restek Rtx-5ms fused silica capillary column (30.0 m x 0.25 mm

internal diameter, 0.25 µm film thickness, Bellefonte, PA, USA). A CTC-Combi PAL auto sampler (PAL LHX-xt) was used to inject 1µl of sample into the gas chromatography (GC) injection port at 220 °C, operating in split-less mode at 241.0 kPa pressure for 39 seconds. After injection, the column oven was held at 35 °C for 3 minutes, then heated to 320°C at 8 °C min^{-1} and held at this temperature for 8 minutes. Helium was used as the carrier gas with the constant linear velocity set at 44.0 cm/sec (1.5 ml min^{-1}) in split mode (30:1) after the high-pressure split-less injection. The mass spectrometer (MS) was operated in electron impact ionization mode with 70eV and mass range of 33 to 550 m/z. The temperature of the capillary interface was 320 °C, with the source temperature set at 230 °C.

Initial identification of detected peaks was made by matching their mass spectra with the spectra of reference compounds found in the databases NIST EPA/NIH Mass Spectral Library database (National Institute of Standards and Technology, NIST11) and Wiley Registry of Mass Spectral Data 10th edition (John Wiley & Sons, Hoboken, New Jersey, USA).

4.2.3 Dual-choice feeding performance tests

The experiment was conducted using Petri dishes (9 cm diameter) lined with damp filter paper (LabServ qualitative filter paper; code: LBS0001.090; size: 90mm). Filter papers were water saturated and then drained until water stopped dripping. Leaves were taken from fungi-infected and uninfected plants and scanned to create a digital image (JPEG format) for the purpose of calculating the whole area of the leaves. Leaves with similar area and age were paired together. Whole leaf area and the infected area were calculated. Both leaves were then placed inside the Petri dish, along with a single adult female beetle. Petri dishes were closed and sealed with laboratory film (Parafilm; PM-992) to be airtight and left at 23 °C, 8/16 hr dark and light period for 2 days. For each fungal pathogen, the experiment was replicated 20 times with 20 Petri-dishes. After 2 days, the leaves were scanned and saved as JPEG image files with 300 dpi resolution and using Paint 6.1 (Windows 7) painted the concerned area (feeding holes) of the leaf image. The image was then converted to bitmap (24bitmap). The digital bitmap images were imported to Surface.exe software (written by Carsten Thiemann for Michael Rostás), which convert pixels into cm^2 (Pus, 2017).

4.2.4 Data analysis

Data were analysed using Minitab version 16 and GenStat version 16. One-sample t-tests were used to compare differences in the amount of feeding between the pathogen infected and the healthy leaves to test the hypothesis of no difference. The olfactometer data were analysed using a generalised linear model (GLM) assuming a Poisson distribution through log-link function. Volatiles were analysed by using the Shimadzu GCMS Solution Postrun analysis software. Principle component analysis was performed with the Multi Variate Statistical Package (MVSP) 3.22, Kovach Computing Services.

4.3 Results

4.3.1 Olfactometer experiments

Cassida rubiginosa demonstrated a significant preference for healthy *C. arvense* leaves when tested against *P. punctiformis*-infected *C. arvense* leaves (Chi-sq. = 25.08, df = 1, P < 0.0001),

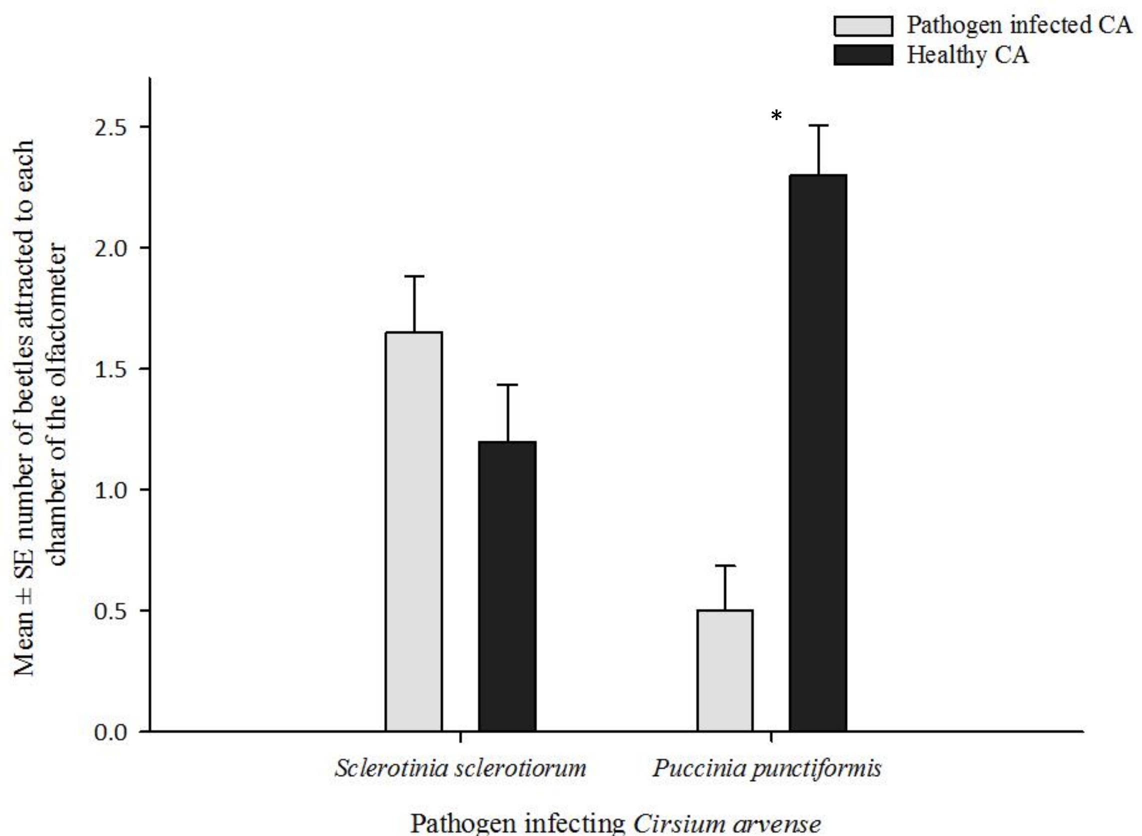

Figure 4.1 Mean ± SE number of *Cassida rubiginosa* that entered either the chamber containing pathogen infected or the healthy leaves of *C. arvense* (CA) during olfactometer experiments. Three adult females per replicate. Statistical comparisons are within groups, *Puccinia punctiformis* and *Sclerotinia sclerotiorum*. N = 20. *P < 0.05. PP: *Puccinia punctiformis* and SS: *Sclerotinia sclerotiorum*

whereas no significant preference (Chi-sq. = 1.43, df = 1, P = 0.232) was exhibited when tested with *S. sclerotiorum*-infected *C. arvense* leaves versus healthy *C. arvense* leaves (Figure 4.1). There was no significant preference (Chi-Sq. = 1.43, df = 1, P = 0.232) towards either chamber when tested with healthy *C. arvense* leaves (1.65 ± 0.21) and healthy *C. arvense* leaves with flowers (1.2 ± 0.21).

4.3.2 Volatile extraction

Volatile profiles showed a unique blend of compounds where the *P. punctiformis*-infected leaves were characterised by the emission of benzenoids and indole, and *S. sclerotiorum*-infected leaves by green leaf volatiles (GLVs) (Figure 4.2). The most abundant compound with the *P. punctiformis* infection was indole and benzene ethanol, whereas in *S. sclerotiorum*-infected *C. arvense* it was β-elemene and 3-hexenal. In fresh, healthy *C. arvense*, the most abundant compound was β-caryophyllene and (E,E)-α-farnesene (Table 4.1).

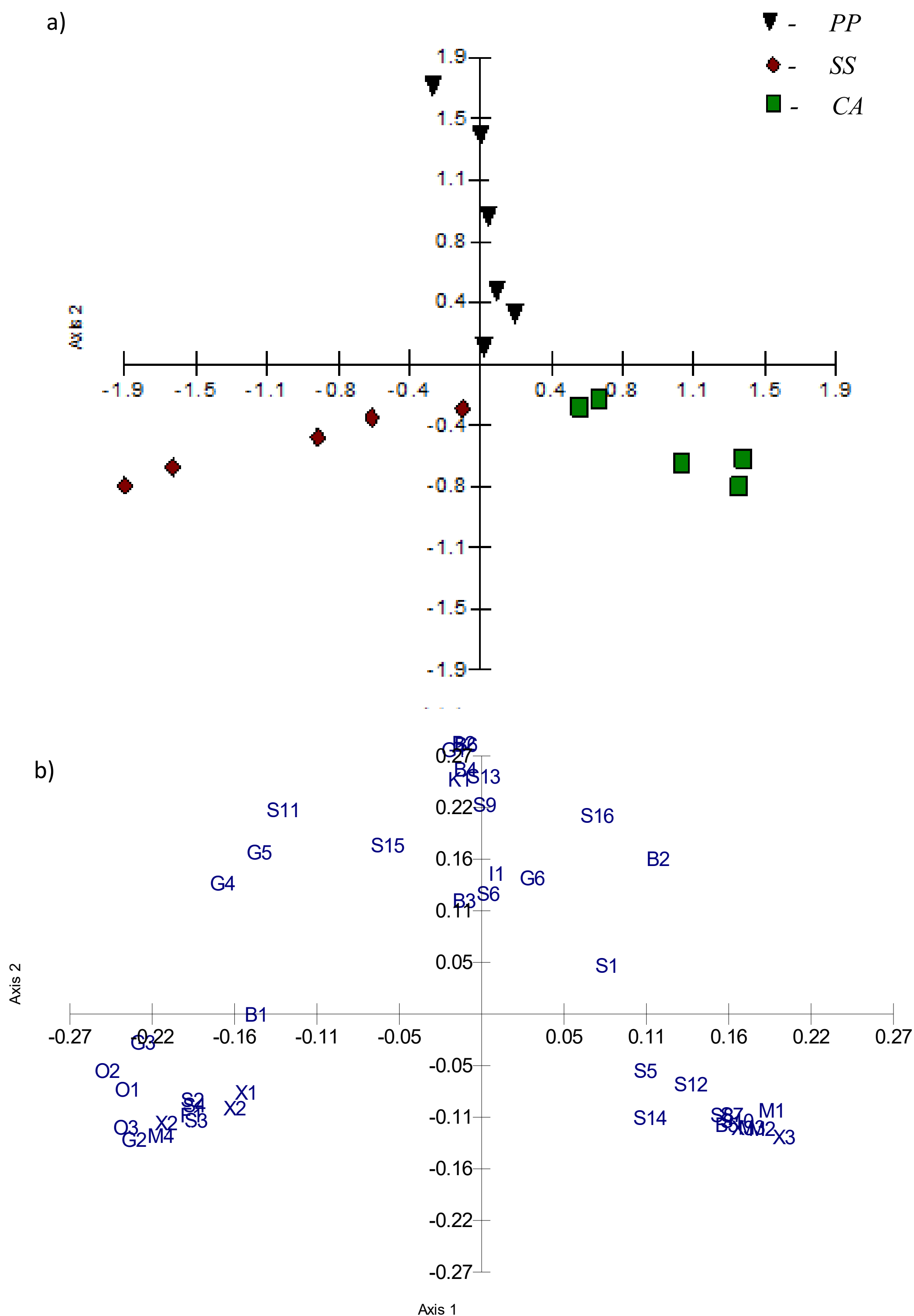

Figure 4.2 Principle component analysis distinguished clusters of emitted volatiles according to the treatment/infection. a) Score plot of the different classes of volatile compounds according to the treatment. b) Loading plot with volatiles analyzed using GC-MS. Axis 1 eigenvalue 13.31 / explained variance 30.2; Axis 2: 8.98 / 20.4; Axis 3: 5.57 / 12.65. PP - *Puccinia punctiformis* infected *Cirsium arvense* plants; SS - *Sclerotinia sclerotiorum* infected *Cirsium arvense* plants; and CA – healthy *Cirsium arvense*. G – green leaf volatiles; B – benzenoids; O – C_8 compounds; M – monoterpenoid; S – mesquterpenoid; I – indole; F – furan; K – ketone and X - unknown

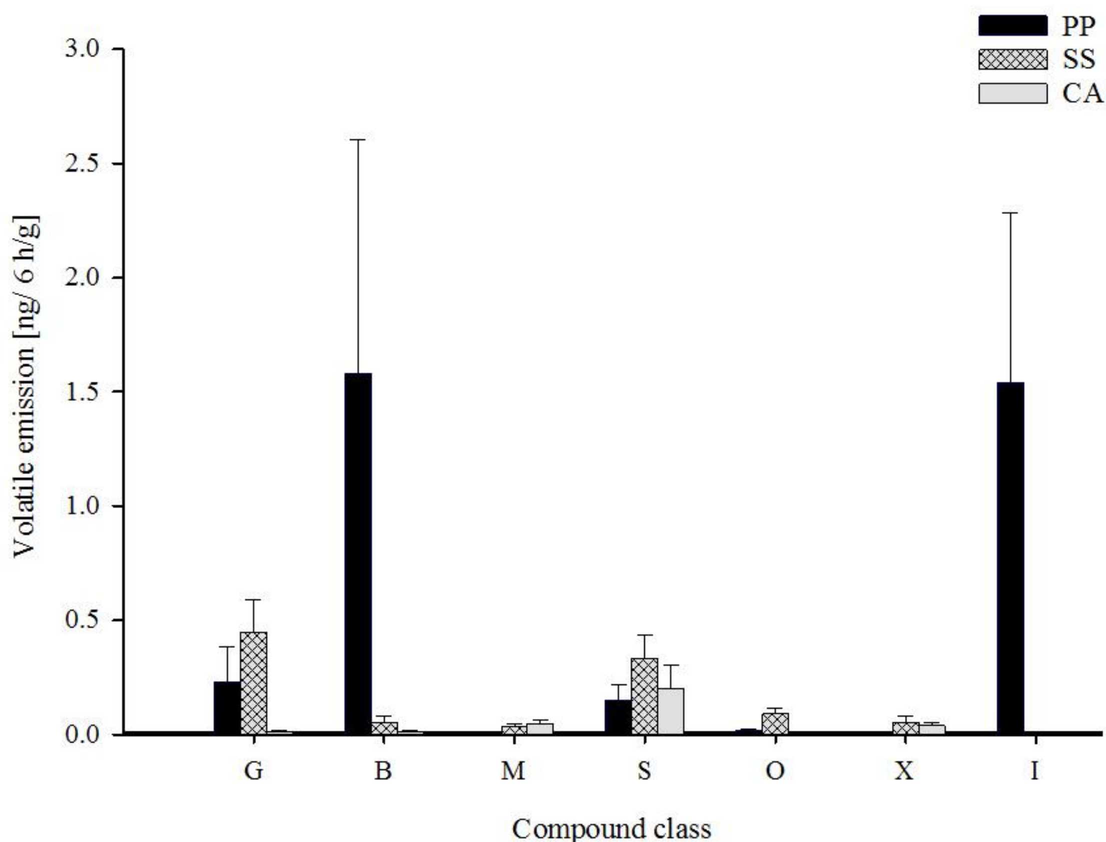

Figure 4.3 Plant volatiles emitted in accordance with the pathogen infections and the healthy *Cirsium arvense*. Mean amount of volatiles taken cumulatively according to the classes for each treatment. Mean ± SE shown. PP – *Puccinia punctiformis*-infected *Cirsium arvense* plants; SS – *Sclerotinia sclerotiorum*-infected *Cirsium arvense* plants; and CA – healthy *Cirsium arvense*. G – green leaf volatiles; B – benzenoids; O – C_8 compounds; M – monoterpenoid; S – sesquiterpenoid; I – indole and X – unknown

Some volatile compounds were found to be emitted by both *S. sclerotiorum*- and *P. punctiformis*-infected *C. arvense* such as 3-hexen-1-ol, 1-hexanol, benzaldehyde, 1-octen-3-ol, 1,5-octadien-3-ol, 3-octanone, benzene acetaldehyde, benzene ethanol, benzyl aldehyde and rosifoliol. Others were present only in *P. punctiformis*-infected leaves such as hexanal, phenol, β-phenethyl acetate, indole, α-bisabolene and 2-pentadecanone, 6,10,14-trimethyl, whereas 3-hexenal, (E)-2-hexenal and 2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl were only detected in *S. sclerotiorum*-infected *C. arvense*.

