UNIVERSIDAD DE LA FRONTERA
Facultad de Ingeniería y Ciencias
Programa de Doctorado en Ciencias de Recursos Naturales

COMPARISON OF FLAVONOID CONTENTS BETWEEN WILD POPULATIONS AND CULTIVATED ECOTYPES OF MURTILLA (*Ugni molinae* Turcz) AND THEIR ROLE ON INSECT-PLANT INTERACTIONS

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES

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TEMUCO- CHILE
2016

“COMPARISON OF FLAVONOID CONTENTS BETWEEN WILD POPULATIONS AND CULTIVATED ECOTYPES OF MURTILLA (*Ugni molinae* Turcz) AND THEIR ROLE ON INSECT-PLANT INTERACTIONS”

This thesis was conducted under the supervision of thesis director Dr. ANDRÉS EDUARDO QUIROZ CORTEZ, in the Departament of Chemical Sciences and Natural Resources at the Universidad de La Frontera and is presented for review by the members of the examining commite.

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… dedicated to my parents, Manuel and Maria

…and my bro Matias

All I have and will accomplish are only possible due to their love and sacrifices

Acknowledgement

First and foremost I would like to thank my advisor Dr. Andrés Quiroz. Without his patient guidance, teaching, insightful ideas and long hours of work, this study could not have been completed. I can not adequately express my recognition for his support and interest in seeing me obtain not only my degree, but succeed in all my endeavors. For all his help and mentoring, I am very grateful. I want to thank Dr. Leonardo Parra, my co-advisor, for his support in this investigation and as a member of my committee. I have rarely encountered such a dynamic organization and people whether in academia or industry. I thank all of you for the help and look forward to continuing a comunication as I move into the academia. I give many thanks to the Laboratorio de Química Ecologica. It was great meeting and working with such a nice group of people. My lab mates, Paulina, Carla, Paola, Stephy, Loreto, Claudia, Jocelyn, Melissa, Cristian, Herbert and Felipe thanks for all the help over the years. The "Friday beers" was a great help toward keeping sane. Good luck guys, I'm sure you'll be successful where ever you go. I need to thank also, Dra. Ana Mutis, Dr. Emilio Hormazabal and Mr. Marcelo Lizama for their help during my Ph.D.

I am deeply thankful of the Graduate Natural Resources Program of the Universidad de La Frontera (Temuco-Chile) and Experimental Station INIA Carillanca. Financial support for this research was supplied by CONICYT scholarship (21110939), FONDECYT (Project 1100812 and 11140608), FONDEF-INIA (DO5I 10083) and DI15-2012 project from Dirección de Investigación de La Universidad de La Frontera.

The most important thanks are for my family. Manuel, Maria and Matias. You all always knew when to call or write at just the right time. I can not begin to thank you for all you have done over the years. Your sacrifices and encouragement have made everything I have done possible. This work is dedicated to you.

Summary and outline of this thesis

Plant domestication is a co-evolutionary process that starts around 13,000 years ago. In this process plants were artificial selected according to the human society needs. In this framework, plant were increasing their productive traits (size, shape and color) in detrimental to their chemical defenses. This effect was so-called domestication syndrome. Currently, there is a native berry from Chile that started a domestication process near to 20 years ago. This shrub is murtilla (*Ugni molinae* Turcz.), this plant have been particularly studied due to their higher antioxidant activity because flavonoid contents. In this sense, murtilla under a domestication process can be affected by domestication syndrome. Hence, we evaluated the effect of domestication on flavonoid concentration and how it affects the insect-plant interaction and the behavior of a native herbivore insect *Chilesia rudis* Butler. In the first step, seven ecotypes cultivated in the Instituto de Investigaciones Agropecuarias (INIA) and their respective wild counterparts were selected for carried out an insect survey and sampling of leaves, stems and fruits sampling for flavonoid analysis by High Performace Liquid Chromatography (HPLC). Furthermore, the feeding preference of *C. rudis* was also evaluated in choice and no-choice assays. In the second step, cutting from the same cultivated and wild plants were growing and acclimating for a year and then, were established in a common garden for standarizing environmental factors. In the third step, a reciprocal transplant experiment was carried out for evaluating the plasticity about flavonoid contents of wild murtilla plants when they were move to a cultivated system and visceversa. Finally, the effect of a specific enzyme flavonol synthase (FLS) was evaluated through enzymatic assays for all seven cultivated plants and their respective wild counterparts. Results obtained in the first step showed a higher number of insects in wild plants than cultivated. Furthermore, a decrease in four flavonoids –quercetin, kaempferol,

rutin and quercetin glycoside- in all seven cultivated ecotypes were observed. In addition, feeding behavior of *C. rudis* developed a preference behavior to wild plants in relation to cultivated ecotypes. Results obtained in the second step (common garden experiment) showed that the flavonoid content was lower in cultivated plants than wild plants. Moreover, the insect assemblages and the feeding behavior of *C. rudis* were higher in cultivated plants than wild plants. In the third experiment (reciprocal transplant), we showed an increase of flavonoid concentrations for cultivated plants when they were transplanted to wild location. On the contrary, when wild plants were transplanted to a cultivated system a decrease in their flavonoid content was observed. Finally, a higher enzymatic activity of FLS was observed in three wild plants than to their respective cultivated counterparts. The first experiment showed that the chemical defense –flavonoids- was decreased in cultivated plants and the insect communities were increased. Hence, the domestication effect can be not determined in this experiment because of the different environmental conditions involved in this approach. Nevertheless, The domestication effect was determined in murtilla plants subjected to a common garden experiment. Finally, murtilla plants show plasticity in the recovering flavonoid contents in a reciprocal transplant experiment. Because of *Ugni molinae* domestication is a dynamic and continuous process, this thesis is useful for enlarge the knowledge and their application in agricultural management and breeding programs.

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CHAPTER I:

General introduction

1.1 GENERAL INTRODUCION

Plant domestication is a fact that began near to 10,000 and 13,000 years ago, close to the time that human have been selecting plants according to their needs. In this sense, it has been possible to observe two relevant facts: 1) humans have been able to select plants based on their phenotypical traits, and 2) plant selection, in part, is responsible for changes in human social structure, from hunter-gatherer to settled agriculture societies (Meyer et al. 2012), this change was so-called the Neolithic revolution (Childe 1949). Currently, around 2,500 plant species have undergone domestication worldwide according to Dirzo and Raven (2003).

In this framework, plant domestication is the genetic modification of a wild species to create a new form of altered plant to meet human needs. For many crops, domestication has made plant completely dependent on humans, as that it is no longer capable of spreading itself in nature (Doebley et al. 2006). However, the domestication is not always immediate; several authors have indicated that it is a process in which plants pass from their wild type to a domesticated one in a long-term (Clement 1999, Meyer and Purugganan 2013, Meyer et al. 2012). According to Meyer and Purugganan (2013) there are several stages or degrees in domestication process. The first one is called initial domestication, sometimes referred to as the improvement phase involving the spread and adaptation of the domesticated species to different agroecological and cultural environments. This phase leads to phenotypic and genetic divergence among domesticated populations, and it can be thought of as having multiple stages that are associated with varying selective pressures (Stage 1). Some key post-domestication stages may include *in situ* amplification of populations that have desirable alleles (Stage 2). Adaptation of a domesticated species to different environments

and human cultural practices that accompany geographical radiation constitute the stage 3); and deliberate breeding to maximize yield, ease of farming and quality is called stage 4 (Fig. 1). Stages 1–3 have been described previously based on domestication history of seed crops; although these stages are often presented sequentially, they may occur simultaneously.

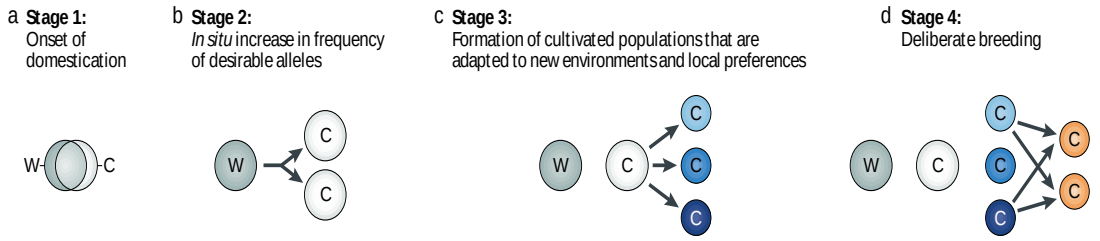


Figure 1. Plant domestication degrees from wild origin to a deliberate breeding. Adapted from Meyer and Purugganan (2013). W = wild plants, C = cultivated plants.

As soon as plant selection began, several species under anthropogenic selection were modified. Hence, plants in wild areas were selected and managed in different places under human cares. These plants grew producing more fruits, grains, leaves and/or increasing the fruit diameter, shape or size. However, these improvements provoked a decrease in other aspects within the nutrient pool of the plants, generating, according to Hammer (1984) the "domestication syndrome". Fuller (2007) indicated that this syndrome could act in different combinations and in several traits -including seed retention (non-shattering), and different changes in branching, height reproductive strategy and in secondary metabolites- (see review, Gross and Olsen 2010). Domestication syndrome may

evolve over thousands of generations, as desirable traits are selected for in the agricultural environment and become fixed within the crop genome. Nevertheless, the domestication syndrome may also evolve within a short time-frame, as in the cases of crops domesticated within the last 100 years or so, such as kiwi or cranberry (Meyer et al. 2012). Hence, the increase of productive characteristics in selected plants altering the levels of secondary metabolites in these last ones. The decrease is because the pool of nutrients in the plant systems is allocated mainly for production, as there is no need of protecting against some kind of biotic or abiotic stresses (Herms and Mattson 1992).

In Chile there are some native species that have begun a domestication process such as, maqui (*Aristotelia chilensis*), calafate (*Berberis mycrophylla*) and murtilla (*Ugni molinae*). Calafate present an interesting composition of anthocyanins and also reach high values (26.13 μmol/g) according to Ruiz et al. (2010). Moreover, this species also has been related to several alkaloid compounds (Manosalva et al. 2014). In this sense maqui is nowadays starting to cultivate at low scale. Murtilla has been domesticated by INIA-Carillanca for 20 years (Seguel and Torralbo 2004). At time, murtilla have an agronomic protocol for its cultivation elaborated by INIA and moreover there is a company involve in the cultivation. Moreover it is in an incipient stage of domestication that includes breeding programs from the original wild material collected before years (Chacón-Fuentes et al. 2015). Hence, this species is adecuate to the addresses of this reseach in comparison to another.

U. molinae Turcz (Myrtaceae), an endemic plant from Chile, is a highly polymorphic perennial shrub reaching heights of over 3 m (Valdebenito et al. 2003, Hoffmann 2005). Researchers at the Experimental Station of the Instituto de

Investigaciones Agropecuarias (INIA) in Carillanca, Región de La Araucanía, have been domesticating these plants (Figure 2). They were originally collected from 100 localities in southern Chile. Through the process of domestication, *U. molinae* cuttings were first grown in greenhouses for 10 years and then transplanted to the field (INIA Experimental Station-Tranapuente in the Región de La Araucanía [South of Chile, 38º 450 S, 73º 210 W]) until now, generating an important number of ecotypes –population that are often best adaptated to local environmental conditions (Knapp and Rice 1997)-. In Chile, there is a strong economic interest in the production of *U. molinae* fruit due to its high antioxidant content.

Figure 2. Murtilla plant. A) whole plant located at INIA-Tranapuente, B) fruits, C) leaves and, D) flowers from *U. molinae*.

This antioxidant activity is attributed to the presence of flavonoid compounds (Avello and Pastene 2005, Rubilar et al. 2006, 2011). For example, several flavonoids such as quercetin, kaempferol, rutin, and myricetin, and their corresponding glycosides, have been identified from *U. molinae* fruit and leaves (Shene et al. 2009, 2012). There are many reports indicating that flavonoids can affect feeding behavior in insects (Abou-Zaid et al.

1993, Simmonds 2001, Takemura et al. 2002, Salunke et al. 2005, Adeyemi et al. 2010, Diaz et al. 2010, Onyilagha et al. 2012). Besides, some flavonoids found in *U. molinae* have been implied in resistance mechanism against herbivores in other plant systems (Chen et al. 2015). For example, Todd et al. (1971) showed that quercetin, a constituent of barley leaves, was toxic to greenbugs, *Schizaphis graminum* (Rondani). Moreover, Dreyer and Jones (1981) reported increased resistance of wheat against *M. persicae* also due to quercetin. However, the inverse mechanisms have been observed with this family compounds. For instance, Takemura et al. (2002) reported increased susceptibility of *Vicia angustifolia* L. against the aphid *Megoura crassicauda* Mordivilko, because of the presence of flavonol glycosides. Specifically, Diaz et al. (2010) reported that quercetin acts as a phagostimulant for beetle *Epilachna paenulata* (Germar) (Coleoptera: Coccinellidae). Moreover, Lin and Mullin (1999) reported stimulant feeding activity by quercetin 3-D-β glucoside in the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (31.3% of consumption). For example, rutin is a phagostimulant to many polyphagous insects including the Lepidoptera *Heliothis virescens* (Blaney and Simmonds 1983). Wink (1988), Nielsen et al. (1998) and Bernays (1991) also reported stimulation of feeding and ovipositional activity by kaempferol, rutin, and glycoside compounds for other herbivores. In addition, isoflavones are a group of secondary metabolites within flavonoids that have been studied due to their activity as phytoestrogens. Some of these substances act as natural antioxidants and their effects on the human organism have been concerning, especially in the cardiovascular system (Pilsakova et al. 2010). Moreover, these compounds have been related to feeding behavior in insects. Sutherland et al. (1980) reported that isoflavonoids such as genistein and biochanin A at doses of 200 mg/mL applied on the 3rd instar of

Heteronychus arator (Coleoptera: Scarabaeidae) affected negatively the feeding behavior. Moreover, Pluempanupat et al. (2013) reported that formononetin at doses of 152 ppm was an active larvicide against the 3rd instar of *Aedes aegypti* (Diptera: Culicidae).

Hence, the domestication effects on the decreasing of secondary metabolites could be related to the preference, performance and behavior of insects (Chen et al. 2015). In this framework, to evaluate the composition of insect communities, their diversity and damage indexes obtained from both wild and cultivated plants could help us figure out the following question: How is the insect assemblages affected under a domestication process? and how is the structure and diversity of them altered? (Chapter II). Briefly Aguilera et al. (2005, 2009) reported a preliminary list of arthropods and insect pests associated with wild murtilla. However, there are no reports comparing two different systems –wild and cultivated- and how the insect communities are altered according to this change. Moreover, few studies on insect diversity and their damage indexes comparing cultivated plants with their wild ancestors have been carried out (Chen and Bernal 2011, Chen et al. 2013). Once the changes in all the variables related to insect communities reported in Chapter II were assessed, the variation in the levels of flavonol compounds (Fig 3) in wild and cultivated plants was analyzed. Leaves, stems and fruits from murtilla were used for flavonol isolation and quantification, this plant material was collected from seven localities and were compared with their respective cultivated one (ecotype). Moreover, *Chilesia rudis* (Lepidoptera; Arctiidae), a native insect hosting murtilla plants, was used for studying their preference and performance in choice and no choice bioassays, comparing leaves from a wild origin versus leaves cultivated one (Chapter III). To mitigate, the possible effect of

environmental differences on the behavioural responses, a common garden was established. Therefore, a similar experiment as described in previous chapters was developed.

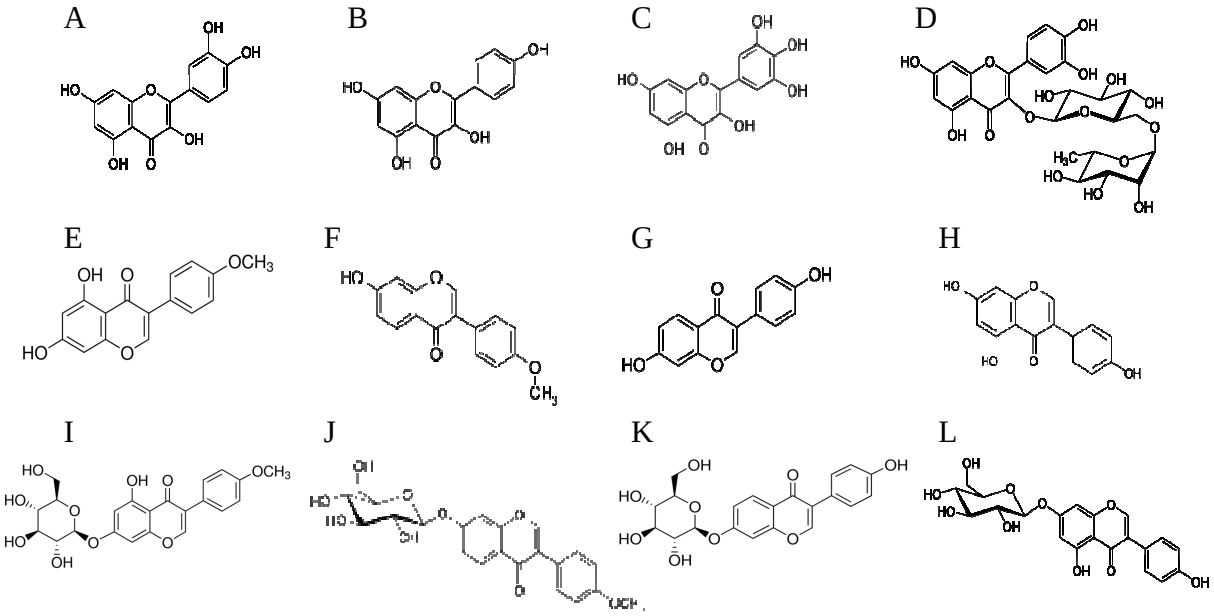


Figure 3. Flavonoid compounds detected and quantified in *U. molinae* plants. A - D, flavonols: A) quercetin, B) kaempferol, C) myricetin and, D) rutin. E - H, isoflavonoids: E) biochanin A, F) formononetin, G) daidzein and, H) genistein. I - L, isoflavonoids (glucosides): I), sissotrin, J) ononin, K) daidzin and, L) genistin.

In Chapter IV, I considered the following question: how is affected the insect plant interaction in murtilla plants subjected to a domestication process in relation to the flavonols and isoflavones content?

Complementarily, in Chapter V the adaptation effect on wild and cultivated plants through a reciprocal transplant experiment was analyzed. Finally, the activity of a single enzyme –flavonol synthase (FLS)- involved in flavonol origin, and particularly in kaempferol production was studied (Chapter VI). This enzyme has the capacity to

transform naringenin into kaempferol. Hence, wild plants that have a higher level of flavonols related to cultivate ones should present a high activity of FLS.

1.2 HYPOTHESES

According to the former information, a domestication process could modify secondary metabolites such as flavonols and isoflavones. Moreover, changes on the composition of these compounds could induce changes in the diversity and behaviour of the insects associated to murtilla plants. Hence, this work proposed the following hypothesis:

"Decreasing of murtilla (*Ugni molinae* Turcz) flavonoids involve in plant chemical defense elicit an increase in both insect numbers and feeding behavior of a native insect due to domestication process"

1.3 GENERAL OBJECTIVE

To determine the effect of *Ugni molinae* domestication process on flavonoid concentrations, insect communities and the feeding behavior of an herbivore insect.

1.4 SPECIFIC OBJECTIVES

1.4.1 To compare quantitatively flavonoids differences between wild populations and cultivated plants and their effect on insect interactions.

1.4.2 To compare qualitatively and quantitatively flavonoid differences between wild and cultivated plants and their effect on insect assemblages in a common garden.

1.4.3 To compare the effect of flavonoid variations in a reciprocal transplant experiment and the activity of FLS in wild and cultivated plants of *U. molinae*.

CHAPTER II:

Insect diversity, community composition and damage index on wild and cultivated murtilla

2.1 INTRODUCTION

Domestication is a process in which plants go from a wild environment to another environment in which they are completely dependent on human care for their survival and reproduction (Turcotte et al. 2014). Furthermore, Evans (1993) reported that there are dramatic changes in plants after the domestication process, and these changes could alter the interactions between insect assemblages. Gepts (2014) reported that in the domestication process, it is possible that there is a development of a "domestication syndrome", in which fruit size, the number of seeds and plant growth are increased according to human requirements. Nevertheless, this process also decreases the natural barriers of plants, such as their chemical defenses, for example, those that help them cope with herbivorous insects (Rodriguez-Saona et al. 2014). Evans (1993) indicated that the domestication process is an anthropogenic and directional selection, and this selection changes the physical or chemical traits of plants that have a strong effect on other plants, insects and their natural enemies. Therefore, crop domestication can affect the structure of insect assemblages (populations in an ecosystem) associated with host-plants and their interactions. For example, Chen et al. (2013) reported that cultivated rice had 50% fewer taxa of associated insects than wild rice and that there were losses in taxonomic species. Moreover, in wild rice, 173 taxa were found that were not found in cultivated rice, whereas cultivated rice supported only 23 taxa. For example, Chen and Bernal (2011) reported that the arthropod diversity was significantly higher in cultivated rice than in wild plants (21.52 ± 0.32 vs. 20.24 ± 0.39 species/plot) when cultivated and wild rice species were compared. Murtilla, *Ugni molinae* Turcz (Myrtaceae), is an endemic and polymorphic shrub from Chile and is distributed from Region del Maule to Region del General Carlos Ibáñez del Campo (Seguel et al. 2000, Seguel and Torralbo 2004). In Chile, there is a strong economic

interest in the production of *U. molinae* due to the presence of antioxidant compounds, specifically flavonoids present in the leaves and fruits, and this plant has an incipient berry used as food (Rubilar et al. 2011). Considering these facts, researchers at the Experimental Station INIA Carillanca (Region of La Araucanía, Chile) have been domesticating this species for approximately 20 years, generating a domestication process from wild to cultivated conditions. There are no studies comparing the insect diversity associated with wild and cultivated murtilla plants. Therefore, the study of insects associated with *U. molinae* plants, both cultivated and wild, could be an excellent biological tool to show changes in the insect community associated with the domestication process. According to the aforementioned information, domestication could increase the taxonomic assemblage and damage index but decrease the insect diversity in cultivated plants. Therefore, the objectives of this report were to compare insect assemblages associated with both wild and cultivated *U. molinae* plants, determine the effects of domestication on herbivory and evaluate the effect of the domestication on insect diversity.

2.2 MATERIAL AND METHODS

2.2.1 Sampling area. Seven cultivated ecotypes of *U. molinae* under the domestication process at INIA-Tranapuente, an experimental field near Puerto Saavedra (Region of La Araucanía, Chile, 38°45`S, 73°21`W), and their respective wild parents populations were considered for the insect survey. The ecotypes selected were 08-1, 12-1, 14-4, 18-1, 19-1, 22-1 and 23-2, and their corresponding wild parents were selected from those showing a similar size (around 1 m tall), architecture, and phenology and were sampled from Caburgua (39°11` S, 71°49`W), Pucón (39°17` S, 71°55`W), Manzanal alto (38°03` S,

73°10`W), Soloyo (38°35` S, 72°34`W), Porma (39°08` S, 73°16`W), Mehuín (39°26` S, 73°12`W), and Queule (39°23` S, 73°12`W). The sampling considered five repetitions of a whole plant per cultivated ecotype and wild locations. The survey and samplings were carried out between December 2012 and October 2013 every two months. Fertilizer was applied annually on cultivated plants according to a soil analysis and consisted of 80, 44, and 43 g per plant of nitrogen, P_2O_5, and K_2O, respectively. Pest control was carried out using Karate (lambda-cyhalothrin; Syngenta, Greensboro, NC, USA), at a dose of 1 to 2 mL L^{-1} of water, or Lorsban 4E (chlorpyrifos; Dow AgroSciences, Indianapolis, IN, USA), at a dose of 1 mL $plant^{-1}$ (one to two applications during the year), according to the incidence of cutworms. To avoid residual toxicity, all samples were collected at least 7 days after insecticide applications.

2.2.2 Insect survey and insect biodiversity indexes. Insect specimens were collected manually with a mouth aspirator between 900 and 1800 h from leaves, stems, flowers and fruits, and each whole plant was visually and manually examined for 5 min. After completing the inspection of each individual plant, the soil surface below the canopy was examined (Knott et al. 2006). The collected insects were those that used the plant as a host and those that visited the plant at the sampling time. The captured insects were stored in Khale's solution (water (56.6%), ethanol (28.3%), acetic acid (3.8%) and formaldehyde (11.3%)), and the species were determined in the laboratory under an optical microscope (Olympus SD 30) using key books reported by Artigas (1994). Furthermore, the relative abundance index was estimated as the number of individuals per plant (Samo et al. 2008), and a relative abundance index was obtained for each sampled species. In addition, the diversity indexes of both wild and cultivated plants were calculated as follows: Margalef index: $D_{mg}=S-1/\ln(N)$ where S = number of species in a sample and N = total number of

organisms in the sample; the Shannon index: $H' = -\Sigma pi \, Log_2(pi)$; and the Simpson index: $D = 1/\Sigma(pi)^2$ where pi = ni/N ni = species abundance and N = total number of organisms in the sample (Samo et al. 2008).

2.2.3 Evaluation of leaf damage. Leaves were collected from both cultivated ecotypes and wild locations (12 leaves per plant) from the four cardinal directions at different heights of the plant. The vegetal material was stored in paper bags, transported to the Laboratory of Química Ecológica of the Universidad de La Frontera and stored at -20 °C until their evaluation. The damage percentage was calculated by evaluating the foliar area using the ImageJ 1.42 software (Wayne Rasband National Institutes of Health, USA). The damage was categorized according to the methodology proposed by Dirzo and Dominguez (1995) as follows: 0= intact; 1= 1-6%; 2= 6-12%; 3=12-25%; 4=25-50%; 5=50-100%. The index damage by plant was calculated by means of the formula reported by Rodriguez-Auad and Simonetti (2001): $DI = \Sigma \, ni \, (ci)/N$, where ni= number of leaves in the i^{th} category of damage, ci= midpoint of each category, and N= total number of leaves.

2.2.4 Statistical analysis. The statistical software Statistix 10 (Tallahassee, Florida, United States of America) was used to analyze the damage index and the total number of insects in both wild and cultivated plants. Damage indexes were analyzed by a fully nested hierarchical random analysis of variance, using domestication degrees as the main factor and temporal variation as a nested factor within domestication degree (wild and cultivated). Posterior LSD Fischer tests were used for comparisons among groups. Finally, for contrasting the damage indexes between cultivated plants and their wild counterparts, *t*-tests were used. To analyze the number of insects, a chi square test was performed. The data were natural-log transformed to meet the assumptions of normality and homogeneity

of variance. Values of $P \leq 0.05$ were considered significant. The results are expressed as means and their corresponding standard errors.

