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Relative Attraction of the Cabbage Looper Moth (Trichoplusia ni (Hübner)) to Wild-type and Transgenic Tomato (Solanum lycopersicum L.)

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Abstract

The cabbage looper moth (CLM), *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) is an agricultural pest that has developed resistance to many frequently used insecticides, so alternative methods are required to reduce greenhouse CLM populations. Host plant volatile organic chemicals (VOCs) are used by female CLMs as cues for host location and oviposition. I hypothesized that changes in host plant VOC production, through genetic modification, could alter host location behaviour by CLMs. These changes in VOCs have potential to give rise to highly attractive transgenic trap crops. Chemical analyses on genetically transformed tomato, *Solanum lycopersicum* L. plants provided evidence of different VOCs relative to wild-type (WT) tomatoes, but CLMs exhibited no preference for transgenic VOCs over WT when virgin or mated females were tested in olfactometer experiments. In conclusion, CLMs do not prefer transgenic tomato VOCs over WT, so the transgenic plants could not be used as effective greenhouse trap crops.

Statement of Co-Authorship

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The author was involved with the insect rearing and olfactometer experimentation of cabbage looper moths (*Trichoplusia ni* (Hübner)) with transgenic and wild-type *Solanum lycopersicum* L. cv. Micro-Tom.

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Table of Contents

Chapters

List of Tables

List of Figures

x

List of Appendices

List of Abbreviations

AAFC	Agriculture and Agri-Food Canada
Bt	*Bacillus thuringiensis*
CCD	Carotenoid Cleavage Dioxygenase enzyme family
CLM	Cabbage Looper Moth
DCM	Dichloromethane
DHS	Dynamic Headspace
GC-MS	Gas Chromatography-Mass Spectrometry
GC-EAD	Gas Chromatography-Electroantennographic Detection
GLMM	Generalized Linear Mixed Model
HPLC	High Performance Liquid Chromatography
IPM	Integrated Pest Management
L1	CCD1a Tomato Line 1
L1OV	CCD1a Tomato Line 1 with *Trichoplusia ni* eggs
L2	CCD1a Tomato Line 2
NCED	9-cis-epoxycarotenoid enzyme family
NPV	Nuclear Polyhedrosis Virus
PCR	Polymerase Chain Reaction
RH	Relative Humidity
VOC	Volatile Organic Chemical
WT	Wild-Type

1.1 Introduction

Globally, approximately 50 000 pathogen, 8000 weed and 9000 arthropod pest species reduce agricultural production by approximately 40% annually (Oerke et al. 1994; Pimentel 1991; Pimentel & Burgess 2014). Even with the use of synthetic pesticides (insecticides, herbicides and fungicides) and other control strategies, economic losses are estimated at $400 x 10^9 a year (Oerke et al. 1994; Pimentel & Burgess 2014) but would be at least $500 x 10^9 in the absence of pest control (Pimentel & Burgess 2014). Currently, synthetic pesticides are the principle means of pest control and globally approximately 3.0 X 10^6 metric tons are applied annually (Pimentel 1991; Pimentel & Burgess 2014; Oerke et al. 1994) at a cost of $30 x 10^9 (Pimentel 2009; Richter 2002).

The use of insecticides began around 2500-1500 BC by Chinese and Sumerian farmers and until the late 19th century involved natural compounds such as sulphur, copper and organic mercury (Oerke 2006), or extracts from plants such as hellebore, tobacco or chrysanthemum (Seiferle & Frear 1948). Subsequently, especially following the second world war, there was a significant shift to the synthetic insecticides, the most common being organochlorines, organophosphates and carbamates (Zhang et al. 2011).

While, as noted above, the broad application of insecticides has limited crop losses, their extensive use has resulted in several significant problems. First, many species have developed resistance to insecticides, thus require higher doses to have any impact on populations, which in turn further selects for resistance (National Research Council 1986; Gould 1984; Knight 1989; Plapp 1976; Roush & McKenzie 1987; Tabashnik 1994). Today > 500 insect species are reported to have evolved resistance to

frequently used insecticides (Georghiou 1990; Kotchen 1999; Liang et al. 2013). Secondly, there have been negative effects on beneficial insects, such as pollinators, parasitoids and predators that occur naturally within the agroecosystems treated (Knight 1989). Third, as the result of drift or run-off, the insecticides contaminate other terrestrial and aquatic ecosystems, negatively affecting many different species, including humans (Eddleston 2016; Liang et al. 2013; Pimentel & Burgess 2014; Richter 2002; WHO 1990; Zhang et al. 2011).

As these negative effects became better known, especially when brought to the attention of the public by Rachel Carson (1962), there was a concerted effort to develop integrated pest management (IPM) programmes with synthetic insecticides only being used as a last resort. The multidisciplinary nature of IPM promoted pest management where the approaches used were based on ecological principles along with socio-economic considerations (Kogan 1998; Pedigo et al. 1986; Stern 1966, 1973; Stern et al. 1959) for selection of suitable methods to prevent pest populations from reaching economic injury levels – theoretical pest population levels at which unacceptable losses are expected (Ehler 2006; Food & Agriculture Organization 1975; Kogan 1998; Prokopy 2003). Integration of IPM works at a minimum of three levels: (1) single organism complex (vertical integration), (2) multiple organism complex (weeds, pathogens, insects) (horizontal integration), and (3) multiple organism complex with ecological system context (Kogan 1988, 1998). It has also been proposed that a fourth level exists – integration at the social, political, legal, and psychological IPM constraint level (Kogan 1998; Prokopy & Croft 1994).

Modern IPM is applied using eight principles: (1) prevention and suppression of pest populations below critical levels, (2) frequent monitoring of pests to alert growers about population growth in order to ensure that strategies used are the most effective, (3) making decisions to determine population threshold levels and when to apply strategies, (4) non-chemical methods specific to the pest to maintain low pest population levels, (5) pesticide selection accounting for pest specificity and problematic environmental impact, (6) reduced pesticide use, (7) anti-resistance strategies, such as use of refuges or the use of multiple pesticides with different modes of action, and (8) evaluation of the effectiveness of the previously applied methods to ensure programme optimization (Barzman et al. 2015). Field monitoring, including record keeping, of pest population economic thresholds are essential in the implementation of IPM principles (Tang & Cheke 2008).

When developing IPM programmes the control methods vary depending on the biology and ecology of the pest species under consideration. The more common methods include (i) biological control - the use of natural enemies, such as parasitoids, predators or pathogens (bacteria, fungi and viruses) to directly reduce pest populations, (ii) plants that have been selected for resistance, including genetically modified plants such as those engineered to express *Bacillus thuringiensis* Berliner (Bt) toxins, (iii) attractant infochemical (particularly pheromone) trap systems, and (iv) habitat management practices (Pimental & Burgess 2014), such as strip cutting *Medico sativa* L. to control *Lygus hesperes* (Knight) in *Gossypium hirsutum* L. fields (Stern et al. 1964).

One strategy within the larger umbrella of habitat management for the control of certain insects is intercropping – the planting of different plant-types in an agricultural

area to disrupt the cues an insect uses to locate and choose host plants (Pitan & Olatunde 2006; Tahvanainen & Root 1972). The resource concentration hypothesis argues that herbivorous insects will find a pure stand of host plants easier than when several different plant species are growing within the system (Root 1973). Furthermore, this increased habitat diversity may also maintain beneficial parasitoids and predators by attracting alternative hosts/prey, as well as provide nectar sources for pollinators (Fitt 2000; Wratten & van Emden 1995). One variation of intercropping is the use of trap crops, where plants more suitable for feeding or oviposition are planted near the cash crop to attract and concentrate the pest, thereby reducing losses to the main crop (Hokkanen 1989, 1991). One variation of trap cropping is the use of dead-end trap crops – using plants preferred by adults for oviposition but on which larvae are unable to survive (Shelton & Nault 2004). Most successful dead-end trapping systems have involved lepidopteran species (Badenes-Perez et al. 2004, 2005a, 2005b; Idris & Grafius 1996), such as using *Barbarea vulgaris* R. Br. var. *arcuata* as a dead-end trap plant for *Plutella xylostella* (Linnaeus) (Lu et al. 2004; Shelton & Nault 2004). A second approach is the "push-pull" (Pyke et al. 1987; Khan et al. 2001), also known as stimulo-deterrent diversion (Miller & Cowles 1990) system. In this case a combination of plant species is used in association with the principle cash crop: repellent plants to deter the pest (push) and attractive trap plants (pull). This approach has provided a cheap and efficient way of controlling stemborer populations in African maize fields, using *Desmodium uncinatum* Jacq. and *Melinis minutiflora* Beauv. as repellent plants, and *Pennisetum purpureum* Schumach. and *Sorghum vulgare* Stapf. var. *sudanense* as trap plants (Khan et al. 2001).

