Universitat de Lleida

ROLE OF ALLYL ESTERS IN PEST CONTROL

MARTA GINER GIL

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Supervisors:

Dr. Jesús Avilla Hernández

Dr. Mercè Balcells Fluvià

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ABSTRACT

The insecticidal properties of a series of allyl esters were tested on different insects using different modes of application. Insecticidal action by topical application was assessed on three Lepidopteran pests (*Cydia pomonella, Grapholita molesta and Lobesia botrana*), on the aphid *Acyrthosiphon pisum* (Hemiptera) and on the beetle *Tribolium castaneum* (Coleoptera). Insecticidal action by ingestion was assessed on *Cydia pomonella, Spodoptera littoralis* (Lepidoptera) larvae and on *A. pisum* nymphs. Additionally, allyl ester action in established cell lines was analyzed in order to discern the mode of action of allyl esters. Finally, the role of allyl esters as insect behaviour modifiers was proven on *A. pisum, T. castaneum, C. pomonella* and *L. botrana,*

Activity by contact application depended on the allyl ester. Allyl cinnamate and allyl naphthoate were the most active compounds against eggs and neonate larvae of *C. pomonella, G. molesta and L. botrana*. Conversely, allyl salicylate produced no mortality even at the highest dose applied (10 mg/mL). Allyl cinnamate was the only one active on *A. pisum* by topical application while all the allyl esters assessed were active against *T. castaneum*.

Loss of cell viability due to allyl ester action was scored on all insect cell lines, assessed by two different methods (MTT and Trypan Blue), and that was produced by cell membrane disruption. Allyl cinnamate was still the most active compound from the series and, interestingly, the most sensitive cells were from *Choristoneura fumiferana* (Lepidoptera) midgut. Then, insecticidal action of allyl cinnamate by ingestion was confirmed on *S. littoralis* and *C. pomonella* larvae, and on *A. pisum* nymphs indicating gut as a target for allyl ester action.

Equal or lower insecticidal activity of corresponding acid and dichloropropylester compared to allyl ester was recorded, and cytotoxicity assays demonstrated that

cell membrane disruption was produced by all of them. Differences in action would be due to differences in compound lipophilicity and its interaction with cell membrane.

All allyl esters assessed produced an effect on chemical communication of *T. castaneum,* but not of *A. pisum*. This could be used to repel *T. castaneum* from stored-products. Regarding *C. pomonella* and *L. botrana,* all allyl esters assessed elicited *C. pomonella* male antennae but only allyl cinnamate did on *C. pomonella* female antennae and *L. botrana* male and female antennae. This activity was not translated in an increase of the number of males flying toward allyl ester mixed with pheromone baits in wind-tunnel assays, whereas in the case of females it did, which could be used to increase the number of females captured in pheromone traps.

These results indicate that allyl esters could have a role in pest control, and point to allyl cinnamate as the best candidate of the series.

Key words: allyl ester, insecticide, cytotoxicity, repellency, attraction, electroantenogram, wind-tunnel.

RESUMEN

La acción insecticida de varios esteres de alilo fue testada en diversos insectos y mediante distintos modos de aplicación. La acción insecticida por aplicación tópica fue testada en tres Lepidópteros plaga (*Cydia pomonella, Grapholita molesta* y *Lobesia botrana*), en el áfido *Acyrthosiphon pisum* (Hemiptera) y en el escarabajo *Tribolium castaneum* (Coleoptera). La acción insecticida por ingestión se testó en larvas de *C. pomonella* y *Spodoptera littoralis* (Lepidoptera) y en ninfas de *A. pisum*. Además, el efecto de los esteres de alilo en líneas celulares de insectos fue evaluado con el fin de conocer su modo de acción. Finalmente, el papel de los esteres de alilo como modificadores del comportamiento de insectos fue evaluado en *A. pisum*, *T. castaneum*, *C. pomonella* y en *L. botrana*.

La actividad por aplicación tópica varió en función del éster de alilo. El cinamato de alilo y el naftoato de alilo fueron los compuestos más activos en huevos y larvas neonatas de *C. pomonella, G. molesta* y *L. botrana*, mientras que el salicilato de alilo no produjo mortalidad a la dosis más alta testada (10 mg/mL). El cinamato de alilo fue el único éster activo por aplicación tópica en *A. pisum* mientras que todos los esteres testados lo fueron para *T. castaneum*.

Los esteres de alilo estudiados produjeron pérdida de viabilidad celular en todas las líneas celulares de insectos cuando dicha viabilidad fue analizada mediante dos metodologías distintas (MTT y Azul de Tripano), y siendo ésta debida a la disrupción de la membrana celular. El cinamato de alilo fue el producto más activo, y las células del aparato digestivo de *Choristoneura fumiferana* (Lepidoptera) las más sensibles. La acción insecticida por ingestión en larvas de *S. littoralis* y *C. pomonella*, y en ninfas de *A. pisum*, fue confirmada y el aparato digestivo fue señalado como principal punto de acción de los esteres de alilo.

Los correspondientes ácidos y dicloropropilesteres presentaron una menor o igual acción insecticida que los esteres de alilo siendo dicha acción también debida a un efecto en la membrana celular. Las diferencias en la acción de los distintos compuestos podrían ser debidas a diferencias en las propiedades lipofílicas de los compuestos y su interacción con las membranas celulares.

Los esteres de alilo produjeron un efecto en la comunicación química de *T. castaneum* pero no en *A. pisum*, lo que podría utilizarse para mantener los productos almacenados libres de *T. castaneum*. En cuanto a *C. pomonella* y *L. botrana*, todos los esteres de alilo probados produjeron una respuesta en las antenas de los machos de *C. pomonella*, mientras que tan solo el cinamato de alilo la produjo en las antenas de hembras de *C. pomonella* y en machos y hembras de *L. botrana*. Esta respuesta no se tradujo en un aumento de la atracción de machos hacia cebos con mezclas de ester de alilo y feromona en ensayos de túnel de viento, pero si aumentó el número de hembras atraídas. Este hecho podría utilizarse par incrementar el número de hembras capturadas en trampas de feromona.

Estos resultados, sugieren el papel de los esteres de alilo en el control de plagas, especialmente del cinamato de alilo.

Palabras clave: esteres de alilo, insecticida, citotoxicidad, repelencia, atracción, electroantenografía, túnel de viento.

RESUM

Les propietats insecticides d'una sèrie d'esters d'al·lil va ésser avaluada en diferents insectes mitjançant diferents modes d'aplicació. L'acció insecticida per aplicació tòpica s'ha avaluat en tres Lepidòpters plaga (*Cydia pomonella, Grapholita molesta* i *Lobesia botrana*), en l'àfid *Acyrthosiphon pisum* (Hemíptera) i en l'escarbat *Tribolium castaneum* (Coleòpter). L'acció insecticida per ingestió es va avaluar en larves de *C. pomonella* i *Spodoptera littoralis* (Lepidòptera), i en nimfes de *A. pisum*. A més a més, l'acció del esters d'al·lil en diverses línees cel·lulars d'insectes va ser testada amb l'objectiu de discernir el seu mode d'acció. Finalment, l'acció dels esters d'al·lil com a modificadors del comportament d'insectes es va provar en *A. pisum*, *T. castaneum*, *C. pomonella* i *L. botrana*.

L'acció per aplicació tòpica va variar en funció de l'ester d'al·lil aplicat. El cinamat i el naftoat d'al·lil van ser els compostos més actius front a ous i larves neonates de *C. pomonella, G. molesta* i *L. botrana*, mentre que el salicilat d'al·lil no va produir mortalitat a la major dosi assajada (10 mg/mL). El cinamat d'al·lil va ser l'únic ester actiu per aplicació tòpica en adults de *A. pisum*, mentre que tots els esters d'al·lil testatsvan ser actius en adults de *T. castaneum*.

Els esters d'al·lil assajats van produir una pèrdua de la viabilitat cel·lular en les línees cel·lulars d'insectes quan va èsser mesurda mitjançant dos metodologies diferents (MTT i Blau de tripà), degut a una disrupció de les membranes cel·lulars. El cinamat d'al·lil va ser el compost més actiu i les cèl·lules procedents de l'aparell digestiu de *Choristoneura fumiferana* (Lepidòpter) les més sensibles. L'acció insecticida per ingestió va ser confirmada en larves de *S. littoralis* i *C. pomonella*, i en nimfes de *A. pisum*, senyalant l'aparell digestiu com a principal punt d'acció dels esters d'al·lil.

Els corresponents àcids i dicloropropilesters van mostrar una menor o igual acció insecticida que els corresponents esters d'al·lil, deguda també, a una acció en la membrana cel·lular. Les diferències en l'acció dels compostos seria deguda a diferències en les propietats lipofíliques dels compostos i la seva interacció amb les membranes cel·lulars.

Pel que fa a l'efecte dels esters d'al·lil en la comunicació química dels insectes, aquesta va tenir lloc en *T. castaneum* però no en *A. pisum*, el que es podria utilitzar per a mantenir els productes enmagatzemats lliures de *T. castaneum*. Pel que fa a *C. pomonella* i a *L. botrana*, tots els esters d'al·lil assajats van produir una resposta en les antenes dels mascles de *C. pomonella*, però tan sols el cinamat d'al·lil la va provocar en les femelles de *C. pomonella* i en mascles i femelles de *L. botrana*. Aquesta acció no es va veure reflexada en un increment de l'atracció de mascles cap a fonts amb l'ester d'al·lil i feromona en assajos en túnel de vent, però si en l'atracció de femelles. Aquest fet podria utilitzar-se per a incrementar el nombre de captures de femelles en trampes de feromona.

Aquests resultats suggereixen un paper dels esters d'al·lil en el control de plagues, especialment del cinamat d'al·lil.

Paraules clau: ester d'al·lil, insecticida, citotoxicitat, repel·lència, atracció, electroantenografia, túnel de vent.

Fruit crops represent an important part of the cultivable area worldwide. In Spain, more than 135,000 ha are dedicated to sweet fruit production and more than 1.000,000 ha to grape production. This is translated in more than 1.130,000 T of peaches and nectarines, 1.100,000 T of apples, 470,000 T of pears (5th largest world producer), and 6.100,000 T of grapes (4th largest world producer) (FAO, 2012). In Catalonia, sweet fruits are present in more than 45,000 ha, most of them (82%) situated in Lleida province. Peach and nectarine orchards take up 43% of the area, followed by pear and apple orchards (27% and 22%, respectively) (DAAM, 2008). Vineyards are also an important crop in Catalonia, proven by the existence of 12 appellations of origin (DOs) (including more than 70,000 ha) that ensure the geographic origin and quality of wines produced (INCAVI, 2012).

Insects, weeds and diseases are responsible of important economic losses in most cultivable areas. Up to 25% of losses in fruit production has been attributed to pests (Pimentel, 2002; Dhaliwal *et al*., 2010); part of them being produced, directly or indirectly, by lepidopteran pests (Moore, 1950; Ramírez-Legarreta *et al*., 2006) which can reduce harvest value due to a depreciation of fruit quality (Bell and McGeoch, 1996; Giliomee and Riedl, 1998).

1. TORTRICIDAE FRUIT KEY-PESTS

Several key-fruit pests belong to the Tortricidae family (order Lepidoptera, suborder Heteroneura, division Ditrysia, superfamily Tortricoidea). Tortricids have a worldwide distribution, especially in temperate and tropical upland regions, and the larvae when feeding can cause great economic damage in a wide variety of crops. Because of this, several control methods have been developed to control them (González, 2003).

In the area of study (Lleida, northeast of Spain), Tortricid key-pests of fruit crops are *Cydia pomonella* (L.), *Grapholita molesta* (Busck) and *Lobesia botrana* (Denis and Schiffermüller) (GenCat, 2012).

Cydia pomonella, codling moth (Fig 1), is the key-pest of apple (*Malus domestica* Borkh.) and pear (*Pyrus communis* L.) orchards in most apple and pear productive areas worldwide, but it is also found in walnut (*Juglans regia* L.), quince (*Cydonia oblonga* Miller), apricot (*Prunus armeniaca* L.) or plum (*Prunus prunus* L.) orchards (ISPI, 2011). *Cydia pomonella* has one to four generations per year, depending on climate conditions (Glen and Brain, 1982; Pitcairn *et al*., 1992; Wearing *et al*., 2001). Eggs are laid on leaves or next to fruits, and larvae start hatching from the third day, depending on temperature and relative humidity (RH), producing damages when feeding on fruits (Fig 1) and favouring the appearance of brown rot (*Monilinia fructigena* Honey) (Moore, 1950; Holb, 2004). Larvae have five instars and overwinter at 5th instar as diapausing larvae. Temperature and photoperiod influence the entrance and the end of diapauses (Riedl, 1983; Russel and Bouzonane, 1989).

Figure 1: *Cydia pomonella* larva, pupa and adult, and apple damaged by the action of *C. pomonella* larvae showing frass at the entry hole.

Grapholita molesta, oriental fruit moth (Fig 2), is a key-pest on stone-fruit orchards in most productive areas and can also feed on apples and pears (ISPI, 2011). Eggs are laid on leaves or near the growing shoots early in the season and on fruits later in the season. Larvae hatch from the third day, depending on the temperature, and complete four or five instars, producing a direct damage when feeding on young shoots and later on fruits (Marí *et al*., 1994; Giraud *et al.*, 1996; Rothschild and Vickers, 1999; González, 2004). Indirectly, feeding wounds can favour fruit infection by brown rot (*Monilia spp*) (Garic *et al*., 2004; Holb, 2006). Three to five generations per year can be observed depending on climate conditions and they overwinter as dormant larvae or prepupae in tree crevices or under the soil (Giraud *et al*., 1996).

Figure 2: *Grapholita molesta* egg, larva and adult.

Lobesia botrana, grapevine moth (Fig 3), is a key-pest on vineyards (*Vitis vinifera* L.) in most productive areas worldwide (ISPI, 2011). Eggs are laid on flower buds and larvae feed on clusters until pupation. Their feeding behaviour causes a direct damage on fruits and favours fungus proliferation, thus reducing quality of grapes and, consequently, wines produced with them (Pearson and Goheen, 1988; Pérez *et al*., 1991). Up to four generations per year can be recorded, and diapauses as pupae determined by climate conditions and photoperiod (Deseo *et al*., 1981; Milonas *et al*., 2001; Roditakis and Karandinas, 2001).

Figure 3: *Lobesia botrana* egg, larva and adult.

2. TORTRICIDAE FRUIT KEY-PEST CONTROL

Due to the larvae feeding behaviour (larvae remain protected by or between fruits), control measures focus basically on eggs, neonate larvae and adults. Chemical and microbial controls are predominantly used against eggs and neonate larvae, while ethological control is mainly used against adults.
Biological control is usually not enough to control Tortricidae pests, but shoud be taken into consideration to reduce the number of chemical treatments along the season.

An important point in fruit pest control is the reduced economic threshold of these crops. This fact is translated in a big effort to achieve the objective of no damage on fruits (< 1%) at the end of the season. The highest price is paid for best quality undamaged fruits while damaged ones are only accepted for fruit processing and, additionally, their market is reduced in certain countries. The quality of fruit also influences quality of fruit based products, then as levels of toxins are higher on damaged fruits and in their products (Sydenham *et al*., 1997; Hasan, 2000). This fact explains that several control methods have been developed against Tortricidae pests, and that several applications (or interventions) are done along the growing season to set no- damages on fruits. **Tolerance thresholds** fixed by the Catalan Council of Integrated Production (2012) are 2 or 3 *C. pomonella*

moths per monitoring trap and week during the first generation, 1 or 2 moths in 2nd and 3rd generation or 0.2% of damaged fruits in pear and apple orchards. In peaches, the tolerance threshold is 15 *G. molesta* moths per monitoring trap and week, 3% of shoots damaged or 1% of fruits damaged. In vineyards, it is recommended to treat from the 2nd generation when 10% of clusters present eggs.

Traditionally, **chemical control** is used. Organophosphates, carbamates, pyrethroids and Insect Growth Regulators (IGR) are successfully used to control Tortricidae species (Charmillot *et al*., 2001; Tunaz and Uygun, 2004; Sáenz-de-Cabezón *et al*., 2005, 2006). However, the uncorrect application of treatments (moment or way) can cause pest control failures (Sauphanor and Bouvier, 1995; Mota-Sánchez *et al*., 2008). Moreover, repeated treatments with the same active ingredient or different ingredients with the same mode of action, step up the appearance of **resistant populations** (http://irac-online.org/). Consequently, resistance has been recorded on Tortricidae species in several regions where chemical control is used as main pest control method (Pree *et al*., 1998; Rodríguez *et al*.,. 2011). Additionally, cross-resistance has been described (Dunley and Welter, 2000; Smirle *et al*., 2002) what have to be into consideration in the election of the active ingredient.

A negative aspect of chemical control is that several compounds that have long been used are not selective and can affect other organisms, including beneficial organisms (Pimentel and Greiner, 1997; Ware *et al*., 2003; Devine and Furlong, 2007). This could reveal other problems, as the appearance of secondary pests that were naturally controlled by them (Shivankar *et al*., 2007).

Chemical control is the most used method to control Tortricidae pests, but mating disruption is implemented with great success reducing the number of chemical treatments per season in several productive areas (Auscher, 1997; Kovanci *et al*., 2005; Ioriatti *et al*., 2011). The number of treatments along the season is still high,

and several effords have been done to improve the control strategy in an environmentally friendly way (Gencat, 2012; www.ruralcat.cat).

Ethological control, based in the use of pheromones, is also used in Tortricidae pest control. **Pheromones** are chemicals (usually mixtures of several compounds) that are emitted by one individual and produce an effect/reaction in another individual from the same species. There are different kinds of pheromones depending on the effect/reaction produced (aggregation, alarm, food trail, sex). In Lepidoteran pest control, sex pheromones, which are emitted, in general, by females to attract males for mating, are the most used. Main compounds of these sex pheromones are straight-chain carbonic compounds with different terminal functional groups (esters, alcohols, acetates, aldehydes) or polyunsaturated hydrocarbons with epoxy derivatives (Ando *et al*., 2004). The main and secondary compounds of the sex pheromones of *C. pomonella*, *G. molesta* and *L. botrana* are described from 60's (George, 1965; Roelofs *et al*., 1971; 1973; Bartell and Bellas, 1981; Arn *et al*., 1985; 1988) and their use in commercial fields started in 90's (Howell *et al*., 1992; Cardé and Minks, 1995). The Catalan Council of Integrated Production recommends their use in front of chemical treatments.

Mating disruption is the usual alternative using pheromones. It is based in the release of pheromone in orchards with the aim of disrupt male orientation to females, so reducing mating and, consequently, population in next generations and damages on fruits. This technique is available for the three Tortricidae species described in this thesis (Kovanci *et al*., 2005; Angeli *et al*., 2007; Dunkelblum, 2007), and field assays started in Lleida region (northeast Spain) at 1988, being established nowadays and having reduced the number of chemical treatments along the season (Barrios *et al*., 2004; www.ruralcat.cat). However, to achieve success when using this technique, some field characteristics are needed and it works better when a huge area, and surroundings, is controlled with this

method after years (Sauer and Karg, 1999; Witzgall *et al*., 1999; Louis and Schirra, 2001).

