Toxicities of Thymol, Citronellal, Eugenol and Rosemary Oil to Control *Agriotes obscurus* (Coleoptera: Elateridae) in Laboratory and Greenhouse Bioassays

By

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ABSTRACT

Toxicities were determined for four naturally occurring monoterpenoid essential oils on late instar larvae of *Agriotes obscurus* in laboratory and greenhouse bioassays. Both contact and fumigant toxicities were determined for thymol, citronellal, eugenol and rosemary oil. Thymol had the greatest contact toxicity (LD_{50}=196.0 μg/larva) while citronellal and eugenol were significantly less toxic (LD_{50}=404.9 μg/larva and 516.5 μg/larva, respectively). Rosemary oil did not show any significant contact toxicity, at the highest dose tested, 1600 μg/larva. In fumigant bioassays, citronellal was the most toxic to wireworm larvae (LD_{50}=6.3 μg/cm^3) followed by rosemary oil (LD_{50}=15.7 μg/cm^3), thymol (LD_{50}=16.9 μg/cm^3), and eugenol (LD_{50}=20.8 μg/cm^3). Phytotoxicities were also evaluated based on corn seed germination and seedling development. In laboratory bioassays, thymol and citronellal significantly inhibited seed germination and development while rosemary oil had only minimal phytotoxic effects.

Based on these laboratory results, four treatments were selected for repellency trials under greenhouse conditions. Citronellal (800 μg/seed), thymol (400 μg/seed), rosemary oil (1600 μg/seed) and eugenol (1600 μg/seed) were evaluated for their ability to protect corn seeds from *Agriotes obscurus* feeding damage. In the bioassay, wireworm health, seed germination, seed damage, and seedling height were evaluated. Rosemary oil treated seeds produced the highest number of distressed wireworms (56%, Odds Ratio = 2.64) compared to all other treatments while not creating any phytotoxic effects. Seeds treated with thymol were the least damaged (90.7% undamaged, Odds Ratio = 0.153) compared to the control while thymol and citronellal retarded seedling height compared to the control. Eugenol produced the lowest seed germination (25.3% germination) while citronellal, rosemary oil and thymol had no negative impact on seed germination.

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For my Family, Parents and Dr. Andrew Riseman.

CHAPTER ONE

1.1 General Introduction

Wireworms are the larval form of click beetles and belong to the family Elateridae. In British Columbia (BC), there are over 150 known species of wireworm (Vernon *et al.*, 2001). However, only two species, *Agriotes lineatus* (L.) and *A. obscurus* (L.), are considered significant pests to British Columbia's (BC) potato industry (Vernon *et al.*, 2001). In BC, these two species have become the single most important potato pest, with damage estimates in 1994 ranging from $500,000 to $800,000 CAD (Vernon, 1998). In addition, they are responsible for significant losses to corn and other field crops.

The larvae are the most destructive stage to agricultural production with subsequent economic impact. In addition to corn (*Zea mays* L.) and potatoes (*Solanum tuberosum* L), larvae are also destructive to crops such as beans (*Phaseolus vulgaris* L.), carrots (*Daucus carota* Hoff.), lettuce (*Lactuca sativa* L.), beets (*Beta vulgaris* L.), strawberries (*Fragaria vesca* L.), and asparagus (*Asparagus officinalis* L.). They cause damage by:

1. feeding on pregerminated seeds causing loss of viability;
2. feeding on roots of young plants causing death; and
3. feeding on below–ground regions of plants, resulting in unmarketable products (i. e., potato tubers)

Wireworms cause most of their damage in the early spring due to both the tender stage of plant development as well as seasonal wireworm movement in the soil profile that brings them closer to the surface.

1.2 Background and Significance

1.2.1 Insect biology

The wireworm life cycle is summarized in Figure 1.1. Adult females emerge from the soil during April to May to mate and then return to the soil in May to June to lay their eggs. A single female can lay between 200-1400 eggs across several locations within a single season. Their eggs are minute, oval and pearly white. After 4-6 weeks, young wireworm larvae hatch and commence feeding. If no food is found, larvae die within 30 days while well-fed larvae of *Agriotes* spp. can live up to 4-6 years (http://www.bayercropscience.co.uk/pdfs/WirewormExpertGuide2005.pdf). Each instar passes through mandible hardening and darkening, feeding, and premolting stages during their life cycle (Furlan 1998).

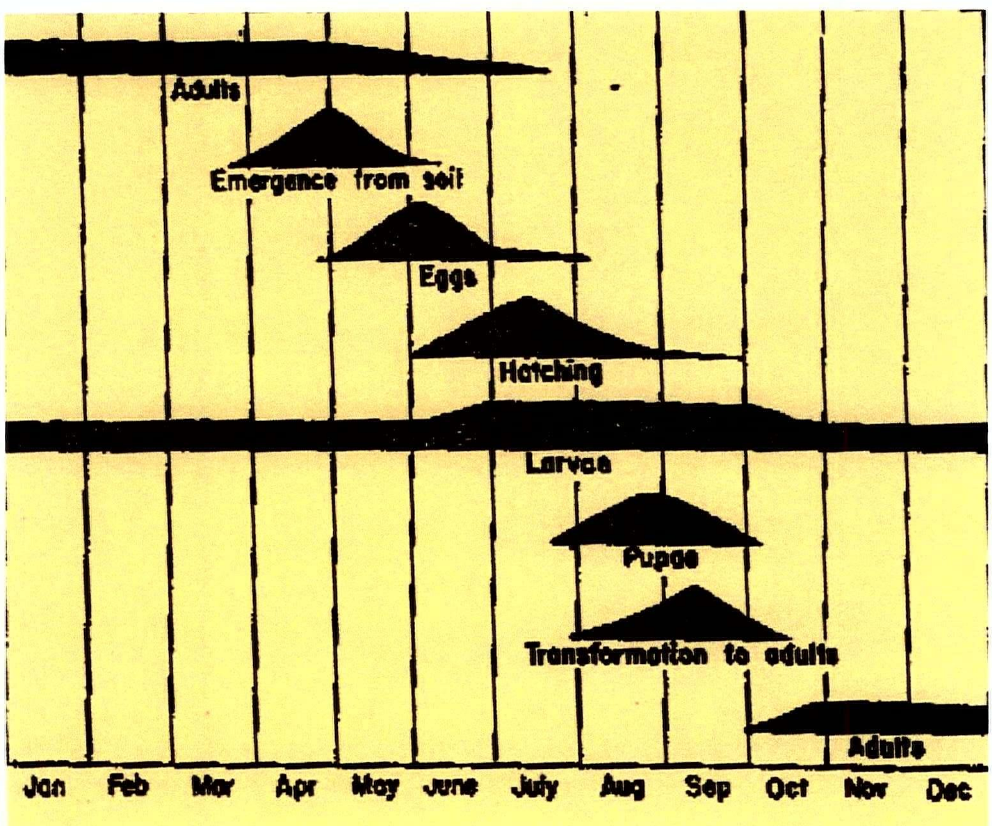

Figure. 1.1. *Agriotes* Life Cycle (Reprinted from PNW Insect Control Handbook, 1992).

Larvae can live between 4-11 years, depending on the species, without reaching adulthood. They typically over-winter in the soil at a depth of 5-25 cm depending on several environmental conditions (e.g., temperature, moisture content). Wireworm larvae are hard, smooth, slender and wire-like. Their body length varies from 2-2.5 cm and bear three pairs of small, thin legs. Typically, the body is yellowish-white to coppery-brown in colour. Typical larval damage by *A. obscurus* is seen on a potato tuber (Figure 1.2).

Figure 1.2. Larval damage on potato tuber by *Agriotes obscurus*.

1.2.2 Effects of soil temperature on larval behaviour

Soil temperatures significantly affect larval behavior. Larvae start to move toward the surface during spring when soil temperatures, at 15 cm depth, reach 10 °C. In late spring and early summer, when soil temperatures reach 26°C and above, larvae tend to move below 15 cm depth. There, the majority of larvae will remain until the following spring (Figure 1.3).

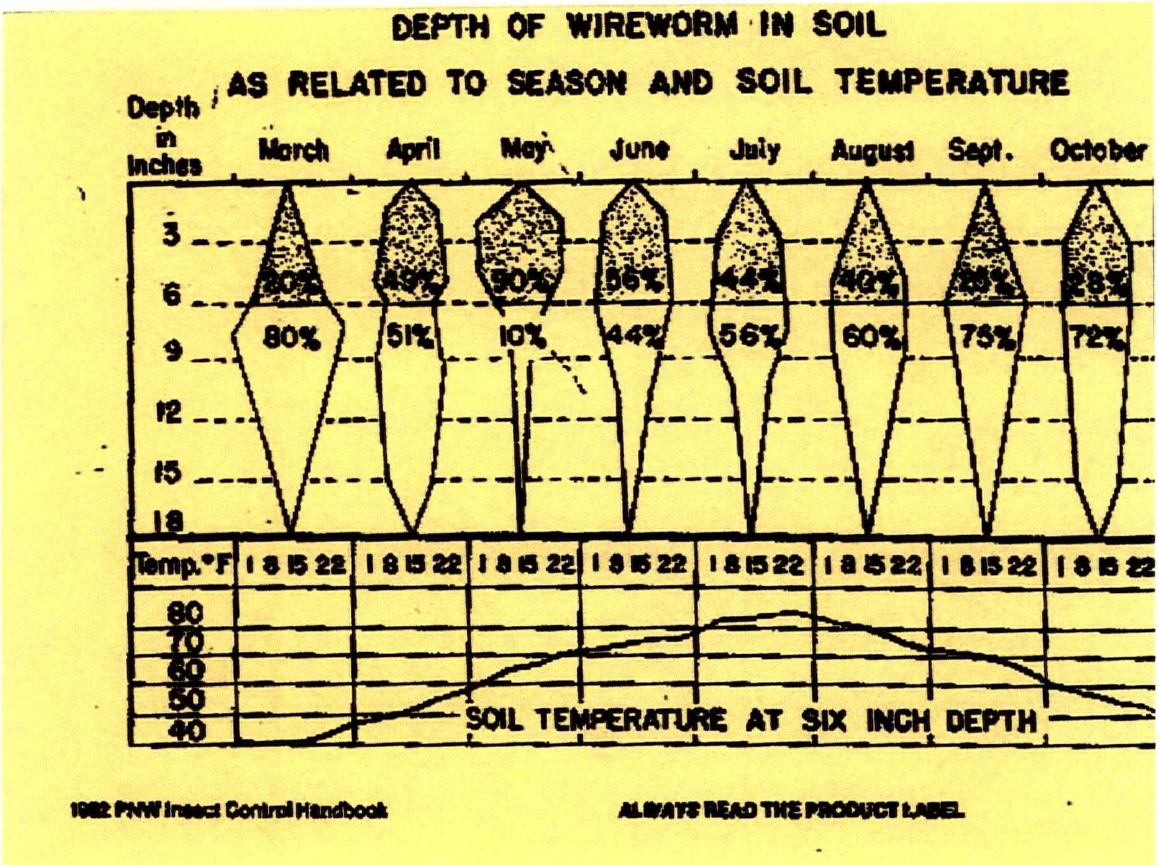

Figure 1.3. Effects of soil temperature on larval behavior (Reprinted from PNW Insect Control Handbook, 1992)

Compared to the immature stages, mature stages (i.e., pupae and adults) can mostly be found in the top 15 cm of the soil, regardless of temperature. Once fully grown (typically in July), the larvae pupate 5-10 cm below the surface. Pupation lasts for less than a month, depending on the species, but adults do not emerge until the following spring.