Table 4.1 Volatile organic compounds emitted by *Puccinia punctiformis*-infected *Cirsium arvense* (PP), *Sclerotinia sclerotiorum*-infected *Cirsium arvense* (SS) and healthy *Cirsium arvense* (CA). S - sesquiterpenoid, M - monoterpenoid, B - benzenoid, F - furans, K - ketones and X - unknown.

Compound	RI	Class	PP ng/h/g	SS ng/h/g	CA ng/h/g
Benzaldehyde	964	B1	0.91 ± 0.09	0.89 ± 0.10	-
Phenol/Benzenol	987	B2	2.77 ± 0.25	-	-
Benzyl alcohol	1038	B3	0.44 ± 0.03	-	0.31 ± 0.03
Benzene acetaldehyde	1043	B4	11.73 ± 1.01	1.26 ± 0.12	-
Benzene ethanol	1111	B5	77.90 ± 8.75	0.50 ± 0.06	-
Methyl salicylate	1196	B6	-	0.04 ± 0.01	0.21 ± 0.02
β-Phenethyl acetate	1256	B7	1.04 ± 0.09	-	-
2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	1537	F1	-	0.43 ± 0.05	-
Hexanal	812	G1	6.01 ± 0.64	-	-
3-Hexenal	814	G2	-	10.26 ± 0.57	-
(E)-2-Hexenal	863	G3	-	5.47 ± 0.38	-
3-Hexen-1-ol	864	G4	4.43 ± 0.48	5.07 ±0.36	-
1-Hexanol	881	G5	1.37 ± 0.15	1.13 ± 0.10	-
(Z)-3-Hexen-1-ol, acetate	1008	G6	0.65 ± 0.07	0.25 ± 0.03	0.50 ± 0.06
2-Pentadecanone, 6,10,14-trimethyl	1841	K1	0.20 ± 0.01	-	-
Sabinene	973	M1	0.03 ± 0.029	0.00096 ± 0.00019	0.09 ± 0.01
β-Myrcene	991	M2	-	-	0.25 ± 0.02
β-Ocimene E	1048	M3	-	0.10 ± 0.01	1.89 ± 0.17
α-Terpineol	1191	M4	-	1.65 ± 0.12	0.06 ± 0.004
1,5-Octadien-3-ol	977	O1	0.12 ± 0.01	0.80 ± 0.05	-
1-Octen-3-ol	982	O2	0.60 ± 0.04	2.37 ± 0.11	-
3-Octanone	988	O3	0.20 ± 0.02	1.25 ± 0.09	-
Indole	1291	S1	92.37 ± 7.43	-	-
γ-Guaiene	1503	S10	1.01 ± 0.05	-	
(E,E)-α-Farnesene	1507	S11	-	-	2.68 ± 0.20
Germacrene A	1510	S12	3.62 ± 0.19	2.27 ± 0.16	-
δ-Cadinene	1526	S13	-	-	0.08 ± 0.01
α-Bisabolene	1541	S14	0.66 ± 0.03	-	-
Caryophyllene oxide	1589	S15	-	0.05 ± 0.01	0.29 ± 0.04
Rosifoliol	1609	S16	0.43 ± 0.04	0.14 ± 0.01	
Neophytadiene	1833	S17	0.31 ± 0.03	1.12 ± 0.13	0.17 ± 0.01
Cyclosativene	1368	S2	0.15 ± 0.02	0.06 ± 0.01	0.25 ± 0.03
α-Copaene	1377	S3	0.31 ± 0.03	0.68 ± 0.01	-
Unknown ST 01	1384	S4	-	0.77 ± 0.05	-
β-Elemene	1392	S5	1.52 ± 0.13	12.20 ± 0.75	0.96 ± 0.09
β-Caryophyllene	1422	S6	0.63 ± 0.06	0.47 ± 0.03	3.82 ± 0.50
Unknown ST 02	1441	S7	0.40 ± 0.05	-	-
(E) β-Farnesene	1455	S8	-	-	0.41 ± 0.04

Germacrene D	1484	S9	0.07 ± 0.01	0.01 ± 0.0014	1.26 ± 0.11
Unknown 1	1026	X1	-	1.70 ± 0.22	-
Unknown 2	1072	X2	-	0.62 ± 0.06	-
Unknown 3	1115	X3	-	-	1.21 ± 0.12
Unknown 4	1557	X4	-	0.19 ± 0.02	-
Unknown 5	1578	X5	-	-	0.67 ± 0.02

4.3.3 Dual choice feeding performance tests

Both pathogens significantly reduced the attractiveness of leaves for *C. rubiginosa* (Table 4.2). By the time of the experiment *S. sclerotiorum* infection level was 30-40% of the total leaf area and *P. punctiformis* infected leaves contained spores in the whole underside of the leaves. Feeding on the infected leaves was confined to infection-free areas and there were remains of uninfected areas in case of *S. sclerotiorum* infected leaves. In *P. punctiformis* infected leaves beetles have fed on the upper surface of the leaves leaving the spore containing under sides.

Table 4.2 *Cassida rubiginosa* responses to feeding performance experiment, offering healthy *Cirsium arvense* leaves and pathogen infected *Cirsium arvense* leaves. N = 20.

Pathogen	Mean feeding (cm^2) + SE		t value	P value
	Pathogen infected leaf	Healthy leaf		
Puccinia punctiformis	0.4290 ± 0.0824	0.7960 ± 0.1340	2.29	0.033
Sclerotinia sclerotiorum	0.4050 ± 0.0909	1.3240 ± 0.2110	4.33	< 0.001

4.4 Discussion

The results of this study demonstrated that phytopathogenic infection of *C. arvense* can influence the long- and short-range host-selection behaviour of the leaf beetle *C. rubiginosa*. The beetles' response differed according to the fungal species. Adults of *C. rubiginosa* preferred the scent of healthy thistle compared with thistle infected by the biotrophic rust *P. punctiformis*. On the other hand, the beetle did not show an olfactory preference for healthy leaves or leaves infected by the necrotroph *S. sclerotiorum*. These results agree with a similar study conducted with the necrotrophic fungus *Botrytis cinerea* on grapevine (*Vitis vinifera*) (Tasin et al., 2012). Infected grapevine and healthy grapevine leaves were used to evaluate the behaviour of *Lobesia botrana* (Lepidoptera), a generalist insect herbivore. *Lobesia botrana*

showed attraction to plants during the first stages of infection, which was similar to our results with *S. sclerotiorum,* whereas in the later stage there was a significant orientation towards healthy grapevine plants (Tasin et al., 2012), which should be considered in future experiments with *S. sclerotiorum*. In this study, *S. sclerotiorum* infection level of a leaf was around 30-40% of the total leaf area. The attraction might have changed if the infection level was higher than this. However, beet armyworm moth (BAW), *Spodoptera exigua*, were attracted to peanut plants (*Arachis hypogaea*) infected with the necrotrophic fungus *Sclerotium rolfsii* both in host-choice and oviposition tests (Cardoza et al., 2003).

Rayamajhi et al. (2006) conducted similar studies using Y-tube olfactometers given the choice of the scent of healthy melaleuca (*Melaleuca quinquenervia*) leaves and *Puccinia psidii*-infected leaves to Australian weevil *Oxyops vitiosa*. The olfactory responses of the weevil was towards non-infected leaves which support our finding with *P. punctiformis*. Avoidance behaviour shown towards *P. punctiformis* during the olfactory experiment is a good adaptive strategy by *C. rubiginosa* that should increase its fitness. There might be a possibility to identify the reduced leaf nutrient quality from the scent of the infected leaves (Hatcher et al., 1995). For example, behavioural studies conducted using two herbivores, a gall making fly, *Asphondylia borrichiae* and a sapsucker, *Pissonotus quadripustulatus*, by manipulating the nutrient quality in sea oxeye daisy, *Borrichia frutescens*, showed that the herbivores were more likely to be attracted to plants that were higher in nutrient quality (Stiling and Moon, 2005). Kluth et al. (2002) have shown reduced performance of *C. rubiginosa* larvae that were fed on *P. punctiformis*-infected *C. arvense* leaves. Therefore, this behaviour conforms to the preference-performance hypothesis (Gripenberg et al., 2010) where the insect chooses to feed on plant material that results in performance of its offspring.

The different long-range, host-finding behaviour of the beetle with the two pathogens may be due to the altered volatile emissions. Cluster analysis and principal component analysis (Figure 4.2) clearly differentiate the volatile profiles of healthy *S. sclerotiorum*-infected and *P. punctiformis*-infected *C. arvense*. Terpenoids were detected as abundant with healthy *C. arvense*. There is a lack of literature regarding the leaf volatiles of *C. arvense.* Volatile compounds such as 1-octen-3-ol, 1, 5-octadien-3-ol, 3-octanone, sabinene, benzene acetaldehyde, benzene ethanol, benzaldehyde and β-caryophyllene were found to be emitted by both *S. sclerotiorum*-infected and *P. punctiformis*-infected *C. arvense* and they have been reported by several researchers as volatiles produced by fungi (Morath et al., 2012; Cardoza et

al., 2003; Cardoza et al., 2002). Predominantly, eight carbon compounds such as 1-octen-3-ol and 3-octanone produce musty odours and are typical fungal volatiles (Morath et al., 2012). 1-octen-3-ol ("mushroom alcohol") has been reported functioning either as a deterrent (Wood et al., 2001) or an attractant (Hedlund et al., 1995) of fungal feeders. Green leaf volatiles were prominent with the *S. sclerotiorum*-infected *C. arvense*. Necrotrophic fungi such as *S. sclerotiorum* cause cell disruption and rapid production of GLVs (Matsui, 2006). Release of C_6-aldehydes, such as 2-hexenal, can be a defensive mechanism of the plant to induce lignin biosynthesis and antifungal protein-gene expressions (Kishimoto et al., 2006; Kishimoto et al., 2005). Peanut plants, *Arachis hypogaea*, infected with *Sclerotium rolfsii*, a related necrotroph fungus, have been shown to emit 3-octanone, (Z)-3-hexenyl acetate and methyl salicylate. Among these, 3-octanone was also identified as a compound released by the fungus itself (Cardoza et al., 2002). However, unlike in this study, Cardoza et al. (2002) did not identify characteristic emission of GLVs by the plant.

Volatiles emitted by *P. punctiformis*-infected leaves were characterised by benzenoids and indole. Benzenoids were also found in *S. sclerotiorum*-infected *C. arvense* but in smaller quantities. Phenol, β-phenlethyl acetate and indole were found only with *P. punctiformis*-infected *C. arvense* leaves. *Puccinia punctiformis*-infected leaves emitted a pungent smell, similar to the smell of the thistle flower. Our olfactory studies testing *C. arvense* leaves with flowers against *C. arvense* leaves without flowers demonstrated that the beetles were not significantly attracted to any chamber. The two dominant components of the volatile blend of *C. arvense* flowers are benzaldehyde and phenyl acetaldehyde (Theis, 2006). In our study, benzaldehyde present in the volatile blend of *P. punctiformis*-infected leaves might be involved in producing the flowery smell (Connick Jr and French, 1991). Leaves serve as flowers with the *P. punctiformis* infection, but the beetles seemed to be deterred by it. The beetle did not repel due to thistle flowery smell inside the olfactometer that showed the beetles' capability of differentiating the thistle flower scent from the *P. punctiformis* scent. Thus, flower scent of *C. arvense* is mainly to attract pollinators and florivores (Theis, 2006). In this study, the volatiles that were detected with *P. punctiformis* infections agree with studies by Naef et al. (2002), Raguso and Roy (1998), and Connick Jr and French (1991), where all three studies highlight the presence of indole. Indole and benzenoids have been reported to be responsible for the scent of many flowers (Theis, 2006; Kite, 1995; Knudsen et al., 1993). Therefore, further experiments are needed to confirm the role of indole in rust-infected *C. arvense*. Since we did not test the response of the beetle to specific volatiles, further electrophysiological experiments coupled

with behavioural experiments are needed to identify the functions of the prominent volatiles that were detected in this study.

Fungus-infected plants were not preferred as food by *C. rubiginosa* when compared to healthy thistles. Our results align with the studies by Kluth et. al. (2001, 2002), which also demonstrated that larvae of *C. rubiginosa* prefer healthy over *P. punctiformis*-infected *C. arvense* leaves. In the nodding thistle, *Carduus nutans*, measurements of feeding, oviposition, longevity, egg production, egg hatch and larval development of *C. rubiginosa* when infected with *Puccinia carduorum* highlighted the adult beetles' preference towards rust-free leaves (Kok et al., 1996). Also, the feeding activity of the adult beetle was confined to uninfected areas of the infected leaves, whereas *C. rubiginosa* larvae have not shown such a behaviour. In contrast, a mutualistic relationship between *Apion onopordi*, a stem-boring weevil, and *P. punctiformis*, on *C. arvense* was reported, where the development of the weevils were found to be better when reared on the infected plant. Moreover, thistle shoot infection with the rust was promoted by the weevil (Friedli and Bacher, 2001). The diverse outcomes in the interactions between thistles, pathogenic fungi and herbivores highlights the specialised and complex nature of each interaction and shows that the consequences of interactions depend on the organisms involved.

Overall, this study showed that fungal biocontrol agents on *C. arvense* reduced the short-range preference of the herbivorous biocontrol agent, *C. rubiginosa* (i.e. it avoided infected portions of leaves). However, given only long-range volatile cues, the beetle was deterred by leaves infected with *P. punctiformis* but showed no difference in preference towards leaves infected with *S. sclerotiorum.* These results suggest that *C. rubiginosa* could complement infection by *S. sclerotiorum*, but would be unlikely to complement *P. punctiformis* since it is unlikely to be encountered. Moreover, fungal effects on *C. rubiginosa* were species specific, as rust infection of thistle leaves not only deterred but also repelled the beetles whereas *S. sclerotiorum* infection reduced the performance, thus highlighting the complexity of plant-fungus-insect interactions.

It is suggested that elucidating the olfactory responses of candidate herbivores to weed targets infected with biocontrol fungi in a pre-released context could increase the accuracy of predictions whether the release of both biocontrol agents will result in improved weed control. A similar approach has been proposed by Park et al. (2018) to improve host-plant specificity tests that are required for estimating non-target risks.

4.5 Acknowledgement

I thank Jason Breitmeyer for technical assistance with GC-MS analyses. This study was funded by the New Zealand Ministry of Business, Innovation, and Employment through AgResearch core funding of pasture weed research. I also acknowledge the Commonwealth scholarship offered by the Ministry of Foreign affairs and Trade to the first author.