"Attract and kill" and **"mass-trapping"** techniques also use sex pheromones to reduce Lepidopteran populations in field, but they are less used than mating disruption. In these cases, pheromone is used to attract moths into traps, where they are killed by the action of an insecticide or simply are trapped in. These methods only reduce the number of male insects, so it is useful the addition of substances to attract females (host-plan aromas, feeding attractants or kairomones) (Light *et al*., 2001; De Cristofaro *et al*., 2004; Knight and Light, 2004; Landolt *et al*., 2007; Fernández *et al*., 2010).

In this context, substances that cause the contrary effect could also be useful in pest control. **Repellents** are chemical substances (or physical stimuli) that produce a dissuasive effect on insects. The use of repellents in insect control helps to prevent pests and to maintain specific areas free from insects. Their use against Lepidopteran pests is not current, but in other insect orders (Trematerra and Lanzotti, 1999; Tapandjou *et al*., 2005; Isman, 2006).

Biological control is also used in Trotricidae pest control (Falcon and Huber, 1991). It focuses in the enhancement of naturally present fauna in field that acts as pest parasite or predator. Several strategies, as use of natural enemy attractants or reduce the number of chemical applications, should be used to take advantage of natural enemy's action against pests (Trimble *et al*., 2001). However, it cannot keep moth populations below economic thresholds without other interventions, and is usually combined with other pest control methods into integrated pest management (IPM) programs, e.g. entomopathogenic nematodes or codling moth granulovirus (CpGV) (Unruh and Lacey, 2001; Lacey and Unruh, 2005; Lacey *et al*., 2005; Riga *et al*., 2006).

A combination of different control methods able to be used in **ecological production** of apples and pears in Catalonia region set good results at the end of the season (Gencat, 2012). Ecologial production includes biological control strategies (natural enemy respect, nematodes, granulovirus, *Bacillus thuringensis*, kaolín, spinosad), good agricultural practices (put cardboards around trunks, remove damaged fruit and shoots), and ethologic control (mating disruption and mass-trapping).

3. RESEARCH ON NEW INSECTICIDES

3.1. NEW INSECTICIDE RESEARCH – chemical control

The number of available insecticides are limited by law in the European Union (http://ec.europa.eu/food/plant/index_en.htm) reducing the **alternatives** of chemical control. This reduces alternatives when selecting **active ingredients** to be used in chemical control and leads to the research of new active ingredients. Additionally, resistant populations have been developed and negative effects in non-target organisms have been observed. These reasons head to the discovery of new insecticidal substances solving problems caused by conventional insecticides.

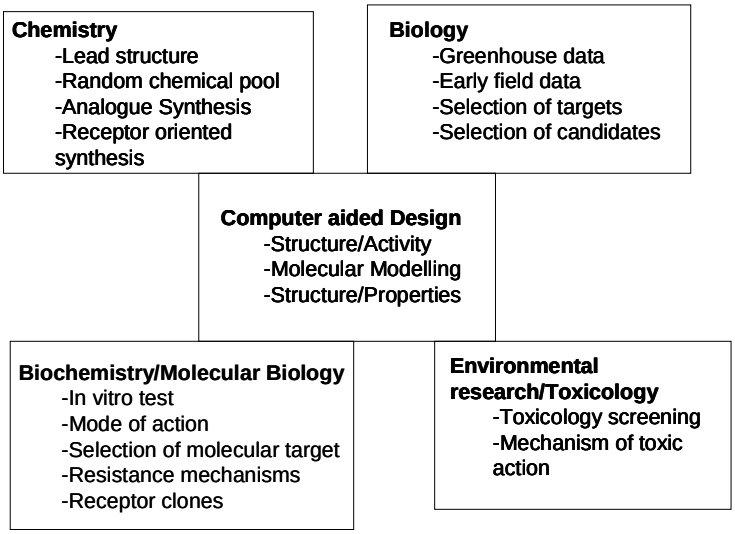
New insecticide research had firstly focused in **natural products**. **Essential oils** are the most studied, being used as a whole or by previously separating compounds, with the aim to find insecticidal substances easy to produce and avoiding (or reducing) problems of conventional insecticides (Isman, 2006). Several compounds from essential oils have shown insecticidal properties against several insect orders (Huang *et al*., 2002; Park *et al*., 2003), and some of them include the **ester chemical group** (Daferera *et al*., 2000; Skaltsa *et al*., 2003; Bakkali *et al*., 2008), as the one targeted in this thesis. However, some problems

were found in essential oils; differences in composition among species, populations or plant habitat (Zygadlo and Juliani, 2003; Oliveira *et al*., 2005; Muñoz-Bartomeu *et al*., 2007) are weak points that need to be solved before introducing them in commercial fields.

The mode of action of these **lipophilic substances** seems to be related to insect cell membrane disruption (Enan, 2005; Bakkali *et al*., 2008; Rattan, 2010), in a similar way of other fatty substances (Kabara, 1987; Sikkemma *et al*., 1995; Najar-Rodriguez *et al*., 2008). Although, other additional mechanisms of toxicity are not rejected (Stenerson, 2004; Enan, 2005; Regnault-Roger *et al*., 2012), and could help to reduce the development of resistant populations (Yang *et al*., 2009; Marchial *et al*., 2010; Perumalsamy *et al*., 2010).

Traditional insecticide research is based in the assessment of chemical compounds directly on insects and the calculation of doses needed to achieve desired pest control. It is frequently based on the screening of several substances that are suggested to have insecticidal properties. Then, the active chemical structure is elucidated and studies focus in the mode of action to know the target where the action takes place. At this point, modifications in chemical structure focused to increase the insecticidal action can be done and insecticidal assays repeated. Finally, ecotoxicological assays are performed (Fig 4).

Figure 4: Steps and knowledge areas used in new insecticide research (From Sjut, 1997; Haskell and McEwen, 1998; Ishaya *et al*., 2007; Yu, 2008).



In the present project, first steps aim to asses allyl ester insecticidal action were done “in vivo”, using different key-fruit pests. As insecticidal properties seem to be not specific, tests on different insect orders with different characteristics were suggested. Because of this, it was essential to choose different insects as models. They have to be easy and cheap mass-reared and be representative of a group of insects for a specific structure, feeding behaviour, metabolic pathway, etc. The most used, representing three different insect orders that are key-pest in several cultivable areas and used to carry out the bioassays described in this thesis, stated below.

Tribolium castaneum Herbst, red flour beetle, is a broad spread pest of stored products (Fig 5). It belongs to the Coleopterans (Tenebrionidae), is easy, cheap and not time-spending to rear (Casadío and Zerba, 1996) and it develops at temperature range 20-38 ºC and 30-70% relative humidity (RH) (Howe, 1956).

Coleopterans have a hard integument which gives specific permeability properties and protection (Merzendorfer and Zimoch, 2003). *Tribolium castaneum* seems to be more tolerant to insecticides than other Coleopteran stored-products pest species (Rozman *et al*., 2007) so it is a good tool for an initial screening of substances able to be used to control other stored-product pests.

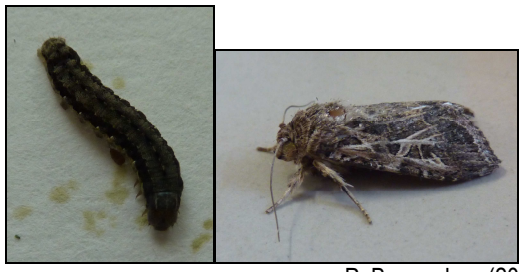
Figura 5: Adults, larvae and exuviae of *Tribolium castaneum.*

Acyrthosiphon pisum (Hemiptera: Aphidade) Harris, pea aphid (Fig 6), is highly used in insecticide research and is also easy to rear if maintained at temperatures from 23-25 ºC and between 60 and 70% RH (Rahbé and Fevbay, 1993; Sadeghi *et al*., 2009). It could be considered as a good representative of soft-bodied insects that feed on plant phloem by inserting their mouth parts. They are not as cosmopolitan as other aphid species (feed on Legumes plants), but they have an active enzymatic system as developed as other cosmopolitan aphids (The International Aphid Genomics Consortium, 2010).

Figure 6: Adults and nymphs of *A. pisum.*

Spodoptera littoralis Boisduval, cotton leafworm, is a phytofagous Lepidopteran (Noctuidae) (Fig 7) that can attack grasses, vegetables, legumes and crucifer plants. It is one of the most destructive agricultural pests within its subtropical and tropical range (www.europe-aliens.org/pdf/Spodoptera-littoralis.pdf). Several resistant populations have been described due to the high insecticidal pressure it has been subjected (Ahmad *et al*., 2007; Masallenejad *et al*., 2009).

Figure 7: *Spodoptera littoralis*, larva and adult.



P. Bengochea (2012).

To improve pest chemical control and to avoid the development of resistant populations it is important to fix the mechanism by which the insecticidal action takes place. There are several insecticidal **mode of action** described (IRAC, 2012). Taking into account the most used pesticides, seven main routes are based on the inhibition of enzymes, the disruption of chemical signal systems, the generation of reactive molecules that destroy cellular components, the degradation of pH gradients across membranes, the dissolution in lipophilic membranes and consequently the disruption of their structure, the disruption of the electrolytic or osmotic balance and the destruction of tissues, DNA or proteins (Stenersen, 2004).

Several methods to discern the insecticidal mode of action are currently described. In the present study, we focus in the **use of insect cell lines** (Smagghe, 2007; Smagghe *et al*., 2009).

Many insect cell lines, from different insects and different tissues, are available (http: //www. Invitrogen.com), or can be established (Schneider, 1972; Vaughn *et al*., 1977) and give information about the cell procedures disrupted by the action of chemicals (Smagghe, 2007). Moreover, the effect of insecticides with known **mode of action** in cell lines have been described (Decombel *et al*., 2004; Masallanejad *et al*., 2008; Shahidi-Noghabi *et al*., 2010) and can be used to compare with effect produced by the new assessed substances.

3.2. NEW INSECTICIDE RESEARCH - Ethological control

Chemical communication plays an important role in insect communication. Many volatile substances emitted by insects or plants produce a specific reaction or response in insects that can be useful in pest control. Because of this, pheromones (as mentioned before), or attractive and repellent substances have interest in pest control. Their application into septa or traps, and the fact that they are usually specific implies a minor effect in non-target organisms and reduces hazards to human health (Welter *et al*., 2005).

To assess substances that can play a **role in insect communication**, the most used methodologies are based in **Electroantennographic** (EAG) (Fig 8) and **wind-tunnel** (Fig 9) assays. EAG apparatus records the different potential produced in an insect antenna when a chemical stimulus is supplied. It shows the possibility of new compounds to be detected by insect antennae, although this is not always translated in an effect in insect behaviour (Ansebo *et al*., 2004). Wind-tunnel assays resembles natural conditions and make it possible to study insect behavioural response to a chemical stimulus.

Figure 8: Electroantennogram apparatus used for EAG assays (Synthech).

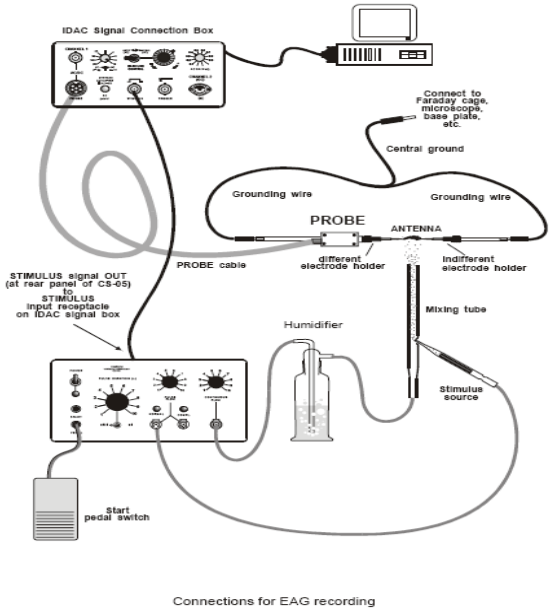


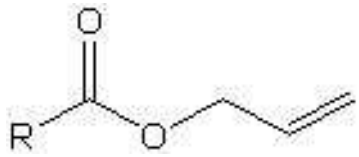
Figure 9: Wind tunnel used on insect behavioral assays.

A variation of wind- tunnel assay is the use of ofactometers, e.g. Y-tube systems. They are also used to discern attractiveness or repellency properties of chemical substances. Insects are released in a tub with two arms where different stimuli are supplied, and election among stimulus and no-stimulus or two different stimuli (each in one arm) are scored.

4. ALLYL ESTERS as candidates for pest control

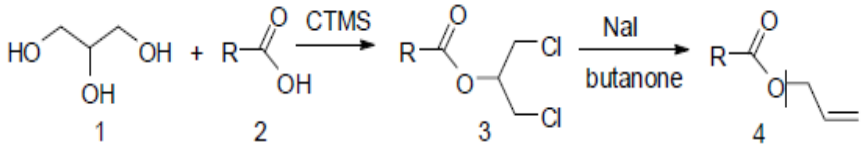
Allyl is the common name of 2-propenyl group (Fig 10) and allyl esters can be obtained by the esterification of prop-2-en-1-ol (allyl alcohol) with an acid. The main inconvenient of this synthetic method is related to the high irritating character of allyl alcohol, so that some alternative synthetic methods have been developed.

Figura 10: Allyl ester - general chemical structure.



One of these alternative synthethic methods for allyl esters synthesis has been developed at the Organic Chemistry group of the University of Lleida (Fig 11). This method uses glycerol (1- Fig 11), the corresponding acid (2- Fig 11) and chlorotrimetylsilane (CTMS) to obtain a dichloroester (3- Fig 11) that is later converted to the coresponding allyl ester (4 – Fig 11) by treatment with sodium iodide (NaI) in butanone.

Figure 11: General procedure for the synthesis of allyl esters (from Escribà *et al*., 2009).



Glycerol is obtained from fat hydrolysis in the biodiesel industry (www.biodiesel.com) in big amounts so that the whole process becomes more profitable if glycerol can be used in other applications. Glycerol can also be used

as starting material in the synthesis of several compounds in food, pharmaceutical, cosmetic and tinctures industries (David, 1996; Pagliaro and Rossi, 2008). Escribà *et al*. (2011) described the former method (Fig 11) using glycerol to obtain allyl esters with interesting biological activity.

Allyl esters have several applications in perfume or cosmetic industry, pharmacy, resin manufacture, production of bioactive materials for agriculture and feeding (Dingfeng *et al*., 1998; Dewis and Hubner, 2006; Yoshinaka *et al*., 2009) and are also used as **food aromas** (Bauer *et al*., 2001; Burdock, 2010; http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:1999:084:0001:0137:ES:PDF).

In a first step, taking in consideration that several substances containgin an allyl group have been described as insecticides (Ojimelukwe and Adler, 1999; Leeleja *et al*., 2007) the assessment of a series of allyl esters from mid-chain fatty acids was successfully done on *C. pomonella* eggs (Escribà *et al*., 2009). This thesis started at this point with the aim to assess the role of allyl esters from different organic acids in pest control.

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New insecticide research has focused in finding substances able to be used in pest control so reducing the problems produced with the use of conventional insecticides (pollution, hazards on no-target organisms, development of resistant populations). Legal reduction of available active ingredients to be used in pest control enhances the relevance of this topic.

When assessing new substances in arthropod pest control, either as insecticides or as behaviour modifiers, several approaches can be used. A first screening of a series of compounds is needed in order to set the most active substances. Then, the study can be focused in the mode of action which is useful to discern the advantages of the substances when considering in pest control management.

The main objective of this thesis is to assess the insecticidal value of a series of allyl esters and try to discern their mechanism of insecticidal action. To achieve this purpose, five specific objectives were set:

❶ Assess the ovicidal and larvicidal action of allyl esters, by contact, on *Cydia pomonella*, *Grapholita molesta* and *Lobesia botrana*.

❷ Assess lethal and sublethal repellent effects of allyl esters, by contact and by ingestion, on *Acyrthosiphon pisum* and *Tribolium castaneum*.

❸ Assess the cytotoxicity of allyl esters on several insect cell lines and the insecticidal action by ingestion on *Spodoptera littoralis* larvae with the aim to get an approach to the mode of insecticidal action of allyl esters.

❹ Assess the insecticidal action of corresponding acids and dicloropropyl esters (precursors and intermediates in the synthesis of allyl esters) and compare these activities with the insecticidal action of the corresponding allyl ester to better understand the insecticidal action of that set of related molecules.

❺ Assess the action of allyl esters on *Cydia pomonella* and *Lobesia botrana* antenna and the effect on insect behaviour in order to discern if some allyl esters are able to be used into ethological control of these pests.

Insecticidal action of five allyl esters on eggs and larvae of three tortricid fruit pests: laboratory tests

Marta GINER, [1,2] Mercè BALCELLS [2,3] and Jesús AVILLA [1,2]

[1] Department of Crop and Forest Sciences, University of Lleida Lleida, Spain.

[2]Crop Protection Department, UdL-IRTA Center for R + D, Lleida, Spain.

[3] Department of Chemistry, University of Lleida, Lleida, Spain

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ABSTRACT

Several substances containing the allyl ester group in their structure have shown insecticidal properties. In the search for new insecticide molecules, five allyl esters from aromatic and heterocyclic acids (allyl 1-naphthoate, allyl 2-thiophenecarboxylate, allyl 2-furoate, allyl salicylate, and allyl cinnamate) were synthesized. Their toxicity on *Cydia pomonella* (L.), *Grapholita molesta* (Busck) and *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera: Tortricidae) eggs of different age, by topical application, and on neonate larvae, by exposition to topically treated diet, has been assessed.

When applied to less than 24-h old eggs, the most active compounds on the three species were allyl cinnamate, allyl 1-naphthoate, and allyl 2-thiophenecarboxylate (LC_{50} range: 2.4 – 7.9 mg/mL), while allyl salicylate was the least active one (LC_{50} range: 13.4 – 17.5 mg/mL). The most active ones killed the eggs in the earlier phases of development (white egg and red ring). In contrast to the action of allyl esters of fatty acids, none of the tested allyl esters affected the duration of the development of treated eggs. The mortality and the duration of development of surviving larvae were not affected. Allyl cinnamate was also the most active compound on neonate larvae (LC_{50} range: 3.1 – 6.1 mg/mL) of the three species.

As conclusion, allyl cinnamate is suggested to be the best insecticide candidate of this series of compounds.

Keywords: Allyl esters, ovicidal action, larvicidal action, *Cydia pomonella*, *Grapholita molesta*, *Lobesia botrana*.