Adult beetles are brown to black in colour, about 8-12 mm long, bullet shaped and hard shelled. They are commonly known as 'click beetles' because they produce clicking sounds when the insect is attempting to right itself after landing on its back. Life stages of A. obscurus are shown in Figure 1.4 A-F.

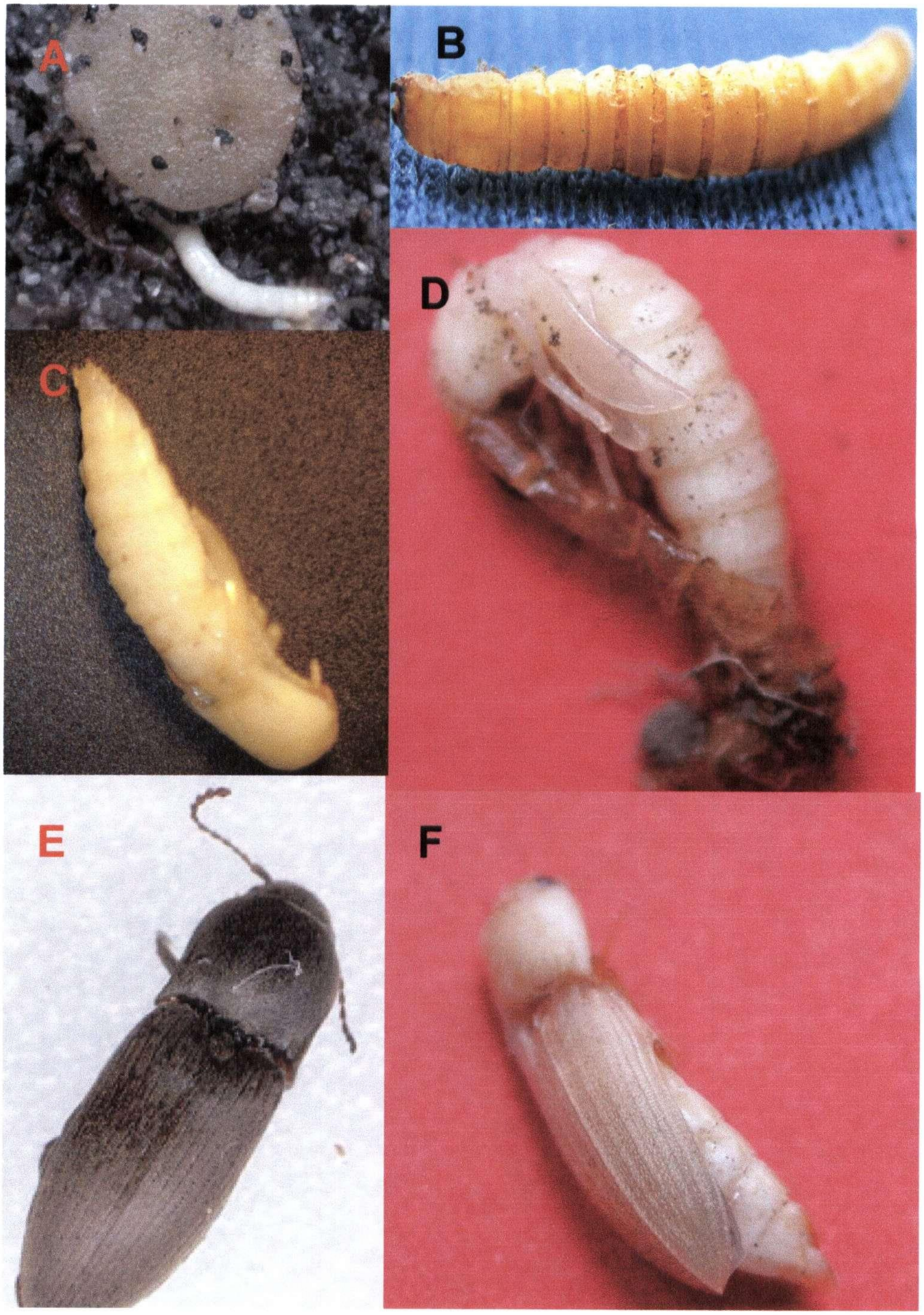

Fig. 1.4. Life stages of *A. obscurus.* A) Larvae just after molting with old cuticle seen beside the newly emerged larvae (2x); B) Late instar larvae before pupation, body is swollen and the cuticle is about to rupture (5x); C) Pupa (10x); D) Pupation with developing elytra and antennae (10.5x); E) Dorsal view of adult (9x) (Courtesy of - http://www.bioimages.org.uk/HTML/R150445.HTM); F) Newly emerged adult (8x).

1.2.3 Current control methods

To date, no crop has been genetically engineered for resistance to wireworms (Pacific Northwest Insect Control Handbook, 1992). Current cultural (e.g., crop rotation) and mechanical (e.g., ploughing) methods used to control infestations have not been effective in reducing populations below the economic threshold levels of affected crops (Pacific Northwest Insect Control Handbook, 1992). Chemical control methods are currently the most effective tool against wireworm infestation but are only effective for a short period of time after application. Due to government restrictions, the number of available insecticides to control this pest has been steadily decreasing. Through 2004, Lyndane, an organochlorine (γ – HCH – Hexa Chloro Cyclo Hexane, Crompton Corporation, Middlebury, CT), was used extensively to control wireworm but has recently been banned in North America due to its potential toxicity to wildlife and mammals (R. Vernon, Personal communication).

1.2.4 Plant essential oils (EOs)

In addition to the synthetic chemicals described above, another class of chemicals that show promise for control of insect pests are the plant essential oils (EOs). These oils are typically volatile compounds distilled from either flowers, roots, shoots, seeds or leaves. They are typically produced in minute amounts where the distillation of an entire plant may produce only a single drop of the desired oil.

Most EOs consist of terpenoids of low molecular weight and are volatile and aromatic (Isman, 1999). They are widely used as fragrances in the perfume industry and as flavoring agents in the food industry. However, in addition to these uses, essential oils have been used as general biocides to kill bacteria, viruses and fungi and recently, have been used to control some insect pests (Lee *et al.*, 1997). There is strong evidence that plant essential oils can be used in agriculture as both insect repellents and fumigants (Lee *et al.*, 1997). One common example of an essential oil used as an insect repellent is citronellal, derived from the citronella plant (*Cymbopogon nardus* L.), and used to repel mosquitoes.

Most EO constituents are monocyclic monoterpenes. They can be absorbed by insects through oral or topical routes, such as inhalation or by direct contact, and typically create symptoms consistent with neurotoxins (e.g., hyperactivity, convulsions) (Isman, 1999). However, their effectiveness was dependant on the ability of the EO to penetrate membranes and reach target sites.

Use of EOs against crop pests has not been thoroughly investigated to date. However, some of these compounds have been tested as greenhouse fumigants to control spider mites (*Tetranychus urticae* Koch) and aphids (*Aphid cerana* L.) (Tuni and Sachinkaya, 1998). In addition, monoterpenes and phenols are reported to be toxic against the western corn rootworm (*Diabrotica virgifera* LeConte) when applied to the soil (Lee, *et al.*, 1997). These same compounds have been tested against the tobacco cutworm (*Spodoptera litura* F.) and have been shown to deter feeding and inhibit larval growth (Isman, 1999).

The use of naturally occurring, plant based chemistry to control pests is gaining greater attention by the scientific community. This is due to the combination of greater analytical capabilities, the ability to dissect complex biochemical systems, and finally, to the overall compatibility of this strategy with the tenets and regulations governing organic farming practices. I believe that the use of plant essential oils will provide farmers an environmentally friendly and sustainable alternative to using synthetic pesticides.

Reasons for the limited use of these chemicals in agricultural settings include phytotoxicity (Schoonhoven, 1982), inability to duplicate laboratory results under field conditions, lack of chemical persistence in the environment (Ladd *et al.*, 1978) and socio-economic constraints (Bernays, 1983). To address these concerns, several criteria have been suggested by Villani *et al.* in (1985) describing the properties these compounds should posses for effective use under field conditions. These include:

1. adequate persistence for crop protection;
2. systemic activity;
3. low cost; and
4. absence of phytotoxicity.

Villani and Gould (1985) suggest that one reason for the lack of success in using EOs as deterrents was that the focus of protection was on only foliage and fruits. They suggest a more successful approach might involve using these compounds to protect germinating seeds and seedlings from soil insects. Reasons cited for this recommendation include.

1. persistence is needed for only a short period;
2. there is decreased need for systemic action;
3. relatively small quantities of extract can protect many seeds; and
4. seeds can be treated before planting.

Based on this reasoning, I developed the following two objectives:

1. To determine contact and fumigant toxicities of thymol, citronellal, eugenol and rosemary oil to late instar larvae of *A. obscurus*; and
2. To evaluate thymol, citronellal, eugenol, and rosemary oil for protective properties on corn seeds/seedlings from feeding injury by *A. obscurus*.

CHAPTER TWO

INSECTICIDAL ACTIVITY OF SELECTED MONOTERPENOIDS AND ROSEMARY OIL TO *Agriotes obscurus* (COLEOPTERA: ELATERIDAE)

2.1 Introduction

Wireworms (*Agriotes* sp. L, Coleoptera: Elateridae), the larval form of click beetles, consist of over 800 species distributed worldwide. They are significant pests wherever they occur, infesting both cereal and forage crops, but have recently become economically significant pests to corn and the single most damaging potato pest in British Columbia (Vernon *et al.*, 2001). These insects are most destructive during their larval stage by feeding on pre-germinated seeds, the radical or young roots of juvenile plants, or directly on tubers, causing unmarketable products. Currently, the most effective control strategies rely on soil–incorporated synthetic pesticides. However, these chemicals often have adverse secondary effects in the environment including long persistence times and human health concerns (U.S. Environmental Protection Agency, 1994). In addition, organic farmers, who cannot use synthetic chemicals, have no effective alternatives for wireworm management. Therefore, alternatives to synthetic pesticides, that are compatible with established control strategies and organic production guidelines, need to be developed.

The use of natural, plant-based chemistry to control insect pests is gaining greater attention by the scientific community due both to increases in analytical capability and enhanced ability to dissect complex biochemical systems. Also, its general compatibility with organic farming practices makes it an attractive topic for agricultural researchers interested in developing sustainable agricultural practices. In line with organic farming practices, identification of production system interactions have allowed for more targeted use of these compounds as crop protection agents. For example, when corn is protected from wireworm damage during the first three weeks of growth, economic impact can be minimized (unpublished data). Therefore, acceptable qualities of these compounds for pest protection include not only direct toxicity but also repellency effects on the pest. However, for this method to be practical in agriculture, potential phytotoxic effects of these compounds, on specific crops, need to be identified.