4.6 References

Ang, B. N., Kok, L. T., Holtzman, G. I., Wolf, D. D., 1994. Competitive Growth of Canada Thistle, Tall Fescue, and Crownvetch in the Presence of a Thistle Defoliator, *Cassida rubiginosa* Müller (Coleoptera: Chrysomelidae). Biological Control. 4**,** 277-284.

Ang, B. N., Kok, L. T., Holtzman, G. I., Wolf, D. D., 1995. Canada Thistle [*Cirsium arvense* (L) Scop.] Response to Density of *Cassida rubiginosa* Müller (Coleoptera: Chrysomelidae) and Plant Competition. Biological Control. 5**,** 31-38.

Asadi, G., Ghorbani, R., Karimi, J., Bagheri, A., Mueller-Schaerer, H., 2013. Host Impact and Specificity of Tortoise Beetle (*Cassida rubiginosa*) on Canada Thistle (*Cirsium arvense*) in Iran. Weed Technology. 27**,** 405-411.

Awmack, C. S., Leather, S. R., 2002. Host plant quality and fecundity in herbivorous insects. Annual Review of Entomology. 47**,** 817-844.

Bacher, S., Schwab, F., 2000. Effect of Herbivore Density, Timing of Attack and Plant Community on Performance of Creeping Thistle *Cirsium arvense* (L.) Scop. (Asteraceae). Biocontrol Science and Technology. 10**,** 343-352.

Barbosa, P., 1991. Plant pathogens and non-vector herbivores. Microbial mediation of plant-herbivore interactions. Wiley, New York. 341-382.

Berner, D., Smallwood, E., Cavin, C., Lagopodi, A., Kashefi, J., Kolomiets, T., Pankratova, L., Mukhina, Z., Cripps, M., Bourdôt, G., 2013. Successful establishment of epiphytotics of *Puccinia punctiformis* for biological control of *Cirsium arvense*. Biological Control. 67**,** 350-360.

Bolton, M. D., Thomma, B. P. H. J., Nelson, B. D., 2006. *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Molecular Plant Pathology. 7**,** 1-16.

Bourdôot, G., Harvey, I., Hurrell, G., Saville, D., 1995. Demographic and biomass production consequences of inundative treatment of *Cirsium arvense* with *Sclerotinia sclerotiorum*. Biocontrol Science and Technology. 5**,** 11-26.

Bourdôt, G., Harvey, I., 1996. The potential of the fungus *Sclerotinia sclerotiorum* as a biological herbicide for controlling thistles in pasture. Plant Protection Quarterly. 11**,** 259-262.

Bourdôt, G. W., Hurrell, G. A., Saville, D. J., Leathwick, D. M., 2006. Impacts of applied *Sclerotinia sclerotiorum* on the dynamics of a *Cirsium arvense* population. Weed Research. 46**,** 61-72.

Bourdôt, G. W., Hurrell, G. A., Trolove, M., Saville, D. J., 2016. Seasonal dynamics of ground cover in *Cirsium arvense* – a basis for estimating grazing losses and economic impacts. Weed Research. 56**,** 179 - 191.

Brosten, B. S., Sands, D. C., 1986. Field trials of *Sclerotinia sclerotiorum* to control Canada thistle (*Cirsium arvense*). Weed Science. 34, 377-380.

Cardoza, Y. J., Alborn, H. T., Tumlinson, J. H., 2002. In vivo Volatile Emissions from Peanut Plants Induced by simultaneous fungal infection and insect damage. Journal of Chemical Ecology. 28, 161-174.

Cardoza, Y. J., Teal, P. E., Tumlinson, J. H., 2003. Effect of peanut plant fungal infection on oviposition preference by *Spodoptera exigua* and on host-searching behavior by *Cotesia marginiventris*. Environmental Entomology. 32, 970-976.

Connick Jr, W. J., French, R. C., 1991. Volatiles emitted during the sexual stage of the Canada thistle rust fungus and by thistle flowers. Journal of Agricultural and Food Chemistry. 39, 185-188.

Cripps, M., Bourdôt, G., Saville, D., Berner, D., Success with the rust pathogen, *Puccinia punctiformis*, for biological control of *Cirsium arvense*. XIV International Symposium on Biological Control of Weeds, 2014, pp. 83.

Cripps, M., Bourdôt, G., Saville, D., Hinz, H., Fowler, S., Edwards, G., 2011a. Influence of insects and fungal pathogens on individual and population parameters of *Cirsium arvense* in its native and introduced ranges. Biological Invasions. 13, 2739-2754.

Cripps, M., Gassmann, A., Fowler, S., Bourdôt, G., McClay, A., Edwards, G., 2011b. Classical biological control of *Cirsium arvense*: Lessons from the past. Biological Control. 57, 165-174.

Cripps, M. G., 2013. Observation of the thistle-feeding tortoise beetle, *Cassida rubiginosa* (Coleoptera: Chrysomelidae). The Weta. 45, 5 - 13.

Cripps, M. G., Edwards, G. R., Bourdôt, G. W., Saville, D. J., Hinz, H. L., Fowler, S. V., 2010. Effects of pasture competition and specialist herbivory on the performance of *Cirsium arvense*. Biocontrol Science and Technology. 20, 641-656.

Cripps, M. G., Edwards, G. R., Waipara, N. W., Bourdôt, G. W., Saville, D. J., Fowler, S. V., 2009. Does transmission of the rust pathogen, *Puccinia punctiformis*, require stem mining vectors? Biocontrol Science and Technology. 19, 447-454.

Cripps, M. G., Jackman, S. D., Rostas, M., Van Koten, C., Bourdot, G. W., 2015. Leaf traits of congeneric host plants explain differences in performance of a specialist herbivore. Ecological Entomology. 40, 237-246.

Crowe, M., Bourchier, R., 2006. Interspecific interactions between the gall-fly *Urophora affinis* Frfld.(Diptera: Tephritidae) and the weevil Larinus minutus Gyll.(Coleoptera: Curculionidae), two biological control agents released against spotted knapweed, *Centaurea stobe* L. ssp. micranthos. Biocontrol Science and Technology. 16, 417-430.

Demers, A. M., Berner, D. K., Backman, P. A., 2006. Enhancing incidence of *Puccinia punctiformis*, through mowing, to improve management of Canada thistle (*Cirsium arvense*). Biological Control. 39, 481-488.

Dicke, M., 2015. Herbivore-induced plant volatiles as a rich source of information for arthropod predators: Fundamental and applied aspects. Journal of the Indian Institute of Science. 95, 35-42.

Dötterl, S., Jürgens, A., Wolfe, L., Biere, A., 2009. Disease status and population origin effects on floral scent: potential consequences for oviposition and fruit predation in a complex interaction between a plant, fungus, and noctuid moth. Journal of Chemical Ecology. 35, 307-319.

Drakulic, J., Bruce, T., Ray, R. V., 2017. Direct and host-mediated interactions between *Fusarium* pathogens and herbivorous arthropods in cereals. Plant Pathology. 66**,** 3-13.

Ehler, L. E., Hall, R. W., 1984. Evidence for Competitive Exclusion of Introduced Natural Enemies in Biological Control: An Addendum. Environmental Entomology. 13**,** 5-7.

Evans, J. A., Eyre, C. A., Rogers, H. J., Boddy, L., Müller, C. T., 2008. Changes in volatile production during interspecific interactions between four wood rotting fungi growing in artificial media. Fungal Ecology. 1**,** 57-68.

Franco, F. P., Moura, D. S., Vivanco, J. M., Silva-Filho, M. C., 2017. Plant–insect–pathogen interactions: a naturally complex ménage à trois. Current Opinion in Microbiology. 37**,** 54-60.

Frantzen, J., 1994. An epidemiological study of Puccinia punctiformis (Str.) Röhl as a stepping-stone to the biological control of *Cirsium arvense* (L.) Scop. New Phytologist. 127**,** 147-154.

Friedli, J., Bacher, S., 2001. Mutualistic interaction between a weevil and a rust fungus, two parasites of the weed *Cirsium arvense*. Oecologia. 129**,** 571-576.

Gripenberg, S., Mayhew, P. J., Parnell, M., Roslin, T., 2010. A meta-analysis of preference–performance relationships in phytophagous insects. Ecology Letters. 13**,** 383-393.

Guo, Z., Döll, K., Dastjerdi, R., Karlovsky, P., Dehne, H.-W., Altincicek, B., 2014. Effect of fungal colonization of wheat grains with *Fusarium* spp. on food choice, weight gain and mortality of meal beetle larvae (*Tenebrio molitor*). PloS One. 9**,** e100112.

Hatcher, P., Paul, N., Ayres, P., Whittaker, J., 1995. Interactions between *Rumex* spp., herbivores and a rust fungus: the effect of *Uromyces rumicis* infection on leaf nutritional quality. Functional Ecology. 97-105.

Hatcher, P. E., 1995. Three-way interactions between plant pathogenic fungi, herbivorous insects and their host plants. Biological Reviews. 70**,** 639-694.

Hatcher, P. E., Paul, N. D., Ayres, P. G., Whittaker, J. B., 1994. The effect of a foliar disease (rust) on the development of *Gastrophysa viridula* (Coleoptera: Chrysomelidae). Ecological Entomology. 19**,** 349-360.

Hedlund, K., Bengtsson, G., Rundgren, S., 1995. Fungal odour discrimination in two sympatric species of fungivorous collembolans. Functional Ecology. 869-875.

Janisiewicz, W., 1996. Ecological diversity, niche overlap, and coexistence of antagonists used in developing mixtures for biocontrol of postharvest diseases of apples. Phytopathology. 86**,** 473-479.

Janzen, D. H., 1977. Why fruits rot, seeds mold, and meat spoils. The American Naturalist. 111**,** 691-713.

Kaye-Blake, W., Bourdôt, G., Dhakal, B., 2010. Valuing potential benefits of research into the biological control of Californian thistle (*Cirsium arvense*): A bioeconomic model. Nong Ye Ke Xue Yu Ji Shu. 4**,** 25.

Kishimoto, K., Matsui, K., Ozawa, K., Takabayashi, J., 2006. Components of C6-aldehyde-induced resistance in *Arabidopsis thaliana* against a necrotrophic fungal pathogen, *Botrytis cinerea*. Plant Science. 170**,** 715-723.

Kishimoto, K., Matsui, K., Ozawa, R., Takabayashi, J., 2005. Volatile C6-aldehydes and allo-ocimene activate defense genes and induce resistance against *Botrytis cinerea* in *Arabidopsis thaliana*. Plant and Cell Physiology. 46, 1093-1102.

Kite, G. C., 1995. The floral odour of *Arum maculatum*. Biochemical Systematics and Ecology. 23, 343-354.

Kluth, S., Kruess, A., Tscharntke, T., 2001. Interactions between the rust fungus *Puccinia punctiformis* and ectophagous and endophagous insects on creeping thistle. Journal of Applied Ecology. 38, 548-556.

Kluth, S., Kruess, A., Tscharntke, T., 2002. Insects as vectors of plant pathogens: mutualistic and antagonistic interactions. Oecologia. 133, 193-199.

Kluth, S., Kruess, A., Tscharntke, T., 2003. Influence of mechanical cutting and pathogen application on the performance and nutrient storage of *Cirsium arvense*. Journal of Applied Ecology. 40, 334-343.

Kluth, S., Kruess, A., Tscharntke, T., 2005. Effects of two pathogens on the performance of *Cirsium arvense* in a successional fallow. Weed research. 45, 261-269.

Knudsen, J. T., Tollsten, L., Bergström, L. G., 1993. Floral scents—a checklist of volatile compounds isolated by head-space techniques. Phytochemistry. 33, 253-280.

Koji, S., Kaihara, K., Nakamura, K., 2012. Stage-specific mortality, fecundity, and population changes in *Cassida rubiginosa* (Coleoptera: Chrysomelidae) on wild thistle. Applied Entomology and Zoology. 47, 457-465.

Kok, L., Abad, R., Baudoin, A., 1996. Effects of *Puccinia carduorumon* Musk Thistle Herbivores. Biological Control. 6, 123-129.

Matsui, K., 2006. Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Current Opinion in Plant Biology. 9, 274-280.

McFadyen, R. E. C., 1998. Biological control of weeds. Annual Review of Entomology. 43, 369-393.

Morath, S. U., Hung, R., Bennett, J. W., 2012. Fungal volatile organic compounds: a review with emphasis on their biotechnological potential. Fungal Biology Reviews. 26, 73-83.

Naef, A., Roy, B. A., Kaiser, R., Honegger, R., 2002. Insect-mediated reproduction of systemic infections by *Puccinia arrhenatheri* on *Berberis vulgaris*. New Phytologist. 154, 717-730.

Ngugi, H. K., Scherm, H., 2006. Mimicry in plant-parasitic fungi. FEMS microbiology letters. 257, 171-176.

Park, I., Eigenbrode, S. D., Cook, S. P., Harmon, B. L., Hinz, H. L., Schaffner, U., Schwarzländer, M., 2018. Examining olfactory and visual cues governing host-specificity of a weed biological control candidate species to refine pre-release risk assessment. Biological Control. 1-13.

Pfunder, M., Roy, B. A., 2000. Pollinator-mediated interactions between a pathogenic fungus, *Uromyces pisi* (Pucciniaceae), and its host plant, *Euphorbia cyparissias* (Euphorbiaceae). American Journal of Botany. 87, 48-55.

Pus, W., Plant-mediated effects of Trichoderma spp. and *Beauveria bassiana* isolates on insect and pathogen resistance. Lincoln University, 2017.

Raguso, R. A., Roy, B. A., 1998. 'Floral'scent production by *Puccinia* rust fungi that mimic flowers. Molecular Ecology. 7, 1127-1136.

Raman, A., Suryanarayanan, T., 2017. Fungus–plant interaction influences plant-feeding insects. Fungal Ecology. 29, 123-132.

Rayamajhi, M., Van, T., Pratt, P., Center, T., 2006. Interactive association between *Puccinia psidii* and Oxyops vitiosa, two introduced natural enemies of *Melaleuca quinquenervia* in Florida. Biological Control. 37, 56-67.

Reed, C. C., Larson, D. L., Larson, J. L., 2006. Canada thistle biological control agents on two south dakota wildlife refuges. Natural Areas Journal. 26, 47-52.

Rizvi, S. Z. M., Raman, A., Wheatle, W., Cook, C., Nicol, H., 2015. Influence of *Botrytis cinerea* (Helotiales: Sclerotiniaceae) infected leaves of *Vitis vinifera* (Vitales: Vitaceae) on the preference of *Epiphyas postvittana* (Lepidoptera: Tortricidae). Austral Entomology. 54, 60-70.

Rostás, M., Cripps, M. G., Silcock, P., 2015. Aboveground endophyte affects root volatile emission and host plant selection of a belowground insect. Oecologia. 177, 487-497.

Rostás, M., Eggert, K., 2008. Ontogenetic and spatio-temporal patterns of induced volatiles in *Glycine max* in the light of the optimal defence hypothesis. Chemoecology. 18, 29-38.

Rostas, M., Hilker, M., 2002. Asymmetric plant-mediated cross-effects between a herbivorous insect and a phytopathogenic fungus. Agricultural and Forest Entomology. 4, 223-231.

Rostás, M., Simon, M., Hilker, M., 2003. Ecological cross-effects of induced plant responses towards herbivores and phytopathogenic fungi. Basic and Applied Ecology. 4, 43-62.

