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A study of volatile organic compounds from transgenic arabidopsis thaliana and solanum lycopersicum plants and analytical characterization of pyrolysis bio-oil

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Graduate Program in Chemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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A STUDY OF VOLATILE ORGANIC COMPOUNDS FROM TRANSGENIC
ARABIDOPSIS THALIANA AND SOLANUM LYCOPERSICUM PLANTS

AND

ANALYTICAL CHARACTERIZATION OF PYROLYSIS BIO-OILS

by

Luis A. Cáceres

Graduate Program in Chemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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Abstract

The two sample matrices investigated in this thesis were originated from tomato plant sources. In the first study, the analysis was performed in complete Solanaceae (*Solanum lycopersicum* cv.) Micro-Tom tomato plants. In the second study, analysis was performed in Solanaceae (*Solanum lycopersicum* L.) greenhouse tomato plant bio-oil.

The first study examined volatile organic compounds (VOCs) from the headspace of Micro-Tom tomato. These plants were genetically transformed to overexpress the *carotenoid cleavage deoxygenase1* (*CCD1*) gene. A wide range of volatile organic compounds were isolated by the means of dynamic and/or static headspace extraction. Development of the analytical method was first conducted using the *Arabidopsis Thaliana* Columbia-0 ecotype as a model plant. The non-targeted analysis of volatiles using a dynamic headspace (DHS) extraction coupled with gas chromatography mass spectrometry (GC-MS) revealed the presence of secondary metabolites including mainly unsaturated hydrocarbons, aromatic derivatives, alcohols, mono and sesquiterpenes. Among the identified volatiles, the apocarotenoid compound β -ionone was also detected in the headspace (HS) of the plants. This molecule was of special interest since it was produced by the action of the *CCD1* gene. The results demonstrated that *Arabidopsis* plants produced the apocarotenoid compound β -ionone and the method also showed the four-fold enhancement of it in the plants overexpressing the *CCD1* gene.

The DHS was applied in the non-targeted analysis of volatiles from Micro-Tom tomato overexpressing the *CCD1* gene. The compounds identified were mainly monocyclic and bicyclic monoterpenes and sesquiterpenes, and aromatic derivatives. In the study there was no evidence of the presence of any apocarotenoid compound, as a consequence a more sensitive strategy using a static headspace (SHS) analysis of tomato flowers only using simultaneously three solid phase microextraction (SPME) fibers was applied.

The non-targeted analysis using SPME fibers revealed the presence of 45 volatiles from transgenic plants and 35 from wild type when all three fibers were used together. Of the total VOCs identified, 30 were common to both types of plants, but 15 were specific to the transgenic and 5 to the wild type plants. The compounds identified from Micro-Tom flowers were mainly monocyclic and bicyclic monoterpenes and sesquiterpenes, and one alkyl benzene compound. The bicyclic monoterpenes, 1R α -pinene, α -pinene and β -pinene were found to be the most abundant molecules present in Micro-Tom tomato flowers. The overall advantage of maximizing the discovery of VOCs based on the selectivity differences with three SPME fibers was evident. Such a benefit was important in the non-targeted analysis of transgenic plants for detecting the production of unexpected compounds.

It is widely understood that chemical pest control involves the use of chemical substances to eradicate insect pest. The use of these synthetic pesticides could not only generate a negative impact to the environment but could affect human health. A complementary part of my work, was to determine the behaviour that common insect pest had when exposed to selected volatile organic compounds. Bioassays using the apocarotenoid compound β -ionone at the concentration found in the headspace of the *Arabidopsis* plants demonstrated to have activity against the adult crucifer flea beetle (*Phyllotreta cruciferae* Goeze) and adult spider mite (*tetranychidae*). Structurally similar molecules, the α -ionone and the dihydro- β -ionone were evaluated. Results indicated that α -ionone had a neutral or no effect, while dihydro-beta-ionone appeared to attract herbivores. Among the volatiles produced by the Micro-Tom tomato the sesquiterpene α -copaene was tested using greenhouse whiteflies (*Trialeurodes vaporariorum*). Results showed that the compound had an attractant effect and promoted the oviposition. This response was corroborated with the Micro-Tom tomato plants in which whiteflies favored plants with higher concentration of the sesquiterpene.

The second challenging sample matrix explored in this thesis was bio-oil from tomato plant residue. The bio-oil was generated from the thermal decomposition of the

tomato biomass in the absence of oxygen in a process called pyrolysis. Bio-oil is complex in composition due to the breakdown of bigger molecules, mainly cellulose, hemicellulose and lignin, as well as the decomposition or conservation of any additional chemicals unique to a specific biomass. One of the many potential applications of the chemicals found in bio-oils is as a pesticide. The search for effective and safe pesticides is a continuing challenge as species quickly adapt to most pesticides that are applied.

In this second part of the research, bio-oil from tomato residue was fractionated based on its pesticidal properties against common pathogens. The distinctive pesticide properties were explored after the chemical complexity of pyrolysis bio-oil from tomato plant waste was reduced through isolation by liquid-liquid extraction, solid phase extraction, and liquid chromatography. Bio-oil from tomato plant residue had a negative effect towards colorado potato beetle (*Leptinotarsa decemlineata*) and the following fungi species: *alternaria alternate*, *fusarium solani*, *phytophthora sojae*, *rhizoctonia solani* and *sclerotinia sclerotiorum*. Neophytadiene, phytol and a number of fatty acids were identified in the most active bio-oil fraction. Bioassays using standards of these compounds determine only a partial feeding deterrent effect with neophytadiene and phytol, suggesting that other unidentified compounds in the bio-oil fractions were also responsible for observed insecticidal activities.

Keywords

Gas chromatography, mass spectrometry, solid phase microextraction, headspace, fibers, β -ionone, coating, gene, transformation, derivatization, bio-oil, pesticide, bacteria, fungi, pyrolysis, phytol, neophytadiene.

Statement of Co-Authorship

1. **L.A. Cáceres**, B.D. McGarvey, I.M. Scott, K. Conn, F. Berruti, C. Briens & K.K.-C. Yeung. Pesticidal properties of tomato plant residue bio-oil, *Pest Management Research Report*, **2011**, *H*, 17-20. (Chapter 5 and 6).

The author was involved in the production of bio-oil from the tomato residue biomass. The Pest Management Research Report (PMRR) is a periodical to facilitate the rapid exchange of information on Integrated Pest management (IPM) among persons involved in research and advisory services on IPM of plant diseases and insect pests in the Agri-food sector of Canada. The publication was written, reviewed and submitted by the author and can be downloaded through the following link <http://phytopath.ca/publication/pmrr/>

2. L. Suqi, **L.A. Cáceres**, K. Schieck, C.J. Booker, B.M. McGarvey, K.K.-C. Yeung, S. Pariente, C. Briens, F. Berruti & I.M. Scott. Insecticidal activity of bio-oil from the pyrolysis of straw from brassica spp, *Journal of Agricultural and Food Chemistry*, **2014**, *62*, 3610-3618. (Referenced in chapter 5).

GC-MS method was developed by the author. Analysis and identification of compounds of the active fractions of bio-oil and biological assays with the Colorado potato beetle were performed by the author. The manuscript was revised by the author in collaboration with I. Scott and K. Yeung.

3. **L.A. Cáceres**, T.W. McDowell, I.M. Scott, A. Hannoufa, B.D. McGarvey, L. Tian, K.K.-C. Yeung & M.W. Sumarah. In vivo extraction of volatile organic compounds (VOCs) from Micro-Tom tomato flowers with multiple solid phase microextraction (SPME) fibers, *Canadian Journal of Chemistry*, **2014**, 1-8. (Chapter 3).

All experiments were designed and performed by the author. Preparation of the first draft and subsequent revisions were conducted by the author.

4. S.E. Khabbaz., L. Zhang, **L.A. Cáceres**, M. Sumarah, A. Wang & P.A. Abbasi. Biological control of Phytophthora and Rhizoctonia root rot and damping-off diseases of cucumber and radish with novel strains of Pseudomonas and Bacillus bacteria and possible mode of actions. *Annals of Applied Biology*, **2014**. (Accepted November 2014, in press).

Experiments and analysis with Solid Phase Microextraction (SPME) and GC-MS were developed and performed by the author. Bacteria cultures were provided by S. Khabbaz and P. Abbasi. Volatile study was written and revised by the author and final submission and reviewed manuscript was performed by S. Khabbaz.

5. Z. Wang, Z. Zhao, M.M. Abou-Zaid, J.T. Anarson, R. Liu, B. Walshe-Roussel, W. Andrew, L. Suqi, A. Saleem, **L.A. Cáceres**, Q. Wei & I.M. Scott. Inhibition of insect glutathione s-transferase (GTS) by conifer extracts. *Archives of Insect Biochemistry and Physiology*, **2014**, 4, 234-249.

Plant material extraction and separation were performed by Z. Wang with the orientation of the author. Draft was written by Z. Wang, and substantial revisions were performed by the author.

6. **L.A. Cáceres**, B.D. McGarvey, C. Briens, F. Berruti, K. K.-C. Yeung & I.M. Scott. Insecticidal properties of pyrolysis bio-oil from greenhouse tomato residue biomass. *Journal of Analytical and Applied pyrolysis* (Accepted **2015**) JAAP-D-14-00350.

Experiments including bio-oil production, extraction and analysis were performed by the author. The first draft was written by the author, and revisions were performed in collaboration with K. Yeung and I. Scott.

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Dedication

To **God** for giving me this opportunity

Preface

This thesis presents analytical strategies for the fractionation and characterization of different sources of highly complex samples. The first strategy deals with the method development that combines the extraction and identification of secondary metabolites released by plants in their headspace. The second project copes with the analytical development for the fractionation and isolation of compounds present in pyrolyzed bio-oil from plants. In addition to compound identification, both projects are complemented with the development of bioassays to evaluate what compounds are active against common insect pests, revealing the importance of using organic (naturally-derived) compounds as pesticides with less toxic and safer use for the environment.

The opening chapter provides background information regarding volatile secondary metabolites released by plants, while the following three chapters discuss the analytical strategies to extract and identify, in a non-targeted approach, compounds found in the headspace of two types of plants. Chapter five reports the work performed during the first two years of my studies and it explains the analytical strategy developed for the fractionation, separation and identification of compounds base on its pesticidal activity.

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List of abbreviations

AAFC	Agriculture and Agri-food Canada
<i>AtCCD1</i>	Carotenoid cleavage deoxygenase 1 <i>Arabidopsis</i> gene
<i>AtCCD4</i>	Carotenoid cleavage deoxygenase 4 <i>Arabidopsis</i> gene
<i>AtCCD8</i>	Carotenoid cleavage deoxygenase 8 <i>Arabidopsis</i> gene
AMDIS	Automated Mass Spectral Deconvolution and Identification System
CAR	Carboxen
CAW	Carbowax
CCDs	Carotenoid cleavage deoxygenases
CFB	Crucifer flea beetle
CI	Chemical ionization
CPB	Colorado potato beetle
DCM	Dichloromethane
DHS	Dynamic headspace
DMAPP	Dimethylallyl diphosphate
DVB	divinylbenzene
EI	Electron impact ionization
EMV	Electron multiplier voltage
FID	Flame ionization detectors
GC-MS	Gas chromatography mass spectrometry
GHW	Greenhouse whitefly
HPLC	High liquid performance chromatography
HS	Headspace
IE	Ionization Energy
HS-GC	Headspace gas chromatography
IPP	Isopentyl diphosphate
IS	Integrated signal matrix
<i>LeCCD1-1</i>	Carotenoid cleavage deoxygenase 1 Tomato gene 1

<i>LeCCD1-2</i>	Carotenoid cleavage deoxygenase 1 Tomato gene 2
LOX	lipoxygenase
MEP	Methylerythritol phosphate
MVA	Mevalonic acid pathway
NIST	National Institute of Standards and Technology
PCA	Principal component analysis
PCR	Protease chain reaction
PDMS	Polydimethylsiloxane
RH	Relative humidity
RPA%	Relative peak area percentage
RTq-PCR	Quantitative reverse transcription PCR
SAS	Statistical analysis system
SHS	Static headspace
SPME	Solid phase microextraction
SWF	Silverleaf whitefly
TIC	Total Ion Chromatogram
TSSM	Two-spotted spider mite
VOCs	Volatiles organic compounds
WT	Wild type

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Chapter 1. Introduction to the Non-targeted Headspace (HS) Analysis of Volatile Organic Compounds (VOCs) from Plants

1.1 Volatile Organic Compounds (VOCs)

Plants have the ability to store and release a wide range of volatile compounds through their specialized organs and tissues and these have a major impact on the earth's atmospheric composition [1]. Volatile compounds released by plants are generally a complex mixture of low-molecular weight organic compounds with high lipophilic properties. These molecules have relatively high vapour pressures and low boiling points at ambient temperatures and are generally referred to as volatile organic compounds (VOCs) [2]. The term "volatile" is usually not well defined, and the vapour pressures of compounds considered to be volatile can vary over several orders of magnitude [3]. The study of the different volatile blends of compounds released by plants has been defined as "volatilome" [4]. The study of plant volatilome is termed "volatilomics" and includes the qualitative and quantitative analysis of VOCs, headspace analysis of volatiles emitted by whole plant systems, organs, or enzymes, as well as advanced on-line analysis of methods for simultaneous measurements of VOC emissions.

VOCs of plant origin diffuse through the troposphere, in which they are very reactive, with life-times from minutes to hours [5] and are responsible for the normal function of many important terrestrial life processes like the regulation of the oxidative capacity of the troposphere in terms of the concentration of carbon monoxide, ozone, and the overall aerosol balance. On an organismal level, the primary functions of airborne VOCs are: 1) semiochemicals that evoke behavioral or physiological responses in other organisms, like repelling herbivore attackers or attracting beneficial insects for pollination and seed dispersion purposes [6]; 2) toxic volatiles used by plants as a defense against predators. These toxins usually make the plants smell or taste bad, and herbivores generally do not want to eat them; 3) "eavesdrop" volatiles released from attacked neighbors to activate defenses before being attacked in a process called plant-to-plant signaling [7]; and 4) active volatile components are used in the manufacturing

of chemicals for fragrances, flavors, preservatives, insecticides, and in drug discovery [8, 9].

Analytical chemists provide the methodological tools and information that may lead to the understanding of the properties, functions, ecology and applications of plant VOCs. These methodological tools should highlight: 1) The evaluation of the most suitable extraction method; 2) The development of a methodical and systematic data analysis and processing; and 3) the interpretation of the results that would lead to the understanding of a specific biological problem.

Depending on the biological problem, HS analysis of volatiles can be a challenge. Some examples of biological problems in which this may be applied include: 1) analysis of disrupted plant cells and/or organs [10]; 2) volatile profiles from detached and not disrupted plant organs [11]; 3) studies in complete plants or plant organs at different development stages [12]; 4) experimentation performed in the wild, garden, laboratory or greenhouse conditions [13] ; 5) volatile studies when plants are under any abiotic or biotic stress [14]; 6) extraction of VOCs at different periods of time or specific times of the day or night [15, 16]; 7) in the establishment of volatile profile differences between plants of the same family but different species or between wild type plants and genetically modified [17]; and 8) gene and pathway correlation to the emission of VOCs [18].

Beside the biological problem to study, there are several challenges in the analysis of VOCs. First, they are highly complex in composition and have a large dynamic range resulting in the masking of compounds in low concentrations by the most abundant ones. Secondly, there is a need for a sensitive method that may provide unbiased information in volatile changes due to biotic and abiotic stress like herbivores and pathogen attacks and environment variations like light intensity, pressure and humidity. Thirdly, in plant biotechnology, where plants are genetically modified, there is the need for a strategy that allows the identification of volatile secondary metabolites or products where the transformation is important in the understanding of gene

function(s) and their impact in the biosynthetic pathway mechanisms. And finally, there is a need to develop improved data analysis. Existing approaches to analyze volatilomic data still do not allow a fast and unbiased comparative analysis of the volatilomic composition of a wide range of genotypes that are often the target of investigations; this is especially true for the non-targeted approaches. Additional to VOC recognition and identification from the thousands of molecular fragments that constitute a typical GC-MS profile, compounds need to be systematically ordered to be able to make comparisons between the volatilomic profiles of the genotypes.

In the following two sections I will describe the principal methods that have been reported in the literature for the HS extraction and analysis of plant VOCs in different areas of research. The advantages and disadvantages associated with each method will be pointed out to facilitate the understanding of the most appropriate method and equipment required for a particular research problem.

1.2 VOC sampling and extraction

Since the objective of the study is the identification *in vivo* of the authentic profile of volatile blends emitted by the plant, selection of the most suitable strategy of plant volatile sampling and extraction become the first and most important step. In general, a complete plant or sample plant needs to be enclosed in a system with or without air circulation. These are termed as “dynamic” or “static” headspace (HS) collection, respectively [19] .

In a dynamic headspace (DHS) method, or purge and trap, ultra-purified inert gas (carrier gas) is pushed into a system containing the plant material and pulled out into a sorbent tube containing an adsorbent matrix [20]. Ideally, the material(s) of the system containing the plant, should not bleed, react, absorb or affect in any way VOCs. Commonly used materials that do not show bleeding include glass vials and containers

which can be connected with metal and/or Teflon devices and tubing [21], although even these materials may not be completely inert. The narrow glass or metal sorbent tubes which can be made in the lab or distributed by the industry are usually packed with the adsorbent suitable for the compounds of interest. Examples of sorbent matrices for the collection of a wide range of VOCs are: silica gel, activated carbon, porous polymers like; 2,6-diphenylene-oxide, ethenylbenzene, styrene-divenylbenzene and vinylpyrrolidone among many others. After a certain period of time, which can range between minutes, hours or days, VOCs can be extracted from the sorbent matrix with pure solvents or mixtures of low point organic solvents such as acetone, n-octane, dichloromethane (DCM), n-hexane and bromodacane or by the means of thermal desorption into the GC instrument's injection port.

In a static headspace (SHS) system, air is not circulated and volatiles from the HS plant material are absorbed in or adsorbed on to the sorbent matrices. An example of a static system is the use of solid phase microextraction (SPME) fibers, which is a simple and fast method for compound extraction [22]. SPME fibers are usually coated with one or a combination of up to three inert matrices such as Polydimethylsiloxane (PDMS), divinylbenzene (DVB), Carboxen (CAR), and carbowax (CAW). With the help of a holder containing a needle, fibers are exposed into the plant HS and after equilibrium between the VOCs and the fiber matrix (from minutes to a few hours) the fiber is retracted into the holder's needle and VOCs are then thermally desorbed in the GC injection port for separation and analysis.

Depending on the plant conditions, the collection can be also performed *in vivo*, *in situ* or *in vitro*. *In vitro* HS analysis refers to extractions performed on cells, tissues, organs or parts removed from plants. Samples are often chopped or ground to maximize recovery, but the VOC profile may differ from that of a live plant. On the other hand, *in situ* and *in vivo* experiments, two concepts that some authors have used interchangeably, refer to the HS collection of volatiles from living undamaged plants or organs. The difference can be established whether or not the volatile collection is performed in the natural plant habitat, in the case of *in situ* or in a laboratory environment, greenhouse,

growth room or garden for the case of *in vivo* experiments. Ultimately, the selection of suitable methods depends on whether the collection is being performed in the field or in the laboratory environment, as well as depending upon the biological questions being addressed [21].

1.2.1 Advantages and disadvantages of DHS systems

Currently, dynamic headspace is the most common sampling strategy used for the extraction of the most natural profile of plant VOCs. There are advantages to using DHS systems. Since plants are enclosed in a system where air is in constant circulation, it is easy to mimic natural plant conditions and experiments can be done on a big scale depending on the size of the plant without causing potential stress that could affect natural volatile profile emissions. Because VOCs are usually trapped into sorbent tubes, experiments can be performed unattended for extended periods of times, from minutes to days, allowing more accurate evaluation of VOC changes. After conditioning by means of solvents or by thermal desorption, sorbent tubes can be reused as many times as required. For the case of solvent extraction of VOCs, samples can be kept or re-injected in the GC-MS instrument.

There are also disadvantages to using a DHS system. The incomplete VOCs adsorbing capabilities for trapping materials and their specific affinities, the ones that trap high polar and high molecular weight components, may be inefficient with molecules of opposite characteristics. In thermal desorption methods the GC instrument is required to be coupled or equipped with a thermal desorption unit which is very costly (thousands of dollars). Injections cannot be repeated, which makes mistakes more costly. Matrix bleeding may occur during extraction procedures leading to artifacts which interfere with volatile analysis. And there are costs associated with solvent disposal and waste management.

1.2.2 Advantages and disadvantages of SHS systems

Static headspace analysis with SPME is the main choice for studies associated with volatiles from small plants, plant organs or disrupted plant tissues. Some of the advantages of using SHS systems with SPME include the following: analysis is fast and eliminates the use of solvents for the extraction of volatiles, avoiding contamination. By careful selection of the physical and chemical fiber properties (polarity and thickness), compounds of different polarity and boiling points (volatiles and semi-volatiles) can be extracted. Extraction efficiency is increased in small HS volumes (1 to 50 mL). According to manufacturer recommendations (Supelco, Bellefonte, PA, USA) after thermal conditioning, fibers can be reused up to 100 times.

There are also disadvantages of using SHS systems with SPME. SPME is more suitable for qualitative analyses and surveys of VOCs profiles at a single time point rather than for quantitative measurements of changing volatile emissions.

Because extraction is based on equilibrium properties, SPME cannot provide a realistic VOC profile of longer periods of time (days). They are not suitable for the extraction of volatiles from complete plant systems in which the HS volume is greater than 50 mL. The static system accumulates heat and humidity, factors that can affect the normal physiological process in VOC emissions. If fibres are not pulled out properly from the GC-MS instrument, they can become damaged and the coating phase can contaminate the GC inlet port.

1.3 GC-MS data acquisition and processing

The instrument used by preference in the separation of VOC is gas chromatography (GC) in which extracted samples can either be injected into the GC inlet port as a liquid solution or by thermal desorption of the adsorbent matrix. In samples that had been extracted by solvents a small part of the solution (0.5 up to 5 μ L) is usually injected in

the heated injector in a split or splitless mode. In thermal desorption, volatiles already extracted by SPME fibers or sorbent tubes are heated in the inlet port at temperatures ranging from 200° to 350°C. Volatiles are commonly separated on fused silica capillary columns [21] with different stationary phases and polarities, as in the case of non-polar dimethyl polysiloxanes, and more polar polyethylene glycol polymers.

The GC an instrument usually used to separate volatile components Separation is performed by gradually increasing the temperature of the oven in which a column is held, and by partitioning properties of the volatiles between the inert carrier gas (Helium or Nitrogen) and the stationary phase, individual components are separated and sent into the GC detector. Detectors can be grouped based on their nature and their desirable characteristic in non-selective, selective and specific. Non-selective detectors respond to all compounds present in carrier gas stream except the carrier gas itself, selective detectors respond with a common physical or chemical characteristic, and specific detectors respond to a single precise component only. GC detectors can also be concentration and mass flow dependent. Some of the desirable characteristics of a detector in general are: Reproducible response to changes in eluent composition in carrier gas stream, high sensitivity, low noise signal, large linear dynamic range, if possible non-destructive and capable of avoiding peak broadening resulting in loss of resolution. There are more than 15 different types of detectors for GC instruments but some of the most common detectors used in plant volatile analysis include: Flame ionization detectors (FID) which are very stable, mass sensitive and suitable in the detection of volatiles in a wide dynamic range, it has no response to inorganic and permanent gases such as CO, CO₂, NH₃, CS₂, N₂, etc.; Photoionization detector (PID) which after careful calibration can be sensitive in the detection of terpenes or molecules having reactive double bonds; The most popular detector in VOC analysis is Mass spectrometry (MS). MS relies in the ionization of individual volatiles usually by electron impact (EI) and the resulting positively charged molecules and molecule fragments are selected according to their mass-to-charge (m/z) ratio by entering a quadrupole ion trap or a quadrupole mass filter. EI involves shooting energetic electrons on a gaseous neutral. When a neutral is hit by an energetic electron carrying several tens

of electron volts (eV) of kinetic energy, some of the energy of the electron is transferred to the neutral. If the electron, in terms of energy transfer, collides very effectively with the neutral, the energy transferred can exceed the *ionization energy (IE)* of the neutral. Then from the mass spectrometric point of view the most desirable process can occur: ionization by ejection of one electron generating a *molecular ion*, a positive *radical ion* $M + e^- \rightarrow M^+ + 2e^-$. Total ion chromatogram (TIC) and mass spectra are obtained as outputs, providing information on compound abundance and retention time and typical ion fragmentation pattern of the molecules as an intensity vs m/z plot. For VOC identification, mass spectral databases such as Wiley and the National Institute of Standards and Technology (NIST) provide possible molecules names based on the matching proximity of the fragmentation pattern of the molecule analysed with the fragmentation patten of a molecule from the database. Figure 1.1 shows an example of a TIC and mass spectrum of the volatile compound humulene extracted from the HS of the *Arabidopsis thaliana* flowers using SPME as a source of extraction.

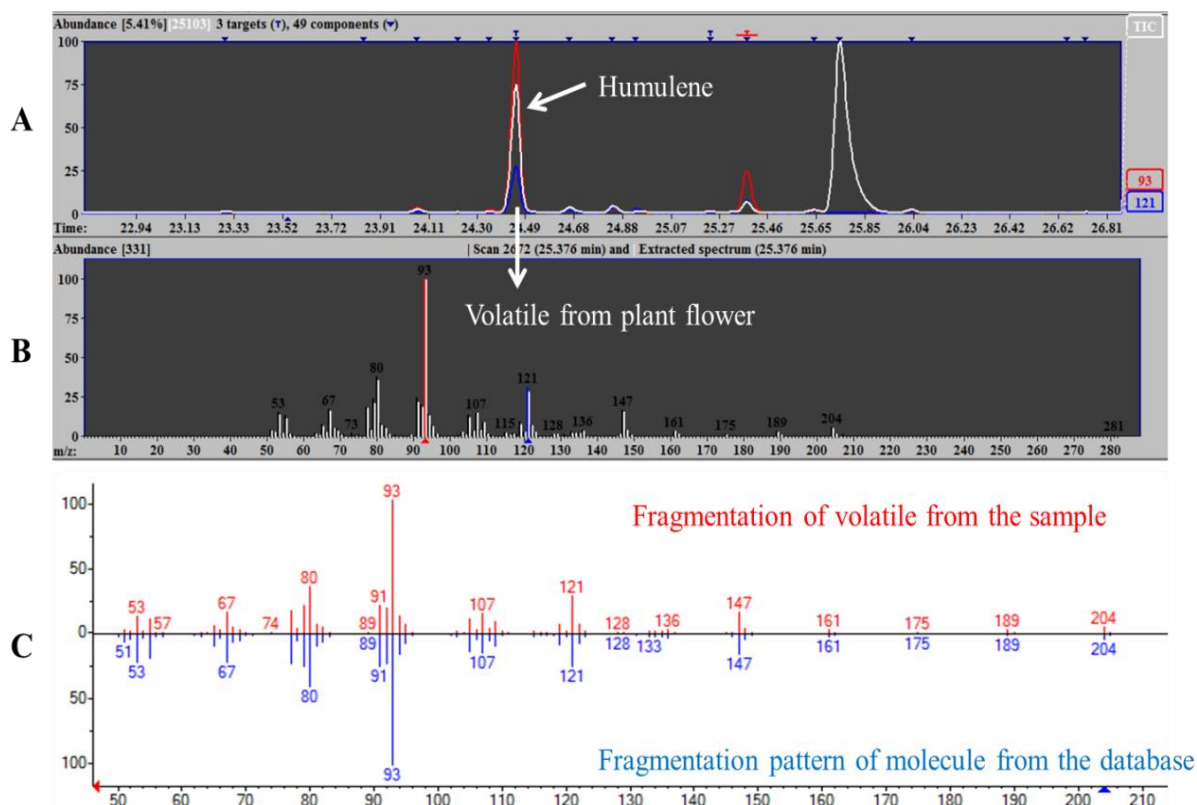


Figure 1.1. (A) Total Ion Chromatogram for *Arabidopsis thaliana* floral headspace; only 4 minutes are shown. (B) Mass spectrum resulting when molecule is fragmented by ionizing energy; the base peak 93 m/z and fragment 121 m/z are shown as coloured lines. (C) The database reveals the mass spectrum of humulene, which is compared by the software with the mass spectrum of the molecule in the sample.

Databases suggest the possible molecules present in a sample, but information needs to be further processed. Different data preprocessing steps should be applied in order to remove possible technical errors and generate the reliable data in the form of normalized peak areas that reflect volatile amounts. These clean data can be used as the input for data analysis. The amount of raw data obtained per sample varies according to the method applied. As an example, GC-MS data recorded could compromise intensity information for 400-800 ions over a separation time of 20-90 min with 20 to 30 scans per second, thus yielding at maximum 1.3×10^8 data points [23]. Standard processing of

these raw data usually includes several steps comprising baseline correction, smoothing and spectrum extraction (deconvolution).

In targeted approaches where molecules are known based on previous experiments or knowledge, the analyses are performed on a rather limited number of metabolites, and samples are often compared using a reference standard which has a predetermined mass spectrum and retention time followed by the export of signal intensities or integrated peak area for further evaluation. In non-targeted approaches, researchers are generally interested in comparing volatiles between plants of different species in the same family, between healthy and diseased plants, or between wild type and genetically modified plants. In all these cases, replication of the samples is important to recognize component signatures present within the data (thus building an unbiased reference per experiment) [24]. Since we are talking about a wide variety of volatiles per sample, in a non-targeted experiment, metabolite levels are not usually quantified as absolute but are relative to each other or to a standard.

Figure 1.2 shows a typical GC-MS-based metabolomics approach for data analysis. First, compounds are extracted from the sample matrix according to the biological problem to solve. Second, and in the case of volatilomic studies, compounds are usually separated using gas chromatography techniques. Third, a fingerprint of the compounds (fragmentation) is obtained by the influence of ionization energy. Fourth, VOCs have to be identified from the thousands of molecular fragments that constitute a typical GC-MS profile (databases and/or analytical standards). Finally, and in order to evaluate differences between samples, comparative analysis using multivariate exploratory techniques dedicated for metabolomics like hierarchical clusters analysis, principal component analysis, organizing maps, etc. are usually used.

Regardless of using gas chromatography for compound separation, volatile metabolites still co-elute prior to being subjected to MS. As a result, overlapping of unique fragmentation patterns can arise which is a main drawback in volatilomic analysis. In addition to the co-elution problem, a high variability in metabolite quantity

within large numbers of biological samples further complicates metabolite identification, and thus limits the entire analysis to a metabolite subset that includes only those compounds that can be reliably identified through all the samples [25].

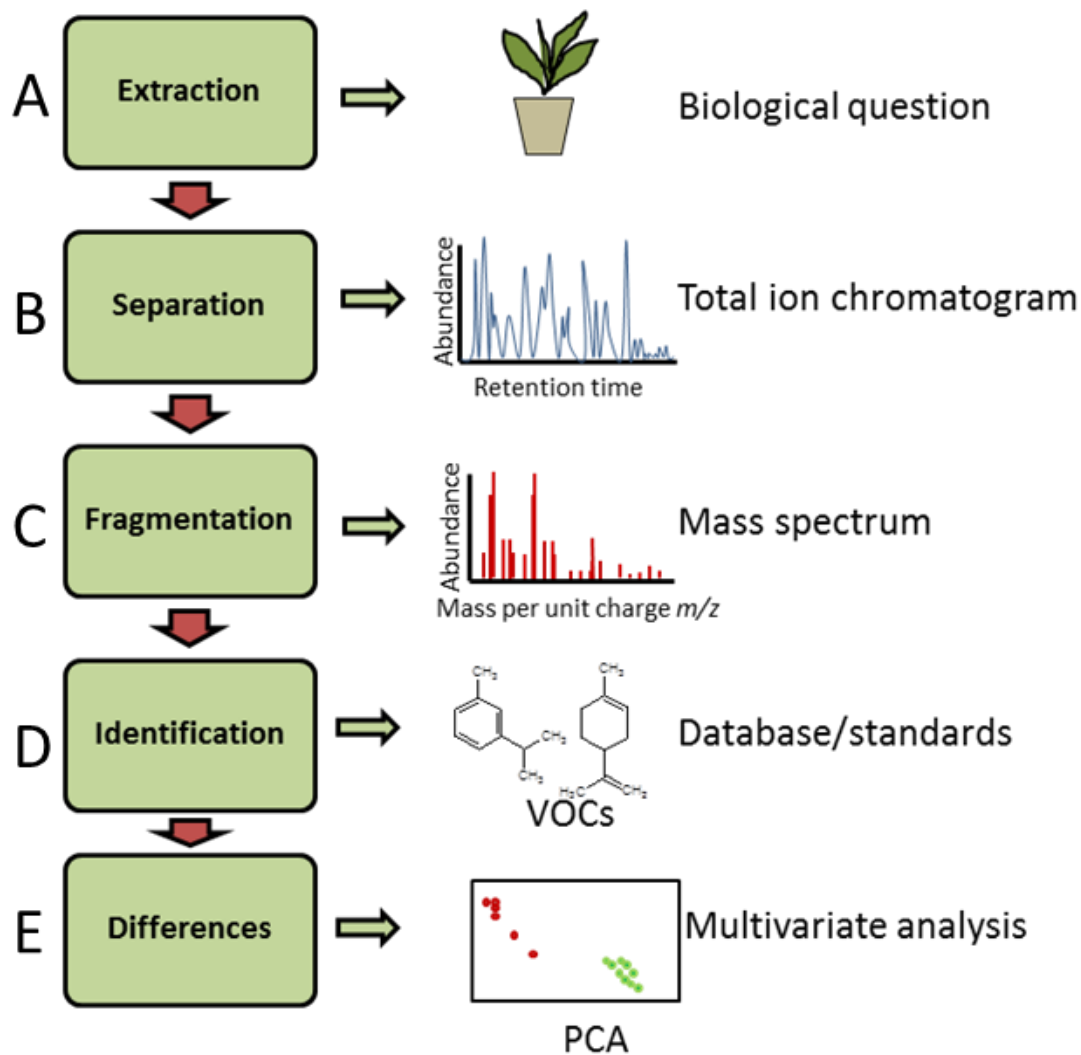


Figure 1.2. Conventional GC-MS-based metabolomics approach; (A) VOC extraction based on the biological problem; (B) Gas chromatography separation of VOCs; (C) Molecular fragments and mass spectrum interpretation; (D) Suggested VOCs identification base on library match and analytical standards; (E) Group clustering base on plant genotype.

1.4 Thesis overview

Researchers at Agriculture and Agri-Food Canada have been working on the generation of transgenic plants such as *Arabidopsis thaliana* and Micro-Tom tomato to generate improved and more resistant crops. My task as an Analytical Chemist is the application of a sensitive HS-GC-MS method in the qualitative, quantitative and semi-quantitative analysis of VOCs from the HS of these plants.

Therefore, this thesis focuses on the volatile profile characterization of wild type and genetically transformed plants overexpressing specific genes. The HS-GC-MS method developed is first applied in the volatile study of the model plant *Arabidopsis thaliana* and second in Micro-Tom tomato plants overexpressing the same class of genes. This research will also investigate the effects of VOCs in plant-insect interactions. The research objectives are as follows:

1. *In vivo* VOCs analysis of transgenic *Arabidopsis thaliana* and Micro-Tom Tomato plants using a non-targeted DHS-GC-MS approach. This objective is explored in chapter two and discusses the difference in volatile emissions between the transgenic and wild type plants.
2. *In vivo* VOCs analysis of transgenic Micro-Tom Tomato plants using a non-targeted SHS-GC-MS approach. This objective is explored in chapter three and discusses the differences in volatile emissions between the flowers of transgenic and wild type plants.
3. Assessment of the biological effects of VOCs produced *in vivo* on herbivores that typically feed on *Arabidopsis* and tomato to determine whether gene overexpression alters plant-insect interactions.

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Chapter 2: Non-targeted Dynamic Headspace (DHS) GC-MS Analysis of Plant Volatile Organic Compounds (VOCs)

2.1 Introduction

Development of strategies that can be used to effectively isolate, extract, detect and semi-quantify volatile organic compounds (VOCs) released by plants in a non-targeted approach is an essential part in studying how plants interact with the environment. Obtaining valid data regarding plant VOCs requires overcoming many challenges including correct experimental design, proper selection of the extraction and separation procedures, and methods to confirm the chemical structures and identities of the VOCs. Furthermore, an unbiased data analysis method is required for correct semi-quantification and the systematic interpretation of results.

In this chapter, an analytical method for the non-targeted *in vivo* analysis of VOCs extracted from whole transgenic and non-transgenic plants is proposed. The non-targeted analytical strategy will lead to understanding not only the differences in volatile profile emissions of the plant types over a fixed period of time, but it will contribute to the understanding of how volatile emissions change due to gene transformation.

Researchers at Agriculture and Agri-food Canada (AAFC) have been working on the generation of transgenic plants. The purpose of the genetic transformation is to overexpress genes that are already in plant's genome. Specific genes, like in the case of the Carotenoid Cleavage Dioxygenase (*CCD*) are enhanced by *Agrobacterium*-mediated transformation [1] (Appendix 1). Once genes are overexpressed Researchers evaluate their function in the formation of secondary metabolites and their relation with the production of better and more resistant crops.

CCD genes are responsible for cleaving a broad range of carotenoids found in plants, such as lycopene, β -carotene, δ -carotene, zeaxanthin, violaxanthin, and neoxanthin, to generate aldehydes and ketones that are volatile aroma compounds [2, 3]. In tomato fruits, for instance, *CCD* generates flavour volatiles such as geranylacetone, pseudoionone, and β -ionone [4]. Some of the volatiles that are produced *in vitro* by the

cleavage action of the *CCD* genes are called “apocarotenoids” and include: β -ionone, α -ionone, 3-hydroxy- β -ionone, pseudoionone, geranylacetone, and 6-methyl-5-hepten-2-one [5, 6]. Figure 2.1 shows the reaction scheme for the production of the apocarotenoid α - and β -ionone by the cleavage action of *CCD1* using α -carotene as a substrate.

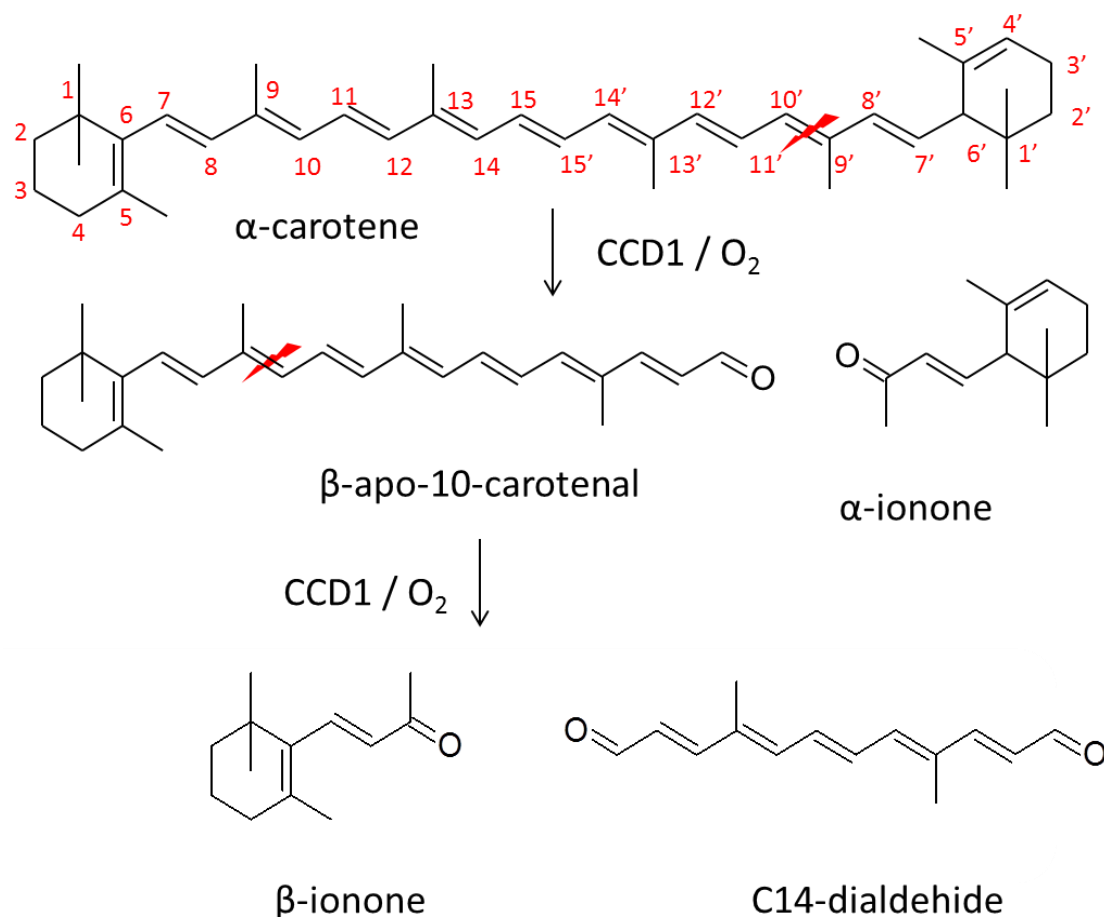


Figure 2.1. Carotenoid cleavage reaction catalysed by the enzyme *CCD1*. Numbering refers to the numbering scheme used in carotenoids to designate main chain carbon atoms and positions of cleavage.

Ultimately, the effect of gene transformation is determined by comparing the VOC expressions between transformed and wild type plants. But there are many important factors to consider in ensuring that experiments are properly and successfully performed. To begin, one has to carefully account for the dynamic nature of VOC

release at various growth stages. The matching of growth can be difficult due to different growth rates among plants, which can also be altered by the gene transformation.

To further complicate the issue, one also has to take into consideration biological variabilities in the plants themselves, as well as the variations arising from the effectiveness of the gene transformation. Finally, another factor that could cause the VOC expression to alter is stress to the plants caused by the VOC sampling procedures.

In an attempt to minimize the biotic and abiotic stress, a “dynamic” headspace (DHS) trapping of plant volatiles is proposed. This is considered dynamic since, instead of allowing the sample to come to equilibrium in a sealed container, the headspace atmosphere was constantly purged out of the sample vessel and through a trap. Analytes were collected, as in a purge and trap analysis, while the carrier gas was vented. The sampling is typically performed for extended periods (hours) to improve the sensitivity of low abundance VOC detection. After the collection step, the sorbent trap was directly back flushed with an organic solvent to transfer the adsorbed compounds to the GC injection port for analysis.

A wide variety of adsorbent traps and various porous polymers, had been reported to collect volatiles components in the plant HS [7, 8]. In this study, a polymer bed made of ethylvinylbenzene-divinylbenzene (Porapak Q) was proposed as it was reported to have minimum retention of water and better overall higher recoveries in the adsorption of a wide range of volatiles and semi volatiles [9].

After extraction and analysis by GC-MS, the raw data needs to be properly managed in order to correctly identify the metabolic differences, and subsequently establish the action of *CCD* gene transformation. In this study a non-targeted strategy for the unbiased analysis of VOCs was developed and tested. This strategy was based on a fully automated alignment of metabolic profiles at the level of individual molecular fragments with assignment to the chemical structures of the metabolites they represent.

Afterwards, a multivariate comparative analysis of individual volatile profiles was performed, which was based on all chemical information derived by the analytical approach. Semi-quantification of volatiles was obtained based on the relative peak area percentage of the components found in the GC chromatogram. In other words, a volatile compound was quantified by determining its peak area as a percent relative to the cumulative area of all peaks.

A DHS-GC-MS collection system with Porapak Q cartridges was developed to extract VOCs from wild type and transgenic *Arabidopsis thaliana* plants overexpressing the *AtCCD1*, *AtCCD4* and *AtCCD8* genes. *Arabidopsis thaliana*, which is a small plant commonly used as a model system for genetic manipulation [10]. It was used in this investigation as a model to test the DHS method for the determination of the authentic profile of plant volatiles especially in the detection of apocarotenoid compounds including β -ionone. The unbiased non-targeted analysis of VOCs proposed in this thesis helped to determine differences in volatile profiles from wild type plants and different lines of transgenic plants overexpressing the *CCD* genes.

Once the extraction strategy and data analysis proposed were evaluated using *Arabidopsis* as a plant model, the next step was the application of the method in an actual agricultural crop. Tomato (*Solanum lycopersicum* cv. Micro-Tom) is a small plant, and like the *Arabidopsis* can be genetically transformed to over express *CCD* genes. Only two *CCD1* genes had been identified in tomato plants the *LeCCD1-1* and *LeCCD1-2* [11]. These two genes are highly similar to each other (83% identity) and to the *Arabidopsis AtCCD1* gene [12]. The method allowed the determination of whether or not the function of the *CCD1* genes in Micro-Tom tomato were similar/different to the *CCD1* in *Arabidopsis*, especially in the production of apocarotenoid compounds or for the creation of a more resistant crop.

2.2 Materials and Methods

2.2.1 Reagents and Standards

Dichloromethane HPLC grade, (Caledon, Georgetown ON, Canada) was used for the extraction of VOCs from sorbent tubes. The following list of analytical standards were provided by Sigma-Aldrich (SAFC, Oakville, ON, Canada); 2- octanone was used as an internal standard in the *Arabidopsis* volatile study; β -ionone, was used to build a calibration curve for quantification purposes in the *Arabidopsis* study; oxoisophorone $\geq 97\%$, isophorone, β -cyclocitral, thespirane mixture $\geq 85\%$, β -damascone, α -ionone and dihydro- β -ionone $\geq 90\%$ were used to evaluate Porapak Q sorbent tubes recoveries. β -ionol, damascenone, (-)-caryophyllene oxide, (R)-(+)-limonene, (-)-trans-caryophyllene, α -pinene oxide, trans-4-carene, β -pinene, α -pinene and (1R)-(+)- α -pinene were used to build a library of known volatile organic compound using AMDIS for the Micro-Tom tomato study.

2.2.2 Plant and Growth Conditions

Wild type *Arabidopsis thaliana* Columbia-0 plants and *Arabidopsis thaliana* Columbia-0 plants over-expressing the *AtCCD1*, *AtCCD4* and *AtCCD8* genes were grown in pots containing Pro-mix-BX soil. Three different lines of each type of gene and two lines of the *CCD8* were studied (Lines are plants that have been transformed with the same procedure but at different time events and are grouped into batches. Lines were labelled as L-1, L-2 and L-3 to indicate the batch difference). The seeds were sown onto pre-moistened soil in 4-inch plastic pots, and the surface of the soil was given adequate moisture. Once the trays containing the pots were covered with a plastic dome to maintain humidity, they were placed at 4 °C in the dark for 3-4 days to stratify the seeds. Subsequently, the trays were transferred to a growth room. The growth room was maintained at a controlled environment of 20 °C \pm 3 °C with a photoperiod of 16 h, light intensity of 95–130 $\mu\text{mol}/\text{m}^2/\text{s}$, and humidity set at 70%. The plastic domes were

removed one week after the seeds germinated. The seedlings were watered three times a week, on every alternate day. Once the plants were two weeks old, they were supplemented with All-purpose Fertilizer 20-20-20 (1 mg/L), which was added to the soil on a bi-weekly basis. Six-week-old plants with flowers were used for the *in vivo* volatile collection.

Wild type Micro-Tom tomato plants and Micro-Tom tomato plants over expressing the *AtCCDI* , *LeCCDI-1* and *LeCCDI-2* genes were grown in pots containing Pro-mix-BX soil. Five different lines of *AtCCDI*, five of *LeCCDI-1* and four of *LeCCDI-2* were studied (Lines represent different generations from the same type of transgenic plant). The seeds were sown onto pre-moistened soil in plastic seed-starting containers (Standard cell inserts, 12 cell, 10.5”W x 20.875”L) with a single seed per container. By direct observation and after approximately 3-weeks, healthy tomato plants were transferred to 4-inch plastic pots (one plant per pot). Plants were transferred to a growth room. The growth room was maintained at a controlled environment of $22\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$ with a photoperiod of 16 h, light intensity of 100–150 $\mu\text{mol}/\text{m}^2/\text{s}$, and humidity set at 75%. Micro-Tom tomato plants were watered two times a week and they were supplemented with All-purpose Fertilizer 20-20-20 (1 mg/L), which was added to the soil on a bi-weekly basis. Six to seven-week-old plants with flowers were used for the *in vivo* volatile collection.

2.2.3 Sorbent Tubes

The Porapak Q sorbent tubes or cartridges used for VOC extraction were purchased from SKC Inc., Eighty four, PA, USA (Figure 2.2). The flame-sealed ends with plastic caps cartridges were made of borosilicate glass tubes (11 cm long, 6-mm OD, 4-mm ID), containing two sections of 50/80 mesh ethylvinylbenzene-divinylbenzene beds (front = 150 mg; back =75 mg) separated by a 7-mm silylated glass wool. A glass wool plug precedes the front section and follows the back section. Porapak Q cartridges were selected based on its adsorption capabilities, the two sections of ethylvinylbenzene-

divinylbenzene beds allows a complete retention during sampling (no breakthrough or back-diffusion) and complete extraction/recovery during analysis for a more efficient trapping of VOCs. The term ‘breakthrough’ refers to an analyte passing completely through the sorbent bed and escaping from the far end of the tube during sampling. Back-diffusion refers to adsorbed analytes going back into the vapour phase and creating a finite concentration of that analyte in the gas-phase near the surface of the sorbent. Porapak Q cartridges were distributed by the manufacturer ready to use and have the advantage over charcoal, which is another matrix commonly used to trap volatile compounds, because of the minimum water retention which is a large component of the headspace of plant tissue.

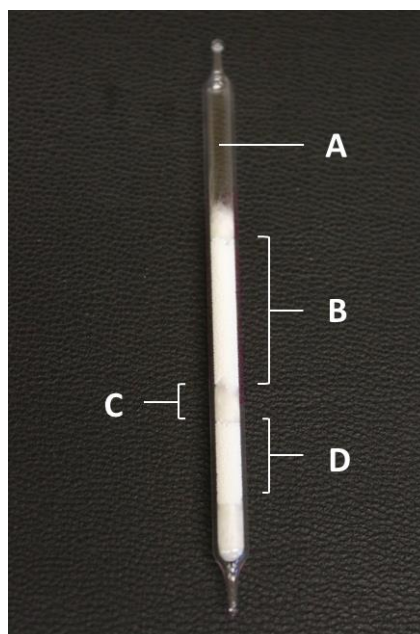


Figure 2.2. Porapak Q sorbent tube for the collection and extraction of VOCs. (A) Borosilicate trap (110 x 4 mm i.d.). (B and D) Two sections of ethylvinylbenzene-divinylbenzene beds (150/75 mg). (C) Borosilicate glass wool. Ends of the tube are cut off before use.

2.2.4 DHS Volatile Collection System

Wild type (WT) and transgenic plants were enclosed in cylindrical 46 cm × 26 cm inert quartz glass chambers with a circular glass base (custom-made). Experiments were done

using six chambers simultaneously (Figure 2.3). Appendix 2 shows the chamber used initially to develop the DHS system. Chambers containing the plants were located in a growth room with the following conditions: 22 ± 3 °C of temperature, 16 h daylight - 8 h darkness, and relative humidity of 55%. During volatile collection, compressed air from a pressurised air tank (air liquide UN 1002 Canada) was passed through an inert Tygon vacuum tubing, 3/8"ID x 7/8"OD (Cole-Parmer, Montreal, QC) at a flow rate of 100 mL/min through a manifold to drive the air into the six chambers. A Porapak Q 75/150 ethylvinylbenzene-divinylbenzene column was connected at the outlet of the chamber to collect plant volatiles.

The volatile profiles of the transgenic plants were compared to that of WT plants by collecting the volatiles from the HS over a 24 h period. In the case of the *Arabidopsis* volatile extraction, every chamber contained one pot with seven to eleven *Arabidopsis* plants. In the case of the tomato plants every pot held one plant. After a 24 h period, the samples were eluted from the columns using 3 mL HPLC grade DCM (dichloromethane). The eluent was then concentrated to exactly 0.25 mL by passing the samples under a stream of nitrogen gas. 2-octanone was added to the samples as the internal standard for the case of the *Arabidopsis* samples with a final concentration of 20 µg/mL. The samples were loaded into the GC-MS instrument from Agilent Technologies.

