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PhD thesis

Characterisation of different hop ecotypes (*Humulus lupulus*, L.) in Central Italy and evaluation of the biological activity of their extracts, EO and components against *Sitophilus granarius* (L.)

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"….Dicono che c'è un tempo per seminare

E uno più lungo per aspettare

Io dico che c'era un tempo sognato

Che bisognava sognare…."

"C'è tempo", Ivano Fossati

TABLE OF CONTENTS

ABSTRACT

The indiscriminate use of synthetic insecticides for several decades has raised long-term human health and environmental concerns, mainly due to their slow degradation in the environment and toxic residues in the products, as well as the evolution of resistance to pesticides in pest populations (Isman, 2006).

The serious problems of insecticide resistance in pests and the contamination of the biosphere associated with large scale use of synthetic pesticides have led to the search for eco-friendly pesticides with greater selectivity to the pests. This awareness has created a worldwide interest in the development of alternative strategies, including the discovery of newer insecticides that are more environmentally friendly than synthetic chemicals (Rajashekar *et al.,* 2012a, b; Miresmailli and Isman 2014).

In this context, the use of insecticides based on botanical extracts is attracting considerable interest among both researchers and consumers.

It's known that various plant compounds bind to some biological macromolecules (mainly proteins), interacting with specific sites inside the cells. Lately, the study on the interactions among natural substances and cellular components has assumed an important role in the development of synthetic medicines/drugs/compounds. This could encourage the identification of new plant compounds/extracts with biological activity since they constitute the basis for new biotechnological approaches to plant defense and its management.

Therefore, in addition to new classes of insecticides, chemicals that have different modes of action are needed to maintain stable food production (Gokce *et al.*, 2012). Most plants defend themselves from herbivory through production of secondary compounds such as terpenes, phenolics and nitrogen-containing compounds (Taiz and Zeiger, 2010). Some of these secondary compounds, including pyrethrin and rotenone, produce direct toxicity, while others cause either physiological disruption as caused by growth regulators or behavioral effects as repellents, attractants or antifeedants (Isman, 1999; Gokce *et al.*, 2006).

Hop (*Humulus lupulus* L.) is a high-climbing, perennial vine, utilized in the brewing industry to add flavor and bitterness to beer (Chadwick *et al.*, 2006) whose production has been estimated at over 100.000 tons worldwide (FAOSTAT, 2014).

However, aerial parts of this plant produces several secondary metabolites as bitter resins, essential oil (EO), tannins and terpenes, proved to have biological activities: xanthohumol was reported to show anticancer and antioxidant effects (Colgate *et al.,* 2010); hop iso-α-acids positively affect glucose metabolism, diet-induced obesity and its relative cognitive decline in rodents (Miura *et al.,* 2005; Yajima *et al.,* 2005; Ayabe *et al.* 2018); main compounds of EO such as α-humulene, α-myrcene and β-caryophyllene showed antimicrobial activities against different strains of Gram-positive and Gram-negative bacteria (Stompor and Zarowska 2016). Moreover, besides medical purposes (Bocquet *et al.,* 2018a), the interest in hop extracts/compounds is increasing in the light of a their possible use also in pest control (Bocquet *et al.,* 2018b). In particular, several papers highlighted activities against insect due to both antifeedant and repellent activities for hop extracts or EO compounds of this plant (DeGrandi *et al* 2012; Powell *et al* 1997).

Aim of the present study is to gain a further insight into wild hop properties. Thus, in a first part the biological activity of ...hop extracts, EO and its principal chemical compounds (α-humulene, α-myrcene and β-caryophyllene) against the granary weevil *Sitophilus granarius* (L.) (Coleoptera Dryophthoridae) was investigated;subsequently, the attention was focused on the characterization of hop ecotypes in different locations of Central Italy (Abruzzo, Molise) in order to identify the best one in terms of polyphenols content associated with a high antioxidant power

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The terpenes, α-humulene, β-myrcene and β-caryophyllene were the main components (77.6%) of EO.

Among all chemical compounds and extracts tested, EO was able to exert the highest contact toxicity with LD_{50} and LD_{90} values of 13.30 and 40.23 µg/adult after 24 h of application, decreasing to 11.77 and 36.80 µg/adult after 48 h, respectively.

Between solvent extracts the most active was the one in acetone while among compounds the greatest contact toxicity was observed for α-humulene.

Moreover, α-humulene was able to exert the highest average RI (-38.89) against *S. granarius* adults.

As regards to inhalation toxicity, the highest fumigant activity was observed for ß-myrcene with LC_{50} and LC_{90} values of 72.78 and 116.92 mg/L in the absence of grain, and 115.78 and 171.42 mg/L in the presence of it.

The highest ingestion mortality was detected in acetone extract while the greatest deterrence was found for methanol extract.

Negligible anticholinesterase activity was found for all substances in the checked range, with the only exception of ß-caryophyllene which showed a dose dependent inhibitory effect, whereas all free fractions of polyphenols showed anticholinesterase activity.

The highest polyphenols content was found for the ecotype collected in 99 Cannelle (78.28 mg/g of dry weight) associated with the highest anticholinesterase activity.

Finally, the highest content of alpha and beta acids was recorded in the sample collected in Bussi.

This study indicate that wild hop is a source of biological substances which are active on *S. granarius* adults by contact, ingestion and fumigant toxicity, to be also utilized as low-cost, eco-friendly pests repellents in the protection of stored food.

Moreover, the results confirm that the examined hop ecotypes show significant differences in the content of polyphenols, which determine different antioxidant and anti-cholinesterase activities. The latter, in particular, could be responsible for the insecticidal action found previously in alcoholic extracts of ecotypes. These results highlight the need to define the chemical composition of the various fractions in order to characterize the various ecotypes and identify the biologically active molecules.

RIASSUNTO

L'uso indiscriminato di insetticidi sintetici per diversi decenni ha sollevato preoccupazioni per la salute umana, principalmente a causa del loro lento degrado nell'ambiente, dei residui tossici da essi rilasciati e dell'evoluzione della resistenza ai pesticidi nelle popolazioni di parassiti (Isman, 2006).

I gravi problemi di resistenza agli insetticidi nei parassiti e la contaminazione della biosfera associata all'uso su larga scala di pesticidi sintetici, hanno portato alla ricerca di prodotti eco-compatibili con maggiore selettività.

Questa consapevolezza ha creato un interesse mondiale nello sviluppo di strategie alternative, compresa la scoperta di nuovi insetticidi più ecologici rispetto alle sostanze chimiche sintetiche (Rajashekar *et al.*,2012a, b; Miresmailli e Isman, 2014).

In questo contesto, l'impiego di insetticidi a base di estratti botanici sta suscitando un notevole interesse sia tra i ricercatori che tra i consumatori.

È noto che vari composti vegetali si legano ad alcune macromolecole biologiche (principalmente proteine), interagendo con siti specifici all'interno delle cellule.

Ultimamente, lo studio sulle interazioni tra sostanze naturali e componenti cellulari ha assunto un ruolo importante nello sviluppo di farmaci sintetici.

Ciò potrebbe incoraggiare la ricerca verso l'identificazione di nuovi composti/estratti con attività biologica in quanto costituiscono la base per nuovi approcci biotecnologici alla difesa delle piante e alla loro gestione.

Oltre alle nuove classi di insetticidi, sono però necessarie sostanze chimiche che presentano diverse modalità di azione, in modo da mantenere una produzione alimentare stabile (Gokce *et al.*, 2012).

La maggior parte delle piante si difende dagli erbivori attraverso la produzione di metaboliti secondari come terpeni, composti fenolici e composti contenenti azoto (Taiz and Zeiger, 2010). Alcuni di questi, tra cui piretrina e rotenone, provocano tossicità diretta, altri causano interruzioni fisiologiche (regolatori della crescita) ed altri ancora vengono utilizzati come repellenti, attrattivi o anti-nutrizionali (Isman, 1999; Gokce *et al.*, 2006).

Il luppolo (*Humulus lupulus* L.) è una pianta perenne e rampicante, utilizzata nei birrifici per aggiungere l'amaro e l'aroma alla birra (Chadwick *et al.*, 2006) la cui produzione è stata stimata di oltre 100.000 tonnellate in tutto il mondo (FAOSTAT, 2014). Tuttavia, parti aeree di questa pianta, producono diversi composti secondari come resine amare, olio essenziale (OE), tannini e terpeni ed hanno dimostrato avere attività biologiche: xantumolo, il principale prenilflavonoide, è stato segnalato possedere effetti antitumorali e antiossidanti (Jacob *et al.*, 2010; Colgate et al ., 2010); gli iso-α-acidi del luppolo influenzano positivamente il metabolismo del glucosio, l'obesità indotta dalla dieta e il relativo declino cognitivo nei roditori (Hiroaki Yajima *et al.,* 2004; Miura *et al.*, 2005; Yajima *et al.*, 2005; Ayabe *et al.*, 2018); i principali composti dell' OE come α-umulene, β-mircene e β-cariofillene hanno mostrato attività antimicrobiche contro diversi ceppi di batteri Gram-positivi e Gram-negativi (Stompor e Zarowska, 2016). Inoltre, oltre agli impieghi in campo medico (Bocquet *et al.*, 2018), l'interesse per gli estratti/composti derivati dal luppolo sta aumentando, alla luce di un loro possibile utilizzo anche nel controllo dei parassiti.

In particolare, diversi articoli hanno evidenziato (Bocquet *et al.*, 2018), attività antinutrizionale e repellente di estratti di luppolo e composti dell'OE di questa pianta (DeGrandi *et al.,* 2012; Powell *et al.*, 1997).

Pertanto, nel presente studio l'attenzione è stata focalizzata sull'utilizzo di luppolo selvatico e sulla valutazione dell'attività biologica di estratti, OE e dei suoi principali composti (α-umulene, β-mircene e β-cariofillene) contro il tonchio del grano *Sitophilus granarius* (L.) (Coleoptera Dryophthoridae). Successivamente, l'attenzione è stata focalizzata sulla caratterizzazione degli ecotipi di luppolo in diverse località dell'Italia centrale (Abruzzo e Molise) al fine di individuarne il migliore in termini di contenuto in polifenoli, associato ad un elevato potere antiossidante.

I terpeni, α-umulene, β-mircene e β-cariofillene sono risultati i componenti principali (77,6%) dell'OE.

Tra tutti i composti chimici e gli estratti testati, l'OE è stato in grado di esercitare la massima tossicità di contatto con valori di LD_{50} e LD_{90} di 13,30 e 40,23 µg/adulto dopo 24 ore dall'applicazione, diminuendo a 11,77 e 36,80 µg/adulto dopo 48 ore, rispettivamente. Tra gli estratti in solvente il più attivo è stato

quello in acetone, mentre tra i composti la maggiore tossicità da contatto è stata riscontrata per l'α-umulene.

Inoltre, l'α-humulene ha mostrato il miglior indice di repellenza (IR) (-38,89) contro gli adulti di *S. granarius*.

Per quanto riguarda la tossicità per inalazione, la massima attività fumigante è stata osservata per il ß-mircene con valori di LC_{50} e LC_{90} di 72,78 e 116,92 mg/L in assenza di grano e 115,78 e 171,42 mg/L in presenza di esso.

La massima mortalità per ingestione è stata rilevata nell'estratto in acetone, mentre la maggiore deterrenza per l'estratto in metanolo.

L'attività anticolinesterasica è risultata trascurabile per tutte le sostanze, con la sola eccezione del ß-cariofillene, che ha mostrato un effetto inibitorio dose-dipendente, ad eccezione, invece, tutte le frazioni libere di polifenoli che hanno mostrato una buona attività anticolinesterasica.

Il più alto contenuto in polifenoli è stato trovato nell'ecotipo raccolto nelle "99 Cannelle" (78,28 mg/g di peso secco) associato alla migliore attività anticolinesterasica.

Infine, il contenuto più elevato in alfa e beta acidi è stato registrato nel campione raccolto in "Bussi".

Questo studio indica che il luppolo selvatico potrebbe rappresentare una fonte di sostanze biologicamente attive verso adulti di *S. granarius*, in quanto ha mostrato una buona tossicità per contatto, ingestione e inalazione, da utilizzare anche come repellente a basso costo ed eco-compatibile nella protezione degli alimenti conservati.

Inoltre, i risultati confermano che gli ecotipi di luppolo esaminati mostrano differenze significative nel contenuto in polifenoli, che determinano diverse attività antiossidanti e anti-colinesterasiche. Quest'ultime, in particolare, potrebbero essere responsabili dell'azione insetticida riscontrata precedentemente negli estratti alcolici di alcuni ecotipi. Questi risultati evidenziano, per cui, la necessità di definire la composizione chimica delle varie frazioni al fine di caratterizzare i diversi ecotipi e identificare le molecole biologicamente attive.

Chapter 1~ Introduction

1.1. AROMATIC PLANTS

Figure 1.1. Some aromatic plants

The growing interest of consumers in substances of natural origin in addition to the increasing concern surrounding potentially harmful synthetic additives has resulted in the use of aromatic plants, their extracts and essential oils, as functional ingredients in the pharmaceutical, food and feed industries (Marshall, 2011; Sacchetti *et al.*, 2005).

Medicinal and aromatic plants (*Figure 1.1*) play a significant role in the life of people and are present in innumerable forms. A simplest definition of the medicinal plant would be "*Medicinal plants are those plants which are used in official and various traditional systems of medicines throughout the world*". Other definition could be "*Medicinal plants are plants that provide people with medicines to prevent disease, maintain health or cure ailments*".

Aromatic plants, also known as herbs and spices, have been used in the Middle East since approximately 5000 BC for their preservative and medicinal properties, in addition to enhancing the aroma and flavor of foods (Chang 2000; Piccaglia *et al.*, 1993).

Additionally, feed additives derived from plants, also called phytogenics or phytobiotics or botanicals, can be included in animal diets to improve their

Ph.D. thesis of Laura De Acutis

productivity and the properties of the resulting feed and animal products (Windisch *et al.*, 2009). Among these natural additives, aromatic plants, their extracts and their essential oils have been examined due to their advantages over the antibiotics as growth promoters. They are residue free and generally recognized as safe.

Medicinal and aromatic plant is obtained both from plants growing in the wild and from cultivated stock. Collection of *ecotypes* plays a vital role in the use of, and trade in, medicinal and aromatic plant material in Europe, since cultivation has not proved to be profitable for the majority of taxa in trade. This is because: many aromatic plants are difficult to cultivate; many are required in small quantities; the quality of some wild- harvested material is supposed superior; the cost associated with obtaining plant material from the wild are relatively low (Lange and Schippmann,1997; Lange, 1998, 2002).

1.1.1. THE ECOTYPES: CONCEPT AND DEFINITION

The process by which one species diverges into two distinct phylogenetic lineages has long fascinated biologists.

Thus, biologists have found that dividing speciation into stages is a useful framework for better understanding the entire process (Wallace, 1865; Turesson, 1922a, b; Clausen, 1951; Grant, 1981; Wu, 2001; Nosilet *et al.*, 2009).

In two papers, published in 1922, Turesson (1922 a, b) introduced the *ecotype concept* and its importance in the study of the constitution and origin of species was almost immediately recognized.

The first term used by Turesson (1922a, p. 102) is *ecospecies*, but this is not immediately defined. In the same paper (p. 112) we read: 'The term *ecotype* is proposed here as [an] ecological unit to cover the product arising <u>as a result of the genotypical response of an ecospecies to a particular habitat</u>. The ecotypes are then the ecological sub-units of the ecospecies, while the genotypes are purely Mendelian sub-units of the genospecies.' In the second, longer, paper of the same year (1922b) we find (pp. 244, 245) the following: 'The term ecospecies

Ph.D. thesis of Laura De Acutis

has been proposed (Turesson, 1922) to cover the Linnean species or genotype compounds as they are realized in nature.'

Hanson and Churchill (1961) define ecotype as a cluster of biotypes, within a species, occupying a particular habitat. A biotype, according to this definition, is a population of a species within a given microhabitat belonging to one genotype. For the most part, an ecotype is designated herein by a species, and biotypes by varieties of that species. Each species (ecotype) in this report may contain as many as six varieties (biotypes) that have similar ecological affinities based on repeated co-occurrence in the study area. Subsequently, an ecotype is identified as a single variety because it is presumed to be ecologically distinct from another variety that is also found in the study area. In some instances, a form or even another closely related species is designated as a biotype; the ecological counterpart for form is ecoform (phenotype) and for another closely related species is ecospecies.

During the 1980s and 1990s, there was less discussion in the literature regarding ecotypes and stages in speciation.

Linhart & Grant (1996), who conducted the most comprehensive review of local adaptation in the 1990s, suggested that 'some characters can vary gradually, others discontinuously, depending on, for example, gene flow, intensity of selection, number of genes involved, and terrain configuration' (Linhart and Grant, 1996). It is true that individual traits may vary in different ways but, ecotypes reflect the composite response of multiple traits to the common selection pressures of ecoregions.

Early in the last decade, Wu (2001) presented a 'genic view of the process of speciation' where he reframed speciation as the product of the accumulation of genic incompatibilities across the genome over time by natural selection. Interestingly, Wu presented the process of speciation as occurring over four stages, which reflected the gradual build up of regions of the genome that could no longer introgress between diverging races or species.

1.2   HOP (*Humulus lupulus* L.)
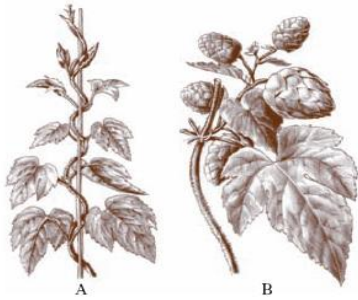
Figure 1.2. Hop plant (A); particular of female cones (B)

Hop (*Humulus lupulus* Linnaeus) (*Figure 1.2*) is a dioecious, hight-climbing and perennial plant belonging to the *Cannabaceae* family of the Urticales order (Roberts and Wilson, 2006). Besides the common hop, the *Humulus* genus includes 2 other species, *Humulus japonicus* Siebold & Zucc and *Humulus yunnanensis* Hu, but only *Humulus lupulus* is of industrial/medical importance (Van Cleemput and others, 2009a).

The centre of origin of the genus is considered to be China, because all *Humulus* species have been found in this area (Neve, 1991; Murakami *et al.*, 2006). *H. japonicuc* is native to Japan, Taiwan and China, while *H. yuannensis* is native to high altitudes of the Yunan Province in China.

H. lupulus, which is almost exclusively cultivated for brewing purposes, was first domesticated in Central Europe in the middle of the ninth century and is currently naturalized throughout the north temperate regions of the world as well as some temperate regions in Australia, South Africa, and South America (Chadwick *et al.,* 2006).

Unlike *H. lupulus L.*, the annual *H. japonicus* Sieb. & Zucc. is a very distinct plant, showing lobed and extremely pubescent leaves. The glands present in the leaves and the cones are smaller than those of *H. lupulus L.* Furthermore, lupulin glands are absent in *H. japonicus* Sieb. & Zucc. (Small, 1978).

Based on geographical locations and leaf morphology the species of *H. lupulus* L. have been classified into five taxonomic varieties: *lupulus* Small for European hops, *cordifolius* Small for Japanese hops, *neomexicanus* Nelson & Cockerell, *pubescens* Small and *lupuloides* Small for hops of North America (Small, 1978).

Ph.D. thesis of Laura De Acutis

1.2.1. TAXONOMY

The common hop (*H. lupulus* L.) is a perennial, climbing, and herbaceous plant which produces new shoots each year in early spring from rhizomes of an underground rootstock. The outgrowing climbing vine grows up to 6-9 m in length and senesces to the perennial rootstock in autumn (Krištín, 1987).

The stems lignify partially, but do not survive winter frosts. The leaves have three to five lobes and have a rough and hairy upper surface and a resinous lower surface (Ghiselli *et al.*, 2015).

As dioecious plant, male and female flowers are on separate plants; however monoecious plants have been found (Verzele and De Keukeleire 1991; Haunold *et al.* 1993). Male plants produce flowers in loose panicles, whereas the female inflorescences develop to a strobile-like structure. Female cones, known as hop cones or hops, are made up of overlapping bracts. The base of each bract is covered with yellow lupulin glands (glandular trichomes). Only female plants are cultivated in hop gardens, while male plants are essential in hop breeding programs for to develop new varieties through controlled hybridization (Neve, 1991).

The cultivated hop is a short-day plant that initiates flowering when it is at a critical size (about 6 m, 20-24 nodes) and critical daylength is about 16 h, beyond which flowering cannot be induced.

H. lupulus L. is a diploid (2n = 20) species with heteromorphic sex chromosomes. The chromosome system for hop plants of European origin is XX for females and XY for males. In *H. japonicus* Sieb. & Zucc. the chromosome system for females plants is 2n = 14+XX and for males is 2n = 14+XY1Y2 (Grabowska-Joachimiak *et al.* 2006).

1.2.2. PHARMACOGNOSY OF HOPS

The resinous inflorescences of hop are used today primarily for their bitter and aromatic properties in the production of beer (Chadwick *et al.*, 2005). In addition, the use of hops as a medicinal plant has more than 2000 y of history (Koetter and Biendl, 2010; Bocquet *et al.*, 2018). The ancient healers used hops

against leprosy, foot odor, constipation, and for blood purification (Karabin *et al.,* 2015). The earliest writings that mention hops date back to the 11th century, when the Arabic medicus Mesue described their anti-inflammatory effects. Later, in 1158, the German abbess and botanist, Hildegard von Bingen, indirectly confirmed the antimicrobial properties of hop when she recommende. In the U.S. hop preparation are used for the treatment of anxiety and insomnia, and are sold in pharmacies in Europe (Anon., 2002) and formerly in pharmacies in the U.S. (Anon., 1946), for these purposes.

To volatile oils is attributed sedative action; alpha and beta-acids, and their derivatives, are recognized properties antibiotic and bacteriostatic (thus favoring the preservation of beer), while the flavonoids of hops possess hormonal properties.

Hops have been shown to contain one of the most potent *in vitro* estrogenic substance known from the plant kingdom, (±)-8-prenylnaringenin (Kitaoka *et al.*, 1998; Milligan *et al.*, 1999).

Xanthohumol (XN) is one of the bioactive substances contributing to its medical applications. Among foodstuffs XN is found primarily in beer and its natural occurrence is surveyed. In recent years, XN has received much attention for its biological effects (Zanoli and Zavatti, 2008), among which are counted:

- Effect on Metabolic Syndrome and Related Disorders;
- Anti-Obesity Activities;
- Hypoglycemic Activities;
- Anti-Hyperlipidemia Activities;
- Cancer Related Bioactivities;
- Cancer Chemo-Preventive Effect (Zanoli and Zavatti, 2008).

Besides these properties, anti-proliferative, anti-oxidative, anti-mycotic, anti-bacterial, and estrogenic effects have been reported in numerous studies (Stevens and Page, 2004; Chadwick *et al.*, 2006; Zanoli and Zavatti, 2008). Therefore, hop was named as "Medical plant of the year 2007" by the Study Group for the Historical Development of Medicinal Plant Science at the University of Würzburg in Germany (Biendl, 2008).

