UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería y Ciencias

Doctorado en Ciencias de Recursos Naturales

Effect of Arbuscular Mycorrhizal Fungi Inoculation and P-Fertilization on Terpene Emitted from Red Clover (*Trifolium pratense* L.) Leaf

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

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TEMUCO-CHILE

2022

“Effect of Arbuscular Mycorrhizal Fungi Inoculation and P-Fertilization on Terpene Emitted from Red Clover (*Trifolium pratense* L.) Leaf”

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Dedicatory

Dedicada a familiares, amigos, profesores y mascota, que me convirtieron en un ser humano más resiliente.

Acknowledgements

Agradecer lo vivido durante estos años, a mi madre Rosa que me enseñó la vida del trabajo y ser una persona de bien. A mis familiares perdidos (Alfonsina, Waldo y Molestoso) que de seguro estarían muy contentos al estar aquí pudiendo ver cómo voy cumpliendo mis expectativas en la vida y que de todos ellos trato de recordar lo que me entregaron para ser un nieto, un sobrino y un compañero del cual se puedan sentir orgullosos. A mi pareja y compañera Yosyua y familia, que me apoyaron siempre en cada paso que emprendo entregándome templanza para tomar las mejores decisiones y donde muchas veces en esta etapa me vio caer, pero ahí estuvo siempre, para darme aliento. A todos mis amigos que día a día ya sea en el trabajo diario o vida después del trabajo se preocuparon por mí y por mis pequeños logros diarios, hasta llegar aquí.

Agradecer al Dr. Quiroz, por apoyarme y entender muchas situaciones respecto a mí, para guiarme en el trabajo de obtención de este gran salto en la vida académica, siempre le estaré agradecido por todo. También, agradecer al director de la carrera de Agronomía, Dr. Bardehle, por todos los buenos consejos, apoyo y comprensión en estos años, gracias a sus palabras que también fueron aplicadas en la cancha. A la Dra. Mutis, que siempre fue una guía tanto en el trabajo como en la vida, siempre agradeceré sus consejos cuando muchas veces perdí el foco. Al Dr. Hormazábal, por apoyarme y guiarme cuando lo necesité de él.

A mis amigos y colegas del laboratorio, Dr. Espinoza, Camilo, Loreto, Washington, Dr. Aranda, Dr. Venthur, Ricardo, Marcelo, Stephanie, Paola, Moraga, Carla por todos los buenos momentos que hemos vivido, lidiando cada uno con sus experiencias, pero a pesar de todo siempre supimos apoyarnos entre todos para mejorar. A los auxiliares del Departamento

de Ciencias Químicas y Recursos Naturales, Don Jorge Celedón y Don Jorge Garcés que siempre estuvieron presentes para ayudarme en variadas tareas y actividades. A Muriel Melo, de INIA-Carillanca por su siempre buena disposición a colaborar y enseñarme, así como también al Dr. Ortega de INIA-Carillanca por su apoyo en emprender el viaje por el trébol rosado, donde cometí muchos errores, pero tuvo paciencia para ayudarme. A la Dra. Aguilera, por su ayuda para comprender los hongos micorrícicos y su siempre buena voluntad y disposición.

Agradecer a mis amigos, Lucho, Eric, Max, Felipe, Guillermo, Johana, Karen, Pilar, que de una u otra forma siempre estuvieron ahí para apoyarme en tiempos complejos.

Agradecer a todos, lo que fueron parte de mi proceso de formación humana y académica, espero cumplir con las expectativas en el futuro y que, desde aquí, no sé qué sucederá con mi vida personal y profesional, pero créanme que seguiré siendo el mismo personaje de siempre y que a pesar de que la vida nos obstaculiza el terreno, siempre estaré ahí para ayudarles y que podamos sobrepasar los obstáculos en conjunto, porque la vida siempre nos tiene una vuelta nueva.

Summary and Thesis Outline

Red clover (*Trifolium pratense* L.) is a perennial legume of nutritional and economic importance in temperate and sub-tropical regions of the world, and it is valuable for animal production and the seed industry. However, red clover declines up the second year of establishment meanly due to the attack of the curculionid *Hylastinus obscurus*. Due to there is no chemical or biological control for this insect, in recent years it has been investigated the chemical ecology involved in the interaction *H. obscurus*-red clover. Currently, two relevant ways to control this pest have been considered. The first corresponds to a plant breeding management developed by the Instituto de Investigaciones Agropecuarias (INIA), resulting in new material with best persistence, such as Quiñequeli-INIA, Redqueli-INIA and Superqueli-INIA. The second correspond to chemical ecology studies of the relationship red clover-*H. obscurus*, resulting in the discovery of attractant (*E*-2-hexenal), repellent (limonene), and antifeedant (formononetin and genistein) semiochemicals. At this respect, Superqueli-INIA cultivar has shown the best agronomic performance and high emission of the monoterpene limonene.

The ability of the plant to produce a large number of terpenoids is supported by the presence of a large number of genes involved in terpene biosynthesis as an outcome of co-evolution with natural enemies. Other terpenoids have non-defensive functions, including signaling to mutualists, such as pollinators or symbionts.

Phosphorus (P) is a macronutrient essential for growth, development, metabolism and regulation of enzymatic processes in plants. Plants are able to take up P from the soil via PHT1 transporters, which are relevant in the mobilisation of P from the soil to the plant. Plants, faced with P deficiency, activate a wide range of morphological, physiological and biochemical

modifications, thus generating a better availability of phosphorus for the plant, which can develop various agronomic characteristics due to P fertilization.

Arbuscular Mycorrhizal Fungi (AMF) represents the most widely distributed microorganism on earth, with a symbiotic interaction with plants since millions of years. The obligate symbiotic relationship to encompass more than 80% of plants. AMF plays a key role in acquiring nutrients for plants, but the plant must deliver carbohydrates to the fungus to maintain the symbiotic relationship. The fungus-plant relationship is strongly associated with an increase in crop productivity associated with the acquisition of nutrients such as nitrogen (N), phosphorus (P) and potassium (K), and improve water acquisition by improving water catchment networks. Moreover, this biotic factor contributes to resistance against pathogens in roots. However, the effect of the plant-fungus relationship on terpene production in red clover has not been investigated.

Considering that, phosphorus (P) and Arbuscular Mycorrhiza Fungi (AMF) have been pointed out as important factors to increase the content of terpenes in plants as a possible control method against *H. obscurus*, this project proposes to increase the production of terpenes due to the joint action of AMF and Phosphorous fertilization, in addition to investigating the biosynthesis of monoterpenes in red clover.

In **Chapter I**, a general introduction of this Doctoral Thesis, showing the introduction, hypothesis, and goals of this study is addressed and a review of the general characteristics of *Trifolium pratense*, and secondary metabolites associated to this legume with their interaction with *Hylastinus obscurus* and the family Curculionidae is addressed.

In **Chapter II**, the effect of AMF, P and AMF + P on monoterpenes emitted by three cultivars of red clover Quiñequeli-INIA, Redqueli-INIA and Superqueli-INIA is addressed. Seeds of the cultivars were germinated and then taken to greenhouse, after which the treatments were applied and after two months monoterpenes were captured from an individual plant. The treatments were carried out in a factorial system using two levels of AMF inoculums (AMF_1: *Claroideoglomus claroideum* and AMF_2: mix inoculum) and two levels of fertilization (basal 11.55 mg/kg and Super Triple Phosphate addition to increase up to 30 mg/kg). Terpenes were trapped for 24 hours by Porapak-Q column and analysed by gas chromatography coupled to a mass spectrometer (GC-MS). Four monoterpenes were identified (α-pinene, sabinene, (*S*)-limonene and p-cymen-7-ol). The results showed that the phosphorous addition elicited an increase of 1500% and 600% in the total emission of terpenes and (*S*)-limonene respectively in Superqueli-INIA cultivar. On the other hand, Quiñequeli-INIA cultivar was present a significant increase for total monoterpene emission for mix inoculum (AMF_2) and P fertilization respect to control. These results suggest that through an adequate phosphorus fertilization, the attack of *H. obscurus* could be controlled.

In **Chapter III**, the effect of AMF inoculation on monoterpenes biosynthesis is addressed. The enzymatic production of monoterpenes from Superqueli-INIA cultivar with two AMF inocula was evaluated via HeadSpace-Gas Chromatography (HS-GC). The enantiospecific production of (*S*)-limonene in red clover was confirmed in this work. A significant increase of (*S*)-limonene was found in plants inoculated with *Claroideoglomus claroideum* as well as with the AMF mix (genera *Scutellospora*, *Acaulospora* and *Glomus*). Moreover, significant increases in other monoterpenes such as (-)-β-pinene, myrcene, linalool, were observed. Results showed higher monoterpene production capacities in the Superqueli-INIA cultivar, suggesting the participation of monoterpene synthases (MTS). The significant rise of (*S*)-limonene in red clover plants inoculated with AMF suggests this strategy could be implemented in an agronomical management for controlling the *H. obscurus*, the primary pest.

In **Chapter IV,** a general discussion of all the results from the thesis word is addressed. Red clover is a plant that has the ability to adapt to a variety of biotic and abiotic conditions. Regarding the emission of captured terpenes of the cultivars, a better adaptation to the response of the factors can be visualized for the cultivar Superqueli-INIA, where in the use of phosphorus (P) fertilization treatments, as in AMF addition, it presented a better response in the emission and biosynthesis of monoterpenes with emphasis on the repellent (*S*)-limonene. The relationship between AMF-P is clearly visualized in chapter III where the emission of monoterpenes is positively influenced when P fertilization was used in the Superqueli-INIA cultivar, increasing its emission significantly, thus generating a possible improvement in the utilization of the nutrient reflected in a better emission of (*S*)-limonene. On the other hand, in Chapter IV of enzyme activity, a significant improvement in biosynthesis was observed using both inoculum without and with the addition of geranyl pyrophosphate (GPP), thus reflecting the potential of AMF in soil nutrient acquisition.

However, the relationship at contrasting phosphorus levels limits mycorrhization due to the exudation of orobanchol, a strigolactone present in the roots of red clover. There is a relationship between P levels and exudation of this metabolite that is relevant to generate colonization. Therefore, if there are high levels of P, mycorrhization will not occur due to the non-exudation of this Strigolactone, however in medium or low phosphorus soil conditions, orobanchol is exuded to generate mycorrhization and improve phosphorus uptake from the soil thanks to the AMF-plant relationship. Finally, P fertilization as well as the use of AMF inoculums are relevant for plant establishment, however, it is important to find the balance of AMF presence and P level in the soil in order to balance biotic and abiotic factors to improve the production of monoterpenes.

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CHAPTER I

General Introduction

1.1 Introduction

The Fabaceae or Leguminosae family plants have economic importance, contributing with 730 genera and over 18,400 species (Ahmed and Hassaballa, 2022) being *Trifolium* one of the genera belonging to the Leguminosae family widely distributed throughout the world. This genus includes annual and perennial species, occupying diverse ecological niches (Scoppola et al. 2018). *Trifolium pratense* L. commonly named as red clover is important in temperate and sub-tropical regions of the world. This plant is originally from Asia Minor and Southeast of Europe (Yan et al. 2022). It is a forage leguminous classified in Chile as perennial or short-lived perennial, having high adaptability to contrasting environmental conditions (Muñoz et al. 2021), growing in a wide range of soil types, pH (range 5.0 – 7.6), as well as the option of growing together with Alfalfa (*Medicago sativa* L.), perennial ryegrass (*Lolium perenne* L.), among others (Tahir et al. 2022). One of the relevant agronomic characteristics of the genus *Trifolium* is its ability to capture atmospheric nitrogen which has been widely studied and this is provoked for the symbiosis with *Rhizobium* bacteria (Furtak et al. 2020), decreasing the requirement of nitrogen fertilizers (Harris and Ratnieks, 2022). Regarding its morphology, it has a zygomorphic crown, with a high number of stems, growing up to 60 cm. This plant has a pivoting root, reaching depths of up to one meter with secondary ramifications, which provides a high resistance to drought (Demanet, 2022).

However, its production declines after 2 years of establishment of the crop. This fact is associated in Chile mainly to the attack of *Hylastinus obscurus* (Marsham), a curculionid that bores the roots of red clover. To date there is no chemical or biological control of this pest (Quiroz et al. 2018), However, there is a recent review of the potential of essential oils in Curculionidae control (Garrido-Miranda et al. 2022). In the last fifteen years through of chemical ecology studies several secondary metabolites with attractant, repellent and antifeedant activities have been identified

(Tapia et al. 2005; Alarcón et al. 2010; Parra et al. 2013; Quiroz et al. 2017; Espinoza et al. 2018; Quiroz et al. 2018). Moreover, red clover produces volatiles organic compounds (VOC), and non-volatiles organics compounds present in leaves, stems, and roots (De Rijke et al. 2005; Figueiredo et al. 2007; Tapia et al. 2007; Quiroz et al. 2018). VOCs could mediate the communication (Ninkovic et al. 2010) with organisms through intra-specific interactions. Red clover has a wide number of organic compounds such as terpenes, green leaf volatile (GLV) (see annex S1.1), isoflavonoids, among others (Figueiredo et al. 2007; Kigathi et al. 2019). Some of them play a repellent and attracting role (Palma et al. 2012; Parra et al. 2013). In the same way, non-volatile organic compounds as isoflavonoids and flavonoids present in red clover possess defensive functions such as anti-feeding like formononetin and genistein or phagostimulant behaviour daidzein and biochanin A (Quiroz et al. 2018).

The production of secondary metabolites in plants and other organisms is modulated as response to biotic and abiotic factors (Jan et al. 2021) such as insects, fungi, bacteria, phosphorus, and nitrogen, among others. One of the biotic factors affecting the production of secondary metabolites is the presence of arbuscular mycorrhizal fungi (AMF), that are microorganism mutualist symbionts colonizing more than 80% of vascular plants roots (Kobae 2019; Chen et al. 2021) belonging to the Phyllum Glomeromycota (Schüßler et al. 2001; Yang et al. 2021). Although the mechanism of symbiosis AMF-plants is gradually being elucidated, it is known that it improves morphological, physiological and biochemical aspects (Leventis et al. 2021) associated with different responses, including production and inhibition of secondary metabolites production with emphasis on terpenes, which play various roles in soil-plant, soil-insect interactions, among others (Carreón-Abud et al. 2015; Sharma et al. 2017; Dowarah et al. 2021).

Phosphorus (P) is an essential plant nutrient required for several events related to plant growing such as carbon metabolism, energy generation, energy transfer, enzyme activation, membrane formation, and nitrogen (N_2) fixation (Verlinden et al. 2022). Moreover, P is a key factor in the formation of biological molecules like ATP, nucleic acids, and phospholipids (Marschner and Rengel, 2012; Zhao et al. 2021; Fall et al. 2022). Phosphorous is a limiting factor and can compromise primary and secondary metabolism (Chan et al. 2021; Etesami et al. 2021). The relationship of AMF-P as biotic and abiotic factors respectively could have effects on the production of terpenes for red clover (*Trifolium pratense*), thus being able to improve the emission response of terpenoids.

Therefore, the following research question arises: Could the presence of AMF and/or P fertilization modify the secondary metabolism associated with monoterpenes in red clover?

1.2 Hypothesis and Research Objectives

1.2.1 Hypothesis

The terpenes emission from the aerial part of red clover will be increased by both their symbiosis with arbuscular mycorrhiza fungi and P-fertilization, producing plants with enhanced level of limonene.

1.2.2 Research Objectives

1.2.2.1 General Objective

- To determinate the effect of arbuscular mycorrhizal fungi inoculation and P-fertilization on terpene emission of red clover (*Trifolium pratense* L.) cultivars.

1.2.2.2 Specific Objectives

- To determine the effect of P-fertilization on the terpene production of red clover cultivars inoculated and non-inoculated with AMF.
- To determinate monoterpene emission *in vivo* and *in vitro* of inoculated and non-inoculated red clover cultivars

1.3 Agronomic Considerations

Red clover (*Trifolium pratense*) is a perennial legume of nutritional and economic importance in temperate and sub-tropical regions of the world. These crops can be grown alone or in the presence of other plant species (e.g., *Lolium perenne*) (Mcelroy et al. 2017). This forage plant is characterized by its versatile adaptation to biotic and abiotic factors (Torres and Dumont 1991) which are considered as relevant in the persistence and field yield of crops. This legume is also valuable for animal production and the seed industry, with exports from 700 tons per year to 100,000 cultivated hectares in the last decade, equivalent to 20% of the prairies sown in Chile (Ortega, 2009). About the biotic and abiotic factors, *Hylastinus obscurus* is the main responsible of the red clover decline (Alarcón et al. 2010; Parra et al. 2013). Plant breeding and chemical ecology studies have allowed to propose a new strategy for controlling this pest. Instituto de Investigaciones Agropecuarias (INIA) has carried out plant breeding of this legume (Ortega et al. 2014) with emphasis on addressing the problem of persistence in field and, therefore, reducing the presence of this insect, generating new experimental lines that may be more resistant to insect attack, and thus more persistent, without compromising the performance. So far, chemical ecology studies of red clover plants have allowed identifying bioactive secondary metabolites against *H. obscurus*. Specifically, formononetin, limonene and (*E*)-2-Hexenal have been reported as antifeeding, repellent and attractant activity on the root borer (Manosalva et al. 2011; Palma et al. 2012; Parra et al. 2013; Quiroz et al. 2017, 2018). On the other hand, red clover is associated to *Rhizobium trifolii* (Furtak et al. 2020), which contributes to the fixation of atmospheric nitrogen by symbiosis plant-bacteria, thus reducing the need of the addition of nitrogen fertilizers to crops. Moreover, arbuscular mycorrhizal fungi (AMF) symbiotic microorganism could influence the production of secondary metabolites (Zeng et al. 2013; Carreón-Abud et al. 2015; Sharma et al.

2017), as well as P fertilization can increase the production of secondary metabolites reported by Zeng et al. (2013).

In summary, reports have established that limonene could be a key factor for controlling *H. obscurus*. However, the effects of biotic and abiotic factors on the concentration of this secondary metabolite have not been studied yet. According to the literature, the use of these factors could collaborate to improve the production of secondary metabolites in plants (Ormeño et al. 2007; Ramakrishna and Ravishankar 2011; Latif et al. 2016). Therefore, the search for the best symbiont fungus as well as the effect of different concentration of P for obtaining red clover plants with higher amount of the repellent monoterpene (*S*)-limonene is the main objective to control *H. obscurus*.

1.3.1 Morphology and Growing Conditions of Red Clover

In Chile, *T. pratense* was introduced in 1869 in the Maule province (Torres and Dumont, 1991), and it has been spread from Santiago to Llanquihue province. This plant has become an important resource for the seed industry, as well as for animal production, as it is a primary food in ruminant diets because of their high nutritional value in comparison to other food resources (Wilkins and Jones, 2000). In agronomy, its primary use is based on short rotation pasture species. Represents approximately 20% of sown pastures and 60% of forage seeds exports (Ortega et al. 2014). INIA, a Chilean governmental institution has been concerned since the eighties to carry out a plant-breeding program for this plant, finding improvements in the production of different varieties of red clover.

Red clover is a plant considered perennial (Sheldrick et al. 1986), however in Chile, it does not persist over two or three years (Ortega et al. 2009). Its morphology possesses a crown-pivoting root with a maximum depth of one-meter, varied ramifications that are beneficial to resist drought periods, and large trifoliate leaves. *T. pratense* plants have a considerable adaptation capacity, resulting in high plant resilience against adverse conditions. Although red clover crop in Chile is extended throughout the country, it is principally in the central-south region, where climatic conditions are not extreme, and precipitations are suitable for the growth of this species. It is usually preferable to sown in autumn and/or spring if irrigation is available. In rainfed conditions, autumn sowing is preferable.

INIA has produced several varieties of red clover, which have been subjected to a breeding program to obtain cultivars with better persistence and forage yield (Ortega, 2009). Since the 1980s, plant breeding began with a focus on the Quiñequeli-INIA cultivar. This cultivar was tested together with others varieties from Europe in New Zealand obtaining the highest yields. (Torres and Dumont, 1991). Plant breeding has developed new cultivars such as Redqueli-INIA and Superqueli-INIA, which have been tested in the field, showing better resistance and forage yield compared to the Quiñequeli-INIA cultivar; the new cultivars shown better results in reducing the infestation by *H. obscurus* (Marsham) (Coleoptera: Curculionidae) commonly named root borer (Ortega et al. 2014).

1.3.2 Insects Associated to Red Clover

Through a series of environmental adaptations and varied factors, plants have had an evolutionary process that has transformed them into the most important organism at the service of the humanity (Molina-Venegas et al. 2021). In ecosystem in which plants are grown, they receive different and diverse simultaneous stimuli from biotic and abiotic factors. A relevant biotic factor in crops is insects and their association with various plants. Figure 1 shows the main pests associated with red clover in the database of European and Mediterranean Plant Protection Organization (EPPO) and reported by Aguilera et al. (1996), being Coleoptera and Lepidoptera the main orders associated with red clover. *Dalaca pallens* (Lepidoptera: Hepialidae) and *H. obscurus* (Coleoptera: Curculionidae) are the main insects in relation to the damage produced on plant. However, this damage does not occur in the same places at the plant. Larva of *D. pallens* feed on vegetation at collar level, consuming leaves and shoots, unlike the curculionid that feeds of the roots (Pruess and Weaver, 1958).

