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EFFECT OF ESSENTIAL OILS ON THE CONTROL OF *Aegorhinus* SPP. AS A POTENTIAL ALTERNATIVE FOR USING IN AN ORCHARD OF EUROPEAN HAZELNUT

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JOCELYNE VIVIANA TAMPE PEREZ

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Effect of essential oils on the control of *Aegorhinus* spp. as a potential alternative for using in an orchard of European hazelnut

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JOCELYNE VIVIANA TAMPE PEREZ

Dr. Francisco Matus
Director Programa de Doctorado en Ciencias de Recursos Naturales

Dr. Andrés Quiroz (Tutor)
Universidad de La Frontera

Dr. Leonardo Parra (Co-Tutor)
Universidad de La Frontera

Dr. Juan Carlos Parra
Director Académico de Postgrado
Universidad de La Frontera

Dra. Miren Alberdi
Universidad de La Frontera

Dr. Eduardo Fuentes
Universidad de Talca

Dra. Graciela Palma
Universidad de La Frontera

Dr. Alejandro Urzúa
Universidad de Santiago de Chile

Dedicada a mis padres, Inés y Víctor

y a mi novio Mario

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Summary and outline of this thesis

Aegorhinus superciliosus (Guérin) and *Aegorhinus nodipennis* (Hope) (Coleoptera: Curculionidae) are two native insects of southern Chile. They are the main pests associated with European hazelnut *Corylus avellana* L. (Betulaceae) and currently, the used control methods show limited effectiveness. The main objective of this thesis was to evaluate a new alternative for controlling these pests. Hence, in Chapter I the characteristics of both weevils and their hosts were described. In addition, the main control methods were reviewed with special emphasis on the use of essential oils (EOs) as repellent and attractant substances of insects. According to the literature reviewed, there is evidence on bioactivity of the EOs suggesting their potential use as alternative methods for the control of *A. superciliosus* and *A. nodipennis*.

In Chapter II, the repellent activity elicited by *Achillea millefolium* (yarrow) EO on *A. nodipennis* was described. Gas chromatography coupled with mass spectrometer was used for the analysis of yarrow EO. Eleven compounds were identified; being β-thujone (96.2%) the main component. This monoterpene exhibited a repellent activity against *A. nodipennis* at the highest doses tested (285.7 ng/cm^2).

In Chapter III, we proved that *A. nodipennis* showed electroantennographic activity elicited by thujone at 285.7 ng/cm^2, supporting the behavioral bioassays showed previously.

In Chapter IV, we found that the *Ruta chalepensis* (rue) EO elicited a repellent effect from both sexes of *A. superciliosus* when high doses were tested. However, when decreasing the

concentration of the oil (285.7 ng/cm^2), only females were repelled. These results allow to suggest that rue EO could be considered as a potential repellent agent to reduce the infestation of this weevil. In addition, nine compounds were identified through GC-MS analysis and two ketones-type compounds; 2-nonanone and 2-undecanone were the main components of the oil.

In Chapter V, we reported that *Eucalyptus* spp. (eucalyptus) EO was attractant for both sexes of the *A. superciliosus,* whereas *Foeniculum vulgare* (fennel) EO was only attractant for female weevil. In addition, the main compounds of these oils, eucalyptol (63.6%) and anethole (50.8%) respectively, were attractant for the *A. superciliosus* females. Subsequently, EAG response of weevils suggested that this curculionid has physiological structures, such as specific receptor for detecting these compounds.

In Chapter VI, we presented a general discussion of this thesis, including all chapters and appendix with complementary graphics of the study, adding concluding remarks and future directions of the thesis.

Resumen

Aegorhinus superciliosus (Guérin) y *Aegorhinus nodipennis* (Hope) (Coleoptera: Curculionidae) son dos insectos nativos del sur de Chile. Ellos son las principales plagas asociadas con el avellano europeo *Corylus avellana* L. (Betulaceae) y actualmente, los métodos de control utilizados muestran una limitada efectividad. El principal objetivo de esta tesis fue evaluar una nueva alternativa para controlar estas plagas. Por lo tanto, en el Capítulo I se describen las características de ambos gorgojos y sus hospederos. Además, los principales métodos de control fueron revisados con especial énfasis sobre el uso de los aceites esenciales (AEs) como substancias repelentes y atractantes de insectos. De acuerdo a la literatura revisada, existe evidencia sobre la bioactividad de los AEs, sugiriendo su potencial uso como un método alternativo para el control de *A. superciliosus* y *A. nodipennis.*

En el Capítulo II, se describe la actividad repelente elicitada por el AE de *Achillea millefolium* (milenrama) sobre *A. nodipennis.* Cromatografía gaseosa acoplada a espectrometría de masa (CG-EM) fue utilizada para el análisis del AE de milenrama. Se identificaron once compuestos, siendo *β*-thujone (96,2%) el principal componente. Este monoterpeno exhibió actividad repelente contra *A. nodipennis* a la más alta dosis testeada (285,7 ng/cm^2).

En el Capítulo III, demostramos que *A. nodipennis* tuvo actividad electroantenográfica elicitada por tujona a 285,7 ng/cm^2, reafirmando los resultados previamente obtenidos en bioensayos conductuales.

En el Capítulo IV, reportamos que el AE de *Ruta chalepensis* (ruda) elicitó un efecto repelente sobre ambos sexos de *A. superciliosus* cuando altas dosis fueron evaluadas. Sin embargo, cuando se disminuyo la concentración del aceite (285,7 ng/cm^2), solo las hembras fueron repelidas. Estos resultados permiten sugerir que el AE de ruda podría ser considerado como un potencial agente repelente para reducir la infestación de este gorgojo. Además, fueron identificados nueve compuestos a través del análisis CG-EM y dos compuestos del tipo cetonas; 2-nonanona y 2-undecanona fueron los principales componentes del aceite.

En el Capítulo V, nosotros reportamos que el AE de *Eucalyptus* spp. (eucalipto) fue atractante para ambos sexos de *A. superciliosus,* mientras el AE de *Foeniculum vulgare* (hinojo) solo fue atractante para hembras. Además, los principales compuestos de estos aceites, eucaliptol (63,6%) y anetol (50,8%) respectivamente, fueron atractantes para las hembras de *A. superciliosus.* Subsecuentemente, la respuesta de EAG de los gorgojos sugiere que estos curculionidos presentan estructuras fisiológicas, tales como receptores específicos para la detección de estos compuestos.

En el Capítulo VI, nosotros presentamos una discusión general de esta tesis, incluyendo todos los capítulos y apéndices con gráficos complementarios del estudio, además de conclusiones generales y futuras perspectivas de la tesis.

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CHAPTER I

General Introduction

General Introduction

The European hazelnut (*Corylus avellana* L.) (Fagales: Betulaceae) is one of the species which has become an alternative to traditional crops in Chile. It is native from Mesopotamy (modern Iran, Iraq and Turkey) and was introduced into Chile in the mid-nineteenth century by European immigrants (Ellena, 2010). During the nineteen nineties, the Instituto Nacional de Investigación Agropecuaria (INIA) Carillanca introduced commercial varieties in order to know their adaptability to the Chilean soils, pointing to the *cv*. Barcelona as the pioneer variety for experimental orchards and then, as the variety for future commercial orchards (Aguilera, 2005).

The European hazelnut is consumed all over the world, not only as a fruit but also in a diversity of manufactured food products. Hazelnut has found its way into non-traditional foods, such as snacks, chocolates, cereals, sauce, ice creams and other dessert formulations due to the recognition of its nutritional properties, because of its special composition of fat (around 60%), most of which are highly rich in monounsaturated fatty acids (mainly oleic acid), protein, carbohydrate, dietary fiber, vitamins (vitamin E), minerals, phytosterols (mainly β-sitosterol), squalene and antioxidant phenols (Ozdemir and Akinci, 2004; Amaral et al., 2006; Alasalvar et al., 2003, 2006). These phytochemicals and phenolic compounds provide protection against harmful effects of oxidative stress, caused namely by free radicals, and are also known to reduce risks of certain type of cancer (Proestos et al., 2005; Oliveira et al., 2008).

In relative to the market in our country, the Centro de Comercio Internacional (CCI), indicated that Chile is in the third place among major exporters of hazelnuts with shell, after the United States of America and China[1], with income margins of USD 131 million in 2011 (Bravo, 2013). Hazelnut market presents an opportunity for Chile as a producer and exporter due to low production costs of fruit and little competition in the Hazelnut market with shell (Gritsko, 2014). Within the comparative advantages which this fruit tree has in Chile, the off-season production on the main importer markets (Turkey, Italy and the United States of America) makes this species be a highly viable alternative to domestic farmers (Grau, 2007). Currently there are more than 10,000 hectares of hazelnut growing in the country, from Región del Maule to Región de Los Lagos, with exponential rates of planting (Cruzat, 2010). If Chile continues with this policy of product commercialization with shell and considering the increase in cultivated area, our country could lead world exports of hazelnuts with shell in the medium term (Bravo, 2013).

With respect to the disadvantages of establish such orchards, the increase of the plantings has provoked a habitat change favorable for native weevils, such as *Aegorhinus superciliosus* and *Aegorhinus nodipennis*, which had before as hosts, shrubs and native trees typical of southern Chile (Aguilera, 1995). Studies about the phytochemistry of plant species indicate that compounds emitted by the plants are used by other organisms, such as insects in their reproductive interactions and in their host location process (Wadhams, 1992). Hueichapán (2011) suggested that host location behaviour by *A. nodipennis* is mediated in part by volatile semiochemicals released by the European hazelnut. In their

[1]Centro de Comercio Internacional (CCI)

chemical identification compounds belonging to different chemical groups were observed, such as; alkenes (4.12%), alkanes (29.90%), carbonyl compounds (18.56%), green leaf volatiles (GLV) (7.22%), terpenes (14.43%), aromatic compounds (8.25%), halogenides (3.09%), hydroperoxide (3.09%), alcohols (3.09%), ethers (1.03%), phenol (2.06%), amines (2.06%) and carboxylic acid (1.03%), many of which can establish interspecific interactions. Some examples show that GLV enhance aggregation pheromone of boll weevil, *Anthonomus grandis* (Coleoptera: Curculionidae) (Dickens, 1989) or that terpenes can act as potential kairomones for the redbay ambrosia beetle, *Xyleborus glabratus* (Coleoptera: Curculionidae) (Niogret et al., 2011).

A. superciliosus (Guérin) (Fig. 1A) and *A. nodipennis* (Hope) (Fig. 1B) (Coleoptera: Curculionidae: Aterpinae) are two phytophagous native insects of Southern Cone of America. In Chile, *A. superciliosus* is distributed from Región del Maule to Región de Los Lagos and *A. nodipennis* from the Región del Maule to Región de Aysén del General Carlos Ibañez del Campo, whereas in Argentina, both insects have been reported in the province of Neuquén (Aguilera et al., 2011; Zavala et al., 2011).

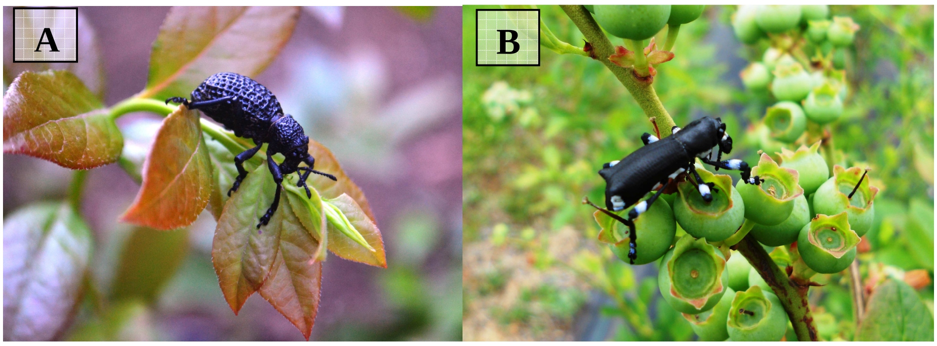

Figure 1. A) *Aegorhinus superciliosus* (Guérin) and **B)** *Aegorhinus nodipennis* (Hope) adults.

The morphology of these insects is divided into four stages, represented as egg, larval, pupal and adult. In the first three stages, both insects exhibit similar morphological characteristics. The eggs are ovoid, pale yellow and about 1 mm in size; their larvae are white and apodous, with sclerotized heads and powerful jaws, which pierce the roots of plants. Pupae are creamy white and exarate (Aguilera & Rebolledo, 2001; Parra et al., 2009a; Aguilera et al., 2011). The *A. superciliosus* adults are dark gray to black and their body is of rugged texture with leg treadmills of 10 mm long each. The head is spherical, with sunken front and convex eyes. The mouthparts are at the apex of the rostrum and the jaws are articulated up and down. Their size is between 1 and 1.7 cm long (Aguilera, 1988; Cisternas et al., 2000; Parra et al., 2009a). In the case of *A. nodipennis*, the adults are matte black, and have a rectangular body, with white scales on the sides up to the first pair of legs. They have white scaly patches or violet blue in the apical part of the femurs. Their elongated body ranges from 1.80 cm long and 0.60 cm wide, and it also has legs of treadmill type (Aguilera et al., 2011).

With respect to their feeding habits, they are similar for both weevils in hazelnut tree. Larvae produce galleries in the main root and neck of their host, damage that is visible in summer with yellowing leaves and early defoliation of branches (Figure 2 A, B). When spring begins, adults emerge and rise to the top of the tree to feed with green twigs and bark of the year, causing a drill which provokes the breakdown of the twigs due to summer winds (Figure 2 C, E). Then, when leaves fall, the adults feed on the catkins (Figure 2 D, F) (Aguilera, 2005). The *A. superciliosus* adults can be found from October to March, although their presence is reported during the whole year. Larvae remain in their host from March to December, being the longest stage of the insect (86% of their life cycle). In the case of *A. nodipennis*, its biological cycle has not been reported (Aguilera, 2005; Aguilera et al., 2011).

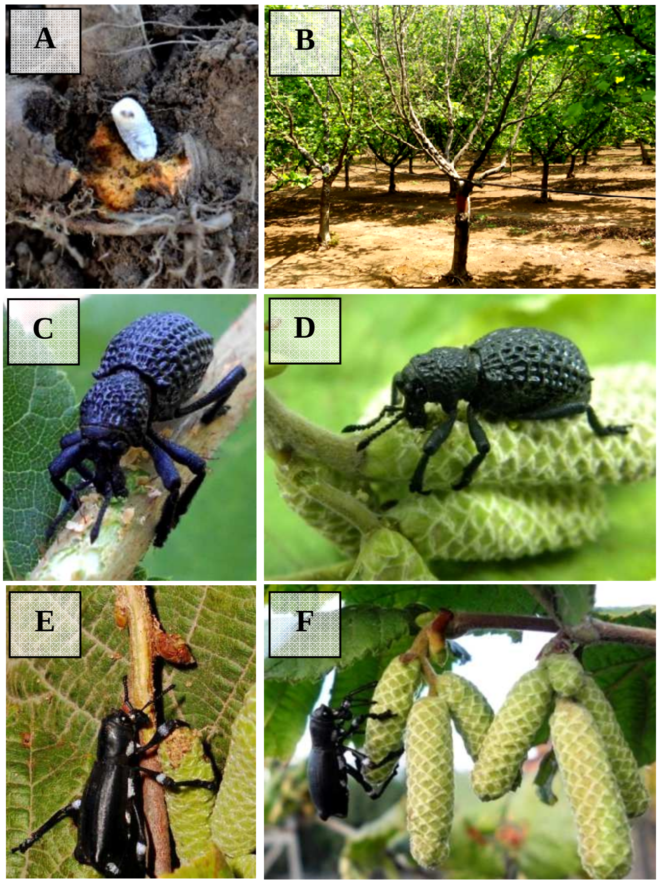

Figure 2. A) Damage of larvae in root of hazelnut B) hazelnut tree defoliated in summer, C) damage of *A. superciliosus* adults in bud of hazelnut, D) damage of *A. superciliosus* adults in catkins of hazelnut, E) damage of *A. nodipennis* adults in bud of hazelnut and F) damage of *A. nodipennis* adults in catkins of hazelnut.

Currently, the main control method for these weevils is guided towards the adult stage of the insects applying insecticides of carbamate family, such as carbofuran and carbaryl (Carrillo, 1993) and of the organophosphate family, such as diazinon and azinfosmetil (Aguilera, 1988; Guerrero & Aguilera, 1989). However, these insecticides have no action on the larval stage, which remains underground in the roots (Aguilera, 1995). The use of the same products applied on *A. superciliosus* has been suggested for *A. nodipennis* (Aguilera et al., 2011).

These insecticides can enter into the body of the insects through inhalation of vapors, gastrointestinal absorption and penetration through the cuticle and mucous membranes (Badii & Varela, 2008) having their toxic action at synaptic level, inhibiting the acetylcholinesterase activity (Legaspy, 1986). Several studies report the high risks on human health and the mammals of this type of insecticides. With respect to their effects on the environment, their half-life in the soil is from 60 to 90 days, affecting water resources, beneficial insects, such as bees and produce residues on harvested fruit (Alpuche, 1990; Parra et al., 2009). Moreover, Roush & McKenzie (1987) indicated that resistance development is given by loss of pesticide effectiveness, which entails increased application frequencies, dosage and costs.

On the other hand, alternative methods such as biological, natural and mechanical control have been described for the *Aegorhinus* species control. However, their use presents diverse inconveniences. There is a limited action in the biological control, because of biotic and abiotic factors (France et al., 2000). In the natural control there are a few natural enemies (Carrillo, 1993) and the mechanical control requires a high demand of manpower (Aguilera, 1995), indicating the chemical control as the most effective method to control these insects. To deal with this situation it is necessary to seek control alternatives that lead to an integrated pest management (MIP) through products of natural origin that do not harm other species, being highly specific and biodegradable in the environment.

Natural products are an alternative to synthetic pesticides as a means to reduce negative impacts to human health and the environment (Isman & Machial, 2006). Many essential oils have shown a broad spectrum of activity against pest insects and are recognized as an important natural source of pesticides. Similar to the synthetic insecticides, the compounds of the essential oils exert their activities through neurotoxic effects involving several mechanisms of action (Regnault-Roger et al., 2012). Therefore, this is a real alternative for insect control and their rapid degradation and specificity, favoring benefic insects and the environment (Pillmoor et al., 1993). According to literature, the biopesticidal potential of essential oils is associated with plants of Myrtaceae, Lamiaceae, Asteraceae, Apiaceae and Rutaceae families, which have been focus of research against specific insects of Lepidoptera, Coleoptera, Diptera, Isoptera and Hemiptera orders (Tripathi et al., 2009).

Repellency and attraction are two biological properties elicited by essential oils, where the term repellent corresponds to substances that act locally or at a distance, deterring an insect from flying to, landing on or biting human or animal skin whereas the term attractant corresponds to a positive displacement movement of the receptor organism (Sendi & Ebadollahi, 2014). For this study, we selected six non-host plant essential oils of both weevils that grown in the Región de La Araucanía, Chile. These species have been reported by their repellent or attractant properties in other pest insects, besides, in their chemical composition, they have bioactive compounds for *Aegorhinus* spp. Eucalyptus essential oils have been described as natural pesticides (Batish et al., 2008) and as attractant to *Anastrepha ludens* (Diptera: Tephritidae) (Robacker, 2007). Similarly, the *Foeniculum vulgare* oil was repellent to *Aedes aegypti* (Diptera: Culicidae) (Kim et al., 2002) and attractive to *A. ludens* (Robacker, 2007), whereas *Thymus vulgaris*, *Ruta chalepensis*, *Achillea millefolium* and *Peumus boldus* essential oils were repellent and insecticides on *Lasioderma serricorne* (Coleoptera: Anobiidae) (Hori, 2003), *Aedes albopictus* (Diptera: Culicidae) (Conti et al., 2013), *A. aegypti* (Thorsell et al., 1998) and *Sitophilus zeamais* (Coleoptera: Curculionidae) (Betancur et al., 2010), respectively. Thus, in the present thesis we focused on the repellent and attractant properties of non-host plant essential oils of *Aegorhinus* spp. outlined above, with the purpose of determining if they may interfere effectively in the behavior of *A. nodipennis* and *A. superciliosus*, as an alternative of monitoring and controlling these insects (Koul et al., 2008).