Phytochemicals have been used for many years to control insect pest damage on agricultural crops (Lee *et al.*, 1997). Plants produce a wide range of secondary metabolites (i.e., terpenoids, alkaloids, phenolics) that often possess insecticidal, fungicidal, bactericidal, antiviral, antifeedant, or insect growth retardant properties (Benner, 1993; Singh *et al.*, 1989; Wilson *et al.*, 1997). Many of these chemicals are attractive alternatives to synthetic chemicals because most are unlikely to leach into ground water or persist in soil or sediments due to their high vapor pressure and relative lipophilicity (Isman, 1999), as well as their reduced impact on beneficial insect populations, an important component in integrated pest management systems used by many organic farmers (Plimmer 1993).

Terpenoids, including the major constituents of most essential oils, are an important class of secondary metabolites possessing insecticidal activity. Essential oils refer to the steam-distillable fraction of plant tissues often responsible for their characteristic scent or odor (Isman, 1999) and which are sometimes incorporated into natural pest control products. For example, citronellal, an acyclic monoterpene has become a popular natural alternative to DEET (*N, N-dethyl-m*-tolumide) for personal protection against mosquitoes and biting flies (Isman, 1999; Karr and Coats, 1988). In addition, rosemary oil, thyme oil, and eugenol are now available in consumer pest control products further supporting the benign effects of these compounds on humans and the environment. Although considered safe for human use, essential oils often display significant biochemical effects on insects. Hough–Goldstein (1990) reported the antifeedent effects of essential oils against the Colorado potato beetle (*Leptinotarsa decemlineata* L.) while Sharma and Saxena (1974) showed their effectiveness as growth inhibitors on houseflies (*Musca domestica* L.).

More specifically, many researchers have reported repellent, antifeedent and toxic properties of selected essential oils against many agriculturally important pests. Thymol showed both repellent and toxic effects against the two-spotted spider mite (*Tetranychus urticae* Koch) (Gengaihi *et al.*, 1996) while individually, thymol and citronellic acid were toxic to the common housefly, western corn rootworm (*Diabrotica vergifera vergifera* LeConte), and the two-spotted spider mite (*Tetranychus urticae*) (Lee *et al.*, 1997). Thyme oil was toxic to tobacco cutworm (*Spodoptera litura*) (Isman *et al.*, 2001) while rosemary oil was toxic to several predaceous mites (*Amblyseius barkeri* Hughes, *A. zaheri* and *Typhlodromus athiasae* Porath) (Momen and Amer, 1999) but also showed repellent properties against the onion aphid (*Neotoxoptera formosana* Takahashi) (Masatoshi and Hiroaki, 1997) and the green peach aphid (*Myzus persicae* (Sulzer) (Masatoshi, 1998). Finally, both contact and fumigant toxicities of eugenol and methyl eugenol were demonstrated on the American cockroach (*Periplaneta americana* L.) (Ngoh *et al.*, 1998). Unfortunately, despite these reports, there is no information on the toxic or repellent properties of these compounds against wireworms or their phytotoxic effects on specific plant species.

The mechanisms of toxicity of essential oils have not been fully identified (Enan, 2001). However, regardless of the method of administration (e.g., oral, topical, or inhalation), insects acutely poisoned by certain essential oils display symptoms consistent with a neurotoxic mode of action (Isman, 1999; Coats *et al.*, 1991; Grundy *et al.*, 1985; Brattstan, 1983), including agitation, hyperactivity, paralysis and quick knockdown.

In this study, I investigated the toxicity of three structurally different (i.e., aliphatic and aromatic) monoterpenoids (thymol, citronellal and eugenol) and one complex essential oil (rosemary oil) on late instar larvae of *Agriotes obscurus* with the following specific objectives:

1) To quantify the contact and fumigant toxicities of selected compounds to *A. obscurus*.
2) To evaluate the phytotoxic effects of selected compounds on corn seed germination and development.

2.2 Materials and Methods

2.2.1 Chemical compounds. The selection of essential oils for inclusion in this study was based on reported efficacy on other insect pests, low mammalian toxicity, and benign effects on the environment. The following technical grade compounds were included: citronellal (>95% purity; EcoSMART Technologies Inc., Franklin, TN, USA), thymol (>98%; Sigma Chemical Co. St. Louis, MO, USA), and eugenol (>95%; Arylessence

Inc., Marietta, GA, USA). Rosemary oil (Lot No. 0213142MB) was provided by EcoSMART Technologies Inc.

2.2.2 Insect rearing and preconditioning. Late instar larvae (19-22 mm in length, 35-48 mg in weight) of *A.obscurus* were collected from fallow fields near the Pacific Agricultural Research Station (Agriculture and Agri. Food Canada) Agassiz, British Columbia, Canada, and stored at 5 ^{0}C in soil-filled containers until use. Twenty-four hours prior to experimentation, the temperature was raised to 20 ^{0}C and slices of potato were placed 2-3 cm below the soil surface to identify actively feeding larvae. Wireworms of similar length (19-22 mm) were collected every 6h under the potato slices for use in the bioassays.

For *in vitro* evaluation of chemical toxicity, it is essential to select wireworms of similar developmental stage and comparable feeding activities. Due to the difficulty in identifying instar stage through morphological observations, I classified wireworm developmental stage through length measurements. All wireworms were 3-4 years old based on length criteria developed by Subklew (1934) for *A. obscurus*. I also selected only actively feeding wireworms for inclusion as these were found to be representative of wireworm behavior in the field (Vernon, personal communication).

2.2.3 Preparation of bioassay arena. Disposable 8 cm petridishes were used for both topical and fumigation bioassays. Twenty grams of dry technical grade sand (pre-washed with running water and autoclaved for 30 min at 120 ^{o}C) and 3.5 ml of distilled water were added to each dish. The sand/water mixture was found previously to allow for normal wireworm behavior (e.g., vigorous movements, molting and pupation) over 4 mo period and was deemed suitable for these bioassays. One untreated corn seed (Pioneer Brand Hybrid # 39T68, Pioneer Hi-Bred Ltd, Chatham, ON), pre-soaked in water for 24 h, was added to the center of each dish as a food source.

2.2.4 Topical bioassays. Five concentrations (100, 200, 400, 800 and 1600 μg/10 μl) of each compound and rosemary oil were prepared in 100% methanol. Wireworms were held with insect handling forceps while applying the chemical with a micropipettor along the entire length of the dorsum. Each insect was treated with 10 μl of the treatment solution (i.e., 100, 200, 400, 800, or 1600 μg/larva) with control insects receiving 10 μl of pure methanol. Immediately following treatment, individual wireworms were placed in petridish arenas that were then sealed with parafilm. Sealed arenas were placed in a dark growth chamber maintained at 15 °C. Toxicity was assessed daily beginning 24 h after treatment. Larval death was confirmed by either probing the thoracic and head areas (if alive, larvae showed movement of body, legs or mouth parts) or by noting changes in body color (once dead, body color changed from their natural color to dark brown to black). These procedures were found to be the most accurate ways to confirm death, as some wireworms appear initially moribund or dead but recovered after a few days. Observations were taken over 15 d in order to allow sublethaly poisoned wireworms to recover or die. During this recovery period, untreated corn seeds were replaced every 3 d. Each chemical/dose combination was tested on seven wireworms. The entire experiment was repeated three times.

Figure 2.1. Topical bioassay arena.

2.2.5 Fumigant bioassays.

Treatment concentrations were 5, 10, 20, 40 and 80 $\mu g/cm^3$ while the arena and wireworm cultures were as described above. Thirty µl of treatment solution were added to a piece of paper towel (2 cm x 1 cm), attached to the lid of the petridish with double sided tape, and immediately sealed with parafilm after placing a wireworm at the center of the petridish (Figure 2.2). Sealed dishes were stored in the dark at 15 ^{0}C. Observations were identical to those in the topical bioassays. Each chemical/concentration combination was tested on seven wireworms with the entire experiment repeated three times.

Figure 2.2. Fumigation Bioassay Arena.

2.2.6 Phytotoxicity test.

Plastic pots (14 cm dia) filled with cleaned sand were used to test the phytotoxicity of the compounds and rosemary oil to corn. Corn seed (Pioneer Brand, Hybrid # 39T68, Pioneer Hi-Bred Ltd, Chatham, Ontario) was pre-soaked in water for 24 hrs, and using a micropipettor, coated with 20 μl of the test solutions described in the wireworm bioassays. Chemical doses delivered to each seed were either 200, 400, 800, 1600, or 3200 μg/seed. After treating seeds, they were planted 2.5 cm deep in the sand. This experiment was conducted in the Horticulture greenhouse, University of British Columbia, which is maintained at 22 ^{0}C. To avoid drying, pots were immersed in trays with 1 cm water every second day. Pure methanol was used as the control treatment. Germination percentage, cotyledon length and radicle length were recorded six days post treatment. Each chemical concentration was tested on 10 corn seeds with the entire experiment repeated three times.

2.2.7 Statistical analyses.

Data from both topical and fumigation bioassays were subjected to probit analysis to estimate LD_{50} or LC_{50} values (Finney 1971, SAS Institute 1991). Analysis of variance procedures were performed by SuperANOVA software (Abacus Concepts Inc. Berkeley, CA). Treatment means were compared by Fisher's Protected LSD with an α value of 0.05 (SAS Institute 1991). For the phytotoxicity assay, analysis of variance procedures were performed by SPSS software (SPSS Inc., Chicago, IL) and treatment means were compared by Fisher's Protected LSD with an α value of 0.05.

2.3 Results and Discussion

2.3.1 Topical bioassays.

Differences in wireworm mortality were observed among the compounds ($F_{3, 30}$ = 6.79, P = 0.0001) and doses ($F_{5, 30}$ = 1.11, P = 0.0001) (Table 2.1). A compound x dose interaction was also noted ($F_{15, 30}$ = 1.28, P = 0.0001) (Table 2.1). Thymol, citronellal and eugenol produced the highest topical mortality (Figure 2.3), with LD_{50} values of 196, 405 and 517 µg/larva, respectively (Table 2.5). Rosemary oil elicited only slight mortality (24%) at the highest dose tested (Table 2.2), and no wireworm mortality or morbidity was observed in the methanol controls.

Table 2.1 ANOVA table for contact toxicity of thymol, citronellal, eugenol, or rosemary oil.

Source	df	SMS	MS	F-Value	P-Value
Block	2	16.98	8.49	0.071	0.09314
E.Oil	3	24316.27	8.11	6.79	0.0001
Concentration	5	6.65	1.33	1.11	0.0001
Block*E.Oil	6	912.65	152.11	1.28	0.298
Block*Concentration	10	2.55	25.51	0.21	0.9931
E.Oil*Concentration	15	22978.62	1531.91	1.28	0.0001
Residual	30	3576.96	119.23		

df-degrees of freedom, SMS-Sums of Squares, MS-Mean Square

Table 2.2 Contact toxicity of selected compounds on late instar larvae of *A. obscurus*.

