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Cory Creed
MSU Graduate Student

Ariel Mollhagen
MSU Undergraduate

Noelle Mollhagen
MSU Undergraduate

Maciej Pszczolkowski
Missouri State University

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ORIGINAL ARTICLE

Artemisia arborescens "Powis Castle" extracts and α-thujone prevent fruit infestation by codling moth neonates

Cory Creed, Ariel Mollhagen, Noelle Mollhagen, and Maciej A. Pszczolkowski

Darr School of Agriculture, Missouri State University, Mountain Grove, MO, USA

Abstract

Context: The codling moth, *Cydia pomonella* L. (Tortricidae), is a major cosmopolitan pest of the apple. The potential of plant-derived semiochemicals for codling moth control is poorly studied.

Objective: To evaluate the potential of crude extracts of five plants from the Asteraceae family: *Artemisia absinthium* L., *Artemisia arborescens* L. "Powis Castle", *Artemisia annua* L., and *Artemisia ludoviciana* Nutt. to prevent apple infestation by *C. pomonella* larvae and to identify the deterrent(s) in these plants.

Materials and methods: *Artemisia* dried leaves were extracted in v/v mixture of 80% ethanol, 10% isopropanol, and 10% of methanol, and the extracts were analyzed using high-performance thin layer chromatography. Preference of fruit treated with test solutions (*Artemisia* extracts or α-thujone) versus fruit treated with solvent was studied using choice assays.

Results: α-Thujone was detected in *A. arborescens* extract at a concentration of 77.4 ± 2.4 mg/g of dry tissue, localized between R_f 0.75 and 0.79 and was absent from crude extracts of remaining *Artemisia* species. Material from each extract in the zone between R_f 0.75 and 0.79 was removed from chromatographic plates and tested for feeding deterrence. Only the material from *A. arborescens* showed feeding deterrent properties. Minimum concentrations that prevented fruit infestation were 10 mg/ml for α-thujone and 1 mg/ml for *A. arborescens* crude extract.

Discussion and conclusions: *Artemisia arborescens* contains chemicals that prevent apple infestation by codling moth neonates. Thujone is one of these chemicals, but it is not the only constituent of *A. arborescens* crude extract that prevents fruit infestation by codling moth neonates.

Keywords

Apple, botanical insecticide, *Cydia pomonella*, fruit feeding, wormwood

History

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Introduction

The codling moth, *Cydia pomonella* L. (Tortricidae) (Figure 1), is the major cosmopolitan pest of apples. In most locations, *C. pomonella* has two or three generations per year, and in unmanaged orchards, fruit infestation by this species may reach up to 80% (Arthurs et al., 2005; Tadic, 1957). This potentially translates to losses exceeding US$2.1 billion in the United States alone (Geisler, 2012).

The grower has limited options to control the codling moth because shortly after hatching larvae burrow into the fruit and stay there until development is complete. This is especially problematic in growing areas that have partial third generations where neonates attempt to penetrate the fruit within days of harvest, the time when insecticides are not permitted to be used.

Correspondence: Maciej A. Pszczolkowski, Missouri State University, Darr School of Agriculture, 9740 Red Spring Road, Mountain Grove, MO 65711, USA. Tel: +1 417 547 7507. E-mail: MPszczolkowski@missouristate.edu

Sprays containing the broad-spectrum organophosphate neurotoxin, azinphos-methyl, are an effective control measure for *C. pomonella*; however, this insecticide must be applied at high levels (1.7 kg/hectare) due to resistance that has accumulated over the years. Due to serious concerns raised by the Environmental Protection Agency, azinphos-methyl is no longer allowed to be used on apples in the USA, has also been banned in European Union, and soon will be banned in New Zealand. The insect ryanodine receptor activator, Rynaxypyr, chloride channel activator, emamectin benzoate, and disruptors of nicotinic/γ amino butyric acid (GABA)-gated chloride channels, such as Spinetoram, have been implemented against the codling moth in the USA and in Europe, but the development of resistance to these insecticides is only a matter of time. Microbial insecticides such as *Bacillus thuringiensis* or codling moth granulosis virus could be used against codling moth neonates, but these insecticides must be consumed in large quantities to exert effects, thus their efficacy is often too low to provide fruit of satisfactory quality.

