Contribution to the development of a biopesticide based on essential oils by tree-injection application in fruit orchards

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Diplôme : Master en bioingénieur : chimie et bioindustries, à finalité spécialisée
Année académique : 2018-2019
URI/URL : http://hdl.handle.net/2268.2/7604

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CLÉMENT BURGEON

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Acknowledgments

I would like to thank my supervisor Pierre-Yves Werrie, for his help with the experiment set-up, his many advices, numerous proofreads and simply the time dedicated to helping me when I was facing difficulties with my work.

I would also like to express my gratitude to my promoter Marie-Laure Fauconnier, for her guidance all along this work and her valuable expertise in the domain.

I am grateful to all the members of the Laboratory of Natural Molecules' Chemistry for hosting during more than 6 months, for the technical support and advices they brought to this work.

Thank you to Hélène Soyeurt and Anne-Catherine Dalcq who helped me in developing the statistical analysis that followed my experiments.

Thank you to Guillaume Le Goff and the Earth and Life Institute, Biodiversity Research Center who have allowed me to perform the experiments in one of their environmental chambers.

Last but not least, I am grateful to my family, friends and Marie for their support throughout my studies.

Abstract

Apple and pear productions are amongst the biggest fruit businesses worldwide. The pear psylla (*Cacopsylla pyri* (L.)) and the rosy apple aphid (*Dysaphis plantaginea* (Passerini)) are two common pests whose resistance to conventional pesticides is continuously increasing. In this research a focus was put on developing ecofriendly alternatives to these pesticides based on essential oils (EOs) for their insecticidal and repulsive properties.

Three EO emulsions were prepared by homogenizing 0.5% of either cinnamon oil (*Cinnamomum cassia* J.Presl), spearmint oil (*Mentha spicata* L.) or clove oil (*Syzygium aromaticum* (L.) Merrill & Perry) with 2% Tween 80 and 20% 100mM EDTA. These were injected in the trunk of two-years old apple trees and the following measurements were performed every 24h over a 96h timespan.

Volatile organic compounds (VOCs) emitted by the leaves were trapped on Tenax TA cartridges. Leaves were also sampled to study the leaf-VOC content. Leaf-emitted and contained VOCs were analyzed by chromatographic techniques coupled to a mass spectrometer (TDU-GC-MS and DHS-GC-MS respectively). Finally, measurements performed with a fluorimeter (Fv/Fm) and infra-red gas analyzer (A) allowed to assess the fitness of the injected plant and in turn understand the phytotoxic effects of the emulsified EOs. PCA and ANOVA also helped to examine the volatiles profiles related to various treatments and over time. Finally plant fitness was also analyzed through an ANOVA.

Results show that EO major components have different behaviors in the leaves. Trans-cinnamaldehyde (from cinnamon oil) accumulated in the leaves (175 $ng/g_{dried\ leaves}$ at t=96h) without being emitted over the 96h. Leaf carvone (from spearmint oil) content increased but released VOCs at a constant rate (approx. 0.2$ng/g_{dried\text{-}leaves}/h$). Eugenol (from clove oil) was absent in both contents and emisions. The overall volatile profiles see most of their variation occurring in terms of terpenic molecules and other stress released molecules.

Finally, no clear phytotoxic effects have been noticed between treatments and blank modalities (average Fv/Fm = 0.83).

To conclude, this study developed a stable, non-phytotoxic emulsions with potential insecticidal and repulsive effects alongside an injection methodology. This study was pioneer in its domain as no trunk-injected EO-based biopesticides have yet been tested.

Key words: EO-based biopesticides, trunk-injection, leaf-emitted volatiles, leaf-contained volatiles, phytotoxicity

Résumé

Les productions de pommes et de poires figurent parmi les plus grandes productions fruiticoles mondiales. Le psylle du poirier (*Cacopsylla pyri* (L.)) et le puceron cendré du pommier (*Dysaphis plantaginea* (Passerini)) sont deux insectes ravageurs dont la résistance aux pesticides conventionnels ne cesse de croître. Ce travail a pour objectif de mettre au point une alternative éco-responsable aux pesticides conventionnels. Cette initiative porte sur l'utilisation des huiles essentielles (HE) en considérant leurs propriétés insecticides et répulsives.

Trois émulsions d'HE ont été préparées en homogénéisant 0,5% d'huile de cannelier de Chine (*Cinnamomum cassia* J.Presl), de menthe verte (*Mentha spicata* L.) ou encore de giroflier (*Syzygium aromaticum* (L.) Merrill & Perry) avec 2% de Tween 80 et 20% 100mM d'EDTA. Ces émulsions ont été administrées par endothérapie végétale à des pommiers de deux ans et les mesures suivantes ont été effectuées toutes les 24h pendant 96h.

Les composés organiques volatils (COVs) émis par la plante ont été prélevés à l'aide de cartouches Tenax TA. Les feuilles ont également été échantillonnées pour étudier leurs contenus en COVs. Les COVs émis et contenus par les feuilles ont été analysés en chromatographie en phase gazeuse couplée à la spectrométrie de masse (« TDU-GC-MS » et « DHS-GC-MS » respectivement). Finalement les mesures effectuées à l'aide d'un fluorimètre (Fv/Fm) et d'un analyseur de gaz infrarouge (A) ont permis d'évaluer la vitalité des arbres traités et ont fourni des informations sur la phytotoxicité des émulsions d'HE. Des analyses statistiques (ACP et ANOVA) ont permis d'examiner les profiles en COVs en lien avec les divers traitements et au cours du temps. Pour finir la vitalité des plantes a également été analysée à l'aide d'ANOVA.

Les résultats ont montré que les composés majeurs d'HE présentent différents comportements dans les feuilles. Le trans-cinnamaldehyde (HE de cannelier) s'est accumulé dans les feuilles (175 $ng/g_{feuilles\ sèches}$ à t=96h) sans être émis sur les 96h. Le contenu en carvone (HE de menthe verte) dans les feuilles a augmenté mais l'émission de cette molécule est restée constante (approx. $0.2ng/g_{feuilles\ sèches}/h$). L'eugenol (HE de giroflier) était absent aussi bien dans le profil contenu que émis. Les profiles en COVs voient la majorité de leur variation en termes de molécules terpéniques et autres molécules indicatrices de stress.

Pour finir, aucun signe évident de phytotoxicité n'était présent entre les traitements et le blanc (Fv/Fm moyen = 0.83).

Pour conclure, cette étude a developpé une émulsion stable et non-phytotoxique avec des effets insecticides et répulsifs potentiels, ainsi qu'une méthode d'endothérapie végétale par injection d'émulsions d'HE.

Mots clés : biopesticides à base d'HE, endothérapie végétale, COVs émis par les feuilles, COVs contenus dans les feuilles, phytotoxicité

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Abbreviations

ANOVA: Analysis of variance

AS: Active substance

BVOC: Biogenic volatile organic compound

DHS: dynamic headspace sampling

EIC: Extracted ion chromatogram

EO: Essential oil

GAP: Good agricultural practice

GC-MS: Gas chromatography- mass spectrometry

GLV: green-leaf volatile

HPH: High pressure homogenization

HSH: High speed homogenization

IGR: Insect growth regulator

IPM: Integrated pest management

IR: Infrared

IRGA: Infra-red gas analyzer

IS: internal standard

LOD: limit of detection

LOQ: limit of quantitation

Fv/Fm: Maximum quantum efficiency of photosystem II

MS: Mass spectrum

PC: Principal component

PCA: Principal component analysis

PI: Polydispersity index

PSII: photosystem II

RH: relative humidity

RI: Retention index

Rt: Retention time

SIM: Single ion monitoring

TDU: thermal desorption unit

VOC: volatile organic compound

1. Introduction

The apple and pear productions are the two biggest fruit businesses in Belgium representing 48.27% and 42.81% respectively of the orchard cultivated land in 2005 (Baert *et al.*, 2009) with productions rising up to 238 244 tons of apples and 331 550 tons of pears in 2016 (*FAOSTAT*, 2016)[1].

This production can be severely impacted by biotic and abiotic factors. In Belgium, the impact of frost (major factor responsible for the drop in apple production by 68% between 2016 and 2017 in Belgium (*FAOSTAT*, 2016)), hail or even droughts on productivity are non-negligeable (Agreste, 2013). Similarly, pests can also be a major problem encountered in fruit orchards. A wide-range of pests exists for both the apple and pear orchards.

Fungal and bacterial diseases are often found in these orchards. Apples orchards for example are affected by fungal diseases like the apple scab (*Venturia inaequalis* (Cooke) Winter)[2] and the powdery mildew (*Podosphaera leucotricha* (Ellis & Everh.) E.S. Salmon) but also by bacterial diseases like the fire blight (caused by *Erwinia amylovora* (Burrill) Winslow et al.) (Kellerhals *et al.*, 2012; Gusberti *et al.*, 2015). Similarly, pear orchards are mainly ravaged by the pear scab (*Venturia pirina* Aderh.), the fire blight (mentioned earlier) and the pear rust (*Gymnosporangium sabinea* Oerst) (Kellerhals *et al.*, 2012).

Insects can also cause severe problems. The codling moth (*Cydia pomonella* Linnaeus.) for example, is found in pear but also apple orchards, where it is considered to be the most important pest. Concerning Belgian pear orchards, the pear psylla (*Cacopsylla pyri* (L.)) is the major insect pest. Lastly, the rosy apple aphid (*Dysaphis plantaginea* (Passerini)) is a pest which seems important to mention given its big impact on apple orchards even when found in small numbers (Peusens, Buntinx and Gobin, 2005; Bangels *et al.*, 2008).

In this document a particular focus on *Cacopsylla pyri* (L.) and *Dysaphis plantaginea* (Passerini) will take place given their devastating effects on pear and apple orchards in Belgium.

1.1. Pests' life cycle

1.1.1. *Cacopsylla pyri* – the pear psylla (Homoptera: Psyllidae)

C. pyri is an insect living on pear trees from which it sucks the phloem sap to use it as an energy source. The phloem sap is interesting for this insect given its content in sugars (e.g. sorbitol and sucrose) which are used as a carbon source and essential amino acids (which it does not synthesize) used as a nitrogen source (Le Goff *et al.*, 2018). At the end of winter, *C. pyri*'s winter-laid eggs hatch, giving therefore the first instar nymphs, which infest new vegetation. These then become mature (summer form adults) in April allowing females to lay eggs, which hatch in May. The first and last generation nymphs emerging from these eggs live on the shoots (where they excrete honeydew) until they are fully developed summer adults (which then cause damage to the fruits). Three to four generations pass until September or October where the winterform appears again (Bovey, 1967; Garcia-Chapa *et al.*, 2005; Civolani, 2012).

[1] FAO data display data up to 2017. This year was not chosen as it is a bad representation of the overall Belgian's apple production given the night frosts still occurring in April.

[2] Known to be the biggest threat to apple orchards in Quebec (Vincent and Rancourt, 1992)

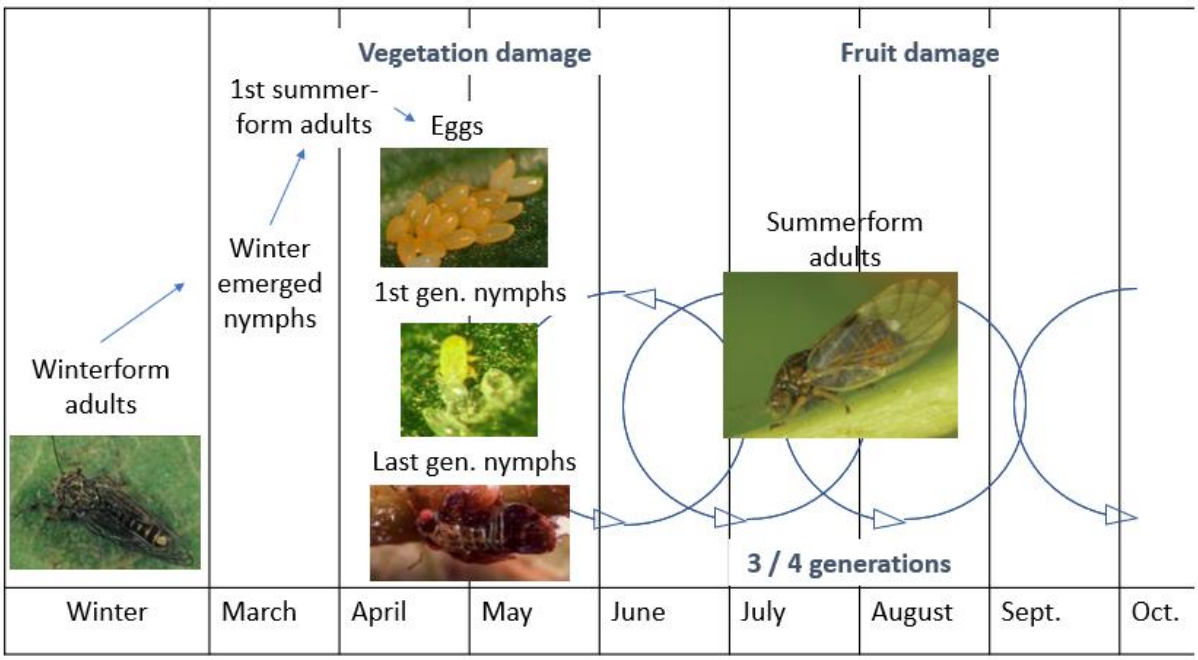


Figure 1. Life cycle of Cacopsylla pyri. Adapated from (Bovey, 1967) with pictures from (Civolani, 2012)

It is mainly during the end-of-summer period, once the 3rd generation reached (increase in the number of eggs laid), that the pear trees are subject to important leaves' loss. In fact, given that *C. pyri* is a phloem feeding insect it weakens the plant's leaves by extracting nutrients from it, often leading to deformed leaves and a premature fall.

It can also indirectly affect the plant through the production of big amounts of honeydew on which sooty molds can develop, hence reducing the leaves photosynthetic activity (Bovey, 1967; Le Goff *et al.*, 2018). Additionally, *C. pyri* is one of the insect vectors responsible for the spreading of the "Pear Decline (PD)", a disease caused by the presence of phytoplasmas in the sieve tubes which can lead to a decline of the pear trees on either a short or long term (few weeks to few years) (Ciancio and Mukerji, no date; Avinent *et al.*, 1997).

1.1.2. *Dysaphis plantaginea* – the rosy apple aphid (Hemiptera: Aphidae)

The rosy apple aphid (also phloem-feeding), is a pest of European origin with a dioecious holocyclic[3] life-cycle with the apple tree as a primary host-plant and *Plantago* species (*P. lanceolata L., P. media L. and P. major L.*) as secondary hosts (Bonnemaison, 1959; Alins, Alegre and Avilla, 2017a).

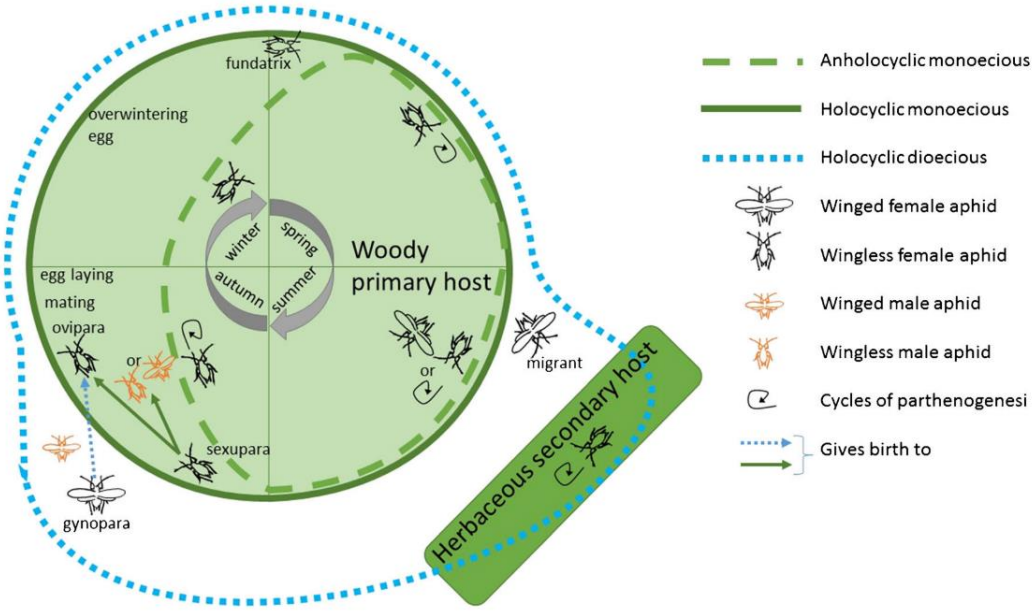


Figure 2. Complete representation of the possible life cycles for the various aphid species. D. plantaginea's cycle is circled in ***dashed blue*** *(holocyclic life cycle)* (Rousselin *et al.*, 2017)

Early April, the eggs hatch freeing the larvae which travel along the branch to enter the burst buds of the apple tree and seek protection in the tightly packed young leaves. Stage two and three larvae are most generally found in the inferior side of young leaves or stipules but can easily travel along the petiole which enables a good repartition of these larvae on the different young leaves. They then undergo the imaginal molt[4] to become adult founders. These do not move anymore and sting the leaves (to feed on) which crisps them.

The founders reproduce to give other virginopara founders (apterae - wingless or *alatae* - winged). The proportion of winged and wingless changes with generations to give a majority of winged after the fourth generation. When the founders' colonies become densely populated, larvae and adults colonize other leaves which eventually leads to their spreading on other trees.

Winged females then infest the secondary host (*Plantago spp.*)[5] on which it starts reproducing one to three days after arrival, to produce, around the end of September 95% of winged sexupara and males. Shortly after the imaginal molt, the winged sexupara fly back to the apple trees where they will lay the sexual females on the inferior side of the leaves. Lastly, before the cold season, the males which have

[3] Dioecious: "having the male and female reproductive organs in separate individuals" (*dioecious | Definition of dioecious in English by Oxford Dictionaries*, no date)
Holocyclic species are species which undergo sexual reproduction at least once in their cycle (Rousselin *et al.*, 2017)

[4] Molt allowing the insect to become an imago – the adult form of an insect.

[5] Very small success rate, given that the aphid cannot detect the Plantago plants (do not detect the plant by sight or smell).

also left the *Plantago* plants will mate with the adult sexual females, which then lay the eggs that will go through winter (Bonnemaison, 1959).

This pest, is known to be amongst the most devastating in apple orchards (Peusens, Buntinx and Gobin, 2005). It mainly damages the leaves in two different ways: the leaves are rolled-up perpendicularly to the central nerve (mature leaves with developed colonies) or bent longitudinally (young leaves with wingless females of the first generation). These damages can account for as much as a 25% of the tree's decrease in productivity, which is correlated to Simon et al. (2011)'s finding that 21.4% of the unmarketable apples issued from organic orchards were affected by *D. plantaginea* (De Berardinis, Baronio and Baumgärtner, 1994).

1.2. Pest management

1.2.1. Current techniques for *Cacopsylla pyri*'s magenement

Regardless of a significant increase in pesticide use at the end of the 20th century, the *C. pyri* population has also rapidly grown. Two main reasons could explain this, first the insect's adaptability to the active substances (AS) used and second the excessive use of broad-spectrum pesticides which have also killed the pear psyllas' natural predators.

Nowadays, integrated pest management (IPM) is used most often to control these populations. IPM is defined as "a systems approach that combines different crop protection practices with careful monitoring of pests and their natural enemies" (Chandler *et al.*, 2011).

IPM programs include general good agricultural practices (GAPs) such as the reduction of nitrogen fertilizers which make the tree less attractive for *C. pyri's* growth and reproduction, but also uses chemical control (mainly active substances of the pyrethroid family) as a key management tool (Horton, 1999; Civolani, 2012). Its effect is however very dependent on the environmental conditions and the time of application.

Various chemical controls, applied at different times of the year, are frequently used against this pest.

An autumn treatment (pyrethroid-based pesticide) can be performed to rid the plant of adult winterforms. For a good action it must be applied on a tree bare from its leaves. This pesticide being broadspectrum, there is a risk for other species which are wished to be kept, like *Anthocoris nemoralis* (Fabricius) present in the early autumn. Secondly, if early frosts appear, the winterforms will already have taken shelter and the spray will not be efficient (Civolani, 2012).

A late-winter treatment also exists which targets the number of egg-laying females therefore greatly reducing the first generation psylla population. This one has also been criticized for the toxicity of its active substance (pyrethroid) and the side-effects on the species which feed on *C. pyri* in spring such as for example *A. nemoralis* for whom food reserves are then scarce (Civolani, 2012; Nin *et al.*, 2012).

Finally, several spring/summer chemical treatments exist. For example, chitin inhibitors (e.g. diflubenzuron and teflubenzuron) aiming at second generations eggs during early May. They are generally used against *C. pomonella* but were proved to impact the psylla. Other products such as the abamectin-based product which aims the young nymphs and the spirodiclofen-based products interfering with the lipids' biosynthesis in *C. pyri* are often used alternately to reduce resistance problems (Civolani, 2012).

As it can be seen above, these chemical controls greatly depend on environmental conditions and the period of application. Additionally, the *C. pyri* populations become resistant to these active substances (resistance rate ranging from a 4.7 fold for the pyrethroids to a 179.7 fold for some organophosphorates when compared to wild populations) (Civolani, 2012).

Alternatives to these chemical based treatments have therefore been suggested: the use of the pest's natural predators, mineral oils, kaolin or even insecticidal soaps used for washing the trees.

A well-known natural control is the use of *A. nemoralis* (introduced earlier) which prevents the psylla's population from expanding (Solomon *et al.*, 1989; Civolani, 2012). The anthocorids often see their population increase only two to three weeks after the pest has developed its own population which they can feed on. This delay is often not tolerated by the farmers, which favors the early-introduction of lab-reared anthocorids (effective with release of 5500 adults *A. nemoralis*/ha in spring) (Sigsgaard, Esbjerg and Philipsen, 2006; Civolani, 2012).