Rostás, M., Ton, J., Mauch-Mani, B., Turlings, T. C. J., 2006. Fungal Infection Reduces Herbivore-Induced Plant Volatiles of Maize but does not Affect Naïve Parasitoids. Journal of Chemical Ecology. 32, 1897.

Simon, M., Hilker, M., 2005. Does rust infection of willow affect feeding and oviposition behavior of willow leaf beetles? Journal of insect behavior. 18, 115-129.

Spring, A., Kok, L. T., 1997. Field studies on the reproductive capacity and development of *Cassida rubiginosa* (Coleoptera: Chrysomelidae) on musk and Canada thistles. Environmental Entomology. 26, 876-881.

Stiling, P., Moon, D. C., 2005. Quality or quantity: the direct and indirect effects of host plants on herbivores and their natural enemies. Oecologia. 142, 413-420.

Tack, A. J., Dicke, M., 2013. Plant pathogens structure arthropod communities across multiple spatial and temporal scales. Functional Ecology. 27, 633-645.

Tasin, M., Bäckman, A.-C., Anfora, G., Carlin, S., Ioriatti, C., Witzgall, P., 2009. Attraction of female grapevine moth to common and specific olfactory cues from 2 host plants. Chemical senses. 35, 57-64.

Tasin, M., Knudsen, G. K., Pertot, I., 2012. Smelling a diseased host: grapevine moth responses to healthy and fungus-infected grapes. Animal Behaviour. 83, 555-562.

Theis, N., 2006. Fragrance of Canada Thistle (*Cirsium arvense*) Attracts Both Floral Herbivores and Pollinators. Journal of Chemical Ecology. 32, 917-927.

Thomas, R. F., Tworkoski, T. J., French, R. C., Leather, G. R., 1994. *Puccinia punctiformis* affects growth and reproduction of Canada thistle (*Cirsium arvense*). Weed Technology. 488-493.

Waipara, N., Barton, J., Smith, L., Harman, H., Winks, C., Massey, B., Wilkie, J., Gianotti, A., Cripps, M., 2009. Safety in New Zealand weed biocontrol: a nationwide pathogen survey for impacts on non-target plants. Journal of New Zealand Plant Protection Society 62**,** 41-49.

Wheeler, G. S., Schaffner, U., 2013. Improved understanding of weed biological control safety and impact with chemical ecology: a review. Invasive Plant Science and Management. 6**,** 16-29.

Wood, W. F., Archer, C. L., Largent, D. L., 2001. 1-Octen-3-ol, a banana slug antifeedant from mushrooms. Biochemical Systematics and Ecology. 29**,** 531-533.

Zwölfer, H., Eichhorn, O., 1966. The host ranges of *Cassida* spp.(Coleoprtera: Chrysomelidae) attacking *Cynareae* (Compositae) in Europe. Zeitschrift für angewandte Entomologie. 58**,** 384-397.

Chapter 5
Impact of the biocontrol beetle, *Cassida rubiginosa,* on the secondary weed target, marsh thistle *(Cirsium palustre)*

Abstract

The folivorous beetle *Cassida rubiginosa* was introduced to New Zealand to control the weed, Californian thistle (*Cirsium arvense*). Although Californian thistle is the primary host, many other thistles are accepted hosts. The objective of this study was to test if the beetle can reduce the fitness of marsh thistle (*Cirsium palustre*). A potted plant experiment was established with four treatments (0, 50, 100 and 200 larvae/plant). Plant growth (width, height, and number of branches) and reproductive performance (number of flowers, seeds, seed weight and % germination) parameters were measured. No significant differences were found for any of the measured parameters, except % germination. Higher larval densities (100 and 200) resulted in approximately 10% less germination compared with lower densities (0 and 50). Under these experimental conditions, *C. rubiginosa* had minimal impact on the performance of marsh thistle. For the beetle to have an impact, it would likely need to attack smaller, non-bolting rosettes, or be combined with additional stressors that might be encountered in a natural field population.
Keywords green thistle beetle, seed viability, alternative host, *Cirsium*.

5.1 Introduction

Herbivorous arthropods released as weed biocontrol agents need to be sufficiently host-specific to ensure safety to non-target plants. The normal practice is to release the most specialised agents, typically those that are monophagous, or restricted to a defined group of host plants closely related to the target weed. Historically, the cases of classical biocontrol agents released against thistle weeds were no exception to this practice, with the most specialised agents selected and released. In New Zealand (NZ), eight biocontrol agents were released from 1973 to 1998 against three thistle weeds: Californian thistle (*Cirsium arvense*), Scotch thistle (*Cirsium vulgare*) and nodding thistle (*Carduus nutans*) (Winston et al., 2014). These historical releases made use of 'transfer projects' whereby the same agents previously released in temperate North America were released in NZ (Fowler et.al., 2010). The most host-specific agents were released in North America to minimise the potential for non-target attack to native thistle species occurring there (Schröder, 1980). The transfer projects were an economical way to safely release biocontrol agents in NZ, but not necessarily the best for releasing the most effective agents (Cripps et al., 2011).

Thistles, in the broad sense, belong to a well-defined taxonomic group (monophyletic tribe), the Cardueae, of which there are no native representatives in NZ (Webb et al., 1988). The absence of native Cardueae species in NZ, and only a few introduced species of economic value within the tribe (e.g. artichoke and safflower), provides a rare opportunity to release oligophagous biocontrol agents specialised at the tribal level. Thus, a new approach, considering the absence of native thistles in NZ, resulted in the release of the oligophagous beetle, *Cassida rubiginosa*, in 2007. The primary target of this biocontrol agent is Californian thistle, although the potential for control of other thistle weeds, which could be considered secondary targets, has been recognised (Bourdôt et al., 2007). At least 63 thistle weed species have been introduced into NZ, of which nine are currently agricultural weeds of economic significance, and the remaining species are considered 'sleeper weeds' with potential to become problematic (Cripps et al., 2013).

Several studies have demonstrated that the beetle can control its primary host, Californian thistle (Ang et al., 1995a; Bacher & Schwab, 2000b; Cripps et al., 2010), but little is known about its potential to control other thistle weeds. The ability for *C. rubiginosa* to control Californian thistle is due, at least in part, to the thistle's unique biology, being a perennial reproducing by seed and by creeping lateral roots. All other thistle weeds reproduce solely by seed. In established populations of Californian thistle, reproduction is primarily by vegetative means, with little recruitment from seed, unless there is soil disturbance (e.g. from cattle pugging, or tillage) (Edwards et al., 2000). Furthermore, the creeping roots are ephemeral, lasting no longer than one year, and the annual formation of new root biomass is directly related to the photosynthetic opportunity of the plant over the growing season (Bourdôt et al., 1998). Thus, defoliation in one growing season can reduce the production of root biomass, and cause subsequent shoot population decline.

With regard to other Cardueae species in NZ, adult *C. rubiginosa* have been observed to feed and oviposit on field populations of nodding thistle, Scotch thistle, winged thistle (*Carduus tenuiflorus*), marsh thistle (*Cirsium palustre*), and artichoke (*Cynara scolymus*) (unpublished data). Utilisation of these plants was predicted by pre-release choice and no-choice host testing (Paynter et al., 2015); however, the potential impact on individual plant, or population performance, is uncertain. As an initial step towards understanding the potential impact of *C. rubiginosa* on other thistle weeds, marsh thistle was selected as a test species, since it is closely

related (congeneric) to Californian thistle, and the survival of the beetle is equivalent on this species (Cripps et al., 2016). The objective of this study was to test the impact of different densities of *C. rubiginosa* larval feeding on growth and reproductive performance of marsh thistle.

5.2 Materials and Methods

5.2.1 Study system

The experiment was conducted at AgResearch (43°38'21.89" S; 172°28'30.00" E), Lincoln from 13 September 2016 to 1 January 2017. Marsh thistle rosettes were excavated from three sites along the Kumara – Inchbonnie Road, Westland (Site 1 - 42º39'42.10" S; 171º25'57.18" E, Site 2 - 42º38'19.12" S; 171º22'52.20" E, Site 3 - 42º38'58.72" S; 171º18'38.06" E) on 29 August 2016. Care was taken to collect plants of similar size (rosette diameter) and same habitat (roadside). Due to the facultative biennial nature of the plant (Falińska, 1997), overwintered rosettes of sufficient size (i.e. >40 cm diameter) were collected to ensure that they would able to flower and produce seeds (Falińska, 1997). The plants were transplanted into 12 L pots containing a standard potting mix (54% aged bark, 45% sand, 1% nutrients, by weight) with added nutrients as Osmocote® 17-11-10 (N-P-K), lime, superphosphate, sulphate of potash and calcium nitrate. The experiment was set up as a randomised block design with five replicate blocks, and four larval density treatments: 0, 50, 100, or 200 larvae per plant. When larvae were applied, there were no significant differences in the width (P=0.291) or height (P=0.407) of plants assigned to the four treatments. The potted plants were spaced 40 cm apart in the blocks to ensure no leaves overlapped. The plants were watered three times per day for 5 minutes with an automatic irrigation system that delivered water directly to the soil of the pot via a Dutch spike.

Adult beetles were collected from a population of Californian thistle near Masterton (Wairarapa) on 20 October 2016. The insects were reared under laboratory condition in 2-L ventilated plastic boxes and fed with *C. arvense* clippings. Eggs laid between 21–25 October were kept at 20 ºC in Petri dishes lined with moist filter papers until larvae hatched. On 1–2 November 2016, naïve first instar larvae were applied to the appropriate treatment plants in the experiment, which matches the time period when larvae would typically begin to hatch in the field (Cripps, 2013). Flowering began on 8 November and by 19 December 2016, all plants in the experiments were flowering. Throughout the flowering period, honeybees and bumblebees

were observed pollinating the flowers. To estimate seed production, 20 flower heads (assumed to be pollinated) from each plant were covered with polythene mesh bags on 19 December. After two weeks, the seeds were collected, air dried and stored at 5º C for 12 months. On 25 December, visual estimates of percent leaf tissue removed from each plant (% herbivory) were recorded. At the end of the experiment on 1 January 2017, plant growth (mature plant height, width, and number of branches) and reproductive performance (number of flowers, number of seeds, seed weight and germination rate) parameters were measured.

Seed germination was tested from a random subset of 100 seeds per plant. The seeds were weighed and placed into Petri dishes (9 cm diameter) with filter paper moistened with 2 mL of distilled water. The dishes were then placed in an incubator at 25º C (van Leeuwen, 1981) and 8:16 hour dark and light condition (Huarte, 2005; Pons, 1984). Every third day, 1 mL of distilled water was added to the Petri dishes to prevent desiccation. Seeds were removed as they germinated over a 21-day period.

5.2.2 Data Analysis

The percent feeding damage was compared between the treatments using one-way analysis of variance (ANOVA). Since there was no damage on control plants (0%) the ANOVA was applied only to the three beetle treatment groups (50, 100 and 200 larvae/plant groups). Pair-wise comparisons were made between each of beetle treatment groups, and then for each beetle treatment against the zero control group using one-sample t-tests. The percent seed germination data was analysed using a generalised linear model assuming a binomial distribution through a logit link function. All other variables were analysed with ANOVA followed by Fisher's protected least significant difference (LSD) test to compare among the four treatment means. The data were analysed using Minitab version 16 and Genstat version 16.

5.3 Results

The percent feeding damage was significantly different among the four beetle treatments ($P<0.001$), and there was no feeding observed on the control plants confirming that larvae were confined to their assigned treatment plants (Table 5.1).

Herbivore treatment density had no significant effect on any of the measured plant traits (mature plant height $P=0.407$; width $P=0.577$; number of branches $P=0.241$; number of flowers

P=0.688; number of seeds P=0.642; seed weight P=0.450), except for seed germination (P<0.001). Seeds from plants treated with 200 and 100 larvae showed significantly reduced germination rates compared with seeds from plants subjected to 0 and 50 larvae (Table 5.1). Compared to control plants without herbivory, the germination rate was reduced by 8% (100 larval treatment) and 10% (200 larval treatment).

Table 5.1 Mean (± standard error) reproductive and growth parameters of marsh thistle (*Cirsium palustre*) after treatment with 0, 50, 100, or 200 larvae per plant of the folivorous beetle, *Cassida rubiginosa*. Treatment means sharing a common letter do not differ at the 5% significance level.

Parameter	**Treatment (larvae per plant)**			
	0	**50**	**100**	**200**
Herbivory (%)	0^{d}	35 ± 1.0^{c}	49 ± 2.7^{b}	63 ± 1.4^{a}
Reproductive				
Germination (%)	84 ± 2.30^{a}	84 ± 2.18^{a}	76 ± 2.89^{b}	74 ± 3.87^{b}
Seed weight (g/100)	0.172 ± 0.01^{a}	0.165 ± 0.01^{a}	0.154 ± 0.003^{a}	0.160 ± 0.006^{a}
Number of seeds[1]	424 ± 56.7^{a}	458 ± 28.6^{a}	382 ± 55.4^{a}	438 ± 14.7^{a}
Flowers/plant	192 ± 61.5^{a}	258 ± 40.3^{a}	251 ± 41.7^{a}	195 ± 52.7^{a}
Growth				
Height (cm)	174 ± 15.2^{a}	147 ± 5.68^{a}	146 ± 24.1^{a}	137 ± 11.5^{a}
Width (cm)	55 ± 0.73^{a}	53 ± 0.92^{a}	54 ± 0.89^{a}	53 ± 2.04^{a}
Number of branches	17 ± 3.65^{a}	10 ± 0.5^{a}	14 ± 3.06^{a}	13 ± 1.46^{a}

[1]The mean number of seeds is based on the total from 20 seed-heads per plant.

5.4 Discussion

This study assessed the impact of the oligophagous beetle *C. rubiginosa* on marsh thistle, a secondary weed target closely related to the biocontrol agent's primary target, Californian thistle. Even at the highest treatment density of 200 larvae per plant, *C. rubiginosa* did not cause sufficient damage to affect the growth or seed production of individual marsh thistle plants. The only detected effect was on seed germination rates, which were significantly reduced in plants that had experienced herbivory from 100 or 200 beetle larvae. However, since the number of seeds and weight of seeds were equivalent between beetle treatments, it is unclear why germination rates might have been different. Furthermore, even though this was a significant effect, it was only 10% different to the control plants, and thus unlikely to scale to population level declines of the weed.

The results of this study suggest that once sufficient rosette size is obtained to initiate bolting, it is unlikely that folivory alone will be able to reduce seed production. A similar result was reported with nodding thistle rosettes, where larval feeding by *C. rubiginosa* caused 23% defoliation, but had no effect on plant growth or seed production (Cartwright & Kok, 1990). Nevertheless, while even a high level of folivory (e.g. 50 to 60%, as imposed here experimentally) might not reduce the performance of a large rosette or bolting plant, it could affect smaller plant size classes. Marsh thistle is a facultative biennial: some individuals grow as a strict biennial, and reproduce in their second year, following a vernalisation period as a vegetative rosette; but many individuals remain in the vegetative state for three to five years before obtaining sufficient size to bolt and produce seeds (Ballegaard & Warncke, 1985; Falińska, 1997). The probability of transitioning to the reproductive stage increases sharply for rosettes over 20 cm in diameter and, at 40 cm, bolting is a near certainty.