2.3 RESULTS

Insect Survey. A total of 243 insects were collected, 188 individuals from wild plants (77.3%) and 55 from cultivated plants (22.7%) (Figure 1A). The several insect orders collected were Coleoptera (28.2%), Diptera (17.9%), Hemiptera (10.2%), Hymenoptera (12.8%), Lepidoptera (10.2%), Neuroptera (2.56%), Orthoptera (5.12%), Homoptera (7.69%), Blattodea (2.56%), and Phasmatidae (2.56%). Coleopterans were represented by Curculionidae (18.18%), Tenebrionidae (12.82%), Carabidae (7.69%), Scarabaeidae and Cerambycidae (5.12%), and finally, Chrysomelidae, Meloidae, Cupedidae, Bostrichidae, Bruchidae and Coccinellidae (2.56%). Dipterans were represented by Asilidae (30%), Tabanidae (20%) and Cecidomyiidae, Dolichopodidae, Muscidae, Tipulidae, and Calliphoridae (10%). For Hemipterans, Lepidopterans and Homopterans, the percentages were distributed equally in the families shown in Table 2. For Neuropterans, Blattodea and Phasmidae, only one family was represented, as shown in Table 2. Orthopterans were represented by Acrididae (66.66%) and Tettigoniidae (33.33%). Finally, Hymenopterans were represented by Formicidae, Ichneumonidae and Apidae (25%) and Pompilidae and Vespidae (12.5%). Insect assemblages were lower in wild parents than in the respective cultivated ecotypes, except for wild plants located in Pucón and Porma (Figure 1A). The dynamics of both cultivated and wild murtilla plants is shown in Figure 2A. The maximum insect assemblages can be observed between October and December for wild plants. In contrast, the assemblages were more stable throughout the year and were lower than that found for wild plants. Moreover, based on field observation (wild species), it was possible

to identify symptoms that indicated the presence of a phytoplasm called witch's broom disease. Symptoms were present in all wild locations.

Damage index evaluation. In general, the wild species presented significantly higher insect assemblages than cultivated plants according to *t*-tests ($P \leq 0.05$) (Figure 1A). For instance, Caburgua, Manzanal Alto, Soloyo, Mehuín and Queule presented insect assemblages higher than their respective cultivated counterparts (Figure 1B). The temporal variation of the damage index that was calculated for wild and cultivated murtilla plants showed a similar pattern throughout the year. The interaction between months and the domestication effect was a significant effect ($F_{12,5446}$= 16.49; $P \leq 0.001$) on the damage index in murtilla plants. Similarly, the domestication effect was a significant effect ($F_{1,5446}$= 28.34; $P \leq 0.001$) on the damage index generated in murtilla plants, as shown in Table 3 and Figure 2B. The highest damage index levels were found in December for cultivated and wild plants (0.9 and 1.2, respectively). However, in this month, the damage index of wild plants was significantly higher ($P \leq 0.05$) than that of their cultivated counterparts.

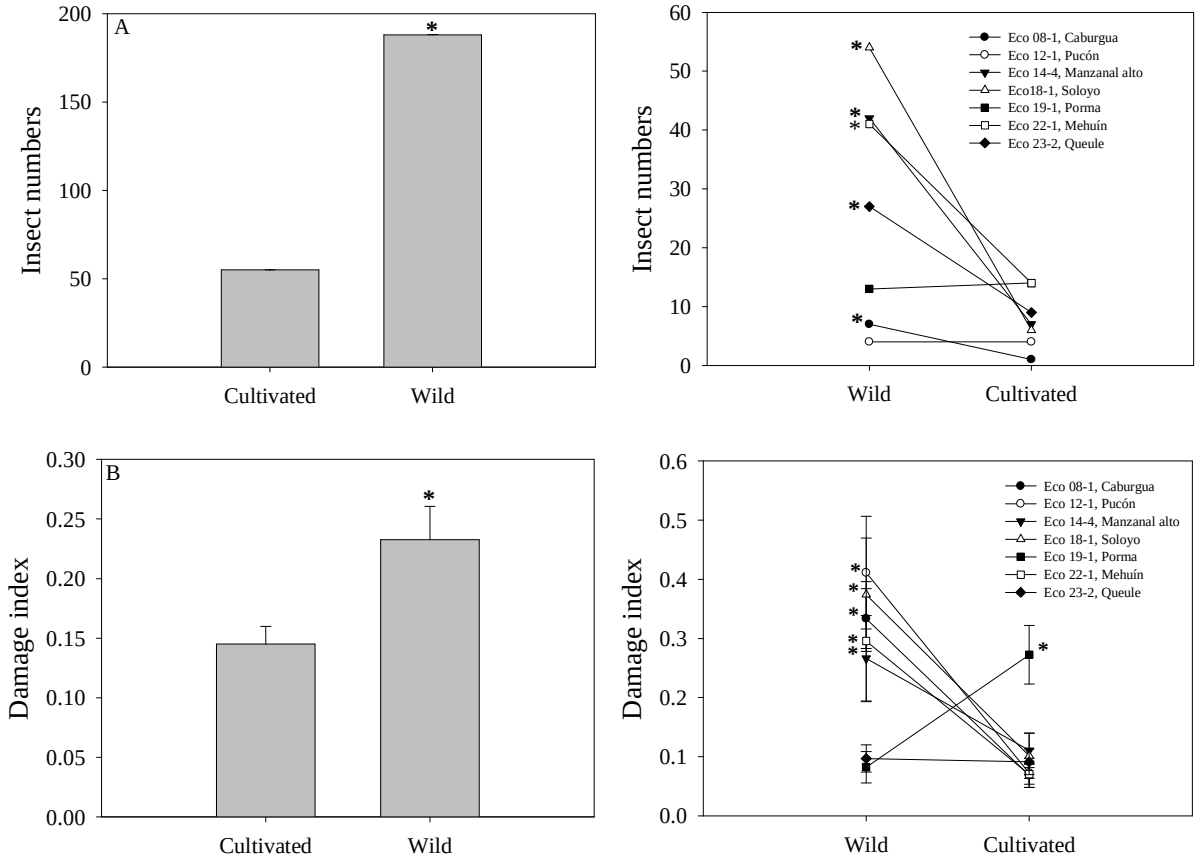


Figure 1. Results from the insect survey: A) insect numbers in wild and cultivated plants (left) and the different agroecological areas (right), damage index in wild and cultivated plants (left) and individual damage index comparison between wild and cultivated plants based on ecotype and geographical area (right). * mean significant differences according to *t*-test ($P<0.05$).

Insect biodiversity. The Shannon index was higher in wild plants (5.15) than in cultivated plants (4.40), suggesting that wild species have greater diversity than cultivated species (Table 1). Moreover, in the Margalef index (Table 1), there was difference between cultivated plants (6.98 vs. 12.98) and wild plants, which indicated that there is greater species richness in wild *U. molinae* plants. However, there was a higher number of insects in wild species than in cultivated species (Figure 1A). Furthermore, differences were observed in wild and cultivated plants according to the Simpson index (19.04 vs. 15.04).

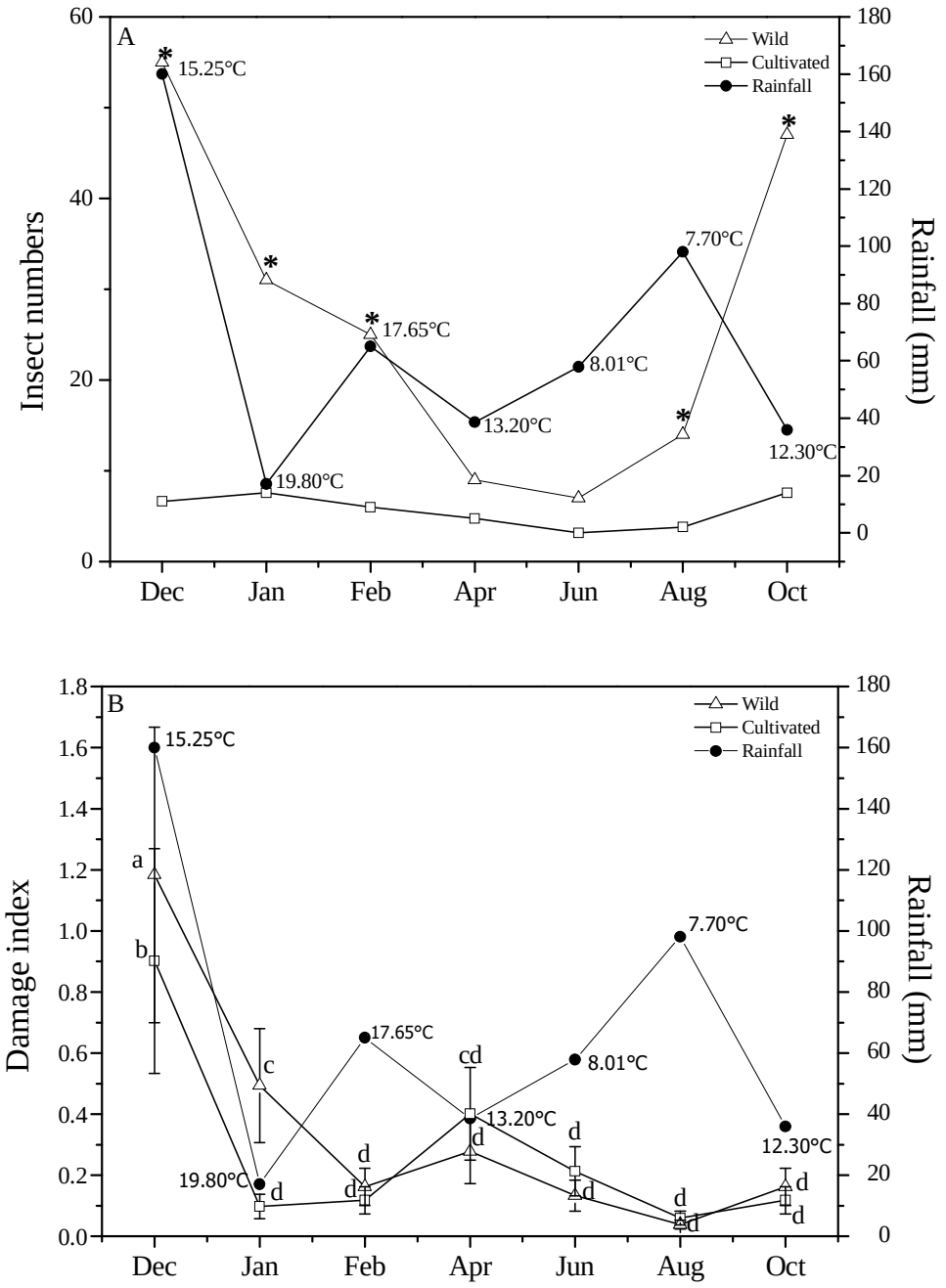


Figure 2. The effect of temporal variation, rainfall and temperatures on insect number (A) and damage index (B) in wild and cultivated murtilla, *Ugni molinae*, from December 2012 to October 2013. Temperatures and rainfall are expressed as the mean between Temuco and Valdivia. *mean differences according to the Chi Square test and different letters mean significant differences based on the LSD Fischer test ($P \leq 0.05$).

Table 1. Diversity parameters evaluated in both wild and cultivated plants of *U. molinae* from December 2012 to October 2013.

Parameters	Wild	Cultivated
Species richness[1]	69	29
Total individuals	188	55
Relative abundance (%)[2]	77.36	22.64
Margalef index	12.98	6.98
Shannon index	5.15	4.40
Simpson index	19.04	15.04

[1]Number of species for wild or cultivated plants.

[2]Percentage of the total number of individuals found in both wild and cultivated domestication stages.

2.4 DISCUSSION

The phytophagous insect associated with *U. molinae* has been studied previously by Aguilera et al. (2005, 2009). Nevertheless, there are no specific reports about insect pests in *U. molinae* related to the domestication process. Aguilera et al. (2005, 2009) reported 22 and 10 species associated with murtilla, respectively. These insects were collected in the 2003-2004 season in the Region of La Araucanía and Region de Los Lagos and correspond to only phytophagous insects. Furthermore, the 2005-2006 season was also evaluated by Aguilera et al. (2009), who added new species to the identified phytophagous insects related to *U. molinae* plants. In the present research, we identified approximately 60 insect species in wild and cultivated murtilla plants from December 2012 to October 2013 that had not been reported previously (Table 2). The results found in this study could be helpful regarding information about the variation in the insect assemblages in crops that are subjected to domestication or anthropogenic intervention. Indeed, despite its short history of domestication (< 20 years), the high number of insects observed in association with *U.*

molinae suggests that once this crop completes its domestication process, it could be affected by a wide spectrum of phytophagous insects. These insects could produce several types of damage due to their defoliating or sucking feeding behaviors, or insects may cause damage when they oviposit, as is the case with *Tettigades chilensis* Amyot & Serville (Hemiptera: Cicadidae) found in this survey. In the last 10 years, several authors have developed a theoretical framework for understanding the evolution of plant defenses that protect against herbivores. Bautista et al. (2012) has suggested that the degree of resistance to herbivores reflects a compromise between the benefits of reduced herbivory and the costs of diverting resources from other functions to resistance. Crawley (1997) reported that plant morphology can influence insect acceptability directly, either by providing a suitable visual cue or by influencing the ability of insects to walk on or bite into the tissue. Furthermore, most phytophagous insects are confined to certain plant parts, which is determined by the physical and chemical attributes to which the insects respond. In addition, the presence (or absence) of chemical barriers, such as secondary metabolites, determines the range of insect attacks. However, this aspect was not addressed in this investigation. Moreover, according to Artigas (1994), *Hylamorpha elegans* is one of the most dangerous species in wheat from the Region of Bío Bío to Region of Los Lagos, Chile, generating plant losses that reach 80%. Although their main hosts are gramineous, *H. elegans* could be using *U. molinae* as a second host. In addition, one highlight in our findings is *Proeulia* spp, which, according to Gonzalez (2003), shows increased presence in fruits related to anthropogenic intervention. Furthermore, species such as *P. chrysopteris* (Butler) are quarantined in the USA and are prohibited in the shipping of kiwi and grapes. Another species that was found in the present report was *Aegorhinus nodipennis*; this curculionid has been reported mainly in association with blueberries, peaches, plums and

apples (Aguilera et al. 2011). *A. nodipennis* could represent a potential threat to *U. molinae* due to its similarity to blueberries. Furthermore, in the present research, we found a specific association between witch's broom disease and murtilla, the most common and destructive disease of the foliage in murtilla plants. The main characteristic of this disease is an uncontrolled branching associated with biotic factors. Moreover, this plant disease is characterized by a reduction in the size of shoots and overgrowth of these; the leaves become smaller and tighten acquiring a reddish to yellowish color, not allowing the development of fruits and when they reach some develop, have a bad taste (Andrade et al. 2009). It is transmitted by Cicadellidae (Hemiptera), particularly by *Carelmapu aureonitens* Linnavuori & De Long and *Carelmapu ramosi* Linnavuori. We found witch's broom in all seven wild locations, making it a decisive factor in determining the presence of *Carelmapu* spp. Moreover, Aguilera et al. (2009) reported that this disease was found in some experimental plots cultivated with *U. molinae* in the Region of La Araucanía. The Margalef index for wild species was higher (12.98) than other reports for cultivated ecosystems. For instance, in barley crops, this index ranged from 0 to 0.96 (Abay et al. 2009). The Margalef index for wild *U. molinae* plants agreed with Lexerod and Eid (2006), where the range varied from 4.09 to 8.47. Nevertheless, the higher richness according to the Margalef index was found in wild plants. This finding could be associated with the loss of chemical defenses due to the domestication process. Overall, for both wild and cultivated plants, the diversity indexes were higher than two considered as moderate. This index is variable from less than 1 (Aslam 2009) to more than 8 (Lexerod and Eid 2006). Therefore, these values are related to a medium level of diversity for cultivated plants and a high level of diversity for wild plants. The Shannon indexes were higher than those reported for farmland wheat (Chateil et al. 2013), where the index ranged from 0.2 to 1.4. In relation to wild plants, the

insect diversity of our data was lower than the Shannon index reported by Bibi and Ali (2013) in a wildlife sanctuary (Pakistan), where the index ranged from 3.31 to 3.39 for the fauna of this landscape. In addition, cultivated plants have a low Shannon index (4.40), meaning that cultivated plants are more affected by anthropogenic management (Takhelmayum and Gupta 2015). Moreover, the Simpson index showed values from 15.04 in cultivated plants to 19.04 in wild plants, meaning that there was high diversity in both sites. As the first approach, the domestication process and the management of a monoculture can be responsible for the loss of or decrease in diversity in cultivated species of *U. molinae*. Seguel and Torralbo (2004) indicated that *Bombus* spp. is the principal pollinators of *U. molinae*, and for this reason, the loss of diversity through the domestication process or monoculture can signify a decrease in pollination. Future research will be focused on the effects of secondary metabolites on the insect assemblages on wild and cultivated *U. molinae* plants. We detected changes in the community and numbers of the insect assemblages, the diversity indexes and also the damage indexes, which could suggest that the domestication of murtilla has altered the insect community in plants under agricultural management compared to plants in wild populations. We think that further experiments should continue to explore how domestication can affect these parameters in a controlled environment through a common garden. This is the first approach relating insect assemblages, diversity and damage indexes in wild and cultivated *U. molinae* plants. Future investigations will determine the effect of domestication on the chemical defenses in murtilla plants. Nevertheless, a single location in an area where all ecotypes were growing together was compared with wild parents in seven different locations. This setup could bias the evaluated diversity and damage variables to detect lower values in the single locality condition. Currently, there are few fields in which this crop is cultivated, and the only

location where all the cultivated plants related to their original counterparts are reported is the Experimental Station INIA-Tranapuente. Therefore, this first approach is subject to environmental factors, which will be avoided in the future through a common garden experiment for both wild and cultivated plants.

Table 2. Insects determined in different ecotypes and localities on wild and cultivated *U.molinae* plants.

Order	Family	Specie	Feeding behavior[1]	Location[2]	Ecotype[2]
Coleoptera	- Curculionidae	*Tartarisius subfasciatus* Bl.	P	Pucón	
		Aegorhinus nodipennis Hope,	P	Manzanal Alto	
		Hybreoleptops tuberculifer Boheman,	P	Manzanal Alto, Queule, Mehuín, Caburgua	
		Megalometis spinifer Boheman	P	Queule	
	- Scarabaeidae	*Hylamorpha elegans* Burmeister,	P	Pucón, Manzanal Alto	Eco 14-4
		Brachysternus prasinus Guérin	P		Eco 22-1
	- Cerambycidae	*Chenoderus testaceus* Blanchard,	P	Soloyo	
		Callideriphus laetus Blanchard	P		Eco 12-1
	- Chrysomelidae	*Kuschelina decorata* Blanchard.	P	Soloyo, Porma	
	- Meloidae	*Epicauta pilme* Molina	P		Eco 19-1, Eco 22-1, Eco 23-2
	- Carabidae	*Ceroglossus valdiviae* Hope,	PR	Manzanal Alto, Queule	
		Helina spp.,	PR	Manzanal Alto	
		Ceroglossus chilensis Escholtz	PR	Manzanal Alto	
	- Cupedidae	*Prolyxoscupes latreillei* Sol.	P	Manzanal Alto	
	- Tenebrionidae	*Oligocara nitida* Gay i Sol,	P	Manzanal Alto, Queule	
		Nycterinus spp.,	P		
		Blaptinus punctulatus Curtis,	P	Queule	Eco 12-1
		Epipedonota spp.,	P	Queule	
		Heliotubus spp.	P	Queule	
	- Bostrichidae	*Neoterius pulvinatus* Blanchard	P	Queule	
	- Bruchidae	*Megacerus eulophus* Erichson	P		Eco 19-1
	- Coccinellidae	*Eriopis connexa* Germar	P		Eco 18-1, Eco 23-2
Diptera	- Cecidomyiidae	*Prodiplosis longifila* Gagné	PR	Pucón, Soloyo	
	- Dolichopodidae	*Dolichopus bipunctatus* Macq.	PR	Manzanal Alto	
	- Asilidae	*Lycomya germaini* Bigot,	PR		
		Eccritosia rubriventris Macquart,	PR	Porma, Queule	Eco 14-4, Eco 19-1, Eco 22-1
		Araiopogon gayi Macq.	PR		Eco 23-2
	- Calliphoridae	*Phaenicia sericata* Meigen	PR	Porma, Queule, Mehuín	
	- Muscidae	*Hylemyia* spp.	D	Porma	
	- Tabanidae	*Scaptia lata* Guérin-Méneville,	H		Eco 12-1, Eco 14-4, Eco 18-1, Eco 19-1, Eco 22-1, Eco 23-2
		Dasybasis chilensis Macquart	H		Eco 18-1, Eco 19-1, Eco 22-1
	- Tipulidae	*Tipula* spp.	P	Mehuín	
Hemiptera	- Pentatomidae	*Acledra haematopus* Spinola	P	Pucón	

	- Lygaeidae	*Lygaeus albornatus* Bl.	P	Soloyo	
	- Scutelleridae	*Missippus spinolai* Sig.	P	Manzanal Alto	
	- Rhopalidae	*Harmostes chilensis* Dallas	P	Caburgua	
Hymenoptera	- Formicidae	*Camponotus chilensis* Spinola,	PR	Soloyo, manzanal Alto, Mehuin	
		Nothidris latastei Em.	PR		Eco 19-1
	- Pompilidae	*Pompilus spinolae* Kohl	PR	Porma	
	- Ichneumonidae	*Chromocryptus* spp,	PT	Mehuín	
		Alophophion chilensis Spinola	P		Eco 14-4, Eco 23-2
	- Vespidae	*Vespula germanica* Fabricius	PR	Mehuín	Eco 08-1, Eco 23-2
	- Apidae	*Apis mellifera* Linnaeus,	PL		Eco 19-1
		Megabombus dalhboni Guérin	PL		Eco 19-1, Eco 22-1, Eco 23-2
Lepidoptera	- Tortricidae	*Proeulia* spp.	P	Soloyo, Porma, Queule, Mehuin	
	- Saturniidae	*Ormiscodes cinnamomea* Feisthamel	P	Porma, Queule	
	- Arctiidae	*Chilesia rudis* Butler	P	Mehuín	
	- Noctuidae	*Feltia malefida* Gueneé	P		Eco 14-4
Neuroptera	- Hemerobiidae	*Gayomyia falcatus* Blanchard in Gay	PR	Soloyo	
Orthoptera	- Acrididae	*Aucacris eumera* Hebard,	P	Manzanal Alto	
		Trimerotropis ochraceipennis Bl.,	P	Manzanal Alto	
		Dichroplus maculipennis Blanchard	P	Porma	
	- Tettigoniidae	*Dichroplus porter* Liebermann,	P	Manzanal Alto, Porma, Mehuín	
		Coniungoptera nothofagi Rentz y Gurney,	P	Manzanal Alto	
		Heteromallus notabilis Brun.	P	Manzanal Alto	
Homoptera	- Cicadellidae	*Ribautiana terrima* Herrich-Schaffer	P	Porma	Eco 22-1
	- Cicadidae	*Tettigades chilensis* Amyot & Serville	P	Mehuín	
	-Diaspididae	*Hemiberlesia rapax* Comstock.	P	Mehuín	
Blattodea	- Blattidae	*Blatta* spp.	O	Queule	
Phasmatidae	- Phasmidae	*Agathemera crassa* Blanchard	P	Queule	

[1]Feeding behavior; P= Phytophagous, PR= Predator, PT= Parasitoids, PL= Pollinator, O= Omnivorous, H= Hematophagous.

[2]Location corresponds to wild plants and Ecotype corresponds to cultivated plants.

Table 3. Summary results of two-way ANOVAs for the effects of temporal variation of the damage index in murtilla, *Ugni molinae*.

Parameter	Variable	*df* [1]	*F*	*P*
Damage index	Domestication degrees	1, 5446	28.34	≤ 0.001
	Domestication × Months (within domestication)	12, 5446	16.99	≤ 0.001
	Residual	5446		
	Total	5459		

[1] Degrees of freedom: numerator, error

CHAPTER III:

Domestication in murtilla (*Ugni molinae* Turcz) reduced defensive flavonol levels but increased resistance against a native herbivorous insect

3.1 INTRODUCTION

The domestication of plants is a process of artificial selection in which wild plants are modified to meet human needs (Meyer and Purugganan 2013, Cornille et al. 2014, Gepts 2014). As a result, plant domestication may generate a so-called "domestication syndrome" (Hammer 1984, Evans 1993, Abbo et al. 2014), where the domesticated plants have features useful for human consumption such as increases in yield, fruit size, number of seeds, and plant growth (Wink 1988). However, certain plant traits such as those associated with defense against herbivores can be negatively affected (Hammer 1984). This may occur particularly in plants domesticated for high yield where a pool of available resources is allocated to fruit production instead of defense (Herms and Mattson 1992, Davila- Flores et al. 2013). Consequently, domesticated plants may be less defended against their enemies as compared with their wild ancestors (Rosenthal and Dirzo 1997, Rodriguez-Saona et al. 2011, Chen and Bernal 2011, see review by Chen et al. 2015). For example, in a recent study, Altesor et al. (2014) found that cultivated potato (*Solanum tuberosum* L.) plants have lower levels of glycoalkaloids and were more susceptible to attack by two generalist herbivores, the green peach aphid, *Myzus persicae* Sulzer, and the potato aphid, *Macrosiphum euphorbiae* Thomas, as compared with their wild ancestors.

Flavonoids are an important group of plant defensive compounds (Harborne 1988, Harborne and Williams 2000), which can be affected by domestication. For instance, Mikulic-Petkovsek et al. (2012) analyzed several species of wild and cultivated berries and found higher levels of the flavonoid quercetin in wild plants than in cultivated ones. Similarly, Giovanelli and Buratti (2009) analyzed four varieties of cultivated blueberries and a wild counterpart and showed that the total phenols and anthocyanin concentrations in

wild fruit were two and three fold higher, respectively, than in cultivated fruit. Flavonoids are known to affect insect feeding behavior (Harborne 1988). For example, Simmonds (2001) reported that rutin, a commonly studied flavonol glycoside, is a phagostimulant to many polyphagous insects such as *Schistocerca americana* Drury and *Heliothis virescens* F.; however, high levels of this compound were deterrent to the noctuids *Helicoverpa zea* Boddie, *Spodoptera littoralis* Boisduval, *Spodoptera exigua* Hubner, and *Spodoptera exempta* Walker. Some flavonoids are also known to reduce larval performance (Elliger et al. 1980). For example, rutin caused 50% mortality and reduced the relative growth rate of *Spodoptera eridania* Cramer (Lindroth and Peterson 1988). Also, Beninger and Abou-Zaid (1997) showed that rutin, quercetin, and a glucoside quercetin isolated from four pine species decrease larval mass and increase mortality of *Lymantria dispar* L.