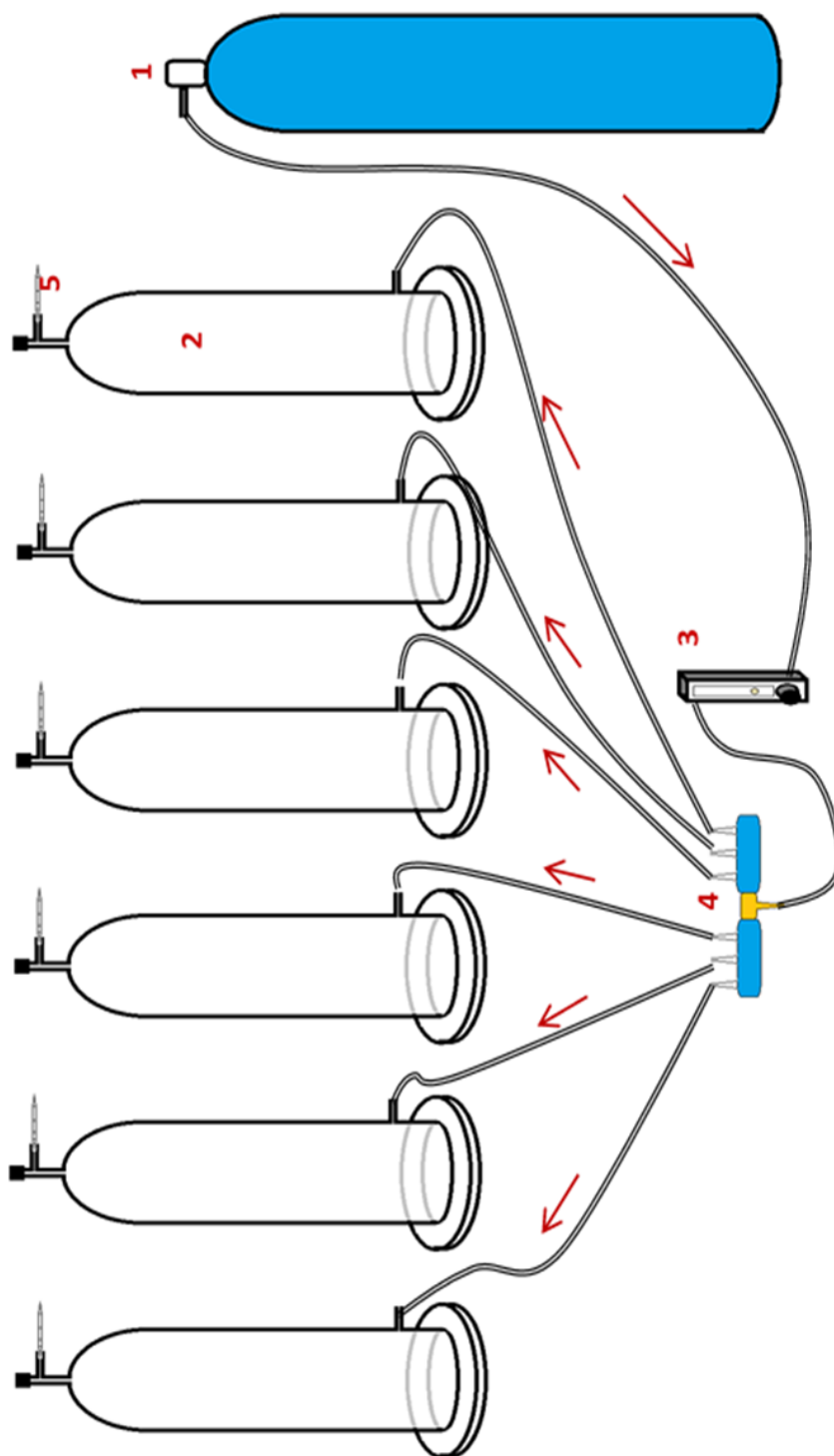


Figure 2.3. DHS collection of volatiles using six glass chambers for the simultaneous collection of VOCs into sorbent tubes. (1) Compressed medical air. (2) Inert glass chambers with double opening. (3) Flowmeter. (4) Metallic manifold to draw air in the chambers at the same flow rate. (5) Porapak Q sorbent tubes to trap VOCs.

2.2.5 GC-MS Parameters

2.2.5.1 *Arabidopsis Thaliana* GC Method

The GC-MS instrument from Agilent Technologies (Santa Clara, CA USA) used for sample injection, separation and detection of VOCs was equipped with an autosampler (7693), gas chromatograph system (7890 A) and inert XL EI/CI MSD with triple-axis detector (5975C) See Appendix 3 for a photo of these instruments.

Two microliters of plant volatile extracts were loaded onto the GC inlet port using the autosampler and components were separated in a 30 m × 0.25 mm i.d. 0.25 μm HP-5MS; 5% (w/v) phenyl dimethylpolysiloxane fused silica capillary column in the pulsed splitless mode (25 psi until 0.5 min; the purge flow to the split vent was adjusted at 40 mL/min for 1 min). The carrier gas used was Helium (99.999%) maintained at a flow rate of 1 mL/min with a column head pressure of 12.445 psi. The oven temperature was maintained at 30 °C for 1 min, then increased at 5 °C/min to 200 °C, and then held for 1 min at this temperature. The total run time for each sample was 36 min. A solvent delay of 10 min was used in every run. The voltage used in the EMV mode was relative and the resulting EMV was 1376. A full scan spectrum was generated between 100 and 350 *m/z* at a rate of 4.51 scans per second.

2.2.5.2 Micro-Tom Tomato GC Method

Two microliters of Micro-Tom tomato volatile extracts were injected into the GC system and components were separated in a 30+10 m Dura-guard × 0.25 mm i.d. 0.25 μm DB-5MS+DG capillary column in the pulsed splitless mode (25 psi until 0.5 min; the purge flow to the split vent was adjusted at 40 mL/min for 1 min). The carrier gas used was Helium (12.445 psi). The resulting EMV was 1424. The oven temperature was maintained at 30 °C for 1 min, then increased at 5 °C/min to 200 °C, and then held for 1 min at this temperature. The total run time for each sample was 36 min. A solvent delay

of 5 min was used in every run. Full scan spectra were generated between 100 and 350 m/z at a rate of 4.51 scans/sec.

Before samples were analyzed, quality control procedures were conducted on the GC-MS to obtain optimal instrumental conditions for high separation efficiencies and sensitive detector responses. See Appendix 4 for details.

2.2.6 Analysis of Volatile Organic Compounds

Volatile organic compounds (VOCs) were identified using Automated Mass Spectral Deconvolution and Identification System (AMDIS) GC-MS analysis (Version 2.71, June 12, 2012), with the Mass Spectral Search Program National Institute of Standards and Technology (NIST) (Version 2.0 g, December 4, 2012). The GC-MS spectra of 40 samples in the case of the *Arabidopsis* and 48 in the case of Micro-Tom tomato were deconvoluted using the freely available AMDIS software. A library of known compounds was compiled within AMDIS using the NIST database as a reference for the *Arabidopsis* plants study (Appendix 5). For the Micro-Tom tomato study, a library of known standard compounds and NIST database reference was built within AMDIS (Appendix 6). The *.ELU files resulting from AMDIS processing were then uploaded to the SpectConnect web tool (freely available at <http://www.spectconnect.mit.edu>) along with the *.MSL library file. SpectConnect generated a matrix of integrated signal (IS) data. The aligned retention times and peak integration from SpectConnect were manually checked in order to eliminate background peaks and silica containing contaminants. Details on the identification of system background signals can be found in Appendix 7.

Principal component analysis (PCA) was used to compare and evaluate and differentiate VOCs from wild type and the corresponding transgenic lines. PCA was performed using Minitab release 14.13 statistical software (1972-2004 Minitab Inc. State College, PA).

Semiquantitative data of 4 replicates in the *Arabidopsis* volatile study is presented as a percentage area, relative to the area of the internal standard 2- octanone. Peak areas from the TIC were first normalized by dividing the area count by the number of plants in each pot, and then the peak area of each component was normalized to the peak area of the internal standard by using the equation:

$$RPA_x = ac_x / ac_{is} \text{ (Equation 2.1)}$$

where RPA_x = relative peak area of component, ac_x = area count of the component and ac_{is} = area count of the internal standard. The relative peak area percentage of each component was then calculated by using the formula:

$$RPA \% = (RPA_x / \Sigma RPA) \times 100 \text{ (Equation 2.2)}$$

where $RPA\%$ = relative peak area percentage of component and ΣRPA = total relative peak area.

Semiquantitative data of 3 replicates in the Micro-tom tomato volatile is presented as a percentage area, relative to the area of the R- α -pinene (Equation 2.2).

To quantify the amount of β -ionone emitted, a calibration curve (TIC peak area vs concentration) with the following concentrations was made; 0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ng/ μ L ($R^2=0.9852$). One-way ANOVA (Proc. GLM, Statistical Analysis System, Server Interface Ver. 2.03, SAS Institute, Cary, NC) was used to test for significant differences in the concentration obtained.

In addition to identifying the volatile compounds using the automated search, the profiles were compared with data of apocarotenoid standards measured by our instruments. Data of the following standards were obtained: β -ionone, oxoisopherone, β -ionol, α -ionone, β -damescenone, theaspirane, isophorone, caryophyllene, limonene, dihydro- β -ionone, and β -cyclocitral.

2.3 Results and Discussion

2.3.1 Construction of the Dynamic Headspace (DHS) Sampling System

This chapter focuses on the VOC sampling of whole plants including soil and pot. The selection of containers with proper dimensions for the extraction process was the first important step. Custom-made quartz glass chambers were chosen to minimize stress imposed on plants as the quartz glass material was chemically inactive, and allowed light to enter without altering the wavelengths associated with plant normal development ($100 \mu\text{mol}/\text{m}^2/\text{s}$). The dimensions of the chambers (46 cm x 26 cm) with a capacity of 24.4 L were designed to accommodate the height reached by six-week-old *Arabidopsis* plants (45 cm including the pot). Medical air coming from a compressed tank was pushed into each one of the chambers containing the plants, at a controlled flow rate of 100 mL/min. When flow rates were above 110 mL/min flower petals appeared to close, likely due to the pressure built up within the chamber.

2.3.2 Ethylvinylbenzene-divinylbenzene Beds Adsorption Capabilities

The second important part of the dynamic headspace (DHS) extraction was the sorbent materials in which VOCs were retained. Before performing actual experiments with the plants, the adsorption capabilities of the ethylvinylbenzene-divinylbenzene beds cartridges (Porapak Q) were evaluate by using volatile standards.

Due to the variation in adsorption capacities, various solid matrices for the capturing and enrichment of VOCs have been reported [8]. The adsorption mechanism is defined as the attachment of a VOC at the surface of a solid. This process generally takes place at the external and internal surfaces of the adsorbents. Most adsorbents have porous structures and, therefore, a high surface area that is often $>100 \text{ m}^2 \text{ g}^{-1}$ [7]. In general, the larger the surface area of the polymer, the better the adsorption capabilities. For this study, Porapak Q cartridges, made of the porous polymer ethylvinylbenzene

divinylbenzene with a surface area of $600 \text{ m}^2 \text{ g}^{-1}$ and pore diameter of 50 nm were used. These cartridges had the advantage of being stable at higher temperatures ($180 \text{ }^\circ\text{C}$), and being able to extract volatile and semi volatile compounds. The cartridges were ready to use without the need of any solvent conditioning. The extraction capability of the cartridges was evaluated using eight apocarotenoid standards (Table 2.1).

Apocarotenoid recoveries of the Porapak Q cartridges were performed in two ways: the direct injection of $10 \text{ }\mu\text{l}$ of $20 \text{ ng}/\mu\text{l}$ solution of the eight apocarotenoids in acetone into the cartridge; and 24 h of DHS sampling of $10 \text{ }\mu\text{l}$ of the same solution placed at the bottom of the glass chamber. In both cases, the analytes were extracted and quantified by GC-MS. The peak areas were compared to that obtained from the direct injection standard of solutions in the GC-MS. The results are shown as % recoveries in the Table 2.1. On average more than 96 and 88% recoveries were achieved when extracting the apocarotenoid compounds from the Porapak Q cartridges from the direct injection into the cartridge and from the DHS collection of the same solution respectively. For the specific case of β -ionone, a molecule of particular interest in later sections, more than 90% recovery was achieved.

Table 2.1. Extraction recoveries of selected apocarotenoid compounds through Porapak Q sorbet tubes. *Two* methods were used: the injection of 10 μl of the 20 ng/ μl apocarotenoid mixture solution; and 24 hours of DHS collection of the 10 μl of the 20 ng/ μl solution with the volatile glass chamber. Data represent the mean percentage and standard error of 5 replicates.

Compound	RT (min.)	Direct Injection % recovery (\pm S.E.)	24 h of DHS % recovery (\pm S.E.)
Isophorone	16.27	96.6 (1.9)	86.6 (5.7)
Oxoisophorone	17.02	99.0 (1.0)	91.8 (2.9)
β -Cyclocitral	19.35	97.0 (2.0)	84.6 (5.8)
Theaspirane A	21.8	94.8 (1.8)	88.6 (4.7)
Theaspirane B	22.27	94.6 (1.5)	89.8 (3.0)
β -Damascone	24.94	95.6 (1.5)	86.6 (4.9)
α -Ionone	25.32	96.2 (1.7)	89.2 (2.7)
Dihydro- β -ionone	25.63	97.4 (1.0)	87.4 (3.6)
β -Ionone	26.87	97.2 (1.4)	90.8 (2.0)

The overall high recoveries (>94%) achieved by the direct injection standards into the cartridge demonstrates that ethylvinylbenzene divinylbenzene had high adsorption capabilities for these apocarotenoids, and there was a roughly 9% reduction in the recoveries resulting from the DHS sampling. The non-quantitative recovery can be attributed to analyte loss due to the non-specific adsorption on surfaces of the chamber and tubing, and/or the unwanted desorption of analytes from the sorbent during the sampling period. Nevertheless, quantitative recovery is not essential to the work presented in this thesis. The recurring focus is to profile the relative differences in VOC between a test subject and a control. In other words, absolute quantitation is unnecessary, and thus non-quantitative recovery is acceptable, providing that the recovery level is kept constant within the data set used for a comparison.

2.3.3 *Arabidopsis thaliana* VOC Extraction

Once acceptable recoveries had been established for the DHS sampling setup, the experiments proceeded to attempt VOC sampling from *Arabidopsis* plants. Studies began with the *Arabidopsis thaliana* (*At*) ecotype-0 plants, and Figure 2.4 shows a photo of the *Arabidopsis* (*At*) plants at the flowering stage enclosed by the glass chambers ready for volatile extraction.



Figure 2.4. Two glass chambers for the dynamic headspace (DHS) collection of volatiles. Every chamber contains a pot with a set of seven to eleven *Arabidopsis thaliana* ecotype-0 plants at flowering stage.

The *in vivo* study of volatiles was performed with wild type (WT) plants and plants overexpressing three *CCD* genes (*AtCCD1*, *AtCCD4* and *AtCCD8*). Different

lines of plants (up to three), from the third generation with overexpressed *CCD* genes, were provided by the molecular biology lab at Agriculture and Agri-Food Canada.

2.3.4 Non-targeted Analysis of *Arabidopsis* VOCs

Based on the appearance of the chromatograms six-week flowering *Arabidopsis thaliana* plants from both transgenic and wild type plants released a similar spectrum of volatiles into the chamber's HS. Figure 2.5 shows the representative gas chromatography (GC) total ion chromatograms (TICs) of a WT and three *AtCCD1 Arabidopsis* plants. The MS data revealed the presence of secondary metabolites including mainly unsaturated hydrocarbons, aromatic derivatives, alcohols, apocarotenoids, mono and sesquiterpenes. Table 2.2 shows the average peak area percent, relative to the internal standard 2-octanone of the 22 VOCs identified by the database of the WT type and the different lines of the corresponding *Arabidopsis* plants overexpressing the *CCD* genes.

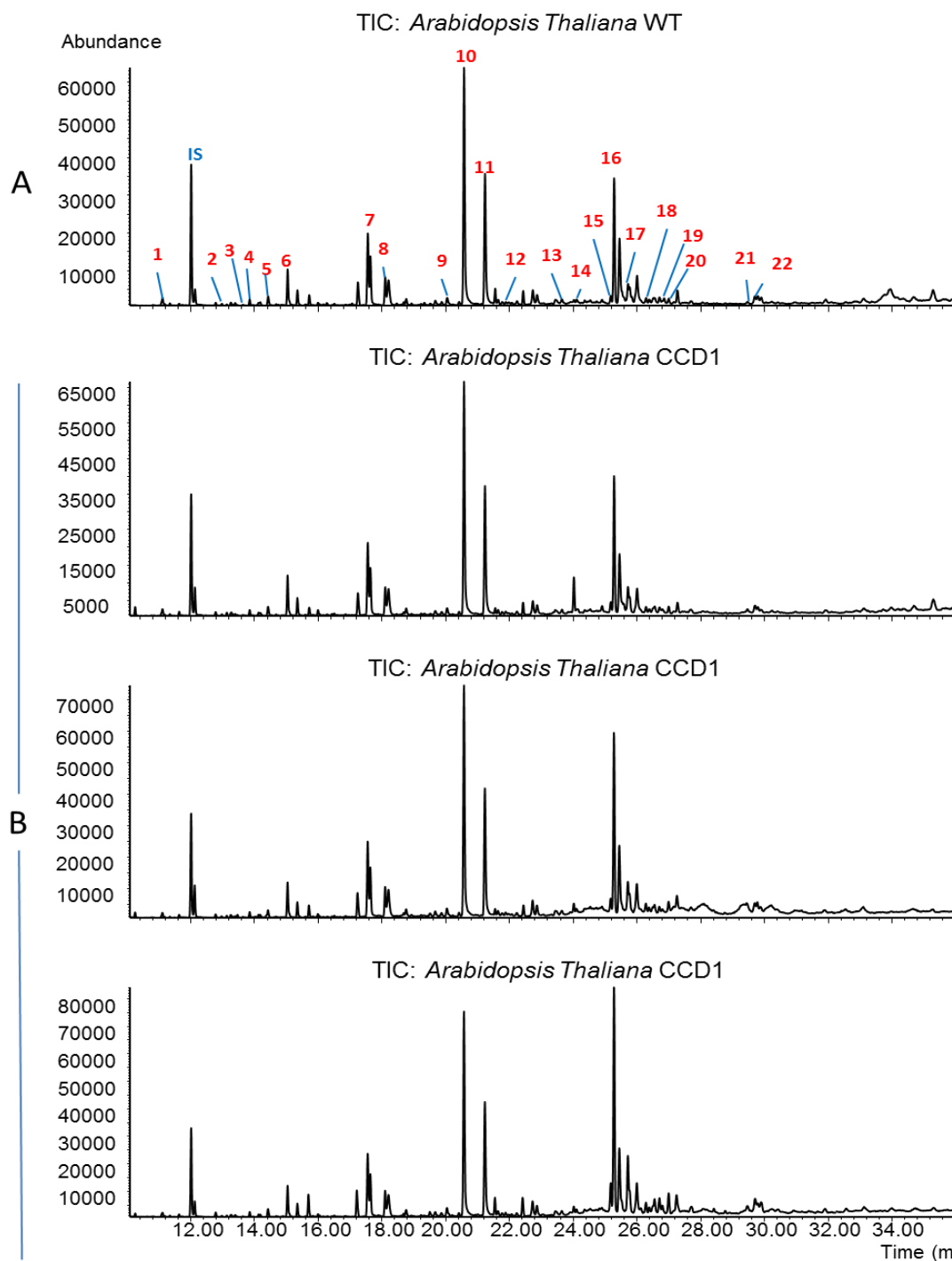


Figure 2.5. Volatile profiles of flowering stage WT and *AtCCD1 Arabidopsis thaliana* (ecotype Col-0) detected by DHS-GC-MS. The figures show the representative TIC obtained from a (A) WT and (B) three *AtCCD1* Lines. Peak numbering refers to that used in Table 2.2.

Table 2.2. Mean relative peak areas and (\pm S.E.). % from four measurements of the volatile compounds collected for WT and *AtCCD* lines of *Arabidopsis thaliana* plants.

No.	RT (min)	Compound	Chemical group	Mean peak area percent relative to internal standard 2-octanone (\pm SE) n=4											
				WT ¹			<i>AtCCDI</i>			<i>AtCCDI</i>			<i>AtCCD8</i>		
				L-1	L-2	L-3	L-1	L-2	L-3	L-1	L-2	L-3	L-1	L-2	L-3
1	11.126	Benzaldehyde	Aromatic	0.65(0.18)	0.58(0.04)	0.76(0.06)	0.55(0.09)	0.60(0.04)	0.66(0.32)	0.51(0.25)	0.45(0.18)		0.51(0.25)	0.45(0.18)	
2	12.992	2,4-Nonadiene	Unsaturated H	0.12(0.07)	0.11(0.06)	tr	tr	tr	tr	0.05(0.01)	0.05(0.01)		0.05(0.01)	0.05(0.01)	
3	13.651	3-undecyne	Unsaturated H	tr	tr	0.06(0.04)	tr	tr	tr	tr	tr		tr	tr	
4	13.859	Benzene,1,4-diethyl	Aromatic	0.41(0.19)	0.41(0.11)	0.36(0.03)	0.3(0.01)	0.32(0.01)	0.26(0.02)	0.38(0.02)	0.31(0.02)		0.38(0.02)	0.31(0.02)	
5	14.442	Acetophenone	Aromatic	0.79(0.22)	0.66(0.15)	0.61(0.06)	0.55(0.04)	0.61(0.04)	0.51(0.04)	0.62(0.05)	0.65(0.04)		0.62(0.05)	0.65(0.04)	
6	15.054	Benzene,1-methyl-3-ethyl	Aromatic	1.88(0.45)	1.89(0.25)	1.92(0.39)	1.86(0.33)	1.80(0.01)	1.76(0.02)	1.77(0.02)	1.69(0.02)		1.77(0.02)	1.69(0.02)	
7	17.573	Benzaldehyde,4-ethyl	Aromatic	5.40(1.24)	5.39(2.06)	3.41(0.42)	4.36(1.03)	4.8(1.06)	4.38(0.16)	4.27(0.89)	4.19(0.43)		4.27(0.89)	4.19(0.43)	
8	18.116	Isoxyalkdehyde	Aromatic	2.07(0.57)	2.10(0.48)	2.56(0.57)	2.50(0.59)	2.08(0.57)	2.1(0.75)	2.01(0.58)	2.03(0.58)		2.01(0.58)	2.03(0.58)	
9	20.063	3-cyclohexene-1-o,5-methylene	Alcohol	0.87(0.12)	0.72(0.18)	0.75(0.26)	0.69(0.02)	0.63(0.38)	0.59(0.48)	0.63(0.01)	0.53(0.02)		0.63(0.01)	0.53(0.02)	
10	20.594	Acetophenone,2,4-dimethyl	Aromatic	13.8(2.36)	12.3(1.83)	11.3(1.02)	10.4(1.08)	13.6(1.02)	14.4(1.05)	15.2(1.03)	15.8(1.03)		15.2(1.03)	15.8(1.03)	
11	21.247	Acetophenone,4-ethyl	Aromatic	9.02(1.05)	9.33(1.24)	6.4(1.09)	8.66(2.37)	10.3(1.15)	10.4(0.89)	10.3(1.28)	10.49(1.05)		10.3(1.28)	10.49(1.05)	
12	21.905	3-buten-2-one,4-phenyl	Aromatic	0.33(0.08)	0.27(0.01)	0.29(0.01)	0.24(0.03)	0.18(0.01)	0.14(0.02)	0.25(0.02)	0.28(0.02)		0.25(0.02)	0.28(0.02)	
13	23.667	Ethanone,1-(2,3-dihydro-1H-inden-5-yl)	Aromatic	0.58(0.16)	0.59(0.13)	0.61(0.09)	0.56(0.05)	0.50(0.03)	0.46(0.03)	0.47(0.04)	0.49(0.03)		0.47(0.04)	0.49(0.03)	
14	24.037	alpha-cubebene	Sesquiterpene	0.70(0.24)	0.78(0.22)	0.83(0.02)	1.05(0.05)	0.83(0.02)	1.03(0.02)	0.83(0.03)	0.92(0.03)		0.83(0.03)	0.92(0.03)	
15	25.198	alpha-thujene	Monoterpene	0.76(0.08)	0.69(0.19)	0.54(0.19)	0.48(0.21)	0.42(0.19)	0.56(0.19)	0.39(0.20)	0.32(0.20)		0.39(0.20)	0.32(0.20)	
16	25.307	Caryophyllene	Sesquiterpene	9.33(1.12)	9.88(1.02)	13.28(1.02)	9.97(1.05)	9.91(0.75)	10.0(1.48)	8.88(1.03)	8.81(1.03)		8.88(1.03)	8.81(1.03)	
17	25.741	Thiopsene	Sesquiterpene	2.18(0.93)	2.26(0.37)	2.28(0.94)	2.27(0.26)	2.17(0.24)	2.27(0.85)	2.14(0.25)	2.07(0.24)		2.14(0.25)	2.07(0.24)	
18	26.307	Humulene	Sesquiterpene	0.07(0.02)	0.05(0.01)	0.08(0.01)	0.02(0.03)	0.04(0.01)	0.08(0.01)	0.05(0.01)	0.05(0.03)		0.05(0.01)	0.05(0.03)	
19	26.873	beta-ionone	Apocartenoid	0.43(0.26)	0.56(0.28)	1.02(0.16)	1.26(0.14)	0.36(0.09)	0.31(0.16)	0.23(0.11)	0.26(0.08)		0.23(0.11)	0.26(0.08)	
20	27.017	beta-chamigrene	Sesquiterpene	0.60(0.04)	0.55(0.03)	0.58(0.13)	0.34(0.12)	0.58(0.02)	0.48(0.03)	0.25(0.03)	0.23(0.03)		0.25(0.03)	0.23(0.03)	
21	29.495	Isocaryophyllene	Sesquiterpene	0.19(0.08)	0.10(0.03)	0.12(0.03)	0.17(0.05)	0.16(0.02)	0.12(0.03)	0.06(0.03)	0.09(0.03)		0.06(0.03)	0.09(0.03)	
22	29.726	Caryophyllene epoxide	Sesquiterpene	0.63(0.07)	0.48(0.03)	0.61(0.17)	0.24(0.09)	0.58(0.02)	0.57(0.07)	0.53(0.23)	0.58(0.18)		0.53(0.23)	0.58(0.18)	

¹ n = 8

tr = trace

L = Line

VOCs identified in the HS of the *Arabidopsis thaliana*, were mainly of isoprenoid origin. Isoprenoids are composed of the C₅ units of active isoprene, which is known as isopentenyl diphosphate (IPP). These isoprenoid compounds are also termed terpenoids with monoterpenes (C₁₀) and sesquiterpenes (C₁₅) as examples. In the majority of plants C₁₀ precursors of monoterpenes are predominantly synthesized within plastids by the (methylerythritol phosphate) MEP pathway, whereas precursors of (C₁₅) sesquiterpenes are produced in the cytoplasm via the classical mevalonic acid (MVA) pathway, these pathways are delineated in plants with differences in the types of compounds produced [13, 14].

The only monoterpene identified with the method was α -thujene (**15**), which is a flower fragrance [15], even though *Arabidopsis* is by nature an auto pollinator plant, compound **15** could play an important role in pollination process, but studies regarding this possible function should be performed in order to confirm this hypothesis. The sesquiterpenes caryophyllene, thujopsene, humelene and beta-chamingrene (**16**, **17**, **18**, **20**) had been previously reported in *Arabidopsis* plants [16]. This group of compounds have diversity functionalities, from plant development and signaling to direct and indirect defense. For instance the compound **16** is released in higher amounts by tomato plants upon whitefly attack and reduced its feeding rates [17].

Compound **18** has a direct defense function against pathogen attack in pepper plants (Solanaceae, *Capsicum annum* L.) [18]. Caryophyllenes, including **16**, **18** and **21** are sesquiterpenes present in various essential oils such as in cloves (Myrtaceae, *Eugenia caryophyllus*), common hop (Cannabaceae, *Humulus lupulus*), Cat thyme (Lamiaceae *Teucrium marum*) and Perennial plant (Verbenaceae, *Lantana camara* L.) [19, 20].

The natural bicyclic **16** and **21** are trans and cis double bond isomers, respectively, while **18** is a ring-opened isomer. In essential oils, **16** is frequently found mixed with **21** and/or **18** [21]. Compound **21** has been very seldom studied and its

toxicity is still unknown. On the other hand, **16** has been well studied due to its use as a cosmetic ingredient and a food flavoring additive [22].

Aromatic compounds (**1**, **4-8**, **10-13**) represent a large class of VOCs which mainly are released by the flowers and were found in all types of plants studied in our DHS analysis. These compounds are exclusively derived from the aromatic amino acid phenylalanine (Phe) [23], which is synthesized via two alternative pathways [24]. According to the structure of their carbon skeleton these compounds can be classified as benzenoids (C₆-C₁) **1**, **8**, phenyl-ethanoid-related compounds (C₆-C₂) **4-7**, **10,11** and phenyl-butanoid-related compounds (C₆-C₄) **12**.

The dynamic HS collection method allowed the detection and identification of a compound with a retention time of 26.873 min and a mass fragmentation pattern identical to that of an authentic β -ionone standard. β -ionone (**19**), which is an apocarotenoid, is not derived directly from MEP and MVA pathways, but instead from cleavage of tetraterpenes such as carotenoids, by the action of *CCDs*.

Even though, the volatile organic compounds released by WT, *AtCCDI*, *AtCCD4* and *AtCCD8 Arabidopsis* plants were similar, differences in emission of particular compounds (relative percentage area) were observed. In the following three sections, a detailed description in the differences and similarities of VOCS is provided. The discussion will be based on the information from Table 2.2 and supported by principal component analysis (PCA).

2.3.4.1 WT and *AtCCDI* Volatile Organic Compounds

VOCs released by both WT and *AtCCDI* lines were similar in profile, but in order to evaluate differences in emissions, a detailed description of the significant changes observed on the three lines of *AtCCDI* plants compared to the WT and to each other was performed.

As it was depicted in Table 2.2 and illustrated in Figure 2.6, volatile organic compound emissions of both WT and *AtCCDI* Line 1 were almost identical according to the relative peak area percentage.

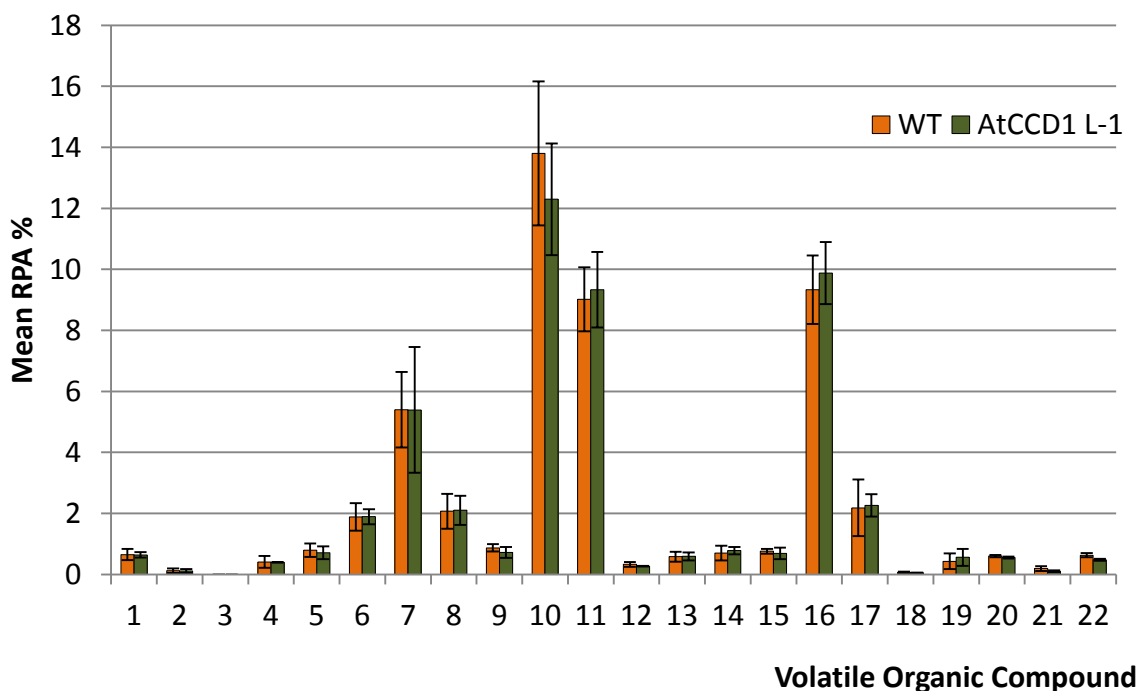


Figure 2.6. Mean relative peak area % (\pm S.E.) of four replicates of the volatile organic compounds identified in the HS of WT and *AtCCDI* L-1 *Arabidopsis thaliana* plants. VOC number refers to Table 2.2.

All of the compounds found in the HS of WT had a near 1:1 ratio when compared to the transgenic plants. The small differences observed in peak intensities are within the level one would expect due to biological variations. Volatile organic compounds of both the WT and *AtCCDI* Line 2 were also mostly similar when comparing the average relative peak area percent (Table 2.2 and Figure 2.7).

Small differences were observed from five compounds; benzaldehyde,4-ethyl (**7**) which was found to be 1.6-fold higher in the WT compared to the transgenic plants; acetophenone-2,4-dimethyl (**10**) with a relative peak area percentage (RPA%) of 13.8 in WT and 11.3 in the transgenic plant; the aromatic compound acetophenone,4-ethyl (**11**)

with a RPA% of 9.02 in WT and 6.40 in transgenic plants; the sesquiterpene caryophyllene (**16**) which was found to be 1.4-fold higher in transgenic plants compared to the WT; and finally, there was difference in the emission of the apocarotenoid compound β -ionone (**19**) with a 2.4-fold difference in transgenic plants compared to the WT.

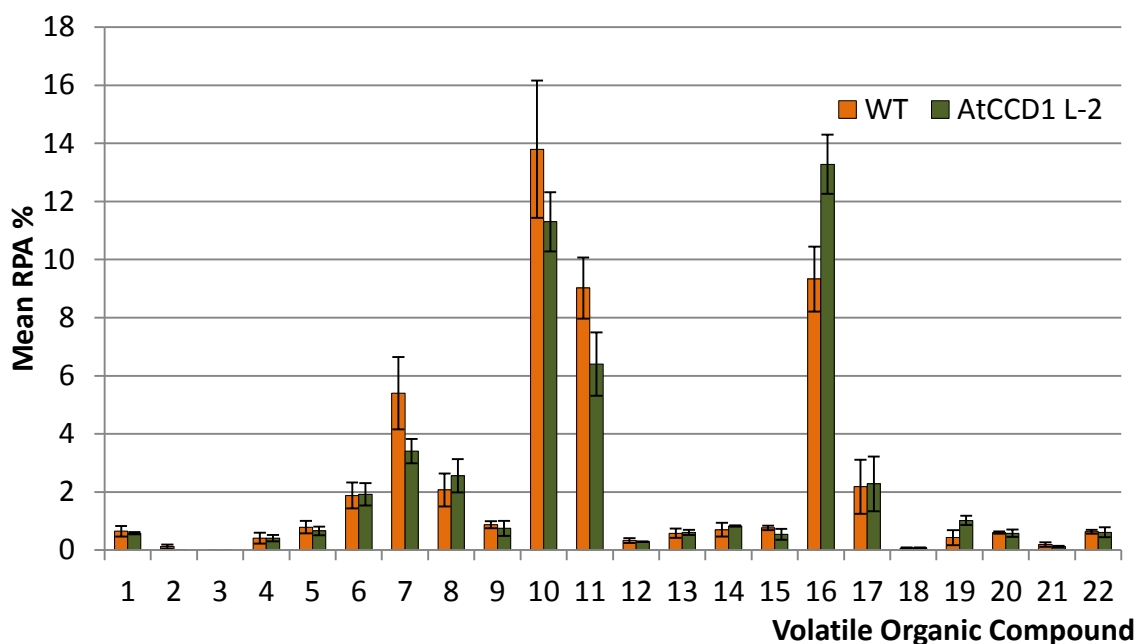


Figure 2.7. Mean relative peak area % (\pm S.E.) of four replicates of the volatile organic compounds identified in the HS of WT and *AtCCD1 L-2 Arabidopsis thaliana* plants. VOC number refers to Table 2.2.

The most abundant volatiles in both types of plants were **10**, **11** and **16**. The sesquiterpene compound **16** was the most abundant VOC in the transgenic (*AtCCD1 L-2*) plant; meanwhile compound **10** was the most abundant in WT type plants. Changes in emission of the sesquiterpene **16** had not been reported to be attributed to the action of the *CCD1* gene, but in previous studies it was demonstrated to be enhanced by *Arabidopsis* flowers as a defense to bacterial attack [25].

Volatile organic compounds of both WT and *AtCCD1* Line 3 were similar according to the relative peak area Table 2.2 and Figure 2.8.

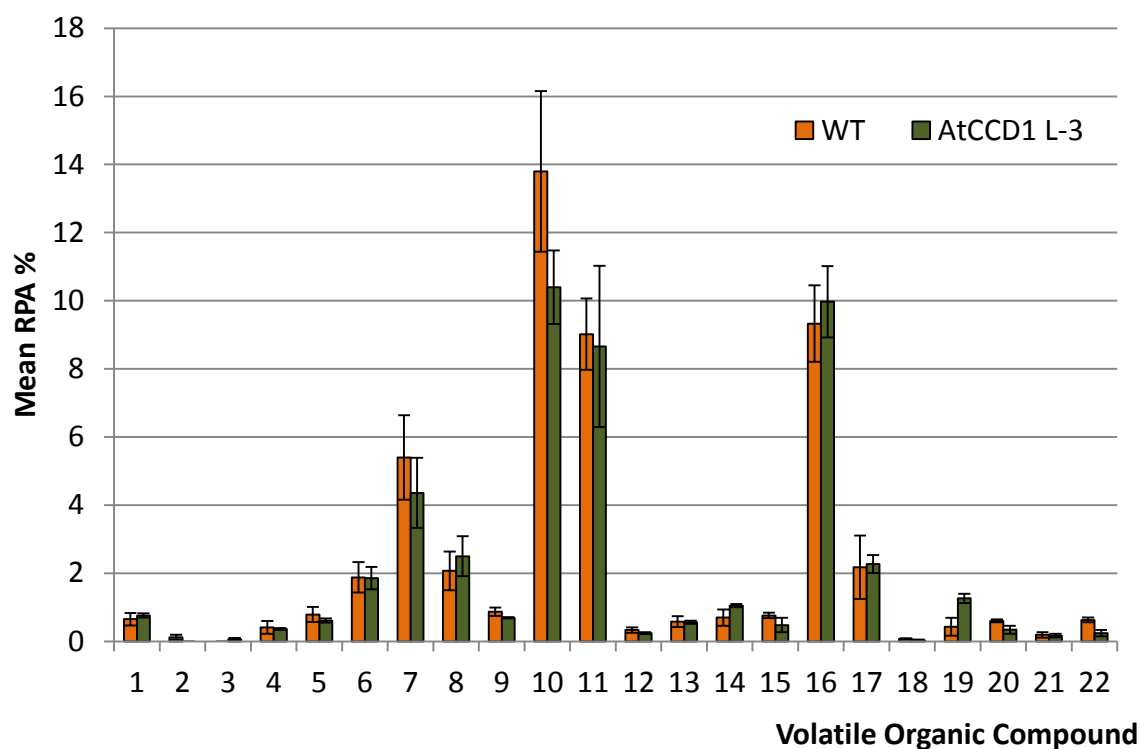


Figure 2.8. Mean relative peak area % (\pm S.E.) of four replicates of the volatile organic compounds identified in the HS of WT and *AtCCD1* L-3 *Arabidopsis thaliana* plants. VOC number refers to Table 2.2.

Compound **10** was the most abundant compound of the two plant strains and the differences were represented by a RPA% of 13.8 in the WT and 10.40 in the transgenic plants. The apocarotenoid compound β -ionone (**19**) was 3-fold higher in the transgenic plants compared to the WT; on the contrary, the sesquiterpene caryophyllene epoxide (**22**) was found to be released by the WT in higher proportions (2.6-fold higher) compared to the transgenic plant. To evaluate whether the subtle differences in the VOC profiles among the various *AtCCD1* lines and WT were of any significance, PCA was applied as an explorative tool to analyze and visualize the VOC emission differences. The score plot was judged by visual inspection for the distance within the cluster and the distance between the clusters. The results are presented as a two-dimensional plot of

sample scores in the space defined by the PCA (Figure 2.9). When plotting the WT and the three lines of the *AtCCD1*, three separate clusters were observed, indicating separation according to the plant type. L-2 and L-3 showed a distinct separation from the WT and L-1. This was attributed to the differences explained in the last three sections and especially to the enhancement of the VOC, β -ionone (**19**). The lower variability in the wild type compared to the *AtCCD1* L1 plants reflects less biological variation of the volatile profile intensities.

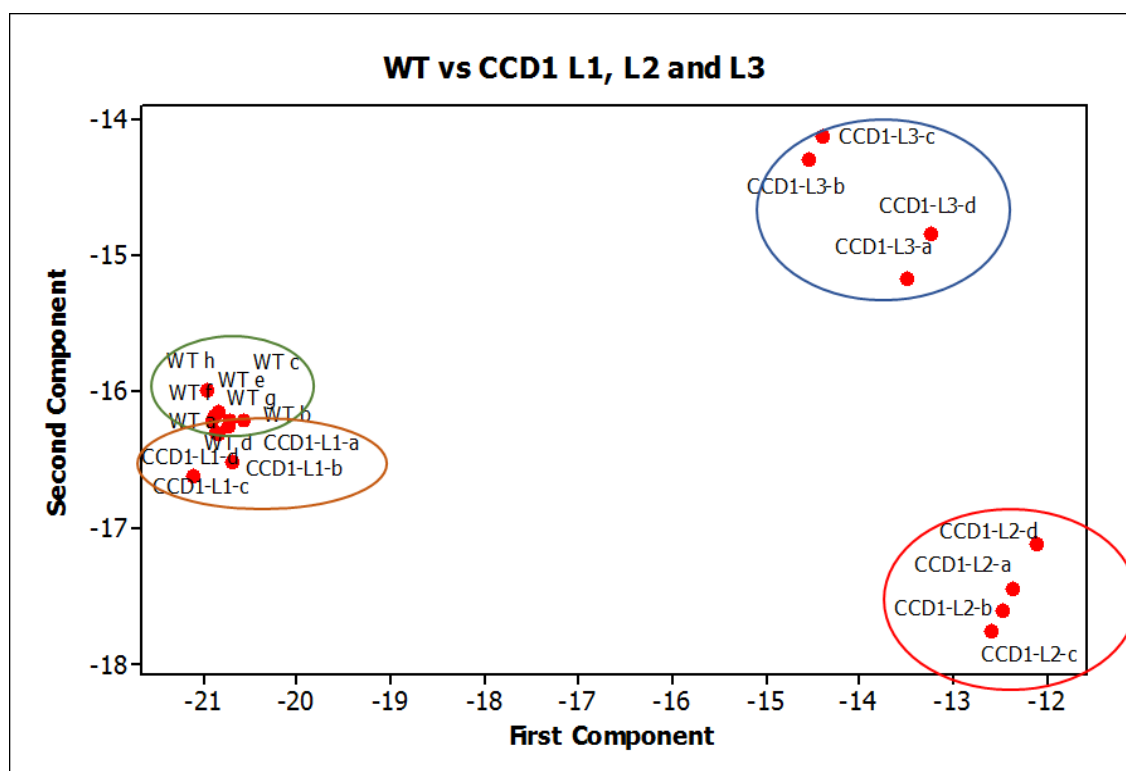


Figure 2.9. PCA plot showing the separation of wild type and *AtCCD1* L-1, L-2 and L-3.

2.3.4.2 WT and *AtCCD4* Volatile Organic Compounds

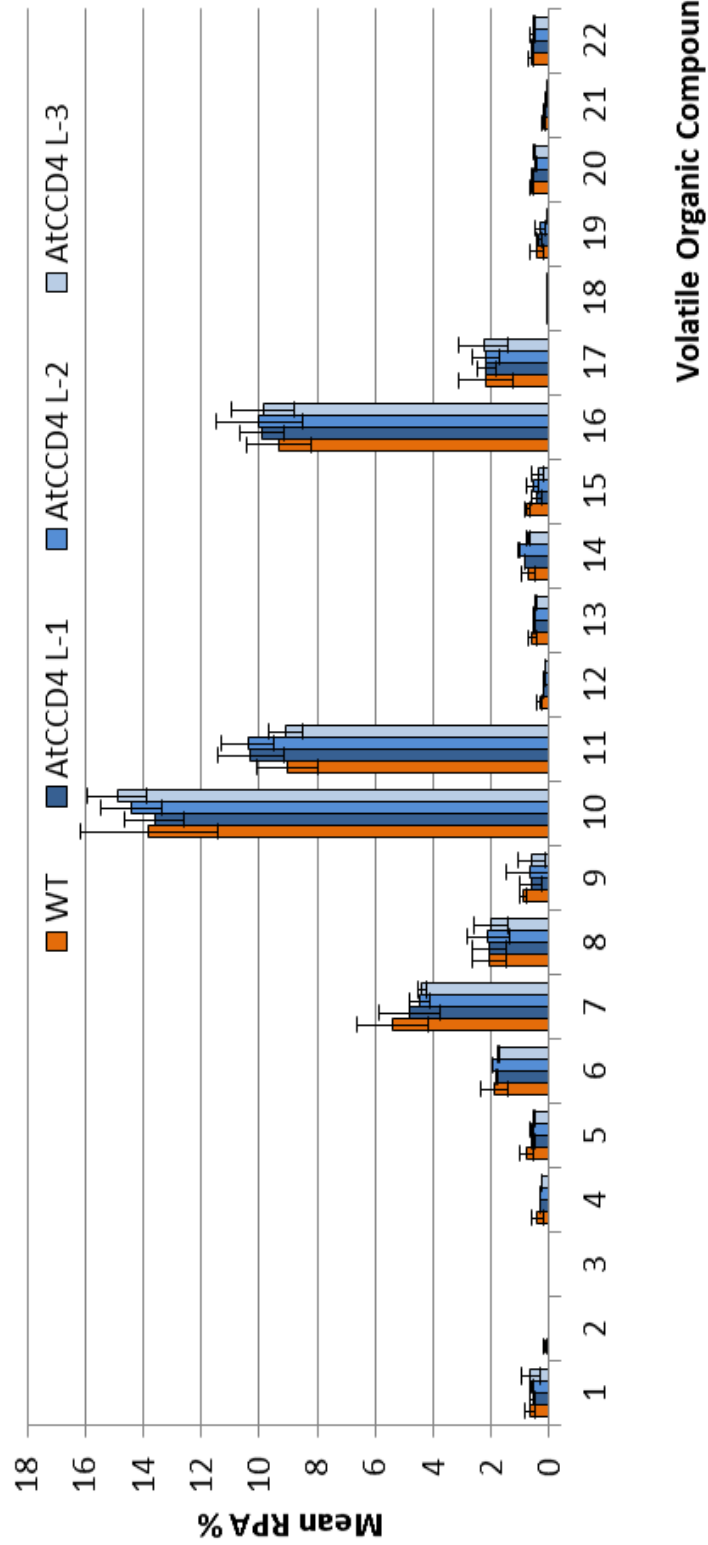
VOCs released by both WT and *AtCCD4* lines were similar in GC-MS TIC profile (data not shown) but in order to evaluate differences in emission levels, a detailed description

of the changes detected on the three lines of *AtCCD4* plants compared to the WT and to each other was performed.

As it was detected in the *AtCCD1* plants, variation on volatile profiles of *AtCCD4* L-1, L-2 and L-3 was associated with the changes in volatile emissions compared to the wild type plant (Table 2.2).

Twenty out of the 22 VOCs found in the WT were within the 1:1 ratio compared to the transgenic plants (Figure 2.10). Some differences were attributed to the following VOCs: Even though the emission percentage of compounds **12** and **21** were low in all plants compared to the total blend of VOCs, the relative peak area percentage of the aromatic compound (**12**) and the sesquiterpene (**21**) were found to be 2-fold higher in WT compared to the transgenic plant lines. The apocarotenoid compound β -ionone (**19**) was elicited in lower proportion in the *AtCCD4* L-3 (0.05%) compared to the WT (0.43%), L-1 (0.36%) and L-2 (0.31%).

Figure 2.10. Mean relative peak area % (\pm S.E.) of four replicates of the volatile organic compounds identified in the HS of WT and *AtCCD4* L-1, L-2 and L-3 *Arabidopsis thaliana* plants. VOC numbers refer to those used in Table 2.2.



Volatile emissions were similar in profile in all *Arabidopsis* plant types. Small differences were attributed to the peak intensities (RPA %) which varied between the plant types. It can be noted that even though the groups according to the plant type are observed in the PCA (Figure 2.11), the distance between the clusters of the WT and *AtCCD4* lines were not as differentiated as the ones observed with the *AtCCD1* L-2, L-3 and WT.

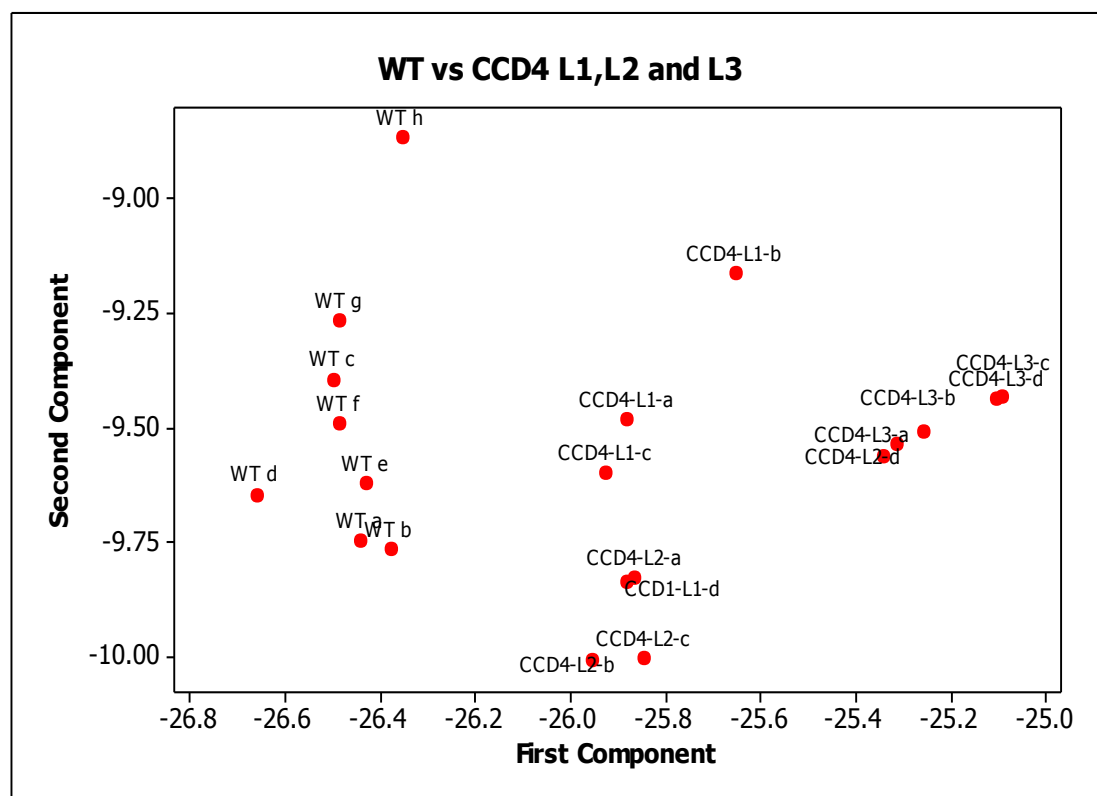


Figure 2.11. PCA plot showing the separation of wild type and *AtCCD4* L-1, L-2 and L-3

2.3.4.3 WT and *AtCCD8* Volatile Organic Compounds

In this section, VOCs differences between the WT and the two *AtCCD8* lines were described and Table 2.2. As it was observed before, VOC profiles were similar with variation in peak intensities only (Figure 2.12).