In addition, hops are counted for their biological activity that include <u>defence against</u> <u>phytophagous insects</u>.

1.3.   TOXICITY AND MODE OF ACTION OF INSECTICIDES

A complete understanding of the mode of action of insecticides requires knowledge of how it affects a specific target site within an organism. The target site is usually a critical protein or enzyme in insects.

Insecticides can be classified according to their mode of entry into insect: stomach poisons, contact poisons, and fumigants. However, another way insecticides can be classified is by their mode of action. Mode of action of the major chemical classes of insecticides involves mainly three target sites in the nervous system: *acetylcholinesterase*, an enzyme of critical importance in the transmission of nerve impulse (<u>organophosphorus</u> and <u>carbamates</u>), voltage-gated sodium channels across the nerve membrane (<u>pyrethoids</u> and <u>DDT</u>), and the acetylcholine receptor (neonicotinoids) (Rajashekar *et al*., 2016; Yu SJ, 2008).

Selective insecticides such as juvenile hormone mimics (<u>fenoxycarb</u> and <u>pyriproxyfen</u>), ecdysone agonists and chitin synthesis inhibitors (<u>Diflubenzuron</u>) act on insect specific targets that disrupt reproduction and development (Chandler *et al*., 2011).

Among the insecticides derived from natural sources (*Table 1.1*), *azadirachtin*, from the Indian neem tree, is a feeding deterrent and an insect growth regulator that suppresses fecundity, moulting, pupation and adult emergence (Morgan DE, 2009). Compounds that selectively act on the insect nicotinic *acetylcholine receptor* (neonicotinoids), such as <u>imidacloprid</u>, <u>acetamiprid</u> and <u>thiomethaxam</u> are among the modern insecticides used in pest management (Kaufman *et al*., 2010).

The botanical insecticides that have primarily been used and are commercially available include pyrethrin, rotenone, sabadilla, ryania, nicotine, and azadirachtin (El-Wakeil, 2013).

Plant phenolics are well known to play crucial roles in plant ecology and physiology and play important roles in the prevention of many chronic diseases.

17

More recently polyphenols such as flavonoids have been investigated for their acetylcholinesterase inhibitory activity (Uriarte-Pueyo, 2011).

1.3.1. INHIBITION OF ACETYLCHOLINESTERASE

Acetylcholinesterase (AChE), an enzyme involved in the termination of impulse transmission, is considered an important target for insecticides and acaricides (Casida and Durkin, 2013).

Acetylcholinesterase plays a key role in cholinergic transmission by catalyzing the rapid hydrolysis of the neurotransmitter acetylcholine into acetate and choline. This enzyme terminates the chemical impulse at a rate similar to the controlled diffusion processes, allowing a rapid and repeatable response. Insect AChE is the main target of insecticides used in agriculture.

Organophosphate and carbamate insecticides act by interfering with the synaptic transmission of the nerve impulse in the insect nervous-system. Because of the inability of phosphorylated AChE to hydrolyze acetylcholine, the concentration of acetylcholine in the synapse builds up, and excessive neuroexcitation occurs because of the prolonged binding of acetylcholine to its postsynaptic receptor. The signs of intoxication include restlessness, hyperexcitability, tremors, convulsions, paralysis, and, finally, death.

The mode of action of the carbamate insecticides is similar to that of the organophosphates on the CNS, and the symptoms of intoxication are similar to those with the organophosphates.

Decarbamylation of acetylcholinesterase is rapid, typically in minutes, and, therefore, the carbamate insecticides are regarded as reversible acetylcholinesterase inhibitors (Von Osten *et al.* 2004; Casida and Durkin 2013; Cespedes *et al.* 2013).

Active principle	Plant species	Insect toxicity	Insect species
Anonaine	*Annona reticulata*	Contact	*C. chinensis*
Azadirachtin	*Azadirachta indica*	Contact/IGR	Stored grain pests, Aphids
E-anethole	*Foeniculum vulgare*	Contact	*S. oryzae*, *C. chinensis*
β-Asarone	*Acorus calamus*	Contact	Stored grain pests
Bornyl acetate	*Chamaecyparis obtusa*	Contact	*S. oryzae*
Camphor	*Ocimum kilimandacharicum*	Contact	*S. oryzae*
(+)-3-Carene	*Baccharis salicifolia*	Contact	*T. castaneum*
Caryophyllene oxide	*Origanum vulgare*	Contact/fumigant	*T. castaneum*
1,8 Cineole	*Eucalyptus*	Contact/fumigant	*R. dominica* *T. castaneum*
Cinnamaldehyde	*Cinnamomum aromaticum*	Contact	*T. castaneum, S. zeamais*
p-Cymene (cymol)	*Chenopodium ambrosioides*	Contact	*S. zeamais* *B. germanica*
Decalesides I and II	*Decalepis hamiltonii*	Contact	*Stored grain and household insects granaries*
Eugenol	*Citrus*	Fumigant	*S. oryzae*
Geraniol	*Pelargonium graveolens*	Fumigant	*T. confusum, T. castaneum, S. Oryzae*
Hexadecane	*Chenopodium ambrosioides*	Contact	*T. castaneum, S. granaries granaries*
Linalool	*Ocimum canum* Sims.	Fumigant	*S. oryzae, R. dominica*
Limonene	*Citrus*	Contact	*T. castaneum*
(−)- limonene	*Baccharis salicifolia*	Contact/fumigant	*T. castaneum*
Nicotine	*Nicotiana tabacum*	Contact	Mites, aphids, thrips, leafhopper
Pyrethrins I and II	*Tanacetum cinerariaefolium*	Contact; stomach Poison	Stored grain pests, crop pests
β-Pinene	*Baccharis salicifolia*	Contact	*T. castaneum*

Table 1.1. List of insecticidal active principles of plants

1.3.2. EO$_S$: DEFINITION AND THEIR IMPORTANT PROPERTIES

According to the Encyclopedic Dictionary of Polymers, essential oils (EOs) are defined "*volatile oils or essences derived from vegetation and characterized by distinctive odors and a substantial measure of resistance to hydrolysis*" (Gooch, 2011).

Ph.D. thesis of Laura De Acutis

These compounds can be isolated from distinct anatomic parts of the plants mainly by distillation and pressing.

The definition of an essential oil excludes other aromatic/volatile products obtained by different extractive techniques like extraction with solvents (concretes, absolutes), supercritical fluid extraction, and microwave-assisted extraction. Essential oils also differ from fixed oils or fatty oils in both chemical and physical properties. Fatty oils contain glycerides of fatty acids and leave a permanent stain on filter paper, whereas essential oils contain volatile compounds and vanish rapidly without leaving any stain (Zuzarte, 2015). Essential oils are secondary metabolites synthesized by plants, and they play very important roles in plant defense (both against biotic and abiotic stresses) and signaling processes, including also the attraction of pollinators and benefcial insects (Pavela, 2015; Zuzarte, 2015; Taiz and Zeiger, 2010).

EOs are synthesized by plants both internally (secretory glands allocated inside the plants) as well as externally (secretory glands placed on the plant surface) (Svoboda and Greenaway, 2003) and they are produced by different plant organs such as flowers, herbs, buds, leaves, fruit, twigs, bark, seeds, wood, rhizomes, and roots and can be accumulated in specific histological structures (Asbahani *et al.*, 2015). Essential oils (EOs) are comprised of volatile mono and sesquiterpenoids that have great importance for the pharmaceutical, food and cosmetic industries (Baser and Buchbauer, 2015). Sesquiterpenes contain 15 carbon atoms, and they are less volatile and have a higher boiling point than monoterpenes. As a consequence, fewer of them contribute to the fragrance of EOs (Hüsnü and Buchbauer, 2015).

EOs interfere with basic metabolic, biochemical, physiological, and behavioural functions in insects and have been demonstrated to possess contact, inhalation and ingestion toxicity, antifeedant activity, capacity to delay development, adult emergence and fertility, deterrent effects on oviposition and arrestant and repellent action (Tripathi *et al.*, 2009; Germinara *et al.*, 2017).

EOs of aromatic plants were traditionally used against economically important pests and some of them have provided potential alternatives to currently used insect control agents (Nerio *et al.*, 2010; Isman *et al.*, 2011).

1.4. COMPOSITION OF SECONDARY METABOLITES IN HOP

Hops, belonging to the category of aromatic plants, could be a source of biological active compounds.

Most of the important components of hop are produced in the glandular trichomes found at the base of the bracts (*Figure 1.7-A*), though, some of them are also synthesized in the trichomes on the underside of young leaves (Stevens and Page, 2004). So far more than 1000 compounds have been identified in hop, including *essential oils*, *bitter acids*, *polyphenols* and *prenylflavonoids.* The composition of these compounds is defined by the genotype and has been used for distinguishing different hop varieties (Wang *et al*., 2008). The typical hop composition is illustrated in Table 1.1.
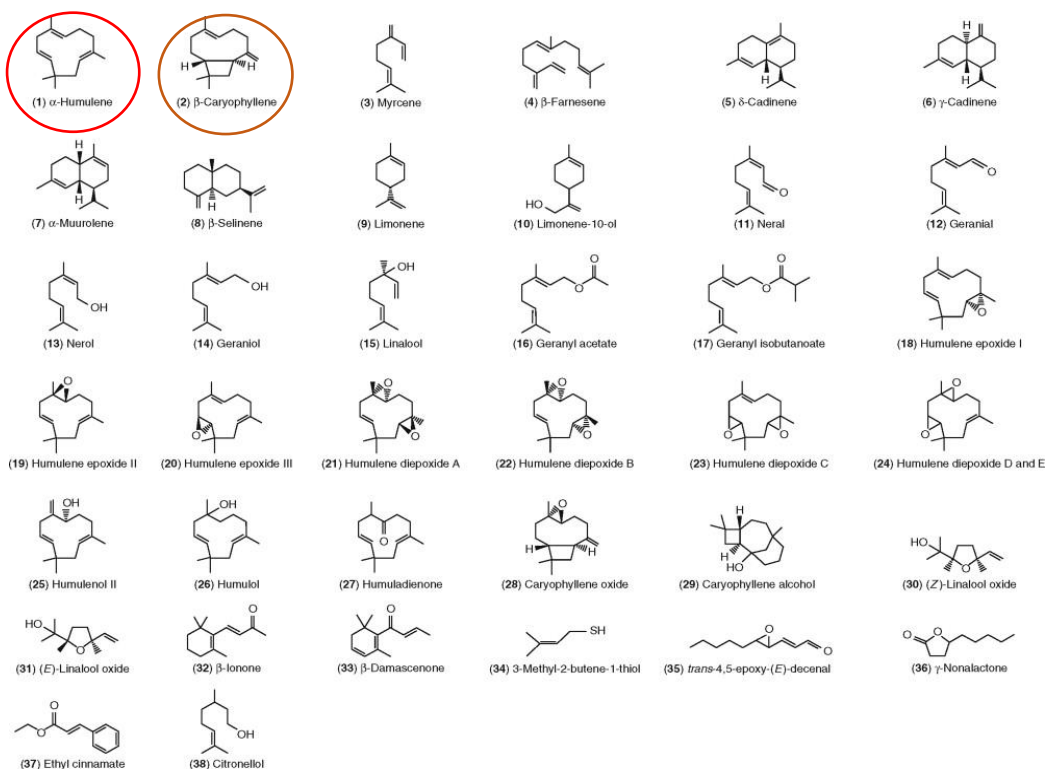
Figure 1.6. Principal compounds of hop EO

Essential oil (EO)

Essential oils represent 0.5% to 3% (v/w) of the weight of the total female cones (Eri *et al*., 2000). The main components of EOs are the *monoterpenes* (β-myrcene) and the *sesquiterpenes* (β-caryophyllene, and β-humulene) (*Figure*

Ph.D. thesis of Laura De Acutis

1.6) (Roberts and Lewis, 2002). The essential oil contributes to the aroma and flavor of beer (Van Cleemput *et al.*, 2009).

<u>Bitter acids</u>

The main hop bitter acids are α- and β-acids, also called *humulones* and *lupulones,* which are present as a mixture of three congeners, co-, n-, and ad-, depending on the side chain in the chemical structure of the molecules.
During brewing, the relatively insoluble α-acids are transformed into their corresponding mixture of beer-soluble diastereomeric pairs of iso-α-acids (cis- and trans-isomers), thus resulting in six iso-α-acids originating from the three main α-acids. The α-acids, particularly humulone (35–70% of total α-acids), cohumulone (20–65%), and adhumulone (10–15%) are regarded as the most important constituents in determining the quality of hops (Verzele and Keukeleire, 1991; Chadwick *et al.*, 2006).

Compounds	% (m/m)
α-acids	2-19
β-acids	2-10
Proteins	15
Ash and salts	10
Cellulose-lignin	40-50
Monosaccharides	2
Oils and fatty acids	1-5
Pectins	2
Polyphenols and tannins	3-6
Ammino acids	0.1
volatile oil	0.4-3.4 (v/m)
Water	8-12

Table 1.2. Typical composition of dried hop

Polyphenols

The dried hop cones contain 4–14% polyphenols, mainly phenolic acids, prenylated chalcones, flavonoids, catechins and proanthocyanidins (Gerhäuser, 2005).
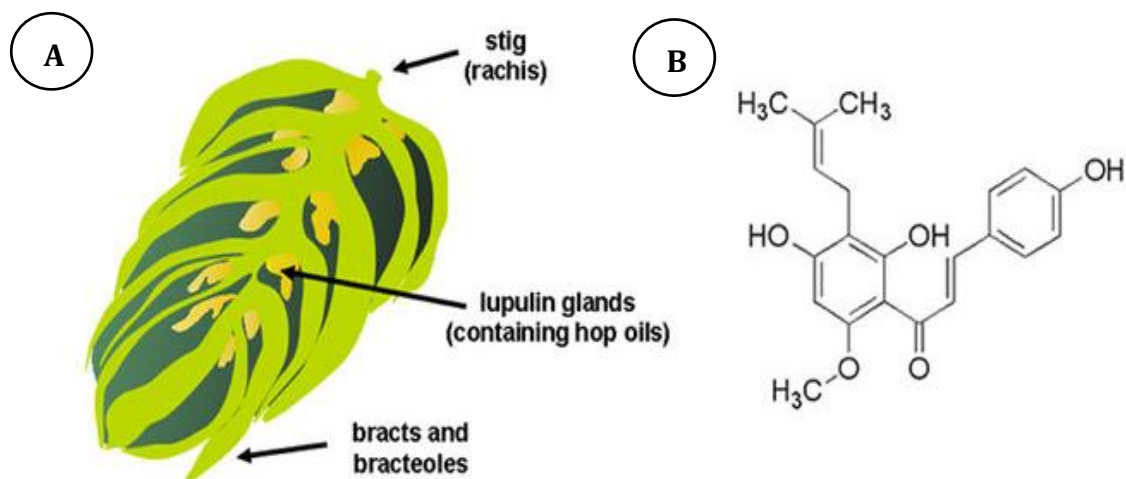
Figure 1.7. Schematic drawing of a female hop cone that is composed of a central spine (strig), bracts (pear-shaped petal that does not contain lupulin glands), bracteoles and the characteristic lupulin glands that are tiny yellow sacs containing the hop oils (**A**); Chemical structure of xanthohumol (3′-[3,3-dimethyl allyl]-2′,4′,4-trihydroxy-6′-methoxychalcone), the major prenylated chalcone of the hop plant (**B**).

The yellow compound (Greek: xantho = yellow) is found in high quantities in the lupulin glands. In fact, the majority of known flavonoids from hops can be considered as derivatives of the compound 2',4,4',6'-tetrahydroxy-3'-prenylchalcone, commonly known as desmethylxanthohumol (DMX) (Chadwick *et al.*, 2006). The most abundant and most important prenylated flavonoid in fresh, and properly preserved hops, is the chalcone xanthohumol (XN) (3′-[3,3-dimethyl allyl]-2′ ,4′ ,4-trihydroxy-6′ -methoxychalcone), present at concentrations of 0.01–0.5% (*Figure 1.7-B*). Since the 1990s, interest in health-promoting activities of XN increased constantly, scientific investigations were initialized worldwide and papers and patents on this topic have increased steadily. Many studies identified XN as a broad-spectrum cancer

Ph.D. thesis of Laura De Acutis

chemopreventive agent acting by multiple mechanisms relevant for cancer development and progression (Gerhauser *et al.*, 2002; Miranda *et al.*, 1999). XN is able to scavenge reactive oxygen species and it modulates many enzymes involved in carcinogen metabolism and detoxification (Gerhauser *et al.*, 2002).

In addition, another prenylated flavonoid of hop, 8-prenylnaringenin (8-PN) was identified as a very potent phytoestrogen (Milligan *et al.*, 1999; Gerhauser and Frank, 2005).

1.4.1. INSECTICIDAL EFFECTS OF HOPS

In the literature, insecticidal activities of the hop and its principal component xanthohumol against some insect species are reported (Gokce *et al.*, 2006a, b, c, 2007, 2012; Eri *et al.*, 2009; Yanar *et al.*, 2011; Cam *et al.*, 2012; Karakoc and Gokce, 2012; Karaca and Gokce, 2014; Bedini *et al.*, 2016; Jackowski *et al.*, 2015; Stompor *et al.*, 2016).

In particular, spent hops has been evaluated as a source of EO and chemicals with repellent activity against *Rhyzopherta dominica* (F.) and *Sitophilus granarius* (L.) (Bedini *et al.*, 2016a).

Contact and ingestion toxicities of *H. lupulus* extracts were examined against *Thaumetopoea solitaria* Frey (Er *et al.*, 2008).

Hop beta acids (HBA) (lupulones) have been mentioned for their effects on mortality of *Varroa destructor* Anderson and Trueman (DeGrandi-Hoffman *et al.,* 2012).

HBA also can reduce two-spotted spider mite oviposition and reduce the survival of adults (*Tetranychus urticae* Koch) (Jones *et al.*, 1996) and hop aphid (*Phorodon humili* Schrank) (Hampton *et al.*, 2002).

Insecticidal effects of the dichloromethane, ethyl acetate, acetone, ethanol and methanol extracts of *Humulus lupulus* L. cones and its principal component, xanthohumol were demonstrated on five stored pests, *Sitophilus granarius* (L.), *Sitophilus oryzae* (L.), *Acanthoscelides obtectus* (Say.), *Tribolium castaneum* (Herbst) and *Lasioderma serricorne* (F.) (Aydin *et al.*, 2017).

Insecticidal properties against the larvae and adults of Colorado potato beetle (*Leptinotarsa decemlineata* L.) (Gokce *et al.*, 2012), as well as an antifeedant action against the adults of the same species for hop extracts was also reported (Alkan *et al.*, 2015).

1.5. INSECTS

1.5.1. COLEOPTERA

The beetles are at once absolutely typical of, and unique among, the Insecta, a paradox of a kind which, thought familiar to any practising systematist, is a costant stumbling block to laboratory experimentalist of the moder school.

The beetles, neverthless, form an isolated and well-characterised taxon, corrently recognised and named by Aristotele as far back as the fourth century (Wentworth Thompson, 1910).

The order Coleoptera, or beetles, is represented by some 350.000 known species (Lawrence *et al.*, 1999), but recent estimates suggest there are hundreds of thousands or even millions of undescribed species.

The name "Coleoptera" derive from Greek, *koleon*, that means "sheath" and *pteron* "wing"; Aristotle already called beetles "koleopteros" (κολεοπτερος) to refer to the hardened front wings protecting the membranous hind wings. English beetle means "the little biter", derived from Old English britain, "to bite". Coleoptera, order also commonly called beetles, weevils or fireflies, comprise 25% of all described animals and plants, and represent the primary contributor to Earth's biodiversity.

Beetles are *holometabolous* insects and their life stages are: *egg – larvae – pupa – adult*.

Egg. The eggs develop in the ovaries of the female and are laid in a sheltered place where the young will have a food supply and favorable conditions for development. Eggs may be laid singly or in masses; hatching usually occurs after several days. The female nearly always goes on her way after egg-laying and leaves the young to care for themselves.

Larvaae. Beetle larvae which inhabit a similarly wide range of situations, but usually occupy different niches from the adults, are more difficult to
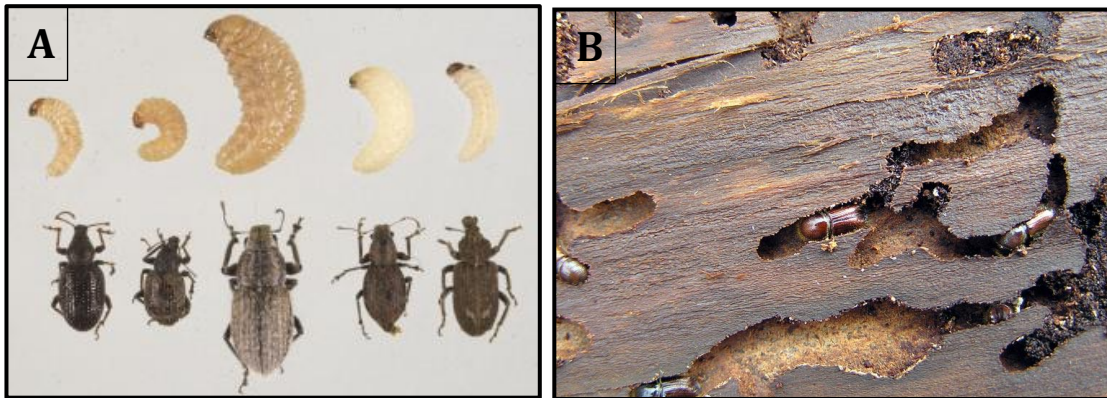
Figure 1.3. A Weevil larvae and corresponding adult (left to right) apple weevil, garden weevil, whitefringed weevil, Fuller's rose weevil, (vegetable weevil). **B**: Bark beetle (*Ips typographus*, L.).

recognise. All have a well-developed head with biting mouthparts, and frequently the maxillary galea and lacinia are partly or completely fused. They lack silk glands and crotchet-bearing prolegs characteristic of lepidopterous

Pupa. The pupa is called the "resting stage". The pupa of most species looks like a pale, mummified adult beetle. In a few species the pupa is covered by a cocoon made by the last instar larva. Beetle pupae are of the exarate type, that is, the appendage free and visible and do not cling to the body. Although the pupa is capable of doing little more than wriggling its abdomen, great changes are taking place internally, for larval tissues are breaking down to form adult structures.