Hypothesis and research objectives

Hypothesis

The use of essential oils and pure compounds isolated from non-host plants of *Aegorhinus* spp. is a potential alternative for monitoring and controlling these weevils.

Research objectives

General objective

To evaluate the attractant or repellent activity of essential oils and pure compounds from non-host plants on *Aegorhinus* spp. in laboratory bioassays

Specific objectives

To identify essential oils and pure compounds eliciting a better attractant effect on *Aegorhinus* spp. in laboratory bioassays

To identify essential oils and pure compounds eliciting a repellent effect of *Aegorhinus* spp. in laboratory bioassays

CHAPTER II

Repellent Effect and Metabolite Volatile Profile of the Essential Oil of *Achillea millefolium* Against *Aegorhinus nodipennis* (Hope) (Coleoptera: Curculionidae)

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Repellent Effect and Metabolite Volatile Profile of the Essential Oil of *Achillea millefolium* Against *Aegorhinus nodipennis* (Hope) (Coleoptera: Curculionidae)

J Tampe [1,2], L Parra [1], K Huaiquil [1], A Mutis [1], and A Quiroz [1]

[1]Laboratorio de Química Ecológica, Departamento de Ciencias Químicas y Recursos Naturales, Universidad de La Frontera, Temuco, Chile.

[2]Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Casilla 54-D. Temuco, Chile.

Abstract

Aegorhinus nodipennis (Hope) (Coleoptera: Curculionidae) is an important native pest in fruit crops that is mainly found in European hazelnut fields in the south of Chile. We investigated the behavioral response of *A. nodipennis* to volatile compounds released from the essential oil of *Achillea millefolium* and its main constituent using olfactometric bioassays. Gas chromatographic and mass spectral analysis of the *A. millefolium* essential oil revealed the presence of 11 compounds. Monoterpene β-thujone (96.2%) was the main component of the oil. Other compounds identified were α-thujone, 1,8-cineole, *p*-cymene, and 4-terpineol, all with percentages below 1%. Both *A. millefolium* essential oil and thujone exhibited a repellent activity against this insect at higher doses tested (285.7 ng/cm^2), demonstrating their potential as repellents for this species.

Key words: *Achillea millefolium,* essential oil, repellent, *Aegorhinus nodipennis*.

Introduction

Aegorhinus nodipennis (Hope) (Coleoptera: Curculionidae) is a polyphagous weevil that is native to South America and is one of the three main fruit pests in Chile, following *Aegorhinus phaleratus* (Erichson) and *Aegorhinus superciliosus* (Guérin) (Zavala et al., 2011). This insect is associated with many vegetal species, such as *Nothofagus dombeyi* (its main host native), *Nothofagus pumilio*, and *Nothofagus obliqua* (Fagales: Nothofagaceae). In addition, the weevil successfully colonizes introduced species, such as *Betula pendula* and *Corylus avellana* (Fagales: Betulaceae), with the latter being its main introduced host plant (Klein & Waterhouse, 2000; Aguilera et al., 2011). Adult female weevils oviposit near the neck of the host tree, mainly under the soil surface. Thus, neonates move to the root system, drilling into the main root, forming galleries and severely damaging their host, which can lead to plant death. Next, the larvae cover the galleries with sawdust and feces to complete their pupal development. Once adults emerge, they feed on the leaves and shoots of the season, thereby affecting vegetal growth (Aguilera et al., 2011). So far, the application of synthetic insecticides (organophosphates and carbamates) has not been able to prevent the damage caused by these weevils (Parra et al., 2009a). Moreover, the use of insecticides involves the selection of resistant populations, environmental problems, health risks, and residues on harvested fruits (Pimentel et al., 1992; Prabakar & Jebanesan, 2004). Thus, new methods based on the use of essential oils (EOs) have been considered as an alternative that can contribute to the reduction of the pest population because they are safer to use, ecofriendly, biodegradable, and more compatible with the environmental components than conventional pesticides (Kalita et al., 2013).

Essential oils are complex mixtures of volatile organic compounds that are classified as secondary metabolites in plants (Guenter, 1972). In general, a large number of EOs extracted from different plant families have been demonstrated to exhibit high repellency against several arthropod species (Isman, 2000; Isman & Machial, 2006), especially stored grain insects (Coleoptera) (Nerio et al., 2009, 2010; Licciardello et al., 2013; Olivero-Verbel et al., 2013). In this context, the *Achillea* genus, with over 120 species described, is one of the most important members of the family Asteraceae. Species of this genus have been widely used in folk medicine due to their ethno pharmacology and phytochemical properties, mainly in species of the *Millefolium* group, such as *Achillea millefolium* (L.).

Commonly referred to as yarrow, *A. millefolium* is widespread in the Northern Hemisphere as well as in South American countries. In Chile, this species inhabits the area from the Región Metropolitana to the Región de Magallanes. *Achillea millefolium* EOs have been intensively studied (Nemeth, 2005; Benedek & Kopp, 2007; Santoro et al., 2007; Nemeth & Bernath 2008). The biological properties of EOs, such as antibacterial, antifungal, and insecticidal activities, have been investigated mainly in the last two decades (Barel et al., 1991; Magiatis et al., 2002; Başer et al., 2002; Simic et al., 2002; Nemeth & Bernath, 2008; Conti et al., 2010; Tozlu et al., 2011). Recent studies have revealed that their use as repellents is effective for controlling pest (Debboun et al., 2006; Nenaah, 2013). Similarly, *A. millefolium* EO was reported as a promising repellent against the aphid *Rhopalosiphum maidis* (Fitch) (Hemiptera: Aphididae) (Halbert et al., 2009), while *Achillea wilhelmsii* C. (Koch) and *A. millefolium* EOs have elicited repellency against *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), suggesting that the *Achillea* EOs are potential agents for pest control (Karahroodi et al., 2009). This background supports the study of *A. millefolium* as a prominent source of molecules with biological properties. Therefore, the aims of this work were to evaluate the repellent activity of yarrow EO against *A. nodipennis*, one of the most important European hazelnut pests in Chile, and to determine the *A. millefolium* EO metabolite profile by GC-MS.

Material and Methods

Insects. *Aegorhinus nodipennis* adults were manually collected from birch trees in the commune of Vilcún (38° 41′ S, 72° 25′ W) and from a commercial European hazelnut plantation in the commune of Gorbea (39° 05′ S, 72° 39′ W), Región de La Araucanía, in southern Chile, between November and October 2012-2013. Insects were collected at least 1 month before the behavioral experiments. During that period, the insects were acclimated and maintained under laboratory conditions in terms of photoperiod (10°C; 16-h photophase) and feeding (leaves and shoots of birch every 3 days and two sprays with distilled water daily). Twenty-four hours before each bioassay, the insects were maintained separately in individual Petri dishes and starved. Afterwards, the insects were observed for 10 min, and those that were active and walking were selected for the olfactometric assays. Each insect was used in a single bioassay (Parra et al., 2009b).

Essential oil. *Achillea millefolium* EO (100% purity) was purchased from Campestre Company, Temuco, Chile. The extraction protocol of EO used by Campestre included steam distillation for 90 min using both flowers and leaves collected from Curacautín (Chile) during the flowering period. The EO yield was 0.03% for 80 kg of plant material.

Chemical. A thujone standard mixture (~10% *β*-thujone basis and ~70% *α*-thujone basis) was obtained from Sigma Aldrich (Steinheim, Germany).

Analysis of the metabolite profile of EO by GC-MS. The identification of *A. millefolium* EO chemical components was performed using GC-MS (Focus DSQ, Thermo Electron Corporation). The separation of the compounds was performed using a capillary column BP-1 (30 m × 0.22 mm × 0.25 μm). Helium was used as the carrier gas (1.5 mL/min). The initial temperature of the oven was 40°C; the temperature increased at a rate of 5°C/min to 280°C and remained at this temperature for 5 min. The temperatures of both the injector and the interface were 250°C; the detector temperature was fixed at 200°C, and the electron impact ionization energy was 70 eV. Acquisition of each mass spectrum was performed in the mass range of 30 to 350 m/z. An aliquot of 1 μL of the commercial essential oil dissolved in dichloromethane was injected at a concentration of 1 μg/μL. The identification of the compounds was performed by searching a library of mass spectra, NIST (Mass Spectral Library Version 2.0), using a matching algorithm with a reverse search technique to verify that the highest peaks of the reference compounds were present in the essential oil mass spectra (Tapia et al., 2007). In addition, a comparison of their Kovats indices (KI) was performed by injecting an alkene series (C_9-C_{26}). The experimental KIs were compared with theoretical KI compounds reported in the database "NIST" (NIST ver. 2.0, Thermo) (Babushok et al., 2007).

Olfactometer bioassay. Behavioral studies were performed in a four-arm olfactometer described by Parra et al. (2009b). This olfactometer consists of three acrylic plates held together with metal screws; the top plate has a hole in the center to introduce the insect, which is subsequently placed in a vacuum adapter. Four holes in each of the four arms are connected to the glass tubes containing filter paper impregnated with the odor stimuli and the control. The olfactometry assay was conducted according to the procedure of Tapia et al. (2005), which consists of observing the movements of the insect into an area called the arena. The observed arena is divided into five sections: a central square zone and four zones corresponding to the arms, two of which were enriched with air containing volatiles from EO or thujone standard as stimuli, and the other two arms contained dichloromethane (HPLC grade, Sigma Aldrich, Steinheim, Germany) as a control. The samples were prepared by applying 50 μL of the EO or thujone standard diluted in dichloromethane on a Whatman N° 1 filter paper (0.5 cm wide by 3.5 cm long). *Achillea millefolium* EO was applied at a dose of 285.7 ng/cm^2 (N = 30), similar to the report by Parra et al. (2009b), whereas the main component, thujone (96.2%), was tested at three different doses (2.8, 28.5, and 285.7 ng/cm^2) (N = 10/dose). The filter paper impregnated with chemical stimuli was placed in a glass tube (7 cm long × 1.5 cm i.d.) in the respective arm of the olfactometer. The insect was placed in the center of the olfactometer, and an air flow (800 mL/min) was generated for carrying the volatile stimuli into the olfactometer. The olfactometric response of each insect to the stimulus was registered for 20 min, and the time spent in each arm was processed by EthoVision 3.1 software (Noldus et al., 2002). The time spent by *A. nodipennis* in the stimulus (yarrow EO/thujone) or the control arms of the olfactometer was compared using the non-parametric Friedman test ($P < 0.05$).

Results

GC-MS analysis of the essential oil of *A. millefolium*

GC-MS analysis indicated the presence of 11 volatile constituents of *A. millefolium*, with *β*-thujone as the main component (96.2%), followed by *α*-thujone (1.2%) (Fig 1). The other compounds identified were 1,8-cineole, *p*-cymene, 4-terpineol, carvotanacetone, *E*-caryophyllene, and *epi*-bicyclosesquiphellandrene, among others, each with percentages below 1% (Table 1).

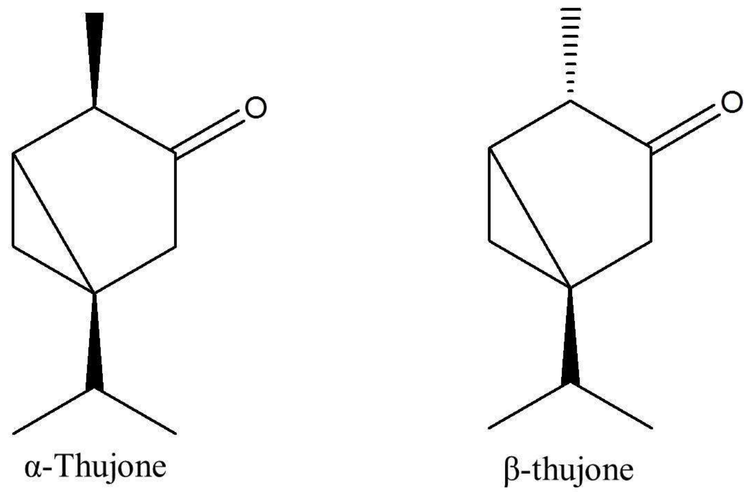

Figure 1 Chemical structure of *α*- and *β*-thujone, both of which are available in the essential oil of *Achillea millefolium.*

Table 1 Chemical composition (%) of *Achillea millefolium* EO

RT	Constituents	KI		*A. millefolium* EO	
		Exp.[a]	Lib.[b]	Area [c] Percentage	Identification [d]
9.61	*p*-Cymene	1013	1013	0.4	I, MS
9.76	1,8-Cineole	1019	1019	0.4	I, MS
11.70	*α*-Thujone	1087	1087	1.2	I, MS, Co-GC
12.22	*β*-Thujone	1104	1103	96.2	I, MS, Co-GC
13.86	4-Terpineol	1164	1164	0.1	I, MS
15.51	Carvotanacetone	1222	1221	0.7	I, MS
19.52	Unidentified	1373	-	0.1	MS
20.55	*E*-Caryophyllene	1412	1412	0.1	I, MS
22.01	*epi*-Bicyclosesquiphellandrene	1472	1471	0.1	I, MS
22.99	*δ*-Cadinene	1511	1511	*t*	I, MS
24.15	Spathulenol	1562	1562	*t*	I, MS
25.75	14-Methyloxacyclotetradecan-2-one	1631	1643	0.1	I, MS
26.20	Unidentified	1652	-	*t*	MS

Notes: [a] Exp. BP-1 column. [b] Lib. NIST Database.

[c] *t* = Trace < 0.1%.

[d] I = Retention index, MS= mass spectrum, Co-GC= co-injection with authentic compound.

Table 1.1 IUPAC names of the compounds from *Achillea millefolium* EO

Common Name	**IUPAC Name**
p-Cymene	1-methyl-4-(1-methylethyl)-benzene
1,8-Cineole	1,3,3-trimethyl-2-Oxabicyclo[2.2.2]octane
α-Thujone	[1*S*-(1*α*,4*α*,5*α*)]-4-methyl-1-(1-methylethyl) Bicyclo[3.1.0]hexan-3-one
β-Thujone	[1*S*-(1*α*,4*β*,5*α*)]-4-methyl-1-(1-methylethyl) Bicyclo[3.1.0]hexan-3-one
4-Terpineol	4-methyl-1-(1-methylethyl)-3-Cyclohexen-1-ol
Carvotanacetone	(*S*)- 2-methyl-5-(1-methylethyl)-2-Cyclohexen-1-one
E-Caryophyllene	(*E*)-(1*R*,9*S*)-(-)-4,11,11-trimethyl-8-methylene-Bicyclo[7.2.0]undec-4-ene
epi-Bicyclosesquiphellandrene	1-Isopropyl-4-methyl-7-methylene-1,2,3,4,4*α*,5,6,7-octahydronaphthalene
δ-Cadinene	[1*S*,cis]-Naphthalene,1,2,3,5,6,8*α*-hexahydro-4,7-dimethyl-1-[1-methylethyl]
Spathulenol	(1*αR*,4*αR*,7*S*,7*αR*,7*βR*)-1,1,7-Trimethyl-4-methylenedecahydro-1H-cyclopropa[e]azulen-7-ol
14-Methyloxacyclotetradecan-2-one	14-methyloxacyclotetradecan-2-one

Olfactometer bioassay with *A. millefolium* essential oil

The results indicated that the average time spent by the insects in the stimulus source was significantly lower (6.9 ± 0.8 min) than in the control (10.6 ± 0.9 min) (F = 23.09; df = 2; P = 0.0458). In addition, the behavior of *A. nodipennis* exhibited a significant response to the decision zone respect to stimulus and control (P < 0.0001) (Fig 2).

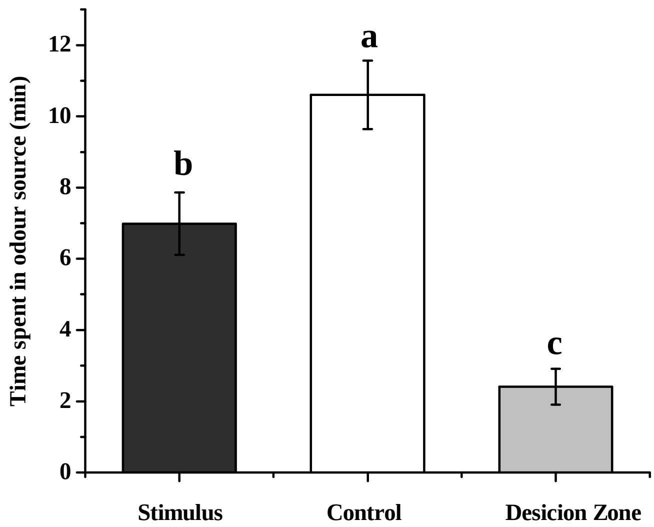

Figure 2 Average time spent (min) (± SE) by *Aegorhinus nodipennis* on yarrow essential oil (285.7 ng/cm^2) during the olfactometer test. Different letters indicate significant differences ($P \leq 0.05$) based on the non-parametric Friedman test, followed by the Conover-Inman test. S= Stimulus, C=Control and DZ=Decision zone. N=30.

Olfactometer bioassay with thujone standard

From the three doses tested, only the highest dose caused repellency (control 14.4 ± 1.2 min/stimuli 4.65 ± 1.2 min) (285.7 ng/cm^2, F = 24.33; df = 2; P = 0.0105). In contrast, the lowest doses (28.5 ng/cm^2, F = 4.65; df = 2; P = 0.1025; and 2.8 ng/cm^2, F = 20.49; df = 2; P = 0.848) were not significantly different compared with the control. However, the response to a dose of 2.8 ng/cm^2 was different compared with the decision zone (P < 0.0001) (Fig 3).

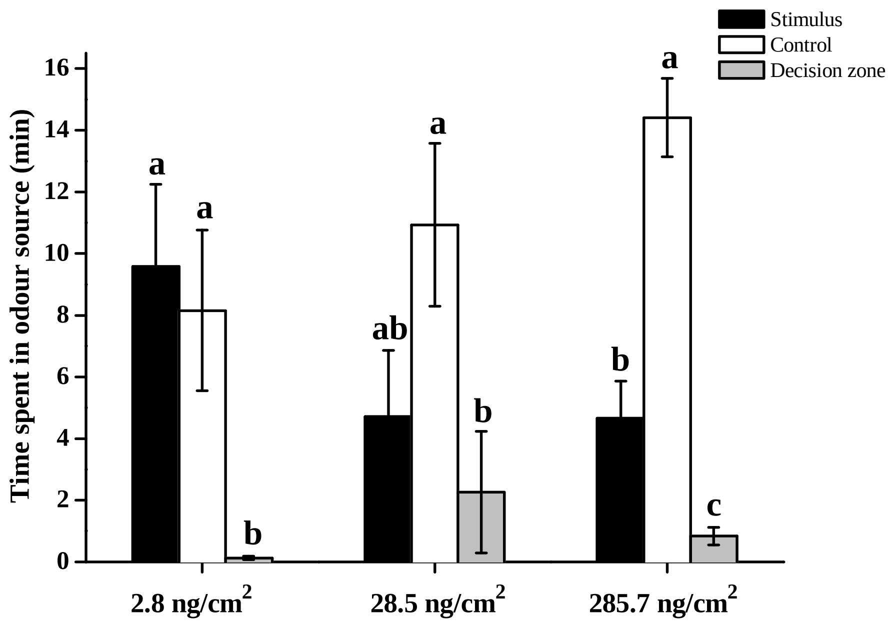

Figure 3 Average time spent (min) (± SE) by *Aegorhinus nodipennis* on the thujone standard mixture during the olfactometer tests performed using three different doses (2.8, 28.5, and 285.7 ng/cm^2). Different letters indicate significant differences based on the non-parametric Friedman test ($P \leq 0.05$), followed by the Conover-Inman test. S= Stimulus, C=Control and DZ=Decision zone. N=10.