Monoterpenoid	n	Mean [z] (% Dead)	Std. Dev.	Std. Error
Thymol 0	21	0[a]	0	0
Thymol 100	21	0[a]	0	0
Thymol 200	21	6.67[d]	1.65	9.52
Thymol 400	21	7.14[de]	2.47	1.42
Thymol 800	21	100[f]	0	0
Thymol 1600	21	100[f]	0	0
Citronellal 0	21	0[a]	0	0
Citronellal 100	21	4.76[a]	8.25	4.76
Citronellal 200	21	4.76[a]	8.24	4.76
Citronellal 400	21	9.52[ef]	1.65	9.52
Citronellal 800	21	85.71[ef]	0	0
Citronellal 1600	21	100[f]	0	0
Eugenol 0	21	0[a]	0	0
Eugenol 100	21	0[a]	0	0
Eugenol 200	21	4.76[a]	8.24	4.76
Eugenol 400	21	4.29[dc]	1.43	8.25
Eugenol 800	21	7.14[de]	1.42	8.25
Eugenol 1600	21	9.52[f]	8.25	4.76
R.Oil 0	21	0[a]	0	0
R.Oil 100	21	0[a]	0	0
R.Oil 200	21	0[a]	0	0
R.Oil 400	21	0[a]	0	0
R.Oil 800	21	4.76[a]	8.24	4.76
R.Oil 1600	21	2.38[b]	2.18	1.26

[z] means with same letter superscripts, within column, were not different by Fisher's LSD procedure at α=0.05.

Previous studies using thymol and citronellal at lower doses (LD_{50} = 25.4 µg/larva and 111.2 µg/larva, respectively) were shown to be toxic to tobacco cutworm (*Spodoptera litura* L.) (Hummelbrunner and Isman, 2001), western corn rootworm (*Diabrotica virgifera virgifera* L.), two spotted spider mite (*Tetranichus urticae* Koch.) and the house fly (*Musca domestica* L.) (Lee *et al.*, 1997). I do not know the reason why higher doses were required in my experiments. Body weight differences between wireworms and those other insects may have changed the effective dose required for mortality. However, a more plausible explanation is that wireworms possess a highly which likely makes them less permeable to chemical penetration. The differences in toxicity to wireworms among the compounds tested herein might be due to differences in their absorption properties (Lee et al. 1997), and/or to differences in the ability of *A. obscurus* to detoxify, and/or excrete these compounds.

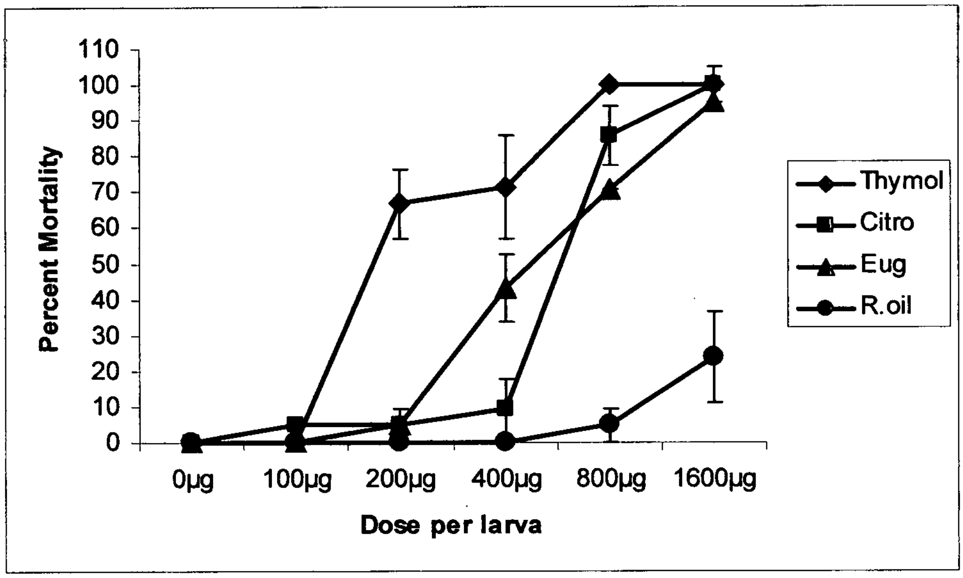

Fig. 2.3. Percent mortality of wireworms (n=21) in the topical bioassay exposed to either thymol, citronellal, eugenol, or rosemary oil; error bars = standard error of the mean.

All compounds tested induced symptoms similar to those characteristic of neurotoxins. For example, thymol induced rapid paralysis at higher doses (800-1600 µg/larva) while eugenol and rosemary oil induced convulsion-like symptoms (e.g., stressful wiggling) or paralysis starting at 800 µg/larva. At lower doses of thymol and citronellal, some wireworms were able to recover from paralysis after a few days post-treatment. The recovery from paralysis observed at lower doses of these EOs suggests that these monoterpenoids may be internally metabolized and/or excreted; however, the exact means of recovery remains unknown (Harwood *et al.*, 1990). Recovery did not occur with higher doses of any of the compounds tested, including rosemary oil.

2.3.2 Fumigant bioassays.

When presented to wireworms in fumigation bioassays, differences in wireworm mortality were observed among the four compounds ($F_{df,\ total\ df} = F_{3,48} = 15.5$, $P = 0.0001$) and doses ($F_{5,\ 48} = 80.3$, $P = 0.0001$) (Table 2.3). A significant compound x dose interaction was again noted ($F_{15,\ 48} = 2.51$, $P = 0.008$) (Table 2.3). In contrast to the topical bioassays, all four compounds induced acute toxic effects (Figure 2.4), with citronellal displaying the highest volatile toxicity (LC_{50} = 6.3 μg/cm^3), followed by rosemary oil (LC_{50} = 15.8 μg/cm^3), thymol (LC_{50} = 17.0 μg/cm^3) and eugenol (LC_{50} = 20.8 μg/cm^3) (Table 2.5). Even though rosemary oil did not produce high contact toxicity, it produced high fumigant toxicity (95% mortality at 80μg/cm^3) (Table 2.4)

Table 2.3 ANOVA table for fumigant toxicity of selected compounds

Source	df	SS	MS	F-Value	P-Value
E.Oil	3	12132.78	4044.26	15.5	0.0001
Concentration	5	104679.5	20935.89	80.29	0.0001
E.Oil*Concentr	15	9807.49	653.84	2.51	0.0081
Residual	48	12516.23	260.75		

Table 2.4 Mortality of late instar *A.obscurus* in fumigation bioassay.

Monoterpenoid	n	Mean ^{Z} (% Dead)	Std. Dev.	Std. Error
Thymol 0	21	0^{a}	0	0
Thymol 5	21	0^{a}	0	0
Thymol 10	21	47.62^{c}	21.82	12.59
Thymol 20	21	95.24^{b}	8.25	4.76
Thymol 40	21	100^{c}	0	0
Thymol 80	21	100^{d}	0	0
Citronellal 0	21	0a	0	0
Citronellal 5	21	23.81^{b}	2.24	4.76
Citronellal 10	21	85.71^{d}	0	0
Citronellal 20	21	100^{d}	0	0
Citronellal 40	21	100^{d}	0	0
Citronellal 80	21	100^{d}	0	0
Eugenol 0	21	0^{a}	0	0
Eugenol 5	21	0^{a}	0	0
Eugenol 10	21	4.76^{b}	8.24	4.76
Eugenol 20	21	42.85^{c}	28.57	16.49
Eugenol 40	21	71.43^{d}	37.79	21.82
Eugenol 80	21	85.71^{d}	24.74	14.29
R.Oil 0	21	0^{a}	0	0
R.Oil 5	21	0^{a}	0	0
R.Oil 10	21	23.81^{b}	21.81	12.59
R.Oil 20	21	52.38^{c}	45.92	26.51
R.Oil 40	21	90.47^{d}	8.25	4.76
R.Oil 80	21	95.24^{d}	8.25	4.76

^{z} means with same letter superscripts, within column, were not different by Fisher's protected LSD procedure at α=0.05.

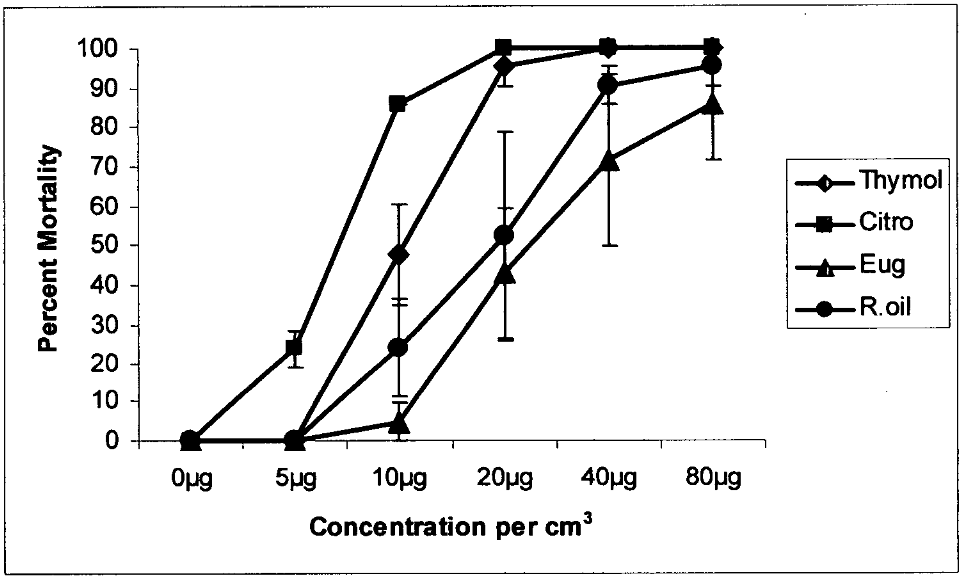

Fig. 2.4 Percent mortality of wireworms in the fumigation bioassay exposed to thymol, citronellal, eugenol, or rosemary oil; error bars = error of the mean.

Table 2.5. Toxicities of the monoterpenoids thymol, citronellal, eugenol and rosemary oil to *A. obscurus* larvae in topical and fumigation bioassays.

Chemical	Topical Bioassay					Fumigation Bioassay				
	n	Slope (±SE)	95% CI	LD_{50} (µg/larva)	χ^2 value	n	Slope (±SE)	95% CI	LC_{50} (µg/cm^3)	χ^2 value
Thymol	7	4.9±1.6	127-291	195.5	2.39	7	5.4±1.7	1.9-8.8	17.1	0.45
Citronellal	7	2.8±0.8	243-675	404.9	3.36	7	5.6±2.3	1.1-10.1	6.4	0.02
Eugenol	7	4.5±1.3	344-777	516.5	1.14	7	3.9±1.1	1.8-6.1	20.9	0.3
Rosemary Oil	7	2.8±1.6	Not determined	2378.7	0.34	7	3.5±1	1.6-5.5	15.9	0.99

CI=Confidence interval.

Differences in acute toxicity via fumigation may be due to differences in the ability of compounds to penetrate wireworms through the respiratory system and/or cuticle. Symptoms induced by the volatile forms of these monoterpenoids were similar to those observed in topical bioassays, suggesting a common mode of action despite different routes of exposure. The ability of wireworms to recover post-exposure varied by chemical and concentration but not by method of exposure. As observed in the topical bioassays, the ability of wireworms to recover decreased with increased concentration of all monoterpenoids tested.

2.3.3 Phytotoxicity bioassays.

Differences in corn seed germination percentage, cotyledon length and radicle length were observed among the four treatments ($P > 0.005$) and doses ($P > 0.0001$). No phytotoxicity was observed with rosemary oil, whereas thymol, citronellal, and eugenol all inhibited germination and reduced cotyledon and radical growth as concentration increased (Table 2.2). In support of these findings, Lee *et al.* (1997) observed damage to leaves and root death after applying thymol and eugenol around corn plants. The phytotoxicities observed following exposure to certain monoterpenoids is not surprising in light of research that has shown many plant-produced secondary compounds are phytotoxic to some degree (Duke, 1991). In addition, monoterpenoids often exhibit variable effects on different plant species that can include overall growth inhibition, prevention of seed germination, or outright plant death (Lee *et al.*, 1997).