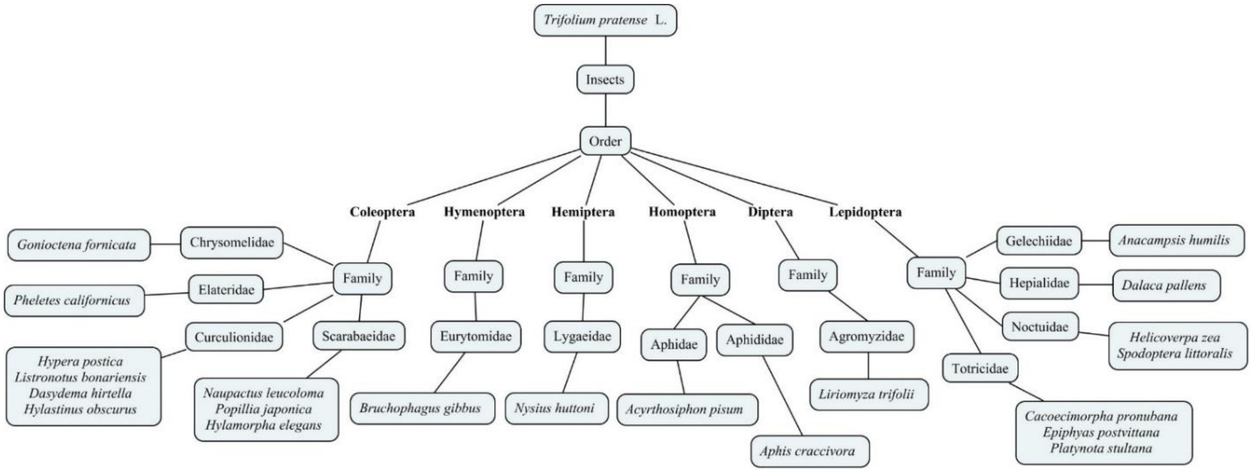

Figure 1. Insects associated with red clover cultivars based on EPPO Global Database and Aguilera et al. (1996).

However, the main cause of red clover decline in Chile is associated with insect *H. obscurus* (Fig. 2) native from Europe and Asia.

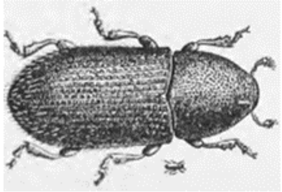

Figure 2. *Hylastinus obscurus* (Marsham) (Webster, 1910).

This monophagous and monovoltine insect, selects its host during the flight period (September to November), lay the eggs on the crown of the plant (November-December) and the larvae hatched introduces into the roots (December-March) building galleries where feed, the adults stay feeding into the roots until September (Fig. 3). Although, adult, larvae, and pupa stages are present all year. This feeding behaviour cause a huge damage to the radical system interfering in the conduction of nutrient and water (Fig. 4). Its destructive presence in red clover crops is evident in the second year, being the summer months the most complex for the growth of this plant, up to 36 insects per plant have been found (Carrillo and Mundaca, 1974). Plantations of this legume infested by *H. obscurus*, reducing the effectiveness of the crops (Quiroz et al. 2018).

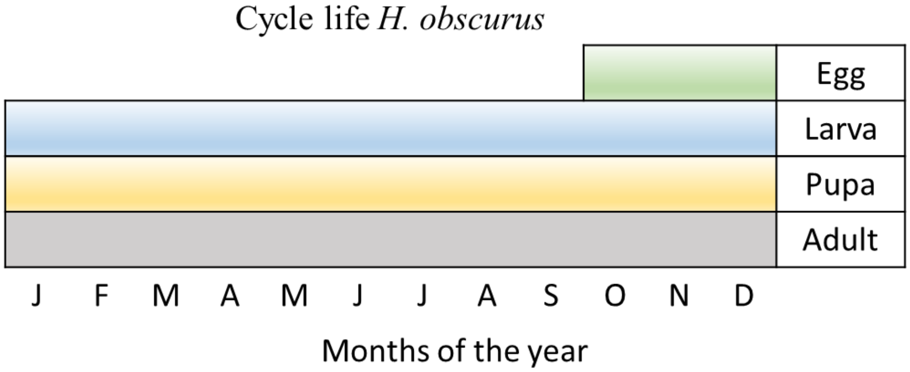

Figure 3. Life cycle of *H. obscurus* in a year (adapted from Carrillo and Mundaca, 1974).

Figure 4. Damage of *H. obscurus* (Marsham) in red clover roots.

Source: Laboratorio de Química Ecológica, Universidad de La Frontera.

Biological, cultural and chemical controls have been ineffective for this curculionid. Gustafson, (1958), collected information regarding the chemical control of this insect, reporting that the chemical application of sodium nitrate ($NaNO_3$), potassium chloride (KCl) and a specialized Kainit (K_2O 11%; MgO 5%; Na_2O 27%; SO_3 10% Cl^- 43%) forage fertilizer did not contribute to the reduction of *H. obscurus* as well as causing adverse effects to plants. Accordingly, the main reason for the ineffectiveness of chemical control is associated with the fact that the life cycle of the insect develops within roots, where the larvae and adults remain all year round. Cultural control is used to prevent infestation in other cultivars, and biological control has not been hardly explored yet (Quiroz et al. 2017; Espinoza et al. 2018).

An alternative control approach emerges from chemical ecology through the investigation of possible secondary metabolites such as semiochemicals associated to the red clover plants as a mitigation measure of this pest. Parra et al. (2013), Quiroz et al. (2017) and Espinoza et al. (2018)

have attempted to search for a form of chemical control using secondary metabolites present in the plant, as well as essential oils from other plants against *H. obscurus*. Parra et al. (2013) reported field use of monoterpene limonene as a repellent against the insect, as well as the (*E*)-2-hexenal compounds as an attractant, both identify in red clover plant. On the other hand, Quiroz et al. (2017) identified the antifeedant activity of two isoflavonoids (genistein and formononetin) present in red clover. Hence and as a first step, is necessary to know the chemical compounds present in red clover plant.

1.4 Secondary Metabolites in Red Clover

Natural products are chemical compounds isolated from living organisms (Claramunt et al. 2013; Anulika et al. 2016; Olivoto et al. 2017). The chemistry associated with natural products includes several aspects: extraction, identification, quantification, and the physical and chemical properties of the reactions involved (Anulika et al. 2016). These chemical compounds are produced by primary and secondary metabolism (Claramunt et al. 2013). Metabolism is defined as a series of biochemical reactions regulated by enzymes for the production of metabolites in the form of catabolism or degradation, and anabolism or synthesis. There are chemicals (e.g. carbohydrates, proteins, fats, among others) that directly intervene in plant growth and reproduction, which are derived from the primary metabolism (Dewick, 2002; Claramunt et al. 2013; Talapatra and Talapatra, 2015; Anulika et al. 2016). The secondary metabolism produces chemical compounds that do not have a direct use in both growth and reproduction of organisms (Claramunt et al. 2013; Anulika et al. 2016), focusing on a function associated with ecology. Many of the compounds generated in the primary metabolism are the starting source to produce secondary metabolites for different biosynthetic pathways. The most important secondary metabolites present in red clover can be classified into two groups: terpenes and (iso)flavonoids. The presence of each of these groups of compounds in a wide diversity differs according to the plant under study, mediated by different biosynthetic pathways, all deriving from the glycolysis process (Yeshi et al. 2022).

A comprehensive review of secondary metabolites identified in red clover plants is shown in Annex S1.7. These secondary metabolites have been identified through diverse instrumental techniques such as high-performance liquid chromatography (HPLC), solid phase micro-extraction-gas coupled to chromatography-mass spectrometry (SPME-GC-MS), among others. Buttery et al. (1984) reported the identification of VOCs from red clover trapped by Tenax adsorbent and analyzed by gas chromatography (GC) coupled to mass spectrometry (MS). The main compounds were: 1) leaves: hexenol (24%), (*Z*)-3-hexenyl acetate (33%) and (*E*)-β-ocimene (22%); 2) flowers: acetophenone (24%), methyl cinnamate (11%) and 1-phenyl-ethanol (8%) and 3) pods: (*E*)-β-ocimene (35%), (*Z*)-β-ocimene (6%) and longifolene (5%). Tapia et al. (2007) studied the Quiñequeli-INIA cultivar used roots between 1.5 and 2.5 years old were subjected to a supercritical fluid extraction and further analysis by gas-liquid chromatography coupled to mass spectrometry; the mayor compounds identified were benzaldehyde (0.79 mg/mL), (*E)*-2-hexenal (0.76 mg/mL), and limonene (0.68 mg/mL) as major compounds. Figueiredo et al. (2007), studied red clover plants in green, hay and silage conditions, capturing with an SPME fiber of divinylbenzene-polydimethylsiloxane (DVB/PMDS) and then analyzed by a GC-MS. The reported compounds in green clover samples were: 3-octanol (3.44%), 6,10,14-trimethyl-2-pentadecanone (3.46%) and benzaldehyde (3.74%). In the case of hay, phenyl ethyl alcohol (2.28%), (*Z*)-β-caryophyllene (2.65%), β-farnesene (10.4%) and 6,10,14-trimethyl-2-pentadecanone (11.8%) were the mayor compounds. Finally, in silage, the most abundant compounds were ethyl hexanoate (co-eluting with 3-octanol) (3.51%), phenyl ethyl alcohol (co-eluting with linalool) (3.69%) and 3-methyl-butyl butanoate (3.77%). Kigathi et al. (2009) captured VOCs emitted by the aerial part of red clover plants via Super-Q trap and analyzed by GC-MS, informing that the main monoterpenes were α-pinene (2.78 ng/g), myrcene (2.13 ng/g), limonene (1.01 ng/g), (*Z*)-β-ocimene (6.64 ng/g), (*E*)-β-ocimene (23.42 ng/g). Additionally, hydrocarbons undecane (7.12 ng/g) and dodecane (13.59 ng/g); and aldehydes decanal (15.06 ng/g) and nonanal (11.74 ng/g) were identified. On another hand, Manosalva et al. (2011) described the presence of fatty acid in red clover roots of Quiñequeli-INIA cultivar obtained by dichloromethane extraction. The GC-MS analysis showed the presence of palmitic acid (33.2%), oleic acid (12.1%), and lauric acid (8.0%), among others. Palma et al. (2012) trapped the volatiles released from red clover roots of the cultivar Redqueli-INIA during 60 min by SPME using a PDMS/DVB fibre and then analyzed by GC-MS. Ethanol, hexanal, *E*-2-hexenal, α-pinene, 3-octane, and limonene were identified. Moreover, the authors informed variations in volatile compounds of roots of red clover. (*E*)-2-hexenal appears until 5-month-old roots, and limonene was detected only in the 6 and 24-month-old roots. 3-octanone appeared in all samples except 24-month-old roots. Ethanol was also present in samples except for 16-month-old roots. Hexanal was detected at the three youngest aged roots (5, 9 and 11-month-old roots), and α-pinene was intermittently determined in the 9 and 24-month-old roots. Vlaisavljević et al. (2014) obtained essential oils from different aerial part of red clover plants, in three growth stages (30 cm, 50 cm and buttoning period), identifying aliphatic and aromatic monoterpenes, sesquiterpenes and diterpenes. In the 30 cm plants ρ-cymene (3.59%), limonene (3.86%), β-ionone (9.46%) were identified. In 50 cm plants, the most abundant compounds were phytol (14.54%) and 2-pentanone (6.66%). Finally, in plants with buttoning only dihydrocarvone (6.47%) was identified.

There are also reports of compounds derived from the phenylpropanoid biosynthetic route, such as flavonoids and isoflavonoids (see Annex S1.7). Klejdus et al. (2001) carried out the identification of isoflavonoids by HPLC-MS of the red clover cv. Start, finding glycosidic and non-glycosidated isoflavonoids such as biochanin A, formononetin and daidzein 7-*O*-glucoside, among others. Krenn et al. (2002) used extracts and plants from Melbrosin International (Vienna, Austria) for the identification and quantification of isoflavonoids. Reporting daidzein (0.10 mg/g), genistein (0.08 mg/g), formononetin (2.85 mg/g) and biochanin A (2.03 mg/g). Lemežienė et al. (2015) studied eight different cultivars and three wild red clover ecotypes for isoflavonoid determination. The extraction of these compounds was carried out using methanol/water (80/20) mixture. An Ultra performance liquid chromatography (UPLC) with a photodiode array (PDA) detector was carried out for the analysis and quantification. The results showed the presence of formononetin (2.61-4.40 mg/g), biochanin A (1.79-3.32 mg/g), daidzein (0.06-0.14 mg/g) and genistein (0,36-0.59 mg/g). Tundis et al. (2015) performed the identification of flavonoids present in an ethanolic extract of flowers, identifying luteolin (16.7 mg/g), kaempferol (0.8 mg/g) and myricetin (0.5 mg/g). Vlaisavljević et al. (2017) determined the profile of red clover phenolic compounds in different growth stages. Isoflavonoids such as apigenin (0.32 µg/g), daidzein (1.527 µg/g), genistein (0.580 µg/g) and quercetin (0.12 µg/g), were identified in methanolic extract of Una red clover variety. Quiroz et al. (2017) reported significant differences in the content of aglycones isoflavonoids in roots of two Chilean red clover cultivars, Quiñequeli-INIA and Redqueli-INIA, such as formononetin (0.098 and 0.195 mg/g dry matter, respectively) and biochanin A (1.575 and 2.668 mg/g dry matter, respectively). Finally, Medina et al. (2022) reported through enzymatic activity of crude extracts of Superqueli-INIA, the presence of 11 monoterpenes such as: β-pinene, myrcene, α-phellandrene, (*S*)-limonene, geraniol, linalool among others, this being the first work on the relationship between the metabolic pathway of terpenes and a biotic factor of incidence for the plant.

1.4.1 Terpene Biosynthesis

Terpenes are the most characteristic secondary metabolites of plants. These compounds are also called terpenoids or isoprenoids and constitute a group of the most numerous natural products. The name terpene derives from the German "*Terpentin*," which is a pine resin. They are compounds derived from isoprene (2-methyl-1,3-butadiene) and are present in higher plants. A wide variety of these compounds are present in essential oils, being widely used as aromas and fragrances in the food industry and perfumery. According to the biosynthesis of these compounds, which has been described in detail, the starting compound, isopentenyl pyrophosphate (IPP), have two different biosynthetic pathways (Dewick, 2002) (Fig. 5), according to the precursor organism in which it is studied. Mevalonate (MEV) and 1-deoxy-d-xylulose-5-phosphate (DXP) pathways are characteristic in plant cells, occurring in the cytosol (pathway MEV) and plastid (DXP pathway) (Eisenreich et al. 2004; Vranová et al. 2013; Zebec et al. 2016). The first stage of the terpene biosynthetic pathway comprises a Claisen-type condensation of two acetyl-CoA molecules to obtain acetoacetyl-CoA. Subsequently, an aldol-type condensation with a third acetyl-CoA molecule produces 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). The conversion to (*R*)–mevalonic acid (MVA) is produced by a reduction in the presence of two molecules of NADPH. The first molecule of NADPH reduces the thioester group to aldehyde and the second one reduces the aldehyde to an alcohol. Then, the production of mevalonic acid is transformed into isoprene phosphorylated by ATP action (adenosine triphosphate). Finally, isopentenyl pyrophosphate (IPP) is isomerized to become a dimethylallyl pyrophosphate (DMAPP); this isomerization is stereospecific and reversible process. The production of terpenes begins from these two formed isomers. In the production of monoterpenes (C_{10}), about one thousand compounds of this type are known. Most of these molecules are present in essential oils and they are volatile. There are three monoterpene types: linear or alicyclic, monocyclic, and bicyclic. The combination of IPP and DMAPP by means of a prenyl-transferase enzyme produces geranyl pyrophosphate (GPP); this molecule is the precursor for the conversion of monoterpenes. This process is formed by the terpene-cyclases enzymes capable of transforming these linear to cyclic compounds. From here, the enzymes can transform from α-terpinyl cation into various cyclical terpenes, e.g., α-pinene and β-pinene, limonene, among others (Zhou and Pichersky, 2020; Qiao et al. 2021).

The enzymatic production of some secondary metabolites such as terpenes shows differences in Chilean cultivars of red clover. Preliminary results have shown that different cultivars of red clover, including Quiñequeli-INIA, Redqueli-INIA, and Superqueli-INIA, showed differential enzymatic activity to produce limonene (Quiroz, personal communication). This compound produces repellent activity against *H. obscurus*. Therefore, there could be differences in the plant-insect interaction according to the cultivar.

1.5 Relationship Between Secondary Metabolites and Curculionidae Order

The great biodiversity of living organisms interact between them by means of chemical compounds. The constant search to identify these chemicals has led ecological chemistry to become involved in these multitrophic relationships, also the relationship between plants and inserts is characterized by a constant exchange of chemical information, where one or both act as emitters and the other as receivers (Camarena, 2009). These chemical compounds are called semiochemicals, and there are many functionalities associated with these interactions (Benelli et al. 2014). The pheromone concept is used when a semiochemical has action in intra-specific relationships. On the other hand, allelochemicals are responsible for interspecific communication. When there is a chemical signal which influences the receptor, it is called kairomones, when there is a benefit to the emitter it is called allomones and finally there are the synomones which generate a benefit for both organisms involved (Cortesero et al. 2016). *H. obscurus* belongs to the order Coleoptera and family Curculionidae, a large family with more than 4,600 genera worldwide and over 50,000 described species. South America has approximately 20% of these species, making it a niche of high interest (Girón and Cardona-Duque, 2018). However, plant-insect interaction is a co-evolutionary relationship that has been represented since the time of the Devonian period (416 million years ago) (Wink, 2018). Since this relationship, the presence of terpene compounds in plants and their relationship with insects has been extensively studied, and this family compounds mediates herbivory attacks, among others (Welling et al. 2016; Boncan et al. 2020).

In the case of the Coleoptera order, there are plant-insect relationships associated with their attraction or repellent effects (Table 2). Tomlin et al. (1997), using *Pissodes strobi* made (ethanol/methanol/water) (EtOH/MeOH/H_2O) (79:29:1) extracts of *Picea sitchensis* clones, analyzed by GC-MS, found terpenes such as α-pinene and β-pinene, myrcene, 3-carene, 1,8-cineole, terpinolene, camphor which had resistance effects against the insect in its behaviour. 11 clones of this Pinaceae were used, with the Haney clone showing the best response in the insect-plant relationship. Another plant-curculionid interaction was evaluated by Lee et al. (2001). They analysed by GC-MS, essential oils of Anise (*Pimpinella anisum*), Arnica (*Arnica montana*), Citronella, Clove (*Syzygium aromaticum*), Eucalyptus, Fennel (*Foeniculum vulgare*), Frankincense, Grapefruit (*Citrus* × *paradisi*), Hypericum, Juniper, Lavender (*Lavendula* spp.), Myrrh, Patchouli (*Pogostemon cablin*), Petitgrain, Rosemary, Tea tree, Thyme, Ylangylang.

Reporting that mortality of *Sitophilus oryzae* was most effective with the compound's camphor, carvacrol, 1,8-cineole, citral, p-cymene, geraniol, limonene, linalool, perilla alcohol, terpinene, terpineol, terpinen-4-ol, thymol and thujone by LD_{50} assessment. Also, the curculionid *Aegorhinus superciliosus* commonly known as "Cabrito del Frambueso" reported attractive effects by olfactometric assays for the monoterpenes Eucalyptol and Limonene emitted by *Vaccinium corymbosum* (Arándano; Blueberry) (Parra et al. 2009). Zhang et al. (2010) evaluated the electrophysiological response of the curculionid to the volatile terpenes present in *Pinus armandi* Fr. by electroantennography (EAG). He reported that females of this insect responded strongly to low and high concentrations of α-phellandrene and only males responded to low concentrations of β-pinene and high concentrations of β-pinene. Thus, it was possible to separate the terpenes by sex of the curculionid by means of the EAG response.

Dendroctonus armandi in interaction of a mixture of terpenes (α-pinene, β-pinene, myrcene, 3-carene, limonene and longifolene) present in *Pinus armandi* Fr. in a field trapping test reported by Chen et al. (2014) had no effect against the insect. Piesik et al. (2016) obtained the same result with Y-tube olfactometric assay using the terpenes (β-ocimene, linalool, β-caryophyllene and β-farnesene) present in *Rumex confertus* Willd against their insect *Hypera rumicis* L. Additionally, Espinoza et al. (2016) reports repellent effect against *A. superciliosus* by essential oil from *Pilgerodendron uviferum* (D. Don) identify sesquiterpenes (δ-cadinol, cubenol, 15-copaenol and δ-cadinene) with the same result Góngora et al. (2020) report repellence against *Hypothenemus hampei* (Ferrari) in Olfactometric Y-tube assay with sesquiterpene β-caryophyllene. Finally, Giunta et al. (2016) evaluated changes in different volatile monoterpenes emitted by *Pseudotsuga menziesii* var. *glauca* (Mirb.) Franco with *Dendroctonus pseudotsugae*

Hopkins infestation. The monoterpenes identify were α-pinene, 3-carene, limonene, camphene, sabinene, α-terpinene, terpinolene, p-cymene, α-phellandrene, β-ocimene and γ-terpinene.