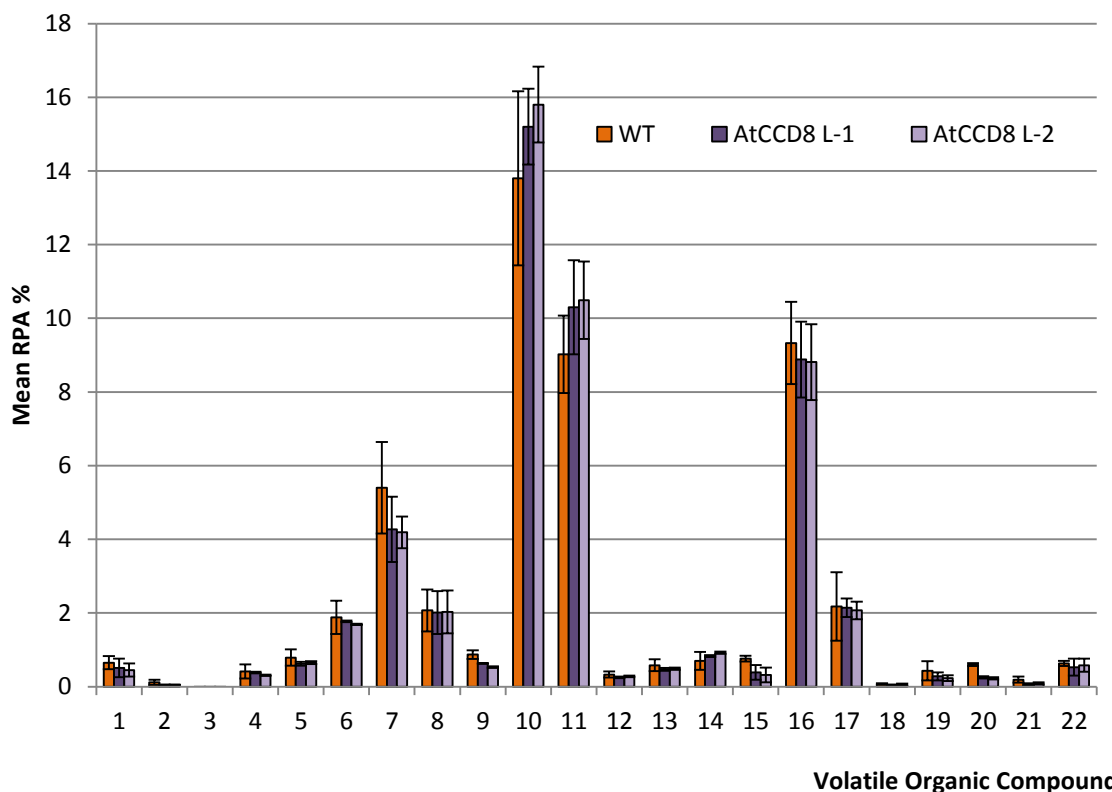


Figure 2.12. Mean relative peak area % (\pm S.E.) of four replicates of the volatile organic compounds identified in the HS of WT and *AtCCD8* L-1 and L-2 *Arabidopsis thaliana* plants. VOC numbers refer to those used in Table 2.2.

Most of the volatiles from transgenic plants were within the 1:1 ratio compared to the wild type. Some differences include a 1.2-fold increase in volatile compound (7) in WT compared the transgenic lines. Additionally, the RPA% of the VOC β -ionone (19) was 0.43 compared to the 0.23 and 0.26% observed in the *AtCCD8* L-1 and L-2. Similarly, there was a reduction in the emission of volatile (20), from 0.60% in the WT to 0.25 and 0.23% in the corresponding transgenic lines.

Even though the ratio of the aromatic derivative compound 11 and the sesquiterpene compound 16 were 1:1, relative peak area percentage demonstrated that in the WT compound 11 was less abundant than compound 16. The opposite was observed in the transgenic plants. The aromatic compound 11, which is a floral

compound, has been reported to be elicited in higher concentrations in pepper plants under biological stress [26].

The PCA in Figure 2.13 shows the clusters of the groups of plants based on the intensities of volatiles released into the HS.

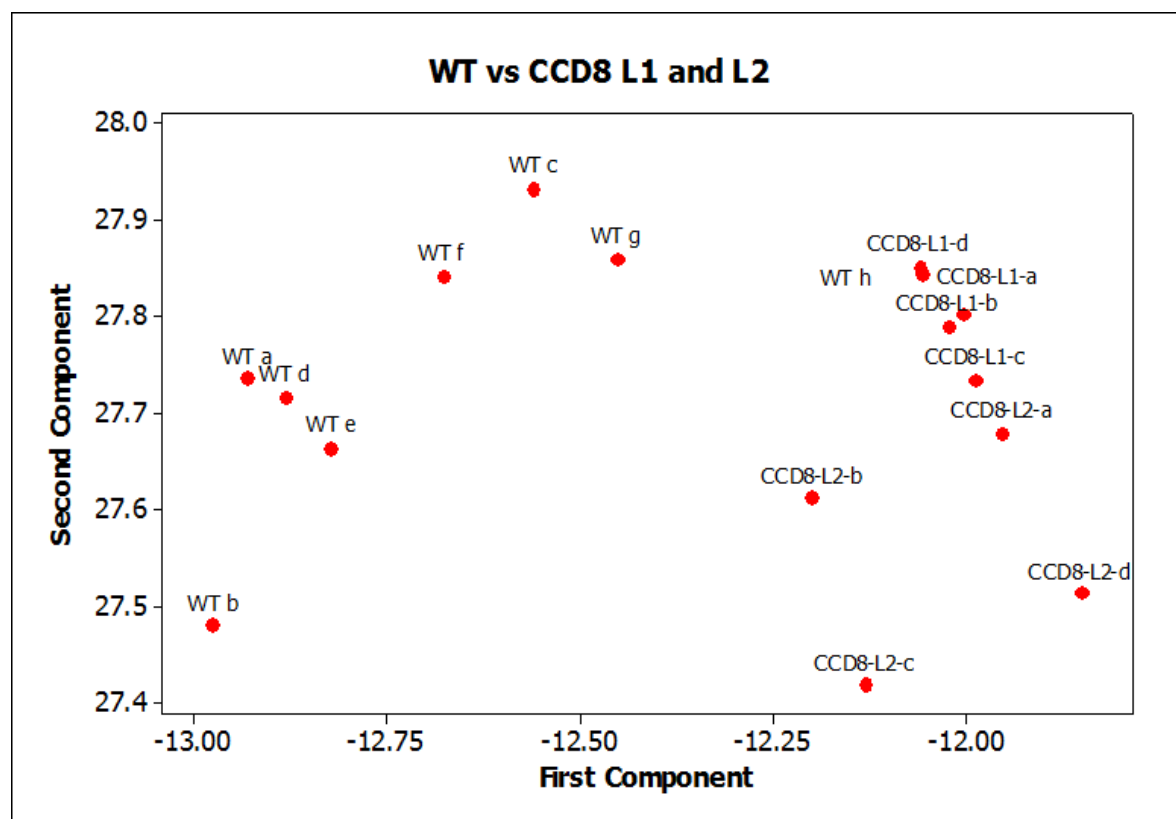


Figure 2.13. PCA plot showing the separation of wild type and *AtCCD8* L-1 and L-2

Like in the *AtCCD4* analysis, the grouping of the *AtCCD8* lines was not as prominent as the ones observed with the *AtCCD1* due the similarity on the volatiles ratios released among all type of plants. Small differences in emission of compounds **7**, **19** and **20** separated WT plants in the PCA plot from the transgenic lines but differences were not substantially different.

2.3.5 β -Ionone Quantification

The DHS collection system allowed the extraction and detection of 22 VOCs from the HS of the *Arabidopsis thaliana* plants. As it was expected, profiles were similar in all nine types of plants (WT, *CCD1* L-1, L-2, L-3, *CCD4* L-1, L-2, L-3 and *CCD8* L-1 and L-2) with variations in peak intensities due to biological variation of the plants.

The DHS-GC-MS method was able to detect the apocarotenoid molecule β -ionone (**19**), but no other apocarotenoid compounds were observed. Figure 2.14 shows the enhancement of a representative *Arabidopsis* TIC.

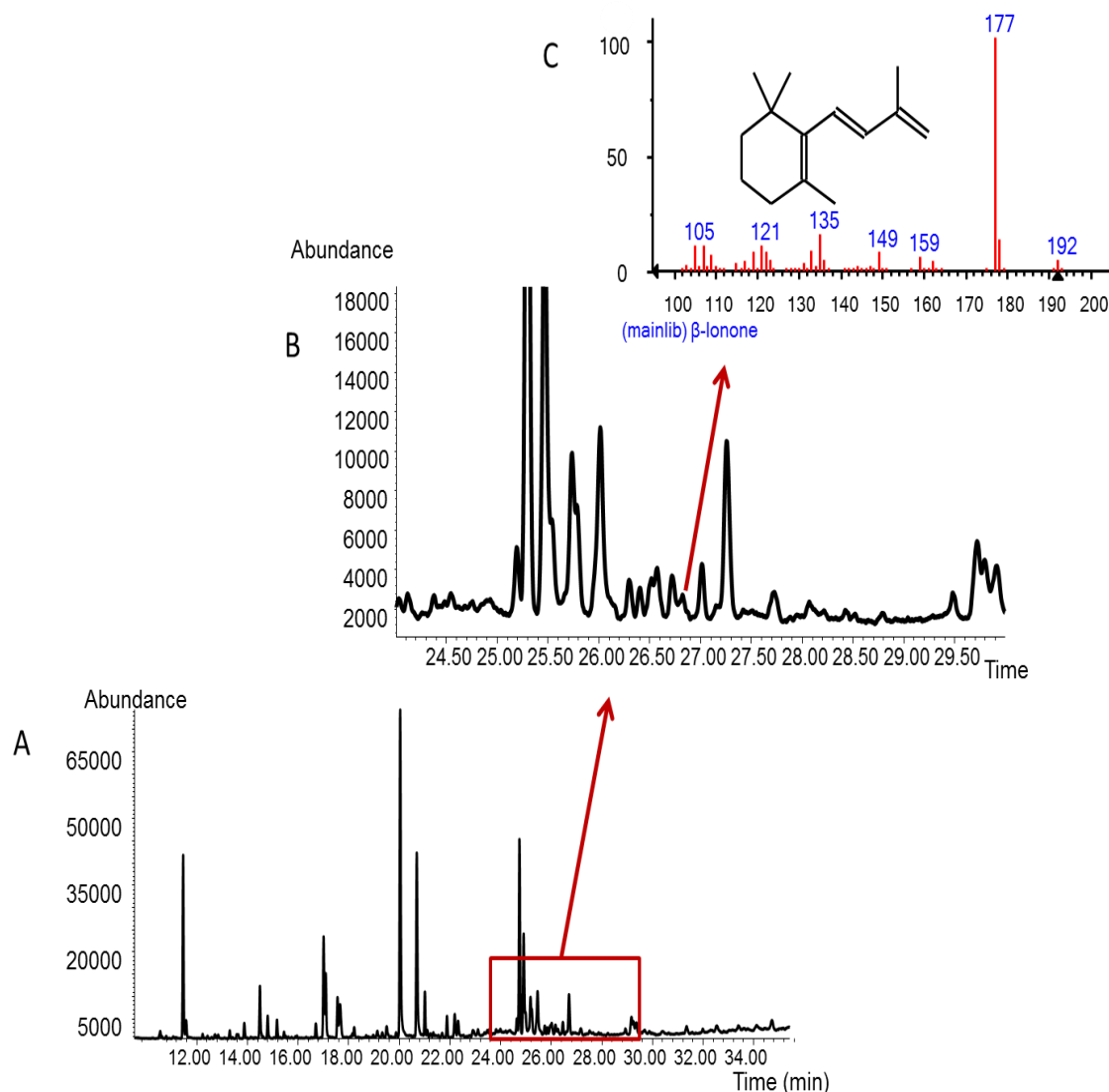


Figure 2.14. (A) Representative TIC of *AtCCDI*; (B) Enhanced TIC (x4) showing β -ionone peak at 26.821 min; (C) Mass spectrum of the apocarotenoid β -ionone identified by the databases and analytical standard.

It was observed that the *AtCCDI*-enhanced plants released a higher level of the apocarotenoid β -ionone (**19**) compared to the wild type and to the other *AtCCD* genes. Quantitative analysis by using a calibration curve showed that the flowering transgenic plants released substantially higher levels (up to 4.5-fold higher) of compound **19** relative to wild-type plants (Figure 2.15). Statistical analysis of the concentration showed significant difference in the production of **19** (One-way ANOVA, d.f=8, 27;

F=21.57; P<0.001). The enhancement of compound **19** confirms the action and selectivity of the *CCD1* gene in the plants. This compound results from the specificity of oxidative cleavage of the 9,10 and/or 9',10' double bond in the C₄₀ β-carotenoid molecule [27] (Figure 2.16). *AtCCD4* and *AtCCD8* did not show significant incremental differences of **19** compared to the wild type; this is a confirmation that the actions of these two genes are different from the *AtCCD1*.

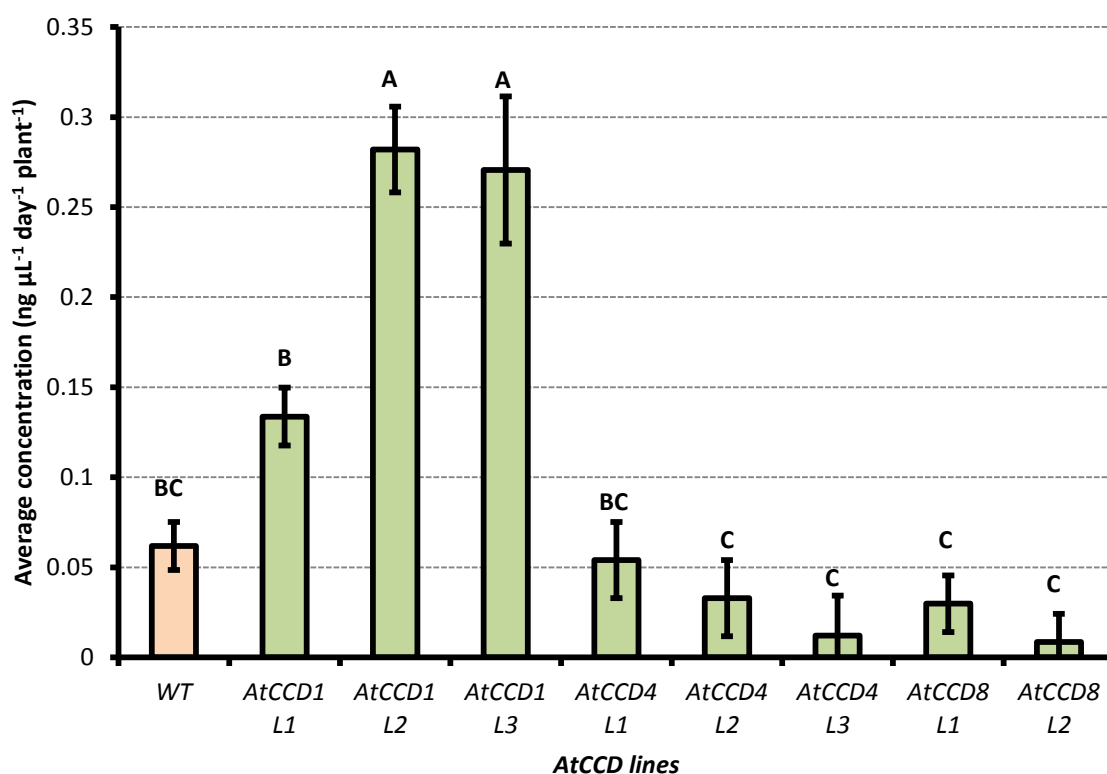


Figure 2.15. Concentration in ng/μL of the apocarotenoid β-ionone (±SE) detected in the WT and *AtCCD* lines from the *Arabidopsis* plants after 24 h HS collection. Peak area obtained from the TIC were divided by the number of plants contained in a pot. Bars with different letters are significantly different (One-way ANOVA, d.f=8, 27; F=21.57; P<0.001).

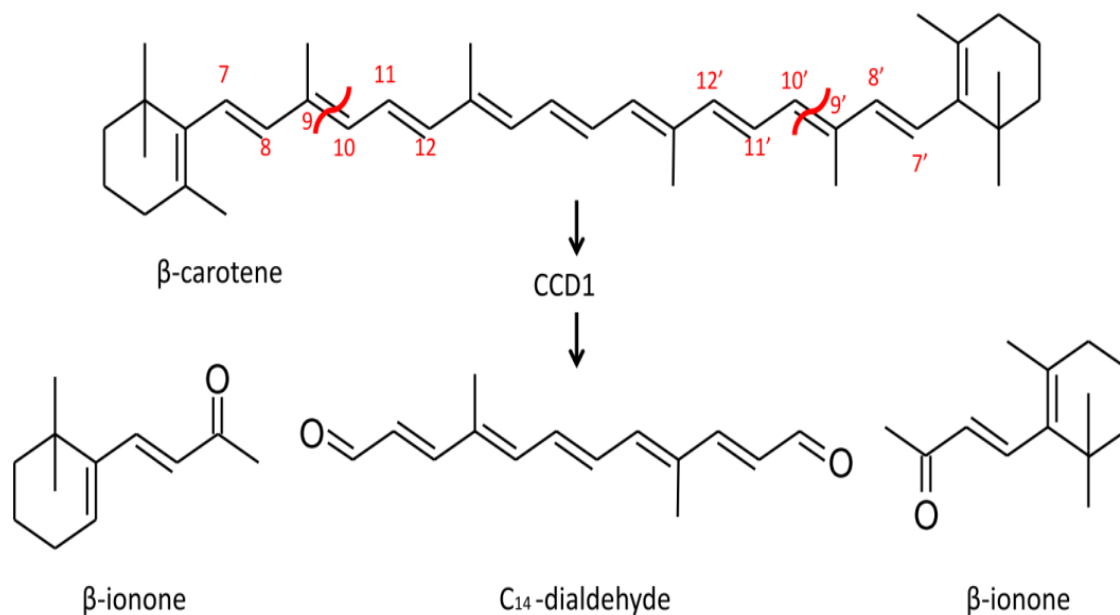


Figure 2.16. Selective oxidative cleavage of the *CCD1* of the β -carotene molecule to produce the apocarotenoid compound β -ionone. Strokes indicate cleavage sites.

The concentration of β -ionone found correlates with the levels of gene expression found in the *AtCCD1* plants L-1, L-2 and L-3. These lines were 11, 49 and 22-fold higher in gene expression, respectively, compared to the WT.

2.3.6. Conclusions from the *Arabidopsis* DHS-GC-MS Study

The DHS-GC-MS method allowed the extraction, separation and detection of 22 VOC from WT and transgenic *Arabidopsis* plants overexpressing the *CCD* genes. VOC profiles were identical for every type of plant with the exception of variation in the intensities of a few VOC compounds due to biological and non-biological factors. Biological factors include plant size and plant development as well as gene expression, the latter of which can even differ among plants of the same type and line. Non-biological factors include the plant response to the glass chamber environment (increment in HS humidity due to plant transpiration and pressure due compressed air flow) and variation in the GC-MS detector sensitivity.

The DHS-GC-MS method also extracted, detected and quantified the volatile compound β -ionone which was found to be 2 to 4-fold higher in the *AtCCD1* lines compared to the WT and other transgenic lines. It is important to note that there were variations in the emission of other volatile organic compounds, as it was described before. These changes can be attributed to biological factors like plant size and number of flowers per plant. There is no evidence of a direct effect of the CCD genes in the production of terpenes and their possible influence for their enhancement.

Since the DHS-GC-MS method and non-targeted data analysis succeeded in the extraction of the most authentic profile of VOCs in *Arabidopsis* plants and in the detection of the apocarotenoid compound β -ionone, the next step was the application of the DHS-GC-MS method for the non-targeted analysis of volatile organic compounds in Micro-Tom tomato plants over expressing the *CCD1* genes.

The following sections show the results of the non-targeted analysis of VOCs found in the HS of the Micro-Tom tomato plants.

2.3.7 Non-targeted Analysis of Micro-Tom VOCs

Micro-Tom tomato plants already have the *CCD1* genes in their genome, but in this study the genes were overexpressed by genetic manipulation in order to evaluate their function in the formation of new compounds. These genes, which can be expressed in different tissues of a plant, are commonly implicated in plant growth regulation, production of plant hormones and in the formation and production of volatiles [28]. It was established, in previous studies, that the *CCD* genes in tomato plants triggered the formation of secondary metabolites responsible for the special fragrance and flavour, especially in their fruits [12], but there are presently no studies of the volatile profiles released *in vivo*. In this section, a non-targeted *in vivo* study was performed similar to the *Arabidopsis* study in order to evaluate the effect of the genes in the production of VOCs, and any differences compared to the wild type.

For the non-targeted analysis of volatiles from Micro-Tom tomatoes, the 48 Micro-Tom tomato chromatograms (six from the WT, fifteen from the *AtCCD1*, fifteen from the *LeCCD1-1* and twelve from the *LeCCD1-2*) were deconvoluted with AMDIS. Metabolites present in each chromatogram were identified by matching their mass spectra to the NIST library. The highest library match was selected, and only matches greater than 800 were considered. The *.ELU files from AMDIS were uploaded to SpectConnect. After automated baseline correction, intensities of the 7,200 molecular fragments with the corresponding retention times were aligned through 48 GC-MS profiles by SpectConnect. Data from the SpectConnect integrated signal (IS) output was manually inspected for molecular fragments originating from contaminants coming from the Porapak Q matrix, soil, pot and/or the injection port septum. The manually cleaned IS matrix data was subjected to transformation;

$$n_t = \text{Log} [n+1] \text{ (Equation 2.3)}$$

Normalization (n_t) was performed to reduce fluctuations in the data due to biological and non-biological variations. PCA plots were used to visualize volatile emission variations across all set of samples.

In the following sections a description of the VOCs identified by the DHS-GC-MS will be performed. Data of individual volatiles identified were presented as a relative peak area percentage relative to the most abundant VOC found in the Micro-Tom tomato plants and compared to the PCA to establish differences and similarities.

2.3.8 VOCs emission from WT and *CCD1* Micro-Tom

The *AtCCD1* gene in the *Arabidopsis thaliana* has high specificity in cleaving symmetrically 9-10 (9'-10') the carotenoid molecule to form β -ionone. This was confirmed by the DHS-GC-MS method which succeeded in the detection and quantification of the compound, especially in the determination of the increment of the

apocarotenoid compound in plants that overexpressed the gene. There was a direct correlation of the *CCD* enhancement with the β -ionone produced in plants. As a next step, the focus was on the non-targeted analysis of VOCs released by transformed Micro-Tom tomato plants.

Like the *Arabidopsis* analysis, profiles of wild type and transgenic Micro-Tom tomato plants were compared. The three genes overexpressed were *AtCCD1* (*Arabidopsis* gene in tomato), and the two types of tomato gene (*LeCCD1-1* and *LeCCD1-2*). The tomato *CCD1* genes are similar in structure and functionality to the *CCD1 Arabidopsis* genes [4], so it was expected to observe a similar function of the gene in the production of the volatile β -ionone and/or other apocarotenoid derivatives. Figure 2.17 shows a wild type Micro-Tom tomato plant enclosed in a glass chamber during volatile collection.



Figure 2.17. Glass chamber for the dynamic headspace (DHS) collection of volatiles. Every chamber contained a pot with one Micro-Tom tomato plant at flowering stage.

Flowering Micro-Tom tomato plants from both transgenic and non-transgenic plants do not present obvious phenotypical differences; the transformations are only at the molecular level. Even though, genetic transformation was performed, all plants (WT, *AtCCDI*, *LeCCDI-1* and *LeCCDI-2*) released a similar spectrum of volatiles into the chamber's HS, which was expected, while the plants share the same basic genotype, the random insertion of a gene with a constitutive promoter, can have unexpected effects. Figure 2.18 and Figure 2.19 show representative TIC of volatiles found in a WT and *CCDI* Micro-Tom tomato plants.

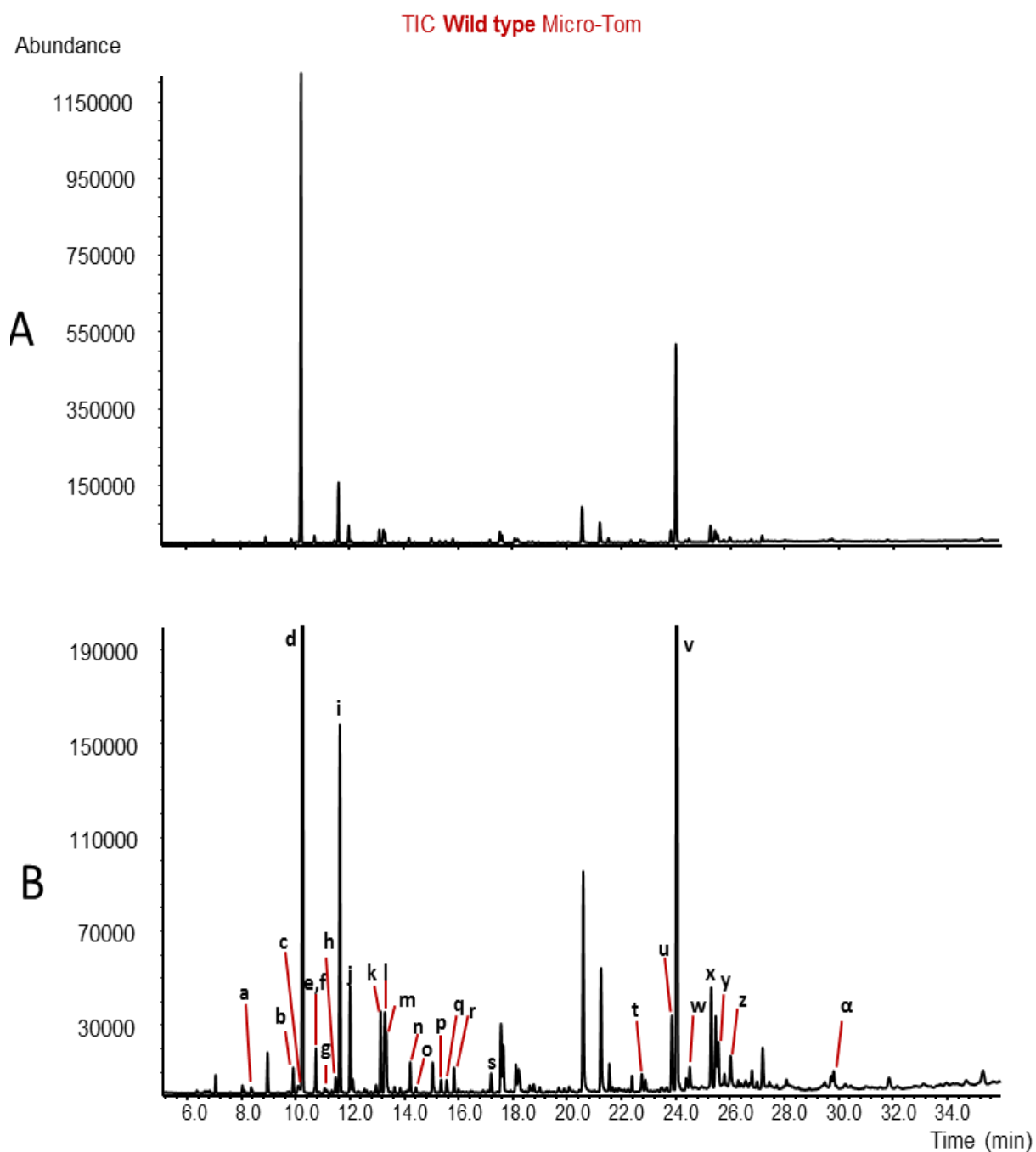


Figure 2.18. A Representative TIC obtained from a wild type Micro-Tom tomato plant showed at (A) full scale, and (B) enhanced scale (x6). Peak labels correspond to Table 2.3, Table 2.4 and Table 2.5.

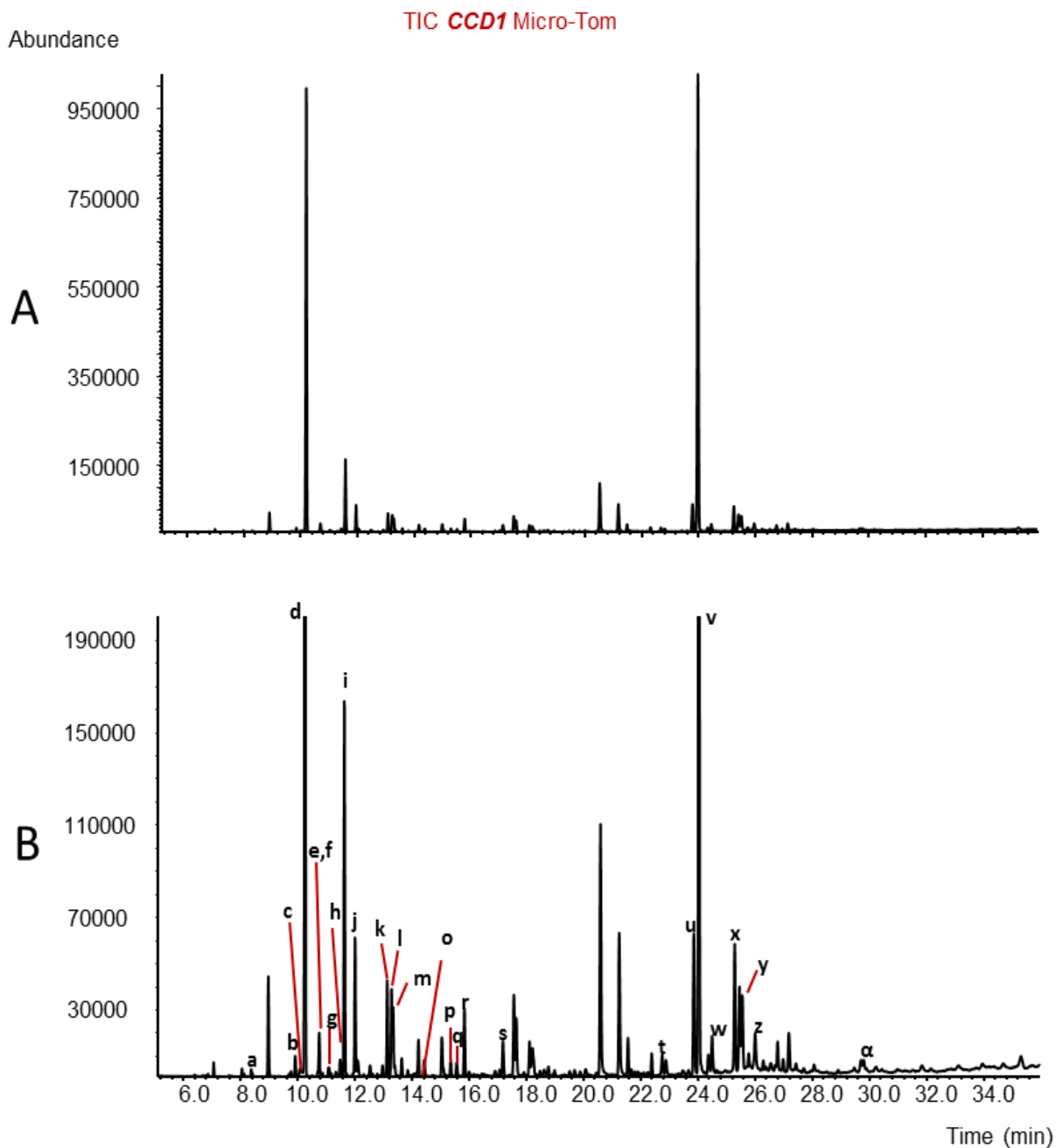


Figure 2.19. A Representative TIC obtained from a *CCD1* Micro-Tom tomato plant showed at (A) full scale, and (B) enhanced scale (x5). Peak labels correspond to Table 2.3, Table 2.4 and Table 2.5.

Twenty seven VOCs were identified by the database consisting of aromatic derivatives, monoterpenes, sesquiterpenes and four unidentified compounds (due to low

NIST match). Table 2.3, Table 2.4 and Table 2.5 show the average relative peak area percent of the compounds extracted and identified in WT and the corresponding lines of the Micro-Tom tomato plants overexpressing the *CCDI* genes. Monoterpenes, which are produced by the methyl-D-erythritol 4-phosphate (MEP) are typical leaf products whereas sesquiterpenes, which precursors have been shown to be derived in part from the MEP and MVA pathways, are typically flower fragrances [29], although the most common single compounds in floral scent are the monoterpenes o-Cymene (**k**), Limone (**l**) and Pinenes (**c**, **d** and **j**) [13].

Among the volatiles identified in the Micro-Tom tomato plants (WT and transgenic) the monoterpenes R- α -pinene (**d**), β -pinene (**i**) and the sesquiterpene α -copaene (**v**) were found to be the most abundants (Figure 2.18 and Figure 2.19). Compounds **d** and **i** are two structural isomers of pinene produced in nature from geranyl diphosphate (GDP), via cyclisation of linalyl pyrophosphate followed by loss of a proton from the carbocation equivalent. Compound **i**, is a common constituent of a number of essential oils described in the literature, as having repellent activity against mosquitos [30].

The secondary metabolite compound **d**, present in the essential oils of aromatic plants, is responsible for the biochemical interaction between plants known as allelopathy. It is used by some plants, like maize (*Poaceae*, *Zea mays*), to control germination and growth [31]. Compound **v**, a tricyclic sesquiterpene with chiral properties, is found in a number of essential oil-producing plants, like from the leaves, stem barks and resins of *Canarium parvum* Leen., and *Canarium tramdenanum* (Burseraceae) [32]. Compound **v** is reported to be attractive to the Mediterranean fruit fly (*Ceratitis capitata* W.) [33].

The second most abundant sesquiterpene extracted in the tomato HS was β -Caryophyllene (**x**). This sesquiterpene was identified in volatile studies in tomato plants [34], and has been associated with induced plants under herbivore attack [35].

The following three sections will provide descriptions of the difference and similarities for volatiles detected in plant expressing the three *CCDI* tomato genes.

2.3.8.1 WT and *AtCCDI* VOCs

The *AtCCDI* is an *Arabidopsis* gene that was introduced into the Micro-Tom tomato genome by genetic transformation. Five lines demonstrated to overexpress the gene and were then tested by the volatile analysis method. Table 2.3 shows the relative peak area percentage of the wild type Micro-Tom tomato plants and five lines of the *AtCCDI*.

Table 2.3. Mean relative peak areas and (\pm S.E.). % from three measurements of the volatile compounds collected for WT and five *AtCCDI* lines of Micro-Tom tomato plants.

Peak	compound	RT(min)	chemical group	Mean peak area percentage relative to R- α -pinene (\pm S.E.). n=3					
				WT	L-1	L-2	L-3	L-4	L-5
a	o-Xylene	8.35	Aromatic	0.31(0.06)	0.12(0.05)	0.11(0.04)	0.14(0.08)	0.27(0.06)	0.23(0.12)
b	Tricyclene	9.90	Monoterpene	0.34(0.07)	0.36(0.17)	0.3(0.1)	0.31(0.18)	0.39(0.06)	0.36(0.17)
c	α -Pinene	10.09	Monoterpene	0.23(0.05)	0.07(0.01)	0.06(0.01)	0.18(0.01)	0.14(0.1)	0.16(0.06)
d	R- α -Pinene	10.26	Monoterpene	38.02(4.24)	23.32(3.42)	24.18(1.28)	23.47(2.24)	35.95(2.78)	31.16(3.01)
e	α -Fenchene	10.70	Monoterpene	0.68(0.02)	0.36(0.07)	0.31(0.13)	0.33(0.12)	0.67(0.02)	0.72(0.16)
f	Camphene	10.71	Monoterpene	0.68(0.07)	0.3(0.08)	0.36(0.13)	0.35(0.22)	0.63(0.06)	0.66(0.37)
g	Unknown	10.78	Unknown	nd	0.08(0.01)	nd	0.03(0.01)	nd	0.11(0.03)
h	Sabinene	11.49	Monoterpene	0.17(0.06)	0.28(0.07)	0.29(0.02)	0.31(0.15)	0.23(0.06)	0.28(0.09)
i	β -Pinene	11.56	Monoterpene	3.84(0.99)	2.34(0.64)	2.38(0.97)	2.31(0.85)	3.83(0.88)	3.14(1)
j	3-Carene	12.01	Monoterpene	1.24(0.23)	0.52(0.04)	0.55(0.19)	0.57(0.23)	1.15(0.21)	1.31(0.52)
k	o-Cymene	13.14	Aromatic	0.97(0.18)	0.63(0.25)	0.68(0.03)	0.62(0.12)	0.94(0.16)	0.95(0.11)
l	Limonene	13.29	Monoterpene	0.82(0.22)	0.71(0.02)	0.73(0.37)	0.85(0.13)	0.79(0.2)	0.96(0.08)
m	β -Phellandrene	13.34	Monoterpene	0.84(0.06)	0.81(0.05)	0.85(0.13)	0.88(0.06)	0.83(0.06)	0.85(0.15)
n	Eucalyptol	14.23	Monoterpene	0.38(0.07)	0.34(0.05)	0.38(0.01)	0.39(0.03)	0.37(0.06)	0.22(0.09)
o	γ -Terpinene	14.43	Monoterpene	0.4(0.04)	0.53(0.13)	0.55(0.13)	0.54(0.37)	0.39(0.03)	0.41(0.15)
p	α -Pinene oxide	15.35	Monoterpene	0.24(0.06)	0.27(0.05)	0.25(0.02)	0.42(0.06)	0.25(0.15)	0.31(0.1)
q	Unknown	15.56	Unknown	0.22(0.08)	0.14(0.06)	0.1(0.05)	0.22(0.16)	0.24(0.15)	0.32(0.13)
r	Unknown	15.84	Unknown	0.16(0.03)	0.12(0.06)	0.18(0.05)	0.18(0.07)	nd	nd
s	Unknown	17.20	Unknown	0.25(0.06)	0.26(0.17)	0.21(0.06)	0.24(0.09)	0.26(0.15)	0.36(0.15)
t	δ -Elemene	22.64	Sesquiterpene	0.68(0.23)	0.58(0.16)	tr	0.52(0.01)	0.69(0.29)	0.29(0.16)
u	Cyclosativene	23.85	Sesquiterpene	1.64(0.29)	0.82(0.24)	0.84(0.04)	0.97(0.12)	1.64(0.26)	1.43(0.22)
v	α -Copaene	24.04	Sesquiterpene	14.2(1.66)	23.45(2.74)	23.79(1.83)	23.58(3.18)	15.1(1.47)	19.22(2.08)
w	Sativene	24.51	Sesquiterpene	0.31(0.09)	0.21(0.08)	0.16(0.03)	0.27(0.09)	0.29(0.09)	0.37(0.07)
x	β -Caryophyllene	25.05	Sesquiterpene	3.16(0.06)	3.03(0.7)	2.84(0.29)	4.12(0.75)	4.08(0.06)	4.22(0.81)
y	β -Cubebene	25.57	Sesquiterpene	0.76(0.17)	0.66(0.39)	0.71(0.06)	0.64(0.17)	0.73(0.15)	0.43(0.22)
z	Humulene	26.02	Sesquiterpene	1.01(0.16)	0.81(0.28)	0.86(0.1)	0.82(0.32)	0.87(0.15)	0.97(0.28)
α	Caryophyllene oxide	29.82	Sesquiterpene	0.55(0.39)	0.28(0.38)	0.28(0.13)	0.23(0.03)	0.43(0.25)	0.7(0.15)

nd = not detected

tr= trace

AtCCDI = *Arabidopsis* gene

L=Line

VOCs released from the HS of WT and transgenic *AtCCDI* Micro-Tom tomato plants lines were similar in profile with variation in the intensities of the individual VOCs. Two differences that can be observed in Figure 2.20 were: 1) the average peak area of compound **d** in WT plants was 38.02%, a 1.6-fold reduction compared to the transgenic plants L-1, L-2 and L-; and 2) on average, compound **v** was found to be 1.6 fold-higher in the same transgenic lines compared to the WT. In other words, the ratio between **d** to **v** in WT was 2:1, while in the transgenic lines it was 1:1. This difference can also be observed in the representative GC chromatograms depicted in Figure 2.18 for the WT and Figure 2.19 for the transgenic plant. There are no reports of the action of the *CCDI* gene in either the enhancement or reduction in the emission of these two volatiles organic compounds, but it was suggested in another study, that the sesquiterpene α -copaene (**v**), which is leaf volatile, can be enhanced by unhealthy tomato plants due to fungal infections [36]. Most of the compounds from L-4 and L-5 were in the ratio 1:1 compared to the WT. Like L-1, L-2 and L-3 plants, small variations were observed especially in the two most abundant compounds (**d** and **v**) (Figure 2.20). There was a small difference on the emission of compound **v** in L-5 (19.22%) compared to the WT (14.20%) and L-4 (15.1%).

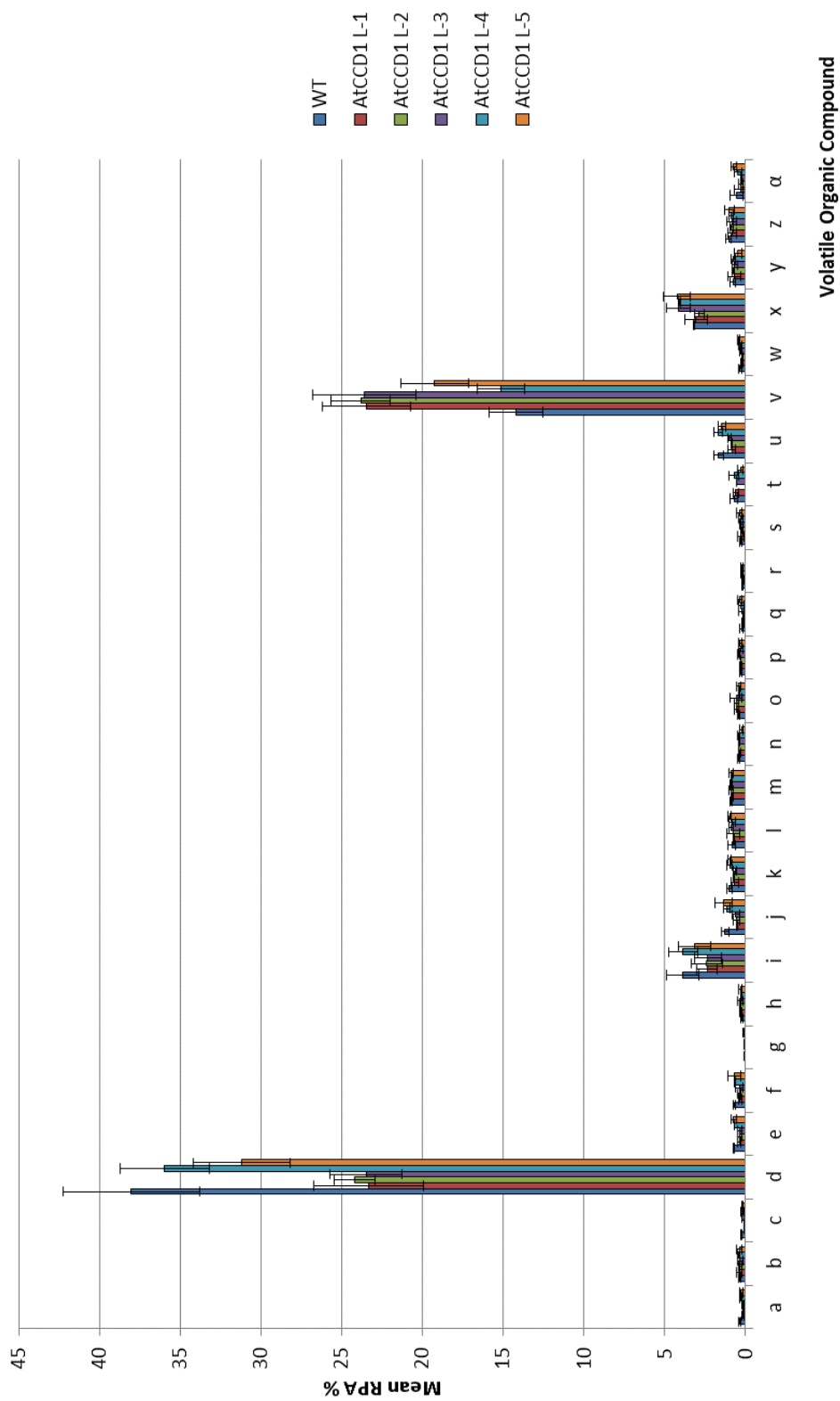


Figure 2.20. Mean relative peak area (\pm S.E.) of three replicates of the volatile organic compounds identified in the HS of WT and *AtCCD1* L-1, L-2, L-3, L-4 and L-5 Micro-Tom plants. VOC label refers to Table 2.3.

Principal component analysis (Figure 2.21) shows the WT and *AtCCD1* Micro-Tom tomato plant lines where three distinguishable clusters were observed.

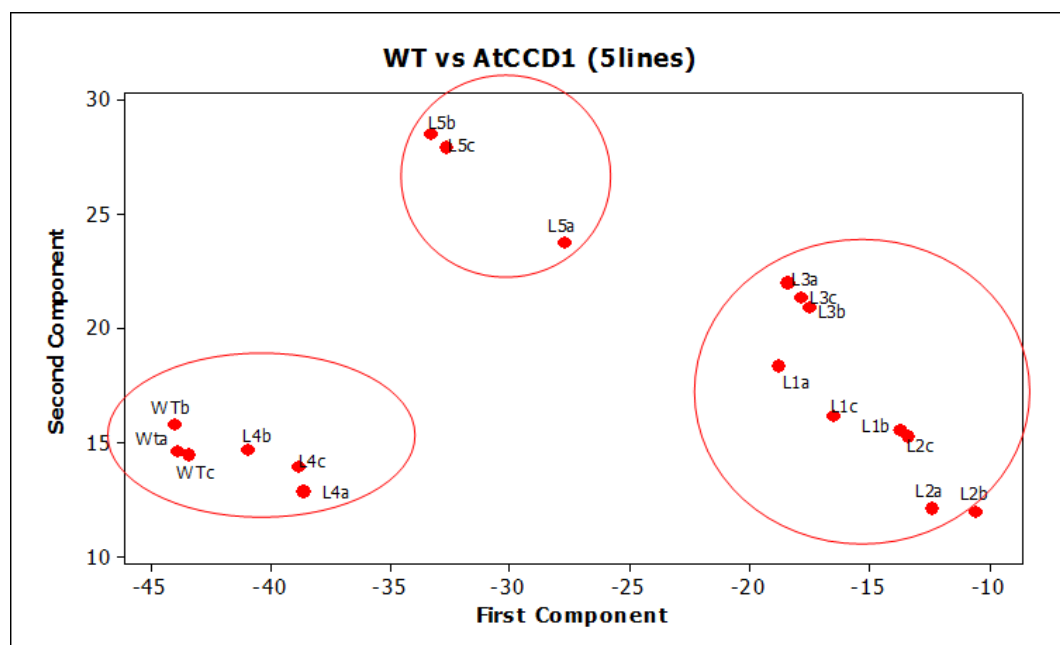


Figure 2.21. PCA plot showing the differentiation of wild type Micro-Tom tomato and 5 lines of *AtCCD1* Micro-Tom Tomato, n=3.

The *AtCCD1* L-1, L-2 and L-3 demonstrated to have a similar emission of volatiles but they were separated from the WT and L-4 group. The reason for this separation was given by the ratio difference between the two most abundant compounds, the monoterpene R- α -pinene (**d**) and the sesquiterpene α -copaene (**v**). *AtCCD1* L-5 was found to make an individual cluster according to the PCA plot. The compounds responsible for this separation were Compound **v** which was found to be 1.4-fold higher in the transgenic line compared to the WT and the sesquiterpenes δ -elemene (**t**) and β -cubebene (**y**) that were found to have two-fold higher emissions in WT plants compared to the transgenic line.

2.3.8.2 WT and *LeCCDI-1* VOCs

The *LeCCDI-1* is one of the two *CCDs* identified in the tomato genome. Five lines demonstrated to overexpress the gene were tested by the volatile analysis method. Flowering WT and *LeCCDI-1* Micro-Tom tomato plants released a similar spectrum of volatiles into the chamber's HS. Volatiles found in *LeCCDI-1-1* were also similar to *AtCCDI*. Table 2.4 shows the relative peak area percentage of the wild type Micro-Tom tomato plants and five lines of the *LeCCDI-1*.

Table 2.4. Mean relative peak areas and (\pm S.E.). % from three measurements of the volatile compounds collected for WT and five *LeCCDI-1* lines of Micro-Tom tomato plants.

Peak	compound	RT(min)	chemical group	Mean peak area percentage relative to R- α -pinene (\pm S.E.). n=3					
				WT	L-1	L-2	L-3	L-4	L-5
a	o-Xylene	8.88	Aromatic	0.15(0.03)	0.2(0.06)	0.36(0.02)	0.31(0.01)	0.27(0.19)	0.28(0.03)
b	Tricyclene	9.80	Monoterpene	0.28(0.01)	0.31(0.04)	0.33(0.03)	0.34(0.02)	0.35(0.08)	0.3(0.12)
c	α -Pinene	9.99	Monoterpene	0.22(0.04)	0.33(0.05)	0.25(0.01)	0.28(0.14)	0.34(0.13)	0.28(0.02)
d	R- α -Pinene	10.22	Monoterpene	33.15(1.42)	35.08(1.23)	38.83(0.79)	38.56(2.08)	32.5(1.98)	37.28(2.18)
e	α -Fenchene	10.59	Monoterpene	0.52(0.18)	0.66(0.15)	0.05(0.03)	0.05(0.01)	0.76(0.31)	0.59(0.05)
f	Camphene	10.67	Monoterpene	0.36(0.08)	0.33(0.04)	0.35(0.04)	0.38(0.01)	0.25(0.03)	0.35(0.05)
g	Unknown	10.78	Unknown	tr	tr	tr	0.04(0.01)	0.12(0.07)	tr
h	Sabinene	11.38	Monoterpene	0.25(0.07)	0.33(0.12)	0.48(0.06)	0.49(0.03)	0.37(0.02)	0.47(0.02)
i	β -Pinene	11.53	Monoterpene	3.15(0.67)	5.74(0.48)	5.61(0.87)	5.78(0.04)	3.27(0.87)	4.45(1.09)
j	3-Carene	12.51	Monoterpene	1.18(0.09)	1.21(0.17)	1.01(0.26)	1.11(0.01)	0.89(0.07)	1.2(0.01)
k	o-Cymene	13.10	Aromatic	0.83(0.04)	0.99(0.15)	0.93(0.03)	0.91(0.05)	0.95(0.12)	0.91(0.31)
l	Limonene	13.20	Monoterpene	0.78(0.12)	0.89(0.13)	0.83(0.27)	0.89(0.09)	0.9(0.09)	0.84(0.03)
m	β -Phellandrene	13.23	Monoterpene	0.82(0.17)	1.04(0.16)	0.95(0.11)	0.93(0.22)	0.94(0.11)	0.93(0.06)
n	Eucalyptol	13.27	Monoterpene	0.31(0.08)	tr	0.45(0.11)	0.48(0.07)	0.43(0.05)	0.16(0.05)
o	γ -Terpinene	14.12	Monoterpene	0.05(0.01)	0.05(0.04)	0.08(0.04)	0.12(0.09)	0.33(0.04)	0.28(0.06)
p	α -Pinene oxide	15.44	Monoterpene	0.22(0.02)	0.28(0.08)	0.2(0.03)	0.19(0.02)	0.34(0.06)	0.25(0.04)
q	Unknown	16.44	Unknown	0.15(0.01)	0.25(0.04)	0.11(0.01)	0.13(0.03)	0.27(0.01)	0.2(0.03)
r	Unknown	16.44	Unknown	0.13(0.01)	0.18(0.03)	0.17(0.08)	0.16(0.01)	0.25(0.03)	0.16(0.05)
s	Unknown	17.55	Unknown	0.19(0.08)	0.21(0.07)	0.23(0.01)	0.25(0.01)	0.31(0.04)	0.2(0.08)
t	δ -Elemene	22.66	Sesquiterpene	0.56(0.03)	0.63(0.05)	0.65(0.01)	0.65(0.01)	0.68(0.01)	0.6(0.07)
u	Cyclosativene	23.62	Sesquiterpene	1.54(0.05)	1.49(0.15)	1.48(0.01)	1.39(0.03)	1.66(0.23)	1.52(0.16)
v	α -Copaene	23.81	Sesquiterpene	11.84(1.76)	8.77(0.61)	7.25(0.79)	7.25(1.3)	4.11(1.84)	5.36(1.24)
w	Sativene	24.27	Sesquiterpene	0.26(0.09)	0.36(0.06)	0.18(0.05)	0.28(0.01)	0.38(0.04)	0.31(0.02)
x	β -Caryophyllene	25.05	Sesquiterpene	3.44(0.89)	2.11(0.44)	2.33(0.35)	2.48(0.48)	3.12(0.28)	2.78(1.04)
y	β -Cubebene	25.39	Sesquiterpene	0.75(0.07)	0.65(0.01)	0.54(0.14)	0.56(0.05)	0.87(0.01)	0.7(0.01)
z	Humulene	26.15	Sesquiterpene	0.95(0.08)	0.88(0.16)	0.89(0.04)	0.87(0.38)	0.87(0.12)	0.92(0.58)
α	Caryophyllene oxide	29.49	Sesquiterpene	0.45(0.04)	0.43(0.08)	0.4(0.01)	0.44(0.04)	0.33(0.04)	0.44(0.08)

tr = trace

LeCCDI-1 = Tomato gene

L=Line

When comparing the emission levels of the individual compounds in the five lines of plants overexpressing the *LeCCDI-1* the following characteristics were observed: 1) the majority of compounds were in the ratio 1:1; the two most abundant monoterpenes in all type of plants were the R- α -pinene (**d**) and the β -pinene (**i**), and 3) the two most abundant sesquiterpenes were the α -copaene (**v**) and the β -caryophyllene (**x**). Pinene is a bicyclic monoterpene and the compounds **d** and **i**, are two structural isomers. This class of compounds are found in the flowers and vegetative parts of plants, including tomatoes. Compound **v** and **x** are both sesquiterpenes, where **v** is a natural tricyclic and **x** is a natural bicyclic found in many essential oils.