Table 2.6 Phytotoxic effects of selected compounds on corn seed germination and development.

Chemical	Dose µg/seed	Seed		Cotyledon		Radical	
		Germination %	95% CL	Length	95% CL	Length	95% CL
Thymol	0	97 [a]	79-100	15.6 [a]	13.8-17.4	21.6 [a]	19.2-24
	200	97 [a]	79-100	15.3 [a]	14.5-16.7	21.9 [a]	19.1-24.9
	400	97 [a]	79-100	15.6 [a]	14.5-16.7	20.6 [a]	17.6-23.5
	800	70 [b]	53-87	14.7 [a]	13.6-15.9	19.5 [a]	16.6-22.4
	1600	77 [b]	59-94	13.9 [b]	12.7-14.9	17.7 [b]	14.8-20.6
	3200	20 [c]	03-37	6.8 [c]	5.7-7.9	8.1 [c]	5.1-10.9
Citronellal	0	97 [a]	79-100	15.6 [a]	13.7-17.4	21.6 [a]	19.2-24
	200	97 [a]	79-100	16.1 [a]	14.9-17.2	23.3 [a]	20.4-26.2
	400	93 [a]	76-100	15.6 [a]	14.5-16.7	21.8 [a]	18.9-24.7
	800	93 [a]	76-100	15 [a]	13.9-16.1	20.7 [a]	17.7-23.6
	1600	83 [a]	66-100	12.8 [b]	11.7-13.9	18.5 [a]	15.6-21.4
	3200	63 [b]	46-81	12.1 [b]	10.9-13.2	18.6 [a]	15.7-21.5
Eugenol	0	97 [a]	79-100	15.6 [a]	13.8-17.4	21.6 [a]	19.2-24
	200	100 [a]	83-100	14.7 [a]	13.5-15.8	20 [a]	17.1-22.9
	400	97 [a]	79-100	16.9 [a]	15.8-18	23.4 [a]	20.5-26.4
	800	90 [a]	73-100	16.9 [a]	15.8-18	21.4 [a]	18.5-24.3
	1600	90 [a]	73-100	13.6 [b]	12.5-14.7	19.2 [a]	16.3-22.1
	3200	57 [b]	39-74	11.9 [b]	10.8-13	16.1 [b]	13.2-19
Rosemary Oil	0	97 [a]	79-100	15.6 [a]	13.8-17.4	21.6 [a]	19.2-24
	200	93 [a]	76-100	16.7 [a]	15.5-17.8	22.9 [a]	20.1-25.9
	400	87 [a]	69-100	16.6 [a]	15.5-17.8	21.9 [a]	18.9-24.8
	800	97 [a]	79-100	16.4 [a]	15.3-17.5	20.4 [a]	17.5-23.3
	1600	93 [a]	76-100	16.4 [a]	15.3-17.6	25.9 [a]	22.9-28.8
	3200	90 [a]	73-100	15.3 [a]	14.1-16.3	21.2 [a]	18.2-24.1

[z] means with same letter superscripts, within column, were not different by Fisher's LSD procedure at α=0.05. CL=Confidence level.

The phytotoxic effects of thymol, citronellal and eugenol on corn germination and growth are of concern, since this effect may extend to other crops and limit their usefulness for wireworm control. However, our results suggest that some of the compounds tested might provide some level of wireworm control if applied to corn seed at doses below that causing phytotoxicity. Thymol, applied to corn seed at 400 μg/seed caused no phytotoxicity, but when presented to wireworms either topically (400 μg/larva) or as a fumigant (20 μg /cm^3), induced 71.4 and 95.2% mortality, respectively. Under laboratory conditions, thymol (0.1 μg/μl in acetone) was also shown to repel *A. obscurus* when applied as a single 10 μl droplet to filter paper substrates (unpublished data). Therefore, thymol applied as a seed treatment to corn might kill, intoxicate and/or repel attacking wireworms long enough for seed germination and establishment to take place. Other non-phytotoxic candidates that may provide some protection to corn seed from wireworm attack are citronellal at 800 μg/seed (topical and fumigant mortality of 85.7% and 100% mortality, respectively), eugenol at 1600 μg/seed (topical and fumigant mortality of 95.2% and 85.7% mortality, respectively), and rosemary oil at 1600 μg/seed (topical and fumigant mortality of 23.8% and 95.2% mortality, respectively).

I conclude that the insecticidal activities of thymol, citronellal, eugenol and rosemary oil on the late instar larvae of *A. obscurus* observed in the laboratory are sufficient to warrant further investigation. Future research should focus on use of thymol, citronellal and rosemary oil in actual field studies and with a broader concentration series to evaluate their toxicities to *A. obscurus* and phytotoxicity to corn seeds/seedlings.

CHAPTER 3

SCREENING OF MONOTERPENOIDS TO GUARD CORN SEEDS/SEEDLINGS FROM *Agriotes obscurus* (Coleoptera: Elateridae) FEEDING INJURY

3.1 Introduction

A. obscurus larvae are responsible for severe economic losses to potato, corn and other tuber, cereal or forage crops planted in infested fields. They cause damage by feeding on seeds, the developing seedlings, or by burrowing into the tubers, thereby reducing the marketability of these products. Together, *A. obscurus* and *A. lineatus* are responsible for significant losses in BC potato production, with damage estimates in 1994 ranging from $500,000 to $800,000 (Vernon, 1997). Unfortunately, the availability of synthetic pesticides to control these pests is becoming more limited due to their adverse effects on the environment. Therefore, there is interest in evaluating naturally occurring compounds as alternatives to these environmentally damaging chemicals.

Natural compounds derived from plants have been used for many years to guard crops from insect pests (Lee *et al.*, 1997). These compounds, which can act either as anti-feedents, repellents, or sub-lethal toxicants, have also been shown to possess phyto-protective activities against many phytophagous insects (Landis *et al.*, 1988). Of these natural compounds, plant essential oils (EOs) are one class that have been extensively evaluated for insecticidal, anti-feedent and repellent effects. EOs are odorous components and/or secondary metabolites that are separated from plant tissues through steam distillation (Isman, 1999) and are normally comprised of mono-, di- tri-, and sesqui-terpenes; however, most EOs are mixtures of mono- and sesqui-terpenes

(Hummelbrunner and Isman, 2001). EOs are quite volatile and are frequently used as flavoring agents in food and fragrances in perfume. However, due to their pesticidal activity and their apparent neutrality on the environment and vertebrates, EOs have garnered significant interest as eco-friendly pesticides.

In recent years, several researchers have reported the acute toxic effects of EOs on plant or human pests (Isman, 1999; Hummulbrunner and Isman, 2001). Of the EOs evaluated, the oil from the citronella plant (*Cymbopogon nardus* L.), and its major constituent, citronellal, has shown the greatest promise for pest control (Isman, 1999). Citronellal is often used as a nontoxic alternative to DEET (N, N-diethyl-m-toluamide) for protection against mosquitoes and other biting insects. In addition to citronellal, Lee et al. (1997) reported the acute toxicities (i.e., acaricidal and larvicidal) of other monoterpenoids including thujone (from *Artemisia absinthum* L.) on western corn rootworm (*Diabrotica virgifera vergifera* LeConte.), eugenol (from *Syzigium aromaticum* L.) on two-spotted spider mite (*Tetranychus urticae* Koch.) and thymol (from *Thymus vulgaris* L.) on the common house-fly (*Musca domestica* L.). In addition, the oils from rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus vulgaris* L.), peppermint (*Mentha piperita* L.), lavender (*Lavandula ungustifolia* Mill.), and spearmint (*Mentha spicata* L.) have all shown repellent activities against two-spotted spider mite (*Tetranychus urticae*) (Hori, 1998) while the EOs from thyme, peppermint, and spearmint have also shown larvicidal activity against the tobacco cutworm (*Spodoptera litura* L.) (Isman *et al.*, 2001). Ngoh *et al* (1998) demonstrated both contact and fumigant toxicities of eugenol against the American cockroach (*Periplaneta americana* L.). In addition, research from our group demonstrated both contact and volatile toxicities from thymol, citronellal, eugenol, and rosemary oil against the late instar larvae of *A. obscurus* (L.). Despite these positive indications, relatively few EOs have been tested for their pesticidal efficacy under greenhouse or field conditions, and none have not gained widespread use (Landis et al. 1988; Bernays 1983).

Recent studies have attempted to identify the mode of action of these EOs. Symptoms of insects acutely poisoned by certain essential oils, or their pure constituents, have shown similarity to neurotoxins, irrespective of the route of administration (Isman, 1999). Insects poisoned with EOs often display hyperactivity, convulsions and tremors followed by paralysis (Isman 1999, Enan 2001). Enan (2001) reported that eugenol, α-terpeniol and cinnamic alcohol were able to block octopamine receptor binding sites in the American cockroach, thereby suggesting these EOs negatively affect the nervous system. Preliminary trials in our laboratory analyzing wireworm behavior following exposure to vapors of thymol, citronellal, eugenol and rosemary oil support these observations (data not shown). Following initial paralysis or distress, affected wireworms often returned to normal behavior indicating these insects were able to either metabolize or excrete the toxic compounds. Another possibility is that the chemicals lost their toxic activity after a certain period of time.

It is suggested that if seeds or seedlings are protected from feeding damage beyond a critical time period of early growth, economic damage to crops can be greatly reduced. In this study, I evaluated the protective effects of four EOs that were previously shown in laboratory bioassays to repel or kill *A. obscurus*. Specific objectives of this research were to evaluate the protective properties of these compounds, as a seed pre-treatment, in terms of inducing wireworm distress and reducing feeding damage to corn seed and seedlings.

3.2 Materials and Methods

3.2.1 Chemical compounds.

The following technical grade compounds were included in this research: citronellal (>95% purity; EcoSMART Technologies Inc., Franklin, TN, USA), thymol (>98%; Sigma Chemical Co., St. Louis, MO, USA), and eugenol (>95%; Arylessence Inc., Marietta, GA, USA). Rosemary oil (Lot No. 0213142MB) was provided by EcoSMART Technologies Inc.

3.2.2 Seed pre-treatments.

Seed treatment solutions were composed of the following compounds diluted in pure methanol and applied at the listed dose: thymol (400 μg/seed), citronellal (800 μg/seed), eugenol (1600 μg/seed) and rosemary oil (1600 μg/seed). These doses, under laboratory and greenhouse conditions, were shown to produce minimal phytotoxic effects on corn seeds while producing high contact and fumigant toxicities to *A. obscurus* (Chapter 2). Control treatments consisted of seeds treated with methanol alone or distilled water.

3.2.3 Insect rearing and preconditioning.

Late instar larvae (16-22 mm length, 33-48 mg weight) of *A. obscurus* were collected from vegetable crop fields at the Pacific Agricultural Research Station (Agriculture and

Agri. Food Canada) Agassiz, British Columbia, Canada, and stored at 5 ^{0}C in soil filled containers until use. Twenty-four hours prior to experimentation, the temperature was raised to 20 ^{0}C and slices of potato were placed 2-3 cm below the soil surface. This procedure was followed in order to select only actively feeding larvae. Wireworms of similar length (19-22 mm) were collected every 6 h from under the potato slices for use in the bioassays.