In the present study, large rosettes (>40 cm diameter) were used in order to ensure that any impact of folivory on seed production could be measured. If smaller rosettes had been used, with lower probability of bolting, folivory by *C. rubiginosa* might have had an effect. Ultimately, *C. rubiginosa* will need to reduce seed output in order to cause population level declines of marsh thistle, or other secondary thistle weeds. This could be achieved by reducing the survivorship of rosettes, delaying the transition to the reproductive stage, or reducing the size of reproductive plants, since plants that bolt from smaller rosettes tend to produce less seed (Falińska, 1997). In the case of nodding thistle, the rosette-feeding weevil (*Trichosirocalus horridus*), was shown to contribute more to population decline than seed feeders since it

reduced the growth and survival of individual plants in the population (Shea et al., 2006). In contrast, nodding thistle seed-feeders did not reduce the survival or vigour of individual plants, and therefore high rates of seed consumption (at least 70%) were necessary to cause significant population declines (Shea & Kelly, 1998).

Biocontrol agents are often more effective when combined with other stress factors, such as complementary biocontrol agents, interspecific plant competition, and resource limitation (e.g. drought or nutrient stress) (Sheppard, 1996). In the current study, marsh thistle plants were grown in large pots, free from competition, and with ample nutrients and water, enhancing the potential for the plants to compensate for the herbivore damage (Hawkes & Sullivan, 2001). Under field conditions, their growth would be constrained by competition and environmental factors (Falińska, 1997). Under these conditions, folivory could have an impact on the weed. The extent to which *C. rubiginosa* might contribute to the control of secondary target weeds, will depend not only on the severity of attack, but on the phenological stages attacked, with younger or smaller stages more likely to be affected. Further studies assessing the impact of this biocontrol agent on different phenological stages of annual and facultative biennial thistle weeds would be of value.

5.5 Acknowledgments

This study was funded by the New Zealand Ministry of Business, Innovation, and Employment through AgResearch core funding of pasture weed research. I thank Prof. John Hampton, Bio-Protection Research Centre, for advice concerning seed germination techniques. I thank Josefina Narciso, Bio-Protection Research Centre for technical assistance.

5.6 References

Ang, B. N., Kok, L. T., Holtzman, G. I., Wolf, D. D., 1995. Canada thistle (*Cirsium arvense* (L.) Scop.) response to density of *Cassida rubiginosa* Müller (Coleoptera: Chrysomelidae) and plant competition. Biological Control. 5**,** 31-38.

Bacher, S., Schwab, F., 2000. Effect of herbivore density, timing of attack and plant community on performance of creeping thistle *Cirsium arvense* (L.) Scop. (Asteraceae). Biocontrol Science and Technology. 10**,** 343-352.

Ballegaard, T. K., Warncke, E., 1985. The age distribution of a *Cirsium palustre* population in a spring area in Jutland, Denmark. Ecography. 8, 59-62.

Bourdôt, G. W., Fowler, S. V., Edwards, G. R., Kriticos, D. J., Kean, J. M., Rahman, A., Parsons, A. J., 2007. Pastoral weeds in New Zealand: Status and potential solutions. New Zealand Journal of Agricultural Research. 50, 139-161.

Bourdôt, G. W., Leathwick, D. M., Hurrell, G. A., Saville, D. J., 1998. Relationship between aerial shoot and root biomass in Californian thistle. Proceedings of the New Zealand Plant Protection Society. 51, 28-32.

Cartwright, B., Kok, L. T., 1990. Feeding by *Cassida rubiginosa* (Coleoptera: Chrysomelidae) and the Effects of Defoliation on Growth of Musk Thistles. Journal of Entomological Science. 25, 538-547.

Cripps, M. G., 2013. Observations on the thistle-feeding tortoise beetle, *Cassida rubiginosa* (Coleoptera: Chrysomelidae). The Weta. 45, 5-13.

Cripps, M. G., Bourdôt, G. W., Fowler, S. V., 2013. Sleeper thistles in New Zealand: status and biocontrol potential. New Zealand Plant Protection. 66, 99-104.

Cripps, M. G., Edwards, G. R., Bourdot, G. W., Saville, D. J., Hinz, H. L., Fowler, S. V., 2010. Effects of pasture competition and specialist herbivory on the performance of *Cirsium arvense*. Biocontrol Science and Technology. 20, 641-656.

Cripps, M. G., Gassmann, A., Fowler, S. V., Bourdôt, G. W., McClay, A. S., Edwards, G. R., 2011. Classical biological control of *Cirsium arvense*: Lessons from the past. Biological Control. 57, 165-174.

Cripps, M. G., Jackman, S. D., Roquet, C., van Koten, C., Rostás, M., Bourdôt, G. W., Susanna, A., 2016. Evolution of Specialization of *Cassida rubiginosa* on *Cirsium arvense* (Compositae, Cardueae). Frontiers in Plant Science. 7, 1261.

Edwards, G. R., Bourdôt, G. W., Crawley, M. J., 2000. Influence of herbivory, competition and soil fertility on the abundance of *Cirsium arvense* in acid grassland. Journal of Applied Ecology. 37, 321-334.

Falińska, K., 1997. Life history variation in *Cirsium palustre* and its consequences for the population demography in vegetation succesion. Acta Societatis Botanicorum Poloniae. 66, 207 - 220.

Fowler, S. V., Paynter, Q., Hayes, L., Dodd, S., Groeteman, R., Biocontrol of weeds in New Zealand: an overview of nearly 85 years. In: S. M. Zydenbos, (Ed.), Seventeenth Australasian Weeds Conference. New Zealand Plant Protection Society, Chirstchurch, New Zealand, 2010, pp. 211-214.

Hawkes, C. V., Sullivan, J. J., 2001. The impact of herbivory on plants in different resource conditions: a meta-analysis. Ecology. 82, 2045-2058.

Huarte, R., & Benech-Arnold, R., 2005. Incubation under fluctuating temperatures reduces mean base water potential for seed germination in several non-cultivated species. Seed Science Research. 15, 89-97.

Paynter, Q., Fowler, S. V., Gourlay, A. H., Peterson, P. G., Smith, L. A., Winks, C. J., 2015. Relative performance on test and target plants in laboratory tests predicts the risk of non-target attack in the field for arthropod weed biocontrol agents. Biological Control. 80, 133-142.

Pons, T. L., 1984. Possible significance of changes in the light requirement of *Cirsium palustre* seeds after dispersal in ash coppice. Plant, Cell & Environment. 7**,** 263-268.

Schröder, D., 1980. The biological control of thistles. Biocontrol News and Information. 1**,** 9-26.

Shea, K., Kelly, D., 1998. Estimating biocontrol agent impact with matrix models: *Carduus nutans* in New Zealand. Ecological Applications. 8**,** 824-832.

Shea, K., Sheppard, A., Woodburn, T., 2006. Seasonal life-history models for the integrated management of the invasive weed nodding thistle *Carduus nutans* in Australia. Journal of Applied Ecology. 43**,** 517-526.

Sheppard, A. W., The Interaction between Natural Enemies and Interspecific Plant Competition in the Control of Invasive Pasture Weeds. In: V. C. Moran, J. H. Hoffmann, Eds.), Proceedings of the IX International Symposium on Biological Control of Weeds. University of Cape Town, Stellenbosch, South Africa, 1996, pp. 47-53.

van Leeuwen, B. H., 1981. The role of pollination in the population biology of the monocarpic species *Cirsium palustre* and *Cirsium vulgare*. Oecologia. 51**,** 28-32.

Webb, C. J., Sykes, W. R., Garnock-Jones, P. J., 1988. Flora of New Zealand. DSIR, Botany Division, Christchurch.

Winston, R., Schwarzländer, M., Hinz, H. L., Day, M., Cock, M., Julien, M., 2014. Biological Control of Weeds: A World Catalogue of Agents and Their Target Weeds. USDA Forest Service, Morgantown, West Virginia.

Chapter 6
General Discussion

The overall aim of the research in this study was to investigate the host selection behaviour of the biocontrol agent *Cassida rubiginosa* with respect to olfaction, feeding and oviposition and in relationship to the phylogeny of the tribe Cardueae. Furthermore, it was anticipated that this study would provide an example of how the combination of quantitative phylogeny data and chemical ecological data (VOCs) could be used to predict the host range of potential biocontrol agents. To investigate the multi-targeting potential, *Cirsium palustre*, closely related, and equally preferred to its primary host, by the beetle during olfactory and host-choice experiments (chapter 2), was used.

The research has extended exiting knowledge of the *C. rubiginosa* beetle, especially regarding its ability to discriminate between more or less suitable host plants by olfaction. In the laboratory, olfactory experiments (chapter 2 and 3) were undertaken to explore how the beetle responded to constitutive and induced volatiles in a phylogenetically designed system. Quantitative measures were used by calculating phylogenetic distance during the experiments. In addition, experiments in chapter 2 investigated the feeding and oviposition preference along the thistle tribe during adult host choice experiments by using whole plants inside cages. Chapter 4 investigated the behaviour of *C. rubiginosa* when its host plant was infected with pathogens: *Puccinia punctiformis* and *Sclerotinia sclerotiorum*. In chapter 2, constitutive volatiles were collected, and in chapter 3 conspecific larvae induced volatiles and chapter 4, fungal pathogen induced volatiles were collected and analysed using GC-MS. From the results obtained from chapter 2 multi-targeting potential was investigated using *Cirsium palustre* in chapter 5. A potted plant experiment was established with four treatments (0, 50, 100, and 200 larvae/plant). Plant growth (width, height, and number of branches) and reproductive performance (number of flowers, seeds, seed weight and % germination) parameters were measured.

6.1 Key objectives and findings

The following sections briefly summarize the key objectives and main findings for each experimental chapter.

1. Host choice, feeding and oviposition of *Cassida rubiginosa* is phylogenetically conserved and the beetle would make host plant choices that are adaptive for the survival of their offspring (Chapter 2).
    - Adult female beetles showed attraction towards *Cirsium vulgare, Cirsium palustre, Carduus tenuiflorus, Carduus nutans, Cynara scolymus*, *Arctium lappa*, *Centaurea macrocephala* and *Carthamus lanatus* when compared with *Cirsium arvense* in olfactory experiments where as it was only attracted to *Cirsium arvense*, *Cirsium vulgare, Cirsium palustre, Carduus tenuiflorus* when tested with a control (section 2.3.1).
    - The beetle's olfactory choice was phylogenetically conserved.
    - A total of 104 constitutive volatile compounds were collected from 16 Cardueae plants used in the experiment.
    - No phylogenetic relationship was found among the constitutive volatiles blends of the tested thistle species.
    - The beetle showed preferences towards nine plant species: *Cirsium vulgare, Carduus tenuiflorus*, *Cirsium palustre, Carduus nutans*, *Silybum marianum*, *Arctium lappa*, *Cynara scolymus, Cynara cardunculus* and *Centaurea nigra,* during host choice experiments (section 2.3.2) where feeding and oviposition was evaluated.
    - Feeding and oviposition decreased with phylogenetic distance.
    - Larval host range is narrower than the adult host range and adults when choosing the host plants have prioritized those plants that are favourable for larval survival.
    - Feeding and oviposition was not observed in tested non-cardueae plant species except for *Tragopogon porri* where one egg mass was found in which no larvae emerged.
    - Olfactory experiments showed similar host range to host choice experiments across the Cardueae tribe, predicting the possibility of using olfactory experiments in host range testing.
2. Conspecific herbivore induced plant volatiles (HIPVs) increase the attractiveness of the beetle towards thistles. This would be evident for both (1) plant genera (*Cirsium* and *Carduus*) that are preferred with constitutive volatiles, and (2) other Cardueae genera that have not showed preference towards constitutive volatiles, thus increasing the host range of thistle species that the beetle is attracted to when exposed to conspecific HIPVs (Chapter 3).

- Following chapter 2: beetles were attracted to constitutive volatiles of *Cirsium arvense*, *Cirsium vulgare, Cirsium palustre, Carduus tenuiflorus*.
- But with conspecific HIPVs they have showed attraction towards *Carduus nutans*, *Silybum marianum, Centaurea nigra, Cynara cardunculus* and *Cynara scolymus*.
- A total of 97 volatiles were collected from the larval damaged leaves of 13 Cardueae plants.
- The herbivore induced volatile bouquet of the plants that are attracted contained higher variety of sesquiterpenes where as the undamaged plant volatile bouquet contained higher variety of monoterpenes.
- There was no phylogenetic relationship of the HIPVs emitted by the 13 Cardueae plant species.
- HIPVs had a role in increasing the olfactory host range of *Cassida rubiginosa*. Thus, it is important to investigate conspecific cues to identify the behaviour towards host plants.

3. Preference of *Cassida rubiginosa* is reduced due to the presence of plant pathogenic fungi (*Puccinia punctiformis* and *Sclerotinia sclerotiorum*) infestations on *Cirsium arvense* leaves.
    - *Cassida rubiginosa* demonstrated significant preference for healthy *Cirsium arvense* leaf volatiles when tested against volatiles of pathogen-infested leaves.
    - The beetles did not show any preference when tested with *Cirsium arvense* leaves together with flowers versus *Cirsium arvense* leaves.
    - Volatile profile showed a unique blend of compounds where the *Puccinia punctiformis* infected leaves were characterized by the emission of benzenoids and indole, and *Sclerotinia sclerotiorum* infected leaves by green leaf volatiles.
    - Both pathogens significantly reduced the attractiveness of the leaves for *Cassida rubiginosa* as the feeding on the infected leaves were confined to infection-free areas.
4. Investigation of different densities of *Cassida rubiginosa* larval feeding on growth and reproductive performance of *Cirsium palustre*, a congeneric thistle species to the primary host of the beetle.
    - Feeding damage was significantly different among the four beetle treatments.
    - Herbivore treatments density had no significant effect on any of the measured plant traits (mature plant height, width, number of branches, number of flowers, number of seeds and seed weight) except for seed germination.

- Seeds from plants treated with 200 and 100 larvae showed significantly reduced germination rates compared with seeds from plants subjected to 0 and 50.
- Compared to control plants without herbivory, the germination rate was reduced by 8% (100 larval treatment) and 10% (200 larval treatment).
- For the beetle to have an impact, it would likely need to attack smaller, non-bolting rosettes, or be combined with additional stressors that might be encountered in a natural field population.

6.2 Direction of future research

Comparison of plant species and herbivore interactions in a phylogenetic context can provide meaningful insight in to predicting the host range. A phylogenetic framework has revealed the patterns of evolution of many morphological and chemical character but not many studies focus on having phylogenetic studies with herbivory. In this study we have given detailed description of one successful case where quantitative phylogeny and olfactory cues are used to determine host range. Furthermore, this study showed that olfactory cues can be used in determining host range of biocontrol agents and recommend using it in similar research. Few studies (Park et al., 2018; Sutton et al., 2017) have emphasised the importance of including olfactory studies in host range testing but studies lack usage of quantitative phylogeny in host range testing. Therefore, more studies using different plant-herbivore systems are needed to conclude the results in general.

This study has shown that the conspecific damage by larvae of *C. rubiginosa* aid in the attraction of adult beetles towards certain plant species. It would be interesting to investigate heterospecific herbivore effect on attraction of the *C. rubiginosa* beetle. Further, testing the changed behaviour of attraction of the adults according to gender, age and the life cycle stage could add useful information explaining host selection behaviour. In chapter 3, only olfactometer experiments were conducted. The research can be expanded including field research with larval damaged plants. For instance, investigations can be conducted in the field, to answer the question "would Cassida rubiginosa be more likely to attack alternative thistle hosts if it is surrounded by *Cirsium arvense*, compared to an isolated patch of an alternative thistle?"