Oily substances represent another alternative tested in the recent years against insects' pest. Oviposition deterrence has been shown on several species, for example the codling moth (Riedl *et al.*, 2015) and the pear psylla (Zwick and Westigard, 1978; Erler, 2004; Marčić *et al.*, 2009). Regardless from the fact that these oils show good results as *C. pyri*'s oviposition deterrent[6] they are derived from fossil fuels and are therefore less sustainable.

Finally, the application of powdered suspension of kaolin products also show deterrent effects on both overwintered and summer forms, supposedly due to a change in color or texture of the tree bark (Puterka *et al.*, 2000).

1.2.2. Current techniques for Dysaphis plantaginea's management

Most pesticides used currently aim at eliminating the fundatrices in spring. In 2005 and until now only two groups of active substances were authorized as fundatrice eliminating aphicides in Belgian non-organic apple orchards. These two are carbamates and the neonicotenoids (Figure 3) (Peusens, Buntinx and Gobin, 2005). They are applied once before flowering and often a second application comes just after (Cross *et al.*, 2007; Alins, Alegre and Avilla, 2017b). In organic productions on the other hand, azadirachtin-based pesticides are, to the author's knowledge, the only pesticides used for the elimination of these spring fundatrices (Cross *et al.*, 2007).

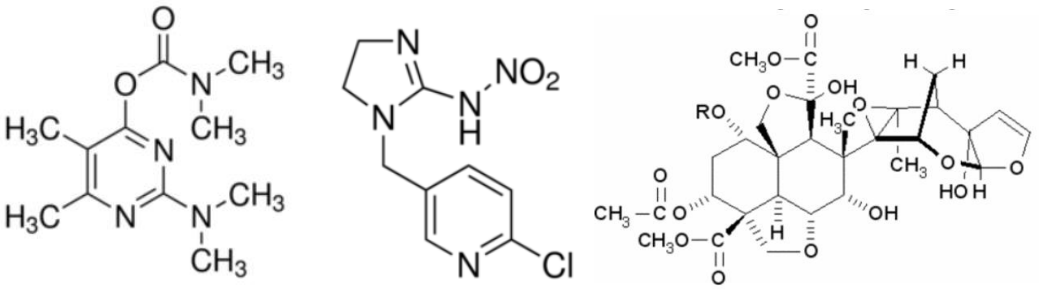


Figure 3. Structural representation of the neonicotenoids pirimicarb (left) and imidacloprid (center) and the carbamate azadirachtin (right) (Sigma-Aldrich).

[6] Oviposition reduced by 98% for overwintering adults and this up to 5 weeks (Zwick and Westigard, 1978).

One of the main problems with *D. plantaginea* is that it can very rapidly develop a resistance to pesticides given its rapid life cycle (Machial, 2010). Alternatives have therefore been investigated to reduce the risk of a resistance development but also to decrease the effects on beneficial arthropods and the vulnerability of farmers to changing legislations (Wyss and Daniel, 2004; Alins, Alegre and Avilla, 2017b).

An example of this is the contact acting insecticide pyrethrum which was proven to be moderately effective against *D. plantaginea*. However, its relative effectiveness was only apparent when several applications were performed (probably due to its low persistence). Given the repeated applications, treatment costs are often greater than for conventional pesticides (where only a single treatment is needed very often) (Cross *et al.*, 2007).

Sowing weed strips between the apple tree rows to increase the amount of aphidophagous species has also been looked as a possible alternative. In fact, this technique led to a significant decrease in *D. plantaginea*. A weed mixture is to be sown in order to maintain a constant flower bed to maintain the presence of other predator insects (e.g. syphrids) which feed on aphids at a certain growth stage and then switch to nectar when mature. Another advantage of having weedstrips include the availability of other harmless aphid species (e.g. *Brevicoryne brassicae L.*) which can be used to feed on when *D.* plantaginea are found in low density on apple trees (Wyss, 1995).

Augmentative releases[7] of *D. plantaginea* predators can also be performed to complement the impact of natural predators in times of high aphid abundance. Wyss et al. (1999) have found that releasing non-specific aphid predators *A. bipunctata* and *E. balteatus* separately or conjointly was effective in reducing the pest's population.

Given the side-effects of conventional pesticides on human health and on the environment and the poor efficicacy of current alternatives, it seems interesting to further investigate into essential oils (EOs) as potential alternatives.

1.3. Essential oils

1.3.1. Essential oils, biological properties

Plant essential oils (EOs) are defined as "complex mixtures of odorous substances obtained from botanical raw materials by water vapor extraction, dry distillation, or mechanical treatment without heating". These essential oils are mainly composed of monoterpenoids, sesquiterpenoids (90% of all molecules combined) and structures with aromatic rings. Terpenoids are structures built with isoprene units (2 isoprene units for monoterpenoids and 3 for sesquiterpenoids) (Blenau, Rademacher and Baumann, 2012).

The secondary metabolites composing EOs are produced and emitted by plants to adapt to biotic and abiotic stress originating from its environment. For example in plant-insect communication, they serve as stimuli to encourage seed dispersion or simply to repel these insects as a defense mechanism (Miguel, 2010). Furthermore EOs are also known to play a role in allelopathic communication given the phytotoxicity some EOs have displayed, for example *Mentha piperita* L. (Mitcham, Mahdavikia and Saharkhiz, 2015).

[7] Augmentative Biological control: "The practice of rearing and releasing biological control agents to effet pest suppression" (Hoy, 1990).

Given these natural properties, EOs represent a promising alternative and have recently received a lot of attention in the scientific community.

EOs have proven to be efficient antimicrobial agents[8]. This property is supposedly due to their hydrophobic nature. In fact, they tend to interact with the lipid membranes and separate them which results in a leakage of the inner cell components and cause the cell's death (Bajpai, Baek and Kang, 2012). EOs are also good antioxidants. Good antioxidant substances are mainly those which contain phenolic compounds, carotenoids, tocopherol and ascorbic acid (Elmastas *et al.*, 2007). In fact, aromatic plant oils such as *Thymus vulgaris* L. (thyme) or *Ocinum basilicum* L. (basil) oils for example showed good antioxidant properties due to their high contents in thymol and eugenol respectively (Wei and Shibamoto, 2010). Several EOs have also been investigated for their anti-inflammatory properties. EOs act on different aspects of the inflammatory response. They can act of the lipoxygenase pathway, they can have an effect as pro-anti-inflammatory cytokines suppressors and finally they are able to modulate pro-inflammatory gene expression (Miguel, 2010; Wei and Shibamoto, 2010).

For the sake of this research, the main interest is the EOs' capacity to play a role as insect biopesticides[9], i.e as repellents but also as insecticides (fumigation, contact and ingestion toxicity). These aspects will be further presented and investigated in the coming sections of this work.

1.3.2. Essential oils for insect pest management

The insecticidal properties of EOs are often linked to their neurotoxic properties, affecting the insect's nervous system in 3 mains ways (Figure 4 – "direct toxicity"):

- By blocking the gamma-aminobutyric acid (GABA) receptors and therefore increasing the chloride current at this location which modifies the action potential of a nerve. GABA is a feeding stimulant and affects the taste cell responses, interfering with its correct delivery induces feeding deterrence. Thymol for example was found to impact the GABA receptors of *Drosophila melanogaster* (Meigen) (Rattan, 2010; Campos *et al.*, 2018).
- By binding to the octopamine receptors. Octopamine is said to be the homologous compound of epinephrine in invertebrates. It is associated with the initiation and maintenance of different movements, interfering with the correct functioning of these receptors hence leads to movements impairments. Eugenol, α-terpineol and carvacrol are molecules which were proved through tests on *Periplaneta americana* (Linaeus.) to bind to octopamine receptors (El-Kholy *et al.*, 2015; Campos *et al.*, 2018).
- By affecting acetylcholinesterase (AChE) enzymes through competitive and non-competitive inhibition. Inhibiting the AChE leads to an accumulation of acetylcholine at the synapse. This causes a constant stimulation of the post-synaptic neuron resulting in ataxia and even death. Geraniol, linalool and γ-terpinene were found to affect AChE through non-competitive inhibition and molecules like camphor, estragole and S-carvone through competitive inhibition (Rattan, 2010; Campos *et al.*, 2018).

[8]For example, Hammer et al. (1999) found that *Cymbopogon citratus (DC.) Stapf* EO is effective against microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica*.

[9] Biopesticides are classified in 3 categories according to the U.S Environmental Protection Agency (EPA): "1. Biochemical pesticides (e.g., certain natural compounds used for pest management). 2. Plant incorporated protectants (PIPs), which are the result of transgenes that impart the synthesis of natural pest management compounds in crops and 3. biocontrol organisms (e.g microbial fungi)" (Seiber *et al.*, 2014).

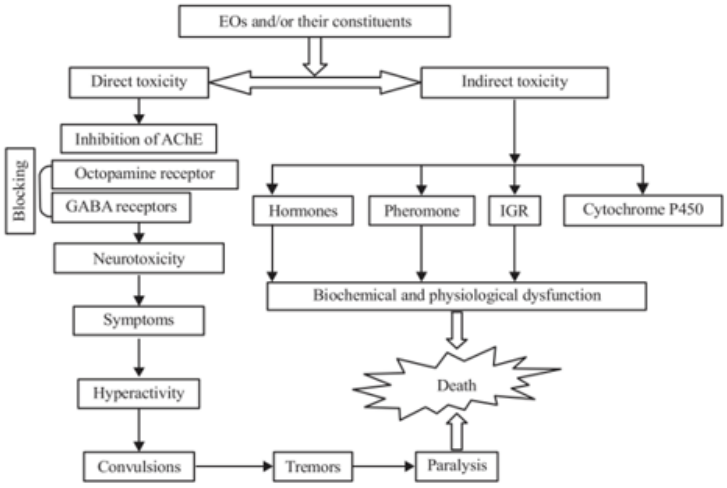


Figure 4. Toxicity mechanisms of essential oils on insects (Mossa, 2016)

Essential oils are also often used as fumigants. This is explained first by their high volatility and second by their rapid penetration in the insect (Singh, 2014; Mossa, 2016). They have been proved effective against stored food pests (e.g *Sitophilus* oryzae (Linnaeus) and *Tribolium* castaneum (Herbst)) through researches like Lee et al. (2002)'s who analyzed the effect of EO monoterpenoids and found for example 100% mortality at 50µg/ml air for ketone derivatives (l-fenchone and pulegone).

Insect growth regulator (IGR) is another potential mode of action. The IGRs tend to disrupt the growth and affect the reproduction of the pests concerned. The growth inhibitions could be explained by the inhibition of several biosynthetic processes happening at the insect's different developmental stages (Singh, 2014). Aziza et al. (2014) showed a significant decrease in the life-span of new *Phthorimaea opercula* (Zeller) adults when, for example, vapors of *Cymbopogon citratus* (D.C.) Stapf were applied on potato tubers.

Furthermore, it is important to state that the mode of application (direct contact, ingestion or fumigation) plays a major role in the intensity of the EO's toxicity to insects.

Essential oils can also act as insect repellents. These are substances that act as vapor barriers locally or at a distance, deterring an insect from flying to, landing on or biting a surface (Nerio, Olivero-Verbel and Stashenko, 2010). They can also act as antifeedants, which are "behaviour-modifying substances that deter feeding through a direct action on peripheral sensilla in insects" (Isman, 2002; Mossa, 2016).

Finally, the attractant effect of certain essential oils (and their components) could be used in the development of insect traps. Hernandez-Sanchez et al. (2001) tested the attractiveness of several mango (*Mangifera indica* L.) terpenes in laboratory cages on fruit flies (*Ceratitis capitate* (Wiedemann)) and found that p-cymene and limonene were good attractants (Hernández-Sánchez *et al.*, 2001).

1.3.2.1. Essential oils in the management of D. plantaginea populations

Aphids (Aphidoidea superfamily) are a large class of pests attacking a wide range of crops worldwide. They have a high capacity at developing resistance to insecticides[10] due to their rapid development and short generation times (Machial, 2010). For these reasons, several EOs have already been tested and their effects analyzed on a number of aphid species (Atanasova and Leather, 2018).

For more clarity, the information concerning the studies found have been summarized in Table 1 and additional comments are added in the text when needed.

[10] Six different types of resistance have been identified for *M. persicae*, the green peach aphid for example (two resistances involve metabolic mechanisms and four others target-site mechanisms) (Simon and Peccoud, 2018)

Table 1. Summary of the mentioned studies on the management of D. plantaginea

Pests concerned	Essential oil tested	Type of test performed	Main results obtained (interesting ones in the case of this research)	Article
Myzus persicae	13 oils, including *Mentha spicata* L., *Mentha piperita* L. , *Thymus vulgaris* L. and *Rosmarinus officinalis* L.	Repellent activity	Spearmint, Thyme and rosemary repelled at a dose of 10µl. Rosemary and thyme oils at a dose of 1 µl also. Linalool, d,l-camphor and α-terpineol had repellent activities.	(Hori, 1998)
		Antifeeding, settling inhibitory and toxic activites	Thyme oil (+++) > spearmint and pennyroyal (++) > peppermint and lavender (+) (Results found for each test can be found Annex 1)	(Hori, 2002)
Myzus persicae and Acyrthosiphon pisum	*Hemizygia petiolata* oil (containing >70% (E)-β-farnesene)	Alarm pheromone bioassay, Olfactometer bioassay (attraction to host plant), settlement bioassays and field experiments	Goal was to see the potential of (E)-β-farnesene rich EOs on insect populations: Populations reduced in field trials. Alarm pheromone bioassay affected by inhibitor compounds in the EO. In plants: EBF production coupled to that of other secondary metabolites. Aphids: EBF production only. Aphids can detect these other secondary metabolites and therefore distinguish plant-based and insect-based EBFs.	(Bruce *et al.*, 2005)
Lipaphis pseudobrassicae	23 plant species including *Bifora radians* Bieberstein, Thymes *(Thymbra sintenisii* Bornm. and *Thymbra spicata* L.)	Insecticidal assay (using microtiter plates with whatmann papers inside)	Great variability in LC_{50} obtained between the oils: Oils with similar compositions: e.g. *T. spicata* and *S. hortensis*: LC_{50} of 0.9 and 1.7 mg/ml respectively. But also between repetitions (due to differences in plant age, proportion of the different parts used in the distillation process, etc…). *B. radians* most aphicidial: LC_{50} of 0.3 mg/ml after 60 min., followed by *T. sintenisii* and *T. spicata*: 0.9 mg/ml after 60 min.	(Sampson *et al.*, 2005)
Brevicoryne brassicae	*Nepeta cataria*, *Lavandula augustifolia, Origanum majorana* and *Rosmarinus officinali*	Fumigation assays (using dessicators as closed environment)	All 4 EOs highly toxic. Highest toxicity (lowest LC_{50}): LC_{50} 1.42 µl/l of air for *Nepeta cataria.*	(Pavela, 2006)
Myzus persicae, *Macrosiphoniella sanborni*, *Aphis fabae* and *Acyrthosiphon pisum*	*Mentha pulegium* L. (from 2 locations), *Mentha piperita* L., *Ocinum basilicum* L. and *Citrus sinensis* L.	Fumigation assays (using gas-tight glass vials)	All 5 EOs were highly toxic: LC_{50} ranging from 0.17 to 1.92 µl/l of air)	(Kimbaris *et al.*, 2010)
Dysaphis plantaginea and *Myzus persica*	17 EOs including *Syzygium aromaticum*, *Melaleuca alternifolia, Mentha piperita, Thymus vulgaris, Pogostemon cablin*, *Cymbopogon citratus* and *Thymus vulgaris*	Insecticidal potential through spray emulsion on adults in preliminary tests. Determination of LC_{50} and LD_{50} for selected EOs (*P. cablin*, *C. citratus* and *T. vulgaris)*	10 out of 17 showed a mortality of at least 50% of *D. plantaginea*'s vs. only 3 on *M. persica at* concentration of 5.0µl/ml. All 3 selected EOs led to paralysis of *D. plantaginea.* Several EOs such as S. aromaticum have a great potential and must be further analyzed. Many EOs gave good results in the screening test and therefore are worth being tested on further tests.	(Machial, 2010)
Dysaphis plantaginea	Hemp (*Cannabis sativa* L.)	Field trial in apple orchard to evaluate insecticidal properties	Hemp EO used at concentration of 0.02, 0.05 and 0.10% and compared to Mospilan 20 SP (acetamiprid) used at a dose of 0.125 kg/ha. The EO at concentrations 0.05 and 0.10% were as effective as the conventional pesticide with a decrease in aphid population of 94.87% and 95.52% at these concentrations.	(Gorski, Szklarz and Kaniewski, 2009)

As mentioned in the table, Hori (1998) found that linalool, d,l-camphor and α-terpineol are responsible for repellent actions. This is a very promising information as these compounds are found in many other EOs and should therefore encourage further research into their application as aphicides. In this context, *Thymus satureioides' EO* (up to 18% α-terpineol and 3% linalool) is a promising candidate (Ramzi *et al.*, 2017). Hori (2002) brought out other interesting plants as candidates (e.g Thyme, spearmint, pennyroyal, peppermint and lavender oils).

Bruce et al. (2005) led a research on EOs rich in (E)-β-farnesene (EBF), the alarm pheromone released by many aphid species when they are under attack (particularly in the subfamily Aphididae). Even though, EBF does not figure amongst the major alarm pheromones released by *D. plantaginea* (Francis *et al.*, 2005), similar researches to that of Bruce et al. (2005)'s could be done in order to find, for example, an EO rich in α-farnesene - a sesquiterpene known to repel *D. plantaginea* (Warneys *et al.*, 2018).

Sampson et al. (2005)'s work was interesting as it reminded that "even minor compounds (of an EO) can have a critical function due to coupled effects, additive action between chemical classes and synergy or antagonism" which is better understood when comparing results for two EOs having same proportions of major compounds: *Satureja hortensis* L. and *Thymbra spicata* L. containing 24% and 30% carvacrol as well as 45% and 38% γ-terpinene respectively.

Even if fumigation will not be the technique applied for the apple and pear orchard given that it needs to be performed in a close environment, Pavela (2006) and Kimbaris et al (2010)'s articles seemed interesting to mention as they remain good indicators of the potential of these EOs as aphicides.

Machial (2010) is the one of only work[11] to the author's knowledge which tested EOs specifically against the aphid aimed by this project, *D. plantaginea*. As it can be seen in the table, a greater number of oils appeared efficient against *D. plantaginea* compared to *M. persicae* in screening tests. This could be explained by the fact that *M. persicae* is a generalist aphid, as it is a host on approximately 30 plant families, compared to *D. plantaginea* specific to apple trees. This means that *M. persicae* has higher detoxicative abilities and is therefore protected against a larger number of toxins. Another reason could be the differences in number and types of neuroreceptors from one species to another leading to different sensitivities (Machial, 2010).

Additionally, thought-provoking observations were found when the 3 EOs selected for further analysis were sprayed on the aphid. Paralysis as well as excess fluid exudation from the aphid's cauda were observed (Figure 5). A hypothesis to explain the latter could be an effect of these oils on the octopaminergic system (which was found responsible for the contractions of the abdomen in *P. Americana* (Washio and Tanaka, 1992)) coupled to a tissue disruption (dorsal lesions visible in Figure 5. c when patchouli oil was applied).

[11] Along with Gorski et al. (2009)

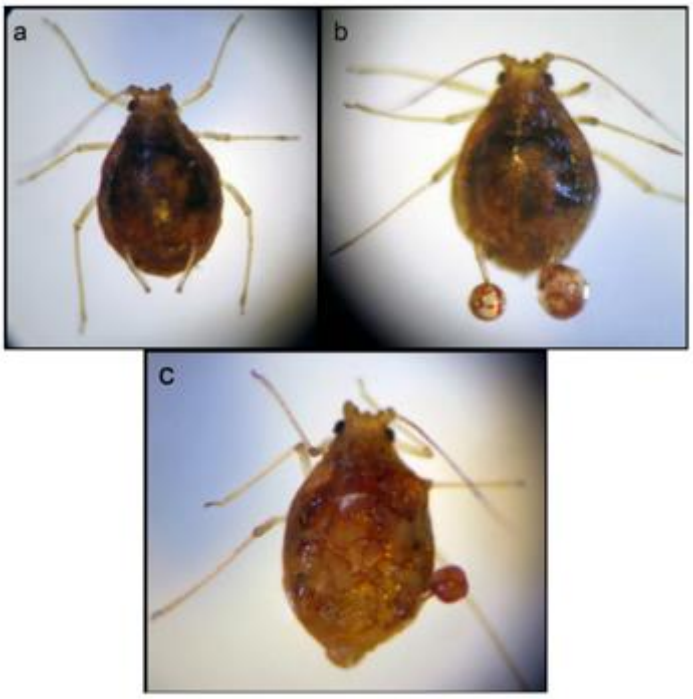


Figure 5. Pictures of *D. plantaginea* : (a) untreated, (b) treated with thyme oil and (c) with patchouli oil (Machial, 2010)

1.3.2.2. Essential oils in the management of C. pyri populations

Only one research was found concerning the application of EOs specifically on *C. pyri*. That is Imrek et al. (2017)'s, which gave promising results regarding the use of fennel (*Pimpinella anisum* L.) and citrus (*Citrus aurantium* L.) oils.

Several researches were found concerning other psyllids belonging to the *Psylloidea* superfamily. In fact, several studies were conducted on the effects of essential oils on the Asian citrus psyllid, *Diaphorina citri* (Kuwayama) and others on *Cacopsylla chinensis*, an important pest of pear in China.

The information found has been gathered in Table 2.

The researches directed on *C. chinensis* were thought to be particularly interesting in the case of this project given that just like *C. pyri*, *C. chinensis* belongs to the same order (implying that they have many morphological similarities) and additionally, both are pear pests.

The research led by Tian et al. (2015) showed promising results when compared to AkseBio2[12] (a botanical natural pesticide used against *C. pyri*) which showed a mortality of 81.10% of young nymphs and 52.70% of older nymphs at 0.7 mg/ml after 7 days (Erler, Yegen and Zeller, 2007; Tian *et al.*, 2015).