Figure 2.22 (A) shows the emission difference of the VOCs extracted in the WT, *LeCCDI-1* L-1, L-2 and L-3. Volatile profiles of L-2 and L-3 were relative close to each other, but wild type plants demonstrated a two-fold reduction on the emission of compound **i** and two-fold incremental increase of compound **v** compared to the same two lines. The ratio **d**:**v** in L-1 was 1:4 while in L-2 and L-3 it was 1:5 and 1:3 in the WT.

Figure 2.22 (B) shows the emission difference of the compounds extracted in WT compared to *LeCCDI-1* L-4 and L-5. There was a three-fold and two-fold reduction in the emission of compound **v** in L-4 and L-5, respectively compared to the WT. The RPA % of compound **d** was similar in WT and L-4 (33.15 and 32.50 % respectively) but L-5 showed a 37.28%.

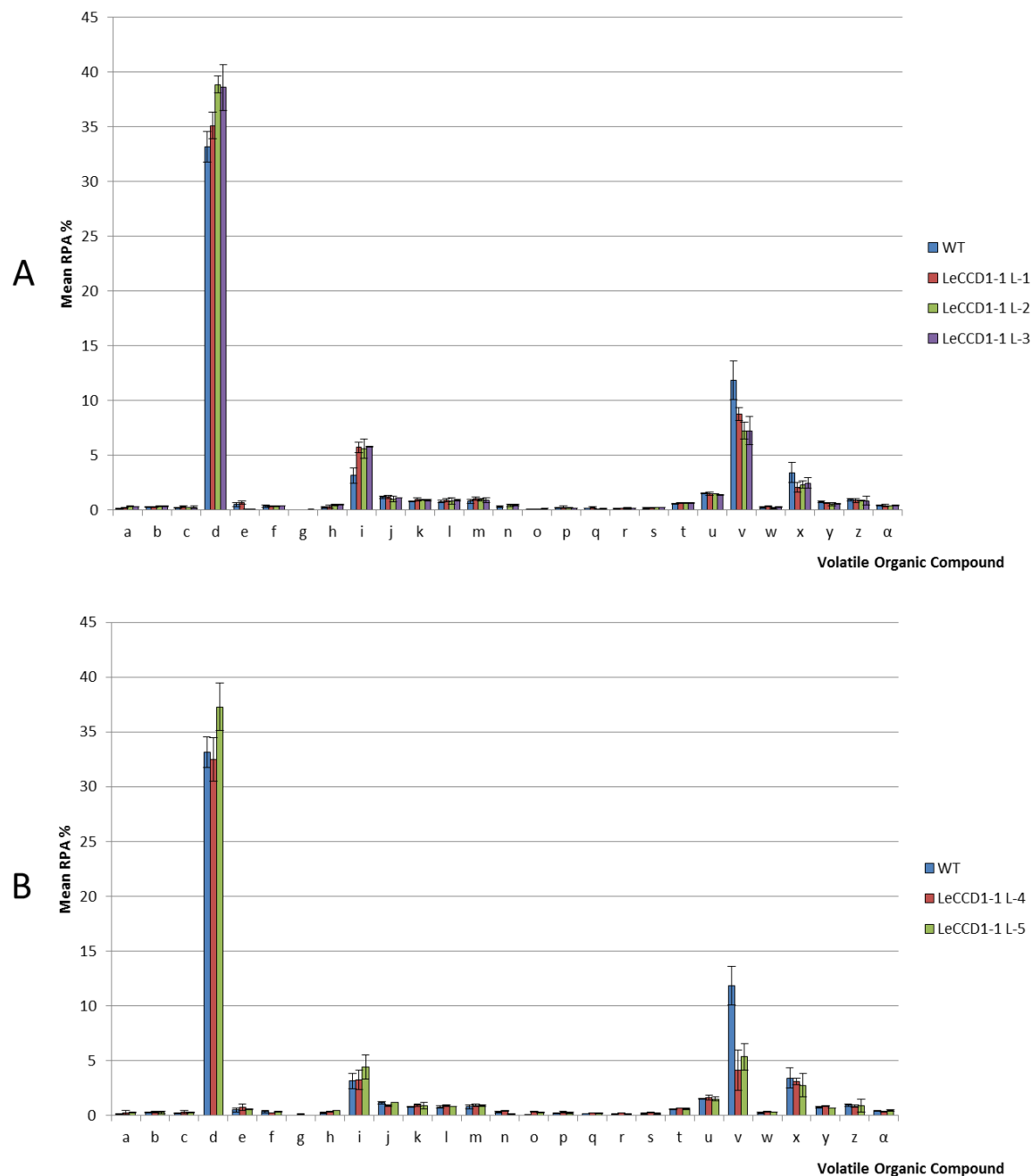


Figure 2.22. Mean relative peak area % (\pm S.E.) of three replicates of the volatile organic compounds identified in the HS for WT and *LeCCD1-1* Micro-Tom plants. (A) Comparison between WT and L-1, L-2 and L-3, (B) Comparison between WT and L-4 and L-5. The VOC label refers to Table 2.4.

Even though plants were releasing similar profiles of volatile organic compounds, small variations in concentration (RPA %) were observed. These differences were mainly due to the changes in emission of the most abundant compounds as it was described before and these variations were denoted in the PCA (Figure 2.23) producing clustering according to the plant type.

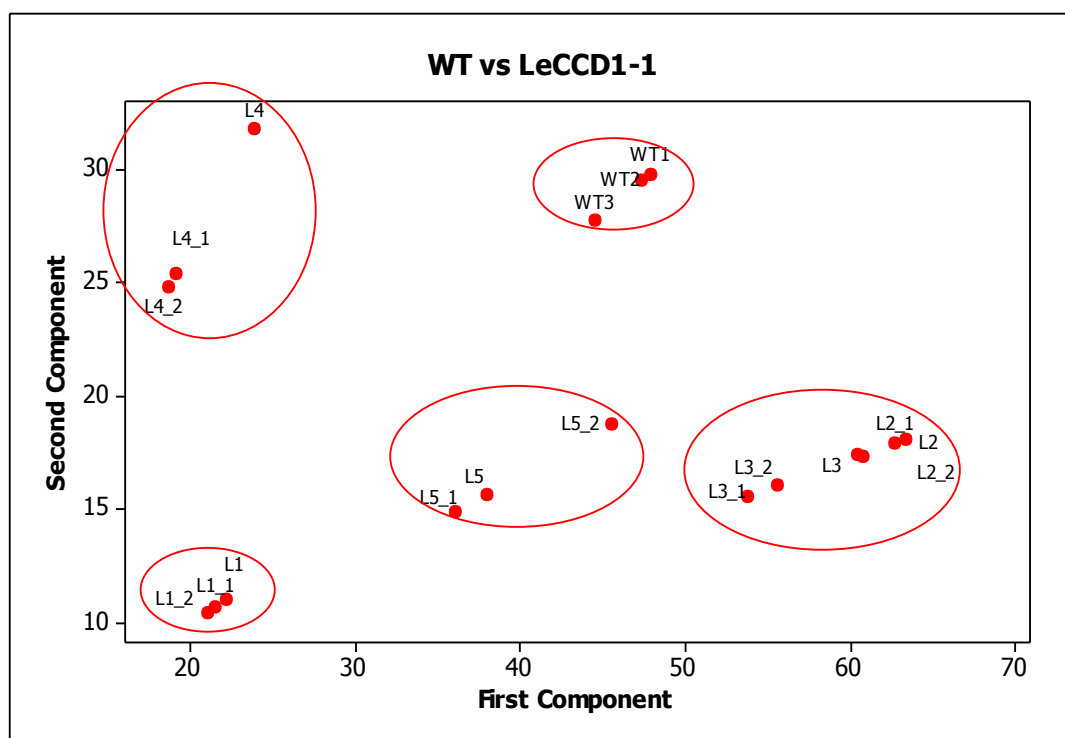


Figure 2.23. PCA plot showing the differentiation of wild type Micro-Tom tomato and 5 lines of *LeCCD1-1* Micro-Tom tomato, n=3.

Even though environmental conditions were kept constant as much as possible, small variability in abiotic factors like light intensity, pressure, humidity, etc, can trigger changes in the natural blend of volatiles. For instance it has been demonstrated that changes in soil, and HS humidity [37], which were factors difficult to control in our experiments, can induce variability in volatiles blends. Additionally, the action of the *LeCCD1-1* gene, of which the level of expression varies among plants, could influence biological pathway mechanisms and as a result variation in volatiles' blend ratios.

2.3.8.3 WT and *LeCCDI-2* VOCs

The *LeCCDI-2* is the other *CCDI* identified in the tomato genome. Four lines demonstrated to overexpress the gene and were tested by the volatile analysis method. Flowering WT and *LeCCDI-2* Micro-Tom tomato plants released a similar spectrum of volatiles into the chamber's HS. Volatiles found in *LeCCDI-2* were also similar to *LeCCDI-1*. Table 2.5 shows the relative peak area percentage of the wild type Micro-Tom tomato plants and four lines of the *LeCCDI-2*.

Table 2.5 Mean relative peak areas and (\pm S.E.). % from three measurements of the volatile compounds collected for WT and four *LeCCDI-2* lines of Micro-Tom tomato plants.

Peak	compound	RT(min)	chemical group	Mean peak area percentage relative to R- α -pinene (\pm S.E.). n=3				
				WT	L-1	L-2	L-3	L-4
a	o-Xylene	8.88	Aromatic	0.15(0.03)	0.13(0.05)	0.12(0.03)	0.11(0.03)	tr
b	Tricyclene	9.80	Monoterpene	0.28(0.01)	0.26(0.04)	0.23(0.03)	0.25(0.02)	0.22(0.08)
c	α -Pinene	9.99	Monoterpene	0.22(0.04)	0.13(0.04)	0.12(0.01)	0.13(0.08)	0.11(0.03)
d	R- α -Pinene	10.22	Monoterpene	33.15(1.42)	33.53(1.23)	35.68(1.15)	32.77(1.18)	33.18(1.98)
e	α -Fenchene	10.59	Monoterpene	0.52(0.18)	0.38(0.15)	0.39(0)	0.41(0.09)	0.35(0.04)
f	Camphene	10.67	Monoterpene	0.36(0.08)	0.32(0.04)	0.35(0.04)	0.38(0.03)	0.33(0.03)
g	Unknown	10.78	Unknown	nd	tr	0.05(0.03)	0.03(0.01)	0.03(0.01)
h	Sabinene	11.38	Monoterpene	0.25(0.07)	0.41(0.12)	0.48(0.06)	0.31(0.08)	0.32(0.04)
i	β -Pinene	11.53	Monoterpene	3.15(0.67)	2.38(0.48)	2.18(0.87)	2.69(0.56)	2.58(0.87)
j	3-Carene	12.51	Monoterpene	1.18(0.09)	0.98(0.17)	0.97(0.26)	1.22(0.09)	1.11(0.07)
k	o-Cymene	13.10	Aromatic	0.83(0.04)	0.77(0.15)	0.89(0.03)	0.75(0.05)	0.68(0.12)
l	Limonene	13.20	Monoterpene	0.78(0.12)	0.99(0.13)	0.87(0.27)	0.88(0.09)	0.72(0.09)
m	β -Phellandrene	13.23	Monoterpene	0.82(0.17)	0.88(0.16)	0.77(0.11)	0.5(0.22)	0.55(0.11)
n	Eucalyptol	13.27	Monoterpene	0.31(0.08)	0.08(0.02)	0.08(0.03)	0.04(0.01)	0.04(0.01)
o	γ -Terpinene	14.12	Monoterpene	0.05(0.01)	0.12(0.04)	tr	0.12(0.03)	0.1(0.04)
p	α -Pinene oxide	15.44	Monoterpene	0.22(0.02)	0.39(0.08)	0.26(0.03)	0.15(0.02)	0.13(0.01)
q	Unknown	16.44	Unknown	0.15(0.01)	0.1(0.03)	0.13(0.01)	0.06(0.03)	0.04(0.01)
r	Unknown	16.44	Unknown	0.13(0.01)	0.08(0.03)	0.16(0.08)	0.09(0.03)	0.06(0.03)
s	Unknown	17.55	Unknown	0.19(0.08)	tr	0.06(0.03)	0.02(0.01)	0.03(0.04)
t	δ -Elemene	22.66	Sesquiterpene	0.56(0.03)	0.62(0.05)	0.67(0.05)	0.68(0.1)	0.71(0.02)
u	Cyclosativene	23.62	Sesquiterpene	1.54(0.05)	1.33(0.15)	1.21(0.13)	1.28(0.14)	1.23(0.23)
v	α -Copaene	23.81	Sesquiterpene	11.84(1.76)	9.06(0.93)	10.94(1.05)	9.89(1.3)	9.32(1.84)
w	Sativene	24.27	Sesquiterpene	0.26(0.09)	tr	0.27(0.05)	0.21(0.08)	0.22(0.04)
x	β -Caryophyllene	25.05	Sesquiterpene	3.44(0.89)	2.98(0.44)	3.58(0.35)	3.31(0.48)	2.94(0.35)
y	β -Cubebene	25.39	Sesquiterpene	0.75(0.07)	0.68(0.05)	0.69(0.14)	0.71(0.1)	0.69(0.16)
z	Humulene	26.15	Sesquiterpene	0.95(0.08)	0.83(0.16)	0.88(0.04)	0.91(0.38)	0.87(0.11)
α	Caryophyllene oxide	29.49	Sesquiterpene	0.45(0.04)	0.33(0.12)	0.39(0.15)	0.35(0.08)	0.38(0.12)

tr = trace

nd = no detected

LeCCDI-2 = Tomato gene

L=Line

27 VOCs were identified in the HS of the flowering stage WT and *LeCCDI-2* Micro-Tom tomato plants. Profiles were similar to the volatiles observed in the *AtCCDI* and *LeCCDI-1* with variations in their relative peak area. Compared to the other two tomato genes, there was less variation in the relative area of the individual components of the corresponding transgenic lines and the WT. This could be due to the fact that this group of plants have less variation in the overall plant size compared to each other Figure 2.24.



Figure 2.24. Flowering stage WT and *LeCCDI-2* Micro-Tom tomato plants.

Figure 2.25 shows the main difference between the volatiles released by the WT and the four transgenic plants. All the volatiles were in the ratio 1:1. Some variations, which were marginal, but still important to note were: 1) the monoterpene sabinene (**h**), was found to be two-fold higher in the transgenic L-2 compared to the wild type; 2) compound **v** was detected among all plant types, but there was a difference between the emission in the WT (11.84%) compared to the transgenic lines, with RPA % of 9.06, 10.94, 9.89 and 9.32 respectively; and 3) the compound **d** which was in the 1:1 ratio in all plant types, had a 35.68% emission in L-2 compared to 33.15, 33.53, 32.77 and 33.18 in WT, L-1, L-3 and L-4 respectively. Compound **h** has been detected in the leaves of tomato (*Lycopersicon esculentum*) plants before [38] and recognized to be increased

among the volatile blend emitted by the tomato plant under biotic stress [39]. It was found to be two-fold higher in the transgenic L-2 compared to the wild type.

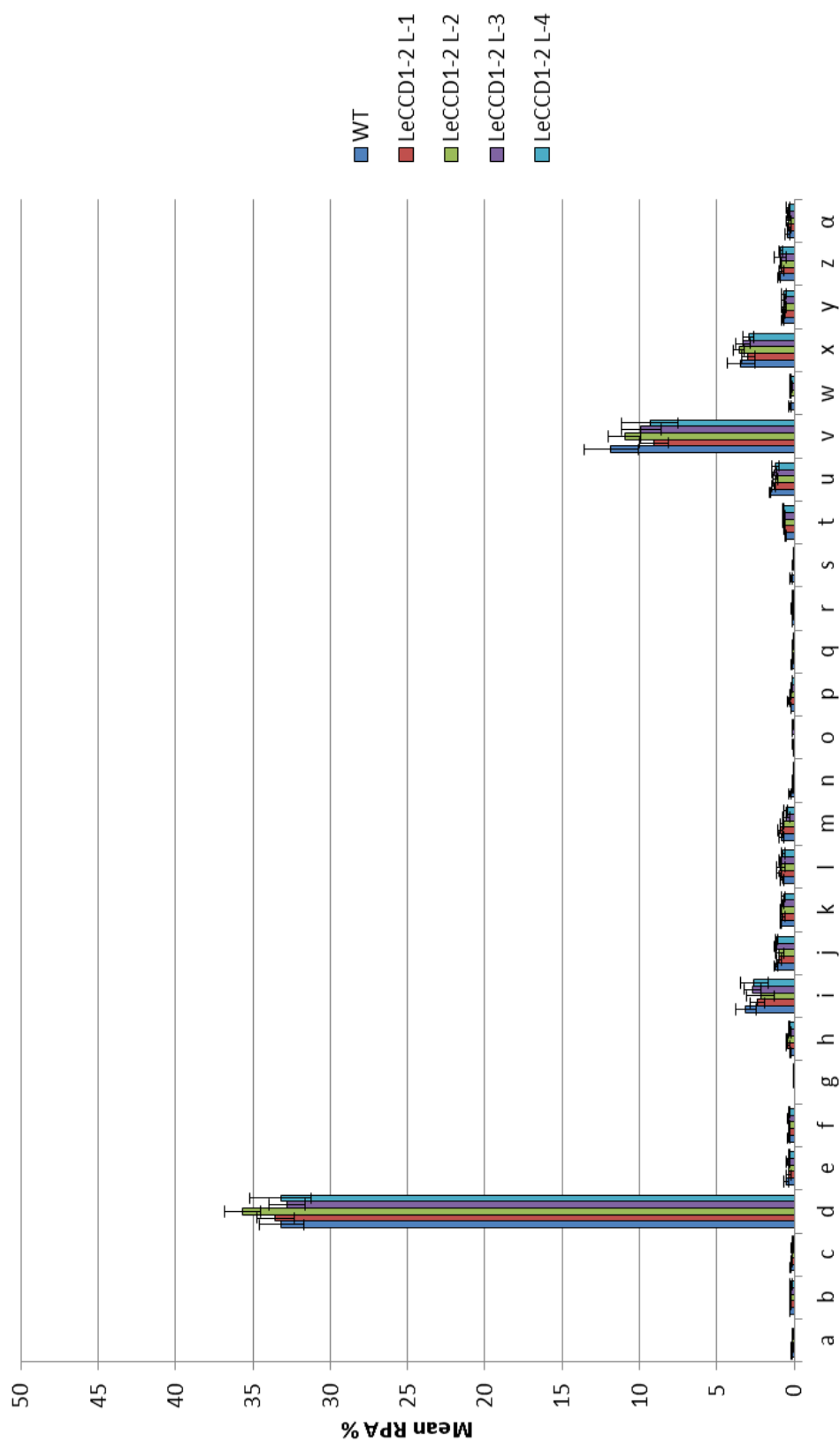


Figure 2.25. Mean relative peak area % (\pm S.E.) of three replicates of the volatile organic compounds identified in the HS for WT and *LeCCD1* L-1, L-2, L-3 and L-4 Micro-Tom plants. VOC label refers to Table 2.5.

The PCA plot (Figure 2.26) was judged by visual inspection for the distance within the cluster and the distance between the clusters. The results were presented as a two-dimensional plot of sample scores in the space defined by the PCA. When plotting the WT and the four lines of the *LeCCD1-2* the clustering of the groups were not as differentiated as the other tomato genes.

The relative peak area of the sesquiterpene α -copaene (**v**) was 11.84% in WT plants, meanwhile transgenic plants *LeCCD1-2* L-1, L-2, L-3 and L-4 were 9.06, 10.94, 9.89 and 9.32 respectively. This reduction in emission grouped the WT plants apart from the other lines. Likewise, the volatile organic compound R- α -pinene (**d**), which was the most abundant in all plants were found to be released in higher proportion in L-2 (35.68%) compared to the WT (33.15%) and the other transgenic lines L-1 (33.53%), L-3 (32.77%) and L-4 (33.18%).

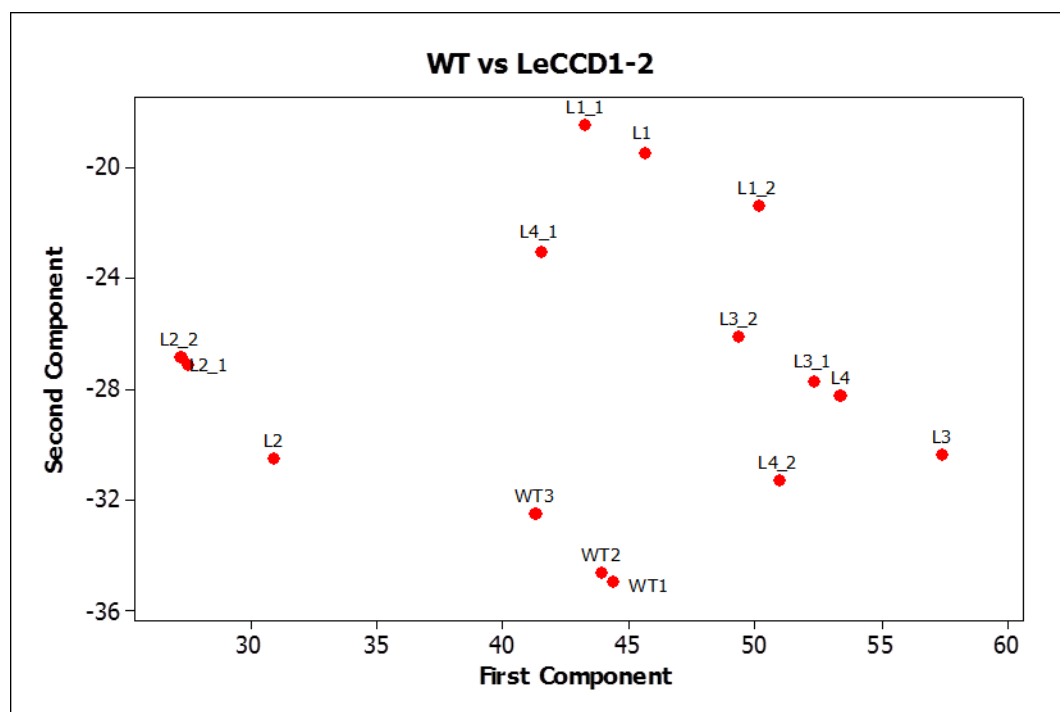


Figure 2.26. PCA plot showing the differentiation of wild type Micro-Tom tomato and 4 lines of *LeCCD1-2* Micro-Tom tomato, n=3.

AtCCDI-2 L-1, L-3 and L-4 did not show distinguishable grouping due to the less variation on the individual VOCs compared to the WT and L-2.

2.3.9 Conclusions from the Micro-Tom Tomato DHS-GC-MS study

Although transgenic Micro-Tom tomato plants over expressed the *AtCCDI*, *LeCCDI-1* and *LeCCDI-2* genes, the DHS-GC-MS method did not detect compound β -ionone (**19**) and/or other apocarotenoid molecules. The presence of β -ionone was also not reported in other *in vivo* HS studies of tomato plants over expressing these genes in the literature [2,4,5]

In vitro studies on ripened tomato fruits (different from Micro-Tom tomato species) over expressing the *LeCCDI-1* and *LeCCDI-2* genes had demonstrated the presence of β -ionone [4]. In that study they reported that these two genes catalyzed the symmetric cleavage of multiple cyclic carotenoids *in vitro* to produce β -ionone and pseudo-ionone. This confirmed that the *CCDI* gene served the same function in tomato plants as in *Arabidopsis* plants, but the resulting apocarotenoid compounds were localized in the tomato fruits and not released as volatiles into the environment.

If transgenic plants were releasing this compound in small ratios and low concentration that were below the extraction capabilities of the DHS system, a more sensitive method should be created in order to evaluate the function of the tomato genes in the formation of any apocarotenoid compound.

Similar to the *Arabidopsis thaliana* analysis, the *in vivo* non-targeted DHS-GC-MS approach developed in this study allowed for the extraction, detection and semi-quantitation of the most realistic volatile profile. Additionally, it helped to illustrate the differences in volatile changes in emissions due to gene transformation and/or biotic and abiotic factors.

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Chapter 3: Non-targeted Static Headspace (SHS) GC-MS Analysis of plant Volatile Organic Compounds (VOCs)

A revised version of this chapter has been published and reproduced in part with permission from **L.A. Cáceres**, T.W. McDowell, I.M. Scott, A. Hannoufa, B.D. McGarvey, L. Tian, K.K.-C. Yeung & M.W. Sumarah. In vivo extraction of volatile organic compounds (VOCs) from Micro-Tom tomato flowers with multiple solid phase microextraction (SPME) fibers, *Canadian Journal of Chemistry*, **2014**, 1-8.

A Dynamic Headspace (DHS), also called “purge and trap”, coupled with GC-MS for the non-targeted analysis of VOCs in whole-plants was described in chapter two. The dynamic system, with a controlled flow of compressed air, was used to carry VOCs onto a sorbent tube where volatiles were accumulated for further desorption and analysis. The extraction method and non-targeted analysis allowed for the *in vivo* characterization and evaluation of volatile profile of the plants. In chapter three a different strategy, called “Static Headspace (SHS)” with Solid Phase Microextraction (SPME) fibers, coupled with GC-MS was developed for the analysis of volatiles from specific plant organs (flowers). SPME, which combines extraction and concentration processes simultaneously, is a simple, fast, sensitive and solvent-free technique and was used to determine VOCs of WT and genetically transformed Micro-Tom tomato plants overexpressing the *LeCCDI* gene. The SHS is a different extraction strategy compared to DHS, but the *in vivo* non-targeted analysis used to identify and evaluate volatile emissions was similar to the one used in chapter two. However, this application of SPME fibres is novel as it is the first time three SPME fibers of different physical and chemical properties were used simultaneously to maximize the extraction selectivity. The extraction and analysis of volatiles from flowers only was used for three reasons: 1) the majority of volatiles from tomato plants are released by the flowers which are easy to concentrate in a small HS (glass adaptor); 2) since the HS is small, SPME extraction efficiency is increased compared to the one used in the dynamic system thus allowing the extraction and detection of VOCs at the trace levels; and 3) since the strategy is expected to be sensitive, it should help determine changes in volatiles due to the overexpression of the *LeCCDI* gene.

3.1 Introduction

Plants interact with the environment by releasing VOCs into the atmosphere. Plant volatiles are produced by several organs of the plant with the majority released by the flowers, mainly to attract pollinators for subsequent reproduction [1]. Studies of floral volatile emission in recent decades has been partly driven by the discovery of new

aromatic notes for the perfume and fragrance industry [2]. More importantly, VOC analysis provides information on plant physiology and how plants interact with their surroundings. VOCs diffuse through the air, directly and indirectly influencing other plants, insects, third trophic level species, and even human beings [3]. An important research area in plant biology is to genetically alter a plant's VOC emission, thereby manipulating its ecological impacts; for example, to enhance the production of natural compounds that can deter insect pests. This approach is advantageous over the conventional use of chemical pesticides, which generally have negative impacts to humans and the environment [4]. Additionally, the overuse of pesticides has led to insecticide resistance and the decline of beneficial insects such as pollinators, predators and parasitoids [5].

Many approaches are available for VOC collection. In general, a sample plant is enclosed in a chamber or vial with or without air circulation. This is respectively termed as “dynamic” or “static” headspace (HS) collection.[6] The collection can be also performed *in vivo*, *in situ* or *in vitro* with plant organs or whole plants. *In vitro* HS analysis refers to extractions performed on organs removed from plants. Samples are often chopped or ground to maximize recovery, but the VOC profile may differ from that of a live plant. On the other hand, *in situ* and *in vivo* experiments, two concepts that some authors have used interchangeably, refer to the HS collection of volatiles from living plants or organs. Ultimately, the selection of suitable methods depends on whether the collection is being performed in the field or in the laboratory environment, as well as the biological questions being addressed [6].

Various sampling approaches for floral VOCs have been reported under different circumstances. In particular, solid phase microextraction (SPME) is often preferred in the HS analysis of flowers because extraction of volatiles is fast, it does not require solvents, and it can capture VOCs at the parts per billion level from the small sampling volume of flowers [6]. SPME is based on adsorption/absorption and desorption of volatiles from an inert fiber coated with various types of ad/absorbents, making selection of the appropriate fiber coating a critical step. In adsorption VOCs are attracted

to the outer surface of the fiber coating, while absorption VOCs interact with the fiber coating's structure. In general, the main criteria used in SPME fiber selection are molecular weight, size, shape, polarity, and concentration of the analytes [7]. These criteria will determine how the volatile compounds will interact and how fast they will move in and out of the fiber matrix to reach equilibrium. Large selections of products are available for specific or targeted VOC extraction applications from the manufacturer, Supelco (Sigma-Aldrich, St. Louis, MO, USA). Fibers recommended by Supelco for static HS collection of volatiles are classified based on thickness, and more importantly, matrix polarity. Common examples include non-polar non-bonded polydimethylsiloxane (PDMS), bipolar cross-linked PDMS-divinylbenzene (DVB), polar polyacrylate cross-linked (PA), bipolar cross-linked carboxen (CAR)-PDMS, polar cross-linked carbowax (CW)-DVB, polar cross-linked carbowax-templated resin (TPR), and bipolar cross-linked DVB-PDMS-CAR) [8]. A number of reports in the literature compared the performance of SPME fibers for several applications related to volatile compounds. For example the HS-SPME analysis of the volatile metabolome from ripe tomato fruits compared the total GC-MS peak areas obtained from six fibers and found that the DVB/CAR/PDMS, CAR/PDMS and DVB/PDMS had better extraction performance over the PDMS, Polyethylene Glycol (PEG) and PA fibers [9]. In another study, three fibers were compared in the *in vivo* HS-SPME analysis of five selected VOCs, from two types of *Osmanthus* flowers (*Osmanthus fragrans* var. *latifolius* and *O. fragrans* var. *thunbergii*). It was determined that the carbowax (CW)/PDMS had better extraction capabilities than the PDMS and PDMS/DVB fibers [10]. The *in vivo* HS-SPME of the Chinese daffodil flower determined that the highest extraction efficiencies were observed for most compounds using the CAR/PDMS fiber, except for the two compounds, α -linalool and benzeneproyl acetate, where PDMS/DVB was the best fiber [11]. In the extraction of VOCs from zucchini flowers (*Curcubita pepo* L.) [12], valeriana flowers (*Patrinia scabiosifolia*) [13], blue bird flowers (*Delphinium elatum* L.) and orange jessamine flowers (*Murray paniculata* L.) [14, 15], the PDMS/DVB fiber was chosen to be the most suitable. Based on these reports, fibers with certain polar characteristics such as PDMS/DVB, CAR/PDMS or CW/PDMS

generally resulted in better extraction performance, but clearly there was not a common consensus on the most suitable selection of SPME fibers for floral VOC analysis.

The discord in fiber selection is likely due to the complexity of the sample matrices and diversity in VOC profile. VOC composition can be highly variable due to floral phenotypes with a range of spatial and temporal dimensions within a flowering plant group. Specifically, VOCs may vary within and between populations of the same plant species and can greatly change based on other abiotic factors such as season, temperature, water, light intensity, pressure, relative humidity and time of day [16]. Generally, plant VOCs encompass a complex mixture of low molecular weight lipophilic compounds derived from different biosynthetic pathways, typically including terpenoids, fatty and amino acid derived products, phenylpropanoids and saturated and unsaturated hydrocarbons with their oxygenated derivatives [17]. Some compounds are difficult to differentiate as they have very similar size and molecular weight; e.g., the isomers of the monocyclic monoterpenes, α , β , γ , and δ -terpinene, the bicyclic monoterpenes, α and β -pinene, and the sesquiterpenes β -caryophyllene and α -humulene. In addition, volatile emissions can vary widely in concentration due to growth stage, plant type and variety. Taking all of these considerations into account, the selection of SPME fibers will critically influence the outcome of the VOCs analysis. Instead of debating the selection of one optimum fiber, the simultaneous use of multiple fibers with different characteristics may be a more effective way to maximize the recovery of VOCs.

In this chapter, the use of multiple fibers simultaneously was examined for the purpose of maximizing the extraction of VOCs in the semi-quantitative, untargeted, analysis of floral VOCs from Micro-Tom tomato plants overexpressing the *LeCCDI*. To my knowledge, such simultaneous use of multiple fibers has not been reported in the literature. A combination of three fibers was selected for this study: PDMS/DVB, PDMS and PDMS/CAR. They are distributed by Supelco as a package for the extraction of VOCs, but their simultaneous use has not been suggested. According to the literature, the hydrophobic surface of the PDMS-coated fibers is most sensitive to non-polar

compounds, the CAR/PDMS is sensitive to small molecules because of the small and uniformly porous size [2], and the bonded PDMS/DVB is most suitable for analytes in high concentration and for samples that contain multiple analytes. The different polarity properties of these fibers should in theory offer complementary characteristics of selectivity.

3.2 Materials and Methods

3.2.1 Plant Material

Transgenic tomato (*Solanum lycopersicum* cv. Micro-Tom) plants with enhanced expression of LeCCD1 gene were generated by *Agrobacterium*-mediated transformation [18]. Seeds of wild type and transgenic tomato were grown in pots containing 58 g of professional growing medium soil PROMIX BX (Premier Horticulture Inc., Brantford, ON). The plants were cultivated at the Greenhouse and Processing Crops Research Centre (Agriculture and Agri-Food Canada, Harrow, ON). Growth conditions were 23 ± 2 °C, 40-70 % RH, and 16:8 h day: night photoperiod with supplemental lighting. During cultivation, plants were protected from biotic and abiotic damage. When plants reached the flowering stage (6 week-old), they were moved to a laboratory room (22 °C ± 2 °C and 30-60 % RH) for VOC collection.

3.2.2 Reagents and Standards

The following analytical standards were purchased by Sigma-Aldrich (SAFC, Oakville, ON, Canada); β -ionone, α -ionone and dihydro- β -ionone $\geq 90\%$ were used to evaluate SPME fiber extraction performances. Dichloromethane HPLC grade, (Caledon, Georgetown ON, Canada) was used to make solutions of the analytical standards.

3.2.3 Solid Phase Microextraction (SPME) fibers

The SPME fiber assembly kit (Supelco Analytical, Bellefonte, PA), containing three 24Ga fused silica fibers (100 μm PDMS, 75 μm CAR/PDMS and 65 μm PDMS/DVB) was selected for this work based on the physical and chemical properties required for the extraction of VOCs. The size of these fibers refers to the diameters, which in turn relates to the thickness of the extraction phases. Generally, thicker phases favour higher recovery, while thinner SPME phases favour faster equilibration. In all three cases, the chosen sizes were intermediate in the ranges available. Fibres were introduced into the HS of the plants and into the GC injector port using a manual SPME holder. Figure 3.1 shows the three SPME fibers used for this study.

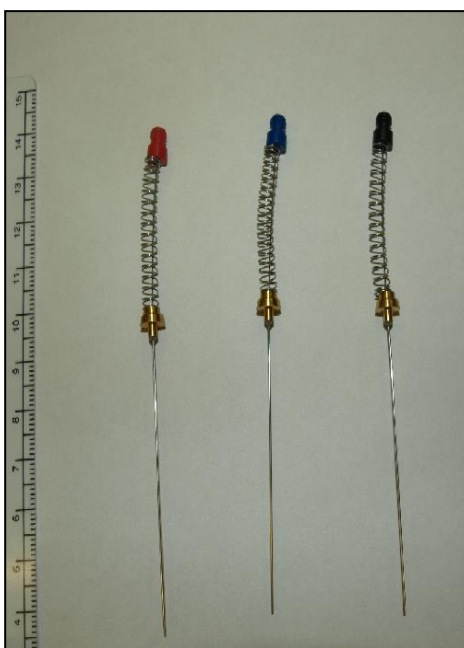


Figure 3.1. Solid Phase Microextraction (SPME) fibers used in the extraction of VOCs from the Micro-Tom tomato flowers HS. Three fibers were used; 100 μm PDMS (red), 65 μm PDMS/DVB (blue) and 75 μm CAR/PDMS (black).

3.2.4 Gas Chromatography Method

The GC-MS instrument from Agilent Technologies (Santa Clara, CA USA) used for sample injection, separation and detection of VOCs was equipped with an autosampler

(7693), gas chromatograph system (7890 A) and inert XL EI/CI MSD with triple-axis detector (5975C).

An inlet temperature of 250 °C was chosen for fast volatilization and desorption of compounds from the fiber. A pulsed/splitless mode using a 0.75 mm i.d. liner (Supelco, Bellefonte, PA) was used to control the pressure at the inlet part of the instrument. The injection pulse pressure was 25 psi to transfer VOCs out of the inlet into the capillary column faster, thereby reducing chance for sample decomposition and discrimination. After 0.5 min the inlet pressure went to normal (12.445 psi). The purge flow to split vent was 40 mL/min at 1 min, which was the flow rate used and the time to sweep away residual vapors after the beginning of the run. Fibers were desorbed in the inlet port for 10 min and VOCs were separated in a DB-5MSDG capillary column (30 m x 0.25 mm, 0.25 µm). The oven gradient was programmed starting with 30 °C for 1 min and then increasing at 5 °C/min to 200 °C where it was held for 1 min. Helium (99.999%) was used as the carrier gas maintained at a flow rate of 1 mL/min with a column head pressure of 12.445 psi. Full scan spectra were generated between 40 and 350 amu at a rate of 4.51 scans/sec.

3.2.5 SPME Fibers Extraction Performance

Prior to use, each SPME fiber was thermally conditioned in the GC injector according to manufacturer guidelines. To determine the best time for the fibers to extract VOCs, 25 µL of a 0.3 ng/µL solution in DCM of three apocarotenoid standards (β -ionone, α -ionone and dihydro- β -ionone) was used. The solution was carefully added into a sealed 15 mL glass adaptor and the three SPME fibers were exposed simultaneously into the adaptor HS for a determined period of time. Three replicates at 5, 10, 15, 20, 25 and 30 minutes were performed and extraction efficiency was determined based on the peak area count in the TIC.

3.2.6 VOCs Extraction from Micro-Tom Tomato Flowers

Four replicates of wild type and transgenic tomato plants overexpressing the *carotenoid cleavage dioxygenase 1* gene (*CCDI*) were used for this study. Inflorescences containing 2 to 4 open flowers were carefully enclosed using a 15 mL glass adaptor which ends were closed with parafilm (“M” laboratory film, Neenah, WI) to ensure proper inflorescence sealing without injuring and stressing the flowers. After a 24-h accumulation period, the three SPME fibers were inserted through the parafilm, and were attached to the adaptor as shown in Figure 3.2. Fibers were exposed to the flower’s HS at laboratory conditions of temperature ($22\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$), with a photoperiod of 16 h, light intensity of 90-100 $\mu\text{mol}/\text{m}^2/\text{s}$, relative humidity of 30-60 % and for an extraction time of 20 min. Preliminary experiments with the fibers and apocarotenoid standards demonstrated that a 20-min extraction time achieved the best overall extraction efficiency. Fibers were extracted at the GC inlet port within a storage time of less than 72 min. According to the manufacturer, VOCs can be kept on fibers up to 24 h without significant loss. Internal standards were not introduced into the HS so that there was no interference with the natural environment of the flowers and to avoid piercing the sealed HS. Background subtraction was achieved by analyzing fibers exposed to empty sealed glass adaptors.



Figure 3.2. Experimental setup for the *in vivo* sampling of Micro-Tom flowers. VOCs were allowed to accumulate in the vial for 24 h prior to the introduction of three SPME fibers (extraction time 20 min).

3.2.7 VOCs Analysis

Compounds were identified using Automated Mass Spectral Deconvolution and Identification System (AMDIS) GC-MS analysis (Version 2.71, June 12, 2012), with the Mass Spectral Search Program from the National Institute of Standards and Technology (NIST) (Version 2.0 g, December 4, 2012).

The GC-MS spectra of 24 samples were deconvoluted using AMDIS with the following parameters: adjacent peak subtraction = 2; Resolution – High; Sensitivity – Very High; Shape Requirements – Medium. A library of known compounds was compiled within AMDIS using the NIST database (Version 2.71, Jun 12, 2012) as a

reference. The *.ELU files resulting from AMDIS processing were then uploaded to the SpectConnect web tool (freely available at <http://www.spectconnect.mit.edu>) along with the *.MSL library file. The SpectConnect processing was performed using the following settings: Elution time threshold – 2 min; Support threshold – 50%; Similarity threshold – 70%; Library similarity threshold – 60%. Based on these parameters, SpectConnect generated a matrix of integrated signal (IS) data. The aligned retention times and peak integration from SpectConnect were manually checked in order to eliminate background peaks and silica containing contaminants from the SPME and septum. The IS metabolite matrix was filtered by adding the area of those compounds sharing similar retention times, m/z or which were identified by the NIST library as a similar match. Finally, a signal-to-noise ratio (S:N) of 50:1 and 50% hit rate for both wild type and transgenic plants were used as common parameter in order to be considered as real peaks. One-way ANOVA (Proc. GLM, Statistical Analysis System, Server Interface Ver. 2.03, SAS Institute, Cary, NC) was used to test for significant differences in the total area peak of the replicates.

Principal component analysis (PCA) on the Log [n+1] transformed data of the combined fibers was used to compare and evaluate fiber performance and to differentiate VOCs from wild type and transgenic Micro-Tom plants. PCA was performed using Minitab release 14.13 statistical software (1972-2004 Minitab Inc. State college, PA). Venn diagrams were created in Microsoft Excel based on repetition of signals; if at least 2 out of 4 samples occurred at greater than S:N of 50:1 for a particular metabolite, the retention time for that metabolite was added to the Venn data pool.

3.3 Results and Discussion

3.3.1 Effect of SPME Sampling Time on VOC Extraction

SPME is an equilibrium extraction technique in which analytes from the HS of the sample matrix are directly adsorbed and concentrated to the extraction fiber. The SPME fiber itself is a thin fused-silica optical fibre coated with a thin polymer film (Figure 3.3) and for this case PDMS/DVB, PDMS or CAR/PDMS.

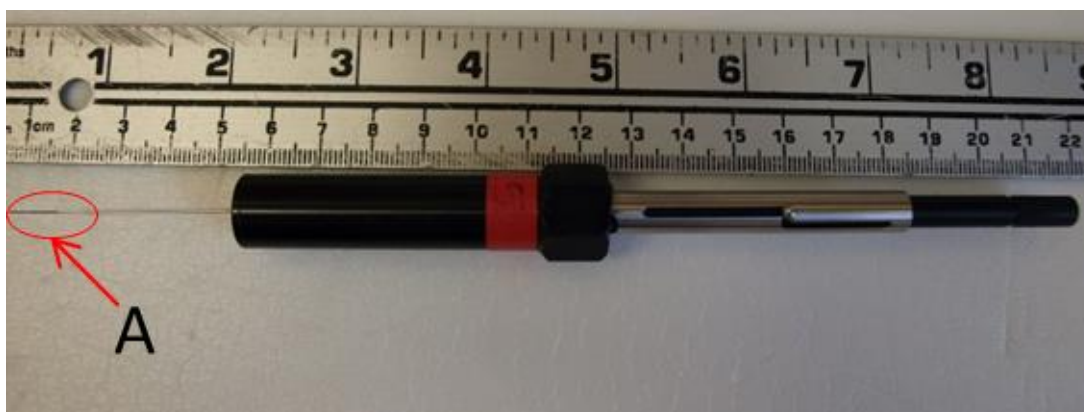


Figure 3.3. SPME holder with an exposed 1 cm long 75 μm CAR/PDMS fiber (A).

Several factors influence the extraction of VOCs, some of which are related directly to the SPME device coating. In this study three fibers with different chemical and physical properties were selected to cover the extraction of a wide variety of compounds. As described in section 3.2.6 experimental conditions of temperature, light intensity and RH were kept constant. To determine the best overall extraction efficiency of the fibers a solution of three apocarotenoids and possible products of the cleavage action of the *CCD1* gene were used. The concentration of the solution containing β -ionone, α -ionone and dihydro- β -ionone (0.3 $\text{ng}/\mu\text{L}$) was chosen based on the concentration of β -ionone found in the DHS study of *Arabidopsis* plants overexpressing the *CCD1*. As it can be depicted in Figure 3.4, the overall higher extraction efficiency using simultaneously three SPME fibers was reached at 20 min for the case of the

PDMS/DVB and PDMS fibers. In the case of the CAR/PDMS the best extraction efficiency of the compounds α -ionone and dihydro- β -ionone was observed at 25 min. Based on the peak area count it was determined that the PDMS/DVB performed better in the extraction of the standards compared to the PDMS and CAR/PDMS respectively.

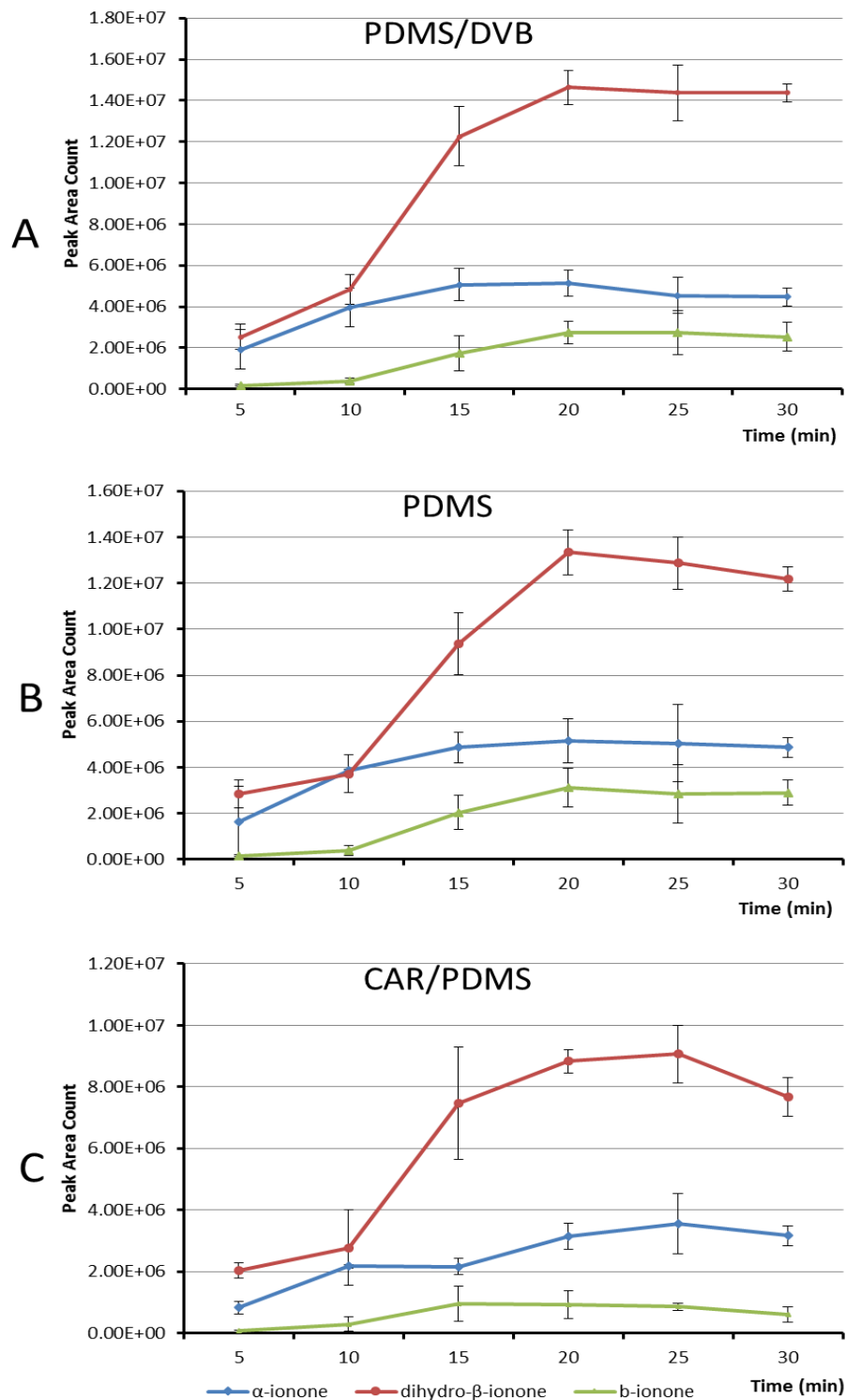


Figure 3.4. Peak area count (\pm S.E.) of three replicates of the compounds α -ionone, dihydro- β -ionone and β -ionone sampled at six different times using three SPME fibers: (A) PDMS/DVB; (B) PDMS; and (C) CAR/PDMS.

Since all fibers were introduced simultaneously, the overall extraction time of 20 minutes was used for the non-targeted analysis of VOC in the tomato flowers.

3.3.2 VOC Extraction Efficiency

The overall efficiencies of the selected three SPME fibers for extracting VOCs were assessed by examining the total GC-MS peak areas (Figure 3.5). Because the amount of VOCs accumulated in each glass adapter is expected to be directly proportional to the number of flowers enclosed within, the peak areas displayed in Figure 3.5 were normalized to the number of flowers present. Results showed that PDMS/DVB was more efficient than the other two fibers in both tomato types with significantly higher peak areas (One-way ANOVA; $d.f.=3$; $F=7.39$; $p<0.0001$). The PDMS/DVB fiber had 5.6-fold higher extraction efficiency than PDMS. Figueira et al. found comparable extraction efficiency between the same two fibers in the HS-SPME analysis of volatile metabolites from ripe tomato fruits [9]. Therein, PDMS/DVB was approximately 7-fold more efficient than PDMS according to the total peak area of the metabolites measured. However, the higher efficiency of PDMS/DVB compared to CAR/PDMS observed in this work (3.4-fold) was not observed in their study [9]. The *in vivo* study of 9 VOCs from daffodil flowers (*Narcissus jonquilla* L. Amaryllidoideae) using 6 SPME fibers also reported that PDMS exhibited lower extraction efficiency than PDMS/DVB and CAR/PDMS [11], but the best overall extraction was attributed to the CAR/PDMS. In a contrasting study, the *in vivo* HS-SPME analysis of 5 VOCs from two varieties of Osmanthus flowers (*O. fragrans* var. *latifolius* and *O. fragrans* var. *thunbergii* Oleaceae) found no difference in the overall extraction efficiency of the PDMS and PDMS/DVB [10].

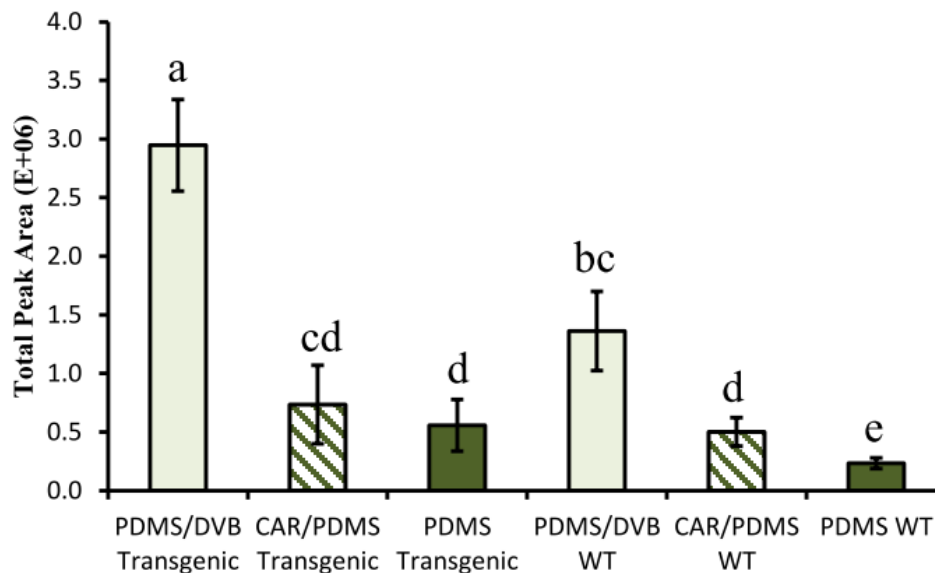


Figure 3.5. Average total peak areas of VOCs detected from transgenic and wild type (WT) plants with the three different SPME fibers used. The error bars represent \pm S.E. based on four replicates. The letters above the error bars indicate whether the data sets are statistically different from one another ($p < 0.0001$).