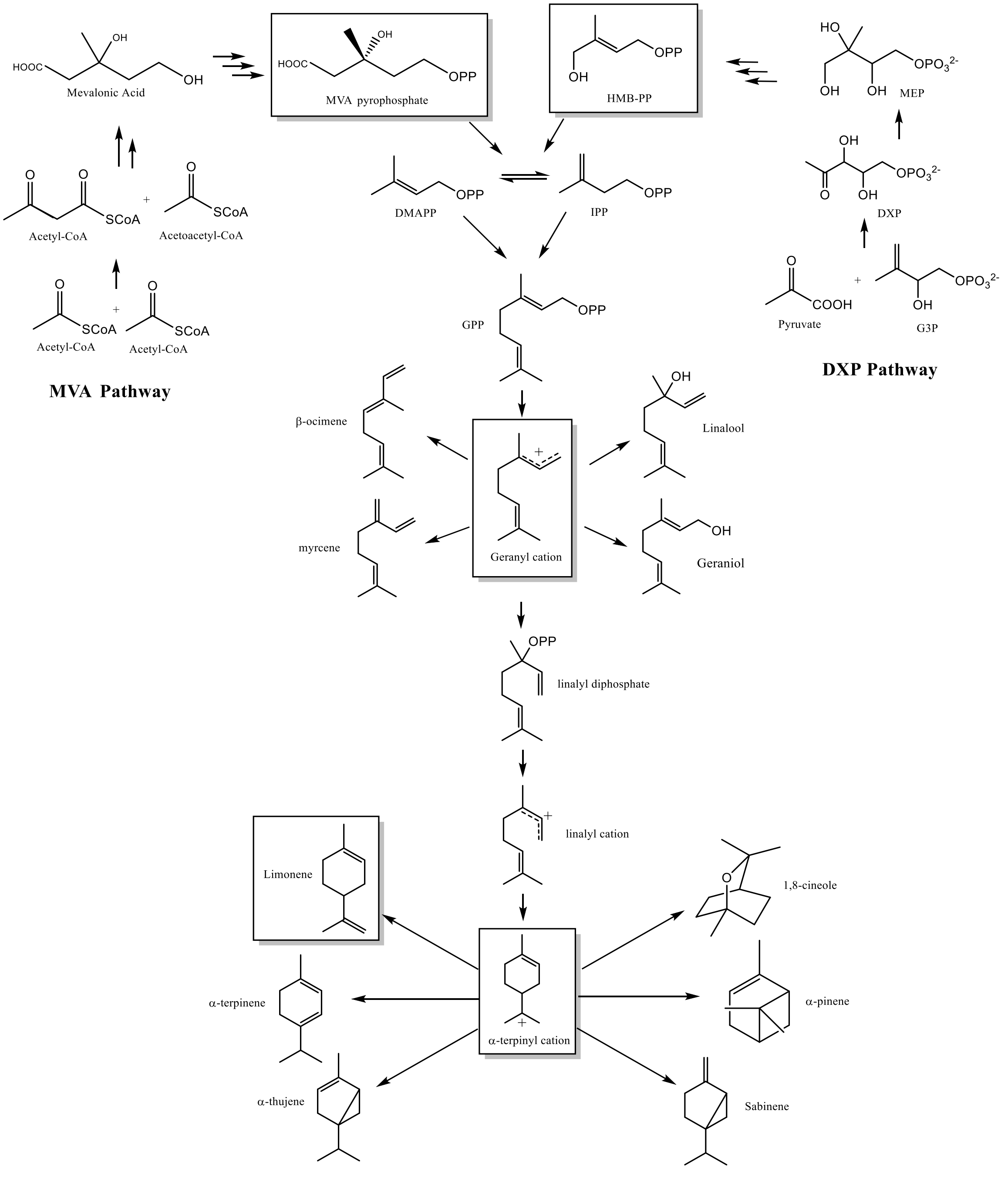

Figure 5. Mevalonate and DXP pathway of monoterpenes in plants (adapted from Eisenreich et al. 2004; Vranová et al. 2013; Zebec et al. 2016)

Table 2. Interaction of terpenes-curculionids by different assays.

Curculionid	**Plant(s)**	**Compound(s)**	**Classification**	**Effect**	**Reference**
Pissodes strobi	*Picea sitchensis*	α-pinene, β-pinene, Myrcene, 3-carene, 1,8-cineole, Terpinolene, Camphor	Monoterpene	Resistance	Tomlin et al. (1997)
Sitophilus oryzae	Anise, Arnica, Citronella, Clove, Eucalyptus, Fennel, Frankincense, Grapefruit, Hypericum, Juniper, Lavender Myrrh, Patchouli, Petitgrain, Rosemary, Tea tree, Thyme, Ylangylang	Camphor, Carvacrol, 1,8-cineole, Citral, p-Cymene, Geraniol, Limonene, Linalool, Perilla alcohol, Terpinene, Terpineol, Terpinen-4-ol, Thymol, Thujene	Monoterpene	Toxicity LD_{50}	Lee et al. (2001)
Aegorhinus superciliosus	*Vaccinium corymbosum* L.	Eucalyptol, Limonene	Monoterpene	Attractive	Parra et al. (2009)
Dendroctonus armandi	*Pinus armandi* Fr	β-pinene, α-phellandrene, α-pinene, (-)-camphene, (*S*)-limonene, (*R*)-limonene	Monoterpenes	Electroantennography	Zhang et al. (2010)

Dendroctonus armandi	*Pinus armandi* Fr	Mix of α-pinene, β-pinene, myrcene, 3-carene, limonene and longifolene	Mono- and sesquiterpenes	Field-trapping bioassay (No attractive)	Chen et al. (2014)
Hypera rumicis	*Rumex confertus* Willd	β-ocimene, linalool, β-caryophyllene and β-farnesene	Mono- and sesquiterpenes	Olfactometric Y-tube assay without effect	Piesik et al. (2016)
Aegorhinus superciliosus	*Pilgerodendron uviferum* (D. Don)	δ-cadinol, cubenol, 15-copaenol and δ-cadinene	Sesquiterpene	Repellent effect	Espinoza et al. (2016)
Dendroctonus pseudotsugae Hopkins	*Pseudotsuga menziesii* var. *glauca* (Mirb.) Franco	α-pinene, 3-carene, limonene, camphene, sabinene, α-terpinene, terpinolene, p-cymene, α-phellandrene, β-ocimene and γ-terpinene	Monoterpene	Insect infestation causes significant variation in monoterpene emitted	Giunta et al. (2016)
Hypothenemus hampei	Not used	α-Terpinene, (*R*)-limonene, farnesene and β-caryophyllene	Mono- and sesquiterpenes	Olfactometric Y-tube assay β-caryophyllene Repellence	Góngora et al. (2020)

The emission of volatile organic compounds in plants has been widely studied (Gounguené and Turlings, 2002; Figueiredo et al. 2007; Sabudak and Goren, 2011; ul-Hassan et al. 2015). The study of chemical compounds emitted by plants to mediate the behaviour of some associated pests that directly affect the economic threshold of the crops have emerged as an interesting tool for developing new insect control strategies. In that sense, a number of studies have shown that red clover emits a series of secondary metabolites with biological activity, affecting the behaviour of *H. obscurus* (Quiroz et al. 2005, 2017, 2018; Tapia et al. 2005; Tapia et al. 2007; Manosalva et al. 2011; Palma et al. 2012; Parra et al. 2013; Toledo et al. 2014) and the insect recognizes its host plant by detecting chemical compounds released from both the aerial and roots using specialized receptor located in their antennae (Palma et al. 2012).

Host search by the root borer is associated with the plant-insect relationship, in which it has a first barrier in limonene repellence in summer months. If this process is not sufficient, the exudation of isoflavonoids represents the second defensive barrier with anti-feedant compounds. Buttery et al. (1984) identified 10 compounds in root extracts that are attractive to the root borer, but none of these was competitive with the respective crude extract. Alarcón et al. (2010) evaluated the population dynamics of *H. obscurus* on different cultivars and experimental lines of red clover, identifying three putative resistant lines, Syn Pre III, Syn Int V and Syn Int VI which exhibited high forage yield. Later, experimental line Syn Int VI became the new cultivar Superqueli-INIA (the newest) (Ortega et al. 2014). Tapia et al. (2007) reported that (*E*)-2-hexenal and limonene, isolated from red clover roots, showed an attractant and repellent respectively effect on adults *H. obscurus*. Moreover, the authors demonstrated that the concentrations of limonene and (*E*)-2-hexenal increased and decreased respectively over time. These results suggest that the activation of a plant defensive mechanism would involve this semiochemical dynamic. Parra et al. (2013)

validated the potential use of (*E*)-2-hexenal as an attractant and limonene as a repellent against *H. obscurus* under field conditions. Toledo et al. (2014) evaluated the effect of dichloromethanolic root extract from cultivars and experimental lines of red clover by comparing the presence of fatty acids with the behaviour of *H. obscurus*. Among the cultivars and experimental lines stuidied, the Redqueli-INIA cultivar, showed a low population of *H. obscurus* per plant (0,1 insect average). This also associated with the highest concentration of palmitic acid (88 µg/g) in the roots of this cultivar. Thus, the concentration of this fatty acid can be related to the infestation of the curculionid. Quiroz et al. (2017) showed that formononetin and genistein, identified in higher amount 0.195 mg/g dry matter and 0.651 mg/g dry matter in the new cultivar Superqueli-INIA, elicited a significant high feeding deterrent activity from *H. obscurus* when they were incorporated in artificial diets. Also, essential oils from other plants have been tested against *H. obscurus*, as reported by Espinoza et al. (2018) performed extractions of essential oil, petroleum ether and dichloromethane from *Pilgerodendron uviferum*, identifying some sesquiterpenes such as: (-)-*trans*-calamenene, cadalene, (-)-cubenol, (-)-epi-cubenol, (-)-torreyol and (-)15-copaenol as majority compounds, and then used in anti-alimentary assays on *H. obscurus*, finding as results weight loss in insects with the essential oil, dichloromethane extract, as well as with the pure compounds (-)-*trans*-calamenene and cadalene.

All these results suggest defensive chemical compounds in aerial and radicular part of red clover can modify the *H. obscurus* behaviour (Fig. 6). Quiroz et al. (2018) report the relationship between isoflavonoids present in red clover cultivars (Sabtoron High, Syn II Int 5, Syn II Int 4, Quiñequeli-INIA, Superqueli-INIA and Redqueli-INIA). Formononetin was analysed in foliage and root by HPLC-UV. Cultivar Sabtoron High had the highest to the total content of the compound, but Superqueli-INIA had the highest content in the root. Feeding trials were carried out with freeze-dried roots of the cultivars analysed, which resulted in a significant decrease in weight gain for the cultivars Superqueli-INIA and Sabtoron High.

However, of the secondary metabolites identified in red clover, the first barrier against the insect's choice as host plant corresponds to the metabolites present in the aerial portion of the plant, providing the first defensive mechanism; therefore, it is important to improve the compounds, especially the monoterpene limonene present in this section of the plant, to avoid infestation.

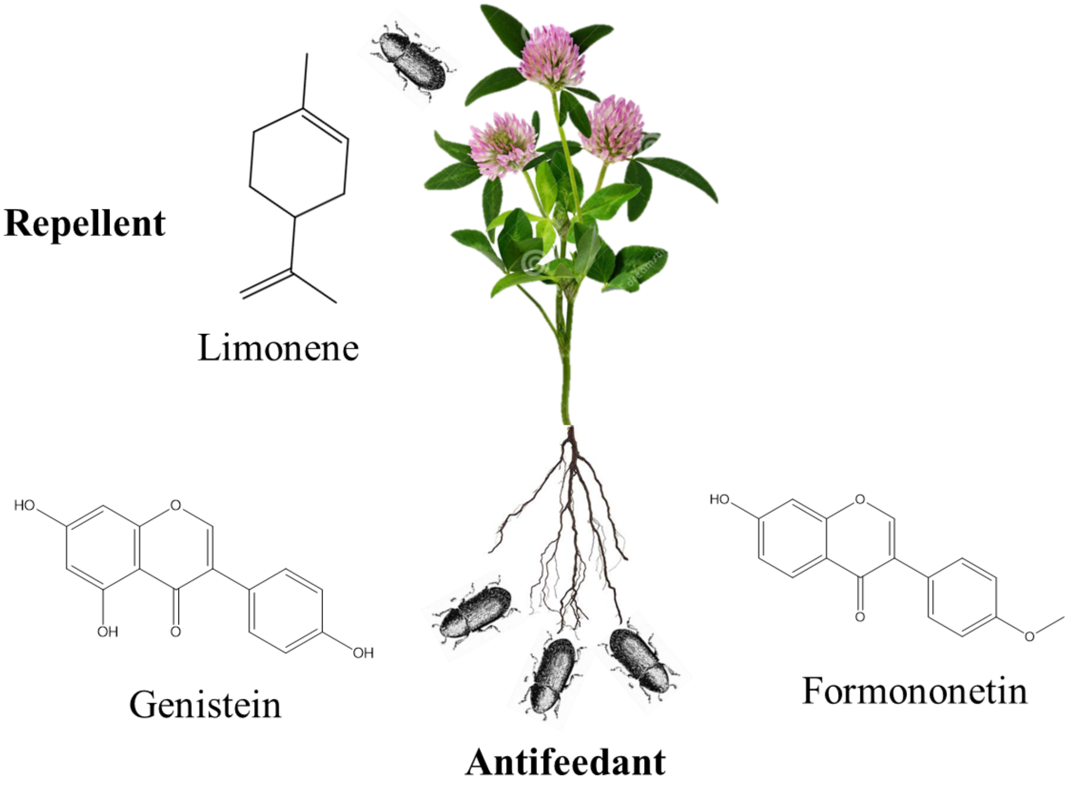

Figure 6. Chemical compounds reported by Parra et al. 2013 and Quiroz et al.(2017) that modify the behaviour of *H. obscurus* (Marsham).

CHAPTER II

Effect of Arbuscular Mycorrhizal Fungi Inoculation and Phosphorus Fertilization on Monoterpene Emission from Red Clover Leaves

Sent to *J. Soil Sci. Plant Nutr*

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Effect of Arbuscular Mycorrhizal Fungi Inoculation and Phosphorus Fertilization on Monoterpene Emission from Red Clover Leaves

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Abstract

Red clover (*Trifolium pratense* L.) is a forage legume widely used in Chile and the world, due to its high forage yield and high nutritional value. Arbuscular mycorrhizal fungi (AMF) are microorganisms that stimulate growth, metabolism, and persistence of plants in the field. Phosphorus (P) is an essential nutrient for plant and is possible to evaluate the incidence of these fungi and P by means of chromatographic identification techniques. To the present, it has been possible to identify the monoterpene (*S*)-limonene with biological activity emitted by red clover against *Hylastinus obscurus* its main pest. The aim of this study was evaluate the effect of two commercial AMF inoculation and P fertilization on monoterpene emission from red clover Quiñequeli-INIA (QQ), Redqueli-INIA (RQ) and Superqueli-INIA (SQ) cultivars were studied in a growth chamber and laboratory condition for 24 hours. The monoterpenes were captured with a Porapak-Q column and identified by gas chromatography coupled to mass spectrometer (GC-MS). The compounds obtained corresponded to α-pinene, sabinene, (*S*)-limonene and p-cymen-7-ol. The results showed that the phosphorous addition elicited an increase of 1500% and 600% in the total emission of terpenes and (*S*)-limonene respectively in Superqueli-INIA cultivar. On the other hand, Quiñequeli-INIA cultivar was present a significant increase for total monoterpene emission for mix inoculum (AMF$_2$) and P fertilization respect to control. These results suggest that through an agronomic management of P fertilization, the attack of *H. obscurus* could be controlled.

Keywords: Arbuscular Mycorrhizal Fungi, Monoterpenes, *Trifolium pratense*, phosphorus, (*S*)-limonene

2.1 Introduction

Red clover is a perennial legume of nutritional and economic importance in temperate and subtropical regions of the world. The persistence of this forage is influenced by various biotic and abiotic factors in field conditions, which determine its duration in two to three years (Ortega et al. 2014). However, this legume is strongly attacked by *Hylastinus obscurus* Marsham affecting the radical system of the plant (Quiroz et al. 2017). There is not a proper control system applied on this curculionid (Quiroz et al. 2005). An alternative approach based on the chemical ecology of *H. obscurus* have been proposed (Parra et al. 2013; Quiroz et al. 2017; Espinoza et al. 2018). Semiochemicals, produced by red clover, such as (*S*)-limonene and formononetin, have been reported for eliciting repellent and antifeedant behaviours from *H. obscurus* (Parra et al. 2013; Quiroz et al. 2018). From these results emerge the question of how the production of these semiochemicals could be increased?

One of the strategies is the stimulation of their production through the management of the agronomic conditions (Jan et al. 2021). Recently, Medina et al. (2022) reported that the enzymatic production of monoterpenes in Superqueli-INIA red clover cultivar was significantly increased in plants inoculated with *Claroideoglomus claroideum* fungi as well as with the AMF mix (genera Scutellospora, Acaulospora and Glomus). Previously, studies carried out by Carreón-Abud et al. (2015) revealed that colonization by *Rhizophagus irregularis* significantly increased the main components of the *Satureja macrostema* essential oil, a Mexican native medicinal plant, such as limonene, β-linalool, menthone, pulegone, and verbenol acetate. Welling et al. (2016) summarized the reports about the knowledge of plant terpenoid accumulation in response to AMF–plant symbiotic relationships, concluding that AMF–plant associations promote the accumulation of plant terpenoids beneficial to human health. AMF are microorganisms belonging to the Phyllum Glomeromycota (Schüßler et al. 2001), mutualist symbionts which colonize the roots of more than 80% of vascular plants (Kobae 2019; Chen et al. 2021).

Phosphorus (P) is an essential plant nutrient required for several events related to plant growing such as carbon metabolism, energy generation, energy transfer, enzyme activation, membrane formation, among others (Schachtman et al. 1998). Moreover, P is a key factor in the formation of biological molecules like Adenosine Triphosphate (ATP), nucleic acids, and phospholipids (Marschner and Rengel, 2012; Zhao et al. 2021). However, P is known to be a limiting factor and can compromise primary and secondary metabolism (Chan et al. 2021). Also, we know that the enzymatic production of terpenes was increased in AMF inoculated red clover plants (Medina et al. 2022). We hypothesized that the joint effect of AMF and P will increase the emission of terpenes, especially limonene, from the aerial parts of Quiñequeli-INIA, Redqueli-

INIA and Superqueli-INIA red clover cultivars. Therefore, the use of AMF and P in red clover plants could be a good tool to increase resistance of cultivars to *H. obscurus* in the field.

The aim of this study was to evaluate the effect of two AMF inocula and P addition on monoterpenes emission from Quiñequeli-INIA, Redqueli-INIA and Superqueli-INIA red clover cultivars with emphasis on (*S*)-limonene production as the main repellent compound reported towards *H. obscurus*.

2.2 Materials and Methods

2.2.1 Experimental design associated with Red Clover cultivars

Red clover seeds (100 units) of Quiñequeli-INIA, Redqueli-INIA and Superqueli-INIA cultivars were allowed to germinate in a chamber for 7 days (>90% germination) at 25° C. Then they were transplanted into individual pots for one month in a greenhouse at 23° C. Afterwards, four plants of similar characteristics were established in 7 L pots filled with a mixture of 1:1 soil: peat (Kekkilä DSM 0, Finland). The experimental design considered two AMF inocula and two levels of P. The AMF were: a) *Claroideoglomus claroideum* (AMF_1), and b) a AMF consortium formed by fungi of the genera Scutellospora, Acaulospora and Glomus (AMF_2), both supplied by Myconativa® (Myconativa, Temuco, Chile). Each pot was supplemented with 3.00 g of inoculum (40 spore per gram). The P levels were a) basal soil level 11.55 mg/kg (P_0), and b) The addition of 1.0 g of Triple Super Phosphate (TSP) up to 30.00 mg/kg (P_1). The experiment arrangement was completely randomized with 3 replicates. All the pots were transferred to a growth chamber in experimental station INIA-Carillanca at 14/10 h of light/darkness and 23° C. Two months later, volatiles trappings were developed under the same conditions (Table 1). Data were analysed by analysis of variance (ANOVA) by SPSS software (IBM SPSS Statistics, Version 23.0. Armonk, NY) followed by multiple comparisons of means using Tukey's honestly significant difference (HSD) test using Statistix V.10 software (Analytical Software, Tallahassee, Florida). All results with $\rho < 0.05$ will be statistically significant.

2.2.2 Volatiles Collection

Monoterpene collection was performed by enclosing 0.40 g of fresh matter of aerial portion of one of the plants in each pot in a 900-mL Pyrex glass chamber (6 cm ID and 30 cm high) with a top outlet with a cartridge containing 100 mg of Porapak-Q column (ethyl vinylbenzene - divinylbenzene polymer) (Waters Associates, Framingham, MA) previously cleaned and activated with 1,00 mL of diethyl ether (GC grade; Merck, Darmstadt, Germany) and conditioned at 150º C for 2 hours in nitrogen stream (70 mL/min). The entrainment was performed by using a positive/negative pressure air system according to the methodology proposed by Agelopoulos et al. (1999). Volatiles trappings were carried out for 24 h, then were desorbed with 1.00 mL of n-hexane (Optima Scientific, Darmstadt, Germany) and concentrated up to 25 µL with N_2 gas.

2.2.3 GC- MS Analysis

From each of extracts obtained from collection, 1 µL of the concentrate was injected. Volatile monoterpenes were analyzed using a Gas Chromatograph (GC) (Thermo Electron, Waltham, MA) coupled to a Mass Spectrometer (MS) (Thermo Electron, Waltham, MA). Separation was performed using a capillary column BP-1 (30 m x 22 mm x 0.25 μm) and Helium as carrier (1.0 mL/min) at initial temperature of 40° C for 2 min and increased until 250° C with 5° C of increment/min. Both injector and interface temperatures were kept at 250° C, while the detector temperature was fixed at 200° C. Moreover, the electron impact ionization energy was set up at 70 eV. The acquisition of each mass spectrum was carried out in the mass range from 30 to

400 m/z. A retention time peaks were compared to mass spectrum library of National Institute of Standards and Technology (NIST) (Mass Spectral Library Version 2.0), using a matching algorithm with a reverse search technique to verify highest peaks from the reference compound. Additionally, Kovats Indexes (KI) (Kovats, 1958) were determined by injection of alkane series ($C_9 - C_{20}$). Furthermore, experimental KI's were compared to theoretical KI´s reported in the NIST database (Babushok et al. 2007). The quantification of identified monoterpenes in ng/g day was performed using a calibration curve of (*S*)-limonene $R^2 = 0.98$ (Sigma-Aldrich, Darmstadt, Germany).

2.3 Results and Discussion

Table 2 shows the monoterpenes identified by mass spectrum comparison, determination of Kovats index (KI) and standard injection. These compounds have been identified previously in red clover, α-pinene and sabinene, has been reported by Kigathi et al. (2009) in leaves. The terpene p-cymen-7-ol has not been previously reported, but Tava et al. (2009) identified p-cymen-8-ol in red clover. (*S*)-limonene has been previously reported as limonene without specifying its chirality Kigathi et al. (2009), Palma et al. (2012) Vlaisavljevic et al. (2014), Kigathi et al. (2019) with exception of Medina et al. (2022), which used a chiral column to confirm the enantiomer *S* is emitted by red clover.