In the last study presented in the table, the garlic chive oil's good toxicity could be explained by the presence of several sulphur volatiles which proved to inhibit *D. citri*'s response to attractive host plant volatiles (Mann *et al.*, 2011).

[12] Standard specifications can be found in Annex 2.

Table 2. Summary of the mentioned studies on the management of D. plantaginea

Pests concerned	Essential oil tested	Test performed	Main results obtained (interesting ones in the case of this research)	Article
Cacopsylla pyri (winterforms)	*Rosmarinus officinalis* L., *Mentha piperita* L., *Pimpinella anisum* L., *Citrus aurantium* L.	Oviposition deterrence and ovicidal efficacy	All were tested at a dose of 120 µl/l. Oviposition deterrence: Citrus oil: only one showing 100% oviposition deterrence three days after application. Fennel oil: only one to show deterrence of 50% 10 days later. Mint: lowest deterrence effect. Ovicidal efficacy: all except mint and rosemary caused mortalities of 87.5 to 89.3% on eggs no older than 48h. All EOs ovicidal efficacy strongly decreased seven to ten days later.	(İmrek *et al.*, 2017)
Cacopsylla chinensis (winterforms)	*Allium* sativum L. and its main constituents (diallyl trisulfide (50.43%) and diallyl disulfide (25.30%))	Insecticidal (contact) activities	Toxicities: *Allium* sativum L.: LC_{50} of 1.42 µg/adult diallyl trisulfide: LC_{50} of 0.64 µg/adult diallyl disulfide: LC_{50} of 11.04 µg/adult	(Zhao *et al.*, 2013)
Cacopsylla chinensis (winterforms)	*Eucalyptus robusta* Smith EO and its main constituents (α-pinene (30.18%) and 1,8-cineole (26.08%))	Insecticidal (contact) activities	Toxicities: *Eucalyptus robusta* Smith: LC_{50} of 10.61 µg/adult α-pinene: LC_{50} of 1.34 µg/adult 1,8-cineol: LC_{50} of 11.76 µg/adult	(Liu *et al.*, 2014)
Cacopsylla chinensis	S*yzygium aromaticum* L. and its main constituents (Eugenol (88.61%), eugenyl acetate (5.62%) and β-caryophyllene (1.39%))	Insecticidal activities in laboratory and field assays	Laboratory: EO and constituents affected both summer form adults and nymphs but remained much less toxic compared to the conventional pesticide abamectin (results displayed in Annex 3). Field assays: After three days: reduction of 46.56%, 66.18% and 73.01% of the *C. chinensis* nymphs (concentrations of 1.20, 2.40 and 4.80 mg/ml)	(Tian *et al.*, 2015)
Diaphorina citri	Garlic chive - *Allium tuberosum* Rottl. (plant, crushed leaves and EO), Wild onion - *Allium canadense* L. (plant and crushed leaves)	Repellent activities (with a T-olfactometer)	Repellent activities observed for all. Best repellency with garlic chive EO (concentration of 5.0 mg/ml)	(Mann *et al.*, 2011)
Diaphorina citri	Coriander (*Coriandrum sativum* L), rose (*Rosa spp.*), thyme (*Thymus vulgaris* L.) , lavender (*Lavandula spp.* L.) and tea tree (*Malaleuca alternifolia*) and Garlic chive *(Allium tuberosum* Rottl.) oils	Repellent (T-olfactometer) and insecticidal (contact) activities	All five EOs had repellent activities (more *D. citri* in release arm compared to EO-treated arm). Toxicities: Lavender oil: highest toxicity: LC_{50} of 0.16 µg/insect Thyme oil: lowest toxicity: LC_{50} of 17.26 µg/insect Garlic chive oil: an LC_{50} of 0.17µg/insect	(Mann *et al.*, 2012)

1.3.2.3. Selected essential oils

Several essential oils, for which good results were obtained as insect repellents or insecticides, have been mentioned in the two previous subsections. Out of all the EOs mentioned above, only seven were selected as part of this project. Indeed, not only must they show good insecticide (and repellent) properties but the product should also be easily available and easily registrable (if the product needs registration at all) (Isman, 2000).

The first two EOs selected belong to the *Mentha* genus: *Mentha spicata* L. (spearmint) and *Mentha piperita* L. (peppermint). Good results were obtained in the tests performed by Hori (1998, 2002) on *M. persicae* for both of these 2 oils. The results found by Machial (2010) for *M. piperita* on *D. plantaginea* (73.3 ± 8.8 %) also encourage even further the research led on this oil.

The following two EOs selected belong to the *Thymus* genus: *Thymus vulgaris* var. *thujanoliferum* and *Thymus satureioides* suggested earlier in the frame of this research. Firstly because of Hori (1998)'s finding concerning linalool and α-terpineol. Secondly, because Hori (1998) found that thyme oil was the only one showing significant activity in all his tests and Machial (2010) found that thyme oil had the lowest LD_{50} when applied topically (2.5 µg/insect) to *D. plantaginea*. Finally, Isman and Machial (2006) mentioned that thyme oil figures along with clove oil , cinnamon oil, mint oil, lemongrass oil, rosemary oil and oregano oil "among the best known essential oils with bioactivity against insects and other pests". This satisfies even more the decision of testing these oils.

Even if no research was found discussing the effect of *Cinnamomum cassia* on any insect of *D.* plantaginea or *C. pyri*'s superfamily, it was still decided to test this EO. The reasons are the following: Firstly, *C.* cassia's oil has been analyzed in Kim et al. (2003)'s work on adults of *S. oryzae* and *Callosobruchus chinensis* (L.) through contact exposure (petri dish) and fumigation exposure. *C. cassia* oil gave good results in contact exposure with 100% mortality on petri dish for both insects after 1 day when a concentration of 0.7 mg/cm^2 was used. It was found that the fumigation activity seems to play an important role in the insecticidal effect. In fact, Kim et al. tested the effect of opening or letting the container closed on fumigant and direct contact tests. A significant difference was observed between the results obtained (e.g. 100% mortality of *S. oryzae* when 0.7mg/cm^2 concentration is used and the insects are put in direct contact in a closed environment, compared to 33% in an open container) (Kim *et al.*, 2003). Given the good results obtained here against insects of the Coleoptera order and given that soft-bodied insects such as aphids may be more vulnerable to some specific essential oil compounds than hard-bodied insects which have a more sclerotized cuticle (Chiasson, Vincent and Bostanian, 2009), it seems interesting to test this oil on the concerned pests.

Secondly, two pesticides with cinnamaldehyde as active ingredient have already been developed and commercialized. Cinnacure (30% cinnamaldehyde, 70% inert ingredients) is a broad spectrum insecticide which also aims at reducing aphid populations (Proguard, 2002). Cinnamite (also 30% cinnamaldehyde) is another pesticide which works by contact action and is labeled for use against mites and aphids in greenhouses (Cloyd, 1999). The development of these natural pesticides proves that cinnamaldehyde is efficient against aphids (and other pests). Therefore, knowing that *C. cassia* is composed of approximately 85% trans-cinnamaldehyde (Ooi *et al.*, 2006), it seems important to test its effect on the rosy apple aphid and the pear psylla.

The tea tree, *Malaleuca alternifolia* oil also seems important to test given Mann (2012)'s finding on this oil's significant repellency of the psyllid *D. citri* and Machial (2010)'s results as an insecticide for *D. plantaginea* (60.0 ± 20.8 %). Even though this last result does not figure as one of the best mortalities

found, the high standard deviation suggests that other results, hopefully higher mortalities, could be found.

The same reasoning applies to the last EO, S*yzygium aromaticum* L. (clove) oil with which Machial (2010) also found a mortality of 60.0 ± 20.8 %. Its potential as a good pesticide is backed up by Tian et al. (2015)'s findings on *C. chinensis* explained in the previous section.

Finally, even if these oils have insecticidal properties that make them worth testing in further trials, for this experiment it must be noted that not all oils are produced in Europe which could at times compromise their availability. In fact, *M. spicata* is native from Central Europe (and hence easily grown) (Almeida, Mezzomo and Ferreira, 2012), *M. piperita*'s culture is readily available (e.g in Latvia, it is cultivated since the 18th century), *Thymus sp.* can easily be grown in the south of Europe. Availability should therefore not be a problem for these three oils. The three others however are not produced in Europe. *Cinnamomum cassia* is produced mainly in China, S*yzygium aromaticum* is produced in Asia and Africa (Indonesia is the biggest producer) and finally, *Malaleuca alternifolia* on the other hand is a plant found almost exclusively in Australia.

Variations in compositions must also be considered when choosing the EO used for the final product. In fact, various chemotypes can exist within one species. For instance, *Melaleuca alternifolia* has six distinct chemotypes (one dominated by terpinen-4-ol, one by 1,8-cineole, one by terpinolene and the other three are dominated by 1,8-cineole and differ in their terpinen-4-ol and terpinolene content) (Homer *et al.*, 2000). Choosing an EO for the development of the final product hence not only implies choosing one species but also paying attention to the chemotype. In fact, various compositions lead to different phytotoxic effects and insecticidal properties.

Furthermore, the price of the EOs is a non-negligible aspect. In fact, these vary largely from one oil to the other, for example prices may range from approximately 40€ kg^{-1} for *Cinnamomum cassia* to 9000€ kg^{-1} for *Rosa damascena oil* (Ultra International B.V., 2018). Although the most expensive oil selected here is relatively affordable compared to the latter (± 120€ kg^{-1} for Thyme oil), the EO chosen for the final product should be selected in order to offer a biopesticide selling price competitive when compared to conventional pesticides.

1.3.2.4. Phytotoxicity of the proposed essential oils

A biopesticide should also show low phytotoxicity, i.e "plant injuries from chemicals" (Ibrahim and Tiilikkala, 2001). This phytotoxicity can appear in different ways such as a burnt appearance of the leaf tips, entire leaf surface or margin of the leaf. The buds and roots can also be burnt in some cases. Located chlorosis appears as spots along margins when general chlorosis appears as a curling, crinkling or cupping of the leaf. Another effect of phytotoxicity is abnormal growth (e.g. the number and size of fruits and flowers are reduced) (Ibrahim and Tiilikkala, 2001).

Taking into account that the active substances in the developed biopesticide would be the molecules constituting the above mentioned EOs, it seems important to analyze their phytotoxicity. The literature found concerning their phytotoxicity is gathered in Table 3, where the articles are listed following the same scheme as the following paragraphs.

S. aromaticum EO (i.e clove oil) and its many constituent, eugenol have been the subject of many studies from which it comes out every time that both clove oil and eugenol have strong phytotoxic effects (Bainard, Isman and Upadhyaya, 2006; Ahuja *et al.*, 2015; de Oliveira *et al.*, 2016).

In Ahuja et al. (2015)'s study it was found once again that at the same concentration applied, the several weeds tested have different susceptibility to eugenol (e.g. *C. occidentalis* always presented better germination rates than *C. benghalensis*).

In addition, it can, not only be said that phytotoxicity differs from one plant to another but it also differs between developmental stages. In fact, all tests explained earlier were led on seeds and all were found to be affected by this oil. O the other hand Tian et al. (2015) found no apparent signs of phytotoxicity when clove EO was sprayed on fully grown pear trees.

This lack of phytotoxicity cannot be generalized to all fully-grown trees as it can be seen through Miller and Tworkoski (2010)'s study summarized in Table 3. Although, they noted that approximately four weeks after the treatment, the apple trees fully recovered. It could therefore be interesting to test clove oil on pear trees (given the absence of phytotoxicity) and on apple trees given that they recover relatively fast. In this last case, a potential application could be against *D. plantaginea* eggs that go through winter so that the EO does not affect flowers and leaves appearing later in the year.

Concerning *Thymus vulgaris*, it appeared to be phytotoxic through seed germination tests but its main constituents have been assessed on apple trees and they appeared to be non-phytotoxic (van Tol *et al.*, 2008; Uremis, Arslan and Sangun, 2009). This proves once more that the EOs do not act in the same way when applied on seeds and grown trees, encouraging the testing of EO with important contents in the tested molecules like for example *T. satureioides* containing about 18% of α-terpineol and 3% of linalool (Ramzi *et al.*, 2017).

C. cassia's phytototoxicity has not been studied much. Cloyd et Cycholl (2002) explain at the end of their study that even if cinnamaldehyde presents the greatest phytotoxicity compared to the other treatments used, it is only a minor damage as it does not affect the marketability of these herbs. It must be kept in mind that unlike these herbs, what would be sold in the case of apple and pear trees are the fruits; and even if no phytotoxicity is visible, the fruit yield could be affected.

An interesting observation done Synowiec et al. (2017) was that the most phytotoxic EOs analyzed in their work were composed of a majority of oxygenated monoterpenes (from 64.1% to 93.3%), which could contribute to their phytotoxic properties. An example of these oxygenated monoterpenes includes carvone present at 63.2% in *C. carvi*'s oil (oil showing the highest phytotoxicity). Given this information as well as Morcia et al. (2016)'s (Table 3), it can be said that *Mentha spicata* (63.59% carvone) could have potential phytotoxic effects above a certain concentration (close to 0.125%).

On another note, the fact that Terzi et al. (2007) found no sign of phytotoxicity up to a concentration of 2% for the tea tree oil (*Malaleuca alternifolia*) is sufficient given that the EO solution used in this project would probably never exceed this limit. Additionally, to support the idea of the tea tree oil being non-phytotoxic comes the patent deposited by Pipko et al. (2005) which claims that this oil has been used in the development of a "very effective biocide against bacteria, fungi and insects" being non phytotoxic (Pipko et al., 2005).

Table 3. Summary of the works found treating with the phytotoxicity of the selected essential oils

Oil or molecule of interest in the study	Plant used to assess phytotoxicity	Test performed	Main results obtained (interesting ones in the case of this research)	Article
Clove – *Syzygium aromaticum* and its main constituent (Eugenol) oil	Purple sprouting broccoli (*Brassica oleracea* L.), common lambsquarters (*Chenopodium album* L.) and redroot pigweed (*Amaranthus retroflexus* L.)	Seedling growth and leaf cell membrane integrity	Clove oil (2.5%) and eugenol (1.5%) significantly reduced all three species seedling fresh weight. Clove oil: great damage and even death on the common lambsquarters and redroot pigweed seedlings (91% and 99% growth reduction on a fresh weight basis). Both greatly increased the electrolyte leakage (an indicator of the cell membrane damage).	(Bainard, Isman and Upadhyaya, 2006)
	Mimosa pudica L. and *Senna obtusifolia* (L.) H.S.Irwin & Barneby	Seed germination assays as well as radicle and hypocotyl elongation	Phytotoxic effect of this oil.	(de Oliveira *et al.*, 2016)
	Echinocloa crus-galli (L.) Beauv., *Phalaris minor* Retz., *Sorghum hapelense* (L.) Pers., *Leptochloa chinensis* (L.) Nees., *Ageratum conyziodes* L., *Commelina benghalensis* L., *Cassia occidentalis* L. and *Bidens Pilosa* L. .	Seed germination assays	Phytotoxic effect of this oil.	(Ahuja, Batish, Singh, & Kohli, 2015
	Pear tree (*Pyrus spp.)*	Spraying of EO on tree	No signs of phytotoxicity (even at 10 mg/ml).	(Tian *et al.*, 2015)
Eugenol and eugenol-based pesticides (Matran 2 EC)	*Malus* x *domestica tree*	Spraying of EO on tree	Matran (containing 50% eugenol) applied at 2 to 8% concentrations: Both showed significant toxicity to flower and leaves already 15 minutes after application. Eugenol completely burnt all exposed flowers and leaves at concentrations of 8 and 10%.	(Miller and Tworkoski, 2010)
Thymus vulgaris	*Xanthium strumarium* L. , *Avena sterilis* L. and *Phalaris brachystachys* L.	Seed germination assays	Highest germination inhibition on two out of the three weeds used for *Thymus vulgaris* compared to *Ocinum basilicum* L. , *Salvia officinalis* L. and *Melissa officinalis.*	(Uremis, Arslan and Sangun, 2009)

Cinnamom camphora (L.) J. Presl , *Juniperus virginiana* L. and *Lavandula angustifolia* P. Mill.. as well as α-terpineol , (R)-(-)-carvone , linalool, (R)-(-)-fenchone and citronellal	*Malus* x *domestica tree*	Analyzed the phytotoxicity at the grafting site where they applied budding strips which had be rubbed in EO (to fight the red bud borer – *Resseliella oculiperda* (Barnes))	No signs of phytotoxicity for any of the treatments tested	(van Tol *et al.*, 2008)
Cinnamomum cassia based pesticide (Cinnamite – 30% cinnamaldehyde)	*Lavandyla stoechas* L. , *Origanum vulgare* L. 'Santa Cruz', *Rosmarinus officinalis* L., *Hypericum perforatum* L. 'Topaz', *Thymus vulgaris* L. 'Wolly' and *Thymus vulgaris* L. 'Nutmeg'	numerical phytotoxicity rating scale from 0 to 3 (assessed visually) (details in Annex 4)	Both rates of cinnamaldehyde (6.61 ml/l and 4.98 ml/l) presented a significantly higher phytotoxicity than the other treatments used (pyrethrin, azadirachtin, potassium salts of fatty acids, paraffinic oil and capsaicin)	(Cloyd and Cycholl, 2002)
Mentha piperita L.	*Tomato (Lycopersicon esculentum Mill.), radish (Raphanus sativus L.), field bindweed (Convolvulus arvensis L.), purslane (Portulaca oleracea L.) and jungle rice (Echinochloa colonum L.)*	Seed germination, root and stem growth and dry weight analysis	As the concentration was increased, inhibition in all the tests increased. Each plant reacted differently to the EOs: For example, at already 0.3 ml/l the tomato seeds' germination was inhibited significantly, compared to the purslane seed germination which was only suppressed at a concentration of 1.8 ml/l.	(Mahdavikia and Saharkhiz, 2015)
	Amaranthus retroflexus, Avena fatua, Bromus secalinus,Centaurea cyanus	Seed germination assays	*M. piperita* amongst the most phytotoxic EOs (along with *C. carvi*, *T. vulgaris* and *S. officinalis)*	(Synowiec *et al.*, 2017)
Carvone	12 barley varieties (*Hordeum vulgare* L.)	Seed germination assays	Starting at a concentration of 0.125%, carvone was toxic for many barley varieties, significantly inhibiting seed germination (e.g. Tidone variety)	(Morcia *et al.*, 2016)
Mentha spicata L.	Chickpea (Cicer arietinum L. var Samrat)	Seed germination assays	All seeds germinated at the concentration tested (0.0125 to 0.1 µl/ml)	(Kedia *et al.*, 2014)
Tea tree oil and main components (terpinen-4-ol, γ-terpinene and 1,8-cineole)	Barley (*Hordeum vulgare* L.)	Seed germination assays	None of the tested molecules severely impacted the barley seeds germination at concentration lower than 2%	(Terzi *et al.*, 2007)

1.3.2.5. Remarks on phytotoxicity

To conclude, almost all the EOs suggested pose a potential risk of phytotoxicity. However, several remarks must be made. Firstly, as mentioned earlier, it must be noted that phytotoxicity is different from one plant species to another. Secondly, it also depends on the mode of application (i.e phytotoxicity will not appear in the same way for plants which have been sprayed or injected). Thirdly, the EOs present diverse modes of action with many different sites of phytotoxins. Examples of these include the inhibition of mitochondrial respiration of isolated organelles (e.g 1,8-cineole and camphor), the inhibition of mitotic stages through the reduction of glutathione levels (e.g Atermisin sesquiterpenes) or even the breaking of the plasma membrane (e.g Dehydrozaluzanin C (DHZ)) (Duke and Oliva, 2004).

Given the many mode of actions of phytotoxic molecules, no established protocols or standard methods have been put in place to analyze phytotoxicity. Methods such as selecting a site of action and testing biologically active molecules on it, or even testing a specific compound on all known target sites have been shown to be effective but can be somewhat time-consuming.

Other easier tests can be performed before finding the exact mechanism and site of action of the phytotoxic molecules. As described in the above findings, dose-response curves can be drawn when analyzing the seed germination. But other methods are also often used (Dayan, Romagni and Duke, 2000) :

Electrolyte leakage, consisting in the measure of cellular content movements into the solution in which the tissue floats or is submerged, can be performed.

Mitotic analysis is simply achieved with a microscope to ease the counting of cells in each mitotic stage and analyze the abnormal mitotic figures.

Metabolite complementation studies can also be performed to help in finding the modes of action of diverse molecules.

Additionally, photosynthetic efficiency can easily be assessed by monitoring the chlorophyll's fluorescence ("indication of the capacity of the photosystem apparatus to return to a ground state after being exposed to a short burst of saturating light"). In fact, fluorescence measures can be elaborated to describe a system in terms of vitality, productivity, sensitivity and resistance to stress (Strasser, Srivastava and Tsimilli-Michael, 2000).

Finally, another method to evaluate the physiological effect of phytotoxins is simply to monitor the carbon dioxide and water exchanges (transpiration rate) between the leaves and the ambient air. In fact, CO_2 absorbs infrared radiation in 3 bandwidths, these exchanges can therefore be determined using an infrared gas analyzers (IRGAs). These can calculate the CO_2 and H_2O concentrations based on the difference in absorption of infrared (IR) between the source and the detector (Verhoef and Campbell, 2005). Having an idea of the transpiration rate therefore gives essential information on the water status of the plant.

Given that the EO-based pesticide would be applied to mature apple and pear trees, the last 2 methods seem particularly interesting as they are non-destructive and highly sensitive. They will be further described in the material and method section.

Appart from being phytotoxic, EOs present the disadvantage of being highly volatile which can therefore reduce their effectivness span, but they are also susceptible to degradation reactions when exposed to abiotic factors such as air, light and high temperatures (Turek and Stintzing, 2013).