In addition to comparing the total peak areas for each fiber, the individual VOCs detected from each of the three SPME fibers was considered (Table 3.1). Firstly, a total of 50 compounds were detected from the two Micro-Tom types, transgenic and wild type. Sixteen of the 50 compounds were identified by the NIST library with AMDIS as plant volatiles with a mass spectra match factor greater than 900 (chemical structures shown in Figure 3.6). The remaining 34 compounds were classified as unknown. As expected from the general trend observed in Figure 3.5, the PDMS/DVB fiber extracted the greatest number of volatile compounds from the transgenic and wild type plants, i.e. 43 and 31, respectively. The CAR/PDMS and PDMS fibers each extracted 22 and 19 compounds from the transgenic plants and 15 and 12 compounds from the wild type plants, respectively. It is noteworthy that β -ionone and other expected apocarotenoid compounds resulting from CCD gene transformation were not present. This allows us to conclude that either the CCD1 gene did not serve to cleave the carotenoid substrates as intended in tomatoes, or the resulting apocarotenoid compounds were indeed produced but not release into the headspace sampled by our methods.

Table 3.1. A list of compounds identified by AMDIS with the NIST library using three fibers from transgenic and wild type Micro-Tom flowers.

RT (min)	VOC	No.	<i>m/z</i>	Transgenic Micro-Tom			Wild type Micro-Tom		
				PDMS/DVB	CAR/PDMS	PDMS	PDMS/DVB	CAR/PDMS	PDMS
5.983	unknown	1	41	+					
6.038	unknown	2	41	+					
8.815	unknown	3	43	+					
9.536	Tricyclene	4	93	+++++	+++++	+++	++		++
9.583	unknown	5	93	+++		+			
9.584	unknown	6	93	++	+++		+	+++++	
9.691	β -Thujene	7	93	+++++++	+++++	+++++	+++++	+++++	+
10.014	(1S)- α -Pinene	8	93	+++++	+++++	+++++	+++++	+++++	+++
10.053	unknown	9	91				++++		
10.180	unknown	10	91	+++++	++			+++++	
10.263	(1R)- α -Pinene	11	93	+++++++	+++++	+++++	+++++	+++++	+++++
10.413	unknown	12	121		+++++			+	
10.489	Camphene	13	92	+++++++	+++	+	+++++	+++++	+
10.549	unknown	14	91				+		
10.560	unknown	15	91	++++					
10.628	unknown	16	91		++++	+++++			
11.203	Sabinene	17	91	+++++	+	++	+		
11.224	unknown	18	92	+					
11.228	unknown	19	137	+			+		
11.231	unknown	20	91	+++			+		
11.394	β -Pinene	21	93	+++++++	+++++	+++++	+++++	+++	
11.479	unknown	22	77	+	+		+		+
11.545	unknown	23	136					+	
11.768	unknown	24	282	+					
12.067	unknown	25	91	+++++			+		
12.332	α -Phellandrene	26	92	+++++++	+++++	+	+++++	+	
12.378	unknown	27	91	+	+				
12.656	α -Terpinolen	28	121	+++	+	+	++		
12.944	unknown	29	119	++++	+		+		
13.002	unknown	30	119	+			+		
13.048	<i>o</i> -Cymene	31	119	+++++	++		+		
13.065	Limonene	32	68	+++++	++++	+++	+++++		
13.095	unknown	33	68	+++++					
13.176	unknown	34	136	+++					
14.080	γ -Terpinene	35	136	+++			+		
14.105	unknown	36	77	+					
14.934	4-Carene	37	91	+++++++	+++++	+	++++	+	
15.597	unknown	38	267	+					
16.746	unknown	39	267	+					
16.753	unknown	40	73	+++	+	+	+		+
21.886	unknown	41	73	+			+		
22.644	δ -Elemene	42	121	+++		++++	+		
22.679	unknown	43	121						+
22.981	unknown	44	97	+					
24.021	unknown	45	57	++		++	+		+
25.058	β -Caryophyllene	46	41	+++++	+++++	+++++	+++	++	++
26.070	unknown	47	93	++		++	+	+	
26.121	Humulene	48	93				+		
27.229	unknown	49	205	+			++	+	++
27.359	unknown	50	205	+++++	+++	+++++	+++++	++	+++++
Number of compounds				43	22	19	31	15	12

Note: Data shows the numbering of compounds, and average peak areas of the components extracted by individual fibers. Conditions: n=4, S:N 50:1 and hit rate of 50%. Average peak area ranges indicated by the symbol (+) between the following numbers: 2.7e03 + 4.0e04 ++ 1.0e05 +++ 2.0e05 ++++ 3.0e05 +++++ 1.5e06 ++++++ 3.5e06 +++++++ 1.0e07 ++++++++ 8.5e07.

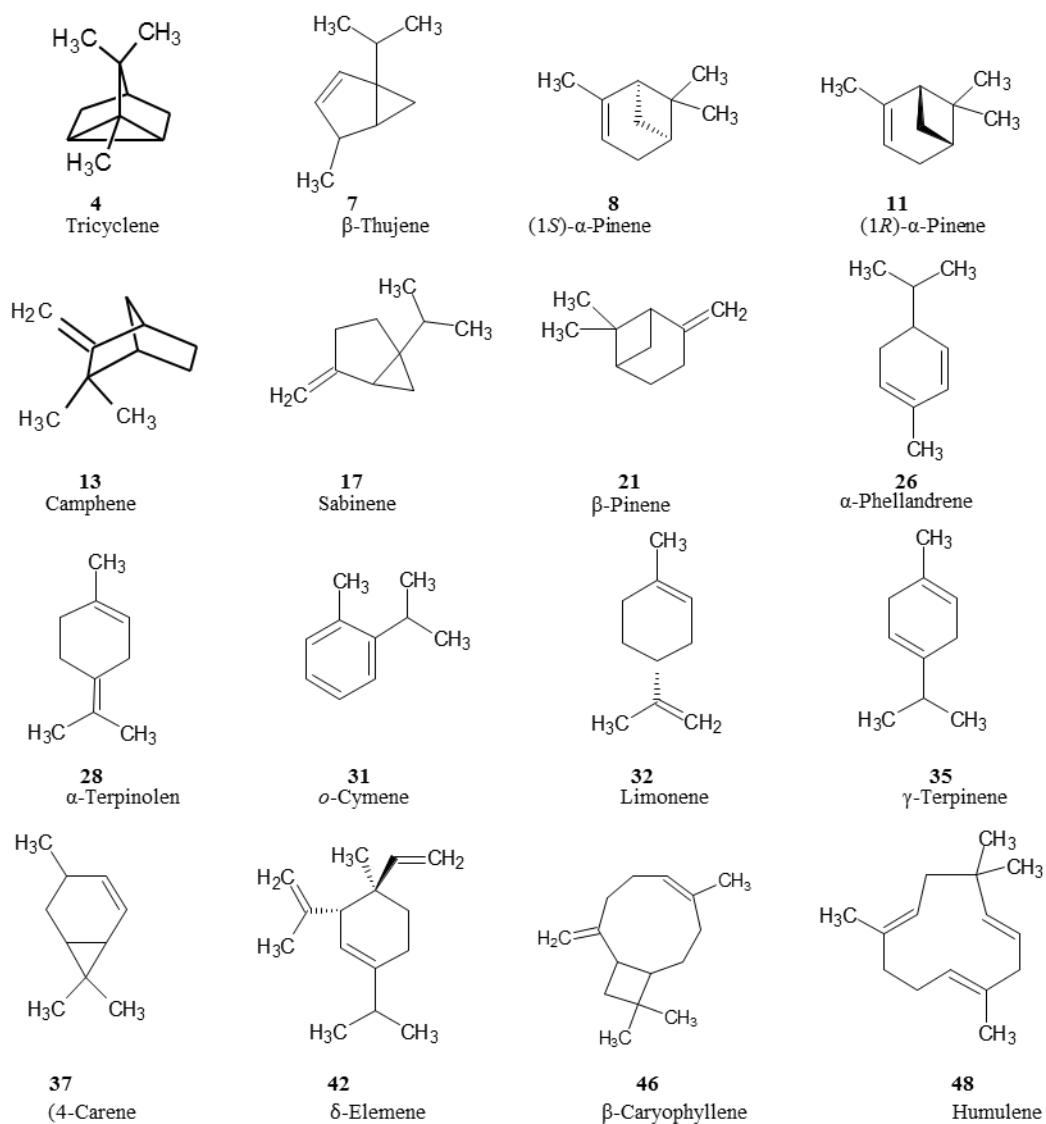


Figure 3.6. Chemical structures of volatile compounds identified by the database with a match factor > 900. Compounds were classified as monocyclic monoterpenes (**26**, **28**, **32** and **35**), bicyclic monoterpenes (**7**, **8**, **11**, **13**, **17**, **21** and **37**), tricyclic monoterpene (**4**), alkyl benzene related to monoterpene (**31**), monocyclic sesquiterpenes (**42** and **48**), and bicyclic sesquiterpene (**46**). The compound numbering is based on that in Table 3.1 and Table 3.2.

3.3.3 Fiber Selectivity

Even though the PDMS/DVB fiber showed the highest extraction efficiency in terms of both total peak areas and the number of VOCs detected, it is interesting and important to examine whether the CAR/PDMS and PDMS fibers exhibited any differences in selectivity or bias toward certain compounds or groups of compounds. According to the results shown in Table 3.1, four VOCs were extracted by the CAR/PDMS and/or PDMS fibers that were not extracted by the most efficient PDMS/DVB fiber. For example low emissions of Compound **43** (m/z 121) were selectively extracted by the PDMS fiber only. This compound was identified by the database as isoterpinolene, which is a hydrocarbon having the same carbon framework as the terpinenes and has been detected as one of the main components in *Warionia saharae* Asteraceae flowers [19]. Similarly, Compounds **12** (m/z 121) and **23** (m/z 136) were only extracted by the CAR/PDMS fiber. Finally, Compound **16** (m/z 91), was extracted by both PDMS and CAR/PDMS, but more efficiently by PDMS according to peak areas. Venn diagrams are presented in Figure 3.7 to help visualizing the selectivity differences of the fibers.

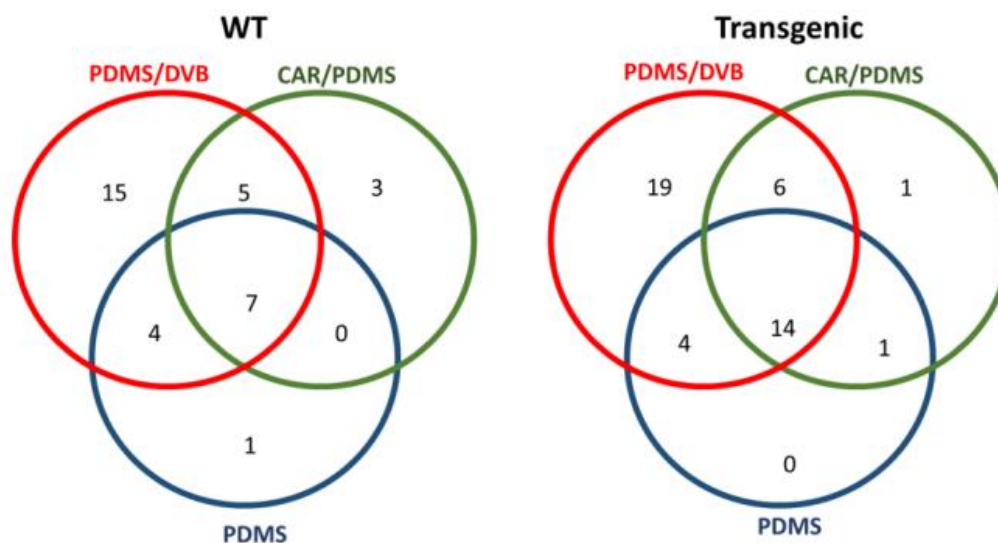


Figure 3.7 Venn diagrams of VOCs detected from the wild type (WT) and transgenic Micro-Tom plants using the three SPME fibers as labeled.

Other examples that showed differences in extraction bias among the fibers are: 1) Compound **4**, monoterpene tricyclene ($C_{10}H_{16}$, m/z 93, 136 Da), a cyclic hydrocarbon with substituted bridged rings, which was more efficiently extracted by the CAR/PDMS fiber compared to the PDMS/DVB and PDMS; 2) Compound **6** (m/z 93), which had a higher peak area using the CAR/PDMS compared to the PDMS/DVB; 3) Compound **50** (m/z 205), which was extracted equally by PDMS and PDMS/DVB, but less efficiently by CAR/PDMS; 4) Compound **8**, (1*S*)- α -pinene, which was extracted by all three fibers, but most efficiently by CAR/PDMS; and 5) Compounds δ -elemene (**42**) and **45** (m/z 57), both of which were extracted by the PDMS and PDMS/DVB only.

Differences in extraction efficiency and selectivity among the three fibers studied were evident from the results presented. The PDMS/DVB generally exhibited the highest recovery for most of the compounds detected. This can be attributed to the “like attracts like” rule, where the polar characteristics of the fiber are compatible to a varying degree with the polarity observed for the VOCs shown in Figure 3.6. In addition, the DVB part of the DVB/PDMS fiber was reported to be composed of wide pores (meso- and macropores) that permit shorter equilibration times [20], and may also contribute to more efficient extraction. Bias towards a small number of compounds was observed for CAR/PDMS and/or PDMS. Unfortunately, as only one of these compounds was identified by AMDIS, it prohibits the drawing of any conclusions regarding the nature of the bias of these fibers. Nevertheless, alternative selectivity was clearly present among the fibers, and the simultaneous use of SPME fibers will be unquestionably beneficial in maximizing the discovery of VOCs in untargeted analyses.

3.3.4 Differences Between Micro-Tom types

The combined results from the three fibers studied reveal differences in floral VOC emission between the wild type and transgenic Micro-Tom plants. According to the results shown in Figure 3.5, the total amount of VOC emissions was higher from the transgenic plants than from the wild type plants. Specifically, the peak areas were 1.5- to 2.4-fold higher for the three fibers. PCA was applied as an exploratory tool to analyze

and visualize the VOC emission differences from the two plant types, based on the data from all three fibers. The score plot was judged by visual inspection of the distances within a cluster and the distances between clusters. The results are presented as a two-dimensional plot of sample scores in the space defined by the PCA (Figure 3.8) and indicate two separated clusters according to the plant type based on the difference in the number of volatiles detected. Wild type plants showed a closer clustering or lower variability compared to the transgenic plants, reflecting less biological variation of the volatile profile intensities. It is noteworthy that the difference in the extracted volatile compounds between the two types of plants correlates with the fact that transgenic plants have higher emissions of volatiles compared to the wild type. This is consistent with the function of *CCDI* in carotenoid catabolism, which when up-regulated in the transgenic Micro-Tom plants, produces an array of volatile and non-volatile products [21].

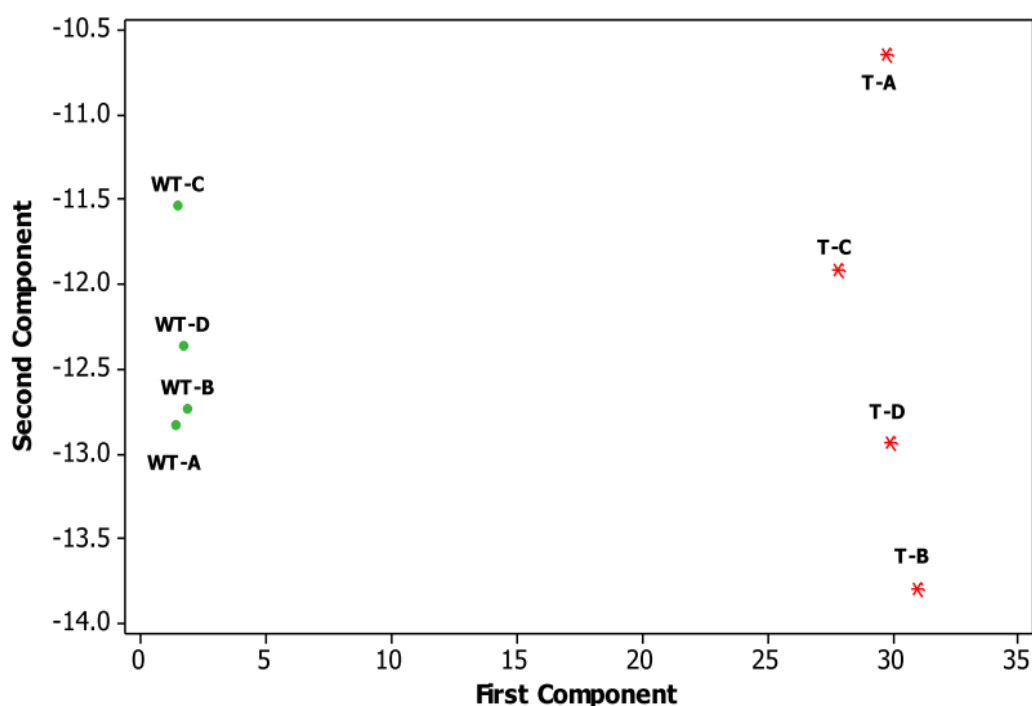


Figure 3.8. PCA score plot showing the differentiation of wild type and transgenic Micro-Tom flower VOCs extracted by three SPME fibers combined.

Of the 50 detected compounds from the three fibers, 45 were from the transgenic plants and 35 from the wild type (Table 3.2). Out of the 45 compounds from the transgenic tomato flowers, 15 were unique. Five out of the 35 were unique for the wild type tomato flowers. The identities of some of these compounds were not fully confirmed by AMDIS due to the low database match numbers; e.g. Compounds **1**, **2** and **3** (with the corresponding m/z base peaks of 41, 41 and 43), which were only found in the transgenic plants. Nevertheless, the database recognized these as three small acyclic compounds that could be derivatives of the isoprene unit ($\text{CH}_2=\text{C}(\text{CH}_3)\text{CH}=\text{CH}_2$), a hemiterpene volatile produced by many plants via the non-mevalonate pathway (DOXP-MEP) [3]. Another two interesting examples were Compounds **15** (m/z 91) and **34** (m/z 136), which were identified by the database respectively as floral volatile 2,4(10)-thujadiene and the naturally occurring monoterpene alcohol, terpineol. These two compounds were also extracted only from the transgenic Micro-Tom tomato plant and may be associated with herbivore attraction and defense [22].

Table 3.2. A list of compounds identified from the transgenic and wild type Micro-Tom flowers combined for all three fibers.

RT (min)	VOC	No.	m/z	Fibers combined	
				Transgenic	Wild type
5.983	unknown	1	41	+	
6.038	unknown	2	41	+	
8.815	unknown	3	43	+	
9.536	Tricyclene	4	93	+	+
9.583	unknown	5	93	+	
9.584	unknown	6	93	+	+
9.691	β -Thujene	7	93	+	+
10.014	(1S)- α -Pinene	8	93	+	+
10.053	unknown	9	91		+
10.180	unknown	10	91	+	+
10.263	(1R)- α -Pinene	11	93	+	+
10.413	unknown	12	121	+	+
10.489	Camphene	13	92	+	+
10.549	unknown	14	91		+
10.560	unknown	15	91	+	
10.628	unknown	16	91	+	
11.203	Sabinene	17	91	+	+
11.224	unknown	18	92	+	
11.228	unknown	19	137	+	+
11.231	unknown	20	91	+	+
11.394	β -Pinene	21	93	+	+
11.479	unknown	22	77	+	+
11.545	unknown	23	136		+
11.768	unknown	24	282	+	
12.067	unknown	25	91	+	+
12.332	α -Phellandrene	26	92	+	+
12.378	unknown	27	91	+	
12.656	α -Terpinolen	28	121	+	+
12.944	unknown	29	119	+	+
13.002	unknown	30	119	+	+
13.048	o-Cymene	31	119	+	+
13.065	Limonene	32	68	+	+
13.095	unknown	33	68	+	
13.176	unknown	34	136	+	
14.080	γ -Terpinene	35	136	+	+
14.105	unknown	36	77	+	
14.934	4-Carene	37	91	+	+
15.597	unknown	38	267	+	
16.746	unknown	39	267	+	
16.753	unknown	40	73	+	+
21.886	unknown	41	73	+	+
22.644	δ -Elemene	42	121	+	+
22.679	unknown	43	121		+
22.981	unknown	44	97	+	
24.021	unknown	45	57	+	+
25.058	β -Caryophyllene	46	41	+	+
26.070	unknown	47	93	+	+
26.121	Humulene	48	93		+
27.229	unknown	49	205	+	+
27.359	unknown	50	205	+	+
Number of compounds				45	35

Note: Conditions are otherwise the same as Table 3.1.

Several interesting compounds were detected in both wild type and transgenic plants, but at a higher concentration in the transgenic plants: Compound **46**, bicyclic monoterpene β -caryophyllene, previously found to play an important role in herbivore attraction [23] and in the indirect defense of kidney bean plants, lima bean leaves and maize [24, 25]; Compound **32**, terpenoid Limonene, reported to be associated with the direct defense of wild tomato (*Lycopersicon hirsutum*) leaves [26]; Compound **35**, γ -terpinene, a natural plant monocyclic monoterpene and one of the most abundant compounds in *Millingtonia Hortensis* (Bignoniaceae) essential oils, and reported to have significant insecticidal activity towards the cotton leafworm *Spodoptera Littoralis* (Lepidoptera-noctuidae) and the black bean aphid *Aphis fabae* (Hemiptera-aphididae) [22]; Compound **12** (m/z of 121), identified as α -fenchene, a bicyclic monoterpene which is a floral volatile, but according to our knowledge, has not been reported as a Micro-Tom tomato plant volatile.

Finally, five compounds were extracted by all three fibres, and from both the transgenic and wild type plants. They are: monoterpenes β -thujene (**7**), (1*S*)- α -pinene (**8**), (1*R*)- α -pinene (**11**), camphene (**13**), β -caryophyllene (**46**), and compound **50** (m/z 205). Of these, **8** and **11** had been reported to play an important role in the pollination process[27] . These compounds captured our attention because they were also found to be the most abundant volatiles from Micro-Tom plants based on GC-MS peak areas.

3.4 Conclusions

The different extraction capabilities of three SPME fibers were combined to take advantage of their wider range of analyte selectivity for the semi-quantitative and untargeted study of tomato flower VOCs. Even though the general extraction efficiency of PDMS/DVB was the highest of the three fibers, the PDMS and the CAR/PDMS offered a slightly different bias in the extraction of VOCs. By combining the use of three fibers, the extraction of VOCs was maximized for both wild type and transgenic Micro-Tom tomato flowers. Results also represent a preliminary assessment of the

differences of VOC emission between the transgenic and wild type plants. The quantity and the number of VOCs detected were higher from the transgenic plants than from the wild type plants. This is consistent with the expected function of *CCD1* in carotenoid catabolism. Unfortunately, the anticipated presence of the β -ionone and other CCD derivatives in the HS of either the transgenic or wild type plants were not observed. To properly evaluate the effect of the gene over-expressions of interest, future experiments analyzing other parts of the tomato plant, beyond the HS, will be conducted. In addition, a larger number of replicates will be acquired to account for variability from the various stages of preparation of transgenic plants.

3.5 References

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Chapter 4: The effect of transgenic plant VOCs on herbivore behaviour

In the last two chapters, a non-targeted approach for the analysis of VOCs from the HS of the *Arabidopsis thaliana* and Micro-Tom tomato flowers and plants was demonstrated. The approach involved three main components: the extraction strategy (DHS or SHS), instrumental analysis and data analysis. All three needed to be as unbiased and comprehensive as possible for the comparative evaluation of VOCs according to the plant type.

A complementary study, which is presented in this chapter, relates to the application of biological assays using *Arabidopsis thaliana*, Micro-Tom tomato plants, and herbivores. The bioassays not only allowed for an evaluation of the attractant/repellent effect of the VOCs released by the plants but also indicated the potential use of volatiles from the transgenic plants as a means of insect pest management and control.

4.1 Introduction

As it was depicted in the *in vivo* non-targeted analysis of volatiles from plants in the last two chapters, the *Arabidopsis thaliana* ecotype columbia-0 (L.) Heynh (Brassicaceae) and *Solanum lycopersicum* cv. Micro-Tom (Solanaceae) plants and flowers released a variety of VOCs. These were mainly aromatic and hydrocarbon derivatives, mono and sesquiterpenes and for the specific case of *Arabidopsis*, the apocarotenoid compound β -ionone. These VOCs are derived mainly from four biosynthetic classes, terpenoids, fatty acid catabolites, aromatics, and amino acids [1]. The emission of apocarotenoid volatiles such as β -ionone, mainly found in the fruits and flowers of several plant species, is associated with *carotene cleavage dioxygenase 1 (CCD1)* expression [2]. In general, all volatile compounds are released through the membranes of epidermal tissue, trichomes, pores, or cells deep within the plant, and normally are created from precursors whose hydrophilic functional groups have been substituted through reduction, methylation and acylation reactions [3].

In addition to their specific physical barriers, like plant organ thickness and trichomes that increase plant fitness, plants also release VOCs to solve fundamental problems that result from their immobility. Some of the roles include the attraction of insects for seed dispersal or pollination processes, direct and indirect herbivore and pathogen defense and for plant to plant communication [1, 4]. A complementary part of my research is to evaluate the attractant/repellent functions of plant volatiles against herbivore species, so a description of plant volatiles in this role will be the principal focus of this chapter.

The biochemical mechanisms of defense against the herbivores are extensive, highly dynamic, and are mediated both by direct and indirect defenses. The defensive compounds are either produced constitutively or in response to plant damage, and affect feeding, growth, and survival of herbivores. In addition, plants also release volatile organic compounds that attract the natural enemies of the herbivores. These strategies either act independently or in conjunction with each other. However, the understanding of these self-protective strategies is still limited [5, 6]. Certain plant volatiles are referred to as herbivore-induced plant volatiles (HIPVs) and act as repellents to herbivores and attractants for parasitoids and predators [7].

Attraction in plant-insect interactions can be defined as the insect's ability to choose and orientate towards a plant or plants that have been releasing VOCs of interest for the insect (feeding, reproduction or oviposition site) [8]. Insects are able to detect these volatiles through specialized receptors which transmit a nerve signal to the brain. For instance, the most abundant volatiles from the ant-pollinated flower *Cytinus hypocistis* L. (Cytinaceae), 4-oxoisophorone, (*E*)-cinnamaldehyde and (*E*)-cinnamyl alcohol had a strong attractant effect only towards ant species that pollinate the flowers, the *Aphaenogaster senilis* Mayr (Hymenoptera: Formicidae) and the *Crematogaster auberti* Emery (Hymenoptera: Formicidae), but not towards other ants found in the same habitat [9]. Similarly, 14 compounds detected in the flower odor of *Silene otites* L. (Caryophyllaceae) including benzaldehyde, eugenol, and linalool, were measured electrophysiologically as attractive to both the northern house mosquito *Culex pipiens*

L. and the yellow fever mosquito *Aedes aegypti* L. (Diptera: Culicidae), acting as potential guides to find the floral nectar [10].

In the case of third trophic level attraction (indirect defense), the volatiles cis-3-hexen-1-ol, linalool, and cis- α -bergamotene were found to be released by *Nicotiana attenuate* Torr. Ex Wats (Solanaceae) plants. These volatiles attracted the generalist predator the western big-eyed bug *Geocoris pallens* Stål (Hemiptera: Geocoridae) of three leaf-feeding herbivore species, the caterpillars of *Manduca quinquemaculata* Haworth (Lepidoptera: Sphingidae), the leaf bug *Dicyphus minimus* Cerruti (Heteroptera: Miridae) and the flea beetle *Epitrix hirtipennis* Melsheimer (Coleoptera: Chrysomelidae) [11]. Field experiments demonstrated that methyl salicylate (MeSA), a herbivore-induced plant volatile (HIPV) had an attractive effect on the aphid and mite predator, green lacewing *Chrysopa nigricornis* Leach (Neuroptera: Chrysopidae) [12].

Repellence, on the contrary, is the physical stimulus that drives the insect away from the plant after the detection of VOCs that produce distress, distaste or aversion [13]. For example, the phenylpropanoid plant compounds methyl salicylate and salicylaldehyde applied to the leaf surface of bean and cucumber inhibited feeding and egg-laying activity by western flower thrips *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) [14]. Among plant volatiles, terpenoids have been well documented to act as toxins, feeding deterrents, or oviposition deterrents in a range of insects [15-17]. In another study it was found that the monoterpene volatiles from rough lemon *Citrus jambhiri* Lush (Rutaceae), including linalool, limonene and β -pinene, increased in emissions under herbivore attack and had insect-repellant effect against wild-type *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) [18].

As it was demonstrated in the *Arabidopsis* HS analysis in chapter two of this thesis, transformed plants overexpressing the *CCDI* gene produced more β -ionone compared to the wild type. This volatile compound was produced by the cleavage of carotene molecules by the *CCDI* gene product instead of directly through the traditional mevalonate and methylerythritol phosphate pathway pathways [19]. Carotene

degradation by oxidative cleavage produces an array of important terpenoid products collectively known as apocarotenoids [20]. A number of these are non-volatile, such as abscisic acid and strigolactones, whereas others are volatiles and comprise C9 to C13 compounds, including β -ionone, α -ionone, β -damascone, β -damascenone, 4-oxo- β -ionone, 4-oxoisophorone, theaspirone and cyclocitral.

The apocarotenoids, α -ionone, β -ionone and dihydro- β -ionone for instance, compose the unique fragrance of violet *Viola arvensis* Murray, *V. odorata* L., and *V. reichenbachiana* Rchb. (Violaceae) flowers and these versatile aroma-chemicals play important roles in the making of perfumes and fragrances [21]. The floral bouquet of *Philodendron adamantinum* Schott (Araceae), which is dominated by dihydro- β -ionone, is responsible for the attraction of the beetles *Erioscelis emarginata* Mannerheim (Coleoptera: Scarabaeoidea) facilitating the pollination process [22]. Production of β -ionone was part of the induced defense by canola flowers *Brassica napus* (Brassicaceae) when plants were wounded by the crucifer flea beetle *Phyllotreta cruciferae* (Goeze) (Coleoptera: Chrysomelidae) [23]. β -ionone together with a range of terpenoid compounds like linalool, myricene and pinene appeared to synergistically attract the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) females in maize fields [7], while α -ionone and α -ionol have been described as effective male attractants for *Bractocera atifrons* Hendel (Diptera: Tephritidae) [24].

The objective of this part of my work is to demonstrate the necessity to consider plants as essential and interactive components of biological control practices by evaluating the behavioral response (attractant/repellant) of selected herbivores, first by exposing them to the complete plants and second by exposing them to selected VOC standards through the application of biological assays.

For the *Arabidopsis thaliana* study, the behavioural response of the crucifer flea beetle (CFB) *P. cruciferae* to the volatile blend of complete WT and transgenic *Arabidopsis* plants overexpressing the *AtCCDI* was monitored. The *AtCCDI* plants

were selected according to the DHS study (chapter two, sections 2.3.4 and 2.3.5), in which the overexpressed *AtCCDI* enhanced the production of the apocarotenoid compound β -ionone compared to wild type plants. To confirm if the repellence/attraction was due to the β -ionone alone, a series of biological assays were performed using standards of β -ionone and two structurally related compounds: α -ionone and dihydro- β -ionone. The standards were tested against CFB, and two additional herbivores: the two-spotted spider mite (TSSM) *Tetranychus urticae* (Koch) (Acari: Tetranychidae) and the Silverleaf whitefly (SWF) *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae). These experiments demonstrated the unbiased effect of the apocarotenoid compounds and confirmed their effectiveness against other herbivores.

For the Micro-Tom tomato study, the greenhouse whitefly (GHW) *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) was used to screen its behavioral response to the volatile blend of complete WT and transgenic Micro-Tom tomato plants overexpressing the *LeCCDI-1* gene. The volatile standards of the sesquiterpenes β -pinene, α -pinene and (1*R*)-(+)- α -pinene were used to determine the oviposition deterrent effect of the GHW.

4.2 Materials and Methods

4.2.1 Reagents

The analytical standards; β -ionone, α -ionone, dihydro- β -ionone $\geq 90\%$, β -pinene, (1*S*)- α -pinene and (1*R*)-(+)- α -pinene, along with dichloromethane (DCM) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Acetone was purchased from Caledon (Georgetown, ON, Canada).

4.2.2 Plants and Growth Conditions

Wild type (WT) *Arabidopsis thaliana* Columbia-0 plants and transgenic plants over-expressing the *AtCCDI* gene (lines 1, 2 and 3) were grown in containers with Pro-mix-BX soil at Agriculture and Agri-Food Canada (AAFC), London ON, Canada. Ten to fifteen seeds were sown onto pre-moistened soil in 4-inch plastic pots, and the surface of the soil was given adequate moisture. Once the trays containing the pots were covered with a plastic dome to maintain humidity, they were placed at 4 °C in the dark for 3-4 days to stratify the seeds. Subsequently, the trays were transferred to a growth room maintained at a controlled environment of 20 °C ± 3 °C, 70% RH with a photoperiod of 16:8 h L:D and light intensity of 95-130 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The plastic domes were removed one week after the seeds germinated. The seedlings were watered three times a week on every alternate day. Once the plants were two weeks old, they were supplemented with all-purpose fertilizer 20-20-20 (1 mg/L), which was added to the soil on a bi-weekly basis. Six-week-old plants with flowers were used in the choice bioassays.

WT Micro-Tom tomato plants and Micro-Tom tomato plants over-expressing the *LeCCDI* gene were grown in pots containing general purpose professional growing medium pro-mix-BX mycorrhizaeTM soil. Tomato seeds were sown onto pre-moistened soil in plastic seed-starting containers containing 12 standard cell inserts, 10.5”W x 20.9”L with a single seed per container. By direct observation and after approximately 3 weeks, healthy tomato plants were transferred to 4-inch plastic pots, one plant per pot and plants were held in a growth room maintained at a controlled environment of 22 °C ± 3 °C, 75% RH, with a photoperiod of 16 h, and light intensity of 100-150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Micro-Tom tomato plants were watered two times a week and were supplemented with all-purpose fertilizer 20-20-20 (1 mg/L) on a bi-weekly basis. Six to seven-week-old plants with flowers were used to set up a choice test and oviposition bioassays.

4.2.3. Plant volatile identification and standard solutions

Volatile extraction and identification for both the *A. thaliana* and the Micro-tom tomato plants were performed by the DHS-GC-MS method depicted in chapter two. To quantify β -ionone emissions in the *A. thaliana* a calibration curve with the following concentrations was made: 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ng/ μ L ($R^2=0.9852$). Similarly, for the Micro-tom tomato the following calibration curves were made: (1S)- α -pinene at 5, 10, 15, 20 and 25 ng/ μ L ($R^2=0.9879$); β -pinene at 30, 35, 40, 45, and 50 ng/ μ L ($R^2=0.9732$); and (1R)- α -pinene at 80, 90, 100, 110 and 120 ng/ μ L ($R^2=0.9709$).

4.2.4 Herbivore Test Species

Four herbivores were selected for our investigation representing different classes, orders, feeding types and host plant preference. The adult crucifer flea beetle (CFB) *P. cruciferae* (Figure 4.1 A) is a specialist herbivore [25] as it only feeds on *Brassicaceae* crops including mustard, canola, cabbage and radish, and was affected by *Brassica* volatiles in previous studies [17, 23]. The generalist, polyphagous, two-spotted spider mite (TSSM) *T. urticae* [26] (Figure 4.1 B), is considered to be one of the most economically important spider mites and it has been reported to feed on over 200 species of plants [27]. Two species of whiteflies, the silverleaf whitefly (SWF) *B. tabaci* and the greenhouse whitefly (GHW) *T. vaporariorum* (Figure 4.1 C) are considered polyphagous insects and have been an economically important insect pests for many years having a host range of around 600 different plant species [28]. Whiteflies are an important pest that feeds on tomatoes, squash, broccoli, cauliflower, cabbage, melons, cotton, carrots, sweet potato, cucumber, pumpkin and ornamental plants [29]. They also excrete sticky honeydew which may spoil some commodities and they may cause leaf yellowing or death of the host [30].

In the following sub-sections descriptions of the different bioassays performed to test the repellent/attractant response using these herbivores are provided. Note that bioassays using the Cabbage loopers moths *Trichoplusia ni* (Noctuidiae) were

performed as well in a feeding test, but not significant effect was observed (Appendix 8)

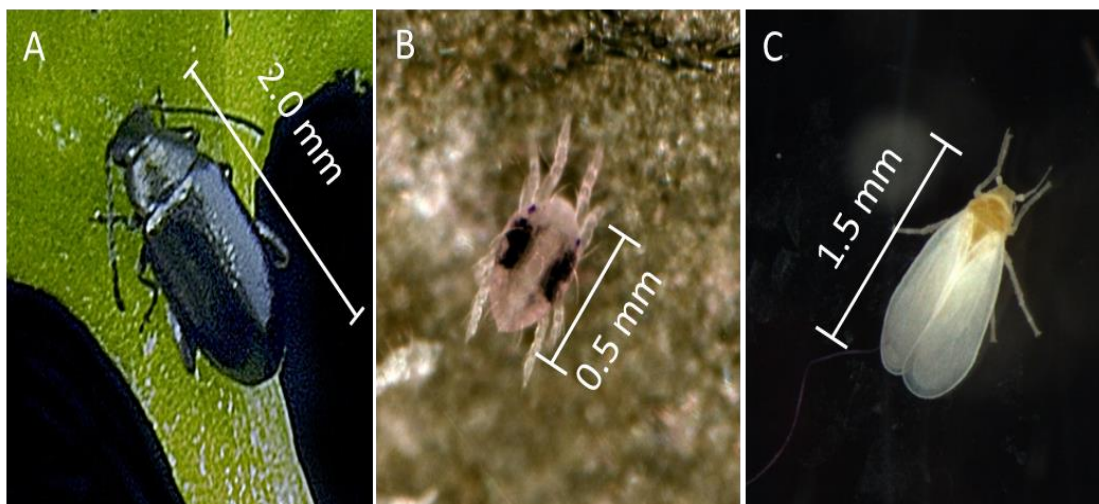


Figure 4.1. Crucifer flea beetle (CFB) *Phyllotreta cruciferae* (Goeze) photo taken at AAFC, Harrow, ON (A), Two spotted spider mite (TSSM) *Tetranychus urticae* (Koch) photo taken at AAFC, London, ON (B), Greenhouse whiteflies (GHW) *Trialeurodes vaporariorum* (Westwood) (C) photo taken at AAFC, London, ON.

4.2.4.1 Crucifer Flea Beetle (CFB) Two Chamber Choice Test

Adult CFBs were collected in sweep nets from canola fields at the Southern Crop Protection and Food Research Centre, AAFC, London ON during the summer of 2012. After field collection, CFBs were transferred into 50 cm³ cages with canola plants for food and the day before the experiment 15 insects were gently aspirated into a 50 mL erlenmeyer. The choice experiments were performed by connecting two volatile collection chambers by an inert glass bridge, 35 cm x 0.8 cm i.d. (Figure 4.2). Each chamber enclosed a pot containing seven to eleven *Arabidopsis* plants, one with WT plants and the other *AtCCDI* plants (three lines were tested). A trial began when 15 adult CFB were introduced at the centre of the bridge between the two chambers. After

24 h the number of insects that had moved into each chamber was counted. Six trials, with new plants each time, were performed.

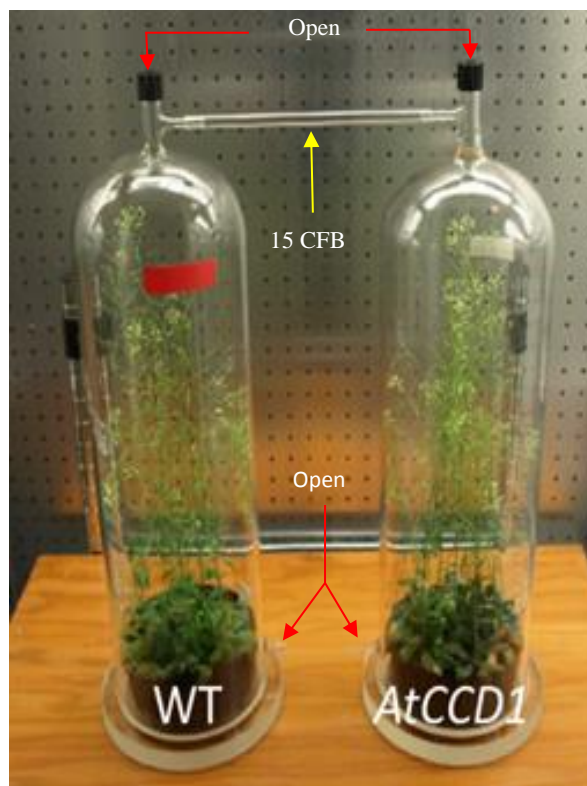


Figure 4.2. The setup for the two chamber CFB choice. One chamber containing WT plants was connected to a chamber containing AtCCD1 plants. Each glass chamber had open ports to allow for air ventilation. In the centre of the glass tube (bridge) 15 CFB were introduced and 24 h after which the number of beetles was counted on each plant type.

4.2.4.2 CFB Y-tube Olfactometer Test

The measure of attraction to solutions of pure standards α -, β - and dihydro- ionone was conducted with individual CFB in a Y-tube olfactometer (Figure 4.3). A concentration of 0.3 ng/ μ L was used as a reference, as this was the average concentration of β -ionone measured in the DHS of two lines of *Arabidopsis* plants overexpressing the *AtCCD1*

gene as described in Chapter two section 2.3.5. The inside diameter of the glass Y-tube was 2.8 cm and the length of the tube, from the base to the Y-juncture, was 15.5 cm. A pump (BOC Edwards E2M 1.5, Mississauga, ON, Canada) was used to push air through a charcoal and water trap, followed by two flow meters to control the air flow (50 mL/min) in two separate lines leading to two 500 mL erlenmeyer flasks, one empty, the other containing 20 μ L of the apocarotenoid solution in a 10 mL beaker. Air that passed through the flask containing the ionone solution went into one arm of the Y-tube. Simultaneously, air that passed through the empty control flask went into the other arm of the Y-tube. An adult CFB was placed 8 cm past the junction of the two arms. The experiment was repeated three times and each experiment used ten CFBs, one at a time, and the direction of movement into either arm was observed for a maximum of five minutes. To eliminate a potential left or right turning bias, the positioning of the treatment and control was switched after each group of 5 CFB had been tested.

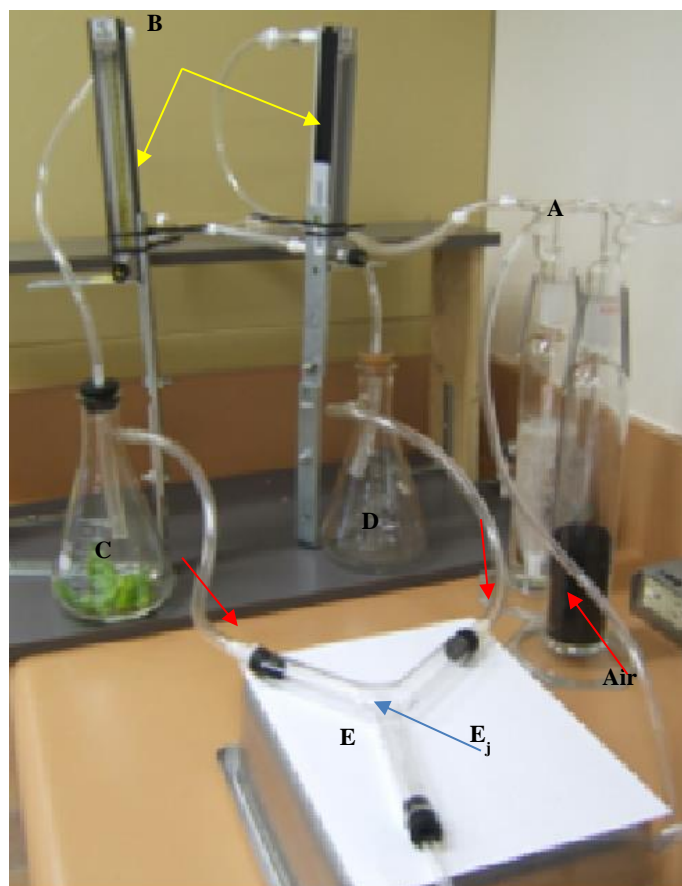


Figure 4.3. The setup for the Y-tube CFB olfactometer included: Air pumps (not shown); water and charcoal traps (A); air flow meters (B); Erlenmeyer with treatment (C) and control (D); Y-tube (E) and tube junction (E_j), below which each CFB was placed one at a time.

4.2.4.3 Two-spotted Spider Mite (TSSM) Paper Clamp Bio-assay

Solutions of α -, β - and dihydro- β -ionone in acetone were used to test the response of the adult two-spotted spider mites. TSSM were reared on California red kidney beans *Phaseolus vulgaris* L. (Fabaceae) plants in a growth room at 24°C, 65% RH and with a photoperiod of 16:8 h L:D at AAFC London. The bioassay conducted was modified from a procedure [31] where the experiments were performed in a sterile fume hood at room conditions of 24±2°C and 35% RH inside a styrofoam box with one open side

through which observations could be made. The apparatus consisted of two strips of Whatman™ qualitative #1 filter paper (1.0 x 0.75 cm), (Sigma-Aldrich, Oakville, ON, Canada) held in a small size paper clamp (Figure 4.4). At each end of the filter paper 20 µL of apocarotenoid solution (0.05, 0.1, 0.3 and 0.5 ng/µL) and 20 µL acetone control were pipetted. An adult female TSSM was placed at the middle of the filter paper bridge (1.5 x 0.5 cm) connecting the two filter papers. For each set of drops, three mites were tested on a bridge, and every treatment was repeated ten times with observations of the mite movement recorded up to 1 min.

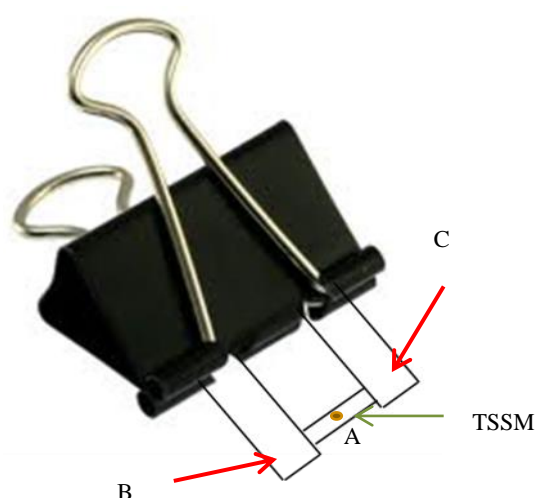


Figure 4.4. The setup for the paper clamp bioassay included: a filter paper bridge, 1.5 x 0.5 cm, in the middle of which was placed an adult TSSM (A); with the bridge connecting two filter papers (B and C), one containing the corresponding 20 µL of the ionone compound treatment and the other one the acetone control.

4.2.4.4 Silverleaf Whitefly (SWF) Oviposition Bioassay

A colony of SWF was reared on greenhouse Poinsettia *Euphorbia pulcherrima* Klotzsch (Euphorbiaceae) plants at the Vineland Research and Innovation Centre, Vineland, ON. Samples received at AAFC London, ON included approximately 200 adult SWF and

were held in a growth chamber at 24°C and 60% RH, 16:8 L:D until experiments were performed. A Potter Precision Laboratory Spray Tower, 120 cm in height, 36 cm in width and 36 cm in depth (Burkard Scientific, Uxbridge, UK) was used to spray detached tomato leaves with the ionone solutions. The tower was operated at 5.0 psi pressure and calibrated by spraying 5mL of concentrated solutions of α -, β - and dihydro-ionone onto 9.5 cm diameter petri dishes. The final concentrations of ionone solutions sprayed were in the range of 0.05 to 0.5 ng/ μ L. Two detached sets of WT *Solanum lycopersicum* cv. Micro-Tom (Solanaceae) leaves (approximately 11 cm² each) were used for the control and treatment as follows: control leaves were sprayed with 5 mL acetone and the treated leaves were sprayed with 5 mL on both sides with the corresponding ionone solution. Leaf stems were put into 3 mL rosette vials with water to preserve leaves during the bioassay period. The control leaf, treated leaf and 20 mixed sex adult SWF were held in a 2 L beaker covered with parafilm “M” laboratory film (Neenah, WI, USA) (Figure 4.5), with several small holes punctured by needle. The beakers were kept in an incubator chamber at 24°C, 60% RH and 16:8 L:D for a 24 h period, after which the SWF were chilled at 4°C. The leaves were examined for eggs and the number on each counted using a stereo microscope (Olympus SZX, Richmond Hill, ON, Canada). The oviposition deterrence index (ODI) was calculated using a formula:

$$\text{ODI} = [(T-C)/(T+C)] \times 100 \text{ (Equation 4.1)}$$

where T is the number of eggs counted on the tomato leaf containing the ionone treatment, and C the number of eggs counted on the leaf treated with acetone [32]. The ODI is considered to reflect attraction if the values are positive up to +100 or deterrence when values are negative down to -100.

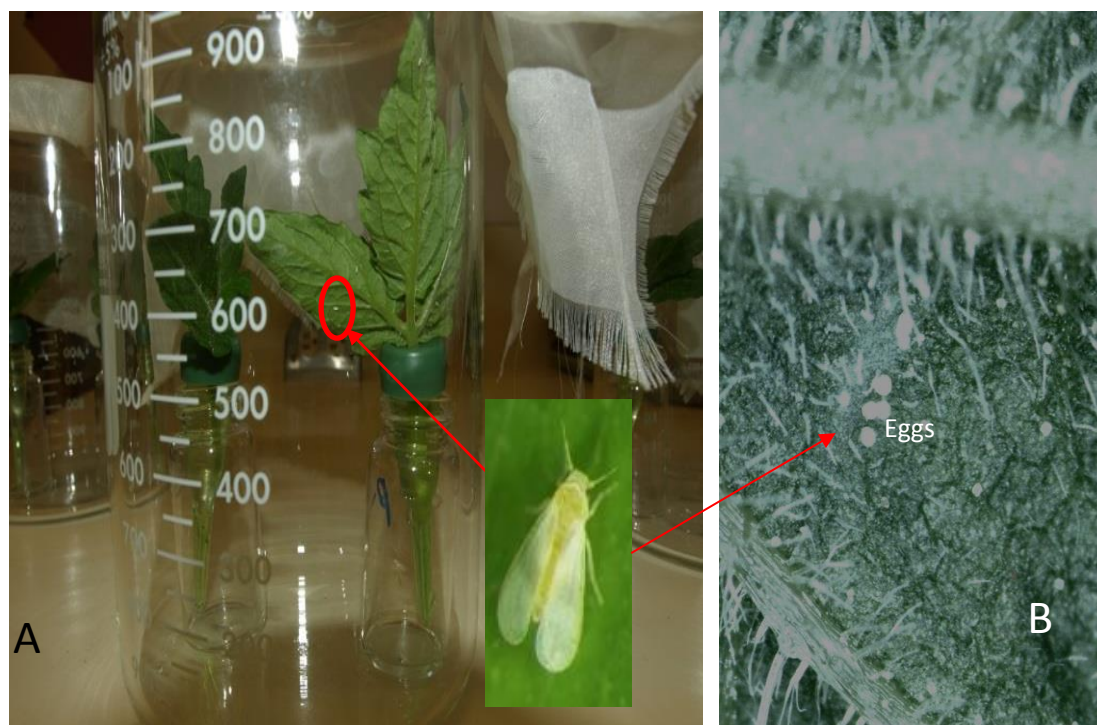


Figure 4.5. Set up of the SWF oviposition bioassay included: 20 mixed sex adult SWF held in a covered 2 L beaker with one set of Micro-Tom leaves sprayed with the treatment and another set of leaves sprayed with the acetone control (A); followed by the counting of eggs laid by the SWF on each treated set of leaves after 24 h (B).