Figure 1 shows the effect of the different treatment (control, AMF_1, AMF_2, P_1, AMF_1-P_1 and AMF_2-P_1) on the total emissions (ng/g day) of the four monoterpenes identify in Quiñequeli-INIA (QQ-INIA), Redqueli-INIA (RQ-INIA) and Superqueli-INIA (SQ-INIA) red clover cultivars. For QQ-INIA the treatments AMF_1 and AMF_1-P_1 and AMF_2-P_1 significantly decreased. AMF_1 was 1.26 ng/g day equivalent to a decrease of 98.70 percent respect to control, the same trend was shown by AMF_1-P_1 (33.76 ng/g, decrease of 34.35 %) and AMF_2-P_1 (1.89 ng/g, decrease of 98.04 %). However, AMF_2 and P_1 were the treatments that significantly increased compared to the control (149.38 ng/g day, increase 55.18 % ; 112.3 ng/g day, increase 16.66 % respectively). The RQ-INIA cultivar was the one that showed an overall negative response to the total emission treatments. With the exception of the AMF treatments, AMF_2 increased its emission by over 1000% (20.64 ng/g) and AMF_1 increased by 430%. Despite the significant increase in total emission for AMF treatments, this cultivar showed the lowest values. SQ-INIA control emitted a total of 18.13 ng/g day. An overall negative response was represented for the treatments, significantly decreasing the emission of total monoterpenes, with a decrease of about 50 or 70 %. Interestingly, the treatment with only phosphorus fertilization P_1 in this cultivar significantly increased and represent the best response of all cultivars and treatments, 270.6 ng/g day was emitted SQ-P_1 increasing by almost 1500 %.

Related to the total monoterpene emission, it is relevant to review because the use of AMF and P do not show a trend in the increase of monoterpenes emission in other plants (Rapparini et al. 2008; Blanch et al. 2012; Bustamante et al. 2020). Therefore, visualisation of total monoterpene content is useful to understand whether factors (biotic and abiotic) modify the secondary metabolism of monoterpene production in red clover. Rapparini et al. (2008) using a Long-Ashton solution with 41 ppm of phosphorus in *A. annua* L. plants reported no significant differences for any of identified terpenes and Bustamante et al. (2020) using leaves of *R. officinalis* quantified terpenes associated with an inorganic fertilizer (NPK; 100:60:73), reporting a low decline with the compounds α-pinene, sabinene and limonene. Interestingly, Blanch et al. (2012) in *P. pinaster* reported higher emissions of total terpenes identified with 10 and 20 ppm of P level respect to low P treatment (2 and 5 ppm). In relation with our results, this is consistent in high P level, the figure

2 shows the emission of (*S*)-limonene and the better emission response associated with QQ-INIA and SQ-INIA cultivars with high P level and without AMF addition.

The results of total monoterpenes content associated with the use of AMF and P as factors are significantly lower than the control and treatments without mixing the AMF and P factors. In relation to this response, Soleimanzadeh (2010) reports a significant decrease in AMF colonization associated with an increase in P fertilization in Sunflower (*Helianthus annuus* L.) inoculated with *G. etunicatum*, *G. mosseae* and *G. intraradices*. Tanwar et al. (2013) reports the same results associated with *Capsicum annum*, colonization of *G. mosseae* and *A. laevis* decreases associated with increased P fertilization. On the other hand, Babikova et al. (2014) using *Vicia faba* plants with a mixed commercial inoculum comprising eight types of AMF (*G. aggregatum*, *G. clarum, G. deserticola, G. monosporus, G. mosseae, R. irregularis, G. margarita* and *P. brasilianum*) and fertilization with a 57.7 ppm phosphate solution, found that the emission of β-pinene control compared to P fertilization and AMF treatments did not present significant differences but was observed a decreasing trend. Velasquez et al. (2020), in *V. vinifera* plants inoculated with *F. mosseae*, determined the volatiles emitted by roots, reporting a significant increase of p-cymen-7-ol production, and finding a non-significant decrease in limonene emission. However, there are some compounds which are influenced by AMF inoculation in our treatments, p-cymen-7-ol shows a positive response to production in QQ-INIA cultivar with mixed inoculum. On the contrary, Balzergue et al. (2013) by formulating treatments using 90 spores of *R. irregularis* and two levels of phosphorus 0.9 and 450 ppm in *M. truncatula* report a mycorrhization of 2 and 62% for low and high of phosphorus used.

An interesting aspect of the relationship between the abiotic and biotic factors mentioned for monoterpene production in red clover is a compound exuded by the roots already reported and isolated by Yokota et al. (1998). This compound Orobanchol is a strigolactone known from the parasitic red clover plant (*Orobanche minor*). This strigolactone plays a relevant role associated with the generation of arbuscular mycorrhizal fungal symbiosis in red clover, because as reported by Olroy (2013) it represents a signal compound to complete colonization. In addition, Yoneyama et al. (2007) in red clover plants associated with different treatments with phosphorus, nitrogen and calcium, identified that at low levels of P fertilization this strigolactone significantly increases its exudation from the roots. Therefore, in the treatments applied with P fertilization and addition of AMF, this strigolactone would decrease its exudation from roots of red clover cultivars QQ-INIA, RQ-INIA and SQ-INIA, thus causing a decrease in AMF colonisation, thus generating a better P flux between soil and red clover, the direct communication pathway mediated by PHT1 transporter (Ferrol et al. 2019), an essential nutrient for the biosynthetic pathway of monoterpenes in plants. The results presented in comparison to references show the variability of the data presented associated with the variables with various types of: monoterpene identified, AMF used and doses and finally P fertilization.

According to Kaur and Suseela, (2020), the use of AMF in plants represents a reprogramming of primary and secondary metabolomics, mediated by different signal compounds. The same authors mention that AMF can take amino acids directly from soil, but this acquisition can increase, decrease, or have no effect for the plant in the formation of protein blocks or enzyme. Finally, our results reveal a modification to monoterpene production due to the aggregation of AMF as a biotic factor, being the responses varied according to terpene identified and cultivar inoculated with AMF. Additionally, a group of enzymes present in plants in general, called monoterpene synthases (MTS) reported and classified by Chen et al. (2011), are the basis of the metabolic machinery derived from primary metabolism. Therefore, with the information gathered from databases (Chang et al. 2021; Kanehisa et al. 2021), we can suggest an approach to the metabolic pathway associated to the production of these monoterpenes in red clover cultivars (Fig. 3).

2.4 Conclusion

From the results obtained in this investigation and contrasted with another authors, we can infer that the use of AMF and P have capacity to modulate the secondary metabolism, obtaining a variable response, be positive, negative, or indifferent according to the monoterpene analyzed. Understanding that the use of these AMF, confers improvement in agronomic properties in field, we can also elucidate the aid of delivery of these AMF to the red clover plants in an improvement of the secondary metabolism of monoterpenes. (*S*)-limonene repellent reported against *H. obscurus* for the cultivar Superqueli-INIA showed better response to a high P dose but no with AMF inoculation. Therefore, the use of P-rich fertilizers should be added without considering the presence of AMF when high levels of P are used. This information is useful for red clover farmers to improve the defensive system of the plants against insect pests. Due to this, as a future perspective, experiments should be test metabolites such as p-cymen-7-ol with its interaction with *H. obscurus* due to the increase in the emission.

Table 1. Treatment characterisation associated with red clover Superqueli-INIA (SQ) cultivar, Arbuscular Mycorrhizal Fungi (AMF$_1$: *C. claroideum*; AMF$_2$: genera *Scutellospora*, *Acaulospora* and *Glomus*, and Phosphorus (P$_1$) addition as TSP. The same treatments apply to the Quiñequeli-INIA (QQ) and Redqueli-INIA (RQ) cultivars.

Treatment per pot	**AMF**	**Phosphorus fertilization**
SQ-P$_0$ (control)	No addition	11.55 mg/kg
SQ-AMF$_1$	3.00 g of inoculum	11.55 mg/kg
SQ-AMF$_2$	3.00 g of inoculum	11.55 mg/kg
SQ-AMF$_1$-P$_1$	3.00 g of inoculum	11.55 mg/kg plus 1.0 g of TSP
SQ-AMF$_2$-P$_1$	3.00 g of inoculum	11.55 mg/kg plus 1.0 g of TSP
SQ-P$_1$	No addition	11.55 mg/kg plus 1.0 g of TSP

Table 2. Compounds identified by GC-MS and KI determination in Red clover cultivars.

RT (min)	Compound	KI Experimental*	KI Library**
8.38	α-Pinene	931	932
9.46	Sabinene	971	969
11.01	*(S)*-Limonene	1023	1022
16.99	*p*-Cymen-7-ol	1228	1232

RT: Retention time; KI: Kovats Index; *=Determination by alkane series; **=Determinate with database NIST.

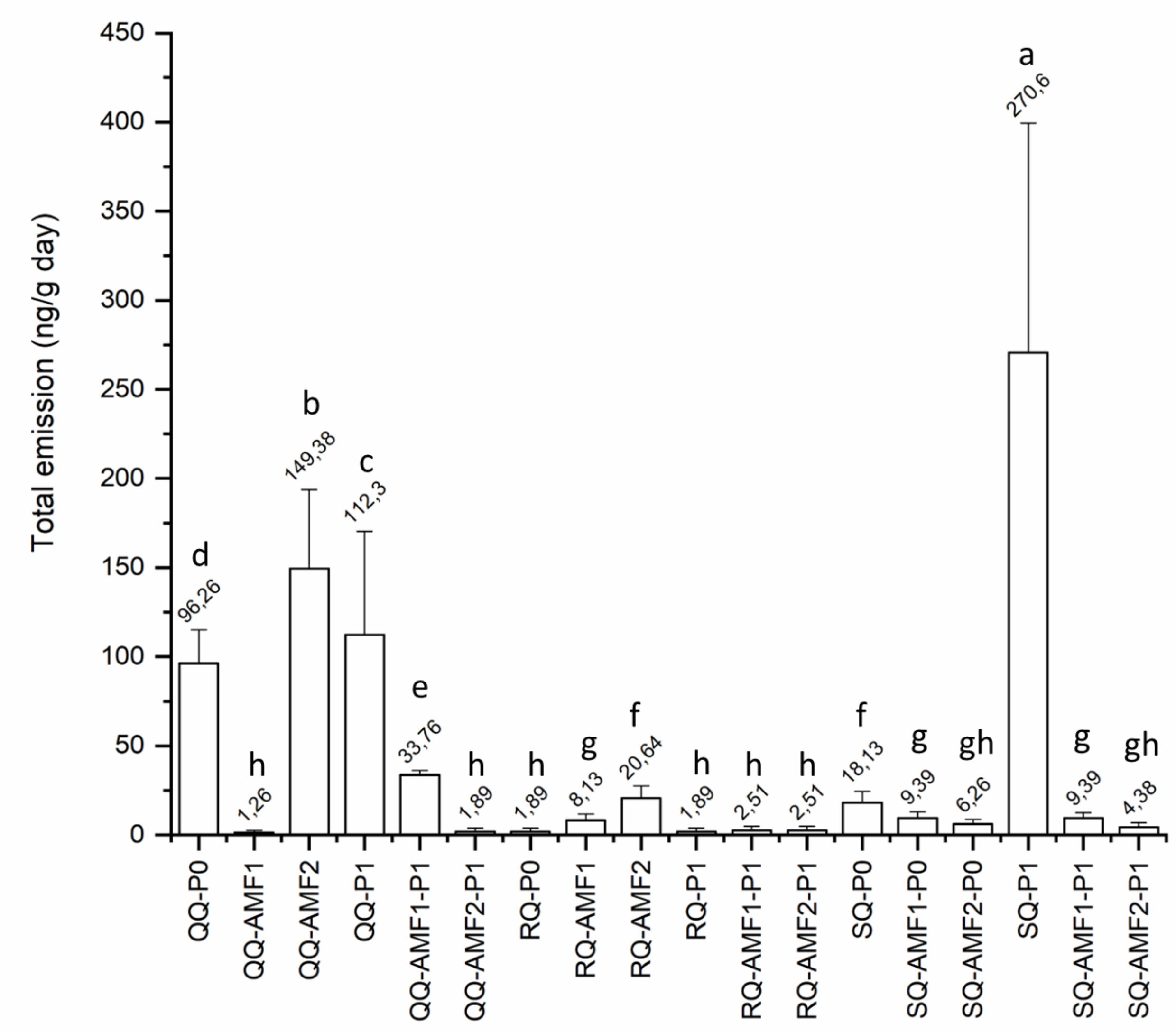

Figure 1. Total terpenes production in nanograms per gram of fresh matter per day (ng/g) identified in Quiñequeli-INIA (QQ), Redqueli-INIA (RQ) and Superqueli-INIA (SQ) red clover cultivars with treatments *Claroideoglomus claroideum* (AMF$_1$) and mixed (AMF$_2$) as well as phosphorus (P$_1$) addition. n=3. Values indicate mean ± standard error. Different letter indicates significant differences based on Tukey's honestly significant difference (HSD) ($p < 0.05$).

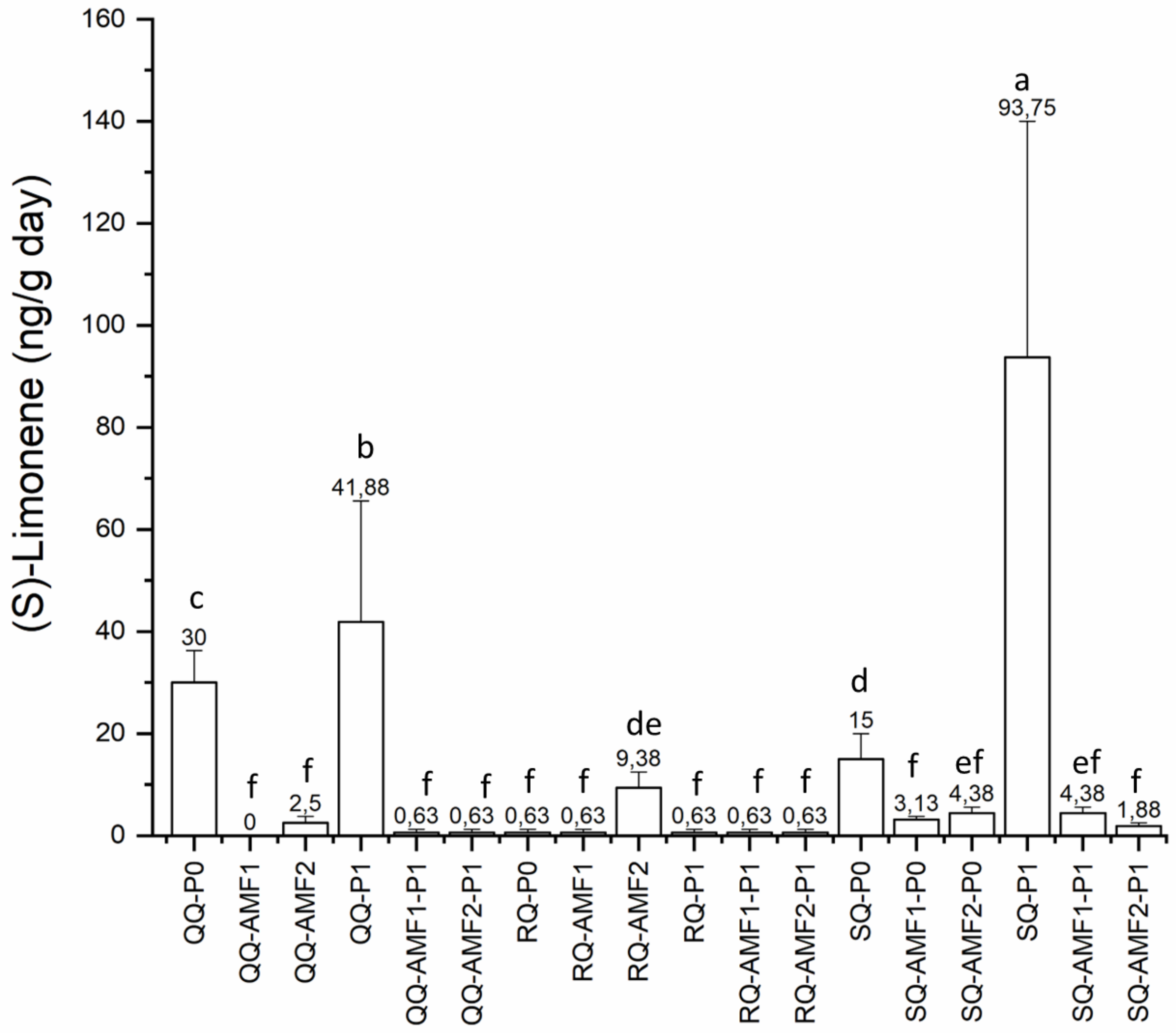

Figure 2. *(S)*-limonene emission in nanograms per gram of fresh matter per day (ng/g) in Quiñequeli-INIA (QQ), Redqueli-INIA (RQ) and Superqueli-INIA (SQ) (*Trifolium pratense* L.) cultivars. n=3. Values indicate mean ± standard error. Different letter indicates significant differences based on Tukey's honestly significant difference (HSD) ($p < 0.05$).

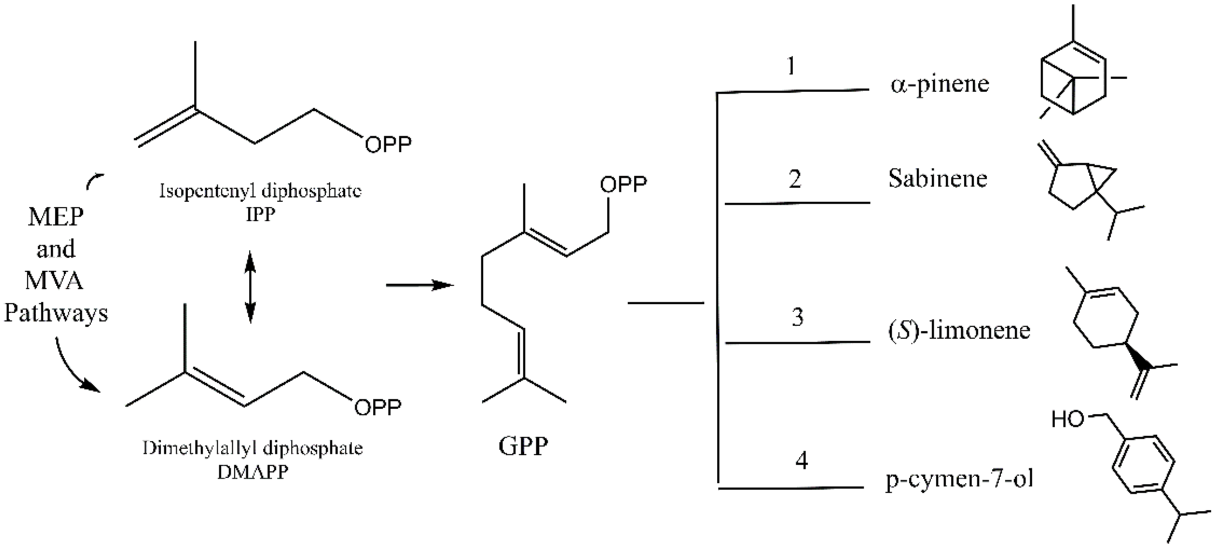

Figure 3. Suggested summarised biosynthetic pathway of monoterpene production in red clover cultivars. 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway and cytosolic mevalonate (MVA) pathway. Enzymes involved (1) (-)-α-pinene synthase (EC 4.2.3.119); (2) sabinene-hydrate synthase (EC 4.2.3.11); (3) (4*S*)-limonene synthase (EC 4.2.3.16); (4) p-cymene methyl-monooxygenase (EC 1.14.15.25). (Information extracted from BRENDA, BRaunschweig ENzyme DAtabase (www.brenda-enzymes.org) (Chang et al. 2021) and KEGG, Kyoto Encyclopedia of Genes and Genomes (https://www.kegg.jp) (Kanehisa et al. 2021).

CHAPTER III

Arbuscular Mycorrhizal Fungi Enhance Monoterpene Production in Red Clover (*Trifolium pratense* L.): A Potential Tool for Pest Control

Published

Medina, C. Mutis, A. Bardehle, L. Hormazabal, E. Borie, F. Aguilera, P. Ortega, F and Quiroz, A. (2022). Arbuscular mycorrhizal fungi enhance monoterpene production in red clover (*Trifolium pratense* L.): A potential tool for pest control. *Nat. Prod. Res.* https://doi.org/10.1080/14786419.2022.2094375

Arbuscular mycorrhizal fungi enhance monoterpene production in red clover (*Trifolium pratense* L.): A potential tool for pest control

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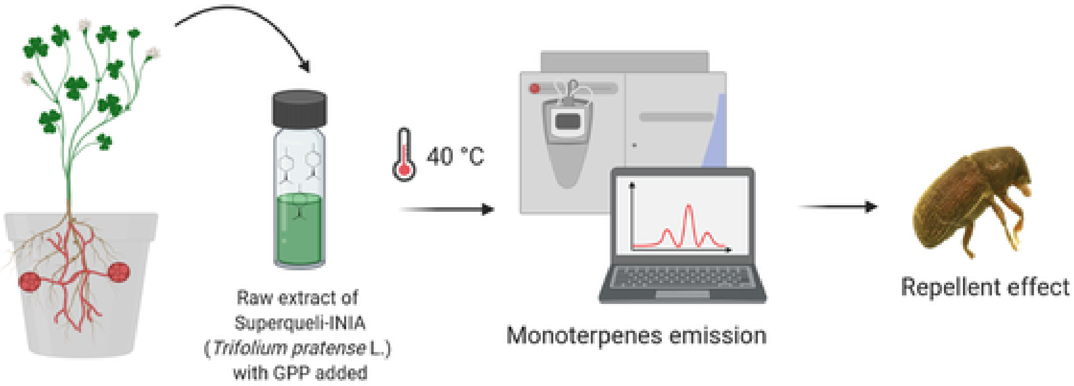
Abstract

The possibility of modifying terpene production in plants is a defensive strategy that has been studied in conjunction with their biosynthetic pathways. A biotic factor such as Arbuscular Mycorrhizal Fungi (AMF) could modify terpene production in red clover (*Trifolium pratense* L.). In this work, the enzymatic production of monoterpenes in Superqueli-INIA cultivar with two AMF was evaluated via HeadSpace-Gas Chromatography (HS-GC). A significant increase of (*S*)-limonene was found in plants inoculated with *Claroideoglomus claroideum* as well as with the AMF mix (genera *Scutellospora*, *Acaulospora* and *Glomus*). Moreover, significant increases in other monoterpenes such as (-)-β-pinene, myrcene, linalool, were observed. Results showed higher monoterpene production capacities in the Superqueli-INIA cultivar, suggesting the participation of monoterpene synthases (MTS). The significant rise of (*S*)-limonene in red clover plants inoculated with AMF suggests this strategy could be implemented in an agronomical manage for controlling the *H. obscurus*, the primary pest.