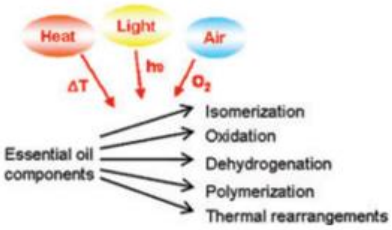


Figure 6. Schematic representation of possible degradation pathways of essential oil components (Turek and Stintzing, 2013)

Solutions to counter these problems include the controlled release of volatiles by working on the formulation aspect of the product (e.g emulsifying the EO) which has already been widely studied. Another idea could be revisiting the application mode of these EOs. Most of the pesticides currently used are sprayed onto the trees but much of the pesticides are wasted through drifts (due to abiotic factors such as wind). In fact, it is estimated that only 29 to 56% of the pesticides distributed with air blast sprayers are deposited on the tree canopy (Wise *et al.*, 2014).

Alternatives therefore exist in order to be more efficient and could also allow, depending on the technique, a slower distribution in the plant. These include : trunk implantation, trunk-injection , soil injection/ drenching and trunk basal spray (Kuhns, 2011).

1.4. Alternative techniques for the application of phytosanitary products

Trunk injection works by injecting in the cambium water-soluble chemicals or emulsified preparations in the case of non-water-soluble products. The cambium is targeted given that it is in this portion of the trunk that new xylem cells are created. These new xylem cells constitute the sapwood capable of conducting water and minerals from the roots to the leaves. The xylem consists mainly of vertical tubes but some rays, i.e horizontal tubes oriented radially, are also present. These rays could play an important role in the spreading of the trunk-injected pesticide.

Aiming at the xylem means that the water contained pesticide will be able to rise up the tree all the way to the leaves which are pierced by sucking insects like aphids (Kuhns, 2011).

In this case the pesticide can either be pressured or not in the tree. Using pressure presents the advantage of fasting-up the uptake of the pesticide but on the other hand, could lead to bubbling effects which would kill the cells and slow the uptake. In fact, a pesticide reservoir would be created under the bubble, which could later be redistributed through rays. Even if a slower rate of distribution is what is searched here, it is not wished in this way as bubbling effects damage the plant (kill the cells) and would not be controlled by the practitioner. Other disadvantages of this pressured technique include a risk of chemical blow-back (given the high pressure) and an elevated cost for non-experts (material and competent main d'oeuvre).

No-pressure on the other hand, depends exclusively on the transpiration rate of the plant, which therefore guides the ascension of water in the xylem. These treatments are particularly efficient in summer, when a maximum transpiration rate is reached. Trunk implantation is considered a no-pressure technique. In this technique, a hole is drilled in order to reach the cambium and a capsule containing the pesticide is then inserted. This capsule can be coated with a material which will dissolve

when the rising water passes through it. For this kind of application, Kuhns advices to put a capsule every 10 cm of the circumference. This kind of technique presents the advantage of being inexpensive and easily installed (Kuhns, 2011).

Another no-pressure (gravitational pressure only) technique is a trunk-injection which consists in drilling a hole in the trunk, putting a plastic tube through it and letting the pesticide drain naturally through this hole. This type of application has already been commercialized in the past to treat iron chlorosis (Figure 7).

Figure 7. Example of no-pressure system for iron chlorosis treatment (Kuhns, 2011).

Pressured techniques on the other hand consist of 2 mains types: low-pressure and high-pressure techniques. Low-pressure techniques present the advantage compared to no-pressure or high-pressure techniques of speeding the uptake by the trees without the bubbling problems. An example of this kind of apparatus is the "Mauget pressurized capsule" (Mauget) used for many product applications, including azadirachtin described earlier for *D. plantaginea*'s management (Figure 8).

Figure 8. Left: Pressurized capsule. Right: Application of such capsules (Mauget)

Other pressured techniques include the use of "Arborjet Tree I.V." equipment (Arborjet) claimed to be a low pressured technique as well by the manufacturer (Figure 9 left). This one is used in the case of high quantities of injected products. "Arborjet Quik-jet" (Arborjet) on the other hand injects lower doses and at higher pressure (Figure 9 right) (Kuhns, 2011).

Figure 9. Left: Arborjet Tree I.V kit. Right: Arborjet Quik-jet kit (Arborjet)

Examples of utilization of the "Abrorjet Tree I.V" include for example Doccola et al. (2007)'s study on the reduction of Hemlock Wooly Adelgid (*Adelges tsugae* (Annand)) infesting hemlocks (*Tsuga spp.*).

"Arborjet Quik-jet" on the other hand, has been used for example by Wise et al. (2014) in their study on the seasonal effectiveness of trunk-injected pesticides in apple trees. Wise et al. (2014) explain that their study was a proof that trunk injection is successful to control pests in apple trees. They have found that a single injection allowed an activity of the pesticide over a period of 40 to 90 days after application compared to approximately 14 days of control for sprayed insecticides, which could be explained by a "reservoir effect" in the xylem. Another study lead by VanWoerkom et al. (2014) even showed that a single injection of certain pesticides (e.g. Ima-jet™) was effective in controlling several pests for two growing seasons. This therefore shows the great potential of such techniques in increasing the release time of pesticides, particularly interesting in the case of highly volatile biopesticides.

Trunk basal spraying works in a similar fashion than trunk-injection. The only difference between the two being that basal spraying relies on the absorption of the chemicals in the bark to allow the entrance of chemicals in the vascular system; where trunk-injection on the other hand directly aims at the xylem. Even if a certain drift can occur with this method, it presents the advantage of requiring no drilling and a simple equipment (reservoir linked to a tube with a spraying nozzle) (Kuhns, 2011).

Trunk-administration therefore seems to be a good technique for the application of the biopesticide developed in the scope of this research. As explained earlier though, the EOs have complex behaviors. Firstly, because they are composed of many active ingredients which interact with each other (synergic or antagonist effects for example) and have an impact on the plant and the aimed insect. Secondly, because EOs are subject to many factors which can alter their composition meaning that their interactions and their effects can change in time.

It therefore seems primordial to monitor these changes in time through an analysis of the volatile organic compounds (VOCs) contained and emitted by the leaves. Additionally, this information seems crucial as it will also give an idea of the efficiency span of the developed EO-based pesticide.

1.5. Instrumental methods for the detection and quantification of volatile organic compounds

To analyze the volatiles contained in the leaves they must first be extracted. The classic way of extracting molecules from a solid is with the help of a solvent. Typical methods use a Soxhlet system where the solvent is continuously recycled through the sample in order to increase the extraction yield. Firstly, this method requires large volumes of both substrate and solvents to ensure a good extraction. In the case of the apple trees used it is not guaranteed that much raw material would be available for each sample. Secondly and most importantly, the problem with this method is that the analyte must be stable in the boiling solvent. Hence, this method is not adapted in this case. As mentioned earlier, EOs are sensitive to high temperatures and would be rapidly degraded, the extract obtained would therefore not represent the VOCs profile of the leaves. Other solvent assisted methods exist (e.g. pressurized liquid extraction, microwave and sonic wave assisted extractions) which require less solvent and are also speeded up. Nevertheless, solvent extraction does not seem appropriate in this case because it tends to separate the molecules depending on their affinity for the solvent compared to the matrix but does not consider the volatility of these molecules. Therefore, in the case of EOs, it is possible that the solvent extracted most of the volatile terpenes constituting the EO but other hydrophobic (non-volatile) molecules would be extracted as well (Smith, 2003).

A more selective way to extract volatile analytes in a solid matrix is by using thermal desorption.

Once the volatiles have been extracted from the matrix and contained in the headspace (HS) they must be trapped before being sent to analysis. Many techniques have been put in place for headspace sampling.

The first and one of the best-known methods is the solid phase micro-extraction (SPME). This technique is very appreciated by researchers as it is relatively simple, fast and offers fairly good detection limits (5 to 50 pg g^{-1} for volatile/ semivolatile and nonpolar/semipolar analytes) (Wardencki, Michulec and Curyło, 2004). The SPME is an adsorption technique relying on the partition coefficient of the analyte between the SPME coating and the sample. In this technique, the sample is placed in a closed vial which is incubated at a given constant temperature (Tat *et al.*, 2005). The SPME fiber is then introduced in the vial to allow the adsorption of the analytes on the fiber. The molecule which have been trapped are then desorbed and sent to the gas chromatograph (GC).

Many techniques have then been created to overcome the limited concentration capability of headspace-solid phase microextraction (HS-SPME), due to its small volume of sorbent on the coating.

In-tube sorptive extraction principles are applied for example through headspace solid-phase dynamic extraction (HS- SPDE). In this technique the analytes are attached to the polymer coating on the inner wall of a needle through its push/pull action (Bicchi *et al.*, 2008). The advantages of this techniques compared to HS-SPME is that the concentration capability can be optimized depending on the amount of analyte in the HS, by adjusting the number of push/pulls (Bicchi *et al.*, 2008).

Another important type of sampling method is the headspace sorptive extraction (HSSE), which works in the same way as the stir bar sorptive extraction (SBSE) used in liquid samples. In HSSE, the volatiles extracted from the matrix (heating and agitation) found in the headspace are accumulated onto a coated magnetic stir bar (often a PDMS coating, which can vary from 25 to 250 µL). The stir bar is then place in a glace tube and sent to the desorption unit (Bicchi *et al.*, 2008).

Other techniques based on the principle of "large surface area high concentration capacity headspace sampling" (HCC-HS) also exist (Bicchi *et al.*, 2008). One technique which seems particularly interesting in the case of plant volatile fractions, and even more when the metabolism of the plant changes, is the sorptive tape extraction (STE). In this technique the analytes are concentrated by sorption on a flexible PDMS tape in the near headspace or directly put in contact with a surface, leaves for example in the case of plants (Figure 10). This tape is then desorbed thermally or by using a solvent and then analyzed by GC-MS. The main advantage of this technique is that it offers high analyte recovery (Bicchi *et al.*, 2008; Sgorbini *et al.*, 2010).

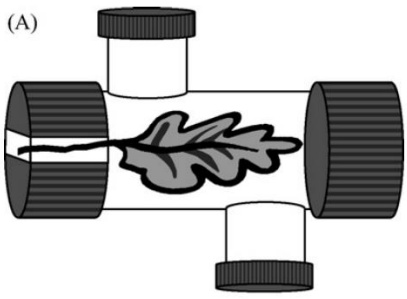


Figure 10. Vial designed for HS-STE from live plants (Bicchi *et al.*, 2007)

Headspace liquid-phase microextraction (HS-LPME) is another headspace sampling method which consists in creating a very small droplet of solvent containing an internal standard (1 µl for example) at the end of a micro-syringe needle in such way that it can then be retracted back in the needle and directly injected into the GC. The advantage of such method is that it is easily automated, selective (given the possibility of choosing the solvent used) and requires small amounts of sample. A good compromise must be made when choosing the solvent given that its vapour pressure must be low to avoid evaporation when sampling but must be compatible with the GC as well.

Finally, another widely used technique is dynamic headspace sampling (DHS). This one is often used given the extra flexibility it offers compared to other methods in terms of sampled volume and the variety of adsorptive phases in the trap. This really allows a perfect adaptation of the method to the studied case. Two main approaches exist: the purge and trap approach (P&T) where the volatiles are stripped from the matrix considered with the help of a gas flowing through it and the dynamic approach in which the analytes are sampled thanks to a gas flowing over the matrix (agitation and temperature of the vial allows a constant renewal of the surface in contact with this gas). This gas then passes through a cold trap or adsorbant phase and is then sent to thermal desorption and GC. Even if a correct comparison between researches using DHS is difficult given that the sampling parameters often vary, DHS is still believed to be the reference technique in the plant field given the possibility of achieving high concentrations if a correct volume is chosen (Bicchi *et al.*, 2008).

DHS can typically be applied both on a leaf-sample from which the volatils are extracted but also for the emitted volatiles on a living plant with the use of Nalophan™ bags to which the traps are attached.

2. Objective and strategies

Numerous key aspects in the development of an EO-based biopesticide must be studied before performing field trials on infested trees.

In this study, a first aspect focuses on the development of an EO emulsion formulation which is stable in time, bio-compatible and which allows a rapid ascension of an aqueous solution in the plant's xylem when injected.

Secondly, the evolution of the VOCs contained and emitted by the leaves of EO-injected *Malus domestica* Borkh (var. Jonagold) trees will be analyzed. A kinetics curve will be drawn for the major components of the selected EOs, which should help in meeting the following objectives:

- Determine the time interval between EO injection and its detection in the leaves.
- Determine the extent to which EOs are accumulated in the leaves in order to evaluate its role in insect ingestion toxicity.
- Determine the emission kinetics of EO molecules in order to evaluate its role in insect repellency.

Finally, the phytotoxicity of selected EOs in the emulsified form on these injected trees will be assessed. The goal is to determine which EOs show no, or little induced stress and give an insight on the tolerable amounts of these natural molecules artificially injected (i.e analyze the phytotoxic effects).

3. Material and method

This part of the work will be structured overall following the main objectives presented in the previous section:

- Characterization of selected EOs.
- Development of an EO emulsion formulation.
- Preparation of the EO emulsion before injection.
- Experimental design.
- Experiment set-up and trunk-injection of the EO emulsion.
- Assessment of these EO emulsions' phytotoxicity.
- Content and emitted VOCs sampling and analysis.

3.1. Characterization of selected EOs

Based on biological tests performed on the rosy apple aphid by the UCLouvain partner, three EOs have been selected for tests on apples trees: cinnamon oil - *Cinnamomum cassia* J.Presl (Pranarôm, batch n°OF31162), spearmint oil - *Mentha spicata* L. (Pranarôm, batch n°OF31966) and clove oil – *Syzygium aromaticum* (L.) Merrill & Perry (Pranarôm, batch n°OF33050)**.**

These oils were analyzed to establish their molecular profile (Annex 5, Annex 6 and Annex 7) and to detect their major components. These are used later in the work to represent the EO's kinetics of accumulation in the leaf and its emission.

A 10mg EO in 10mL n-hexane (CAS 110-54-3, Merck Millipore) solution was first prepared and then analyzed using a GC-MS system (7890A-5975C, Agilent Technologies Inc.) equipped with a HP-5 MS capillary column (30m x 250 µm x 0.25 µm, Agilent Technologies Inc.).

1 µL of this solution was injected in splitless mode at 250°C. Helium was used as the carrier gas at a flow rate of 1 mL/min. The GC oven temperature program was: 40°C for 2 min, followed by an increase to 100°C at a rate of 5°C min^{-1}, an increase to 120°C at a rate of 3°C min^{-1}, a maintenance at 120°C for 3 min, an increase to 220°C at a rate of 5°C min^{-1} and finally an increase to 310°C at a rate of 15°C min^{-1}. The quadrupole temperature was fixed at 150°C and the MS source at 230°C. The mass spectra have been recorded in EI mode at 70 eV. The scanned mass ranged from 30 to 400 m/z. The components identification was based on a comparison of the obtained spectra with the NIST 14 reference database and on a comparison of the calculated retention indices (RI)[13] to RIs from the literature.

[13] Using a Kovats (C7 to C30 alkanes') solution analyzed in the same conditions.

3.2. Development of the EO emulsion formulation

In this section, the various tests performed to come up with a stable and non-phytotoxic EO emulsion (which also shows enhanced ascension rates up the xylem) are described.

3.2.1. Addition of EDTA

To reduce occlusion of the sieve plate pores[14], adding EDTA (Tritriplex III, Merck Millipore) to the formulation was considered (Tetyuk, Benning and Hoffmann-Benning, 2013). Its effect on the ascension rate of a water-based solution was assessed by injecting two trees (injection protocol in section 3.4.2):

- One with a methylene blue dye solution containing EDTA (0.1% w/v methylene blue, 20% v/v 100mM EDTA).
- One with methylene blue only (0.1% w/v methylene blue).

The trees were left injected for 48 hours and then cross sections were prepared to compare the ascension rate of the colored solution.

3.2.2. Choice of emulsifier and homogenization process used

Four bio-compatible emulsifying agents were tested to evaluate which lead to the best emulsion stability. Table 4 describes the emulsifier used, the concentration of the emulsifier solution, the EO to emulsifier solution ratio respected and finally the reference article. Note that all the emulsions were prepared with 1% cinnamon oil and 20% (v/v) 100 mM EDTA.

Table 4. Surfactant used, concentration of the surfactant solution, EO to emulsifier ratio and reference article.

Surfactant used	Concentration of emulsifier solution (%, w/v)	EO to emulsifier solution ratio (%, v/v)	Reference article
Tween® 20 (CAS 9005-64-5, Merck Millipore)	Concentrated	1:4	(Ghosh *et al.*, 2013b)
Arabic gum (CAS 9000-01-5, Merck Millipore)	20	1:4	(Fernandes, Borges and Botrel, 2014)
Native pea protein	10	1:3	(Jiang *et al.*, 2014)
Structurally modified pea protein[15]	10	1:3	(Jiang *et al.*, 2014)

[14] Structure allowing the connection of individual sieve elements which constitutes the sieve tube for phloem transport (Kalmbach and Helariutta, 2019)

[15] A 20% (w/v) pea protein solution was prepared and titrated to pH 12 with a 2M NaOH solution. The solution is then kept for 1h at this pH and titrated back to neutral pH with a 2M HCl solution. A 10% (w/v) solution is then prepared.

Two repetitions of each emulsion were prepared so that both homogenization processes could be tested on each of these. The two homogenization processes used are the following:

- A high-speed homogenization (HSH) at 9 500 rpm for 6 min (Ultra-Turrax T25) followed by a high-pressure homogenization (HPH) with 8 cycles at 5 000 psi (FMC) (adapted from (Jo *et al.*, 2015)).
- Ultrasonication (13 mm probe, 30 min with 1 s/1 s on/off pulse , 20 kHz, 750 W, amplitude 40%, $T°_{solution}$=24°C) (Sonics) (Ghosh *et al.*, 2013b).

After homogenization, the emulsions' stability was evaluated by analyzing the EO particle sizes using a particle sizer (Beckman Coulter – Delsa^{TM} Nano C Particle Analyzer).

3.2.3. Phytotoxic effect of EDTA and of a stable surfactant (Tween 20)

The phytotoxic effect of EDTA, of Tween® 20 and of Tween® 80 were assessed through chlorophyll fluorescence measurements (described in section 3.5.1). The solutions injected in the trees (injection system described in section 3.4.2) before fluorometric measurements were the following (relative concentrations are expressed in % (v/v)):

- Water and Tween® 20 (99.6% and 0.4 % respectively).
- Water and Tween® 80 (99.6% and 0.4 % respectively).
- Water, Tween® 20 and EDTA (79.6% water, 0.4% Tween and 20 % 100mM EDTA respectively).
- Water, Tween® 80 and EDTA (79.6% water, 0.4% Tween and 20 % 100mM EDTA respectively).

EO was not added to any of the solutions given that the idea of this test was to analyze the effect of all the other compounds part of the formulation.

3.3. Preparation of the EO emulsion before injection

To prepare 100mL of an EO-emulsion: 15mL of water was put under constant agitation at 1 250 rpm with a magnetic stirrer. Tween 80 was then added to respect a 1:4 (v/v) EO to Tween 80 ratio considering that concentrations of 0.5% EO were tested (selected EOs described in section 3.1). 20mL of a 100mM EDTA solution was added to the mix, which was then brought to the final volume of 100mL. After 5 minutes of constant agitation at 1 250 rpm, the solution was stabilized using the HSH and HPH with the parameters described above (section 3.2.2). The solution was then stored in a fridge to prevent any degradation of the EO by light or temperature.

3.4. Experiment set-up and trunk-injection of the emulsion

3.4.1. Experimental design

Two factors were studied in this experiment that is, the type of treatment applied to the apple trees and the evolution with time. Five different modalities were tested for the treatment applied, three treatments are the EO emulsions injected. The fourth treatment is a negative control (i.e. trees are injected with the formulation exempt of EO). Finally, the last modality is the blanc (i.e the trees are left without injection but found in the same environmental conditions as the others).

Table 5. Description of each modality of the first factor studied – the treatment applied.

Modality	Treatment
1	Cinnamon EO emulsion injected
2	Spearmint EO emulsion injected
3	Clove EO emulsion injected
4	Negative control (i.e. emulsion with no EO)
5	Blanc (i.e. no injection)

The second factor studied was time, with five different modalities as well. In fact, the experiment took place over a 96h time-lapse and data was collected at t=0h, 24h, 48h, 72h and 96h.

Two types of apple trees were used overall in this work:

- One-year old apple trees were used exclusively for formulation of the emulsion section (3.2 section). The exact variety is unknown as they were grown from collected seeds after consumption at the UCLouvain.
- Two-years old apple trees were used for all tests studying the two factors described above. The two years old trees are *Malus domestica* Borkh (var. Jonagold) which have been grafted on M26 rootstocks.

These trees were confronted to various pests during their storage in greenhouses. The phytosanitary products applied by the technicians to fight these pests can be found in Annex 8 and Annex 9.

Experiments on the two-year-old apples trees took place in environmental chamber at the UCLouvain over the month of June and July 2019. Each treatment was tested on three replicates (three trees injected each time). The trees used had developed mature and fully expanded leaves but were free of flowers or fruits. These were watered every day with 500mL of water. The environmental chamber operated in the following conditions: 21 ± 0.5°C, 62 ± 10 % relative humidity and 16:8 h light: dark periods.

3.4.2. Administration

3.4.2.1. Injection systems used

A standardized and easily reproductible injection method had to be developed here given the trunks' small diameter (e.g. one year old: 4 mm diameter). A no-pressure system based on a drip pocket (e.g. Baxter) and needles for medical practices was used.

The number of injection points had to be determined to better conceptualize the device. Kuhns (2011) suggests when using implantation techniques to set up implants every 10 cm in circumference (Kuhns, 2011). Additionally, it was found during the EDTA formulation tests, that a water-based product injected at one injection site had the capacity of diffusing over approximately 180° on small diameter trees (±4 mm).

Based on these findings, one drip with two distinct conducts and needles was used to treat small one-year old trees (Figure 11) and with three distinct conducts and needles for two-years old trees (Figure 12). An extra conduct was added to improve the distribution in the xylem.