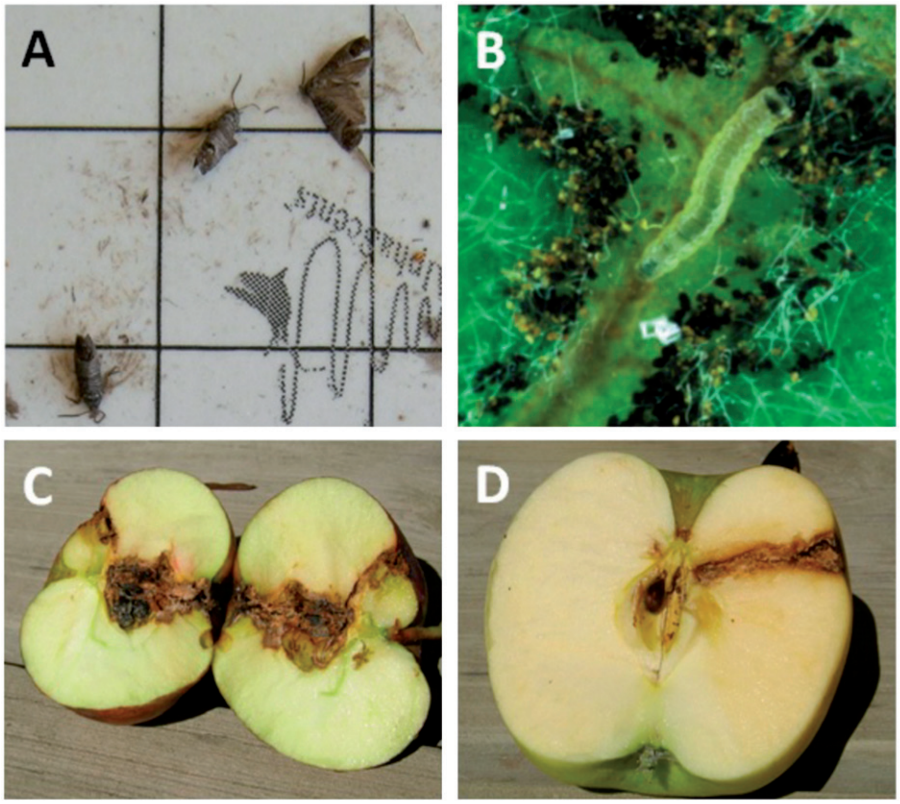

Figure 1. The codling moth and two examples of fruit damage caused by this pest. (A) The moths immobilized on the sticky board of monitoring trap. The wingspan of the moth equals approximately 2 cm. (B) Codling moth neonate larva, about 1.5 mm long, is the invasive stage. (C) Fruit damage caused by infestation early in the season. (D) Fruit damage caused by infestation late in the season, about 30 d before harvest.

Pheromone- or kairomone-based strategies such as attract-and-kill and mating disruption are expensive and do not resolve problems caused by dense codling moth populations, and migration of gravid moths from adjacent unmanaged areas in mosaic landscapes typical of small-scale apple production (Witzgall et al., 2008) or insecticide resistance (Poullot et al., 2001). Recent studies using microencapsulated kairomones against codling moth showed promising results (Light & Beck, 2010). However, further research on alternative strategies of controlling the codling moth is needed. Preventing fruit infestation by neonate larvae with feeding deterrents of plant origin is a novel strategy, and may be useful especially prior to harvest when all insecticides are restricted.