Adult beetles. The adults (*Figure 1.3-A*) may be recognised by the *elytra*, hardened forewings which protect the membranous hind-wings, and when closed, cover a cavity over the posterior thorax and abdomen. The spiracles open into this cavity, an adaptation which reduces water loss during respiration, and protects the abdomen from insolation and desiccation. Elytra differ from hardened fore-wings of some other insects (cockroaches, bugs etc.) in being usually convex, embracing the abdomen laterally, and meeting dorsally in a straight longitudinal line (suture) when closed. The striking evolutionary success of beetles is almost certainly related to the effective protection afforded

Ph.D. thesis of Laura De Acutis

by the elytra in the open and in almost every conceivable terrestrial and freshwater habitat.

caterpillars. Most beetle larvae have legs; in the suborder Polyphaga the last two segments are fused as a tarsungulus. Weevil and many longhorn beetle larvae are legless, and some others have reduced legs. The body is usually subcylindrical but may be flattened (especially in subcortical larvae), or C-shaped at rest (scarabs and weevils).

Many beetles are serious pests: bark beetles (*Figure 1.3-B*) can cause great harm to trees, some other species can infest foodstuffs whilst chafers and leaf beetles can cause serious harm to crops. However, many beetles are considered useful insects and play an important role as nutrient recyclers returning organic matter through multitrophic interactions, which contribute to soil fertility. This great diversity and ability to adapt, along with being a key component in the workings of the biosphere, make beetles one of the most abundant and successful of all insects.

1.5.2. CLASSIFICATION OF BEETLES

They are subdivided into four suborders: *Adephaga*, *Archostemata*, *Myxophaga*, and *Polyphaga*.

Adephaga is a suborder of highly specialized beetles and the second-largest suborder of the order Coleoptera. Members of this suborder are adephagans, a term which notably include ground beetles, tiger beetles, predacious diving beetles, and whirligig beetles. The majority of the species belongs to the family of *carabids,* or ground beetles (Carabidae).

Archostemata is the smallest <u>suborder</u> of Coleopetera, that consisting of fewer than 50 known species organised into five families. They are an ancient lineage with a number of primitive characteristics. Their morphology is to the first beetles, which appear in the fossil record about 250 million years ago.

Myxophaga is the second smallest suborder of the Coleoptera after Archostemata, consisting of roughly 65 species of small to minute beetles in four families. The members of this suborder are aquatic and semiaquatic, and feed on algae (Kirejtshuk and Poinar, 2006).

The major families of the beetles are:

Curculionidae. They are one of the largest animal families. They include the bark beetles as subfamily Scolytinae.

The family also includes the ambrosia beetles, of which the present day subfamily Platypodinae was formerly considered the distinct family Platypodidae.

Dryophthoridae. It is a family of beetles belonging to the *Curculionoidea* superfamily. Some insects belonging: *Cosmopolites sordidus*, banana root borer, *Sitophilus granarius*, granary weevil, *Sitophilus oryzae*, rice weevil, *Rhynchophorus ferrugineus,* red palm weevil.

Staphylinidae (rove beetles); scavengers and herbivores; elytra are characteristically shorter than the abdomen.

Carabidae (ground beetles), predators; many beneficial species including the fiery hunter, *Calosoma calidum*.

Chrysomelidae (leaf beetles), herbivores; includes many pests of agricultural crops. Most species have distinctive shapes or color patterns (e.g., Colorado potato beetle, *Leptinotarsa decemlineata*).

Scarabaeidae (lamellicorn beetles, June beetles, scarab beetles), herbivores; robust beetles with heavy spines on femur and tibia. Distinctive lamellate antennae. Usually live in the soil as larvae and feed on plant roots. Includes many pest species, including the Japanese beetle, *Popillia japonica*.

Tenebrionidae (darkling beetles), herbivores; found in flowers, rotting wood, and occasionally as pests of stored grain. Most abundant in arid climates.

Cerambycidae (longhorned beetles), herbivores; all larvae are wood borers. Adults have distinctively long antennae. A few species are pests of wood and wood products.

Elateridae (click beetles), herbivores; larvae are known as wireworms. Some species feed destructively on the roots of crop plants. When adults are turned on their back, they can snap the head and abdomen against the substrate to right themselves.

Buprestidae (metallic wood borers), herbivores; larvae are known as flat-headed wood borers. Some species are forestry pests.

Coccinellidae (lady beetles), most adults and larvae are predators of aphids and scale insects, but a few species are pests of agricultural crops (e.g., Mexican bean beetle, *Epilachna varivestis*).

Cicindellidae (tiger beetles), predators.

Dytiscidae (predaceous diving beetles), large aquatic predators.

Gyrinidae (whirligig beetles), aquatic predators.

Hydrophilidae (water scavenger beetles), scavengers and predators.

Silphidae (carrion beetles), scavengers.

Lampryidae (fireflies), herbivores.

Dermestidae (carpet beetles), scavengers and herbivores.

Nitidulidae (sap beetles), scavengers and herbivores.

Meloidae (blister beetles), larval parasites, adult herbivores.

Passalidae (wood-boring beetles), herbivores.

1.5.3. *DRYOPHTHORIDAE* FAMILY

Weevil, also called *snout beetle*, true weevil of the insect order Coleoptera (beetles and weevils), are the largest family of animals and organism group in general.

These insects can be easily recognised by the long rostrum or snout, an elongated part of the front portion of the head. Quite often *rhynch-* (meaning nose) is part of the species or generic name. The *snout* is sometimes mistaken as the piercing-sucking proboscis, however, the tip of the rostrum bears tiny little chewing mouthparts. The rostrum can be of considerable length, sometimes as long as the body of the weevil and is housed in an abdominal groove during rest. The antennae are usually clubbed and mostly 'elbowed'. The weevils' general body form is variable, some species are quite long and slender, whereas others are small, stout and nearly spherical. The legs can be quite long and well developed for running and can be retracted in many species against the very hard body. Upon disturbance weevils usually drop and feign death. Most Curculionidae larvae are cryptic miners or borers that feed within plant tissue. Therefore weevils are severe pests of agricultural and forestry crops and of stored products (as well as *Sitophilus granarius* L.). There is hardly any plant that cannot be

29

infested by at least one weevil species. These beetles in general are difficult to control due to their cryptic way of life, their tough and sclerotized cuticle and the long life span of the adults. However, there is also a number of good bugs that are in use as biocontrol agents, like the introduced *Cyrtobagous salviniae*, that is used to control the noxious water fern Salvinia molesta on the middle and lower Sepik. This family includes also some extremely destructive pests (the granary weevil (*Figure 1.4-A*) *Sitophilus granarius,* the rice weevil (*Figure 1.4-B*) *Sitophilus orizae,* and the maize weevil (*Figure 1.4-C*) (*Sitophilus zeamais*).
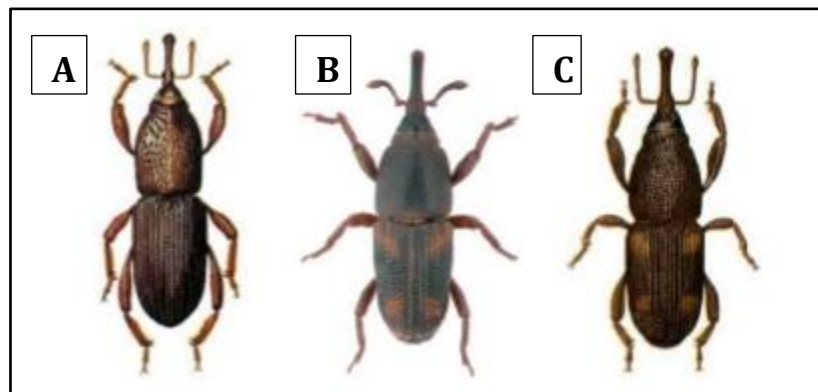
Figure 1.4. *Sitophilus granarius* (**A**); *Sitophilus orizae* (**B**); *Sitophilus zeamais* (**C**).

1.5.4. THE GRANARY WEEVIL: *SITOPHILUS GRANARIUS*

The granary weevil is a small black-brown weevil. Its size, which depends primarily on its food supply and the size of the developmental grain (Surtees, 1965), varies between 3.8 and 5.1 mm (including the rostrum).

The granary weevil, *Sitophilus granarius* (L.) (Coleoptera Dryophthoridae), is one of the most widespread and is found throughout the temperate regions of the world and in cool upland areas of the tropics. It is a pest of stored maize and a variety of stored products. Control of this insect population around the world is primarily dependent upon insecticides and fumigants, which resulted in undesirable effects on non-target organisms, fostered environment and human health concern (Champ and Dyte, 1976; White and Leesch, 1995).

Synonyms*: Calandra granaria* (Fabricius) Gistl, 1848; *Curculio contractus* Geoffroy, 1785; *Curculio granarius* Linnaeus, 1758.

Ph.D. thesis of Laura De Acutis

The developmental stages of *S. granaries* are*: Eggs, Larvae and Pupae (*Figure 1.5-A*).

Larvae are white and apodous and there are four larval instars. The general appearance of the larva and pupa is similar to that of *S. zeamais* and *S. oryzae*.

Adult. Adults of *S. granarius* can vary considerably and are between 2.5-5.0 mm in length, but 3 to 4 mm is usual. They have a characteristic rostrum and the *antennae* have eight segments and are often carried in an extended position when the insect is walking. The body is sparsely covered with short, stout yellow hairs. The head is prolonged into a slender snout. Sexual dimorphism is evident in the dorsal surface, which is more tightly and strongly perforated in males than in females. The prothorax has distinctly oval punctures. Adults do not have wings and cannot fly.

The life spa of the granary beetle is 7-8 months generally. Females usually lay around 150-300 eggs, throughout their lives. Eggs are laid individually in cavities that the female makes in the grain kernels. Cavities are sealed by a waxy plug, which the female secretes. Eggs incubate for about 4-14 days before hatching, depending on temperature and humidity. One larva develops in each infested kernel.

There are four larval stages tha all develop in the grain and also pupation occurs inside the grain. The adult emerged chews its way out of the grain, leaving a characteristic rectangular exit hole (*Figure 1.5.-B*).
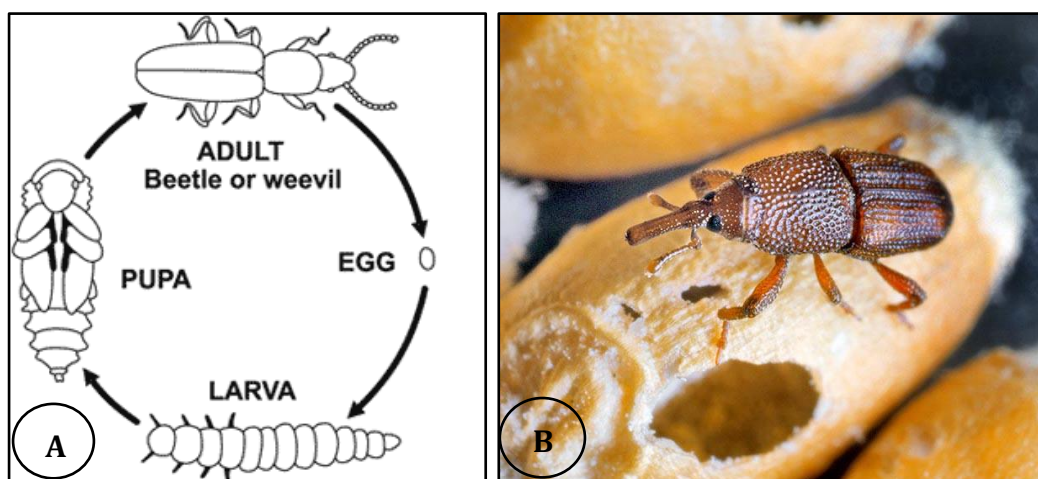

Figure 1.5. Life cycle of *Sitophilus granarius* (**A**); weevil adult emerged out of the grain leaving a characteristics hole (**B**)

Having left the kernel the female releases a sex pheromone to attract males for mating. In warm conditions the life cycle can be completed in 4-6 weeks, but this can up to 21 weeks in the winter. Adults can survive for a month or more without food if there are cooler conditions. This species is flightless but can walk fairly long distances and can be dispersed further afield in infested grain.

1.5.5. REGION OF ORIGIN AND DIFFUSION OF *SITOPHILUS* SPECIES

Due to their economic impact worldwide as storedproduct pests, as well as to that fact that they are an easily bred object for study, the written records are exceptionally numerous for at least those *Sitophilus* species that cause damage to stored grains (Plarre, 2010). The granary weevil primarily causes economic damage in the Mediterranean area, in Middle Europe, Asia, North America while the rice and maize weevils are often found in the warm and humid lowlands and tropical areas.

The diffusion of *S. linearis* is related to its appearance in its host and forage plant, the tamarind.

As far as is known in the breeding biology, all species of *Sitophilus* develop from egg to pupa inside the kernel of their host plants. The female deposits the eggs with her ovipositor in an oblong hole in the kernel, which she prebores with her mouthparts. Then the egg cavity is sealed with a clear and sticky secretion. For *S. linearis* and *S. glandium*, it is known that the host can hold multiple eggs (Cotton, 1920; Kaushal *et al.*, 1993). With only a few exceptions, for the stored grain pest species *S. granarius*, *S. oryzae* and *S. zeamais*, each kernel only hosts one egg. During the larval period of the beetle, the kernel is completely hollowed out and later the adults remains for a while inside the wheat kernel and then comes out. While both the rice and maize weevils have been observed feeding on stored grain, as well as on ripe fruit and mature grain in field (Taylor, 1971).

It is possible to hypothesize that the extant *Sitophilus* species can be considered to be endemic to the forested areas of the Oriental Region. The stored-grain pest species *S. granarius*, *S. oryzae*, and *S. zeamais*, whose worldwide propagation is the result of the global grain trade, and *S. linearis*, whose pantropic incidence can

also be traced to the human trading of its host plant, are with great likelihood also of Oriental origin (Buckland, 1981; Weidner, 1983).

1.5.6. PREVENTION AND CONTROL OF *S. GRANARIUS*

Weevils are classified in the most important primary pests of stored wheat, whose adults damage grains, and larvae inhabit and feed inside the grain (Rees, 2004; Beckett *et al.*, 2007). As chemical measures for pest insect control in storages, fumigants and contact insecticides are stil being used (Kljajić, 2008) as an essential part of integrated pest management. Among fumigants, methyl-bromide (in use until 2015) and phosphin are used, and among contact insecticides organophosphorus compounds (dichlorvos, malathion, chlorpyrifos-methyl and pirimifos-methyl) and pyrethroids (deltamethrin, bioresmetrin and cyfluthrin) are used (Marijana *et al.,* 2011).

However, their indiscriminate use has raised long-term human health and environmental concerns, mainly due to their slow degradation in the environment and toxic residues in the products, and the development of resistance to pesticides in pest populations (Isman, 2006). Resistance to lindan, malathion, dichlorvos, fenitrothion, chlorpyrifos-methyl, pirimifos-methyl, deltamethrin and cypermethrin was found in *S. granarius*, in *S. oryzae* to na lindan, malathion, DDT, fenitrothion, pirimifos-methyl and deltamethrin, and in *S. zeamais* to lindan, malathion, DDT, fenitrothion, pirimifos-methyl, permethrin, deltamethrin and cypermethrin (Kljajić and Perić, 2005; Busvine, 1980). For these reasons, the development of alternative strategies to synthetic chemicals is very important in pest control.

The practice of using plant derivatives, or botanical insecticides as we now know them, in agriculture dates back at least two millennia in ancient China, Egypt, Greece, and India (Isman, 2006).

It's also important to take preventive and fight techniques against the granary weevil. The rooms used as storage must be perfectly impenetrable by insects and a good storage hygiene, play an important role in limiting infestation by *S. granarius*.

In addition, there are known a number of natural enemies capable of predating or parasiting *S. granarius*, for example *Bacillus thuringiensis thuringiensis* Kurstaki, *Lonchaea corticis* Taylor, *Cephalonomia tarsalis* Ashmead, *Acaropsellina docta* Zaher, *Anisopteromalus calandrae* Howard, and many others.

Aim of research activity carried out here has been to add some knowledge on wild hop, by investigating two different aspects:

1. **biological activity of wild hop germoplasm** collected in Molise (Bojano), during the flowering stage of the female cones, was evaluated through bioassays developed in the Entomology laboratory of the Department of Agricultural, Environmental and Food Sciences at the University of Molise. Extracts of dried hop cones obtained by maceration with different solvents (hexane, methanol, acetone) and hydrodistillation were carried out and biological activity of extracts, EO and its main compounds was evaluated.

   These biological activities were observed by several bioassays to determine contact and fumigant toxicities, repellent, antifeedant and nutritional effects against insect. As insect test it was used the granary weevil, *S. granarius*, one of the most damaging pest of stored cereals worldwide that causes major quantitative and qualitative losses by its feeding activity and excretory products.

2. Subsequently, in collaboration with the Department of Agricultural Production and Environment Sciences of the University of Firenze, the attention was focused on the **characterization of hop ecotypes** collected in September 2017 in different locations of Central Italy (Abruzzo, Molise) in order to identify the best one.

   Following a first study on chemical composition of spontaneous hop in Northern Italy (Mongelli *et al.*, 2015), in which only one characterization of accessions from Central Italy (Ghiselli *et al.*, 2015) was present, here 7 ecotypes were investigated for their content in α and β-acids, as well as the polyphenolic fraction and its inhibitory effect of these compounds on acetylcholinesterase (AChE) activity.

Ph.D. thesis of Laura De Acutis

Chapter 3~Materials and methods

3.1. EVALUATION OF BIOLOGICAL ACTIVITY

3.1.1. PLANT MATERIALS

Aerial parts of plants (hop cones) (*Figure 3.1-A*) were collected during the flowering stage in Bojano (Molise region, Italy) (**N** 41°47'840" **E** 14°49'428") and from the same plants were taken the rhizomes planted in the garden of University of Molise (Campobasso, Italy). The dry flowers (*Figure 3.1-B*) were obtained after oven drying at 35 °C for 72 h.

Bojano is located at 482 m a.s.l. at the northern edge of the Matese massif. The area has an average annual rainfall of 700 mm, and mean annual temperature of 14-15 °C.

Figure 3.1. Hop plant collected in Bojano (**A**), dried female flowers (**B**)

The soil where hop plants were harvested, is presented with a neutral reaction, **pH**: 7.25, sandy texture, **fine sand**: 54%, **coarse sand**: 23%, low **organic carbon content**, 10.7 g/Kg. There is also a low **C/N**, 5.9, so there is a deficiency of available nitrogen for the plant. It is also a strongly calcareous soil, $\mathbf{C_aCO_3}$: 37.26%, with a very low content of available phosphorus, $\mathbf{P_2O_5}$: 5.14 mg/kg.

3.1.2. PLANT EXTRACTS

The <u>extracts</u> were prepared by solvents with different polarity (methanol, acetone and hexane) and by hydro-distillation in a Clevenger-type apparatus (*Figure 3.3-B*) for 3 h and stored in a refrigerator until use.

The extracts were prepared first drying samples of collected hop at 35 °C for 72 h before grinding with an electric grinder. Crude extracts were obtained by extractions with solvents dissolving 50 g of powder obtained from dried flowers (*Figure 3.2-A*) in 250 mL of methanol, acetone and hexane (*Figure 3.2-B*). A manual agitation was done for 10 min. Then, were filtered the mixtures. These three filtrates were subsequently concentrated by rotary evaporator.
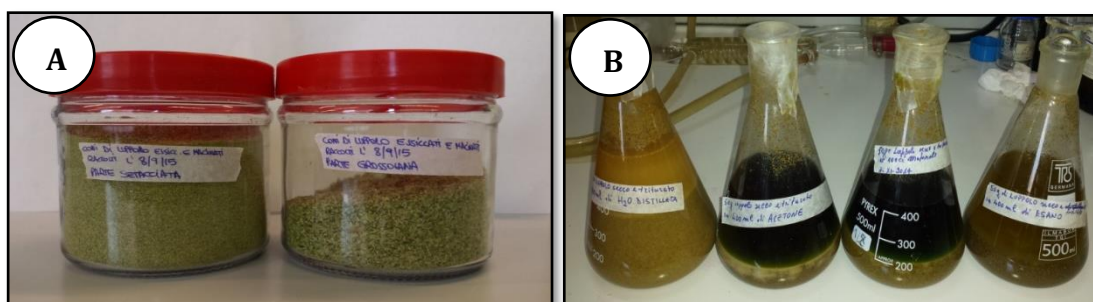
Figure 3.2. Hop flowers dried and ground (**A**); different extracts (**B**).

About <u>essential oil extraction</u>, samples of hop essential oil were isolated by hydrodistillation for 3 h using a Clevenger-type apparatus, according to the method recommended in the current European Pharmacopoeia (2010).

Shortly, 50 g of ground hop cones were weighed and dissolved with a 700 ml of distillated water into a distillation glass. The distillation time was 3.5 hours since the distillation begins. At the end of extraction the obtained essential oil was collected and measured. The oil (*Figure 3.3-A*) was stored at 4 °C in the dark until it was tested and analyzed. The EO density of wild hop was **0.875 g/L**.

Ph.D. thesis of Laura De Acutis
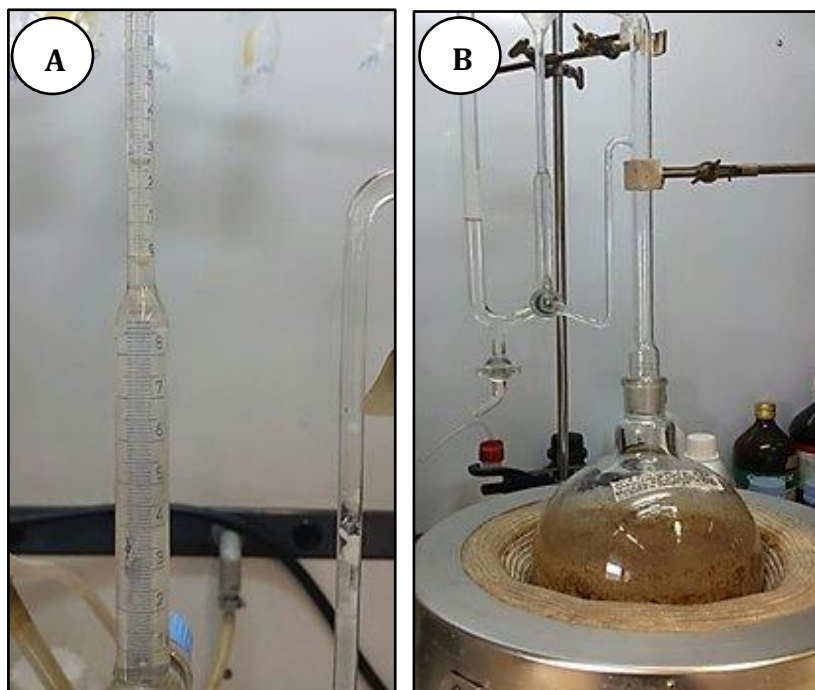
Figure 3.3. EO extracted in condenser (**A**); Essential oil extraction by Clevenger- type apparatus (**B**).

3.1.3. COMPOUNDS TESTED

Myrcene, α-humulene, β-caryophyllene were purchased from Sigma-Aldrich (Italy). In details, myrcene with a purity ≥ 90%; β-caryophyllene with a purity ≥ 80%; α-humulene with a purity ≥ 96%.