Murtilla, *Ugni molinae* Turcz. (Myrtaceae), an endemic plant from Chile, is a highly polymorphic perennial shrub reaching heights of over 3 m (Valdebenito et al. 2003, Hoffmann 2005). Researchers at the Experimental Station of the Instituto de Investigaciones Agropecuarias (INIA) in Carillanca, Región de La Araucanía, Chile, have been domesticating this species for the past 20 years for high productivity (Seguel and Torralbo 2004); these plants were originally collected from 100 localities in southern Chile. Through the process of domestication, *U. molinae* cuttings were first grown in greenhouses for 10 yr and then transplanted to the field (INIA Experimental Station-Tranapuente in the Región de La Araucanía [South of Chile, 38º 450 S, 73º 210 W]) until now. In Chile and worldwide, there is a strong economic interest in the production of *U. molinae* fruit due to its high antioxidant content. This antioxidant activity is attributed to the presence of flavonoid compounds (Avello and Pastene 2005, Rubilar et al. 2006, 2011). For example,

several flavonoids such as quercetin, kaempferol, rutin, and myricetin, and their corresponding glycosides, have been identified from *U. molinae* fruit and leaves (Shene et al. 2009, 2012).

Aguilera et al. (2009) reported, for the first time, larvae of *Chilesia rudis* Butler (Lepidoptera: Arctiidae) attacking *U. molinae*. *C. rudis* is a polyphagous, univoltine insect, native to Chile (Vargas and Parra 2003), and one of the most serious pests of grasslands, acting as a severe defoliator of several plants (Ángulo and Ruiz 1974). The life cycle of *C. rudis* in the Región de La Araucanía, Chile, has been described by Ángulo and Ruiz (1974): the larval stage lasts 6–8 months, from May until December, while the pupal stage lasts 2 months; the adults emerge in February and live for 8–14 d. The larvae feed on different plant parts but prefer the leaves.

In this study, we hypothesized that *U. molinae* domestication has decreased chemical defenses and resistance against herbivores. Specifically, we compared the levels of four major flavonols—quercetin, kaempferol, quercetin 3-D-β glucoside, and rutin—in wild and domesticated *U. molinae* plants. We also determined seasonal differences in flavonol content as well as differences among various plant tissues. Finally, we studied *C. rudis* larval growth and feeding on wild and domesticated *U. molinae* leaves, and tested the effects of each of the four identified flavonols on larval leaf consumption.

3.2 MATERIAL AND METHODS

3.2.1 Sampling Area. Seven cultivated ecotypes, i.e., geographically distinct

populations, of *U. molinae* from the INIA Experimental Station-Tranapuente were used for experiments. These ecotypes have been maintained in a field at the Experimental Station for almost 10 yr, as indicated above. We selected these ecotypes based on their agronomic characteristics such as size, growth, and productivity (Seguel and Torralbo 2004), as well as the availability of their wild parents in the original collection areas. The ecotypes were sampled from two geographical regions: five ecotypes were originally sampled from the Región de La Araucanía (ecotypes 08-1, 12-1, 14-4, 18-1, and 19-1), and the other two ecotypes were originally sampled from the Región de Los Ríos (ecotypes 22-1 and 23-2). Fertilizer was applied annually according to soil analysis, and consisted of 80, 44, and 43g per plant of nitrogen, P_2O_5, and K_2O, respectively. Pest control was carried out using Karate (lambda-cyhalothrin; Syngenta, Greens- boro, NC) at a dose of 1 to 2 ml/liter of water or Lorsban 4E (chlorpyrifos; Dow AgroSciences, Indianapolis, IN) at a dose of 1 ml/plant (one to two applications during the year), according to the incidence of cutworms. To avoid residual toxicity, all samples for chemical and biological assays (see below) were collected at least 7 d after insecticide applications. Each cultivated ecotype was paired with its wild ancestor, which was located in the same geographical area where its cultivated counterpart originated. For the wild plants, the following sampling areas were used: Caburgua (39° 110 S, 71° 490 W), Pucón (39° 170 S, 71° 550 W), Manzanal Alto (38° 030 S, 73° 100 W), Soloyo (38° 350 S, 72° 340 W), and Porma (39° 080 S, 73° 160 W) from the Región de La Araucanía; and Mehuín (39° 260 S, 73° 120 W) and Queule (39° 230 S, 73° 120 W) from the Region de Los Ríos. Therefore, the following seven cultivated, wild ecotypes, geographical areas were paired for chemical and biological assays: Eco 08-1, Caburgua, Eco 12-1, Pucón, Eco 14-4, Manzanal Alto, Eco 18-1, Soloyo, Eco 19-1, Porma, Eco 22-1, Mehuín, and Eco 23-2, Queule.

3.2.2 Plant Material and Insects. Leaves, stems, and fruits (when available) of *U. molinae* were sampled monthly (from December 2012 until October 2013) from both cultivated and wild plants. Five plants were sampled for each cultivated ecotype and at each wild site (N 1⁄4 70 plants). Samples were taken from all four cardinal directions and at different heights of each plant, and were standardized by age to control for phenological variation. After this, samples were stored in paper bags, placed in a cooler, and then transported to the Laboratorio of Química Ecológica, Universidad de La Frontera (Temuco, Chile). Samples were stored at -20°C (Zeraik 2010, Yi et al. 2012) until used in chemical analysis (see below), while samples for bioassays were used within 24 h after collection. *C. rudis* larvae (older instars; size 30–40 mm) used for bioassays were collected manually in late spring (December) from grasses in Temuco, Padre Las Casas, Chile (38° 460 S, 72° 360 W); thus, these larvae likely had no prior experience on *U. molinae*. Larvae were deprived of food for 3 d before the experiments.

3.2.3 U. molinae Extract. Throughout a year, 735 samples from leaf, stem, and fruit of both cultivated and wild plants were collected. Samples were rapidly frozen in liquid nitrogen for 5s (Mikulic-Petkovsek et al. 2012), and then milled in a grinder. After this, samples (5g) were placed in a flask where ethanol HPLC grade (Sigma-Aldrich, St. Louis, MO) was added to the samples (50% v/v in water, solvent-to-solid ratio of 5:1). These flasks were placed in a magnetic stirrer for 20 min at 30 °C and 170 rpm. After this time, samples were filtered in darkness through a Whatman number 1 filter paper (Whatman International Ltd., Maidstone, United Kingdom). The filtrate was concentrated in a rotary evaporator at 45 °C and lyophilized for 8 h (Rubilar et al. 2011). Finally, each sample was suspended in 10 ml of ethanol and left for 5 min in a Branson 3510 sonicator. Samples

were stored at -20 °C in amber flasks (25 ml) until their use in High Performance Liquid Chromatography (HPLC) analysis.

3.2.4 Chromatographic Separation and Quantification by HPLC Analysis. The ethanolic extracts obtained from leaves, stems, and fruits were filtered through a 0.22 mm membrane and these were analyzed by HPLC. Twenty microliter of each sample was injected into a Shimadzu HPLC (Model LC-20A Prominence, Kyoto, Japan) equipped with a C-18 column (150/4.6 mm I.D.; particle size 5 mm) maintained at 40 °C. The analysis was performed using a linear solvent gradient consisting of 1% formic acid (A) and acetonitrile (B) as follows: 0–5 min, 5% A/95% B; 5–10 min, 30% A/70% B; 10–20 min, 55% A/45% B; 20–30 min, 5% A/95% B at a flow rate of 1 ml/min (Simirgiotis et al. 2009). Flavonols were monitored at 280 nm; UV spectra from 190 at 700nm were used for peak characterization. The identification of flavonols was based on the peak retention time in comparison with that of a standard. To construct calibration curves for flavonols, standard solutions were dissolved in methanol (Sigma-Aldrich) at 1,000 mg/liter. The stock solutions of each standard were used to prepare a serial concentration between 0.05 to 500mg/liter (Kumar et al. 2009). All the standards were stored at 4 °C until their injection into the HPLC (Zu et al. 2006). To determine the limits of detection (LOD) and quantification (LOQ), the stock solution of each standard was diluted in MeOH to provide serial dilutions. Each solution was injected in the HPLC until obtaining the 3-r signal to noise (S/ N) ratio for LOD of each flavonol and a value of 10-r for LOQ (Olszewska 2008).

3.2.5 No-Choice Bioassay. This study evaluated *C. rudis* larval performance on cultivated versus wild *U. molinae* leaves. One *C. rudis* larva was placed in a Petri dish (94 mm in diameter by 16 mm high) containing either a cultivated or a wild *U. molinae* leaf.

The bioassay lasted 2 d, and leaves were replaced after 24 h. Ten replicates were performed for each of the seven cultivated ecotypes and their wild counterparts. The amount of feeding was measured in cm^2 by scanning each leaf and then measuring the area consumed using theImageJ 1.42j software (Wayne Rasband National Institutes of Health). In addition, fresh larval mass was obtained prior to the bioassay and after 48 h, as described in Carpinella et al. (2003), and the mass gained was calculated.

3.2.6 Choice Bioassay. We also conducted experiments using a leaf choice test (Carpinella et al. 2002) to determine *C. rudis* larval preference for cultivated versus wild *U. molinae* leaves. Two leaves, one of a cultivated and one of a wild *U. molinae* plant, were placed in a 10 mm diameter Petri dish, with two 1 cm diameter holes on the top covered by a fine mesh. Larvae of *C. rudis* were placed in a position equidistant from both leaves and allowed to feed for 48 h. Ten replicates were run for each of the seven cultivated ecotypes and their wild counterparts. Leaf consumption was measured as described for no-choice bioassays. Relative amounts (in percentages) of leaf area eaten for each cultivated ecotype and wild location were calculated based on a feeding index, FI% = (W-C) / (C+W) X 100, where C and W represent consumption on cultivated and wild leaves, respectively (Mazoir et al. 2008).

3.2.7 Bioassay with Individual Flavonols. This experiment was conducted to determine if the identified flavonols act as phagostimulants, and also to rule out whether the observed effects were due to environmental factors such as pesticides. A cultivated *U. molinae* leaf (1.2 cm^2) was placed on a Petri dish, as described above, and 0.5 ml of pure compound of quercetin, kaempherol, quercetin 3-D-β glucoside, or rutin at concentrations of 0.1, 1, 5, 50 mg/liter were applied over each leaf using a micropipette. Leaves for this

experiment were collected in January from one of the cultivated ecotypes (Eco18-1), which contain higher flavonol levels than the other ecotypes (see results). To test for concentration dependent effects, we used a range of flavonol concentrations that were comparable with those found in the leaves. All compounds tested were dissolved in ethanol (solvent), purchased from Sigma-Aldrich, and had purities > 98%. Controls (0 mg/liter) had ethanol only. One *C. rudis* larva was then placed inside the Petri dish. After 24 h the consumed area was recorded using the ImageJ 1.42j software. This experiment was replicated 10 times for each flavonol concentration and controls.

3.2.8 Statistical Analysis. The statistical software R (R 3.0.2; the R foundation for statistical computing, Vienna, Austria) was used to analyze the data. The effects of domestication (i.e., wild versus cultivated plants) and location (i.e., cultivated ecotype, wild geographical area of collection) on total flavonol content and larval mass and consumption were analyzed using fully nested hierarchical random analysis of variance (ANOVA), with domestication nested within location and location used as a random effect. The effects of domestication, time of year, or plant part, and their interaction on total flavonol content were analyzed using a two-way ANOVA. ANOVA was also used to test for the effects of different concentrations of individual flavonols on leaf consumption. Scott–Knott tests were used for comparisons among groups. For each individual flavonol, *t*-tests were used for paired comparisons between wild and cultivated plants. Data were natural-log transformed to meet the assumptions of normality and homogeneity of variance. We used arcsine square-root transformation for percent data. Values of $P \leq 0.05$ were considered as significant. Results are expressed as means and their corresponding standard errors.

3.3 RESULTS

Chromatographic Analysis. Overall, across all tissues (leaves, stems, and fruit), the total amount of flavonols in wild *U. molinae* plants was ~20% higher than in the cultivated plants, showing a significant domestication effect (Table 1A; Fig. 1A), which was dependent on the ecotype or geographical area (Table 1A; Fig. 1B), such that wild plants from Manzanal Alto, Caburgua, Mehuín, and Queule had significantly higher flavonol concentrations as compared with their cultivated counterparts (Scott–Knott test, $P \leq 0.05$), while other sites were not significant ($P > 0.05$). There was temporal variation in flavonol content (significant time of year effect: $F_{6,84} = 15.41$, $P < 0.001$). For both wild and cultivated plants, total flavonol content increased from December to April (which coincides with adult emergence and oviposition), then decreased from April to June (coinciding with young larval development), and increased again from June to October (which coincides with older larval development; Fig. 2). Throughout the year, wild plants had higher levels of flavonols than cultivated plants (significant Domestication effect: $F_{1,84}$, $P = 0.029$; Fig. 2). There was, however, no Domestication x Time of Year effect ($F_{6,84} = 0.418$, $P = 0.865$), indicating that the differences in flavonol content between wild and cultivated plants were consistent throughout the year. Within plants (spatial variation), *U. molinae* fruit had higher flavonol content than leaves and stems (significant Plant Part effect: $F_{20,168} = 63.163$, $P < 0.001$; Fig. 3). However, differences in flavonol content between wild and cultivated plants were significant only for leaves (significant Domestication x Plant Part interaction: $F_{20,168} = 1.869$, $P = 0.018$; Fig. 3). The analysis of *U. molinae* extract showed the presence of four flavonols -rutin, quercetin-3-D-β glucoside, quercetin, and kaempferol. The amount of flavonol was higher in wild plants ($P \leq 0.05$) than cultivated plants (Table 2; Fig. 4).

No-Choice Bioassay. In no-choice tests, *C. rudis* larvae gained almost twice as much mass when fed foliage from wild *U. molinae* plants compared with those fed cultivated plants; however, the effect of domestication on larval mass gained depended on location (Table 1B; Fig. 5A). In all cultivated and ecotype and wild and geographical area combinations, except for Eco 12-1, Pucón, *C. rudis* larvae gained more mass when they were fed wild *U. molinae* plants than those fed cultivated plants (Fig. 5B). *C. rudis* larvae also consumed 67% more when fed wild *U. molinae* leaves as compared with those fed cultivated leaves (Table 1C; Fig. 5C). Similar to mass gained, *C. rudis* larvae across all cultivated and ecotype and wild and geographical area, except for Eco 12-1, Pucón, ate more foliage when fed wild *U. molinae* plants as compared with those fed cultivated plants (Table 1C; Fig. 5D).

Choice Biossay. *C. rudis* larvae consumed 61% more of the wild *U. molinae* leaves than the cultivated leaves (Table 1D; Fig. 5E). As in the no-choice test, there was a significant domestication nested within location effect (Table 1D), indicating that the effect of domestication on *C. rudis* leaf area consumption was affected by ecotype and geographical area (Fig. 5F).

Table 1. Summary results of nested models for the effects of domestication and location on flavonol content in murtilla, *Ugni molinae*, and *Chilesia rudis* larval mass gained and food consumption in no-choice and choice tests.

	Parameter	Source of Variation	*df* [1]	*F*	*P*
A	Flavonols (mg/L)	Domestication	1, 476	33.66	≤ 0.001
		Location (within Domestication)[2]	12, 476	4.69	≤ 0.001
		Residual	476		
		Total	489		
B	Mass Gain (g)	Domestication	1, 126	153.32	≤ 0.001
		Location (within Domestication)[2]	12, 126	40.23	≤ 0.001
		Residual	126		
		Total	139		
C	Consumed leaf area (%) No-choice test	Domestication	1, 126	159.02	≤ 0.001
		Location (within Domestication)[2]	12, 126	28.23	≤ 0.001
		Residual	126		
		Total	139		
D	Consumed leaf area (%) Choice test	Domestication	1, 126	22.47	≤ 0.001
		Location (within Domestication)[2]	12, 126	14.20	≤ 0.001
		Residual	126		
		Total	139		

[1] Degrees of freedom: numerator, error.
[2] Location was used as a random effect in the model.

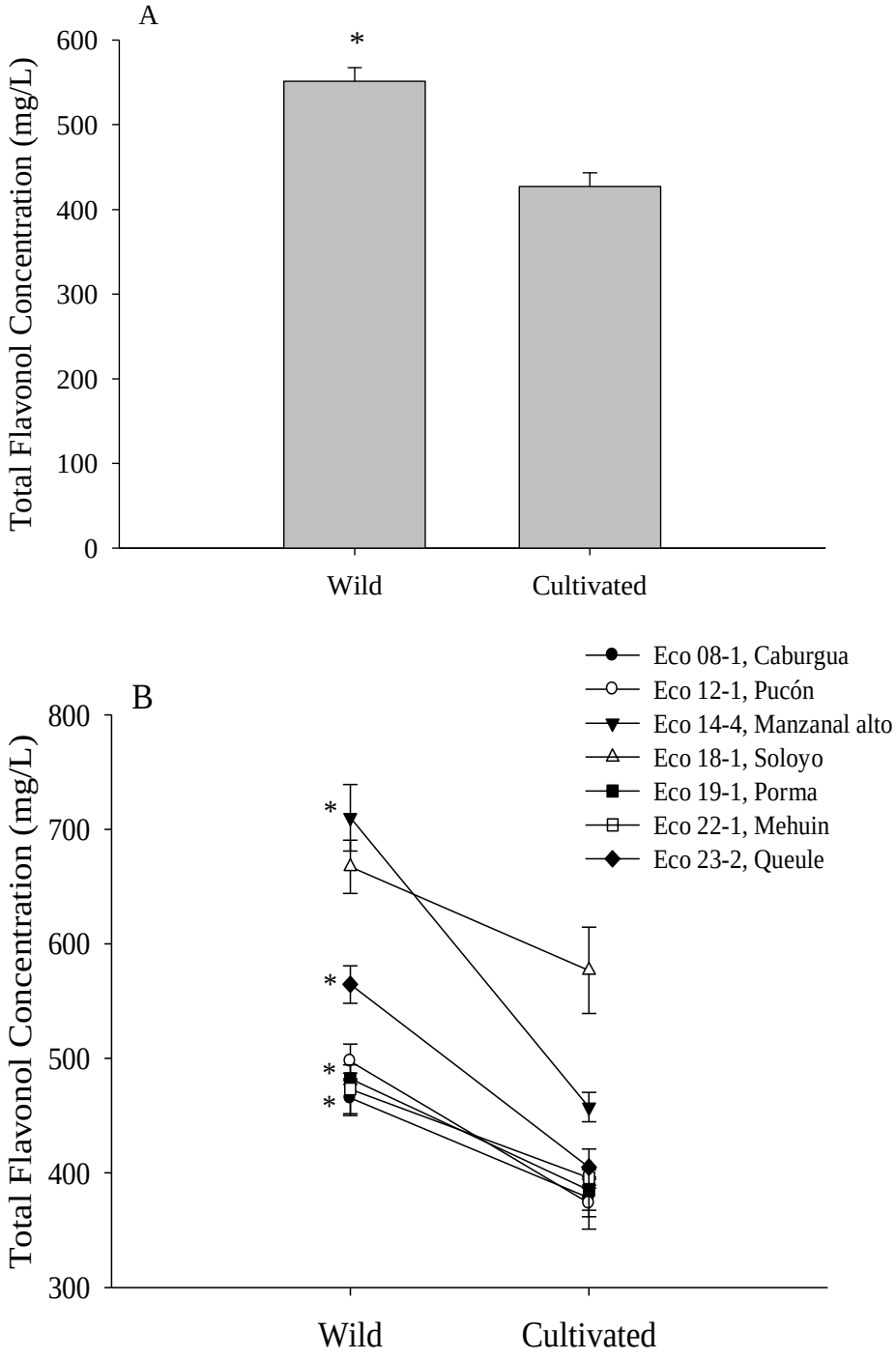


Fig. 1. Comparative amounts (mean ± SE) of total flavonols among all the collected plants in wild and cultivated murtilla, *Ugni molinae* (A), and amounts of flavonols based on each ecotype, geographical area (B). * = significant difference (Scott-Knott test, $P \leq 0.05$).

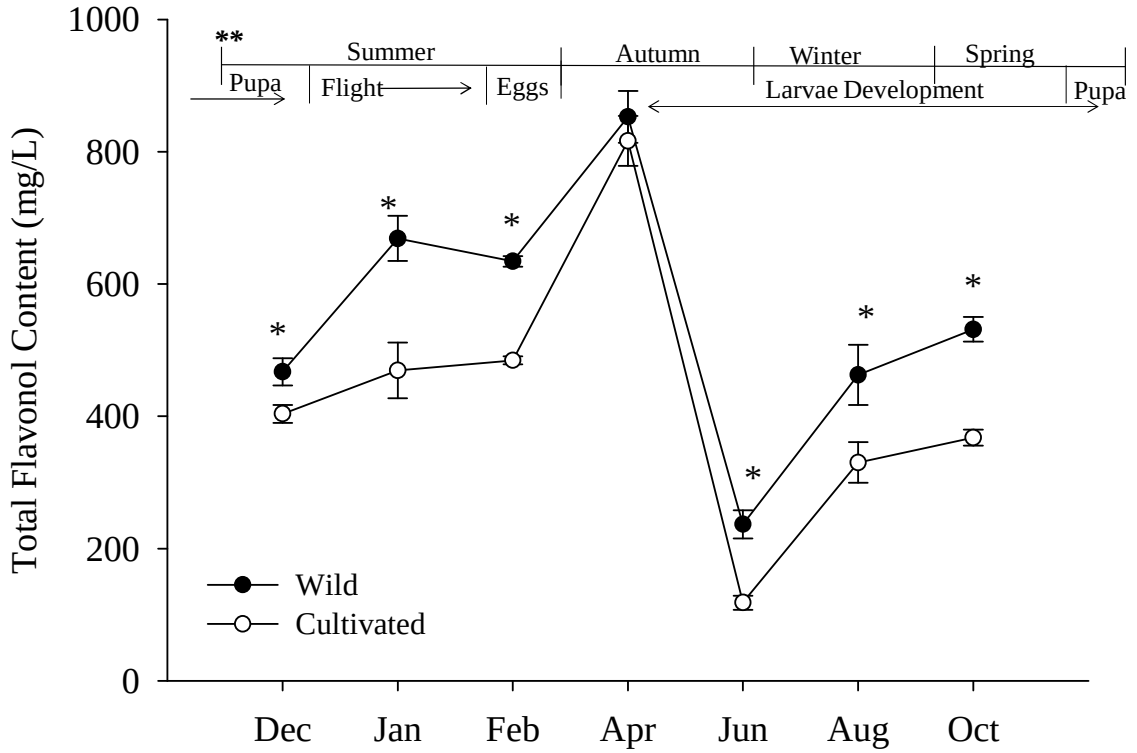


Fig. 2. Temporal variation in flavonol concentration (mean ± SE) in all collected tissues (leaves, stems, and fruit) of wild and cultivated murtilla, *Ugni molinae*, plants from December 2012 to October 2013. * = significant differences (Scott-Knott test, $P \leq 0.05$). Top: *Chilesia rudis* life cycle.

Assays with Individual Flavonols. *C. rudis* larval consumption increased with increasing concentrations of all tested flavonols ($F_{3,64}$= 38.207, $P < 0.05$). However, there was no effect of flavonol type ($F_{3,64}$ = 1.454, P = 0.235) or interaction between flavonol type and concentration ($F_{9,64}$ = 0.243, $P < 0.987$), indicating that the effect of all flavonols on larval consumption was similar instead of all concentrations. *C. rudis* consumed 40% of cultivated *U. molinae* treated with a concentration of 0.1 mg/liter of any flavonol; however, when higher concentrations were applied to leaves, *C. rudis* larvae increased their consumption to 80–90% with increasing concentrations, indicating that these compounds acted as phagostimulants (Fig. 6).

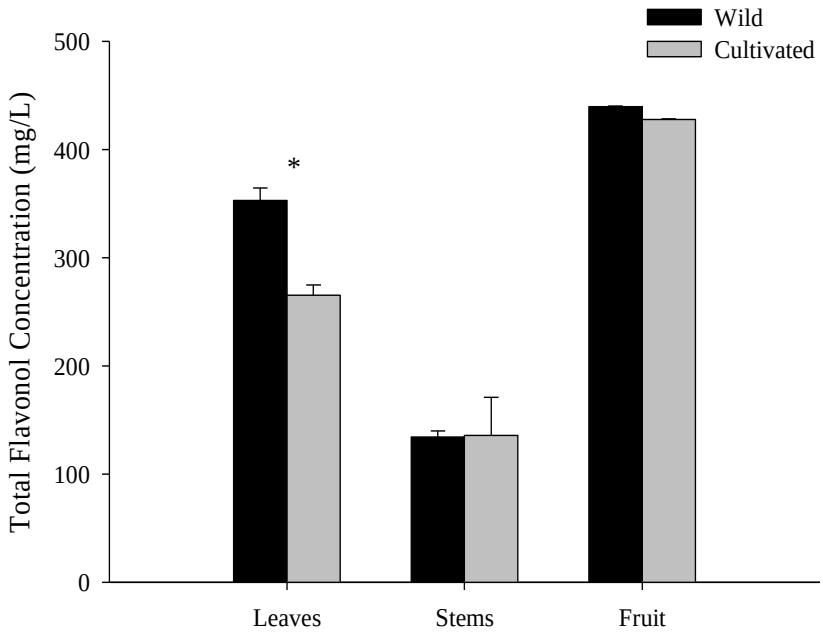


Fig. 3. Spatial variation in flavonol concentration (mean ± SE) among leaves, stems, and fruit of wild and cultivated murtilla, *Ugni molinae.****** = significant difference (Scott-Knott test, $P \leq 0.05$).

3.4 DISCUSSION

Crop domestication can affect plant defenses and resistance against herbivores in unpredictable ways (Meyer et al. 2012). For example, in a recent study, Turcotte et al. (2014) found that domestication across 29 crop species resulted in reduced resistance to a generalist leaf-chewing herbivore, *S. exigua*, but had no effect on the generalist aphid, *M. persicae*. In this regard, our study demonstrates higher levels of flavonols in wild as compared with cultivated *U. molinae* leaves. However, the performance and preference of the native caterpillar *C. rudis*, a generalist folivore, was higher on wild as compared with cultivated *U. molinae*, despite the fact that the former plants have higher amounts of flavonols, indicating that these chemical compounds may be acting as phagostimulants in this insect–plant interaction.

Table 2. Concentrations of individual flavonols from wild and cultivated murtilla, *Ugni molinae*.

Compound number[a]	RT (m)	Compound Name	Mean ± SE (mg/L)		LOQ (mg/L)	LOD (mg/L)
			Wild	Cultivated		
1	13.793	Rutin	231.51 ± 7.11*	190.73 ± 7.44	0.05	0.015
2	14.112	Quercetin-3-D-β-Glucoside	316.12 ± 10.72*	238.77 ± 9.97	0.025	0.0075
3	16.680	Quercetin	2.34 ± 0.12*	1.93 ± 0.10	0.05	0.015
4	17.916	Kaempferol	1.01 ± 0.05*	0.87 ± 0.06	0.05	0.015

LOQ: Limit of quantification.
LOD: Limit of detection.
RT: Retention time.
* Significant differences between wild and cultivated plants (*t*-test, $P \leq 0.05$).
[a] See Figure 4 for compound names.