Discussion

Eleven volatile compounds were identified in the EO obtained from *A. millefolium*, with *β*-thujone (96.2%) being the main constituent. Our results differ in chemical composition from those obtained from *A. millefolium* grown in Estonia, Kosovo and India. EOs from these localities consisted of 66, 33, and 30 components; and *β*-pinene (14.9-29.2%), 1,8-cineole (22%), and sabinene (17.58%) were reported as their main components of *A. millefolium* EO grown in Estonia, Kosovo and India, respectively (Orav et al., 2001; Haziri et al., 2010; Nadim et al., 2011). On the other hand, the volatile composition of *A. millefolium* from Canada was similar to the one we reported, but the content of *β*-thujone was much higher in our study (96.2 v/s. 21.7%) (Hachey et al., 1990). Similarly, high contents of *α* and *β* thujone (0-26.6% and 0-11%) were found in three *A. millefolium* EOs obtained from Belgium, Russia, and Uural. Other compounds, such as 1,8-cineole (0.8-20.3%), *p*-cymene (0.2-5.6%), 4-terpineol (0.2-4.0%), and *E*-caryophyllene (0-12%) were similar to our EO, but at higher percentages (Table 1) (Orav et al., 2007). The differences observed in the composition of *A. millefolium* can be attributed to biotic and abiotic factors during the development of the plant (Perry et al., 1999; Isman & Machial, 2006; Nemeth & Bernath, 2008; Harb & Mahmoud, 2009). All the oils discussed above were obtained from plants that grew in different continents therefore being exposed to different biotic and abiotic conditions (Hachey et al., 1990; Orav et al., 2001, 2007; Haziri et al., 2010; Nadim et al., 2011).

Our results demonstrated that the EO of *A. millefolium*, which was composed mainly of monoterpenes, was repellent to the adults of *A. nodipennis* (Fig 2). The repellency observed could be attributed to the EO major constituent, β-thujone. Similar results were obtained in bioassays realized with thujone standard (~10% β-thujone and ~70% α-thujone), where the repellency could be influenced by the interaction with its isomer α-thujone (Fig 3). Generally, β-thujone is less toxic than α-thujone, and the content of β-thujone is often higher than that of α-thujone (Rice & Wilson, 1976), which is consistent with our results. Similarly, Hwang et al. (1985) reported that α-β thujone functioned as repellents to the yellow fever mosquito *Aedes aegypti* (L.) (Diptera: Culicidae) at doses higher ($\geq$ 0.28 mg/cm^2) than the three doses used in our study. The insecticidal properties of Thujone have also been demonstrated against *Corythucha ciliata* (Say) (Hemiptera: Tingidae) (Rojht et al., 2009), and the insecticidal activities of the EOs of *Lippia turbinata* and *Lippia polystachya* against *Culex quinquefasciatus* (Say) (Diptera: Culicidae) have been attributed to their high thujone concentration (48 and 69%, respectively) (Gleiser & Zygadlo, 2007). There are several indications that the insecticidal and repellent properties of EOs are associated with the presence of terpenes (Coats et al., 1991; García et al., 2005; Kiran & Devi, 2007; Abdel-Sattar et al., 2010), which are related to the plant defense mechanisms against generalist and specialist insects (Langenheim, 1994). Terpenes can be perceived by odorant binding proteins (OBPs) located in the sensilla of insects and are involved in the transmission of airborne chemical signals from plants to insects (Regnault-Roger et al., 2012).

Monoterpenes present in EOs were reported to act by a reversible competitive inhibition on acetylcholinesterase in house fly, cockroach (Grundy & Still, 1985; Ryan & Byrne, 1988; Miyazawa et al., 1997) and stored product insects (Kostyukovsky et al., 2002). In addition, visible symptoms, such as hyperactivity, convulsions, and tremors, followed by paralysis indicated a neurotoxic mode of action for these molecules (Coats et al., 1991; Abdelgaleil et al., 2009). The octopamine receptors can also be considered as a primary target for some monoterpenoids (Evans & Robb, 1993). *α*-terpineol was demonstrated to have a high binding affinity with the octopamine receptor in the American cockroach (Enan, 2001; Enan, 2005). Moreover, 1,8-cineole was reported to act as an acetylcholinesterase inhibitor and blocked the octopamine receptor pathway in protein models of *A. aegypti* (Linnaeus) (Diptera: Culicidae), determining that their mode of action is mediated through both mechanisms (Khanikor et al., 2013). Likewise, the union with gamma amino butyric acid (GABA)-gated chloride channels was suggested (Priestley et al., 2003). GABA is the most important inhibitory neurotransmitter in the central nervous system of insects. Terpenes interfere with GABA-gated chloride channels, provoking hyper-excitation, convulsions, and insect death due to neuronal inhibition (Bloomquist, 2003; Priestley et al., 2003). The *α*-thujone was reported to be a potent neurotoxin affecting the GABA system (Höld et al., 2000), being classified as a neurotoxic insecticide (Ratra et al., 2001; Duke, 2004). Therefore, it is suggested that EOs comprise a mixture of metabolites that interfere with the insect physiology through different mechanisms and at various target sites. The repellency produced by the thujone mixture on *A. nodipennis* may be largely influenced by the isomer *α*-thujone and could be a consequence of the neurotoxicity produced by this monoterpene. Detailed studies are required to determine the specificity of these compounds and their mode of action in curculionids. In the present study, our results provided a first report on the potential of the EOs of *A. millefolium* and the isomers *α-β* thujone as repellent to *A. nodipennis,* an important curculionid pest in southern Chile. Moreover, the information generated in this research will allow a better understanding of the chemical ecology of this insect and its interaction with secondary metabolite terpenes. Future work should aim to evaluate the synergistic effects among the constituents of EOs, determine the electrophysiological response of *A. nodipennis* to the individual constituents of yarrow EOs, and evaluate the mode of action of thujone using molecular and biological techniques.

Acknowledgments

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CHAPTER III

Antennal Response of Weevil *Aegorhinus nodipennis* (Hope) (Coleoptera: Curculionidae) to Thujone Monoterpene

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Antennal Response of Weevil *Aegorhinus nodipennis* (Hope) (Coleoptera: Curculionidae) to Thujone Monoterpene

J Tampe[1, 2], L Parra[1] and A Quiroz[1]

[1]Laboratorio de Química Ecológica, Departamento de Ciencias Químicas y Recursos Naturales, Universidad de La Frontera, Temuco, Chile. [2]Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Casilla 54-D. Temuco, Chile.

*E-mail: andres.quiroz@ufrontera.cl

Abstract

Electroantennographic responses were recorded from the weevil, *Aegorhinus nodipennis* Hope (Coleoptera: Curculionidae) to the thujone monoterpene. Seven tested doses showed a dependent-dose response, where the highest EAG response (1.6 to 2.2 mV) was found in the highest doses (2.86 $x10^2$ to 10^4 ng/cm^2) and generally weak responses (0.07 to 0.6 mV) at the lower doses. The EAG method allowed us identify thujone as a biologically active compound for *A. nodipennis*, able to be used in a possible control strategy for this weevil.

Keywords: EAG, *Aegorhinus nodipennis*, thujone, repellent.

Introduction

Insect behavior is regulated by volatile stimuli that are detected by olfactory sensory neurons (OSNs) found mainly in the antennae and maxillary palps (Andersson et al., 2012). In order to know to which compounds insects respond, the electroantennographic techniques have been widely used. They recorded the potential changes of the antennae as a result of chemical stimulation produced by varieties of odorants present in the environment (Chung-Park, 2002), and delivered information on the sensitivity and specificity during the activation of olfactory receptors (Beck et al., 2012). In this way, parameters are established for the olfactory investigation and to develop alternative control methods based on the manipulation of the behavior through the identification and description of behavioral relevant molecules for the insects (Martin et al., 2011).

Recently, some essential oils of the Lamiaceae, Asteraceae and Cupressaceae families have showed a high biological and insecticidal activity due to the presence of thujone in large amounts (over 44%) (Perry et al., 1999; de la Cruz et al., 2008; Srivastava et al., 2012). Thujone is a monoterpene ketone presenting two stereoisomers (*α*- and *β*-thujone) and naturally, both isomers are present as a mixture (Szolyga et al., 2014). The content of *β*-thujone often exceeds content of *α*-thujone depending on the plant source, but the *β*-thujone is generally of lower toxicity than *α*-thujone, which has been described as a potent neurotoxin (Höld et al., 2000) and it is classified as a neurotoxic insecticide (Ratra et al., 2001; Duke, 2004).

In this context, we have demonstrated that the main component of essential oil isolated from *Achillea millefolium,* thujone, was repellent against *Aegorhinus nodipennis* (Hope) (Coleoptera: Curculionidae) an important native pest of fruit crops in the south of Chile (Tampe et al., 2015). Therefore, this short communication has the aim of evaluating the antennal sensitivity of *A. nodipennis* to several concentrations of thujone for determining the bioactivity of this compound.

Material and Methods

Insects. *A. nodipennis* adults were manually collected from birch trees in the commune of Vilcún (38°41′S, 72°25′W), Región de La Araucanía, in southern Chile, between September and December 2014, according to description of Tampe et al. (2015).

Electroantennographic assays (EAGs). Dose-response recordings of the antenna of *A. nodipennis* adults were conducted using the thujone standard mixture (~ 10% *β*-thujone basis and ~ 70% *α*-thujone basis) which was purchased from Sigma Aldrich (Steinheim, Germany).The antennal sensitivity of *A. nodipennis* was determined by EAG, according to the methodology described by Mutis et al. (2010). The antennae were excised from their heads and were mounted between two gold wire electrodes using glass capillary tubes filled with Dicardio-gel (Difem Pharma). A volume of 50 µL standard solutions diluted in hexane at seven doses (2.86 x 10^{-2} to 10^{4} ng/cm^2) of thujone was placed on a piece of filter paper (1.75 cm^2) and exposed to air for 20 s to allow solvent evaporation, and then, it was positioned inside Pasteur pipette. The control was 50 µL of hexane, also impregnated in filter paper. During electrophysiological recordings, the release of the compound from pipette was performed in pulses of 1 s with continuous airstream of 500 mL/min^{-1}. The signals generated by the antennae were passed through a high impedance amplifier 10x (model Syntech, Hilversum, The Netherlands) and displayed onto a monitor using Syntech version 2.2a software for processing EAG signals. Each dose was tested on 10 different antennae using one antenna per weevil. The stimulation series consisted of the injection of thujone followed by hexane as control, repeating the series for each antenna three times. An interval of 40 s between puffs was waited to ensure antennal recovery. The EAG response elicited from hexane was subtracted from the responses obtained by the stimuli in order to normalize the responses and obtain the net response to thujone standard.

Data analysis. The EAG responses to different doses of thujone were expressed as average from three consecutive puffs and analyzed using non-parametric statistics with the Kruskal-Wallis test followed by the Dunn test ($P < 0.05$).

Results

Electroantennographic response of *A. nodipennis*

The antennae of *A. nodipennis* adults showed responses to all tested thujone doses. Significant differences in the EAG responses were observed among the three highest doses (2.86 x 10^2 to 10^4) respect to lower doses (2.86 x 10^{-2} to 10^0) (F = 34.15; df= 6; $P \leq 0.05$). The highest EAG response was recorded at the 2.86 x 10^3 ng/cm^2 dose with 2.2 ± 0.15 mV, then, the EAG response (1.62 ± 0.18 mV) declines with higher doses (2.86 x 10^4 ng/cm^2). The lowest EAG response was recorded at 2.86 x 10^{-2} ng/cm^2 (0.07 ± 0.07 mV) and subsequently the response grew according to the increase of the applied dose (Fig 1).

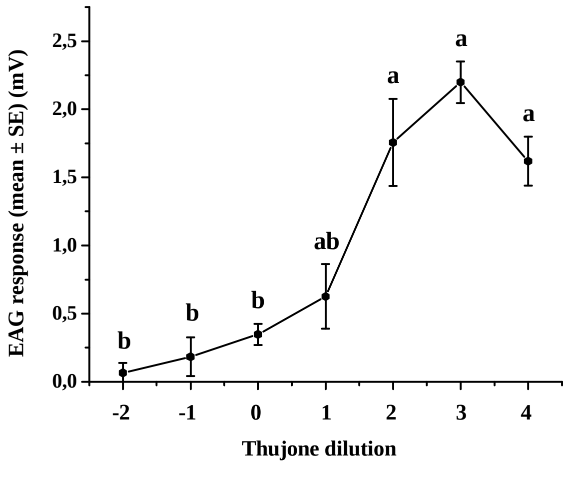

Fig 1 EAG dose-response curves of the antennae of *Aegorhinus nodipennis* adults to seven concentrations of thujone standard. EAG amplitudes of stimulus were adjusted to the control (hexane) and were given as means ± SE. The X axis describes the thujone dilution from 2.86 x 10^{-2} to 10^4 ng/cm^2 (w/v). Each dose was tested on ten individuals.

Discussion

The electroantennographic techniques have demonstrated that curculionids phytophagous, such as *A. nodipennis* have the capacity of detecting certain monoterpenes olfactively. In our study, the EAGs recorded from thujone evoked response (0.07 to 2.20 mV) at all the doses tested on antenna of this insect (2.86 $x10^2$ to 10^4 ng/cm^2), which would be perceived by neuron receptors located mainly on the club of the antenna of the weevils (van Tol et al., 2002). Similarly, Mutis et al. (2010) showed that other curculionid, *Aegorhinus superciliosus*, also have olfactory receptors for monoterpenes at the same doses evaluated in our study (2.86 x 10to 10^2 ng/cm^2), supporting the technique and doses used in this research. In addition, Barata et al. (2000) proposed a classification for the electroantennographic responses of weevil, *Ceutorhynchus assimilis* (Coleoptera: Curculionidae) ($\leq$ 0.3 mV = small response, 0.3 to 0.5 mV = medium response and $\geq$ 0.5 mV = large response), indicating that our results are according to those reported in the literature. Furthermore, our data suggest that the antennae of *A. nodipennis* possess large numbers of receptors and a high sensitivity to thujone, due to the intensity of EAG observed (increase in response) that can be taken into account as a measure of the relative number of responding receptor cells (Payne, 1975).

The repellent properties described for thujone has been reported by several authors. The antennal response of *Cimex lectularius* (L.) (Hemiptera: Cimicidae) to chemical and botanical repellents, showed that the insect was sensitive to derived terpenes, such as (-)-*α*-thujone, where the response of the olfactory sensilla to these compounds were dose-dependent (Liu et al., 2014). Similar observations were reported in the olfactory receptor neurons (ORNs) of *Culex quinquefasciatus* (Say) (Diptera: Culicidae) which were stimulated in a dose-dependent manner with 0.1, 1 and 10 µg of α + β-thujone, doses higher than the used in our study (Syed & Leal, 2008). Previously, we reported thujone as repellent for *A. nodipennis* at doses of 2.86 x 10^2 ng/cm^2 (Tampe et al., 2015). However, we had no knowledge of the olfactory sensitivity of the insects to this compound. Our results demonstrate clearly that the weevil showed a strong and consistent response to thujone at the same dose reported as repellent. Nonetheless, a lower dispersion of the values (mV) obtained at the dose of 2.86 x 10^3 ng/cm^2 suggests us a more homogeneous perception of thujone and maybe a more effective repellent response (Sánchez-Osorio et al., 2007). This study provides the first evidence of electroantennographic responses of *A. nodipennis* to a monoterpene reported as repellent. Therefore, the use of natural compounds could result in the development of a possible botanical insecticide allowing decrease of the use of synthetic insecticides, thus reducing the human health risk and environmental through alternative control strategies for weevils.

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CHAPTER IV

Potential Repellent Activity of the Essential Oil of *Ruta chalepensis* (Linnaeus) from Chile against *Aegorhinus superciliosus* (Guérin) (Coleoptera: Curculionidae).

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Potential Repellent Activity of the Essential Oil of *Ruta chalepensis* (Linnaeus) from Chile against *Aegorhinus superciliosus* (Guérin) (Coleoptera: Curculionidae).

Jocelyne Tampe[1, 2,] Leonardo Parra[1], Karen Huaiquil[1] and Andrés Quiroz[1*]

[1]Laboratorio de Química Ecológica, Departamento de Ciencias Químicas y Recursos Naturales, Universidad de La Frontera, Temuco, Chile.

[2]Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Casilla 54-D. Temuco, Chile.

*E-mail: andres.quiroz@ufrontera.cl

Abstract

The objective of this study was to evaluate the repellent effect of the essential oil of the rue (*Ruta chalepensis*) against the weevil *Aegorhinus superciliosus,* an important pest of fruit crops in Chile. Rue essential oil was obtained by steam distillation, and its components were identified by GC-MS. Their effect on adult *A. superciliosus* insects was evaluated using four-arm olfactometric bioassays. The extraction process had a yield of 0.3% on a dry weight basis, and a chromatographic analysis showed the presence of nine compounds, which represented 89.3% of the total components. The major compounds were 2-nonanone (41.7%) and 2-undecanone (40.1%). Behavioral bioassays showed that the rue essential oil elicited a repellent effect against male and female *A. superciliosus* ($P \leq 0.05$) at a concentration of 1.92×10^7 ng/cm^2. However, at a lower concentration of the oil (285.7 ng/cm^2), only females were repelled ($P \leq 0.05$). The repellency observed against *A. superciliosus* could be attributed to high concentrations of both ketones, suggesting that rue essential oil can be considered as a potential repellent that could reduce the infestation of this weevil. The role of the compounds identified and the repellent activity of this evergreen shrub are discussed.

Keywords: Essential oil, *Ruta chalepensis*, *Aegorhinus superciliosus*.

Introduction

Ruta chalepensis (Rue) is an aromatic evergreen shrub that belongs to the family Rutaceae. It is native to the Mediterranean and is currently distributed worldwide (Akkari et al., 2015). In Chile, it is traditionally cultivated for its pharmacological uses; infusions of its fresh leaves are widely used as treatment for gastric disorders, headache and rheumatism, as well as for their diuretic, anti-inflammatory and anti-spasmodic properties. Analysis of the chemical composition of *R. chalepensis* extracts indicates that the leaves and stems contain alkaloids, phenols, flavonoids, amino acids, saponins and furocoumarins, some of which are responsible for the reported activities (Kacem et al., 2015). Furthermore, rue essential oil is a valuable source of active metabolites used in different industries, including cosmetics, perfumes and phytotherapy. Ketones, acyclic alkenes, monoterpenes hydrocarbons, sesquiterpenes, esters and aldehydes have been identified as the main chemical groups present in the essential oil, and the ketone 2-undecanone is a characteristic compound of the Ruta species (Haddouchi et al., 2013; Ferhat et al., 2014). These compounds are produced during secondary metabolism in the plants, and their synthesis and accumulation might vary by species (Conti et al., 2013). In addition, both intrinsic and environmental factors influence this process (Ferhat et al., 2014; Da Silva et al., 2014). Recent studies have revealed several biological properties of rue essential oil. Insecticidal activity has been the focus of great interest for the potential use of this oil as an alternative botanical pesticide (Conti et al., 2013). In fact, insecticidal effects against ticks, mosquitoes, curculionids pest of stored grain and moths (Bissinger & Roe, 2010; Majdoub et al., 2014; Akkari et al., 2015) and a repellent effect on *Cydia pomonella* Linnaeus (Lepidoptera: Tortricidae) and *Aedes aegypti* Linnaeus (Diptera: Culicidae) (Landolt et al., 1999; Tabanca et al., 2012) have been reported. Therefore, the bioactivity of rue essential oil would be useful for controlling pests of economic importance, such as the raspberry weevil, in Chile.