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3.1.4. GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

The oil was diluted 1:100 with dichloromethane-hexane (2:3) and a 2 μL sample was injected in the gas chromatographic system. A 6890N series gas chromatograph (Agilent Technologies) with an Agilent 5973 mass selective detector (MSD) and equipped with a HP-INNOWAX capillary column (60 m × 0.25 mm I.D, 0.25 μm film thickness, J&W Scientific Inc., Folsom, USA) was used. The carrier gas was helium at a flow rate of 1.0 mL/min.

The injection was made in the splitless mode, the injector temperature was 250 °C. The column oven temperature was initially held at 40 °C, then it was

programmed to 230 °C at 2.5 °C/min, with a final holding time of 20 min. Spectra were recorded in the electron impact mode (ionization energy, 70eV) in a range of 30-500 amu at 3.2 scans/s. A solvent delay time of 10 min was used to avoid overloading the mass spectrometer with solvent.

The identification of the volatile compounds was achieved by comparing mass spectra with those of the data system library (NIST 98, P > 90%) and retention indexes with published data. Component relative percentages were calculated based on GC peak areas.

3.1.5. INSECTS REARING

Sitophilus granarius adults (*Figure 3.4*) were reared in laboratory on wheat grains for several generations in glass cylindrical containers (Ø 15 × 15 cm) closed by metallic net (1 mm) and maintained in the dark at 25 ± 2 °C and 60 ± 5% R.H. Adult beetles, 2-4 weeks old, were used for the experiments.

Figure 3.4. *Sitophilus granarius* adults reared on wheat grain.

3.1.6. CONTACT TOXICITY

The contact toxicity of β-myrcene, α-humulene, β-caryophyllene, hop EO and extracts to granary weevil adults were determined by topical application (*Figure 3.5*).

All of compounds and EO were dissolved in hexane to obtain serial dilutions.

For each dilution an aliquot (0.5 μL) was applied on the pronotum of *S. granarius* adults in thanatosis through a microsyringe (2 μL). Each dilution was assayed on 5 unsexed adults of *S. granarius* and an equal number of individuals was treated with a solvent control. Insects were confined to each Petri dish within covered with plastic net to prevent insects escape, with 5 wheat kernels and maintained under controlled condition (26 ± 2 °C and 60 ± 5% R.H) in the dark. Mortality of adults was observed after 24 and 48 h. The percentage mortalities were transformed to arcsine square-root values for repeated measures analysis of variance (ANOVA). Treatment means were compared and separated by Tukey HSD test. The Lethal dose 50 (LD_{50}) and 90 (LD_{90}) values, the confidence upper and lower limits, regression equations and chisquare ($\chi 2$) values were calculated using probit analysis (Finney, 1971).
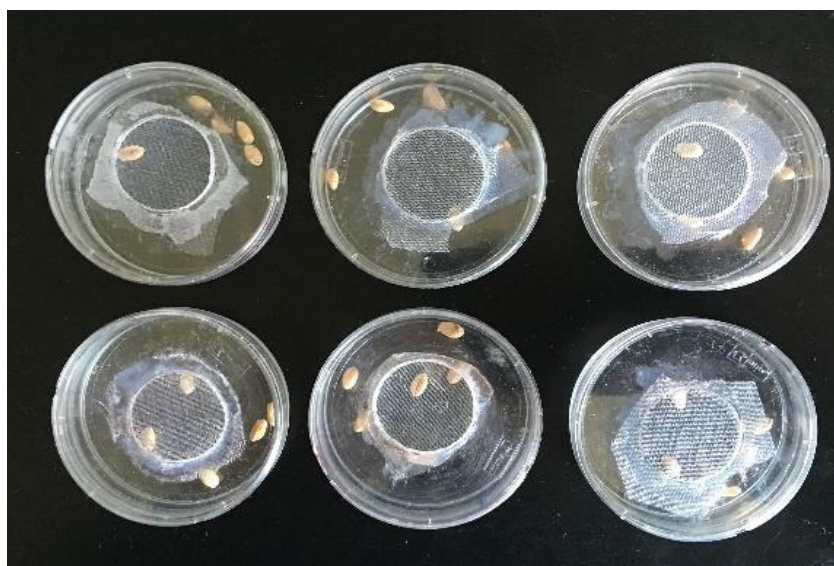
Figure 3.5. Petri dishes containing kernels of grain and *S. granarius* adults treated.

3.1.7. REPELLENCY IN ARENA

Behavioral response of *S. granarius* adults (n. 30) to **α- humulene, ß-myrcene, ß-caryophyllene** and EO, was evaluated by a circular olfactometer solutions to granary weevil adults and their ability to disrupt insect orientation to odors of wheat grains were evaluated in a two-choice pit-fall bioassay similar to that described in previous study (Germinara *et al.*, 2008) (*Figure 3.7*).

The test arena was a steel container (Ø 32 cm × 7 cm height) with two diametrically opposed holes (Ø 3 cm) located 3 cm from the side wall. A filter paper disc (Ø 0.7 cm) was suspended at the center of each hole by a cotton wire taped to the lower surface of the arena.

Glass flasks (500 mL), assigned to collect the responding insects, were positioned under each hole. The inside necks of the collection flasks were coated with mineral oil to prevent insects from returning to the arena. Thirty insects of mixed sex, left for at least 4 h without food, were placed under an inverted Petri dish (Ø 3 cm x 1.2 cm high) at the center of the arena and allowed 30 min to acclimate prior to release. During the assay, the arena was covered with a steel lid to prevent insects from escaping.

Insects were presented with a given dose of EO and compounds (10 μL) adsorbed onto a filter paper disc and solvent control (10 μL) adsorbed onto the opposed paper disc as control. In a second set of experiments, insects were given a choice between the odors emitted by wheat grains (200 g; 14.5% moisture content) left in a collection flask alone or plus a set dose of EO and compouds (10 μL), adsorbed onto the overlying filter paper disc, and control (10 μL) adsorbed onto the opposed paper disc as control.

Tests lasted 3 h and were carried out in the dark at 26±2 °C and 60±5% r.h.

There were five replicates of each assay, and insects were only used once.

In each experiment, a response index (RI) was calculated by using RI = [(T - C)/Tot] × 100, where T is the number responding to the treatment, C is the number responding to the control and Tot is the total number of insects released (Phillips *et al.*, 1993). For each bioassay, the mean numbers of insects in the treatment and control were compared by Student's t-test for paired

41

comparisons. The mean numbers of insects found in the treatment and in the control and the mean RIs at different doses of EO and compounds were subjected to ANOVA and ranked according to Tukey's HSD test.
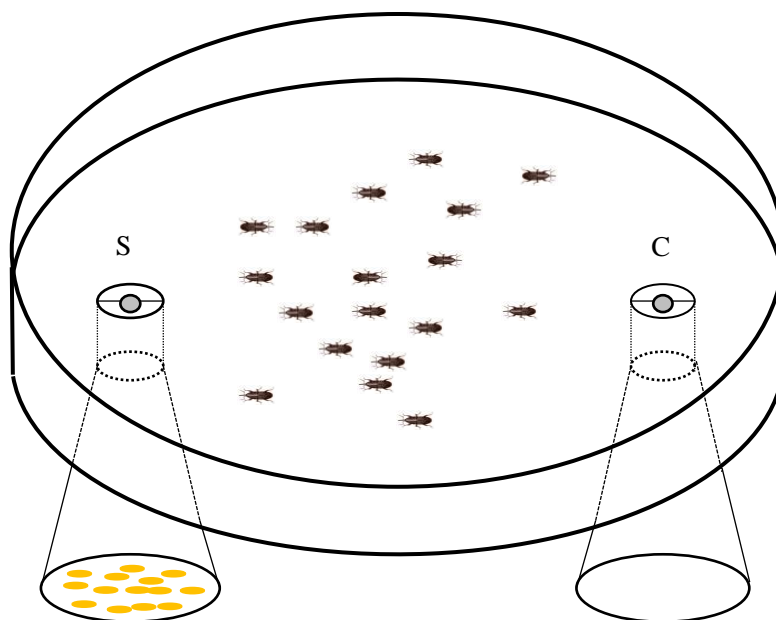
Figure 3.7. Arena bioassay where **S**= Stimulus with wheat grain; **C**= Control.

3.1.8.   FUMIGANT TOXICITY

The fumigant toxicity of myrcene, α-humulene, β-caryophyllene and hop essential oil to granary weevil adults in presence and absence of wheat grains was assessed using the method described in previous studies (Germinara *et al.*, 2007; 2012a).

A glass container (600 mL) (*Figure 3.8*) was used as a fumigation chamber. A filter paper (Whatman No.1) disc (Ø 2.0 cm) was suspended in the centre of the chamber by an iron wire attached to the under surface of its aluminium screw cap. Ten adult insects were placed in the chamber, the paper disc treated with an appropriate volume of the substances tested and the glass container tightly

Ph.D. thesis of Laura De Acutis

closed. In tests with wheat grains, intact kernels (200 g) were placed on the base of the fumigation chamber together with the insects. An untreated paper disc was used as a control. Three replicates of each dose and the control were set up. Bioassays were carried out in the dark at 26 ± 2 °C and 60 ± 5% R.H. for 24 h. Dead insects were counted after exposure to fresh air in Petri dishes after 24h. The percentage mortalities were submitted to two-way ANOVA with substrate presence or absence and dose as the two subjects factors. For each set of experiments, treatment means were separated by Tukey's HSD test.

The LC50 and LC90 values, expressed as mg/L volume, the confidence limit of upper and lower confidence levels, regression equations and χ^2 values were calculated by probit analysis (Finney, 1971).

Figure 3.8. Glass container for fumigant bioassay

3.1.9. ANTIFEEDANT AND NUTRITIONAL EFFECTS

Ingestion test, on which aliquots (200 μL) of a wheat flour and distilled water (10 g in 50 mL) mixture were dropped on a plastic Petri dish to form flour discs (Xie *et al.*, 1996). Discs were dried overnight and then loaded with 5 μL of different extracts solutions or solvent control. Discs were left under hood for 2 h to allow solvent evaporation.

Ph.D. thesis of Laura De Acutis

In a pre-weighed glass vial (Ø 2.5 x 4.0 cm) two flour disks and 10 group-weighed weevil adults were introduced (*Figure 3.9*).

Each vial was then re-weighed and maintained at 26±2°C, 60±5% r.h. for 3 days. The glass vials with flour disks and live insects were weighed again and the number of dead insects recorded. Glass vials containing treated flour disks but without insects were prepared to determine any decrease in weights due to evaporation of acetone and essential oil. For each EO concentration and control 5 replicates were set up.

The following nutritional indices were calculated:

Relative Growth (RG) = (A-B/(B= n. day), when A = growth of living insects at III and V day (mg/n. insect), B = middleweight of insects originally (mg/n. insect);

Relative Consumption (RC) = D/(B for n. day), when D = biomass ingested by living insects at III and V day (mg/n. living insects);

Conversion efficiency of food added (ECI)% = (RG/RC) x 100;

Deterrence food (DF)% = (C-T) x 100/C, dove C = consumption of control discs after 3 and 5 d (Farrar *et a*l., 1989; Huang and Ho, 1998).

Figure 3.9. Ingestion test: vials with *S. granarius* adults and flour disks.

3.1.10. AChE ASSAY

Being the impairment of nervous system function the main mechanism by which plant metabolites toxicity occurs (Casida, 2010; Rattan, 2010), the effect of hop EO and its main components on the AChE, the most conserved mechanism in nervous transmission, was investigated.

AChE activity was detected photometrically (λ=412 nm, 25°C) by means of a Jasco V-570 spectrophotometer (Tokyo, Japan) according to (Ellman *et al.* 1961) by using 5,5'dithio bis(2-nitrobenzoic) acid (DTNB). Briefly, about 0.01 EU of enzyme (from *Electrophorus electricus*, SIGMA) were incubated in phosphate buffer (0.1 M, pH 8.00) plus DTNB (0.2 mM) either in the absence or in the presence of different aliquots of either EO or pure compounds. Reaction was started by the addition of saturating concentration (2.5 mM) of acetylthiocholine iodide and the rate of absorbance change was obtained as tangent to the initial part of the progress curve. Results were expressed as % of the control (reaction rate measured in the absence of plant EO/pure compounds).

Data were submitted to ANOVA followed by Tukey's HSD test for mean comparisons.

3.2.   CHARACTERISATION OF HOP ECOTYPES

3.2.1. PLANT ECOTYPES

Aerial parts of wild hops were collected (*Figure 3.10*) during the flowering stage in the areas of Bojano (Campobasso province, 480 m asl), 99 Cannelle (L'Aquila province, 700 m asl), Bussi 1 and 2 (Pescara province, 344 m asl), Onna (L'Aquila province, 571 m asl) and Roio (L'Aquila province, 714 m asl) in September 2017 (*Table 3.1*). The dry flower heads were obtained after oven drying at 35 °C for 72 h.

Ph.D. thesis of Laura De Acutis

ORIGINS	REGION	ALTITUDE (M a.s.l.)	COORDINATES	DATE OF HARVEST
1-1A Bojano (CB)	MOLISE	482	**N** 41°47'840" **E** 14°49'428"	26th September 2017
2-1C Bojano (CB)	MOLISE	482	**N** 41°47'840" **E** 14°49'428"	26th September 2017
3-Onna (AQ)	ABRUZZO	581	N 42°19'517'' E 13°28'441''	22th September 2017
4-AQ Roio (AQ)	ABRUZZO	810	N 42°20'469'' E 13°23'203''	22th September 2017
5-99 cannelle sx (AQ)	ABRUZZO	721	N 42°20'528'' E 13°23'253''	22th September 2017
6-Bussi (PE)	ABRUZZO	344	N 42°11'291'' E 13°50'590''	22th September 2017
7-Bussi near river (PE)	ABRUZZO	344	N 42°14'206'' E 13°48'506''	22th September 2017

Table 3.1. Different locations where hop ecotypes are collected
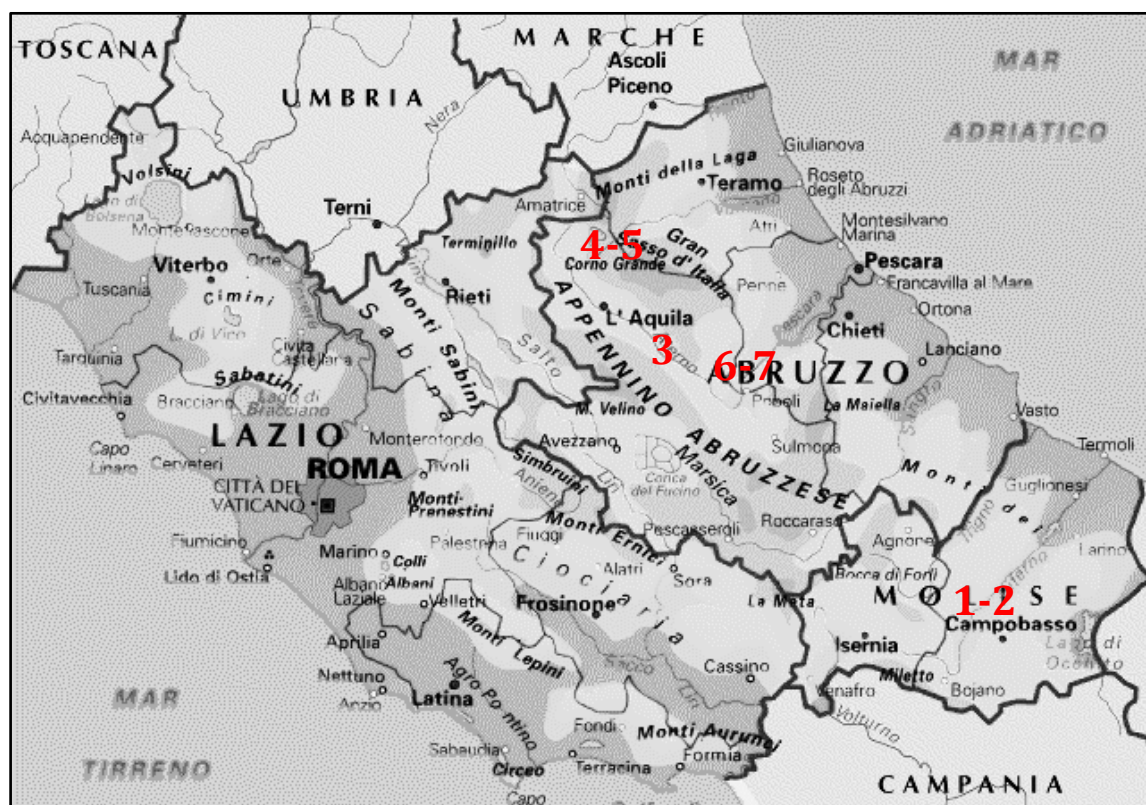
Figure 3.10. Different areas of collection of hop ecotypes.

3.2.2. EXTRACTION OF POLYPHENOLS

The secondary metabolites that have been analyzed belong to the family of phenolic compounds (phenolic acids and flavonoids); were extracted from freeze-dried, milled and measured hops by UV/VIS spectrophotometer to determine its total content.

The analyzes were carried out at the DISPAA laboratory at the University of Study of Firenze. The phenolic compounds were extracted from hop samples (n.3) according to the method of Adom and Liu (2002), which provides a first extraction of free polyphenols in ethanol at 80%, followed by an extraction of the bound phenols first using an acid solvent and subsequently an alkaline solvent. Extractions were repeated in triplicate for each hop sample (n.3) and for each sample both the leaves and the resin have been extracted.

The extraction is divided into three phases:

I.   EXTRACTION OF FREE POLYPHENOLS;

II.   ALKALINE EXTRACTION OF BOUND POLYPHENOLS;

III. ACID EXTRACTION OF BOUND POLIPHENOLS

Determination of polyphenols content

For the determination of total polyphenols content, the absorbance of the ethanol extracts at a wavelength (λ) of 765 nm was measured at the UV/VIS spectrophotometer in 0.5-2 mL MICRO PS cuvettes.

By measuring separately the free and bound (*Figure 3.11*) fractions, the total was obtained by summing the two values. For the analysis of bound polyphenols, samples were prepared by joining 0.5 mL of alkaline extract and 0.5 of acid extract, obtaining a total volume of 1 mL of total bound extract.

A solution of gallic acid in pure Et-OH at a concentration of 1 mg/mL was initially prepared, called "working-stock".

In order to obtain six different standard gallic acid concentrations in pure ethanol, the aliquots of 0.025 mg/mL, 0.050 mg/mL, 0.075 mg/mL, 0.1 mg/mL, 0.15 mg/mL were taken from the previous solution 0.2 mg/mL, which were used to prepare the calibration line to express the total polyphenols content in mg/g of equivalents of gallic acid on dry weight.

As standard (white), was pure ethanol.
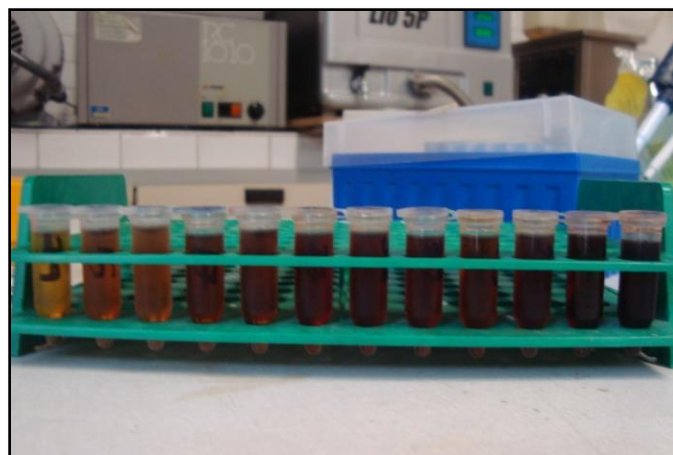
Figure 3.11. Extraction of bound polyphenols

Determination of flavonoids content

For the determination of the total flavonoids the absorbance was measured at a wavelength (λ) of 510 nm to the UV/VIS spectrophotometer according to the method developed by Khodaie and collaborators (2012), in cuvettes from 0.5-2 mL.

The free and bound extracts were measured separately and their total value was obtained by adding these two values. The same method was used for both types of extract.

For the determination of the bound flavonoid fraction, the samples were prepared by combining 0.5 mL of alkaline extract with 0.5 mL of acid extract, obtaining a total of 1 mL.

A catechin solution in pure ethanol has been provided at a concentration of 1 mg/mL, called *working-stock*. To obtain 6 different standard concentrations of gallic acid in pure ethanol, equal to 0.012 mg/ml, 0.025 mg/ml, 0.050 mg/ml,

0.075 mg/ml, 0.1 mg/ml, 0.15 mg/ml, are aliquots from this solution have been withdrawn.

The concentrations were used to prepare the calibration curve of the spectrophotometer to express the total flavonoid content of the samples in mg/g equivalents of catechins. Pure ethanol has been tested as a white standard. Standards for calibration, sample extracts and white solutions were all the same for spectrophotometer reading, adding: 0.2 mL of sample for the bound extract or standard solutions or pure ethanol, or 0.4 ml for 0.833 mL or 0.633 mL of deionized water, 0.062 mL of 5% sodium nitrite with a reaction time of 6 ', 0.062 mL of aluminum chloride, 10% hexahydrate (with a reaction time of 5') and finally 0.833 mL of 1M sodium hydroxide to stop the reaction.

White has been detected in duplicate. They are centrifuged at room temperature for 20 'at 9168 x g. It was therefore possible to observe a color change towards yellow/orange depending on the concentration in catechins.

The absorbance before the white, the standards and the samples was then measured, comparing them with the linear regression line and therefore it was possible to detect the concentrations.

Antioxidant activity (DPPH)

For the determination of antioxidant activity the antiradical activity of the bioactive compounds present in the extracts was evaluated, using the stable radical 2,2-diphenyl-1-picrylhydrazyl or DPPH$^.$ according to the method of Brand-Williams *et al.,* (1995).

The efficient concentration (EC) represents the amount of antioxidant (AO) in the sample necessary to decrease the initial DPPH concentration by half (EC_{50} = (mol/L) AO/(mol/L) DPPH).

EC_{50} was calculated for each sample from a calibration curve by linear regression, using solutions with different concentrations of DPPH.

The higher the EC_{50}, the less efficient the antioxidant activity of the sample. For clarity, the EC_{50} value for each of the sample was converted into ARP, using the following equation:

ARP = (1/EC_{50}) × 100.

The higher the antiradical power value, the more efficient the radical scavenging activity of the sample.

3.2.3. DETERMINATION OF THE CONTENT OF ALPHA AND BETA ACIDS

The dried cones were ground in liquid nitrogen at -195 ° C to avoid normal replacement operations.

From each sample of appropriately ground cones, an aliquot of 0.040 g was weighed, within 2 ml vials, subjected to extraction with 1.5 mL of organic solvent thus constituted: 80% of methanol (CH_2OH), 20% of distilled water and 0.5% of formic acid (HCOOH). The vials were shaken with an ultrasonic stirrer (Bandelin Sonorex Super RK 102H) for 15 min, then incubated at 30°C with a rotary shaker at 10 rpm for 24 h.