Domestication and breeding for high-yielding crops are expected to reduce chemical defenses in plants because of potential trade-offs between growth or reproduction and defense (Wink 1988, Herms and Mattson 1992, Rodriguez-Saona et al. 2011). Domestication in *U. molinae* focused mainly on selection of traits associated with increased productivity, such as bigger plants, more fruit, and larger fruit size (Seguel and Torralbo 2004). As a result, we would expect that cultivated *U. molinae* might allocate resources toward defense, growth, and reproduction differently than wild plants because they are more vigorous, productive plants, they may be less defended against their enemies. Indeed, despite its short history of domestication (< 20 yr), our study shows that domestication in *U. molinae* has led to decreases in flavonol levels, an important class of defensive secondary metabolites in plants. This is in accordance with our hypothesis that domestication has reduced chemical defenses in *U. molinae*. In fact, amounts of four

flavonols—rutin, kaempferol, quercetin, and quercetin 3-D-β glucoside—were lower in cultivated *U. molinae* than in their wild ancestors. Although the trend was the same, i.e., reduction in flavonol levels in cultivated plants, the strength of the effect of domestication varied among populations (Fig. 1B), with some populations responding more strongly than others. Future common garden studies from our group will aim to separate the genetic from the environmental factors responsible for this population level variation. If cultivated plants are less defended (Chen et al. 2015), we predicted that domestication in *U. molinae* would make plants more susceptible to herbivores. In fact, some flavonols found in *U. molinae* have been implicated with resistance against herbivores in other plant systems. For example, Todd et al. (1971) showed that quercetin, a constituent of barley leaves, was toxic to greenbugs, *Schizaphis graminum* (Rondani). Moreover, Dreyer and Jones (1981) reported increased resistance of wheat against *M. persicae* also due to quercetin. This was, however, not the case for the herbivore *C. rudis*, an important defoliator in the ecosystem (Ángulo and Ruiz 1974), which showed lower performance and preference for cultivated *U. molinae* plants than their wild counterparts. In fact, our study shows that flavonol content stimulates feeding in *C. rudis*. Takemura et al. (2002) also reported increased susceptibility of *Vicia angustifolia* L. against the aphid *Megoura crassicauda* Mordivilko, and attributed it to the presence of flavonol glycosides.

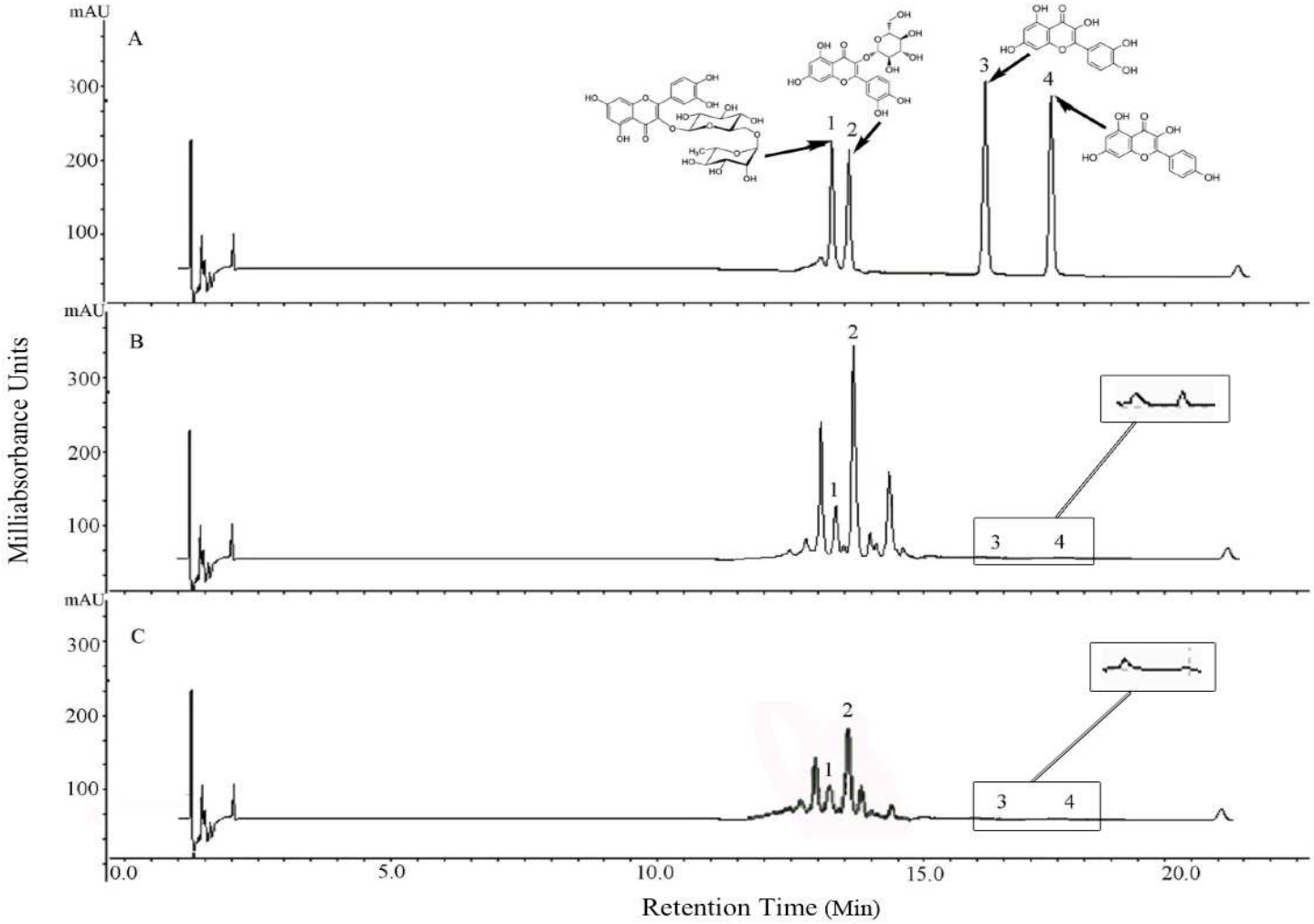


Fig. 4. Representative HPLC chromatograms of synthetic standards (A) and wild (B) and cultivated extracts (C) of murtilla, *Ugni molinae*, leaves. 1) rutin, 2) quercetin-3-D-*β*-Glucoside, 3) quercetin, and 4) kaempferol.

Diaz et al. (2010) reported that quercetin acts as a phagostimulant for the beetle *Epilachna paenulata* (Germar) (Coleoptera: Coccinellidae). Moreover, Lin and Mullin (1999) reported stimulant feeding activity by quercetin 3-D-β glucoside in the western corn rootworm, *Diabrotica virgifera* virgifera LeConte. Wink (1988), Nielsen et al. (1998), and Bernays (1991) also reported stimulation of feeding and ovipositional activity by kaempferol, rutin, and glycoside compounds for other herbivores. Even though *C. rudis* is a generalist herbivore, preference for wild *U. molinae* might be due to the fact that both plant and insect are native to the region and it has likely evolved to exploit its host plant's defenses. In the future, it will be interesting to test the effects of domestication on other native as well as on non native herbivores. There was substantial temporal (monthly) and spatial (within plant) variation in flavonol content in *U. molinae* (Figs. 2 and 3). Concentrations of flavonols peaked in April and were lowest in June, and they were higher in the fruit and leaves than in the stem. Based on *C. rudis* life cycle (Ángulo and Ruiz 1974), larvae are present from May until December, which coincides with an increase in flavonol levels (Fig. 2). They also prefer to feed on leaves, which contain high quantities of these flavonols. This evidence suggests that the herbivore *C. rudis* is well adapted to feed on host plants at times of the year when concentrations of these secondary metabolites in tissues are high. This is further supported by the fact that wild plants, which are preferred by *C. rudis*, have greater amounts of these compounds throughout most of the year and in the leaves. The performance and preference of *C. rudis* was, in general, consistent across most *U. molinae* populations the herbivore grew and ate more foliage from wild hosts than in cultivated hosts except for one geographic site (corresponding to the Eco 12-1, Pucón cultivated, wild pairing; Fig. 5),

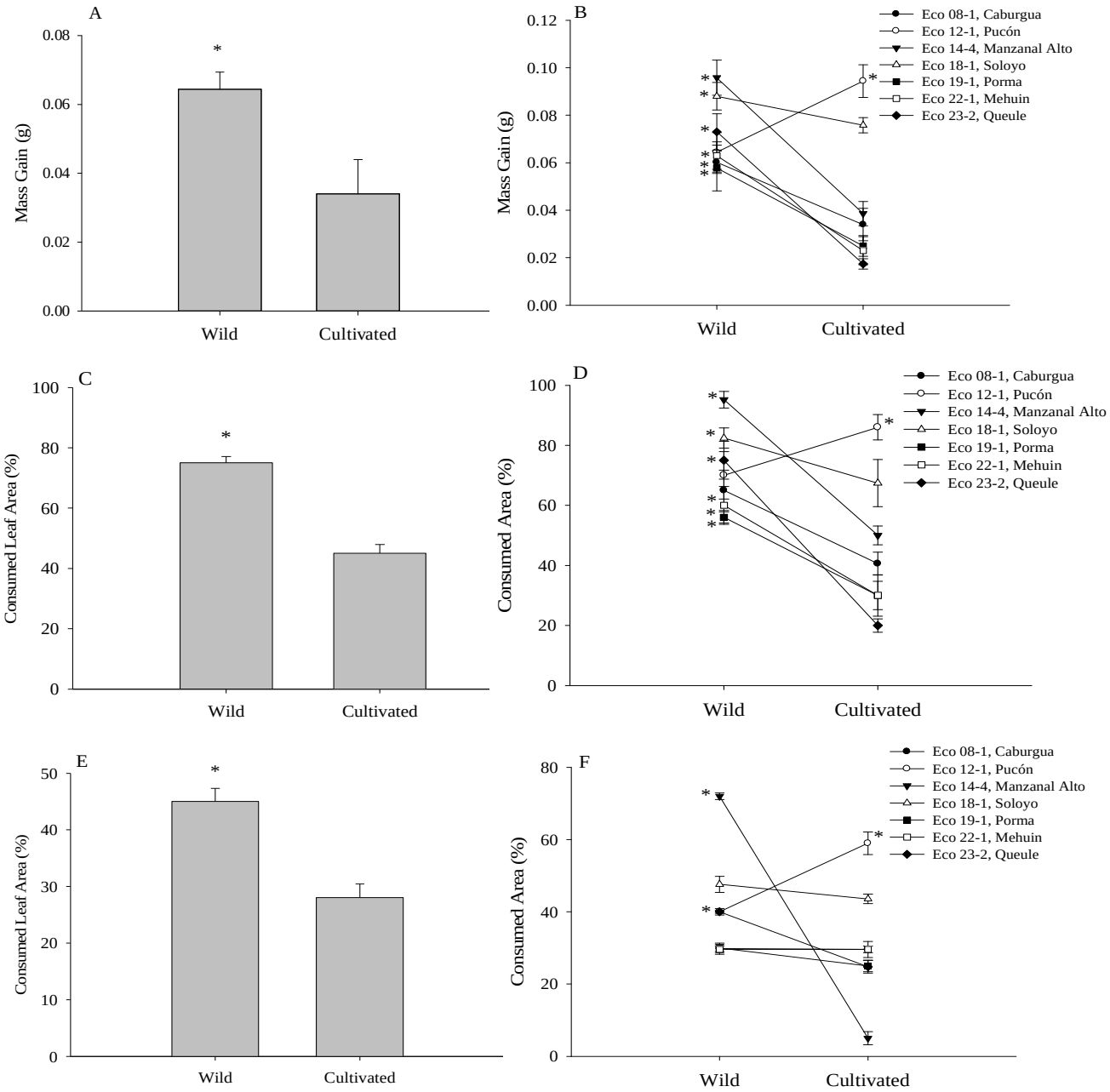


Fig. 5. Results from no-choice assays (A-D): *Chilesia rudis* larval mass gained (A) and food consumption (C) when fed wild and cultivated murtilla, *Ugni molinae*, leaves. And, mass gained (B) and food consumption (D) based on ecotype, geographical area. Results from choice assays (E-F): *C. rudis* larval food consumption (E) when fed wild and cultivated *U. molinae*, leaves. And, food consumption (F) based on ecotype, geographical area. ***** = significant difference (Scott- Knott test, $P \leq 0.05$).

where the opposite was observed, i.e., *C. rudis* gained more mass and consumed more foliage from cultivated (Pucón region) plants as compared with its cultivated (ecotype Eco 12-1) counterpart. Many factors, both physical and chemical, often contribute to resistance of plants against herbivores, which may act alone or interact with each other (Agrawal 2007). In our study, we tested a single class of secondary metabolites, the flavonols, and the individual effects of some of them — rutin, kaempferol, quercetin, and quercetin 3-D-β glucoside — on the feeding behavior of *C. rudis*, and found that all are feeding stimulants (Fig. 6). It is possible that other factors of resistance, unmeasured in this study, were responsible for the small inconsistencies reported here on the effects of domestication on *C. rudis*. The effects of domestication on other classes of secondary metabolites in *U. molinae* and their interactive effects on herbivores require future examination. It is worth noting that we only tested older instars in our study and that younger larvae could be more susceptible to higher flavonol levels.

In conclusion, although domestication and selective breeding have had great positive influences on food availability through increased crop yield and quality (Wink 1988), it has often had a cost for resistance against herbivores (Chen et al. 2015), which may lead to increased use of pesticides. While in a number of crop plants, domestication had reportedly led to lower levels of defensive compounds and therefore lower resistance to pests (e.g., Rosenthal and Dirzo 1997, Rodriguez-Saona 2011, Chen and Bernal 2011, Altesor et al. 2014), in the system studied here, domestication has led to lower levels of chemical defenses in the Chilean native crop *U. molinae* but increased resistance against one of its native herbivores, *C. rudis*. In fact, *C. rudis* uses these compounds as feeding stimulants. These findings show that we cannot generalize the effects of crop domestication

on resistance to herbivores from a number of plant species studied so far to all systems. Moreover, our study highlights the fact that there is some specificity in the response of herbivores to domestication and that not only herbivore identity does matter in these types of studies, but also the measured type of defense.

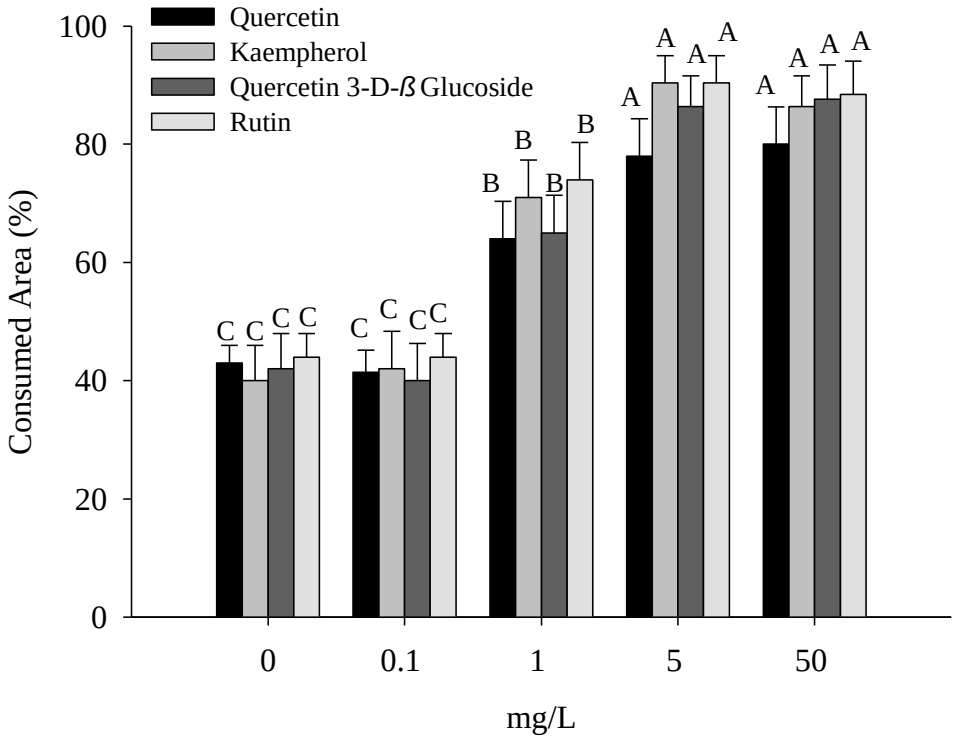


Fig. 6. *Chilesia rudis* consumption on cultivated murtilla, *Ugni molinae*, leaves treated with different concentrations of pure flavonol compounds. Bars with different letters were significantly ($P \leq 0.05$) based on the Scott-Knott test.

The results reported here have important implications for the cultivation of *U. molinae*, a crop that is highly valued due to the antioxidant activity of flavonols in its fruit (Rubilar et al. 2011, Alfaro et al. 2013). In our study, we showed that the levels of flavonols in the leaves were reduced due to domestication but this process did not affect levels in the fruit; thus, domestication should not have jeopardized its economic value. However, if the

focus of domestication in *U. molinae* shifts from higher productivity, i.e., increase in yield, to higher levels of antioxidant compounds, these berries could become more susceptible to certain native herbivores like *C. rudis*. Altogether, the reported findings provide the first insights on the impact of domestication on plant defenses and resistance against herbivores in *U. molinae*. It may also guide future breeding programs by highlighting the potential risks of breeding or high flavonol content on susceptibility of fruit against native, adapted herbivores.

CHAPTER IV:

How the insect plant interaction in Murtilla (*Ugni molinae* Turcz.) subject to a domestication process is affected in relation to the flavonoids content?

4.1 INTRODUCTION

Plant domestication is a process in which plants allocate their nutrients for production or improvement of some characteristics according to human needs. In this framework, the selection and breeding for reaching high-yielding plants have been a priority for farmers (Cock et al. 1979, Peng et al. 1999). Therefore, artificial selection is a principal factor in this process (Bautista et al. 2012). However, the anthropogenic improvements can be negative generating a decrease in other physiological traits, such as the secondary metabolism (Bazzaz et al. 1987, Herms and Mattson 1992, Rosenthal and Dirzo 1997). Hammer (1984) proposed that this change in the nutrient allocation within the plant (domestication syndrome) could be a useful tool for studying the trade off between wild and cultivated systems in the domestication process. Research about domestication process and its consequences on plants with high/low domestication degrees (Clement 1999) have been important for linking information related to pest management or breeding programs in the field as well as developing a new understanding about production, defenses and their insect plant interaction. Furthermore, changes in the secondary metabolism are not the only result of the domestication effect. Insect diversity, community composition and damage index in plants are aspects that can be indirectly affected by plant domestication (Rosenthal and Dirzo 1997, Chen and Bernal 2011, Chen et al. 2013). Hence, secondary metabolites act directly in the feeding behavior on insects, and the domestication effect could be associated with it as follows; wild plants with higher level of secondary metabolites must be more resistant to cope with pests than cultivated plants with lower levels of secondary metabolites. Currently, there are few studies comparing insect community and biodiversity in wild and cultivated systems (Chen and Bernal 2011).

Murtilla, *Ugni molinae* Turcz (Myrtaceae), an endemic plant from Chile, is a highly polymorphic shrub reaching heights of over 3 m (Rubilar et al. 2006, Shene et al. 2012) with significant antioxidant activity attributed to the presence of flavonol compounds, such as quercetin, kaempferol, rutin, and myricetin, and their corresponding glycosides in fruit, steams and leaves (Rubilar et al. 2006, Rubilar et al. 2010, Rubilar et al. 2011, Shene et al. 2012, Augusto et al. 2014, Chacón-Fuentes et al. 2015). There are many reports indicating that flavonols can affect the feeding behavior in insects (Abou-Zaid et al. 1993, Simmonds 2001, Takemura et al. 2002, Salunke et al. 2005, Adeyemi et al. 2010, Diaz et al. 2010, Onyilagha et al. 2012). For example, rutin is a deterrent to many polyphagous insects at $1x10^{-3}$ µg/mL including moth *Heliothis virescens* (Lepidoptera: Noctuidae) (Blaney and Simmonds 1983). In addition, isoflavonoids are a group of secondary metabolites within flavonoids (Hegnauer and Gpayer-Barkmeijer 1993) and these compound have been studied because some of these substances act as natural antioxidants and their effects on human organism have been concerning, especially in cardiovascular system (Pilsakova et al. 2010). Moreover, isoflavonoids have been related to feeding behavior in insects (Sutherland et al. 1980, Pluempanupat et al. 2013). Several isoflavonoids as daidzin, genistin, ononin, daidzein, sissotrin, genistein, formononetin and biochanin A, among others, have been detected and identified in plants belonging to family Myrtaceae such as *Acca sellowiana*, *Psidium guajava* and *P. littorale* (Lapčík et al. 2005) and the evidence of isoflavonoids in non-leguminous taxa has also been studied (Mackova et al. 2006, Lapčík 2007). Researchers at the Experimental Station of the Instituto de Investigaciones Agropecuarias (INIA) in Carillanca, Región de La Araucanía, Chile, have been domesticating *U. molinae* for the past 20 years (Seguel and Torralbo 2004). Hence, *U. molinae* could present levels of isoflavonoids in their leaves, stems or fruits according to

chemotaxonomy and their concentration and phytodiversity could be affected by domestication process affecting the insect plant interaction. The same author reported that leaves, stems and fruits obtained from *U. molinae* collected in wild areas were 20% significantly higher in flavonol concentration than in cultivated ones, showing a significant domestication effect. However, the same author noted that in each one of the locations the main disadvantage of this approach is related to the different environmental conditions where the plants were growing. The environmental plasticity presented within wild *U. molinae* species reported in Chacón-Fuentes et al. (2015) could be influencing the variation in the analyzed flavonoids (Losos et al. 2000). Hence, developing a common environment for evaluated these parameters could find a new and more precise answer to the flavonoids variation between cultivated and wild plants. The common garden allows avoid the environmental plasticity, comparing flavonoid variation under same environmental conditions. In this study, we carried out a common garden for growing and analyze the effect of domestication in *U. molinae* plants. We hypothesized that *U. molinae* domestication decreased chemical defenses and resistance against herbivores and affected the insect diversity, community composition and foliar damage index. Finally, we studied *Chilesia rudis* larval feeding on wild and cultivated *U. molinae* leaves. This insect is a polyphagous, univoltine insect, native from Chile (Vargas and Parra 2003), one of the most serious pests of grasslands, acting as a severe defoliator of different plants (Ángulo and Ruiz 1974) among them, murtilla (Aguilera et al. 2005, 2009).

4.2 MATERIAL AND METHODS

4.2.1 Collection Zones. For the establishment of the common garden two kind of sampling for the cutting obtaining were developed. The first one was carried out from seven *U. molinae* ecotypes (08-1, 12-1, 14-4, 18-1, 19-1, 22-1, 23-2) cultivated at the Experimental Station-Tranapuente of the Instituto de Investigaciones Agropecuarias (INIA) in the Región de La Araucanía (south of Chile, 38° 45`S, 73° 21`W). The second was carried out from the respective wild plant, which was sampled in the original geographical area where its cultivated counterpart had been collected. The following sampling areas were used for the wild cutting collection: Caburgua ($39^{o}11$` S, $71^{o}49$`W), Pucón ($39^{o}17$` S, $71^{o}55$`W), Manzanal Alto ($38^{o}03$` S, $73^{o}10$`W), Soloyo ($38^{o}35$` S, $72^{o}34$`W), and Porma ($39^{o}08$` S, $73^{o}16$`W) from the Región de La Araucanía; and, Mehuín ($39^{o}26$` S, $73^{o}12$`W) and Queule ($39^{o}23$` S, $73^{o}12$`W) from the Región de Los Ríos. Both kinds of cuttings were grown in a greenhouse at INIA Tranapuente for one year (March 2013) until their use in the common garden experiment.

4.2.2 Common Garden Experiment: After one year of acclimation in a greenhouse, all the plants (cuttings) were transplanted to a common garden established at INIA Experimental Station-Tranapuente in the Región de La Araucanía, south of Chile. Both, cultivated and wild plants were putted in eight liter pots and maintained under a greenhouse during the experiment. Only cultivated plants were fertilized annually, with 80 g/plant of nitrogen (calcium ammonium nitrate-27), 44 g/plant P_2O_5 and 43 g/plan K_2O.

4.2.3 Plant Material and Insects. Leaves of *U. molinae* from both cultivated and wild plants kept in the common garden were sampled monthly (March 2014 to January 2015). Five plants were sampled for each cultivated and wild plants (N = 70 plants).

Samples were collected from all four cardinal directions and the samples were placed in a cooler and transported to the Laboratorio of Química Ecológica, Universidad de La Frontera (Temuco, Chile). Samples were stored at -20°C until their use (Yi et al. 2012). *Chilesia rudis* larvae (30-40 mm) used for bioassays were collected manually in late spring of 2014 from grasses in Temuco, Chile. Larvae were deprived of food for 3 days before the experiments.

4.2.4 U. molinae Leaf Extract. Leaves material of both cultivated and wild plants were collected every two months from March 2014 to January 2015. Samples were rapidly frozen in liquid nitrogen for 5 s (Mikulic-Petkovsek et al. 2012), and then milled in a grinder. Later, 0.5 g of each one was placed in a flask where methanol HPLC grade (Sigma-Aldrich, St. Louis, MO) was added (50% v/v in water, solvent-to-solid ratio of 5:1). These flasks were placed in a magnetic stirrer for 20 min at 30 °C and 170 rpm. After this, samples were filtered in darkness through a Whatman N°1 filter paper (Whatman International Ltd., Maidstone, U.K.). The filtrate was lyophilized (Rubilar et al. 2011). Finally, each sample was suspended in 10 mL of methanol and left for 5 min in a Branson 3510 sonicator. Samples were stored at -20 °C in amber flasks (25 mL) until their use in High Performance Liquid Chromatography (HPLC) analysis.

4.2.5 HPLC ESI-MS/MS: The chromatographic separation was carried out using a RP-C18 ODS-3 column (2.1 x 150 mm, 3 μm), injecting 10 μL at 0.2 mL/min and 35 °C. The chromatographic separation was performed using a linear gradient solvent system consisting of 0.1% formic acid/water (A) and 0.1% formic acid/methanol (B). The linear gradient was composed of 0–10 min 5% B, 10-40 min 95% B, 40-50 min 95% B, 50-55 min 5% B, and finally, 55-60 min 5% B. Each sample was injected with an electro spray ionization (ESI) source into the mass spectrometer (LC-MS MS Shimadzu Prominence LC-

20AD coupled at mass spectrometer Applied Biosystems/MDS Sciex3200 Qtrap, Massachusetts, USA). The ion source temperature was set at 380º C, and the capillary voltage was 4500 v (positive polarity) and -4500 v (negative polarity). For phenolic compounds determination, data were collected as positive and negative ion spectra by means of Enhanced Mass Scan (EMS) over a *m/z* 100-1000 Da range at 1000 Da/s and Enhanced Product Ion (EPI) over a *m/z* 50-1000 Da range at 4000 Da/s. The CUR gas was 20 psi, GS1 60 psi and GS2 30 psi. The ion intensities were extracted at the *m/z* values of the molecular (M+) or pseudo-molecular $(M+H)^+$ ions of the corresponding detected compounds. The relative ion peak area of each compound from the sample was compared with the relative ion peak area of the total phenolic compounds.