The selection or avoidance of plants by insects involves the integration of visual, olfactory, and gustatory cues (Landolt & Molina 1996; Miller & Strickler 1984). Host plant volatile organic chemicals (VOCs) are usually involved in the first, longer distance, step of selecting or avoiding a plant as they may be detectable up to 100 m from the source (Evans & Allen-Williams 1993; Finch & Collier 2012; Judd & Borden 1989): their detection resulting in positive or negative chemotaxis (Couty et al. 2006; Palaniswamy et al. 1986; Pivnick et al. 1990, 1994; Reddy et al. 2003; Reddy & Guerrero 2000). In contrast, visual cues, such as colour and shape, are generally detected over shorter distances (Finch & Collier 2000; Foster et al. 1997; Rojas & Tristram 1999; Städler 1974). However, the decision to oviposit or feed may require repeated contact with the plant, known as the appropriate/inappropriate landing behaviour or enhanced searching (Finch & Collier 2000; Finch & Collier 2007; Thorsteinson 1960), at which time the physical (e.g presence or absence of trichomes) and chemical properties of the substrate are assessed.

As a general rule, although final percentage depends upon the insect of interest and crop type, approximately 10% of the agroecosystem is used for the trap plants (Hokkanen 1991), so trap plants must be significantly more attractive than the main crop for this approach to be economically effective. One way of making the trap crops more attractive is through selective breeding, insertion of transgenes, or through genetic modification of existing genes that alter the characteristics known to be used in host selection (Åhman 2013; Åhman et al. 2010; Hokkanen 1991; Pickett et al. 1997).

Recent research at Agriculture and Agri-food Canada (AAFC) modified the genomes of *Arabidoposis thaliana* Heynh. and *Solanum lycopersicum* L. cv. Micro-Tom

(tomato) to upregulate the production of carotenoid cleavage dioxygenases (CCDs), enzymes that function in breaking down carotenoid substrates to produce VOC components with the intention of changing the VOC profiles to influence insect host selection behaviour. The transgenic CCD1a plants of both species had different VOC profiles relative to the wild-type (WT) (Caceres 2015; Challa, 2015), and in two-choice ovipositional cage bioassays the cabbage looper moth (CLM) females deposited a greater number of eggs on CCD1a plants than the control.

The cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), is a widespread, polyphagous species, whose larvae (Fig. 1A) are a serious pest of *Brassica* crops and to greenhouse crops such as tomato, cucumber, and bell pepper (Sarfraz et al. 2011). It is a multivoltine species with overlapping generations during the growing season in the field (Ehler & van den Bosch 1974), and throughout the year under greenhouse conditions. In the past, control in greenhouses was achieved with repeated treatments of synthetic insecticides like carbaryl, parathion, and methomyl (Hill 2008), or with biopesticides like Bt, or nuclear polyhedrosis viruses (NPV) (Li & Liu 2015; Liu et al. 2003). However, these frequent treatments have led to the development of resistance to synthetic insecticides (Akhtar et al. 2012; Li & Liu 2015), as well as to Bt and transgenic plants expressing the Bt toxin. Thus, there is a need for the development of new, non-chemical methods for the control of CLM populations (Li & Liu 2015). The fact that moths prefer to oviposit more eggs on CCD1a plants over WT (Challa 2015),
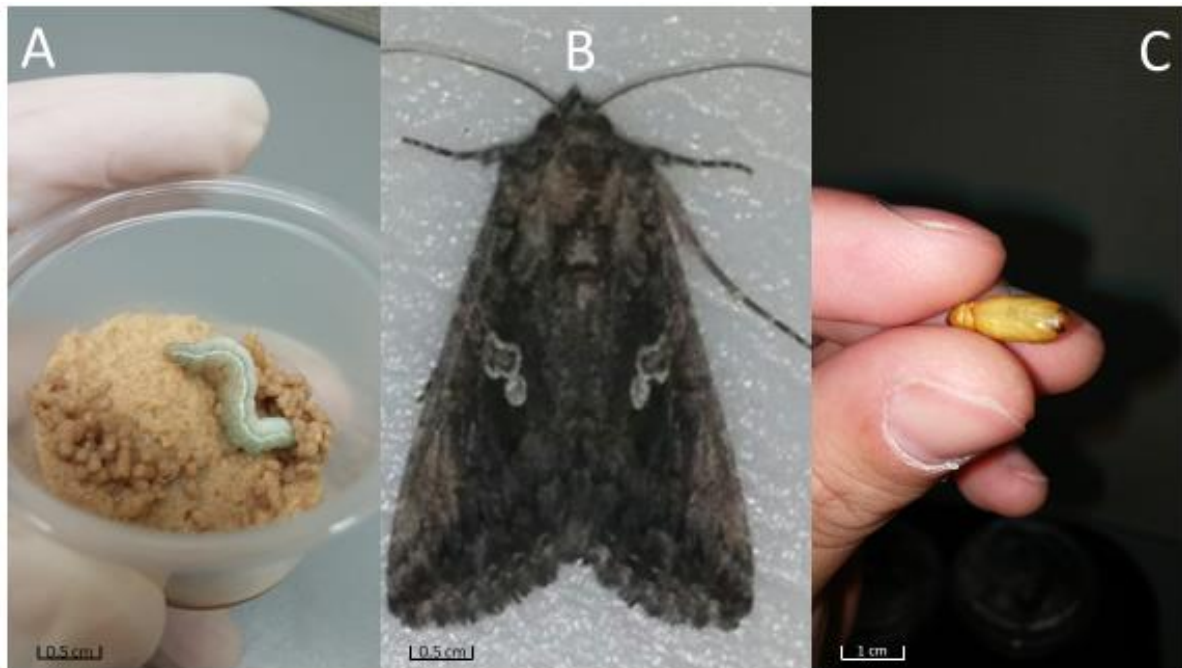
Figure 1. The cabbage looper moth (*Trichoplusia ni*) fifth instar larva (A), mature adult moth (B), and pupa (C).

raises the possibility that transgenic plants might be used as trap crops for insect population control. However, the protocol used by Challa (2015) did not allow one to determine if this was due to changes in the VOCs affecting upwind flight of the moths or if it was related to changes in traits that were detected once physical contact was made with the plant. Consequently, VOC-based attraction must be established first as CLM adult females (Fig. 1B) use a combination of VOCs, leaf shape and plant colour to locate suitable hosts (Jallow et al. 2004; Li & Liu 2015).

I hypothesized that the preference of CLMs to oviposit on CCD1a tomatoes (Challa 2015) was due to a change in the VOCs of the transformed plants, therefore, the first objective of this study was to determine if the VOCs emitted by CCD1a Micro-Tom tomatoes were more attractive to CLMs than those of WT tomatoes. If females do not preferentially locate the genetically modified plants, then they would only find them among the commercial crops by chance, eliminating the possibility of CCD1a plants being effective trap crops.

A second objective was to determine if CCD1a transformed plants that had previously been used as an oviposition site were more or less attractive to CLM females than clean CCD1a transformed plants, as the presence of eggs can result in changes of the profile of VOCs emitted and alter host plant suitability (Coleman et al. 1997; Heath et al. 2013).

A third objective was to analyse the headspace volatiles using dynamic headspace collection and gas chromatography-mass spectrometry (DHS GC-MS) to confirm that the

transgenic tomato plants used had different VOC profiles relative to WT plants, as previously reported by Challa (2015).

1.2 Materials and Methods

Insect Rearing

The insects used in the assays came from a CLM colony maintained at the London Research and Development Centre (LoRDC), Agriculture and Agri-Food Canada (AAFC), at $25\pm2°C$ and $50\pm5\%$ relative humidity (RH) under a 16:8 L:D photoperiod. Fifty pairs of adults were held in 2 litre (L) plastic containers with a layer of moist vermiculite on the floor. In nature moths feed on nectar, so the CLM adults were provided with 10% sucrose solution as a food source. Sheets of paper towel were placed securely over the opening of the container as oviposition sites and changed every three days. To surface sterilize the eggs, the sheets were soaked for 20 minutes (min.) in an 8% formic acid solution followed by a 20 min. water rinse, air dried and then held in large plastic storage bags under the same rearing conditions. A moist paper towel was placed in each bag to ensure that the eggs did not desiccate. Two freshly emerged larvae were transferred with a wet brush from the egg sheets into 1 fluid ounce (fl. oz) plastic cups (Fig. 1A) containing a pinto bean diet (Shorey & Hale 1965) - the cups were arranged 50 to a tray, and reared at the previously mentioned conditions. After approximately 7 days male larvae have an evident, yellow dorsal spot (the germinal testes) and this characteristic was used to sex individuals. Upon pupation (Fig. 1C) females were placed into individual 500 millilitre (mL) containers, ensuring they were of known age and mating status when used in assays. The sex was verified a second time before Y-tube olfactometer experiments using male genital claspers as the defining characteristic.