INTRODUCTION

Cydia pomonella (L.), *Grapholita molesta* (Busck) and *Lobesia botrana* (Denis and Schiffermuller) (Lepidoptera: Tortricidae) are important pests of many fruit crops worldwide. *Cydia pomonella* is a key pest of apple, pear and walnut orchards; *G. molesta* is a key pest mainly of stone and pome fruit orchards; and *L. botrana* is a key pest of vineyards (ISPI, 2009).

Because these species are direct pests of high-value crops, several control methods have been developed against them (Charmillot *et al.*, 2001; Kovanci *et al.*, 2004; Angeli *et al.*, 2007; Dunkelblum, 2007; Srivostava *et al.*, 2009; Ioriatti *et al.*, 2009a, 2009b). Due to their economic importance and low tolerance levels, several insecticide treatments, either alone or in combination with other control methods, are usually needed each season to keep their populations below economic threshold levels.

Larvae of *C. pomonella* develop in fruits, those of *G. molesta* develop in shoots and fruits and those of *L. botrana* feed on fruits, so the larvae remain protected against insecticides inside the fruit or by clusters during most of their development. As the eggs of the three species are laid on leaves and fruits, chemical treatments against these pests target eggs and neonate larvae.

Organophosphates, carbamates, pyrethroids and other insecticides are successfully used to control these pests. However, many of these chemicals are harmful to humans and beneficial organisms, and can cause ecological disturbances (Devine and Furlong, 2007). Furthermore, the development of resistant populations (Pree *et al.*, 1998; Rodríguez *et al.*, 2011; http://www.irac-online.org/) implies the study of new compounds and more ecologically acceptable methods for controlling insect pests as part of integrated pest management (IPM) programs. Research on new insecticides includes the use of

extracts from plants (Isman, 2006), that are usually a mixture of several compounds. Some of them, including several esters, have shown insecticidal properties (Park *et al*., 2003). Moreover, some substances containing an allyl group are toxic, antifeedant or repellent against insects (Ojimelukwe and Adler, 1999; Peterson *et al.*, 2000; Huang *et al.*, 2002; Leelaja *et al.*, 2007).

Allyl esters can be synthesized from bio-diesel industry wastes with low economical value (Escribà *et al.*, 2011). In a previous study, some allyl esters of several fatty acids were synthesized (Escribà *et al.*, 2009) and their action on *C. pomonella* eggs was assessed. The action of the allyl esters was related to the length of the fatty acid alkyl chain. The most active ones produced 100% mortality at 10 mg/mL and increased the duration of the egg development (Escribà *et al.*, 2009). As continuation of this research, the aim of this work was to know the action of five allyl esters from aromatic and heterocyclic acids (allyl 1-naphthoate, allyl 2-thiophenecarboxylate, allyl 2-furoate, allyl salicylate, and allyl cinnamate) on *C. pomonella*, *G. molesta* and *L. botrana* eggs and neonate larvae.

MATERIALS AND METHODS

Insects

A *C. pomonella* population was collected from unsprayed apple tree orchards in 1993 in Lleida (northeast Spain). It has been kept since then as a laboratory population at the UdL-IRTA Center for R + D.

Grapholita molesta and *L. botrana* populations were obtained from mass-reared laboratory cultures from IEPVFA (Piacenza, Italy) and INRA (Bordeaux, France), respectively. Both have been reared for more than 10 years in their laboratories of origin and they have been reared at the laboratory of the UdL-IRTA Center for R + D since 2005 and 2007, respectively. Each species was reared on agar-based semisynthetic diets (Ivaldi-Sender, 1974, for *G. molesta*; Pons *et al.*, 1994, for *C.*

pomonella and *L. botrana*) at room temperature (22 ± 2 ºC) under a 16:8 (L:D) h photoperiod. Adults were kept in cylindrical rearing cages using wax paper as the egg-laying substrate.

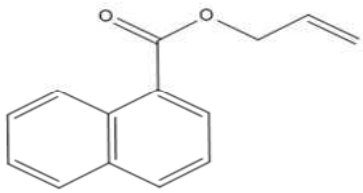
Allyl Esters

Allyl 1-naphthoate (**1**), allyl 2-thiophenecarboxylate (**2**) and allyl salicylate (**4**) (Fig 1) were synthesized following the general procedure described by Escribà *et al*. (2009) and identified by NMR spectroscopy using a VARIAN® AS400 spectrometer (400 MHz for ^{1}H). Allyl 2-furoate (**3**) and allyl cinnamate (**5**) (Fig 1) were purchased from Sigma-Aldrich (Madrid, Spain) (purity 98% and 99%, respectively).

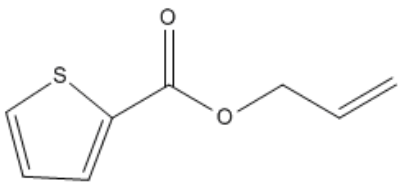
Mortality on less than 24-h Old Eggs

C. pomonella, *G. molesta* and *L. botrana* adults were allowed to oviposit during less than 24 h on wax paper. The eggs were then topically treated with 0.1 μL of the allyl ester dissolved in pure acetone (JR Baker, Deventer, Holland). An injection pump (Harvard Apparatus Model 11, Holliston, MA) equipped with 10 μL syringe (Hamilton, Reno, NV) with a fused silica needle was used for the application. Five concentrations of each allyl ester, in the interval needed to obtain mortality between 5 and 100%, were tested. Three replicates (30 eggs per replicate) were carried out at each concentration. A control (no treated eggs) and an acetone-control (treated with 0.1μL of pure acetone) were also done. For a replicate to be validated, the mortality of both controls had to be < 20%. Each replicate was kept in plastic Petri dishes (Ø= 9 cm, H = 2 cm) with a wet filter paper on the bottom, sealed with Parafilm to prevent egg drying, and kept in the same conditions as the insect stock culture. Eggs were checked daily for 10 days and egg mortality and egg developmental phase at death (white egg, red ring or black head) (Fig 2) was recorded.

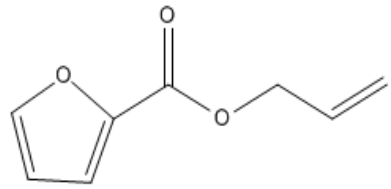
Figure 1: Chemical structures of the allyl esters tested.



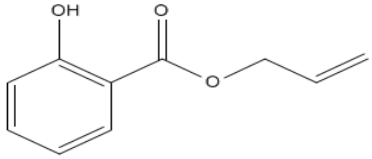
Allyl 1-naphthoate [**1**]



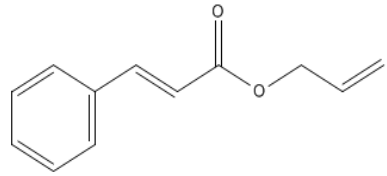
Allyl 2-thiophenecarboxylate [**2**]



Allyl 2-furoate [**3**]



Allyl salicylate [**4**]



Allyl cinnamate [**5**]

A probit analysis of the mortality vs. concentration was carried out using the Polo Plus® program, version 1.0 (Robertson *et al.*, 2003). Lethal concentrations (LC_{50} and LC_{90}) and their confidence intervals (CI) were calculated (95%). The

comparison of LC_{50} and LC_{90} was done using the overlapping CI as a criterion. Hypothesis of equality (equal slopes, equal intercepts) and hypothesis of parallelism (equal slopes) were tested using the same software to compare concentration–response lines among compounds and among species.

Duration of Egg Development

Less than 24-h old *C. pomonella*, *G. molesta* and *L. botrana* eggs were treated with species and ester respective LC_{50}, calculated in previous experiment. If the LC_{50} was higher than 10 mg/mL, a concentration of 10 mg/mL was used. Acetone-treated eggs were used as controls. Treated larvae were kept at 15 ± 0.5 ºC, to allow the enhancement of possible differences in the duration of egg development (Rock and Shaffer, 1983; Chaudhry, 1956; Tobin *et al*., 2001). For each allyl ester and species, three replicates of 30 eggs each were carried out. The eggs were checked daily to record the duration of their development. The mean development time of allyl ester-treated and acetone-treated eggs were compared by a Student *t*-test ($P < 0.05$) (SAS® Version 8; SAS Institute, Cary, NC, USA).

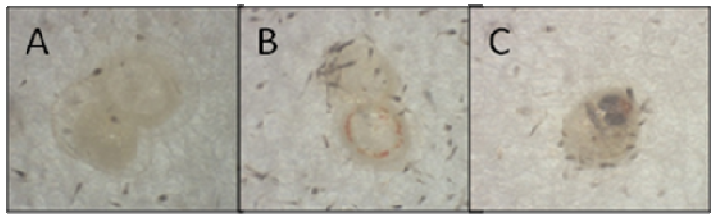
Delayed Effects

A minimum of 20 neonate larvae that had emerged from eggs treated with the LC_{50} or 10 mg/mL and from the respective control were transferred into plastic boxes (Ø = 5 cm, H = 2.5 cm) using a fine paintbrush and maintained on a piece of semisynthetic diet in the same conditions as insect stock culture. Elapsed time until adult emergence and percent of adults emerging from allyl ester-treated eggs were compared with those of the controls by a Student *t*-test ($P < 0.05$) (SAS® Version 8; SAS Institute, Cary, NC).

Mortality on Eggs of Different Phases

Aforementioned methodology was used to apply 0.1 µL of each allyl ester at a concentration of 10 mg/ml to *C. pomonella*, *G. molesta* or *L. botrana* eggs in three different egg developmental phases (white egg, red ring and black head) (Fig 2). Three replicates of 30 eggs each, were used for each allyl ester and egg phase for the three tested species. A binocular microscope was used to recognize the egg phases. Acetone-treated eggs were used as controls. Corrected mortality (Abbot, 1925) was analyzed by means of an ANOVA followed by Duncan's Multiple Range Test ($P < 0.05$) (SAS® Version 8; SAS Institute, Cary, NC).

Figure 2: Cydia pomonella egg development phases (A: white egg, B: red ring; C: black head).



Mortality on less than 24-h old larvae

U-shaped 0.5 ml wells of 96-well plates were half filled with solidified abar based semisynthetic diet. A metal tube was used to press on the diet to eliminate crevices between the diet and the wall of the well. Five µL of a solution of each allyl ester was applied on the top of the diet using a micropipette. Plates were kept at room temperature during 2 h for solvent evaporation. Then, a less than 24-h old larva was put in each well, the plate was sealed with Parafilm and kept in the same conditions as the insect stock cultures. Three replicates with 12 larvae per replicate were used for each allyl ester and species. A minimum of five concentrations were tested for each product. Wells treated with 5 µL of acetone were used as controls and the mortality recorded on them had to be less than

20% to validate the replicate. Mortality was checked 24 h and three days after treatment. A probit analysis was performed following the aforementioned procedure.

RESULTS

Synthesis of Allyl Esters

Yields obtained in the synthesis of allyl esters **1**, **2** and **4** were between 72 and 86%. The structures were confirmed by their NMR spectra [**1:** ^{1}H-NMR (400 MHz, $CDCl_3$): 4.84 (dt, *J* = 5.86 Hz, *J* = 1.56 Hz, 2H, O–C**H**$_2$), 5.25 (d, *J* = 10.55 Hz, 1H, **H**–C= *trans*), 5.38 (d, *J* = 17.2 Hz, *J* = 1.56 Hz, 1H, **H**–C= *cis,* C16), 6.03 (m, 1H, **H**C= *gem,* C15), 7.52 (m, *J* = 8.2 Hz, *J* = 6.6 Hz, 2H, **H**–C arom), 7.55 (m, *J* = 6.6 Hz, *J* = 1.56 Hz, 1H, **H**–C arom), 7.8 (d, *J* = 8.2 Hz, 1H, **H**–C arom), 7.94 (d, *J* = 8.2 Hz, 1H, **H**–C arom), 8.14 (dd, *J* = 7.03 Hz, *J* = 1.56 Hz, 1H, **H**–C arom), 8.85 (d, *J* = 8.6 Hz, 1H, **H**–C arom). **2**: ^{1}H-NMR (400 MHz, $CDCl_3$): 4.72 (d, *J* = 5.47 Hz, 2H, O–C**H**$_2$), 5.22 (d, *J* = 10.55 Hz, 1H, **H**–C= *cis*), 5.34 (d, *J* = 17.2 Hz, 1.56 Hz, 1H, **H**–C= *trans*), 5.95 (m, 1H, **H**–C= *gem*), 7.04 (t, *J* = 3.9 Hz, 1H, **H**–C ring), 7.49 (d, *J* = 5.1 Hz, 1H, **H**–C ring), 7.76 (d, *J* = 2.3 Hz, 1H, **H**–C ring). **4**: ^{1}H-NMR (400 MHz, $CDCl_3$): 4.78 (dt, *J* = 5.86 Hz, *J* = 1.56 Hz, *J* = 1.17 Hz, 2H, O–C**H**$_2$), 5.25 (d, *J* = 10.16 Hz, 1H, **H**–C= *cis*), 5.36 (d, *J* = 17.2 Hz, *J* = 1.56 Hz, 1H, **H**–C= *trans*), 5.96 (m, 1H, **H**–C= *gem*), 6.81 (t, *J* = 7.03 Hz, 1H, **H**–C arom, *para* to OH), 6.91 (d, *J* = 7.03 Hz, 1H, **H**–C arom, *ortho* to OH), 7.38 (m, *J* = 5.47 Hz, 1H, **H**–C arom, *meta* to OH), 7.81 (dd, *J* = 6.25 Hz, *J* = 1.56 Hz, 1H, **H**–C arom, *meta* to OH), 10.66 (s, 1H, OH)].

Mortality on less than 24-h Old Eggs

For each species, the hypotheses of equality and parallelism of the probit lines were rejected [*C. pomonella* (χ^2 = 585, d.f.= 8, *P* < 0.001 and χ^2 = 38.67, d.f.= 4, *P* < 0.001, for the hypotheses of equality and of parallelism, respectively); *G.*

molesta (χ^2 = 577, d.f.= 6, P < 0.001 and χ^2 = 92.21, d.f.= 3, P < 0.001); and *L. botrana* (χ^2 = 491, d.f.= 8, P < 0.001 and χ^2 = 30.62, d.f.= 4, P <0.001)]. For each allyl ester, the hypotheses of equality and parallelism of the probit lines were also rejected (**1**: χ^2 = 102, d.f.= 4, P < 0.001 and χ^2 = 55.96, d.f.= 2, P < 0.001; **2**: χ^2 = 66.49, d.f.= 4, P < 0.001 and χ^2 = 11.57, d.f.= 2, P = 0.003; **3**: χ^2 = 39.11, d.f.= 4, P < 0.001 and χ^2 = 44.57, d.f.= 2, P < 0.001; **4**: χ^2 = 13.97, d.f.= 4, P = 0.007 and χ^2 = 9.01, d.f.= 2, P = 0.011); **4**: χ^2 = 59.13, d.f.= 4, P < 0.001 and χ^2 = 10.01, d.f.= 2, P = 0.007).

Table 1 shows the results of all the probit analyses carried out. With only one exception, allyl cinnamate (**5**) and allyl 1-naphthoate (**1**) were the most active allyl esters tested, followed by allyl 2-thiophenecarboxylate (**2**). Allyl 2-furoate (**3**) and allyl salicylate (**4**) were the least active ones.

Table 2 shows the mortality of less than 24-h old eggs treated with 10 mg/mL of each allyl ester and the percentage of eggs that died at each developmental phase. Untreated eggs, acetone-treated eggs and eggs treated with the least active compound (allyl salicylate, **4**) died mostly in the black head stage. By contrast, the most active allyl esters (allyl cinnamate, **5**, and allyl 1-naphthoate, **1**) caused egg death in an earlier developmental phase (white egg or red ring phase).

Duration of Egg Development

A significant increase in the duration of egg development respect to acetone-treated control was only observed in *C. pomonella* eggs treated with allyl 2-thiophenecarboxylate [control: 13.0 ± 0.4 d; allyl 2-thiophenecarboxylate: 14.5 ± 0.1 d (t = 2.74, d.f. = 4, P = 0.01)]. No significant differences in duration of egg development of treated eggs compared with control (*G. molesta* = 10.8 ± 0.2

days, *L. botrana* = 10.5 ± 0.4 days) were observed in the rest of the cases ($P > 0.05$).

Table 1: Results of the probit analyses for < 24-h-old *C. pomonella*, *G. molesta* and *L. botrana* eggs topically treated with 0.1µL of allyl ester solutions. For the numbering of allyl esters see figure 1.

Species	Allyl ester	N	Slope ± SE	LC_{50} (CI) mg/mL	LC_{90} (CI) mg/mL	χ^2	HF
C. pomonella	1	630	5.5 ± 0.5	4.7 (4.4 - 5.1) **a**	8.1 (7.4 -9.1) **a**	16.5	0.8
	2	540	4.4 ± 0.5	7.9 (7.1 - 8.7) **b**	15.4 (13.4 - 19.0) **bc**	13.8	0.9
	3	540	8.6 ± 1.3	14.7 (13.3 - 16.0) **c**	20.6 (18.5 - 25.5) **c**	18.3	1.5
	4	450	3.6 ± 0.5	17.5 (14.4 - 25.8) **c**	47.1 (30.1 - 138.6) **d**	10.1	0.8
	5	450	3.3 ±0.3	3.8 (2.7 - 4.9) **a**	8.5 (6.4 - 15.5) **ab**	47.4	3.9
G. molesta	1	630	2.9 ± 0.3	4.4 (3.6 - 5.2) **a**	11.9 (8.9 - 20.8) **ab**	50.2	2.6
	2	720	3.5 ± 0.3	6.2 (5.5 - 6.8) **b**	14.3 (12.1 - 18.1) **a**	20.3	1.6
	3	450	8.9 ± 1.3	15.3 (14.2 - 16.6) **c**	21.4 (19.2 - 26.0) **bc**	11.2	1.1
	4	450	5.0 ± 0.6	14.1 (12.9 - 15.6) **c**	26.8 (21.1 - 38.2) **b**	10.3	0.9
	5	540	2.5 ± 0.2	3.3 (2.6 - 4.2) **a**	10.9 (8.4 - 15.9) **a**	33.2	2.2
L. botrana	1	630	2.1 ± 0.2	6.8 (5.9 - 7.9) **c**	27.2 (20.7 - 41) **b**	7.0	0.4
	2	450	2.4 ± 0.3	4.1 (3.2 - 5.0) **b**	13.8 (10.4 - 21.6) **b**	17.6	0.4
	3	450	4.4 ± 0.5	11.0 (8.7 - 15.2) **d**	21.6 (15.9 - 66.9) **b**	77.9	5.9
	4	450	4.0 ± 0.7	13.4 (11.2 - 17.8) **d**	28.0 (20.2 - 63.6) **b**	12.3	1.4
	5	450	3.5 ± 0.3	2.4 (2.1 - 2.7) **a**	5.6 (4.9 - 6.8) **a**	6.8	0.5

n= total number of treated eggs; CI= confidence intervals (95% probability) HF= heterogeneity factor. For each species, values followed by the same letter in the same column are not significantly different because of the overlapping of the confidence intervals ($P < 0.05$).