In Chile, *Aegorhinus superciliosus* or the raspberry weevil is a native insect and one of the most important pests of fruit crops, such as the European hazelnut (*Corylus avellana*) (Fagales: Betulaceae), blueberries (*Vaccinium corymbosum*) (Ericales: Ericaceae) and raspberries (*Rubus idaeus*) (Rosales: Rosaceae) (Aguilera et al., 2011). This polyphagous insect begins to colonize the host plant when female adults oviposit near the neck of a tree. Neonate larvae move to the radical zone to feed and continue their development within this zone, while the adult weevil feeds on seasonal leaves and shoots. Currently, the widespread use of synthetic insecticides, especially organophosphates and carbamates, has not been able to prevent the damage caused by these pests. Because the application of insecticides is associated with many problems, such as high cost, residues on harvested fruit, environmental degradation and resistance development (Parra et al., 2009b), there is growing interest in the search for new control alternatives. In this context, natural products as well as essential oils can be alternatives for pest control because they contain bioactive substances that have low toxicity and a lesser impact on human health and the environment (Regnault-Roger et al., 2012). Currently, no reports about the repellent effect of the rue essential oil in this curculionid exist. However, there is evidence that show the insecticidal potential of the essential oil of *Drimys winteri* on adult *A. superciliosus* (Rebolledo et al., 2012), indicating that essential oils can be effective alternatives for the control of these insects. Therefore, the main objective of this study was to evaluate the repellent efficacy of the *R. chalepensis* essential oil against *A. superciliosus*.

Materials and methods

Plant material and essential oil isolation procedure. The aerial parts of the rue plant were collected during the flowering stage (October 2012) from an experimental field located at the commune of Puerto Montt (41°28' South, 72°57' West) Región de Los Lagos, Chile. The plant identity was confirmed by a comparison of macroscopic and microscopic morphological characteristics to Flora de Chile and specimens in the Herbarium of the Universidad de Concepción (CONC), Chile. The extraction process for the rue essential oil followed the methodology described by Meccia et al. (2009). A sample of 1298 g containing rue leaves, stems and flowers was cut into small pieces and air-dried at room temperature for six days and then oven-dried for 24 h at 20ºC. A 250 g dry matter sub-sample was subjected to steam distillation in a Clevenger-type apparatus with 500 mL distilled water for 4 h. The oil was dried over sodium sulphate and stored at 4ºC in an amber bottle prior to chromatographic analysis.

Analysis of volatile compounds by GC-MS. Volatile compounds (1 µL) of *R. chalepensis* essential oil dissolved in dichloromethane (HPLC grade, Sigma Aldrich, Steinheim, Germany) were injected at a concentration of 1 µg/µL into a gas chromatograph (Focus GC; Thermo Electron Corporation) coupled to a mass spectrometer (DSQ; Thermo Electron Corporation). The capillary column was BP-1 (30 m x 0.22 mm x 0.25 µm) and used helium as the carrier gas at a flow rate of 1.5 mL/min. The injector and interface were operated at 250°C by setting the detector temperature at 200ºC, with an electron impact ionization of 70 eV. The initial oven temperature was programmed to 40ºC for 5 min and increased (5ºC/min) until 280ºC was reached. Mass spectroscopy was performed in a mass range from 30 to 350 *m/z*. Component identification was performed by searching a library (Mass Spectral Library Version 2.0) of mass spectra using a matching algorithm with a reverse search technique and by the injection of an alkene series (C_9-C_{26}) that was used as a reference for calculating the Kovats indices (KI). Experimental KI were compared with the theoretical KI of compounds reported in the NIST database (NIST ver. 2.0, Thermo), as described in Tampe et al. (2015).

Insects. *Aegorhinus superciliosus* adults were manually collected between November 2012 and February 2013 from a blueberry plantation in the commune of Collipulli (37º50' South, 72º08' West), La Araucanía, Chile. Both sexes of *A. superciliosus* were collected at least one month before being used in the olfactometric bioassays. During that time period, the insects were acclimated and maintained under laboratory conditions. They were fed blueberry leaves and shoots and distilled water, and the photoperiod was a 16 h photophase at 20°C. Twenty-four hours before each bioassay, the insects were separately maintained in individual Petri dishes (5 cm i.d and 2 cm height) according their sex and deprived of food. The methodology used to identify the sex of the insects, consisted of examining the femur length of the third pair of legs; if this length exceeds the posterior suture of the fourth sternite, the insect is male, and if it does not, the insect is female (Reyes, 1993). Then, the insects were observed for 10 minutes, and those that were active and walking were selected for the olfactometric assays. A different individual was used in each separate experimental replicate (Parra et al., 2009b).

Olfactometric bioassay. The olfactometric bioassays were performed in a four-arm olfactometer (40 by 40 by 2.5 cm), which was described by Parra *et al*. (2009b). The behavioral response of *A. superciliosus* to rue essential oil was studied according to the methodology described in Tampe et al. (2015), which consisted of an observation of the movement of each insect into the olfactometer during a 20 min period. In addition, EthoVision 3.1 software was used to determine the time the insect spent in each section of the olfactometer, which was divided into 5 areas: a central square zone where the vacuum bomb, with an air flow (800 mL/min) that carried the volatile stimuli into the olfactometer, was connected and four zones corresponding to the arms, two of which were enriched with air containing volatile components released from the essential oil (S), and the other two contained dichloromethane (HPLC grade, Sigma Aldrich, Steinheim, Germany) as a control (C). Bioassays were performed by applying 1 µL of rue essential oil on Whatman Nº 1 filter paper (0.5 cm wide by 3.5 cm long) that was placed into glass tubes (7 cm long × 1.5 cm i.d.) in two opposite arms of the olfactometer (S), while the two opposite arms of the olfactometer contained only paper (C). The dose used was 1.92×10^7 ng/cm^2. To evaluate the diluted oil, 50 µL of the oil diluted in CH_2Cl_2 was applied to Whatman Nº 1 paper filters in two opposite arm of the olfactometer (S) under the same conditions as in the previous bioassay, while the other two arms contained only dichloromethane (C). The dose used was 285.7 ng/cm^2 because it was previously determined as an effective repellent in behavioral bioassays in *A. nodipennis* (Tampe et al., 2015). Twenty repetitions were performed for each sex.

Statistical analysis. A statistical analysis was performed using Stats Direct software, version 2.7.2. The data obtained in the bioassays were expressed as the average time spent in each arm of the olfactometer ± standard error and were compared by using a non-parametric Friedman test ($P \leq 0.05$) followed by a Conover-Inman test.

Results

Analysis of volatile compounds by GC-MS

Steam distillation of the aerial parts of *R. chalepensis* yielded 0.3% (mL/g) of a yellowish color oil and strong odor. In the GC-MS analysis of the sample of rue essential oil, nine compounds were identified, and among them were ketones and esters (Table 1), representing 89.3% of the total components. The main constituents were the two aliphatic ketones 2-nonanone (41.7%) and 2-undecanone (40.1%). In addition, 7 unidentified compounds were present in the sample, representing 10.7% of the total components. The mass spectral data are shown in Table 2.

TABLE 1 Percentage of compounds identified in the *Ruta chalepensis* essential oil.

Rt	Compound	Identification	Percentage (%)
11.08	2-Octanone	MS, KI	0.51
14.34	**2-Nonanone**	**MS, KI**	**41.71**
15.84	Unknown 1	MS	0.5
16.04	Unknown 2	MS	2.76
16.80	Unknown 3	MS	0.73
17.27	2-Decanone	MS, KI	2.18
18.68	Unknown 4	MS	5.63
20.2	**2-Undecanone**	**MS, KI**	**40.11**
22.07	2-Dodecanone	MS, KI	0.97
25.41	2-Tridecanone	MS, KI	0.91
28.75	Unknown 5	MS	0.39
35.84	Unknown 6	MS	0.24
36.12	Ethyl palmitate	MS, KI	1.97
39.06	Unknown 7	MS	0.42
39.33	Ethyl oleate	MS, KI	0.22
39.77	Ethyl stearate	MS, KI	0.77

Rt= Retention time, MS= mass spectrum, KI= Kovats indices (KI) was performed by injecting an alkenes series (C_9-C_{26}). The experimental KIs were compared with theoretical KI compounds reported in the database “NIST”

Table 1.1 IUPAC names of the compounds from *Ruta chalepensis* EO

Common Name	**IUPAC Name**
2-Octanone	Methyl hexyl ketone
2-Nonanone	Methyl heptyl ketone
2-Decanone	Methyl octyl ketone
2-Undecanone	Methyl nonyl ketone
2-Dodecanone	Methyl decyl ketone
2-Tridecanone	Methyl undecyl ketone
Ethyl palmitate	Ethyl hexadecanoate
Ethyl oleate	(*Z*)-9-Octadecenoic acid ethyl ester
Ethyl stearate	Ethyl octadecanoate

Table 2 Mass spectral data of the unknown volatile compounds from *Ruta chalepensis*

Rt	Compound	(%)	*m/z*
15.84	unknown 1	0.5	32(100), 79(30.2), 43(19.9), 94(13.9), 41(10), 51(10.0), 77(9.9), 39(7.7), 105(6.3), 78(5.4), 40(5.3), 91(4.2), 93(4.0), 31(3.5), 70(3.4).
16.04	unknown 2	2.76	79(100), 94(49.3), 77(28.0), 51(23.7), 32(18.5), 39(16.6), 41(15.7), 93(12.3), 105(10.9), 91(8.8), 106(7.7), 53(7.4), 80(6.0), 147(5.8), 120(5.4).
16.80	unknown 3	0.73	128(100), 32(72.3), 51(34.2), 127(16.0), 50(14.8), 129(11.0), 126(9.5), 102(7.0), 101(6.0), 75(5.3), 64(4.6), 74(3.8), 40(3.79), 77(3.6), 76(3.4).
18.68	unknown 4	5.63	43(100), 41(16.2), 70(12.6), 55(11.5), 126(11.4), 69(10.8), 97(10.0), 87(9.4), 56(8.7), 42(7.1), 71(6.0), 98(4.6), 57(3.7), 84(3.7), 39(3.7).
28.75	unknown 5	0.39	157(100), 115(53.1), 43(41.6), 129(21.1), 203(19.6), 42(17.4), 111(13.7), 130(10.4), 31(9.9), 69(8.1), 158(7.6), 47(7.3), 32(6.7), 45(6.6), 56(5.6).
35.84	unknown 6	0.24	55(100), 41(94.2), 69(63.2), 97(53.8), 96(40.1), 70(36.7), 98(35.3), 101(33.9), 95(31.2), 83(30.4), 67(29.1), 73(24.5), 81(23.9), 68(23.6), 43(23.4).
39.06	unknown 7	0.42	32(100), 67(17.6), 41(15.4), 95(12.4), 55(11.3), 81(10.2), 69(9.5), 43(9.3), 96(9.2), 68(8.6), 54(6.3), 40(5.8), 109(5.7), 79(5.6), 97(5.5).

Rt = Retention time, *m/z* = mass-to-charge ratio

Olfactometer bioassay with *R. chalepensis* essential oil

The olfactometric response of *A. superciliosus* toward rue essential oil indicated that both females and males were repelled by the essential oil (P < 0.0001). The average time spent by the females in the stimulus source was significantly less (1.69 ± 0.62 min) than in the control (16.47 ± 0.7 min; F = 46.11; df =2; P < 0.0001) (Fig 1A). Similar behavior was observed in males; where the male insects spent less time in the stimulus (2.41 ± 1.0 min) than in the control (16.48 ± 1.0 min; F = 39.91; df =2; P < 0.0001) (Fig 1B). In bioassays using diluted rue essential oil (285.7 ng/cm^2; Fig 1C and 1D), males did not show a behavioral difference between the stimulus and the control (P > 0.05), while females were repelled by the tested dose of oil (F = 71.47; df = 2; P = 0.0105) remaining for an average of 11.21 ± 0.7 min in the control compared to 7.22 ± 0.6 min in the stimulus.

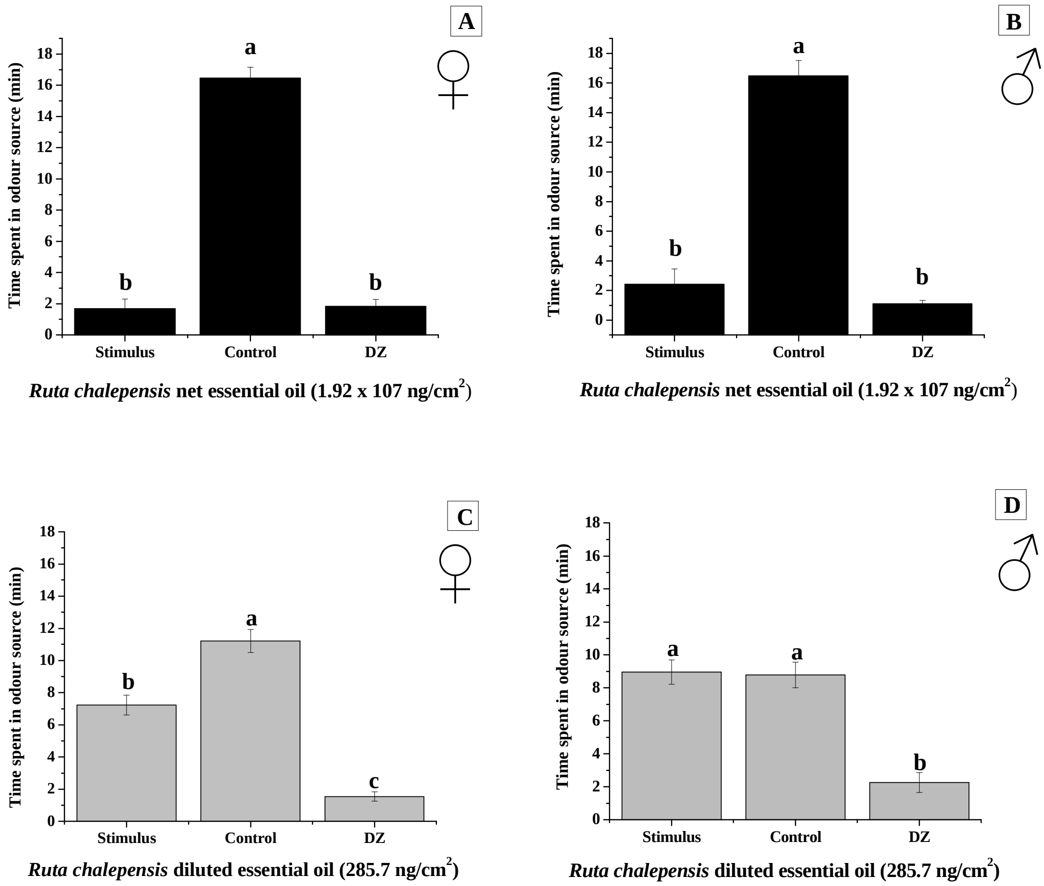

Fig 1 Average time spent (min) (± SE) of *Aegorhinus superciliosus* in rue essential oil (Figure A and B) and in the dilution of rue essential oil (285.7 ng/cm^2) (Figure C and D) in the olfactometer test. Different letters indicate significant difference ($P \leq 0.05$) based on the non-parametric Friedman test followed by Conover-Inman test. S= Stimulus, C= Control and DZ= Decision zone. N=20 per sex.

Discussion

Steam distillation usually produces yields less than 1% (Regnault-Roger et al., 2012). Our essential oil yield was 0.3% (v/w), which is in line with a report by Dob et al. (2008), who indicated that the essential oil of *R. chalepensis* obtained from Algeria by hydro distillation had a yield of a 0.27%, based on sample dry weight. On the other hand, the yield of our sample was lower than that reported by Tounsi et al. (2011), who showed an increasing trend (0.39% to 2.46%) of essential oil obtained from the leaves, stems, flowers and fruits of *R. chalepensis.* Similarly, Mejri et al. (2010) showed a higher yield (5.51%) of *R. chalepensis* essential oil from plants growing in Tunisia and indicated that the drying process had a significant effect on the proportions of main components. The differences in the oil yield might depend on the developmental stage of the plant itself or the different organs used. Some examples have demonstrated that the net essential oil content has been associated with the early growth period in a plant or with senescence (Sangwan et al., 2001). Our plant sample could have been extracted during a period of low accumulation of oil, which would explain the observed differences. Moreover, our essential oil was obtained from pooled organs (leaves, stems and flowers), but the yield was lower than in other studies that used specific *R. chalepensis* organs (Tounsi et al., 2011). In addition, the methods employed to obtain the oil (hydro and steam distillation), together with the diverse weather conditions in the growth habitat, could influence the obtained yield (Mejri et al., 2010; Tounsi et al., 2011; Regnault-Roger et al., 2012).

Interestingly, this essential oil contained a 2-ketone series from C_8 to C_{13} (Table 1). The essential oils from the genus *Ruta* are known for having two methyl-2-ketones (2-

nonanone and 2-undecanone) in their chemical profile; reaching 80% of the oil composition (Haddouchi et al., 2013), which is consistent with our results (81.8%). Similarly, the ketone-type compounds identified as main constituents in our study were consistent with those reported by other authors in other species from the same genus (Dob et al., 2008; Da Silva et al., 2014), indicating that the presence of 2-undecanone does not changed with respect to the geographical area where plant grows, but its proportions vary, ranging from 28.2% to 67.8%. The proportions of 2-nonanone range from 5.2% to 53.1%. The nature and proportions of other constituents of our essential oil were not the same as in other reports. We identified nine compounds, with ketones and esters among them (Table 1). These results disagree with the report by Mejri et al. (2010; 2012), who found oxygenated compounds in aerial parts of *R. chalepensis* essential oil; among them were alcohols, monoterpenes and acetates. Moreover, Tounsi et al. (2011) reported the presence of monoterpenes and fatty acids from different organs of cultivated and wild rue plants. According to Wang et al. (2005), the proportion of water used and the extraction time influences the content of oxygenated compounds in the essential oil because the water affects the oxidation or hydrolysis of these compounds. Similarly, Stashenko et al. (2000) and Mejri et al. (2012) reported that the amount of oxygenated compounds increases with the duration of hydro-distillation. However, our distillation time was higher than both of these studies (240 compared to 210/120 min), and we did not observe the presence of these compounds. Other authors have indicated that the proportion of terpenes is influenced by the circadian rhythm, plant stage and environmental temperature. Therefore, the physiological expression of the secondary metabolism of a plant could present constant changes in some of its metabolites according to the biotic and abiotic factors to which it is subjected (Regnault-Roger et al., 2012).