Plant Transformation and Growth Conditions

Two independent lines (L1 and L2) of CCD1a overexpressing tomato plants (*S. lycopersicum* cv. Micro-Tom) were created via *Agrobacterium*-mediated transformation by Dr. Ali Hannoufa's lab at AAFC using previously established protocols (Cruz-Mendívil et al. 2011; Sun et al. 2006). Challa (2015) genotyped transgenic tomato plants with the CCD1a transgene using PCR with a forward primer, 35S-For, and the gene-specific primer, CCD1-11Rev, and expression was determined by RT-qPCR.

Seeds were germinated on medium made with granulated agar and deionized water (8 grams/litre (g/L)). After approximately one week seedlings were potted in general purpose mycorrhizal growing medium, grown at $25\pm2°C$ and $50\pm5\%$ RH under a 16:8 L:D photoperiod, and watered every two days. Once plants were two weeks old, they were fertilized every other week with standard fertilizer (20:20:20 – Nitrogen: Phosphorous: Potassium) until approximately six weeks old and flowering. Plants used in the assays had similar leaf and stem sizes, flower number, and no notable morphological differences.

Y-tube Olfactometer Experiments

All females were between 3-7 days post-emergence when tested to ensure sexual maturity. When mated females were required, a mature male was placed within a cage with a 3 day old female and left for two days before being used in assays. Females that had laid a large number of eggs (>100) on the walls of the enclosures were considered mated, as virgins deposit very few eggs (<10). As the CLM is nocturnal, the assays were carried out during the scotophase under dim red light (Fig. 5B) at $25\pm2°C$ and $50\pm5\%$

RH. Moths were acclimatised to experimental conditions for 10 min. prior to being assayed.

The flowering plants (L1, L2, WT) were placed into chambers, and the Y-tube apparatus (Fig. 2A) turned on 30 min. before experiments so that pressure and air-flow equilibrium was reached. Smoke from a burning cotton wick was fed into the apparatus before experiments to ensure the equipment was working properly. When experimenting with plants previously used as oviposition sites (L1OV), plants were placed in 35 x 32 x 32 centimetre (cm) mesh enclosures with 2 mated female CLM individuals for 48 hours, and used on the following day.

The Y-tube olfactometer, similar to those used in previous volatile studies (Ngi-Song et al. 1996; Steinberg et al. 1992), was manufactured out of inert, 4cm inner diameter quartz glass, with two 18 cm long arms and a 34 cm stem (Fig. 2C). An air supply was connected via regulator to the Y-tube system using Tygon tubing. The air first moved through a humidifier (Fig. 2G) before being split into two separate lines at the flowmeter (Volatile Assay Systems, Rensselaer, NY) (Fig. 2F) which then fed air into flasks containing activated carbon (Fig. 2E) at a rate of 500 mL/min. Purified air then passed into the treatment chambers (Fig. 2D) connected to each respective Y-tube arm, while a vacuum line, also controlled by the olfactometer air kit, pulled air through the end of the Y-tube at 1000 mL/min to prevent back pressure and turbulent flow.

A randomized complete block design included plant treatments assigned to glass headspace Y-tube chambers with testing conducted in four replicate blocks including 10 moth pseudoreplicates in each block. Treatments were blocked by time, as time-related

pressure changes may potentially lead to variation in insect behaviour (Pellegrino et al. 2013; Wellington 1946). Treatments were arranged in single-plant choice experiments (Plant vs. Air), or 2-plant choice comparisons (CCD1a vs. WT). Experimental females, mated or virgin, were placed into the stem of the Y-tube, and observed for 10 min. They were considered to have made a choice if the moth travelled 10 cm into either arm, or considered as making no choice if they remained in the stem. The olfactometer was rinsed with deionized water and dried between each insect pseudoreplicate, and arm positions were switched to eliminate positional bias after five moths were tested.

Once experiments were complete, activated carbon was removed, all components rinsed with deionized water, and fresh activated carbon was placed within all glassware for 24 hours to remove any residual volatiles.
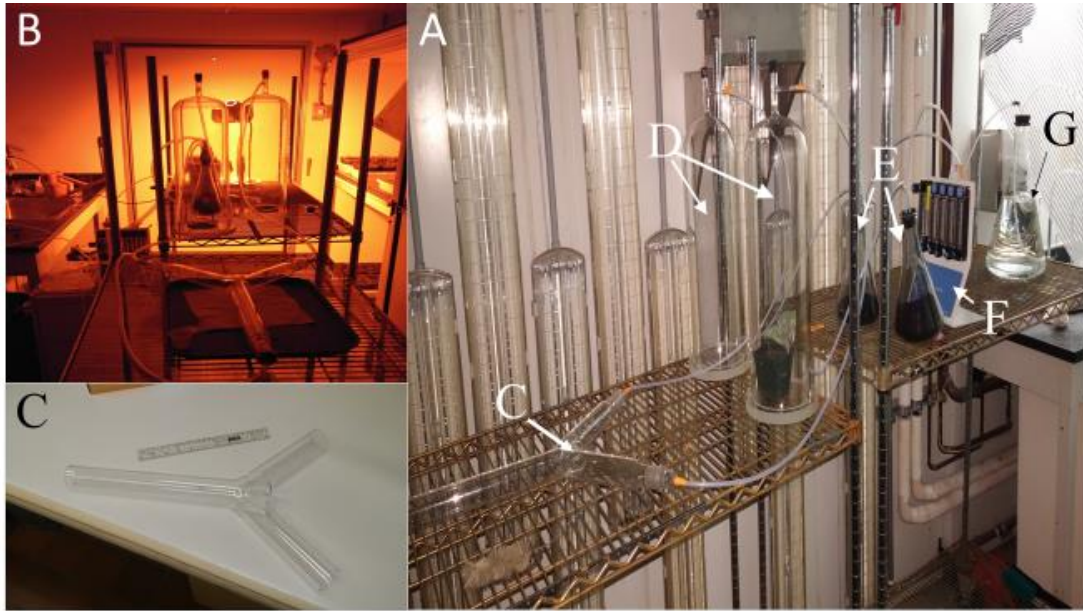
Figure 2. Y-Tube apparatus in climate controlled room (A), and the apparatus under red light (B). Setup includes a Y-Tube (C), plant chambers (D), flasks containing activated carbon (E), flowmeter (F), and humidifier (G).

Figure 3. Dynamic Headspace collection chambers containing two plants of each Micro-Tom tomato genotype – WT, CCD1a L1, and CCD1a L2.

In vivo DHS Collection and GC-MS Analyses

CCD1a and WT plant VOC samples were collected, prepared and analysed with DHS collection/GC-MS analysis protocols and settings developed by Caceres (2015) with one modification - a 5 min. solvent delay was added to the GC-MS method. Plants used for oviposition-related VOC change underwent the preparatory treatment previously mentioned for olfactometer experiments. Individual plants were placed within cylindrical 46×26 cm inert quartz glass DHS collection chambers with base (Fig. 3). Regulator controlled air was fed to a flowmeter that maintained a flow rate of 10 mL/min through Tygon tubing into each chamber before exiting through a Poropak Q 75/150 polydivinylbenzene sorbent column (Cat. #226-115; SKC Inc., USA), and each collection ran for 24 hours.

Collection columns were rinsed with high performance liquid chromatography (HPLC)-grade dichloromethane (DCM), evaporated to 250 microlitres (μL) under nitrogen gas (N_2), and 5 μL of 2-octonone ($C_8H_{16}O$) was added as an internal standard. GC-MS analysis was used to separate and distinguish differences in volatile profiles of tomato plants. The GC-MS (Agilent Technologies – Santa Clara, CA, USA) included an inert XL EI/CI MSD with triple axis detector, an autosampler, and gas chromatograph. A 30+10 m Dura-guard x 0.25 mm i.d. 0.25 μm DB-5MS+DG capillary column in pulsed split-less mode (25 psi. until 0.5 min; Split vent purge flow adjustment was 40 mL/min for 1 min), with Helium as a carrier gas (12.445 psi), was used to separate 2 μL injections of each plant extract of collected VOC emissions into components. Oven temperature was held at 30°C for 1 min, increased to 200°C at 5°C/min, followed by a 20°C/min ramp up

to 280°C which was held for 3 min, for a total run time per sample of 45 min. In addition, a 5 min. solvent delay was used in every sample run. The full scan spectra were produced at a rate of 1.95 scans/sec and between 30 and 350 *m/z*.

Statistical Analysis

As the data collected from the Y-Tube olfactometer experiments deviated from normality, a generalized linear mixed model (GLMM) with log-odds (logit) data transformation was used to determine if there was significant preference by the CLMs for CCD1a VOCs over clean air or WT VOCs. This model accounted for time-dependent random effects, and plant treatment-dependent fixed effects that may have contributed to variation. A Mann-Whitney U test was used to assess significant differences between mated and virgin female choice datasets for each Y-tube treatment. All data were analyzed with R statistical software version 3.3.2. (R Core Team, 2016).