The sample thus extracted was centrifuged at 3000 rpm for 5 minutes. Subsequently the supernatant was taken ready to be analyzed with HPLC using the Varian POLARIS C-18A 15x2 mm column at a wavelength λ of 326 nm.

In the analysis with HPLC was used the ICE-3 standard (International Calibrations Extract), specific for the quantitative analysis of alpha and beta acids in the hops, distributed in Europe by the Swiss company Labor Veritas AG in Zurich. The standard, approved from the International Hop Standards Committee (IHSC) and obtained from the Hallertau variety and extracted at the concentration of supercritical CO, is composed of 44.64% of alpha-acids (13.88% coumulone and 30.76% of umulone and adumulone) and 24.28% from beta-acids (13.44% colupulone and 10.84% of lupulone and adlupulone). The working solution was obtained with 1000 ppm of standards dissolved in distilled water, and distributed in the extracting solution used for the samples, in scalar concentrations of 25, 50, 100, 200, 300, 400 and 500 ppm.

In order to highlight and separate in the chromatogram all the peaks of the amaricants: coumulone, umulone and adumulone for alpha-acids and colupolone, lupulone and adlupulone for beta-acids (*Figure 3.12*), the concentrations of the solvents have been modulated over time.
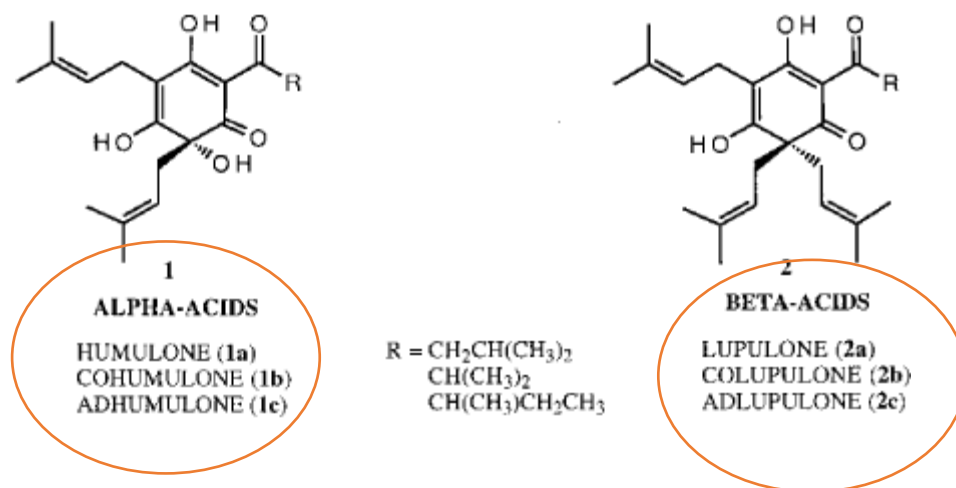
Figure 3.12. The main components of alpha and beta acids respectively.

3.2.4. Anticholinesterase effect of FREE AND BOUND FRACTIONS

By the same method explained previously, the effect of free and bound fractions of polyphenols on the AChE activity was investigated.

As previously highlighted in the aim of the work, the first part of the research activity has dealt with the biological activity of hop extracts, EO and its main compounds by evaluating their toxicity (both contact and fumigant), repellency and nutricional effects on *Sitophilus granarius* adults.

4.1. EO CHARACTERIZATION

In the wild hop collected were identified, by GC-MS analysis, 29 constituents (*Figure 4.1*), accounting for 98.23 % of the whole EO (*Table 4.1*). The main components (92.93 %) were: sesquiterpenes (α-humulene, 37.012 %; β-caryophyllene, 13.74 %; α-selinene 8.69 %; β-selinene 6.63 %) and monoterpenes (β-myrcene, 26.85 %).
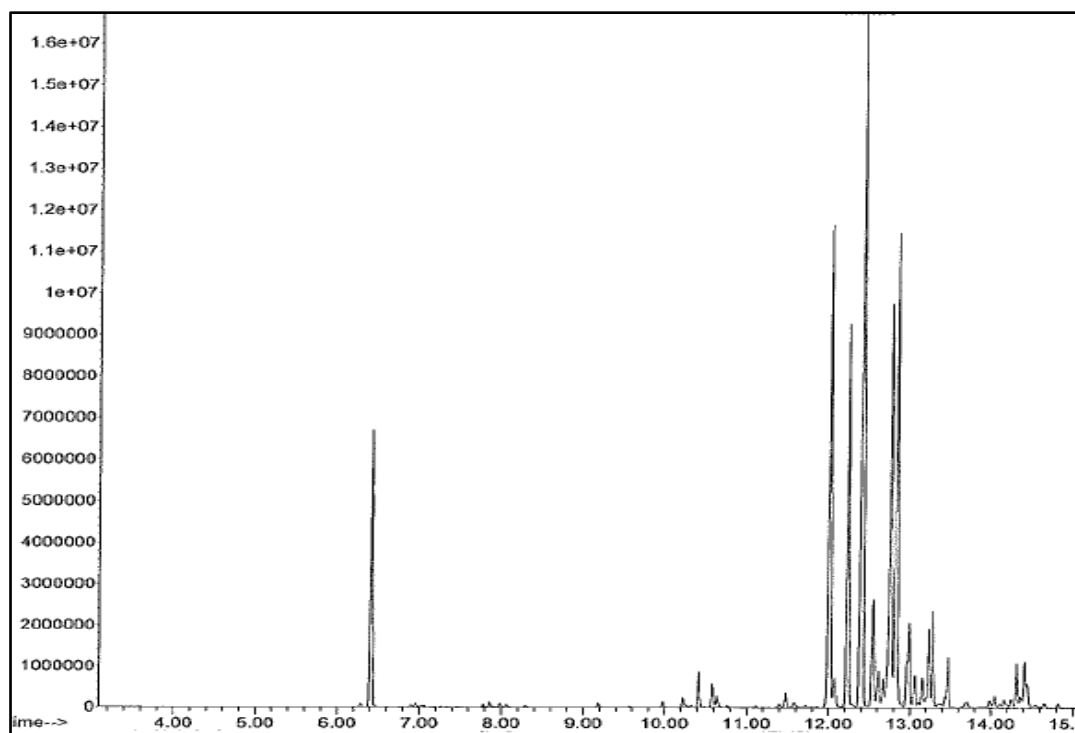
Figure 4.1. Chromatogram of hop EO

Ph.D. thesis of Laura De Acutis

COMPOUNDS	R.T.	% TOTAL
(1S)-(-)-β-Pinene	12.54	0.40
β-Myrcene	13.20	26.85
Methyl heptanoate	14.64	0.21
Methyl 6-methyl heptanoate	17.59	0.14
2-Nonanone	17.80	0.31
β-Linalool	18.17	0.72
Methyl octanoate	19.33	0.20
2-Decanone	22.47	0.23
3-Octen-1-ol, acetate	26.25	0.24
2-Undecanone	26.99	13.63
Methyldec-4-enoate	27.65	1.05
Metholene	28.28	0.13
α-Copaene	30.57	0.21
2-Dodecanone	31.26	0.16
β-Caryophyllene	32.47	13.74
β-Copaene	32.78	0.26
γ-Elemene	32.93	0.59
α-Humulene	33.84	37.01
4,5-di-epi-Aristolochene	34.40	0.37
γ-Selinene	34.66	1.80
β-Selinene	35.14	6.63
2-Tridecanone	35.31	1.09
α-Selinene	35.50	8.70
(E,E)-α-Farnesene	35.81	0.69
γ-Cadinene	36.14	0.75
δ-Cadinene	36.49	1.38
β-Panasinsene	36.95	0.57
Selina-3,7(11)-diene	37.20	0.72
γ-Gurjunene	41.34	0.60
Total		**98.23**

Table 4.1. Chemical composition (%) of hop EO.

4.2. CONTACT TOXICITY

EO and solvent extracts showed an increasing mortality with the dose. Contact mortalities induced by EO were significantly higher than that of control starting from 13.67 and 6.83 μg/adult after 24 h and 48 h, respectively, and reached 100% mortality at 54.68 μg/adult in both cases. EO contact toxicity showed LD_{50} and LD_{90} values of 13.30 and 40.23 μg/adult after 24 h of application, decreasing to 11.77 and 36.80 μg/adult after 48 h, respectively (*Table 4.2*).

As for solvent extracts, mortalities 24 h after application were significantly higher than that of control starting from the dose of 9.37 μg/adult for methanol extract (*Table 4.3*), and 18.75 μg/adult for both acetone (*Table 4.4*) and hexane (*Table 4.5*) extracts; at 48 h after application only acetone extract showed a decrease in the lower active dose which was 9.37 μg/adult. Contact toxicity calculated 24 h after application for the three extracts resulted in LD_{50} values of 16.17, 25.77 and 31.07 μg/adult and LD_{90} values to 33.20, 42.64 and 49.48 μg/adult for acetone, methanol and hexane, respectively; these values slightly decreased after 48 h.

Among the synthetic compounds investigated, β-myrcene (*Table 4.6*) and α-humulene (*Table 4.7*) have reached about 100% mortality 24 h after application at dose of 200.25 and 111.12 μg/adult, respectively; a significant mortality respect to dose control started from 50.06, 27.78 μg/adult, respectively. β-caryophyllene did not reach 100% mortality at the maximum dose checked and showed a significant activity at the dose of 56.37 μg/adult (*Table 4.8*). No significant differences in mortality values among 24 and 48 h after application were found for all the compounds, with the only exception of α-humulene which at 48 h decreased both the lowest active dose, 13.89 μg/adult, and the one causing 100 % mortality, 55.56 μg/adult. The highest toxicity was found for α-humulene which showed LD_{50} and LD_{90} values of 41.87 and 73.51 μg/adult at 24h and 26.83 and 49.49 μg/adult 48h after application, respectively (*Table 4.7*). LD_{50} and LD_{90} values for β-myrcene were respectively 75.91 and 126.05 μg/adult at 24 h and 73.77 and 123.42 μg/adult 48h after application (*Table 4.6*).

Ph.D. thesis of Laura De Acutis

The lowest toxicity was calculated for β-caryophyllene due to LD_{50} and LD_{90} values of 138.51 and 241.27 µg/adult at 24 h, respectively, with no differences at 48 h after treatment (*Table 4.8*).

Dose (µg/adult)	Exposure time (h)	% Mortality (mean ± S.E.)		Regression equation	χ2	LD_{50} (95% CL, µg/adult)	LD_{90} (95% CL, µg/adult)
109.37		100.00±0.00	a				
54.68		93.33±4.22	ab				
27.34		76.67±6.15	bc				
13.67		60.00±5.16	c				
6.83	24h	16.67±6.15	d	$y= 2.666x-2.996$	2.04	13.30 (10.66-16.44)	40.23 (30.53-59.99)
3.42		6.67±4.22	d				
0.00		0.00±0.00	d				
F		90.40					
d.f.		6					
P		<0.001					
109.37		100.00±0.00	a				
54.68		96.66±3.33	ab				
27.34		76.67±6.15	bc				
13.67		60.00±5.16	c				
6.83	48h	30.00±6.83	d	$y= 2.588x-2.771$	1.419	11.77 (9.35-16.60)	36.80 (27.74-55.73)
3.42		6.67±4.22	e				
0.00		3.33±3.33	e				
F		76.18					
d.f.		6					
P		<0.001					

Table 4.2. Contact toxicity of different concentrations of **EO** against *S. granarius* adults 24 and 48 h after topical application. For each exposure time, mean mortality values followed by same letter are not significantly different at P≤ 0.05 (Tukey HSD test).

Ph.D. thesis of Laura De Acutis

Dose (μg/adult)	Exposure time (h)	% Mortality (mean ± S.E.)		Regression equation	χ^2	LD_{50} (95% CL, μg/adult)	LD_{90} (95% CL, μg/adult)
75.00		100.00±0.00	a				
37.50		77.50±4.50	b				
18.75		32.50±7.50	c				
9.37		22.50±5.90	c				
4.69	24h	2.50±2.50	d	$y= 3.33x-4.24$	8.77	25.77 (20.34-34.50)	42.64 (34.05-61.18)
2.34		0.00±0.00	d				
0.00		0.00±0.00	d				
F		95.88					
d.f.		6					
P		<0.001					
75.00		100.00±0.00	a				
37.50		85.00±3.27	a				
18.75		37.50±4.53	b				
9.37		27.50±6.50	b				
4.69	48h	5.00±3.27	c	$y= 0.087x-2.027$	10.60	22.94 (17.79-31.08)	38.69 (30.67-56.18)
2.34		0.00±0.00	d				
0.00		0.00±0.00	d				
F		128.68					
d.f.		6					
P		<0.001					

Table 4.3. Contact toxicity of different concentrations of **methanol** extract against *S. granarius* adults 24 and 48 h after topical application. For each exposure time, mean mortality values followed by same letter are not significantly different at P≤ 0.05 (Tukey HSD test).

Dose (µg/adult)	Exposure time (h)	% Mortality (mean ± S.E.)	Regression equation	$\chi 2$	LD$_{50}$ (95% CL, µg/adult)	LD$_{90}$ (95% CL, µg/adult)
75.00		100.00±0.00 a				
37.50		97.50±2.50 a				
18.75		57.50±4.53 b				
9.37		7.50±3.66 c				
4.69	24h	5.00±3.27 c	$y= 4.10x-4.38$	16.37	16.17 (9.65-28.85)	33.20 (20.96-157.85)
2.34		0.00±0.00 c				
0.00		0.00±0.00 c				
F		290.48				
d.f.		6				
P		<0.001				
75.00		100.00±0.00 a				
37.50		97.50±2.50 a				
18.75		60.00±5.34 b				
9.37		15.00±3.27 c				
4.69	48h	7.50±3.66 cd	$y= 3.84x-4.51$	6.21	14.91 (12.82-17.41)	32.14 (26.29-42.77)
2.34		0.00±0.00 d				
0.00		0.00±0.00 d				
F		241.28				
d.f.		6				
P		<0.001				

Table 4.4. Contact toxicity of different concentrations of **acetone** extract against *S. granarius* adults 24 and 48 h after topical application. For each exposure time, mean mortality values followed by same letter are not significantly different at P≤ 0.05 (Tukey HSD test).

Dose (µg/adult)	Exposure time (h)	% Mortality (mean ± S.E.)	Regression equation	$\chi 2$	LD_{50} (95% CL, µg/adult)	LD_{90} (95% CL, µg/adult)
75.00		100.00±0.00 a				
37.50		67.50±5.26 b				
18.75		17.50±4.53 c				
9.37		7.50±3.66 cd				
4.69	24h	5.00±3.27 cd	$y= 0.07x-2.163$	2.25	31.07 (27.33-36.03)	49.48 (43.19-59.09)
2.34		2.50±2.50 d				
0.00		0.00±0.00 d				
F		137.14				
d.f.		6				
P		<0.001				
75.00		100.00±0.00 a				
37.50		72.50±3.66 b				
18.75		22.50±5.90 c				
9.37		12.50±3.66 cd				
4.69	48h	5.50±3.27 d	$y= 0.06x-1.89$	0.64	28.66 (25.01-33.52)	48.08 (41.71-57.81)
2.34		5.50±3.27 d				
0.00		2.50±2.50 d				
F		118.39				
d.f.		6				
P		<0.001				

Table 4.5. Contact toxicity of different concentrations of **hexane** extract against *S. granarius* adults 24 and 48 h after topical application. For each exposure time, mean mortality values followed by same letter are not significantly different at P≤ 0.05 (Tukey HSD test).

Dose µg/adult	Exposure time (h)	% Mortality (mean ± S.E.)	Regression equation	X^2	LD_{50} (95% CL, µg/adult)	LD_{90} (95% CL, µg/adult)
200.25		100.00±0.00 a				
100.12		70.00±3.78 b				
50.06		37.50±4.53 c				
25.03		7.50±3.66 d				
12.51	24	5.00±3.27 d	$y= 0.026x-1.94$	5.05	75.91 (66.42-88.26)	126.05 (109.63-151.83)
0.00		0.00±0.00 d				
F		169.44				
d.f.		5				
P		< 0.001				
200.25		100.00±0.00 a				
100.12		72.50±3.66 b				
50.06		37.50±4.53 c				
25.03		10.00±3.78 d				
12.51	48	5.00±3.27 d	$y= 0.026x–1.90$	4.04	73.77 (64.48–85.79)	123.42 (107.33–148.57)
0.00		0.00±0.00 d				
F		170.04				
d.f.		5				
P		<0.001				

Table 4.6. Contact toxicity of different concentrations of **ß-myrcene** against *S. granarius* adults 24 and 48 h after topical application. For each exposure time, mean mortality values followed by same letter are not significantly different at P≤ 0.05 (Tukey HSD test).

Ph.D. thesis of Laura De Acutis

Dose (μg/adult)	Exposure time (h)	% Mortality (mean±S.E.)	Regression equation	$\chi 2$	LD$_{50}$ (95% CL, μg/adult)	LD$_{90}$ (95% CL, μg/adult)
222.25		100.00±0.00 a				
111.12		97.50±2.50 a				
55.56		77.50±2.50 b				
27.78		32.50±5.26 c				
13.89	24h	12.50±5.26 d	$y= 0.041x-1.696$	11.09	41.87 (27.89-66.07)	73.51 (54.58-138.17)
0.00		0.00±0.00 d				
F		170.80				
d.f.		5				
P		<0.001				
222.25		100.00±0.00 a				
111.12		100.00±0.00 a				
55.56		97.50±2.50 a				
27.78		42.50±2.50 b				
13.89	48h	27.50±7.50 b	$y= 0.057x-1.518$	2.57	26.83 (22.57-31.77)	49.49 (42.62-60.49)
0.00		7.50±5.26 c				
F		108.11				
d.f.		5				
P		<0.001				

Table 4.7. Contact toxicity of different concentrations of **α-humulene** against *S. granarius* adults 24 and 48 h after topical application. For each exposure time, mean mortality values followed by same letter are not significantly different at P≤ 0.05 (Tukey HSD test).

Dose (µg/adult)	Exposure time (h)	% Mortality (mean ± S.E.)	Regression equation	X^2	LD$_{50}$ (95% CL, µg/adult)	LD$_{90}$ (95% CL, µg/adult)
225.50		87.50±3.66 a				
112.75		35.00±3.27 b				
56.37		17.50±2.50 c				
28.19		10.00±3.78 cd			138.51	241.27
14.09	24	10.00±3.78 cd	$Y= 0.013x-1.73$	3.61	(120.41-161.77)	(209.65-288.77)
0.00		0.00±0.00 d				
F		104.28				
d.f.		5				
P		<0.001				
225.50		87.50±3.66 a				
112.75		40.00±6.55 b				
56.37		20.00±3.78 c				
28.19		10.00±3.78 cd			134.72	238.69
14.09	48	10.00±3.78 cd	$Y= 0.012x-1.64$	3.62	(116.68-157.92)	(207.68-288.05)
0.00		0.00±0.00 d				
F		62.72				
d.f.		5				
P		<0.001				

Table 4.8. Contact toxicity of different concentrations of **ß-caryophyllene** against *S. granarius* adults 24 and 48 h after topical application. For each exposure time, mean mortality values followed by same letter are not significantly different at P≤ 0.05 (Tukey HSD test).

4.3. REPELLENCY IN ARENA

The mean adult response index (RI) of *S. granarius* at 200 g of wheat kernels was 79.33.

The OE, at all the doses tested (54.68 ÷ 875.00 µg), induced negative and significative mean RI (-18.33 ÷ -34.17), indicative of a real repellent effect towards the adults of *S. granarius*, also in the presence of the attractive substrate (*Table 4.9*).

The results in solvent (acetone, methanol and hexane) have reduced the attractiveness of wheat kernels with average values (33.33 ÷ 1.67) at low doses (0.09 ÷ 0.75 mg) and negative (-4.17 ÷ -17.50) at the highest dose (1.50 mg) (*Tables 4.10-4.12*).

Among the solvent extracts, methanol showed the lowest average RI (-17.50) (*Table 4.12*).

In the dose range tested (0.50 ÷ 4.51 mg), all chemical compounds (ß-myrcene, ß-caryophyllene, α-humulene) also elicited negative mean response index (*Tables 4.13-4.15*).

Among the chemicals compounds, α-humulene showed the lowest average RI (*Table 4.15*).

In the presence of increasing doses of each terpene, the mean RI to wheat grain odours (79.84) was significantly reduced and ranged from 4.17 to -23.33 for ß-myrcene (*Table 4.13*), from 0.00 to -34.17 for ß-caryophyllene (*Table 4.14*), and from (-8.89 ÷ -38.89) for α-humulene (*Table 4.15*).

EO

	0,000	54,680	109,370	218,750	437,500	875,000
IR	77,50	-18,33	-21,67	-22,50	-32,50	-34,17

Table 4.9. Behavioral response of *S. granarius* adults to the odors of wheat kernels and/or in the presence of increasing doses of EO from *H. lupulus*. The values of the IR marked with different letters are significantly different for P = 0.05 (Tukey test). Significant differences between the number of insects that have chosen the stimulus and that of the individuals in the control are indicated: * P = 0.05; ** P = 0.01. (T-test).

Acetone extract

mg	0,000	0,094	0,188	0,375	0,750	1,500
IR	77,50	15,00	6,67	6,67	1,67	-4,17
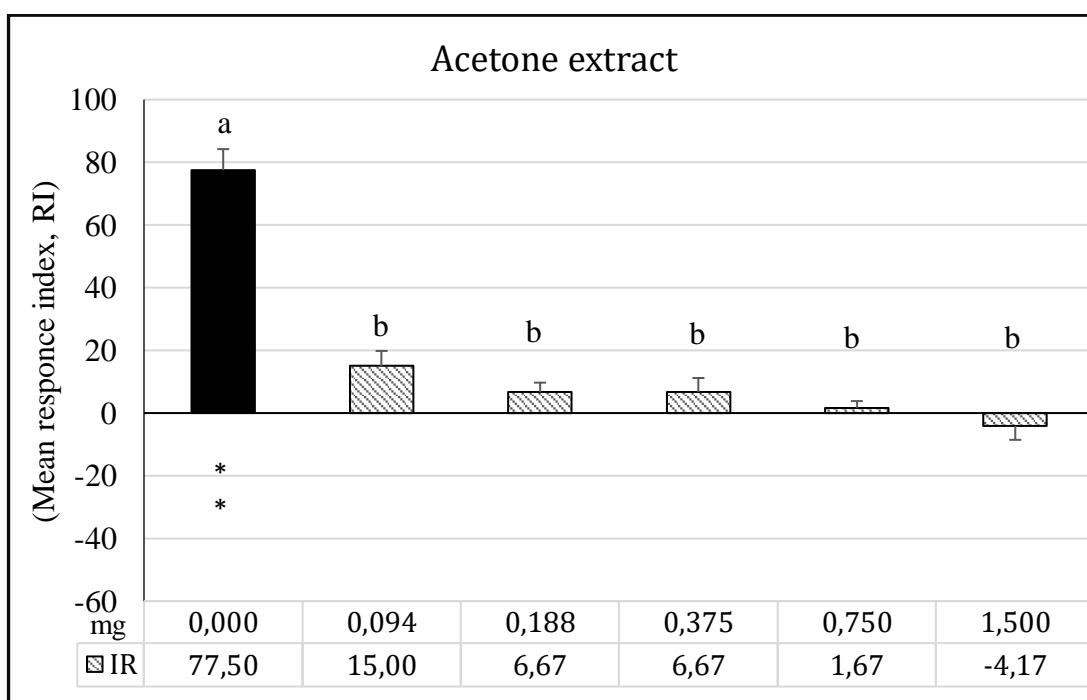
Table 4.10. Behavioral response of *S. granarius* adults to the odors of wheat kernels and/or in the presence of increasing doses of acetone extract from *H. lupulus*. The values of the IR marked with different letters are significantly different for P = 0.05 (Tukey test). Significant differences between the number of insects that have chosen the stimulus and that of the individuals in the control are indicated: * P = 0.05; ** P = 0.01. (T-test).