Some plants belonging to the Asteraceae family and *Artemisia* genus exhibit arthropod-repellent properties (Abad et al., 2012). Terpinen-4-ol isolated from *Artemisia vulgaris* L. repelled the yellow fever mosquito *Aedes aegypti* L. (Hwang et al., 1985). Essential oils from *A. vulgaris* and *Artemisia annua* L. repel the red flower beetle, *Tribolium castaneum* Herbst (Tripathi et al., 2000; Wang et al., 2006). Coumarin and thujyl alcohol from *Artemisia abrotanum* L. repel the tick *Ixodes ricinus* L. (Tunón et al., 2006).

There are also reports that chemicals from plants belonging to the *Artemisia* genus inhibit feeding in insects, including codling moth larvae. Phenylacetylenes from growing buds of *Artemisia capillaris* Thunb. are insect anti-feedants (Yano, 1983, 1987). 1,8-Cineole from *A. annua* is a feeding deterrent in *T. castaneum* (Tripathi et al., 2001). Ethanolic extracts from *A. annua* as well as artemisinin, a sesquiterpene lactone isolated from this plant, inhibit feeding of the South American ladybird beetle, *Epilachna paenulata* Germar, and caterpillars of the southern armyworm *Spodoptera eridania* Stoll (Maggi et al., 2005). Crude extracts (single doses of 10 mg/ml) from *Artemisia absinthium* L. lowered apple infestation by codling moth neonates in preliminary experiments by Suomi et al. (1986); however, the methodology of their bioassay did not allow accurate evaluation of *Artemisia's* repellent properties (due to oversized test arena, 33% of tested larvae never found either treated or control apples in their study). Single doses (10 mg/ml) of crude extracts from *A. arborescens* and *Artemisia ludoviciana* Nutt. had deterrent effects in experiments of Durden et al. (2008, 2009) who used improved bioassay for feeding deterrence of the extracts. In a more recent study (Durden et al., 2011), crude extracts from *A. annua*, and two of its metabolites, artemisinin or 1,8-cineole, were shown to prevent apple infestation by codling moth neonates.

Concentration-dependent effects of *A. arborescens* extracts and thujone, a metabolite found in some species belonging to the *Artemisia* genus, on feeding by codling moth neonates, have not been previously studied. Here, we show that *A. arborescens* extracts, and a thujone stereoisomer, α-thujone, prevent fruit infestation by codling moth neonates.

Materials and methods

Plant material

The aerial parts of *A. arborescens* ''Powis Castle'' were collected in Ozark Arboretum, Mountain Grove, MO, in May

2009 and were air dried at room temperature. A sample was authenticated by S. Lallemand, and vouchered, as MSFES/CIP 006, in the Collection of Insects and Plants at Missouri State Fruit Experiment Station in Mountain Grove, MO.

Insects

Codling moths from USDA-ARS Yakima Agricultural Research Laboratory, Wapato, WA, were held at 25 °C, 70–80% relative humidity (RH), under a 16 h light, 8 h dark (16 L:8D) light-dark regime. Female moths were allowed to oviposit on strips of polyethylene. Neonates, up to 1 h old, were collected and used for the experiments.

Chemicals and crude plant extracts

Dehydration alcohol (a v/v mixture of 80% ethanol, 10% isopropanol, and 10% of methanol) was purchased from EMD (Gibbstown, NJ). All other chemicals, including methanol, toluene, ethyl acetate, glacial acetic acid (99.9% purity), and α-thujone (96% purity), were from Sigma-Aldrich (St Louis, MO). The powdered air-dried leaf matter (150 mg) was placed in a 2-ml Eppendorf tube with 1 ml dehydration alcohol, vortexed briefly, allowed to rest for 10 min at room temperature, and centrifuged at $2000 \times g$ for 10 min. The liquid fraction was removed with a pipette, transferred to a pre-weighed Eppendorf tube, and evaporated in a SpeedVac rotary evaporator. After desiccation, the tubes were re-weighed to determine the final mass of the residue which was subsequently resuspended in dehydration alcohol to the desired concentration.