GC-MS analysis of collected VOCs included only chemical features detected in 2/3 of technical replicates, followed by chemical features found in 2/3 of biological replicates from 24 L1, 22 L2, 23 WT, and 12 L1OV individual samples – this was done to remove any features present due to experimental error. Peaks were adjusted to the internal standard of 2-octonone, and principle component score plot analyses (PCA) were conducted on log-transformed peaks for the comparison of transgenic and WT genotypes. Plots were scrutinized visually for within-cluster and between-cluster data point distances. MS Excel (Microsoft Corporation, 2016) was used for processing of chemical peaks, and R statistical software version 3.3.1. (R Core Team, 2016) was used for peak alignment and construction of PCA plots.

The raw data from the Challa (2015) study were not available for direct comparison, so from my samples I identified those compounds previously reported to have shown significant changes. These were identified using either the mass spectral search program chemical databases via the National Institute of Standards and Technology (NIST version 2.0, 2012) (β-phellandrene, α-copaene, δ-elemene, sabinene) or directly with available standards (1-R-α-pinene, β-pinene, 3-carene, β-caryophyllene) using the Automated Mass Spectral Deconvolution and Identification System (AMDIS version 2.71, 2012). A library of deconvoluted WT, CCD1a L1, CCD1a L1OV, and CCD1a L2 chromatograms was built in AMDIS, and were compared to a library of known compounds in NIST. The metab R script was used in R Statistical Software version 3.3.1. (R Core Team, 2016) to calculate mean peak area, and overall change for the different compounds between plant types, as well as to run two-tailed t-tests to determine significance of the change.

1.3 Results

Y-tube Olfactometer Experiments

Assays testing mated female CLM responses in an empty Y-tube showed that the experimental space did not affect moth behaviour. A mean proportion of 0.44 (C.I. = 0.29, 0.60) chose right over left: there was no significant directional preference (Z = -0.665, P = 0.51), or significant random block effects contributing to the variation (Z = 0.633, P = 0.26).

In single-plant choice experiments, both virgin (**L1** Z=2.012, P=0.04; **L2** Z=2.795, P=0.005) and mated (**WT** Z=2.628, P=0.009; **L1** Z=2.198, P=0.03) CLM females generally showed a significant preference for plant volatiles over clean air, regardless of plant type (Fig. 4). Only mated females with **L2** (Z=1.359, P=0.17) and virgin females with **WT** plants (Z=1.885, P=0.06) showed no significant preference. Blocks contributed no significant variation in plant versus air experiments with virgin (**WT** Z=0.417, P=0.34; **L1** Z=0.770, P=0.22; **L2** Z=1.280, P=0.10) or mated females (**WT** Z=0.840, P=0.20; **L1** Z=2.198, P=0.22; **L2** Z=0.547, P=0.29), and there was no significant influence of mating status (**WT** W=12.00, P=0.30; **L1** W=7, P=0.88; **L2** W=2, P=0.11).

In the two-plant choice experiments neither mated (**L1** Z=1.357, P=0.18; **L2** Z=0.0140, P=0.99) nor virgin (**L1** Z=1.357, P=0.18; **L2** Z=0.790, P=0.22) females showed significant preference when given a choice between volatiles from WT and either L1 or L2 transgenic plants (Fig. 5). Blocks contributed no significant variation in
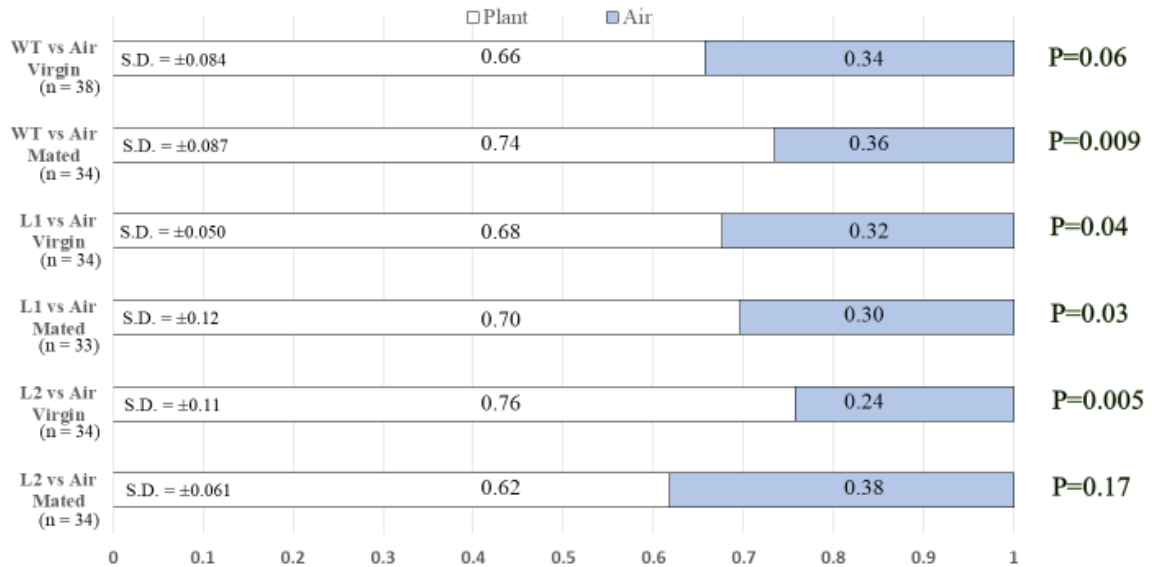
Figure 4. Mean choice proportions ±S.D. and significance of choice by virgin and mated cabbage looper moth (*Trichoplusia ni*) females for air containing CCD1a line 1 (L1), line2 (L2), or wild-type (WT) Micro-Tom tomato plant VOCs relative to air without plant VOCs in Y-tube olfactometer experiments.
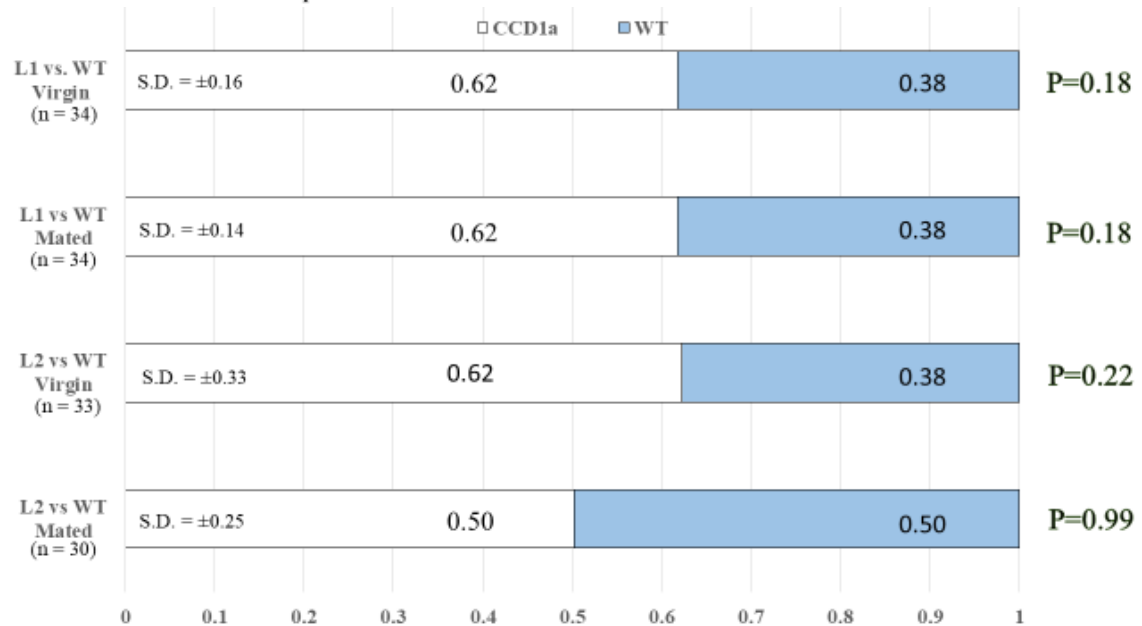
Figure 5. Mean choice proportions ± S.D. and significance of choice by virgin and mated cabbage looper moth (*Trichoplusia ni*) females for air containing CCD1a line 1 (L1) or line 2 (L2) Micro-Tom tomato plant VOCs relative to air containing VOCs from wild-type (WT) Micro-Tom tomato plants in Y-tube olfactometer experiments.
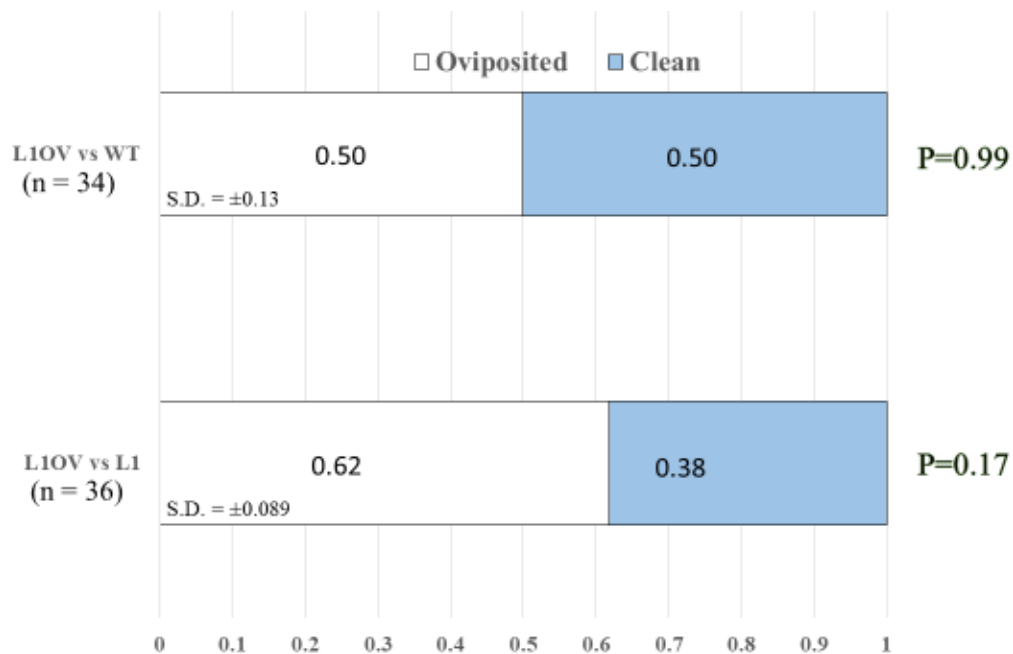
Figure 6. Mean choice proportions ±S.D. and significance of choice by mated cabbage looper moth (*Trichoplusia ni*) females for air containing VOCs from CCD1a line 1 Micro-Tom tomato plants with eggs (L1OV) relative to air containing VOCs from clean line 1 (L1) or wild-type (WT) Micro-Tom tomato plants in Y-tube olfactometer experiments.