Keywords: Red clover, *Claroideoglomus claroideum*, monoterpenes, (*S*)-limonene, arbuscular mycorrhizal fungi.

Graphical abstract



3.1 Introduction

Trifolium pratense L., commonly named as red clover, is an important leguminous forage used for livestock. It is classified as perennial plant, but its survival does not exceed three years because it is strongly affected by the red clover root borer, *Hylastinus obscurus* Marsham (Ortega et al. 2003). Adult and larval states of *H. obscurus* feed on the main root of this plant, affecting plant development and growth. Hitherto, biological, and chemical strategies for controlling this pest are limited. Interestingly, studies have shown that secondary metabolites can mediate the chemical communication between plants and insects (Chen et al. 2011). For instance, (*S*)-limonene, a monoterpene released from *T. pratense* has a natural repellent effect on *H. obscurus* (Parra et al. 2013). The biosynthesis of these compounds can be affected by biotic factors, such as the arbuscular mycorrhizal fungi (AMF). In fact, the AMF is known to induce morphological, physiological, and biochemical changes, including secondary metabolites (Kobae 2019). The relationship between *H. obscurus* and (*S*)-limonene present in red clover, stimulates the following question: Will it be possible to increase the (*S*)-limonene production in AMF-inoculated red clover plants? We hypothesize that symbiosis associated with AMF *C. claroideum* (AMF_1) and mix (genera *Scutellospora*, *Acaulospora*, *Glomus*) (AMF_2) positively affect the biosynthetic pathway of monoterpene production in red clover (Superqueli-INIA cultivar). To date, there are no studies associating the effect of AMF on terpenoids by enzyme bioassays. The use of AMF in red clover will supply information on the effect on monoterpene production as a relevant biotic factor.

3.2 Experimental

3.2.1 Treatments and Experimental Design

The experimental design included a control and two treatment groups. The control group was untreated Superqueli-INIA (SQ-INIA) cultivar. The treatments, red clover plants were inoculated with either *Claroideoglomus claroideum* **(**Myconativa®, Temuco, Chile**)** (SQ-AMF$_1$) or with a commercial inoculum consortium formed by fungi of the genera *Scutellospora*, *Acaulospora* and *Glomus* (Myconativa®, Temuco, Chile) (SQ-AMF$_2$). Twenty pots were established for each treatment distributed in a randomised design inside the growth chamber.

3.2.2 Plant Samples

Red clover (*Trifolium pratense* L.; SQ-INIA cultivar) seeds were obtained from Instituto de Investigaciones Agropecuarias (INIA) at Carillanca Station (38°41'S, 72º25'W) in La Araucanía Region, Chile. The seeds were germinated (90% effective) for 2 weeks in a chamber with a photoperiod of 14/10 hours (light/dark) at 23°C, then the seedlings obtained were subjected to same conditions also controlling humidity (40 - 45%) in individual plastic pots. After one month, four plants were transferred to 7 L pots with a mixture of 1:1 soil of INIA-Carillanca (Andisol) (pH = 6.23; Organic matter: 15.09 %; Nitrogen 10 ppm; Phosphorus 11.55 ppm) with peat (DSM 0, Kekkilä, Finland) and inoculated with 120 propagules (hyphae and spores) and determinate by gridline intersection method (Giovannetti and Mosse 1980) of either AMF$_1$ or AMF$_2$ (Myconativa®, Temuco, Chile), and the controls were untreated plants. After 30 days of establishment, stems and leaves were cut off up to 5 centimetres above the ground with sterile scissors and sterilised with ethyl alcohol (70%) and stored at -80°C for later analysis.

3.2.3 Monoterpene Synthase Bioassays

The enzymatic activity protocol was adapted from Fischbach et al. (2000). Each sample was ground in a mortar in the presence of liquid nitrogen, then 1.5 g were transferred to a 15 mL centrifuge tube. 5.6 mL HEPES (4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid) buffer (Merck KGaA, Darmstadt, Germany) (pH 7.3) was added along with 3.0 µL 10% v/v glycerol (Merck KGaA, Darmstadt, Germany), 3.0 µL 5.0 mM ascorbic acid (Merck KGaA, Darmstadt, Germany), 3.0 µL dithiothreitol (Merck KGaA, Darmstadt, Germany), and 3.0 µL 0.1 M phenylmethylsulphonyl fluoride (Merck KGaA, Darmstadt, Germany). The samples were vortexed for 2 minutes and sonicated for 5 minutes at 4°C and centrifuged at 11,000 *g* for 15 minutes at 4 °C, after which 5 mL of supernatant was extracted from each sample and transferred to a 22-mL glass vial (Perkin Elmer, Waltham, Massachusetts, USA). To the supernatant, 3 µL 5 mM sodium phosphate, 75 µL 5 mM Mn^{+2}, and 400 µL 40 mM geranyl pyrophosphate (Cayman Chemical, Michigan, USA) were added, achieving a final concentration of 2.92 mM. When processing controls, GPP was replaced by ammonium carbonate. Samples were shaken at 150 rpm for 1 hour at 40 °C transferred in an orbital shaker, then the volatile terpenes were analyzed by means of head space-gas chromatography (HS-GC).

3.2.4 Analysis of Monoterpenes Using HS-GC

Vials containing the supernatant were placed in the TurboMatrix Headspace Sampler (Perkin Elmer, Waltham, Massachusetts, USA) at 70 °C for 30 minutes for subsequent analysis using a gas chromatograph (GC) (Clarus 680; Perkin Elmer, Waltham, Massachusetts, USA) equipped with a 30 m x 0.25 mm x 0.25 µm chiral column (Cyclodex-β; Agilent, Santa Clara, California, USA). The injection port and flame ionization detector were set at 250 °C, while the oven temperature was maintained at 35 °C for 0.5 minutes, and then increased at 30 °C min^{-1} to 78 °C, which was maintained for 4 minutes. Temperature was then increased at 9 °C min^{-1} to 160 °C, then at 35 °C min^{-1} to 250 °C (Fischbach et al. 2000). Helium was the carrier gas (2 mL min^{-1}). Compounds were identified and quantified using the Kovats Index (KI) by means of a series of C_9-C_{18} alkanes and pure standards (Supelco™). The experimentally obtained KI's were then searched and compared with the NIST Chemistry WebBook database (National Institute of Standards and Technology). Chiral column chromatography allowed to determine that red clover produces (*S)*-limonene only. This result was corroborated by co-injecting the sample with the respective *R* and *S* pure standards.

3.2.5 Determination of Total Protein in Crude Extracts of Red Clover

The total protein content of the crude extracts (sample) of the control and treatments were determined according to Bradford's methodology (Bradford 1976). First, the standard protein curve of serum albumin (BSA) (Merck KGaA, Darmstadt, Germany) was prepared at 5 dilution factors ($R^2 = 0.98$) and measured at 595 nm absorbance. The Bradford assay was carried out to determine total protein content in 100 µL raw extract (see annex 1.4) then comparing results to the calibration curve.

3.2.6 Statistical Analysis

Means were compared using analysis of variance with factorial design followed by a Tukey Honestly-Significant-Difference (HSD) all-pairwise comparisons test using Statistix statistical software v. 10.0 (USA).

3.3 Results and Discussion

3.3.1 Identification and Quantification of Monoterpenes by Enzymatic Activity.

Enzymatic activities, expressed as µg/mg total protein per hour (µg/mg P^{-1} h^{-1}), were performed to evaluate the effect of AMF on monoterpenes production. A total of 10 monoterpenes were identified (see annexes S1.2 and S1.3): geraniol, linalool, β-pinene, dihydromyrcenol, myrcene, α and β-phellandrene, (*S*)-limonene, (-)-isopiperitenone and perillyl alcohol. All the monoterpenes identified are generated by the action of monoterpene synthases (MTSs), a group of key enzymes transforming geranyl pyrophosphate (GPP) to different monoterpenes (Chen et al. 2011). The MTSs (or cyclases) are involved in the conversion of the acyclic isoprenoid diphosphate precursor to cyclic hydrocarbon (Gao et al. 2012). This reaction depends on Mn^{+2} or Mg^{+2} and it is initiated by the ionization of GPP to generate an allylic carbenium ion intermediate (terpinyl cation) (Lesburg et al. 1988).

Table 1 describes the effect of the use of AMF_1 and AMF_2 on monoterpene biosynthesis in red clover. The formation of the monoterpenes depended only on endogenous precursor (-) GPP. Seven monoterpenes were identified, providing information on their natural production in red clover. A significant increase of the total monoterpenes production was observed from control SQ (0.99 µg/mg P^{-1} h^{-1}) to 8.28 (SQ-AMF_1) and 8.67 (SQ-AMF_2). When plant was inoculated with either AMF_1 or AMF2 a significant increase in the concentration of (-)-β-pinene (7.40 and 8.34 µg/mg P^{-1} h^{-1}) and (*S*)-limonene (0.40 and 0.33 µg/mg P^{-1} h^{-1}) (see annex S1.5) were observed in comparison to the control (SQ: 0.73 µg/mg P^{-1} h^{-1} and 0.07 µg/mg P^{-1} h^{-1} respectively). It is remarkable the fact that only the action of the AMFs causes a huge increase in the concentration of these monoterpenes, between 371 and 1,042%. On the other hand, β-phellandrene production increased only when plants were inoculated with AMF_1 (1,450%). The effect of the mycorrhization was increased when GPP was added to the enzymatic bioassay (Table 2). AMF_1 and AMF_2 elicited the increase of the total monoterpene production in 916 and 1,151% respectively respect to the control without mycorrhization. Moreover, 70% of the identified compounds significantly increased their production in the presence of AMF compared to the control. Although most of these increases are relevant, the production of myrcene stands out showing the largest increase compared to the control, 4,277% and 4,655% for AMF_1 and AMF_2 respectively. The evidence provided by these results allows us to understand that the SQ cultivar inoculated with AMF promotes an increase in monoterpene production, thus finding a beneficial response in the promotion of the secondary metabolism of red clover (see annex S1.6). The addition of GPP to the SQ extracts treated with AMF accounts for the production potential of these monoterpenes and their maximum expression of the enzymes involved in biosynthesis as evidenced in the significant increase of (*S*)-limonene production when treated with AMF and GPP (Figure 1). These results are supported by Shrivastava et al. (2015) who proposed that the enhanced biosynthesis of terpenoid can be explain at two levels: (1) by increase in substrates through the induction of the pathways, and/or (2) by activation of terpene synthase enzymes Our results show that the enzyme activity increased significantly with the addition of GPP and AMF.

The AMF-plant symbiotic relationships play a key role in more than 80% of terrestrial plants resulting in an enhancing of their mineral nutrition (Smith and Read, 2008; Kobae 2019). The effect of AMF in increasing terpenoids production has been well stablished (Zeng et al. 2013; Welling et al. 2016; Kapoor et al. 2017). However, most of the studies have been focalized in increasing the terpenoids components of essential oil from medicinal plants. Kapoor et al. (2017) reported the analysis of essential oils from 27 plants inoculated with AMF, where 89% of these species showed an increase in the essential content obtained from fruits, seeds, leaves, roots, shoots, and rhizome. We report huge increment of individual monoterpenes, such as β-pinene (1,042%), myrcene (4,655%), limonene (471%) among others. Perusal literature reveals the AMFs *Glomus macrocarpum and G. fasciculatum* increase the content of β-pinene, myrcene, limonene, α-phellandrene, linalool and geraniol in 29, 221, 76.9, 123, 9.1 and 408.7% respectively (Kapoor et al. 2002a, b; Habeeb et al. 2020). Different AMFs species can change the metabolome of the host plant (Zeng et al. 2013; Kaur and Suseela 2020). The relative percentage of terpenes present in dill, carum and coriander essential oil (Kapoor et al. 2002a, b) was dependent of the *Glomus* species inoculated, and moreover in some cases negative effects were observed related to the controls. This effect has been related to genetic composition of specific AMF genotypes (Welling et al. 2015). These results suggest that the AMF_1 and AMF_2 genotypes are ecologically compatible, reducing the variation in their symbiosis with red clover and the subsequent biosynthesis of monoterpenes. Finally, the literature indicates that the increase in the content of secondary metabolites emerge as a defense response to fungal colonization (Zeng et al. 2013), as is the case of herbivory by insects (Shrivastava et al. 2015). Our results show that the increase in limonene, myrcene and β-pinene concentration by AMF colonization could provide protection to the red clover against insect attack. Yildirim et al. (2013) reported that myrcene presents an insecticidal effect on the curculionid *Sitophilus zeamais*, finding 33% mortality, whereas the treatment with β-pinene showed 53% mortality after 96 hours of exposure. We believe that a potential toxic effect of myrcene or β-pinene would represent an opportunity for controlling *H. obscurus* in addition to the repellent (*S*)-limonene (Parra et al. 2013).

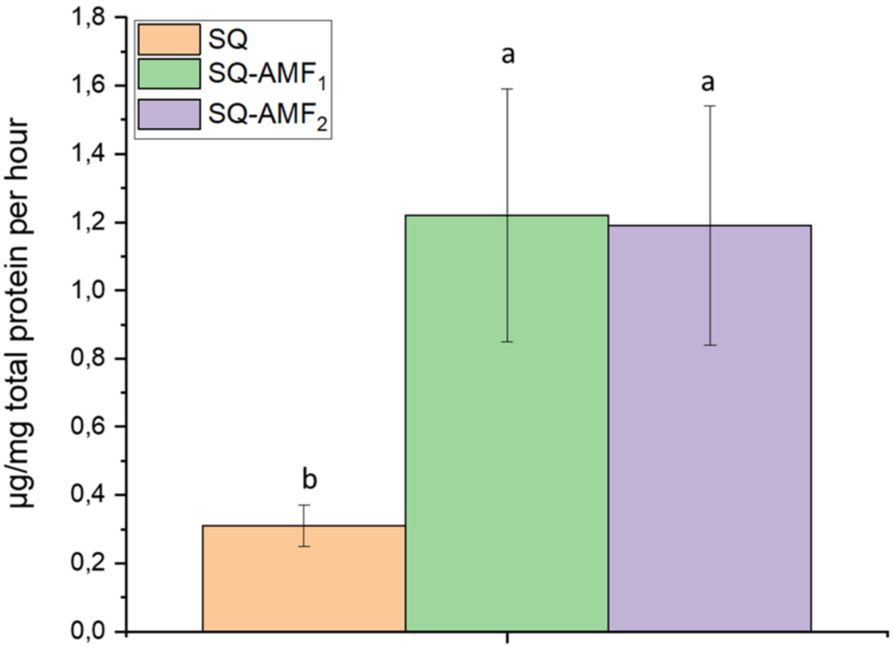


Figure 1. Enzymatic (*S*)-limonene production (μg/mg total protein per hour) in Superqueli-INIA (SQ) INIA cultivar inoculated with the arbuscular mycorrhizal fungi *Claroideoglomus claroideum* (AMF$_1$) or a commercial mixture of arbuscular mycorrhizal fungi (AMF$_2$). Letters denote different groups using the Tukey test ($p < 0.05$).

Table 1. Concentration of monoterpenes (µg/mg P^{-1} h^{-1}) produced in Superqueli-INIA (SQ) (control), SQ plus AMF_1 and AMF_2 treatments without (-) GPP addition.

	Treatments		
Monoterpene	**SQ**	**SQ-AMF_1**	**SQ-AMF_2**
(-)-β-Pinene	0.73 ± 0.13 b	**7.40 ± 0.63 a**	**8.34 ± 0.48 a**
Myrcene	N. D	0.12 ± 0.04 a	N. D
(*S*)-Limonene	0.07 ± 0.02 b	**0.40 ± 0.08 a**	**0.33 ± 0.11 a**
β-phellandrene	0.02 ± 0.00 b	**0.31 ± 0.11 a**	N. D
Linalool	0.09 ± 0.03 a	0.05 ± 0.00 a	N. D
Geraniol	0.04 ± 0.00 a	N. D	N. D
Isopiperitenone	0.02 ± 0.01 a	N. D	N. D
Perillyl Alcohol	0.02 ± 0.01 a	N. D	N. D
Total production	0.99 ± 0.20	8.28 ± 0.86	8.67 ± 0.59

n=3. S.E = Standard Error; N. D = non-detected. Values in bold represent significant increases.

Table 2. Concentration of monoterpenes (µg/mg P^{-1} h^{-1}) produced in Superqueli-INIA (SQ) (control), SQ plus AMF_1 and AMF_2 treatments with (+) GPP addition as precursor.

	Treatments		
Monoterpene	**SQ**	**SQ-AMF_1**	**SQ-AMF_2**
(-)-β-Pinene	0.74 ± 0.50 c	**5.66 ± 0.09 b**	**7.57 ± 0.41 a**
Myrcene	0.47 ± 0.10 b	**20.57 ± 1.70 a**	**22.35 ± 3.36 a**
α-Phellandrene	N. D	0.41 ± 0.05 b	**0.56 ± 0.04 a**
(*S*)-Limonene	0.31 ± 0.06 b	**1.22 ± 0.37 a**	**1.19 ± 0.35 a**
β-phellandrene	0.11 ± 0.04 b	0.12 ± 0.01 b	0.14 ± 0.01 b
Dihydromyrcenol	0.06 ± 0.04 c	0.12 ± 0.01 b	**0.15 ± 0.01 a**
Linalool	0.96 ± 0.06 b	**2.46 ± 0.19 a**	**2.73 ± 0.25 a**
Geraniol	0.29 ± 0.02 c	**0.70 ± 0.19 b**	**1.87 ± 0.11 a**
Isopiperitenone	N. D	N. D	0.38 ± 0.07 a
Perillyl Alcohol	0.20 ± 0.02 c	**0.65 ± 0.21 b**	**2.34 ± 0.21 a**
Total production	3.14 ± 0.84	31.91 ± 2.82	39.28 ± 4.82

n=3. S.E = Standard Error; N. D = non-detected. Values in bold represent significant increases.

3.4 Conclusions

Our results show that the SQ cultivar, when inoculated with AMF, significantly increases the total production of the identified monoterpenes, with emphasis on the production of (*S*)-limonene and other monoterpenes, such as myrcene and β-pinene. This suggests that the respective enzymatic complexes associated with MTS are improved in the same extension by AMF_1 and AMF_2 symbioses. Future studies will be carried out to propose or identify the action mechanism of monoterpene synthase in *T. pratense* and to establish the roles of myrcene and β-pinene in the behaviors of *H. obscurus*, as well as to assess the toxicity and environmental impact of these metabolites.

Acknowledgements. Laboratorio de Química Ecológica and INIA-Carillanca. Financial support was provided by FONDECYT N°1181697.

Conflicts of Interest: The authors declare no conflict of interest.

CHAPTER IV

General Discussion, Concluding Remarks and Future Directions

4.1 General Discussion

Chemical Ecology is a discipline that has its beginnings in the 1950s, associated with the discovery of Bombykol, a chemical compound produced by *Bombyx mori* (Lepidoptera: Bombycidae). This background laid the foundations of knowledge and openness to the scientific world in this discipline that contains various areas of knowledge (biochemistry, physiology, entomology, chemistry). This area of science is defined by the interaction of various organisms at the chemical level (Wortman-Wunder and Vivanco, 2011). From this point on, it is understood that the plant-insect interaction could be mediated by various chemical signals which could present various responses in the insect's behaviour. In general, Chemical Ecology has applications in controlling pests without altering the ecosystem, but in making control chemically friendly to the environment. Plants in general can biosynthesize a variety of chemical compounds (terpenes, flavonoids, phenylpropanoids, and green leaf volatiles) which are formed by specific metabolic pathways and mechanisms within plant (Chang et al. 2021; Kanehisa et al. 2021). The most interesting and relevant groups since the beginning of chemical ecology are associated with terpenes, more specifically monoterpenes. Monoterpenes has a broad spectrum of structures derived from two metabolic pathways (DXP and MVA) (Chen et al. 2011; Kumari et al. 2013; Shrivastava et al. 2015; Sharma et al. 2017; Ninkuu et al. 2021). Monoterpene production in plants and its relationship with insects in pest control can be mediated by biotic and abiotic factors (soil pH, insects, nutrients, light, water availability, soil type, among others) (Zeng et al. 2013; Mota et al. 2020; Balestrini et al. 2021; Rizaludin et al. 2021). For red clover (*Trifolium pratense*) an important forage crop in Chile and in various regions of the world, monoterpenes (limonene, myrcene, ocimene, α, β-pinene, among others) are biosynthesised by its metabolic enzymatic battery (Medina et al. 2022).

Two approaches were carried out in this work. In the first, monoterpene productions evaluated by a direct volatile trapping of volatiles from alive plants using Porapak Q. The effect of AMF inoculation and phosphorous fertilization on monoterpene emission from alive plants of Superqueli-INIA was studied using Porapak-Q. Four compounds were identified: α-pinene, sabinene, (*S*)-limonene and *p*-cymen-7-ol. The more relevant result stated that the addition of phosphorous elicited an increase of 500% in the emission of (*S*)-limonene respectively, and the addition of two AMF had no significant effect on the terpene content. Literature shows contrasting results, some author indicated that the addiction of P did not have effect on the amount of monoterpenes in plants of *A. annua* (Rapparini al. 2008) or decreased in plants of *R. officinalis* (Bustamante et al. 2020). Similar to our results, an increase of the total emission of monoterpenes was reported by Blanch et al. (2012) in *P. pinaster*, when plants were supplemented 10-20 ppm of P level. In this approach, the addition of *Claroideoglomus claroideum* (AMF_1) or a consortium formed by fungi of the genera *Scutellospora*, *Acaulospora* and *Glomus* (AMF_2) did not affected the monoterpenes emission. An increase in P fertilization could affect AMF colonization and hence monoterpene production as it was pointed out by Soleimanzadeh (2010) when *H. annuus* plants were inoculated with *G. etunicatum*, *G. mosseae* and *G. intraradices*. Tanwar et al. (2013). However, both factors separately significantly enhanced the total monoterpene production in Superqueli-INIA cultivar, for treatment only with P fertilization increase 1500% respect to control (Chapter II, Fig 1) and enzymatic (Chapter III, Table 1) around to 800% in total production. This response had been scarcely reported in other plants (Welling et al. 2015; Carreón-Abud et al. 2015; Kapoor et al. 2017; Avio et al. 2018) understanding that terpene metabolism is affected for plants containing high levels available of phosphorus in soil and presence of mycorrhizae, because the high presence of the element generates a decrease of mycorrhization in the plant (Oliveira et al.