Table 2: Mortality (%) and percent distribution of the developmental phase at death of < 24-h-old *C. pomonella*, *G. molesta* and *L. botrana* eggs topically treated with 0.1 µL of a 10 mg/mL solution of allyl esters. For the numbering of allyl esters see figure 1.

Species	Treatment	Mortality (%)	Egg developmental phase at death (%)		
			White egg	Red ring	Black head
C.pomonella	Control	1.5 ± 0.5	15.0 ± 8.1	10.0 ± 4.3	75.0 ± 9.7
	Acetone	10.1 ± 0.6	20.8 ± 6.6	16.2 ± 6.1	63.0 ± 8.0
	1	95.5 ± 1.3	0.0 ± 0.0	58.0 ± 3.0	42.0 ± 8.0
	2	69.4 ± 4.0	0.0 ± 0.0	45. 3 ± 11.1	54.7 ± 11.1
	3	13.3 ± 1.1	17.9 ± 9.5	71.0 ± 17.0	11.1 ± 7.5
	4	15.1 ± 7.6	0.0 ± 0.0	10.0 ± 0.0	90.0 ± 0.0
	5	100.0 ± 0.0	29.6 ± 10.4	64.3 ± 7.1	6.1 ± 3.8
G. molesta	Control	4.4 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
	Acetone	7.6 ± 0.8	18.2 ± 12.5	7.6 ± 5.5	74.2 ± 12.7
	1	96.6 ± 0.1	27.5 ± 4.0	72.5 ± 4.6	0.0 ± 0.0
	2	65.3 ± 7.7	54.9 ± 17.5	23.6 ± 12.1	21.5 ± 8.5
	3	10.0 ± 2.9	0.0 ± 0.0	42.7 ± 26.0	58.3 ± 26.0
	4	24.2 ± 5.8	4.2 ± 2.0	15.0 ± 7.0	80.8 ± 10.8
	5	100.0 ± 0.0	91.3 ± 2.5	7.6 ± 3.4	1.1 ± 1.0
L. botrana	Control	5.0 ± 1.0	0.0 ± 0.0	1.0 ± 1.0	99.0 ± 4.9
	Acetone	5.2 ± 1.2	15.5 ± 12.1	1.5 ± 1.9	83.0 ± 12.0
	1	52.1 ± 3.1	24.2 ± 10.2	44.1 ± 14.2	31.6 ± 8.9
	2	51.3 ± 9.2	19.3 ± 7.4	40.4 ± 4.5	40.3 ± 6.4
	3	25.6 ± 1.7	44.8 ± 12.4	32.4 ± 3.8	22.9 ± 8.6
	4	42.5 ± 5.3	0.0 ± 0.0	3.3 ± 3.3	96.7 ± 3.3
	5	100.0 ± 0.0	77.6 ± 5.5	16.7 ± 2.6	5.7 ± 3.8

Delayed Effects

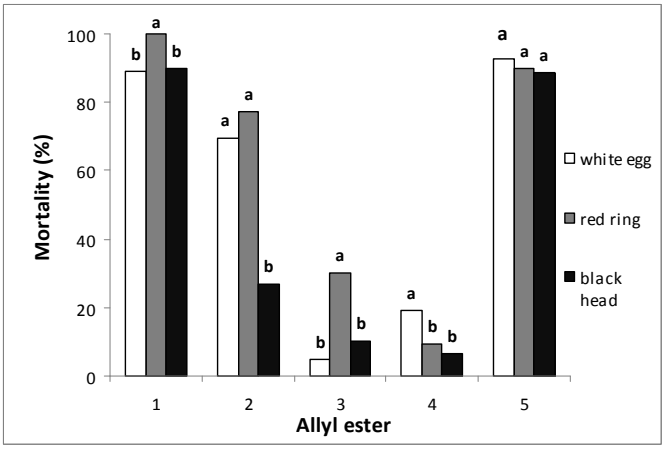
A significant reduction in percentage of adults emerging from surviving eggs was only observed when *L. botrana* eggs were treated with allyl 2-furoate (acetone-treated control: 78.5.5 ± 2.4 %; allyl 2-furoate: 58.0 ± 3.5 %; t = 3.93, d.f. = 15, P = 0.001). No significant differences were observed in the rest of the cases ($P \geq$ 0.05, percent of emergence in the controls: *C. pomonella* = 77.2 ± 1.2 %; *G. molesta* = 69.3± 1.7 %).

A significant reduction in time until adult emergence was observed only in *G. molesta* when treated with allyl 2-furoate [control: 23.9 ± 0.2 days; allyl 2-furoate: 21.6 ± 0.1 days (t = 4.57, d.f. = 28, P < 0.001)], whereas no significant differences were observed in the rest of the cases (P > 0.05, time to adult emergence: *C. pomonella*: 31.5 ± 0.4 days; *L. botrana*: 29.4 ± 0.8 day).

Mortality on Eggs of Different Phases

No general trend was observed in mortality produced by allyl esters when eggs were treated at different egg phases (Fig 2, 3, and 4). *Cydia pomonella* egg mortality was independent from the egg phase at which the allyl esters were applied only in the case of compound **5**; mortality was significantly higher in the white egg phase for compound **4,** and in the red ring phase for compounds **1** and **3**. The activity of compound **2** decreased significantly when it was applied in the black head phase (Fig 3).

Figure 3: Mean corrected mortality of *C. pomonella* eggs treated in different developmental phases with 0.1 µL of a 10 mg/mL solution of allyl esters. (3 replicates, n = 30 eggs). For the numbering of allyl esters see figure 1.

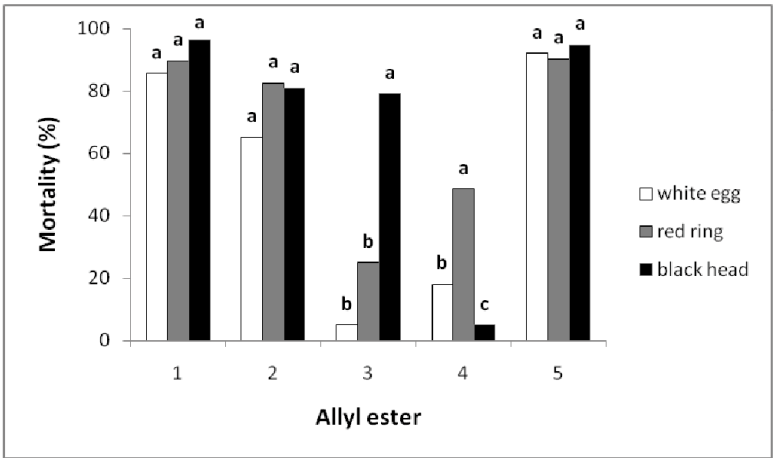


For each allyl ester, columns with the same letter were not significantly different (Duncan's multiple range test, $P < 0.05$).

Grapholita molesta egg mortality was also independent from the egg phase in the case of compounds **1**, **2** and **5**, but the action of compound **4** increased significantly when it was applied in the red ring phase, and the action of compound **3** increased significantly when it was applied in the black head phase (Fig 4).

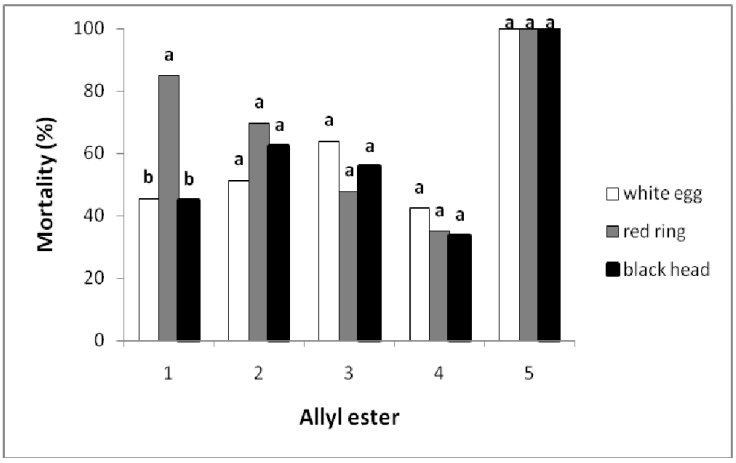
Lobesia botrana egg mortality was independent from the egg phase in the case of compounds **2**, **3**, **4** and **5,** but the action of compound **1** increased significantly if applied in the red ring phase (Fig 5).

Figure 4: Mean corrected mortality of *G. molesta* eggs treated in different developmental phases with 0.1 µL of a 10 mg/mL solution of allyl esters. (3 replicates, n=30 eggs). For the numbering of allyl esters see figure 1.



For each allyl ester, columns with the same letter were not significantly different (Duncan's multiple range test, $P < 0.05$).

Figure 5: Mean corrected mortality of *L. botrana* eggs treated in different developmental phases with 0.1 µL of a 10 mg/mL solution of allyl esters. (3 replicates, n=30 eggs). For the numbering of allyl esters see figure 1.



For each allyl ester, columns with the same letter were not significantly different (Duncan's multiple range test, $P < 0.05$).

Mortality on less than 24-h Old Larvae

Table 3 shows the results of the probit analysis when mortality was recorded at 24 h from treatment. The most active allyl ester was allyl cinnamate, whose LC_{50}'s for the three species were significantly lower than those of the other allyl esters tested, with the exception of the LC_{50} of allyl 2-thiophenecarboxylate for *L. botrana*. The probit line of allyl salicylate was not calculated, as it did not produce a significant mortality when tested at 10 mg/mL.

Hypotheses of equality and parallelism were rejected when all concentration–response lines of active allyl esters were compared (χ^2 = 495.0, d.f. = 24, P < 0.001 and χ^2 = 88.36, d.f. = 12, P < 0.001).

When species concentration–response lines were compared for each allyl ester, the hypothesis of equality was rejected in all cases (**1**: χ^2 = 33.17, d.f. = 4, P < 0.001; **2**: χ^2 = 30.49, d.f. = 4, P < 0.001; **3**: χ^2 = 78.96, d.f. = 4, P < 0.001; **5**: χ^2 = 37.86, d.f. = 4, P < 0.001). The hypothesis of parallelism was not rejected for compounds **1** and **5** (χ^2 = 2.04, d.f. = 2, P = 0.361; χ^2 = 0.78, d.f. = 2, P = 0.676, respectively) but it was rejected for compounds **2** and **3** (χ^2 = 22.76, d.f. = 2, P < 0.001 and χ^2 = 16.44, d.f. = 2, P < 0.001, respectively). When the comparisons were done per species, the hypotheses of equality and parallelism were rejected in *C. pomonella* (χ^2 = 196.0, d.f. = 6, P < 0.001 and χ^2 = 23.32, d.f. = 3, P < 0.001) and in *L. botrana* (χ^2 = 66.13, d.f. = 8, P < 0.001 and χ^2 = 16.31, d.f. = 4, P = 0.003) but not parallelism in *G. molesta* (χ^2 = 20.88, d.f. = 6, P = 0.002 and χ^2 = 4.54, d.f. = 3, P = 0.209).

No significant differences were observed when comparing LC values recorded 24 h and three days after the treatment, with the exception of allyl furoate on *C. pomonella,* where a significant reduction on LC values at 3 days respect 1 day was observed.

Table 3: Results of the probit analysis for < 24-h-old *C. pomonella*, *G. molesta* and *L. botrana* larvae exposed to diet topically treated with 5µL of allyl esters.

Species	Allyl ester	n	Slope ± SE	LC_{50} (CI) mg/mL	LC_{90} (CI) mg/mL	χ^2	HF
C. pomonella	1	250	2.5 ± 0.4	12.5 (10.1 - 16.1) **b**	41.6 (28.2 -88.8) **b**	22.3	1.2
	2	280	3.2 ± 0.5	9.4 (7.3 - 11.9) **b**	23.6 (16.8 - 55.2) **ab**	62.6	2.8
	3	360	7.6 ± 1.1	17.9 (16.3 - 19.5) **c**	26.4 (23.3 - 34.0) **b**	43.4	1.5
	5	320	3.4 ±0.4	6.1 (5.2 - 7.0) **a**	14.6 (12.5 - 18.2) **a**	20.9	0.8
G. molesta	1	180	3.0 ± 0.5	5.7 (4.2 - 7.3) **b**	15.1 (11.2 - 25.8) **b**	14.1	1.1
	2	180	3.1 ± 0.6	9.3 (7.5 - 11.5) **c**	23.9 (17.7 - 41.4) **b**	10.5	0.9
	3	280	5.1 ± 1.1	11.5 (9.0 - 13.1) **c**	20.6 (17.2 - 33.8) **b**	32.2	1.5
	5	210	3.3 ± 0.5	3.1 (2.5- 3.7) **a**	7.5 (6.1 - 10.3) **a**	16.4	0.9
L. botrana	1	180	2.2 ± 0.6	12.5 (10.0 - 27.4) **c**	48.7 (29.3 - 184.1) **b**	4.4	0.6
	2	210	1.3 ± 0.2	5.9 (3.5 - 9.5) **ab**	13.8 (10.4 - 21.6) **a**	17.0	1.2
	3	252	2.4 ± 0.4	10.8 (7.7 - 15.6) **bc**	31.8 (19.5 - 70.0) **ab**	69.2	3.1
	5	250	2.7 ± 0.4	4.8 (3.7 - 6.0) **a**	14.1 (9.9 - 29.8) **ab**	41.3	2.1

n= total number of treated larvae; CI= confidence intervals (95% probability); HF= heterogeneity factor. For each species, values followed by the same letter in the same column are not significantly different because of the overlapping of the confidence intervals ($P < 0.05$).

DISCUSSION

The allyl esters of aromatic or heterocyclic acids had not been previously tested as pesticides. As a whole, the ovicidal activity of the tested allyl esters followed the same trend in the three pest species tested: allyl cinnamate and allyl 1-naphthoate were more effective than the other three allyl esters. As the probit lines of all the allyl esters for the same species were not parallel, the results of the

comparison of their toxicity depend on the concentration. At the LC_{50} level, the toxicity of allyl cinnamate was 5 times higher than the toxicity of the less active one (allyl salicylate). The comparison of the slopes of the probit lines also suggests that the mode of action of the compounds may differ, what would be explained by differences in chemical structure of tested compounds. When compared to allyl esters of fatty acids, the substitution in the structure of the molecule of alkyl chains for aromatic or heterocyclic moieties entails decreased the activity on *C. pomonella* eggs, as the LC_{50} of the most active allyl esters of fatty acids was 0.71 mg/mL (Escribà *et al*., 2009). The less active compound also took longer to kill the eggs than the most active ones did, as the death occurred at the black head phase in the former ones, and in the earlier phases (white egg or red ring) in the latter ones. The same trend was observed in the case of allyl esters from fatty acids (Escribà *et al*., 2009). For the three species, the toxicity of the allyl esters was not clearly produced in a specific phase suggesting that the tested compounds do not act as IGR, as IGR compounds mainly kill eggs in the red ring or black head phase (Canela *et al.*, 2000; Charmillot *et al.*, 2001; Sáenz-de-Cabezón *et al.*, 2005; 2006).

In general, all compounds had a similar activity on the three developmental egg phases tested. This fact would eventually facilitate its application in the field. It was more evident in the case of the most active compound, allyl cinnamate (Fig 3, 4 and 5), suggesting a kind of suffocation produced in few time from application of allyl ester, as observed by other authors when using horticultural oils (Wins-Purdy *et al*., 2009).

In general, the duration of the development of treated eggs was not significantly different to that of the control ones, while allyl esters of fatty acids increased egg developmental time on *C. pomonella* (Escribà *et al.*, 2009). However, in the case of fatty acids allyl esters, although significantly different, the observed differences

were < 1 d, and they had not any biological importance. No delayed effects in the surviving larvae from treated eggs were observed, reinforcing the hypothesis that allyl esters have not any IGR action.

The larvicidal effect of the tested allyl esters showed the same trend discussed for the ovicidal effect: allyl cinnamate was the most active one, and allyl 2-furoate and allyl salicylate the least active ones for the three species. LC_{50} of allyl cinnamate to eggs or larvae was very similar, what would facilitate its use in the field. The methodology used simulated field conditions, because only the surface of the diet was treated, as the leaf and fruit surface would be, so the amount of compound needed to act by ingestion had to be low. Only significant increase of LC_{50} of allyl naphthoate larvicidal action respect ovicidal action on *C. pomonella* and *L. botrana* was recorded suggesting that main action on larvae took place by contact.

Concentrations needed to kill eggs and larvae of the three species, even in the case of the most active compounds, indicate that high amounts of compound are needed in all cases. So, the possibility of synthesizing this family of products from biodiesel industry wastes with low economical value, such as glycerol, could be an advantage (Escribà *et al*., 2011). Yields obtained in the synthesis were high, and in the same range of those reported for other allyl esters (Escribà *et al*., 2009; Eras *et al*., 2009). Additionally, most of the tested allyl esters have been described as food aromas and fragrances (Bhatia *et al*., 2004; Burdock, 2010), so low toxic effects on vertebrates may be expected. Another possible advantage would be the lack of cross-resistance. In a preliminary experiment, we tested the toxicity of allyl cinnamate on a *C. pomonella* population selected for resistance to Chlorpyrifos-ethyl, and it was not significantly different from its toxicity to the laboratory population, what suggests that cross-resistance is not probable to occur.

In conclusion, allyl cinnamate was the best candidate of the serie of allyl esters assessed against the three tortricid pests studied. It acted as ovicide and larvicide, at similar concentrations on the three species, what could be an advantage in areas where the species cohabit. However, more assays were required about allyl ester fate in environmental conditions.

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Insecticidal and repellent action of allyl esters against *Acyrthosiphon pisum* (Hemiptera: Aphididae) and *Tribolium castaneum* (Coleoptera: Tenebrionidae) by contact and ingestion

Marta GINER,[1,2] Jesús AVILLA,[1,2] Mercè BALCELLS [2,3] and Guy SMAGGHE [4]

[1] Department of Crop and Forest Science, University of Lleida, Lleida, Spain.

[2] Department of Crop Protection, UdL-IRTA Center. Lleida, Spain.

[3] Department of Chemistry, University of Lleida, Lleida, Spain.

[4] Department of Crop Protection, Ghent University, Gent, Belgium.

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ABSTRACT

In this project, three allyl esters from mid-chain fatty acids (allyl octanoate, allyl heptanoate and allyl hexanoate) and two from aromatic acids (allyl cinnamate and allyl 2-furoate) were investigated against two different pests. Lethal and sublethal repellent effects by topical and ingestion exposure were tested against the pea aphid *Acyrthosiphon pisum* (Harris) and the red flour beetle *Tribolium castaneum* (Herbst) as these represent two insect pest orders of Hemiptera and Coleoptera each with a specific feeding behaviour using piercing-sucking and biting-chewing mouthparts, respectively.