4.2.4.5 Greenhouse Whitefly (GHW) Micro-Tom Tomato Choice Bioassay

Adult greenhouse whiteflies (GHW) *T. vaporariorum* were collected at the Greenhouse and Processing Research Centre, Harrow, ON. Approximately 200 adult GHW were received at AAFC London, ON and held in 2 vials containing tomato plant stems. The tomato stems containing the GHW were enclosed in a plastic bag with small perforations for air ventilation. Insects were kept in a growth chamber at 24 °C, 60% RH and 16:8 h L:D until experiments were performed. Two potted flowering stage Micro-tom tomato plants, one WT and one *LeCCDI-1* line three, with a pot separation of 10.5 cm, were placed into hexagonal vented plastic chambers (47 cm ht x 35.8 cm dia) with

20 adult GHW (Figure 4.6). Observations were made after 24 h after which the number of whiteflies was counted according to the plant chosen.

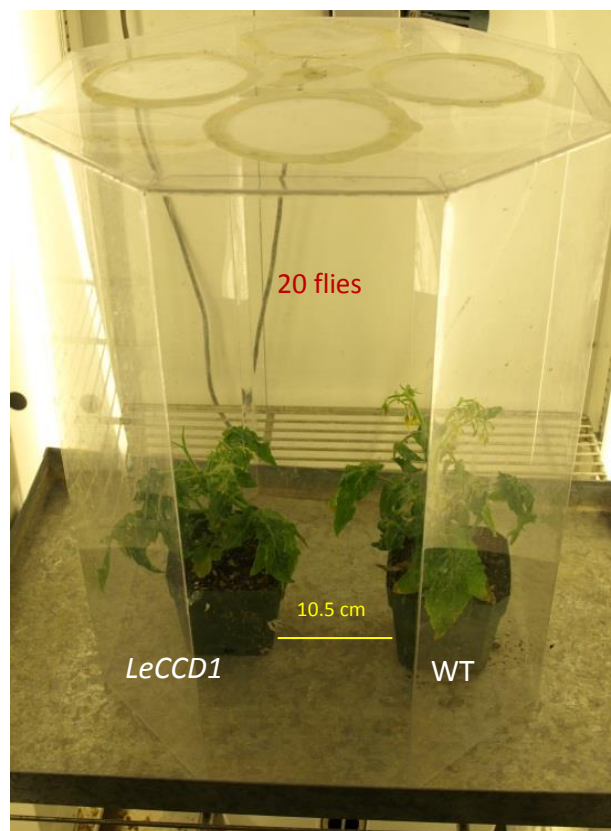


Figure 4.6. The setup of the adult GHW choice test included: a hexagonal plastic vented chamber covering one WT and one LeCCD1-1 line three Micro-tom tomato plant with 20 adult GHW and held in a controlled environmental chamber for 24 h after which flies were counted on each plant. Six replications were performed.

4.2.4.6 Greenhouse Whitefly (GHW) Oviposition Bioassay

GHW oviposition deterrent test was performed by using the tomato volatile standards, β -pinene (40 ng/ μ L), α -pinene (10 ng/ μ L) and (1*R*)-(+)- α -pinene (100 ng/ μ L). For this test the WT tomato leaves were treated by direct application of 200 μ L (100 on the top and 100 on the bottom) of the corresponding VOC standards and acetone control using a

P1000 micropipette (Gilson Pipetman, Middleton, WI, USA) . The calculation of the ODI was performed as mentioned in section 4.2.4.4

4.2.5 Statistical Analysis

Statistical differences in the concentration of β -ionone released by the WT and transgenic plants and between treatment groups in the herbivore bioassays were determined by one-way analysis of variance (ANOVA) using Proc GLM with Tukey's Studentized Range (HSD) test. Statistical differences for the transformed oviposition deterrence index (ODI_t) using the following equation,

$$ODI_t = A \sin (ODI/100)^{1/2} \text{ (Equation 4.2)}$$

were determined by two-way ANOVA. All analysis were performed using the Statistical Analysis System (SAS) (Server Interface Ver. 2.03, SAS Institute, Cary, NC).

4.3 Results and Discussion

4.3.1 *Arabidopsis* Volatiles and Gene Expression

Volatile organic compounds extracted and identified in the *Arabidopsis thaliana* WT and *AtCCDI* L-1, L-2 and L-3 were depicted in Chapter 2 sections 2.3.4. Volatiles were mainly of isoprenoid origin (monoterpenes and sesquiterpenes), unsaturated hydrocarbons and aromatic derivatives. The DHS-GC-MS system was able to detect the presence of β -ionone which is not derived directly from MEP and MVA pathways but from the symmetrical cleavage of β -carotene molecules through the action of the *AtCCDI* gene. It was found that on average the concentration of β -ionone in WT plants was 0.062 ng/ μ L and that *CCDI* lines L-1, L-2 and L-3 were 2.2, 4.5 and 4.3-fold higher. These higher levels released over a 24 h period correlate to the higher levels of

gene expression by *Arabidopsis* plants after transformation (data provided by A. Hannoufa, Agriculture and Agrifood Canada, London ON). Protease chain reaction (PCR) was performed to confirm the presence of the corresponding transgene in eight lines of the first generation of plants (T1) (Figure 4.7 A). A 1 Kb DNA marker was used to “flag” the position of the corresponding *CCDI* gene. The size of the *CCDI* gene is 1700 base pairs (bp) which are present as intensified bands on the gel. PCR amplified gene bands only confirm the presence of the overexpression. In order to confirm the transcript levels of the *CCDI* transgenes, quantitative reverse transcription PCR (RT-qPCR) was performed on the third generation (T3) of the transgenic plants. The transcript levels of independent lines for each transgene were tested and the transcript levels were compared to that of WT plants. The three lines having higher levels of expression were labeled as L-1, L-2 and L-3.

AtCCDI plants L-1, L-2 and L-3 were demonstrated to have 11, 49 and 22-fold higher levels of gene expression, respectively, compared to the WT ($df=3,8$; $F=34.59$; $P < 0.0001$) (Figure 4.7 B). The same lines released higher amounts of β -ionone compared to the WT. These lines were used to evaluate the behaviour of CFB in a two chamber choice test.

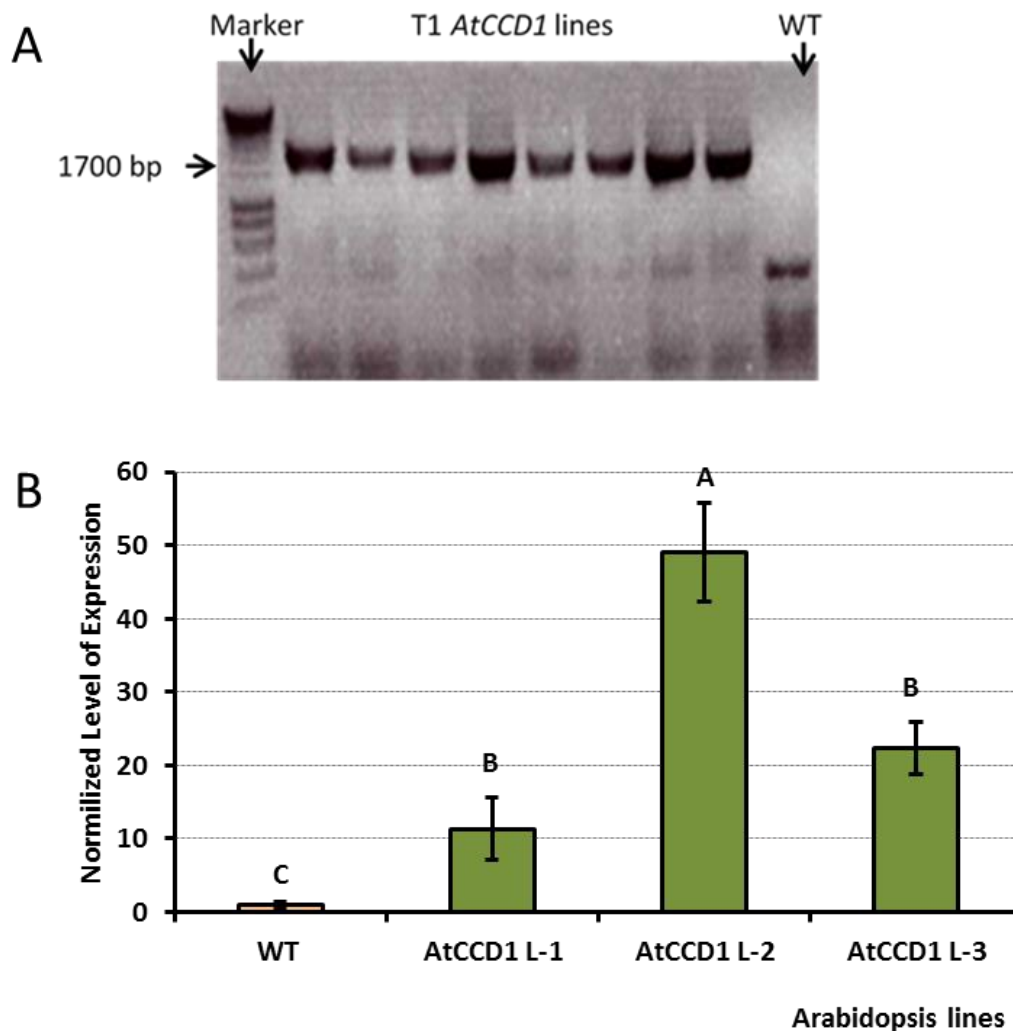


Figure 4.7. PCR gel for eight lines of T1 *AtCCD1* (A) and the expression level of T3 *AtCCD1* by RTq-PCR (B). Error bars represent the standard error of four technical replicates. Bars with different letters are significantly different (one-way ANOVA, Tukey's Test $P < 0.0001$).

4.3.2 CFB Two Chamber Choice Test

The CFB preference between the *AtCCD1* L-1 and the wild type plants was not significantly different ($df=1,10$; $F=1.16$; $P=0.30$) (Figure 4.8 A). In contrast, more CFB moved towards the WT rather than to the *AtCCD1* L-2 plants ($df=1,10$; $F=16.38$; $P=0.002$) (Figure 4.8 B). Similar behaviour was observed with *AtCCD1* L-3, but was

not significantly different compared to the wild type ($df=1,10$; $F=2.26$; $P=0.16$) (Figure 4.8 C).

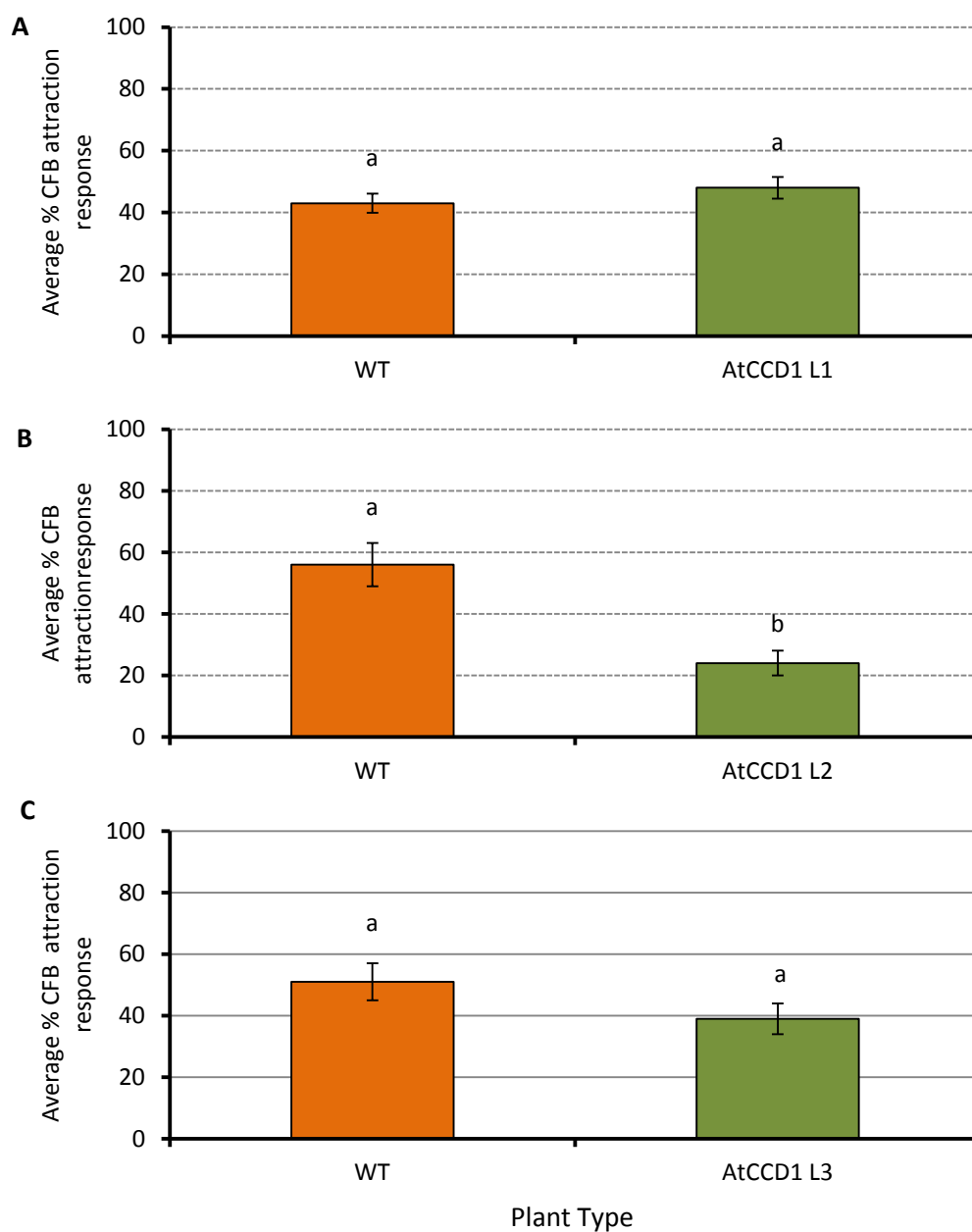


Figure 4.8. Average percent CFB attraction response (\pm S.E.) between a set of WT and transgenic plant lines *AtCCD1 L1* (A), *AtCCD1 L2* (B) and *AtCCD1 L3* (C) in a two chamber choice test. Six replicates were performed per line. Error bars with different letters are significantly different (one-way ANOVA, Tukey's Test $P<0.05$).

When CFB were allowed to move between WT and *AtCCD1* plants in a choice test, it was difficult to determine whether the insects were attracted by the WT or repelled by the *AtCCD1* L-2 plants. *AtCCD1* L-2 were demonstrated to have higher transcription levels of the gene and as a consequence higher levels of β -ionone (0.28 ng/ μ L) compared to the WT (0.06 ng/ μ L). However, it was demonstrated through *in vivo* non-targeted analysis of VOCs from the *Arabidopsis thaliana* (Chapter 2 section 2.3.4) many different volatile compounds with varying concentrations were released, perhaps masking the real effect of β -ionone. To conclude that the deterrence or attractiveness of the plants towards the CFB is due to the release of β -ionone alone would be premature.

To eliminate other plant volatiles as factors in the observed preference towards the WT plants, a series of bioassays using representative concentrations of β -ionone standards was performed. Two other compounds with similar chemical structure, α -ionone and dihydro- β -ionone, were tested at the same concentration in order to determine the specificity of the ionone molecule to the observed response. α -ionone, β -ionone and dihydro- β -ionone (Figure 4.9) are each composed of a cyclohexene ring with a butyl side-chain and bearing a carbonyl group. The difference between the isomers, α -ionone and β -ionone, is the location of the endocyclic double bond, which is at the C-2 position of the ring for the α -, and C-1 for the β -ionone. This difference makes the α -ionone to have a chiral center in the C-1 position. The dihydro- β -ionone shares the same structure as β -ionone, but without the double bond at the C-3 position on the alkyl side-chain.

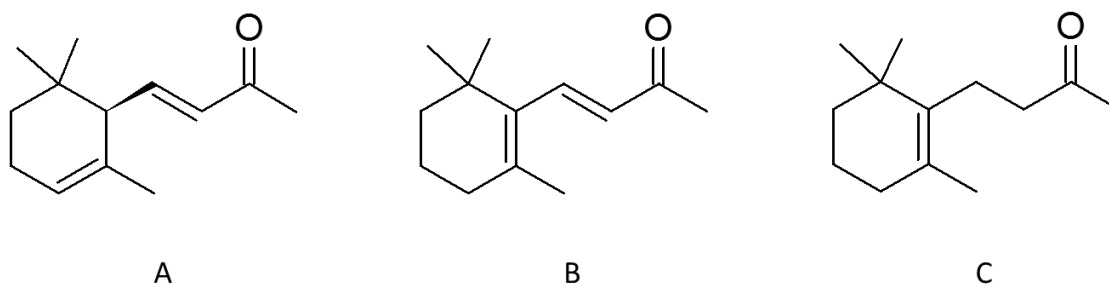


Figure 4.9. Chemical structures of compounds α -ionone (A), β -ionone (B) and dihydro- β -ionone (C).

4.3.3 CFB Y-tube Olfactometer Test

The Y-tube olfactometer test allowed for the evaluation of an attractant or repellent effect for α -, β - and dihydro- β ionones using the crucifer flea beetle (CFB). The concentration of the pure compounds used for this test was 0.3 ng/ μ L which was the concentration of β -ionone found in the two *Arabidopsis* plants with a repellent effect in the choice test (*AtCCDI* L-2 and L-3). The CFB consistently moved towards the control Y-tube arm (air) rather than toward the arm with the β -ionone ($df=1,4$; $F=52.76$; $P=0.002$) (Figure 4.10 B). The CFB response when given the choice between α -ionone and air was not significantly different ($df=1,4$; $F=1.66$; $P=0.260$) (Figure 4.10 A), but significantly more CFB moved toward the dihydro- β -ionone than toward the control arm ($df=1,4$; $F=12.84$; $P=0.023$) (Figure 4.10 C).

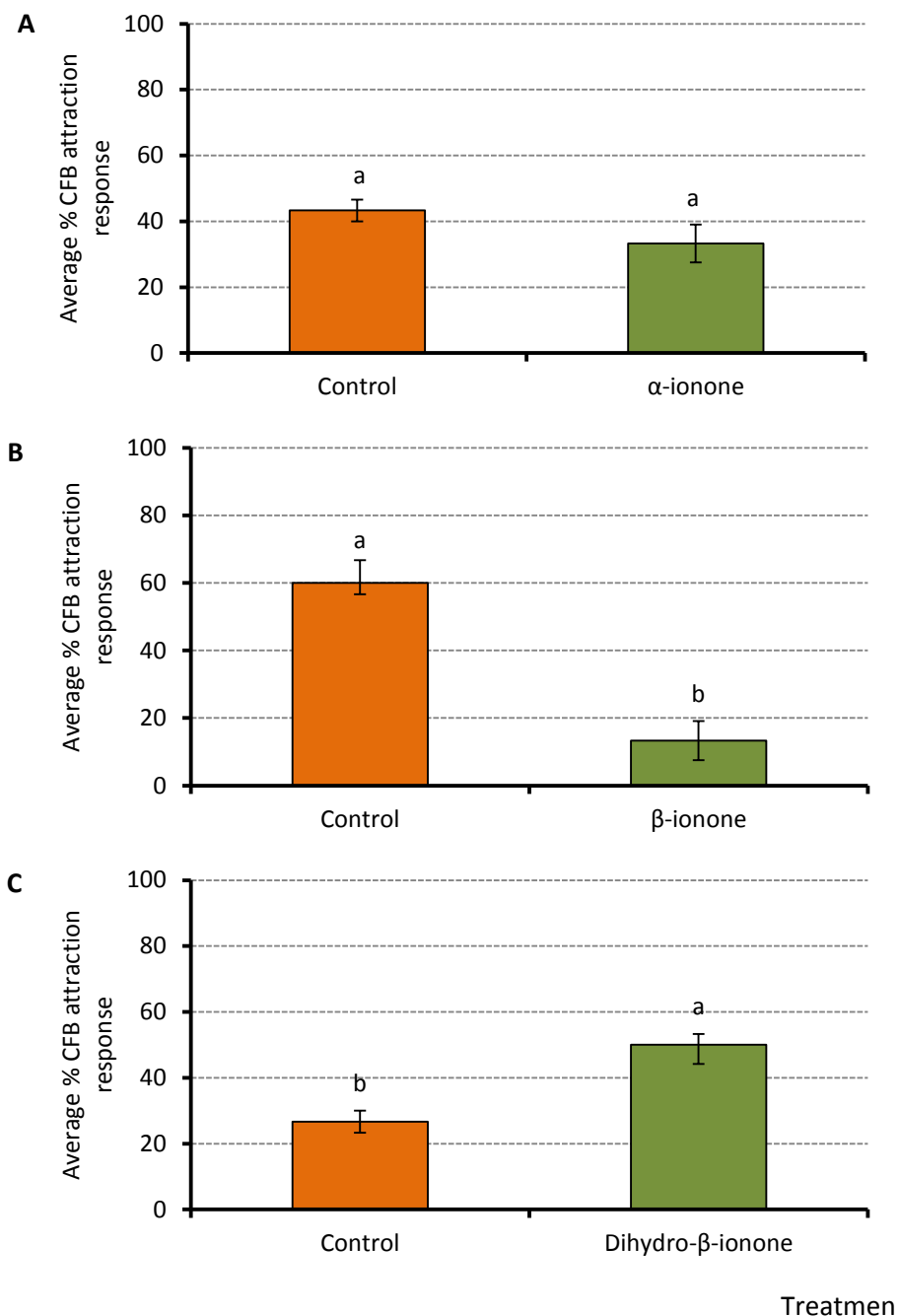


Figure 4.10. Average percent CFB attraction response (\pm S.E.) toward the control (air) or the 0.3 ng/ μ L α -ionone(A), β -ionone (B) or dihydro- β -ionone (C) Y-tube olfactometer arm. Three replicates were performed. Error bars with different letters are significantly different (one-way ANOVA, Tukey's Test $P < 0.05$).

There was a significant preference by CFB to move towards the arm with air only, rather than air containing β -ionone, which corroborates the behaviour observed with the *Arabidopsis* plant choice test. CFB did not have the same response to the structurally similar α -ionone and dihydro- β -ionone at the same concentration indicating a clear differentiation in the distinction of each molecule by CFB. An opposite behavioural response between dihydro- β -ionone and β -ionone was due to the difference in the molecule's alkyl side-chain double bond, while between the α -ionone and β -ionone the difference was the presence of a chirality center in the C-1 of the cyclohexene for the case α -ionone and the location of the molecule's endocyclic double bond which for the case of β -ionone is in the C-1 position meanwhile for the α -ionone is in the C-2 position of the cyclohexene ring.

Experiments performed in a vertically oriented, narrow-bore glass, T-shaped olfactometer to test the CFB behavior to a series of volatiles found in canola *Brassica napus* variety AC Excel observed the strongest response with β -ionone compared to E-caryophyllene, indole, (\pm)-linalool, (+)-limonene, E-geraniol, and (-)- β -pinene [23]. The same behaviour response was observed in the Y-tube olfactometer between the three ionones. As the absence or relocation of the double bond in the ketone compound and the chiral center in the case of the α -ionone, were demonstrated to affect the behavioural response of CFB, bioassays to compare the three ionones within a concentration range of 0.05 and 0.5 ng/ μ L was performed with two other herbivores to determine if this was a conserved response.

4.3.4 Two-spotted Spider Mites (TSSM) Paper Clamp Bioassay

Static choice tests like the paper clamp bioassay are useful in that they rely on diffusion to transport the test sample toward the receiving herbivore. This bioassay allowed for an evaluation of each compound in close proximity to the two-spotted spider mite without the effect of other stimulus [34]. A dose-dependent negative effect was observed with the adult TSSM exposed to β -ionone in the 0.05 to 0.5 ng/ μ L range ($df=3,36$; $F=3.58$;

$P=0.02$) (Figure 4.11). In contrast, there was no significant difference observed with α -ionone, ($df=3,36$; $F=0.75$; $P=0.53$) or dihydro- β -ionone, ($df=3,36$; $F=0.50$; $P=0.69$).

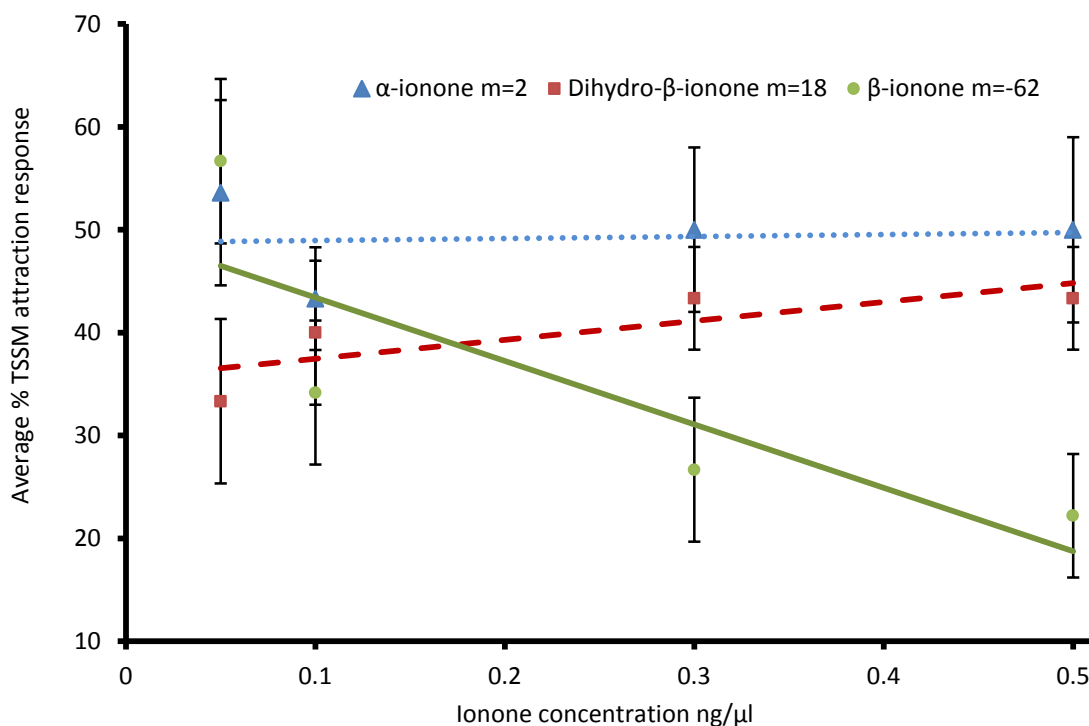


Figure 4.11. Average percent TSSM attraction response (\pm S.E.) towards α -, β - and dihydro- β ionone solutions and linear trends (m =slope) between 0.05 and 0.5 ng/ μ l using a paper clamp bio-assay. Ten replicates were performed for each ionone concentration.

The response of the spider mites was similar to that of the CFB in that β -ionone from 0.5 to 5.0 ng/ μ L had a repellent effect, while α -ionone and dihydro- β -ionone did not. Dihydro- β -ionone may be considered slightly attractive towards TSSM, but this was not a significant effect. This is the first evidence that β -ionone has a repellent effect against the TSSM, however it was demonstrated that another species of mite, the red-legged earth mite *Halotydeus destructor* Tucker (Acari: Penthalidae) fed less on *Trifolium glanduliferum* Boiss (Fabaceae) extracts that had high levels of β -ionone and other terpenes [35]. These results suggest that β -ionone could be an important pest

management tool for the control of the TSSM, which has been demonstrated to adapt to a wide variety of environments and developed resistance to many pesticides [36].

Integrated pest management of spider mites currently uses biological control as one strategy to control population growth [37], for instance, it has been shown that common bean plants *Phaseolus vulgaris* var. Taylor's (Fabaceae) infested with spider mites induces the volatiles β -cymene and β -caryophyllene which in turn attract the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Mesostigmata: Phytoseiidae) [38]. In my research the attractant effect of the compound dihydro- β -ionone and neutral effect of the α -ionone was demonstrated. Interesting complementary work could be the testing of dihydro- β -ionone as an attractant for other species that in turn would feed on the undesired pest. However, uncertainty lies in whether the transgenic plants would produce these volatiles in place of β -ionone.

4.3.5 SWF Oviposition Deterrence

The purpose of the oviposition test was to study the preference of the herbivore for an environment that is suitable for egg and nymph development. The insect preference for different plants includes physical and chemical factors, or even a combination of both [39]. The selection process could comprise different stages, visual, olfactory, and gustatory stimuli [40, 41]. Olfactory evaluation is the best indicator of oviposition preference since herbivores have more time to detect which one of the two options given (treated leaf vs control leaf) was the most suitable. The whitefly species used for this bioassay had not been exposed to α -ionone, dihydro- β -ionone or β -ionone prior to the testing.

The ODI value is based on the comparison of eggs laid on control and treated leaves and values vary from + 100 (very attractive) to - 100 (complete deterrence). It was determined that β -ionone acted as a deterrent at 0.05, 0.1 and 0.5 ng/ μ L treatments, with an average ODI value of -55, -65 and -34, respectively. The β -ionone ODI was significantly different compared to α -ionone and dihydro- β -ionone ($df=2,27$; $F=17.04$;

$P < 0.0001$) (Figure 4.12). The average ODI observed with α -ionone were -9.5, 12.13 and -10.9, and 9.5, 8.0 and 13.7 with dihydro- β -ionone for the 0.05, 0.1 and 0.5 ng/ μ L treatments, respectively.

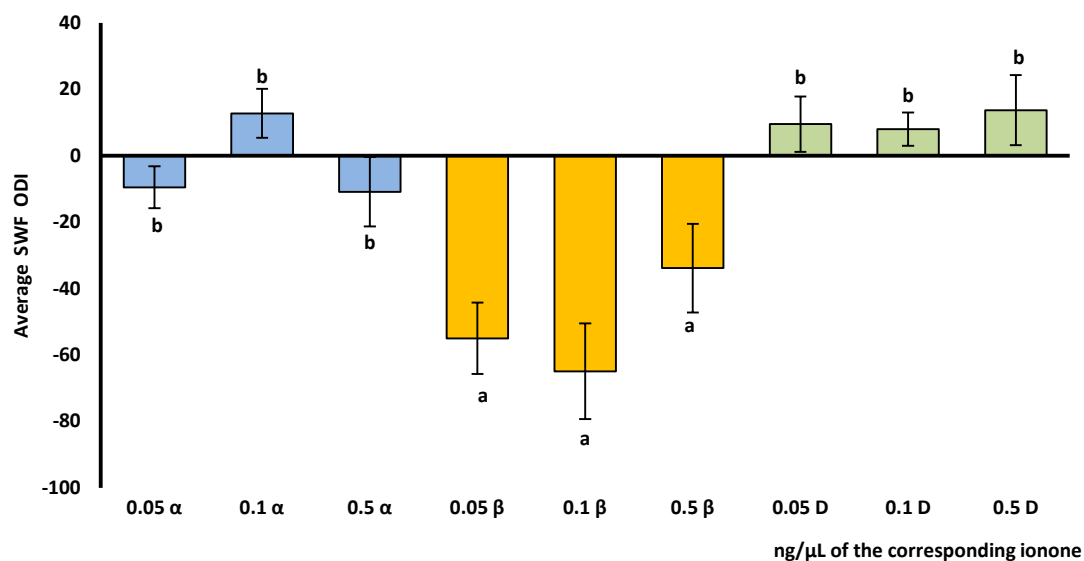


Figure 4.12. Average SWF ODI (\pm S.E.) for a series of solutions of α -ionone (blue bars), β -ionone (yellow bars), and dihydro β - ionone (green bars). Positive ODI values mean attraction, negative ODI values mean deterrence. Three replicates were performed per concentration. Means with different letters are significantly different (two-way ANOVA, Tukey's test $P < 0.0001$).

These results demonstrated that the HS concentration of β -ionone produced by *Arabidopsis* (Chapter 2 section 2.3.4) should be sufficient to act as to oviposition by SWF. Leaves treated with the other two components did not have a negative effect. When the oviposition of SWF on tomato leaves was studied, it was demonstrated that the essential oils of *Artemisia camphorata* Vill. (Asteraceae), *Ageratum conyzoides* L. (Asteraceae), *Foeniculum vulgare* Mill. (Apiaceae), *Lippia alba* Mill. (Verbenaceae), *Plectranthus neochilus* Schltr (Lamiaceae), and *Tagetes erecta* L. (Asteraceae) had repellent and oviposition-deterrent effects [32]. (E)-Caryophyllene (30.67 %) and the monoterpenes α -pinene (15.02 %) and α -thujene (11.70 %) were identified as the major

constituents of the essential oil of *P. neochilus*. It was demonstrated that oil extracts from plant sources can significantly affect egg oviposition and can be used as a strategy to control the pest. This procedure requires extraction by solvents and application of the extract on to the leaves, which is impractical, especially for field trials. SWF has been recognized as an important pest in soybeans *Glycine max* L. (Fabaceae), and current approaches for management is through the development of more resistant crops [42]. Resistant genotypes represent an important tool for integrated pest management. For example, of 10 different soybean genotypes, one genotype (JAC-17) was the least attractive for egg deposition, indicating a non-preference for oviposition by SWF [43]. No volatile or secondary metabolites were associated with the oviposition deterrence, but low trichomes density was positively correlated with the oviposition site and may be associated with the resistance of 'JAC-17' to infestation.

In the SWF bioassay performed in my research a negative effect on SWF oviposition was demonstrated with β -ionone. A repellent effect was observed as well with the TSSM in the paper clamp bioassay. These results are promising considering that β -ionone, which was the apocarotenoid compound produced by *Arabidopsis plants* (Chapter two of the thesis), can be enhanced by gene manipulation and can act as an alternative tool to control the pests instead of the application of chemical pesticides. This demonstrates the potential value of plant volatile compounds that can directly influence insect behaviour.

4.3.6 GHW Micro-Tom Tomato Choice Bioassay

A choice test where two Micro-tom tomato plants, a WT and *LeCCDI-1*, from the third line were caged with twenty mixed sex adult greenhouse whiteflies (GHW) allowed for a measure of whitefly preference. The choice test setup was different compared to the one with the *Arabidopsis* plants held in two separate chambers connected by a bridge (Figure 4.2). In this case, the two types of plants were enclosed with the GHW in one chamber in which flies were able to fly freely between the two plants (Figure 4.6). Even though more than half of the flies on average seemed to go to the transgenic plant, there

was no significant difference as to the preference of the insects between the *LeCCD1-1* and the wild type ($df=1,10$; $F=2.57$; $P= 0.14$) (Figure 4.13).

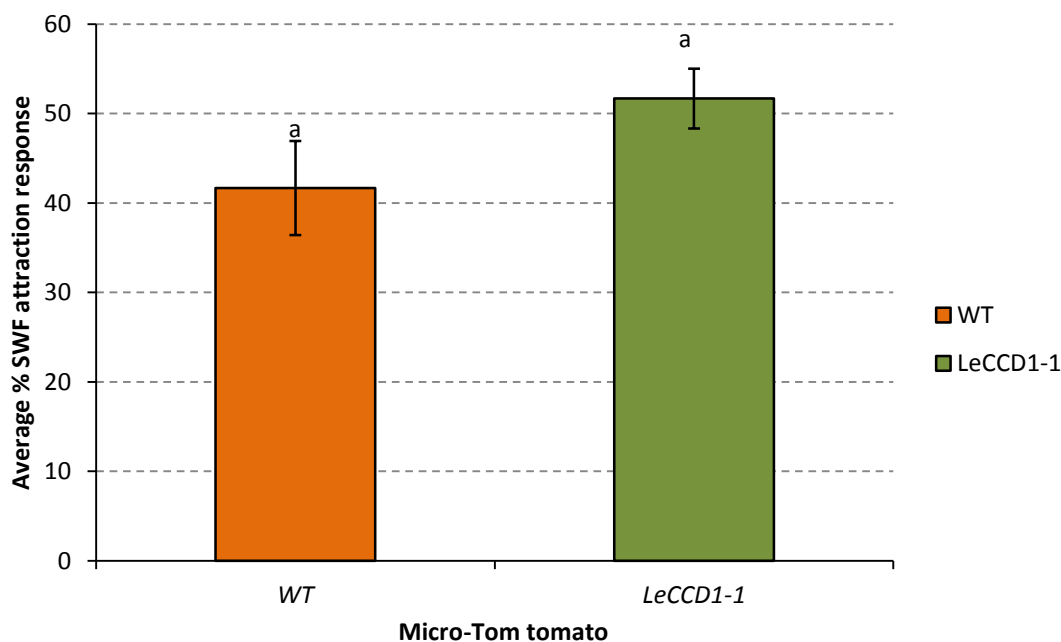


Figure 4.13. Average percent GHW attraction response (\pm S.E.) to wild type and *CCDI-1* Micro-Tom tomato plants. Six replicates were performed. Error bar with same letters are not significantly different (one-way ANOVA, Tukey's test $P>0.05$).

Similar choice experiments using GHW and tomato plants (WT and *LeCCD1-1*) were conducted at AAFC Harrow, ON. In those choice tests, paired comparisons between WT and three groups of *LeCCD1-1* at two plant stages (vegetative and flowering) examined oviposition differences. Results of these trials showed that the whiteflies oviposited more on the plants that contained the *CCDI* gene for both plant stages (personal communication, Dr. Les Shipp AAFC-AAC Harrow, ON). These results indicated that Micro-tom tomato plants overexpressing the *LeCCDI* might attract oviposition of greenhouse whiteflies. The differences between the two trials included the time period, 24 vs. 48 h, and the GHW presence on plants vs. egg oviposition. Both

factors may have led to a more significant effect being measured for the *LeCCDI* plants in the AAFC Harrow trials.

According to the non-targeted analysis depicted in Chapter two section 2.3.8.2 of this thesis, Micro-tom tomato plants released a variety of VOCs including monoterpenes, sesquiterpenes and aromatic derivatives. When comparing the emission levels of the individual compounds of the WT and *LeCCDI-1* line three the following characteristics were observed: 1) the majority of compounds released by the WT were in the ratio 1:1 compared to the *LeCCDI-1*; 2) the two most abundant monoterpenes in the plants were the R- α -pinene and the β -pinene. These compounds were 1.2-fold and 2-fold higher in the transgenic plants compared to the WT; and 3) the most abundant sesquiterpene was the α -copaene which was 2-fold higher in the WT compared to the *LeCCDI-1*. The structural isomers α and β -pinene are bicyclic monoterpenes usually found in the flowers and vegetative parts of plants, including tomatoes. The compound α -copaene is a sesquiterpene that is usually released by the leaves in tomato plants. These VOCs could act as cues for the flies. It is not clear if the insects recognize the complete blend of VOCs or just a few of them, but odour recognition and positive response depends on how VOCs bind to the odour-binding proteins present in the specialized cells found in the antenna (sensilla) of the whiteflies. Once molecules are bound the signal is then transported to the insect's brain, informing its next move [44].

The α -copaene was not commercially available to test whether or not at the concentrations found in the Micro-Tom tomato plants had any repellent or attractant effect against the GHW. In previous studies, it was reported that α -copaene was an attractant to the Mediterranean fruit fly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) [45, 46]. When mature male flies were directly exposed to angelica seed oil *Angelica archangelica* L. (Apiaceae) and ginger root oil *Zingiber officinale* Roscoe (Zingiberaceae), extracts containing α -copaene at concentrations of 0.9% and 0.4% accordingly, the rate of fly mating increased by two-fold compared to the control. Furthermore, trimedlure [t-Butyl-2-methyl-4-chlorocyclohexanecarboxylate] is a synthetic substitute for α -copaene and still widely used as a powerful lure for the C.

capitata as it was employed to detect emerging manifestations of the insect [47]. Also, α -copaene was reported to be 2 to 5 times more attractive than trimedlure, but difficulties in obtaining α -copaene in quantities sufficient for large scale trap deployment has prevented its use in field trials [48].

In the choice bioassay performed with the GHW, insects were exposed to complete plants, in which a blend of VOCs were released in similar ratios. Any differences in the behavior of the flies can be attributed to the significant difference in volatiles, in this case possibly the 1R- α -pinene, β -pinene and the α -copaene. The oviposition bio-assays with 1R- α -pinene and its isomers, α -pinene and β -pinene at concentrations of 100, 10 and 40 ng/ μ L respectively, measured a positive ODI for all three monoterpenes tested. The whiteflies were attracted to the leaves treated with the 1R- α -pinene and the β -pinene, but not as much so with the α -pinene ($df=2,15$; $F=2.75$; $P= 0.096$) (Figure 4.14). These results may be concentration dependant as the 2 former compounds were 4 to 10- fold higher in concentration.

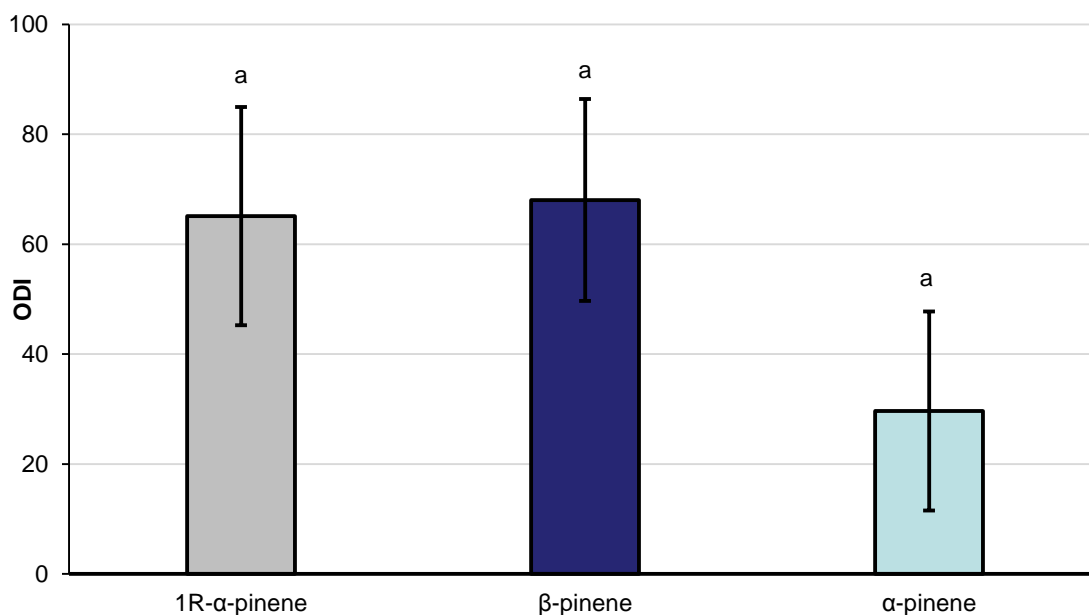


Figure 4.14. Average GHW ODI (\pm S.E.) on Micro-Tom tomato leaves treated with the monoterpenes 1R- α -pinene (100 ng/ μ L), β -pinene (40 ng/ μ L), and α -pinene (10 ng/ μ L). Positive ODI values mean attraction. Six replicates were performed. Means with same letters are no significantly different (one-way ANOVA, Tukey's test $P < 0.096$).

These results in part confirm the attraction by whiteflies for these compounds in higher concentration in the HS of *CCDI* tomato. Further research should determine whether or not increments of α -copaene could act as a semiochemical to attract beneficial insects, such as the wasp *Encarsia formosa* Gahna (Hymenoptera: Aphelinidae), considered as a biological control to control GHW [49].

4.4 Conclusions

This chapter described a strategy to evaluate the effect of transgenic plant volatiles towards selected herbivores. The strategy, which started with the extraction and identification of VOCs from the HS of *Arabidopsis thaliana* and Micro-tom tomato plants overexpressing the *CCDI* genes, included the following steps: 1) the screening by

a choice bioassay to identify the preference of a herbivore towards two types of plants (WT vs. *CCDI*); 2) the identification by GC-MS of the HS plant volatiles that could be responsible for the insect response; and 3) the performance bioassays with volatile standards identified to confirm the biological effect and to avoid the influence of other VOCs from the plants. A complementary step that could be included but was not performed in this thesis is the correlation of the repellent/attractant response for its possible usage in pest management and control in place of synthetic insecticides. This last step requires transformation of crops in a large scale (greenhouse environment).

In the *A. thaliana* study, it was observed that the crucifer flea beetle (CFB), an important Brassica insect pest, was significantly repelled by the *AtCCDI* L-2 volatiles. This transgenic line showed a 49-fold higher expression of the gene compared to the WT. Consequently, the higher levels of gene expression correlated with the higher production of the volatile compound β -ionone, 4.5-fold greater than the WT. Bioassays with β -ionone and two similar ionones, α -ionone and dihydro- β -ionone, demonstrated each produced a different behavioural response based on the ionone chemical structure that was consistent with three different herbivores, the two-spotted spider mite (TSSM), the silverleaf whitefly (SWF) and the CFB.

For instance, it was revealed that β -ionone at 0.3 ng/ μ L, the concentration typically measured in the HS of transgenic plants, had a significant and strong repellent effect to TSSM and CFB and between 0.05 to 0.5 ng/ μ L reduced oviposition by the SWF. Experiments with the isomer α -ionone at the same concentrations observed neither repellent or attractant effect towards the same pests. Interestingly, the other isomer dihydro- β -ionone was found to act as an attractant, especially towards the CFB in a Y-tube bioassay. The difference between these three molecules is attributed to the location, and in the case of the dihydro- β -ionone, the absence of a π bond. According to the observations made, these differences at the molecular level affected the behavioral response of the herbivores. The bioassays with chemical standards confirmed the observations with the whole plant choice test. This study supports the over-expression of CCD genes as a tool for the enhancement or creation of volatiles that might counteract

the attack of important herbivore pests, and result in the reduction of pesticides that cause a detrimental effect to the environment.

In the Micro-Tom tomato plant study, the following discoveries were made: 1) since transgenic plants overexpressing the *CCD1* gene did not release β -ionone, no repellent affect was observed; 2) choice bioassays with the greenhouse whitefly (GHW) revealed that even though there was no significant difference in the attraction/repellent effect, more than half of the fly population landed on the transgenic plants while the preference of the GHW to oviposit on the transgenic plants was observed by others. Both WT and CCD1 plants revealed a similar profile of VOCs, the main difference was from the flowers where the bicyclic monoterpenes, 1R- α -pinene and β -pinene, were released in greater concentrations from the *CCD1*, and from the leaves, the tricyclic sesquiterpene α -copaene was more abundant in the WT. Oviposition bioassays with the standard 1R- α - pinene and its isomers, α -pinene and β -pinene, demonstrated an attractive response indicating the preference of the insect for the transgenic plants. These components are part of the natural volatile profile of the tomato plants and increasing their levels serves to attract more GHW, even though α -copane is considered to be an attractant to more than nine different types of insects [50] and was higher in the HS of the WT tomato. Further research should use the performance of biossays with the pinenes and copaene standards to evaluate the possible attraction of beneficial insects that could feed on either whiteflies or whiteflies eggs, for example the whitefly parasite (*Encarsia formosa*). It is widely understood that chemical pest control involves the use of chemical substances to kill or disrupt the life cycle of an insect pest. However, as it was demonstrated in this chapter, plants can produce a wide variety of secondary metabolites that can be used as an alternative management approach. Although conventional pesticides are still used, they are gradually being replaced by less toxic compounds that disrupt insect development or modify behaviour. Some of these new chemicals are much safer for the environment and more species specific than most conventional insecticides.

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Chapter 5: Investigation into the Analytical Characterization of Pyrolyzed Tomato Residue Bio-oil and its Pesticide Applications

Reproduced in part with permission from **L.A. Cáceres**, B.D. McGarvey, I.M. Scott, K. Conn, F. Berruti, C. Briens & K.K.-C. Yeung. Pesticidal properties of tomato plant residue bio-oil. *Pest Management Research Report*, **2011**, 4, (H), 17-20.

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As stated in the preface section of this thesis, chapter five describes the work performed during the first two years of my graduate studies and it explains the analytical strategies developed in order to simplify a complex sample matrix called bio-oil. Greenhouse tomato plant residue was the biological matter used in this study to produce bio-oil by thermal decomposition in a process called “pyrolysis”. Bio-oil fractionation and analysis was performed along with bioassays in order to evaluate its pesticides properties against the Colorado potato beetle (CPB) a common insect pest that causes a lot of detrimental to important crops in North America.

5.1 Introduction

The pyrolytic conversion of agricultural residual biomass into bio-oil represents a potentially attractive technology for the removal and processing of agricultural waste from farms and greenhouses and its conversion into an alternative source of green energy and value-added chemicals [1]. Researchers at the Institute for Chemicals and Fuels from Alternative Resources (ICFAR) at the University of Western Ontario designed a highly automated fast pyrolysis fluidized bed pilot plant to convert solid biomass into bio-oils, gases and bio-char at temperatures from 250 to 800 °C under near-atmospheric pressure [2], a schematic of the pyrolysis processes is presented in the method section figure 5.1. Biomass previously dried and ground to < 1 mm particle size is delivered to a bed of hot sand fluidized with inert gas (typically nitrogen) operating at temperatures between 300 and 550 °C and with gas residence times of the order of seconds. Through thermal cracking, the biomass is transformed first into solid bio-char residues, gases and vapors. They exit the reactor through a hot filter which retains the bio-char within the sand bed. This bio-char is valuable for use as a fertilizer [1]. The gases and vapors are directed to a condenser followed by an electrostatic precipitator (ESP) where the condensables are collected as bio-oil while the permanent gases are vented. Such gases, including carbon monoxide, methane, hydrogen, carbon dioxide and inert nitrogen, have energy value that can be utilized for industrial processes [1]. The bio-oil accumulated in the ESP is soluble in acetone and has a higher density and

viscosity than the aqueous fraction collected in the condenser. The difference in viscosity and density values can be attributed to the water content in the bio-oils [3].

Our group has been characterizing the biopesticide properties of bio-oils by testing the entire content and its fractions against selected insects, bacteria and fungi [4-8]. In the last few years, we attempted to isolate and identify the active chemicals responsible for the activities in bio-oils obtained from various biomasses. Bio-oils are complex in chemical composition, but in general, are mainly composed of water, organic compounds and a small amount of ash. According to Bridgwater, bio-oil is a miscible mixture of polar organics (about 75–80 wt. %) and water (about 20-25 wt. %). It is obtained in yields of up to 80 wt% in total of dry feed [1]. The general analytical approach begins with the fractionation of bio-oils with solvent extractions followed by liquid chromatographic separations. Bioassays against an organism of interest are used to select the most active fraction for GC-MS identification. Our earlier work examined bio-oils from tobacco, coffee grounds, and grape seeds and skins. Briefly, nicotine, a chemical present in tobacco and a known pesticide, was found to remain intact during the pyrolysis process. While bio-oil from tobacco leaves has been examined for its pesticide properties against a variety of species of fungi and bacteria, and an insect, the Colorado potato beetle (*Leptinotarsa decemlineata*), it was found that the nicotine in tobacco bio-oil was not solely responsible for the observed pesticide activity and that nicotine-free fractions of the bio-oil demonstrated pesticide activity. According to the GC-MS data, this fraction was found to be rich in phenolic compounds [6]. Coffee ground bio-oils produced at 500 and 550 °C were the most active against two species of bacteria, whereas the 400 and 450 °C bio-oil samples were the most active against the CPB.

After fractionation of the active fractions, it was found that both phenolic and non-phenolic containing fractions were active, likely due to the collective presence of various molecules [4]. Even though no pesticide bio-assays were performed with bio-oils from grape skins and grape seeds, the organic compounds octadecanoic acid, reported to have insecticidal effects against the fall armyworm (*Spodoptera frugiperda*)

[9], and ethyl ester acids, reported to have strong bactericidal effects [10], were present in the aqueous phase [2].

Bio-oils obtained from the basic plant biomass components of lignin, cellulose, hemicellulose were analysed by our group as well [7]. The lignin bio-oil, collected in the ESP, was found to be the most insecticidal when tested individually. Collectively, the cellulose and hemicellulose components of the biomass were found to enhance the insecticidal activity of the lignin fraction when pyrolyzed altogether. Most recently, we expanded our studies to the bio-oils from canola and mustard straws [8]. These *Brassicaceae* crops were chosen for their content of glucosinolates and isocyanates, compounds with recognized anti-herbivore activity. These two groups of compounds did not survive the pyrolysis process, but it was reported that the main compounds identified within the most active fractions were hexadecanoic and octadecanoic fatty acids.