4.2.6 Chromatographic Separation and Quantification by HPLC. The methanolic extracts obtained from leaves were filtered through 0.22 µm membrane and then were analyzed by HPLC. 20 µL samples were injected into a Shimadzu HPLC equipped with a C-18 column (150 x 4.6 mm I.D.; particle size 5 mm) maintained at 40°C. The analysis was performed using a linear solvent gradient consisting of 1% formic acid (A) and acetonitrile (B) as follows: 0-5 m, 5% A/ 95% B; 5-10 m, 30% A/70% B; 10-20 m, 55% A/45% B; 20-30 m, 5% A/95% B at a flow rate of 1 mL/m (Simirgiotis et al. 2009). Flavonols and isoflavonoids were monitored at 260 nm and UV spectra from 190 at 800 nm were used for peak characterization. The identification of flavonols and isoflavonoids was based on peak retention time in comparison with the respective standards. To construct calibration curves for flavonols and isoflavonoids, standard were dissolved in methanol (Sigma-Aldrich, St. Louis, MO) for obtaining a concentration of 1000 mg/L. The stock solutions of each standard were used to prepare a serial concentration between 0.05 and 500 mg/L (Kumar et al. 2009).

4.2.7 Insect Survey and Insect Biodiversity Indexes. Insect specimens were collected manually between 09:00 and 18:00 from the whole plants established in the common garden. Each plant was examined for 5 min. After completing inspection of each individual plant, the soil surface below canopy was examined (Knott et al. 2006). Captured insects were stored in Khale solution and determined in the laboratory under optical microscope (Olympus SD 30) using key books reported by Artigas (1994). In addition, for both, wild and cultivated plants diversity indexes were calculated as follow; Margalef index: D_{mg}=S-1/ln(N) where, S = Number of species in a sample and N = Total number of organisms in the sample. Shannon index: H´=-Σpi Log_2 (pi), and Simpson index; D=1/Σ $(pi)^2$ where, pi = ni/N ni = Species abundance and N =Total number of organisms in the sample (Samo et al. 2008).

4.2.8 Leaf Damage Evaluation. Leaves were collected from both cultivated and wild plants (6 leaves per plant) using all the four cardinal directions at different heights of the plants established in the common garden. The vegetal material was stored and transported to the Laboratorio de Química Ecológica of the Universidad de La Frontera and stored at -20 °C until analysis. Damage percentage was calculated evaluating the foliar area using Imagej 1.42 software (Wayne Rasband National Institutes of Health, USA). The damage was categorized according to methodology proposed by Dirzo and Dominguez (1995), as follows: 0= intact; 1= 1-6%; 2= 6-12%; 3=12-25%; 4=25-50%; 5=50-100%. Damage index by plant was calculated by means of the formula reported by Rodriguez-Auad and Simonetti (2001) DI = Σ ni (ci)/N, Where: ni= number of leaves in the i^{th} category of damage, ci= midpoint of each category, N= total number of leaves.

4.2.9 Choice Bioassay. A leaf choice test experiments was developed (Carpinella et al. 2002) to determine *C. rudis* larval feeding preference for cultivated versus wild *U. molinae* leaves. Two leaves, from a cultivated and a wild *U. molinae* plant were placed in a 10-mm diameter Petri dish with two 1-cm^2 diameter holes on the top covered by a fine mesh. Larvae of *C. rudis* were placed in an equidistant position from both leaves and allowed to feed for 48 hr. Ten replicates were run for each of the 7 cultivated ecotypes and their wild counterparts. Leaf consumption was measured similar to no–choice bioassay. Relative amounts (in percentages) of leaf area eaten by each cultivated ecotype and wild locations were calculated based on a feeding index, FI% = (W-C)/(C+W) × 100 where C and W represent consumption of cultivated and wild leaves, respectively (Mazoir et al. 2008).

4.2.10 No-choice Bioassay. This experiment evaluated *C. rudis* larval preference of cultivated versus wild *U. molinae* leaves. One *C. rudis* larva was placed on a Petri dish (94 X 16 mm) containing either a cultivated or a wild *U. molinae* leaf. The bioassay lasted two days and leaves were replaced after 24 h. Ten replicates were performed for each of the 7 cultivated ecotypes and their wild counterparts. The amount of feeding was measured in cm^2 by scanning each leaf and then measuring the consumed area using ImageJ 1.42j software (Wayne Rasband National Institutes of Health, USA).

4.2.11 Statistical Analysis. The statistical software Statistix 10 (Tallahassee, Florida, United States of America) was used to analyze the damage index, insect biodiversity index data, flavonoids comparison and *C. rudis* consumption. Damage indexes, the effects of domestication, time of year or plant part, and its interaction on total flavonoids content were analyzed using an analysis of variance (ANOVA). Tukey test was used for

comparisons among groups. For each individual biodiversity index, two Samples *t*-tests were used for paired comparisons between wild and cultivated plants. Finally, chi square test was performed for analysis of total insect assemblages between wild and cultivated plants. Values of $P \leq 0.05$ were considered as significant. Results are expressed as means and their corresponding standard errors.

4.3 RESULTS

Analysis through HPLC-ESI-MS/MS of wild and cultivated murtilla plants showed the presence of phenolic compounds in each one of cultivated plants. 16 compounds were found for cultivated and wild plants (Table II, Table III). An important number of compounds in these tables belong to flavonols such as kaempferol, quercetin, myricetin and rutin. Moreover, and isoflavonoids, daidzin was also found.

Flavonoids Content. Eight isoflavonoids and five flavonols were identified and quantified in both cultivated ecotypes and the corresponding wild parents of murtilla plants. Daidzin was the most abundant flavonoid found in both wild and cultivated plants with 335.59 ± 8,79 and 374.35 ± 23.01 μg/g, respectively (Table IV). There was a significant variation of flavonols and isoflavonoids content depending on location ($F_{6, 4364}$=28.81; $P \leq 0.001$); domestication degree ($F_{1, 4364}$=49.90; $P \leq 0.001$); months ($F_{5, 4364}$=1245.13; $P \leq 0.001$), and compounds ($F_{12, 4364}$=1225.56; $P \leq 0.001$). Furthermore, interactions among location x domestication ($F_{6, 4364}$=22.00; $P \leq 0.001$); domestication x months ($F_{5, 4364}$=23.41; $P \leq 0.001$); and domestication x compounds ($F_{12, 4364}$=6.08; $P \leq 0.001$) were significant according to analysis of variance (Table I).

Table I. Summary ANOVA results for the effects of domestication, location and months in flavonol content and damage index in murtilla, *U. molinae* and consumption in no-choice and choice test by *C. rudis*.

Parameter	Source of variation	*df*[a]	*F*	*P*
Flavonols (µg/g)	Domestication	1,4364	49.90	< 0.001
	Location	6,4364	28.81	< 0.001
	Months	5,4364	1245.13	< 0.001
	Compounds	12,4364	1225.56	< 0.001
	Domestication x Location	6,4364	22.00	< 0.001
	Domestication x Months	5,4364	23.41	< 0.001
	Domestication x Compounds	12,4364	6.08	< 0.001
	Residual	4364		
	Total	5459		
Damage Index	Domestication	1,332	118.37	< 0.001
	Location	6,332	1.94	= 0.074
	Months	5,332	52.21	< 0.001
	Domestication x Location	6,332	2.26	= 0.037
	Domestication x Months	5,332	11.75	< 0.001
	Residual	332		
	Total	419		
Consumed leaf area (%) No-choice test	Domestication	1,117	18.01	< 0.001
	Location	6,117	2.39	= 0.032
	Domestication x Location	6,117	0.52	= 0.78
	Residual	117		
	Total	139		
Consumed leaf area (%) choice test	Domestication	1,117	14.62	< 0.001
	Location	6,117	2.32	= 0.037
	Domestication x Location	6,117	0.66	= 0.68
	Residual	117		
	Total	139		

[a] Degrees of freedom: numerator, error

[b] Estimate population variance

Moreover, daidzein (55.23 μg/g), genistin (114.47 μg/g), myricetin (62.63 μg/g), quercetin (124.35 μg/g) and quercetin 3-D-β-glucoside (85.91 μg/g) were significantly higher in wild plants than the respective cultivated ones (Table IV) according to *t*- test ($P \leq 0.05$). In Figure 1A wild plants showed significantly higher amounts of flavonols and isoflavonoids (78.9 μg/g) than cultivated counterparts (67.8 μg/g) ($P \leq 0.05$). The flavonoid amounts for Pucón (99.7 μg/g), Caburgua (82.8 μg/g), Soloyo (79.9 μg/g) and Manzanal Alto (72.2 μg/g) were significantly higher than in cultivated plants ($P \leq 0.05$). On the other hand, ecotypes 22-1 (72.1 μg/g) and 23-2 (77.8 μg/g) were higher compared with their wild relatives (Fig. 1B). Flavonoid content had a similar temporal variation from March 2014 to January 2015 in both wild and cultivated murtilla plants (Fig. 1C). The highest total level in flavonoids was observed in March for wild (221.6 μg/g) and for cultivated ones (180.8 μg/g) (Fig. 1C). From May to November, the temporal variation was similar through time. Regarding the content of flavonols between wild (89.8 μg/g) and cultivated (75.2 μg/g) plants, significant differences were observed ($P \leq 0.05$). Nevertheless, there were no differences between isoflavonoids in wild or cultivated plants (Fig. 1D).

Table II. Mass spectral data and retention time (Rt) of phenolic compounds identified from *U. molinae* cultivated leaves by HPLC-ESI-MS/MS.

Compounds	Rt (min)	Cultivated		Cultivated			References
		Molecular formula	Molecular weight (g/mol)	Precursor ion $[M+H]^+$	Precursor ion $[M-H]^-$	*m/z* fragment ion	
Quinic acid	3.160	$C_7H_{12}O_6$	192.17	-	191	173, 109, 136, 126, 84, 92, 109, 107, 110, 80, 86, 70	Abu-Reidah et al. (2015)
Quercetin glycoside	3.433	$C_{21}H_{20}O_{12}$	464.38	465	-	303, 429, 285, 277, 235, 218, 176, 238, 259	respect* PT104650
Procyanidin B1-B2	20.938	$C_{30}H_{26}O_{12}$	578.52	579	-	409, 127, 427, 435, 287, 247, 301, 291, 271, 259, 139, 151	reSpect PS045802
(+)- Epicatechin	22.411	$C_{15}H_{14}O_6$	290.26	-	289	161, 245, 203, 205, 109, 136, 122, 151, 121, 162	Stintzing et al. (2004)
Levoglucosan gallate	29.130	$C_{13}H_{14}O_9$	314.07	315	-	153, 193, 171, 125, 109	Abu-Reidah et al. (2015)
Myricetin 3-xyloside	31.523	$C_{20}H_{18}O_{12}$	450.34	451	-	319, 415, 385, 290, 273, 245, 165, 153, 115, 343, 331	reSpect PS093001
Myricetin	31.723	$C_{15}H_{10}O_8$	318.23	319	-	273, 245, 179, 165, 153, 137, 111, 301, 290, 263, 217, 127, 109, 69, 147, 189, 161, 177, 199, 221, 219	Biesaga and Pyrzynska (2009)
Myricitrin	31.859	$C_{21}H_{20}O_{12}$	464.37	-	463	316, 317, 271, 319, 320, 325, 329, 321	Michodjehoun-Mestres et al. (2009)
Quercetin 3-beta-O-galactoside	32.093	C21H20O12	464.09	-	463	300, 271, 319, 321	MassBank PR100948

Quercetin 3-arabinoside	33.123	$C_{20}H_{18}O_{11}$	434.36	-	433	300, 301, 271, 255, 302, 305, 255	reSpect PT209320
Quercetin	33.590	$C_{15}H_{10}O_7$	302.23	303	-	285, 229, 153, 137, 165, 257, 247, 201, 173, 239, 219	Biesaga and Pyrzynska (2009)
Quercitrin	34.110	$C_{21}H_{20}O_{11}$	448.38	-	447	301, 300, 284	Hvattum and Ekeberg (2003)
Kaempferol	34.429	$C_{15}H_{10}O_6$	286.23	287	-	153, 121, 258, 231, 213, 203, 197, 165, 163, 153	Biesaga and Pyrzynska (2009)
Rutin	35.544	$C_{27}H_{30}O_{16}$	610.52	-	609	608, 463, 300	Abu-Reidah et al. (2015)
Caffeic acid	36.609	$C_9H_8O_4$	180.16	181	-	163, 145, 135, 121, 107, 105, 93 79, 91, 77, 81, 67	Parveen et al. (2008)
N.A.	46.917	$C_{22}H_{22}O_{10}$	446.40	447	-	151, 152, 149, 121, 85, 71	reSpect PS086806

N.A.: No assignated

***** Mean information compared with the following spectral databank: Respect for phytochemicals (Spectra.psc.riken.jp).

Table III. Mass spectral data and retention time (Rt) of phenolic compounds identified from *U. molinae* wild leaves by HPLC-ESI-MS/MS.

Compounds	Rt (min)	Molecular formula	Molecular weight (g/mol)	Wild			References
				Precursor ion		*m/z* fragment ion	
				$[M+H]^+$	$[M-H]^-$		
(+)- Epicatechin	21.509	$C_{15}H_{14}O_6$	290.26	207	-	139, 123, 147,161,119	reSpect* PS045604
Daidzin	26.167	$C_{21}H_{20}O_9$	416.38	-	415	414	reSpect PS043807
Kaempferol	26.311	$C_{15}H_{10}O_6$	286.23	287	-	167	reSpect PT104020
Levoglucosan gallate	28.740	$C_{13}H_{14}O_9$	314	315	-	153	Abu-Reidah et al. (2015)
Myricetin galloyl-hexoside	28.910	$C_{29}H_{28}O_{16}$	632	-	631	317	Abu-Reidah et al. (2015)
Gossypin	30.447	$C_{21}H_{20}O_{13}$	480.38	481	-	319	reSpect PT108480
Myricetin 3-xyloside	31.225	$C_{20}H_{18}O_{12}$	450.34	-	449	316	reSpect PS093008
Myricitrin	31.956	$C_{21}H_{20}O_{12}$	464.37	-	463	464, 316, 317	Hvattum and Ekeberg (2003)
Ellagic acid	32.187	$C_{14}H_6O_8$	302.19	303	-	257, 229, 201, 173	Abu-Reidah et al. (2015)
Quercetin glycoside	32.347	$C_{21}H_{20}O_{12}$	464.38	465	-	303	reSpect PT104650
Quercetin-3-*D*-xyloside	32.716	$C_{20}H_{18}O_{11}$	434.35	435	-	303	reSpect PT111670
Luteolin	33.512	$C_{15}H_{10}O_6$	286.24	287	-	153	Biesaga and Pyrzynska (2009)
Quercetin	33.802	$C_{15}H_{10}O_7$	302.23	303	-	285, 257, 247, 229, 195, 165, 153	Biesaga and Pyrzynska (2009)
Myricetin	33.911	$C_{15}H_{10}O_8$	318.23	319		245, 273, 153,	Biesaga and

						165	Pyrzynska (2009)
Hesperetin	33.912	$C_{16}H_{14}O_6$	302.07	303	-	153, 137	reSpect PS078003
Quercitrin	33.969	$C_{21}H_{20}O_{11}$	448.1	-	447	448, 300, 301	Sanchez-Rabaneda et al. (2003)
Linarin	50.593	$C_{28}H_{32}O_{14}$	592.17	594	-	447	reSpect PS085202

***** Mean information compared with the following spectral databank: Respect for phytochemicals (Spectra.psc.riken.jp).

Table IV. Flavonoid concentrations in wild and cultivated murtilla plants. Different letters in columns indicate significant differences according to ANOVA and post-hoc Tukey test ($P \leq 0.05$). ***** Indicates significant difference (*t* -test, $P \leq 0.05$) between cultivated and wild plants per compound.

Compounds	**Flavonoid group**	**Retention time (m)**	**Cultivated plants (µg/g)**	**Wild plants (µg/g)**
Rutin	Flavonol	11.024	138.58 ± 7.81 b	175.60 ± 4.07 b
Daidzin	Isoflavonoid	11.406	335.59 ± 8.79 a	374.35 ± 23.01 a
Genistin	Isoflavonoid	12.010	92.31 ± 3.77 bcd	114.47 ± 10.64 cd *
Myricetin	Flavonol	13.406	48.89 ± 4.12 def	62.63 ± 4.30 def *
Ononin	Isoflavonoid	13.924	27.80 ± 1.39 ef	26.36 ± 2.63 fg
Daidzein	Isoflavonoid	14.417	43.07 ± 2.37 def	55.23 ± 1.77 efg *
Quercetin 3-O-*β*-glucoside	Flavonol	14.627	75.99 ± 1.22 cde	85.91 ± 3.55 cde *
Quercetin	Flavonol	14.797	112.24 ± 3.38 bc	124.35 ± 5.93 bc *
Sissotrin	Isoflavonoid	15.384	0.49 ± 0.09 f	0.46 ± 0.10 g
Genistein	Isoflavonoid	15.568	1.10 ± 0.09 f	1.30 ± 0.01 g
Kaempferol	Flavonol	16.752	0.55 ± 0.01 f	0.74 ± 0.01 g
Formononetin	Isoflavonoid	16.937	2.66 ± 0.20 f	3.80 ± 0.05 g
Biochanin A	Isoflavonoid	18.461	2.57 ± 0.02 f	1.05 ± 0.01 g

Insect survey and Biodiversity Indexes. A total of 65 insects were collected from wild (14; 21.53%) and cultivated (51; 78.46%) murtilla plants (Table V; Table VI, Fig 2A). Five different insect orders for cultivated plants such as Coleoptera, Lepidoptera, Orthoptera, Homoptera and Diptera were determined, whereas four orders were found for wild plants (Coleoptera, Lepidoptera, Orthoptera, and Diptera). The high level of insect was found for ecotypes 08-1 (16) and 14.4 (12), and for wild plants Pucón was the location with the highest number of insects (7) compared with the other locations (Fig 2B). Moreover, the temporal insect variation showed a high number for wild plants (35) compared with ecotypes (6) in January (Fig. 2E). Simpson (10.97), Shannon (3.75) and Margalef (4.32) indexes were higher in cultivated plants (Table VI).

Damage Index Evaluation. Domestication effect was significant ($F_{1, 332}$ = 118.37; $P \leq 0.001$) on damage index according to ANOVA. These data showed that domestication effect is involved on damage on *U. molinae* plants, in contrast to location. Moreover, the interaction between domestication x locations showed a significant difference ($F_{6, 332}$ = 2.26; $P = 0.0377$) on damage index. The effect domestication x months was significant ($F_{5, 332}$=11.75; $P \leq 0.001$) on damage index in the year (Table I). Figure 2C shows that cultivated plants were more affected by herbivores than their wild parents ($P < 0.05$), according to a *t*-test with a damage index of 1.72 ± 0.31 vs 0.47 ± 0.09, respectively. Moreover, all seven cultivated ecotypes were significant higher than the wild plants ($P < 0.05$) according to *t*-test (Fig 2D). Temporal variation through the year also showed a significant difference ($F_{5, 332}$= 52.21; $P \leq 0.001$) among the months on the damage index (Fig. 2F). Finally, temporal variation on damage index reached their maximum levels in November for both cultivated (4.27 ± 0.09) and wild (1.41 ± 0.12) plants (Fig. 2F).

Table V. Order, family and species of insects found on wild and cultivated *U.molinae* plants, and their geographical distribution.

Order	Family	Species	Location	Ecotype
Coleoptera	Meloidae	*Epicauta pilme* (Molina)	Pucón	14-4
	Chrysomelidae	ND		18-1, 23-2
	Elateridae	ND	Queule	19-1
	Carabidae	ND	Pucón	22-1
	Coccinellidae	*Eriopis connexa* (Germar)	Pucón	23-2
	Cerambycidae	*Callisphyris macropus*		23-2
		(Newman)	Pucón	
	Scarabaeidae	ND		08-1
Lepidoptera	Hesperiidae	*Hylephila fasciolata*	Caburgua	
		(Blanchard)		
	Noctuidae	ND		14-4
	Nymphalidae	*Vanessa carye* (Hubner)		08-1
Diptera	Muscidae	ND		14-4
	Tipulidae	ND	Pucón	14-4
Orthoptera	Gryllidae	*Acheta assimilis* (Fabricius)	Soloyo, Pucón	08-1, 12-1
Homoptera	Cicadellidae	*Carelmapu ramosi*	Caburgua	14-4
		(Linnovuori)		

ND: No determinated

Table VI. Biodiversity parameters evaluated in both wild and cultivated plants of *U. molinae* Turcz from March 2014 to January 2015.

Parameters	Cultivated	Wild
Species Richness	18	10
Number of individuals	51	14
Relative Abundance (%)	78.46	21.53
Simpson Index	10.97	7.53
Margalef Index	4.32	3.41
Shannon Index	3.75	3.12

No-Choice Test. ANOVA test showed that domestication ($F_{1, 117} = 18.01$; $P \leq 0.001$) and location ($F_{6, 117} = 2.39$; $P = 0.032$) had, independently, a significant effect on *C. rudis* preference. Nevertheless, domestication x location was not significant on *C. rudis* preference ($F_{6, 117} = 0.52$; $P = 0.789$) according to ANOVA test (Table I). Figure 3A indicate that cultivated plants were more consumed by *C. rudis* 46.17% compared with their wild relatives (21.12%). Furthermore, ecotypes 12-1, 22-1, 18-1 and 19-1 were more preferred by *C. rudis* than their wild plants with 62%, 37%, 40% and 31% respectively (Fig 3B).

Choice Test. Similar to no-choice test, domestication and location effects were significant ($F_{1, 117} = 14.62$; $P \leq 0.001$ and $F_{6, 117} = 2.32$; $P = 0.037$ respectively) on *C. rudis* preference (ANOVA). Nevertheless, domestication x location was not significant on preference of *C. rudis* ($F_{6, 117} = 0.66$; $P = 0.683$) as is shown in Table I. Similarly to no choice assays, Figure 3C shows that cultivated plants (23.4%) were more preferred by *C. rudis* than their wild plants (5.7%). Ecotypes 08-1, 22-1, and 23-2 were higher than their wild plants with 15%, 24% and 40.7% respectively (Fig. 3D).

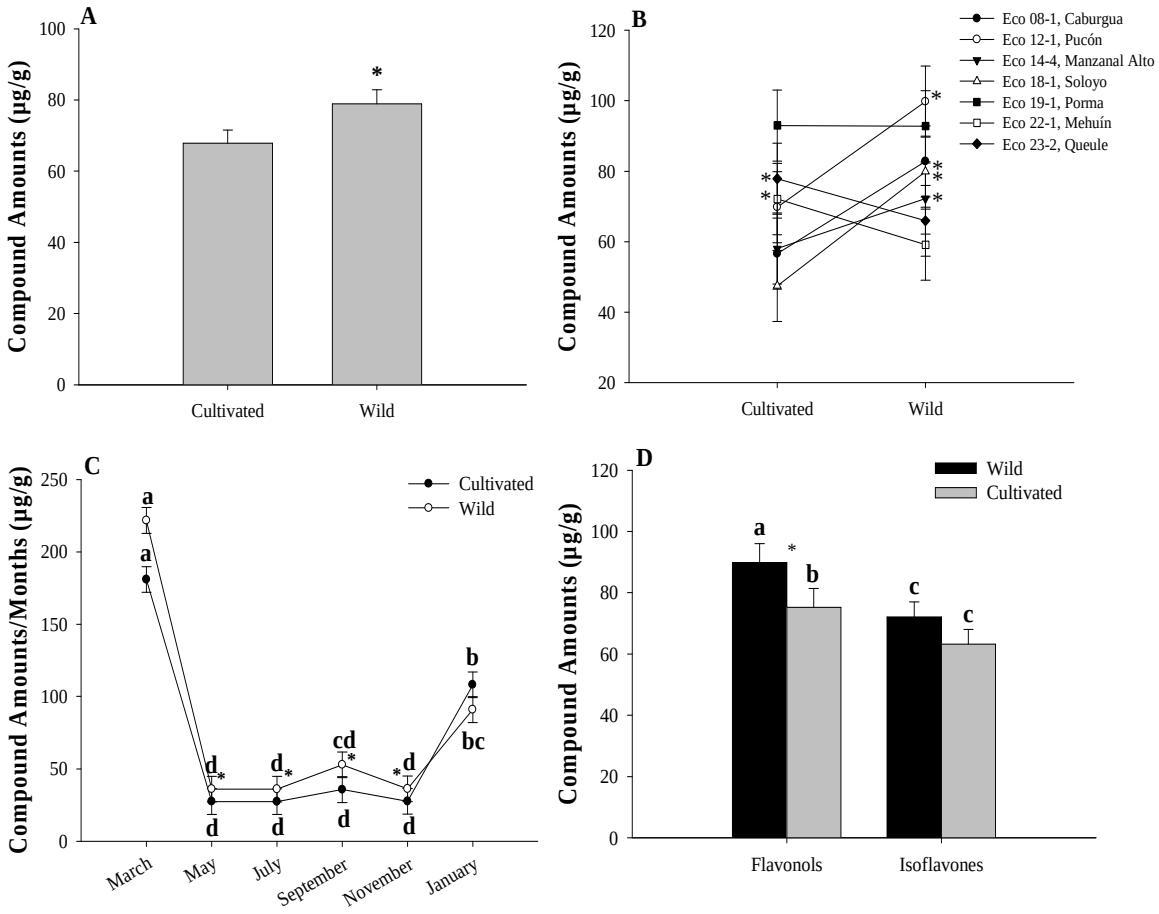


Figure 1. Comparative amounts (mean ± SE) of flavonols and isoflavones between wild and cultivated murtilla, *U. molinae* (A), amounts of flavonols and isoflavones based on ecotype, geographical area (B), temporal variation in flavonol and isoflavones concentration in leaves of wild and cultivated murtilla plants from March 2014 to January 2015 (C), and amounts of flavonols and isoflavones between wild and cultivated murtilla plants (D). ***** = significant difference (*t* -test, $P \leq 0.05$). Different letters indicate significant differences (Tukey test, $P \leq 0.05$).