3.2.4 Greenhouse evaluations.

Black plastic pots (15 x 18 cm) were used in order to give sufficient space for seedling growth and wireworm movement. Drainage holes of the pots were blocked using fiber-fill to prevent wireworm escape. Pots were filled to 2/3 capacity with well-cleaned sand (Technical grade 1). Five corn seeds (Pioneer Brand, Hybrid # 39T68, Pioneer Hi-Bred Ltd, Chatham, Ontario), pre-soaked in distilled water for 24 h, treated with 20 μl of treatment solution using a micropipetter, were planted with equal distances between seeds and covered with the same medium. Each pot was watered to field capacity with clean water. Ten randomly selected wireworms were then placed on the top of the sand. This experiment was conducted at the University of British Columbia Horticulture Greenhouse. Temperature was maintained at 19 ^{0}C/16 ^{0}C day/night, respectively. Pots were watered as needed with clean water. Control treatments included seeds treated with 20 μl methanol or distilled water and pots with and without wireworms.

Three weeks after sowing, the following data were recorded: germination percentage, seedling height, seed damage (visually assessed), number of distressed wireworms, and wireworm position in the pot (localized to either the top, middle or bottom portion). Seedling height was measured from the seed to the end of the longest leaf. A wireworm was considered distressed if:

1. Once removed from the pot and placed in an arena (8 cm dia. petriplate with 10 g pre-moistened sand), it was incapable of directed movements but capable of slight movement forwards or backwards; or

2. wireworms twisted or writhed (Bob Vernon and Wim Vanherk, personal communication).

3.2.5 Statistical analyses.

Each treatment was evaluated using 15 pots in a completely randomized design. Data were analyzed using logistic regression and analysis of variance (SPSS Inc., Chicago IL). Significance level and odds ratios (i.e., effectiveness of a treatment compared to the control treatment) were calculated for seed germination, seed damage and wireworm distress.

3.3 Results

3.3.1 Wireworm distress

Of the four treatments evaluated, citronellal (800 µg/seed), rosemary oil (1600 µg/seed) and thymol (400 µg/seed) induced >45% distressed wireworms (Table 3.1). These treatments were significantly different compared to the controls but not different from each other. Out of 900 wireworms, 61.1% of the distressed wireworms were found in these three treatments (Table 3.1). Based on the odds ratio (Expected β value), rosemary oil was 2.6 times, thymol 2.4 times and citronellal 1.8 times more effective than the control in inducing wireworm distress (Table 3.1) Eugenol created 3.1% more distressed wireworms compared to the control, but this difference was not statistically significant (p = 0.902; odds ratio = 1.031).

Table 3.1 Logistic regression of wireworm distress and wireworm position; RC=Reference Category (95% C.L for odds ratio).

Chemical (µg/seed)	Wireworm Distress				Wireworm Position		
	Percent	*p* Value	Odds Ratio	95% CL	% Bottom	% Middle	% Top
Citronellal (800µg)	46.7	<0.001	1.81	1.13,2.9	32.6	31.1	36.4
Eugenol (1600µg)	33.3	0.9	1.03	0.64,1.7	34.7	40.7	24.7
R. Oil (1600µg)	56	<0.001	2.64	1.65,4.2	27.4	34.9	37.7
Thymol (400µg)	53.3	<0.001	2.37	1.48,3.8	28.2	43	28.9
Methanol	33.3	0.9	1.03	0.64,1.67	21.8	38.8	39.5
D. Water	32.7	RC	RC	RC	32.4	36	31.7

C.L=Confidence level

3.3.2 Seed feeding damage

The three treatments that created the highest proportion of distressed wireworms (i.e., thymol, rosemary oil, and citronellal) were also the three most effective treatments for protecting corn seed from feeding damage (Table 3.2; Figure 3.1). All three significantly reduced damage compared to the control. Seeds treated with thymol displayed 85% less damage while seeds treated with rosemary oil or citronellal displayed 71% less seed damage compared to the control (Table 3.2). Eugenol not only failed to prevent seed damage (as compared to the control) but actually produced greater seed damage than the control. Perhaps conditions in the sand allowed the eugenol residue to volatilize and dissipate below effective concentrations during the three week interval between seed treatment and data collection, thus allowing wireworms to feed on the seed.

Table 3.2. Logistic regression of seed damage and seed germination; RC=Reference Category (95% C.L for odds ratio).

Chemical (µg/seed)	Seed Damage				Seed Germination			
	Percent	*p* Value	Odds Ratio	95% C.L	Percent	*p* Value	Odds Ratio	95% C.L
Citronellal (800µg)	16	<0.001	0.29	0.13,0.63	98.7	<0.001	0.09	0.03,0.24
Eugenol (1600µg)	72	<0.001	4.37	2.16,8.8	25.3	0.47	1.71	0.4,7.45
R. Oil (1600µg)	16	<0.001	0.29	0.13,0.63	96	<0.001	0.12	0.04,0.33
Thymol (400µg)	9.3	<0.001	0.15	0.06,0.39	98.7	<0.001	0.02	0.01,0.07
Methanol	44	0.5	1.26	0.65,2.46	54.7	0.13	5.24	0.6,45.68
D. Water	38.7	RC	RC	RC	62.7	0.13	5.24	0.6,45.69
Methanol w/o wireworms	0	<0.001	0	0	98.9	<0.001	0.01	0.03,0.24
D. Water w/o wireworms	0	<0.001	0	0	98.5	<0.001	0.02	0.02,0.08

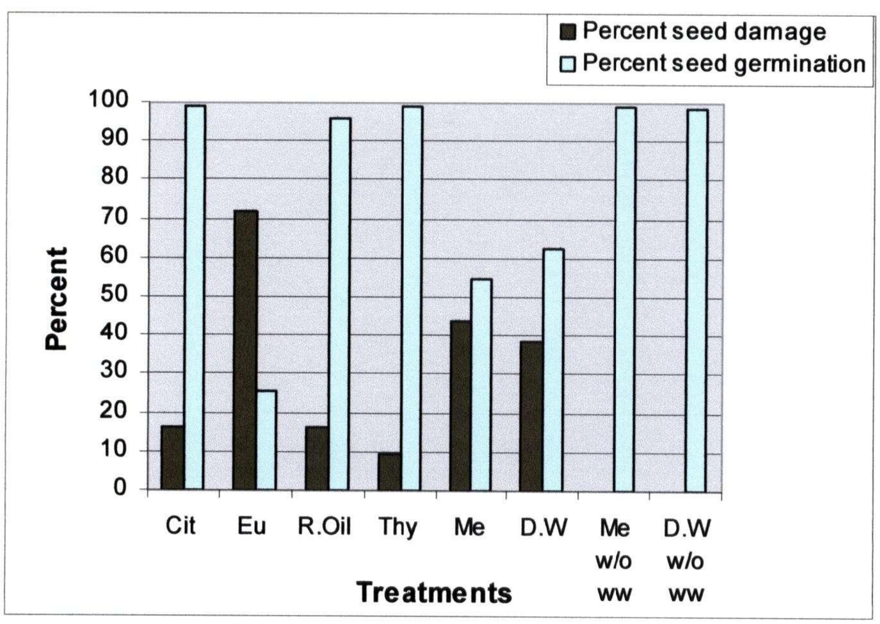

Figure 3.1. Graphical representation of % seed damage and % seed germination of corn seeds (Cit-Citronellal 800µg, Eu-Eugenol 1600µg, R.Oil-Rosemary oil 1600µg, Thy-Thymol 400µg, Me-Methanol, D.W-Distilled water, Me w/o ww-Methanol without wireworms, D.W w/o ww-Distilled water without wireworms).

3.3.3 Seed germination

There were significant treatment effects on seed germination (Table 3.2) with germination significantly reduced in the eugenol treatment as compared to all other treatments (Table 3.2). However, there were no significant differences in seed germination between thymol, citronellal, or rosemary oil. Seed germination in control treatments (i.e., distilled water or methanol) was significantly lower than in the thymol, citronellal, or rosemary oil treatments. This may be due to direct feeding damage to the seeds as these were the same treatments that had the 2nd and 3rd highest percent of damaged seeds as assessed through visual observation (Table 3.2). Visual inspection of the seeds showed that seeds treated with eugenol had the most damage caused by wireworm feeding. However, average cotyledon length (see Cotyledon length below) in the eugenol treatment was similar to the length of the control plants. Therefore, I conclude that the poor germination observed was due to feeding damage and not due to phytotoxic effects of eugenol.

3.3.4 Seedling height

There was a significant treatment effect on seedling height (Table 3.3). Seedlings from thymol, citronellal or eugenol seed pre-treatments displayed significantly reduced height compared to the controls while rosemary oil did not produce this effect (Table 3.4). This indicates that at the doses used, these compounds were either phytotoxic to corn seedlings or wireworm feeding on roots indirectly affected seedling growth. Analysis of variance indicated a significant pot effect on final plant height (Table 3.3). This may be due to either differences in seedling emergence time (i.e., seedlings that emerged earlier grew taller), or due to the growth inhibition caused by wireworm feeding differences on developing cotyledons or radicals. Seeds treated with distilled water (without wireworms), produced the highest seedling height (16.7 cm) followed by distilled water (with wireworms) (15.1 cm) and rosemary oil (14.7cm); the latter two not significantly different from each other (Table 3.4). This indicates that the selected dose of rosemary oil did not have phytotoxic effects on corn seedling development and the reduction of height was due to wireworm feeding on roots. Seedling height from the methanol (with wireworms), thymol, eugenol, and citronellal treatments were all significantly reduced as compared to the controls. Previous experiments on evaluating the phytotoxicities of these compounds reported that at the doses used in the present study, no phytotoxicity should have occurred. Therefore, I conclude that reduction in plant height in these treatments is due to wireworm feeding damage post-germination.

Table 3.3 Results from analysis of variance of seedling height of corn seedlings following seed treatment.

Source	Type III SS	df	MS	F Value	*p* Value
Model	98273.67	116	847.187	71.907	<0.001
Treatment	1610.122	7	230.017	19.523	<0.001
Pot	483.917	14	34.566	2.934	<0.001
Treatment*Pot	2978.072	94	31.682	2.689	<0.001
Error	4158.921	353	11.782		
Total	102432.6	469			

Table 3.4. Mean effects of selected compounds and controls on plant height of corn seedlings; means with different letters were significantly different as determined by two factor ANOVA with α=0.05.

Treatment(µg/seed)	Mean Height (cm)	n^z	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Citro (800)	12.18 [a]	74	0.4	11.39	12.97
Thymol (400)	11.34 [a]	74	0.396	10.56	12.11
Eugenol (1600)	11.91 [a]	19	0.864	10.21	13.61
R.OIL (1600)	14.70 [c]	72	0.406	13.9	15.5
d.Water+WW	15.12 [c]	47	0.524	14.08	16.15
Methanol+WW	13.73 [b]	41	0.553	12.64	14.81
dWater Only	16.67 [d]	70	0.415	15.86	17.49
Methanol Only	15.61 [d]	73	0.403	14.82	16.49

z = number of surviving corn plants out of 75.