Significant mortalities against aphid nymphs were produced when fed via diet containing allyl cinnamate, allyl 2-furoate and allyl heptanoate at 0.1 mg/mL, and in dose-response tests allyl cinnamate was the most active with an LC_{50} of 0.03 (0.02-0.05) mg/mL. Although topical application on aphids was only effective at doses of 0.1 mg/ aphid for allyl cinnamate, beetles of *T. castaneum* adults were sensitive to all allyl esters assessed with LC_{50}'s ranging between 0.1 – 0.2 mg/insect.

In binary choice bioassays, none of the allyl esters produced repellent effects on *A. pisum* aphids, neither by ingestion (0.1 mg/mL in the diet) nor by contact (100 µg/cm^2 on filter paper). In contrast, all allyl esters, except allyl hexanoate, showed high to moderate repellence effects by contact on *T. castaneum* beetles at 10 µg/cm^2. Interestingly, the repellent activity of allyl cinnamate was equal to the commercial insect repellent *N,N*-diethyl-*m*-toluamide (DEET). The data obtained suggest a possible role of allyl esters in insect pest control.

Key words: allyl esters, repellence, toxicity, *Acyrthosiphon pisum*, *Tribolium castaneum*

INTRODUCTION

Acyrthosiphon pisum Harris (Hemiptera: Aphididae), the pea aphid, is a soft-bodied insect that feeds by sucking the phloem of plants on several species of legumes (Fabaceae). They can cause crop damage directly by feeding, and indirectly by transmission of pathogenic plant viruses and also favour the development of fungi on honeydew excreted by them (Dixon, 1998; The International Aphid Genomics Consortium, 2010).

Tribolium castaneum Herbst (Coleoptera: Tenebrionidae), the red flour beetle, can cause damage in stored grain and flour directly by feeding (Rees, 2004), and indirectly by contamination of stored products by own exuviate and by the secretion of several quinones (Hodges *et al*., 1996).

Use of chemicals is the most important method to control insect pests (Griffiths *et al*., 1989; Zettler and Arthur, 2000), but the intensive use of conventional insecticides has produced problems as development of resistant populations and negative effects on non-target organisms, the environment and human health (Zettler, 1991; Unal and Jepson, 1991; Ware *et al*., 2003; Andrews *et al*., 2004; Cao *et al*., 2008). These facts added to restrictions in use of insecticides (http://ec.europa.eu/food/plant/index_en.htm) support the importance of finding new substances able to be used in pest control in an environment-friendly way.

Plant derivatives have traditionally been used as insecticides, and new insecticide research is focusing on plant origin substances in order to avoid some negative aspects associated with conventional insecticides (Isman, 2006; Koul *et al*., 2008). Essential oils were successfully assessed as aphid and beetle toxicants and/or repellents (Petterson *et al*., 1994; Rozman *et al*., 2007; Zapata and Smagghe, 2010), but differences in composition depending on part of plant used, harvest time, and population origin is a problem to be solved before practical application (Oliveira *et al*., 2005; Muñoz-Bertomeu *et al*., 2007). Essential oils are mainly mixtures of several compounds that interfere in

several cell functions (Enan, 2005; Bakkali *et al*., 2008; Abdelgaleil *et al*., 2009; López and Pascual-Villalobos, 2010), which is described as a way to minimize the development of resistant populations (Yang *et al*., 2009; Machial *et al*., 2010). Some of these compounds belong to the ester chemical group (Daferera *et al*., 2000; Skaltsa *et al*., 2003; Bakkali *et al*., 2008), which is also present in several substances with insecticidal properties (Petterson *et al*., 1994; Ojimelukwe and Adler, 1999; Puterka *et al*., 2003, Rajendran and Muralidhoran, 2005).

As a part of an ongoing project aimed to re-use glycerol coming as a by-product from the biodiesel industry (www.biodiesel.org), several allyl esters were synthesized and their insecticidal properties on three Lepidopteran species were successfully assessed (Escribà *et al*., 2009; Chapter 1) and insect cell membrane fixed as major target for these chemistries (Chapter 3). Interestingly, these findings may open new opportunities to employ these compounds in the control of important pest insects also other than Lepidoptera.

This paper describes for the first time the toxicity and the repellent effect of allyl octanoate, allyl heptanoate, allyl hexanoate, allyl cinnamate and allyl 2-furoate against the pea aphid *A. pisum* and the red flour beetle *T. castaneum* as these represent two important insect pest orders of Hemiptera and Coleoptera each with a specific feeding behaviour using piercing-sucking and biting-chewing mouthparts, respectively. In the repellency experiments, we compared the potency with the commercial repellent DEET.

MATERIAL AND METHODS

Chemicals

Allyl octanoate, allyl heptanoate, allyl hexanoate, allyl cinnamate and allyl 2-furoate (≥97% purity), acetone of analytical grade and DEET (*N,N*-diethyl-*m*-toluamide, 98%)

were purchased from Sigma-Aldrich (Bornem, Belgium).

Insects

Insects were reared in the Department of Crop Protection at Ghent University (Belgium).

A continuous colony of the ***A. pisum*** was maintained on young plants of *Vicia faba* L. (Fabales: Fabaceae) at 23-25ºC and 65±5 % relative humidity (RH) under a 16:8 h L: D photoperiod (De Geyter *et al*., 2012). Less than 24-h old nymphs were used in toxicity and repellency bioassays by ingestion, while adults were used in toxicity and repellency bioassays by contact.

All stages of ***T. castaneum*** were reared on a 10:1 (w/w) mixture of wheat flour and brewer's yeast at standard conditions of 25-27°C and 65±5 % RH under a 16:8 h photoperiod and unsexed adults were used to carry out all the bioassays (Vandenborre *et al*., 2011).

In all toxicity bioassays, insects were considered dead when no leg or antennal movements were observed when gently prodded with a brush.

Insect bioassays

Toxicity by feeding

Toxicity of allyl esters to *A. pisum* by feeding was assessed using the aphid feeding apparatus described by Sadeghi *et al*. (2009a). Ten newborn (< 24-h old) first-instar nymphs were transferred to each aphid feeding apparatus and mortality and number of moults were daily recorded during three days, removing dead aphids. Three replicates were carried out for each concentration and at least five different concentrations per each allyl ester were done. In the control series the diet was supplemented with an equal amount of carrier solvent (acetone); the mortality needed to be < 20% to validate the replicate.

Probit analysis of mortality vs. concentration using POLO-Plus program version 1.0 (LeOra software, CA, USA) was carried out and lethal concentrations (LC_{50}, LC_{90}) and their corresponding 95% confidence intervals (95% CI) were calculated and LC's were considered to be significantly different when the 95% CI's did not overlap.

The mean number of moults of *A. pisum* at sublethal dose (0.01 mg/mL for allyl cinnamate and 0.1 mg/mL for the rest of allyl esters) was compared with the control by one-way ANOVA followed by Dunnett's test, $P < 0.05$) using the JMP 8.0.1 software (SAS Institute, Cary, NC).

Toxicity by contact

In the toxicity assays by contact, 0.1 µL of allyl ester solution was directly applied on the thorax of a *A. pisum* adult using a 10 µL syringe (Hamilton, Reno, NV) connected to an injection pump (Harvard apparatus model 11, Holliston, MA). The same methodology was used for *T. castaneum* adults, but then 0.5 µL of allyl ester solution was applied. An adequate control with acetone was done in both cases, and less than 20% mortality was required to validate the replicate. In all experiments with aphids and beetles, three replicates of ten insects per allyl ester and concentration were used, and at least five different concentrations were assessed per each active allyl ester. After the treatment, aphids were maintained in the aphid feeding apparatus containing non-treated diet and mortality was recorded 2 and 4 h after the application. After *T. castaneum* treatment, adults were maintained in glass Petri dishes (Ø=9 cm, H= 1.5 cm) provided with wheat flour, and mortality was recorded at 2 h, 24h and daily during a week.

Probit analysis of mortality vs. Concentration was done using POLO-Plus program version 1.0 (LeOra software, CA, USA) as abovementioned. LT_{50} (median lethal time) for the lowest dose producing 100% mortality at the seventh day was calculated using the abovementioned program.

Repellency by ingestion and contact

Two binary choice bioassays were carried out in order to discern whether allyl esters had a repellent effect on *A. pisum* by feeding or by contact.

The experimental unit used to assess the repellency on aphid nymphs caused by feeding consisted of two joined aphid feeding apparatus (Sadeghi *et al*., 2009). The methodology was similar to the one described by De Geyter *et al*. (2012). Five aphids were put in each part of aphid feeding apparatus, one containing untreated diet (aphid diet + acetone) and the other containing treated diet (aphid diet + allyl ester solution). Percentages of repellence (PR) were scored at 1, 2, 4 and 24 h from the beginning of the assay using the following formula: PR = [(C–T)/(C+T)] x 100; where C=number of insects in untreated area and T=number of insects in treated area (Zapata and Smagghe, 2010). One experiment consisted of six replicates per allyl ester and per concentration (0.01, 0.1 and 0.5 mg/mL), and the experiment was repeated three times.

The experimental unit used to assess the repellency on aphid adults caused by contact consisted of glass Petri dishes (Ø= 5.0 cm, H= 1 cm) covered with filter paper (Ø= 4.5 cm) cut in two halves. One filter paper was treated with 100 µL of allyl ester solution and the other one with the same volume of acetone. Petri dishes were kept into an air flume during 5 min for solvent evaporation. Then ten aphid adults were released on the centre of the Petri dish. The PR was calculated at 1 and 2 h from the beginning of the assay as abovementioned. One experiment consisted of six replicates per allyl ester and dose (10, 100 and 1000 µg/cm^2), and the experiment was replicated three times.

Repellence by contact on beetle adults of *T. castaneum* was evaluated by a binary choice bioassay, that was similar to the one used for *A. pisum* but we used here a 9-cm-diameter glass Petri dish as described before by Zapata and Smagghe (2010). In brief, each half of a filter paper (Ø= 8.0 cm) was treated with 0.5 mL allyl ester solution dissolved in acetone and the other half with solvent alone. Ten unsexed adults were

transferred into the centre of each Petri dish after evaporation of solvent and PR was scored at 2, 4, 8 and 24 h from the beginning of the assay. One experiment consisted of six replicates per allyl ester and dose (1, 10, 100 and 1000 µg/cm^2), and the experiment was repeated three times.

In both contact bioassays, a positive control using the known insect repellent DEET (Watson and Barson, 1996; Ditzen *et al*., 2008) at 10 µg/cm^2 was performed.

Mean PR (n=3) per treatment and concentration per each species were compared by one-way ANOVA, followed by Tukey HSD test (P = 0.05) and the same was done per each treatment by time. Mean PR values of *T. castaneum* at 10 µg/cm^2 were compared to DEET by one-way ANOVA followed by Dunnett's test (P = 0.05). JMP 8.0.1 software (SAS Institute, Cary, NC) was used to carry out the statistical analysis.

RESULTS

Toxicity by ingestion

Allyl cinnamate, allyl furoate and allyl heptanoate were active against *A. pisum* nymphs by ingestion. Based on LC_{50} and LC_{90} values, allyl cinnamate was the most active being 10 times (ranging between 6 and 16) more toxic than allyl furoate and allyl heptanoate (Table 1).

Mortality produced by allyl octanoate or allyl hexanoate was < 20%, even at the maximum concentration tested (1 mg/mL) and no more assays were performed.

Table 1: Toxicity of allyl esters on newborn (0-24 h old) nymphs of the pea aphid (*Acyrthosiphon pisum*) after feeding for 3 days on artificial diet supplemented with different concentrations of allyl esters.

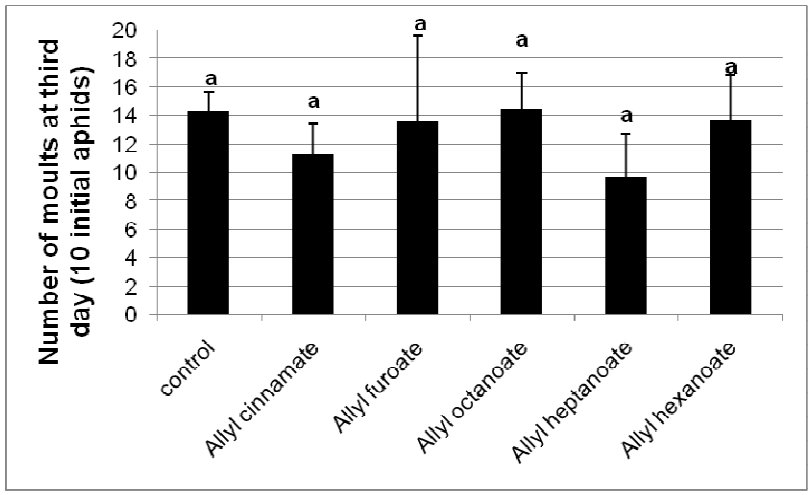
Allyl ester	LC_{50} (95%CI) (mg/mL)	Ratio	LC_{90} (95%CI) (mg/mL)	Ratio	Slope ± SE	χ^2	HF
Allyl cinnamate	0.03 (0.02 - 0.05) **a**	-	0.18 (0.10 - 0.48) **a**	-	1.66 ± 0.21	24.9	1.66
Allyl 2-furoate	0.21 (0.06 - 0.42) **b**	7	2.90 (1.1 - 29) **b**	16	1.14 ± 0.25	34.4	2.15
Allyl heptanoate	0.31 (0.21- 0.43) **b**	10	1.16 (0.68 - 5.7) **b**	6	2.23 ± 0.43	17.8	1.62

Data are given as 50% (LC_{50}) and 90% (LC_{90}) lethal concentration values together their respective 95% confidence interval (95%CI) (both in mg/mL) and the slope ± SE of the toxicity-concentration curve, and the χ^2 and heterogeneity factor HF (χ^2/d.f.) as accuracy of data fitting to probit analysis in POLO-PC. Different letters in the same column indicate significant differences due to overlapping of 95%CI.

Interestingly, mortality was produced rapidly. Most of the lethal effects were obtained during the first day. For instance, mortality was already 87% with 0.7 mg/mL of allyl cinnamate after 1 day and this increased to 100% at day 3; and similarly for allyl furoate (52±12% and 68±11%, respectively) and for allyl heptanoate (53±13% and 72±13 , respectively).

In addition when aphids were exposed to sublethal doses, no apparent effects were observed. There were no differences ($P > 0.05$) in the numbers of moults in treated aphids as compared to the controls, indicating that there was no evident retardation of aphid development after feeding of allyl esters (Fig 1).

Figure 1: Number of pea aphid (*A. pisum*) moults during a period of three days when newborn nymphs (< 24 h old) were feeding on diet containing allyl esters at sublethal dose.



Error bars indicate SEM (n=3). Different letters indicate significant differences between treatment and control (one-way ANOVA, Dunnett's test, $P < 0.05$)

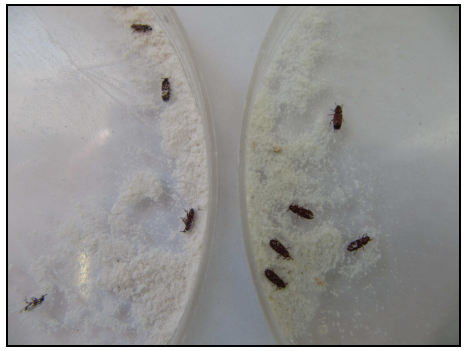
Toxicity by topical application

In this bioassay when the five allyl esters were dosed topically at 30 µg per adult pea aphid, only allyl cinnamate produced a significant toxicity with 75±7% mortality. Then in dose-response bioassays, the LC_{50} was calculated to be 27 (25-29) µg/aphid (χ^2= 18.1; d.f.= 12; HF= 1.51) at 4 h after topical application. The other four allyl esters did not produce a significant toxicity (<20%) when dosed at 30 µg per adult aphid, and therefore no other insect dose-response assays were performed.

With the adult beetles of *T. castaneum*, the allyl esters produced significant effects when dosed topically. Significant high mortalities of 45-100% were scored at 24 h from application of 0.24 mg per beetle adult. Based on the calculated LC_{50} and LC_{90} values, the most active was compound was allyl octanoate (0.10 and 0.16

mg/insect, respectively), but allyl cinnamate and the two aromatic allyl heptanoate and allyl hexanoate posed the same order of activity (Table 2). It was observed that flour in contact with *T. castaneum* previously treated with allyl esters turned pinkish, but this was not the case with the flour being in contact with untretaed *T. castaneum* in the controls (Fig 2).

Figure 2: Flour colour changed when contact with *T. castaneum* topically treated with allyl esters but not with no-treated *T. castaneum*. Left side: treated *T. castaneum* (pinkish flour); Right side: untreated *T. castaneum* (yellow flour).



The LC_{50} at 24 h was significantly smaller than the LC_{50} at 2 h for allyl esters of aromatic acids [LC_{50} at 2h, allyl cinnamate: LC_{50}= 0.34 (0.30-0.40) and allyl furoate: LC_{50}= 0.60 (0.46-1.18) mg/insect] (see Table 2), but no significant differences were observed from 24 h onwards.

The LT_{50} (time needed to produced 50% mortality) at 0.25 mg/insect doses were lower than 2 h for allyl cinnamate and allyl heptanoate. To allyl furoate, allyl octanoate and allyl hexanoate a higher dose was needed to produce 50% mortality in less than two hours.

Table 2. Toxicity of allyl esters on adults of the red flour beetle (*Tribolium castaneum*) at 24 h after topical application.

Allyl ester	LC_{50} (95%CI) (mg/insect)	Ratio	LC_{90} (95%CI) (mg/insect)	Ratio	Slope ± SE	χ^2	HF
Allyl cinnamate	0.20 (0.16 - 0.22) **b**	2	0.28 (0.24 - 0.30) **ab**	1.8	9.57 ± 2.38	7.93	0.61
Allyl 2-furoate	0.20 (0.14 - 0.26) **b**	2	0.46 (0.34 - 0.94) **b**	3	3.43 ± 0.52	27.12	2.09
Allyl octanoate	0.10 (0.08 - 0.12) **a**	-	0.16 (0.14 - 0.26) **a**	-	5.34 ± 1.00	16.85	1.40
Allyl heptanoate	0.14 (0.10- 0.18) **ab**	1.4	0.32 (0.24 - 0.70) **ab**	2	3.47 ± 0.49	27.85	2.12
Allyl hexanoate	0.20 (0.18 - 0.24) **ab**	2	0.30 (0.26 - 0.44) **ab**	1.9	7.33 ± 1.36	20.77	1.84

Data are given as 50% (LC_{50}) and 90% (LC_{90}) lethal concentration values together their respective 95% confidence interval (95%CI) (both in mg/insect) and the slope ± SE of the toxicity-concentration curve, χ^2 and heterogeneity factor HF (χ^2/d.f.) as accuracy of data fitting to probit analysis in POLO-PC. Different letters in the same column indicate significant differences due to overlapping of 95%CI.