2015; Trejo et al. 2020). These results indicate the sensitivity of the system to a dual application of AMF and P. The right equilibrium between two factors is an important issue to consider in the future.

In the second approach, monoterpene enzymatic activity was determined in leaves of Superqueli-INIA, allowing to elucidate the potential of this red clover cultivar for producing monoterpenes, specifically the repellent (*S*)-limonene towards *H. obscurus*. Enzymatic conditions such as pH, metal cofactor, addition of the terpene precursor Geranyl Pyrophosphate (GPP), among others, were determined considering the report by Fischbach et al. (2000). It could be determined that through the determination of the enzymatic activity a greater diversity of the number of monoterpenes associated with red clover was observed (Table 1, chapter IV) compared to the capture of volatiles by Porapak Q (Chapter III). This gives us certainty of the important activity of the monoterpene synthases (MTS) in red clover, and that their precursor GPP represents a production limiting factor in biosynthetic pathways (DXP and MVA). MTS are a group of key enzymes transforming GPP to different monoterpenes (Chen et al. 2011). The MTSs (or cyclases) are involved in the conversion of the acyclic isoprenoid diphosphate precursor to cyclic hydrocarbon (Gao et al. 2012). This reaction depends on Mn^{+2} or Mg^{+2} and it is initiated by the ionization of GPP to generate an allylic carbenium ion intermediate (terpinyl cation) (Lesburg et al. 1988). The addition either of the two AMF significantly increased the emission of monoterpenes, between 371 and 1,042% and when GPP was added this increment was remarkable, reaching percentages around 1,150%. These results show that the inoculation with AMF promotes secondary metabolism of terpenes and the addition of GPP maximizes the expression of the enzymes involved in biosynthesis as evidenced in the significant increase of (*S*)-limonene production when treated with AMF and GPP. These results are supported by Shrivastava et al.

(2015) who proposed that the enhanced biosynthesis of terpenoid can be explain at two levels: (1) by increase in substrates through the induction of the pathways, and/or (2) by activation of terpene synthase enzymes Our results show that the enzyme activity increased significantly with the addition of GPP and AMF. Zeng et al. (2013) reported that the increase in the content of secondary metabolites emerge as a defense response to mycorrhizal fungal colonization, as is the case of herbivory by insects.

Nevertheless, the use of AMF associated with monoterpene production showed qualitative and quantitative effects, allowing to determine a metabolic pathway of the use of GPP in the production of monoterpenes in red clover (Table 2, chapter IV). Regarding the production of the most important monoterpene with insect repellent activity, a significant increase in the emission of (*S*)-limonene was observed, thus being able to determine that under ideal conditions the plant has a better metabolic capacity to produce such compound.

It is known that the ability of plants, in this case red clover, to produce a large number of terpenoids can be supported by the presence of a large number of genes involved in terpene biosynthesis as an outcome of coevolution with natural enemies (Pichersky and Raguso 2018). In this direction, the monoterpenes biosynthesized in red clover must be related with some ecological role. The fact that the monophagous monovoltine curculionid (*H. obscurus*), which causes great economic damage to red clover, has developed an ancestral association with red clover, would indicate that an intrinsic defense mechanism would have been generated through biosynthesis of defensive monoterpenes such as (*S*)-limonene. It is very striking that by means of the enzymatic methodology a greater quantity of monoterpenes was identified, validating results previously stated, that is, the ductility of this plant to generate more monoterpenes when it is bio-stimulated with AMF and when is fertilized with phosphorous, turning this tripartite interaction in a large platform for the evolution of “new terpenes” via mutation and selection.

The knowledge of the phytochemistry, Chemical Ecology, agronomic manage, growth conditions, among others of red clover is essential to know the possible guidelines for controlling the insect associated with the presence of secondary metabolites, thus being able to identify and recognize the importance of the monoterpene (*S*)-limonene in the *T. pratense*-*H. obscurus* interaction because its repellent activity.

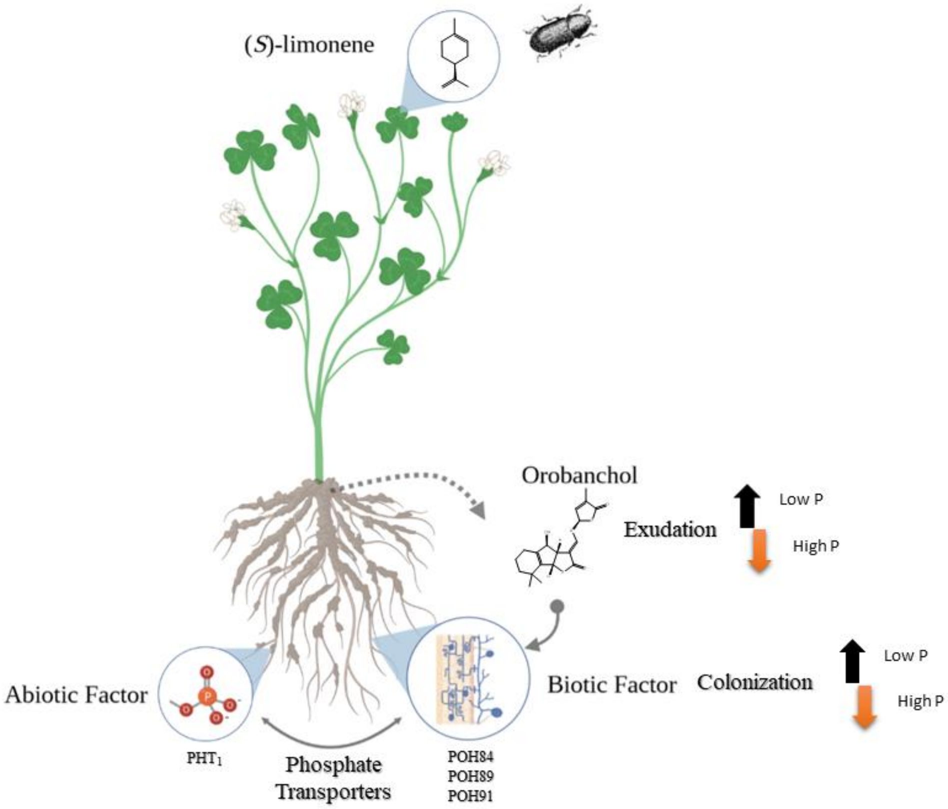


Figure 1. Schematic summary of the relationship between Arbuscular Mycorrhizal Fungi (AMF) and Phosphorus (P) fertilization associated with the Strigolactone Orobanchol at P levels associated with secondary metabolism of monoterpenes of Red clover. (Yoneyama et al. 2007; Ferrol et al. 2019; Das et al. 2022)

4.2 Concluding remarks and future directions

The results allow to confirm the hypothesis addressed in the thesis work. In summary, the application either AMF, P or AMF + P significantly increase monoterpene production in red clover Superqueli-INIA cultivar.

The use of inoculation with *Claroideoglomus claroideum* and mix inocula allowed to enhance monoterpene emission. This effect was reflected at the enzymatic level, establishing the red clover capacity for increasing monoterpene production in the presence of AMF as a relevant biotic factor in the transport of different nutrients between soil-plant with AMF as carrier. The results showed a significant effect on the production of (*S*)-limonene product of the P supplementation, thus being able to determine that phosphorus collaborates in the biosynthetic route of monoterpenes for red clover. However, the enzymatic battery for monoterpene production was activated when AMF, P and GPP were added together, showing a genomic plasticity for the generation of these secondary metabolites.

The contrasting results of the effects between AMF and P addition on terpene production suggest that there is a subtle relationship between both factors. Therefore, deeper studies focused on establishing a balance between both factors are necessary in pursuit of an increase in terpenes that allow red clover to develop a more effective defensive system.

Finally, the knowledge generated contributes to a better understanding of the secondary metabolism of red clover by recognising the metabolic capacity of this legume to generate a greater diversity of compounds that could be tested against *H. obscurus* by means of MTS.

This doctoral thesis not only contributes to broaden the spectrum of knowledge of the role of biotic and abiotic factors in red clover and other legumes in the production of secondary metabolites that interact with pest organisms, but also to the development of new methods and mechanisms to improve the production of monoterpenes for crop protection purposes. Future work could focus on characterising and evaluating the expression of monoterpene synthases present in red clover and their interaction with biotic and abiotic factors to improve and guide the production of their repellent monoterpene (*S*)-limonene or other monoterpenes present in red clover.

Furthermore, the results obtained in this thesis provide relevant information for the establishment of red clover crops by growers using biotic and abiotic factors for reducing *H. obscurus* population.

Further studies are needed to deep investigate the production mechanism of geranyl pyrophosphate (GPP), the main precursor of the monoterpene pathway. Knowledge of the genetics and metabolic pathways (DXP and MVA) of red clover cultivars is the essence of improving monoterpene production parameters to produce plants that are more resistant to field conditions and less infested by *H. obscurus*.

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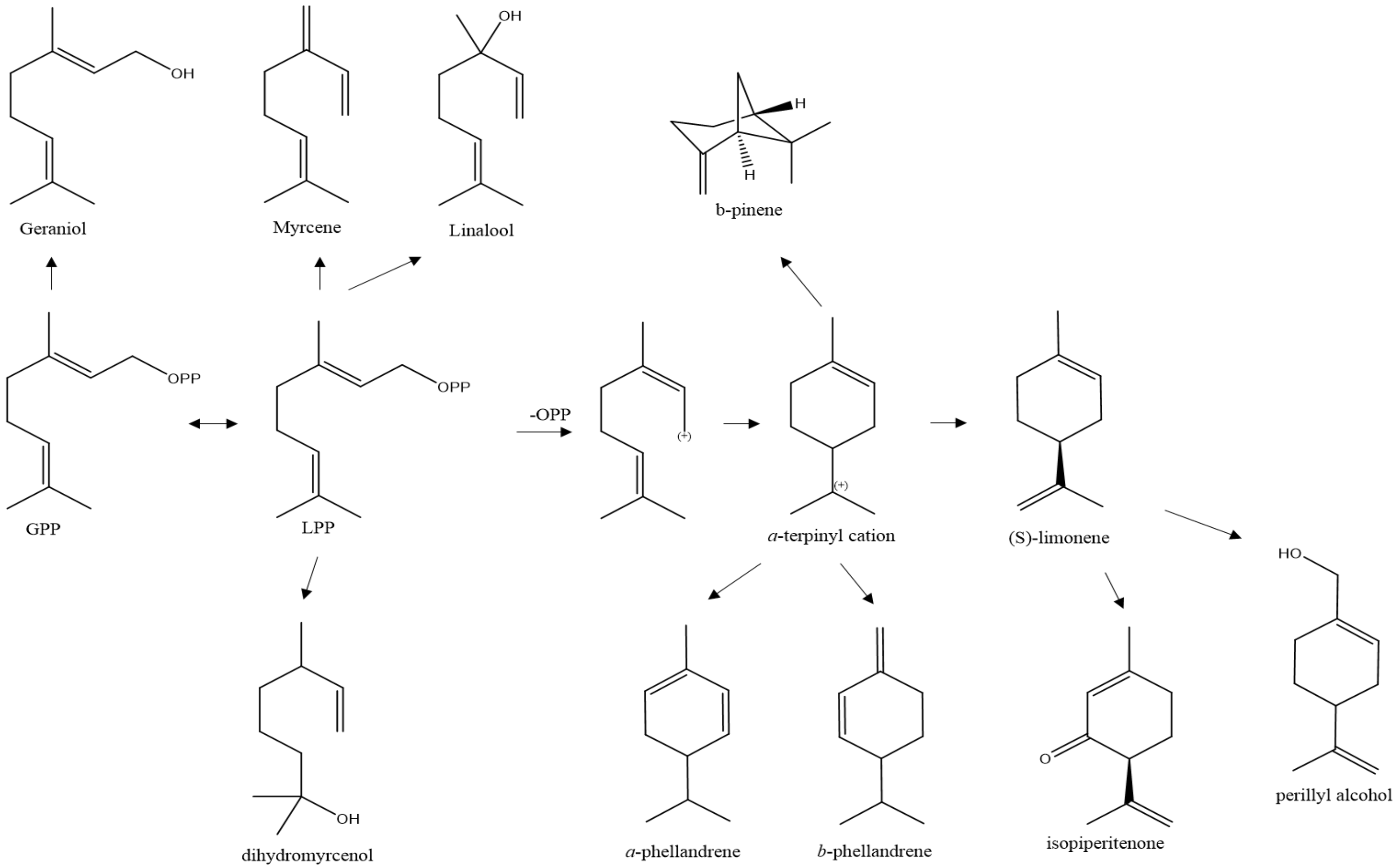
Annexes

Supplementary Table S1.1. Emission (μg/g h^{-1}) of different profile of green leaf volatile (GLV) in cultivars QQ, RQ y SQ via GC-MS. Quiñequeli-INIA (QQ), Quiñequeli-INIA plus Pure AMF (QQ-AMF_1), Quiñequeli-INIA plus Mix AMF (QQ-AMF_2), Quiñequeli-INIA plus Phosphorus (QQ-P_1), Quiñequeli-INIA plus AMF_1 plus Phosphorus (QQ-AMF_1-P_1) and Quiñequeli-INIA plus AMF_2 plus Phosphorus (QQ-AMF_2-P_1). The same combination of treatments was used for RQ and SQ. n=4. Used standard internal. Values show mean ± SE. Different letter indicate significant differences based on the LSD test ($p < 0.05$)

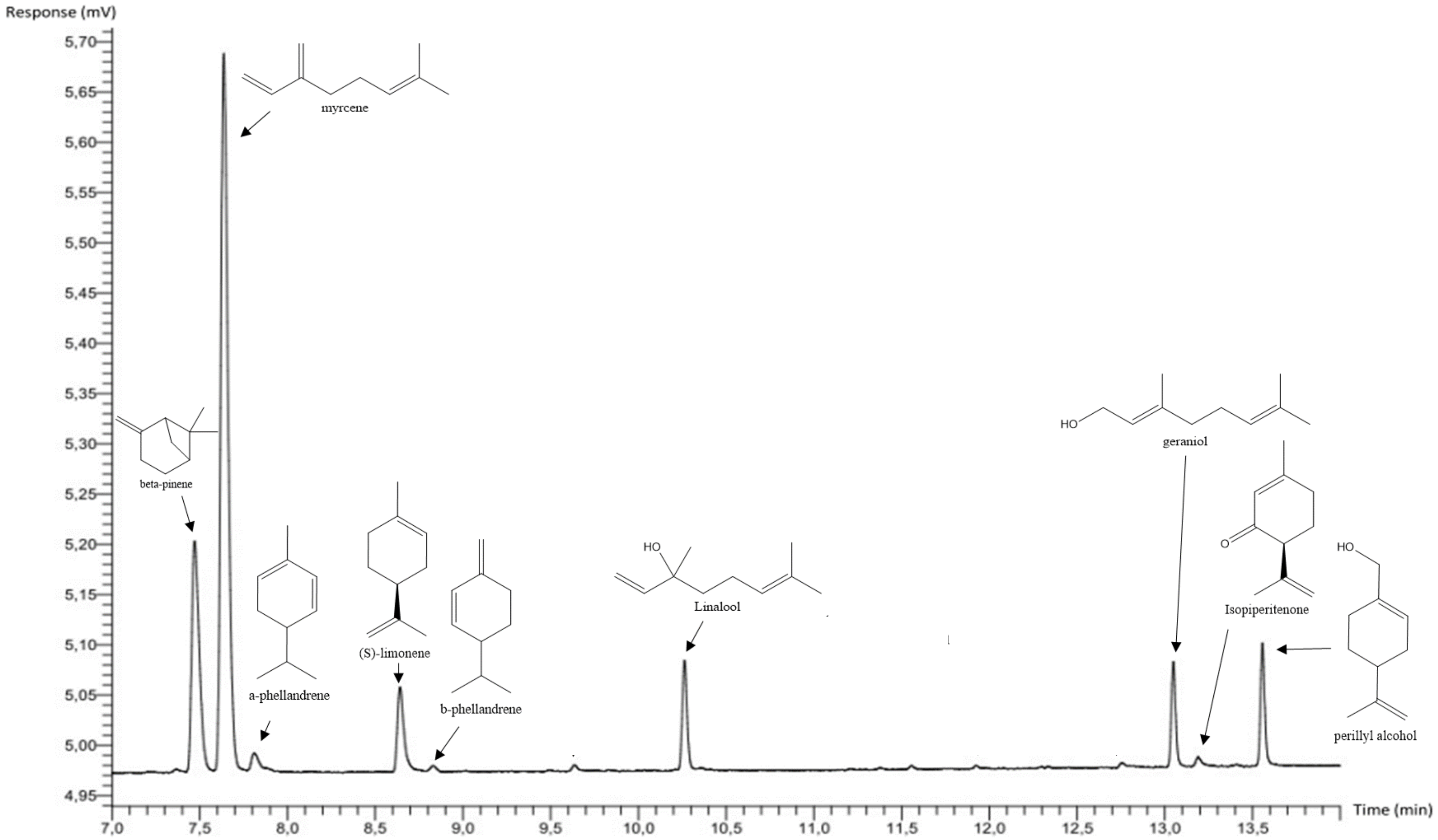
Compound	**μg/g h^{-1}**					
	QQ	**QQ-AMF_1**	**QQ-AMF_2**	**QQ-P_1**	**QQ-AMF_1-P_1**	**QQ-AMF_2-P_1**
3-Hexanone	8.40 ± 0.80 ab	7.00 ± 3.00 ab	22.40 ± 6.00 a	13.40 ± 2.20 ab	11.60 ± 2.80 ab	N. D
2-Hexanone	7.80 ± 0.40 ab	3.80 ± 2.40 b	20.40 ± 5.60 a	8.20 ± 1.60 ab	7.20 ± 2.20 ab	N. D
3-Hexanol	N. D	2.20 ± 0.80 bc	2.00 ± 1.20 abc	6.80 ± 1.00 a	5.80 ± 1.60 ab	N. D
2-Hexanol	N. D	1.20 ± 0.40 ab	0.60 ± 0.40 b	3.20 ± 0.40 a	3.20 ± 1.00 a	N. D
Z-3-Hexenyl Acetate	6.80 ± 3.40 a	N. D	N. D	0.80 ± 0.80 b	ND	N. D
m-diethyl benzene	6.00 ± 1.80 b	N. D	12.80 ± 2.60 a	N. D	ND	N. D
m-Ethyl acetophenone	81.60 ± 9.80 ab	6.20 ± 2.60 b	232.00 ± 75.00 a	28.00 ± 10.80 b	19.20 ± 8.40 b	N. D
p-Ethyl acetophenone	105.00 ± 49.00 a	9.20 ± 2.80 b	117.00 ± 27.20 a	23.00 ± 8.40 ab	5.40 ± 3.80 b	N. D

Compound	**µg/g h^{-1}**					
	RQ	**RQ-AMF$_1$**	**RQ-AMF$_2$**	**RQ-P$_1$**	**RQ-AMF$_1$-P$_1$**	**RQ-AMF$_2$-P$_1$**
3-Hexanone	15.00 ± 2.00 ab	27.20 ± 7.00 a	N. D	16.40 ± 0.60 ab	9.20 ± 1.60 b	15.40 ± 3.00 ab
2-Hexanone	22.40 ± 2.40 a	23.20 ± 6.80 a	11.40 ± 4.20 a	12.00 ± 0.40 a	6.60 ± 1.80 a	10.80 ± 3.20 a
3-Hexanol	4.40 ± 2.20 ab	10.20 ± 2.80 a	7.20 ± 3.80 ab	5.40 ± 0.60 ab	3.60 ± 1.00 b	5.80 ± 1.20 ab
2-Hexanol	2.20 ± 1.20 ab	7.40 ± 2.60 a	4.20 ± 2.20 ab	3.40 ± 0.20 ab	1.80 ± 0.60 b	3.00 ± 1.00 ab
Z-3-Hexenyl Acetate	N. D	N. D	N. D	3.80 ± 3.80 a	N. D	N. D
m-diethyl benzene	N. D	N. D	N. D	N. D	N. D	N. D
m-Ethyl acetophenone	31.40 ± 6.40 ab	17.80 ± 7.40 bc	44.00 ± 8.00 a	N. D	N. D	12.20 ± 4.20 bc
p-Ethyl acetophenone	4.60 ± 1.00 ab	6.60 ± 3.80 ab	14.20 ± 4.20 a	N. D	N. D	4.20 ± 2.20 ab