3.3.5 Wireworm position

Based on logistic regression, there were no treatment effects on wireworm position within the pots. Wireworms were equally distributed in all three sections (Table 3.1).

3.4 Discussion

In this study, rosemary oil, thymol and citronellal protect corn seeds from *A. obscurus* feeding damage. However, of these three treatments, only rosemary oil lacked phytotoxic effects on corn. Despite this, these compounds can protect crops from wireworm damage through reductions in wireworm fitness or by acting as feeding deterrents or repellents. However, further evaluations are required to refine the use of these compounds for crop protection and should also determine the sensitivity of early instar larvae to these compounds. Early instar larvae may be more sensitive and therefore, lower doses may be able to achieve the same level of control.

The use of feeding deterrents has been suggested as one of the better methods for protecting germinating seeds and seedlings from soil insects (Villani *et al.* 1985). In addition, Landis *et al.* (1988) introduced the term 'phyto-protectants' to encompass plant-derived chemicals that display this effect (e.g., guarding plants from yield-reducing insect injury) without regard to mode of action. In corn production, the seedling (e.g., first 3 week period post-germination) is the most vulnerable stage for wireworm attack with resulting economic loss. However, if feeding is delayed until after this period, the plant is better able to withstand subsequent wireworm attack without economic impact (B. Vernon, personal communication).

In previous laboratory bioassays, I observed both contact and volatile toxicities of these compounds on wireworms (Chapter 2). However, in the present study, I did not observe the same degree of mortality. Several factors may have lead to this difference. In soil, insects may contact or perceive chemicals differently than under laboratory conditions or have modified behaviors that effect exposure to the test compounds (Landis *et al.* 1988). In addition, toxicities may change due to the compounds binding to soil particles or undergoing deactivation reactions through hydrolysis or microbial action (Landis *et al.* 1988). Any of these possibilities may have contributed to the reduced mortality to wireworms observed in the current study. However, absolute lethality aside, it was important that some of our treatments (i.e., citronellal, thymol and rosemary oil) were able to protect developing tissues (i.e., radicals and cotyledons) from wireworm damage post-germination. This indicates that either the compounds had a systemic protective activity or they were persistent around the immediate environment of the seedling.

In summary, the tested monoterpenoids were capable of protecting corn seeds and seedlings from feeding injury by *A. obscurus*. The tested dose of rosemary oil protected seeds without inducing any phytotoxic effects. However, the tested concentrations of thymol and citronellal, while offering protection from wireworm feeding, induced slight phytotoxic effects.

CHAPTER 4: SUMMARY AND CONCLUSIONS

Natural products derived from plants, including plant essential oils, have been used for centuries to protect crops from harmful insects. However, their use in modern agricultural production has been undermined by the convenience and perceived greater efficacy of synthetic pesticides. In order for these natural compounds to be considered and incorporated into modern production, several criteria have been suggested detailing their general attributes. They should have:

- adequate persistence for crop protection
- some systemic activity
- low cost
- absence of phytotoxicity

In addition to these general criteria, researchers have suggested that a more successful approach for incorporating natural products into modern agriculture might involve using these compounds as seed protectants, primarily protecting germinating seeds and seedlings from soil-borne insects. Reasons cited for this recommendation include:

- persistence is needed for only a short time period
- seedlings have a decreased need for systemic action compared to mature plants
- relatively small quantities of extract can protect many seeds
- seeds can be treated before planting

The research described in this thesis evaluated the contact and fumigant toxicities of thymol, citronellal, eugenol (monoterpenoid essential oil constituents) and rosemary oil (a complex essential oil) on late instar larvae of *A. obscurus*, in order to evaluate their utility as seed protectants for use in modern agriculture.

Based on the mode of application (i.e., contact, fumigant, or seed pre-treatment), the essential oils tested displayed varied toxicities and repellent effects. For example, thymol produced the highest contact toxicity and greatest seed protection but ranked only third as a fumigant. Citronellal produced the highest fumigant toxicity and second best seed protection while also ranking second in the contact assays. These results indicate that while all of the essential oils tested displayed some level of toxicity, their use in agricultural settings must be carefully tested and paired with appropriate application methods and uses. For example, thymol may be the most appropriate replacement for use as a traditional pesticide spray or seed pre-treatment but citronellal may be the most appropriate replacement for fumigation. Future research should continue evaluating these compounds for absolute toxicity across a broader range of pests but should also evaluate them as alternatives to the most damaging pesticides currently used including organophosphate-based pesticides and methyl bromide as a soil fumigant.

In addition to direct effects on wireworms, these compounds displayed varied phytotoxicities when tested on corn seed. Rosemary oil was the only essential oil that had minimal phytotoxic effects, even at the highest doses. However, rosemary oil ranked second in the fumigation assay and only fourth in the contact assay. The most toxic contact essential oil, thymol, produced the greatest phytotoxicity, further complicating the decision making process. Minimizing these secondary phytotoxic effects will be one of the greatest challenges when using these compounds in agricultural settings. However, phytotoxic effects can often be minimized through adjustments to the mode of delivery and formulation. For example, granular formulations of these compounds can be applied as bands along the side of seed rows to repel and guard the seeds from wireworm attack. Granular formulations are thought to provide protection for a longer time period compared to spray applications. Future research should further evaluate these compounds for phytotoxic effects across a wider range of plant species as well as under a broader range of field conditions and modes of application.

In envisioning how these results are best applied to agricultural settings, two issues should be kept in mind: mode-of-exposure and the development of a broad-based cost:benefit analysis. The contact and fumigation mortality results clearly support continued development of some of these compounds for use in crop protection. However, in practical terms, not all modes of exposure are equal. For example, the high contact mortality observed with thymol would suggest some type of soil drench or spray application. Since wireworms are soil-borne pests, spray applications are not a viable option (though they are for other pests) while drenching large production fields is inappropriate. The high fumigation toxicities observed with citronellal or rosemary oil suggest development of a soil fumigation treatment. Using citronellal as an example, we calculate approximately 64 kg ha^{-1} would need to be applied in order to achieve the desired concentration to 1 m depth. While this may be technically feasible, it would not be economically viable.

The strong repellent properties of some of these compounds may indicate the most appropriate use of these compounds in agricultural settings. A seed pre-treatment, which is able to prevent wireworm attack for an initial growth period, appears to be the most straightforward and simplest application. Based on our results, 400 μg/seed thymol, 800 μg/seed of citronellal, or 1600 μg/seed of eugenol or rosemary oil would be required for adequate protection. These amounts are similar to quantities used for synthetic pesticides. Compared to the recommended rate of 125-1250 μg/seed for thiamethoxam (Cruiser 5FS, Syngenta Crop Protection, Greensboro, NC, USA), essential oils offer a viable alternative.

In addition to mode of exposure, a broad-based cost:benefit analysis should be completed for each essential oil. These calculations should include both economic factors (e.g., cost per ha, anticipated yield increase) as well as less-quantifiable factors including environmental impact (e.g., persistence and residual effects), human health (e.g., acute and chronic effects), and utility in organic farming systems (e.g., collateral mortality on non-target organisms). Economic factors aside (due to significant site- and operation specific variation), all of the essentials oils tested are biodegradable and photodegradable thereby greatly reducing their persistence in the environment (Isman, 1999).

Regarding human health issues, acute oral LD_{50} values are available for eugenol (500 mg/kg rat) and thymol (980 mg/kg rat) with both possessing relatively high values. However, all compounds (i.e., thymol, citronellal, eugenol and rosemary oil) are listed in the US FDA's GRAS (Generally Recognized As Safe) list (US FDA, 2005) indicating their safe use in food products. Unfortunately, inclusion on this list does not guarantee human safety due to different evaluation criteria between the various governmental agencies (Trumble, 2002). However, based on the available information, the selected compounds do not display carcinogenic, hepatotoxic or teratogenic activity in human or any other tested animals. Furthermore, rosemary oil has been shown to have both hepatoprotective and antimutagenic effects on laboratory rats (Fahim *et al.*, 2000) while eugenol has been evaluated as both an anti-inflammatory and a cancer chemopreventive agent (Kim *et al.*, 2003).

Several companies have already developed pest control products that contain thymol, citronellal, eugenol, or rosemary oil as active ingredients. For example, California Organic Fertilizers Inc., is marketing Phyta-guard, a combined insecticide and fungicide containing 10% rosemary oil and 20% clove oil. EcoSMART (Franklin, USA) has introduced several pest control products containing plant essential oils as active ingredients (Ecotrol -10% rosemary oil as a broad-based insecticide; Sporan – 16% rosemary oil as a fungicide; and Home and Garden Spray - 0.45% thyme oil as a garden insecticide). Therefore, these compounds are already being widely distributed and used without any environmental or toxicological problems identified to date.

Based on these findings, plant essential oils can be used as pest control agents and seed protectants. However, their efficacy to control insect pests and minimize seed damage, as well as indirect effects on beneficial organisms and on crop plants, must be evaluated under a broader range of conditions and applications. Finally, binary mixtures of these compounds should be evaluated to identify any synergies that may be present that can further enhance wireworm control.

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APPENDIX 1

VIDEO ANALYSIS OF WIREWORM BEHAVIOR FOLLOWING EXPOSURE TO THE VOLATILE FORMS OF THYMOL, CITRONELLAL, EUGENOL AND ROSEMARY OIL

Materials and Methods:

The experimental arenas for these experiments consisted of 14 cm diameter petridishes with 40 g of pre-cleaned horticulture grade #1 sand. Two corn seeds (same cultivar as used in previous experiments), pre-soaked for 24 hours, treated with either 20 µl of a test solution (i.e., final doses equaled 0, 200, 400, 800, 1600 or 3200 µg/seed) or 20 µl of methanol (as control) were placed at opposite sides of the arena.

After preparing an arena, it was placed under a video camera (Panasonic CCTV Camera, Model Number WV –BP 330, Matsushita Communication Industrial Corporation of the Philippines, Lagana, Philippines) that was attached to a Panasonic time-lapse video cassette recorder (same company). Five wireworms, selected using the same criteria as described in Chapter 2, were released to the arena and enclosed with the lid. Just after wireworm release, video recording was started and continued for 24 hours.

Statistics:

Each treatment was observed using one replicate. Due to the difficulty in coding observed movements into quantitative data, only the visual observations are presented.

Observations:

All four chemicals applied at either 1600 or 3200 μg/seed, strongly repelled wireworms. Many wireworms turned back as they moved closer to the treated corn seed. A few wireworms appeared to have touched the treated corn seed and soon began stressful wiggling/convulsions for 3-4 minutes. At these doses, most wireworms displayed paralysis at the end of the 12 hr period. When wireworms touched the methanol treated seed, their speed of movement increased but they did not display typical distress reactions.

Thymol and citronellal (200 or 400 μg/seed) or rosemary oil (400 or 800 μg/seed) also displayed repellent activity. However, wireworm wiggling and number of turn-overs were less when compared to either the 1600 and 3200 μg/seed treatments. Eugenol and rosemary oil, at 200 or 400 μg/seed, did not repel wireworms and did not clearly induce stressful wiggling. In addition, wireworms displayed neutral behaviors in relation to the methanol treated corn seeds. They did not display any distress movements when close to the control seed nor display any stressful wiggling after touching these seeds.