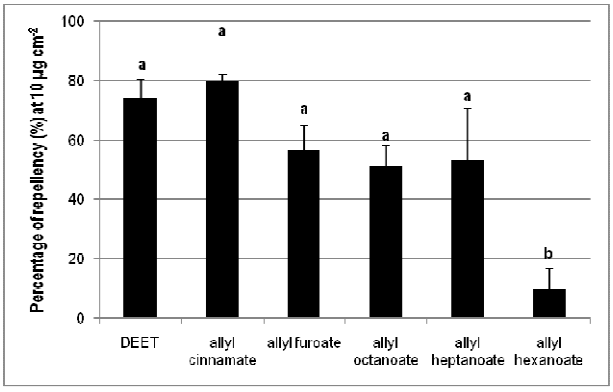
Repellent action

When aphid adults of *A. pisum* were exposed to 0.1 mg/mL in the diet, the repellency was low with PR values of < 20%. The same fact was observed by contact and only with allyl cinnamate the repellency was somewhat significant (PR= 34±12%). In any case PR increased with the increase in time of exposure ($P < 0.05$). Conversely, DEET produced a significantly higher PR of 50±10% at 10 µg/cm^2 ($P < 0.05$).

In contrast to aphids, we scored significant repellent activities with the beetle adults of *T. castaneum* by the five allyl esters at 10 µg/cm^2 after 4 h from the beginning of the assay (Fig 3). No significant differences in PR between DEET and four of the allyl esters were recorded. The strongest repellent effect was scored with allyl cinnamate demonstrating a PR value of 80±2% (Fig 3). In

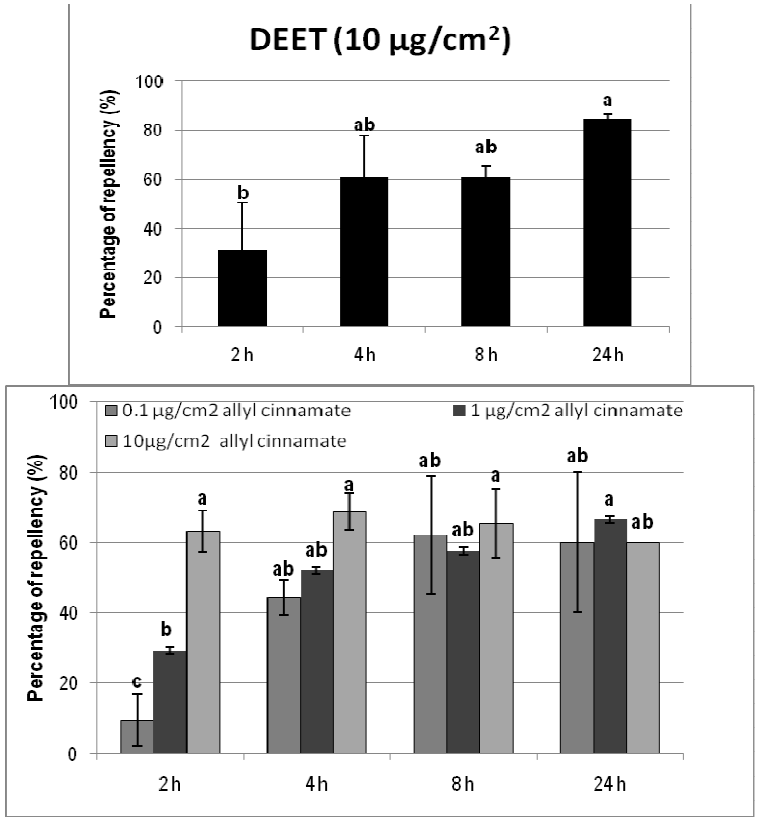
addition, it was of interest that the effect was rapid. As shown in Figure 4, maximum repellency was obtained already after 2 h with a concentration of 10 µg/cm^2 and this was after 8 h with the lowest concentration tested (0.1 µg/cm^2) (*P*=0.003, d.f.=3; F=16.9). For the commercial repellent DEET, the data confirmed its potency with a PR value of 60±10% at 4 h after the application of 10 µg/cm^2 (Fig 3). Hence, the repellency by DEET at 10 µg cm^{-2} increased to 84±2 % at 24 h, which was significantly equal (*P*>0.05) to the repellency effect produced by allyl cinnamate at 0.1, 1 and 10 µg/cm^2 (Fig 4).

Figure 3: Mean percent of repellency (PR) in red flour beetle adults (*Tribolium castaneum*) after 4 h in a binary choice bioassay by contact with surfaces treated with allyl esters and DEET at 10 µg/cm^2.



Error bars indicate SEM (n=3). Different letters indicate significant differences between PR produced by DEET and each allyl ester (one-way ANOVA, Dunnett's test, $P < 0.05$).

Figure 4: Repellency produced by DEET (10 µg/cm²) and allyl cinnamate at different concentrations (0.1, 1 and 10 µg/cm²) in red flour beetle adults (*Tribolium castaneum*) after 2 to 24 hours from the start of the bioassay.



Different letters indicate significant differences between percentages of repellency (PR) recorded at different time (DEET) and PR between doses of allyl cinnamate at different time (Tukey HSD test, $P < 0.05$).

DISCUSSION

To our knowledge, this is the first report about insecticidal action of allyl cinnamte, allyl furoate, allyl octanoate, allyl heptanoate and allyl hexanoate on *A. pisum* and *T. castaneum*. This action includes effects on mortality and repellency, depending on allyl ester and mode of application.

While only allyl cinnamate showed a significant topical insecticidal action on adults of *A. pisum*, all of the allyl esters assessed were active against *T. castaneum*. Interestingly, beetle defensive reaction after allyl ester application was observed; only flour in contact with treated *T. castaneum* became pinkish, what is a fact described by the reaction of quinones from defensive substances segregated by *T. castaneum* with starch (Alexander and Barton, 1943; Hodges *et al*., 1996). This fact was also observed by García *et al*. (2005) after the monoterpene β-pinene application. The topical insecticidal action took place in few time from application, similarly as observed when assessing allyl esters on Lepidopteran species (Escribà *et al*., 2009; Chapter 1). This rapid effect is related with a direct action on cell membranes (Cohen and Quistad, 1998) which was previously ascertained on insect cells after allyl ester application (Chapter 3).

By contrast, high toxicity of allyl esters by ingestion was recorded on *A. pisum*. LC_{50} values scored after aphid feeding were in the same range as obtained by other authors when assessing protease inhibitors, saponins, kinins, lectins or pyrokinin analogs using the same methodology (Sadeghi *et al*., 2009b; Carrillo *et al*., 2011, Smagghe *et al*., 2010a; De Geyter *et al*., 2012; Nachman *et al*., 2012). This fact gives relevance to the allyl ester action by ingestion, what is in relation to higher cytotoxicity observed on insect midgut cells (Chapter 3).

As no differences in moulting were observed among treated and control aphids from feeding bioassay, no antifeedant activity or an effect in aphid development was suggested to be produced by allyl esters, as observed on Lepidopterans after allyl ester ingestion (Chapter 1). A direct effect in the insect gut would be suggested as target of allyl ester action, similarly as produced by saponins on aphids (De Geyter *et al*., 2012).

Regarding to repellent properties of allyl esters, these were not active against *A. pisum* by contact or ingestion. The significant high values of PR scored to DEET

and the fact that PR did no increase along time, as observed by other authors when assessing aphid repellents (Bruce *et al*., 2005; Lowery and Isman, 1993; Smagghe *et al*., 2010b), validate the methodology used and reaffirmed the lack of deterrent effect produced by allyl esters on *A. pisum*. Lack of deterrency would limit the allyl ester use in aphid control, as they would not prevent virus transmission. Even though a lower amount of allyl cinnamate was needed to cause insect death by ingestion, the insect gut tissue is an interestingly target for pest control (Hakim *et al*., 2010).

On the contrary, a strong repellent effect by allyl esters was observed against *T. castaneum*. Although not before proven, it can be said that this could be expected due to the fact that some straight short-chain fatty acids and some monoterpenoids have previously been described as beetle repellents (Cohen *et al*., 1974; Ukeh and Umoetok, 2011). Allyl cinnamate was the most active allyl ester assessed, being active in the same dose-ranges as some essential oils (García *et al*., 2005; Zapata and Smagghe, 2010) and DEET. These results joined to the fact that the action increased with longer exposures, suggests a potential role of allyl cinnamate to control *T. castaneum* pest insects.

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Toxicity of allyl esters in insect cell lines and in *Spodoptera littoralis* larvae

Marta GINER,[1,2] Jesús AVILLA,[1,2] Mercè BALCELLS,[2,3] Silvia CACCIA[4] and Guy SMAGGHE[4]

[1] Department of Crop and Forest Sciences, University of Lleida, Lleida, Spain.

[2] Department of Crop Protection, UdL-IRTA Center, Lleida, Spain.

[3] Department of Chemistry, University of Lleida, Lleida, Spain.

[4] Department of Crop Protection, Ghent University, Ghent, Belgium.

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ABSTRACT

We investigated the effects of five allyl esters, two aromatic acids (allyl cinnamate and allyl 2-furoate) and three fatty acids (allyl hexanoate, allyl heptanoate and allyl octanoate) in established insect cell lines derived from different species and tissues. We studied embryonic cells of the fruit fly *Drosophila melanogaster* (S2) (Diptera) and the beet armyworm *Spodoptera exigua* (Se4) (Lepidoptera), fat body cells of the Colorado potato beetle *Leptinotarsa decemlineata* (CPB) (Coleoptera), ovarian cells of the silkmoth *Bombyx mori* (Bm5) and midgut cells of the spruce budworm *Choristoneura fumiferana* (CF203) (Lepidoptera). Cytotoxicity was determined with use of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and trypan blue. In addition we tested the entomotoxic action of allyl cinnamate against *Spodoptera littoralis*.

The median cytotoxic concentrations (EC_{50}s) of the five allyl esters in the MTT bioassays ranged between 0.25-27 mM with significant differences among allyl esters ($P = 0.0012$), cell lines ($P < 0.0001$), and the allyl ester - cell line interaction ($P < 0.0001$). Allyl cinnamate was the most active product, and CF203 the most sensitive cell line. In the trypan blue bioassays, cytotoxicity was produced rapidly and followed the same trend observed in the MTT bioassay. In first instars of *S. littoralis*, allyl cinnamate killed all larvae at 0.25% in the diet after 1 day, while this happened in third instars after 5 days. The LC_{50} in first instars was 0.08%. In addition, larval weight gain was reduced ($P < 0.05$) after 1 day of feeding on diet with 0.05%.

In conclusion, the data provide evidence of the significant but differential cytotoxicity among allyl esters in insect cells of different species and tissues. Midgut cells show high sensitivity, indicating the insect midgut as a primary target tissue. Allyl cinnamate caused rapid toxic effects in *S. littoralis* larvae at low concentrations, suggesting further potential for use in pest control.

Key words: allyl esters, cytotoxicity, entomotoxicity, *Spodoptera littoralis*

INTRODUCTION

Insects cause important economic damage in crops worldwide. Many methods are available to control pests with chemical insecticides being the main alternative used to minimize losses caused by them. However, problems associated to the application of chemical insecticides, such as development of resistant populations, environmental disturbances, and health concerns are increasing over time. In addition, the number of available insecticides has been reduced by law in many countries with the consequence that it is now essential to find new compounds able to be used as insecticides (http://www.irac-online.org; http://ec.europa.eu/food/plant/index_en.htm; Pimentel and Greiner, 1997; Alford, 2000; Paoletti and Pimentel, 2000; Ware *et al*., 2003).

In the last decades, research towards the development of new insecticides has focused on substances of plant origin; for example essential oils have shown good insecticidal properties (Isman, 2006). However, their practical application is reduced by differences in composition of essential oils between plant species and populations and phase of plant growth (Zygadlo and Juliani, 2003; Oliveria *et al*., 2005; Muñoz-Bertomeu *et al*., 2007).

Essential oils are mainly mixtures of different compounds, some of which belong to the ester chemical group (Daferera *et al*., 2000; Skaltsa *et al*., 2003; Bakkali *et al*., 2008) which had shown insecticidal properties (Ojimelukwe and Adler, 1999; Peterson *et al*., 2000; Leelaja *et al*., 2007; Escribà *et al*., 2009). Moreover, no adverse effects towards human health or the environment are expected because several of these products are already approved for use as fragrances and food flavors (Bauer *et al*., 2001; Burdock, 2010). In addition, it seems that the mode of action is based on disruption of several cell functions which is described as a way to control resistant pest insect populations (Yang *et al*., 2009; Marchial *et al*., 2010; Perumalsamy *et al*., 2010).

The aim of this project was to better understand the toxicity of five allyl esters on five different insect cell lines from different insect orders (Lepidoptera, Diptera and Coleoptera) and tissue origins (embryo, ovary, fat body and midgut). We employed two differential measures of cell viability with use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and trypan blue, in order to screen these products as new anti-insect compounds and to better understand their cytotoxicity for use in pest control. In addition we tested the entomotoxic action of allyl cinnamate against larval stages of the cotton leaf worm, and investigated the lethal and sublethal effects in first and third instars when mixed into the diet. The cotton leafworm *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) was used here as a representative of the major group of Lepidoptera as pest insects in agriculture, but also itself as an important cosmopolitan pest, causing high losses in agriculture due to its polyphagous characteristics with >40 host plants and because many populations show high levels of resistance to multiple all insecticide groups (Alford, 2000).

MATERIALS AND METHODS

Chemicals

Allyl cinnamate, allyl 2-furoate, allyl octanoate, allyl heptanoate and allyl hexanoate (all ≥97% pure) were purchased from Sigma-Aldrich (Madrid, Spain). Other products were of analytical grade unless otherwise mentioned.

Insect Cell Lines

All insect cell lines were maintained at 27°C and 80% relative humidity (RH) in the Laboratory of Agrozoology at Ghent University (Belgium). S2 cells [Schneider 2; embryonic cells of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae)] were grown in SFX-Insect medium (HyClone® Thermoscientific, South Logan, UT), Se4 cells [BCIRL-SeE-CLG4; embryonic cells of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae)] in IPL41 medium (GIBCO-Invitrogen, Paisley, UK)

supplemented with 10% of heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich, Bornem, Belgium), Bm5 cells [ovarian cells of *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae)] in ExCell 420 medium (SAFC™ Biosciences, Hampshire, UK) with 10% FBS, CPB cells [BCIRL-Lepd-SL1, fat body cells of *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae)] in ExCell 420 medium with 5% of FBS, and CF203 cells [FPMI-CF-203; midgut cells of *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae)] in InsectXpress medium (BioWittacker, Verviers, Belgium) with 2.5% of FBS, as previously described (Swevers *et al*., 2003; Decombel *et al*., 2004; Mosallanejad, 2008; Soin *et al*., 2009; Shahidi-Noghabi *et al*., 2010).

Treatment of Insect Cells with Allyl Esters

For determining the effects of allyl esters on insect cells, the cells were treated with allyl esters at final concentrations ranging from 0.0001 to 50 mM (prepared in acetone concentrations, 1%, w/v). In the controls, cells were exposed to the corresponding acetone concentration responded similar to untreated controls. Cell solutions were prepared at a density of $4x10^5$ cells per mL for S2, Se4 and CPB cells, and $2x10^5$ cells per mL for Bm5 and CF203 cells. In brief, 50 µL of cell culture medium together with 1 µL of the compound solution were added per well in a 96-well microtiter plate (Greiner Labortechnik, Frickenhausen, Germany). Subsequently, 50 µL of the respective cell suspension was added into the well. The plates were sealed with parafilm and incubated for 24 h or 96 h at 27°C. Three replicates were done for each concentration, and each experiment was repeated three times.

MTT Cell Viability Bioassay

The viability of the treated cells was determined in accord to Decombel *et al*. (2004). Briefly, after treatment and incubation for 24 or 96 h as described above, the 100 µL of cell suspension in the well was transferred to an Eppendorf microtube and 100 µL of a 1 mg/mL-MTT solution (Sigma-Aldrich, Bornem,

Belgium) was added. After 3 h incubation at 27°C, the formazan crystals were collected by centrifugation for 7 min at 20,000 g at 4°C, the supernatant was removed and the formazan crystals were dissolved in isopropanol. For the next 30 min, the microtubes were rotated using a test tube rotator (Labinco, Breda, the Netherlands). After centrifugation of the resulting solution for 7 min at 20,000 g, 200 µL supernatant out of each Eppendorf tube was transferred into a transparent 96-well plate (one sample per well) and the absorbance was measured at 560 nm in a microtiter plate reader (PowerWare X340; Bio-Tek Instruments Inc., Winooski, VT).

Results are presented as percentage of loss of cell viability compared to the control series with each allyl ester and cell line and then compared by two-way ANOVA followed by a *post hoc* Bonferroni's multiple comparison test ($P \leq 0.05$) using Prism v4 (GraphPad Software Inc., La Jolla, CA). In addition, the significance of difference between percentages of cell viability loss per cell and allyl ester after 24 and 96 h of incubation was analyzed with a Student's *t*-test ($P \leq 0.05$).

In cases where > 50% loss of cell viability at one day was observed at 50 mM, then a dose response curve was estimated from a minimum of five different concentrations in order to calculate median (50%) inhibitory concentrations (EC_{50}) and corresponding 95% confidence intervals (95% CI) in Prism v4; the accuracy of data fitting to the sigmoid curve model was evaluated through examination of R^2 values and EC_{50} comparisons were done using the overlapping of 95% CI as a criterion.

Trypan Blue Cell Viability Bioassay

The trypan blue method was performed in accord to Oh *et al*. (2004). Briefly, after treatment and incubation as described above, 10 µL of cell solution was mixed with 10 µL 0.4%-trypan blue solution (Sigma-Aldrich) and incubated for 3 min. The number of blue (dead) and white (live) cells were counted in a Bürker cell

chamber counter, and the percentage of dead cells was calculated. In control series, the loss of cell viability was <10%. Three replicates were done for each concentration.

The percentage of loss of cell viability was determined for the five allyl esters at 50 mM in the five insect cell lines. Data were analyzed with two-way ANOVA followed by a Bonferroni's multiple comparison test ($P < 0.05$). In addition, the significance of difference between percentages of cell viability loss per cell and allyl ester as determined with trypan blue and the corresponding MTT data after 24 h of incubation was analyzed with a Student's *t*-test ($P < 0.05$).

In case >90% cytotoxicity was scored at 50 mM, the percentage of dyed cells at different short incubation periods, for instance ranging between 1 min and 24 h, was investigated. The median (50%) response time (ET_{50}) which is the time of incubation needed to induce cytotoxicity in 50% of the cells exposure to a specific concentration of compound and the corresponding 95%CI were calculated with use of Prism v4; the accuracy of data fitting to the sigmoid curve model was evaluated through examination of R^2 values and the comparison of ET_{50} values was done using the overlapping of 95%CI as a criterion.