Experimental design

First, crude alcoholic extracts of aforementioned *Artemisia* species were compared by qualitative high-performance thin-layer chromatography (HPTLC), in an attempt to discern chemical(s) of differences among the particular species. Differential bands were isolated by scraping off the appropriate stationary phase area on the TLC plate from each of the lanes representing a different *Artemisia* species. The samples were then extracted with dehydration alcohol, evaporated, re-dissolved at 10 mg/ml, and their potential for preventing apple infestation by codling moth neonates was evaluated as described below. Because only the sample from *A. arborescens* prevented fruit infestation, the band of difference was present only in the lane representing *A. arborescens*, and the band of difference co-localized with α-thujone standard, we further concentrated on bioassaying α-thujone alone and *A. arborescens* crude extract in attempt of producing concentration-dependent response curves. Thujone was bioassayed at concentrations of 1, 3, 10, 30, and 100 mg/ml. *Artemisia arborescens* crude extract was bioassayed at concentrations of 0.1, 0.3, 1, 3, and 10 mg/ml.

HPTLC

HPTLC plates (10×10 cm; Silica gel 60 F_{254}, purchased from EMD, Gibbstown, NJ) were washed in methanol and dried under a fume hood. Crude *Artemisia* extracts were spotted using a CAMAG Nanomat 4 dispenser at 50 μg per lane. Thujone (Figure 2) standard was spotted at 25 or 50 μg per lane. The plates were allowed to air dry for 5 min and developed in a CAMAG Horizontal Developing Chamber using a mobile phase consisting of toluene, ethyl acetate, and glacial acetic acid (8:2:0.2 v/v). The plates were then air-dried for 5 min, derivatized in vanillin reagent, and heated for 10 min on a CAMAG TLC Plate Heater III at 100 °C. The plate was then scanned in white light using a Canon CanoScan 5200F flatbed scanner for documentation and thujone quantification was performed by densitometry as described in Valverde and This (2007). Briefly, the image was scanned and registered as a JPEG compressed file. TLC Analyzer (Massachusetts Institute of Technology, Cambridge, MA) a public domain computer program (Hess, 2007) was used to analyze digital images of HPTLC plates and generate multispectral scans, densitograms, and calibration curves for thujone.

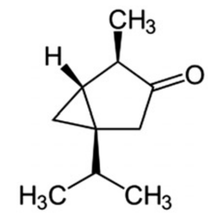

Figure 2. Chemical structure of a secondary metabolite of *Artemisia arborescens* ''Powis Castle'', α-thujone, tested for codling moth deterrence in this study. α -Thujone exhibited weak deterrent effects, which do not explain the level of deterrent activity in *A. arborescens* ''Powis Castle'' crude extracts toward codling moth neonates, suggesting the presence of other codling moth deterrent compounds in this plant.

Bioassays

Test solutions were examined for deterrent effects on codling moth neonates at concentrations ranging from 0.1 to 100 mg/ml. For each dose, four plugs were taken from the same Golden Delicious apple, using a length of plastic soda straw, such that the straw covered the pulp, but not the epidermis of the apple. The crevice between the plug and the edge of the straw was sealed with paraffin wax applied with a warm spatula. The straws were then placed in a holder, apple plug facing up, and 5 μl of test solution were applied to each plug. The plugs were allowed to air dry, and four plugs were placed in a 60×15 mm polystyrene Petri dish, held in place by small pieces of modeling clay. New clay was used for each assay. A glass rod (1.3 mm diameter, 25–27 mm long) was positioned such that each end of the rod touched both the control and the treated member of the plug pair. One neonate was placed, using a camel-hair artists brush, in the middle of the glass rod and the Petri dish was covered with a lid. The entire assembly was covered with a one half of a white plastic RipBall (TM & Enor Corp., Northvale, NJ) to provide a white, slightly opaque cupola and placed on the testing bench illuminated by fluorescent tubes and Soft White 60 general purpose bulbs (General Electric Canada, Cleveland, OH). Such an arrangement provided dispersed, non-directional light of uniform luminosity (900–920 lux) over each test arena, which is important because codling moth neonates exhibit mild phototropism (Jackson, 1982). Assays were evaluated after 20 h to determine which plug was fed upon.