In this work, the biomass of particular interest is greenhouse tomato plant waste. In 2011, Canada produced 540 ha of greenhouse tomato [11], and a typical vegetable greenhouse operation in Canada produces 40-60 tons of organic residues per hectare per year [12]. Similar to canola and mustard straws, tomato plants contain a variety of phytochemicals that provide natural defenses against pathogens and pests including fungi, viruses, bacteria, insects, and nematodes [13-16]. Specifically, tomatoes accumulate phenolic compounds, phytoalexins, protease inhibitors, carotenoids, lycopenes, and glycoalkaloids. For example, the glycoalkaloid tomatine is present in all parts of the tomato plant and has antibiotic properties against a variety of fungi and bacteria [17]. To date, however, there have been no reported studies of insecticidal activity of greenhouse tomato waste bio-oil. A positive use of the tomato plant residue through pyrolysis technology is therefore worthwhile. The objective of the research was therefore to assess the insecticidal activity of the bio-oils obtained from tomato plant waste, and isolate and identify the main active compounds. As in previous studies, the approach focuses on the bio-oil fractions that exhibit significant levels of pesticidal activity. Many separation techniques suitable for bio-oils have been reported in the literature [18-21]. For this study, liquid-liquid extraction with dichloromethane (DCM)

and water was used to separate the bio-oil components based on polarity. Next, fractionation by amino solid phase extraction and reversed phase LC were performed. Fractions with significant insecticidal activities were characterized using GC-MS to identify the responsible compounds.

5.2 Materials and Methods

5.2.1 Reagents

Neophytadiene (Santa Cruz Biotechnology, Dallas, TX) and phytol (97%, from Sigma-Aldrich, Oakville, ON, Canada) were used for confirmation and quantification purposes. Pesticide bioassays were performed using acetone, both as a carrier solvent and as a control (99.9%, Sigma-Aldrich). The reagents, methoxyamine-HCl in pyridine (MOX) and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (Thermo Scientific, Ottawa, ON, Canada) were used in the derivatization and GC-MS analysis of active fractions. Acetonitrile HPLC grade, 99.8% (Caledon Laboratories, Brockville, ON, Canada) was used in HPLC as a mobile phase.

5.2.2 Biomass and Bio-oil Production

Approximately 30 kg of fresh greenhouse tomato plant waste, including leaves, roots, stems and some tomatoes, was collected from Pyramid Farms greenhouse (Leamington, ON, Canada) in April, 2010. Material was dried using a Johns Scientific Watlow Oven drier for 24 h at 70 °C. Dried material was ground to < 1 mm particle size using a GE Motors & Industrial System grinder (Thomas Scientific, Swedesboro, NJ). Bio-oils were produced in a fluidized bed pyrolyser at the Institute for Chemicals and Fuels from Alternative Resources (ICFAR, London, ON, Canada). The reaction took place in an electrically heated fluidized bed pilot plant reactor, 7.8 cm in diameter, with a 52.4 cm long cylindrical section (Figure 5.1) containing 1.5 kg of silica sand as inert bed material. An actual picture of the reactor is shown in Appendix 9. Pyrolysis was

performed at 300 and 500 °C with fluidizing and carrier nitrogen volumetric flow rates adjusted to maintain a nominal vapor residence time of 5 seconds. Bio-oil products were collected from both an ESP and a condenser and were weighed before and after each run to obtain an accurate liquid yield. The bio-oils were stored at 4 °C prior to using them in the bioassays.

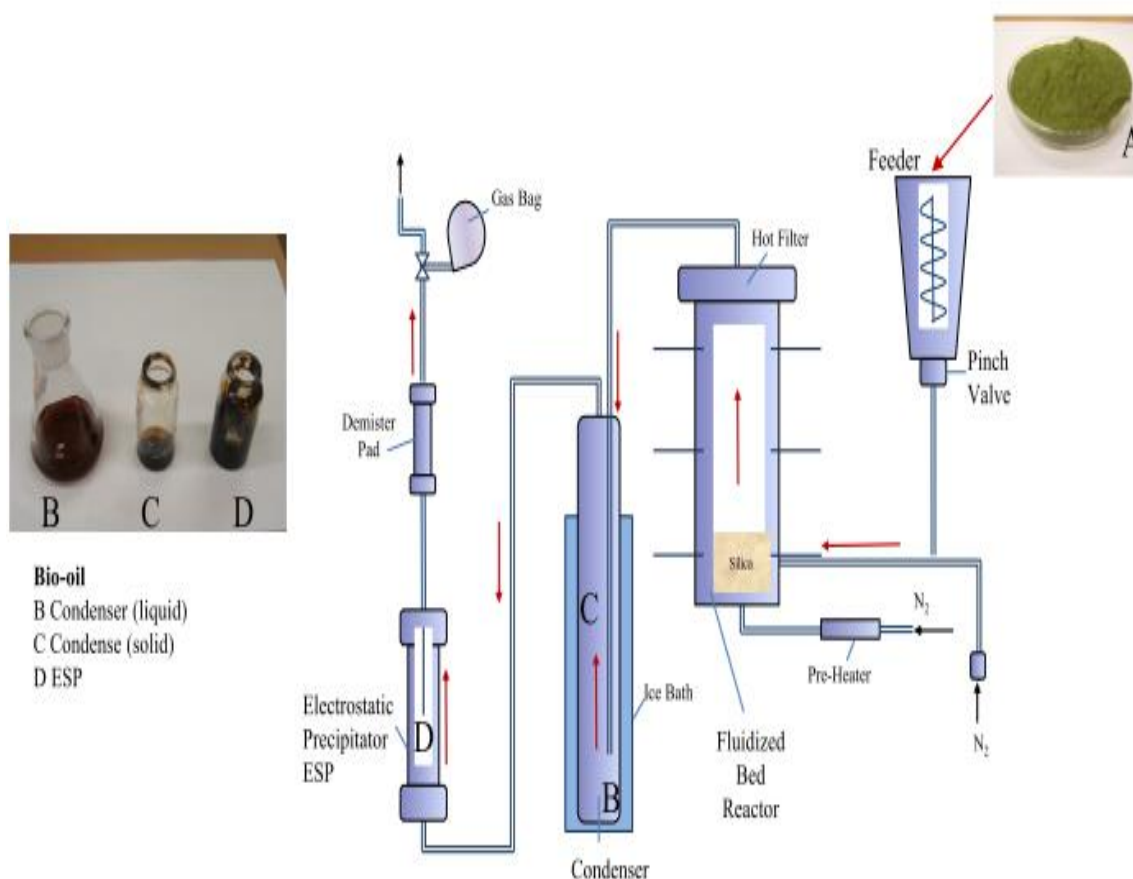


Figure 5.1. Schematic process flow diagram of the ICFAR fluidized bed reactor used to convert tomato plant residue biomass into bio-oil. Dried and ground tomato biomass (A) is added into the feeder and pushed into the fluidized bed reactor by nitrogen pulses. Reactions occurred in the reactor, containing inert silica, due to the high temperatures reached (300 to 500 °C). Sample's gases are then cooled down in the condenser where bio-oils (B) and (C) are collected. Uncondensed gases are then collected in the electrostatic precipitator as a bio-oil (D).

5.2.3 Biological Assays

The organic ESP fraction of tomato waste bio-oil was soluble in a 2:1 acetone/water solvent, the aqueous fraction collected in the condenser was soluble in water, and the solid part collected in the condenser was partially soluble in the 2:1 acetone/water solvent. Two concentrations of each of the bio-oil fractions, 3 and 30 mg/mL, were tested against colorado potato beetle (CPB) and several species of bacteria and fungi.

5.2.3.1 Colorado Potato Beetle Leaf Disc Bio-assay

The CPB bio-assay, was similar to the procedure applied by Bedmutha et al. [4]. Insecticide-susceptible 2nd instar larvae CPB were fed potato *Solanum tuberosum* var. Kennebec and held in an environmental chamber, 25 °C, 50% RH with a photoperiod of 16:8 L:D, at the Southern Crop Protection and Food Research Center, Agriculture and Agri-Food Canada (London, ON). Potato leaf discs were cut to a 4-cm diameter and 50 µL of the bio-oil solution was applied on top and 100 µL on the bottom. The difference in the amount added in each side, is because there is more absorption of the treatment in the bottom compared to the top. The disc was then dried for approximately 15 min in a fume hood before being transferred to a 4.25-cm Gelman Petri dish containing filter paper moistened with distilled water. Five 2nd instar CPB larvae were placed on each treated leaf disc. Larval mortality and potato leaf feeding damage were recorded after 24 and 48 h with each test performed in triplicate. Controls consisted of leaf discs treated with the same volume of acetone or water only. One-way and two way ANOVAs (Proc. GLM, Statistical Analysis System, Server Interface Ver. 2.03, SAS Institute, Cary, NC) were used to test for significant differences in CPB mortality and feeding damage between fractions.

5.2.3.2 Bacteria and Fungi Assays

A 96-well plate bioassay was used to test the effect of tomato bio-oils on the growth of bacteria and fungi. Sterile liquid growth media Potato Dextrose Broth (PDB) was used to prepare controls and dilute bio-oils designated as treatments. Each well was filled with 100 μ L of the treated or untreated PDB and inoculated with either bacteria or fungi, except PDB control wells. All plating was completed under sterile conditions in a biohazard hood. Ten fungal species (*Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium solani*, *Monilinia fructicola*, *Pythium aphanidermatum*, *Phytophthora sojae* race 2, *Pythium ultimum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*) and seven bacterial species (*Acidovorax avenae*, *Clavibacter michiganensis* sub. sp. *Michiganensis*, *Erwinia carotovora* pv. *Atroseptica*, *Pseudomonas syringae* pv. *Tomato*, *Streptomyces scabies*, *Xanthomonas campestris* pv. *Vesicatoria* and *Xanthomonas gardneri* group D) were used. Bacteria were grown on Tryptic Soy Agar (TSA) plates for 3-4 days before a loop-full of the culture was suspended in a 1.5 mL Eppendorf tube containing 1.0 mL of phosphate buffer and vortexed. Twenty microlitres of the specific bacterial solution was used to inoculate each well in the 96-well plate. Fungi were grown on Potato Dextrose Agar (PDA) plates for 7-10 days before fungal plugs, approximately half the diameter of the individual well, were cut out with a scalpel and transferred to the appropriate wells. One fungal plug was transferred per well. Each 96-well plate included the following controls: 1. Potato Dextrose Broth (PDB) control (no bacteria or fungi inoculated), 2. Negative control (bacteria or fungi inoculated with no antibiotics or bio-oils present) 3. Positive control (bacteria or fungi inoculated with the addition of antibiotics and no bio-oils present) and 4. Acetone control (bacteria or fungi inoculated with the addition of acetone to reach 3% concentration in each well and no bio-oils present). A mixture of antibiotics Chloramphenicol and Streptomycin, a detergent (Tergitol) and 95% ethanol were added to PDB to prepare a positive control solution for the bacteria. The final concentration of each of the antibiotics in an individual well was 33 mg/L. Two antibiotics were added to PDB to make a negative control solution for the fungi. The final concentrations of the antibiotics in each well were: cycloheximide 3200mg/L and

nystatin 80mg/L. All of the ingredients were previously mixed in 70% (cycloheximide) or 95% ethanol (nystatin) before being added to PDB and distributed to individual wells. The treatments consisted of bio-oils diluted with an equal volume of sterile PDB solution distributed as 100- μ L aliquots per well. Therefore, each 96-well plate consisted of controls and treatment wells that were inoculated with either fungi or bacteria, except PDB controls. Depending on the number of bio-oils tested, the plates had either one species or were split to accommodate two species. Bacteria and fungi were always tested separately. Each control and treatment had 4 reps and each experiment was repeated twice. After inoculation, the 96-well plates were incubated at 21 °C in a clean culture incubator. The incubation time for bacteria was 3-5 days and for fungi, 5-7 days. The incubation time allowed for sufficient growth of bacteria and fungi in control wells so that growth rates in the treatment wells could be compared, read and recorded. In the case of bacteria a turbid solution indicated growth, whereas a clear solution would indicate inhibition.

In the case of fungi, the wells had to be observed more closely and sometimes with the help of a dissecting microscope. The amount of mycelia production and comparison of treated with control wells would give an indication of the amount of inhibition present.

5.2.4 Characterization of Bio-oil

The bio-oil was fractionated based on an insecticidal/feeding damage bioassay-guided method starting with a concentrated solution of the tomato bio-oil obtained from the ESP as a result of the pyrolysis carried out at 500 °C (30 mg/mL TW ESP 500 °C or Fraction A) (Figure 5.2).

5.2.4.1 Liquid-liquid Extraction

The 30 mg/mL Fraction A was extracted in a separatory funnel based on relative solubility in equal parts (400 mL) of water and DCM. Each part was dried using a rotary

evaporator (Rotavapor-R BÜCHI, Switzerland) and then re-dissolved in acetone for the DCM sub-fraction (Fraction B) and in water for the polar sub-fraction (Fraction C) to maintain the original concentration of 30 mg/mL.

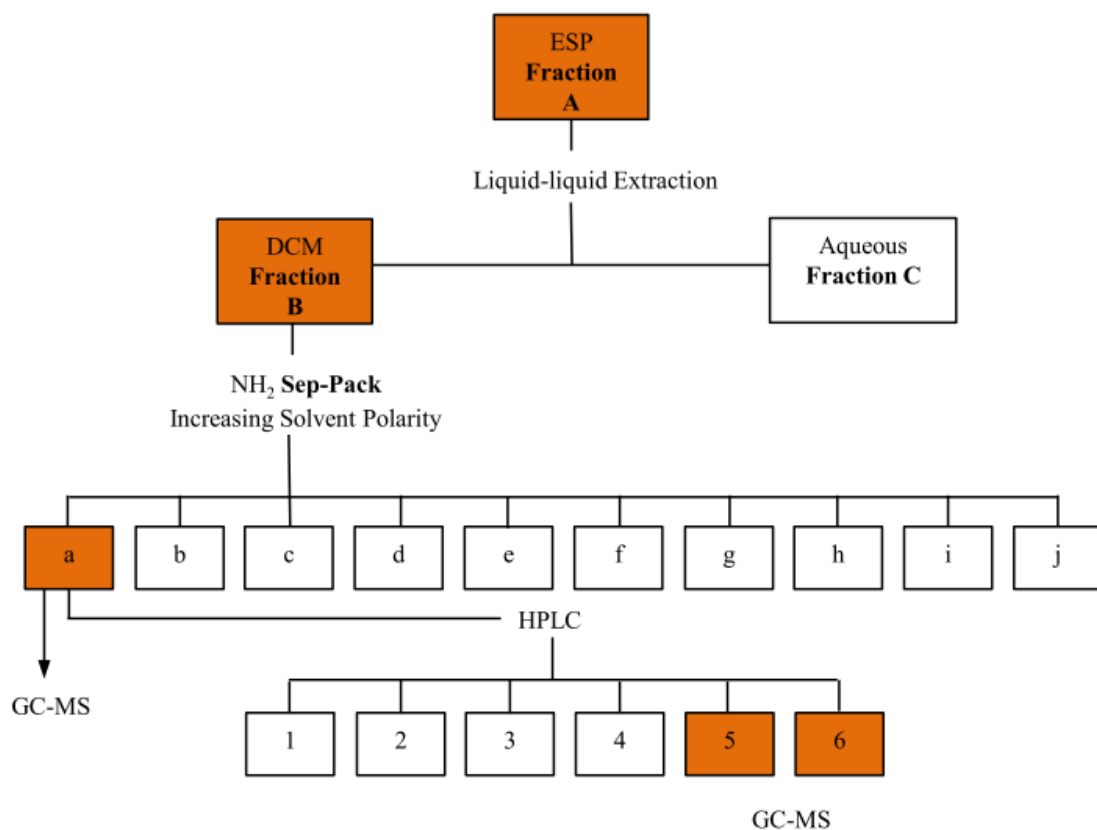


Figure 5.2. Fractionation scheme of the tomato plant residue bio-oil based on CPB mortality and reduced leaf consumption (fractions with highest activity indicated by coloured rectangles).

5.2.4.2 Solid-phase Extraction

The more active DCM fraction of the bio-oil (Fraction B) was further separated into 10 sub fractions using amino solid-phase extraction cartridges (Sep-Pak®Plus, Waters Chromatography Division, Millipore Corporation, Milford, MA). The cartridges were

conditioned with 10 mL of acetone before use. Two milliliters of the 30 mg/mL Fraction **B** was added into the cartridges, and the separation was performed with the following sequence of solvents (10 mL each): hexane (a), DCM (b), 3:1 DCM/MeOH (c), 1:1 DCM/MeOH (d), 1:3 DCM/MeOH (e), MeOH (f), 1:3 H₂O/MeOH (g), 1:1 H₂O/MeOH (h), 3:1 H₂O/MeOH (i) and H₂O (j).

5.2.4.3 Liquid Chromatography Conditions and Fraction Collection

LC separation and fraction collection of the most active hexane sub-fraction, Fraction **a**, was performed on an Agilent 1200 Series liquid chromatographic system (Agilent Technologies Canada Inc., Mississauga, ON, Canada). The system is equipped with a quaternary pump, a G1315D diode-array UV detector scanning from 205 to 400 nm, a column temperature controller set at 23 °C and a G1367B Hip-ALS autosampler.

The chromatographic separation and fraction collection was performed on a SymmetryPrep™ C18 7 µm, 7.8 x 300 mm column (Waters Limited, Mississauga, ON, Canada). The mobile phase was composed of Milli-Q water (A) and HPLC grade Acetonitrile-190 (99.8%) (B), at a flow rate of 2.9 mL/min. The injection volume was 100 µL and the collection trigger mode was time-based, every 5 min starting at 5 min. The gradient was performed as follows: Initial 90% A and 10% B; from 0-5 min B was increased to 70% and held for 15 min; from 20-25 min B was increased to 100% and held for 5 min; from 30-31 min B was decreased to 10% and held until 35 min. Once all sub-fractions were collected, solvents were evaporated using a rotary-evaporator and re-dissolved in acetone to make a 30 mg/mL solution (equivalent to what it was in the original extract).

5.2.4.4 Sample Preparation and GC-MS Conditions

Derivatization with MOX and MSTFA was used to increase volatility of the compounds, and thus improve the detection and identification of compounds in the most active sub fractions of bio-oil (Fractions **a**, **5** and **6**). Fraction **B** at 30 mg/mL and

Fraction **A** diluted 2:1 in acetone/water were derivatized and analyzed as well. A 300- μL aliquot of each of the sub-fractions was transferred into a 1.5-mL sample vial and dried under nitrogen gas. MOX reagent (150 μL) was added to each vial and heated at 50 °C for 1.5 hours. MSTFA (150 μL) was then added to each vial and heated for 0.5 hours at 50 °C, followed by GC-MS analysis. A 5975C inert XL EI/CI MSD with triple-Axis detector and a 7890 A GC system (Agilent Technologies) was used to analyze the bio-oil sub-fractions. A 1- μL sample was injected (splitless mode) into a 30 m x 250 μm x 0.25 μm HP-5MS column (Agilent Technologies). The injector temperature and transfer line temperature were maintained at 270 °C. The oven temperature was set initially at 70 °C for 5 min, and then increased at 5 °C/min to 300 °C and held for 5 min. The m/z scan range was 40-550, and spectra were scanned at a rate of 2.98 scans/s. Identification of chemical components was carried out using the Automated Mass Spectral Deconvolution and Identification System (AMDIS), and the NIST MS search program for the NIST/EPA/NIH mass spectral library (Version 2.0f, Oct 8, 2008, USA). A calibration curve with the following range of concentrations in ppm (6.25, 12.5, 25.0, 50.0 and 100) was used to determine the concentration of neophytadiene ($R^2 = 0.9998$) and phytol ($R^2 = 0.9963$) standards.

5.3 Results and Discussion

5.3.1 Bio-oil Yield

The total yield by weight of bio-oils collected from the condenser and ESP were 25.9 and 37.8 % for the 300 and 500 °C pyrolysis, respectively. For the 300 °C pyrolysis bio-oil, 4.1 % was from the ESP, while 13.2 and 8.6 % were the soluble (aqueous) and solid phases from the condenser, respectively. After pyrolysis at 500 °C, 6.7 % was from the ESP, while 19.8 and 11.3 % were the aqueous and organic phases from the condenser, respectively. The overall trend in yield distribution was consistent with the literature [1] and with previous results using grape residues, coffee grounds and tobacco leaves as biomass feedstocks [2, 4, 6].

5.3.2 Assays for the TW Bio-oils at 300 °C and 500 °C

CPB is a major horticultural pest that has developed resistance to many natural and synthetic chemicals due to enhanced metabolism involving esterases, carboxylesterases and monooxygenases, target site insensitivity, reduced insecticide penetration and increased excretion [22-24]. It therefore represents an excellent model insect and a worthy target for new biopesticide discovery. The CPB leaf disc bioassays were performed on the ESP and the two condenser bio-oil fractions obtained at both temperatures at two concentrations, 3 and 30 mg/mL. Out of the total of twelve fractions tested, the 30 mg/mL solution of TW ESP 500 °C and the 30 mg/mL solution of TW Condenser solid 500 °C produced 64.2 and 43.3 % mortality respectively, which was significantly higher than the solvent control (Two-way ANOVA $d.f.=15, 112$; $F=11.47$; $p < 0.0001$) (Figure 5.3). There were no significant differences between the remaining bio-oil treatments including all of those produced at 300 °C pyrolysis ($p > 0.05$). These results confirm the potential uses of selected bio-oil fractions as an insecticide. Since the highest insecticidal activity was observed in the TW bio-oil, ESP 500 °C (Fraction A).

A preliminary screening of the activity of the same 12 bio-oil sub fractions was performed as well, in order to determine a bactericidal and fungicidal affect (Table 5.1). It was found that the TW ESP 500 °C or Fraction A also inhibited the growth of the fungi, *Alternaria alternate*, *Fusarium solani*, *Phytophthora sojae*, *Monilinia fructicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*, and the bacteria *Streptomyces scabies*. This results show the potential uses of the bio-oil fraction as a fungicide and bactericide. On the contrary, the 30 mg/mL solution of TW Condenser solid 500 °C which was active in the CPB bioassays, showed mild growth inhibition of seven types of fungi and growth of all types of bacteria. The other 10 TW bio-oil sub fractions did show mild and/or no inhibition. Since the highest insecticidal, fungicidal and bactericidal activity was observed in the TW bio-oil, ESP 500 °C (Fraction A), further CPB bioassay-guided fractionation of this fraction using liquid-liquid extraction was pursued to isolate and identify the active compounds.

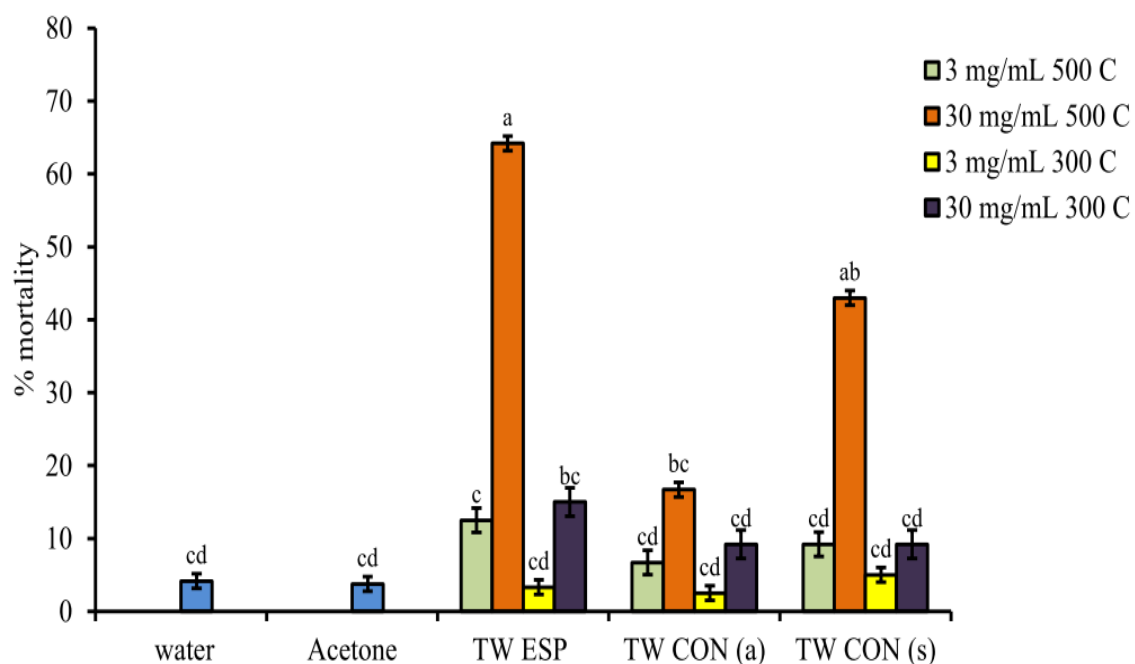


Figure 5.3. Mean percent mortality (\pm SE) of Colorado potato beetle larvae after exposure to leaf discs treated with TW bio-oil solution fractions (12 treatments) for 48 h. Mortality with different letters are significantly different ($p < 0.0001$).

Table 5.1. Activity of tomato waste bio-oils fractions against bacteria and fungi in 96-well plates. Acetone was used as control. Data represents growth inhibition n=3.

		Tomato waste Bio-oil											
Bio-oil fraction		ESP				CON (a)				CON (s)			
Pyrolysis temp (°C)		300		500		300		500		300		500	
Concentration (mg/mL)		3	30	3	30	3	30	3	30	3	30	3	30
BACTERIA	<i>Acidovorax avenae</i>		■		■								
	<i>Clavibacter michiganensis</i> sub.									■	■		
	<i>Erwinia carotovora</i> pv.												
	<i>Pseudomonas syringae</i> pv.				■								
	<i>Streptomyces scabies</i>	■		■	■	■					■	■	
	<i>Xanthomonas campestris</i> pv.												
	<i>Xanthomonas gardneri</i> Group D												
FUNGI	<i>Alternaria alternata</i>				■								■
	<i>Botrytis cinerea</i> (isolate 2)				■		■						■
	<i>Fusarium oxysporum</i> (isolate 1)				■								
	<i>Fusarium solani</i> (isolate 1)				■								
	<i>Monilinia fructicola</i>				■						■		■
	<i>Pythium aphanidermatum</i>				■						■		
	<i>Phytophthora sojae</i> Race 2				■						■		■
	<i>Pythium ultimum</i>				■						■	■	■
	<i>Rhizoctonia solani</i>	■	■	■	■						■		■
	<i>Sclerotinia sclerotiorum</i> (isolate		■		■						■		■

Strong growth inhibition ■

Mild growth inhibition ■


growth ■

5.3.3 Bioassay-guided Separation of Fraction A

Fraction **A** was separated by liquid-liquid solvent extraction using 1:1 water/DCM separation of the yielding a non-polar fraction that dissolved in acetone (Fraction **B**). Fraction **B** produced 62.2 % CPB mortality, which was similar to the mortality observed with Fraction A (Table 5.2) By comparison, exposure to the aqueous fraction (Fraction **C**) resulted in only 13.3 % CPB mortality. Percent leaf consumption on discs treated with Fraction **A** and **B** ranged from 11.8 to 13.9 %, which was significantly lower (One-way ANOVA *d.f.* =3; $F=26.83$; $p < 0.0002$) compared to Fraction C and controls. While both methods led to the same general observations in this case, the CPB mortality was

found to be more useful in revealing the effects of low activity samples. After liquid-liquid extraction, no inhibition of bacteria or fungi growth was noted for any of the fractions. The higher sensitivity of the insect bioassay supported the further testing of the bio-oil fractions with the Colorado potato beetle bioassay.

Table 5.2 Mean percent mortality and consumption (\pm S.E.) of Colorado potato beetle larvae and potato leaf respectively, after exposure to leaf discs treated with Fraction A, B and C tomato waste bio-oil for 48 h. CPB mortality and leaf consumption with different letters are significantly different ($P < 0.0002$) for mortality and ($P < 0.0001$) for potato leaf consumed.



Fraction	Mean % mortality (S.E.)	Mean % potato leaf consumed (S.E.)
A	64.2 (0.30)a	11.8 (1.7)B
B	62.2 (0.35)a	13.9 (1.39)B
C	13.3 (0.24)b	82.2 (1.69)A
Water control	4.4 (0.15)b	88.4 (1.82)A
Acetone control	2.2 (0.11)b	82.2 (0.88)A

5.3.4 Insecticide bioassay-guided solid phase extraction of the DCM fraction (Fraction B)

Fraction **B** was further separated with amino solid-phase extraction. Of the ten fractions obtained, the hexane fraction, Fraction **a**, produced 52.3 % mortality, which was significantly higher (One-way ANOVA $d.f. = 3$; $F = 167.06$; $p < 0.0001$) than all other sub-fractions Figure 5.4.

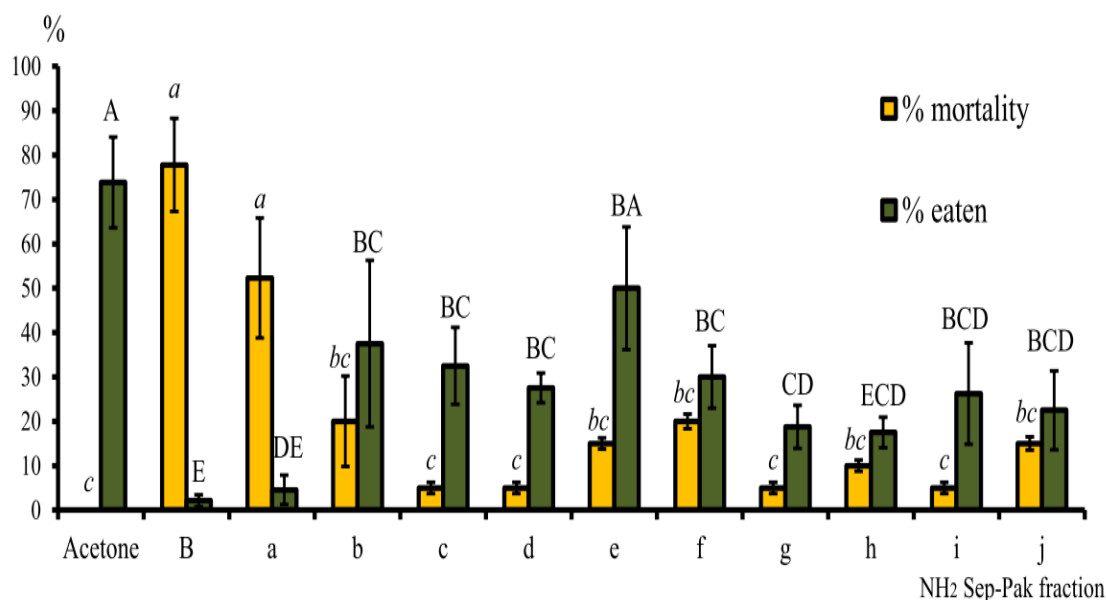


Figure 5.4. Mean percent mortality and consumption (\pm S.E.) of Colorado potato beetle larvae and potato leaf, respectively, after exposure to leaf discs treated with fractions **a** to **j** of TW bio-oil for 48 h. Fraction **B** and acetone were used as a positive and negative control, respectively. CPB mortality and leaf consumption with different letters are significantly different ($p < 0.0001$).

Fraction **a** also limited potato leaf feeding to 4.5%. Analysis of Fraction **a** by GC-MS after derivatization with MOX and MSTFA detected 407 compounds. Three major groups; aromatic, oxygenated and unsaturated (C_5 - C_{20}) derivatives, were identified using AMDIS and the NIST library as the most abundant molecules (data not shown). Among these compounds the diene, neophytadiene, and the acyclic diterpene alcohol, phytol, were found to be the most abundant based on peak area. Phytol, an oily acyclic diterpene C_{20} -alcohol of high-boiling point, is found in all photosynthetic plants. Neophytadiene can also be found in some plants, and can be produced by the dehydration of phytol [25], a process that could occur in the reactor during the cracking process of the TW bio-mass. Aromatic derivatives like o-cresol, m-cresol, and indole which were found in Fraction **a** are important precursors of commercial pesticides, herbicides and fungicides [26]. Phenols including 2,4-dimethylphenol and 4-ethylphenol were identified as well, not only in the TW bio-oil, but also in the active fractions of

bio-oils from tobacco leaves and coffee grounds [4, 6]. In those previous reports, the observed pesticide characteristics were mostly attributed to the synergetic effect of multiple phenols present at various concentrations. Unfortunately, phenols and phenol derivatives are toxic to humans and animals due to the formation of phenoxy radicals [27]. Hence, bio-oil sub-fractions consisting of mainly phenols are not ideal as biopesticides. Another group of pesticidal compounds found in Fraction **a** is benzonitriles. This group of molecules is an integral part of dyes, herbicides, agrochemicals, pharmaceuticals, and natural products. The nitrile group also serves as an important intermediate for a multitude of possible transformations into other functional groups [28]. Finally, linear alpha-olefins (LAOs), C₁₅ – C₁₉, were found in fraction **a**. LAOs comprise a family of valuable commodity chemicals with versatile applications as polymers, detergents and other specialty chemicals [29]. It is possible that other potent chemicals are simply not detected by GC-MS as only 40% of chemicals in bio-oil are normally detectable by this analytical technique.

With all of the above groups of compounds identified in Fraction **a**, it is apparent that further separation is necessary to reduce its complexity in composition. Semi-preparative RP-HPLC was therefore conducted to resolve it into six sub fractions.

5.3.5 Insecticide Bioassay-guided HPLC Fractionation of TW ESP Bio-oil Fraction (a)

From the six HPLC sub-fractions tested the fifth and sixth were observed to be the most active, producing 35 and 30% CPB mortality, respectively. These fractions also exhibited an anti-feeding effect compared to the other fractions with 38.8 and 35.0% feeding damage, respectively (Table 5.3).

Table 5.3. Mean percent mortality and consumption (\pm S.E.) of Colorado potato beetle larvae and potato leaf, respectively, after exposure to 6 HPLC sub-fractions of the hexane fraction (Fraction a) for 48 h. CPB mortality and leaf consumption with different letters are significantly different ($P<0.0004$) for mortality and ($P<0.0001$) for potato leaf consumed.

Fraction (a) sub-fractions ¹	Mean % mortality (S.E.)	Mean % potato leaf consumed (S.E.)
Acetone control	0.0 (0.0)b	93.3 (1.2)A
1	5.0 (0.25)b	82.5 (1.4)B
2	15.0 (0.25)ab	82.5 (2.5)B
3	5.0 (0.25)b	90.0 (0.0)AB
4	5.0 (0.25)b	90.0(2.9)AB
5	35.0 (0.25)a	38.8 (4.3)C
6	30.0 (0.29)a	35.0 (3.5)C

¹ HPLC fraction collection based on time (5 min intervals)

Based on GC-MS analysis, Sub-Fraction **6** contained mainly low abundance fatty acids (Figure 5.5, Table 5.4), nonanoic, hexadecanoic and octadecanoic acid. This may in part explain the observed toxicity of this fraction since fatty acids (C₇-C₂₀) have recognized insecticidal, acaricidal, herbicidal and plant growth regulatory-activities [30]. In Sub-Fraction **5**, the same three fatty acids were also identified along with two additional compounds, neophytadiene and phytol by the NIST library and later corroborated with standards. To our best knowledge, neither neophytadiene nor phytol was previously reported to have insecticidal activity against CPBs. Nevertheless, their presence in various bio-oils has been reported in the literature. Neophytadiene and phytol were the most abundant molecules in the crude bio-oil recovered from the hydrothermal liquefaction of the green microalga *Nannochloropsis sp* [31] and in the bio-oil produced from high temperature liquefaction of the same algae [32]. Likewise, neophytadiene has been reported as a major component of the bio-oil produced during pyrolysis of macroalgae from 537 to 873 K [33]. In our TW bio-oils, the concentrations

of neophytadiene and phytol in Sub-Fraction **5** were determined to be 1.0 mg/mL and 0.8 mg/mL respectively by GC-MS.

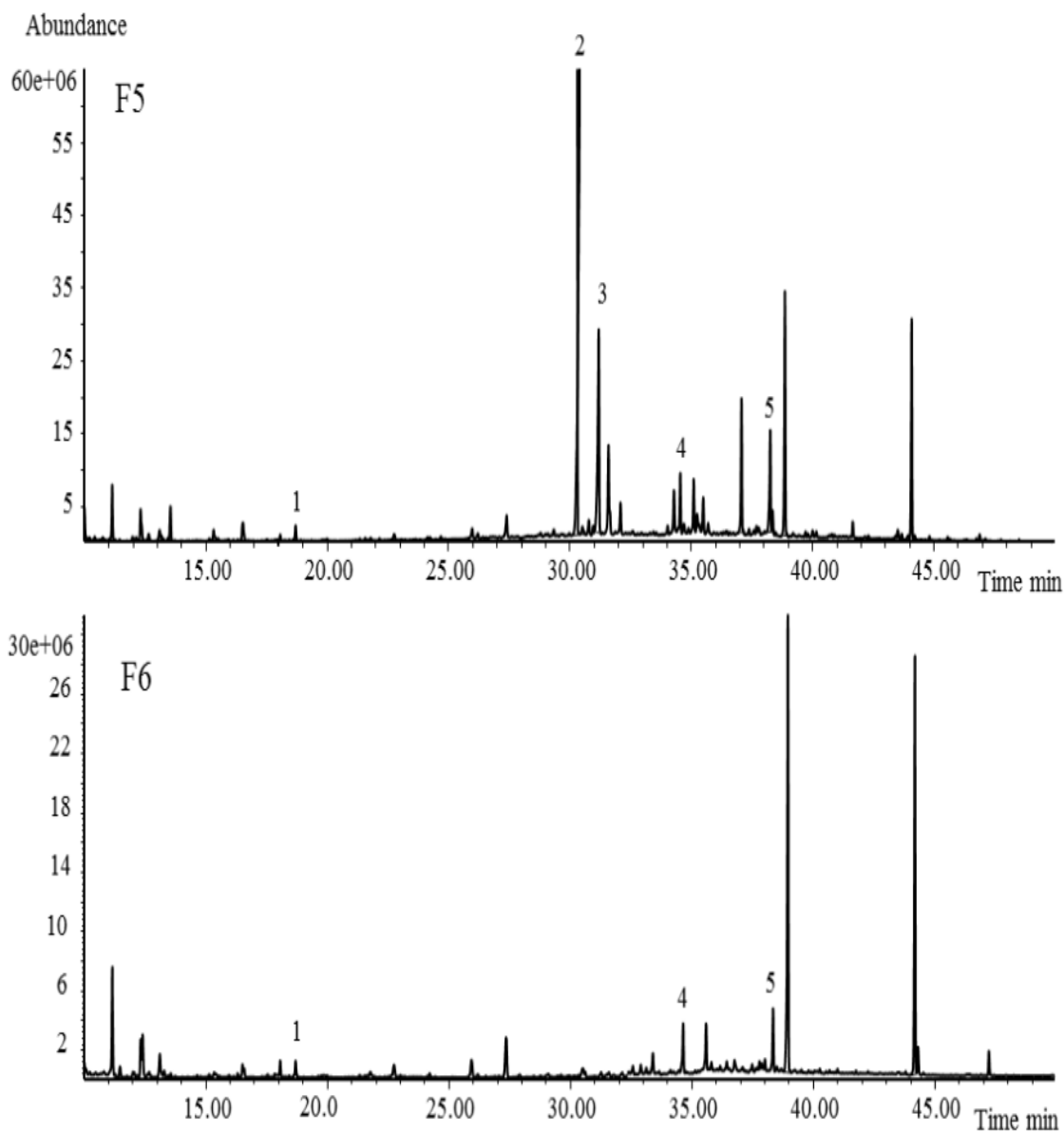


Figure 5.5. GC-MS total ion chromatograms of Fractions **5** (top) and **6** (bottom).

Table 5.4. GC-MS characterization and NIST assignment of Sub-Fractions **5** and **6**.

Peak	RT (min)	NIST assignment	Match factor	RI ^a	% Total Peak Area	
					F5	F6
1	18.71	Nonanoic acid	870	1358	0.68	1.35
2	30.43	Neophytadiene	929	1774	36.41	np
3	31.32	Phytol	949	2119	11.42	np
4	34.66	Hexadecanoic acid	900	2236	2.29	4.08
5	38.36	Octadecanoic acid	959	2385	4.64	4.31

^a Estimated non-polar retention index (n-alkane scale)

np = not present

One hypothesis on why the greenhouse tomato plant residue bio-oil contains neophytadiene and phytol is that TW biomass is composed of leaf and stalk parts of the tomato plant, as well as some fruits and roots, therefore the presence of phytol in the TW bio-oil is likely related to the fact that phytol is a component of chlorophyll found ubiquitously in green vegetables [34]. Chlorophyll molecules have several different side chains, one of which is a long phytol chain. Phytol can undergo elimination of water by rearranging the double bond from the 2-position, so it has been assumed that neophytadiene is produced by dehydration of phytol [35]. Phytol may also be produced by the breakdown of the C-45 fatty alcohol, solanesol, found in the Solanaceae family; e.g., tomato, potato, eggplant, and pepper [36]. Due to the chemical complexity of the pyrolysis products of biomass, it is challenging to acquire detailed information on the transformations of the molecules and to identify pathways of specific products. More studies regarding the mechanisms and pathways need to be performed in order to determine whether these assertions are true.

To determine whether neophytadiene and phytol have any significant activity toward CPB, bioassays were performed with these compounds at the concentrations found in bio-oil Sub-Fraction **5**, individually and combined. In addition, each compound was tested individually at a higher concentration of 30 mg/mL. All test samples containing neophytadiene and/or phytol exhibited some degree of toxicity toward CPB

(Table 5.5). At the concentrations identified in Sub-Fraction 5, neophytadiene (1.0 mg/mL) and phytol (0.8 mg/mL), individually produced relatively low CPB mortality of < 10%, but leaf feeding was significantly reduced compared to the control (*d.f.* =6; *F*=28.16; *p*<0.0001). At 30 mg/mL, neophytadiene and phytol were significantly more toxic, causing 40.0 and 33.3% mortality, respectively, but still below that of Fraction A (64.2%). These results confirmed that neophytadiene and phytol only partially account for activity observed in Fraction A of the TW bio-oil. When neophytadiene and phytol were combined at both the low and high concentrations studied, there was no difference compared to the sum of mortalities obtained individually, indicating little synergistic effect. Importantly, there was a significant reduction in the amount of potato leaf consumed with the concentrated combined solution of neophytadiene and phytol, 6.9% compared to the diluted combined solution, 55.3% (*d.f.* =6; *F*=166.02; *p*<0.0001). The presence of low intensity fatty acids, in terms of peak areas, may contribute to the activity observed in both fractions, as it has been demonstrated by our group [8] that fatty acids are not solely responsible for the insecticidal activity observed in bio-oils produced from Brassica plant species.

Table 5.5. Mean percent mortality and consumption (\pm S.E.) of Colorado potato beetle larvae and potato leaf, respectively after exposure to chemicals identified as the most abundant peaks in fraction 5 for 48 h. CPB mortality and leaf consumption with different letters are significantly different (*P*<0.0001).

Treatment	Concentration (mg/mL)	Mean % mortality (S.E.)	Mean % potato leaf consumed (S.E.)
Acetone	---	0.0 (0.0)b	80.3 (0.9)A
Neophytadiene	30.0	40.0 (4.7)a	8.0 (1.2)D
Neophytadiene	1.0	6.7 (3.3)b	68.9 (3.4)B
Phytol	30.0	33.3 (4.7)a	27.8 (3.3)C
Phytol	0.8	4.4 (2.9)b	70.6 (2.3)B
Neophytadiene + Phytol	30.0+30.0	45.0 (4.7)a	6.9 (0.9)D
Neophytadiene + Phytol	1.0+0.8	12.0 (4.8)a	55.3 (1.8)B

These interesting findings further promote the use of pyrolysis bio-oils as a source of biopesticides, not only from tomato plant residue, but other agricultural biomass. The fractionation method described in this work was developed for isolation and identification of the active components, but separation on a commercial scale will utilize a more direct and efficient process.

5.4 Conclusions

Plant waste from agricultural activities (tomato plant from greenhouses) is another important source of material that was thermally transformed into bio-oils by pyrolysis process. The bio-oil collected from the electrostatic precipitator was found to have valuable pesticide characteristics towards the colorado potato beetle, a serious pest that causes a negative impact on the agricultural sector. Bio-oil is a complex mixture containing a large number of components which can be separated and extracted to supply many chemicals and new products.

During the insect bioassay-guided fractionation of the bio-oil in search of active compounds, we identified oxygenated molecules, phenol and nitrile derivatives, long chain (C9-19) fatty acids, fatty acid esters, diterpenes, and unsaturated hydrocarbons. Of these, neophytadiene and phytol were found to be the most abundant in the active sub-fraction of the TW bio-oil, but still require other compounds from the bio-oil to act synergistically. The yield of neophytadiene may in fact be affected by the pyrolysis conversion of phytol, suggesting higher phytol content biomass is a better starting material for insecticide products. Further research is therefore required to identify which active compounds, potentially produced by the pyrolysis of tomato waste, can further contribute to the insecticidal activity.

5.5 References

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Chapter 6: Conclusions and Future Work

6.1 Conclusions

This thesis overall reported the analytical characterization of small organic compounds extracted from complex systems originated from plants. In Chapters 1 to 4, the focus was on the volatile organic compounds released by the wild type and transgenic plants of *Arabidopsis thaliana* and Micro-Tom tomato, with proper sample extraction and preparation, I demonstrated how a non-targeted analysis using gas chromatography mass spectrometry as a tool could be used as a strategy for the identification of VOCs according to the plant type. In Chapter 5, the focus was on a second type of sample, bio-oil from agricultural waste, where pesticidal properties were explored using various analytical strategies to search for the compounds responsible for the activity against common insect pest.

Since the objective of the first study was to identify the authentic profile of volatile blends emitted *in vivo* by plants, selection of the most suitable strategy for plant volatile sampling and extraction became the first important step. The dynamic headspace (DHS) collection system used in this study was demonstrated to be suitable as it enabled the enclosure of complete plants in glass chambers in which air was pushed in and pulled out into sorbent tubes. Since plants were enclosed in a system with constant air circulation, I was able to mimic the natural growth conditions and minimize stress that could alter the volatile profile emissions. Volatiles released by the plants for a 24 h period were extracted and enriched by the ethylvinylbenzene-divinylbenzene sorbent tubes. When the DHS system was tested with analytical standards, high recoveries were obtained (>88% in average).

After the extraction and separation of VOCs by GC-MS, a non-targeted analysis was performed for the unbiased semi-quantification and identification. This non-targeted analysis was first based on a fully automated alignment of metabolic profiles at the level of individual molecular fragments with assignment to the chemical structures. Subsequently, a multivariate comparative analysis of individual volatile profiles was

performed, which was based on all chemical information derived by the analytical approach. The results concluded that all of the six-week flowering *Arabidopsis* plants examined (WT, *CCD1*, *CCD4* and *CCD8*), overall, released very similar profiles of volatiles. This was expected since all plants belonged to the same family (Brassicaceae), genus (*Arabidopsis*), species (*thaliana*) and ecotype (Columbia-0). In total, twenty two VOC were identified including aromatic, alcohol, unsaturated hydrocarbons derivatives, monoterpenes, sesquiterpenes, and one apocarotenoid compound. The apocarotenoid compound identified in the HS of the plants was the β -ionone. Most importantly, it was observed that the *Arabidopsis* plant overexpressing the *CCD1* gene released a higher level of the apocarotenoid β -ionone compared to the wild type and to the other *AtCCD* genes. Quantitative analysis showed that the flowering transgenic plants released substantially higher levels (up to 4.5-fold higher) of β -ionone relative to wild-type plants.

With the HS analysis of the *Arabidopsis thaliana*, the action of the *CCD1* gene was confirmed by detecting the enhancement of the apocarotenoid compound β -ionone. Plants overexpressing the *CCD4* and *CCD8* neither enhanced β -ionone emissions nor produced other apocarotenoid derivatives. This led to the conclusion that these genes did not have the same cleavage selectivity of carotene substrates as the *CCD1* did. Cleavage of the carotene substrates by *CCD* genes depend upon the availability of the substrate which can fluctuate between plants. An interesting future research project would be to study the correlation of the carotene compound content in the plants and its effect in the production of secondary metabolites including VOCs.

As in the *Arabidopsis* study, the non-targeted analysis of volatiles by the DHS-GC-MS method allowed for the determination of the most authentic blend of volatiles released *in vivo* by wild type Micro-Tom tomato plants and Micro-Tom tomato plants over expressing the *AtCCD1*, *LeCCD1-1* and *LeCCD1-2* genes. Twenty seven VOCs were identified by the database, consisting of aromatic derivatives, monoterpenes, sesquiterpenes, and four unidentified compounds. Among the volatiles identified in the Micro-Tom tomato plants (WT and transgenic), the monoterpenes R- α -pinene, β -pinene

and, the sesquiterpene α -copaene were found to be the most abundant. Even though profiles were similar in all type of plants, principal component analysis (PCA) showed grouping of the different plant lines due to small changes in the emission of VOCs, especially by the ratio difference between the two most abundant compounds, the monoterpene R- α -pinene and the sesquiterpene α -copaene. For instance, the non-targeted semi-quantitative analysis revealed that the ratio R- α -pinene to α -copaene in WT was 2:1, while in the transgenic lines (*AtCCDI*) it was 1:1. In the case of the *LeCCDI-1* and *LeCCDI-2* lines, profiles were close to the WT. Although transgenic Micro-Tom tomato plants over expressed the *AtCCDI*, *LeCCDI-1* and *LeCCDI-2* genes, the DHS-GC-MS method did not detect the compound β -ionone or any other apocarotenoid molecules. No prior reports of the *in vivo* HS study of Micro-Tomato tomato plants over expressing these genes had been found in the literature; instead it was only demonstrated to be found in tomato fruits after extraction with solvents. For this reason, I speculated that the transgenic plants were releasing this compound in low concentration below the detection limit of the DHS system, hence a more sensitive method using static headspace (SHS) analysis with solid phase microextraction (SPME) fibers was performed and reported in Chapter 3. For this analysis only *AtCCDI* plants were used as it was expected that the *Arabidopsis* gene in the tomato would generate apocarotenoid derivatives.

SPME is often preferred in the HS analysis of flowers because extraction of volatiles is fast, does not require solvents, and can capture VOCs at the parts per billion level from the small sampling volume of flowers [1]. SPME is based on adsorption/absorption and desorption of volatiles from an inert fiber coated with various types of ad/absorbents, making selection of the appropriate fiber coating a critical step. In general, the main criteria used in SPME fiber selection are molecular weight, size, shape, polarity, and concentration of the analytes [2]. These criteria will determine how the volatile compounds will interact and how fast they will move in and out of the fiber matrix to reach equilibrium. To increase sensitivity and selectivity the different extraction capabilities of three SPME fibers (100 μ m PDMS, 75 μ m CAR/PDMS and 65 μ m PDMS/DVB) were combined to take advantage of their wider range of analyte

selectivity. According to my knowledge, the simultaneous extraction capabilities of three SPME fibers have not been reported before. Results demonstrated that even though the general extraction efficiency of PDMS/DVB was the highest of the three fibers, the PDMS and the CAR/PDMS offered a slightly different bias in the extraction of VOCs. By combining all fibers, a total of 50 VOC were extracted. Compounds were classified as monocyclic monoterpenes, bicyclic monoterpenes, tricyclic monoterpene, alkyl benzene related to monoterpene, monocyclic sesquiterpenes, and bicyclic sesquiterpene. There was no evidence of the presence of the compound β -ionone and other apocarotenoid derivatives in either transgenic or wild type plants. If the action of the *CCDI* in tomato plants is the production of these compounds by the cleavage of carotenoid molecules, in this study it was evident that apocarotenoids were not released as part of the HS of the plants. An interesting research question would be to determine whether or not apocarotenoid compounds are being stored in the fruits of WT and transgenic Micro-Tom tomato plants over-expressing the *CCDI* genes, previous studies in tomato plants overexpressing the *CCDs* demonstrated the correlation of β -ionone with carotenoid content in the tomato fruits [3]. This study can be accomplished by the SPME-GC-MS strategy presented in my study, but with a targeted approach. Doing so would help to understand the function of the genes in the formation of these compounds, and establish differences according to the plant type.

The DHS and the SHS were used in the non-targeted analysis of VOCs of WT and *AtCCDI* Micro-Tom tomato plants. Both strategies extracted volatile secondary metabolites released in the HS of the plants. Some of the differences according to the method of extraction are depicted in Table 6.1. Since three SPME fibers were used simultaneously, the extraction capabilities were enhanced by taking advantage of their wider range of analyte selectivity that is why more VOCs were detected in the SHS compared to the DHS. In order to evaluate and establish unbiased differences between the DHS and SHS according to the extraction capabilities, all characteristics of extraction and processing presented in table 6.1 should be the same.