Ph.D. thesis of Laura De Acutis

Hexane extract

	0,000	0,094	0,188	0,375	0,750	1,500
IR	77,50	33,33	24,17	32,50	6,67	-9,17

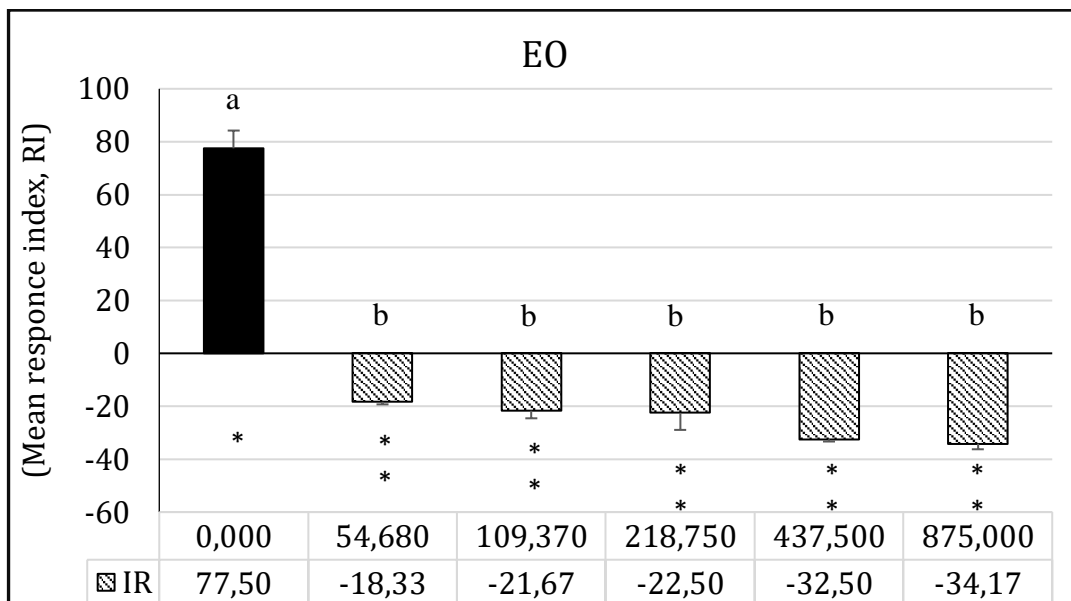**Table 4.11.** Behavioral response of *S. granarius* adults to the odors of wheat kernels and/or in the presence of increasing doses of hexane extract from *H. lupulus*. The values of the IR marked with different letters are significantly different for P = 0.05 (Tukey test). Significant differences between the number of insects that have chosen the stimulus and that of the individuals in the control are indicated: * P = 0.05; ** P = 0.01. (T-test).

Methanol extract

	0,000	0,094	0,188	0,375	0,750	1,500
IR	77,50	15,83	14,17	6,67	5,00	-17,50
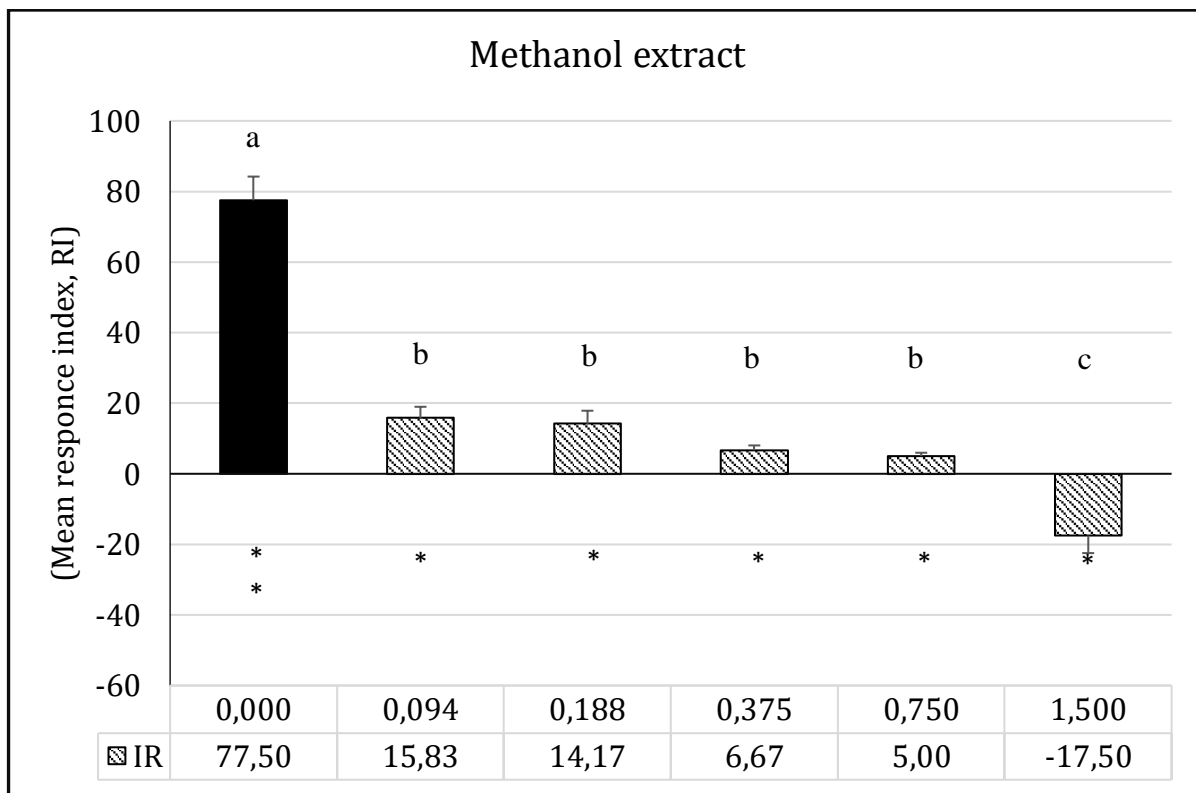
Table 4.12. Behavioral response of *S. granarius* adults to the odors of wheat kernels and/or in the presence of increasing doses of methanol extract from *H. lupulus*. The values of the IR marked with different letters are significantly different for P = 0.05 (Tukey test). Significant differences between the number of insects that have chosen the stimulus and that of the individuals in the control are indicated: * P = 0.05; ** P = 0.01. (T-test).

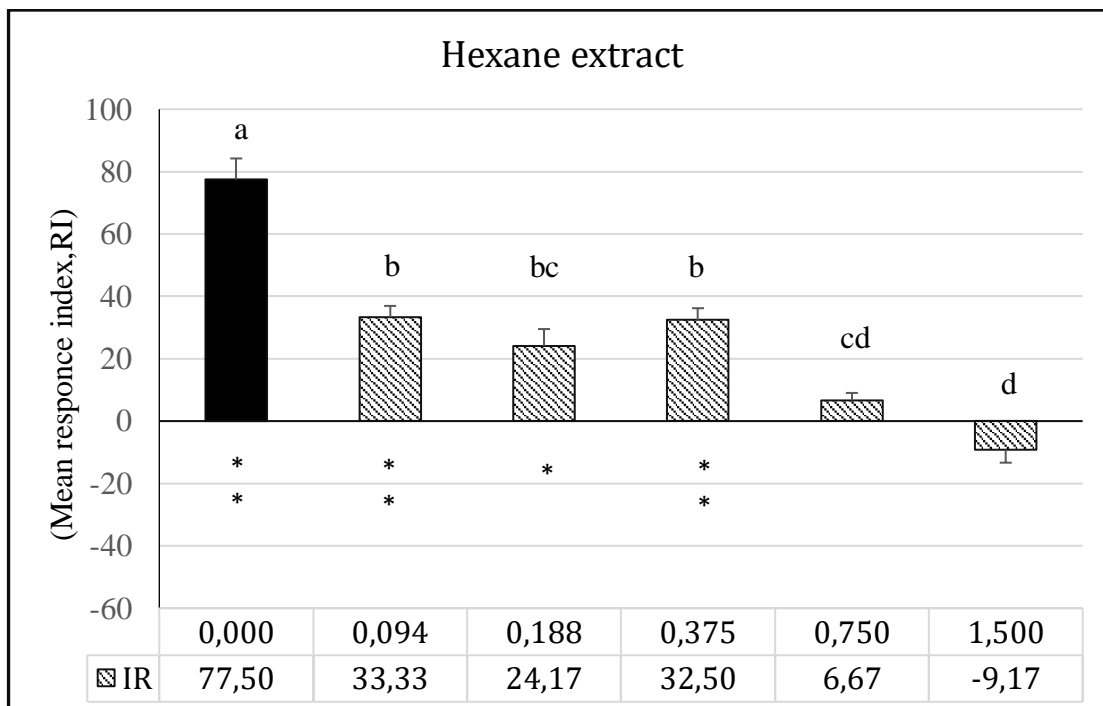**β-Myrcene**

	WG	WG + 0.50 mg	WG + 1.00 mg	WG + 2.00 mg	WG + 4.00 mg
RI	79,84	4,17	-1,67	-5,83	-23,33

Table 4.13. Responses of *S. granarius* adults to odours of grain alone and in the presence of increasing dose of β-Myrcene. Mean with no letters in common are significantly different (P=0.05; Tukey's test)

Table 4.14. Responses of *S. granarius* adults to odours of grain alone and in the presence of increasing dose of β-Caryophyllene. Mean with no letters in common are significantly different (P=0.05; Tukey's test)

α-Humulene

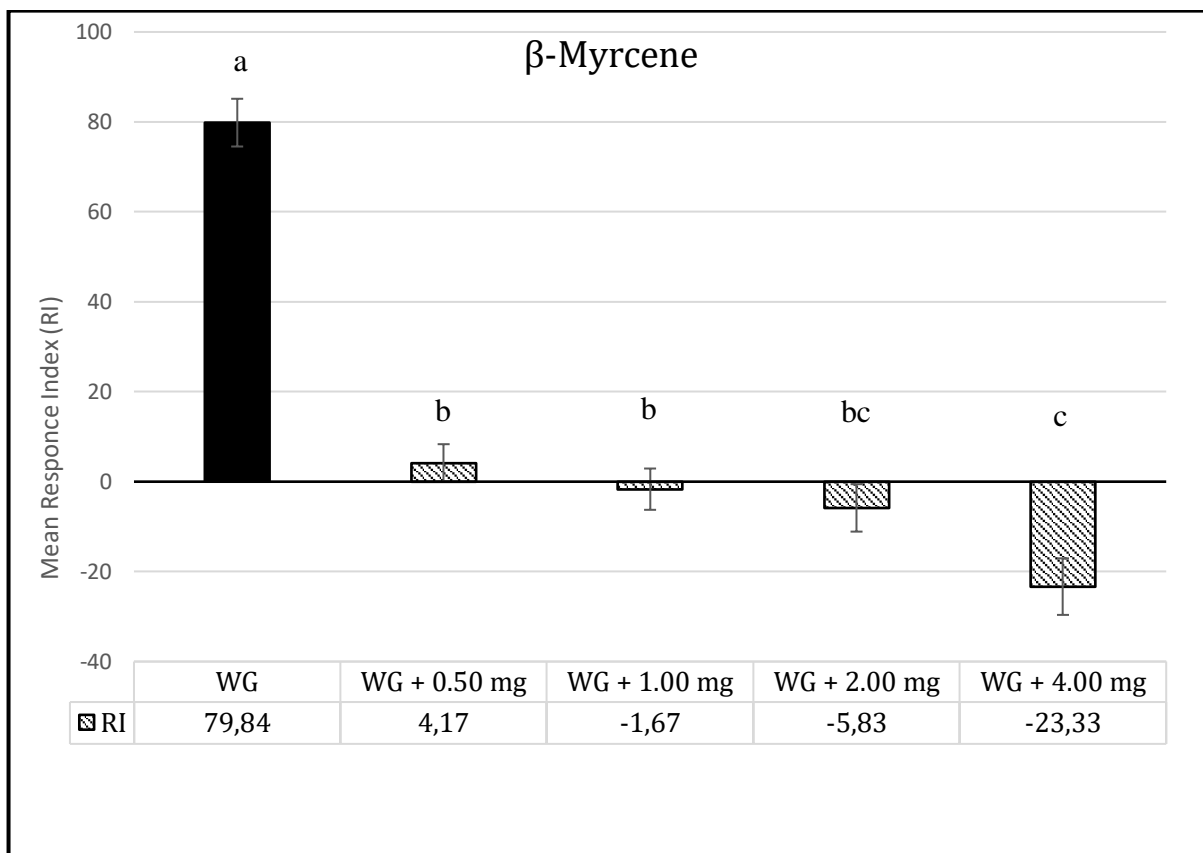|	WG	WG + 0.55 mg	WG + 1.11 mg	WG + 2.22 mg	WG + 4.44 mg
RI	79,84	-8,89	-22,22	-24,44	-38,89

Table 4.15. Responses of *S. granarius* adults to odours of grain alone and in the presence of increasing dose of α-Humulene. Mean with no letters in common are significantly different (P=0.05; Tukey's test)
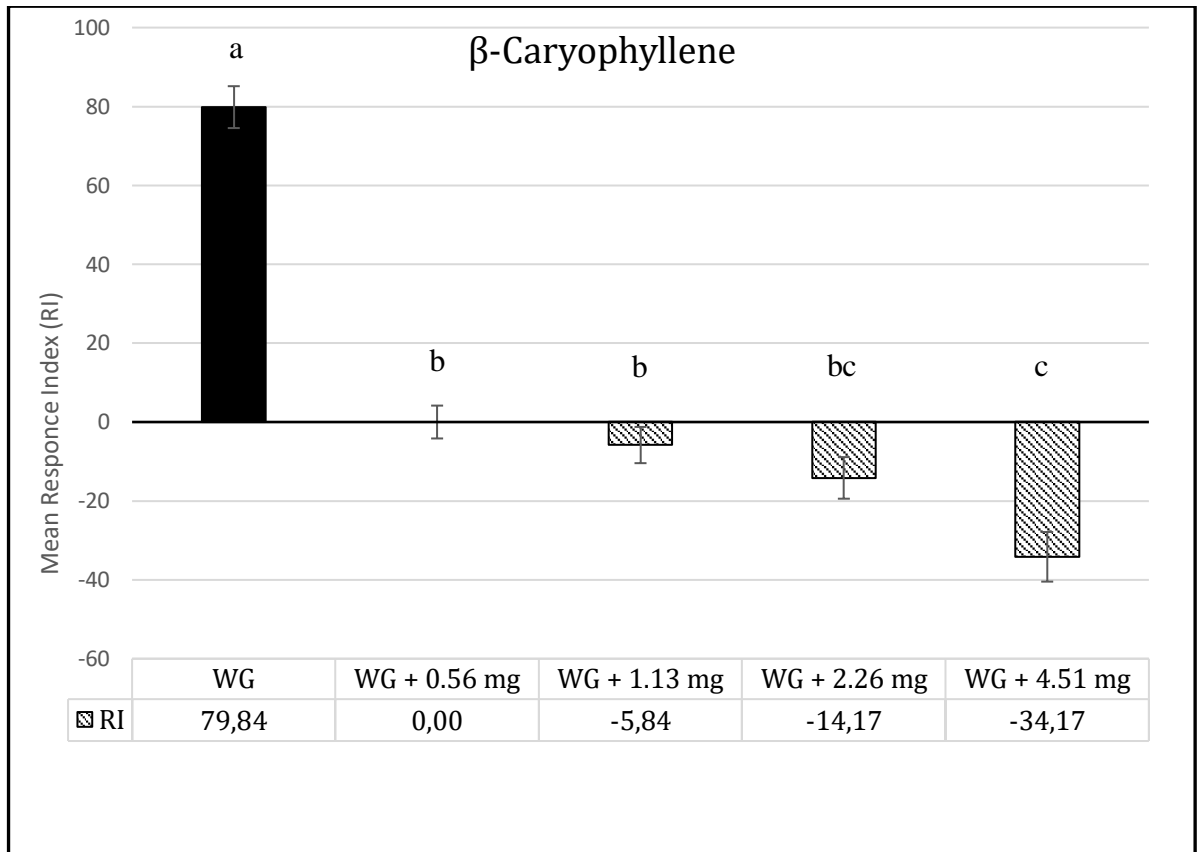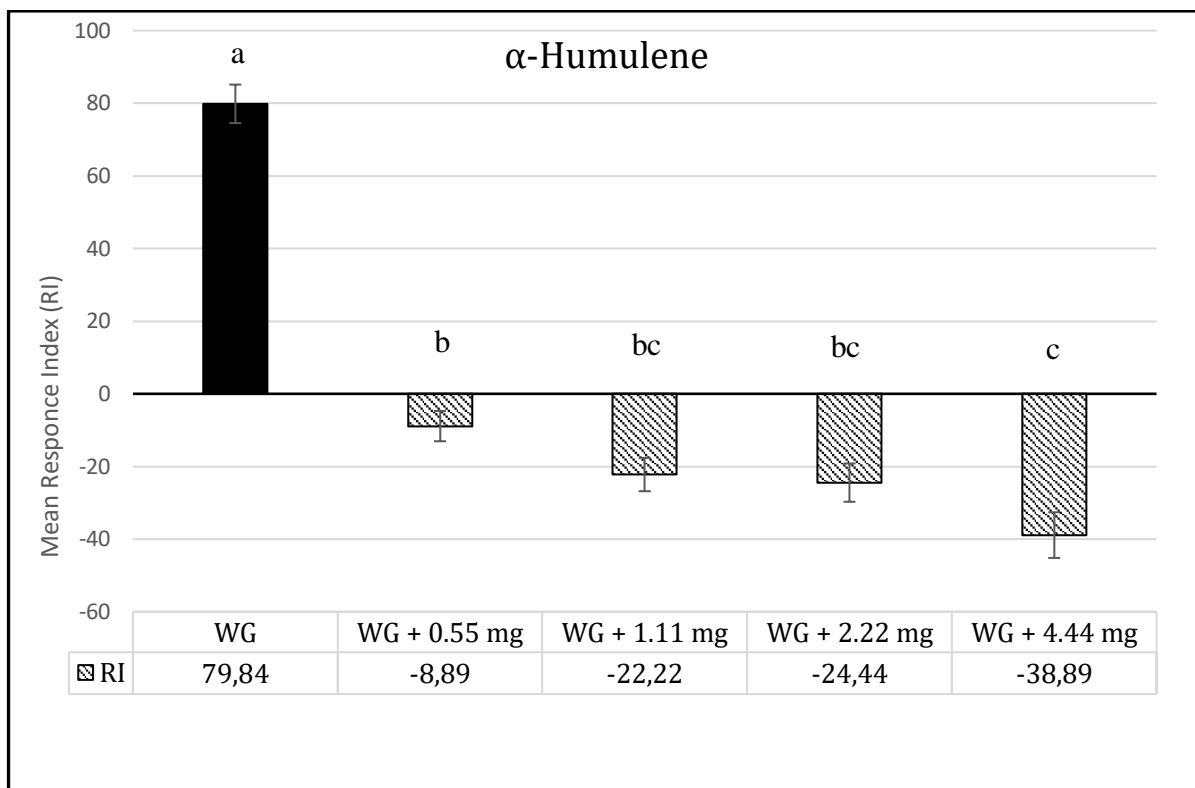
Ph.D. thesis of Laura De Acutis

4.4.   INHALATION TOXICITY

Fumigant mortality of EO increased with the dose (*Table 4.16*). The lowest dose showing significant differences compared to control was 93.33 and 122.50 mg/L volume in the absence and in the presence of grain, respectively, whereas 100% of mortality was obtained in both cases at 210.00 mg/L volume. The LC_{50} and LC_{90} values were respectively 132.41 and 198.98 mg/L volume in the absence of wheat grains, and 136.37 and 201.48 mg/L volume in the presence of grains.

As for pure compounds, in the absence of grain significant differences in mortality values compared to control started from 124.46 and 120.27 mg/L for α-humulene (*Table 4.17*) and ß-caryophyllene (*Table 4.18*), respectively, whereas for ß-myrcene (*Table 4.19*) such a dose was 53.40 mg/L; 100% mortality was reached at 183.73 and 210.47 and 146.85 mg/L for the three compounds, respectively.

The presence of wheat grain led to an increase of doses showing 100% mortality which were 213.36, 240.53 and 186.90 mg/L for α-humulene, ß-caryophyllene and ß-myrcene, respectively; in the presence of wheat the lowest effective dose increased for α-humulene (154.09 mg/L) and ß-myrcene (106.80 mg/L).

The highest fumigant toxicity was observed for ß-myrcene with LC_{50} and LC_{90} values of 72.78 and 116.92 mg/L in the absence of grain, and 115.78 and 171.42 mg/L in the presence of it (*Table 4.19*).

Without grain LC_{50} and LC_{90} values were 127.23 and 188.49 mg/L for α-humulene, respectively, and 128.15 and 205.67 mg/L for ß-caryophyllene; for both compounds these values slightly increased (about 20-30 %) in the presence of grain (*Tables 4.17-4.18*)

Table 4.16. Fumigant toxicity of different concentrations of **E.O** either in the absence or in the presence of wheat grain (100 g) against *S. granarius* adults after 24 h from exposition. For each exposure time, mean mortality values followed by same letter are not significantly different at P≤ 0.05 (Tukey HSD test).