Insects

A continuous colony of the cotton leafworm *S. littoralis* was maintained on an agar-based artificial diet (Iga and Smagghe, 2011) under standardized conditions of 23-25ºC; 60-70% relative humidity and a 16:8 (light:dark) photoperiod at the Laboratory of Agrozoology (Ghent University, Belgium).

Insect Bioassay with Allyl Cinnamate

Newborn (0-6 h) first and third instars of *S. littoralis* were fed on Stonefly Heliothis artificial diet (Ward's Natural Science, NY) containing different concentrations of allyl cinnamate (at 0.05, 0.1, 0.25, 1 and 5%; w/w) for five days; control series were fed with untreated diet. A total of 30 first instars were used per

concentration, and for sublethal effects on weight gain and development groups of 10 third instars were used. At the start of the experiment, the mean fresh weight of the third instars over the different series was 6.0±0.6 mg ($P > 0.1$). Insects were followed at daily interval, and data were analyzed as before with a Student's *t*-test (Smagghe and Degheele, 1994). In addition, the median (50%) toxicity concentration (LC_{50}) which is the concentration needed to kill 50% of the insects treated and the corresponding 95% CI were calculated with use of Prism v4 as above; the accuracy of data fitting to the sigmoid curve model was evaluated through examination of the R^2 value.

RESULTS

MTT Cell Viability Bioassay

The five allyl esters, when tested at 50 mM, caused > 50% of cell viability loss in all cell lines by 24 h of incubation (Table 1). There were five exceptions: allyl heptanoate in S2 (37 ± 17%) and in Se4 (46 ± 6%) cells, allyl 2-furoate and allyl hexanoate in Se4 (33 ± 11% and 44 ± 19%, respectively) and allyl octanoate in CPB cells (15 ± 8%). ANOVA analysis demonstrated significant differences between the five allyl esters ($P < 0.0001$, d.f. = 4; F = 16.7) and the five insect cell lines ($P < 0.0001$; d.f. = 4; F = 8.6), and also for the allyl ester - cell line interaction ($P < 0.0001$; d.f. = 16; F = 13.5). In general, it was seen that allyl cinnamate was the most active product in the five cell lines, and the midgut CF203 cells were the most sensitive to all allyl esters tested.

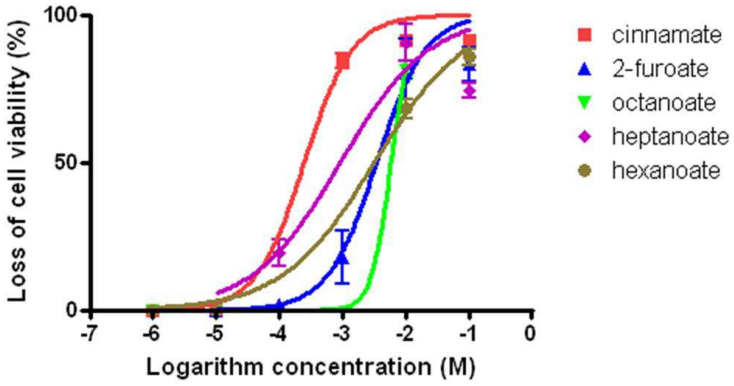
As exemplified with the midgut CF203 cell line in Fig 1, the cytotoxicity by the allyl esters was concentration-dependent in all cases. The EC_{50} of cinnamate in CF203 cells was 0.25 mM, and this was the lowest median effective concentration estimated at 24 h of incubation (Table 2). In addition, the different EC_{50}s varied with a range of 2 logs for the different allyl esters and cell lines (minimum 0.25 mM and maximum 27 mM).

Table 1: Biological activity of five allyl esters in a MTT bioassay after 24 or 96 h of incubation and in a Trypan Blue assay after 24 h of incubation. Cells were treated at 50 mM to the *embryo D. melanogaster* S2 and *S. exigua* Se4, fat body *L. decemlineata* CPB and ovary *B. mori* Bm5 cells, and at 10 mM to the midgut *C. fumiferana* CF203 cells.

		Loss of Cell Viability (%)		
Insect cell line	**Allyl ester**	**MTT-24 h**	**MTT-96 h**	**Trypan blue-24 h**
S2	Allyl cinnamate	90 ± 9	94 ± 3	99 ± 2
	Allyl 2-furoate	59 ± 5	89 ± 10*	91 ± 3*
	Allyl hexanoate	80 ± 3	75 ± 3	55 ± 5*
	Allyl heptanoate	37 ± 17	49 ± 3	10 ± 7*
	Allyl octanoate	88 ± 4	58 ± 1*	100 ± 0*
Se4	Allyl cinnamate	89 ± 3	59 ± 14*	95 ± 3
	Allyl 2-furoate	33 ± 11	61 ± 6*	100 ± 0*
	Allyl hexanoate	44 ± 19	49 ± 3	23 ± 4
	Allyl heptanoate	46 ± 6	46 ± 24	82 ± 6
	Allyl octanoate	77 ± 3	43 ± 10*	52 ± 2*
CPB	Allyl cinnamate	83 ± 9	88 ± 2	88 ± 2
	Allyl 2-furoate	91 ± 4	89 ± 1	100 ± 0*
	Allyl hexanoate	76 ± 11	84 ± 10	85 ± 0*
	Allyl heptanoate	52 ± 14	87 ± 4*	70 ± 0*
	Allyl octanoate	15 ± 8	90 ± 3*	42 ± 10
Bm5	Allyl cinnamate	73 ± 1	69 ± 17	63 ± 7
	Allyl 2-furoate	81 ± 6	31 ± 17*	100 ± 0*
	Allyl hexanoate	69 ± 5	32 ± 1*	17 ± 7*
	allyl heptanoate	77 ± 13	15 ± 13*	35 ± 5
	Allyl octanoate	51 ± 8	88 ± 13*	8 ± 3*
CF203	Allyl cinnamate	91± 2	93 ± 3	88 ± 4
	Allyl 2-furoate	80 ± 12	86 ± 7	100 ± 0
	Allyl hexanoate	68 ± 3	82 ± 12	63 ± 3
	Allyl heptanoate	91 ± 6	92 ± 3	83 ± 3
	Allyl octanoate	82 ± 3	86 ± 7	85 ± 3

Data are given as means±SEM. Three eplicates per concentration and this was repeated 3 times. *Significant difference between MTT-96-24h, andTrypan blue-MTT-24h, Student's *t*-test ($P < 0.05$).

Figure 1: Dose-response curves on the biological activities after 24 h of incubation for loss of cell viability in a MTT bioassay by the five allyl esters: allyl cinnamate, allyl 2-furoate, allyl hexanoate, allyl heptanoate and allyl octanoate, in the midgut *Choristoneura fumiferana* CF203 cell line.



In a separate series, when the cells were incubated over a longer period, i.e. 96 h, then it was clear that generally there were no significant increases in cytotoxicity ($P > 0.05$) as compared to incubations over 24 h, which is indicative that the loss of cell viability happened in the first 24 h of incubation (Table 1). There were, however, a few exceptions with allyl 2-furoate in S2 and Se4 cells, allyl heptanoate in CPB, and allyl octanoate in CPB and Bm5 cells. In addition, it was also noticed that in a few cases the loss of cell viability was reduced as these cultures showed a higher cell numbers at 96 h as compared to 24 h after incubation with allyl esters because the surviving cells after 24 h had rescued cell proliferation during the longer treatment. Some examples of these occurrences are with allyl 2-furoate in S2 and Se4 cells and with allyl octanoate in CPB and Bm5 cells (Table 1).

Table 2: Biological activity of the five allyl esters: allyl cinnamate, allyl 2-furoate, allyl hexanoate, allyl heptanoate and allyl octanoate, in five insect cell lines: embryo *Drosophila melanogaster* S2 and *Spodoptera exigua* Se4, fat body *Leptinotarsa decemlineata* CPB, ovary *Bombyx mori* Bm5, and midgut *Choristoneura fumiferana* CF203, for cell viability in a MTT Assay (EC_{50}) after 24 h of incubation.

Allyl ester	EC_{50} (95%CI; R^2) (mM)				
	S2	**Se4**	**CPB**	**Bm5**	**CF203**
Allyl Cinnamate	3.6 (2.2-6.0; 0.85)	1.0 (0.7-1.5; 0.90)	0.5 (0.3-1.0; 0.88)	73±1%	0.25 (0.10-0.59; 0.97)
Allyl 2-Furoate	59±5%[a]	33±11%[a]	8.9 (4.7-17.3; 0.85)	1.2 (0.3-4.1; 0.76)	3.5 (1.9-6.3; 0.90)
Allyl Hexanoate	3.9 (2.0-7.6; 0.86)	43.7±18.7%[a]	27 (18-40; 0.92)	6.8 (2.2-21; 0.76)	3.2 (1.9-5.5; 0.99)
Allyl Heptanoate	37±17%[a]	46±6%[a]	52±14%[a]	1.6 (0.2-10.2; 0.51)	0.9 (0.2-4.0; 0.88)
Allyl Octanoate	4.7 (2.3-9.5; 0.78)	0.6 (0.2-2.2; 045)	15±8%[a]	51±8%[a]	5.88 (---; 0.90)

Data are given as median (50%) response values together the 95% confidence interval (both in mM) and the R^2 as accuracy of data fitting to the sigmoid curve model after Prism v4 fitting. [a]The highest concentration tested (50 mM) resulted in the given mean ± SEM % loss of cell viability.

Trypan Blue Cell Viability Bioassay

When the five allyl esters were treated at 50 mM during 24 h, there were significant differences between the allyl esters tested ($P < 0.0001$; d.f. = 4; F = 140.0), insect cell lines ($P < 0.0001$; d.f. = 4; F = 62.0), and the interaction of these two factors ($P < 0.0001$; d.f. = 16.0, F = 34.0). As demonstrated by Table 1, the highest cytotoxicity percentages were generally scored by allyl furoate and allyl cinnamate action to all cell lines. In addition, allyl octanoate also cause high loss of cell viability in S2 and CF203 cells, as was also the case for allyl heptanoate in

Se4 and CF203 cells, but their activities were lower in the other cell lines.

In cases that >90% mortality was observed at 50 mM, the cytotoxicity was followed at different time points from 1 min to 24 h of incubation. The different time-response curves with 50 mM of allyl cinnamate and allyl furoate fitted to a sigmoid curve with $R^2 \geq 0.86$. The lowest ET_{50}s, being the time needed to induce cytotoxicity in 50% of the cells incubated with a specific concentration, were scored with allyl furoate in Se4 and CF203 cells (10-14 min) followed by S2 cells (28 min) (Table 3). For allyl cinnamate, longer incubation times were needed to kill 50% of the treated cells: 25, 87, 157 and 449 min in Se4, CF203, S2 and CPB cells, respectively. Although differences in ET_{50}s were observed, these experiments generally confirm the rapidness of the cell toxicity effects.

Table 3: Time of incubation needed to produce 50% loss of cell viability in a Trypan Blue Assay (ET_{50}) by 50 mM of allyl cinnamate and allyl 2-furoate in five insect cell lines: embryo *Drosophila melanogaster* S2 and *Spodoptera exigua* Se4, fat body *Leptinotarsa decemlineata* CPB, ovary *Bombyx mori* Bm5, and midgut *Choristoneura fumiferana* CF203.

	ET_{50} (95%CI; R^2) (min)	
Cells	***Allyl cinnamate***	***Allyl 2-furoate***
S2	157 (142-173; 0.91)	28 (20-38; 0.94)
Se4	25 (20-31; 0.92)	10 (7-14; 0.86)
CPB	449 (383-517; 095)	48 (42-56; 0.93)
Bm5	--	65 (60-90; 0.89)
CF203	87 (61-123; 0.90)	14 (11-18; 0.89)

Data are given as median (50%) response values together the 95% confidence interval (both in min) and the R^2 as accuracy of data fitting to the sigmoid curve model after Prism v4 fitting. --: not determined

Insect Bioassay with Allyl Cinnamate

When first instars were fed with allyl cinnamate at 0.25, 1 and 5% in the diet, there was 100% mortality by 24 h. A lower concentration of 0.1% caused 10% mortality after 1 day but this increased progressively to over 60% at day 2 to 73% at day 5, while with the 0.05% concentration there was no increased mortality as in the control series. After sigmoid curve fitting, the LC_{50} for allyl cinnamate in first instars of *S. littoralis* was estimated to be 0.08% (95% CI: 0.03-0.25%; $R^2 = 0.84$) after 5 days of feeding on treated diet.

With third instars, allyl cinnamate at 0.25% killed all insects progressively from 0% at day 1, 60% at day 2, and 100% at day 5. At 1% all insects were killed after 1 day, while with 0.1% and 0.05% there was no toxicity as in the controls. In addition, the weight of third instars was negatively ($P < 0.05$) affected and this happened even with the lowest concentration of 0.05%. After 1 day of feeding on diet with 0.05 and 0.1% the reduction ($P < 0.05$) in weight gain was 24 and 53%, respectively, as compared to that in the controls. After 5 days of feeding on treated diet, the average individual fresh larval weight for the 0.1% concentration was only 110 ± 8 mg ($P < 0.001$), while this was 136 ± 5 mg ($P = 0.017$) with 0.05%, and 155 ± 7 mg in the controls. Here the treated larvae showed signs of retardation of development since only 50% of the specimens had molted in the fourth instar at day 2 with 0.1% concentration and none with 0.25%. The molting percentage was 100% for the 0.05% concentration and control treatments. With higher concentrations all insects were dead.

DISCUSSION

Because of insecticide resistance against most commonly used groups of insecticides, there is much interest to develop new pesticides to slow down the trend towards insecticide resistance development. Botanical insecticides are often

seen as good alternatives because they often have lower mammalian toxicity and environmental persistence, and therefore pose fewer risks to non-target organisms and human health (Isman, 2006). Allyl esters are known to have detrimental effects on insects, causing mortality and delays in growth and development in different developmental stages; for example, in the codling moth *Cydia pomonella* (L.) (Escribà *et al*., 2009). In this paper we provide experimental evidence that the aromatic allyl cinnamate causes rapid and high mortality with 100% kill of cotton leafworm caterpillars at 0.25% in the diet, with an LC_{50} of 0.08%. In addition, with lower concentrations, larvae of *S. littoralis* showed sublethal effects with a reduction in weight gain and retardation in development. Therefore, based on previous data together with data presented here, we can confirm that allyl esters, and especially allyl cinnamate, have potential uses in pest control. However, to date there is no information available on the mechanism(s) behind the insecticidal action of allyl esters.

To our knowledge, this is the first report on cytotoxic effects by allyl esters in insect cell lines. We investigated cell lines of different insect species and tissue origin. It was very clear in the MTT bioassay that the allyl esters caused reductions in cell viability. To explain more in detail the mechanism behind this loss of cell viability, we tested the effects of the allyl esters in a trypan blue assay. Trypan blue dye can permeate cells in disrupted cell membranes, which results in dead cells taking up the blue color. Here our experiments with short incubations of the insect cells ranging between 1 min and 24 h, confirmed the rapidness of the cytotoxic effects. Although there is no information on the potency of allyl esters to kill and permeabilize cell membranes, we believe that the rapidness by which they exerted their cytotoxicity, may be caused by membrane perturbation at different concentrations for the tested compounds. It is possible that differences in lipid, protein and enzyme composition of the respective cell membranes may explain the sensitivity differences that we observed; however, these possibilities were not

investigated. Based on information obtained from other cell membrane permeabilizing compounds as saponins, there can be a general mechanism via the formation of non-specific 'pores' and an extra effect by membrane rearrangements. But in many cases, different saponins also showed variable effects (Sung *et al.* 1995; Levavi-Sivan *et al.* 2005).

In our results, the cell viability MTT tests indicated that the aromatic allyl cinnamate ester exerted strong cytotoxic effects on the midgut CF203 cells from concentrations of 0.01 mM, and its biological effect took place rapidly, within 24 h of exposure. The current trypan blue experiments confirmed that exposure to active allyl esters caused a strong loss of cell viability because the dye could enter the cells. These impacts took effect very quickly and could be perceived after a few minutes with ET_{50}s of 10-14 minutes, killing 50% of the Se4 and CF203 cells for the most active, aromatic allyl ester compounds. Interestingly, similar rapid cytotoxic effects have also been reported by other research groups for a diversity of compounds; several of which were to some extent chemically related to allyl cinnamate (Cohen *et al.,* 1996; Cohen and Quistad, 1998; Etzenhouser *et al*., 2001; Baziramakenga *et al*., 1995; Kim *et* al., 2004; Esteves *et al*., 2008). Kabara (1987) and Najar-Rodríguez *et al*. (2008) also confirmed a rapid strong activity in "*in vivo*" experiments with insecticidal oils. However, it should be mentioned here that, although in some, but not in all cases, there was a link between cell/insect effects and membrane disruption, which supports the idea that different mechanisms may contribute in the allyl ester (cyto)toxicity. Previously, Sikkemma *et al.* (1995), Enan (2005), Bakkali *et al*. (2008) and Rattan (2010) hypothesized on a multi-target function for hydrocarbons and essential oils. More recently, Wang *et al*. (2010) observed that methyl palmitate, which is a long-chain fatty acid ester, affected mitochondria in their "*in vivo*" mite experiments. Nonetheless, it can potentially be hypothesized that allyl esters have a mode of action that is different from all existing insecticide groups, which is important if cross-resistance

development is to be avoided with existing active ingredients already in the market. However, mammalian toxicity and environmental safety characteristics need to be determined before exploitation of these potential alternatives in pest control in practice can be considered.

It is evident that more research is necessary to better understand the reasons way by allyl esters cause cell toxicity. However, although the exact mechanism of allyl esters remains enigmatic, the current data reveal the interesting observation that the insect midgut CF203 cells show high sensitivity. This may confirm the high entomotoxic action by ingestion observed "*in vivo*" on aphids when allyl esters were added to the aphid artificial liquid diet (Chapter 2). Similarly in the current insect bioassays with larvae of *S. littoralis*, ingestion of allyl cinnamate, when mixed in the diet, posed high and rapid toxicity. However, additional information on other insecticidal effects is currently lacking. Nonetheless, we believe that the current data provide a strong indication of the potency of allyl esters, particularly aromatic ones, in the control on pest insects, and additionally point to the insect midgut epithelium as a primary target tissue. Indeed the insect midgut is an interesting target tissue as any detrimental effect on the midgut epithelial cells will lead to starvation, implying lower insect damage, as well as death of the intoxicated insect (Hakim *et al*., 2010). In addition, although aphid cell lines were not used in this study as these are not available, we believe that the current data also suggest that allyl esters can represent important leads in the development of alternate, environmentally sound aphid control agents since the cell lines from the different tissues and different insect orders as used in this study were all susceptible to at least some of the allyl esters and because aphids are not sensitive to *Bacillus thuringiensis* (*Bt*) toxins (Sharma *et al*., 2004).