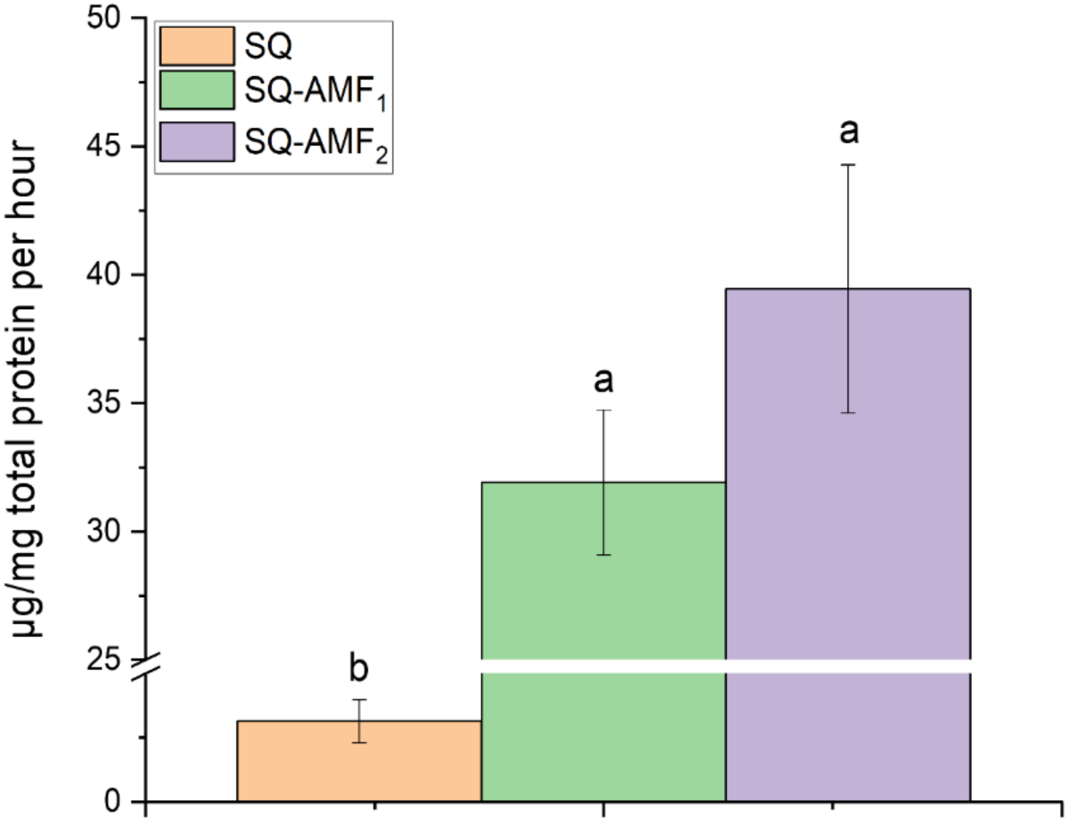
Compound	**µg/g h^{-1}**					
	SQ	**SQ-AMF$_1$**	**SQ-AMF$_2$**	**SQ-P$_1$**	**SQ-AMF1-P$_1$**	**SQ-AMF$_2$-P$_1$**
3-Hexanone	N. D	18.80 ± 3.80 ab	21.80 ± 3.80 ab	27.80 ± 13.20 ab	36.80 ± 6.60 a	28.60 ± 5.40 ab
2-Hexanone	24.80 ± 12.40 a	14.40 ± 0.40 a	18.80 ± 4.00 a	58.00 ± 29.60 a	28.60 ± 6.00 a	22.60 ± 5.60 a
3-Hexanol	N. D	7.20 ± 1.40 ab	6.20 ± 0.80 ab	18.00 ± 8.20 a	12.40 ± 3.20 ab	10.20 ± 2.00 ab
2-Hexanol	N. D	4.20 ± 1.00 ab	2.60 ± 0.60 ab	15.80 ± 8.40 a	8.20 ± 2.80 ab	6.60 ± 1.60 ab
Z-3-Hexenyl Acetate	N. D	5.20 ± 2.20 a	N. D	50.60 ± 47.80 a	N. D	N. D
m-diethyl benzene	N. D	N. D	N. D	N. D	N. D	N. D
m-Ethyl acetophenone	26.00 ± 10.40 a	12.40 ± 3.60 a	232.00 ± 75.00 a	32.20 ± 11.60 a	10.40 ± 3.40 a	12.80 ± 3.20 a
p-Ethyl acetophenone	7.80 ± 3.20 b	5.20 ± 2.20 b	117.20 ± 27.20 a	12.20 ± 8.80 b	N. D	3.60 ± 1.20 b



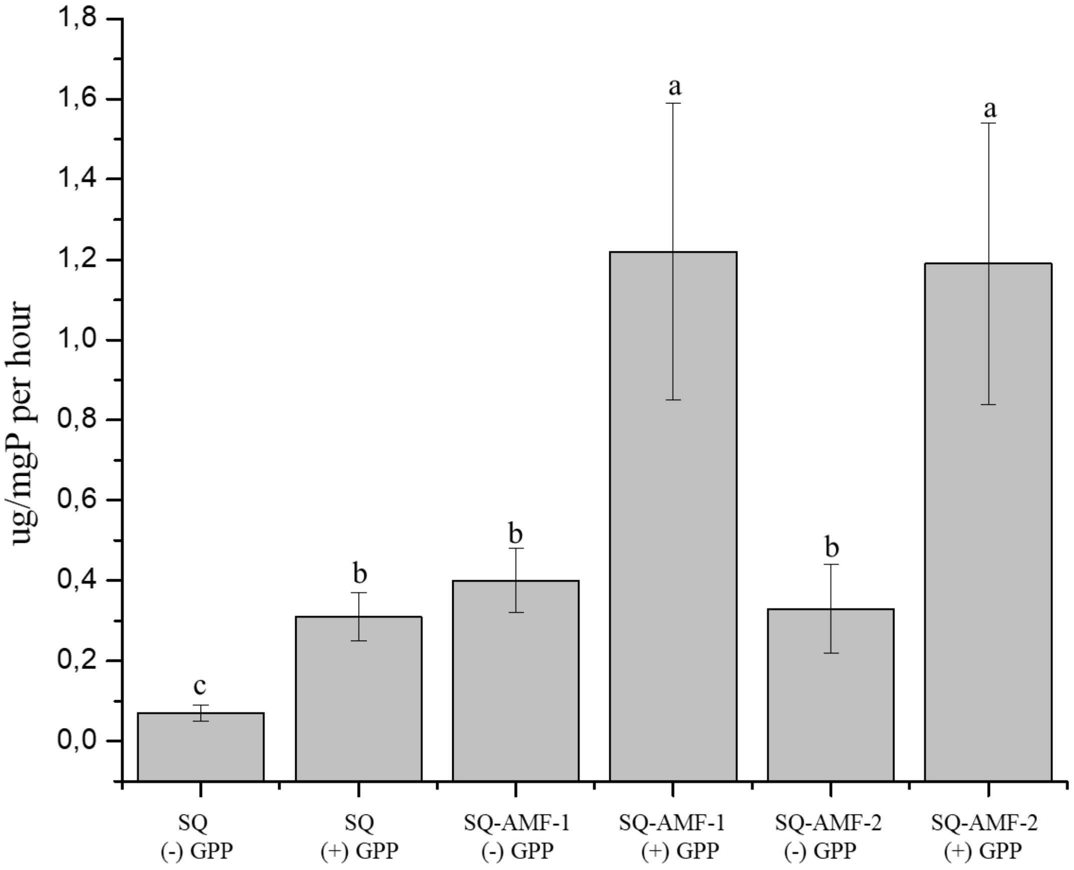
Supplementary Figure S1.2. Proposed biosynthetic pathway of monoterpenes in Superqueli-INIA



Supplementary Figure S1.3. Characteristic chromatogram in Superqueli-INIA cultivar by HS-GC

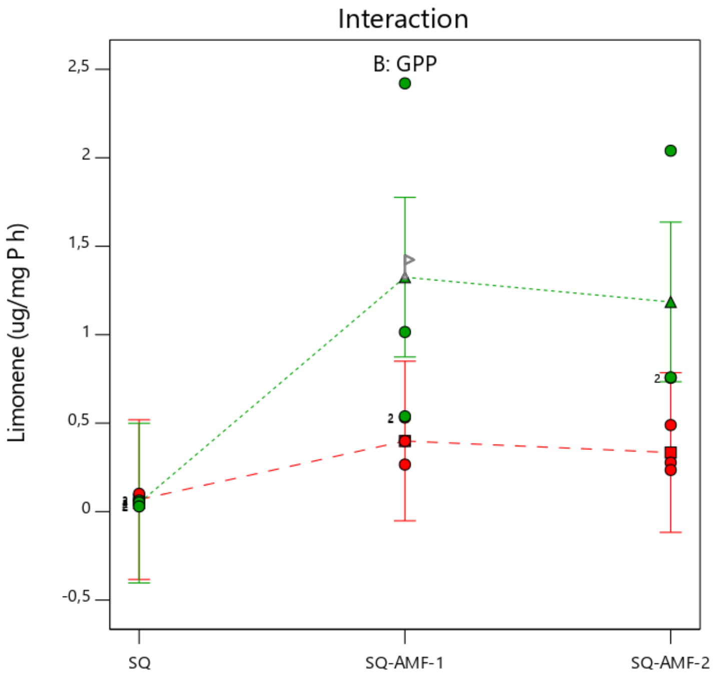


Supplementary Figure S1.4. Total protein content in Superqueli-INIA crude extract analyzed with BSA curve.

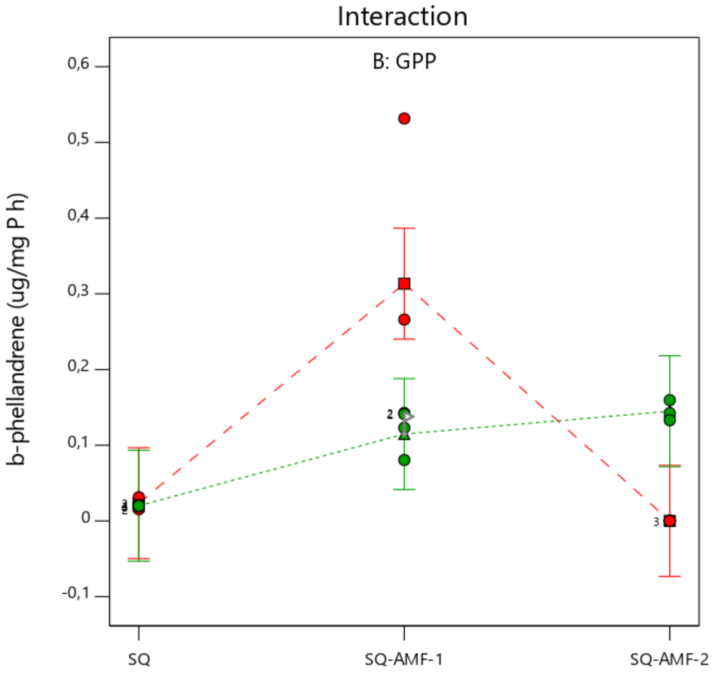


Supplementary Figure S1.5. Enzymatic production in µg/mg $P^{-1}*h^{-1}$ of (*S*)-limonene in treatments with AMF_1 and AMF_2 with and without geranyl pyrophosphate (GPP) added. Different letters show different groups using Tukey test ($p < 0.05$)

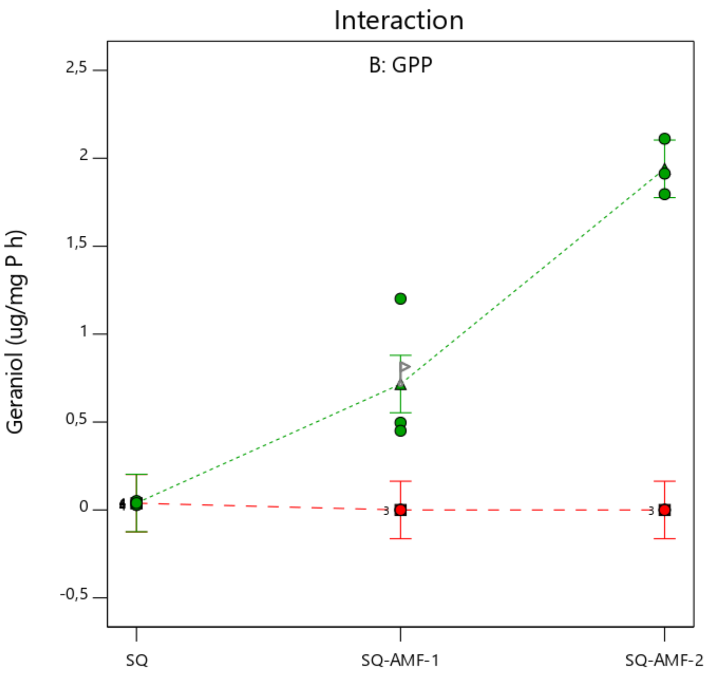
a)


Interaction
B: GPP
Limonene (ug/mg P h)
2,5
2
1,5
1
0,5
0
-0,5
2
2
SQ
SQ-AMF-1
SQ-AMF-2

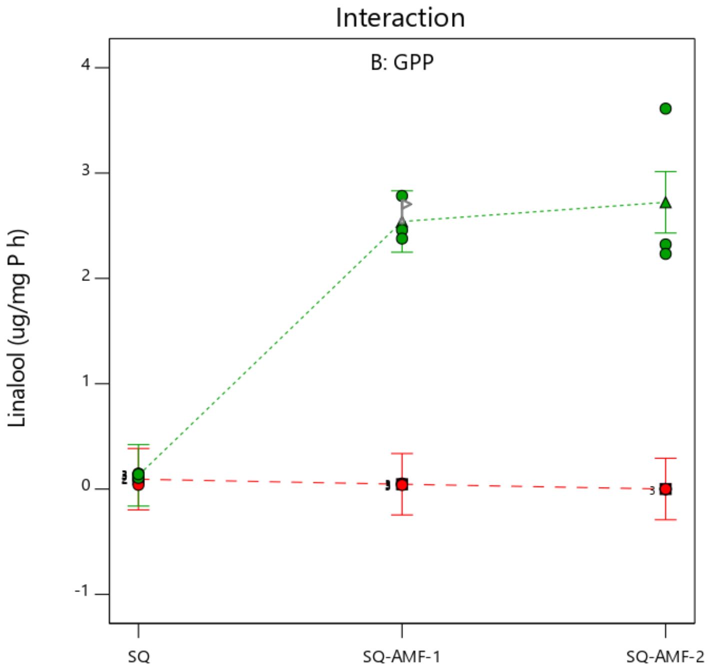
b)


Interaction
B: GPP
b-phellandrene (ug/mg P h)
0,6
0,5
0,4
0,3
0,2
0,1
0
-0,1
2
3
SQ
SQ-AMF-1
SQ-AMF-2

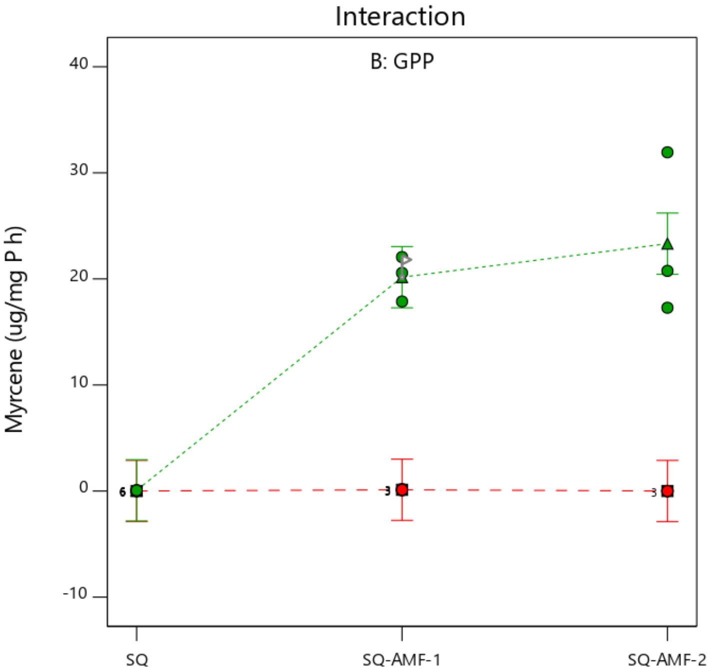
c)


Interaction
B: GPP
Geraniol (ug/mg P h)
2,5
2
1,5
1
0,5
0
-0,5
SQ
SQ-AMF-1
SQ-AMF-2

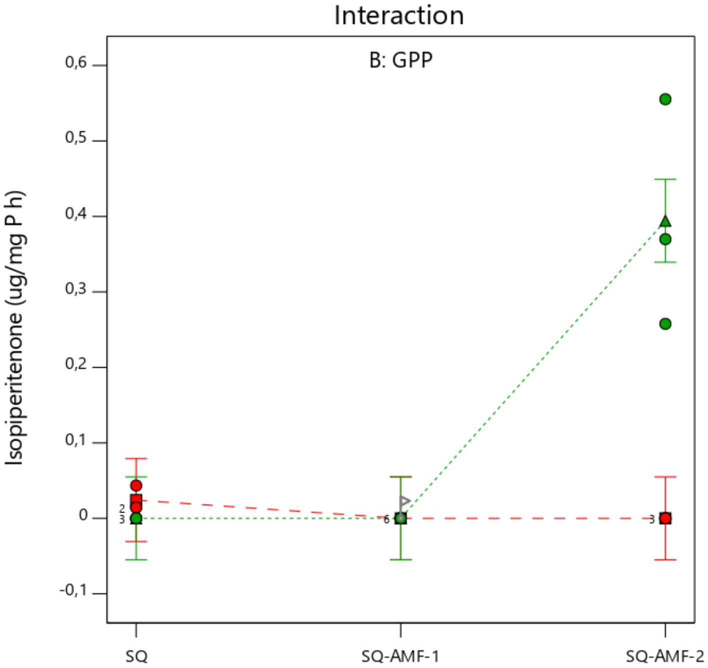
d)


Interaction
B: GPP
Linalool (ug/mg P h)
4
3
2
1
0
-1
SQ
SQ-AMF-1
SQ-AMF-2

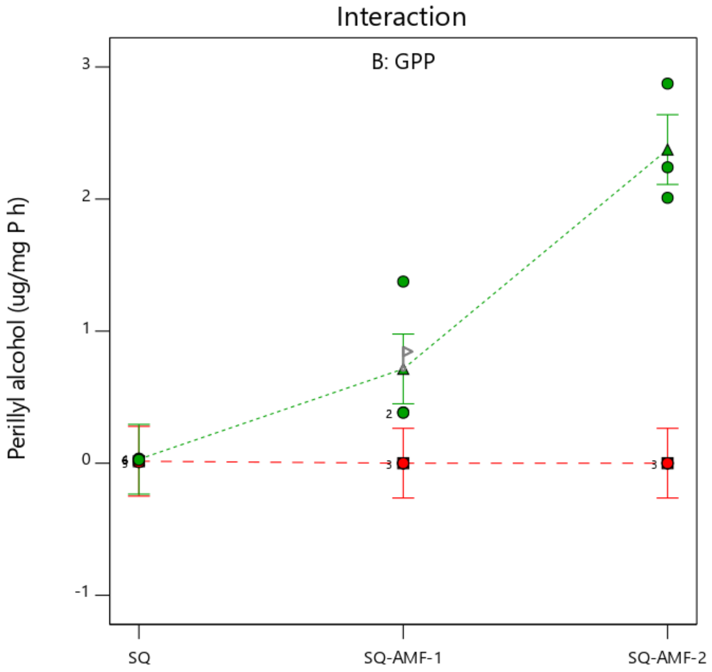
e)


Interaction
B: GPP
Myrcene (ug/mg P h)
40
30
20
10
0
-10
6
3
3
SQ
SQ-AMF-1
SQ-AMF-2

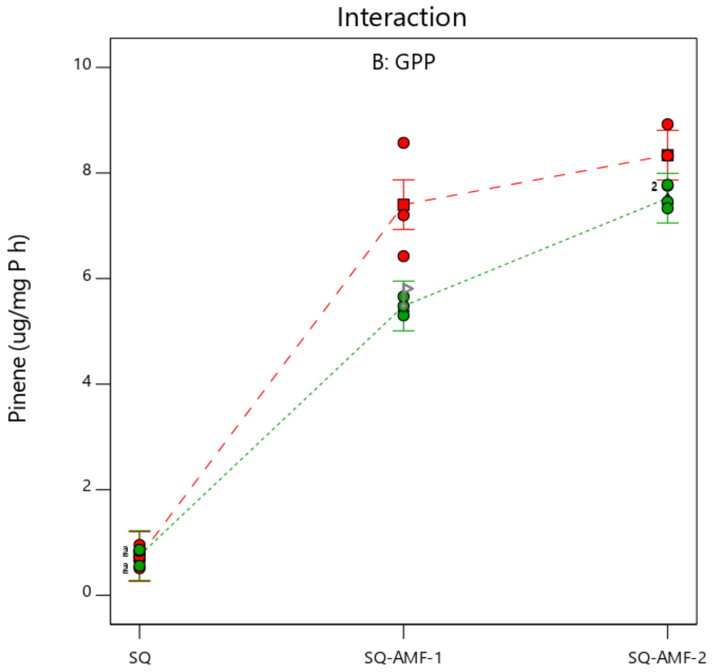
f)


Interaction
B: GPP
Isopiperitenone (ug/mg P h)
0,6
0,5
0,4
0,3
0,2
0,1
0
-0,1
2
3
6
3
SQ
SQ-AMF-1
SQ-AMF-2

g)

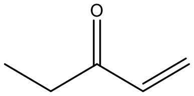
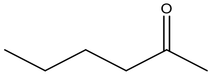
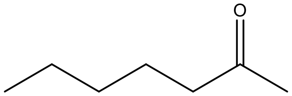
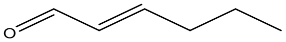
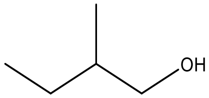
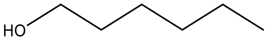


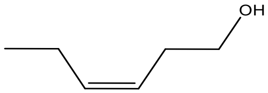
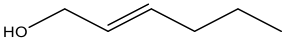
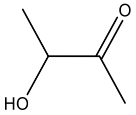
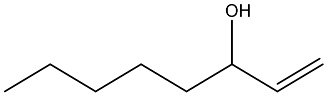
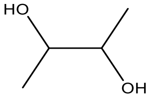
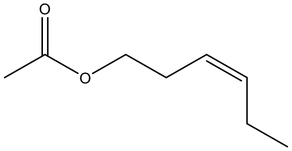
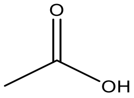
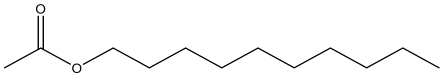
h)

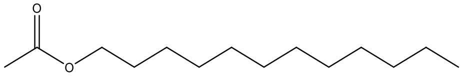
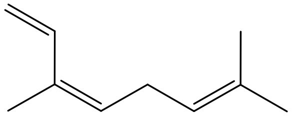
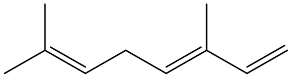
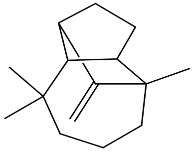
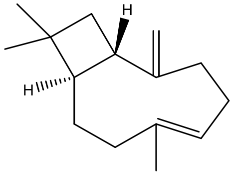


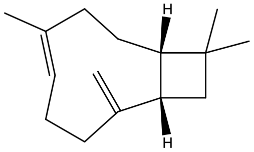
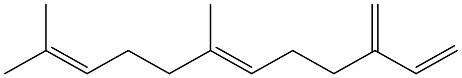
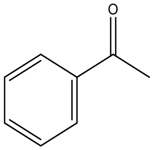
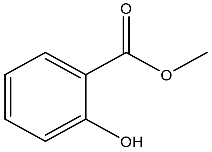
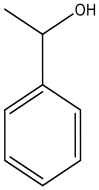
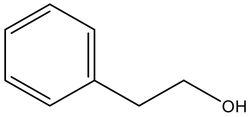
Supplementary Figure S1.6. Interaction plot between SQ (control), SQ + AMF$_1$ (*Claroideoglomus claroideum*) and SQ + AMF$_2$ (mix) treatments to produce monoterpenes: a) (*S*)-limonene, b) β-phellandrene, c) geraniol, d) linalool, e) myrcene, f) isopiperitenone, g) perillyl alcohol and h) α-pinene. Red colour shows without GPP aggregation, green colour represents with GPP aggregation.