APPENDIX 2

MANUAL TRACKING OF WIREWORM MOVEMENTS FOR A TWELVE HOUR PERIOD

Materials and Methods:

Experimental arenas and seed treatments were similar to the arenas used in videotape analysis. However, in this experiment, only one late instar larva of *A. obscurus* was introduced to the center of the arena. Wireworm movements were recorded using a transparency that was attached to the lid of the arena. Manual tracking was continued for a single 12 hr period.

Results:

Tracked wireworm movements from the thymol 1600 μg/seed and 3200 μg/seed treatments showed clear repellent effects (Fig. A.1). Throughout the 12 hour monitoring period, wireworms clearly avoided the areas around the treated seeds.

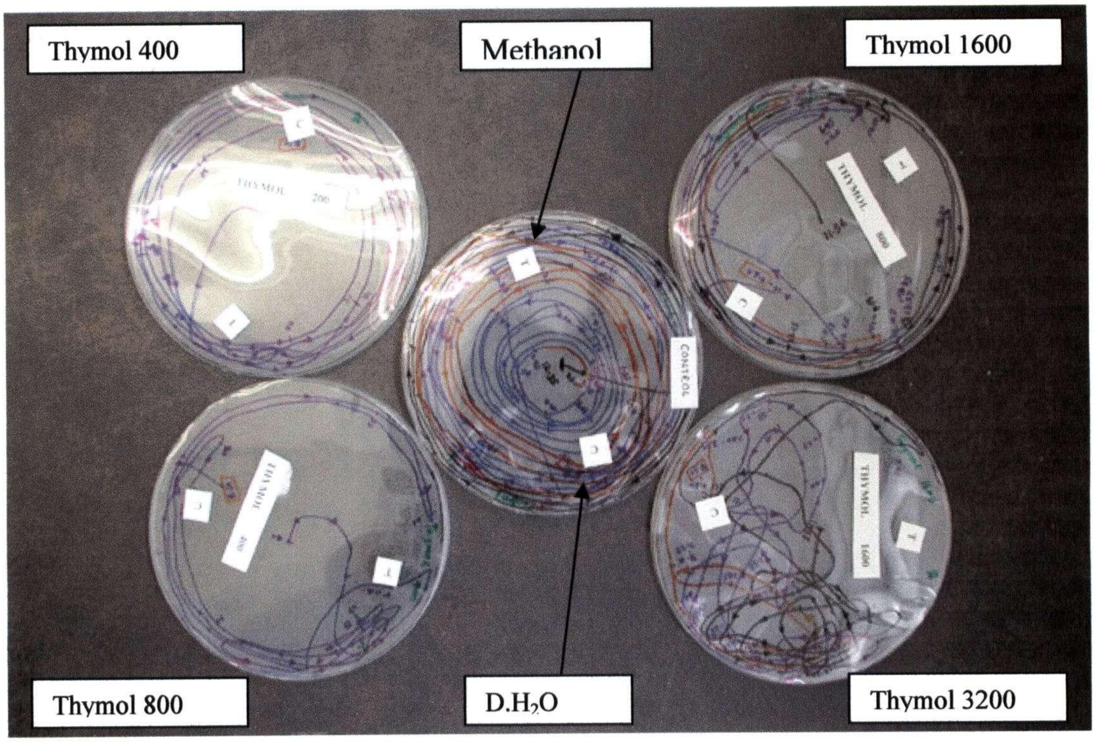

Figure A.1. Tracks of wireworm movement in thymol treatments (400, 800, 1600 or 3200 µg/seed)

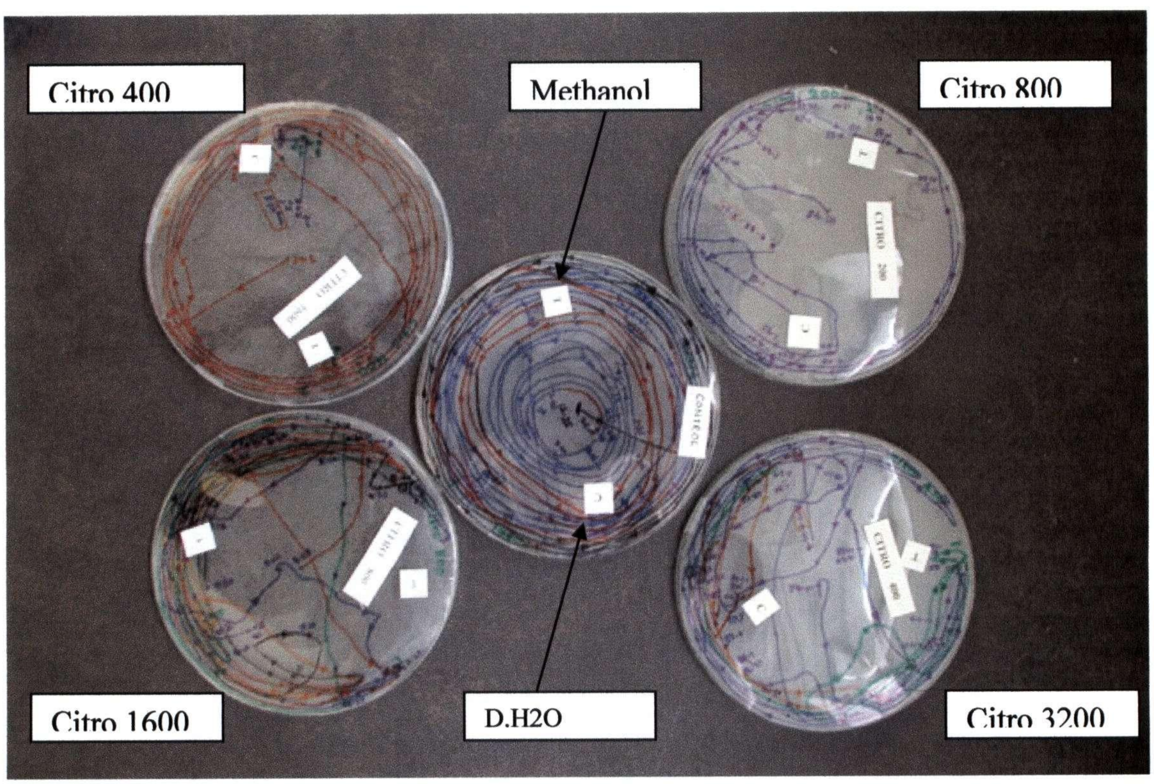

Figure A.2. Tracks of wireworm movement in citronellal Treatment (Dose = µg/seed).

APPENDIX 3

MONITORING OF ADULT *A. obscurus* and *A. lineatus* SPECIES DISTRIBUTION AT THE UBC SOUTH CAMPUS FARM USING PHEROMONE TRAPS

Introduction:

Species distribution surveys are essential in order to estimate population structure and tailor control strategies to specific infested fields. Surveys also allow for the evaluation of interspecific hybridization between these two species in natural environments. This is important because control measures may be either positively affected (i.e., the same control agents useful on a single species may be effective on hybrid populations) or negatively affected (i.e., hybrids may receive/develop insecticide resistant from one species or have modified behaviors that could limit chemical exposure) by species interactions.

Materials and Methods:

Pheromone traps (Phero Tech Company Ltd.) for *A. obscurus* and *A. lineatus* were used to monitor the beetle populations at the UBC South Campus Farm from 16th of April 2004 to June 2004. Four traps per species were placed approximately 20 m apart.

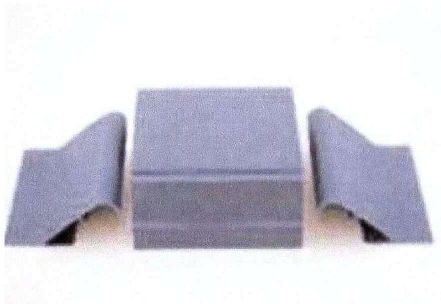

Figure A. 3. Vernon trap (box and two ramps)

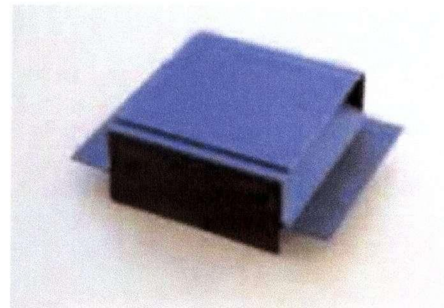

Figure A. 4. Assembled trap

Figure A. 5. Vernon trap in field (protruded part of the two ramps are covered with soil)

(Curtsey of http://www.pherotech.com/vernon_beetle_trap.html)

Results:

The number of adult click beetles trapped changed with respect to catch date and species (Table A.1).

Table A.1. *A. obscurus* and *A. lineatus* counts from 4 pheromone traps

Date of Collection	Number Adult A. lineatus	Number Adult A. obscurus	Ratio of *A. lineatus* to *A. obscurus*
16.04.2004	390	28	14
21.04.2004	690	92	7.5
23.04.2004	707	18	39
05.05.2004	967	91	10.6
15.05.2004	177	40	4.4
02.06.2004	43	8	5.4
TOTAL	2974	277	10.7

Overall, greater numbers of *A. lineatus* were collected than *A. obscurus*. This may be due to either real population density differences, temporal differences in emergence between the two species, or differences in trap efficacy between the two species. Normally, *A. obscurus* adults emerge from the soil earlier than *A. lineatus* adults (Vernon, 2001). Based on this survey, *A. lineatus* appears to be the dominant species at the UBC farm.

APPENDIX 4

INSECT COLLECTION METHODS

1. Corn Seed Method:

Corn and wheat seeds were mixed in 1:1 ratio, by volume, using vermiculite as the filler medium. The mixture was used to fill 10 cm dia plastic pots that were then watered to field capacity before being buried to a depth of 10-15 cm (Figure A.6). Holes for the traps were dug using a posthole auger. After placing a trap in a hole, soil was firmed around the pot to ensure good contact between the pot and surrounding soil. Once positioned, the trap was covered with loose soil and a plastic tray was placed over it. The cover helps to increase trap temperature thus facilitating seed germination. Following placement, the trap site was marked with a surveyor's flag. In this study, traps were placed every two meters for a total of 75 traps and collected after 2 weeks. However, only 14 wireworms were collected using this method. The low number of insects in the catch may be due to the lower density of wireworms in the selected field.

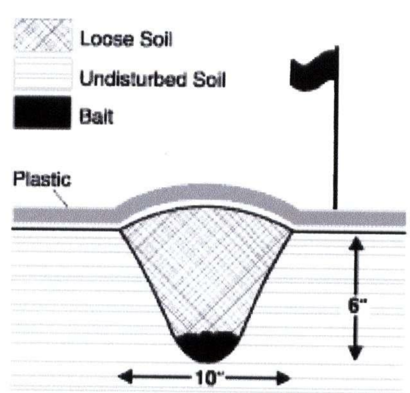

Figure A.6 Sketch of a corn seed method (Pacific Northwest Insect Control Handbook, 1992)

2. Soil Sampling Method:

This is a simple method that involves hand digging the soil. Hole depth varies with soil temperature. Most of the wireworms can be found approximately 15 cm deep when the soil temperature is approximately 15-20 ^{0}C. If the soil temperature is below 15-20 ^{0}C, the wireworms will be deeper. After digging, wireworms must be carefully searched for as the first instars are very small. Once found, wireworms should be stored in soil filled tubs. Most of the wireworms used in this study were collected by this method.