We found in this project that allyl cinnamate showed the highest activity and the insect midgut CF203 cells the highest sensitivity, but here it should be noticed that

for some allyl esters relatively high concentrations were needed to cause an effect. However, it is of interest to mention that Escribà *et al*. (2011) previously noted that these compounds can be produced in large volumes from industrial fat wastes. In addition, we observed that the aromatic allyl cinnamate caused rapid toxicity in cotton leafworm larvae, which is a cosmopolitan pest that is causing high economic losses in agriculture. Therefore, we believe this information provides further support for allyl esters as new candidate insecticides for use in agricultural pest control. However, before claiming firm conclusions, trials under more field-related conditions are necessary to verify their applicability in the control of pest insects, as well as an evaluation of potential hazards on beneficial organisms and natural enemies within managed agricultural environments.

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Insecticidal action of cinnamic and octanoic acids, and their corresponding 2,3-dichloropropyl and allyl esters

Marta GINER,[1,2] Jesús AVILLA,[1,2] Guy SMAGGHE[3] and Mercè BALCELLS [2,4]

[1] Department of Crop and Forest Sciences, University of Lleida, Lleida, Spain.

[2] Crop Protection Department, UdL-IRTA Center for R + D, Lleida, Spain.

[3] Crop Protection Department, Laboratory of Agroozology, University of Gent, Gent, Belgium.

[4] Department of Chemistry, University of Lleida, Lleida, Spain.

ABSTRACT

Some lipophilic compounds, such as allyl esters, have insecticidal properties due to a direct effect on cell membranes. In order to ascertain the chemical characteristics of the molecules responsible for the insecticidal activity and to better understand their mode of action, the aim of this study was to assess the insecticidal activity of cinnamic and octanoic acid and their corresponding allyl and 2,3-dichlopropyl esters.

Insecticidal bioassays were conducted by topical application on eggs, larvae and adults of insects of three different orders [*Cydia pomonella* (Lepidoptera: Tortricidae), *Acyrthosiphon pisum* (Hemiptera: Aphididae) and *Tribolium castaneum* (Coleoptera: Tenebrionidae)] and via ingestion on *C. pomonella* larvae and *A. pisum* nymphs. Finally, cytotoxicity was measured on embryonic cells of *Drosophila melanogaster* (S2 cells) using the MTT and the Trypan blue methods.

Differences in insecticidal action were observed depending on the compound, the insect and the mode of application. Octanoic acid showed the same contact insecticidal activity on *T. castaneum* and cytotoxicity against S2 cells as the corresponding allyl ester. In contrast, lower activity was recorded on *C. pomonella*, both by contact or ingestion. Cinnamic acid was less cytotoxic and less active by contact than the corresponding allyl ester and no action was recorded by ingestion. In both cases (contact and ingestion), corresponding dichloropropyl esters did not show high insecticidal activity; no sublethal effects were observed in any case. The same results were obtained when a resistant strain of *C. pomonella* was used for the bioassays.

All active compounds disrupted cell membranes, suggesting that insecticidal action is due to the lipophilic characteristics of these substances and that lipophilic properties play a key role in insecticidal action.

Results indicate that esterification increases the activity of these compounds.

Key words: allyl ester, dichloropropylester, cytotoxicity, codling moth, pea aphid, red flour beetle, insect cell line

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Antennal and behavioural response of virgin and mated males and females of *Cydia pomonella* and *Lobesia botrana* to allyl esters

Marta GINER,[1,2] Mercè BALCELLS [2,3] and Jesús AVILLA [1,2]

[1] Department of Crop and Forest Sciences, University of Lleida, Lleida, Spain.

[2] Crop Protection Department, UdL-IRTA Center for R + D, Lleida, Spain.

[3] Department of Chemistry, University of Lleida, Lleida, Spain.

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ABSTRACT

Electroantennographical (EAG) responses to allyl esters used as fruity aromas (allyl cinnamate, allyl octanoate, allyl heptanoate and allyl hexanoate) were assessed on virgin and mated adults of *Cydia pomonella* and *Lobesia botrana* to determine whether they could be used in pest control. Adult behavioural reaction was later assessed in a wind tunnel, with and without the main compound of the corresponding sex pheromone.

Antennae of *C. pomonella* males responded to all allyl esters but antennae of females reacted only to allyl cinnamate. A higher EAG response to allyl cinnamate than to pheromone was only observed in mated *C. pomonella* females. Higher response to allyl cinnamate than the rest of allyl esters were recorded in virgin *L. botrana* while no differences between treatments were recorded after mating.

In wind tunnel assays, the presence of allyl cinnamate did not interfere with pheromonal action on *C. pomonella* males when number of contacts was compared, though lower contacts were recorded when allyl octanoate, allyl heptanoate or allyl hexanoate were in the blend. In the case of females, a higher proportion of codling moths moved towards the source when allyl cinnamate was in the wind tunnel plume. The same response to allyl cinnamate was observed in *L. botrana*.

Results suggest that allyl cinnamate is the best candidate, but more assays in field conditions are required to determine its role in insect communication and its feasibility for inclusion in an integrated pest management programme, especially for *C. pomonella*.

Key words: allyl ester, codling moth, grape berry moth, EAG, wind-tunnel.

Giner M, Balcells M, Avilla J. Antennal and behavioural response of virgin and mated males and females of Cydia pomonella and Lobesia botrana to allyl ester. Bull Entomol Res. Submitted 2012.

To our knowledge this is the first report on the insecticidal action of allyl esters. Results presented in this thesis show the potential use of allyl esters as insecticides, pointing out the different insecticidal action depending on the assessed allyl ester, the insect and the way by they are supplied/applied to insects.

Toxicity by **contact** and **ingestion** was studied using insects from different orders and different feeding behaviour, and taking into consideration different developmental stages. Additionally, the potential role of allyl esters as ethologic modifiers was assessed since their aromatic properties might influence insect behaviour. Results show that the allyl ester group was not the only responsible of the insecticidal action. The chemical structure of the rest of the molecule and its interaction with each insect/target play a major role.

Focusing on the mode of application, higher insecticidal action of allyl esters was scored when allyl ester was ingested by insects than when allyl ester contact with insect (Escribà *et al*., 2009; Chapter 1, 2 and 3). LC_{90}s obtained from feeding bioassays are in the same range than LC_{90} of insecticidal substances used against aphid nymphs and Lepidopteran larvae (Sadeghi *et al*., 2009; Pineda *et al*., 2004; Chapter 2). Added to the fact that midgut cells are more sensitive to allyl esters than the rest of insect cells from different tissue origin suggests that the gut is a target for allyl ester action (Chapter 3).

Toxicity by contact was also produced, probably as a consequence of allyl ester non-specific mode of action. Disruption of cell membrane was described as the main mechanism of allyl esters insecticidal action (Chapter 3). Differences in the degree of cytotoxicity among allyl esters and cell lines would be explained by differences in allyl ester lipophilic properties, composition of insect cell membranes and a differential degree of interaction between them. However, other

mechanisms of action can not be rejected as several authors have indicated that multiple targets may be affected by lipophilic compounds (Sikkerma *et al*., 1995; Rattan, 2010). This possibility would be practical in pest control as a way by to reduce the development of resistant populations (Yang *et al*., 2009; Machial *et al*., 2010).

Reduced insecticidal activity of corresponding free fatty and aromatic acids by ingestion (and also by contact) on aphids (data not shown), and both on a sensible and a resistant strain of *Cydia pomonella* (increased enzymatic system in the resistant strain) (Chapter 4) were recorded, indicating that there is no need for a previous hydrolysis of allyl ester bond, and that the insecticidal effect is due to the allyl ester itself. Insecticidal properties of aromatic and middle-chain fatty acids were reported in this thesis (Chapter 4), reasserting the results of Imai *et al*. (1995) and Ramsewak *et al*. (2001), but in all cases a higher amount of acid than of the corresponding allyl ester was needed to kill insects. The mechanism of insecticidal action of acids is the same than corresponding allyl esters, but acting in slower way translated in to lower insecticidal action (Chapter 3 and 4). For the corresponding dichloropropylesters, the same effect (**disruption of cell membrane**) was observed in S2 cells (Chapter 4) but lower cytotoxicity and insecticidal action was recorded. The lower insecticidal action might be due to changes in lipophilicity of the compounds in comparison to allyl esters. Interestingly, no sublethal effect was observed in eggs or larvae of *C. pomonella*, opposite to the results of several authors when assessing insecticides that include halogens in the molecule (Prestwich, 1986; Yoshida and Toscano, 1994). Neither an effect in insect development was suggested by acid or allyl ester action (Chapter 1, 2 and 4), even though, an effect in *C. pomonella* eggs by allyl esters of fatty acids was reported (Escribà *et al*., 2009).

Regarding to insecticidal properties as insect behaviour modifiers due to aromatic properties, different activity of allyl esters was observed depending on the allyl ester and insect assessed. Allyl cinnamate was in all cases the most active compound, but it produced a different response depending on species assessed (Chapter 2 and 5). From literature about fruit aromas it is considered that both repellency and attractiveness could be expected (Light *et al*., 2001; Vallat and Dorn, 2005; Caballero-Gallardot *et al*., 2011) and both effects could be used in ethologic control of several pests.

A modification on insect behavior mediated by allyl esters was recorded in *T. castaneum* (Coleoptera), and in *C. pomonella* and *L. botrana* (Lepidoptera), but not in *A. pisum* (Hemiptera). **Repellence** was observed in *T. castaneum* adults in a similar way than that scored to for some essential oils or their constituents (García *et al*., 2005; Zapata and Smagghe, 2010). Indeed the action of allyl cinnamate was in the same range than the commercial insect repellent DEET (*N, N*- diethyltoluamide) what pointed allyl cinnamate as a candidate in stored products pest control (Chapter 2). By contrast, **no effect** of allyl esters in aphids was suggested from results of choice-assays by contact or by ingestion (Chapter 2) and **attractiveness** was scored on females of *C. pomonella* and *L. botrana* (Chapter 5). An elicitation of *C. pomonella* antennae was recorded in virgin adults, but only females were more attracted to sources baited with allyl cinnamate. Although, an increased fluttering was recorded in males when allyl ester was present in the wind-tunnel what could cause an effect able to be used in pest control. The same response was observed on *L. botrana* (Chapter 5). Reduced antenna elicitation was produced in mated females, when compared to virgin ones, what suggested an allyl cinnamate role previous to mating (Chapter 5). As ethyl cinnamate is described as component emitted by *Grapholita molesta* male moths during courtship moth behavior (Birch and Hefez, 1987; Löfstedt *et al*., 1990), a similar effect of allyl cinnamate would be hypothesized, by similarities in

chemical structure. However, no data was found about male sex pheromone in *C. pomonella* or *L. botrana*, and assays conducting to know the effect in *G. molesta* are on course.

Despite that insecticidal action was recorded, difficulties in field application are expected. Regarding to Tortricidae species, high amounts of allyl esters are needed to kill insects by contact, especially when compared with commercial insecticides (Rodríguez *et al*., 2011). This is an inconvenient in practical field application and reduces their commercial prospects. Even more, Tortricidae larvae are only exposed to insecticides for a short time due to their internal feeding behavior making difficult the allyl ester action. Furthermore low doses (sublethal in most cases) would be ingested before the larvae are introduced into fruits, so the insecticidal effect would not be produced. However, a strong point about allyl ester election is the fact that active allyl esters would be produced from biodiesel production by products with low cost (Escribà et al., 2011). Also, allyl esters are used as accepted fruity aromas (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:1999:084:0001:0137:ES:PDF) so low negative effects in human health or environment would be suspected. In addition, similar effect in different Tortrididae species was recorded, independently of egg phase, thus increasing their ratio of activity or when several species cohabit. In any case, studies aim to find a correct formulation for allyl esters would be needed to be determined and also allyl ester fate in field conditions.

Focusing in ethologic control, allyl cinnamate would be an interesting candidate in Tortricidae fruit pest control, especially on *C. pomonella*, as would increase the number of female catches in pheromone field traps with no reduction of male captures. The increased fluttering and activation of males in front of allyl esters would also be translated in increased attractiveness or an increased mating

disruption effect, but this needs to be deeply studied and assessed in field conditions.

The study of allyl ester action on *C. pomonella* in field conditions will be on-course aim to discern if the attractiveness could be used in pest control. Moreover, knowing the effect of allyl esters on a wild population of *C. pomonella* also would give information about the chemical communication among wild and laboratory strains aim to discern if a kind of “domestication” or a reduction of chemical communication capacities is produced in laboratory strains.

Regarding to aphids, a correct formulation to allow aphid mortality by ingestion is needed, but their use when risk of virus transmission exists would limit their practical use, as no antifeedant or repellent effect was suggested from repellence bioassays (Chapter 2).

Regarding to *T. castaneum* both toxicity and repellency was produced by allyl esters. Owing to stored products pest behavior (into ensile or packet products) the action as repellent is more interesting, specially in the case of allyl cinnamate due to lower doses are needed. Allyl esters could be used to maintain stored products free from pest.

Ideally, allyl esters could be useful when several insect pests cohabit due to the similar insecticidal action among insect species are suspected, as can be seen from results described in this thesis.

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❶ Allyl naphthoate, allyl 2-furoate, allyl thiophenecarboxylate and allyl cinnamate are toxic by contact on eggs and larvae of *Cydia pomonella*, *Grapholita molesta* and *Lobesia botrana,* while no action is produced by allyl salycilate.

❷ Allyl cinnamate, allyl 2-furoate and allyl heptanoate are toxic by ingestion on *Acyrthosiphon pisum* nymphs, but only allyl cinnamate is toxic by contact application. No effect in aphid development was caused by allyl esters.

Tribolium castaneum adults are sensible to contact application of allyl cinnamate, allyl 2-furoate, allyl octanoate, allyl heptanoate and allyl hexanoate and also repellence are produced by all of these products, but allyl hexanoate.

❸ Allyl cinnamate, allyl 2-furoate, allyl octanoate, allyl heptanoate and allyl hexanoate are cytotoxic to several insect cell lines causing cell membrane disruption, and being midgut cells from *Choristoneura fumiferana* the most sensible ones. Lethal effect on *Spodoptera littoralis* larvae after allyl cinnamate ingestion points the gut as a target for allyl ester action.

❹ Corresponding acids and dichloropropyl esters are less (or equal) active as insecticides than allyl esters, acting also by cell membrane disruption and without producing any detrimental effect on surviving treated insects.

❺ Allyl cinnamate, allyl octanoate, allyl heptanoate and allyl hexanoate elicite *C. pomonella* male antenna, while only allyl cinnamate does on *C. pomonella* females and *L. botrana* males and females. The presence of allyl cinnamate into pheromonal blends could improve ethologic pest control of these Tortricidae.

❶ El naftoato de alilo, el 2-furoato de alilo, el tiofencarboxilato de alilo y el cinamato de alilo son tóxicos por contacto en huevos y larvas de *Cydia pomonella*, *Grapholita molesta* y *Lobesia botrana*, mientras que el salicilato de alilo no es activo.

❷ El cinamato de alilo, el 2-furoato de alilo y el heptanoato de alilo son tóxicos por ingestión en ninfas de *Acyrthosiphon pisum*, pero solo el cinamato de alilo es tóxico por aplicación tópica. Los esteres de alilo no produjeron ningún efecto en el desarrollo de lo áfidos.

Los adultos de *Tribolium castaneum* son sensible a la aplicación tópica de cinamato de alilo, 2-furoato de alilo, octanoato de alilo, heptanoato de alilo y hexanoato de alilo, produciendo también todos repelencia por contacto, exceptuando el hexanoato de alilo.

❸ El cinamato de alilo, el 2-furoato de alilo, el octanoato de alilo, el heptanoato de alilo y el hexanoato de alilo son citotóxicos para varias líneas celulares de insectos, causando disrupción en la membrana celular, y siendo las células del aparato digestivo de *Choristoneura fumiferana* las más sensibles. El efecto letal del cinamato de alilo por ingestión en larvas de *Spodoptera littoralis* señala al aparato digestivo como punto de acción de los esteres de alilo.

❹ Los correspondientes ácidos y dicloropropilesteres son menos (o igualmente) activos como insecticidas que los esteres de alilo, causando también la disrupción de la membrana celular y sin producir efectos subletales en los insectos que sobrevivieron al tratamiento.

❺ El cinamato de alilo, el octanoato de alilo, el heptanoato de alilo y el hexanoato de alilo son reconocidos por las antenas de los machos de *C. pomonella*, pero tan solo el cinamato de alilo por las antenas de las hembras de

C. pomonella y de los machos y las hembras de *L. botrana*. La presencia del cinamato de alilo en mezclas feromonales puede contribuir a la mejora del control etológico de dichos Tortricidos.

❶ El naftoat d' al·lil, el 2-furoat d' al·lil, el tiofencarboxilat d' al·lil i el cinamat d' al·lil són tòxics per contacte en ous i larvas de *Cydia pomonella*, *Grapholita molesta* i *Lobesia botrana*, mentre que el salicilat d' al·lil no ho és.

❷ El cinamat d' al·lil, el 2-furoat d' al·lil i l' heptanoat d' al·lil són tòxics per ingestió en nimfes d' *Acyrthosiphon pisum,* però només el cinamat d' al·lil ho és tòxic per aplicació tòpica. Els esters d' al·lil no van produir cap efecte en el desenvolupament dels àfids.

Els adults de *Tribolium castaneum* van ser sensible a l' aplicació tòpica de cinamat d' al·lil, 2-furoat d' al·lil, octanoat d' al·lil, heptanoat d' al·lil i hexanoat d' al·lil, produint també tots ells, excepte l'hexanoat d'al·lil, repelencia per contacte.

❸ El cinamat d' al·lil, el 2-furoato d' al·lil, l' octanoat d' al·lil, l' heptanoat d' al·lil i l' hexanoato d' al·lil són citotòxics per a diverses línees cel·lulars d' insectes, causant una disrupció de la membrana cel·lular, i èssent les cél·lules de l' aparell digestiu de *Choristoneura fumiferana* les més sensibles. L' efecte letal del cinamat d' al·lil per ingestió en larves de *Spodoptera littoralis* señala l' aparell digestiu com a punt d' acció dels esters d' al·lil.

❹ Els corresponents àcids i dicloropropilesters van ésser menys (o igual) actius com a insecticides que els esters d' al·lil, causant també la disrupció de la membrana cel·lular i sense produir efectes subletals en els insectes que van sobreviure al tractament.

❺ El cinamat d' al·lil, l' octanoat d' al·lil, l' heptanoat d' al·lil i l' hexanoat d' al·lil són reconeguts per les antenes dels mascles de *C. pomonella*, però tan sols el cinamat d' al·lil va ésser reconegut per les antenes de les femelles de *C. pomonella* i dels mascles i les femelles de *L. botrana*. La presència del cinamat d' al·lil en mescles feromonals pot contribuir a la millora del control etològic d'aquests Tortricids.

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