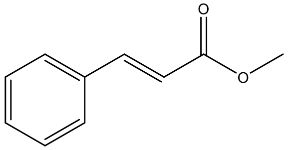
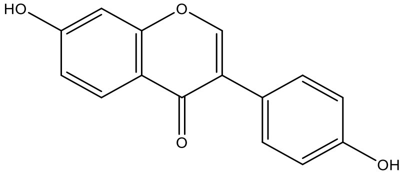
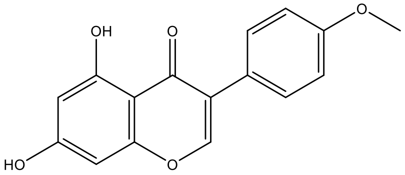
Supplementary Table S1.7. Secondary metabolites of red clover (*Trifolium Pratense* L.) in different organs of plant by different chromatographic analysis.

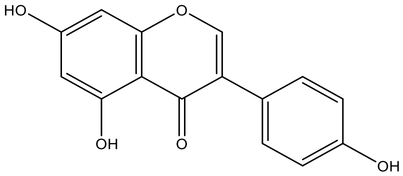
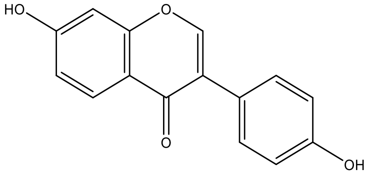
Compound	Chemical Structure	Part	Technique	Reference
1-Penten-3-one		Pods	GC-MS	Buttery et al. (1984)
2-Hexanone		Flowers	GC-MS	Buttery et al. (1984)
2-Heptanone		Flowers	GC-MS	Buttery et al. (1984)
(*E*)-2-Hexenal		Flowers Pods	GC-MS	Buttery et al. (1984)
		Roots	SFE-GC-MS	Tapia et al. (2007)
		Roots	SPME-GC-MS	Palma et al. (2012)
2-Methyl-butanol		Pods	GC-MS	Buttery et al. (1984)
Hexanol		Leaves Flowers	GC-MS	Buttery et al. (1984)
		Aerial part	SPME-GC-MS	Figueiredo et al. (2007)

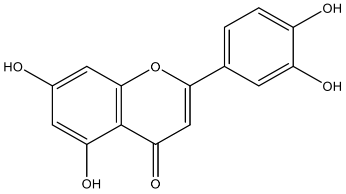
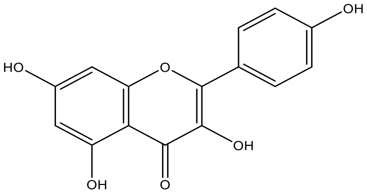
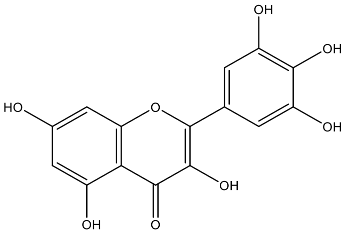
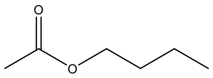
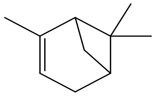
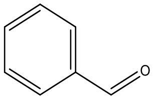
(*Z*)-3-Hexenol		Leaves Flowers	GC-MS	Buttery et al. (1984)
(*E*)-2-Hexenol		Leaves Flowers	GC-MS	Buttery et al. (1984)
2-Hydroxybutan-3-one		Flowers Pods	GC-MS	Buttery et al. (1984)
1-Octen-3-ol		Leaves Flowers Pods	GC-MS	Buttery et al. (1984)
2,3-Dihydroxybutane		Flowers	GC-MS	Buttery et al. (1984)
(*Z*)-3-Hexenyl acetate		Leaves Flowers Pods	GC-MS	Buttery et al. (1984)
		Whole plant	GC-MS	Kigathi et al. (2019)
Acetic acid		Pods	GC-MS	Buttery et al. (1984)
Decyl acetate		Pods	GC-MS	Buttery et al. (1984)

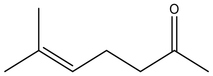
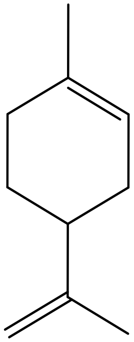
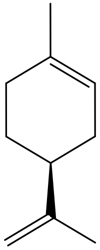
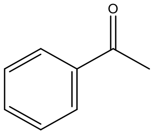
Dodecyl acetate		Pods	GC-MS	Buttery et al. (1984)
(*Z*)-β-Ocimene		Leaves Flowers Pods	GC-MS	Buttery et al. (1984)
		Whole plant	GC-MS	Kigathi et al. (2009)
(*E*)-β-Ocimene		Leaves Flowers Pods	GC-MS	Buttery et al. (1984)
		Whole plant	GC-MS	Kigathi et al. (2009)
		Whole plant	GC-MS	Kigathi et al. (2019)
Longifolene		Pods	GC-MS	Buttery et al. (1984)
Caryophyllene		Leaves Flowers Pods	GC-MS	Buttery et al. (1984)

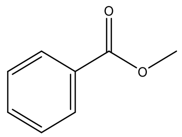
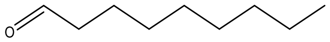
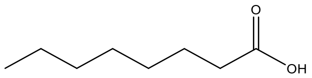
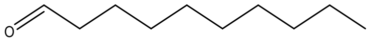
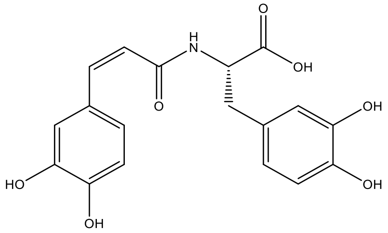
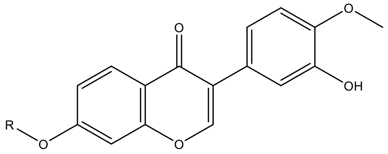
(*Z*)-β-Caryophyllene		Aerial part	SPME-GC-MS	Figueiredo et al. (2007)
(*E*)-β-Farnesene		Leaves pods	GC-MS	Buttery et al. (1984)
		Aerial part	SPME-GC-MS	Figueiredo et al. (2007)
Acetophenone		Flowers Pods	GC-MS	Buttery et al. (1984)
Methyl salicylate		Leaves	GC-MS	Buttery et al. (1984)
1-Phenyl ethanol		Flowers	GC-MS	Buttery et al. (1984)
2-Phenyl ethanol		Flowers	GC-MS	Buttery et al. (1984)

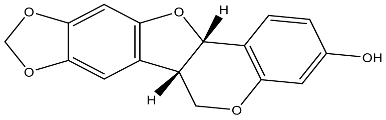
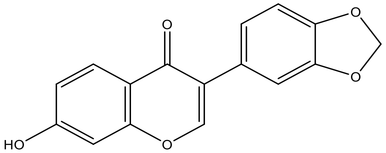
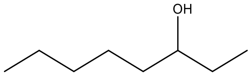
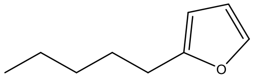
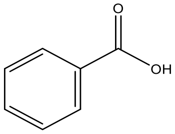
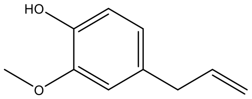
Methyl cinnamate		Flowers	GC-MS	Buttery et al. (1984)
Formononetin		Leaves Stems Flowers	UPLC	Lemežienė et al. (2015)
		Roots	HPLC-MS	Saviranta et al. (2010)
		Whole plant	LC-UV	Klejdus et al. (2001)
		Whole plant	HPLC	Krenn et al. (2002)
		Whole plant	UPLC-DAD-MS	Taujenis et al. (2015)
Biochanin A		Leaves Stems Flowers	UPLC	Lemežienė et al. (2015)
		Whole plant	UPLC-DAD-MS	Taujenis et al. (2015)
		Roots	HPLC-MS	Saviranta et al. (2010)
		Whole plant	LC-UV	Klejdus et al. (2001)
		Whole	HPLC	Krenn et al. (2002)

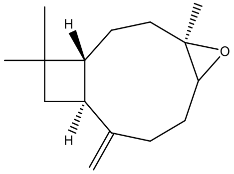
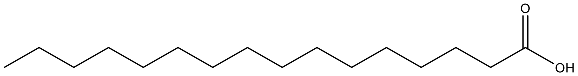
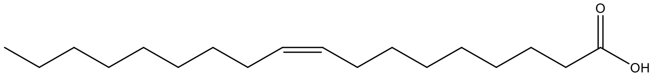
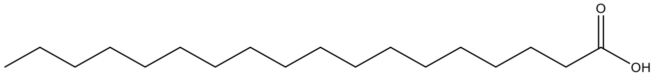
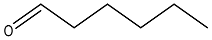
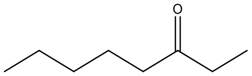
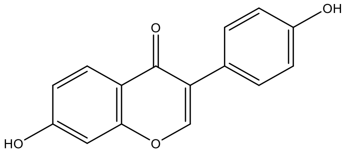
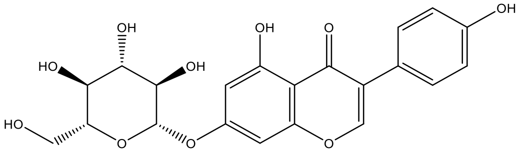
		plant		
Genistein		Leaves Stems Flowers	UPLC	Lemežienė et al. (2015)
		Whole plant	UPLC-DAD-MS	Taujenis et al. (2015)
		Whole plant	LC-UV	Klejdus et al. (2001)
		Whole plant	HPLC	Krenn et al. (2002)
Daidzein		Leaves Stems Flowers	UPLC	Lemežienė et al. (2015)
		Whole plant	HPLC	Krenn et al. (2002)
		Whole plant	UPLC-DAD-MS	Taujenis et al. (2015)
		Whole plant	HPLC-MS/MS	Vlaisavljević et al. (2017)

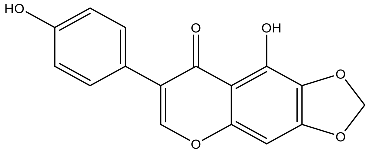
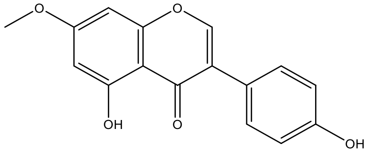
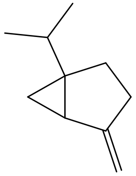
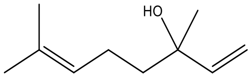
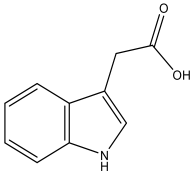
Luteolin		Flowers	HPLC	Tundis et al. (2015)
Kaempferol		Flowers	HPLC	Tundis et al. (2015)
Myricetin		Flowers	HPLC	Tundis et al. (2015)
n-Butyl acetate		Roots	SFE-GC-MS	Tapia et al. (2007)
α-Pinene		Roots	SFE-GC-MS	Tapia et al. (2007)
		Root	SPME-GC-MS	Palma et al. (2012)
Benzaldehyde		Roots	SFE-GC-MS	Tapia et al. (2007)
		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)

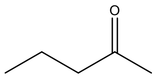
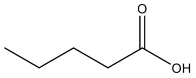
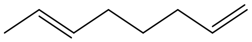
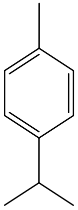
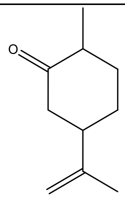
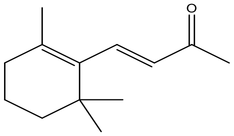
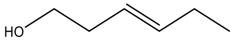
		Aerial part	SPME-GC-MS	Figueiredo et al. (2007)
6-Methylhept-5-en-2-one		Roots	SFE-GC-MS	Tapia et al. (2007)
Limonene		Roots	SFE-GC-MS	Tapia et al. (2007)
		Root	GC-MS	Manosalva et al. (2011)
		Whole plant	GC-MS	Kigathi et al. (2009)
		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
(*S*)-limonene		Aerial part	HS-GC	Medina et al. (2022)
Acetophenone		Roots	SFE-GC-MS	Tapia et al. (2007)
		Aerial part	SPME-GC-MS	Figueiredo et al. (2007)
Methyl benzoate		Roots	SFE-GC-	Tapia et al. (2007)

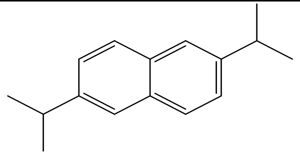
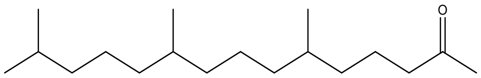
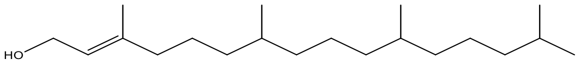
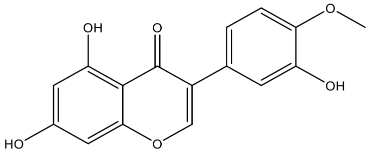
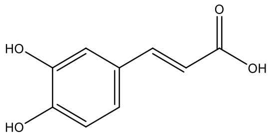
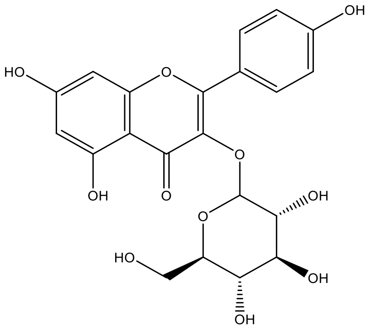
			MS	
		Aerial part	SPME-GC-MS	Figueiredo et al. (2007)
Nonanal		Roots	SFE-GC-MS	Tapia et al. (2007)
		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
Octanoic acid		Roots	SFE-GC-MS	Tapia et al. (2007)
Decanal		Roots	SFE-GC-MS	Tapia et al. (2007)
		Aerial part	SPME-GC-MS	Figueiredo et al. (2007)
cis-Clovamide		Roots	HPLC-MS	Saviranta et al. (2010)
Calycosin-G	 R: Is glucoside or galactoside	Roots	HPLC-MS	Saviranta et al. (2010)

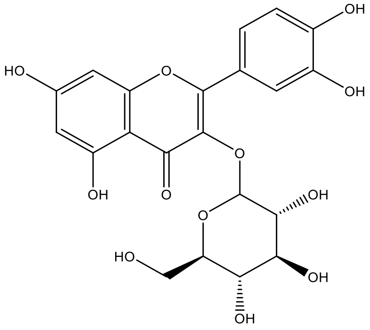
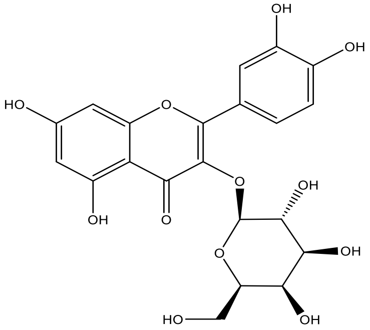
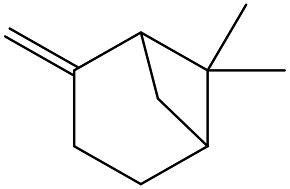
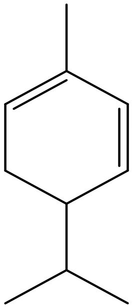
Maackiain		Roots	HPLC-MS	Saviranta et al. (2010)
Pseudobaptigenin		Roots	HPLC-MS	Saviranta et al. (2010)
		Whole plant	LC-UV	Klejdus et al. (2001)
1-Nonene		Root	GC-MS	Manosalva et al. (2011)
3-Octenol		Root	GC-MS	Manosalva et al. (2011)
2-Pentyl furan		Root	GC-MS	Manosalva et al. (2011)
Benzoic Acid		Root	GC-MS	Manosalva et al. (2011)
Eugenol		Root	GC-MS	Manosalva et al. (2011)
Caryophyllene oxide		Root	GC-MS	Manosalva et al. (2011)

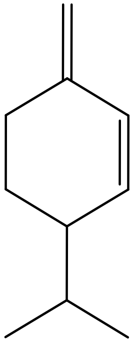
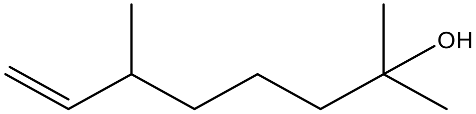
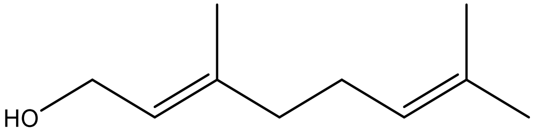
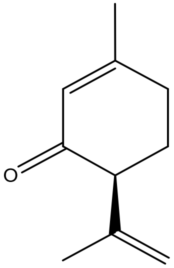
		Aerial part	SPME-GC-MS	Figueiredo et al. (2007)
Palmitic acid		Root	GC-MS	Manosalva et al. (2011)
		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
Oleic acid		Root	GC-MS	Manosalva et al. (2011)
Stearic acid		Root	GC-MS	Manosalva et al. (2011)
Hexanal		Root	SPME-GC-MS	Palma et al. (2012)
3-Octanone		Root	SPME-GC-MS	Palma et al. (2012)
Daidzin		Whole plant	LC-UV	Klejdus et al. (2001)
Genistin		Whole plant	LC-UV	Klejdus et al. (2001)

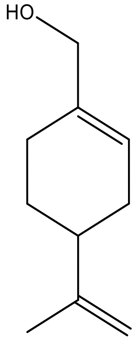
Irilone		Whole plant	LC-UV	Klejdus et al. (2001)
		Whole plant	UPLC-DAD-MS	Taujenis et al. (2015)
Prunetin		Whole plant	LC-UV	Klejdus et al. (2001)
		Whole plant	UPLC-DAD-MS	Taujenis et al. (2015)
Sabinene		Whole plant	GC-MS	Kigathi et al. (2009)
Linalool		Whole plant	GC-MS	Kigathi et al. (2009)
		Aerial part	HS-GC	Medina et la. (2022)
Indole		Whole plant	GC-MS	Kigathi et al. (2009)

2-pentanone		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
Pentanoic acid		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
β-Myrcene		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
		Aerial part	HS-GC	Medina et al. (2022)
ρ-Cymene		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
Dihydrocarvone		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
β -Ionone		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
		Aerial part	SPME-GC-MS	Figueiredo et al. (2007)
3-Hexen-1-ol		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)

2,6-Diisopropylnaphthalene		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
Hexahydrofarnesyl acetone		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
Phytol		Whole plant	MAHD-GC-MS	Vlaisavljević et al. (2014)
Pratensein		Whole plant	UPLC-DAD-MS	Taujenis et al. (2015)
Caffeic acid		Whole plant	HPLC-MS/MS	Vlaisavljević et al. (2017)
Kaempferol-3-O-glucoside		Whole plant	HPLC-MS/MS	Vlaisavljević et al. (2017)

Quercetin-3-O-glucoside		Whole plant	HPLC-MS/MS	Vlaisavljević et al. (2017)
Hyperoside		Whole plant	HPLC-MS/MS	Vlaisavljević et al. (2017)
(β)-pinene		Aerial part	HS-GC	Medina et al. (2022)
α-phellandrene		Aerial part	HS-GC	Medina et al. (2022)

β-phellandrene		Aerial part	HS-GC	Medina et al. (2022)
Dihydromyrcenol		Aerial part	HS-GC	Medina et al. (2022)
Geraniol		Aerial part	HS-GC	Medina et al. (2022)
Isopiperitenone		Aerial part	HS-GC	Medina et al. (2022)

Perillyl alcohol		Aerial part	HS-GC	Medina et al. (2022)

Description of the different techniques

UPLC: Ultra Performance Liquid Chromatography; **HPLC**: High Performance Liquid Chromatography; **SFE-GC-MS**: Supercritical Fluid Extraction- Gas- Chromatography-Mass Spectrometry; **HPLC-MS**: High Performance Liquid Chromatography-Mass Spectrometry; **GC-MS**: Gas- Chromatography-Mass Spectrometry; **SPME-GC-MS**: Solid Phase Microextraction- Gas- Chromatography-Mass Spectrometry; **LC-UV**: Liquid Chromatography-Ultraviolet; **MAHD-GC-MS**: Microwave-Assisted Hydrodistillation- Gas- Chromatography-Mass Spectrometry; **UPLC-DAD-MS**: Ultra Performance Liquid Chromatography-Diode Array Detector- Mass Spectrometry; **HPLC-MS/MS**: High Performance Liquid Chromatography-Electrospray Ionization Triple-Quadrupole. **HS-GC:** Headspace-Gas Chromatography.