A feeding deterrence index was calculated as described in Jones (1952) by dividing the number of neonates feeding on apple plugs treated with *Artemisia* extracts by the number of neonates feeding on the plugs treated with dehydration alcohol only, subtracting this figure from 1, and multiplying the result by 100. Prior to every experiment, glass rods and Petri dishes were washed sequentially in tap water, double-distilled water, alcohol, and then dried. In contrast with the bioassay of Suomi et al. (1986) where one-third of tested larvae did not find the fruit, our method assures that 100% of tested larvae feed on apple plugs. See Durden et al. (2008) for a detailed description of this bioassay.

Statistics

Exact Fisher's test ($\alpha = 0.05$) was used in all bioassays to test the null hypothesis that neonates do not discriminate between plugs or apples treated with plant extract and those treated with alcohol (i.e., 50% of the neonates choose treated plugs or apples and 50% of the neonates choose control plugs or apples).

Results

HPTLC analysis of *Artemisia* extracts

In order to test the ability of various *Artemisia* species to deter codling moth neonates from apples, we initially compared crude alcohol extracts of *Artemisia* leaves by qualitative HPTLC. Derivatized α-thujone produced a pink band that located at R_f 0.75–0.79 in the lane containing the crude extract from *A. arborescens*. Noteworthy, α-thujone was absent from the *A. absinthium*, *A. annua*, and *A. ludoviciana* crude extracts. The α-thujone concentration in the *A. arborescens* extract averaged 77.4 ± 2.4 mg/g ($n = 16$).

Feeding deterrent effects of crude *Artemisia* extracts isolated from HTPLC plates

Each of the crude *Artemisia* extracts was examined in a deterrent assay (Durden et al., 2008) with codling moth neonates. Only the material from *A. arborescens* showed feeding deterrent properties at 10 mg/ml (Table 1, $n = 24$, $p < 0.01$, Fisher's exact test). The calculated deterrence index was 90.9. The remaining crude extracts, isolated from HPTLC plates at R_f 0.75–0.79, did not have any detectable feeding deterrent properties.

Concentration-dependent, feeding deterrent effects of crude *Artemisia arborescens* extract and α-thujone alone

A pure preparation of commercially available α-thujone prevented fruit infestation by codling moth neonates at concentrations of 10, 30, and 100 mg/ml (Table 2, $n = 34$–44, $p < 0.05$, Fisher's exact test) and exhibited moderate deterrence indexes ranging from approximately 70 to 78. The crude extract from *A. arborescens* was more effective and prevented fruit infestation by codling moth neonates at concentrations as low as 1 mg/ml (Table 3, $n = 46$, $p < 0.01$, Fisher's exact test), with higher deterrence indices, in the 80–90 range.

Table 2. Concentration-dependent feeding deterrence of α-thujone against codling moth neonates.

		Number of neonates feeding on apple		
Concentration tested (mg/ml)	Number of neonates tested	Treated with extract	Treated with solvent	Deterrence index
100	44	8**	36	77.8
30	38	7**	31	77.4
10	34	8*	26	69.2
3	34	16	18	11.1
1	40	20	20	0

**$p < 0.01$, *$p < 0.05$, Fisher exact test.

Table 3. Concentration-dependent feeding deterrence of the crude extract from *Artemisia arborescens* ''Powis Castle'' against codling moth neonates.

		Number of neonates feeding on apple		
Concentration tested (mg/ml)	Number of neonates tested	Treated with extract	Treated with solvent	Deterrence index
10	48	4***	44	90.9
3	38	6**	32	81.3
1	46	8**	38	78.9
0.3	38	12	26	53.8
0.1	38	15	23	34.8

***$p < 0.001$, **$p < 0.01$, Fisher exact test.

Table 1. Comparative feeding deterrence of extracts isolated from *Artemisia absinthium*, *Artemisia arborescens*, *Artemisia annua*, and *Artemisia ludoviciana* chromatograms.