transgenic plant versus WT plant experiments with virgin females (**L1** Z=0.836, P=0.20; **L2** Z=0.790, P=0.25) or mated females (**L1** Z=0.806, P=0.21; **L2** Z=0.797, P=0.21), and there was no significant influence of mating status (**L1** W=10, P=0.66; **L2** W=5, P=0.49).

Females CLMs did not discriminate between a clean plant (no eggs), regardless of whether it was a **WT** (Z=0.00200, P=0.99) or transgenic **L1** (Z=1.359, P=0.17), over a transgenic plant that had previously been used as an oviposition site (**L1OV**) (Fig. 6). There were no significant block effects for experiments with clean **WT** (Z=0.371, P=0.36) or **L1** (Z= -0.024, P=0.98) plants. All raw data for the Y-tube olfactometer experiments are in Appendix A.

Chemical Analysis

The PCA scatterplot (Fig. 7) depicts relationships between chemical profiles of individual plants from the three plant lines: the closer the data points, the closer the resemblance. L2 is clearly quite different from WT and L1, while there is still some degree of overlap between WT and L1.

It is evident that the VOC profile of L1 with eggs (L1OV) differs significantly from the other three lines without eggs (Fig. 8).

δ-elemene was the only compound previously reported by Challa (2015) that was not detected in any of my samples, while 3-carene and α-copaene were only seen in the profiles of certain lines (Table 1 - 4).

While there were some changes observed between the VOCs of transgenic lines (with or without eggs) and the WT control, such as declines in β-phellandrene, and

increases in caryophyllene and 1-R-α-pinene (Tables 1-4) there were no significant differences (two-tailed t-test, P>0.05).

Individuals factor map (PCA)
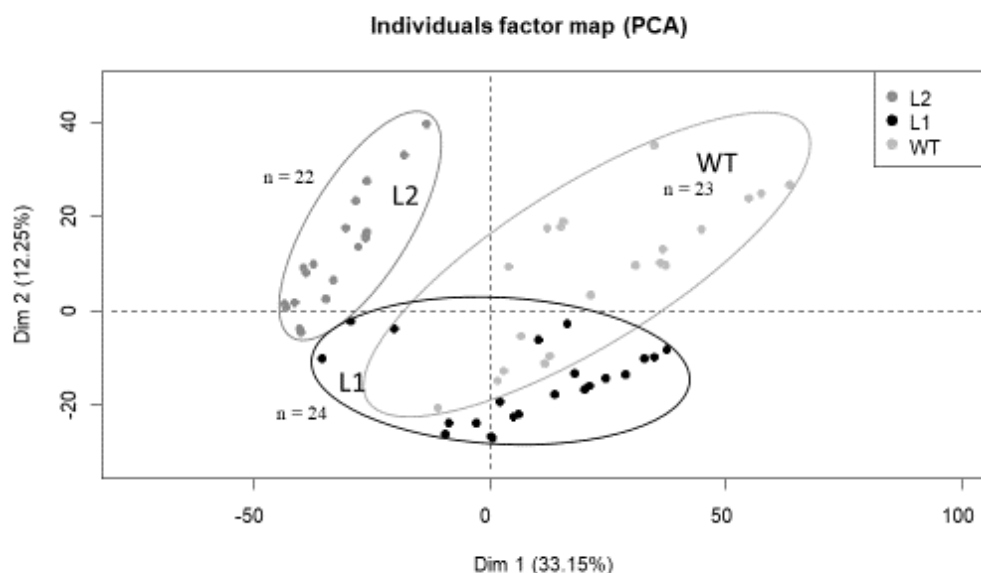
Figure 7. PCA scatterplot illustrating relative differences and similarities in volatile organic chemical profiles between individual plants from CCD1a line 1 (L1), CCD1a line 2 (L2), and wild-type (WT) Micro-Tom tomato genotypes. Each point represents the profile of a single plant.

Individuals factor map (PCA)
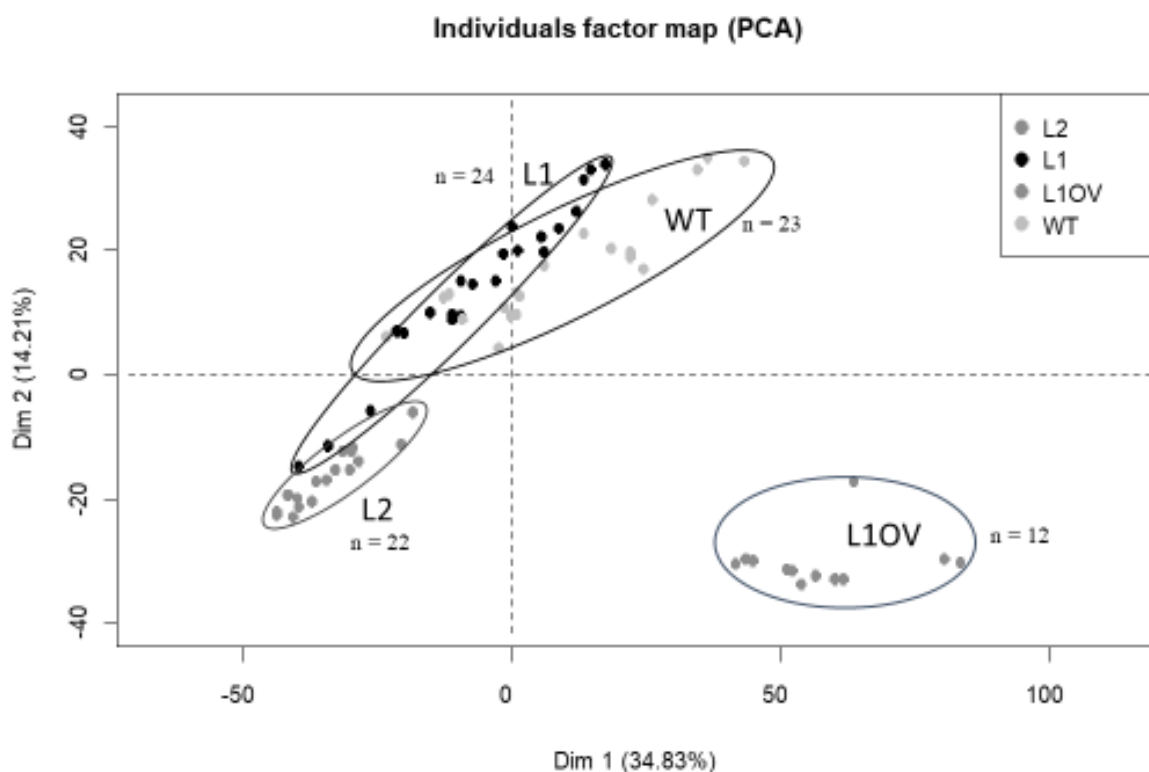
Figure 8. PCA scatterplot illustrating relative differences and similarities of volatile organic chemical profiles between individual plants from different genotypes − CCD1a line 1 (L1), CCD1a line 1 + cabbage looper moth (*Trichoplusia ni*) eggs (L1OV), CCD1a line 2 (L2), and wild-type (WT) Micro-Tom tomato. Each point represents the profile of a single plant.