The behavioral response produced by the rue essential oil on both *A. superciliosus* sexes (Figure 1A; 1B) indicates its potential value as a natural repellent. However, this effect was absent for males when the concentration of the oil was lowered to 285.7 ng/cm^2 (Figure 1D). These results was not consistent with the reported by Tampe et al. (2015), who determined this concentration as effective in weevil other of the same genus *Aegorhinus nodipennis* (Hope) using the *Achillea millefolium* L. essential oil and its main constituent, thujone. Behaviorally, both males and females of *A. superciliosus* responded differently when subjected to volatile stimuli. Apparently, females are more sensitive than males because the females must find a suitable host for themselves and their offspring. Similar observations were also reported by Palma et al. (2012), who indicated that in behavioral assays, a stronger response to the volatile compounds of their host plant, red clover, was observed in *Hylastinus obscurus* (Coleoptera: Curculionidae) females than in males. Moreover, in agreement with our observations, Conti et al. (2013) reported that *R. chalepensis* essential oil was mainly composed of 2-nonanone (37.4%) and 2-undecanone (20.5%) and was an effective repellent against the hematophagous mosquito *Aedes albopictus* Linnaeus(Diptera: Culicidae) at an RD_{50} of 0.000215 µL/cm^2 for skin and at an RD_{90} of 0.007613 µL/cm^2. Similarly, *R. graveolens* essential oil and its main compound, 2-undecanone (43.2% ± 0.8) repelled *A. aegypti* L. at a dose of 0.187 mg/cm^2 for the oil and 0.109 mg/cm^2 for 2-undecanone (Tabanca et al., 2012), which indicated that *A. superciliosus* females are more susceptible to this essential oil. Other authors have determined that methyl ketones (C_7 - C_{15}) protected against the malaria mosquito, *Anopheles gambiae* Giles (Diptera: Culicidae) (Innocent et al., 2008) and that the repellent BioUD®, whose active ingredient is 2-undecanone, was at least 2-4 times more repellent than DEET (*N*,*N*-Diethyl-meta-toluamide) against three species of ixodid ticks (Bissinger et al., 2009; Bissinger& Roe, 2010). On the other hand, 2-nonanone and 2-undecanone was shown to be attractive to the foreign grain beetle, *Ahasverus advena* (Waltl) (Coleoptera: Cucujidae) at a concentration of 59 and 131 ng/µL, respectively, in behavioral bioassays (Wakefield et al., 2005) and to both sexes of the olive bark beetle *Phloeotribus scarabaeoides* (Bernard) (Coleoptera: Scolytidae) using 10 µL of these ketones in an olfactometer bioassay (Szauman-Szumski et al., 1998). Likewise, 2-nonanone has been reported by eliciting an attractant behavioral response in the same weevil, *A. superciliosus* at concentrations of 10 and 100 µg/mL (Parra et al., 2009b).

Currently, the modes of action and molecular targets of the ketones in curculionids are not well understood. However, there is evidence that the ketone, 2-undecanone can activate and inhibit the odorant receptors (ORs) of *A. aegypti* L. (Diptera: Culicidae) (Bohbot & Dickens, 2010). The ORs are located in the dendritic membrane of the olfactory sensory neurons (OSNs) of the insects. They are responsible for triggering olfactory transduction by changing the action potentials as a message sent to the brain (Kaupp, 2010). The insect repellent 2-undecanone activates and inhibits the $AaOR_8$ and $AaOR_2$ receptors respectively; *i.e.*, this compound can act as an olfactory agonist or antagonist in *A. aegypti*, modulating receptor activity that is behaviorally expressed by different insect responses (Bohbot & Dickens, 2010). This finding suggests that the repellent effect produced by rue oil in both sexes of *A. superciliosus* could be attributed to 2-undecanone. The potential use of this substance can be explored in the future for developing biodegradable alternatives for synthetic pesticides against raspberry weevils.

In conclusion, the chemical analysis of the essential oil obtained from the aerial part of *Ruta chalepensis* did not show great differences to others studies reported in the literature. The ketones 2-nonanone and 2-undecanone were the main compounds identified in the oil and did not vary much with respect the geographic area where the plants grew. The repellency observed against *Aegorhinus superciliosus* can be attributed to high concentrations of both ketones. Our results suggest that rue essential oil can be considered as a potential repellent that might reduce the infestation of this weevil. Future studies should aim to (1) evaluate the effects of 2-nonanone and 2-undecanone in behavioral bioassays, (2) determine the electrophysiological response of *A. superciliosus* to these compounds and (3) evaluate the effectiveness of both the essential oil and the two ketones under field conditions.

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CHAPTER V

Attractant Activity Elicited by *Eucalyptus* spp. and *Foeniculum vulgare* Essential Oils and their Main Constituents from Adult Raspberry Weevil, *Aegorhinus superciliosus* (Guérin) (Coleoptera: Curculionidae)

In preparation

Attractant Activity Elicited by *Eucalyptus* spp. and *Foeniculum vulgare* Essential Oils and their Main Constituents from Adult Raspberry Weevil, *Aegorhinus superciliosus* (Guérin) (Coleoptera: Curculionidae)

JOCELYNE TAMPE [1,2] AND ANDRÉS QUIROZ [1,2]

[1]Laboratorio de Química Ecológica, Departamento de Ciencias Químicas y Recursos Naturales, Universidad de La Frontera, Temuco, Chile.

[2]Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Casilla 54-D. Temuco, Chile.

ABSTRACT

The use of essential oils as semiochemicals for behavioral manipulation of the raspberry weevil *Aegorhinus superciliosus* Guérin (Coleoptera: Curculionidae) is being investigated for its potential use in capture traps as a control strategy for this pest, which damages fruit tree and shrubs in the south of Chile. Essential oils of two non-host plants, *Eucalyptus* ssp. (eucalyptus) and *Foeniculum vulgare* (fennel) were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) and evaluated in olfactometric and EAG bioassay in order to establish whether they have any effect on the insect behavior. The GC-MS analysis indicated the presence of 17 compounds in the *Eucalyptus* spp. essential oil and its major constituents were eucalyptol (63.6%), α-pinene (18.9%) and cymene (2.9%). For *F. vulgare* essential oil, 16 volatile constituents were identified, being anethole (50.8%), estragole (19.5%), fenchone (11.1%) and α-pinene (7.16%) the dominant compounds. The olfactometric bioassays revealed that eucalyptus essential oil was attractive for both sexes of *A. superciliosus* whereas fennel essential oil was only attractive for the weevil females ($P < 0.05$). In addition, eucalyptol and anethole were electrophysiologically active for both sexes of the insect. The females antennae showed higher EAG response than males and were attracted to different concentrations of these compounds in a two-arm olfactometer ($P < 0.05$); whereas the males were not attracted at any tested doses ($P > 0.05$). The attractant semiochemicals determined in the present study could be the starting point for the development of effective attractant lures for controlling the raspberry weevil.

Key words: Essential oils, *Aegorhinus superciliosus*, behavioral bioassays, electroantennographic response.

Introduction

Semiochemicals properties, such as attractant and repellent activity of the essential oils and their constituents, are increasingly used for insect control in integrated pest management programs in order to reduce the application of pesticides (Koul et al., 2008; Isman & Machial, 2006). Essential oils are oleaginous substances composed of chemical mixtures of different types of molecules, usually, monoterpenes, sesquiterpenes and a wide variety of aromatic phenols, alcohols, oxides, ethers, esters, aldehydes and ketones which determine the characteristic aroma of the plant (Tripathi et al., 2009). In nature, they are produced by secondary metabolism of higher plants and play an important protective role against phytophagous insects (Bakkali et al., 2008). In fact, they may act as attractant or repellent, constituting effective alternatives to synthetic pesticides without producing adverse effects on humans and environment (Regnault-Roger et al., 2012; Isman, 2000; Isman & Machial, 2006).

Aegorhinus superciliosus (Guérin) or raspberry weevil (Coleoptera: Curculionidae), is a native pest of the south of Chile associated with many vegetal species such as raspberry, *Rubus idaeus* L. (Rosaceae), blueberry, *Vaccinium corymbosum* L. (Ericaceae) and European hazelnut, *Corylus avellana* L. (Betulaceae) (Klein & Waterhouse, 2000; Aguilera et al., 2011; Zavala et al., 2011). Both larvae and adults damage their host plant. The larvae drill thin tunnels from the surface towards the inside of the roots while the adults that emerged from the soil feed the leaves and shoots of the season, coinciding with the flowering and fructification state of their hosts. Currently, control of *A. superciliosus* is difficult, because the insecticides cannot be applied during flowering without affecting pollination agents or their beneficial. Therefore, the development of safer alternatives to conventional synthetic insecticides is highly desirable.

In this regard, we have recently investigated the effect of essential oils from non-host plants on behavior of *A. superciliosus* (unpublished data). In this study we selected two essential oils, eucalyptus and fennel, which proved to be attractive towards adults of this weevil. Both oils are well known by exhibited pesticides properties against many insects (Batish et al., 2008; Cosimi et al., 2009; Kim & Ahn, 2001). However, there are a few reports in the literature on their attractant effects. Some evidences links the attraction behavior of weevil *Gonipterus scutellatus* Gyllenhaal (Coleoptera: Curculionidae) with eucalyptus essential oils (Tooke, 1953). On the other hand, Robacker (2007) conducted experiments to evaluate the attractiveness of 26 plant essential oils, including eucalyptus and fennel oils on Mexican fruit fly, *Anastrepha ludens* Loew (Diptera: Tephritidae). Additionally, attractant properties of the main compounds described for these oils have been well documented. Eucalyptol, the main constituent of eucalyptus essential oil has been reported as attractant to *Oxyops vitiosa* Pascoe (Wheeler, 2015), *Xyleborus glabratus* Eichhoff (Kuhns et al., 2014) and *Cosmopolites sordidus* Germar (Ndiege et al., 1996) (Coleoptera: Curculionidae). Similarly, anethole, the main constituent of fennel essential oil has shown attractive activity on *Epicometis* (*Tropinota*) *hirta*, *Cetonia aurata aurata*, *Oxythyrea funesta* Poda (Tóth et al., 2003), *Anomala marginata* Robinson and *Trigonopeltastes delta* Foster (Cherry et al., 1996) (Coleoptera: Scarabaeidae).

Due to the interest for natural product like essential oils, it is important to develop a better understanding of their mode of biological action for new application in agriculture, specifically in controlling pest such as *A. superciliosus*. Thus, mass trapping of adults using natural attractant compound could be an efficient alternative for reducing pest populations through trapping system, more security for the human health and the environment (Aguilera et al., 2011; Schmera et al., 2004). Therefore, in this study we investigated: 1) the attractant effect of eucalyptus and fennel essential oils and their main compounds, eucalyptol and anethole on raspberry weevils, 2) their electrophysiologically activities, and 3) the metabolite profile of both oils by gas chromatography coupled to mass spectrometry (GC-MS) associated with this behavior.

Materials and methods

Experimental insects. The populations of adults *A. superciliosus* were collected manually from blueberry (*V. corymbosum*) plantation in the commune of Collipulli (37º50'South, 72º08' West), Región de La Araucanía, Chile, between November and December of the periods 2012, 2013 and 2014. The acclimatization of the insects was performed according to methodology described by Tampe et al. (2015) changing feeding by blueberry plant shoots. Before the olfactometric bioassay (24 hours), each insect was maintained separately according to its sex in individual Petri dishes and starved. Only those insects that were walking and active 10 min before each bioassay were selected. Each insect was a replica of the experiment and used only once (Parra et al., 2009b).

Essential oils. The *Eucalyptus* spp. and *Foeniculum vulgare* essential oils were purchased from Campestre Company, Temuco, Chile (100% purity).

Essential oil analysis. The chemical composition of *Eucalyptus* spp. and *F. vulgare* essential oils was determined by GC-MS using a Focus DSQ, Thermo Electron Corporation. A 1 µL aliquot of the essential oils diluted in dichloromethane was injected at a concentration of 1 µg/µL separately in a capillary column BP-1 (30 m x 0.22 mm x 0.25 µm) with helium as carrier gas (1.5 mL/min).The analytical conditions were programmed to remain at 40ºC, which increased in a gradient of 5ºC min up to 280ºC, remaining at this temperature for 5 min. The temperatures of both injector and interface were 250°C; detector temperature was fixed at 200ºC and the electron impact ionization was 70 eV, with mass spectra from 30 to 350 *m/z*. Compounds identified in both samples were confirmed by comparison of their Kovats indices (KI) obtained by injecting an alkene series (C_9-C_{26}). The experimental KIs were compared with theoretical KI compounds reported in the database "NIST" (NIST ver. 2.0, Thermo) (Babushok et al., 2007). In addition, a comparison of their mass spectra with available NIST mass spectral library according to reverse search technique described by Tapia et al. (2007) was carried out. Quantification of essential oil components was expressed in relative percentage on total area of chromatogram and was carried out by peak area normalization measurements. In addition, calibration curves based on peak area ratio were constructed using the standards at disposition for quantification of volatile compound identified, which were expressed in (ng/µL) (Mutis et al., 2010).

Synthetic chemicals. Eighteen compounds identified from the essential oils were purchased from commercial sources. *α*-pinene (Sigma Aldrich, 97%), camphene (Sigma Aldrich 95%), (-)-*β*-pinene (Fluka ≥ 98.5%), *β*-myrcene (Fluka ≥ 95.0%), *α*-phellandrene (Aldrich 95%), cymene (Fluka ≥ 99.5%), limonene (Sigma Aldrich 97%), eucalyptol (Safc 99%), *γ*-terpinene (Fluka ≥ 98.5%), camphor (Fluka ≥ 95.0%), 4-terpineol (Fluka ≥ 98.5%), terpineol (Sigma Aldrich 95%), anethole (Fluka ≥ 99.5%), terpinyl acetate (Safc 95%), aromadendrene (Aldrich 97%), globulol (Sigma Aldrich 98.5%), palmitic acid (Cayman chemical company > 98%), oleic acid (Cayman chemical company ≥ 98%). Standard solutions of 1, 10, 50, 100 and 200 ng of all compounds were diluted in hexane for the quantification of each essential oil component. In addition, standard solutions of 5, 50 and 500 ng from eucalyptol and anethole were diluted in hexane (GC grade; Merck, Darmstadt, Germany) for EAG and two-arm olfactometer assays.

Behavioral Bioassays. A four-arm olfactometer was used to determine behavioral response of both male and female *A. superciliosus* produced by each essential oil. The olfactometer is formed by 3 acrylic plates (40 by 40 by 2.5 cm) tied together by metal screws. Five sections, divided in four arms (two arms for the stimuli and two for the control) and one central square zone named as decision zone, comprising the olfactometer (Parra et al., 2009b). In each extreme of the arm, there is a hole where it connects the glass tubes (7 cm long x 1.5 cm i.d.) containing the Whatman Nº1 filter paper (0.5 cm wide by 3.5 cm long). In the center of the top plate there is a hole for connecting the vacuum adapter with an air flow of 800 mL/min that generates an entrainment of volatile stimuli into the olfactometer. The methodology used was according to those described by Tapia et al. (2005), consisting of observing the movements of the insect into the olfactometer arena during 20 min. The data obtained were processed by EthoVision 3.1 software (Noldus et al., 2002). Each oil was evaluated independently at one concentration (500 ng) according to reported by Tampe et al. (2015) and twenty replicates for each essential oil and sexes were conducted.

For evaluating the behavioral response to the main compounds identified from eucalyptus and fennel essential oils was used a two-arm olfactometer described by Rojas et al. (2002). The olfactometer consisted of a Y-shaped glass tube; the base and each arm of the olfactometer were 16 cm long and 3.0 cm i.d. The connection of the vacuum pump was performed in the tube base which produced air flow through the arm and stem of the tube at a rate of 800 mL/min. Olfactometry was conducted according to the methodology described by Mutis et al. (2010), which consists of introducing one male or female of *A. superciliosus* into the base of the Y-tube giving the insect 5 min to walk freely toward the end of one of the arms of the olfactometer, being considered as successful when the weevil walked 3 cm past the Y-junction and remained there for at least 20 s. After each assay, the Y-tubes were cleaned and exchanged to avoid positional bias. Each Y-tube arm end was connected with a Pasteur pipette containing 50 µL of eucalyptol or anethole diluted in hexane and the other arm containing 50 µL hexane as control, both applied on paper Whatman Nº 1 (1.75 cm^2). Eucalyptol and anethole were evaluated independently at three different doses (5, 50 and 500 ng) (N ≥ 25/dose/sex).

Electroantennography (EAG). Electrophysiological responses of both sexes of *A. superciliosus* adults to eucalyptol and anethole was determined by EAG, according to the methodology described by Mutis et al. (2010) consisting in excised the antennae from the head of the weevil (base until distal part of the terminal segment) and mounted between two glass capillary electrodes. Each capillary was filled with Dicardio-gel (Difem Pharma) into which gold wires were inserted. The stimulus lasted 1 s and was released into a purified airstream for 500 mL min^{-1}. The signal originated by the antenna was conducted through a high impedance amplifier (10 X) (Syntech, Hilversum, The Netherlands) and displayed onto a monitor using Syntech version 2.2a software for processing EAG signals. Each standard was prepared in hexane to three doses (5, 50 and 500 ng). A volume of 50 µL of the stimuli was placed on strips of filter paper (1.75 cm^2) and exposed to air for 20 s to allow solvent evaporation. Then, it was positioned inside the Pasteur pipette. As control 50 µL hexane were used also impregnated on filter paper. The puffs series were stimuli followed by hexane, repeating three times the series for each antenna. Then, interval of 40 s between each puffs were waited to ensure antennal recovery. The EAG response provoked by hexane was subtracted to the responses obtained by the stimuli in order to normalize the responses and obtain the net response to each compound. Each dose was tested on 10 different antennae according to sexes and one antenna was used per weevil.

Statistical Analysis. Data obtained from behavioral bioassays in four-arm olfactometer were expressed as average of the time spent (min) in each arm of the olfactometer ± standard error and were analyzed by the non-parametric Friedman test ($P \leq 0.05$) followed by Conover test. Simultaneously, the comparison between sexes was analyzed by non-parametric Wilcoxon test ($P \leq 0.05$). The data obtained from the two-arm olfactometer were analyzed by the significance obtained from X^2 test, where (*) $P \leq 0.1$ (**) $P \leq 0.05$ and (***) $P \leq 0.01$. The data obtained from the EAG response were analyzed by non-parametric Kruskal-Wallis test followed by Conover-Inman test ($P \leq 0.05$) and the comparison between sexes was analyzed by non-parametric Wilcoxon test ($P \leq 0.05$).

Results

Essential oils analysis. The constituents of eucalyptus and fennel essential oils, percentage composition and retention index are shown in Table 1. In *Eucalyptus* spp. essential oil, 17 compounds were identified (92.2%). The major constituents were eucalyptol (63.6%), *α*-pinene (18.9%) and cymene (2.9%). Other minor components did not exceed 1%. In addition, 1 unidentified compound was present in the sample, representing 7.8% of the total components. Their mass spectral data are observed in Table 2. The GC-MS analyses of fennel essential oil indicate the presence of 16 volatile constituents, representing 100% of the total components. The main constituents were anethole (50.8%), estragole (19.5%), fenchone (11.1%), *α*-pinene (7.16%), *α*-phellandrene (4.4%), cymene (1.81%) and limonene (1.82%). Other compounds with percentages below 1% were also identified.

Table 1 Constituents of essential oils of *Eucalyptus* spp. and *Foeniculum vulgare*.

No	Component	Retention index	*Eucalyptus* spp.		*F. vulgare*		Method of identification
			Area (%)	Concentration (ng/µl)	Area (%)	Concentration (ng/µl)	
1	α-Pinene	8.66	18.9	70.4	7.1	39.8	S, MS, KI
2	Camphene	8.99			0.2	1.8	S, MS, KI
3	Monoterpene	9.71			0.2	-	L
4	(-)-β-Pinene	9.78	0.5	1.9	0.6	3.7	S, MS, KI
5	β-Myrcene	10.30			0.9	7.2	S, MS, KI
6	α-Phellandrene	10.64			4.4	74.9	S, MS, KI
7	Cymene	11.13	2.9	22.3	1.8	20.7	S, MS, KI
8	Limonene	11.38			1.8	9.2	S, MS, KI
9	Eucalyptol	11.40	63.6	176			S, MS, KI
10	γ-Terpinene	12.22	0.5	1.7	0.5	2.6	S, MS, KI
11	Monoterpene	13.10	0.16	-			L
12	Fenchone	12.80			11.1	-	MS, KI
13	Isopentyl isovalerate	13.52	0.4	-			MS, KI
14	Camphor	14.26			0.4	4.5	S, MS, KI
15	Pinocarveol	14.39	0.4	-			MS, KI
16	Pinocarvone	14.80	0.1	-			MS, KI
17	4-Terpineol	15.54	0.2	0.5			S, MS, KI
18	Terpineol	15.90	0.7	4.0			S, MS, KI
19	Estragole	16.05			19.5	-	MS, KI
20	Fenchyl acetate	16.79			0.06	-	MS, KI
21	Anethole	18.56			50.8	583.11	S, MS, KI
22	Terpinyl acetate	20.22	1.1	4.8			S, MS, KI
23	Sesquiterpene	22.09	0.2	-			L
24	Aromadendrene	22.78	0.8	3.1			S, MS, KI

Table 1 *Continuation*

No	Component	Retention index	*Eucalyptus* spp. Area (%)	*Eucalyptus* spp. Concentration (ng/µl)	*F. vulgare* Area (%)	*F. vulgare* Concentration (ng/µl)	Method of identification
25	Sesquiterpene	23.74			0.06	-	L
26	*β*-Cadinene	24.71			0.04	-	MS, KI
27	Globulol	26.09	0.4	4.6			S, MS, KI
28	Palmitic acid	34.15	0.4	21.1			S, MS, KI
29	Oleic Acid	37.32	0.6	2.4			S, MS, KI

S: standard, MS: mass spectra, KI: Kovats index and L: Literature, both matched with NIST data (2015).