Table 6.1. Differences between the dynamic headspace (DHS) and the static headspace (SHS) strategies used in the non-targeted analysis of VOC in WT and *AtCCDI* Micro-Tom tomato plants.

Characteristic	DHS	SHS
Sample material	Whole plants	Flowers only
Headspace type	Circulating air flow rate of 100 mL/min	Static air
HS volume	24.4 L glass chamber	15 mL glass adaptor
Extracting matrix	Sorbent tubes made of ethylvinylbenzene-divinylbenzene beds (Porapak Q)	Three SPME fibers; 100 μ m PDMS, 65 μ m PDMS/DVB and 75 μ m CAR/PDMS
Extraction time	24 h	24 h concentration 20 min extraction
Extraction location	Laboratory conditions	Growth room conditions
Extraction strategy	VOC are trapped into the sorbent tubes by the high affinity of the sorbent beds for lipophilic to medium polarity organic compounds of intermediate molecular weight.	VOC are extracted by adsorption into the three SPME fibers of different physical and chemical properties after equilibrium is reached
Desorption from extracting matrix	Dichloromethane (DCM)	Thermal desorption in the GC inlet
AMDIS parameters	Adjacent peak subtraction = 2; Resolution – High; Sensitivity – Very High; Shape Requirements – Medium.	Same as DHS
VOCs detected	27	50
Apocarotenoid compounds	Not detected	Not detected

One of the main goals in any project of pest management and plant protection is to find strategies for protecting important crops against pest attack. One traditional

solution is the use of pesticides, which is not desirable due to the negative impact on the environment and other living organisms. An alternative to the use of pesticides is the creation of more resistant crops, which means that the plants can produce the necessary secondary metabolites to defend themselves while being attacked. The *in vivo* volatile analysis of *Arabidopsis thaliana* demonstrated the production and 2.2 to 4.6-fold enhancement of the apocarotenoid compound β -ionone in the HS of plants overexpressing the *CCDI* gene compared to that of the wild type. In Chapter 4, biological assays showed that the apocarotenoid compound at the concentration of 0.3 ng/ μ L in the *CCDI* plants had a strong repellent effect against the crucifer flea beetle (*Phyllotreta cruciferae* Goeze) and two-spotted spider mite (*Tetranychus urticae* Koch), and significant oviposition deterrence against silverleaf whiteflies (*Bemisia tabaci* Gennadius).

The current research therefore offers a way to reduce the use of pesticides. Four strategies employed in this study can be used as important tools in pest management and crop protection: 1) enhancement of the existing genes in plants to increase the production of volatiles that can protect plants from insect attack, 2) analytical strategy for the extraction, identification and quantification of volatiles present in the HS of transformed plants, 3) the correlation between one gene and according volatile formation and 4) design of biological assays to determine the effect of the volatile compound against the insect pest.

An important complementary research work could be the application of the strategy for the non-targeted analysis of volatiles produced by the same type of plants as they are exposed to herbivory. This could demonstrate the production or enhancement of VOCs due to the biotic stress, and their potential uses as biopesticides.

The last part of my thesis (Chapter 5) explored a second way to reduce pesticide use. This was accomplished by the fractionation of bio-oil sample according to its pesticidal properties. Plant waste from agricultural activities (tomato plants from greenhouses) was an important source of material that was thermally transformed into

bio-oils through a pyrolysis process. The bio-oil collected from the electrostatic precipitator was found to have valuable pesticide characteristics towards the Colorado potato beetle, a serious pest that causes a negative impact on the agricultural sector. During the insect bioassay, guided fractionation of the bio-oil in search of active compounds, oxygenated molecules, phenol and nitrile derivatives, long chain (C9-19) fatty acids, fatty acid esters, diterpenes, and unsaturated hydrocarbons were identified. Of these, neophytadiene and phytol were found to be the most abundant in the active sub-fraction of the tomato waste bio-oil. The activities of phytol and neophytadiene were below the activity observed with the original bio-oil, suggesting a synergistic relationship with other compounds present in the sample. The yield of neophytadiene may in fact be affected by the pyrolysis conversion of phytol, suggesting higher phytol content biomass is a better starting material for insecticide products. The analytical strategy used to fractionate the bio-oil presented in this study successfully simplified the complex bio-oil matrix and can be used not only for the determination of molecules responsible for the pesticidal activity, but can be used in conjunction with a wide range of assays for the extraction of compounds with a particular activity.

6.2 References

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Appendices

Appendix 1: The *Agrobacterium*-mediated transformation for CCD in plants

There are a few methods to transfer the genes, but for the purpose of this thesis only a general view of the “*agrobacterium*” mediated transformation technique will be explained, since this was the method performed by the molecular biology lab at Agriculture and Agri-Food Canada. The *Agrobacterium*-mediated transformation process performed in the plant material is shown in figure A3 and involved the following steps: (a) extraction of a transferred gene (T-DNA) of interest from a young plant called generation 0 (T0); (b) regions of the gene were cloned into a strand of a nucleic acid called “primer” that served as a starting point for DNA synthesis; (c) insertion of the transgene into a circular piece of DNA called “Ti-plasmid” (d) introduction of the T-DNA-containing-plasmid into *Agrobacterium* using a vector; (e) mixture of the transformed *Agrobacterium* with plant cells by dipping flowers into the *Agrobacterium* solution to allow transfer of T-DNA into plant chromosome; (f) seeds from transformed plants, first generation (T1) were screened using antibiotic selection and polymerase chain reaction (PCR); (g) positive screened seeds were grown and new plants, second generation (T2), were then tested for gene quantification by real-time PCR.

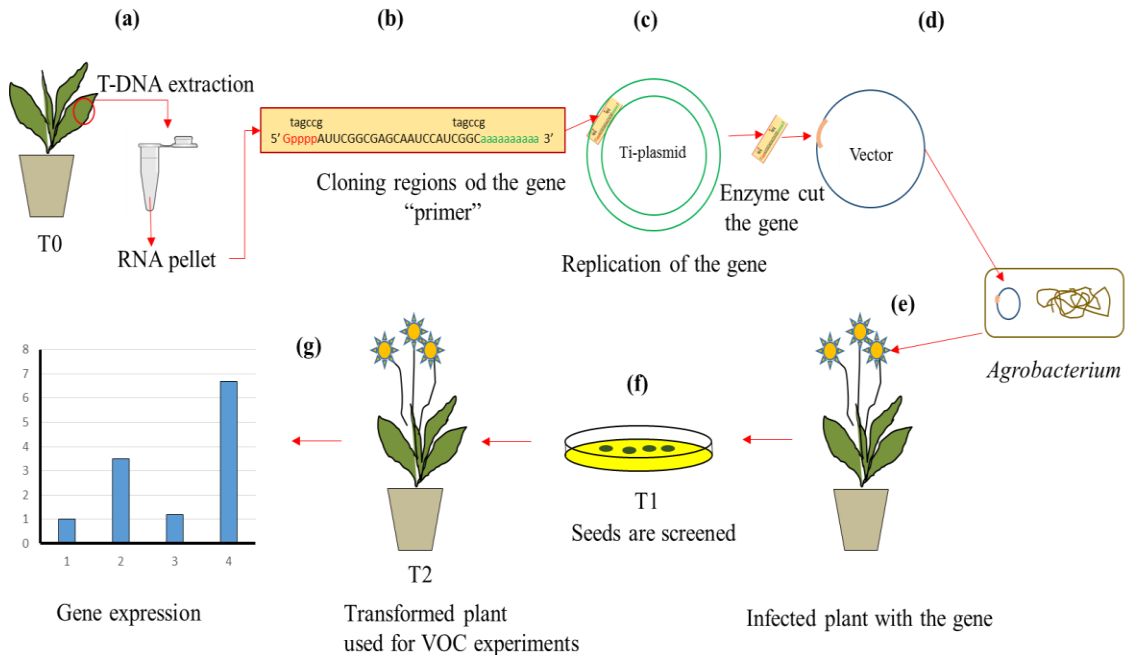


Figure A1 An *Agrobacterium*–mediated transformation method is used to insert new genes in plants as follows: gene is extracted from a plant (a); gene is inserted into a primer (b); replication of the gene using a Ti-plasmid (c); introduction of the replicated gene into the agrobacterium using a vector (d); flower plants are dipped into a *Agrobacterium* solution having the new gene (e); seeds that survive an antibiotic are grown to generate transformed plants (f); transformed plants are used for VOC experimentation and then are tested for gene expression by qRT-PCR (g).

Appendix 2: Cylindrical glass chamber

A 29.56 mL glass chamber used initially to test the extraction of volatile organic compounds.



Figure A2 Cylindrical glass chamber (48 cm height X 28 cm diameter) with a compressed air tank used initially to test collection of plant volatiles. Inlet (A), gauge to monitor HS pressure (B), air outlet tubing with a cartridge to trap volatiles (C), and flowmeter (E).

Appendix 3: Gas chromatography mass spectrometry instrument

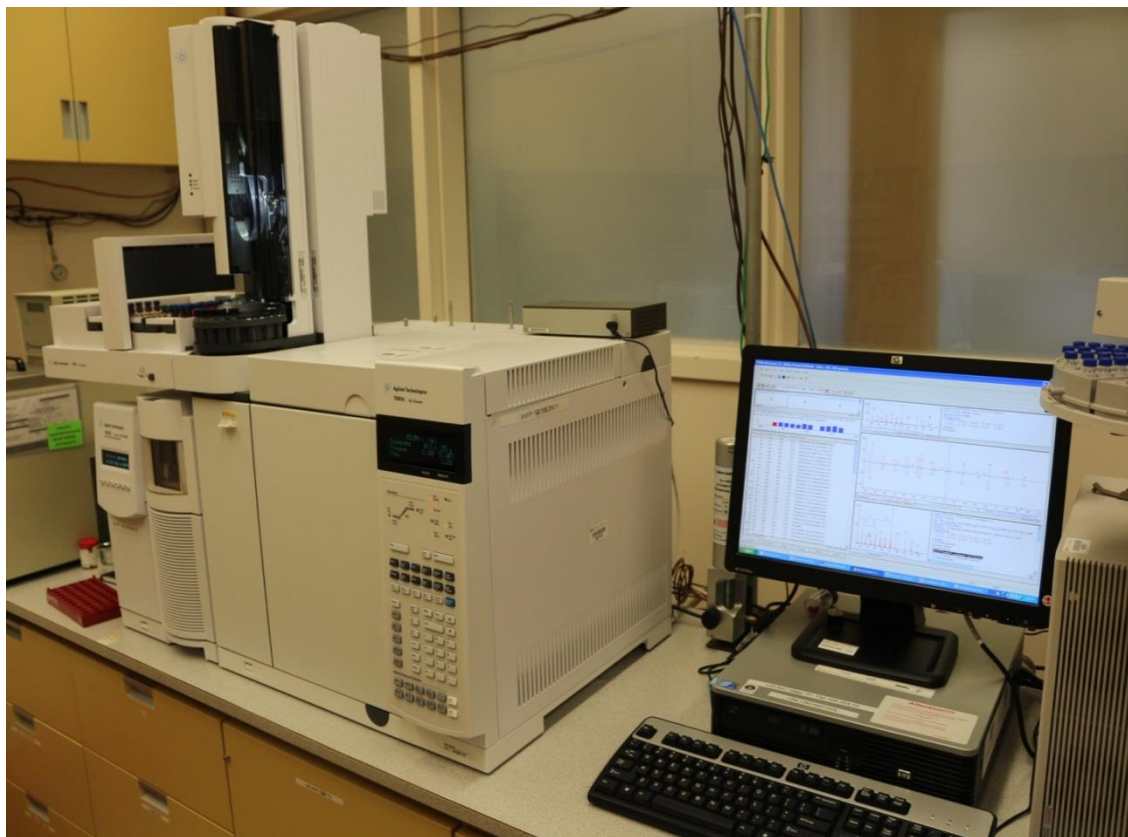


Figure A3 The GC-MS instrument from Agilent Technologies (Santa Clara, CA USA) used for sample injection, separation and detection of VOCs was equipped with an autosampler (7693), gas chromatograph system (7890 A) and inert XL EI/CI MSD with triple-axis detector (5975C).

Appendix 4: Quality Control and Preparation of GC-MS Instrument Prior to Analyses

Before separation of the VOCs extracted from the plants using the GC-MS system, my task as an analytical chemist, was to determine the optimal instrumental conditions for an efficient separation and sensitive response.

Four essential components encompass the chromatography system: 1) the injector system that drove the sample into the GC; 2) the oven containing the column for compounds separation; 3) The detector which responded to compounds eluting from the column, and 4) the data system.

The injection port was verified for air leak periodically. This was accomplished by switching the injection port purge on and off and checking the ions associated with leaks (primarily O₂ 32 *m/z* and N₂ 28 *m/z*). If the abundance of these ions with the purge off were much greater (x100) than the abundance of these ions with the purge on, a leak was confirmed. Leaks were easily corrected by changing the syringe, the septum and the liner. The injection system was tested by loading 2 μL of DCM solvent before samples were injected. This evaluated not only the injector performance, but allowed me to determine that the interface to the GC column was contaminant- free, inert and without leaks.

An inlet temperature of 250 °C was chosen for fast volatilization of compounds in the mixture. A pulsed splitless mode was selected to control the pressure at the inlet part of the instrument. The injection pulse pressure was 25 psi at the beginning of the run to transfer the 2 μL of the sample out of the inlet into the capillary column faster, thereby reducing chance for sample decomposition and discrimination. After 0.5 min the inlet pressure went to normal (12.445 psi). The purge flow to split vent was 40 mL/min at 1 min, which was the flow rate used and the time to sweep away residual vapors after the beginning of the run.

Compounds from the sample mixture were separated in a GC-column base on their distribution between the mobile and stationary phase. Therefore, selection of the suitable GC-column for selective separation of VOCs was an important step. The columns for this study were chosen base on the chemistry and thickness of the stationary phase as well as column length and internal diameter. Since plant volatiles are lipophilic in nature, the non-polar HP-5MS and DB-5MS columns (5% phenyl, 95% methyl polysiloxane) were considered to be selective for this group of compounds. The HP-5MS was used for the *Arabidopsis* and the DB-5MS for the Micro-Tom tomato study. The stationary thickness was selected according to sample volatility. Low boiling point compounds are better retained in thicker columns (5 μ m), on the contrary high boiling point compounds are better retained in thinner coatings (0.1 μ m) and since a wide range of VOCs was expected (40 – 150 °C) a 0.25 μ m in thickness was selected. The inner diameter of the columns was 0.250 mm.

A single oven ramp temperature program was used to separate VOCs from the plant extracts. A initial temperature of 30 °C (below the boiling point range of VOCs) was selected and held for 1 min. Oven temperature was linearly increased at 5 °C/min to 200 °C (above the boiling point range of VOCs) and held for 1 min.

To optimize the performance of the detector (MSD) the GC-MS instrument was tuned in a weekly basis using electron ionization (EI) as mode of operation at 70 eV. The autotune mode used Perfluorotributylamine (PFTBA) as a tuning standard and the report (Figure A4) was evaluated based on the following parameters; Three tuning masses (69, 219, and 502 m/z) were identified, which were within $\pm 0.1 m/z$. The peak widths of these three peaks were $0.60 \pm 0.1 m/z$. The peak at 69 m/z was the largest with 100% relative abundance. Relative to the peak at 69 m/z the peak at 219 m/z was in the range of 50-250% and the peak 502 m/z was greater than 3%. The isotope mass assignment was 1 m/z greater than the mass assignments of the parent peaks. The isotope ratio figures (indicating the relative abundances of the naturally occurring isotopes) were close to the theoretical values 1.08 for 69 m/z , 4.32 for 219 m/z and 10.09 for 502 m/z .

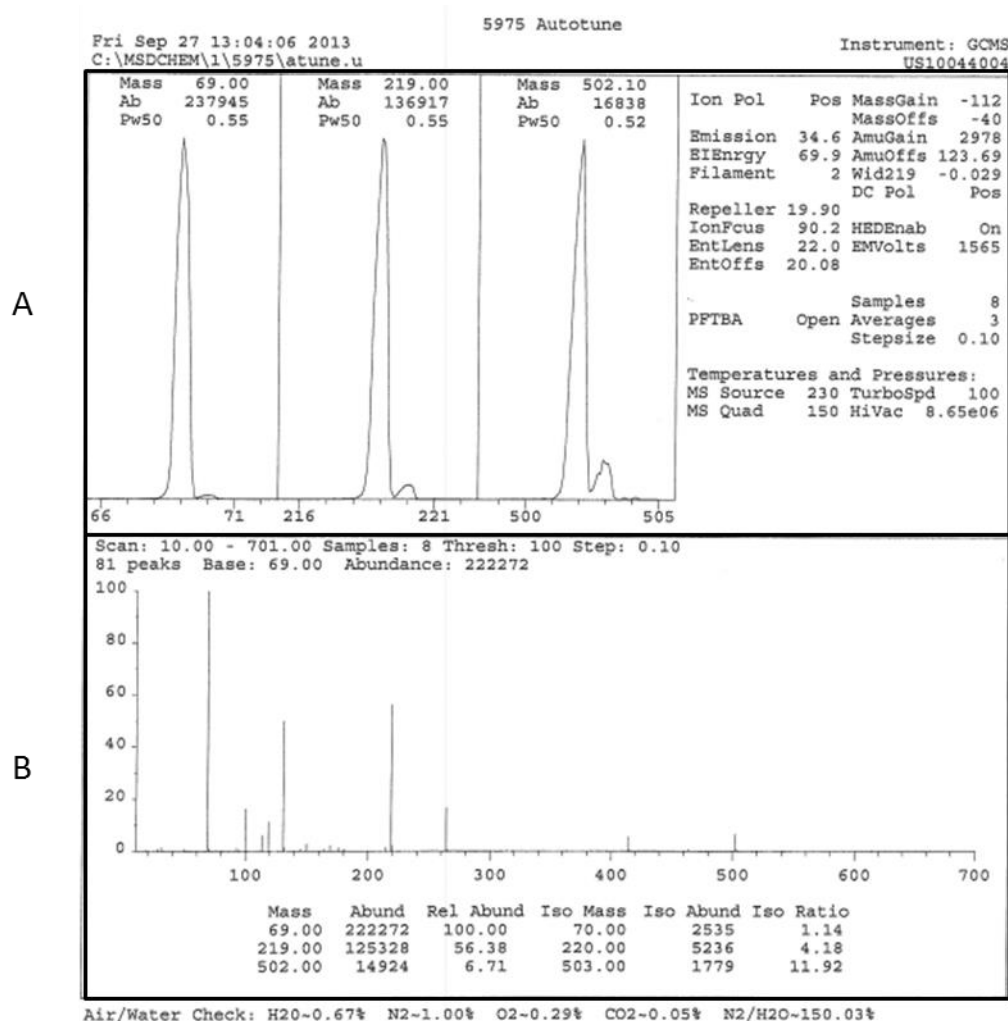


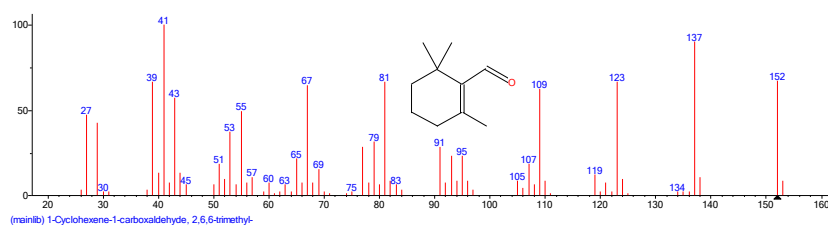
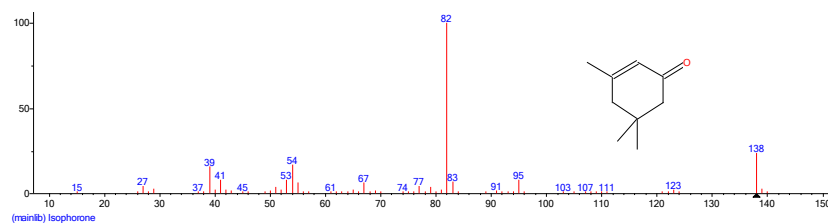
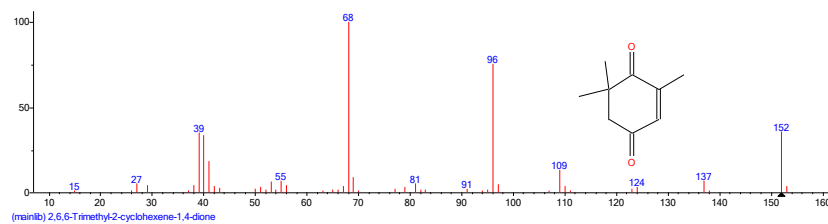
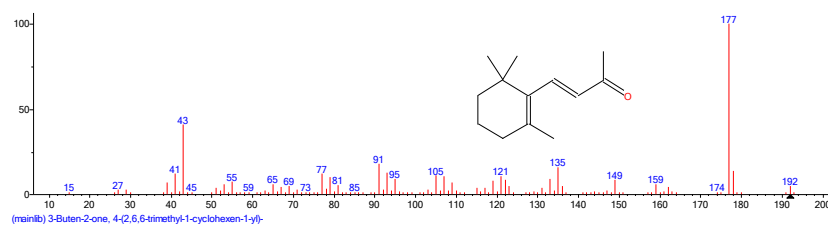
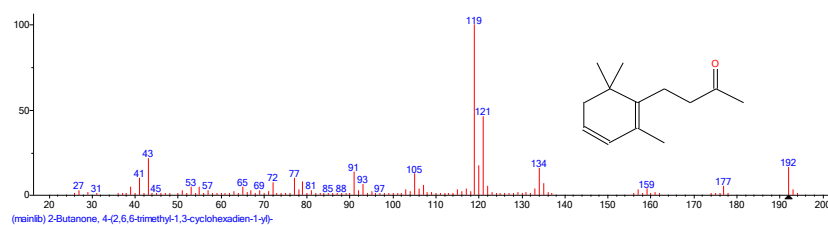
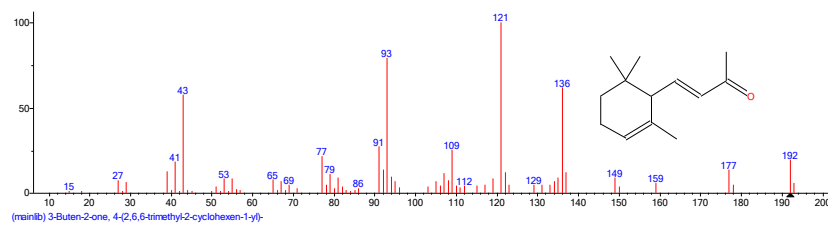
Figure A4 Autotune report example using the PFTBA standard and EI mode; (A) Symmetrical smooth peak shapes with mass assignments and peak identification (69, 219 and 502 m/z); (B) Scan showing correct mass assignments, typical relative abundance and proper isotope ratios.

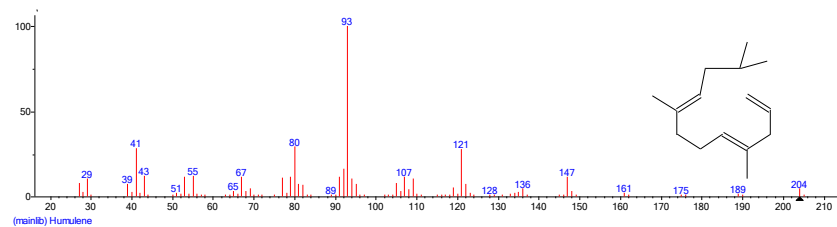
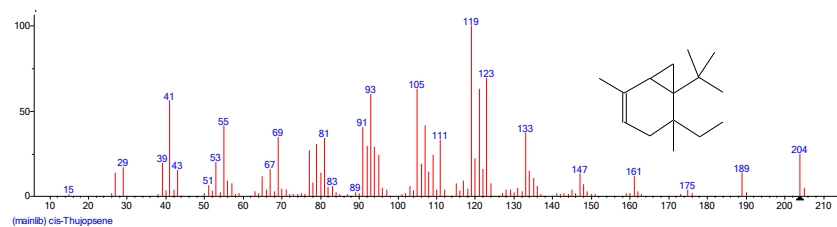
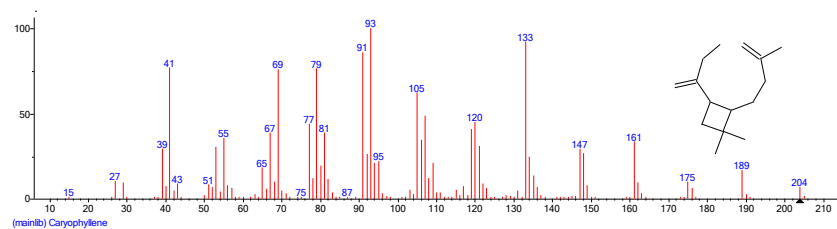
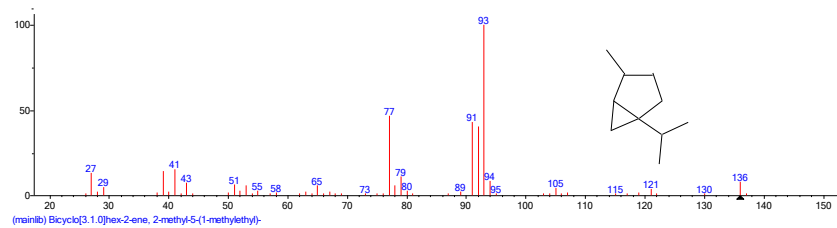
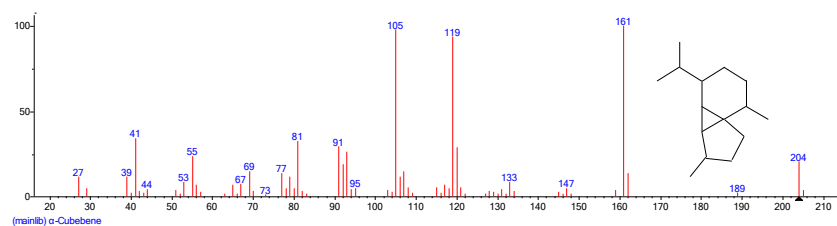
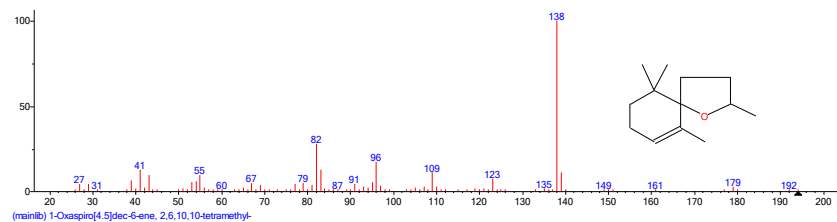
Air and water leaks in the MSD were confirmed when the abundance of the 28 m/z (N_2) and 18 m/z (H_2O) relative to 69 m/z were greater than 5 and 10% respectively. When tuning parameters were within the specifications, the instrument was ready to use for VOCs study.

Since the analysis performed in this study was non-targeted a Full Scan acquisition mode was utilized instead of the Selective Ion Monitor (SIM). The Full Scan

mode was quite useful identifying unknown compound in the plant extracts, because it allowed monitoring a wide range of masses. The mass range selected for this study covered from 100-350 m/z 4.51 times per second.

Appendix 5: Mass spectra of compounds used to build a library for the *Arabidopsis thaliana* study





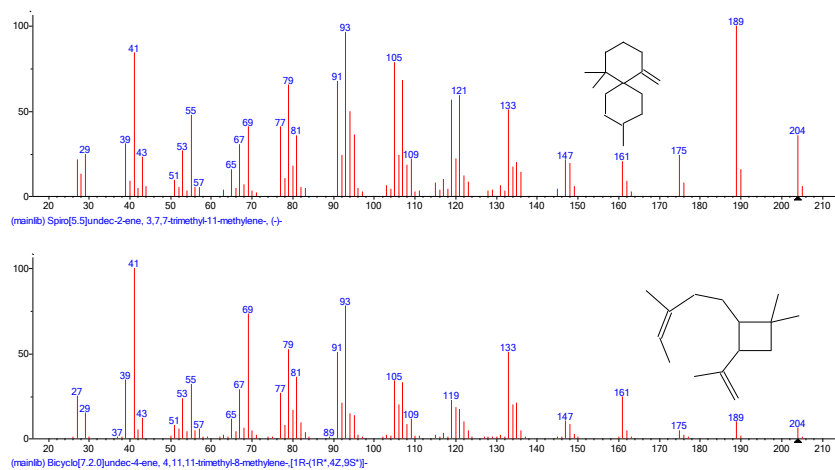
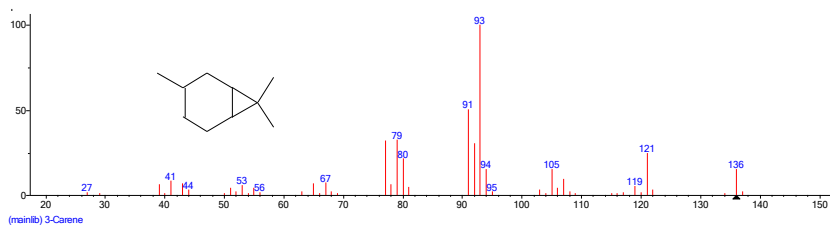
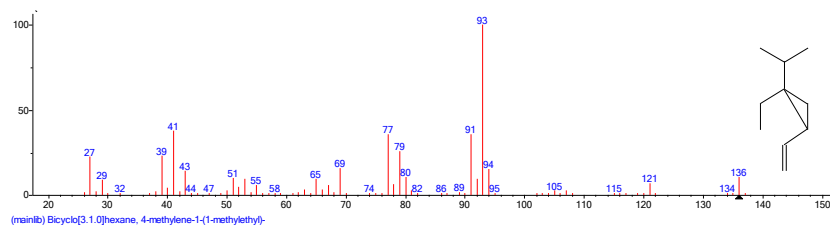
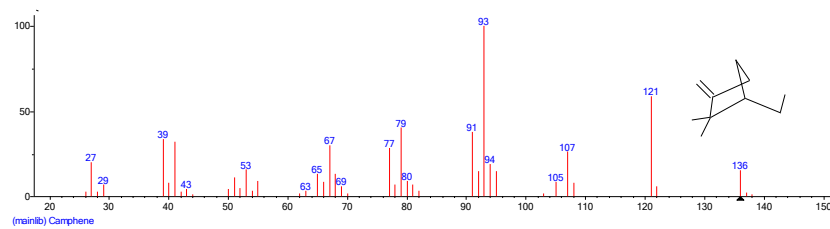
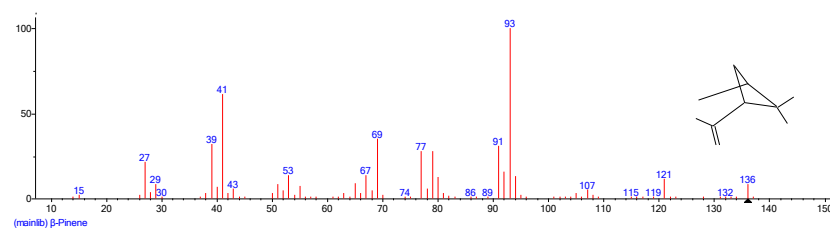
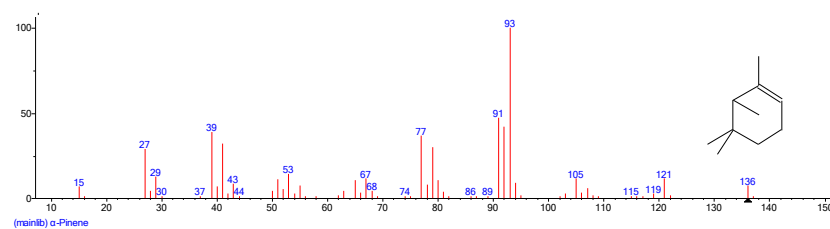
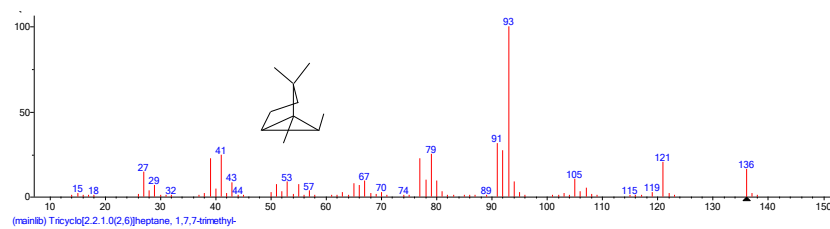


Figure A5 Mass spectra of 14 compounds used to build a library with AMDIS for the VOC study in *Arabidopsis thaliana* plants. X-axis: m/z (mass-to-charge ratio), Y-axis: signal intensity (relative abundance %).

Appendix 6: Mass spectra of compounds used to build a library for the Micro-Tom Tomato study



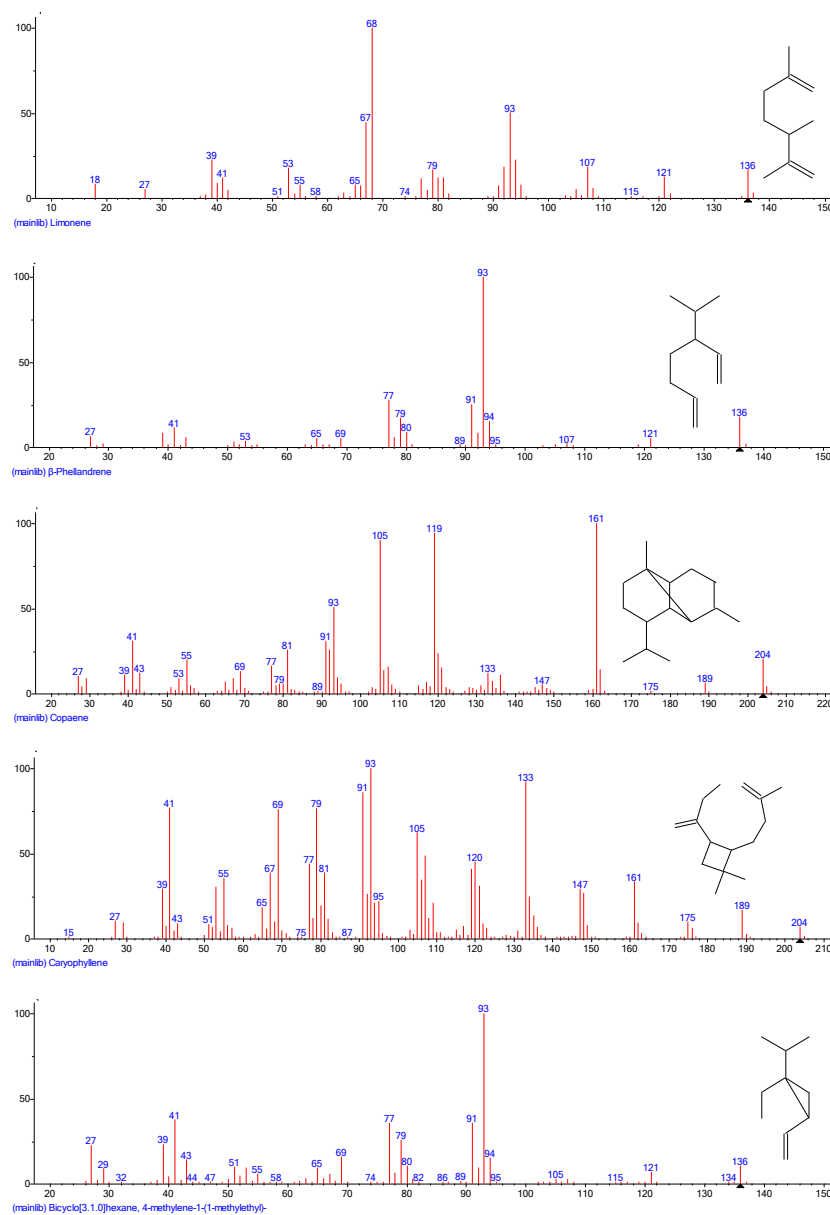


Figure A6 Mass spectra of 11 compounds used to build a library with AMDIS for the VOC study in the Micro-Tom tomato study. X-axis: m/z (mass-to-charge ratio), Y-axis: signal intensity (relative abundance %).

Appendix 7: Background Analysis

A common problem in DHS analysis is the identification of background fragments, false positives and zero values that could lead to data analysis misinterpretation. SpectConnect integrated signal (IS) output was manually inspected in order to identify molecular fragments of contaminants coming from the Porapak Q matrix and/or the injection port septum. These molecular fragments had typical patterns different from plant volatile organic compounds, and were eliminated manually from the data set. An example of a common contaminant found was Pentasiloxane, dodecamethyl ($C_{12}H_{36}O_4Si_5$) with a typical fragmentation pattern shown in Figure A5.

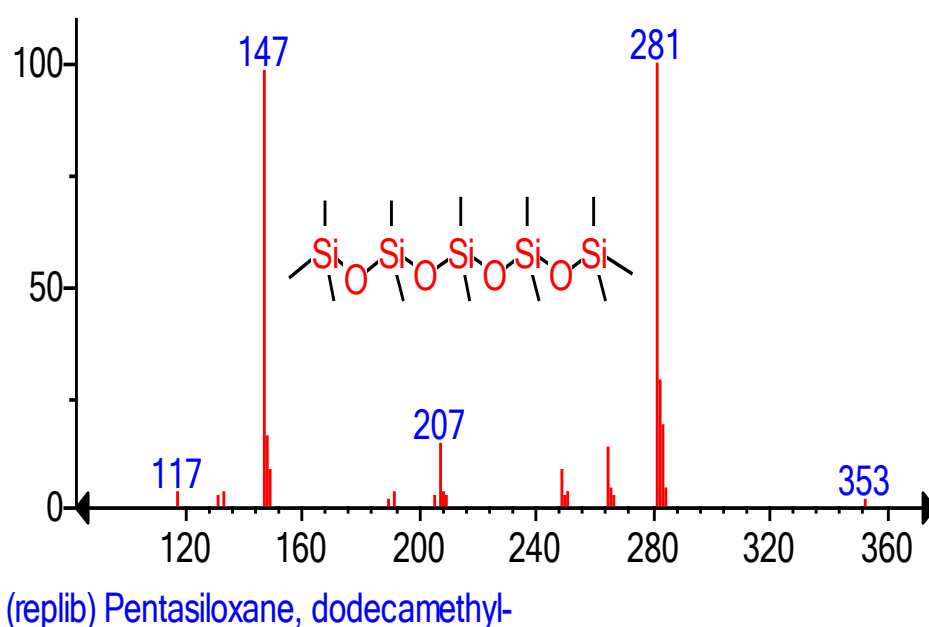


Figure A7 Mass spectra of a contaminant dodecamethyl pentasiloxane identified by the NIST database. Fragmentation patterns of contaminants were manually removed from the data set.

Molecular fragments that were highly correlated with contaminants and related to siloxanes, such as m/z 147, 267, 341, 281, etc, were recognized from the SpectConnect data set and were easily removed from it before further analysis.

Fragments of molecules with low NIST match <800 and not recognized as plant volatiles were eliminated as well from the data set. An example of this is the compound identified as naphthalene, 2,6-bis(1,1-dimethylethyl) and shown in Figure A6.

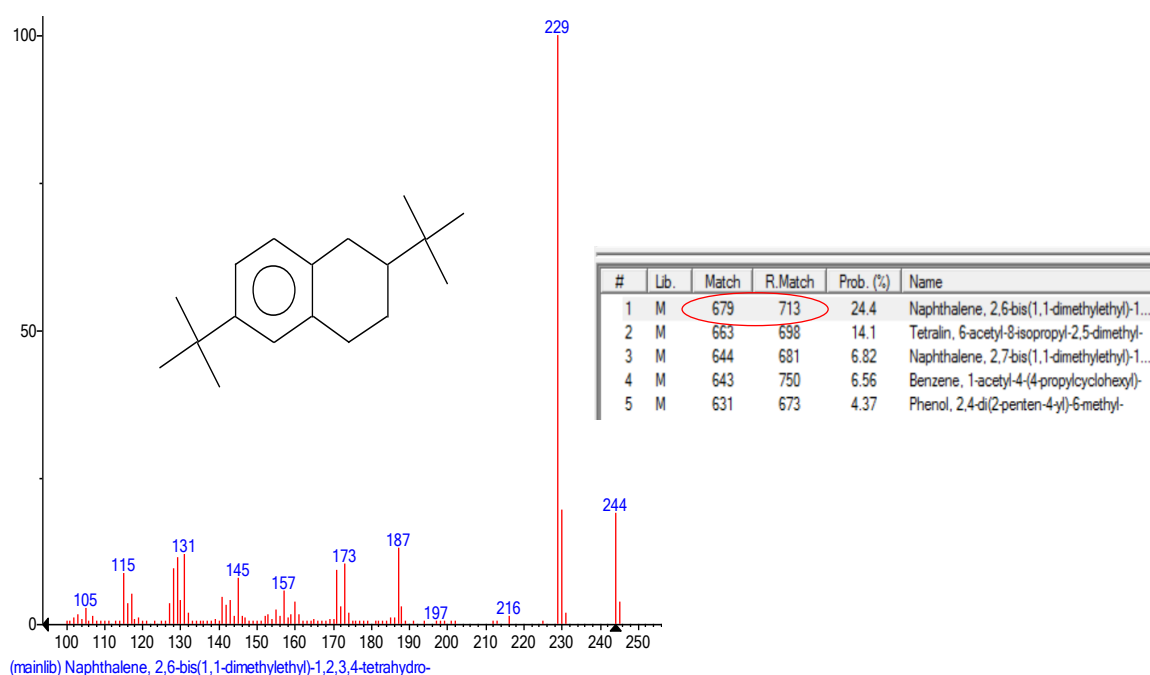


Figure A8 Fragmentation pattern of compound identified by the database with a match number of 679 and not recognized as a volatile from plant volatile headspace.

Elimination of zeros from the data set, background fragments and no plant volatile peaks due to lower NIST match and difficulty to verify was an essential step before effective comparison of the plant-specific data can be made. Because every volatile collection was performed in using one pot containing from seven to eleven

plants, integration values from the SpectConnect integrated signal (IS) of the corresponding group of plants were divided by the number of plants.

The manually cleaned matrix data was subjected to Log [n+1] transformation. This data normalization process was performed to reduce fluctuations in the data due to biological and non-biological variations. Examples of biological variation include plant size development and number of flowers. Non-biological variations can include differences in GC-MS sensitivity.

Appendix 8: Feeding bioassay of WT and CCD1 Arabidopsis plants with Cabbage Loopers Moth

The Cabbage Looper *Trichoplusia ni* is a member of the moth family Noctuidae and it was used to determine a feeding effect with the Arabidopsis plants. In the bioassay a set of WT and *AtCCD1* Arabidopsis plants were infested with 60 2nd instar cabbage loopers for 24 hours (Figure A8). Leaves were removed from the plant and screened for feeding evaluation using coral Draw program. Pixels of leaf consumed were determined as a percentage by using the formula $(TP_c \times 100/TP_l)$ in which TP_c is the total pixels consumed by the herbivores and TP_l the total pixels of the leaf.

Cabbage looper was another herbivore used to investigate the attractant repellent effect of the *AtCCD1* lines and wild type plants. Results were no significant different, and it was found that the herbivore fed on all types of *Arabidopsis* plants. Figure A9 shows the average percent eaten after 24 h.

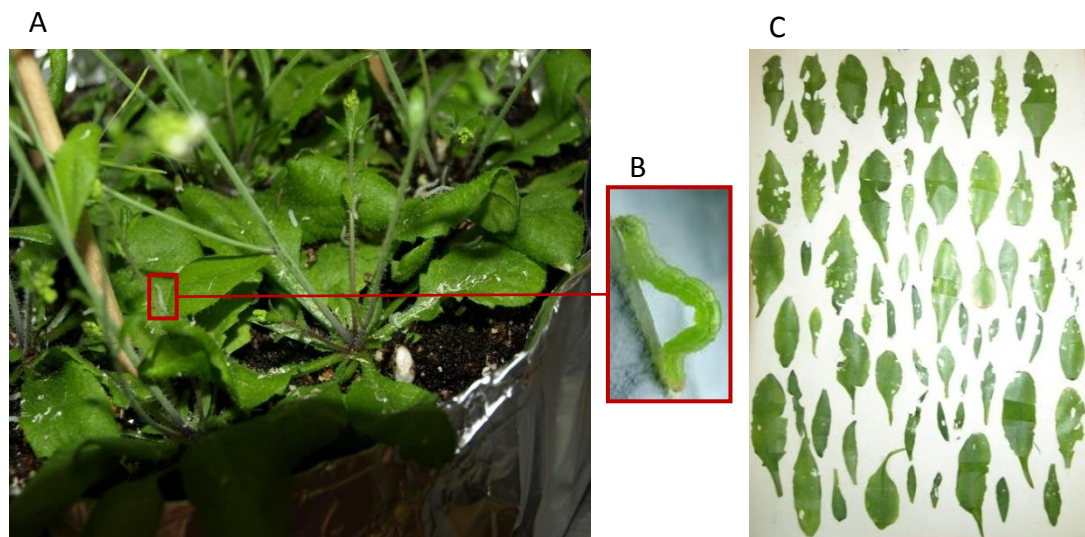


Figure A9 A close up of a set of *AtCCD1* plants (A) infested with 2nd instar cabbage loopers *Trichoplusia ni* (Noctuidae) (B) for 24 hours. Leaves were removed and screened for feeding evaluation

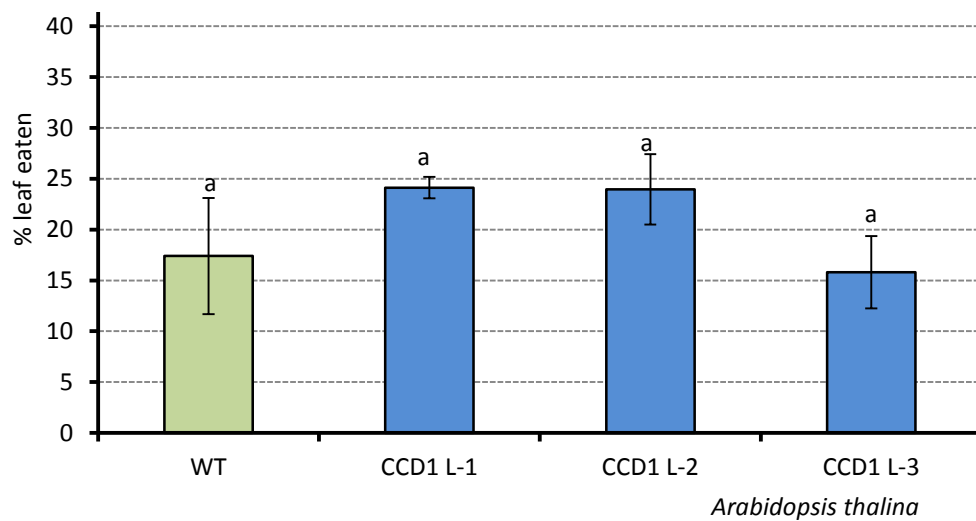


Figure A10 Percent leaf eaten (\pm S.E.) by 2nd instar cabbage loopers *Trichoplusia ni* (Noctuidae). Error bars with the same letter were not significantly different ($d=3.4$, $F=1.47$, $P=0.35$).

Appendix 9: Fluidized bed reactor



Figure A11 Fluidized bed reactor at the Institute for Chemicals and Fuels from Alternative Resources (ICFAR) from the University of Western Ontario. The reactor was used to obtain bio-oil from tomato plant residue biomass by pyrolysis process.

Appendix 10: Liquid chromatography Instrument



Figure A12 LC separation and fraction collection of the most active hexane sub-fraction, Fraction a, was performed on an Agilent 1200 Series liquid chromatographic system Agilent Technologies Canada Inc.

Curriculum Vitae

of Luis A. Cáceres

EDUCATION

Ph.D. Candidate, University of Western Ontario 20015

- Analytical Chemistry
- Expected completion date: February 13, 2015

Graduate studies in Chemical Analysis, Javeriana University 1998

- Specialization in Chemical Analysis
- Evaluated by World Education Services (WES) as equivalent to a master's degree in Chemical analysis at a recognized Canadian university

B.Sc. Degree in Chemistry and Biology, La Salle University 1995

- Evaluated by World Education Services (WES) as equivalent to a bachelor's degree in Science at a recognized Canadian university

PROFESSIONAL EXPERIENCE

Research Associate (Ph.D. Student)

Agriculture and Agrifood-Canada London, Ontario 2009-2015

- Method development for the identification and quantification of small molecules responsible for the pesticidal activity of pyrolyzed tomato plant waste bio-oil
- DHS-GC-MS analysis of plant volatiles and their uses as a pest control
- Comparison of three SPME fibers for the SHS-GC-MS analysis of volatiles from transgenic micro-tom tomato plants

Teaching Assistant (First year chemistry)

University of Western Ontario London, Ontario 2009-2014

Research Scientist I (Analytical support)

Parmalat R&D London, Ontario 2005-2008

- Determination of total nitrogen content in dairy products through the kjeldahl method

- Analysis of fat, moisture, pH, total acidity, Iodine test, Karl fisher titrations and fatty acids in food products

Research Scientist (8-week placement)

Chemistry Lab Trojan UV London, Ontario 2005

- Analysis of water samples through HPLC systems for the identification of pollutants.
- Improvement of water quality through UV technologies

VOLUNTEER EXPERIENCE

Spanish Teacher

London Public Library – Central Branch London, Ontario 2010-2011

Guest Speaker

WIL Employment Connections London, Ontario 2005

- Talk “The genetic wheel” a description of the human phenotypic traits

PRESENTATIONS

- Insect repellent and attractant activities of Brassicaceae apocarotenoid volatiles α - β - and dihydro- β -ionone. Oral presentation at the Entomological Society of Ontario (ESO) Annual Meeting. October 3-5, Toronto ON
- Apocarotenoid emission study in wild type and transgenic Arabidopsis plants over expressing CCD1 and their feeding effects against cabbage loopers. Poster. 51st annual meeting of the Phytochemical Society of North America. August 11-15, 2012
- Pesticidal properties and chemical composition of tomato plant residue bio-oil. ECI Canary Islands, Spain, May 22-27, 2011
- MS Characterization of the Pesticide Properties of Bio-oils from Pyrolysis, Poster. 58th ASMS conference Salt Lake City Utah. May 23-28, 2010
- Evaluation of the active components of pyrolysed bio-oils from tomato waste and sunflower seed hulls”. Oral presentation. Conference of the Agricultural Biorefinery Innovation Network (ABIN) for Green energy, Fuels & Chemical. March 14-16, 2010
- Evaluation of the active components of pyrolysed bio-oils from tomato waste and sunflower seed hulls. Oral presentation ICFAR group. Niagara falls February 28- March 1, 2010

PUBLICATIONS

- **Luis A. Cáceres** et al. (2011). Pesticidal properties of tomato plant waste bio-oil. *Pest Management Research Report*, (H) 17.
- Suqi L., **Luis A. Cáceres** et al.(2014). Insecticidal Activity of Bio-oil from the Pyrolysis of Straw from *Brassica spp.* *Journal of Agricultural and Food Chemistry* 62, 3610-3618.
- **Luis A. Cáceres** et al. (2014) In vivo extraction of VOCs from Micro-Tom tomato flowers with multiple SPME fibers. *Canadian Journal of Chemistry* (Accepted) cjc-2014-0269.
- **Luis A. Cáceres** et al. (2015) Insecticidal properties of pyrolysis bio-oil from greenhouse tomato residue biomass. *Journal of Analytical and Applied pyrolysis* Manuscript accepted.
- Zhiling W., **Luis A. Cáceres** et al. (2014) Inhibition of insect glutathione S-transferase (GST) by conifer extracts. *Archives of Insect Biochemistry & Physiology* (Accepted) AIBP-14-0105.
- Salah E. Khabbaz., Liang Zhang, **Luis A. Cáceres**, Mark Sumarah, Aiming Wang, and Pervaiz A. Abbasi. 2014. Biological control of Phytophthora and Rhizoctonia root rot and damping-off diseases of cucumber and radish with novel strains of Pseudomonas and Bacillus bacteria and possible mode of actions. *Annals of Applied Biology* 166, 00 00. (Accepted November 2014, in press).

AWARDS

- 2014 Entomological Society of Ontario traveling award 2014
- Dr. Joseph Soltys Graduate Award in Chemistry 2014
- Parmalat Recognition Award in Food Chemical Analysis 2008