Table 1. Mean peak area ± S.D., fold change, and p values for identified volatile organic compounds in collected headspace air from line 1 CCD1a (L1) Micro-Tom tomatoes relative to wild-type (WT) Micro-Tom tomatoes. P values were obtained with a two-tailed t-test to investigate significance of fold change.

Volatile Organic Compound	L1 Peak Area (n=24)	WT Peak Area (n=23)	Fold change	P Value
1-R-α-Pinene	$6.19 \times 10^6 \pm 1.47 \times 10^7$	$5.46 \times 10^6 \pm 1.46 \times 10^7$	+0.18	0.88
3-Carene	$8.34 \times 10^5 \pm 9.59 \times 10^5$	$7.95 \times 10^5 \pm 7.64 \times 10^5$	+0.08	0.95
β-Phellandrene	$1.14 \times 10^5 \pm 1.22 \times 10^5$	$1.12 \times 10^6 \pm 1.12 \times 10^7$	-3.30	0.54
β-Pinene	$1.51 \times 10^6 \pm 1.39 \times 10^7$	$2.25 \times 10^6 \pm 5.18 \times 10$	-0.57	0.64
Caryophyllene	$8.59 \times 10^5 \pm 8.52 \times 10^5$	$1.02 \times 10^6 \pm 1.41 \times 10^6$	-0.25	0.89
α-Copaene	0.00	$9.22 \times 10^4 \pm 7.61 \times 10^4$	Absence	--
δ-Elemene	0.00	0.00	Not Detected	--
Sabinene	$5.76 \times 10^5 \pm 7.60 \times 10^5$	$8.0 \times 10^5 \pm 6.86 \times 10^5$	-0.47	0.75

Table 2. Mean peak area ±S.D., fold change, and p values for identified volatile organic compounds in the collected headspace air from line 2 CCD1a (L2) Micro-Tom tomatoes relative to wild-type (WT) Micro-Tom tomatoes. P values were obtained with a two-tailed t-test to investigate significance of fold change.

Volatile Organic Compound	L2 Peak Area (n=22)	WT Peak Area (n=23)	Fold change	P value
1-R-α-Pinene	$1.67 \times 10^7 \pm 3.00 \times 10^7$	$5.46 \times 10^6 \pm 1.46 \times 10^7$	+1.61	0.16
3-Carene	0.00	$7.95 \times 10^5 \pm 7.64 \times 10^5$	Absence	--
β-Phellandrene	$2.52 \times 10^5 \pm 4.05 \times 10^5$	$1.12 \times 10^6 \pm 1.12 \times 10^7$	-2.15	0.65
β-Pinene	$5.21 \times 10^6 \pm 9.23 \times 10^6$	$2.25 \times 10^6 \pm 5.18 \times 10$	+1.21	0.29
Caryophyllene	$2.95 \times 10^6 \pm 3.01 \times 10^6$	$1.02 \times 10^6 \pm 1.41 \times 10^6$	+1.53	0.24
α-Copaene	0.00	$9.22 \times 10^4 \pm 7.61 \times 10^4$	Absence	--
δ-Elemene	0.00	0.00	Not Detected	--
Sabinene	$1.81 \times 10^6 \pm 2.27 \times 10^6$	$8.0 \times 10^5 \pm 6.86 \times 10^5$	+1.18	0.49

Table 3. Mean peak area ±S.D., fold change, and p values for identified volatile organic compounds in the collected headspace air from line 1 CCD1a (L1) Micro-Tom tomatoes relative to line 1 with *Trichoplusia ni* eggs (L1OV) Micro-Tom tomatoes. P values were obtained with a two-tail t-test to investigate significance of fold change.

Volatile Organic Compound	L1 Peak Area (n=24)	L1OV Peak Area (n=12)	Fold change	P Value
1-R-α-Pinene	$6.19 \times 10^6 \pm 1.47 \times 10^7$	$2.02 \times 10^6 \pm 2.97 \times 10^6$	+1.62	0.44
3-Carene	$8.34 \times 10^5 \pm 9.59 \times 10^5$	0.00	Presence	--
β-Phellandrene	$1.14 \times 10^5 \pm 1.22 \times 10^5$	$1.21 \times 10^7 \pm 1.90 \times 10^7$	-6.74	0.25
β-Pinene	$1.51 \times 10^6 \pm 1.39 \times 10^7$	$2.54 \times 10^6 \pm 5.17 \times 10^6$	-0.75	0.58
Caryophyllene	$8.59 \times 10^5 \pm 8.52 \times 10^5$	$1.31 \times 10^5 \pm 1.75 \times 10^5$	+2.71	0.36
α-Copaene	0.00	$2.04 \times 10^5 \pm 2.28 \times 10^5$	Absence	--
δ-Elemene	0.00	0.00	Not Detected	--
Sabinene	$5.76 \times 10^5 \pm 7.60 \times 10^5$	$8.62 \times 10^5 \pm 1.15 \times 10^6$	-0.58	0.78

Table 4. Mean peak area ±S.D., fold change, and p values for identified volatile organic compounds in the collected headspace air from wild-type (WT) Micro-Tom tomatoes relative to line 1 with *Trichoplusia ni* eggs CCD1a (L1OV) Micro-Tom tomatoes. P values were obtained with a two-tailed t-test to investigate significance of fold change.

Volatile Organic Compound	WT Peak Area (n=23)	L1OV Peak Area (n=12)	Fold change	P values
1-R-α-Pinene	$5.46 \times 10^6 \pm 1.46 \times 10^7$	$2.02 \times 10^6 \pm 2.97 \times 10^6$	+1.44	0.52
3-Carene	$7.95 \times 10^5 \pm 7.64 \times 10^5$	0.00	Presence	--
β-Phellandrene	$1.12 \times 10^6 \pm 1.12 \times 10^7$	$1.21 \times 10^7 \pm 1.90 \times 10^7$	-3.44	0.12
β-Pinene	$2.25 \times 10^6 \pm 5.18 \times 10^6$	$2.54 \times 10^6 \pm 5.17 \times 10^6$	-0.18	0.90
Caryophyllene	$1.02 \times 10^6 \pm 1.41 \times 10^6$	$1.31 \times 10^5 \pm 1.75 \times 10^5$	+2.96	0.44
α-Copaene	$9.22 \times 10^4 \pm 7.61 \times 10^4$	$2.04 \times 10^5 \pm 2.28 \times 10^5$	-1.15	0.57
δ-Elemene	0.00	0.00	Not Detected	--
Sabinene	$8.0 \times 10^5 \pm 6.86 \times 10^5$	$8.62 \times 10^5 \pm 1.15 \times 10^6$	-0.11	0.94

1.4 Discussion

The two lines of Micro-Tom transgenic CCD1a over-expressing tomato plants used in these experiments were more attractive to CLM females than air (Fig. 4) but overall were no more attractive than WT plants in a two-plant choice test (Fig. 5). Furthermore, the mating status of females did not affect preference (Fig. 4 and 5), nor did the presence of deposited eggs (Fig. 6) when L1OV was compared with WT. The fact that CLM females showed no preferences, despite differences in the VOCs from both the transformed lines compared to the WT, as well as those with or without eggs, (Figs. 7 and 8) suggests that the specific compounds used to locate host plants are always present at sufficiently similar concentrations/ratios to elicit positive chemotaxis. The lack of preference, despite differences in VOC profiles, is similar to that observed in two-plant Y-tube olfactometer experiments looking at female preferences to the volatiles from a primary (cabbage) and secondary (cotton) host plant (Jallow et al. 2004; Li & Liu 2015).

Both the PCA scatterplots (Figs. 7 & 8), and changes in mean peak areas of several specific compounds (Tables 1-4) observed (generally in keeping with the trends reported previously by Challa (2015)), indicated that there are changes in VOC emissions of CCD1a lines compared to WT. However, in the case of the specific compounds investigated, no significant differences were detected due to the high sample to sample variability. VOC profiles can be affected by temperature and water stress (Gouinguené & Turlings 2002), as well as individual plant genetics (Vickers et al. 2014), and slight differences in these might have contributed to the between sample variability observed.

I also observed that certain compounds, such as α-copaene and 3-carene, were present in the VOC profiles of some lines and not others. However, it is evident from the results that overall changes in the VOC profiles and/or the presence/absence of certain compounds did not influence the upwind chemotactic flight responses of the CLM. Therefore, one would conclude that none of the chemical cues used by CLMs to locate suitable host plants were altered enough in transformed plants to significantly modify female foraging behaviour.