Dose (mg/L)	Wheat grain	% mortality (mean ± S.E.)	Regression equation	X^2	LC$_{50}$ (95% CL, mg/L)	LC$_{90}$ (95% CL, mg/L)
210.00		100.0±0.0 a				
180.83		76.7±6.7 b				
151.67		63.3±3.3 b				
122.50		36.7±3.3 c				
93.33		20.0±5.7 cd				
46.67		10.0±5.7 de				
23.33		6.7±6.7 de				
11.67	Absence	0.0±0.0 e	$y= 0.019x-2.55$	10.34	132.41 (121.89-143.89)	198.98 (183.32-219.86)
5.83		0.0±0.0 e				
2.92		0.0±0.0 e				
1.46		0.0±0.0 e				
0.73		0.0±0.0 e				
0.00		0.0±0.0 e				
F		86.688				
d.f.		12				
P		<0.001				
210.00		100.0±0.0 a				
180.83		73.3±8.8 b				
151.67		60.0±5.7 b				
122.50		36.7±3.3 c				
93.33		16.7±3.3 d				
46.67		10.0±5.7 d				
23.33		3.3±3.3 d				
11.67	Presence	0.0±0.0 d	$y= 0.020x-2.68$	8.64	136.37 (125.88-147.79)	201.48 (185.91-222.51)
5.83		0.0±0.0 d				
2.92		0.0±0.0 d				
1.46		0.0±0.0 d				
0.73		0.0±0.0 d				
0.00		0.0±0.0 d				
F		84.021				
d.f.		12				
P		<0.001				

Ph.D. thesis of Laura De Acutis

Table 4.17. Fumigant toxicity of different concentrations of **α-humulene** either in the absence or in the presence of wheat grain (100 g) against *S. granarius* adults after 24 h from exposition. For each exposure time, mean mortality values followed by same letter are not significantly different at P≤ 0.05 (Tukey HSD test).

Dose (mg/L)	Wheat grain	% mortality (mean ± S.E.)	Regression equation	X^2	LC$_{50}$ (95% CL, mg/L)	LC$_{90}$ (95% CL, mg/L)
183.73		100.0±0.0 a				
154.09		56.7±8.82 b				
124.46		26.6±3.3 c				
94.83		13.3±3.3 cd				
47.41		10.0±5.7 cd				
23.71		6.7±6.6 d				
11.85		3.3±3.3 d			127.23	188.49
5.93	Absence	0.0±0.0 d	$y= 0.021x-2.66$	23.55	(108.94-150.14)	(162.78-234.61)
2.96		0.0±0.0 d				
1.48		0.0±0.0 d				
0.74		0.0±0.0 d				
0.00		0.0±0.0 d				
F		113.336				
d.f.		11				
P		<0.001				
213.36		100.0±0.0 a				
183.73		63.3±3.3 b				
154.09		43.3±3.3 c				
124.46		13.3±3.3 cd				
94.83		10.0±5.7 cd				
47.41		0.0±0.0 d				
23.71		6.7±3.3 d				
11.85	Presence	0.0±0.0 d	$y= 0.018x-3.04$	23.02	164.95	234.39
5.93		0.0±0.0 d			(145.95-190.06)	(205.43-289.39)
2.96		0.0±0.0 d				
1.48		0.0±0.0 d				
0.74		0.0±0.0 d				
0.00		0.0±0.0 d				
F		61.049				
d.f.		12				
P		<0.001				

Table 4.18. Fumigant toxicity of different concentrations of **ß-caryophyllene** either in the absence or in the presence of wheat grain (100 g) against *S. granarius* adults after 24 h from exposition. For each exposure time, mean mortality values followed by same letter are not significantly different at P≤ 0.05 (Tukey HSD test).

Dose (mg/L)	Wheat grain	% mortality (mean ± S.E.)	Regression equation	X^2	LC$_{50}$ (95% CL, mg/L)	LC$_{90}$ (95% CL, mg/L)
210.47		100.0±0.0 a				
165.37		66.6±8.8 b				
120.27		26.6±6.7 c				
60.13		23.3±3.3 cd				
30.07		13.3±8.8 cd				
15.03	Absence	3.3±3.3 cd	$Y= 0.017x-2.12$	16.07	128.15 (103.85-161.07)	205.67 (170.39-272.64)
7.52		0.0±0.0 d				
3.76		0.0±0.0 d				
0.00		0.0±0.0 d				
F		49.813				
d.f.		8				
P		<0.001				
240.53		100.0±0.0 a				
210.47		63.3±3.3 b				
165.37		36.7±3.3 c				
120.27		20.0±5.7 d				
60.13		6.7±3.3 e				
30.07		0.0±0.0 e				
15.03	Presence	0.0±0.0 e	$Y= 0.018x-3.06$	9.88	172.60 (159.15-187.08)	244.78 (225.37-272.90)
7.52		0.0±0.0 e				
3.76		0.0±0.0 e				
0.00		0.0±0.0 e				
F		177.704				
d.f.		9				
P		<0.001				

Ph.D. thesis of Laura De Acutis

Table 4.19. Fumigant toxicity of different concentrations of **ß-myrcene** either in the absence or in the presence of wheat grain (100 g) against *S. granarius* adults after 24 h from exposition. Mean mortality values followed by same letter are not significantly different at P≤ 0.05 (Tukey HSD test).

Dose (mg/L)	Wheat grain	% mortality (mean ± S.E.)	Regression equation	X^2	LC$_{50}$ (95% CL, mg/L)	LC$_{90}$ (95% CL, mg/L)
146.85		100.0±0.0 a				
106.80		73.3±3.3 b				
53.40		46.6±3.3 c				
26.70		6.6±3.3 d				
13.35		3.3±3.3 d			72.78	116.92
6.68	Absence	3.3±3.3 d	$y= 0.029x-2.11$	9.17	(63.91-83.35)	(103.50-135.95)
3.34		0.0±0.0 d				
0.00		0.0±0.0 d				
F		113.336				
d.f.		7				
P		<0.001				
186.90		100.0±0.0 a				
146.85		63.3±6.7 b				
106.80		40.0±5.7 c				
53.40		13.3±6.7 d				
26.70		3.3±3.3 d				
13.35	Presence	0.0±0.0 d	$y= 0.023x-2.67$	5.61	115.78 (104.79-127.91)	171.42 (155.90-193.13)
6.68		0.0±0.0 d				
3.34		0.0±0.0 d				
0.00		0.0±0.0 d				
F		87.938				
d.f.		8				
P		<0.001				

Ph.D. thesis of Laura De Acutis

4.5.   ANTIFEEDANT AND NUTRICIONAL EFFECTS

Nutritional studies showed that the different extracts (methanol, acetone and hexane) had significantly effects on growth rate, food consumption and food utilization (Tables 4.20-4.22).

The greatest deterrence, 74% after 5 days from bioassay, was observed for methanol extract (*Table 4.20*).

The highest mortality, 52% at the concentration of 750 µg/disk after 5 days from treatment, was registered by acetone extract (*Table 4.21*), whereas the lowest activity was observed for hexane extract with deterrence of 24% at the concentration of 750 µg/disk and maximum mortality of 38% (*Table 4.22*).

Concentration (µg/disk)	RGR	RCR	ECI	Mortality (%)	FDI (%)
750.00	-0.011±0.008 a	0.066±0.047 a	-31.915±11.695 a	16.00 a	74.000±20.199 b
375.00	-0.020±0.006 ab	0.188±0.009 b	-10.363±2.465 b	4.00 b	27.951±7.782 a
187.50	-0.0130±0.007 b	0.199±0.007 b	-6.304±2.620 b	0.00 c	21.222±7.185 a
93.75	-0.003±0.003 c	0.230±0.015 b	-1.134±1.173 b	0.00 c	12.621±4.576 a
46.87	-0.001±0.002 c	0.224±0.018 b	-0.416±0.780 b	0.00 c	15.780±4.405 a
Control	0.012±0.003 c	0.278±0.039 b	6.973±0.513 b	0.00 c	

Table 4.20. Nutritional indices, mortality and food deterrence of *S. granarius* adults of different concentrations of **methanol extract**. Means in the same column with the same letter are not significantly different at the 0.05 level determined by the Tukey's test.

Ph.D. thesis of Laura De Acutis

Concentration (μg/disk)	RGR	RCR	ECI	Mortality (%)	FDI (%)
750.00	-0.089±0.018 a	0.109±0.101 ab	-52.026±12.226 a	52.00 a	41.033±13.712 a
375.00	-0.035±0.009 ab	0.183±0.034 a	-27.759±12.944 abc	34.00 b	55.427±9.801 a
187.50	-0.107±0.005 a	0.037±0.129 ab	-45.364±16.115 ab	40.00 ab	44.223±21.273 a
93.75	-0.048±0.016 ab	0.204±0.060 ab	-39.071±14.959 abc	16.00 bc	48.037±16.664 a
46.87	0.008±0.007 bc	0.354±0.029 ab	1.936±1.934 bc	4.00 c	18.489±5.192 a
Control	0.039±0.013 c	0.404±0.033 b	9.028±2.166 c	4.00 c	

Table 4.21. Nutritional indices, mortality and food deterrence of *S. granarius* adults of different concentrations of **acetone extract**. Means in the same column with the same letter are not significantly different at the 0.05 level determined by the Tukey's test.

Concentration (μg/disk)	RGR	RCR	ECI	Mortality (%)	FDI (%)
750.00	-0.025±0.003 a	0.022±0.015 a	-11.596±1.410 a	38.00 a	24.463±6.509 b
375.00	-0.014±0.007 a	0.268±0.006 ab	-5.248±2.352 ab	4.00 b	21.816±2.537 ab
187.50	-0.053±0.019 ab	0.310±0.026 bc	-0.529±6.876 ab	2.00 b	4.827±5.844 a
93.75	0.011±0.003 ab	0.315±0.016 bc	3.337±1.064 b	0.00 b	4.616±3.320 a
46.87	0.009±0.004 ab	0.316±0.012 bc	2.756±1.426 b	0.00 b	7.376±3.119 a
Control	0.027±0.004 b	0.341±0.016 c	7.735±0.854 b	0.00 b	

Table 4.22. Nutritional indices, mortality and food deterrence of *S. granarius* adults of different concentrations of **hexane extract**. Means in the same column with the same letter are not significantly different at the 0.05 level determined by the Tukey's test.

Ph.D. thesis of Laura De Acutis

4.6.   AChE ASSAYS

In order to gain a first insight into the toxic mechanism of hop EO and its main compounds, the effect of different doses of EO, α-humulene, ß-caryophyllene and ß-myrcene on AChE was checked (*Figure 4.2*).

Negligible anticholinesterase activity was found for all substances in the checked range, with the only exception of ß-caryophyllene which showed a dose dependent inhibitory effect starting from 9.02 mg/ml (10 µL) and reaching about 50 % inhibition at the highest dose checked (36.08 mg/mL, 40 µL) (*Figure 4.2*).
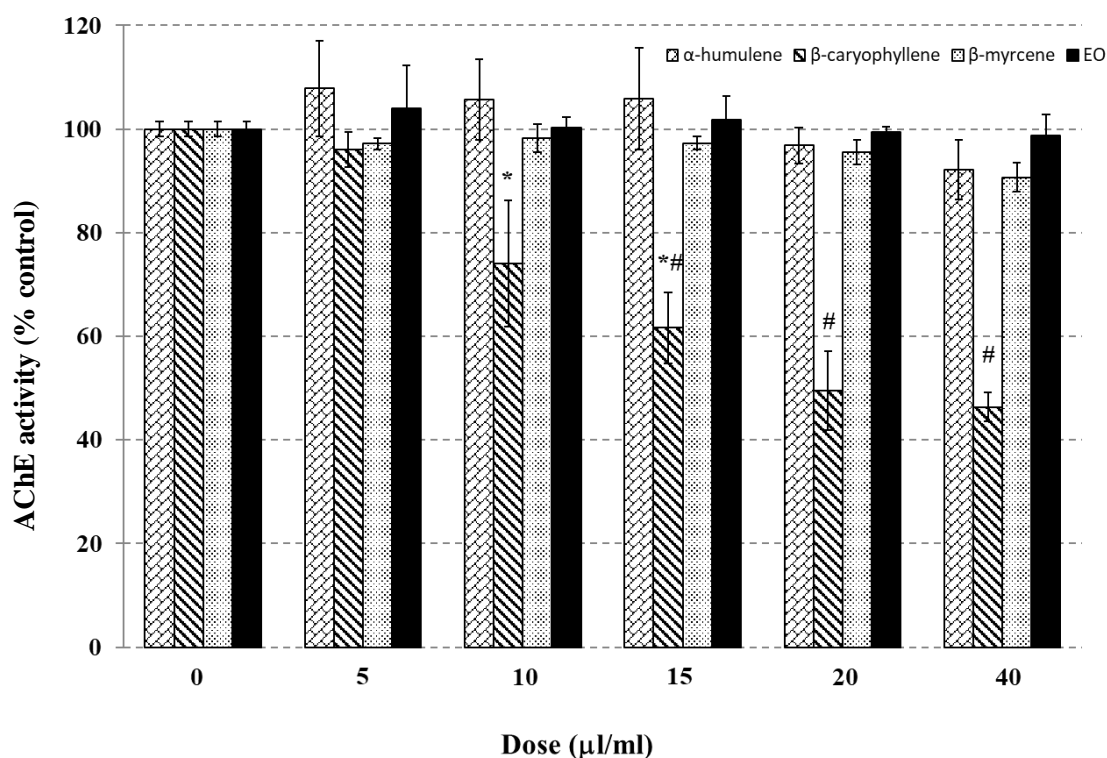

Figure 4.2. The effect of different doses of EO and its main compound, α-humulene, ß-caryophyllene and ß-myrcene on AChE activity (%).

Ph.D. thesis of Laura De Acutis

Chapter 5~Results/2 characterization of hop ecotypes.

Having investigated the biological activity of the ecotype collected in Bojano towards adults of *Sitophilus granarius*, in the second part of the research activity, a first step into the characterization of hop ecotypes in Central Italy was carried by collecting germoplasm in different areas of Abruzzo and Molise regions and analysing their phenolic fraction and its antioxidant and anticholinesterase activity, as well as α- and β-acids content.

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5.1. POLYPHENOLS CONTENT

The total polyphenol content (*Table 5.1*) ranged from 33.91±0.78 (Onna) and 78.28±1.36 (99 Cannelle) mg/g dry weight (DW), with statistically significant differences (p <0.05) among the various ecotypes collected, differentiable in 4 groups. With the exception of the sample collected in Molise (Bojano sample), the bound polyphenols, ranging between 10.99±0.18 (Onna) and 29.26±0.79 (Bojano) mg/g DW, were on average lower than the free ones, varied between 22.92±0.91 (Onna) and 50.32±0.31 (99 Cannelle) mg/g.

Samples	Polyphenols			
	Free (mg/g DW) ± SE	Bound (mg/g DW) ± SE	P	Total (mg/g DW) ± SE
Bojano	24.03±0.82 d	29.26±0.79 a	**	53.30±1.59 c
99 Cannelle	50.32±0.31 a	27.96±1.62 a	**	**78.28±1.36 a**
Bussi 1	32.75±1.67 c	27.39±1.44 ab	**	60.14±2.66 b
Bussi 2 (river)	29.70±0.30 c	19.94±0.12 c	**	49.64±0.21 c
Onna	22.92±0.91 d	10.99±0.18 d	**	**33.91±0.78 d**
L'Aquila-Roio	38.74±1.48 b	21.85±1.76 bc	**	60.59±0.39 b

Table 5.1. Content of free, bound and total polyphenols, expressed in mg/g DW, in samples of female hops flowers collected in central Italy. ** P <0.01. Different letters in the same column indicate a significant difference (P <0.05).

Ph.D. thesis of Laura De Acutis

The **total flavonoids content** (*Table 5.2*), ranging between 9.50±0.30 (Bussi 2) and 17.87±0.34 (L'Aquila-Roio) mg/g DW, accounted for 20-30% of phenolic fraction, with significant differences ($p < 0.05$) among the ecotypes. The highest content of free flavonoids was recorded for the Roio sample (11.61±0.79 mg/g DW) while the lowest for the Bojano sample (2.12±0.13 mg/g DW). The bound flavonoids were found between 4.31±0.15 (Onna) and 9.40±0.93 (Bojano) mg/g DW.

SAMPLES	Flavonoids			
	Free (mg/g DW) ± SE	Bound (mg/g DW) ± SE	P	Total (mg/g DW) ± SE
Bojano	2,12±0,13 d	9,40±0,93 a	**	11,52±1,05 cd
99 Cannelle	8,33±0,31 b	8,93±0,94 ab	ns	17,27±1,24 ab
Bussi 1	5,58±0,13 c	8,38±0,16 abc	**	13,96±0,27 bc
Bussi 2 (river)	3,79±0,19 d	5,71±0,15 cd	**	9,50±0,30 d
Onna	5,57±0,07 c	4,31±0,15 d	**	9,88±0,21 d
L'Aquila- Roio	11,61±0,79 a	6,26±0,67 bcd	**	17,87±0,34 a

Table 5.2. Content of free, bound and total flavonoids, expressed in mg/g DW, in samples of female hops flowers collected in central Italy. ** $P < 0.01$. Different letters in the same column indicate a significant difference ($P < 0.05$).

Ph.D. thesis of Laura De Acutis

5.2.   ARP AND ANTICHOLINESTERASE ACTIVITY

The maximum antioxidant capacity (*Table 5.3*), measured as an antiradical power (ARP), was found for the Bussi 1 sample (301.63±1.52 mg/g) while the minimum for that of Roio (240.96 ± 3.92 mg/g).

The contribution to ARP of the free polyphenol fraction was on average higher than that deriving from the bound fraction, with significant differences (p <0.01) for the samples Bojano, Onna and Roio (*Table 5.3*).

The all samples showed anticholinesterase activity (*Tables 5.4-5.9*), identified mainly in the <u>free polyphenol fraction</u>. In particular, the ecotype collected at the 99 Cannelle showed the maximum inhibition capacity (62.55%) (*Table 5.4*), while the samples collected in Molise (Bojano), the lower anticholinesterase activity (26.51%) (*Table 5.5*).

Moreover, a good correlation (0.835 Pearson's correlation coefficient; P < 0.05 levels - two-tailed) was found between free <u>free polyphenol content and anticholinesterase activity.</u>

Samples	ARP				AChE
	Free (mg/g DW) ± ES	Bound (mg/g DW) ± ES	P	Total (mg/g DW) ± ES	(% inibition)[a]
Bojano	155,74± 3,01 a	120,88±4,52 b	**	276,63±1,52 b	**26,51±1,66 c**
99 Cannelle	143,42±10,42 a	133,92±7,22 ab	ns	277,34±9,44 b	**62,55±2,24 a**
Bussi 1	143,27±6,78 a	**158,36±5,46 a**	ns	**301,63±1,52 a**	43,43±4,58 b
Bussi 2 (fiume)	148,80±1,38 a	122,65±7,70 b	ns	271,45±6,32 b	36,76±0,73 bc
Onna	151,51±0,67 a	120,88±0,37 b	**	272,39±0,94 b	35,56±3,32 bc
L'Aquila-Roio	148,39±0,10 a	92,57±3,95 c	**	240,96±3,92 d	33,89±5,98 bc

Table 5.3. Antioxidant activity and anticholinesterase activity of phenolic fraction of the different hops ecotypes. ** P <0.01; ns, not significant. Different letters in the same column indicate a significant difference (P <0.05).

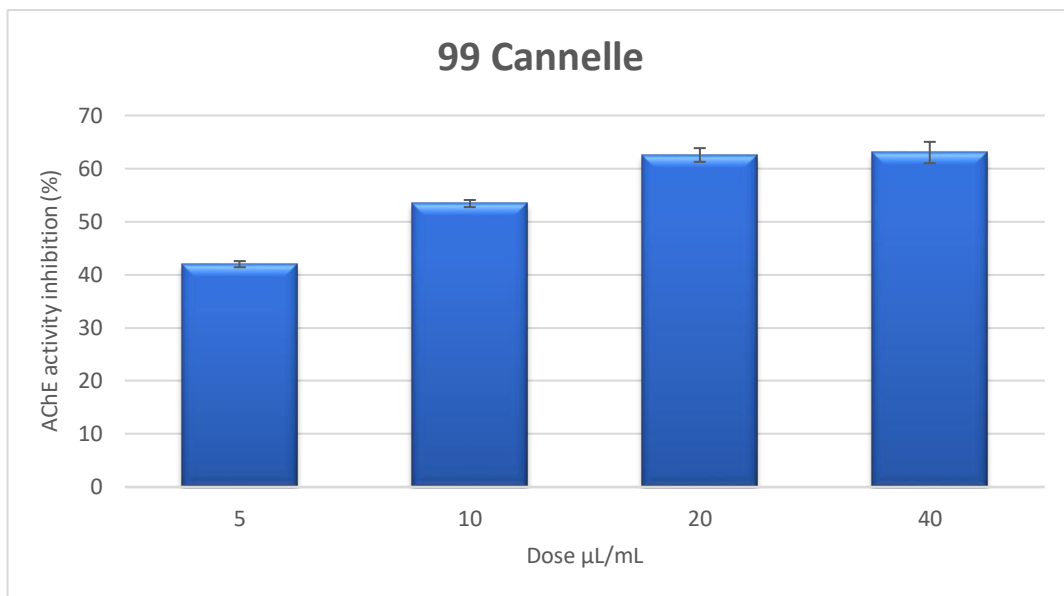**[a] only AChE inhibition due to free polyphenol fraction is reported.**
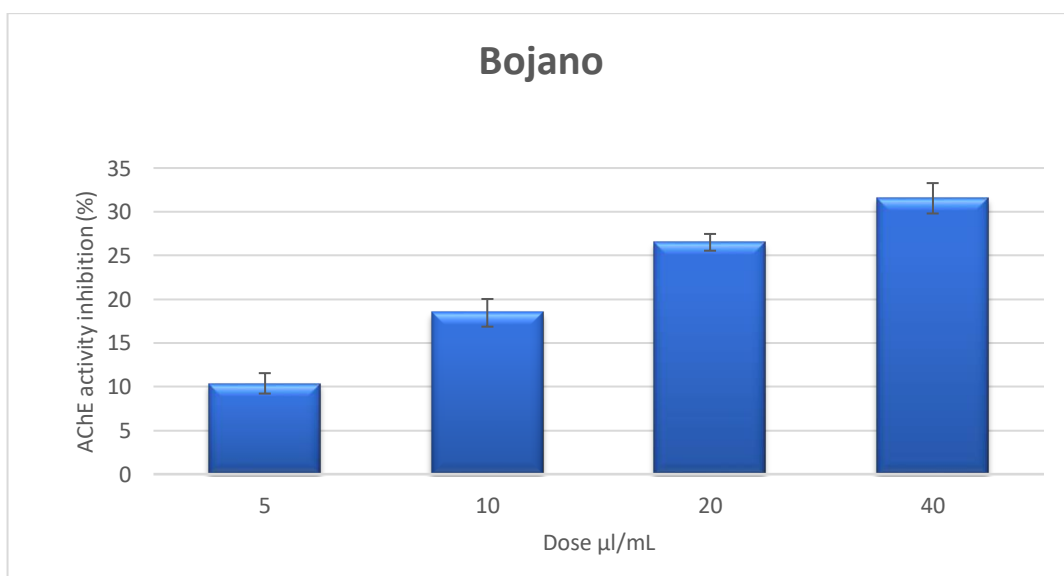
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Table 5.4. AChE activity of the sample collected in 99 Cannelle that showed the maximum inhibition capacity.