Table 1.1 IUPAC names of the compounds from *Eucalyptus* spp. and *F. vulgare* EO

Common Name	IUPAC Name
α-Pinene	2,6,6-trimethyl-bicyclo [3.1.1] hept 2-ene
Camphene	2,2-dimethyl-3-methylene-bicyclo [2.2.1] heptane
(-)-*β*-Pinene	(*S*)-6,6-dimethyl-2-methylenebicyclo [3.1.1] heptane
β-Myrcene	7-methyl-3-methylene-1,6-octadiene
α-Phellandrene	2-methyl-5-(1-methylethyl)-1,3-cyclohexadiene
Cymene	1-methyl-4-(1-methylethyl)-benzene
Limonene	1-methyl-4-(1-methylethenyl)-cyclohexene
Eucalyptol	1,3,3-trimethyl-2-Oxabicyclo[2.2.2]octane
γ-Terpinene	1-methyl-4-(1-methylethyl)-1,4-Cyclohexadiene
Fenchone	1,3,3-trimethyl-bicyclo[2.2.1]heptan-2-one
Isopentyl isovalerate	3-methyl-3-methylbutyl ester, butanoic acid
Camphor	1,7,7-trimethyl-bicyclo[2.2.1]heptan-2-one
Pinocarveol	6,6-dimethyl-2-methylene-bicyclo[3.1.1]heptan-3-ol
Pinocarvone	6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptan-3-one

Table 1.1 *Continuation*	
4-Terpineol	4-methyl-1-(1-methylethyl)-3-Cyclohexen-1-ol
Estragole	1-Allyl-4-methoxybenzene
Fenchyl acetate	1,3,3-trimethyl-acetate-bicyclo[2.2.1]heptan-2-ol
Anethole	(*E*)-1-Methoxy-4(1-propenyl)benzene
Terpenyl acetate	*α*, *α*, 4-trimethyl- acetate-3-Cyclohexene-1-methanol
Aromadendrene	[1*αR*-(1*αα*,4*αα*,7*α*,7*αβ*,7*βα*)]-1H-Cycloprop[e]azulene,decahydro-1,1,7-trimethyl-4-methylene
β-Cadinene	[1*S*-(1*α*,4*α*β,8*αα*)]-1-Isopropyl-4,7-dimethyl-1,2,4*α*,5,8,8*α*-hexahydronaphthalene
Globulol	(1*αα*,4*α*,4*αα*,7*α*,7*αβ*,7*βα*)-1,1,4,7-Tetramethyldecahydro-1H-cyclopropa[e]azulen-4-ol
Palmitic acid	n-Hexadecanoic acid
Oleic Acid	(*Z*)-9-Octadecenoic acid

Table 2 Mass spectral data of unknown volatile compounds from *Eucalyptus* spp. EO.

Rt	**Compound**	**(%)**	***m/z***
47.45	unknown 3	7.8	386(100), 275(67.22), 301(64.94), 213(53.04), 145(52.85), 368(50.81), 107(46.39), 105(45.50), 95(41.94), 353(38.46), 159(38.24), 255(37.61), 161(36.64), 81(36.44), 163(36.01)

Rt = Retention time, *m/z* = mass-to-charge ratio

Behavioral bioassays with essential oils.

In four-arm olfactometer test, both females and males of *A. superciliosus* were more significantly attracted to eucalyptus essential oil (500 ng) than the control (Fig. 1A). Females remained 12.62 min ± 0.72 in the stimuli source v/s 6.21 min ± 0.61 in the control (F = 50.72; df = 2.02; P = 0.01) whereas males remained 10.82 min ± 1.21 in the stimuli v/s 7.8 min ± 1.18 in the control (F = 41.40; df = 2.02; P = 0.04). There was no significant difference between sexes in any evaluated zones (Fig. 1A). For the second oil in study, only females were attracted to fennel essential oil (500 ng), with a permanency time of 12.19 ± 1.29 min in the stimuli v/s 6.63 ± 1.23 min in the control (F = 18.81; df = 2.02; P = 0.02). Moreover, difference between both sexes were found in stimulus source (P = 0.02) (Fig. 1B).

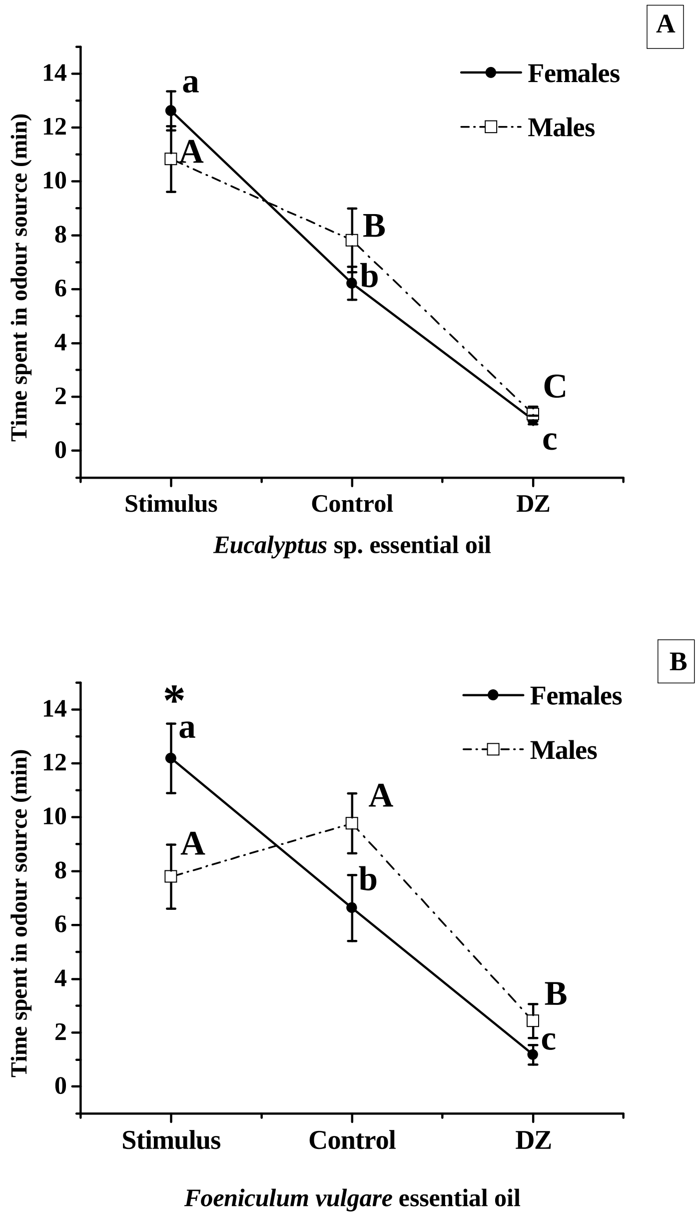


Fig. 1 Average time spent (min) (± SE) by both sexes of *Aegorhinus superciliosus* on (A) *Eucalyptus* spp. and (B) *Foeniculum vulgare* essential oil (500 ng) in four-arm olfactometer bioassay. Different letters indicate statistical difference among zones (stimulus, control and decision zone) ($P \leq 0.05$) based on the non-parametric Friedman test followed by Conover test, letters in square correspond to statistics of males and (*) = indicate statistical

difference between sexes in the same zone ($P \leq 0.05$) based on the non-parametric Wilcoxon test. N=20 per sex.

Behavioral Bioassays with standards.

The responses of both sexes of the weevil expressed in percentages in two-arm olfactometer are shown in Figure 2. At evaluating eucalyptol, females of *A. superciliosus* showed attraction at the dose of 500 ng ($X^2_{3.33}$, $P = 0.03$). Lower doses were not significant ($P > 0.05$). There were no significant differences in male responses at any tested dose (5, 50 and 500 ng) ($P > 0.05$) (Fig. 2A). In the case of anethole, only females were attracted at the dose of 50 and 500 ng ($X^2_{2.13}$, $P = 0.07$; $X^2_{6.53}$, $P = 0.005$). Males showed no preference for this monoterpene at any evaluated dose (5, 50 and 500 ng) ($P > 0.05$) (Fig. 2B).

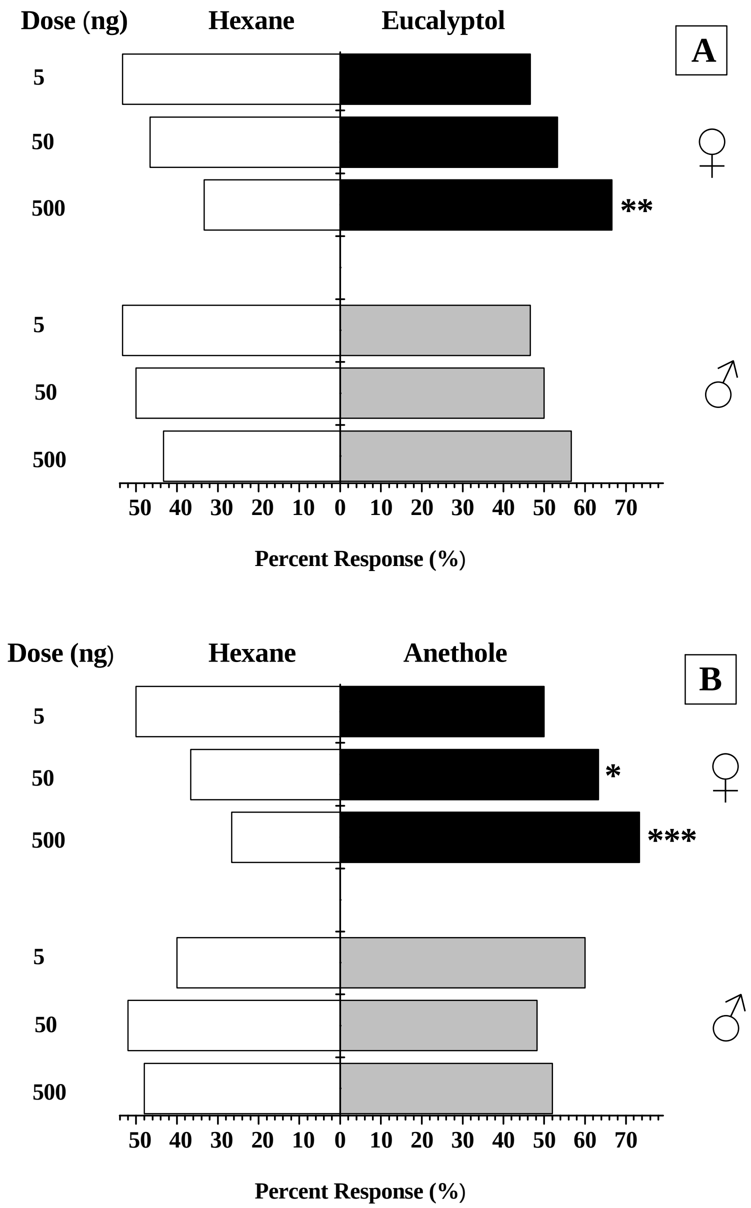
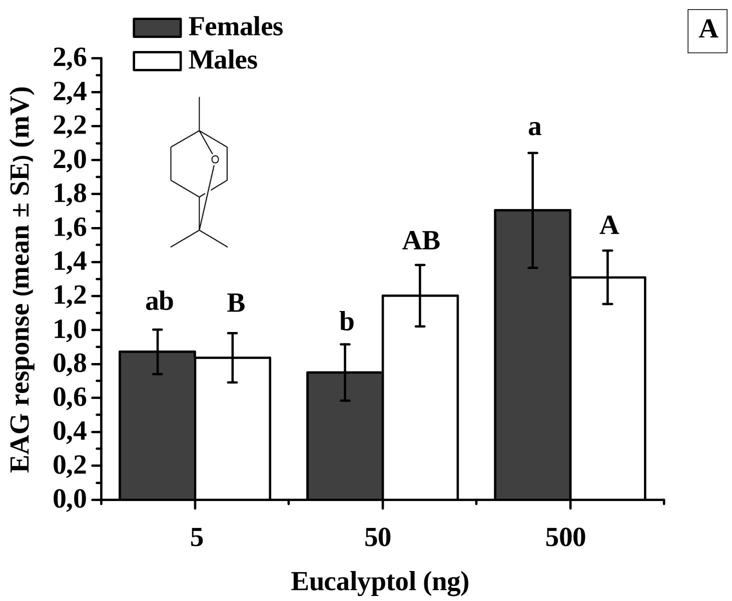


Fig. 2 Response (%) of both sexes of *A. superciliosus* to three doses (5, 50 and 500 ng) of eucalyptol (A) and anethole (B) in a two-arm olfactometer. One, two and three asterisks indicate statistical significance according to X^2 for (*) $p \leq 0.1$ (**) $p \leq 0.05$ and (***) $p \leq 0.01$, respectively, with $N \geq 25$.

EAG response.

Male and female antennae of *A. superciliosus* were sensitive to both monoterpenes in the three evaluated doses (5, 50 and 500 ng) (Fig. 3). Female electroantennographic responses to eucalyptol were significative between the two highest doses, with a mean intensity of 0.75 mV ± 0.17 (50 ng) v/s 1.70 mV± 0.34 (500 ng) (T = 5.3; df = 2.05; P = 0.02). Similarly, male electroantennographic responses only were statistically different between the highest and the lowest dose, with a mean response of 1.31 mV ± 0.16 (500 ng) v/s 0.84 mV ± 0.15 (5 ng) (T = 7.06; df = 2.05; P = 0.006). There were no differences between male and female antennal response to any tested doses ($P > 0.05$) (Fig. 3A). In the case of anethole, there was a dose effect on the female electroantennographic response, observing significant difference between the highest and two lower doses. The mean intensities were 2.19 mV ± 0.35 (500 ng) v/s 1.04 mV ± 0.20 (50 ng) (T = 13.32; df = 2.05; P = 0.0047) and 0.64 mV ± 0.10 (5 ng) ($P < 0.0001$). Male antennae showed a similar response at the three tested doses (5, 50 and 500 ng) ($P > 0.05$). Differences between sexes were found only at 500 ng (P = 0.02) (Fig. 3B).



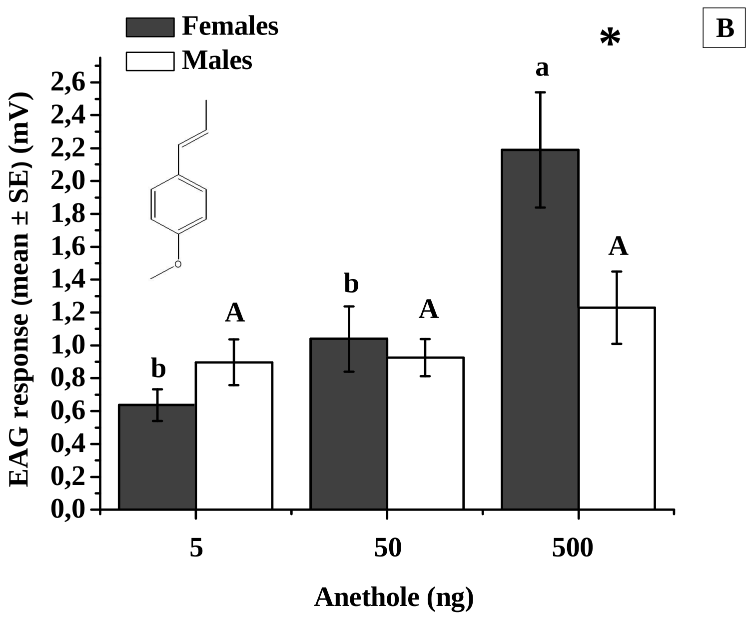


Fig. 3 EAG dose-response curves of the antennae of *Aegorhinus superciliosus* adults to three concentrations (5, 50 and 500 ng) of eucalyptol (A) and anethole (B). The EAG amplitudes were adjusted to the control (hexane) and were given as means ± SE. Different letters indicate statistical difference among doses ($P \leq 0.05$) based on the non-parametric Kruskal-Wallis test followed by Conover-Inman test, letters in square correspond to the

statistics of the males and (*) = indicates statistical difference between sexes at the same dose ($P \leq 0.05$) based on the non-parametric Wilcoxon test. Each dose was tested on ten individuals.

Discussion

The chemical analysis of the *Eucalyptus* spp. essential oil by GC-MS showed that eucalyptol was the major component identified followed by α-pinene and cymene (Table 1). These compounds have been reported by Maciel et al. (2010), Cheng et al. (2009) and Pagula et al. (2000) as the main components in eucalyptus species essential oils, such as *Eucalyptus globulus*, *E. camaldulensis* and *E. urophylla* obtained from Brazil, Taiwan and Mozambique, in the following proportions: eucalyptol (83.9% - 58.3% - 40%), α-pinene (4.1% - 6.25% - 4.6%) and cymene (2.9% - 0.4% - 4.7%), respectively. Moreover, our results differ from Gilles et al. (2010) who reported that the main constituent of *Eucalyptus olida* obtained from Australia was (*E*)-methyl cinnamate in absence of eucalyptol. Therefore, we suggest that there is a variation in the oil compositions of the same genus associated with species as well as to biotic and abiotic conditions of growing in each country (Regnault-Roger et al., 2012). *F. vulgare* essential oil presented 14 compounds, mainly terpenes. Anethole, an oxygenated monoterpene, was found as the main component of oil with a rate of 50.8%, followed by estragole (19%) and fenchone (11%). Barazani et al. (2002) and Miraldi (1999) indicated that anethole and estragole were often the main components in the fennel essential oil, which agrees with our study. Though the main compounds identified in our sample are consistent with those previously reported, there is a quantitative difference in its proportion. Anethole content was lower in our study (50.8%

v/s < 69%), whereas that estragole content was higher than previous records (19% v/s 5%). Similar proportions of fenchone (11%) and higher contents of α-pinene (7.16% v/s > 2.7%) and α-phellandrene (4.4% v/s > 1%) were observed in our sample (Anwar et al., 2009; Telci et al., 2009; Mimica-Dukié et al., 2003). Moreover, our results differ from those obtained by Özcan et al. (2006) who studied the oil composition of fennel from Turkey, in which estragole (> 50%) following by fenchone (> 13% and < 24%) revealed to be dominant in absence of anethole. Comparison between our results and the results of other reports showed differences, probably due to the plant varieties or sites, as well as the time of harvesting.