The decision to lay eggs or not occurs once the female locates the plant and is determined by leaf morphology and contact chemical cues (Landolt 1993; Ramiswamy et al. 1987). Thus, the ovipositional preference for CCD1a plants reported by Challa (2015) could be due to differences in these short-range cues between transformed and WT plants. The importance of these short-range cues could be tested by examining the leaf morphology in greater detail (scanning electron microscopy, colour spectral analysis) to determine if there are subtle differences not evident to the naked eye, as well as testing the response of chemoreceptors on the tarsi/ovipositor (Städler et al. 1995; Wallace et al. 2004) to the leaf surface chemistry of the WT and transgenic lines.

The general rule for the successful use of trap crops as an economically viable control method is that they should not represent more than 10% of all plants present (Hokkanen 1991; Pickett et al. 1997). Consequently, if as proposed, the oviposition preferences observed by Challa (2015) only occurred once the female moths contacted the plant, then the absence of preference by upwind chemotaxis I observed suggests that the transgenic lines tested would not be suitable as trap crops within a greenhouse setting, as CLM females would only find the trap plants by chance. Even then, if CLM females

showed a preference for the VOCs of transgenic plants, it would have to be very close to absolute preference for the transgenic plants to be effective trap crops. The lack of preference observed in my experiments indicate that the CCD1a Micro-Tom tomato plants currently available would not help reduce the CLM pest pressure in greenhouse crops.

1.5 Conclusion

The purpose of this thesis was to determine if CLM females exhibited preferential chemotactic upwind flight to the VOCs from two transgenic Micro-Tom tomato lines with the upregulated CCD1a gene over those from the untransformed WT. While the DHS GC-MS data found detectable differences in the VOC profiles between genotypes, the results of Y-tube olfactometer choice assays found that neither virgin or mated females showed any significant preference between lines, and their preference was not affected by the presence of eggs. This would indicate that the chemical change in the VOCs did not sufficiently alter the chemical cues that are responsible for olfactory chemotaxis in the CLM. Thus, the CCD1a tomato plants tested would not be effective as dead-end or push-pull trap crops in an IPM program for controlling the CLM populations in greenhouses.

Future Directions

In order to modify plants as effective trap crops, the first step would be to determine the specific components of the VOC blend that are used by CLM females when foraging for suitable oviposition sites. This could be accomplished by first identifying the VOC compounds that are detected by the CLM female antennae using GC-EAD (Gas chromatograph linked with electroantennographic detection) (Scheidler et al. 2015). Secondly, one would have to conduct assays to determine which compounds actually influence foraging behaviour. Once the ideal profile has been determined then the appropriate transformations could be undertaken to develop suitable trap crops. To date, one has looked at the CCD1a enzyme, but this is only one of many that are

implicated in the pathways involved in the production of VOCs and secondary plant metabolites (Aharoni et al. 2004; Lee & Chappell 2008). Thus the genetic manipulation of enzymes affecting other pathways, such as other CCDs or 9-cis-epoxycarotenoid enzymes (NCEDs), should be considered. Furthermore, in my study I only tested CCD1a in the flowering stage and in future studies one should test different phenological stages as VOC constituents can change with plant phenology (DellaPenna & Pogson 2006; Hirschberg 2001; Ilg et al. 2014; Walter & Strack 2011).

Furthermore, since visual, gustatory, and morphological cues are known to influence insect host finding and oviposition behaviour, these parameters should also be examined at the same time as the VOCs when developing trap crops as an effective alternative to synthetic insecticides for insect pest management in greenhouses.

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Appendix A– Raw Data Tables

Table 5. Proportions of virgin and mated female cabbage looper moths (*Trichoplusia ni*) tested in the olfactometer that chose air containing wild-type (WT) Micro-Tom tomato plant VOCs over clean air.

Treatment	Mating Status	Rep	n	Plant	Air	Choice Proportion
WT v Air	Virgin	1	9	6	3	0.66
WT v Air	Virgin	2	10	5	5	0.60
WT v Air	Virgin	3	10	8	2	0.80
WT v Air	Virgin	4	9	6	3	0.67
WT v Air	Mated	1	9	6	3	0.67
WT v Air	Mated	2	9	7	2	0.77
WT v Air	Mated	3	7	6	1	0.85
WT v Air	Mated	4	9	6	3	0.67

Table 6. Proportions of virgin and mated female cabbage looper moths (*Trichoplusia ni*) tested in the olfactometer that chose air containing CCD1a line 1 (L1) Micro-Tom tomato plant VOCs over clean air.

Treatment	Mating Status	Rep	n	Plant	Air	Choice Proportion
L1 v Air	Virgin	1	9	6	3	0.67
L1 v Air	Virgin	2	8	5	3	0.63
L1 v Air	Virgin	3	9	6	3	0.67
L1 v Air	Virgin	4	8	6	2	0.75
L1 v Air	Mated	1	8	7	1	0.88
L1 v Air	Mated	2	8	5	3	0.63
L1 v Air	Mated	3	8	5	3	0.63
L1 v Air	Mated	4	9	6	3	0.67

Table 7. Proportions of virgin and mated female cabbage looper moths (*Trichoplusia ni*) tested in the olfactometer that chose air containing CCD1a line 2 (L2) Micro-Tom tomato plant VOCs over clean air.

Treatment	Mating Status	Rep	n	Plant	Air	Choice Proportion
L2 v Air	Virgin	1	8	5	3	0.63
L2 v Air	Virgin	2	7	5	2	0.71
L2 v Air	Virgin	3	9	8	1	0.88
L2 v Air	Virgin	4	9	7	2	0.77
L2 v Air	Mated	1	9	6	3	0.67
L2 v Air	Mated	2	9	6	3	0.67
L2 v Air	Mated	3	7	4	3	0.57
L2 v Air	Mated	4	9	5	4	0.56

Table 8. Proportions of virgin and mated female cabbage looper moths (*Trichoplusia ni*) tested in the olfactometer that chose air containing CCD1a line 1 (L1) Micro-Tom tomato plant VOCs over air containing wild-type (WT) Micro-Tom tomato plant VOCs.

Treatment	Mating Status	Rep	n	Plant (L1)	Plant (WT)	Choice Proportion
L1 v WT	Virgin	1	10	7	3	0.70
L1 v WT	Virgin	2	8	3	5	0.38
L1 v WT	Virgin	3	7	5	2	0.71
L1 v WT	Virgin	4	9	6	3	0.67
L1 v WT	Mated	1	8	6	2	0.75
L1 v WT	Mated	2	8	6	2	0.75
L1 v WT	Mated	3	10	5	5	0.50
L1 v WT	Mated	4	8	4	4	0.50

Table 9. Proportions of virgin and mated female cabbage looper moths (*Trichoplusia ni*) tested in the olfactometer that chose air containing line 2 (L2) Micro-Tom tomato plant VOCs over air containing wild-type (WT)Micro-Tom tomato plant VOCs.

Treatment	Mating Status	Rep	n	Success	Failure	Choice Proportion
L2 v WT	Virgin	1	9	7	2	0.77
L2 v WT	Virgin	2	9	6	3	0.66
L2 v WT	Virgin	3	8	1	7	0.125
L2 v WT	Virgin	4	7	6	1	0.85
L2 v WT	Mated	1	7	1	6	0.14
L2 v WT	Mated	2	8	4	4	0.50
L2 v WT	Mated	3	8	5	3	0.625
L2 v WT	Mated	4	7	5	2	0.71

Table 10. Proportions of mated female cabbage looper moths (*Trichoplusia ni*) tested in the olfactometer that chose air containing VOCs from line 1 Micro-Tom tomato plants with *T. ni* eggs oviposited upon them (L1OV) over air containing wild-type (WT) or clean line 1 (L1) Micro-Tom tomato plant VOCs.

Treatment	Mating Status	Rep	n	Success	Failure	Choice Proportion
L1OV v L1	Virgin	1	9	5	4	0.56
L1OV v L1	Virgin	2	8	6	2	0.75
L1OV v L1	Virgin	3	8	5	3	0.62
L1OV v L1	Virgin	4	9	5	4	0.56
L1OV v WT	Mated	1	9	6	3	0.66
L1OV v WT	Mated	2	9	3	6	0.33
L1OV v WT	Mated	3	8	4	4	0.50
L1OV v WT	Mated	4	8	4	4	0.50

Table 11. Proportions of cabbage looper (*Trichoplusia* ni) moths that chose left or right in an olfactometer without plant VOCs present.