Table 5.5. AChE activity of the sample collected in Molise that showed the minimum inhibition capacity.
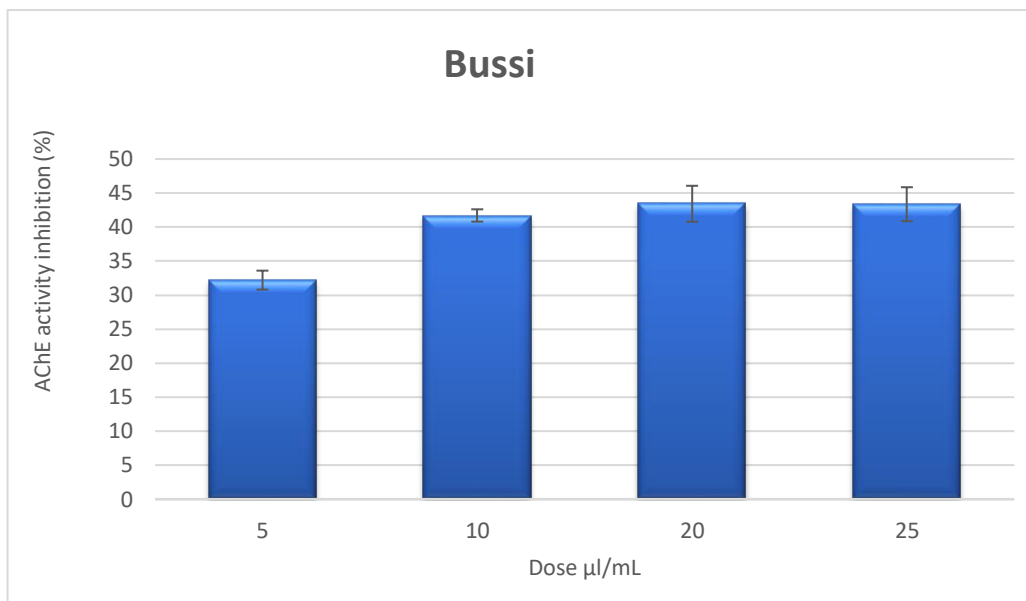
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Table 5.6. AChE activity of the sample collected in Bussi (Pescara province).

Table 5.7. AChE activity of the sample collected in Bussi river (Pescara province).
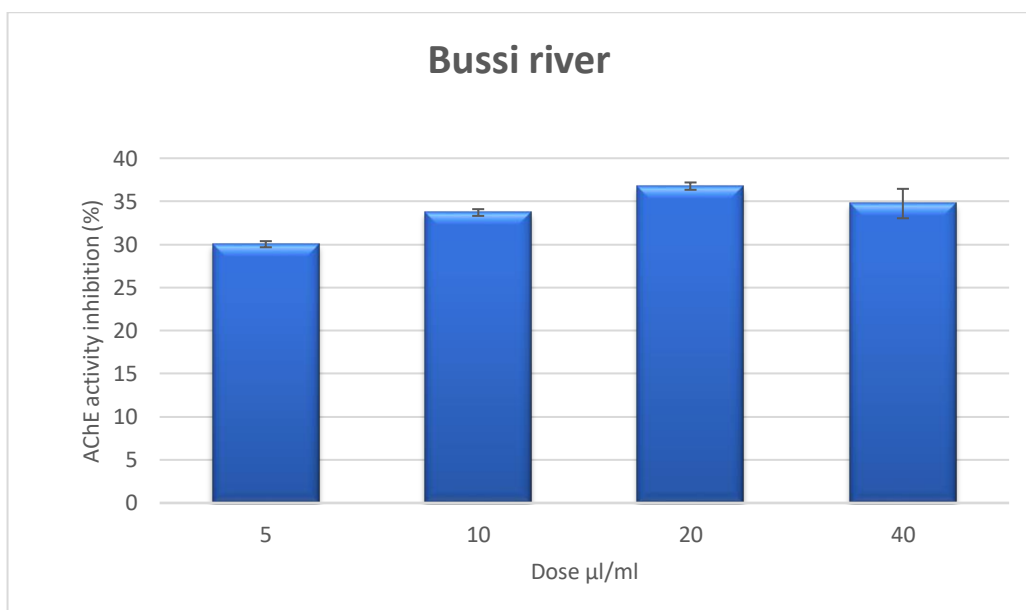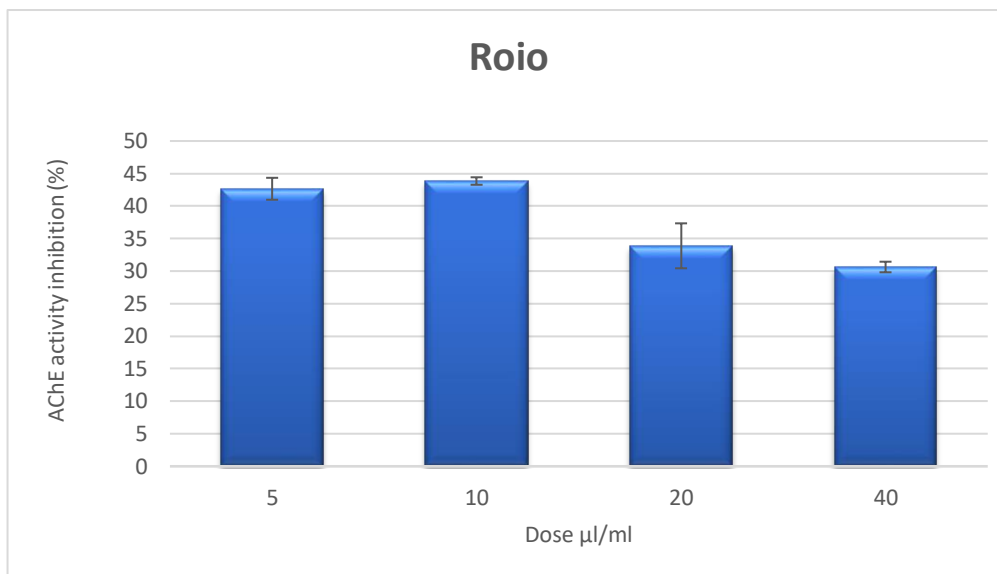
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Table 5.8. AChE activity of the sample collected in Roio (L'Aquila province).

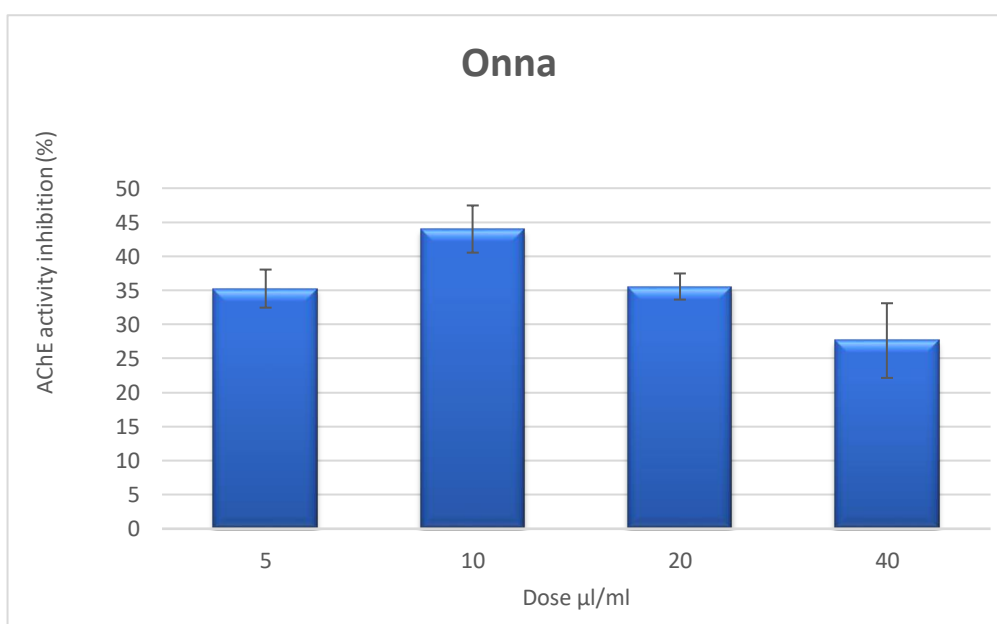**Table 5.9.** AChE activity of the sample collected in Onna (L'Aquila province).

Ph.D. thesis of Laura De Acutis

5.3. CONTENT OF ALPHA AND BETA ACIDS

The average α-acids content was lower in germplasm of Central Italy if compared to previous determinations of both Northern Italy and European wild accessions: the average obtained was 0.38 g/100g DM against a reported 3.82 g/100 g DM (Mongelli *et al.*, 2015) and 2.38 g100g DM (Patzak *et al.*, 2010a).

Specifically, the total alpha acids content (Co-Humulone, Humulone, Ad-Humulone) ranged from 0.2016 (in Onna sample) to 0.7193 g/100g dry matter (DM) in Bussi sample *(Table 5.10)* with significant differences (p <0.01) among the various ecotypes collected.

The same trend was found for β-acids content; in fact, the average of our samples was 0.19 against what is reported for national (2.59 g/100 g DM) and European (3.66 g/100 g DM) samples (Mongelli *et al.,* 2015; Patzak *et al.,* 2010b).

The total content of beta acids (Co-lupulone, Lupulone, Ad-Lupulone) lower than that of alpha acids, ranged from 0.1076, in the sample collected in Molise, to 0.3303 g/100g DM in Onna ecotypes, with statistically significant differences (p <0.001) among all samples, as shown in *Table 5.10*.

Samples	Co-Hum (g/100 g DM)	Hum (g/100 g DM)	Ad-Hum (g/100 g DM)	TOT AA (g/100 g DM)	Co-Lup (g/100 g DM)	Lup (g/100 g DM)	Ad-Lup (g/100 g DM)	TOT BA (g/100 g DM)
1A Bojano (CB)	0.0497 ± 0.0007 f	0.2196 ± 0.0005 c	0.0493 ± 0.0002 c	0.3187 ± 0.0007 c	0.0337 ± 0.0002 f	0.0697 ± 0.0006 d	0.0172 ± 0.0003 cd	0.1207 ± 0.0099 e
1C Bojano (CB)	0.0432 ± 0.0016 g	0.1831 ± 0.0002 e	0.0429 ± 0.0005 d	0.2693 ± 0.0023 e	0.0302 ± 0.0005 f	0.0627 ± 0.0012 e	0.0147 ± 0.0002 d	0.1076 ± 0.0010 f
Onna (AQ)	0.0788 ± 0.0004 c	0.0893 ± 0.0004 g	0.0334 ± 0.0003 e	0.2016 ± 0.0001 g	0.1277 ± 0.0012 a	0.1633 ± 0.0006 a	0.0392 ± 0.0008 a	0.3303 ± 0.0006 a
AQ Roio (AQ)	0.0634 ± 0.0007 d	0.1571 ± 0.0003 f	0.0409 ± 0.0005 d	0.2615 ± 0.0006 f	0.0954 ± 0.0011 c	0.1064 ± 0.0007 c	0.0260 ± 0.0007 b	0.2280 ± 0.0024 c
99 cannelle sx (AQ)	0.1192 ± 0.0006 b	0.3666 ± 0.0006 b	0.0916 ± 0.0004 b	0.5776 ± 0.0014 b	0.0596 ± 0.0011 d	0.0673 ± 0.0007 d	0.0195 ± 0.0013 c	0.1465 ± 0.0028 d
Bussi (PE)	0.1432 ± 0.0012 a	0.4685 ± 0.0012 a	0.1075 ± 0.0005 a	0.7193 ± 0.0005 a	0.1197 ± 0.0024 b	0.1355 ± 0.0012 b	0.0364 ± 0.0012 a	0.2917 ± 0.0037 b
Bussi near river (PE)	0.0565 ± 0.0005 e	0.2007 ± 0.0007 d	0.0505 ± 0.0006 c	0.3078 ± 0.0007 d	0.0400 ± 0.0004 e	0.0680 ± 0.0011 d	0.0185 ± 0.0009 cd	0.1266 ± 0.0014 e
F	1643,64	41535,69	3749,98	27625,98	1203,74	1900,09	127,52	1799,25
d.f	6	6	6	6	6	6	6	6
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 5.10. Content of alpha and beta acids in different hop ecotypes collected

Ph.D. thesis of Laura De Acutis

Chapter 6~ Discussion

First goal of this work has been to evaluate biological activity of wild *Humulus lupulus* L. against the weevil *S. granarius*. Thus, preliminarly EO composition was determined. The major constituents of EO extracted from hop flowers collected in Campobasso province (Bojano area) were sesquiterpenes (α-humulene, 37.012 %; β-caryophyllene, 13.74 %; α-selinene 8.69 %; β-selinene 6.63 %) and monoterpenes (β-myrcene, 26.85 %).

Humulene, caryophyllene, selinene and myrcene have been already recorded as major components of flower hop EOs (Bedini *et al.*, 2015; Mongelli *et al.,* 2015; Dušková *et al.*, 2016) even if in different proportions. It is possible to ipotize that the high variability of EOs components depending on cultivation area and plant genotypes (Tucker *et al.*, 1984; Dušková *et a*l., 2016).

It must be noted that, at present, a comprehensive and unequivocal evaluation of intraspecific diversity for European wild hop is lacking and data are scattered in different papers, in which analyses were performed on plant material collected alternatively from wild and field grown plants, with obvious differences due to the changeable behavior of hop under different geoclimatic conditions. Therefore, a rational and straightforward phenotypic comparison is often difficult to obtain (Mongelli *et al.*, 2015). Literature also suggests that the composition of the EOs for a given ecotype offers limited variability from year to year but may be characteristically different for different accessions.

Topical application of hop extracts, EO and principal chemical compounds to granary weevils adult induced dose-dependent contact mortality that slightly increased with the exposure time.

The toxicity of hop extracts, was higher than those reported by Aydin *et al.* (2017) against five stored pest, *S. granarius*, *S. oryzae*, *Acanthoscelides obtectus*, *Tribolium castaneum* and *Lasioderma serricorne*. In particular, the LD_{50} of acetone extract towards *S. granarius* obtained in the present work was 14.9 μg/adult, a value lower than   25.7 μg/adult reported in (Aydin *et al.*, 2017). The

Ph.D. thesis of Laura De Acutis

same trend was observed for methanol extract, with 48 h LD_{50} of 22.94 µg/adult against the reported >40 µg/adult (Aydin *et al.*, 2017).

The LD_{50} of EO was 11.77 µg/adult after 48 h, comparable to the values reported for xanthohumol, the most important prenilflavonoid of hop, considered more active than solvent extracts (Aydin *et al.*, 2017).

The contact toxicity of EO to *S. granarius* was also comparable with those observed against the congener *S. zeamais* for the EOs of other aromatic plants including various *Artemisia* species (Liu *et al.*, 2010; 2014; Chu *et al.*, 2012, 2013) but lower than that obtained using a pyrethrum extract (Liu *et al.*, 2010).

To date, in the literature there are no studies concerning the activity by fumigation of wild hop against *S. granarius* adults. In this study, the highest fumigant toxicity was observed for ß-myrcene with LC_{50} and LC_{90} values of 72.78 and 116.92 mg/L in the absence of grain, and 115.78 and 171.42 mg/L in the presence of substrate. This value was lower than those recorded for other plants, as lavender EOs against stored-product insect beetles including *O. surinamensis* (LC50 11.3 mg/L air), *R. dominica* (LC50 11.4 mg/L air), *S. oryzae* (LC50 >15 mg/L air), *T. castaneum* (LC50 >15 mg/L air) (Abdelgaleil *et al.*, 2009; Pugazhvendan *et al.*, 2012) and *S. granarius* itself (Laznik *et al.*, 2012).

About the mechanism by which hop toxicity takes place, a first evaluation was also carried out here by checking anticholinesterase activity of EO, and its main compounds. However, the negligible inhibition obtained for all sample, with the only exception of ß-caryophyllene, suggests that other should be the target of hop EO.

The repellent activity of hop EO and its main compounds to granary weevil adults was checked using arena bioassay. The lowest average RI (-38.89) was found for α-humulene, the principal component of EO. In the arena, EO, extracts and all compounds exhibited repellency even in the presence of wheat grains indicating the capability to effectively disrupt granary weevil orientation to the attractive host substrate. A remarkable repellent activity of spent hops was

reported (Bedini *et al.*, 2015) for β-myrcene, α-humulene and β-caryophyllene against *R. dominica* and *S. granarius*.

In two-choice pitfall bioassay the insects are not in direct contact with the repellent compound and they are subjected to the attractive presence of food (Bougherra *et al.* 2015; Bedini *et al.*, 2015).

An influence of the presence of food on the efficacy of chemicals such as the synthetic pyrethroid cyfluthrin (Arthur, 2000) and the macrocyclic lactone spinetoram (Vassilakos *et al.*, 2014) was previously observed. In addition, an interaction between the chemicals and the food such as a differential volatiles sorption can not be excluded.

Interestingly, the two-choice pitfall bioassay allowed to highlight the presence of individuals that did not make a choice remaining in the arena at the end of the experiment (Non-choosing Individuals). Such behavior was previously observed also by Bougherra *et al.* (2015) and is probably a characteristic response of the species to the environmental conditions of the arena bioassay.

In the nutritional experiments, sublethal concentrations of solvent extracts (methanol, acetone and hexane) had significantly effects on growth rate, food consumption and food utilization. Ingestion toxicities of plant extracts have been reported on other insects, e.g*., Thaumetopoes solitaria*, Colorado potato beetle, plum curculio (Gokce *et al.,* 2006; 2007; 2012), proving that hop methanol extract was the most active causing 83% mortality after 48 h (Mehmet *et al.,* 2009).

This showed that *Humulus lupulus* is also acting as a stomach poison.

The results reported in the second part of the study showed that the hop germplasm collected in Central Italy had good levels of polyphenolic compounds associated with a high antiradical power.

The differences among localities in polyphenols and flavonoids content and in ARP were generally smaller, but statistically significant.

The ARP was associated with the polyphenol content, both free and bound. This trend has been observed for other plant species (Parr and Bolwell, 2000).

Results were comparable to the contents observed in plant materials rich in phenolic substances, such as tea, coffee, blackberries, blueberries (Balasundram *et al.*, 2006), and herbs such as oregano and marjoram (Zheng and Wang, 2001).

The average α-acids content was lower in Central of Italy germplasm if compared to previous determinations of both Northern Italy and European wild accessions: the average obtained was 0.38 g/100 gDM against a reported 3.82 g/100 g DM (Mongelli *et al.*, 2015) and 2.38 g/100 g DM (Patzak *et al.*, 2010a). The same trend was found for β-acids content; in fact, the average of our samples was 0.19 against what is reported for national (2.59 g/100 g DM) and European (3.66 g/100 g DM) samples (Mongelli *et al.*, 2015; Patzak *et al.*, 2010b). These notable differences could be due to the variability attributed to the genotype and to environmental one, as well as to the collection time which greatly influences the accumulation of these substances.

Ph.D. thesis of Laura De Acutis

Chapter 7~ Conclusions

The wild hop (*Humulus lupulus* L.) exhibited fumigant and high contact toxicity against granary weevil adults. Therefore, a product based on hop extracts or its main active chemical compounds may have potential to control this destructive pest.

The highest contact toxicity was registred for EO.

The large availability of spontaneous hop plants and its good content of essential oil makes wild hop an excellent low-cost resource for the production of eco-friendly alternative to synthetic insecticides in the protection of stored food-stuff from insect pests.

In addition, this plant showed a good repellent activity able to disrupt granary weevil orientation to an attractive host substrate, indicating a possible applications to flush out insect infestation from empty stores before fresh grain is introduced.This could create a chemical barriers able to mask grain odours to insects, and to incorporate it into packaging materials to prevent insect infestation of packaged foods (Hou *et al.*, 2004; Germinara *et al.*, 2012b; 2015).

In addition to contact toxicity, plant extracts have been screened for activity as insect antifeedants. In some instances, the bioactivity of crude plant extracts on insects is comprised of both toxic and antifeedant effects. Azadirachtin, for example, derived from the neem tree (*Azadirachta indica*), is both a toxicant and antifeedant and has been one of the most widely tested and successfully implemented botanical insecticides over the past two decades (Schmutterer, 1990, 1992, 1995). In the current study, *H. lupulus* exhibited antifeedant activity on *S. granarius* adults in addition to contact toxicity.

These results suggest that potential future application of these extracts or their active components for granary weevil control may exploit more than one mode of action. However, future experiments should focus on determining whether prolonged exposure of *S. granarius* adults to these plant extracts decreases antifeedant effects over time due to habituation of response.

Ph.D. thesis of Laura De Acutis

H. lupulus contains alpha and beta acids, polyphenols, prenylflavanoids, and proanthocyanidins (Taylor, 2003; Bocquet *et al*., 2018). The beta acid derivative of *H. lupulus* repels both chewing and sucking insect pests of plants and the two-spotted spider mite, *Tetranychus urticae* Koch (Jones, 1996, 2003).

Future studies will need to be conducted to determine whether the biological activity of the hop ecotypes collected in this study against granary weevil adults is mediated by the beta acid component. Furthermore, identification of the bioactive components of wild hop may allow development of botanical insecticides with greater potency than the crude plant extracts evaluated here.

The anticholinesterase activity was found only in free fraction of polyphenols whereas negligible anticholinesterase activity was found for all chemical compounds of EO in the checked range, with the only exception of ß-caryophyllene. The potential of polyphenols to act as acetylcholinesterase inhibitors is known (Kennedy, 2011; Roseiro, 2012).

The ecotype with a highest content of polyphenols was the one collected in L'Aquila province. The germoplasm of this second characterization showed significant variability, probably due to the environment and to the genotype. The variability is the basis to constitute improvement.

The high content in polyphenols associated with antioxidant and anti-cholinesterase activity makes this second characterization very interesting in view of other possible applications, in addition to alternative to synthetic insecticides, such as in pharmaceutical and beer production sectors.

Moreover, having constituted a first bank of the germplasm partially "*in situ*" of these species in Abruzzo e Molise, will allow to have an indispensable material for further studies and characterizations, in order to make a rational cultivation of hops possible.

The material requires further investigation, in particular for the content of resin and alpha and beta acids and for the quantity and quality of the aromatic components in order to evaluate a possible application also in the production of beer.

This assessment must be made from samples taken from different accessions grown in the same environment (Ghiselli *et al.*, 2015).

Moreover, the content of the polyphenols in the free fraction was high, an aspect of particular interest in the light of a greater bioavailability.

In conclusions, the results of this study indicate that wild *H. lupulus* has a potential for development as a commercial insecticide against *S. granarius.* Moreover, the good content of phenolic compounds makes wild hop suitable for applications in herbal and pharmaceutical sectors, as well as, in the production of beer after having arranged genetic improvement programs.

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Ph.D. thesis of Laura De Acutis

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