As it can be seen in Figure 11, this system has the advantage of working with a three-way valve. This one allows an easy-stopping of the flow while filling up the other drips used (for the two other replicates). Nevertheless, the time-consuming elaboration of this design remains its main drawback. In fact, the three-way valve used in medical practices has two inlets and one outlet. Hence, the second conduct linked to this valve must be hand-prepared to allow its connection to an inlet.

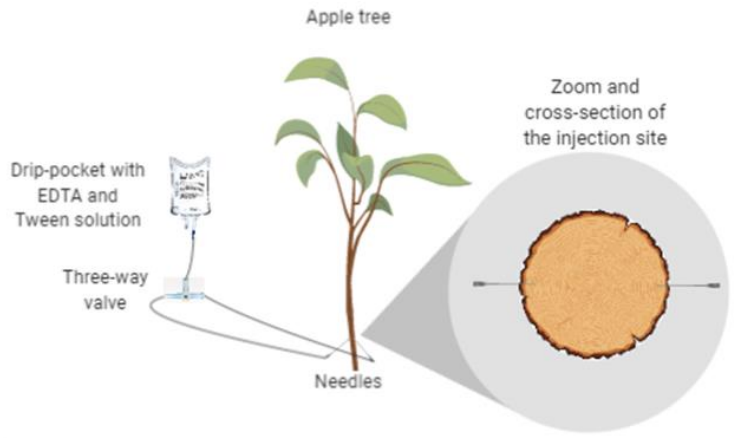


*Figure 11. Injection apparatus developed for **one-year old trees***

Figure 12 illustrates a second set up used on the two-year old trees. The advantage of this system is that it is easier to set-up and takes up less space compared to the first one, allowing it to be directly attached to the tree. A small disadvantage of this technique is that it has currently no flow-stopping system, the tubes must therefore be pinched with a tube pincher (Hoffman).

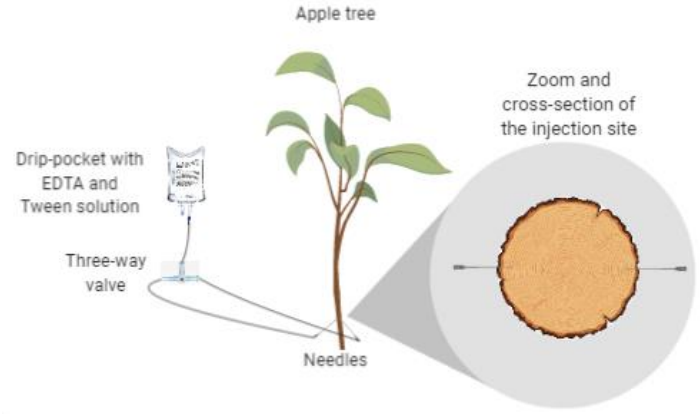


*Figure 12. Injection apparatus developed for **two-year old trees***

This new design only requires a drip which has been carefully emptied through the injection orifice of the drip pocket and three double-needled tubes (BD Vacutainer Safety-Lok). The advantage of this system is that it is easier to set-up and takes up less space compared to the first one, allowing it to be directly attached to the tree. A small disadvantage of this technique is that it has currently no flow-stopping system, the tubes must therefore be pinched with a tube pincher (Hoffman).

3.4.2.2. Injecting two-years old apples trees

Before injection, the drip pocket was filled up with 60 ± 2 mL of the solution (modality 1, 2, 3 or 4 from Table 5) using a syringe through the pocket's injection port. This drip pocket was then connected to the various parts explained above (Figure 12). The emulsion flow was allowed through the tubes until liquid comes out of the needles in a continuous manner and until all the bubbles have been removed from the tubing system. This step was performed in order to avoid cavitation in the tree vascular system. The flow was then stopped (with a tube pincher). Finally, the pocket was wrapped up in aluminum foil to prevent any degradation of EO by light.

The following aspects must be cared for when inserting the needles[16] in the trunk:

- A 1cm-deep, 1mm wide hole was drilled at all injection points (zoom in Figure 12). The hole was drilled with an upward orientation along a 60° angle from a vertical basis (the trunk).
- The injection points were located right above the grafting site.

Once the injection system was in place, the emulsion flow was opened to allow the emulsion to rise up the xylem (based on the plant's transpiration rate).

[16] BD vacutainer® Luer adapter ref 367300.

3.5. Assessment of these EO-emulsions' phytotoxicity

Phytotoxicity was assessed using two different types of measurements. The first one measured the maximum quantum efficiency of photosystem II (Fv/Fm) using a fluorimeter. The second one measured the net CO_2 assimilation rate / net photosynthetic rate (A, expressed in μmol_{CO2} m^{-2} s^{-1}) using an infrared gas analyzer (IRGA).

3.5.1. Fluorimeter

3.5.1.1. Theoretical aspects

When a chlorophyll molecule is excited by light energy, it turns into an excited configuration for a short period of time before losing its energy through several dissipation processes (photochemical or non-photochemical processes) (Hansatech Instruments, 2006).

Non-photochemical processes dissipate this energy in other ways than by activating photosynthesis – often in the form of infra-red radiation and red/far red radiation, known as chlorophyll fluorescence. Any form of biotic or abiotic stress influences the photosynthetic performance of the leaves and hence also inversely alters the chlorophyll fluorescence emission (i.e. photosynthetic performances decrease with stress and in turn increase the energy dissipation by chlorophyll fluorescence) (Hansatech Instruments, 2006).

Given that photosystem II (PSII) is the first photosystem to be activated when photosynthesis occurs following a light stimulus, it is mainly its fluorescence which is observed when taking the measurement. PSII electron acceptors (chlorophyll *a*) are re-oxidized when the leaf sample is dark adapted[17] (using a leaf clip) and react when a rapid illumination of the sample occurs after this dark-adaptation. When illumination occurs, a rapid polyphasic rise[18] (microsecond range) in fluorescence occurs followed by a slow decrease. This is called the Kautsky induction and can be graphically represented (Figure 13).

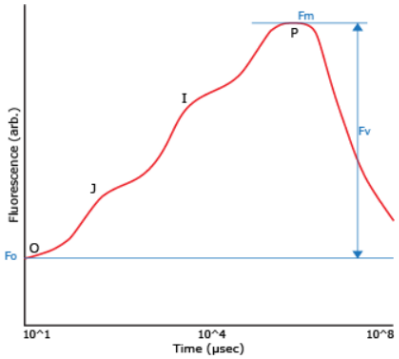


Figure 13. The Kautsky fluorescence curve (Hansatech Instruments, no date)

Several elements are displayed:

- O, J, I and P represent each phase's maximum.
- Fo is the fluorescence origin.
- Fm is the fluorescence maximum.
- Fv is the variable fluorescence, i.e difference between Fm and Fo.

[17] i.e adapated to a zero-light intensity
[18] Explained by a progressive saturation of the PSII reaction centers.

Therefore, based on fluorescence measurements taken every 10 µs during one second to retrace this graph, the fluorimeter displays several parameters including the Fv/Fm ratio (Strasser, Srivastava and Tsimilli-Michael, 2000). Fv/Fm is a widely used parameter to evaluate the photochemical efficiency of PSII (i.e. the maximum quantum yield) and is in other words an indication of the sample's stress (the lower the value the higher the stress). A healthy sample typically achieves a Fv/Fm value close to 0.83. Plants which have been stressed usually show lower values and are considered dead when showing values lower than 0.3 (Maxwell and Johnson, 2000; Bresson *et al.*, 2017).

3.5.1.2. Practical aspects

The fluorimeter used here was a portable Plant Efficiency Analyser (PEA) (Hansatech Instruments). Three leaves were randomly chosen at t= 0h on each of the three replicate trees. These leaves were placed in the dark for 20 minutes (Hansatech Instruments, 2006). Following this, a measurement of the chlorophyll fluorescence was taken for each dark-adapted leaf (light intensity= 3000 µmol m^{-2} s^{-1}). This measurement was then taken on the same leaves at t= 0h, 24h, 48h, 72 and 96h.

3.5.2. Infrared gas analyzer

3.5.2.1. Theoretical aspects

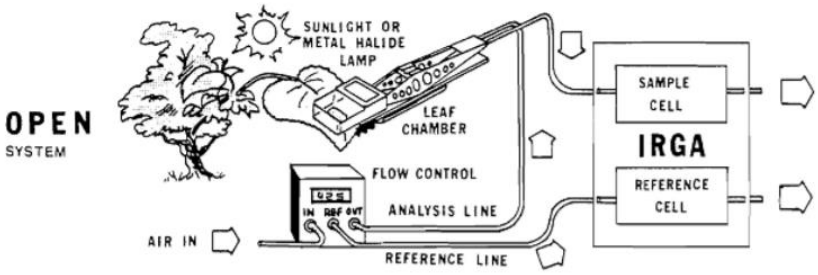


Figure 14. Schematic representation of the functioning of an open system infra-red gas analyzer (IRGA) (Mulkey and Smith, 1988).

This device is used to monitor gaseous (CO_2 and H_2O) transfers between the leaf and its environment. Both molecules are hetero-atomic gas molecules and therefore absorb at specific infrared (IR) wavelengths resulting in a characteristic absorption spectrum. The principle behind the determination of these gases concentrations in the leaf chamber relies on the Beer-Lambert Law: $\alpha_w = 1 - e^{(-l*M*k_w)}$, where α_w is the absorption of radiations by the hetero-atomic molecule, l is the radiation path length and M is the air's specific gas molar concentration.

Air is pumped from an open environment, passed on calcium sulphate (removes water) or a mixture of calcium oxide and sodium hydroxides (CO_2 to carbonates) when CO_2 and H_2O measurements are performed respectively[19] (Hall et al., 1993; Pandey, Paul and Singh, 2017). This air is then transferred to the reference line and to the analysis line (to which the leaf chamber is attached). Finally, air from both lines is pumped to the double-beam IRGA, containing the sample and the reference cells. Both cells are through-fall cells, meaning that there is a continuous air flow through the cell (Figure 15) (Hall *et al.*, 1993).

[19] In fact, CO_2's major absorption band is at 4.25µm, with others at 2.66, 2.77 and 14.99 µm. Water (vapor) molecules also absorb in the 2.7 µm region. This explains why water is removed from the air for CO_2 measurements and *vice versa*.

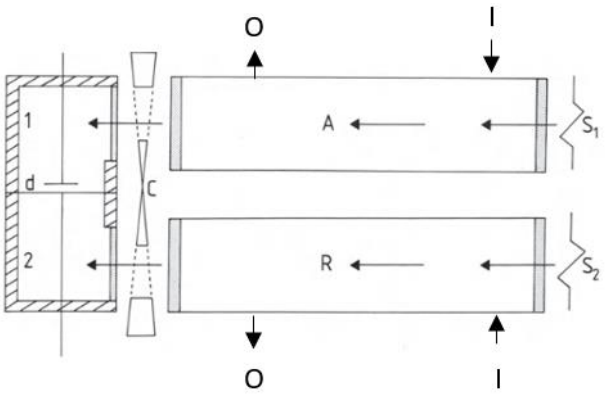


Figure 15. Example of a double beam IRGA with the detector's absorption cells in parallel (adapted from (Hall *et al.*, 1993)*). Infra-red radiation (IR) is generated by the sources (S_1 and S_2) and absorbed by the gas which passes through the cell (enters by I and leaves by O). The IR passes through a bandpass filter which chops the signal simultaneously (C). It is then detected (d), rectified and amplified* (Hall *et al.*, 1993)*.*

The net photosynthesis and transpiration rate are calculated as follows (Pandey, Paul and Singh, 2017):

$$Net\ photosynthesis = \left(\frac{CO_{2\,ref} - CO_{2\,sample}}{Leaf\ area}\right) * Flow\ (\mu mol_{CO2} m^{-2} s^{-1})$$

$$Transpiration = \left(\frac{Vapour\ dens._{sample} - Vapour\ dens._{ref}}{Leaf\ area}\right) * Flow\ (mmol_{H2O} m^{-2} s^{-1})$$

3.5.2.2. Practical aspects

The IRGA used here is composed of one ASUM2 air supply unit linked to a LCA2 gas analyzer and a PLC leaf chamber (12 cm^2 leaf chamber) (ADC). Three leaves were randomly chosen at t= 0h on each of the three replicate trees. The leaf-chamber was one is closed on the leaf, the ventilation mode was activated, and the following parameters were recorded: ΔC (vpm), the temperature (°C) and the relative humidity (RH, %). These measurements were then taken on the same leaves at t= 0h, 24h, 48h, 72 and 96h.

3.6. Leaf VOCs content and emission

In this section, the sampling of both leaf VOCs content and emission will be first explained. Later, the sample preparation (when necessary) and the exact gas chromatography mass spectrometry (GC-MS) techniques used for their analysis are presented.

3.6.1. Sampling

3.6.1.1. Leaf-content VOCs sampling

At t= 0h, 24h, 48h, 72h and 96h, ten leaves were randomly sampled from each replicate tree. These leaves were directly immerged in liquid nitrogen to prevent the formation of wounding green-leaf volatiles (GLVs). These could in fact, saturate the Tenax cartridge used during DHS (explained in the next section) but also lead to an over quantification and misrepresentation of the GLVs emitted by the tree under EO stress. A dry mass measurement was performed on a 1g leaf sample dried at 60°C until constant weight (three dried mass per tree) (Llusià and Peñuelas, 2001). This allowed, after dynamic headspace sampling GC-MS (DHS-GC-MS) analysis, to establish the content kinetics curve of the EOs major components in the leaves (µg $g_{dried\ leaves}^{-1}$).

3.6.1.2. Emitted VOCs sampling

The branch was placed inside a 60L polyethylene terephthalate (PET) bag[20] (Nalophan, Odometrics). On one end, the bag was closed, with a colson clamp, around the branch to which an activated charcoal tube was jointed to purify the air entering the bag (Charleston *et al.*, 2006). On the other end, a inox tube correctly enclosed inside the bag was screwed to a stainless steel tube connector (Swagelok) (Helsper *et al.*, 2006). The latter allowed an easy connection of the bag to trapping cartridges.

Tenax TA (Camsco) cartridges were used to trap the VOCs emitted by the apple trees. Tenax TA was chosen as it is known to show best properties for sampling of complex mixtures of volatiles (Vallat and Dorn, 2005). Two of these were placed in series to ensure that the sample adsorption was within the breakthrough volume each time. Air coming from the bag was passed through them at a constant flow rate of 50 mL min^{-1} for 24h with Gilian air sampling pumps (Sensidyne) (Figure 16).

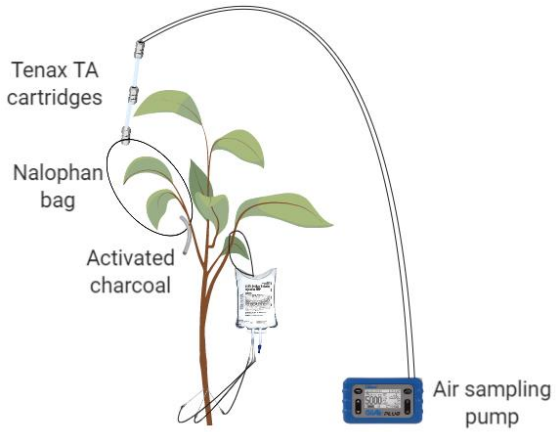


Figure 16. Emitted VOC sampling system represented for a one-year old apple tree.

At t= 24h, 48h, 72h and 96h, the cartridges were removed and replaced (except for t=96h) by clean Tenax cartridges. The removed Tenax TA were capped with stainless-steel caps (Swagelok) and stored at -80°C until analysis. Additionally, the exact volume of air pumped was recorded. At the end of the 96h experiment, all the leaves found on the "emission" branch were sampled and weighed. This was performed each time for the three replicate trees.

A dry mass measurement was also performed on a 1g leaf sample dried at 60°C until constant weight (three dried mass per tree) (Llusià and Peñuelas, 2001).

The collection of this data allowed after thermal desorption of the cartridges and GC-MS analysis (TDU-GC-MS), to establish the emission kinetics curve of the EOs major components (µg $g_{dried\ leaves}^{-1}$ h^{-1}) (Llusià and Peñuelas, 2001; Padhy and Varshney, 2005; Lamb *et al.*, 2008).

[20] The inner surface of these bags were cleaned with a gentle stream of gaseous nitrogen before each use.

3.6.2. Chromatographic analysis

3.6.2.1. Calibration curves

Based on the characterization of the selected EOs, calibration curves using pure standards were established for each EO major component:

- For cinnamon oil: Trans-cinnamaldehyde (CAS 14371-10-9, Sigma- Aldrich, ≥ 99% purity).
- For spearmint oil:
    - (R)-(+)-Limonene (CAS 5989-27-5, ≥ 90% purity, Sigma-Aldrich).
    - (+)-Carvone (CAS 2244-16-8, 99.9% purity, Supelco).
- For clove oil: Eugenol (CAS 97-53-0, 99% purity, Sigma-Aldrich).

A 400µg mL^{-1} (standards in hexane) solution was initially prepared and later diluted 1.25, 1.67, 2.5 and 5, 50 and 200 times to establish the calibration curves. 1-phenyloctane (CAS 2189-60-8) was used as internal standard for each dilution (constant concentration of 400 µg mL^{-1}). 1µl of solution was injected on a clean Tenax placed in the thermal desorption unit. The linearity range and the regression coefficient (R^2) are two validation parameters that were looked at for each of these calibration curves.

The analyses were performed using a TDU/CIS unit coupled to a GC-MS system (7890A-5975C, Agilent Technologies Inc.) equipped with a HP-5 MS capillary column (30m x 250 µm x 0.25 µm, Agilent Technologies Inc.). Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The thermal desorption unit's (TDU) temperature program was as follows: 40°C for 1 min, followed by an increase to 280°C at a rate of 100°C min^{-1} and a maintenance at 280°C for 5 min. The cooled injection system (CIS), mounted with a baffled glass liner, operated in solvent vent mode and respected the following temperature program: -60°C for 0.10 min, then an increase to 250°C at a rate of 12°C s^{-1} and finally maintained at 250°C for 2 min. The GC oven program on the other hand was: 40°C for 2 min, followed by an increase to 220°C at a rate of 5°C min^{-1}, an increase to 310°C at a rate of 15°C min^{-1} and a maintenance at 310°C for 3 min. The quadrupole temperature was fixed at 150°C and the MS source at 230°C. The mass spectra have been recorded in EI mode at 70 eV. The scanned mass ranged from 30 to 400 m/z. The SIM search was led on ion 82 ((+)-Carvone) starting at 17.5 min, ions 103 and 131 (Trans-cinnamaldehyde) starting at 18.4 min and ion 164 (Eugenol) between RT 20.6 and 21.6 min.

3.6.2.2. Leaf-content VOCs analysis

Once out of the -80°C freezer, the leaves sample was kept frozen due to liquid nitrogen poured onto it. This frozen sample was grinded (IKA grinder), 1g of leaves was weighted and 2 mL of a 20% (w/v) NaCl solution was added in the vial. The addition of saline solution allowed a salting out effect to take place. This technique leads to a shift of the partition equilibrium of neutral organic solutes toward nonaqueous phases (Endo, Pfennigsdorff and Goss, 2011). In other words, salting-out leads to an increase in concentration of the leaf-contained hydrophobic molecules (hence also the EO components) in the non-aqueous phase. The increased concentration of such molecules in the sample's headspace impacts its quantitation during DHS-GC-MS analysis.

The vial containing the crushed leaves was then inserted in the dynamic headspace sampler and left incubating for 20 min at 35°C. Following this, 1200 ml of helium carrier gas were passed onto the sample's headspace at a 30 mL min^{-1} flow rate and captured onto a Tenax cartridge. A dry purge phase[21] then took place. 200 mL of nitrogen was blown through the cartridge at 50mL min^{-1}.

[21] Excess water captured on the cartridge could form an ice block in the CIS and prevent a correct functioning.

The Tenax was then placed in the TDU, where 1 µL of a 400µg ml^{-1} 1-phenyloctane solution (used as internal standard (IS)) was injected. The rest of the analysis took place using the same parameters as explained in the previous section (last paragraph of the 3.6.2.1 Calibration curves section). The components identification was based on a comparison of the obtained spectra with the NIST 14 reference database and on a comparison of the calculated RIs to RIs from the literature.

3.6.2.3. Leaf-emitted VOCs analysis

The analysis of leaf-emitted VOCs was performed by analyzing the Tenax cartridges which were stored at -80°C using the method described in the last paragraph of the 3.6.2.1 section. Once more, 1 µL of a 400µg ml^{-1} 1-phenyloctane solution (used as internal standard) was injected on the cartridge prior to thermal desorption. The components identification was here also based on a comparison of the obtained spectra with the NIST 14 reference database and on a comparison of the calculated RIs to RIs from the literature.

3.7. Statistical analysis

All the data were gathered on excel and processed using the R 3.5.2 software (R-Development-Core-Team) or the Minitab 19 software (Minitab, LLC). The statistical procedures performed on the data obtained were a principal component analysis (PCA) (multivariate analysis) and an analysis of variance (ANOVA). For more clarity, the various statistical analyses performed are explained separately for data obtained after GC-MS analysis for both VOCs emitted and contained and for phytotoxicity data obtained with the fluorimeter (Fv/Fm) and the IRGA (A).

3.7.1. Phytotoxicity measurements

ANOVAs were performed on Fv/Fm and A data and at each time independently. This means that these were simple one-way ANOVAs where the factor was the treatment applied.

The application conditions for such analysis were the following: normal distribution of the studied parameters and of equal variance. The number of repetitions being equal to three implies that the normal distribution was respected. The equality of variances on the other hand was verified using Levene's test ($P < 0.05$). All samples were independent from one another, allowing the use of one-way ANOVA ($P < 0.05$).

The null hypotheses in these tests implies that there is no significant difference between the means of all treatments applied. In the case of a p-value lower than 0.05, the null hypothesis was rejected and Dunnett's test was performed. This one allowed to compare each treatment applied with a control treatment, i.e. the Blanc treatment performed in this study.