In chapters 2 and 3, volatiles were identified. Future research could be extended towards electrophysiological studies to identify the responses of beetle towards each of the identified compound. And also further research should focus on using much more sensitive methods such as thermo-desorption of VOCs using GC-MS where the sample mixture is heated to promote desorption into the headspace of the tube before putting it onto GCMS (Hatipoglu et al., 2016) or silicone tubing method (Kallenbach et al., 2015) and should try to do the collection outside in the field. From the volatile data collected during the study, further analysis focusing on terpenoids is recommended. It is also interesting to investigate differential attractions to plant induced volatiles due to herbivory of different genders of the beetle. During the experiments I have conducted some trials to induce feeding on other materials. I extracted secondary metabolites adapting the methods used by Müller and Renwick (2001) and Reifenrath and Müller (2008), using methanol, dichloromethane and hexane and applied the extraction to lettuce and oblaten (thin wafer) and tried to induce feeding in *Cassida rubiginosa*. At the time beetles were not fully active (nearly diapaused) and due to time constrain I did not do further. But further research can direct towards investigating role of secondary metabolites in eliciting feeding behaviours of the beetle.

The temporal aspects of the diseased plant have an effect on the herbivore behaviour (Rostás et al., 2003). Development stage of the fungus and the physiological state of the plant are bounded to the time researcher choose to do the experiment. For example, during the latter stage of the fungal infection nutrient level of the plant might be much lowered than the earlier stages of the infection. This can have an effect on the volatiles and can alter the insect behaviour. A study conducted by Tasin et al. (2012) showed that olfactory behaviour of *Lobesia botrana* (Lepidoptera), a generalist insect changed according to the infection stage of necrotrophic fungus *Botrytis cinerea* on grapevine (*Vitis vinifera*). Further research should lead on investigating the olfactory attraction of the beetle and emitted volatiles during different stage of the fungal establishment. During this study, affect to the weed was not considered. Further research will be needed to quantify the impact of the attack of several natural enemies on *Cirsium arvense*, alone and together,

The effect of plant herbivory can change with the timing of leaf damage (Akiyama and Ågren, 2012). In chapter 5, we used bolting marsh thistle plants. Future research should be directed towards evaluating the herbivore effect on timing of the attack by using plant species that were equally preferred by the beetle during the experiments conducted during this research. This can

be conducted using different stages (e.g. seedling stage, small sized rosettes) of the plant exposing to different densities of the beetle larvae. Can measure the nutrient levels of the plant leaves, height, width, number of seeds, seed weights and seed germination. Similar can be done using other alternative hosts such as *Carduus* species. Furthermore, research can be expanded with Cardueae plants that have different life cycles (biennial, annuals) involving field studies.

6.1 Conclusion

The work has demonstrated that quantitative phylogenetic tools and chemical basis of plants can be used in host range testing which agrees with Briese (1996). By doing so it is possible to identify the plants that are likely to be attacked by the potential biological control agent with less effort than the traditional methods proposed by Wapshere (1974). This is because the number of plant species to be tested will reduce. In my study, olfactometer experiments proved to be effective in identifying the plants that the larvae survived on best. However, similar experiments with other biological control agents are needed before olfactory tests are incoroporated into normal host-specificity testing procedure. Moreover, incorporating chemical ecological aspects into weed biological control can improve the understanding of safety and the impact of biological control agents. Fundamental information present in this thesis can be used in future biological control perspectives specifically in determining the host range of a potential biocontrol agent.

6.2 References

Akiyama, R., Ågren, J., 2012. Magnitude and timing of leaf damage affect seed production in a natural population of *Arabidopsis thaliana* (Brassicaceae). PLoS One. 7**,** e30015.

Briese D 1996. Phylogeny: can it help us to understand host choice by biological weed control agents. Proceedings of the 9th International Symposium On Biological Control of Weeds. Pp. 19-26.

Hatipoglu, S. D., Zorlu, N., Dirmenci, T., Goren, A. C., Ozturk, T., Topcu, G., 2016. Determination of Volatile Organic Compounds in Fourty Five Salvia Species by Thermal Desorption-GC-MS Technique. Records of Natural Products. 10.

Kallenbach, M., Veit, D., Eilers, E. J., Schuman, M. C., 2015. Application of silicone tubing for obust, simple, high-throughput, and time-resolved analysis of plant volatiles in field experiments. Bio-protocol. 5.

Müller, C., Renwick, J. A. A., 2001. Different phagostimulants in potato foliage for *Manduca sexta* and *Leptinotarsa decemlineata*. Chemoecology. 11**,** 37-41.

Park, I., Eigenbrode, S. D., Cook, S. P., Harmon, B. L., Hinz, H. L., Schaffner, U., Schwarzländer, M., 2018. Examining olfactory and visual cues governing host-specificity of a weed biological control candidate species to refine pre-release risk assessment. BioControl. 1-13.

Reifenrath, K., Müller, C., 2008. Multiple feeding stimulants in *Sinapis alba* for the oligophagous leaf beetle *Phaedon cochleariae*. Chemoecology. 18**,** 19-27.

Rostás, M., Simon, M., Hilker, M., 2003. Ecological cross-effects of induced plant responses towards herbivores and phytopathogenic fungi. Basic and Applied Ecology. 4**,** 43-62.

Sutton, G., Paterson, I., Compton, S., Paynter, Q., 2017. Predicting the risk of non-target damage to a close relative of a target weed using sequential no-choice tests, paired-choice tests and olfactory discrimination experiments. Biocontrol Science and Technology. 27**,** 364-377.

Tasin, M., Knudsen, G. K., Pertot, I., 2012. Smelling a diseased host: grapevine moth responses to healthy and fungus-infected grapes. Animal behaviour. 83**,** 555-562.

Appendices

Appendix I: Constitutively emitted, tentatively identified, volatile compounds that were found to be present in more than 2 samples (out of 5 samples) of each Cardueae plant species (ng/h/kg). Mean±SE is present. N=5.

Chemical compound	*Cirsium arvense*	*Cirsium palustre*	*Cirsium vulgare*	*Carduus nutans*	*Carduus tenuiflorus*	*Silybum marianum*
Benzenoids						
Benzaldehyde						
Benzyl alcohol	61.66 ± 30.90					
Benzeneacetaldehyde						87.41 ± 40.32
Acetophenone			4.58 ± 2.76			
Methyl salicylate	43.79 ± 20.78					
Benzeneacetic acid 1-methylethyl ester						
Green Leaf Volatiles						
3-hexanone						
3-hexanol			351.81 ± 296.26			
3-hexen-1-ol						
3-hexen-1-ol, acetate	99.90 ± 60.67					
Monoterpenes						
α-thujene						
Cymene					20.98 ± 0.74	
1,8-cineole						
Unknown MT 01						

β-cis-ocimene	377.64 ± 165.04	11.93 ± 5	47.66 ± 16.32	13.36 ± 4.25		279.83 ± 175.13
ɣ-terpinene					2.06 ± 0.66	
Linalool oxide						
Sabinene hydrate					7.69 ± 0.78	
Linalool						
Menthol					4.79 ± 2.32	
Pinocamphone						
α-pinene			8.10 ± 3.71		4.41 ± 2.1	
α-terpineol	11.52 ± 4.7					
Camphene						
2,4(10)-thujadien						
Sabinene	18.98 ± 6.51			393.35 ± 239.63		
β-pinene						
β-myrcene	49.63 ± 16.55					
α-phellandrene						
D-limonene	35.19 ± 11.7	26.62 ± 11.21				
Sesquiterpenes						
Unknown ST 01						
β-bourbonene (m/z 204,81)						
β - elemene	191.33 ± 85.84	50.04 ± 16.28				9313.38 ± 5580.46
Unknown ST 02						
Unknown ST 03					313.01 ± 90.47	

Unknown ST 04						
α-cedrene						20.83 ± 7.62
α-gurjunene						
β-caryophyllene	763.06 ± 496.7	228.19 ± 124.62	77.26 ± 21.63	66.22 ± 22.44	27.47 ± 4.07	174.27 ± 101.14
Unknown ST 05						
α-cubebene			14.33 ± 5.07			
Trans-α-bergamotene	11.48 ± 9.18	3.26 ± 1.32				
Unknown ST 06			10.99 ± 4.07	14.54 ± 9.15		
α-guaiene						
β-farnesene	81.99 ± 43.47					
α-humulene		17.22 ± 9.58	10.91 ± 2.87			
Alloaromadendrene						
Unknown ST 07						
Unknown ST 08						1395.80 ± 1074.29
Unkown ST 09					86.40 ± 37.63	
ɣ-muurolene			88.50 ± 43.28			
Unknown ST 10						
Germacrene D	251.46 ± 110.37	13.40 ± 7.10		39.45 ± 27.18	35.84 ± 14.07	
α-amorphene			122.87 ± 47.65			
β-selinene						1564.58 ± 815.39
Unknown ST 11						1241.12 ± 642.25
α-selinene					162.72 ± 50.59	
α-farnesene E,E	536.12 ± 199.73					

Unknown ST 12						
Unknown ST 13						
α-murolene			293.86 ± 125.73	68.14 ± 45.76	74.78 ± 26.42	322.38 ± 251.57
α-bulnesene		188.96 ± 74.83				
α-longipinene				18.33 ± 7.51		
Unknown ST 14						
Germacrene A			37.72 ± 24.32	52.20 ± 46.26	1011.21 ± 217.12	2573.65 ± 1841.96
Unknown ST 15					10.54 ± 3.15	
ɣ-cadinene						
Cubebol			17.01 ± 7.24			
δ-cadinene	16.41 ± 6.86		67.36 ± 24.53	32.37 ± 17.38	11.30 ± 2.41	47.40 ± 39.3
Unknown ST 16					4.29 ± 1.85	
Unknown ST 17						117.71 ± 56.33
Caryophyllene oxide	58.45 ± 39.78	9.82 ± 5.95			5.00 ± 2.35	
α-tetralol					17.87 ± 1.98	
Neophytadiene	33.78 ± 8.17					104.68 ± 43.01
Cyclosativene	50.67 ± 26.25	6.16 ± 1.68	24.61 ± 7.67	103.83 ± 30.96	33.76 ± 7.96	275.39 ± 112.43
α-copaene		64.71 ± 27.57	174.66 ± 48.29	120.67 ± 85.72	113.44 ± 33.17	939.30 ± 397.79
Longicyclene				52.10 ± 25.57		
Unknown ST 18					35.89 ± 9.34	1037.36 ± 750.41
Other Volatiles						
Pentanal, oxime	20.73 ± 10.05					

2,2,4-trimethyl-1,3-pentanediol diisobutyrate		34.57 ± 18.83			
3-hepten-1-ol, (Z)-				16.97 ± 5.75	
(E)-2-ethyl-2-hexenal			5.37 ± 2.21	223.88 ± 28.12	
1-hexanol, 2-ethyl					
1-undecene					
Undecane					
Naphthalene				3.60 ± 0.96	
Decanal				5.26 ± 0.87	
Glutaric acid, butyl isobutyl ester				30.20 ± 4.48	
Unidentified volatiles					
Unknown 01					
Unknown 02				10.28 ± 2.84	
Unknown 03					
Unknown 04					
Unknown 05					235.63 ± 104.69
Unknown 06				121.30 ± 68.9	
Unknown 07					
Unknown 08					
Unknown 09	242.22 ± 117.04				
Unknown 10					

Unknown 11					15.96 ± 4.42	
Unknown 12						
Unknown 13						
Unknown 14						
Unknown 15						
Unknown 16	134.30 ± 24.36					

Cont…..

Chemical compound	***Ptilostemon afer***	***Arctium lappa***	***Cynara cardunculus***	***Cynara Scolymus***	***Carthamus lanatus***	***Centaurea cyanus***
Benzenoids						
Benzaldehyde						116.3 ± 77.49
Benzyl alcohol						21.72 ± 14.74
Benzeneacetaldehyde						1518 ± 923.53
Acetophenone						
Methyl salicylate						74.65 ± 19.72
Benzeneacetic acid 1-methylethyl ester						
Green Leaf Volatiles						
3-hexanone						
3-hexanol			54.60 ± 32.45	44.42 ± 20.9		
3-hexen-1-ol						
3-hexen-1-ol, acetate						

Monoterpenes						
α-thujene						
Cymene	9.05 ± 2.87					
1,8-cineole					54.29 ± 37.09	
Unknown MT 01	37.53 ± 15.75			17.17 ± 12.92		
β-cis-ocimene	558.45 ± 234.38	5.30 ± 2.56	29.78 ± 11.54	80.79 ± 43.63		
ɣ-terpinene						
Linalool oxide						10.86 ± 5.47
Sabinene hydrate						
Linalool						28.01 ± 13.07
Menthol						
Pinocamphone						
α-pinene		9.08 ± 1.76	10.69 ± 4.12		117.71 ± 62.14	8.44 ± 4.56
α-terpineol						
Camphene						
2,4(10)-thujadien						
Sabinene				4.41 ± 2.15	75.33 ± 52.47	
β-pinene		5.03 ± 0.98	19.49 ± 5.03		93.08 ± 37.9	
β-myrcene			21.22 ± 2.94	35.03 ± 9.56		
α-phellandrene						4.84 ± 3.42
D-limonene			19.84 ± 5.69	9.89 ± 6.36		
Sesquiterpenes						

Unknown ST 01		134.47 ± 79.84	399.14 ± 388.2			
β-bourbonene	147.34 ± 42.82					
β-elemene						343.8 ± 132.59
Unknown ST 02		32.82 ± 9.84			31.13 ± 9.66	
Unknown ST 03						
Unknown ST 04						
α-cedrene						
α-gurjunene		26.23 ± 7.02				
β-caryophyllene	124.18 ± 31.64	181.81 ± 34.74	5.84 ± 2.62		47.59 ± 10.53	547.43 ± 185.22
Unknown ST 05						37.52 ± 12.29
α-cubebene						48.58 ± 19.96
Trans-α-bergamotene						
Unknown ST 06	14.37 ± 3.77					
α-guaiene						
β-farnesene	8.14 ± 3.97					
α-humulene	39.3 ± 12.25	20.92 ± 9.01				340.27 ± 133.09
Alloaromadendrene		3.15 ± 0.98				
Unknown ST 07		3.85 ± 1.8				
Unknown ST 08						
Unknown ST 09						
ɣ-muurolene						116.31 ± 47.77
Unknown ST 10						
Germacrene D	2937.14 ± 804.63	8.42 ± 3.42				1252.2 ± 343.35

α-amorphene	143.38 ± 59.83					
β-selinene						4464.02 ± 1447.5
Unknown ST 11						
α-selinene						
α-farnesene E,E						
Unknown ST 12						
Unknown ST 13						55.31 ± 26.8
α-murolene	196.07 ± 48.09	9.47 ± 5.78			34.29 ± 13.79	494.99 ± 120.74
α-bulnesene						
α-longipinene						
Unknown ST 14	528.63 ± 159.06					
Germacrene A		67.72 ± 43.63				
Unkown ST 15						
ɣ-cadinene		10.15 ± 2.11				
Cubebol						
δ-cadinene	77.39 ± 23.16	33.72 ± 9.59			5.05 ± 2.19	191.82 ± 37.93
Unknown ST 16						
Unknown ST 17						
Caryophyllene oxide	15.37 ± 6.81					26.57 ± 12.47
α-tetralol						
Neophytadiene						
Cyclosativene					157.68 ± 35.87	146.3 ± 35.45
α-copaene	62.53 ± 16.21	93.10 ± 29.20			382.57 ± 109.88	904.75 ± 281.7

Longicyclene				
Unknown ST 18				
Other Volatiles				
Pentanal, oxime				
2,2,4-trimethyl-1,3-pentanediol diisobutyrate				
3-hepten-1-ol				
(E)-2-ethyl-2-hexenal				
1-hexanol, 2-ethyl	15.17 ± 6.55			13.59 ± 5.14
1-undecene	29.72 ± 11.35			
Undecane	34.62 ± 9.78			
Naphthalene	10.52 ± 1.70		5.23 ± 0.73	
Decanal				
Glutaric acid, butyl isobutyl ester		11.33 ± 4.93		
Unidentified volatiles				
Unknown 01				
Unknown 02				
Unknown 03		20.25 ± 7.28		
Unknown 04		12.03 ± 4.63		
Unknown 05				
Unknown 06				

Unknown 07					154.47 ± 49.91
Unknown 08	12.11 ± 4.46				
Unknown 09					
Unknown 10					
Unknown 11					
Unknown 12					
Unknown 13				1827.10 ± 990.91	
Unknown 14				31.71 ± 4.59	
Unknown 16				16.12 ± 2.75	
Unknown 17					552.06 ± 231.5

Cont…..