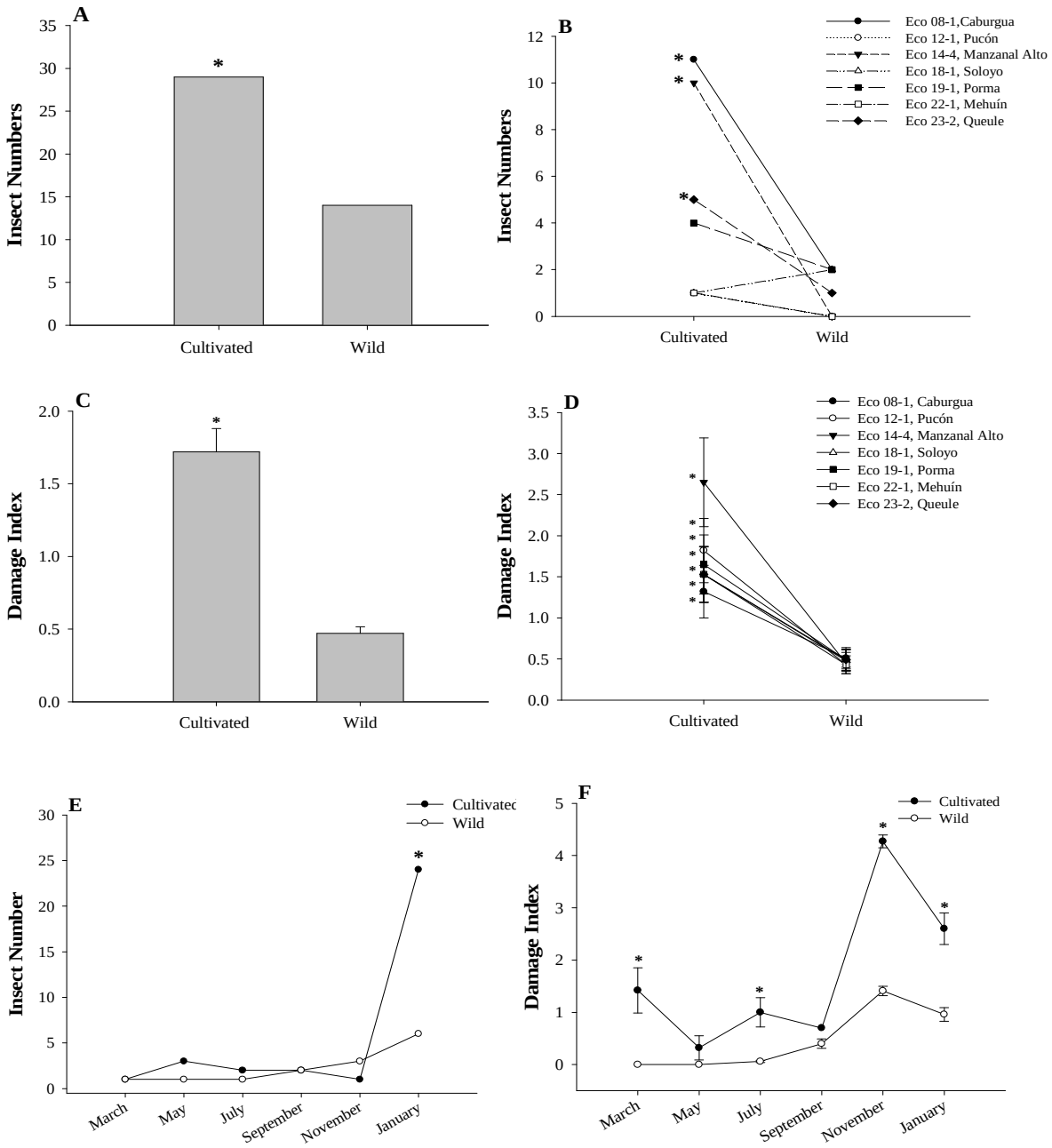


Figure 2. Comparative amounts (mean) of insects between wild and cultivated murtilla, *U. molinae* (A), amounts of insects based on ecotype, geographical area (B), Comparative amounts (mean ± SE) of damage index between wild and cultivated murtilla, *U. molinae* (C), amounts of insects based on ecotype, geographical area (D), temporal variation in insects of wild and cultivated murtilla plants from March 2014 to January 2015 (E), and levels of damage index between wild and cultivated murtilla plants (F). ***** = significant difference (*t* -test, $P \leq 0.05$). Different letters indicate significant differences (Tukey test, $P \leq 0.05$).

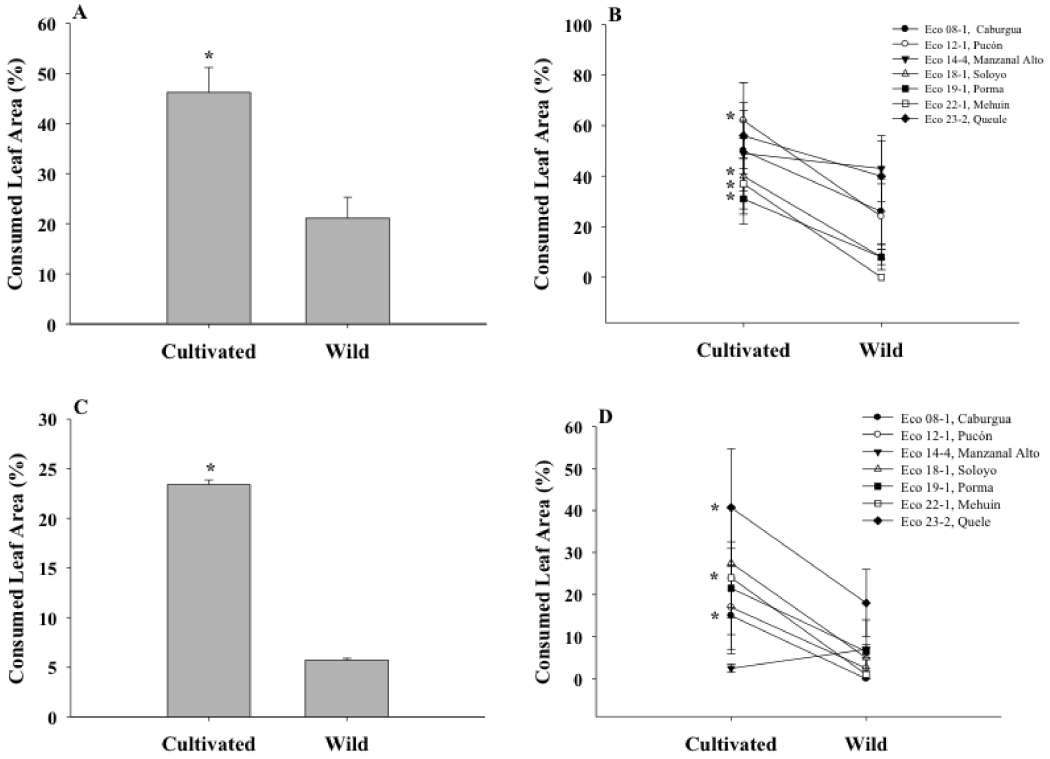


Figure 3. Results from no-choice bioassays (A-B): *Chilesia rudis* food consumption (A) when fed wild and cultivated murtilla, *Ugni molinae*, leaves. And, food consumption (B) based on ecotype, geographical area. Results from choice bioassays (C-D): *C. rudis* larval food consumption (C) when fed wild and cultivated *U. molinae*, leaves. Food consumption (D) based on ecotype, geographical area. ***** = significant difference (*t*-student test, $P \leq 0.05$).

4.4 DISCUSSION

Crop domestication can affect plant defenses and their resistance against herbivores in unpredictable ways (Meyer et al. 2012). Moreover, domestication and breeding for high-yielding crops are expected to reduce chemical defenses in plants because of potential trade-offs between growth/reproduction and defense (Wink 1988, Herms and Mattson 1992, Rodriguez-Saona et al. 2011, Altesor et al. 2014). In this context, domestication in *U. molinae* has focused mainly on selection of traits associated with increased productivity,

such as bigger plants, more fruit, and larger fruit size (Seguel and Torralbo 2004). We could expect that cultivated *U. molinae* might allocate resources towards defense, growth, and reproduction in a different way to wild plants. Indeed, despite its short history of domestication (< 20 years), Chacón-Fuentes et al. (2015) showed that domestication in *U. molinae* has led to decreases of flavonol content, an important class of defensive secondary metabolites in plants present in this species. However, that work did not consider the possible role of environmental factors in the comparison of wild locations (seven different areas) with cultivated plants (a unique area). In the current research and in order to determine the flavonoid variations minimizing environmental effects, we carried out a common garden experiment. Overall, common garden experiments have been used as a tool for analyzing diversity, assemblages of insects, chemical defenses and yield comparing different plant domestication degrees as was reported by Rosenthal and Dirzo (1997). In this sense, common garden experiments offer a way to standardize biotic and abiotic factor for wild and cultivated plants. Comparative analysis performed here showed that the same environmental factors contrasted with the previous report from Chacón-Fuentes et al. (2015). Moreover, in this research all seven wild locations presented domestication effects on cultivated plants, contrasting with the previous report where four wild localities and two ecotypes were affected by domestication process. In this work, the concentration of both rutin (138-175 μg/g) and kaempferol (0.55-0.74 μg/g) were no different between cultivated and wild plants (Table IV), respectively. Spite of temporal flavonoid variation observed in cultivated and wild plants established in a common garden was different from that reported by Chacón-Fuentes et al. (2015), wild plants showed higher amounts of flavonoids than cultivated ones. The contrasting environmental factors involved in the investigation developed by Chacón-Fuentes et al. (2015), such as the comparison between different wild

locations with a unique cultivated area, could influence the flavonoids production in the time. For that reason, in the present research a common garden design experiment was established for avoiding biotic or abiotic pressure differences that could affect the flavonoid production. Overall, both studies were agreed with our hypothesis that domestication has reduced chemical defenses in *U. molinae*. In fact, amounts of flavonoids were lower in cultivated *U. molinae* than their wild parents. Therefore, if cultivated plants are less defended (Chen et al. 2015) we predicted that domestication in *U. molinae* would make plants more susceptible to herbivores foraging. In fact, some flavonoids found in *U. molinae* have been implicated in resistance mechanism against herbivores in other plant systems (Chen et al. 2015). Todd et al. (1971) showed that quercetin, a constituent of barley leaves, was toxic to greenbugs, *Schizaphis graminum* (Rondani) (Hemiptera: Aphididae) interfering with reproduction, weight gain and survival at doses of 3.75 X 10^{-4} M. However, the inverse mechanisms have been observed with this family of compounds. For instance, Diaz et al. (2010) reported that quercetin acted as a phagostimulant for beetle *Epilachna paenulata* (Germar) (Coleoptera: Coccinellidae). Moreover, Lin and Mullin (1999) reported stimulant-feeding activity elicited by quercetin 3-D-β glucoside at doses of 9.7 μmol/g from western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) (31.3% of consumption). Wink (1988), Nielsen et al. (1998) and Bernays (1991) have also reported stimulation of feeding and ovipositional activity by kaempferol, rutin, and glycoside compounds for other herbivores. These results suggest that domesticated plant could express or inhibit the production of a particular compound resulting plants more attacked by insects. In addition to the flavonols reported in murtilla by Chacón-Fuentes et al. (2015), we detected for the first time the presence of eight

isoflavonoids compounds in *U. molinae* leaves trough HPLC analysis. In general, berries do not have higher amounts of these compounds (Mazur 1998). However, according to studies of Lapcik (2005, 2007), it is possible to find isoflavonoids in Myrtaceas. Therefore, the presence of these compounds in *U. molinae* –Myrtaceae- is according to the chemotaxonomy reported for this species (Lapcik 2005, 2007). Furthermore, the presence of these kinds of compounds is important due to their use as phytoestrogens and for coping to insect pests (Mackova et al. 2006).

The results reported here have important implications for the cultivation of *U. molinae*, a crop that has been highly valued due to the antioxidant activity of flavonols in its fruit (Rubilar et al. 2011, Alfaro et al. 2013). In our study, we showed that the levels of flavonols in the leaves were reduced due to domestication process. However, if the focus of domestication in *U. molinae* shifts from higher productivity, i.e., increases in yield, to higher levels of antioxidant compounds, these berries could become more susceptible to certain native herbivores such as *C. rudis*. Altogether, the reported findings provide first insights on the impact of domestication on plant defenses and resistance against herbivores in *U. molinae*. It may also guide to future breeding programs by highlighting the potential risks of breeding for high flavonol and isoflavonoids content on susceptibility of fruit against native herbivores. Furthermore, we detected changes in community and numbers of the insects, biodiversity and damage indexes, showing cultivated plants a significant insect numbers and damage by herbivores in leaves in relation to their wild counterpart. It is suggesting that domestication has elicited a decrease of defense secondary metabolites such as flavonols and isoflavonoids in murtilla plants. For example, Chen and Bernal (2011) reported that all herbivores have a response to domestication effects, finding more insects in cultivated plants than cultivate ones. However, those authors did not found differences in

the total of arthropods between wild and cultivated rice species, suggesting that domestication has altered the arthropod capacity for controlling herbivores. Moreover, Chen et al. (2013) reported nearly 50% lower taxa in cultivated rice plants than wild plants, 173 unique taxa were found in wild plants, whereas only 23 were found in cultivated plants establishing that changes in the arthropod community and structure are associated with plant domestication. These data agree with our results reporting 51 insects for cultivated plants and only 14 for wild *U. molinae* plants. In addition, our results indicated that there were 6 unique insect families in cultivated plants –Cicadellidae, Chrysomelidae, Cerambycidae, Noctuidae, Muscidae and Nymphalidae, and there was only one unique insect family for wild plants –Scarabaeidae- (Table V). Besides, Margalef index for cultivated species was higher (4.32) than other reports for cultivated ecosystems such as barley crops whose index ranged from 0 to 0.96 (Abay et al. 2009). Margalef index for wild *U. molinae* plants is lower than Lexerod and Eid (2006) where the range varied from 4.09 to 8.47, showing that cultivated plants have a higher insect abundance than wild plants. Moreover, Simpson (10.97) and Shannon (3.75) indexes were higher in cultivated plants, indicating a major number of insect assemblages that could be related to a decrease of chemical defense –flavonoids- in cultivated plants. For a better understanding of insect assemblages on murtilla plants subjected to domestication process is necessary a detailed study of each one of the insect reported. Finally, *C. rudis* showed to be a proper biological indicator of the negative effect of domestication on chemical defense in murtilla. Moreover, we observed that the feeding behavior of *C. rudis* was increased in murtilla cultivated ecotypes. However, Chacón-Fuentes et al. (2015) showed that the *C. rudis* preferred wild plants than cultivated in no-choice (80%) and choice test (45%) bioassays. Nevertheless, these results could be explained due to the effect of environmental variation in that study.

In the present study, the environmental factors were avoided because the common garden experimental design developed, where cultivated and their respective wild ones were subjected to the same environmental conditions. Is this a product of flavonoids content decreasing? To state this is risky, but our results strongly suggest that the answer goes in that direction.

In conclusion, although domestication and selective breeding have had great positive influences on food availability through increased crop yield and quality (Wink 1988), it has often come at a cost for resistance against herbivores (Chen et al. 2015), which may lead to increased use of pesticides because the decreasing of secondary metabolites with defensive role. Overall, domestication process effects are reported in this study. Flavonoid levels were significantly higher in wild plants than the respective cultivated. We detected changes in community and numbers of the insect assemblages, biodiversity index and also in damage index that could suggest that the domestication has elicited a decrease of defense secondary metabolites such as flavonols and isoflavonoids in murtilla plants. Finally, *C. rudis* showed to be a proper biological indicator of the negative domestication effect on murtilla chemical defense. In conclusion, an experimental design based in a common garden experiment was a useful tool for the standardization of environmental pressures that are exposed plants in an incipient domestication stage versus their wild counterparts located in several areas.

CHAPTER V:

Chemical defense recovery is affected by ecotype and locality on a reciprocal transplant experiment

5.1 INTRODUCTION

Murtilla, *Ugni molinae* Turcz (Myrtaceae) is an endemic and polymorphic shrub from Chile distributed from Región del Maule to Región del General Carlos Ibáñez del Campo (Seguel and Torralbo 2004, Rodriguez et al. 2015). In Chile, there is a strong economic interest in the production of *U. molinae* due to the presence of antioxidant compounds, specifically flavonoids in their leaves, stems and fruits (Chacón-Fuentes et al. 2015). Briefly, these plants were originally collected from 100 localities in southern Chile. *U. molinae* cuttings were first grown in greenhouses for 10 years and then transplanted to the field (INIA Experimental Station-Tranapuente in the Región de La Araucanía (south of Chile, 38° 450 S, 73° 210 W) beginning a domestication process (Seguel et al. 2000). This experimental field is located near Puerto Saavedra, a coastal area near the Pacific Ocean and its weather is characterized as being a moderate oceanic climate with marine influence (Scheuermann et al. 2008). Besides, researches at this institution are developing breeding programs among the different phenotypes and ecotypes from *U. molinae*. Currently there are two different varieties available that have been patented in 2008 and 2010 (USA: 21, 273P3), the former is named South Pearl INIA (Seguel and Montengro 2008) and the latter is named Red Pearl INIA (Seguel and Montenegro 2010). *U. molinae* has an average production of 1 Kg/plant of fruits (1.1 Kg/plant for Red Pearl INIA and 0.9 Kg/plant for South Pearl INIA), from 1.0 cm diameter for Red Pearl INIA to 1.1 for South Pearl INIA, fruit weight from 0.8 to 0.9 g for Red Pearl INIA and South Pearl INIA respectively, ºbrix degrees and ORAC were higher in Red Pearl INIA varieties compared with South Pearl INIA (15 ºbrix and 11.811 ORAC vs 14 ºbrix and 9.734) in relation to reports by Seguel et al. (2000).

Plant domestication is a process in which plants may increase their productive traits according to human needs detriment of chemical defense (Herms and Mattson 1992, Gross and Olsen 2010, Meyer and Purugganan et al. 2013, Milla et al. 2015). This process begins from a wild population, where plants are selected at first instance due to their useful traits generating a selection of one above others. However, the potential gene flow in a wild conspecific population could affect the expression of some selected plant characteristics (Wyngaard 1998). Plant domestication is directly related to defenses in plants through the secondary metabolite production (Rosenthal and Dirzo 1997, Benrey et al. 1998, Rodriguez-Saona et al. 2011, Turcotte et al. 2014). Particularly flavonoids in *U. molinae* plants have been evaluated for showing changes in flavonol concentrations reaching significant differences from 450 mg/L in cultivated to 550 mg/L in wild murtilla plants (Chacón-Fuentes et al. 2015). Moreover, same authors reported that in different wild locations the flavonol concentrations were higher than cultivated ones. Furthermore, the study of the temporal variation of these compounds through the year indicated that during December, January, February, June, August and October its concentration was higher in wild than cultivated plants. The distribution of these compounds trough the plant indicated that in leaves the total flavonols in wild murtilla plants (350 mg/L) was higher than in cultivated plants (250 mg/L). Finally, these authors found 4 major flavonol compounds in the extract of *U. molinae* leaves, such as rutin (231.00 mg/L), quercetin glucoside (316.00 mg/L), quercetin (2.34 mg/L) and kaempferol (1.01 mg/L) in all the cases the concentrations were higher in wild plants than in cultivated ones. The *U. molinae* is a plant that grows under a wide range of natural conditions and has distinguishable forms of associating with different habitats (Hiesey 1940). The examination of local and geographically based life history variation is an approach to examine how different

selective forces may have molded life histories (Wyngaard 1998). Reciprocal transplant experiments are an effective design for analyzing plant populations revealing that environmental influences on plants could be large or small relative to genetic (Schoen et al. 1986). The present study is built upon earlier studies in domestication (Chacón-Fuentes et al. 2015). Here, we carried out a reciprocal transplant experiment to compare the level of flavonoids in cultivated plants transferred to wild areas, and wild plants transferred to a cultivated sites and how it may have molded life histories. If each population is adapted to the environment in which it has presumably evolved, any differences between the resident and transferred population may reflect adaptations to selective forces in *U. molinae* and moreover could be an indicator of the chemical defenses recovery against a new environmental stresses.

5.2. MATERIAL AND METHODS

5.2.1 Collection Zones. Cuttings from cultivated plants at the Experimental Station-Tranapuente of the Instituto de Investigaciones Agropecuarias (INIA) in the Región de La Araucanía (south of Chile, 38° 45`S, 73° 21`W) were obtained from two ecotypes (22-1 and 23-2) of *U. molinae*. Cuttings of the respective wild plants were sampled from the original geographical area where their cultivated counterparts were collected originally around 20 years ago. The following sampling areas were used for the wild cutting collection: Mehuín (39^{o}26` S, 73$^{o}$12`W) and Queule (39^{o}23` S, 73$^{o}$12`W) located in the Región de Los Ríos, Chile. All the cuttings were grown in greenhouse in INIA for one year and then established in a common garden in INIa-Tranapuente for one year until their use in the reciprocal transplant experiments.

5.2.2 Reciprocal Transplant Experiment. After a year of climate in a greenhouse and one more year in a common garden, cuttings obtained from cultivated plants were transplanted to the repective wild areas (Mehuín and Queule), and at the same way cuttings obtained from wild plants were transplated in the Experimental Station INIA-Tranapuente. After one year, leaves of both wild and cultivated transplanted plants were collected for flavonoids analysis

5.2.3 Plant Material. Five plants were sampled for each cultivated and wild plants (*N* = 20 plants). Leaves were taken from all four cardinal directions and at different heights of the plant according to the methodology proposed by Chacón-Fuentes et al. (2015). Then, samples were placed in a cooler and transported to the Laboratorio de Química Ecológica at Universidad de La Frontera, Temuco, Chile. Samples were stored at -20°C until their chemical analyses.

5.2.4 U. molinae Leaf Extracts. Leaves were rapidly frozen in liquid nitrogen for 5 s (Mikulic-Petkovsek et al. 2012), and then, milled in a grinder. Later, 0.5 g from milled leaves was placed in a flask and 25 mL of methanol HPLC grade (Sigma-Aldrich, St. Louis, MO) was added to the samples (50% v/v in water). These flasks were placed in a magnetic stirrer for 20 min at 30 ^{o}C and 170 rpm. After this time, the samples were filtered in darkness through a Whatman n^{o}1 filter paper (Whatman International Ltd., Maidstone, U.K.). The filtrate was lyophilized for 8 h. Finally, each sample was suspended in 10 mL of methanol and left for 5 m in a Branson 3510 sonicator. Samples were stored at -20^{o}C in amber flasks (25 mL) until their use for High Performance Liquid Chromatography (HPLC) analysis.

5.2.5 Quantification by HPLC. Methanolic extracts obtained from both wild and cultivated leaves were filtered through a 0.22 µm membrane. 20 µL of sample were

injected into a Shimadzu HPLC equipped with a C-18 column (150 x 4.6 mm I.D.; particle size 5mm) maintained at 40 °C. The analysis was performed using a linear solvent gradient consisting of 1% formic acid (A) and acetonitrile (B) as follows: 0-5 m, 5% A/ 95% B; 5-10 m, 30% A/70% B; 10-20 m, 55% A/45% B; 20-30 m, 5% A/95% B at a 1 mL/min flow rate (Chacón-Fuentes et al. 2015). Flavonoids were monitored at 260 nm; UV spectra from 190 at 800 nm were used for peak characterization. The identification of flavonoids was based on peak retention time in comparison with a standard. To construct calibration curves for flavonoids, standard solutions were dissolved in methanol (Sigma-Aldrich, St. Louis, MO) at 1000 mg/L. The stock solutions of each standard were used to prepare a serial concentration between 0.05 and 500 mg/L.

5.2.6 Statistical analysis. The statistical software Statistix 10 (Tallahassee, Florida, United States of America) was used to analyze the flavonoid levels. Total flavonoid content was analyzed using an *t*-test analysis, and an analysis of variance (ANOVA) were performed for analyses differences between ecotypes and localities, Fischer test was used for comparisons among groups. Values of $P \leq 0.05$ were considered as significant. Data were natural-log transformed to meet the assumptions of normality and homogeneity of variance. Results are expressed as means and their corresponding standard errors.

5.3. RESULTS

The total amount of flavonoids in wild plants (140 μg/g) was higher than cultivated (82 μg/g) (Fig. 1) according to ANOVA test ($P < 0.05$). This difference is mainly supported by the ecotype 22-1 (Fig. 2A) ($F_{3, 252} = 2.68$ $P = 0.0473$). Cultivated ecotypes reached total flavonoid concentration of 71 μg/g and 94 μg/g for ecotypes 22-1 and 23-2 respectively. However, for wild plants the total flavonoid concentration reached 169 and 111 μg/g

respectively. Surprisingly, the total amount of flavonoid in cultivated plants increased when they were transplanted to the respective wild origin area (Fig. 1) and in wild plants decreased the amount of flavonoid when they were moved to a cultivated area (Fig. 1) (ANOVA; $P < 0.05$). Cultivated plants exposed to wild environments reached higher flavonoid concentrations (120 μg/g) in relation with wild plants exposed to a cultivated managements (42 μg/g) as is showed in the Figure 1. Finally, in Figure 2B two ecotypes and localities were analyzed in relation to the total flavonoid concentrations showing significative differences according to ANOVA test ($F_{3,256} = 5.20$, $P < 0.001$), Table I. Original cultivated plants transfer to wild location reached concentrations of 108 μg/g and 132 μg/g for ecotype 22-1 and 23-2 respectively. Wild plants transfer to cultivated systems reached values of 33 and 50 μg/g for Mehuín and Queule respectively.

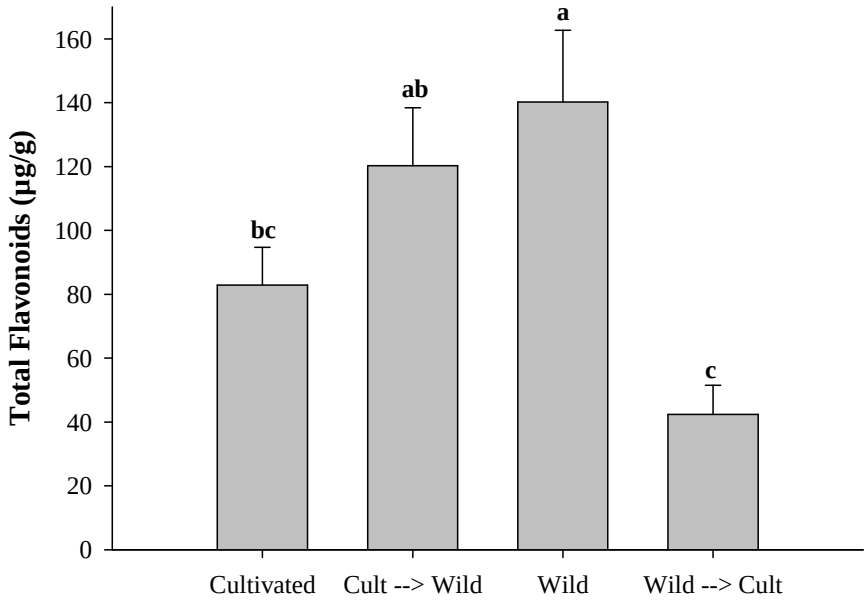


Figure 1. Comparative amounts (mean ± SE) of flavonoids in cultivated and wild plants, and cultivated plants exposed to wild environments (Cult → Wild) and wild plants exposed to a cultivated system (Wild → Cult). Different letters show a significant difference according to ANOVA test and Tukey post-hoc comparison ($P < 0.05$).