3.7.2. Content and emission VOCs profiles

In the case of VOCs profiles obtained after GC-MS analyses, a PCA was performed. In fact, such analyses are interesting in the case of large data sets as it transforms these observations of possibly correlated variables into a small set of values of uncorrelated variables (i.e. the principal components[22] - PCs). Each PC explains a certain percentage of the variance observed between the individuals. Additionally, each PC is composed of all the molecules in the original profile, but which have a different contribution to the creation of each PC.

By using PCAs, the goal was to identify chemical families or specific molecules responsible for variations in volatile profiles from one treatment to another as well as over time.

For content profiles, scatter plots of individuals (i.e. one specific treatment and time sample), where the x- and y-axis are two PCs, have been drawn. A qualitative interpretation of these graphs was performed. No quantitative analysis was performed in this case due to insufficient number of repetitions.

For the emission profiles, scatter plots of individuals were first analyzed. For each dimension and at each specific time, a linear model was created with the mean coordinate values for each treatment. These models were then analyzed with an ANOVA.

The application conditions here were also: normal distribution of the studied parameters and of equal variance. The null hypothesis for this test implies that there is no significant difference between the mean coordinate of each treatment. In the case of a significant difference, the top five contributors to each dimension were analyzed.

[22] These principal components are also known as dimensions.

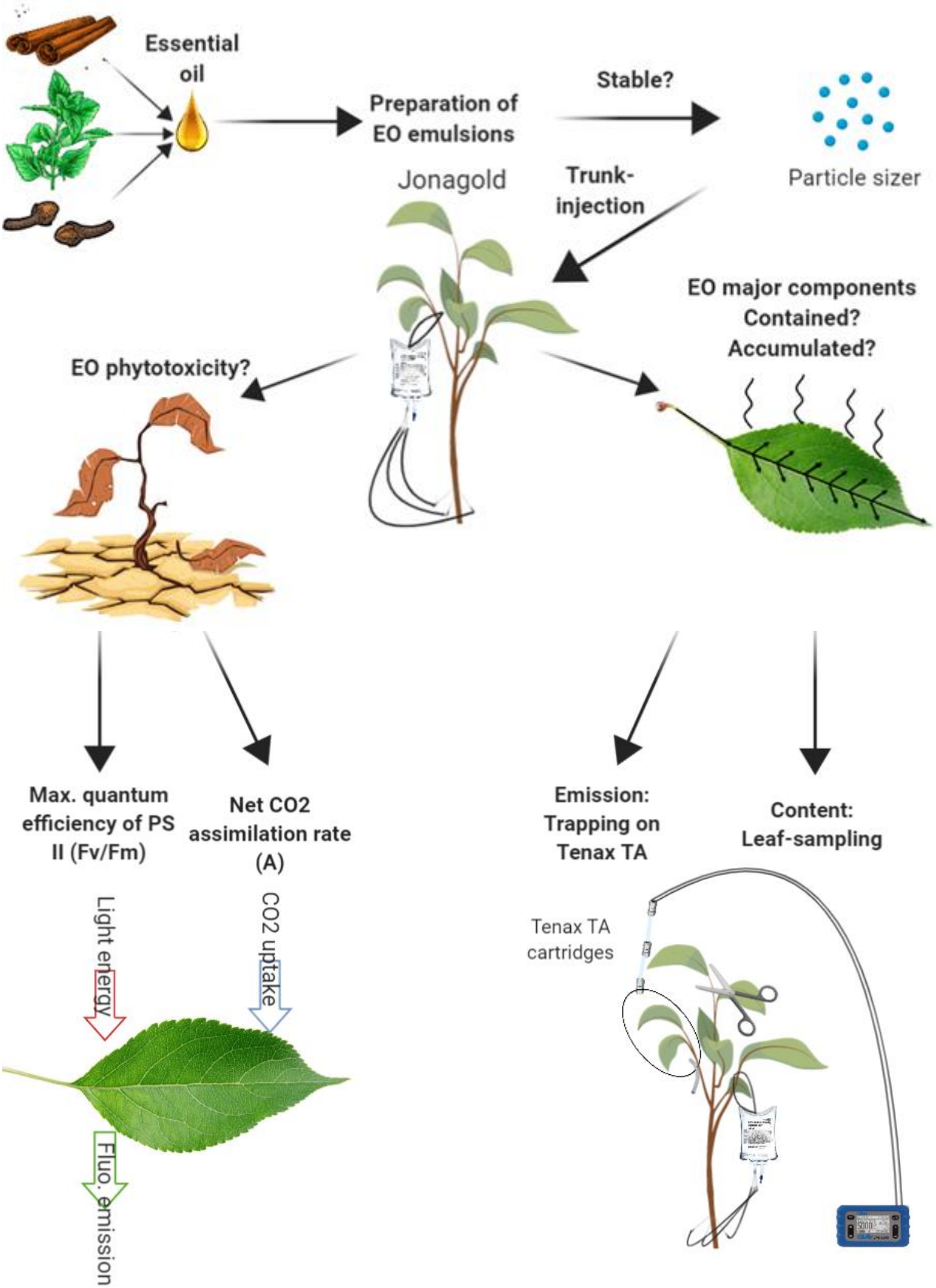


Figure 17. Schematic representation of objectives and method

4. Results

This following section will be subdivided into three major parts. Firstly, particle size distribution data and photosynthetic efficiency measurements, gathered during the EO-emulsion formulation, will be analyzed.

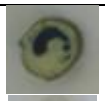
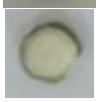
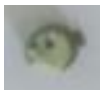
The second section will focus on presenting the volatile profiles obtained when analyzing the leaf content and emission.

Thirdly, the last section will present the data gathered to evaluate the plant's fitness in response to the various treatments applied.

4.1. Formulation

The first step of this study aimed at assessing whether injecting a solution in the xylem sap at the trunk's base is detectable higher in the trunk after a certain time-lapse. Furthermore, the effect of EDTA additions to improve the ascension rate of the solution was also evaluated. A visual test was hence performed, comparing two methylene blue dye solutions (0.1%) containing 20% 100mM EDTA or no EDTA.

Table 6. Visual interpretation of differences in ascension rate after 48h of injection, between a methylene blue solution and an EDTA-methylene blue solution. The injection site is located at 14cm from the trunk-base. Illustration are cross sections of the tree-trunk.

	0cm	5cm	10cm	14cm injection site	19cm	24cm	34cm
With EDTA							
Without EDTA							

The cross sections suggest that the ascension rate of the solution is increased in the presence of EDTA. In fact, with EDTA the solution has dispersed as far as 20cm from the injection site and only 5cm without. EDTA is said to reduce occlusion of the sieve plate pores. In fact, EDTA slows down callose deposition which usually seals sieve elements when these are injured (Thompson and van Bel, 2012). Given that water transport exists between the phloem and the xylem (through the symplast of horizontal ray parenchyma cells (Pfautsch *et al.*, 2015)), reducing the flow in the phloem due to injury reduces in turn the transfer to the xylem.

Methylene blue was also found lower than the injection site. Two reasons can explain this: EDTA's diffusion to counter the difference in concentration and its presence in the phloem.

Given the results obtained here, it was decided to add EDTA to the solution in order to allow the product's transport all the way to the leaves.

The next important step in the development of the product's formulation was to create an emulsion stable in time. This aspect was covered by comparing the particles sizes distribution (and average particle sizes) of various emulsions prepared. As a reminder, cinnamon oil was emulsified with four biocompatible emulsifying agents using two different homogenization processes.

The emulsifying agents tested were: Tween 20, arabic gum, native pea proteins and structurally modified pea proteins. The emulsification processes used, on the other hand, were an HPH and an ultrasonication.

Only the emulsions prepared with Tween 20 and arabic gum were later analyzed with the particle sizer given that both pea protein emulsions were unstable right after homogenization (a white deposit appeared minutes after homogenization, Figure 18).

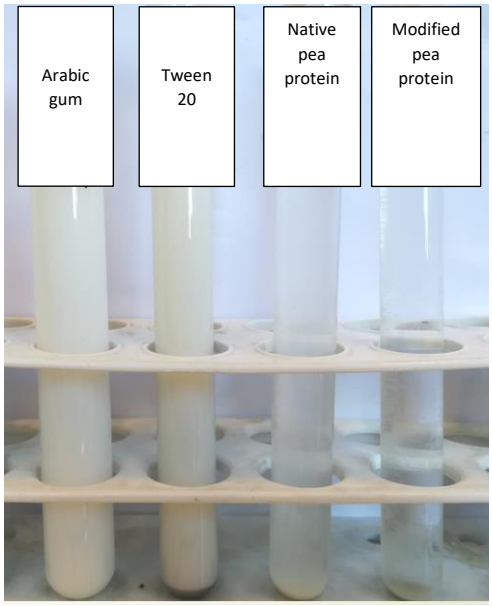


Figure 18. Visual representation of the emulsions' stability prepared with the high-pressure homogenizer. From left to right: Arabic gum, Tween 20, native pea protein and modified pea protein.

Both ultra-sonicated and high-pressure homogenized Tween 20 preparations gave good droplet size (Table 7). Both fit in the nano-emulsion domain, i.e emulsions exhibiting sizes lower than 300 nm and which are thermodynamically unstable but kinetically stable (these take months to destabilize) (Anton and Vandamme, 2011). Table 7 provides polydispersity indexes (PI), giving information on the deviation of oil droplets from the average size (Tadros *et al.*, 2004). All emulsions prepared are monodispersed (PI < 0.1).

Table 7. Droplet diameter and polydispersity index for the emulsion preparation processes analyzed with the particle sizer.

	Ultra-sonicated Cinn. oil + Tween 20	**HPH Cinn. oil + Tween 20**	**Ultra-sonicated Cinn. oil + arabic gum**	**HPH Cinn. oil + arabic gum**
Average droplet diameter (d, in nm)	228.83 ± 15.04	139.33 ± 10.53	846.43 ± 191.35	924.40 ± 16.97
Polydispersity index (PI)	0.08 ± 0.02	0.16 ± 0.03	0.27 ± 0.00	0.29 ± 0.00

It was decided to prepare the future emulsions using the HPH and Tween 20 because this combination shows the smallest droplet size and hence the best stability.

Finally, the phytotoxicity of EDTA and Tween 20 containing solution was tested. As it can be seen in Figure 19, the EDTA and Tween 20 combination caused a major drop in the Fv/Fm ratio from 0.819 ± 0.031 at t= 24h down to 0.005 ± 0.009 at t= 96h.

A healthy plant is considered to have a Fv/Fm ratio close to 0.83 (Maxwell and Johnson, 2000). On the other hand, leaves for which the Fv/Fm ratio is below 0.3 are considered dead (Bresson *et al.*, 2017). Injecting the EDTA and Tween 20 combination caused a healthy plant to die in 96h. Tween 20 injected by itself also seemed to cause a decrease in the Fv/Fm ratio but remained lower than the combination described previously. This finding was surprising given that Tween 20 is mentioned as biocompatible in the literature (Flores-Villaseñor *et al.*, 2016).

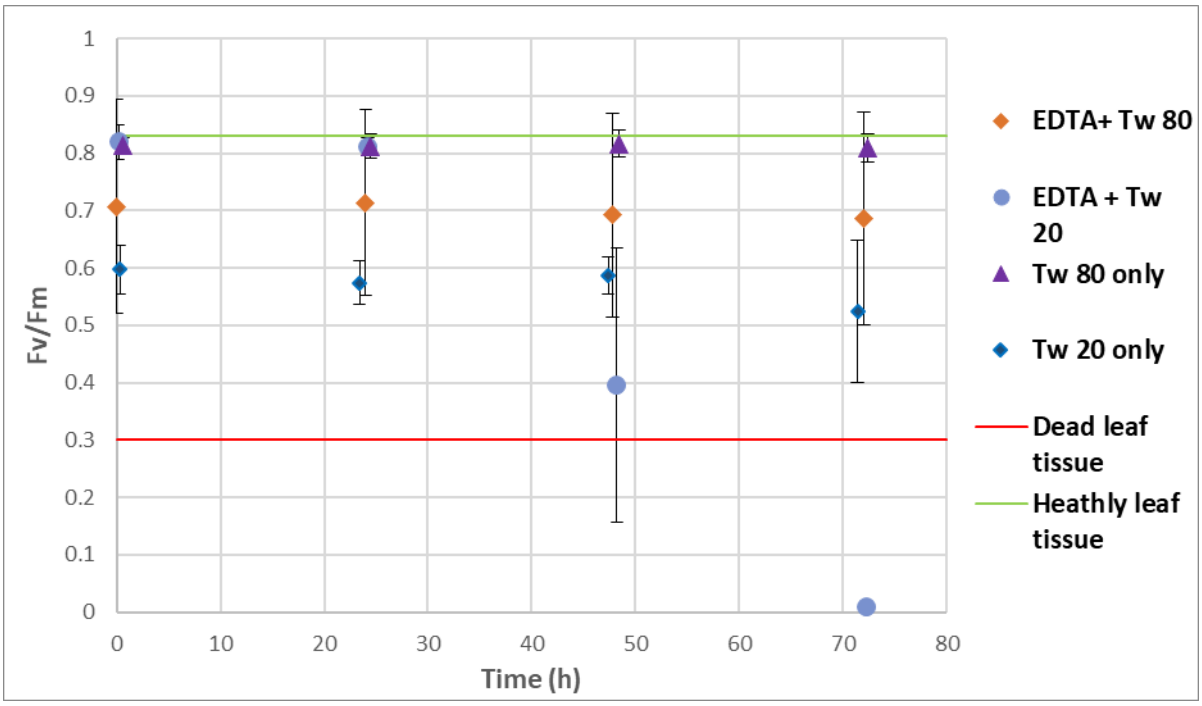


Figure 19. Maximum efficiency of photosystem II over time for 4 different solutions: one containing EDTA and Tween 80, one with EDTA and Tween 20, one with Tween 80 only and finally Tween 20 only (n=3). In the first two cases, these were made of 0.4% Tween (20 or 80) and of 20% 100mM EDTA.

Therefore, another biocompatible surfactant, similar to Tween 20, was tested. Two other trees were injected: one with an EDTA and Tween 80 solution and the other one with a solution containing Tween 80 only. Promising results were obtained this time, with Fv/Fm ratio relatively stable over the 96h period (Figure 19).

Good stability results were also found when cinnamon oil (0.5%) was added to an EDTA and Tween 80[23] combination: d = 115.2 nm and PI = 0.147[24].

This preliminary worked enabled the development of a good base formulation permitting a good conductivity up the xylem as a result of EDTA addition. It should also allow the transport of hydrophobic essential oils in the xylem due to its emulsification with Tween 80. Lastly, the phytotoxicity appearing following injection of emulsified EO, if any, should only be caused by the EO itself.

[23] Emulsion prepared as described in material and method section 3.3.
[24] No standard deviation because only one repetition was performed here.

These hypotheses have therefore been tested by confronting two years old apple trees to five different treatments: three emulsified oils at 0.5% - solution containing EDTA, tween 80 and either 0.5% of cinnamon, spearmint or clove oil- one negative control (i.e injected only with a solution containing EDTA and tween 80) and one blanc (no injection).

4.2. Apple tree volatiles contained and emitted during the treatment

This part of the project was subdivided into two main subsections. Firstly, identifying and quantifying when possible the EO's major components in the leaves and emitted by them to establish kinetic curves. As explained previously, the product's efficiency against the aimed pests as well as its mode of action (ingestion toxicity and/or repellent effects) strongly depends on this. The second goal consisted in analyzing the volatiles' profile contained and emitted by the leaves. The purpose was to detect injection-induced changes and to detect variations in profiles from one EO injection to another.

4.2.1. Essential oil major components identification and quantification

Table 8 reports the equations of the calibration curves used for the quantitation of the EOs major compounds found in the leaves and emitted by them. Y is expressed in relative area and x in relative concentration[25].

Results show a good linear correlation in the ranges considered with a regression coefficient (R^2) greater or equal to 0.985 for all four compounds. In this first section, the graphs displaying the kinetic curves show the values obtained for each repetition.

Table 8. Ions used as quantifiers (in bold) and qualifiers, calibration curves, regression coefficients R^2 and linearity range for the major compounds found in the tested EOs.

Compound	Ions	Equation	R^2	Linearity range (ng / mL)
Limonene	**68**, 93, 67	y=0.365x+0.004	0.990	678657 – 583
D-carvone	**82**, 108, 93	y=0.527x+0.020	0.985	861049 – 1503
Trans-cinnamaldehyde	**131**, 132, 103	y=0.628x+0.018	0.989	954490 – 623
Eugenol	**164**, 103, 149	y=0.465x+0.013	0.990	1325150 – 4570
Limonene	**68**, 93, 67	y=0.365x+0.004	0.990	678657 – 583

[25] i.e. Y in area component per area internal standard and x in concentration component per concentration internal standard.

4.2.1.1. Cinnamon oil treatment – Detecting and quantifying trans-cinnamaldehyde

The first test performed consisted in injecting a cinnamon oil emulsion in the tree. Figure 20 which gives the cinnamaldehyde content found in the leaves indicates an overall increasing trend in time. This proved therefore that the injection system functioned correctly and that the oil droplets were able to rise up the xylem.

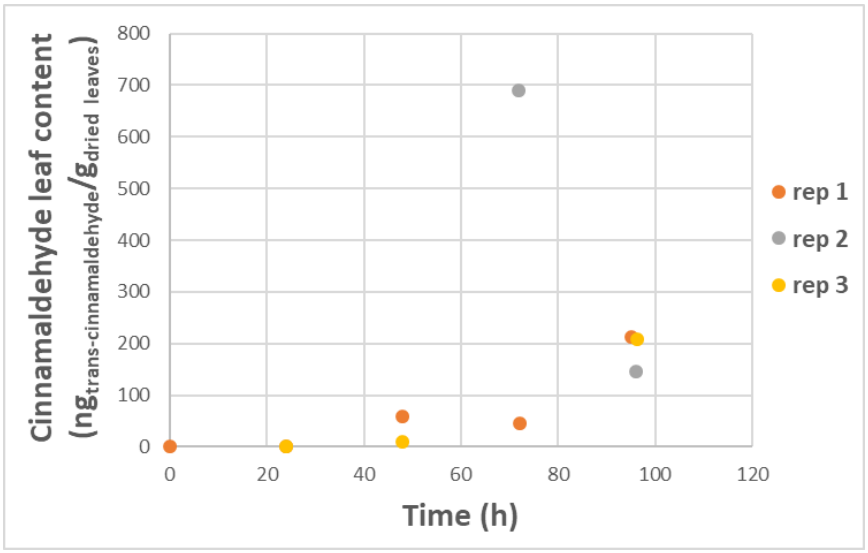


Figure 20. Cinnamaldehyde content in the leaves of trees injected with cinnamon oil emulsion (0.5%). At t= 0h and t= 24h, leaf content is equal to 0 $ng_{trans\text{-}cinnamaldehyde}/g_{dried\ leaves}$ because inferior to the limit of detection

Figure 21 (left) shows that the ion 131, major ion in the trans-cinnamaldehyde mass spectrum displays a large peak at a retention time (Rt) of 18.646 min. Secondly, the retention index (RI) obtained from this Rt (RI = 1272) is close to the literature RI of 1271 (Babushok, Linstrom and Zenkevich, 2011)**.** Finally, the mass spectrum (MS) obtained in scan mode around this Rt displays important ions of this molecule's mass spectrum (such as ion 131, 132 and 103). These factors ensure the identification of this peak was correct.

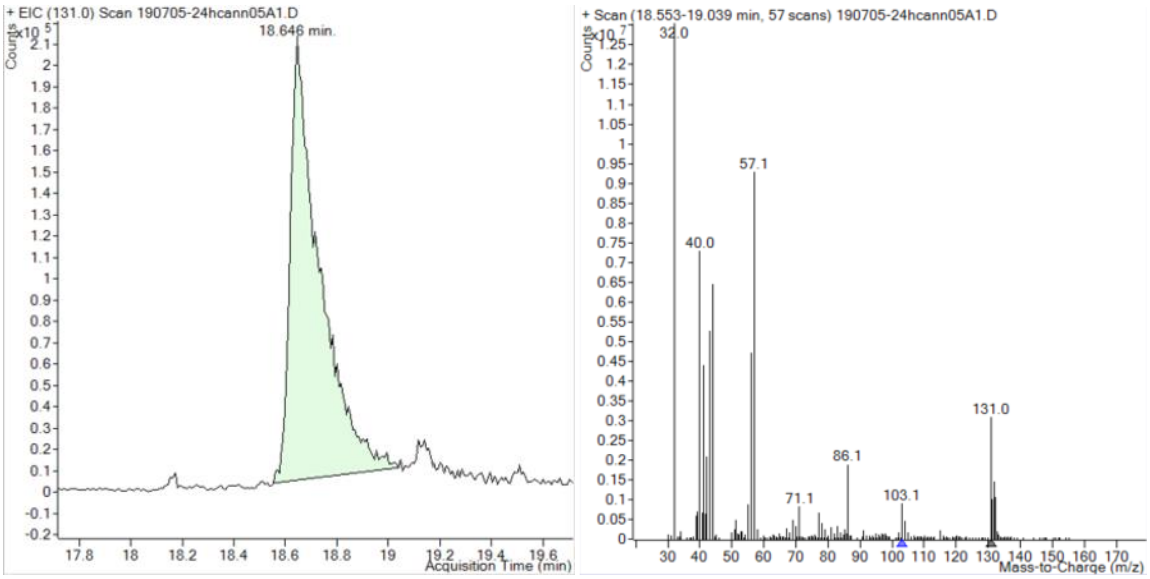


Figure 21. Left: Ion 131 extracted-ion chromatogram (EIC), Right: mass spectrum found within this time range for cinnamaldehyde in the 24h-Tree n°1 leaf content sample.

Zhao et al. (2014)[26] established that the lower limit for their cinnamaldehyde calibration curve was equivalent to 20 ng/mL. As opposed to this project, Zhao et al. (2014) led the quantification in single ion monitoring (SIM) mode which lowers the detection threshold. Mentioning this value remains interesting as it reminds that cinnamaldehyde could also be quantified here if the linearity range was extended to much lower concentrations.