Chemical compound	***Centaurea macrocephala***	***Centaurea nigra***	***Onopordum acanthium***	***Echinops ritro***
Benzenoids				
Benzaldehyde				
Benzyl alcohol				
Benzeneacetaldehyde				
Acetophenone				
Methyl salicylate				
Benzeneacetic acid 1-methylethyl ester	19.50 ± 8.70			
Green Leaf Volatiles				
3-hexanone			45.93 ± 22.19	

3-hexanol	107.16 ± 31.36			
3-hexen-1-ol			335.31	335.31 ± 159.51
3-hexen-1-ol, acetate			4335.61	4335.61 ± 2417.32
Monoterpenes				
α-thujene		48.37 ± 21.15		3.60 ± 1.69
Cymene		23.12 ± 10.34	19.64 ± 9.96	22.02 ± 7.49
1,8-cineole		275.01 ± 119.59		
Unknown MT 01	257.91 ± 93.01			13.14 ± 7.03
β-cis-ocimene	175.05 ± 68.61		242.06 ± 180	196.49 ± 74.81
ɣ-terpinene				15.07 ± 7.8
Linalool oxide				
Sabinene hydrate				
Linalool				
Menthol				
Pinocamphone		49.68 ± 28.05		
α-pinene		1698.13 ± 718.92		119.31 ± 17.44
α-terpineol				
Camphene		84.50 ± 36.37		106.87 ± 22.98
2,4(10)-thujadien		18.90 ± 8.42		
Sabinene				142.54 ± 49.23
β-pinene		4214.37 ± 1934.76		92.88 ± 31.58
β-myrcene	23.11 ± 9.57	120.89 ± 51.99		
α-phellandrene				

D-limonene	10.64 ± 3.71			
Sesquiterpenes				
Unknown ST 01	20.55 ± 10.92		335.42 ± 80.27	
β-bourbonene				
β-elemene				
Unknown ST 02				
Unknown ST 03	28.72 ± 12.27			
Unknown ST 04				
α-cedrene				
α-gurjunene				
β-caryophyllene	180.49 ± 74.84	57.57 ± 10.27	250.83 ± 43.56	21.23 ± 6.89
Unknown ST 05				
α-cubebene				
Trans-α-bergamotene			43.94 ± 16.65	
Unknown ST 06				
α-guaiene	906.39 ± 463.45			
β-farnesene	210.12 ± 143.28			
α-humulene			79.67 ± 27.84	
Alloaromadendrene				
Unknown ST 07	19.65 ± 6.79			
Unknown ST 08				
Unknown ST 09				
ɣ-muurolene				32.62 ± 18.47

Unknown ST 10			101.81 ± 33.14	
Germacrene D			137.67 ± 50.26	
α-amorphene		236.75 ± 93.34		
β-selinene				
Unknown ST 11				
α-selinene				20.59 ± 13.74
α-farnesene E,E				
Unknown ST 12	306.44 ± 105.22			
Unknown ST 13				
α-murolene			450.62 ± 180.63	
α-bulnesene	210.22 ± 75.44			
α-longipinene				
Unknown ST 14				
Germacrene A			16.00 ± 8.84	12.54 ± 5.05
Unknown ST 15				
ɣ-cadinene				60.45 ± 13.93
Cubebol				
δ-cadinene			164.84 ± 38.38	
Unknown ST 16				
Unknown ST 17				
Caryophyllene oxide				
α-tetralol				
Neophytadiene				

Cyclosativene	17.89 ± 4.23		106.13 ± 39.24	9.59 ± 4.98
α-copaene	114.64 ± 65.52	811.23 ± 471.17	182.11 ± 79.44	252.83 ± 87.34
Longicyclene				
Unkown ST 18				
Other Volatiles				
Pentanal, oxime				
2,2,4-trimethyl-1,3-pentanediol diisobutyrate				
3-hepten-1-ol				
(E)-2-ethyl-2-hexenal				
1-hexanol, 2-ethyl			37.72 ± 12.09	
1-undecene				13.91 ± 9.94
Undecane				
Naphthalene			7.19 ± 2.25	12.12 ± 2.78
Decanal			40.18 ± 17.58	
Glutaric acid, butyl isobutyl ester				
Unidentified volatiles				
Unknown 01	213.24 ± 64.53			
Unknown 02	134.36 ± 75.62			
Unknown 03				
Unknown 04				

Unknown 05				
Unknown 06				
Unknown 07				
Unknown 08				
Unknown 09	223.05 ± 68.63	75.98 ± 31.07		52.11 ± 16.09
Unknown 10	309.16 ± 112.06			
Unknown 11				
Unknown 12	19.87 ± 10.77			
Unknown 13				
Unknown 14				
Unknown 16				
Unknown 17				

Appendix II: Feeding (number of holes), oviposition (number of egg masses) and number of larvae emerged from egg masses of *Cassida rubiginosa* on different plant species in a no-choice experiment. Plant species sharing a common letter do not differ at the 5% significant level for a given column

Plant species	Feeding		Oviposition		No. of larvae hatched		Larvae pre egg mass	
	Mean ± SE		Mean ± SE		Mean ± SE		Mean ± SE	
Cirsium arvense	72.3 ± 14.5	*bcdef*	6.8 ± 1.3	*ab*	37.5 ± 8.0	*ab*	9.0 ± 1.5	*a*
Cirsium palustre	64.8 ± 13.0	*def*	9.7 ± 1.8	*a*	38.7 ± 9.5	*ab*	7.0 ± 1.5	*abc*
Cirsium vulgare	44.0 ± 9.1	*eh*	5.3 ± 1.3	*ab*	25.3 ± 6.5	*abc*	7.7 ± 1.6	*abc*
Carduus nutans	58.3 ± 11.8	*de*	5.0 ± 1.1	*b*	21.5 ± 4.8	*bc*	6.8 ± 1.3	*abc*
Carduus tenuiflorus	68.3 ± 13.7	*cdef*	6.3 ± 1.3	*ab*	25.0 ± 5.5	*abc*	8.3 ± 1.4	*abc*
Silybum marianum	131.8 ± 25.9	*a*	5.8 ± 1.2	*ab*	19.8 ± 4.5	*bc*	7.5 ± 1.4	*abc*
Ptilostemon afer	22.8 ± 5.0	*g*	1.8 ± 0.7	*c*	0.5 ± 0.4	*e*	0.3 ± 0.3	*e*
Arctium lappa	117.5 ± 23.2	*abc*	7.3 ± 1.6	*ab*	41.3 ± 10.1	*a*	8.7 ± 1.7	*ab*
Cynara cardunculus	107.0 ±21.1	*abcf*	7.3 ± 1.3	*ab*	25.8 ± 5.7	*abc*	5.3 ± 1.1	*bc*
Cynara scolymus	124.3 ± 24.5	*ab*	9.3 ± 1.5	*a*	26.5 ± 5.8	*abc*	5.8 ± 1.2	*abc*
Carthamus lanatus	24.0 ± 5.2	*g*	1.8 ± 0.7	*c*	4.5 ± 1.4	*d*	2.0 ± 0.7	*d*
Centaurea cyanus	30.0 ± 6.4	*gh*	7.0 ± 1.3	*ab*	29.5 ± 6.4	*ab*	6.8 ± 1.3	*abc*
Centaurea macrocephala	48.5 ± 9.9	*deh*	5.0 ± 1.1	*b*	14.8 ± 3.5	*c*	4.8 ± 1.1	*c*
Centaurea nigra	76.5 ± 15.3	*abcdef*	7.8 ± 1.4	*ab*	41.0 ± 8.7	*a*	6.8 ± 1.3	*abc*
Onopordum acanthium	79.8 ± 15.9	*abcdf*	6.8 ± 1.3	*ab*	30.5 ± 6.6	*ab*	6.8 ± 1.3	*abc*
Echinops ritro	0	*i*	0	*c*	0	*e*	NA	
Tragopogon porri	0	*i*	0.5 ± 0.4	*c*	0	*e*	0	*e*
Inula hookeri	0	*i*	0	*c*	0	*e*	NA	
Taraxacum officinale	0	*i*	0	*c*	0	*e*	NA	

Appendix III: Comparison of feeding (number of feeding hole), oviposition (number of egg masses), number of larvae hatched from egg masses (total number of larvae emerged from egg masses) and larvae per egg masses (number of larvae emerged/ number of egg masses) from each test plant compared with *Cirsium arvense* in dual-choice experiments. P values are from ANOVA and zero values were compared using one-sample t-test. **Bold** numbers indicates a statistically significant difference at 5% level (=95% confidence level).

Test plant	Phylogenetic distance from *C. arvense*	Feeding		P values for feeding	Oviposition		P values for oviposition
		Test Plant species	*C. arvense* (CA)		Test Plant species	*C. arvense* (CA)	
Cirsium palustre	4.66	28.7 ± 11.9	23.4 ± 9.7	0.728	0.8 ± 0.3	0.5 ± 0.2	0.512
Cirsium vulgare	4.66	123.1 ± 44.1	19.8 ± 7.2	**< 0.001**	1.5 ± 0.6	0.6 ± 0.3	0.164
Carduus nutans	9.43	24.5 ± 7.9	27.7 ± 8.9	0.787	1.0 ± 0.4	1.6 ± 0.6	0.506
Carduus tenuiflorus	9.43	40.5 ± 6.8	22.4 ± 3.9	**0.015**	0.6 ± 0.3	0.5 ± 0.2	0.961
Silybum marianum	28	31.1 ± 8.2	23.2 ± 6.2	0.434	0.3 ± 0.2	0.8 ± 0.3	0.121
Ptilostemon afer	50.37	1.9 ± 0.7	31.8 ± 8.5	**< 0.001**	0.0	1.1 ± 0.4	**0.010**
Arctium lappa	53.12	33.7 ± 11.3	34.3 ± 11.5	0.970	0.2 ± 0.2	1.0 ± 0.4	0.091
Cynara cardunculus	53.12	33.7 ± 7.3	40.3 ± 8.6	0.557	0.3 ± 0.2	1.8 ± 0.6	**0.011**
Cynara scolymus	53.12	30.0 ± 7.9	33.2 ± 8.7	0.785	0.6 ± 0.3	0.7 ± 0.3	0.860
Carthamus lanatus	55.97	3.7 ± 1.2	38.9 ± 11.2	**< 0.001**	0.1 ± 0.1	1.4 ± 0.4	**0.003**
Centaurea cyanus	55.97	5.9 ± 1.7	34.9 ± 9.3	**< 0.001**	0.2 ± 0.1	1.8 ± 0.6	**0.003**
Centaurea macrocephala	55.97	12.4 ± 4.6	35.0 ± 12.8	**0.047**	0.0	0.7 ± 0.3	**0.010**
Centaurea nigra	55.97	28.1 ± 6.2	35.4 ± 7.7	0.456	0.2 ± 0.1	1.2 ± 0.7	0.236
Onopordum acanthium	58.25	12.8 ± 2.6	36.4 ± 7.0	**< 0.001**	0.2 ± 0.1	1.8 ± 0.7	**0.012**
Echinops ritro	71.17	0.8 ± 0.4	55.4 ± 15.0	**< 0.001**	0.0	2.1 ± 0.5	**< 0.001**
Tragopogon porri	NA	0.0	42.8 ± 5.9	**< 0.001**	0.0	2.0 ± 0.4	**< 0.001**
Inula hookeri	NA	0.0	48.1 ± 8.1	**< 0.001**	0.0	1.3 ± 0.6	**0.029**
Teraxacum officinalee	NA	0.0	36.8 ± 7.4	**< 0.001**	0.0	1.0 ± 0.4	**0.028**

Appendix III: Cont..

Test plant	Phylogenetic distance from *C. arvense*	No. of larvae hatched from egg masses		P values for no. of larvae hatched	Larvae per egg masses		P values for larvae per egg masses
		Test Plant species	*C. arvense* (CA)		Test Plant species	*C. arvense* (CA)	
Cirsium palustre	4.66	6.6 ± 2.7	3.7 ± 1.7	0.537	4.2 ± 1.4	3.0 ± 1.2	0.575
Cirsium vulgare	4.66	11.6 ± 5.8	4.9 ± 2.8	0.145	4.9 ± 1.1	2.4 ± 1.2	0.104
Carduus nutans	9.43	7.0 ± 2.8	9.8 ± 3.0	0.563	3.6 ± 1.2	4.3 ± 1.3	0.683
Carduus tenuiflorus	9.43	5.1 ± 2.9	4.1 ± 2.0	0.879	2.5 ± 1.3	3.2 ± 1.3	0.683
Silybum marianum	28	2.0 ± 1.1	7.0 ± 2.3	0.103	2.0 ± 1.1	5.2 ± 1.5	0.136
Ptilostemon afer	50.37	0.0	8.5 ± 3.1	**0.007**	0.0	4.6 ± 1.3	**0.005**
Arctium lappa	53.12	1.9 ± 1.9	8.7 ± 8.7	0.071	1.0 ± 1.0	4.5 ± 1.5	0.056
Cynara cardunculus	53.12	2.5 ± 1.8	14.4 ± 4.3	**0.014**	1.7 ± 1.1	5.3 ± 1.2	**0.025**
Cynara scolymus	53.12	3.3 ± 1.6	5.1 ± 2.3	0.761	2.1 ± 0.9	2.9 ± 1.2	0.818
Carthamus lanatus	55.97	0.5 ± 0.5	10.7 ± 2.9	**0.002**	0.5 ± 0.5	5.3 ± 1.2	**0.002**
Centaurea cyanus	55.97	1.2 ± 0.8	13.9 ± 4.2	**0.002**	1.2 ± 0.8	6.0 ± 1.0	**0.003**
Centaurea macrocephala	55.97	0.0	11.4 ± 4.0	**0.006**	0.0	4.7 ± 1.3	**0.005**
Centaurea nigra	55.97	1.2 ± 0.8	8.8 ± 5.1	0.304	1.2 ± 0.8	2.4 ± 1.2	0.544
Onopordum acanthium	58.25	1.6 ± 1.1	13.6 ± 4.7	**0.012**	1.6 ± 1.1	5.4 ± 1.2	**0.026**
Echinops ritro	71.17	0.0	15.6 ± 3.3	**< 0.001**	0.0	5.8 ± 1.0	**< 0.001**
Tragopogon porri	NA	0.0	13.6 ± 3.4	NA	0.0	5.7 ± 0.8	NA
Inula hookeri	NA	0.0	7.5 ± 4.4	**0.024**	0.0	2.9 ± 1.1	**0.023**
Teraxacum officinalee	NA	0.0	7.2 ± 3.2	NA	0.0	3.6 ± 1.2	NA

Appendix IV: Tentatively identified volatile compounds collected from more than 2 samples (total 5 samples) of each plant species (ng/h/kg) while *Cassida rubiginosa* larvae were feeding on them.