Additionally, Table I showed a significative differences according to ANOVA test between compounds and differents ecotypes and/or wild localities ($F_{103, 416} = 108.11$, $P < 0.001$). The major compounds found in leaves of wild *U. molinae* plants were rutin (786 μg/g) and daidzin (732 μg/g) for Mehuín in wild plants and in the reciprocal transplant experiment the high concentration was reached for daidzin (718 μg/g) in ecotype 23-2 transplanted to Queule as is shown is Table II. Moreover, there was an increase in daidzin amounts from ecotype 22-1 (91.05 μg/g) when it was exposed to wild environments (648 μg/g), the same compound showed a decreased from 424.34 μg/g to 72.27 μg/g when it was transplant from a wild environment to cultivated one for Queule plants. For genistin, ecotypes 22-1 (138.74 μg/g) and 23-2 (57.20 μg/g) showed an increase when they were exposed to a wild environment (327.31 μg/g and 345.01 μg/g, respectively). In contrast, this compound presented a decrease when it was exposed to cultivated management in both localities Mehuín and Queule from 24.70 μg/g and 46.62 μg/g to 4.94 μg/g and 7.79 μg/g. Furthemore, myricetin showed the same trend Mehuín and Queule reached values of 631 μg/g and 445.12 μg/g but, when they were exposed to a management system their values decreased to 125.34 μg/g and 87.90 μg/g respectively. Additionally, ecotype 23-2 presented an increase from 7.44 μg/g to 190.04 μg/g when it was transplanted in a wild location. Finally, quercetin and sissotrin showed a decrease in ecotypes 22-1 and 23-2 when they were exposed to their respective wild environments from 128.32 μg/g and 122.34 μg/g to 0.64 μg/g and 0.38 μg/g for quercetin respectively and, from 351.02 μg/g and 562.67 μg/g to 225.62 μg/g and 422.05 μg/g respectively for sissotrin (Table II).

5.4. DISCUSSION

Plant domestication is a co-evolutionary process in which is affected the flavonoid concentrations according to the domestication degree (Gepts 2014, Chacón-Fuentes et al. 2015). Therefore, cultivated plants could be losing chemical defenses –flavonoids- in comparison to wild plants. In this sense, the total phenolic compounds can be used as indicator of chemical defense capacity according to Gong and Zhang (2014). For example, Chacón-Fuentes et al. (2015) reported in a recent study that domestication in *U. molinae* reduced the concentration of rutin (231 vs 190 mg/L), quercetin (2.34 vs 1.93 mg/L) and kaempferol (1.01 vs 0.87 mg/L) from wild plants to cultivated ones. In this sense, domestication affects flavonoid concentrations. The most important ecological interactions are between plants and herbivores (Gong and Zhan 2014). Plant domestication is a co-evolutionary process of selection for adaptation to environmental factors (Gepts 2014). Hence, environmental changes, for example the change from cultivated to wild systems or conversely, can alter the chemical defense in a sense to improve the interaction to the environment and particularly could be triggering a variation in the secondary metabolites, in our case the amount of flavonoids. These compounds have been studied due to their deterrent activity in some insect pests (Simmonds 2001). For instance, Salunke et al. (2005) reported that flavonols –rutin, myricetin, quercetin, fisetin and quercitrin- were toxic to adults and eggs of *Callosobruchus chinensis* (L) (Coleoptera: Bruchidae) at doses from 0.1 mg/mL to 10 mg/mL. Moreover, Onyilagha et al. (2012), tested the effects of flavonoids from *Camelina sativa*, on flea beetle, *Phyllotreta cruciferae* (Coleoptera: Chrysomelidae), glycosides from quercetin were deterrent at several doses.

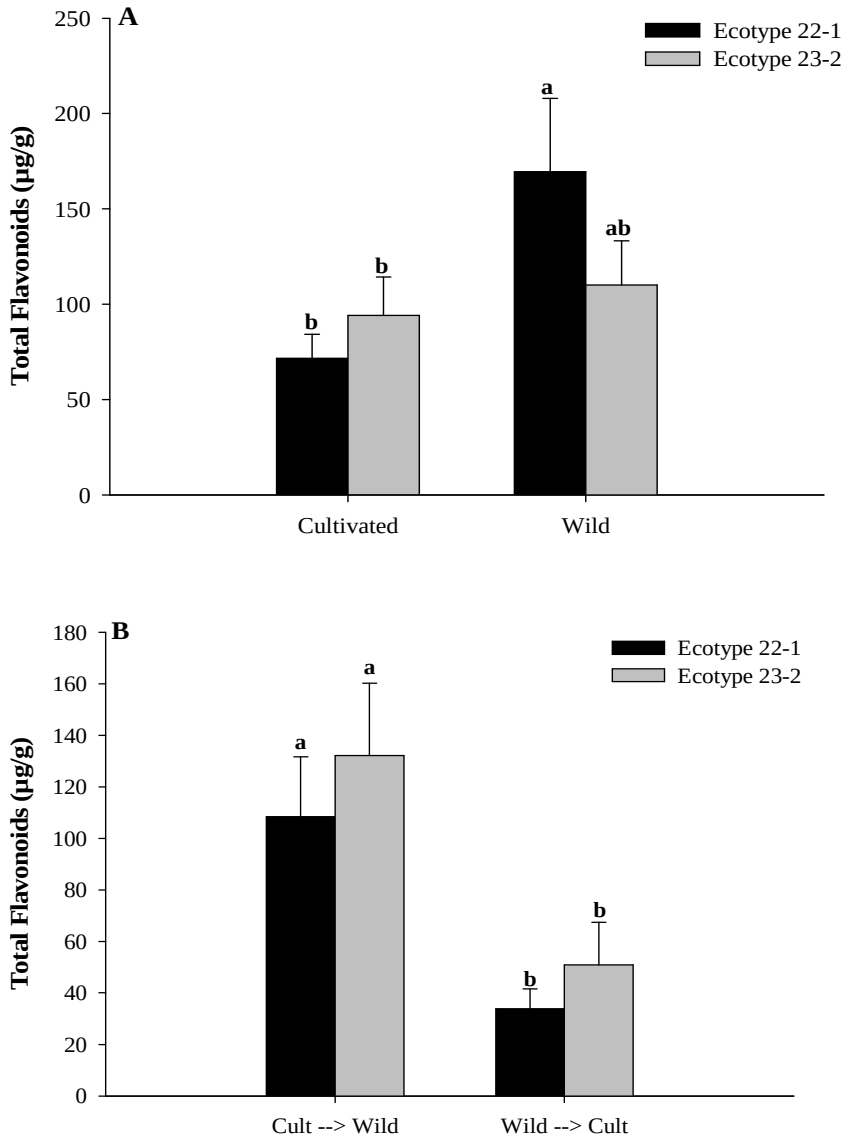


Figure 2. Comparative amounts (mean ± SE) of flavonoids based on ecotype and geographical area (A). Amounts of flavonoids between wild and cultivated murtilla plants (B), in a reciprocal transplant experiment. Different letters indicate significant differences (ANOVA; Tukey test, $P \leq 0.05$).

Finally, reports by Augusto et al. (2014) for deshidrate fruit of murtilla, compared cultivated ecotype 14-4 growing in INIA-Tranapuente with their respective wild plant located in Puerto Saavedra, Región de La Araucanía, showed that total phenolic compound, measured trough gallic acid equivalent, was higher in cultivated plants (40.28 mg) than

wild ones (19.35 mg). Moreover, antioxidant capacity, evaluated trough as 2,2 diphenyl 1-picrylhydrazyl (DPPH), trolox equivalent antioxidant capacity (TEAC) and 2.2´ azino-bis (3-ethylbenzothiazoline 6- sulphonic acid) (ABTS) assays showed that cultivated plants presented a better antioxidant capacity than the wild ones (Augusto et al. 2014).

Table I. Summary results of ANOVA for the effect of domestication and compounds in murtilla plants.

Parameter	Source of variation	*df*[a]	*F*[b]	*P*
Flavonoid concentration (µg/g)	Domestication	3,252	2.68	$P = 0.047$
	Residual	252		
	Error	259		
Flavonoid concentration (µg/g) reciprocal transplant experiment	Domestication	3,256	5.20	$P < 0.001$
	Residual	256		
	Error	259		
Compound concentration (µg/g)	Compounds	103,416	108.11	$P < 0.001$
	Residual	416		
	Error	519		

[a] Degrees of freedom: numerator, error.

[b] Estimate population variance

Nevertheless, there are two possible explanations related to these data; the former corresponds to the different environmental system in which plants were collected (Tranapuente versus Puerto Saavedra). According to Gepts (2014), domestication is an evolutionary process that responds to induce adaptation related to the change in the environmental conditions. Hence, wild and cultivated plants have a differents ways to use their nutrient pool in response to the environmental factors. The second reason has to do with the genetic relation between wild and cultivated plant, for instance the wild ancestor of

the ecotype 14-4 was originally collected in Manzanal Alto (Inland zone), and surprisingly wild plants for being used in that study were collected in Puerto Saavedra, coastal zone. Therefore, wild and cultivated plants were not directly related in an evolutionary view. Hence, changes within the plant could be based on genetic or environmental factors that induce variation when the plant adaptation is studied in wild and cultivated plants in different environments. According to Wyngaard (1998), comparisons of populations living in different habitats can be particularly difficult if both the populations and environments differ in an assortment of variables. This case is exemplified by the life history variation exhibited by *U. molinae* in the nature. Our results indicate that plants under reciprocal transplant experiment change their flavonoid concentrations, e.g., cultivated plants transplanted in a wild location increase their flavonoid concentration in contrast to wild plants under cultivated managements. This agrees with the theories reported by Ross-Ibarra et al. (2007) indicating that adaptation is the first instance, where domestication can occur. Moreover, these results suggest that there were chemical defense recuperation, probably generated by chemical defense induction due to biotic and abiotic stresses in cultivated plants exposed to wild environment. In contrast, wild plants exposed to a cultivated system showed a decrease in their flavonoid concentrations. Both results suggest that could be an enzyme that under somes stresses –biotic and abiotic- is overexpressed generating a higher flavonoid concentrations in cultivated plants exposed to wild environments or, *U. molinae* plants have the capacity for reallocate the nutrients quickly for develop a defense mechanism modifying the trade-off between yield and defense. Therefore, cultivated plants could recover chemical defense to detriment yield in wild conditions through trade-off process. Our results suggest strongly that in *U. molinae* the adaptation process occurs at a brief time as can be seen in Figure 1A, where all the cultivated ecotypes –22-1 and 23-2-

showed a higher flavonoid concentrations than wild plants transplanted in locations under agricultural management (INIA-Tranapuente). Hence, these results support the idea that in a reciprocal transplant experiment, the adaptation in *U. molinae* is presented in at last one year from the change of environmental conditions, indicating that domestication process in *U. molinae* is broadly subjected to an adaptation process.

Table II. Reciprocal Transplant experiment and their total concentration of flavonoids in leaves for cultivated and wild plants. Different letters indicate significant differences according to ANOVA and post-hoc Tukey test ($P \leq 0.05$).

	Control (µg/g)				Reciprocal transplant experiment (µg/g)			
Compound names	**Ecotype 22-1**	**Ecotype 23-2**	**Mehuín plants**	**Queule plants**	**Ecotype 22-1**	**Ecotype 23-2**	**Mehuín plants**	**Queule plants**
Biochanin A	0.00 ± 0.00 ^{n}	0.00 ± 0.00 ^{n}	1.50 ± 0.69 ^{n}	1.52 ± 0.43 ^{n}	0.15 ± 0.05 ^{n}	0.23 ± 0.11 ^{n}	0.82 ± 1.10 ^{n}	0.30 ± 0.08 ^{n}
Daidzein	65.58 ± 2.51 ^{klmn}	12.00 ± 3.53 ^{n}	1.32 ± 0.91 ^{n}	75.06 ± 163.53 ^{klmn}	68.80 ± 9.33 ^{klmn}	38.40 ± 22.99 ^{lmn}	0.26 ± 0.18 ^{n}	0.34 ± 0.13 ^{n}
Daidzin	91.05 ± 5.22 ^{jklmn}	318.00 ± 28.63 ^{gh}	732.30 ± 181.00 ^{ab}	424.34 ± 112.71 ^{efg}	648.00 ± 14.83 ^{bc}	718.00 ± 46.58 ^{ab}	146.46 ± 36.20 ^{ijkl}	72.27 ± 19.59 ^{klmn}
Formononetin	0.00 ± 0.00 ^{n}	0.00 ± 0.00 ^{n}	5.44 ± 0.00 ^{n}	7.10 ± 0.00 ^{n}	1.02 ± 0.00 ^{n}	0.42 ± 0.00 ^{n}	1.08 ± 0.00 ^{n}	1.42 ± 0.00 ^{n}
Genistein	0.00 ± 0.00 ^{n}	0.00 ± 0.00 ^{n}	0.14 ± 0.00 ^{n}	0.08 ± 0.00 ^{n}	0.08 ± 0.00 ^{n}	0.03 ± 0.00 ^{n}	0.02 ± 0.00 ^{n}	0.01 ± 0.00 ^{n}
Genistin	138.74 ± 14.49 ^{ijkl}	57.20 ± 8.31 ^{klmn}	24.70 ± 6.41 ^{mn}	46.62 ± 19.65 ^{lmn}	327.31 ± 70.79 ^{gh}	345.01 ± 62.03 ^{fg}	4.94 ± 1.28 ^{h}	7.79 ± 1.27 ^{h}
Kaempferol	0.00 ± 0.00 ^{n}	0.00 ± 0.00 ^{n}	2.16 ± 0.00 ^{n}	0.82 ± 0.00 ^{n}	9.28 ± 0.00 ^{n}	0.57 ± 0.00 ^{n}	0.43 ± 0.00 ^{n}	0.16 ± 0.00 ^{n}
Myricetin	24.47 ± 5.88 ^{mn}	7.44 ± 2.75 ^{n}	631.20 ± 80.49 ^{bc}	445.12 ± 30.12 ^{ef}	129.75 ± 41.07 ^{ijklm}	190.04 ± 48.90 ^{ij}	125.34 ± 15.11 ^{ijklm}	87.90 ± 5.15 ^{jklmn}
Ononin	40.57 ± 5.68 ^{lmn}	50.56 ± 3.17 ^{klmn}	1.86 ± 0.52 ^{n}	1.44 ± 1.59 ^{n}	0.11 ± 0.04 ^{n}	1.50 ± 1.33 ^{n}	0.37 ± 0.10 ^{n}	0.20 ± 0.26 ^{n}
Quercetin	128.32 ± 10.68 ^{ijklm}	122.34 ± 12.66 ^{ijklm}	3.08 ± 0.47 ^{n}	3.46 ± 2.70 ^{n}	0.64 ± 0.21 ^{n}	0.38 ± 0.36 ^{n}	0.61 ± 0.09 ^{n}	0.51 ± 0.25 ^{n}
Quercetin 3-β-Glycoside	93.21 ± 3.00 ^{jklmn}	94.77 ± 1.87 ^{jklmn}	2.56 ± 2.34 ^{n}	4.36 ± 1.07 ^{n}	0.18 ± 0.08 ^{n}	0.48 ± 0.33 ^{n}	0.51 ± 0.46 ^{n}	0.78 ± 0.15 ^{n}
Rutin	351.02 ± 131.96 ^{fg}	562.67 ± 65.14 ^{cd}	786.10 ± 58.49 ^{a}	426.00 ± 40.37 ^{efg}	225.62 ± 19.17 ^{hi}	422.05 ± 178.21 ^{efg}	157.22 ± 11.69 ^{ijk}	488.00 ± 100.84 ^{de}
Sissotrin	0.00 ± 0.00	0.00 ± 0.00	0.16 ± 0.00	2.40 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.03 ±0.00	0.01 ± 0.00

	n	n	n	n	n	n	n	n

5.5. CONCLUSION

Reciprocal transplant experiment showed that the murtilla adaptation into different wild – Mehuín, Queule- and cultivated areas -Tranapuente- can affect the flavonoid concentrations in response to the new ecological interactions. Moreover, our results strongly suggest that changes generated in almost one year in which plants could develop variations in their defense mechanism -flavonoid profiles-. Furthermore, our results suggest that cultivated plants exposed to wild environments can recover their chemical defenses due to abiotic and biotic –herbivores- interactions. Finally, phenotypic changes due to domestication process are not only co-evolutionary examples. In fact, they can be used as a tool for developing mechanisms for facilitating, breeding programs at present and future in investigation of/on adaptive genes in *U. molinae* plants offering a new alternative for improving actual agricultural managements.

CHAPTER VI:

Flavonol synthase (FLS) enzymatic activity is decreased in cultivated *Ugni molinae* plants subjected to a domestication process

6.1 INTRODUCTION

Flavonoids have been associated to a broad range of applications such as antioxidant, deterrence for insects and even related to human health in cancer prevention (Ruiz et al. 2010). Moreover, according to Xu et al. (2012) flavonoids are a family of over 8,000 secondary plant metabolites characterized by their C6-C3-C6 skeleton that can be classified into eight subgroups: flavanones, dihydroflavonols, flavones, flavonols, flavan-3,4-diols, flavan-3-ols, anthocyanidins, and proanthocyanidins, according to the oxidation state and substitution pattern of their C-ring structure. Flavonoids can be conjugated to sugar molecules occurring most of them naturally in plant tissue. Within flavonoids, flavonols are one of the most abundant, acting as auxin transport regulators, modulation of flower color, protection against ultraviolet radiation, prevention against microorganism and pest invasion, and signaling interactions with insects and microbes have been attributed to these compounds. Moreover, flavonols have been also studied due to their defensive characteristics against insect pests in plants (Bohm et al. 1998, Harborne and Williams 2000, Winkel-Shirley 2001). These compounds are formed from dihydroflavonols, as illustrated in Figure 1, by the introduction of a double bond between C-2 and C-3 through the action of flavonol synthase (FLS) (Forkmann 1991). Moreover, the B-ring of dihydrokaempferol can be hydroxylated at the 3´position by flavonoid 3´- hydroxylase (F3´H), or at the 3´ and 5´ positions by flavonoid 3´5´-hydroxylase (F3´5´H) to produce dihydroquercetin and dihydromyricetin, respectively. The oxidation reaction introducing the C-2/C-3 double bond was considered to be specific for dihydroflavonol substrates (Lukacin et al. 2000, Preub et al. 2009). Furthermore, FLS has been reported, as a bifunctional enzyme capable of transform not only dihydrokaempferol into kaempferol but

also is efficient to transform naringenin into kaempferol (Lukacin et al. 2003). Related to the pest management, there are studies about deterrent and anti-feeding properties of these compounds. Onyilagha et al. (2012) reported that flavonols such as kaempferol, quercetin and isorhamnetin were deterrent against flea beetle, *Phyllotreta cruciferae* (Coleoptera: Chrysomelidae) in *Camelina sativa* leaves. Besides, Beninger and Abou-Zaid (1997) reported that quercetin, rutin and a quercetin glycoside reduced the growth of 2nd instar of gypsy moth *Lymantria dispar,* (Lepidoptera: Erebidae) when they were incorporated into a diet. Finally, Abou-Zaid et al. (1993) showed that flavonols quercetin, rutin, rhamnetin and quercitrin reduced the growth of *Ostrinia nubilalis* (Lepidoptera: Crambidae) larvae at doses of 1, 10 and 100 mg/g of fruit. For the past 20 years, a highly polymorphic perennial and wild native shrub from Chile, named "Murtilla", *Ugni molinae* (Valdebenito et al. 2003, Hoffmann 2005) has been domesticated and studied by Instituto de Investigaciones Agropecuarias Carillanca in Región de La Araucanía, Chile. 100 localities were originally selected in South of Chile for collecting wild *U. molinae* plants and cuttings were first grown in greenhouses for 10 years and then transplanted to the field until now (Chacón-Fuentes et al. 2015). In Chile and worldwide, there is a strong economic interest in the production of *U. molinae* fruit due to its high antioxidant content given particularly by flavonol compounds. Furthermore, a recent investigation by Chacón-Fuentes et al. (2015) reported differences in the flavonols concentration present wild and cultivated plants of *U. molinae* showed a domestication effect on the flavonol productions. Considering that the flavonols reported by those authors are derived of naringenin through the same biosynthetic pathway and previous analysis when this substrate was used in our research, the only quantifiable peak by high performance liquid chromatography was kaempferol. This compound was selected in this study as enzymatic activity parameter (see chromatogram in

annex, Fig. 1). Moreover, kaempferol has been particularly associated to deterrent activity in insect by those last authors and also they showed a relation between domestication degree and kaempferol concentration in *U. molinae* plants. However, to our knowledge, there is scarce information about FLS in berries (Flores et al. 2014) and currently, there is no information about flavonol synthase in *U. molinae*. Therefore, the hypothesis of this research was: There is a lower amount of FLS involved in kaempferol production in leaves of cultivated than wild *U. molinae* plants. For dealing with that, we analyzed kaempferol extract from leaves of both wild and cultivated plants using naringenin as substrate for FLS enzyme.

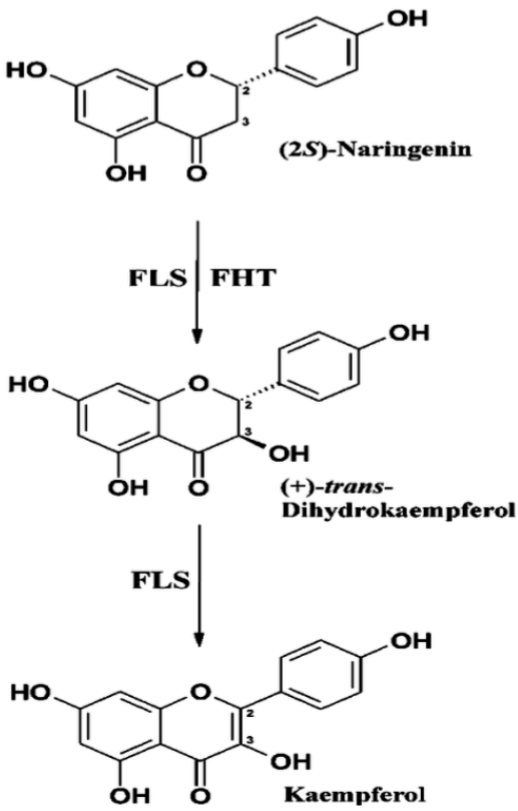


Figure 1. Scheme of the kaempferol biosynthetic pathway from naringenin into kaempferol through the action of the enzyme flavonol synthase. FLS: flavonol synthase. FHT: flavanone 3ß-hydroxilase.

6.2 MATERIAL AND METHODS

6.2.1. Cultivated Material collection. Seven cultivated plants (Ecotypes: 08-1, 12-1, 14-4, 18-1, 19-1, 22-1 and 23-2) of *U. molinae* were used for collected cuttings at the Experimental Station-Tranapuente of the Instituto de Investigaciones Agropecuarias (INIA) in the Región de La Araucanía (south of Chile, 38° 45`S, 73° 21`W). Cuttings were grown in greenhouse in INIA Tranapuente. Then, after 1 year of acclimation in a common garden, leaves were sampled and stored until their use for kaempferol analysis and enzyme assay.

6.2.2. Wild Material Collection. Wild plants were sampled from the original geographical area where their cultivated counterparts were collected originally around 20 years ago. The following sampling areas were used for the cutting collection: Caburgua ($39^{o}11$` S, $71^{o}49$`W), Pucón ($39^{o}17$` S, $71^{o}55$`W), Manzanal Alto ($38^{o}03$` S, $73^{o}10$`W), Soloyo ($38^{o}35$` S, $72^{o}34$`W), Porma ($39^{o}08$` S, $73^{o}16$`W) from Región de La Araucanía and Mehuín ($39^{o}26$` S, $73^{o}12$`W) and Queule ($39^{o}23$` S, $73^{o}12$`W) from Región de Los Ríos, Chile. Each cultivated plant was paired with their respectively wild plants as follow: Eco 08-1/Caburgua; Eco 12-1/Pucón; Eco 14-4/Manzanal alto; Eco 18-1/Soloyo; Eco 19-1/Porma; Eco 22-1/Mehuín and Eco 23-2/Queule.

6.2.3. Kaempferol Extraction. Leaves of both cultivated and wild plants were collected. Samples were rapidly frozen in liquid nitrogen for 5 s (Mikulic-Petkovsek et al., 2012), and then milled in a grinder. Then, samples (5 g) were placed in a flask where 25 mL of methanol HPLC grade (Sigma-Aldrich, St. Louis, MO) was added to the samples (50% v/v in water, solvent-to-solid ratio of 5:1). These flasks were placed in a magnetic stirrer at 170 rpm for 20 min at 30 ^{o}C. After this time, samples were filtered in darkness

through a Whatman n°1 filter paper (Whatman International Ltd., Maidstone, U.K.). The filtrate was lyophilized. Finally, each sample was suspended in 10 mL of methanol and left for 5 min in a Branson 3510 sonicator according to Chacón-Fuentes et al. (2015). Samples were stored at -20°C in amber flasks (25 mL) until their analysis by High Performance Liquid Chromatography (HPLC).

6.2.4. *Enzyme Preparation. U. molinae* leaves were ground in a mill up to a fine powder. 1.5 g of the powder obtained, 0.25 g quartz sand, 0.25 g Polyclar AT and 4 mL of 0.1 M Tris/HCl (containing 0.4% Na-ascorbate, pH 7.25) were properly homogenized (Halbwirth et al. 2009). Then, the resulting homogenate was centrifuged at 5000g for 10 min at 4 ºC. Finally, the supernatant was taken to accomplish FLS assays. This preparation was performed in triplicate for statistical analysis.

6.2.5. *Enzymatic Activity*. Once obtained the enzyme extract, FLS activities were measured according to Halbwirth et al. (2009) with slight modifications. Briefly, 20 µL of 50 mM of naringenin, 50 µL enzyme extract obtained above, 5 µL of 3.48 mM 2-oxoglutarate, 5 µL of 2.01 mM $FeSO_4$-$7H_2O$, and 60 µL of buffer (0.1 M Tris/HCl + 0.4% Na-ascorbate, pH 7.25) were mixed. Then, the enzyme assay was incubated for 60 min at 30 °C. The assay was finished by addition of 140 µL of ethyl acetate, 10 µL of acetic acid and 10 µL of 0.1 mM EDTA. Finally, the organic phase was taken to carry out the HPLC analysis as detailed below. In addition, the reaction in absence of substrate (so-called blank) was also performed for comparison. Enzyme activity was expressed as pkatal, where one katal is defined as the enzyme activity transforming 1 mol of compound per second (Flores et al. 2014).