		Number of neonates feeding on apple		
Artemisia species	Number of neonates tested	*Treated with extract*	*Treated with solvent*	Deterrence index
A. absinthium	26	13	13	0
A. arborescens ''Powis Castle''	24	2**	22	90.9
A. annua	26	13	13	0
A. ludoviciana ''Valerie Finnis''	24	11	13	0

The areas of the chromatograms that co-localized with α-thujone standards were extracted with dehydration alcohol, prepared as described in methods and tested in the bioassay for apple-feeding deterrence against codling moth neonates.
**$p < 0.01$, Fisher exact test.

Discussion

The botanical insecticide, repellent, and deterrent field has grown tremendously in the past decade. Plants of the *Artemisia* family have been one the area of interest in these studies. However, recent reports have concentrated mainly on the effects of *Artemisia* on stored product beetles such as *T. castaneum*, *Callosobruchus maculatus*, *Sitophilus oryzae, Sitophilus granaries,* or *Rhyzopertha dominica* (Kordali et al., 2006; Liu et al., 2006; Negahban et al., 2006a,b; Sharifian et al., 2012; Wang et al., 2006) or in actual and potential malaria vectors such as mosquitoes (Devi et al., 2010; El-Sheikh, 2009; Panneerselvam et al., 2012; Tonk et al., 2006; Tunón et al., 2006). The same trend can be observed in recent studies on the effects of thujone on insects (Hill et al., 2009; Ghaninia et al., 2007; Lee et al., 2003; Liu et al., 2010; Syed & Leal, 2008; Sener et al., 2009). In all aforementioned species, *Artemisia* extracts had toxic and/or repellent properties.

Only recent studies have intensively examined the feeding deterrent or repellent effects that *Artemisia* extracts or essential oils have in economically important caterpillars such as *Plodia interpunctella* Hübner (Karahroodi et al., 2009), *Glyphodes pyloalis* Walker (Khosravi et al., 2010), *Plutella xylostella* L. (Sharma et al., 2006), *Pieris rapae* L. (Hasheminia et al., 2011), or *Spodoptera frugiperda* J. E. Smith (Knaak et al., 2013). In all cases, *Artemisia* extracts or essential oils exhibited either larvicidal or repellent and anti-feedant properties. Reports on the effects of dietary thujone on caterpillars are scarce; we are aware of only one paper (Lee et al., 1999) that shows larvicidal activity and growth inhibitory effect of thujone against the European corn borer, *Ostrinia nubilalis* Hübner. To the best of our knowledge, our current study demonstrates for the first time that *A. arborescens* and thujone prevent fruit infestation by internal fruit feeding larvae.

We did not find α-thujone in the crude extracts from *A. absinthium*, *A. annua*, and *A. ludoviciana*. This finding is consistent with recent reports of the chemical composition of plants from the *Artemisia* genus, which often occur as chemotypes that are morphologically indistinguishable, but chemically different. *Artemisia absinthium* is a good example here: chemotypes from Siberia, Bashkir, or Morocco may contain significant amounts of α-thujone ranging from 16% to almost 40% of essential oil (Blagojevic et al., 2006; Derwich et al., 2009), whereas those from Italy contain only trace amounts of α-thujone (Blagojevic et al., 2006). Similarly, *A. absinthium* from Northern Iran contains negligible amounts of α-thujone (Rezaeinodehi & Khangholi, 2008).

Recent analysis by GC/MS (Radulović et al., 2013) showed the absence of α-thujone in essential oil from Serbian *A. annua.* Earlier works of Holm et al. (1997) and Goel et al. (2008) yielded similar results: thujone has not been found in any of seven *A. annua* chemotypes originating from regions as diverse as China, Tasmania, Hungary, France, the former Yugoslavia, Italy, and India.

Blust and Hopkins (1987) found α-thujone in essential oil from *A. ludoviciana* collected in Kansas. Although the concentration was not reported, visual inspection of gas chromatograms published in their paper suggests that α-thujone was a minor component of the oil in their study. A newer study by Lopes-Lutz et al. (2008) on the chemical composition of Canadian *A. ludoviciana* demonstrated absence of thujones in essential oil obtained from foliage of this plant.