Cinnamaldehyde has also been found in the emissions but only at t= 24h (Figure 22). The Rt obtained is slightly different than the one obtained for leaf content samples. Nevertheless, this peak still corresponds to cinnamaldehyde given the ions 131 and 103 at this Rt.

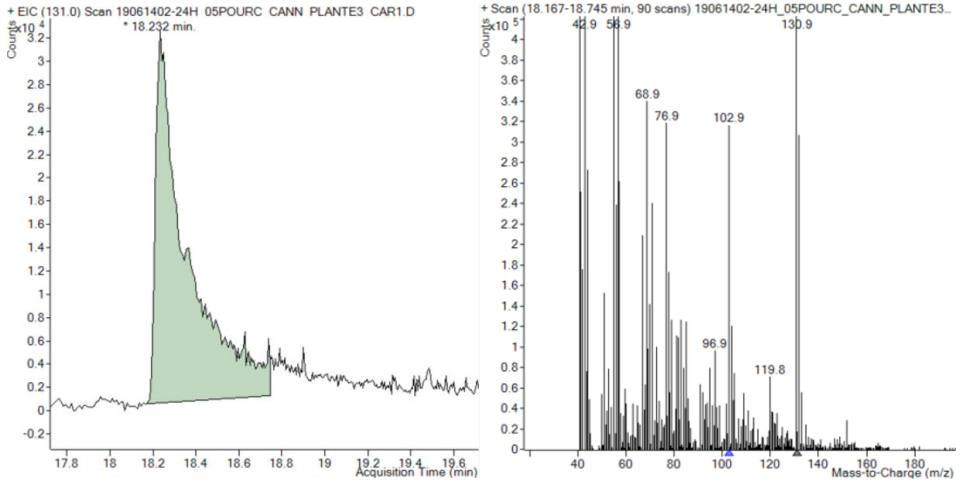


Figure 22. Left: Ion 131 EIC, right: mass spectrum found within this time range for cinnamaldehyde in the 24h- Tree n°3 emission sample.

The presence of cinnamaldehyde in the emission at t= 24h could only be explained by the fact that the air enclosed in the Nalophan bag at t= 0h was contaminated by traces of cinnamaldehyde from previous "set-up" tests. The absence of cinnamaldehyde in the blanc indicates that this compound is not produced by apple trees and its absence from the emissions at t=48h indicates that this molecule is not brought by the EO emulsion injected[27].

[26] This study, also conducted using a GC-MS (Trace Ultra GC ISQ MS), aimed at analyzing the kinetics of cinnamaldehyde in rats. The cinnamaldehyde was hence diluted in rat plasma to consider the matrix effect. Even if the preparation of the calibration dilutions differs, this article reminds that a lower boundary for this calibration curve can be found.

[27] Air is pumped for 24h through the bag at a rate of 3L/h. Given that the closed environment around the plant is of approximately 60L, the air is entirely regenerated every 20h. Therefore, after the first 24h the volatiles found in the profile are exclusively biogenic or due to the EO injected.

4.2.1.2. Spearmint oil treatment – Detecting and quantifying limonene and carvone

Spearmint oil emulsions have also been injected in apples trees. Two major components were used to determine whether spearmint was found in the leaves: limonene and carvone.

Limonene

Limonene was found in the leaves of spearmint treated trees, however it is difficult to known whether injecting spearmint oil caused an increase in the limonene leaf content or if this concentration is due to its natural production by apple trees.

In fact, when analyzing Figure 23, the spearmint treatments do not stand out compared to the others. On the other hand, a difference between trees treated with EO emulsions and the blanc and negative control appears. The blanc and negative control repetitions fluctuate (apart from "Blanc rep 1" at t=24h) between 2.5 and 7.5$ng_{limonene}/g_{dried\ leaves}$. Repetitions for treated trees rise all the way to up to 24.5 $ng_{limonene}/g_{dried\ leaves}$ ("spearmint rep 1 at t=72h).

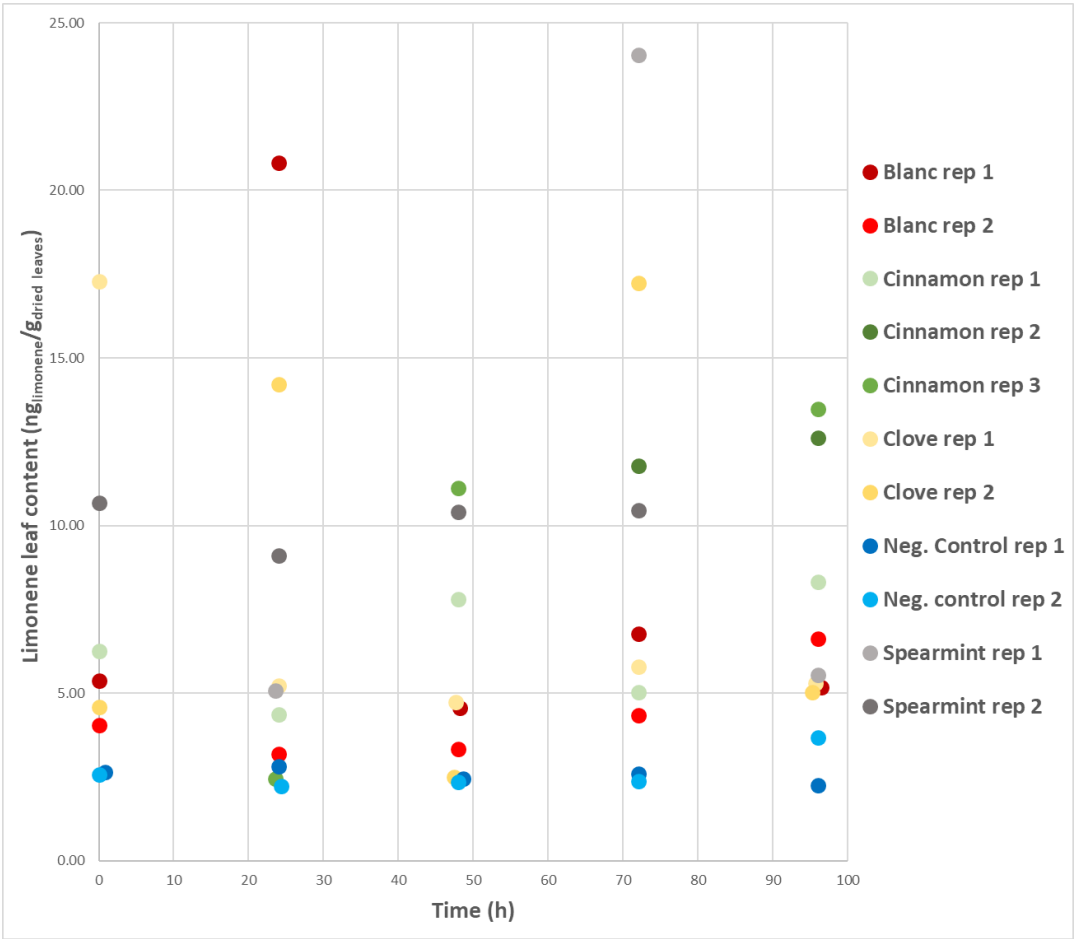


Figure 23. Limonene content in leaves of all treatments over time. Legend: Red = Blanc, Green = cinnamon treat. , Yellow = clove treat. , Blue = spearmint treat. and Grey = Negative control. Note that the points are dispersed around their corresponding times to allow a better visualization of all points (i.e. to prevent stacking of points for a given time).

As opposed to what has been found for cinnamaldehyde, the trees in which limonene was present also emitted this molecule[28]. Similarly to the leaf content described previously, it can be noticed in Figure 24 that overall the negative control and blanc repetitions (except for "blanc rep 2") are the two treatments which tend to emit the smallest amount of limonene over time.

Secondly, it can also be noticed on this graph that spearmint treated trees (rep 1 and rep 2) show the highest limonene emission values at t = 48h and t = 96h. Unfortunately, no data is available for the spearmint treatment at t= 72h due to malfunctioning of all three pumps used between t=48h and t=72h.

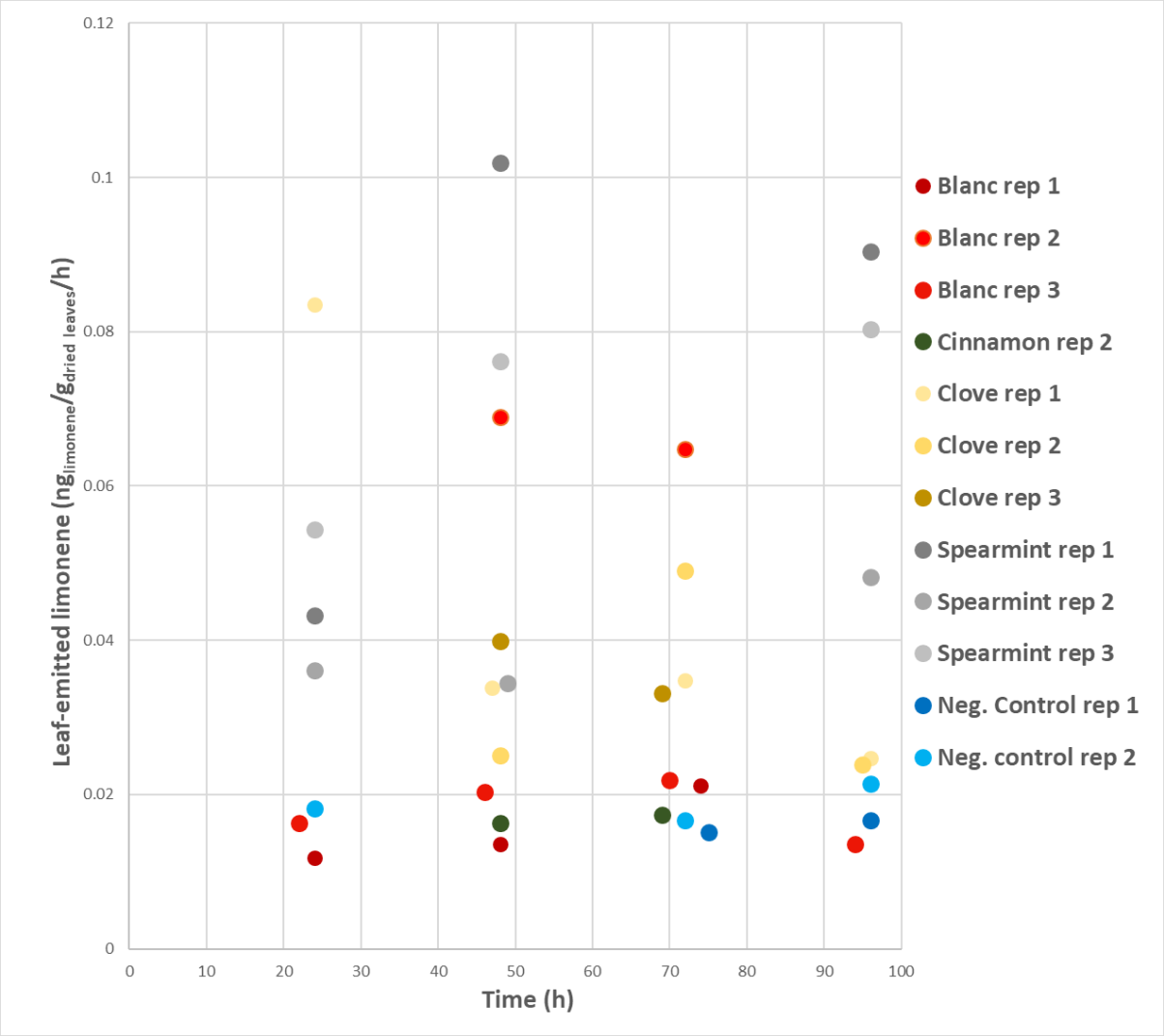


Figure 24. Limonene emitted by leaves over time for each treatment applied. Note that the points are dispersed around their corresponding times to allow a better visualization of all points (i.e. to prevent stacking of points for a given time).

An ANOVA would have been interesting to understand whether there is a significant difference between the treatments. The number of repetitions being lower than three in some cases, the test of homogeneity of variances cannot be performed and subsequently neither can the ANOVA.

[28] Part of the limonene found in the emission at t=24h could have been explained by contamination of the enclosed air. Although, the concentrations found at t=24h do not stand out compared to concentrations at other times.

Carvone

Carvone was found in both the leaves and the emissions of spearmint treated trees, suggesting that this emulsified oil was also efficiently conducted in the xylem. Carvone visibly accumulates in the leaves given that its content increases (Figure 25 left) when the emissions remain constant over time (Figure 25 right).

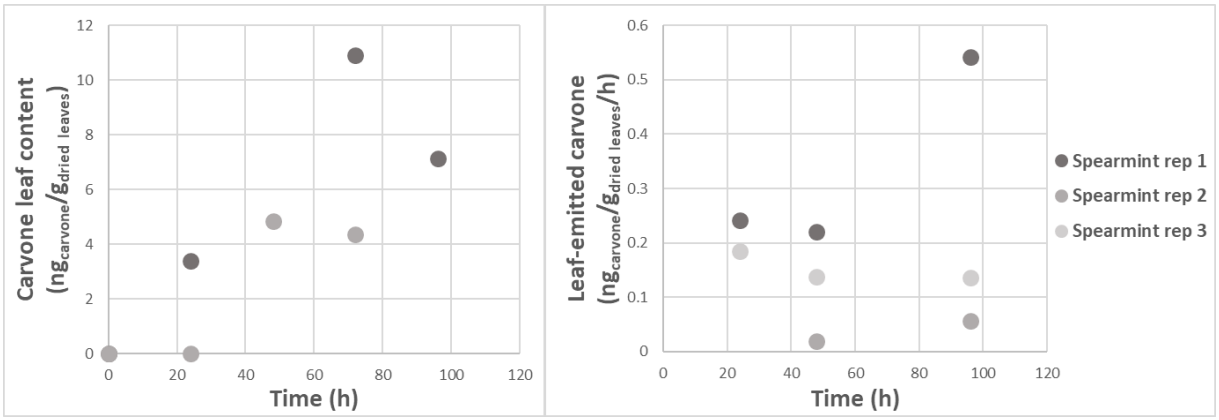


Figure 25. Left: Carvone leaf content, right: Leaf emitted by spearmint (0.5%) treated trees over time

4.2.1.3. Clove oil treatment – Detecting and quantifying eugenol

Eugenol could not be detected and quantified in either the leaf content or the emissions of clove treated trees. Obstruction of the needles used when placing them in the trunk could explain the absence of eugenol but the main explanation remains the poor analytical performances obtained for this compound, i.e a low response factor and very high lower limit for the linearity range (Table 8). This explanation is strengthened when analyzing the EIC for ion 164 (major ion of the eugenol molecule) of a t=96h leaf content sample (Figure 26). In fact, an increase in the peak area can be noticed at a retention time of approximately 20.8 min. This Rt corresponds to a RI of 1360, very close to eugenol's literature RI value of 1357 (Babushok, Linstrom and Zenkevich, 2011). The presence of eugenol remains uncertain as an exact identification was not possible.

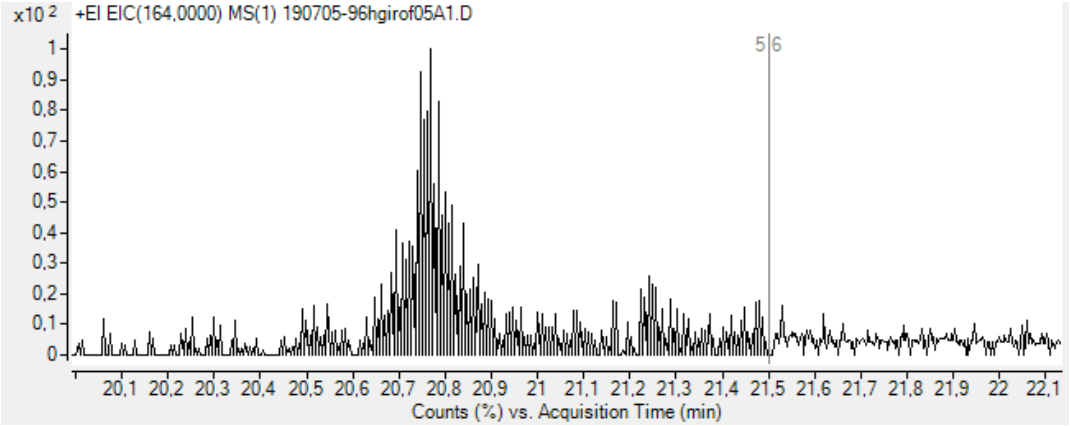


Figure 26. Ion 164 EIC zooming on a retention time window corresponding to that of eugenol.

4.2.2. Discussion regarding leaf content and emission of aimed compounds

As observed in this section, all three compounds identified do not show the same behaviors in terms of content or emissions:

- Cinnamaldehyde content increased in the leaves but was not released in the emissions.
- Limonene showed constant concentrations in both leaf content and emission over time.
- Carvone seemed to increase in the leaf content and was emitted at a constant rate.

Emission results from the diffusion of VOC along a vapor pressure gradient guiding them from the cells to the atmosphere surrounding the leaves (Peñuelas and Llusià, 2001). In fact, part of the difference in the trends mentioned just above could be explained by their different vapor pressure:

Table 9. Vapor pressure (PubChem) of three major components analyzed in the leaf contents and emissions.

	Trans-cinnamaldehyde	**D-carvone**	**Limonene**
Vapor pressure at 25°C (mm Hg)	2.89×10^{-2}	1.15×10^{-1}	1.55

As shown by Table 9, trans-cinnamaldehyde is the molecule with the smallest vapor pressure. This molecule therefore has a smaller tendency to volatilize and hence accumulates in the leaves.

Another factor affecting the release of VOCs is the ability of some species to store the VOCs they produce (known as biogenic volatile organic compounds (BVOCs)) in plant secretory organs, such as glandular trichomes and resin ducts (Kesselmeier and Staudt, 1999). Glandular trichomes are “epidermal outgrowths characterized by the presence of a head made of cells that have the ability to secret and store large quantities of specialized metabolites” (Huchelmann, Boutry and Hachez, 2017).

Given that these three molecules have been introduced following trunk-injection, they are not concerned by storage in such organs. In this case, they diffuse slowly through aqueous phases in the mesophyll, lipid bilayer membranes and internal air space (in the substomatal cavity) before release through the stomata.

The aqueous and lipid phase that the VOCs must cross can be seen as two temporary storage phases for newly synthesized compounds and for injected molecules in this case. A specific partitioning therefore exists between the aqueous and lipidic phase of the leaf. This depends on the affinity of the compound for one phase or the other and on the quantity of this phase in the leaf.

The extent to which a molecule dissolves in the lipid phase is given by its octanol/water partition coefficient ($K_{o/w}$), i.e the “ratio of solubility of a compound in octanol (a non-polar solvent) to its solubility in water (a polar solvent)”, limonene for example which has a $K_{o/w}$ of 30.550 mol mol^{-1} will more likely be found in this lipid storage (Niinemets and Monson, 2013).

In other words, the rate at which molecules are released by a leaf is affected by several upstream mechanisms (Figure 27). The first one is the release rate from these storages to the leaf intercellular air space (k_a and k_l). This release rate depends on the diffusion path, the octanol water partition (for k_l) and Henry’s law constant and diffusion constant (for k_a). Secondly, once in the intercellular air space, the flux out of the leaf k_g is affected gas phase conductance from the outer surface of cell walls to the substomatal cavity but also by the conductance between this cavity and the ambient air (Niinemets and Monson, 2013).

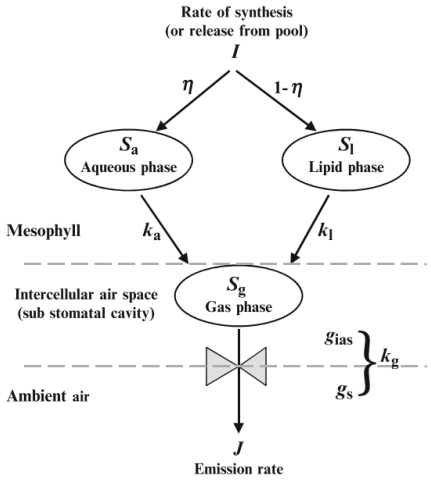


Figure 27. Potential role of non-specific storage in BVOC emissions (Niinemets and Reichstein, 2003)

Concerning carvone's emission, the non-specific phase storage seems to be the most plausible. Whether this one will tend towards an "aqueous phase" or "lipid phase" storing should depend on whether spearmint oil is still in the emulsified form when found in the leaves. In fact, if it is still emulsified, the Tween 80 should protect the globules from interaction with the plant and the globules hence act as water soluble particles and are found in the fast-release pool – the aqueous phase.

It must be noted though that even if constant releases are observed for both limonene and carvone over the 96h period using 24h intervals, variations within the 24h time lapse can occur. In fact, if the production of one VOC has not been negatively feedbacked and that stomata conductance has dropped (due to abiotic factors for example), the VOC gas phase concentration in the substomatal cavity will increase at first with no or little emission. Finally, a steady-state will be reached, where the partial pressure will compensate the increased resistance. The flux will therefore return to its steady state (Niinemets and Monson, 2013). Another type of variation within the 24h period could be for example an emission bursts following the 6h dark period upon stomatal reopening. Such behavior was observed by Niinemets and Reichstein (2003) with methanol.

Now that the kinetics of the oil's major components has been assessed, it seems important to analyze the effect of injecting these oils on the global apple tree VOCs profile.

4.2.3. VOC profile analysis

This section will firstly focus on describing the molecules found in both leaf content and emission profiles, regardless of the treatments applied to have an idea of the molecules present and their origin. A brief explanation of heatmaps created to try and have a better overview of the different variations occurring in time and from one treatment to another follows. This part is then followed by the PCA performed on both leaf content and emission profiles and finally when possible an ANOVA on the dimensions generated by the PCA.