Currently, the most attractive aspect of the study of the essential oils and their constituents are their biological properties mainly guided to the crop protection, as an alternative to conventional insecticides (Batish et al., 2008). The results from bioassays showed that non-host plants essential oils can effectively interfere with the behavior of *A. superciliosus*. Both sexes of raspberry weevil were attracted to the odor emanating from eucalyptus essential oil (500 ng) and only female weevils were attracted to fennel oil (500 ng) (Fig. 1). There are few studies that report the attractant effect of these oils. However, Tooke (1953) reported that the curculionid *G. scutellatus* showed preference to essential oils of different eucalyptus species and associated this behavior with the presence of eucalyptol in its composition. Moreover, Robacker (2007) showed that fennel and eucalyptus essential oils were attractive to tephritid *A. ludens*, but they were much less attractive than AFF lures (*Anastrepha* fruit fly). We suggest that the attraction observed on *A. superciliosus* could be influenced by any compounds present in both oils, such as α-β pinene, limonene and eucalyptol, which have been reported as constituents of the

Vaccinium corymbosum L. host-plant during population peak of the insect (Parra et al., 2009b), besides anethole, main compound of fennel essential oil. Even though the males were no attracted by the fennel oil, the females were attracted by both essential oils ($P <$ 0.05) (Fig. 2). It is of greater importance to control the females within a pest control strategy, as they cause a more severe damage because of oviposition. The attractant activity observed with both essential oils is apparently related to its terpene type components since there is a relationship between the chemical structures of the most abundant compounds and their attractant activities (Silvério et al., 2013).

A. superciliosus is frequently found to be clustered in blueberry shrubs during the blue pink phenological stage. This behavior has been attributed to volatile compound blend released in this stage, which contains eucalyptol (8%) in its composition (Parra et al., 2009b). Our results show an attractant response of females *A. superciliosus* to this compound at the highest dose tested (500 ng, $P \leq 0.05$) in two-arm olfactometer bioassays. This is consistent with the report by Parra et al. (2009b) who also observed that only the females were attracted to eucalyptol at 50 ng. Therefore, we can suggest that the female weevils are capable of perceiving this compound in a range from 50 to 500 ng. Moreover, the electroantennographic results indicated that both sexes of *A. superciliosus* antennae have receptors for the eucalyptol with significant difference among doses ($P < 0.05$). The mean responses of the females ranged from 0.75 ± 0.17 to 1.70 ± 0.34 mV, while the male responses ranged from 0.84 ± 0.15 to 1.31 ± 0.16 mV. Similarly, eucalyptol mean response in eucalyptus woodborer *Phoracantha semipunctata* (Coleoptera: Cerambycidae) has been greater than 0.8 mV (Barata et al., 2000). Ndiege et al. (1996) and Wheeler (2015) reported to eucalyptol as an electrophysiologically active compound acting as an attractant for the banana weevil *Cosmopolites sordidus* Germar and for *Oxyops vitiosa* Pascoe (Coleoptera: Curculionidae) in laboratory bioassays. Furthermore, the antennal olfactory system of *Thyrinteina arnobia* (Stoll) (Lepidoptera: Geometridae) showed sensitivity to this monoterpene obtained from essential oils of seven eucalyptus species (Batista-Pereira et al., 2006). Eucalyptol was also electrophysiologically active in female *Gonipterus* spp. (Bouwer et al., 2014) and cabbage seed weevil *Ceutorhynchus assimilis* Paykull (Coleoptera: Curculionidae) (Blight et al., 1995) and acted as attractant for the weevil *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae), suggesting this may be useful for managing this insect (Kuhns et al., 2014).

Similar results were obtained with anethole, where two-arm olfactometer signals, showed that this component elicited an attraction response in the adult females of this weevil at the highest tested dose (500 ng, $P < 0.05$). Additionally, olfactory system of both sexes of *A. superciliosus* can perceive this compound electrophysiologically. A dose-dependent EAG response was obtained in female weevils, ranging from 0.64 ± 0.10 to 2.19 ± 0.35 mV, whereas male response was similar at three evaluated doses (0.90 ± 0.14 to 1.23 ± 0.22 mV). Differences between sexes were observed at the highest dose tested of anethole (500 ng) and in general terms, it has been demonstrated that females showed greater EAG response than males, but this was not always significant (EAG-eucalyptol). An EAG higher response of bruchid female *Callosobruchus maculatus* (Fabricius) than males was observed at being stimulated with the seed-extract of *Vigna unguiculata* (L.) Walp., it could be explained by the fact that the cowpea seed beetles lay their eggs on the external surface of dry seed in Fabaceae species (Adhikari et al., 2002). This behavior may be found in the females which need find a suitable host that provides food and safety for the progeny

(Paukku & Kotiaho, 2008). According to our results, anethole produced a dose-dependent EAG (0.6 to 4.4 mV) and attractant behavioral response on *Proagopertha lucidula* (Faldermann) (Coleoptera: Scarabaeidae) at the dose of 10 μg/μL, concentration higher than the three used in our study (Chang-Kuan et al., 2009) and it was electrophysiologically active in the antennae of both sexes of *Oxythyrea funesta* (Coleoptera: Scarabaeidae) (Vuts et al., 2008). Likewise, anethole is known to be attractive to scarabs *Cetonia aurata aurata* and *Oxythyrea funesta* (Coleoptera: Scarabaeidae) in seasonal monitoring (Schmera et al., 2004; Toth et al., 2003), for the weevil *Hylobius pales* (Coleoptera: Curculionidae) (Thomas & Hertell, 1969) and for the moth *Argyresthia conjugella* Zeller (Lepidoptera: Argyresthiidae) (Bengtsson et al., 2006). On the other hand, evidences of field trapping systems showed that traps baited with anethole were effective to capture adults scarab of *Anomala marginata* Robinson, *Trigonopeltastes delta* Foster (Cherry et al., 1996) and *Epicometis* (*Tropinota*) *hirta* (Toth et al., 2003) (Coleoptera: Scarabaeidae). Based on these reports, we must guide our future trapping systems to evaluate anethole and eucalyptol individually and in combination for a possible synergistic effect between them.

Odor perceptions in the raspberry weevil involve monoterpenes, in particular eucalyptol and anethole. These oxygenated monoterpenes are most probably attractive, since they are associated with susceptible hosts (Leufveâ & Birgersson, 1987). According to chemical nature of these compounds, our results suggest that both sexes of *A. superciliosus* are generalist neuron receptors able to detect volatile compounds from essential oils (Boeckh, 1984), which could be considered as foraging kairomones for the species, contributing to host-selection and oviposition behavior (Parra et al., 2009b; Ruther et al., 2002). The identification of semiochemicals from non-host plants of *A. superciliosus* could be used to develop monitoring systems or to lure them into traps in the field test. Some studies showed the effectiveness of kairomones as tools potential of capture for insect pest (Brockerhoff et al., 2006; Light et al., 2001; Lingren, 2000). However, more studies are needed to evaluate the effect of these compounds or combinations of them at different doses on the behavior of our insect. This study is a starting point to develop efficient kairomones to be used in monitoring and Integrated Pest Management (IPM) programs for raspberry weevil.

In conclusion, it was possible to identify fifteen and fourteen compounds from *Eucalyptus* spp. and *F. vulgare* essential oils respectively, which were mainly monoterpenes hydrocarbons and oxygenated. Although both essential oils showed capacity to attract weevils, mainly females, the results suggest that eucalyptol and anethole might be a more viable alternative and effectiveness for the insect control, according to the results obtained in EAG and behavioral bioassays. In addition, the biological activity of the essential oils is directly related to their chemical composition and if this varies, it can alter the effect produced. Future studies should be guided to assess the effectiveness of these monoterpenes under field conditions as possible forage kairomones for monitoring and controlling this curculionid.

Acknowledgments

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CHAPTER VI

General discussion, concluding remarks and future directions

General discussion

In the last decades, the study and use of essential oils (EOs) derived from aromatic plants as low-risk insecticides, have increased considerably owing to their popularity among organic growers and environmentally conscious consumers (Regnault-Roger et al., 2012). The EOs are a major source of highly active and potent metabolites, affecting biology, behavior and physiology of insects (Isman & Machial, 2006). Plant oils are generally considered broad-spectrum and safe for the environment because the array of compounds they contain quickly biodegrade in the soil and are safer for humans (Rajendran & Sriranjini, 2008; Devi & Maji, 2011; Sendi & Ebadollahi, 2014). They can be constituted by complex natural mixtures of about 20 to 60 compounds at different concentrations, and they are characterized commonly by two or three major components at fairly high concentrations compared with other components present in trace amounts. Generally, the major constituents determine the biological activity of EOs (Sendi & Ebadollahi, 2014), which is agrees to results obtained in this research. Moreover, it is important to emphasize the intraspecific variability of EOs chemical composition, because their bioactivity is directly related to their composition. This factor is determined by the rind of the extracted plant organ, phenological state, time of year and isolation method used as well as climatic and soil conditions (Masotti et al., 2003; Angioni et al., 2006; Isman & Machial, 2006). At this respect, we evaluated two *A. millefolium* EOs from different localities and with different chemical compositions. We observed that *A. nodipennis* showed a differential behavior depending on the EO. Specifically, yarrow oil, whose main component was thujone, elicited repellency in the insect, whereas yarrow oil, whose main component was chamazulene, was not repellent (See Appendix 1). Similarly, Tabanca et al. (2011) reported the same effect when five *A. biebersteinii* samples collected from different zones of Central Turkey were evaluated. In their study, they could observe than only the sample obtained from Ankara showed a notable larvicidal effect on *A. aegypti*, which was attributed to different chemical composition among samples. These results showed the importance of knowing the chemical composition of the EO associated with a biological activity, which could be affected qualitative and quantitatively according to factors mentioned above.

EOs and their derivatives show a broad spectrum of activity against harmful insects and are considered as an alternative for pest control (Pillmoor et al., 1993). In this aspect, several studies have documented the ability of EOs for their repellent and attractant properties against pest insects (Landolt et al., 1999; Tripathi et al., 2000; Papachristos & Stamopoulos, 2002; Wang et al., 2006; Kendra et al., 2011). According to this, *A. millefolium* and *R. chalepensis* EOs were repellents for *A. nodipennis* and *A. superciliosus* respectively in our research, where the repellent activity was dependent on the applied concentration. Commonly, EOs can be inhaled, ingested or absorbed through the cuticle by insects (Regnault-Roger, 1997). In our study, they were perceived by inhalation in olfactometer bioassays acting as a blend of different small-volatile molecules that interfere with the insect physiology. As the active compounds belong to monoterpene family, we can assume that their mode of action is indicative of a neurotoxic action associated with the inhibition of the acetylcholinesterase activity (Coats et al., 1991; Abdelgaleil et al., 2009). These kinds of compounds were the first inhibitors obtained from plants with anticholinesterasic properties described in studies on chemical interactions between plant volatiles and insects (Houghton et al., 2006). In addition, their symptoms are similar to those produced by organophosphate and carbamate insecticides (Isman, 2000). Ryan and

Byrne (1988) reported that some terpenoids are inhibitors of acetylcholinesterase (AChE), provoking paralysis and death in insects. There is also evidence that monoterpenes interfere with the neuromodulator octopamine (Kostyukovsky et al., 2002; Enan, 2005; Isman et al., 2007) and with GABA-gated chloride channels (Priestley et al., 2003). In the chapter II, we suggest that thujone monoterpene affect the GABA system (Höld et al., 2000) as a neurotoxic insecticide (Ratra et al., 2001; Duke, 2004), and according to literature, the tested EOs in this study may cause, repellent activity via one or more of these modes of action. Its use as volatile allelochemicals from EOs may be more effective with a better understanding of these mechanisms.

Terpenoids also play a vital role in plant-plant interactions and serve as attractants for some insects such as pollinators (Sendi & Ebadollahi, 2014). Attraction activity on insects by EOs and specific components is demonstrated in many studies, mainly oriented to development lure for trapping systems (Hammack, 1996; Gorski, 2004; Katerinopoulos et al., 2005) indicating that they can be used in control pest. In the present study, EOs with attractant properties for *A. nodipennis* were not found (See Appendix 2). However, two EOs with attractant properties were identified for *A. superciliosus* (See Appendix 3 and 4). In Chapter 5, we showed that eucalyptus EO was an attractant for both sexes of *A. superciliosus*, whereas their main component, eucalyptol, only was attractant for the females of the species. Moreover, the fennel EO and its main compound anethole were attractant only for the female weevils. Based on these laboratory results, both EOs were evaluated under field conditions in an orchard of European hazelnut. Despite the caught insect number was small (11 insects), the eucalyptus oil was able to attract and capture both sexes of insect with a higher female proportion than male (3 females/1 males), whereas the fennel oil only captured females (4 females), supporting the laboratory assay data (See Appendix 5). We suggest that the attraction produced for both EOs is strongly influenced by their main constituents, in this case, eucalyptol and anethole. The effectiveness of attractant EOs under field conditions has also been evaluated by other authors. Hanula & Sullivan (2008) showed that *Leptospermum scoparium* (Myrtaceae) (manuka) and *Phoebe porosa* (Lauraceae) (phoebe) EOs were attractive baits for field monitoring of *Xyleborus glabratus* (Coleoptera: Curculionidae) in South Carolina, with daily catch mean of 1.3 and 3.5 insects per day, respectively. In addition, they hypothesized that the main components of these oils, two sesquiterpenes, *α*-copaene and calamenene, were probably the primary attractants for this insect. Similarly, Kendra et al. (2011) indicated that traps baited with phoebe oil lures captured more *X. glabratus* beetles than those with manuka oil lures and according to published chemical analyses of manuka oil have shown a high degree of variability among extracts from trees of different geographic regions in New Zealand (Porter & Wilkins, 1998), which may contribute to variability in attraction of *X. glabratus*. Other evidences showed that EOs can act synergizing an attraction, as *Juniperus oxycedrus* EO that enhances the attraction of *α*-ionol to male *Bactrocera latifrons* (Diptera: Tephritidae) (McQuate & Peck, 2001). Moreover, EOs can mask attractant lures and disrupt the olfaction of the insects, as wintergreen and peppermint EOs that produced the reductions in Japanese beetle *Popillia japonica* (Coleoptera: Scarabaeidae) trap counts relative to commercial attractant (phenethyl propionate: eugenol: geraniol, 3:7:3 by volume) (PEG) - baited traps (Youssef et al., 2009).

EOs based commercial products are being developed for a wide range of human and animal uses, including pest control. Some American companies have developed EOs based pesticides in a far shorter time period than would normally be required for a conventional pesticide. For example, Mycotech Corporation produces ValeroTM, as a miticide/fungicide for use in grapes, berry crops, citrus and nuts and CinnamiteTM, as an aphidicide/miticide/fungicide for greenhouse and horticultural crops. Both products are based on cinnamon oil, with cinnamaldehyde (30% in formulations) as the active ingredient (Tripathi et al., 2009). Moreover, EOs based repellents are designed as topical preparations or combustible products that are able to protect the user or environment from harmful insects (Oyedele et al., 2000). Many commercial products like Buzz Away® (containing oils of citronella, cedarwood, eucalyptus and lemongrass), Green Ban® (containing oils of citronella, cajuput, lavender, safrole free sassafrass, peppermint and bergaptene free bergamot oil) and Sin So-Soft® (containing various oils and stearates).

Concerning to the pesticide activity, the creating synergistic combinations is to reduce the dose of polluting substances and reduce the risk of developing resistance. A broad array of pest-repellent products, including herbal teas, plant extracts and fermentation products and industrial clay and rock powder products (e.g. kaolin) are authorized for use in organic agriculture. Nevertheless, the use of these products has declined in recent years because of the commercialization of standardized industrial products (Isman, 2006). Sinzogan et al. (2006) reported that damage to cotton by the bollworm, *Helicopverpa armigera* (Hubner) (Lepidoptera: Noctuidae) can be minimized by mixtures of conventional insecticides at one half the recommended rate by combining extracts of three local plants (*Azadirachta indica*, *Khya senegalensis* and *Hyptis suaveolens*) which provided greater efficacy than the conventional products alone at their recommended rate. Moreover, essential oils also showed some usefulness for building materials. A wood preservative solution mixed eucalyptus EOs with pyrethroids and borax (Urabe, 1992). However, none of the plant extracts alone provided adequate protection (Isman, 2008). The synergistic rationale for using combination products is oriented in producing a dynamic product which acts through multiple modes of action, respecting the principle that the action of the combined product is greater than the sum total of known and unknown chemical components. Both positive and negative synergism can occur between the EO or their components and the other ingredients present in the total formulation (Tripathi et al., 2009).

Nowadays the combination of EOs and active molecules is attracting special attention of the scientific in order to optimize the activity of natural products as new tools for the agriculture. The application and using of many bioactive EOs against pest insects has been limited in the agriculture and food packaging industries because their susceptibility to oxygen, light, and easy volatility at high temperatures (Asbahani et al., 2015; Chung et al., 2013). An alternative is the microencapsulation technology that has been applied to improve the sustained release effect, stabilization from environmental damage and for easy handling through solidification of essential oil (Chung et al., 2013). Controlled released formulations allow deliver smaller quantities of a substance to be used more effectively over a given period of time (Kydonieus, 1980). It can maintain constant concentration of active compounds at target (Lakkis, 2007). In initial stage, the amount of released oil is large and then slowly become constant (Chang & Dobashi, 2003; Chang et al., 2006). Encapsulation process is suitable method for entrapping EOs of different chemical composition. This method reduces loss of the active principles, leading to high loaded microparticles that offer protection against environmental agents and also offers the possibility of controlled substance release (Moretti et al., 2002). Microcapsules are small particles with a size between 1 and 1000 µm comprising an active agent surrounded by a natural or synthetic polymeric membrane. Microcapsules are composed by two parts, namely the core (the internal part) contains the active agent (e.g., an essential oil) and the shell (the external part) protects the core from the outer environment (Ghosh, 2006). There are numerous possibilities to use microencapsulation as a technique to obtain products with high added value. However, only the 2% of application is used in the agriculture (Martins et al., 2014). Some EOs formulations described by their potentiality for insect pest control are the microencapsulation of *Rosmarinus officinalis* and *Thymus herba-barona* EOs, where the microcapsules had toxic effects on *Limantria dispar* (Lepidoptera: Lymantridae, gypsy moth) larvae (Moretti et al., 2002); nanoparticles coated with polyethylene glycol (PEG) and loaded with garlic EO with insecticidal activity against adult *T. castaneum* (Yang et al., 2009) and microcapsules with *R. officinalis* and *Thymus vulgaris* EOs were insecticidal on *Plodia interpunctella* larvae (Sanna-Passino et al., 2004). In addition, microencapsulated thyme EO had long-lasting repellency (over 90% for 4 wk) on larvae of *P. interpunctella*, showing their great potential in the application of food packaging and processing (Chung et al., 2013). Moreover, monoterpenes with insecticide activity also have been encapsulated because it's rapid volatilization, pointing out remarkable differences in the release behavior of linalool depending on matrix composition and the method of encapsulation (Lopez et al., 2012).

Finally, it should be mentioned that certain EOs and their constituents are effective attractants for some insects. An attractant substance could be oriented to develop trapping systems (Katerinopoulos et al., 2005; Copping & Duke, 2007). For example, geraniol and eugenol are used as lures in traps for the Japanese beetle, and methyl eugenol was used to trap Oriental fruit fly *Dacus dorsalis* (Diptera: Tephritidae) (Vargas et al., 2000). These substances can be applied as attractant adhesive films with EOs prepared for the control of agriculture and horticulture pest (Klerk, 1990). Likewise, the repellent substances could be oriented to development of natural insecticides or repellents to prevent insect infestation, representing another potential use of EOs. It is important to point out that repellents could be useful tools in integrated pest management of agricultural crops, particularly in the context of a stimulus deterrent diversionary strategy (SDDS) or push-pull strategy (Miller & Cowles, 1990).

Concluding remarks

From the selection of non-host plants essential oils evaluated in this thesis it was possible to identify essential oils and pure compounds with either repellent or attractant properties on *Aegorhinus* spp. in laboratory bioassays.

Essential oil extracted from *Achillea millefolium* and its main compound thujone elicited a repellent effect on *Aegorhinus nodipennis* in olfactometric bioassay. Similarly, volatile compounds emitted from *Ruta chalepensis* essential oil were repellent to both sexes of *Aegorhinus superciliosus*. On the other hand, both sexes of this weevil were attracted to the odor released from *Eucalyptus* spp. essential oil and only female weevils were attracted to *Foeniculum vulgare* oil, while eucalyptol and anethole were only attractant for *A. superciliosus* females.