Tucker et al. (1993) reported about 2.5% of α-thujone in essential oil of *A. arborescens* ''Powis Castle''. Our estimation that crude extract of the same cultivar contains approximately 7.5% of α-thujone is generally consistent with these findings.

In our experiments, only the material from *A. arborescens* ''Powis Castle'' contained in the area of chromatograms between R_f 0.75 and 0.79, where α-thujone standards localized, showed feeding deterrent properties (Table 1). This finding strongly suggested that α-thujone produced *by A. arborescens* prevents fruit infestation by the codling moth. Indeed, α-thujone alone, tested with our bioassays, showed feeding deterrent properties (Table 2). However, comparison of deterrent activities of various α-thujone concentrations (Table 2) with those obtained by dilutions of *A. arborescens* ''Powis Castle'' crude extracts (Table 3) indicates that α-thujone is not the only insect deterrent chemical that these extracts contain; the crude plant extract is deterrent at concentration 10 times lower than minimum effective concentration of α-thujone.

The crude extract from *A. arborescens* ''Powis Castle'' prevented apple infestation by codling moth neonates in a dose-dependent manner (Table 3), and with a efficacy similar to that exhibited by *A. annua* in our earlier study (Durden et al., 2011), with a minimal effective concentration of 1 mg/ml in both cases. Similar to crude extract from *A. annua,* the crude extract from *A. arborescens* ''Powis Castle'' had more pronounced deterrent properties against codling moth neonates than crude extract from *A. absinthium* that we investigated earlier (Durden et al., 2009). Crude *A. absinthium* extract showed deterrent properties only at concentrations of 10 mg/ml and higher (Durden et al., 2009).

Because both *A. arborescens* ''Powis Castle'' crude extract and α-thujone exert their codling moth deterrent properties at relatively high concentrations, their use as sprays in codling moth control would probably be impractical. Perhaps small-scale organic or environmentally conscious growers could use extracts of *A. arborescens* ''Powis Castle'' as a technique supplementary to mating disruption. Also, it should be noted that the possible use of sprays containing α-thujone may raise some objections. α-Thujone has been found to act as a GABA receptor antagonist both in insects and mice and is regarded as detrimental to human health (Höld et al., 2000).

In our experiments, we used apple plugs of standardized surface area (28 mm^2) and a standard volume of α-thujone (5 μl at 10 mg/ml) had to be applied to each apple plug to prevent fruit infestation. This allowed for a calculation how much of α-thujone would have to be applied to apples at the time close to harvest. Assuming that the apples are of 8 cm diameter each, and there are 6 apples per kilogram, the amount of α-thujone deposit resulting from direct spray would equal approximately 200 mg/kg of fruit, well above the levels of α-thujone presently permitted in food or beverages in the USA and European Union (Demyttenaere, 2012; Pelkonen et al., 2013). However, the fact that α-thujone is a GABA receptor antagonist acting similar to many insecticide prototypic substances (Casida & Palmer, 1988) and insecticides (Casida & Durkin, 2013) suggests screening insecticides that block GABA receptors for their feeding deterrent properties as a new direction of future research on codling moth-feeding deterrents. It may be that some commercially available insecticides prevent apple infestation by the codling moth at concentrations much lower than those required for killing the insect. This way the grower could possibly greatly reduce the field rates of insecticide application (and detrimental impact on the environment) and still maintain satisfactory levels of codling moth neonate control.

Conclusions

Our research showed that *A. arborescens* ''Powis Castle'' contains substances that prevent fruit infestation by internal fruit-feeding larvae and identified α-thujone as one of these substances. Our data indicates that there are additional insect feeding deterrents present in *A. arborescens* ''Powis Castle''. Based on this study, we argue that using this plant or α-thujone as large-scale sprays in codling moth control would probably be impractical. However, our findings delineate a new area of research on codling moth neonate-feeding deterrents based on the screening of insecticides that block GABA receptors for their potential of preventing fruit infestation.

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Declaration of interest

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