Chemical compound	*Cirsium arvense*	*Cirsium palustre*	*Cirsium vulgare*	*Carduus nutans*	*Carduus tenuiflorus*
Benzenoids					
Benzaldehyde					
Ethylbenzaldehyde					
m-ethylacetophenone		72.14 ± 7.71		49.64 ± 20.95	
Green Leaf Volatiles					
3-Hexenal	44.07 ± 30.73				
3-Hexanol	149.07 ± 111.48				
Hexanal		42.59 ± 19.19	162.66 ± 54.14		147.29 ± 78.78
3-Hexen-1-ol		64.59 ± 38.39	425.82 ± 241.46		
1-Hexanol					
2-Hexenal, 2-ethyl					
3-Hexen-1-ol, acetate					
3-Hexen-1-ol, 2-ethyl					
Monoterpenes					
α-Pinene		28.82 ± 8.97			
Sabinene hydrate					11.10 ± 3.10
Linalool oxide					
Linalool					48.47 ± 18.06
Sabenene		55.10 ± 18.44			9.25 ± 2.63

β-myrcene					
α-phellandrene					5.85 ± 2.72
D-Limonene			32.44 ± 13.36		
Cymene					
Unknown MT 01		265.47 ± 150.29	106.54 ± 37.26	40.64 ± 9.98	92.82 ± 39.62
β -cis-ocimene	248.14 ± 166.34	1332.86 ± 697.80	904.66 ± 347.69	282.31 ± 35.15	660.80 ± 246.52
ɣ-terpinene					8.69 ± 3.39
C8 compounds					
1-octene					
1-octen-3-ol		64.34 ± 14.22			
Octanal			36.44 ± 16.04		
Sesquiterpenes					
Unknown ST 01					
Unknown ST 02				91.15 ± 27.55	
Unknown ST 03					
Unknown ST 04					
α-gurjunene					
α-cedrene					
Unknown ST 05					
β-caryophyllene	34.77 ± 26.65	1632.00 ± 617.41	1512.57 ± 367.55	4312.52 ± 1372.18	757.76 ± 115.46
Unknown ST 06				881.40 ± 276.50	
Aromadendrene					

Unknown ST 07			130.41 ± 50.86		
α-cubebene					
trans-α-Bergamotene		678.13 ± 359.26			
Unknown ST 08					
β-gurjunene					
Unknown ST 09				101.63 ± 31.14	
Unknown ST 10					
α-humulene		213.96 ± 97.24	216.17 ± 71.32	828.53 ± 325.22	649.34 ± 370.16
Unknown ST 11					
β-farnesene					
Alloaromadendrene					293.75 ± 157.68
Unknown ST 12				154.62 ± 71.07	
Longifolene				27.60 ± 8.02	
Unknown ST 13					
ɣ-gurjunene					904.41 ± 433.62
Unknown ST 14					
Unknown ST 15		245.72 ± 101.85			
ɣ -muurolene				3827.30 ± 1172.74	
α-amorphene			800.26 ± 303.04		
Germacrene D		990.99 ± 470.37	977.73 ± 259.35	1967.55 ± 633.82	470.88 ± 184.62
α-selinene		572.61 ± 135.62			
β-selinene					

α-muurolene			1898.00 ± 505.15	5590.37 ± 1851.55	
Cyclosativene	13.19 ± 5.77	28.93 ± 16.49	371.78 ± 148.44	2823.65 ± 641.97	86.75 ± 24.50
α-farnesene	233.57 ± 107.05	9509.85 ± 2559.67			
α-bulnesene				3029.29 ± 1102.03	5254.93 ± 1356.46
Germacrene A					
ɣ-cadinene					63.48 ± 22.35
δ-cadinene		58.95 ± 33.29	454.00 ± 137.62	1449.66 ± 437.96	83.40 ± 19.75
α-cedrene					
Unknown ST 16		56.45 ± 15.78			25.46 ± 9.06
Unknown ST 17		69.63 ± 19.56			
Unknown ST 18					278.96 ± 114.04
Germacrene B				153.63 ± 69.53	
α-copaene				6229.24 ± 1700.80	397.92 ± 140.68
Trans-nerolidol		2416.35 ± 2337.50	149.29 ± 51.83		88.12 ± 34.52
Caryophylene oxide		336.21 ± 105.17	475.24 ± 123.12	412.56 ± 218.45	400.79 ± 106.87
Cubenol			137.24 ± 105.17		
Unknown ST 19				198.57 ± 75.39	
β-bourbonene					162.59 ± 53.92
Unknown ST 20					
β-elemene	26.08 ± 10.11	1095.93 ± 250.65	340.06 ± 88.44	1440.02 ± 525.20	1895.52 ± 571.79
Other Volatiles					
Heptanal			13.23 ± 5.77		

2(3H)-furanone, 5-ethenyldihydro-5-methyl-		75.61 ± 44.87			
1-undecene	11.74 ± 5.77				
Decanal		33.74 ± 11.82			
Glutaric acid, butyl isobutyl ester					
Unidentified volatiles					
Unknown 01					
Unknown 02				100.28 ± 68.52	
Unknown 03				189.68 ± 81.23	
Unknown 04			74.63 ± 19.97		
Unknown 05					
Unknown 06					
Unknown 07					
Unknown 08					136.41 ± 57.48
Unknown 09					
Unknown 10		205.53 ± 116.48			
Unknown 11					21.71 ± 9.18
Unknown 12					
Unknown 13					
Unknown 14	64.12 ± 20.41				

Cont…

Chemical compound	*Silybum marianum*	*Arctium lappa*	*Cynara cardunculus*	*Cynara Scolymus*	*Centaurea cyanus*
Benzenoids					
Benzaldehyde					
Ethylbenzaldehyde				12.20 ± 10.16	
m-ethylacetophenone	20.94 ± 10.58		27.53 ± 15.13	30.62 ± 16.56	
Green Leaf Volatiles					
3-Hexenal	113.80 ± 78.40	1184.95 ± 937.43	452.95 ± 154.10	82.82 ± 40.82	
3-Hexanol					
Hexanal					
3-Hexen-1-ol	38.11 ± 16.00	816.06 ± 406.69	194.07 ± 89.48		132.15 ± 87.89
1-Hexanol					
2-Hexenal, 2-ethyl	100.02 ± 41.89				
3-Hexen-1-ol, acetate		1888.77 ± 372.60	211.33 ± 75.25		183.23 ± 97.53
3-Hexen-1-ol, 2-ethyl	30.57 ± 19.82				
Monoterpenes					
α-Pinene					
Sabinene hydrate					
Linalool oxide					71.27 ± 14.98
Linalool		35.63 ± 20.35			72.16 ± 37.70
Sabenene					

β-myrcene			40.80 ± 17.46	53.89 ± 19.76	
α-phellandrene					
D-Limonene			23.53 ± 8.53		
Cymene					
Unknown MT 01		125.14 ± 64.38			
β -cis-ocimene	46.54 ± 18.01	1611.76 ± 571.54	265.17 ± 135.73	36.55 ± 27.07	
ɣ-terpinene					
C8 compounds					
1-octene	12.22 ± 5.78				
1-octen-3-ol					
Octanal				26.09 ± 12.97	
Sesquiterpenes					
Unknown ST 01	89.32 ± 48.53				
Unknown ST 02					
Unknown ST 03	640.41 ± 310.23				
Unknown ST 04					
α-gurjunene			36.09 ± 7.75		
α-cedrene					
Unknown ST 05					
β-caryophyllene	141.69 ± 40.94	4801.12 ± 2021.99	1184.72 ± 676.92	65.14 ± 23.97	1635.81 ± 523.27
Unknown ST 06					
Aromadendrene					29.44 ± 3.01

Unknown ST 07					
α-cubebene					64.16 ± 18.21
trans-α-Bergamotene					
Unknown ST 08	27.59 ± 21.45				
β-gurjunene					
Unknown ST 09					
Unknown ST 10					
α-humulene		1021.31 ± 558.38	100.16 ± 44.52		327.43 ± 86.61
Unknown ST 11	37.23 ± 22.48				
β-farnesene					
Alloaromadendrene		540.88 ± 513.54			
Unknown ST 12					
Longifolene					
Unknown ST 13		173.37 ± 90.56			
ɣ-gurjunene					
Unknown ST 14					
Unknown ST 15					
ɣ-muurolene					156.90 ± 48.02
α-amorphene					
Germacrene D	52.40 ± 23.96		133.70 ± 66.64		1912.17 ± 546.30
α-selinene	486.83 ± 416.58				
β-selinene		9924.53 ± 6380.86			4918.91 ± 845.65

α-muurolene	415.16 ± 197.27				512.52 ± 73.76
Cyclosativene	220.66 ± 76.43				175.54 ± 12.73
α-farnesene		3136.68 ± 1476.30			
α-bulnesene					
Germacrene A	2477.15 ± 1231.35				39.37 ± 18.11
ɣ-cadinene					
δ-cadinene	79.81 ± 40.58	228.04 ± 103.00	70.11 ± 21.65		198.67 ± 39.75
α-cedrene					49.66 ± 24.70
Unknown ST 16					
Unknown ST 17					
Unknown ST 18					
Germacrene B					
α-copaene	372.20 ± 109.12		90.04 ± 39.96		928.62 ± 93.44
Trans-nerolidol			90.77 ± 34.03		
Caryophylene oxide	42.96 ± 18.24	453.39 ± 282.09	75.68 ± 42.94		87.02 ± 28.04
Cubenol					
Unknown ST 19					
β-bourbonene					
Unknown ST 20					136.01 ± 40.14
β-elemene	35.16 ± 15.55	876.41 ± 408.57			
Other Volatiles					
Heptanal					

2(3H)-furanone, 5-ethenyldihydro-5-methyl-					
1-undecene					
Decanal				25.47 ± 15.86	
Glutaric acid, butyl isobutyl ester	33.85 ± 15.76				
Unidentified volatiles					
Unknown 01		338.13 ± 171.41			
Unknown 02					
Unknown 03					
Unknown 04					
Unknown 05		2706.91 ± 1759.23			
Unknown 06		1575.06 ± 1042.51			
Unknown 07					
Unknown 08					
Unknown 09	68.02 ± 33.01				
Unknown 10		1391.63 ± 740.56	1306.13 ± 646.46		
Unknown 11					
Unknown 12					
Unknown 13					
Unknown 14	64.12 ± 20.41				

Cont….

Chemical compound	***Centaurea macrocephala***	***Centaurea nigra***	***Onopordum acanthium***
Benzenoids			
Benzaldehyde			38.63 ± 12.75
Ethylbenzaldehyde	10.53 ± 5.46		
m-ethylacetophenone	125.75 ± 15.14		39.72 ± 19.50
Green Leaf Volatiles			
3-Hexenal	6043.71 ± 2053.74		303.28 ± 68.54
3-Hexanol			
Hexanal			
3-Hexen-1-ol	2341.12 ± 783.49	81.01 ± 22.50	400.21 ± 170.87
1-Hexanol			14.68 ± 11.19
2-Hexenal, 2-ethyl			
3-Hexen-1-ol, acetate	841.31 ± 451.94		
3-Hexen-1-ol, 2-ethyl			
Monoterpenes			
α-Pinene	7.56 ± 3.39		
Sabinene hydrate			
Linalool oxide			
Linalool			
Sabenene			
β-myrcene	81.21 ± 43.94		

α-phellandrene	135.01 ± 95.43		
D-Limonene	54.97 ± 18.05	6.31 ± 2.68	8.64 ± 3.77
Cymene			10.54 ± 6.44
Unknown MT 01	429.19 ± 124.78	10.59 ± 5.76	
β-cis-ocimene	1214.23 ± 310.24	47.14 ± 18.94	202.43 ± 166.74
ɣ-terpinene			
C8 compounds			
1-octene			
1-octen-3-ol			
Octanal			17.71 ± 4.76
Sesquiterpenes			
Unknown ST 01			
Unknown ST 02			
Unknown ST 03			
Unknown ST 04		159.41 ± 29.88	
α-gurjunene			
α-cedrene		25.27 ± 8.84	
Unknown ST 05			24.48 ± 8.79
β-caryophyllene	2639.25 ± 662.26	1752.13 ± 316.14	609.70 ± 146.71
Unknown ST 06			
Aromadendrene	578.47 ± 531.62		
Unknown ST 07			

α-cubebene		55.08 ± 16.12	
trans-α-Bergamotene	353.35 ± 333.49	220.24 ± 71.96	205.29 ± 45.19
Unknown ST 08			
β-gurjunene		202.72 ± 26.22	
Unknown ST 09			
Unknown ST 10		4.91 ± 1.23	
α-humulene		485.71 ± 82.45	
Unknown ST 11			
β-farnesene	878.55 ± 423.56		242.18 ± 143.27
Alloaromadendrene		7.62 ± 4.19	
Unknown ST 12	75.42 ± 49.56		
Longifolene			
Unknown ST 13			
ɣ-gurjunene			
Unknown ST 14	2984.94 ± 738.13		
Unknown ST 15			
ɣ-muurolene			
α-amorphene			
Germacrene D	4146.20 ± 2384.28	4505.42 ± 1025.68	685.70 ± 227.74
α-selinene			
β-selinene			
α-muurolene	1179.30 ± 457.39	2422.14 ± 312.10	1539.10 ± 507.50

Cyclosativene	297.69 ± 56.20	676.04 ± 214.47	479.11 ± 129.26
α-farnesene			
α-bulnesene	1607.77 ± 748.22		250.00 ± 98.93
Germacrene A			
ɣ-cadinene			
δ-cadinene	209.34 ± 89.97	616.00 ± 51.78	508.09 ± 165.00
α-cedrene			
Unknown ST 16			
Unknown ST 17			
Unknown ST 18			
Germacrene B			
α-copaene	3971.13 ± 678.88	6284.00 ± 656.17	773.50 ± 181.89
Trans-nerolidol		202.10 ± 45.98	
Caryophylene oxide	177.29 ± 46.92	207.85 ± 51.21	
Cubenol			
Unknown ST 19			
β-bourbonene	96.84 ± 51.32	134.58 ± 35.85	
Unknown ST 20			
β-elemene	586.28 ± 257.18		102.69 ± 24.06
Other Volatiles			
Heptanal			

2(3H)-furanone, 5-ethenyldihydro-5-methyl-			
1-undecene			
Decanal			
Glutaric acid, butyl isobutyl ester			
Unidentified volatiles			
Unknown 01			
Unknown 02			
Unknown 03			
Unknown 04			
Unknown 05			
Unknown 06			
Unknown 07	225.23 ± 141.52		
Unknown 08			
Unknown 09			
Unknown 10	4660.44 ± 2819.00		
Unknown 11			
Unknown 12	138.40 ± 57.31		
Unknown 13		14.93 ± 6.27	
Unknown 14			