Treatment	Mating Status	Rep	n	Air (A1)	Air (A2)	Choice Proportion
Air v Air	Mated	1	8	4	4	0.50
Air v Air	Mated	2	10	5	5	0..50
Air v Air	Mated	3	9	3	6	0.33
Air v Air	Mated	4	9	4	5	0.44

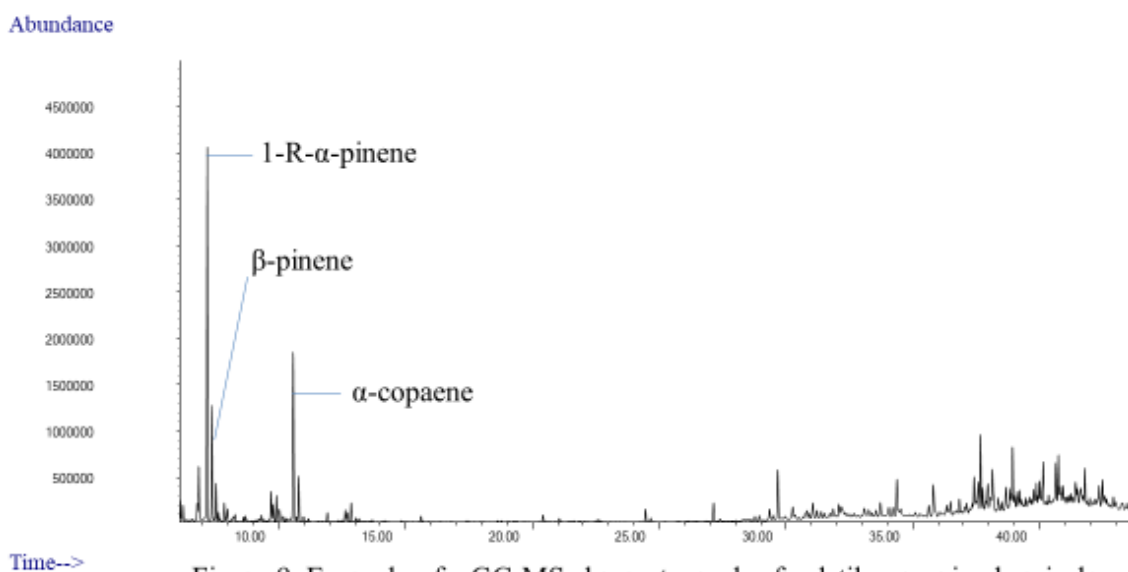# Appendix B – Gas Chromatography-Mass Spectrometry Chromatographs for Tomato VOC Analyses

Figure 9. Example of a GC-MS chromatograph of volatile organic chemicals (VOCs) present in the headspace of an individual wild-type (WT) Micro-Tom tomato plant with three major peaks putatively identified.
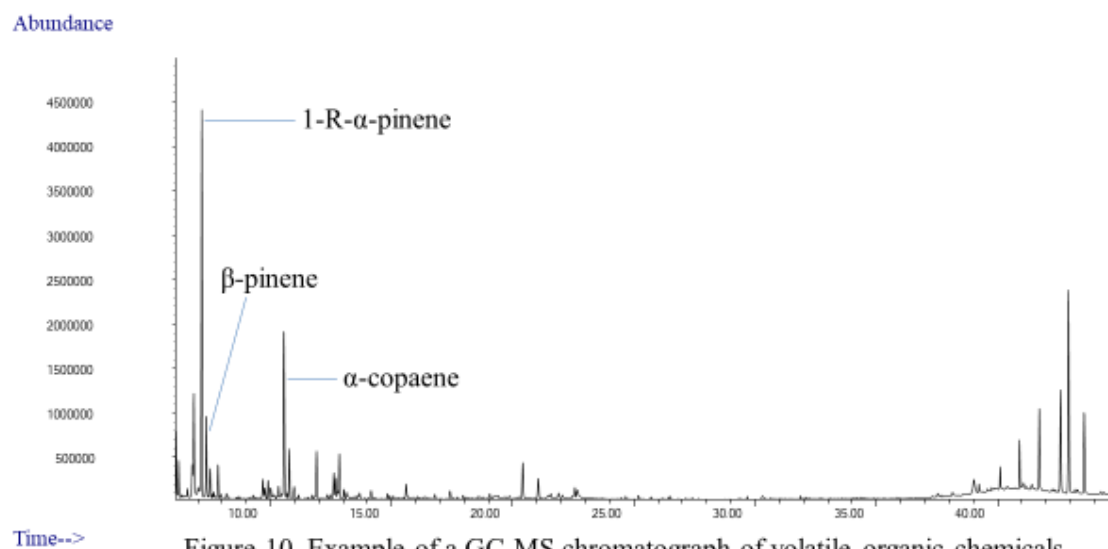

Figure 10. Example of a GC-MS chromatograph of volatile organic chemicals (VOCs) present in the headspace of an individual line 1 CCD1a (L1) Micro-Tom tomato plant with three major peaks putatively identified.
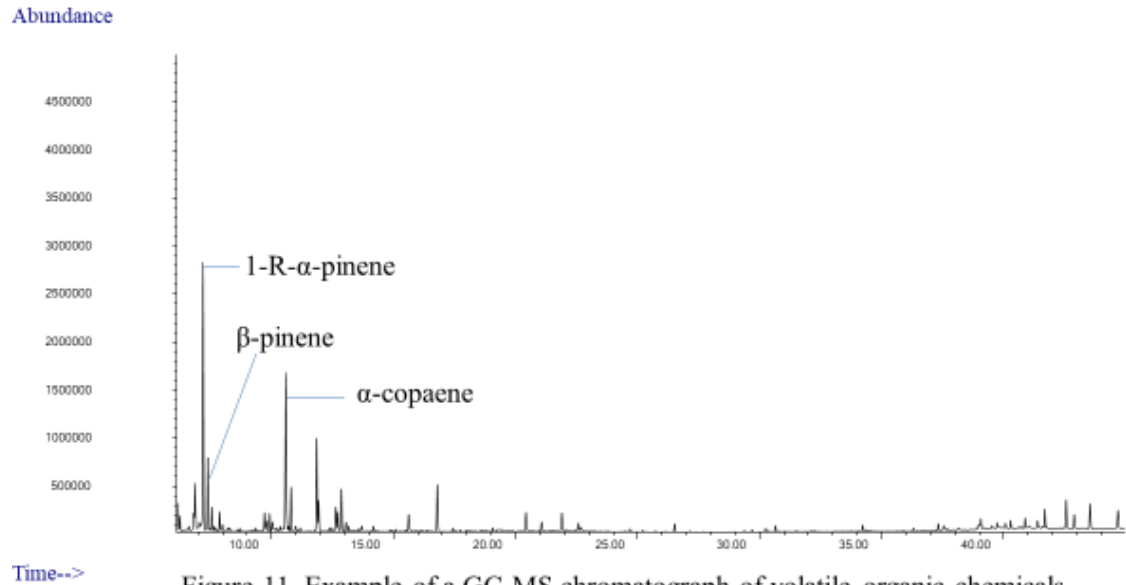
Figure 11. Example of a GC-MS chromatograph of volatile organic chemicals (VOCs) present in the headspace of an individual line 2 CCD1a (L2) Micro-Tom tomato plant with three major peaks putatively identified.
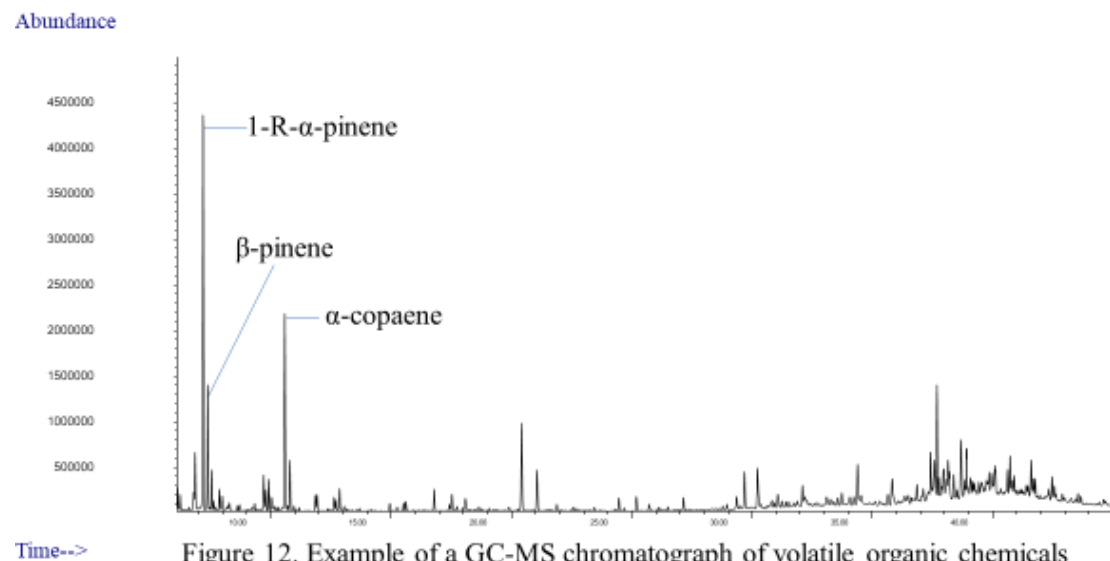

Figure 12. Example of a GC-MS chromatograph of volatile organic chemicals (VOCs) present in the headspace of an individual line 1 CCD1a with *Trichoplusia ni* eggs (L1OV) Micro-Tom tomato plant with three major peaks putatively identified.

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