The chemical identification carried out for the essential oils in a GC-MS allowed know qualitative and quantitative composition of each. Discrepancies were found with those reported in the literature for all oils, which have been attributed to agronomic management, techniques used during their extraction as well as biotic and abiotic variations associated with growing plants.

The electroantennographic bioassays allowed establish that *A. nodipennis* antennae have olfactory receptors for thujone, whereas antennae of both sexes of *A. superciliosus* have olfactory receptors for eucalyptol and anethole. In addition, we suggest that the bioactivity produced in the weevils is associated with compounds of terpene type, mainly monoterpenes. Therefore, we suggest that these results have practical implications for management of *Aegorhinus* spp. associated with behavioral manipulation of the weevils, accepting the hypothesis of this thesis.

Future directions

The knowledge given in this Doctoral Thesis can be orientated to the development of attractant lure for *A. superciliosus* females or it could be the basis for the development of natural origin pesticides against *A. nodipennis* and *A. superciliosus*, where both strategies based on essential oils and their derivatives could be applied in an integrated pest management system for these weevils.

Under this context, future studies of essential oils and their compounds with attractant properties should be focused on optimizing trap design, their number and allocation in the crop fields (height, position, and density), adjusting the dosage of chemicals used and determining a possible synergic effect among them.

In the case of essential oils and their main compounds with repellent properties, future studies should be focused on evaluating the mode of action of thujone using molecular and biological techniques on *A. nodipennis* and evaluating the effects of 2-nonanone and 2-undecanone in behavioral bioassays, determining its electrophysiological effect and mode of action on *A. superciliosus*.

Scientific Production

Paper published

Tampe, J., Parra, L., Huaiquil, K., Mutis, A. & Quiroz, A. 2015. Repellent Effect and Metabolite Volatile Profile of the Essential Oil of *Achillea millefolium* Against *Aegorhinus nodipennis* (Hope) (Coleoptera: Curculionidae). Neotropical Entomology, 44 (3): 279-285.

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Rodríguez Beraud, M., Carrillo López, R., Chacón Fuentes, M., Hormazábal Vásquez, N., Tampe Pérez, J. & Tighe Neira, R. 2015. In vitro and ex vitro rooting of *Ugni molinae* Turcz. Microshoots, an endemic species to Chile. Gayana Botanica, 72 (1): 78-84.

Rodríguez Beraud, M., Hormazábal Vásquez, N., Araneda Durán X., & Tampe Pérez, J. 2016. Effects of gibberellic acid, benzylaminopurine and fluridone on the in vitro germination of *Ugni molinae* Turcz. Gayana Botanica, Nº 73 (1).

Submitted papers

Tampe, J., Parra, L., & Quiroz, A. 2016. Antennal Response of the Weevil *Aegorhinus nodipennis* (Hope) (Coleoptera: Curculionidae) to Thujone Monoterpene. Sent to Neotropical Entomology

Paper in preparation

Tampe, J., Pacheco, B., Parra, L., & Quiroz, A. Attractant Activity Elicited by *Eucalyptus* spp. and *Foeniculum vulgare* Essential Oils and its Main Constituents Against Adult of the Raspberry Weevil, *Aegorhinus superciliosus* (Guérin) (Coleoptera: Curculionidae).

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2010. Tampe J., A., Quiroz & F., Pardo. Fatty acid present in the roots of two cultivars and one experimental line of red clover (*Trifolium pratense* L.). 2nd International workshop "Advances in Science and Technology of Natural Resources", October 27-29, Pucón, Chile.

2007. Tampe, J., M. Rodríguez & G. Cabrera. Efecto de la quitosana sobre la organogénesis y crecimiento "in vitro" de dos especies vegetales; papa (*Solanum tuberosum* L.) y begonia (*Begonia rex*). Poster. IV Simposium de la Sociedad Iberoamericana de Quitina y Quitosana (SIAQ), Brasil.

Scientific Conferences

2013. Explora Conicyt. Participación en el programa "1000 científicos 100o aulas"

2012. Explora Conicyt. Participación en el programa "1000 científicos 100o aulas"

Appendix 1

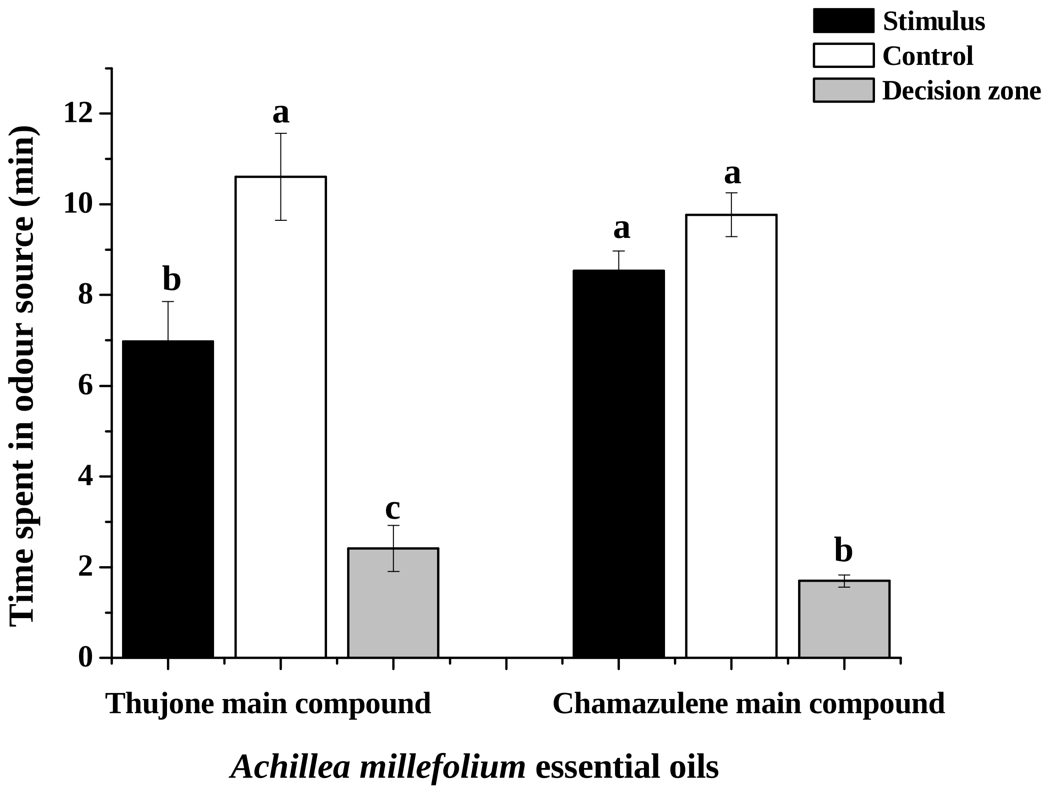

Figure 1. Average time spent (min) (± SE) by *Aegorhinus nodipennis* on two *Achillea millefolium* essential oils of different chemical composition in olfactometer bioassays. Different letters indicate statistical difference ($P \leq 0.05$) based on the non-parametric Friedman test followed by Conover-Inman test, N > 20.

Appendix 2

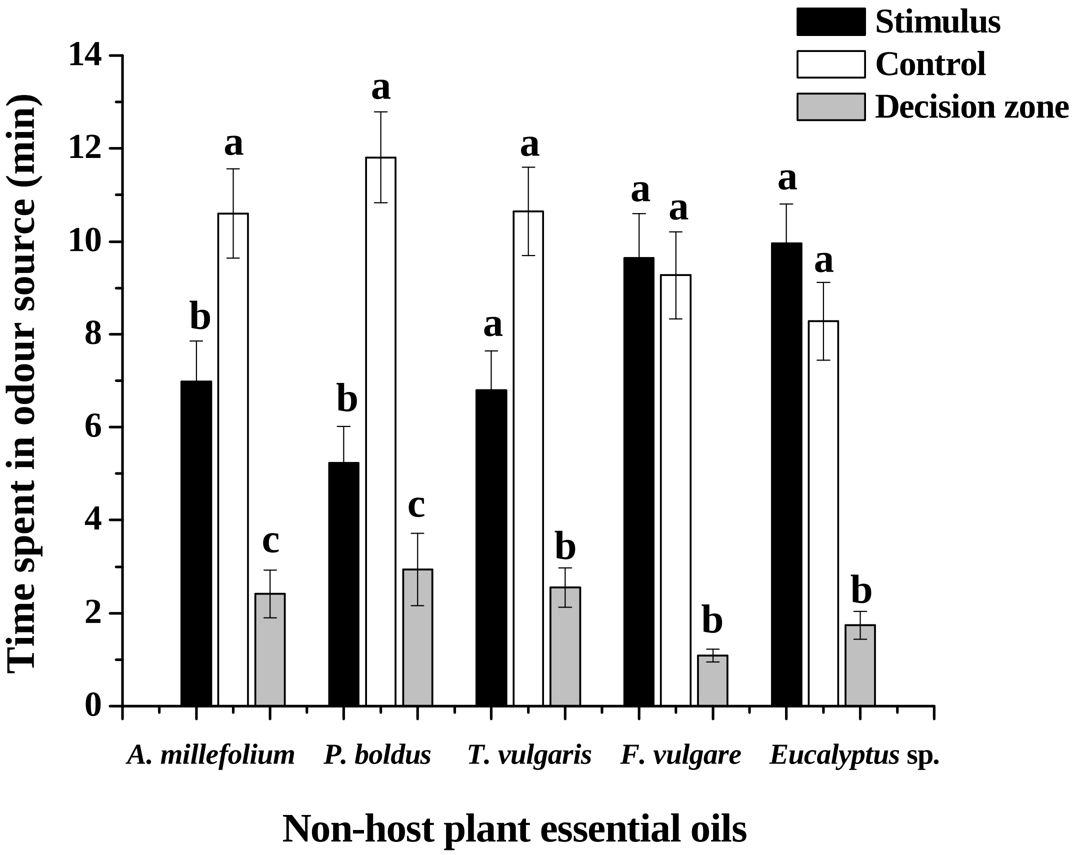

Figure 2. Average time spent (min) (± SE) by *Aegorhinus nodipennis* on non-host plants essential oils (500 ng) in olfactometer bioassays. Different letters indicate statistical difference among zones (stimulus, control and decision zone) ($P \leq 0.05$) based on the non-parametric Friedman test followed by Conover-Inman test, N = 30.

Appendix 3

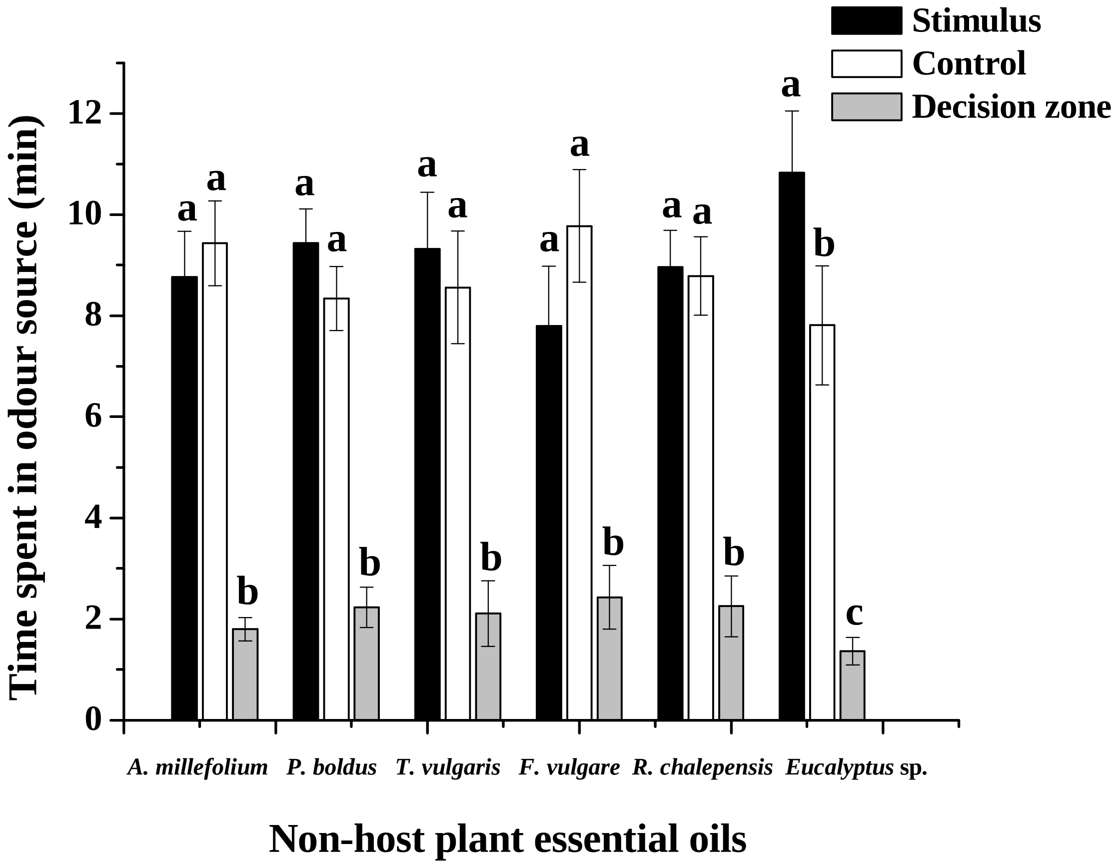

Figure 3. Average time spent (min) (± SE) by *Aegorhinus superciliosus* males on non-host essential oils (500 ng) in four-arm olfactometer bioassay. Different letters indicate statistical difference among zones (stimulus, control and decision zone) ($P \leq 0.05$) based on the non-parametric Friedman test followed by Conover test, N=20 per sex.

Appendix 4

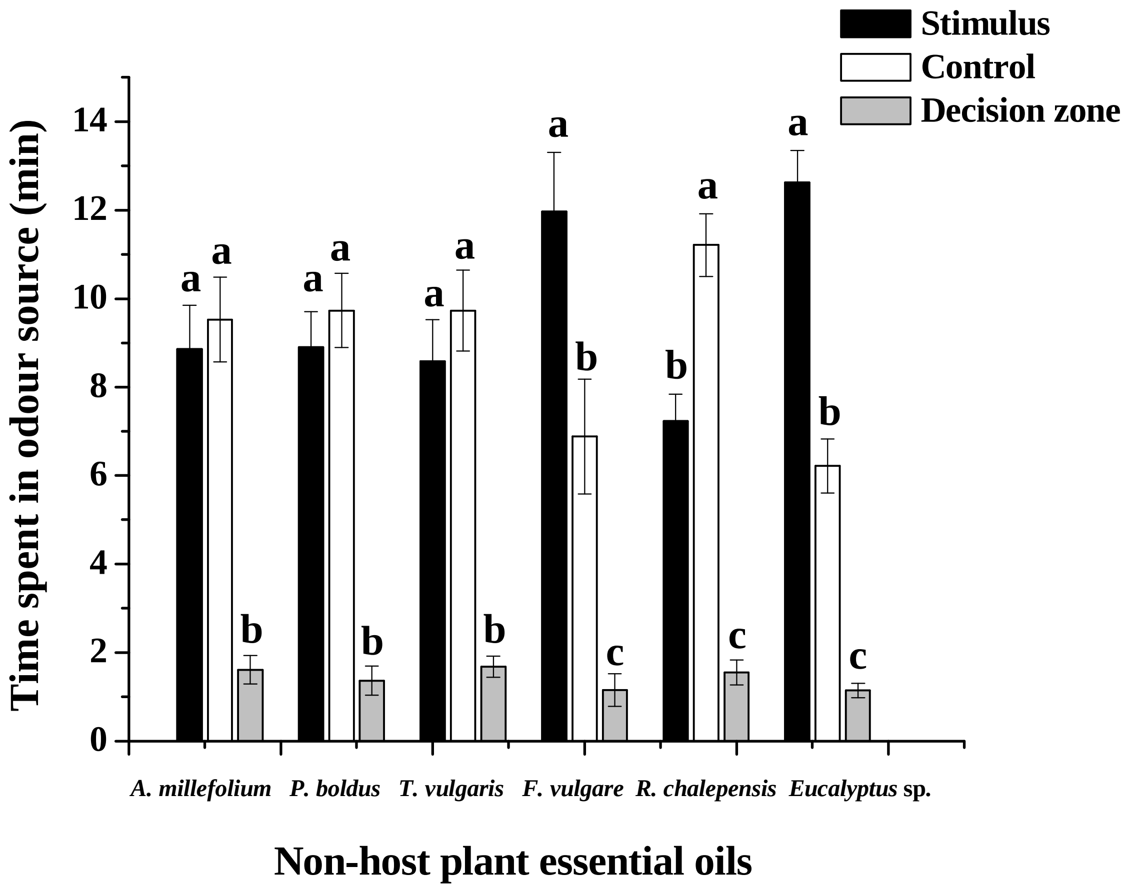

Figure 4. Average time spent (min) (± SE) by *Aegorhinus superciliosus* females on non-host essential oils (500 ng) in four-arm olfactometer bioassay. Different letters indicate statistical difference among zones (stimulus, control and decision zone) ($P \leq 0.05$) based on the non-parametric Friedman test followed by Conover test, N=20 per sex.

Appendix 5

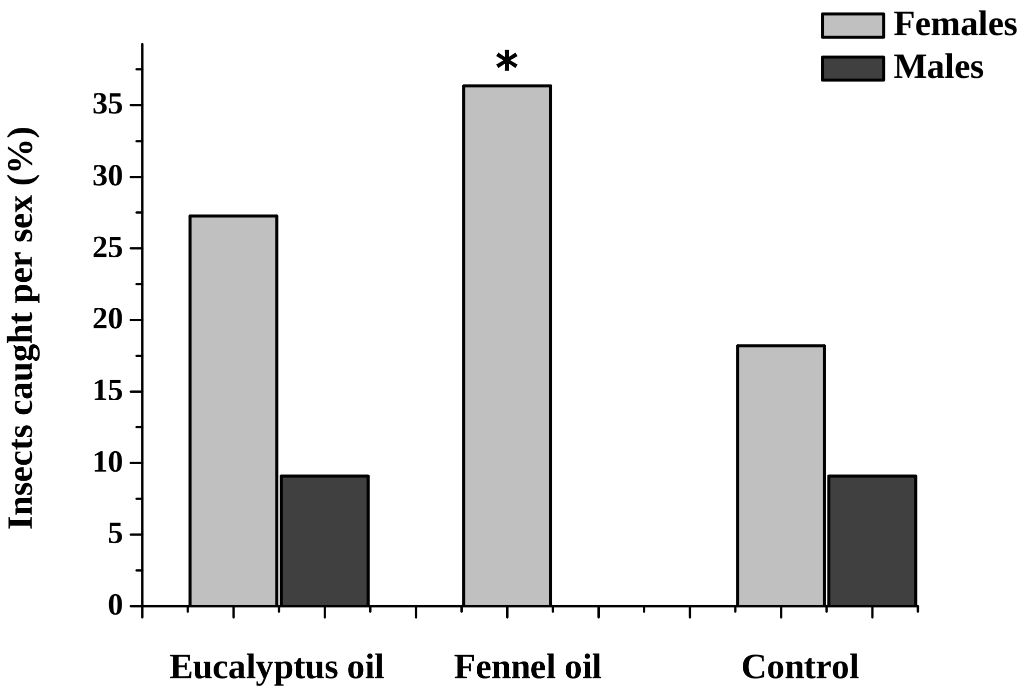

Figure 5. Percentage of insects captured in the field experiment according to sex in each treatment baited with 200 µl of eucalyptus, fennel essential oil and control. Different letters indicate statistical difference ($P \leq 0.05$) based on the non-parametric Friedman test followed by Conover-Inman test and asterisk (*) indicates significant differences between sexes based on X^2 test ($P < 0.05$, N=11).

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