6.2.6. HPLC Analysis. The methanolic extracts obtained from leaves and the enzyme preparations were filtered through a 0.22 µm membrane and they were analyzed by HPLC. 20 µL of sample were injected into a Shimadzu HPLC equipped with a C-18 column (250 x 4.6 mm I.D.; particle size 5 mm) maintained at 40 °C. The analysis was performed using a linear solvent gradient consisting of 1% formic acid (A) and acetonitrile (B) as follows: 0-5 min, 5% A/ 95% B; 5-10 min, 30% A/70% B; 10-20 min, 55% A/45% B; 20-30 min, 5% A/95% B at a flow rate of 1 mL/min (Simirgiotis et al. 2009). Kaempferol was monitored at 280 nm. The identification of kaempferol was based on the peak retention time in comparison with its standard. To construct calibration curves for flavonols, standard solutions were dissolved in methanol (Sigma-Aldrich, St. Louis, MO) at 1,000 mg/L. The stock solutions of each standard were used to prepare a serial concentration between 0.05 to 500 mg/L (Kumar et al. 2009).

6.2.7. Statistical Analysis. The statistical software Statistix 10 (Tallahassee, Florida, United States of America) was used to analyze the kaempferol concentration and FLS enzyme activity data. The *t*-tests were used for paired comparisons between wild and cultivated plants. Data were natural-log transformed to meet the assumptions of normality and homogeneity of variance. Values of $P \leq 0.05$ were considered as significant. Results are expressed as means and their corresponding standard errors.

6.3 RESULTS

Kaempferol Content. Kaempferol concentration was evaluated per each particular ecotype and compared with their respective counterpart. Differences between the ecotypes compared with their wild counterpart are shown in Figure 2, significant differences between cultivated and their respective wild counterparts, such as Eco 18-1/ Soloyo from 0.04 μg/g for cultivated plants to 0.14 μg/g for wild ones ($F_{1, 5}$ = 61.40; P = 0.0159); Eco 22-1/Mehuín from 0.03 μg/g to 0.18 μg/g ($F_{1, 5}$ = 40.97; P = 0.0235) and Eco 23-2/Queule from 0.08 μg/g to 0.25 μg/g ($F_{1,5}$ = 84.28; P = 0.0117) were found according to *t*-test (P < 0.05).

Incubation to Assay FLS Activity. Kinetic studies were carried out to select the best incubation time to the enzymatic assays for FLS. Figure 3 shows the correlation in the amounts (nmol) of naringenin consumed as well as kaempferol produced with four reaction times; 30, 60, 120 and 240 minutes at 30 ºC as the temperature in all cases. The time where the high level of kaempferol was detected corresponded to 60 minutes (1.13 $x10^6$ nmol), coinciding with a low level of naringenin (2.79 $x10^4$ nmol).

Enzymatic Activity. Significant differences in FLS activity were observed for Eco 14-4/Manzanal Alto from 73 pkatal in cultivated plants to 134 pKatal in wild ones ($F_{1, 5}$=7.11; P= 0.0500), Eco 18-1/Soloyo from 66 pKatal to 96 pKatal ($F_{1, 5}$=12.45; P= 0.0243) and Eco 22-1/Mehuín from 38 pKatal to 119 pKatal ($F_{1, 5}$=11.81; P= 0.0264) according to *t*-student test (Table 1). Nevertheless, for all the other comparisons there were no significant differences between cultivated and wild plants ($P \leq 0.05$).

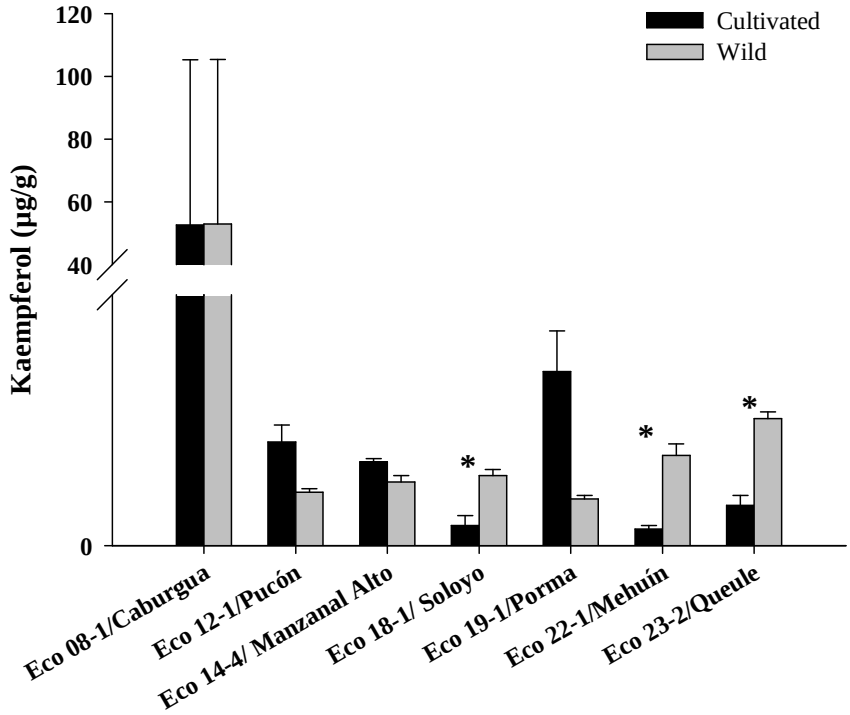


Figure 2. Kaempferol leaf concentrations compared ecotypes and their respective wild counterpart. * mean significant difference between cultivated and wild plants (*t*-test $P \leq 0.05$) (N=5).

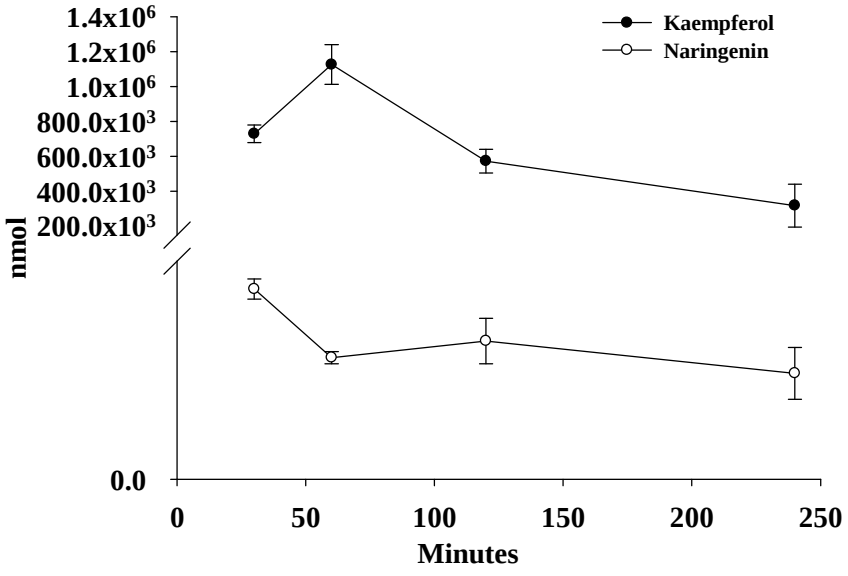


Figure 3. Correlation of the amount of naringenin consumed and kaempferol produced (nmol) in reaction time. Data are expressed as means and their correspondent standard errors.

Table 1. Total FLS activity and their standard deviation (SD) in leaves of cultivated and their respective wild murtilla. In all cases the enzyme was incubated for 60 minutes. * mean significant difference between cultivated and wild plants (*t*-test $P \leq 0.05$) (N=3).

Ecotypes/Locations	Cultivated plants (pKatal)	Wild plants (pKatal)
Ecotype 08-1; Caburgua	83.64 ± 74.28	61.11 ± 2.80
Ecotype 12-1; Pucón	105.28 ± 91.18	70.66 ± 14.29
Ecotype 14-4; Manzanal Alto	73.30 ± 34.66	134.79 ± 19.87 *
Ecotype 18-1; Soloyo	66.42 ± 13.09	96.48 ± 6.79 *
Ecotype 19-1; Porma	85.15 ± 75.67	54.09 ± 3.94
Ecotype 22-1; Mehuín	37.97 ± 33.01	119.97 ± 24.85 *
Ecotype 23-2; Queule	73.91 ± 68.46	66.64 ± 10.53

6.4 DISCUSSION

Plant domestication can affect the flavonols production, generating a decrease in these metabolites and an increase in productive traits in the so-called domestication syndrome (Meyer et al. 2012). Nonetheless, in this syndrome, chemical defenses – flavonols- can be affected in unpredictable ways. For example, the trade off between yield and production can be modified in detriment to the flavonols responsible of the chemical defense in plants that acting on insects feeding behavior. In this framework, Chacón-Fuentes et al. (2015) reported a decrease in flavonol compounds as quercetin, rutin and kaempferol in cultivated murtilla plants in comparison with their respective wild counterparts. In addition, flavonols particularly, have been studied due to their defensive characteristics, Chacón-Fuentes et al. (2015) tested several concentration of kaempferol on the feeding behavior of *Chilesia rudis* (Lepidoptera: Arctiidae) and found that all the

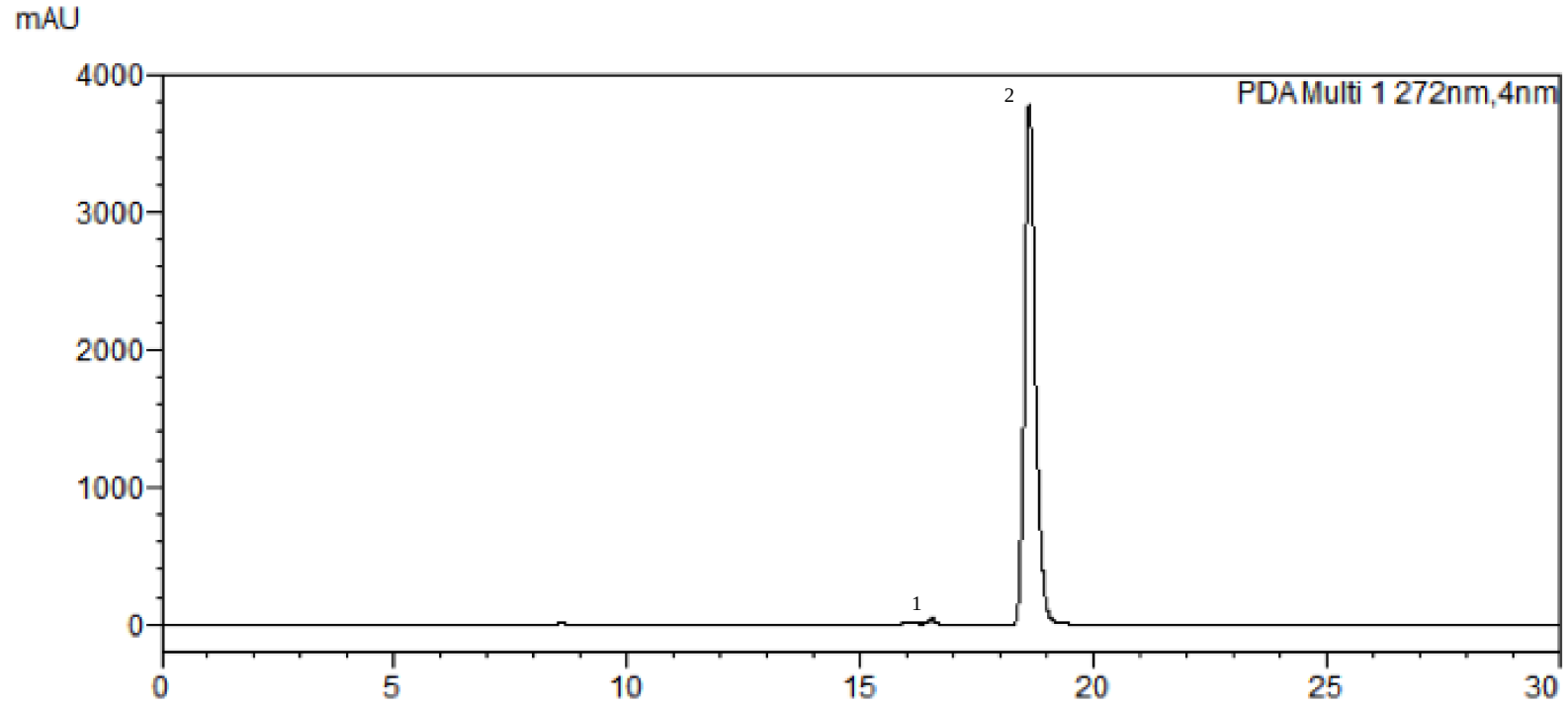
concentration used (0.1, 1, 5 and 50 mg/L) were feeding stimulants. Hence, kaempferol could be modified over the domestication process in murtilla plants, generating changes in its concentrations that could affect the feeding behavior in insect pests due to changes in the enzyme flavonol synthase (FLS), transforming a dihydroflavonol into flavonol, e.g. dihydrokaempferol into kaempferol. For instance, Fang et al. (2013) showed a positive correlation between kaempferol concentration and enzymatic activity of FLS in grape berries, *Vitis vinifera* (cv: Cabernet sauvignon). Hence, our results indicated the enzymatic activity by FLS from naringenin to kaempferol, but FLS could be acting from naringenin as bifunctional enzyme. Our results showed that changes in kaempferol concentration were related to the amount of FLS presented in murtilla plants (Soloyo and Mehuín), showing in cultivated plants a decrease of kaempferol concentration due to a low enzymatic activity (Eco 18-1 and 22-1) in comparison to respective wild plants. Moreover, differences in the total kaempferol concentration were found for Soloyo, Mehuín and Queule. These results were agreed with the activity of FLS where Eco 18-1 and Eco 22-1 presented a lower activity than their respective wild counterpart.

6.5 CONCLUSION

In general, wild plants are exposed to high environmental pressures –biotic and abiotic factors- having a high defensive compounds pool to cope against these several stresses. Hence, our results showed a higher kaempferol concentration in wild location as Soloyo, Mehuín and Queule, indicating a decrease in their cultivated counterparts, this reduction in kaempferol concentration could be related with the FLS Activity. Therefore, these results

suggest a decrease of enzyme key as FLS and for that a reduction of kaempferol production in cultivated plants. Kaempferol concentrations and the FLS activity could be correlated with the domestication in *U. molinae* plants, indicating that the activity of FLS is affected in some ecotypes by domestication process according to our results. Furthermore, these results suggest that murtilla plants under cultivated managements could be experimenting a decrease in the kaempferol production due to the human cultivation showing a loss in the pool of defenses because the low activity of FLS in comparison to wild counterparts (Manzanal Alto, Mehuín and Queule).

Annex



Representative HPLC chromatogram of enzymatic activity of FLS in murtilla, *Ugni molinae*, leaves. 1) Naringenin, and 2) kaempferol.

CHAPTER VII:

General discussion

GENERAL DISCUSSION

Plant domestication is a co-evolutionary process of selection for adaptation to environmental condition changes (Gepts 2014, Machado et al. 2014) that frequently occurs gradually (Machado et al. 2014). One of the most important characteristics for domestication take place is the “cultivation”. According to Chen et al. (2015) cultivation describe the agronomic activities that promote crop growth, pest control activities and tillage. In general, the main focus of the agronomic managements is to improve traits on plants for human needs. In this sense, there are different characteristics that difference a wild plant with a cultivated one. For instance, in natural environments there is a strong competition of plants for light, water or soil nutrients in contrast to the agrosystems (Gepts 2014). Moreover, the need of developing a more efficient production system, in terms of biomass per unit of surface and time in cultivated plants is, according to Gepts (2014), an important difference between wild and cultivated plants. Finally, this author indicated that the human necessities for novelties such as new fruit colors or size and shape of seed are decisive differences in cultivated plants related to wild ones. In the same framework, plants artificial selection is based on criteria dictated by the human society. Mainly, the characteristics proposed for domesticating plants are according to Maag et al. (2015), in a first place, reach an increase in nutritive quality, and in a second one is reduce toxic metabolites for enhance palatability. Finally, to promote plant growth can affect however the relationship insect plant, increasing the performance and fitness of herbivores as a consequence of the increase in nutritive traits and decrease in constitutive chemical defenses (Rodriguez-Saona et al. 2011, Maag et al. 2015). Hence, crop domestication can affect plant defenses and resistance against herbivores in unpredictable ways (Meyer et al.

2012). As is indicated above, domestication and breeding for high-yielding crops are expected to reduce chemical defenses in plants because of potential trade-offs between growth or reproduction and defense (Wink 1988, Herms and Mattson 1992, Rodriguez-Saona et al. 2011). Plant domestication can also affect flavonoid production, generating a decrease in these compounds (Chacón-Fuentes et al. 2015) and changing both diversity and community of insects (Chacón-Fuentes et al. 2016). In the present research, we identified around 60 insect species in wild and cultivated murtilla plants from December 2012 to October 2013 that had not been reported previously (Table 2; Chapter II). The high number of insects reported in association with *U. molinae* suggests that once this crop completes its domestication process could be affected by a wide spectrum of phytophagous insects, producing different kinds of damage due to their defoliating or sucking feeding behavior or insects that generate damage when they oviposited as is the case of *Tettigades chilensis* Amyot & Serville (Hemiptera: Cicadidae) found in this survey. As the first approach, domestication process can be responsible for loss or decrease of biodiversity in cultivated species of *U. molinae*. Seguel and Torralbo (2004) indicated that *Bombus* spp. is the principal pollinator of *U. molinae*. Therefore, loss of biodiversity through domestication process can induce a decreasing in pollination. In the last years several authors have developed a theoretical framework for understanding the evolution of plant defenses against herbivores. Rhoades (1979) suggested that the degree of resistance to herbivores reflects a compromise between the benefits of reduced herbivory and the costs of diverting resources from other functions to resistance. Crawley (1997) reported that plant morphology can influence insect acceptability directly either by providing suitable visual cue, or by influencing the ability of insects to walk on or bite into the tissue. Furthermore, most phytophagous insects are confined to certain plant parts determining the physical and

chemical attribute to which the insects respond. In this framework, domestication in *U. molinae* has focused mainly on selection of traits associated with increased productivity, such as bigger plants, more fruit, and larger fruit size (Seguel and Torralbo 2004). Overall, we detected changes in insect assemblages, damage and diversity indexes that could suggest murtilla domestication has altered the insect assemblages. Furthermore, flavonoid composition was also affected for domestication process in murtilla agreed with previous data related to insect diversity and assemblages (Chapter II). We reported that domestication in *U. molinae* reduced the concentration of rutin (231 mg/L vs 190 mg/L) quercetin (2.34 mg/L vs 1.93 mg/L) and kaempferol (1.01 mg/L vs 0.87 mg/L) from wild plants to cultivated ones. Despite its short history of domestication, theses data suggest that domestication in *U. molinae* has led to decreases in flavonoid concentrations, an important class of defensive secondary metabolites in plants. This agrees with our hypothesis that domestication has reduced chemical defenses in *U. molinae*. Although the trend was the same, i.e., reduction in flavonoid concentrations in cultivated plants, the strength of the effect of domestication varied among populations (Fig. 1B; Chapter III), with some of them responding more strongly than others. Hence, if cultivated plants are less defended (Chen et al. 2015), we predicted that domestication in *U. molinae* would make plants more susceptible to herbivores. In fact, some flavonoids found in *U. molinae* have been implied in resistance against herbivores in other plant systems. For example, Todd et al. (1971) showed that quercetin, a constituent of barley leaves, was toxic to greenbugs, *Schizaphis graminum* (Rondani). Moreover, Dreyer and Jones (1981) reported increased resistance of wheat against *M. persicae* also due to quercetin. However, this was not the case for the herbivore *C. rudis* (Ángulo and Ruiz 1974), an important defoliator in the ecosystem associated to murtilla (Aguilera et al. 2009), which showed higher performance and

preference for wild *U. molinae* plants than their cultivated counterparts. In fact, our study shows that flavonoid content stimulates feeding in *C. rudis*. Even though *C. rudis* is a generalist herbivore, preference for wild *U. molinae* might be due to the fact that both plant and insect are native from the region and it has likely evolved to exploit its host plant defenses. In this regard, our study demonstrates higher levels of flavonoids in wild than cultivated *U. molinae* leaves. Despite that these chemical compounds may be acting as phagostimulants in this insect–plant interaction, *U. molinae* is a crop that is highly valued due to the antioxidant activity of flavonols in its fruit (Rubilar et al. 2011, Alfaro et al. 2013). However, the conclusions presented here could be risky in the sense that the determination of flavonoids was conducted from samples from different agro-ecological zones. This was corrected, and in Chapter IV we showed the flavonoids determination carried out in a common garden with the aim of minimizing the environmental variations. The results were similar to those obtained previously. For example, the concentration of rutin, quercetin glycoside, kaempferol and quercetin varied from 175, 85, 0.74 and 124 μg/g respectively in wild plants to 138, 75, 0.55 and 112 μg/g in cultivated ecotype. Overall, this agrees with our hypothesis that domestication has reduced chemical defenses in *U. molinae*. These results suggest that during the domestication process and because the content of flavonoids change, the domesticated plant could express or inhibit the production of a particular compound resulting plant more susceptible to insect attack. In addition, we detected for the first time the presence of eight isoflavone compounds in *U. molinae* leaves (Table I; Chapter IV. According to Lapcik (2005, 2007) these secondary metabolites seem to be typical in Myrtaceas, Therefore, the presence of these compounds in *U. molinae* – Myrtaceae- is according to the chemotaxonomy reported for this species. Furthermore, we detected changes in community and numbers of the insect assemblages, biodiversity index

and also in damage index that could suggest that the domestication has elicited a decrease of defense secondary metabolites such as flavonols and isoflavonoids in murtilla plants. Finally, *C. rudis* showed to be a proper biological indicator of the negative effect of domestication on murtilla chemical defense. We observed that the feeding behavior was increased in cultivated ecotypes. However, reports by Chacón-Fuentes et al. (2015) showed that the *C. rudis* preference was higher for wild plants in no-choice (80%) and choice test (45%). Nevertheless, these results could be explained due to the high environmental variation in that study. In the present study, the environmental factors were minimizing because of the establishment of a common garden as it was explained above. Therefore, this is a product of the decrease of the flavonoid content? Ensuring this is risky, but our results strongly suggest that the answer goes in that direction. According to Machado et al. (2014) artificial selection is considered as the main evolutionary force. In this framework, we analyzed the effect of the human influence on *U. molinae* domestication in a reciprocal transplant experiment. Plants under reciprocal transplant experiment changed their flavonoid concentrations showing in cultivated plants exposed to wild locations an increase in the flavonoid defenses and visceversa. Therefore, a recovery chemical defenses capacity in cultivated plants exposed to wild environments could be suggesting a physiological plasticity in murtilla plants. Furthermore, wild plants exposed to cultivation presented a decrease in their flavonoid levels, suggesting a quick adaptation to an agricultural management. This agrees with the theories reported by Ross-Ibarra et al. (2007) that indicated adaptation is the first instance where domestication can occur. Furthermore, this recovery capacity suggests a possible increase or over-production of enzymes involve into flavonoid pathways. On the other hand, we report for the first time the relationship between domestication and enzyme involve in plant defense. Thus, we showed that enzymes

responsible of flavonoids production are also affected for a domestication process. Specifically, FLS activity seems to be higher in *U. molinae* for producing kaempferol. Our results showed a higher kaempferol concentration in wild location as Soloyo (0.14 μg/g), Mehuín (0.18 μg/g) and Queule (0.25 μg/g), indicating a decrease in their cultivated counterparts with 0.04, 0.03 and 0.08 μg/g respectively. This reduction in kaempferol concentration was related to the FLS activity, which showed values from 96 and 119 pKatal for Soloyo and Mehuín, in general higher than their cultivated counterparts with 66, and 38 pKatal for Eco 18-1 and Eco 22-1. Therefore, these results showed a decrease of a key enzyme for flavonoid production such as FLS as is presented in ecotypes 18-1 and 22-1. In summary, the concentration of kaempferol and the activity of flavonol synthase could be correlated according to the domestication in *U. molinae* plants, indicating that the activity of FLS as well as the flavonoid concentrations is affected by domestication process. Overall, first chapters showed a domestication effect on cultivated plants, decreasing their defense pool in comparison to wild plants sampled in original wild locations. Nonetheless, the developing of a common garden experiment was agreed with these previous data – affected for environmental factors- showing a decrease of flavonoids in cultivated plants. In conclusion, although domestication and selective breeding have had great positive influences on food availability through increased crop yield and quality (Wink 1988), it has often had a cost for resistance against herbivores (Chen et al. 2015), which may lead to increased use of pesticides. Whereas in a number of crop plants, domestication has been reportedly to lead to lower levels of defensive compounds hence lower resistance to pests (e.g., Rosenthal and Dirzo 1997, Rodriguez-Saona 2011, Chen and Bernal 2011, Altesor et al. 2014). In the system studied here, domestication has led to lower levels of chemical defenses in the Chilean native crop *U. molinae*. The study of *U. molinae* in this incipient

stage of domestication would make possible to analyze it as an evolutionary continuum and in places where cultivated and wild plants coexist. Finally, in areas where wild relatives and cultivated plants coexist it is possible to identify continuous gradients of states or degrees of domestication and this fact is useful for study the effect of adaptation in cultivated plants and use as tool for redomestication, neodomestication (Gepts 2014) or superdomestication of crops (Vaughan et al. 2007).

CONCLUDING REMARKS

Ugni molinae domestication effect was showed in three different experiments. In the first approach, a comparison between wild and cultivated *U. molinae* plants in relation to flavonoid contents, insect diversity and feeding behavior of *Chilesia rudis* was developed. In the second approach the same aspects were developed in a common garden. In the third experiment the plasticity response from *U. molinae* to the flavonoid content changes were tested in a reciprocal transplant. Finally, the role of flavonol synthase in the *U. molinae* domestication process was evaluated. Hence, the main conclusions from this thesis are:

- The first experiment showed that the chemical defense –flavonoids- was decreased in cultivated plants and the insect communities were increased. Hence, the domestication effect can be not determined in this experiment because of the different environmental conditions involved in this approach.

- The domestication effect was determined in murtilla plants subjected to a common garden experiment.

- Finally, murtilla plants show plasticity in the recovering flavonoid contents in a reciprocal transplant experiment.

FUTURE DIRECTIONS

- Because of *Ugni molinae* domestication is a dynamic and continuous process, the evaluation of the insect-plant interaction and the trade-off between growth and chemical defenses on the following domestication stages of domestication could be useful for increasing the knowledge and their application in agricultural management and breeding programs.

- Biosynthetic pathways can be altered in cultivated plants due to the plant domestication process. In this sense, to analyze the variation of key enzymes in the flavonoid pathways in plants under domestication effects could be useful for find a mechanism for increase or stimulate their production in cultivated plants subjected to the domestication process.

- The plasticity found in *U. molinae* plants suggests that their flavonoid contents can be handled. Hence, to study the effect of shadings, mechanical damage, fertilization or hydric deficit on the content of these defensive metabolites.

- In areas where wild relatives and cultivated plants coexist it is possible to identify continuous gradients of states or degrees of domestication. This fact is a solid base for understanding the effect of adaptation in cultivated plants. This knowledgements

can be applied for develop a powerful tool for the future processes of redomestication, neodomestication or superdomestication in crops.

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