4.2.3.1. Description of the contained and emitted VOC profiles

Table 10 and Table 11 display all the molecules found in the leaf content and emission profiles, regardless of the treatment used.

*Table 10. CAS number, calculated retention index and retention index found in the literature for molecules found in the leaf content profile. The calculated RI followed by a * signifies that the difference between the calculated and the literature RI is greater than 5%. In this case the identification is questionable. Calc. is for calculated and Lit. for literature*

Molecule name	CAS NUMBER	Calc. RI	Lit. RI
Alcohols/Phenols			
1-Octanol	111-87-5	1071.9*	1272.1
(Z)-2-Penten-1-ol	1576-95-0	669.5*	771.2
Aldehydes			
β-Homocyclocitral	472-66-2	1255.3	1237.5
(E,E)-2,4-Heptadienal	4313-03-5	1013.1	1011.5
2,4-Hexadienal	142-83-6	913.6	913.2
(E)-2-Decenal	3913-81-3	1261.9	1263.4
(E)-2-Heptenal	18829-55-5	958.4	960.5
(E)-2-Hexenal	6728-26-3	796.3*	853.0
(E)-2-Nonenal	18829-56-6	1160.4	1162.2
(E)-2-Octenal	2548-87-0	1058.9	1060.2
(E)-2-Pentenal	1576-87-0	656.7*	720.0
3-Hexenal	4440-65-7	690.6*	769.0
2-Methyl-4-pentenal	5187-71-3	686.7*	776.0
Decanal	112-31-2	1205.7	1205.4
Heptanal	111-71-7	903.5	902.0
Hexanal	66-25-1	734.2*	799.9
Nonanal	124-19-6	1104.8	1103.3
Octanal	124-13-0	1004.1	1002.8
Aliphatic ester			
(Z)-3-Hexen-1-ol acetate	3681-71-8	1006.1	1004.0
Alkanes			
Dodecane	112-40-3	1199.9	1200.0
Heptadecane	629-78-7	1699.1	1700.0
Hexadecane	544-76-3	1599.0	1600.0
Tetradecane	629-59-4	1399.5	1400.0
Tridecane	629-50-5	1300.3	1300.0
Alkenes			
3-Ethyl-1,5-octadiene	NA	941.9	949.0
1-Ethylcyclohexene	4313-03-5	998.5	1011.5
Aromatic aldehydes			
Benzaldehyde	100-52-7	961.9	969.0
Aromatic esters			
3-Hexen-1-ol benzoate	25152-85-6	1569.2	1569.5
Methyl salicylate	119-36-8	1191.4	1192.9

Molecule name	CAS NUMBER	Calc. RI	Lit. RI
Aromatics			
p-Cymene	99-87-6	1024.1	1024.3
Benzeneacetaldehyde	122-78-1	1044.4	1045.9
Carboxylic acids			
Acetic acid	64-19-7	611.9	622.3
Fatty acid esters			
Ethyl benzoate	93-89-0	1169.9	1171.3
Ethyl heptanoate	106-30-9	1097.9	1096.0
Methyl hexanoate	106-70-7	923.7	924.1
Homoterpenes			
(E)-4,8-Dimethylnona-1,3,7-triene	19945-61-0	1113.3	1107.5
Ketones			
1-Octen-3-one	4312-99-6	978.2	978.0
2,2,6-Trimethylcyclohexanone	2408-37-9	1035.4	1013.0
Monoterpenes			
α-Ionone	127-41-3	1423.0	1425.6
β-Cyclocitral	432-25-7	1219.1	1218.3
β-Ionone	14901-07-6	1478.2	1485.9
δ-3-Carene	13466-78-9	1046.4	1011.3
1,8-Cineole	470-82-6	1032.4	1031.8
β-Pinene	127-91-3	987.9	977.7
D-Limonene	5989-27-5	1027.7	1029.5
Isomenthone	1196-31-2	1160.5	1150.5
Linalool	78-70-6	1099.7	1084.4
Phenylpropanoids			
trans-Cinnamaldehyde	14371-10-9	1272.3	1271.3
Sesquiterpenes			
α-Farnesene	502-61-4	1495.6	1504.1
Caryophyllene	87-44-5	1420.2	1420.1
Unknowns			
Unknown A	NA	947.3	NA
Unknown B	NA	920.2	NA
Unknown C	NA	617.2	NA
Unknown D	NA	624.1	NA

Note that there is a >5% difference between the calculated RI and literature RI for several compounds for the content profile. The reason behind this is that the chromatogram obtained for the Kovats solution gave a bad resolution for the octane peak (first peak appearing).

*Table 11. CAS number, calculated retention index and retention index found in the literature for molecules found in the leaf emission profile. The calculated RI followed by a * signifies that the difference between the calculated and the literature RI is greater than 5%. In this case the identification is questionable. Calc. is for calculated and Lit. for literature*

Molecule name	CAS NUMBER	Calc. RI	Lit. RI
Alcohols			
2-Hexyl-1-decanol	2425-77-6	1772.8	1790
1-Hexanol	111-27.3	853.2	869.7
2-Ethyl-1-hexanol	104-76-7	1012.5	1030.6
1-Octen-3-ol	3391-86-4	963.3	962
(Z)-3-Hexen-1-ol	928-96-1	839.3	856.6
l-Menthol	89-78-1	1153.7	1150.0
Aldehydes			
(E)-2-Decenal	3913-81-3	1243.2	1263.4
Decanal	112-31-2	1186	1205.4
Dodecanal	112-54-9	1385.8	1408.1
Nonanal	124-19-6	1086.1	1103.3
Undecanal	112-44-7	1285.7	1306.5
Aliphatic esters			
(Z)-3-Hexenyl acetate	2497-18-9	993.3	1011.5
Alkanes			
Dodecane	112-40-3	1180.3	1200
Tetradecane	629-59-4	1377	1400
Tridecane	629-50-5	1279	1300
Undecane	1120-21-4	1082	1100
Alkenes			
7-Tetradecene	10374-74-0	1371.3	1374
Aromatic esters			
(Z)-3-Hexenyl benzoate	25152-85-6	1550.1	1550
Methyl salicylate	119-36-8	1177.4	1192.9
Furanes			
trans-Linalool oxide	11063-78-8	1070.6	1071
Homoterpenes			
(3E,7E)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	62235-06-7	1557.7	1566
(E)-4,8-Dimethylnona-1,3,7-triene	19945-61-0	1098.3	1105
Ketones			
l-Menthone	14073-97-3	1136.3	1136

Molecule name	CAS NUMBER	Calc. RI	Lit. RI
Monoterpenes			
β-Ocimene	3338-55-4	1033.7	1037.8
D-Carvone	99-49-0	1178.7	1242
Linalool	78-70-6	1084.6	1099
Terpinen-4-ol	562-74-3	1159.6	1177.1
Phenylpropanoids			
trans-Cinnamaldehyde	14371-10-9	1254.4	1271.3
Sesquiterpenes			
α-Farnesene	26560-14-5	1484.2	1490.9
γ-Muurolene	30021-74-0	1457.9	1476.2
α-muurrolene	31983-22-9	1494.9	1498.3
Caryophyllene	118-65-0	1398	1406.5
δ-Cadinene	483-76-1	1503.7	1523.2
Dihydrocarvone	5948-04-09	1179.2	1201.4
Germacrene D	23986-74-5	1459.3	1480.6
Unknowns			
Unknown 1	NA	1040.2	NA
Unknown 10	NA	1441.6	NA
Unknown 11	NA	1471.6	NA
Unknown 12	NA	1475.2	NA
Unknown 13	NA	1477.4	NA
Unknown 14	NA	1575.7	NA
Unknown 15	NA	1675.3	NA
Unknown 16	NA	1681.2	NA
Unknown 17	NA	1686.2	NA
Unknown 18	NA	1782.9	NA
Unknown 2	NA	1056.7	NA
Unknown 3	NA	1174.9	NA
Unknown 4	NA	1196.1	NA
Unknown 5	NA	1273.7	NA
Unknown 6	NA	1276.5	NA
Unknown 7	NA	1334.7	NA
Unknown 8	NA	1357.2	NA
Unknown 9	NA	1367.5	NA

Although some molecules are found in both profiles (e.g. (E)-2-Decenal, decanal, α-farnesene, caryophyllene, etc…), it can be noticed that these two profiles differ qualitatively. The following observations can be made.

Firstly, the diversity of aldehydes is much greater in the leaf-content profile than in the emitted. This can be partially explained by the creation of green leaf volatiles (GLVs) when the leaf is crushed during sample preparation (e.g. 2-hexenal and 3-hexenal). Green leaf volatiles' (GLVs) are "C_6 aldehydes, alcohols and their esters formed through the hydroperoxide lyase pathway of oxylipin metabolism" (Matsui, 2006). In healthy plants, the amount of GLVs is low but rapidly increases when the tissues are disrupted. GLVs are formed from linolenic and linoleic acids. These are initially catalyzed by lipoxygenase to form reactive hydroperoxides (e.g. linolenic acid 13-hydroperoxide) which is then further metabolized to create (E)-2-hexenal (from linolenic acid) and n-hexanal (from linoleic acid). Once these are created, alcohol dehydrogenase transforms them into their corresponding C_6 alcohols and acetyltransferases catalyze the formation of their ester forms from the alcohol reacting with acetyl CoA. The presence of these C_6 aldehydes in the leaf content profile is hence explained by the fact that

upon homogenization of the leaves, galactolipids (and other fat substances) are released and put in contact with galactolipase which triggers the formation of free fatty acids (Matsui, 2006).

Other C_6 GLVs are present in the emission profile (1-hexanol and 3-Hexen-1-ol) but absent in the leaf content profile. When analyzed deeper the presence of these alcohols, they were only found in some trees at t=24h.

A high variety of linear saturated aldehydes are also found in the leaf content compared to the emissions (e.g. heptanal, octanal, nonanal and decanal). These molecules were found in the apple trees' emissions in the literature (Bengtsson *et al.*, 2001). The presence of these in the leaf content and not in the emission in this case is probably explained by the fact that the molecules were present in the emissions but at lower concentrations hence not detected when analyzing the chromatograms[29].

Other interesting remarks can be made, for example concerning the terpenic profiles. Firstly, a much greater diversity of sesquiterpenes are found in the emission profile compared to the leaf content profile. The opposite is observed for monoterpenes.

These terpenes are produced from different prenyldiphosphates (e.g geranyl diphosphate - the monoterpene precursor and farnesyl diphosphate – the sesquiterpene precursor) which are assembled and converted into the large diversity of terpenes by terpene synthase enzymes (Nieuwenhuizen *et al.*, 2013). Mono- and sesquiterpenes are produced by different pathways in different locations, monoterpenes through the methylerythritol phosphate pathway (MEP pathway) taking place in the plastids while sesquiterpenes are produced through the mevalonic acid pathway (MVA pathway) in the cytosol (Jux, Gleixner and Boland, 2001).

A first explanation to this higher monoterpenic diversity in the leaf content is that crushing leaves during the preparation of the leaf samples breaks down the various parts of the plant, including the plastids which allows to release a whole set of molecules which are not found in the emissions. Secondly, preparing the leaves for analysis (crushing, incubation in the DHS, etc…) not only released readily produced molecules which were stored in the plastids but also created new molecules. This is the case for example with short-chain apocarotenoids (e.g. α- and β-ionone) produced by exposing carotenoids to UV light and heat (during DHS incubation for example) (Walter and Strack, 2011).

On the other hand, monoterpenes found in the emissions have mainly two origins. Firstly, D-carvone, as explained previously, originated from the injection of spearmint in the trees. The other monoterpenes found in the emissions (β-ocimene and linalool) are typically produced or at least increase following as a response to herbivore attack (Holopainen and Gershenzon, 2010; Giacomuzzi *et al.*, 2016).

Methyl salicylate is another molecule produced in stress situations, insect attacks but also mechanical wounding (explaining its presence in the leaf content as well). Methyl salicylate is produced from salicylic acid, a phytohormone used in the defense response signaling cascades (Schmelz *et al.*, 2003; Morkunas and Mai, 2011).

[29] To come up with a reasonable number of molecules to analyze, a filter extracting only the compounds with at least "1% of the biggest area" was used. The biggest area was the internal standard's one. This method was therefore applicable from one sample to another. In the emission case, these aldehydes were probably below this 1% limit.

Now that a brief explanation of the molecules found in leaf content and emission has been performed this discussion will develop a deeper analysis of the impact of treatments and time on the patterns observed.

4.2.3.2. Heatmaps

Firstly, heatmaps were created to detect trends for each components of the VOC profiles depending on the treatments applied and over time (two heatmaps for the leaf content: at t=0h and t= 96h, and two for the emissions, at t=24h and t=96h - Annex 10, Annex 11, Annex 12, Annex 13). These heatmaps display normalized values[30] and should help to better detect differences between the treatments at different times. Dendrograms have also been created on these heatmaps to group the observations by their level of similarity. These were interesting as they allowed to understand without going in depth that the cinnamon oil treatments seem to have similar impact (between the repetitions) on the VOCs leaf content after 96h when compared to the other treatments where it is hard spot trends with the dendrogram (Annex 11). The same can be said for spearmint treatments which are grouped together for the VOCs emission compared to the other treatments (Annex 13).

Due to the large number of molecules used for the creation of these heatmaps, trends for specific molecules between treatments and over time are hard to establish. Different options were possible to tackle this problem. The first one was to perform heatmaps for the molecules grouped by chemical families. The second was to perform a PCA with these different chemical families. Finally, the third option was to perform a PCA on all molecules (regardless of the chemical family). In this work, the last option was chosen.

4.2.3.3. Principal component analysis

This kind of statistical analysis allows an easier analysis and visualization of a dataset as large as this one (i.e a multivariate analysis). A PCA extracts important information from the dataset to create a few principal components (PCs), dimensions representative of all the variables in the original dataset[31] but allowing an easier visual interpretation.

The first step of this PCA is the analyze the eigenvalues of all the dimensions created. Eigenvalues quantify the percentage of the total variance (found between the samples) explained by each PC (Figure 28 and Figure 29).

In the leaf content case, only the first 9 dimensions were kept given that all further dimensions explain less than 3% of the total variance.

[30] $(conc._{molecule,sample\ x} - conc_{molecule,\ all\ samples})/\ stdev$

[31] i.e. the PCs are a linear combination of the original variables.

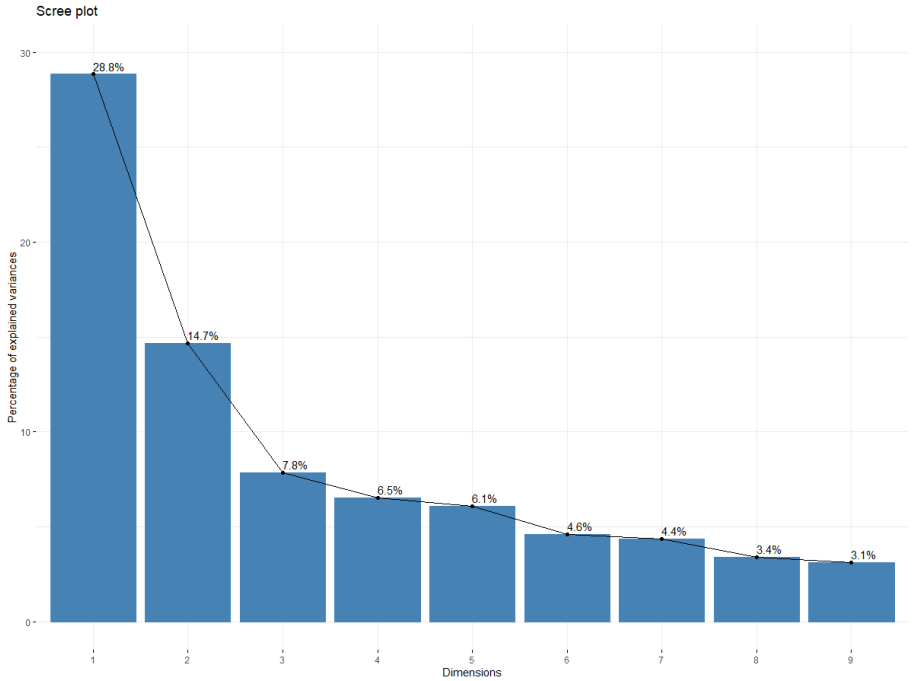


*Figure 28. Eigenvalues for each of the principal components created from all the **leaf content** variables.*

In the emission case on the other hand, 15 dimensions are considered (the following dimensions represent less than one percent of the total variance).

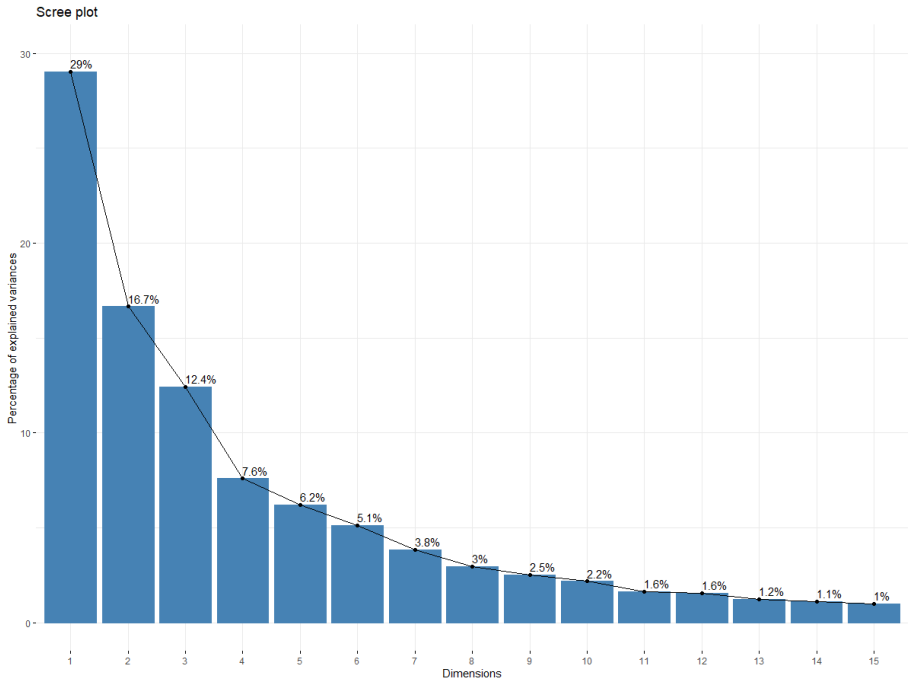


*Figure 29. Eigenvalues for each of the principal components created from all the **leaf emission** variables.*

This means, for example when looking at Figure 28 that 28.8% of the total variance present between the leaf content samples is explained by the first dimension created. This first dimension is composed

of all the molecules in the original dataset but with a different “contribution” to it. For example, from Figure 30, it can be observed that nonanal contributes for more than 5% of the first dimension.

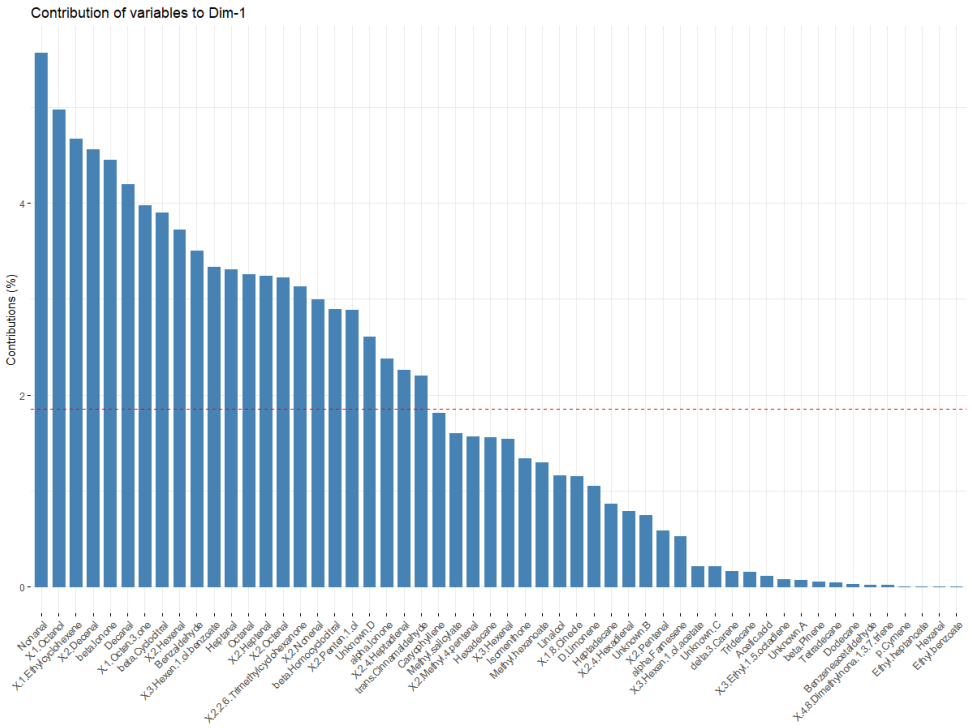


*Figure 30. Contribution of the different molecules to dimension 1 of the **leaf content** ACP. Note: some names are found with an X. infront. This is automatically generated by the program, probably to better recognize that molecule names are not numerics. The red line indicates the average contribution of each variable.*

The samples analyzed can then be plotted on graphs where the axes are two dimensions chosen out of the nine in the leaf content’s case. Given that dimension 1 and 2 for both the leaf content and emission explain the largest part of the variance, plotting the samples on graphs with these two dimensions seemed the most relevant.

The PCA performed here (similarly to heatmaps) are performed on a reduced centered variable (average = 0 and variance = 1). The center of the axes in Figure 31 and Figure 32 represent the distribution’s average. In both graphs, the negative control (blue and pink ellipses in each graph respectively) and the blanc (red ellipse) treatments are the two treatments closest to the center of the graph, indicating that this PCA is not majorly impacted by these treatments. In other words, the negative control and the blank are not major contributors to the variance’s explanation.

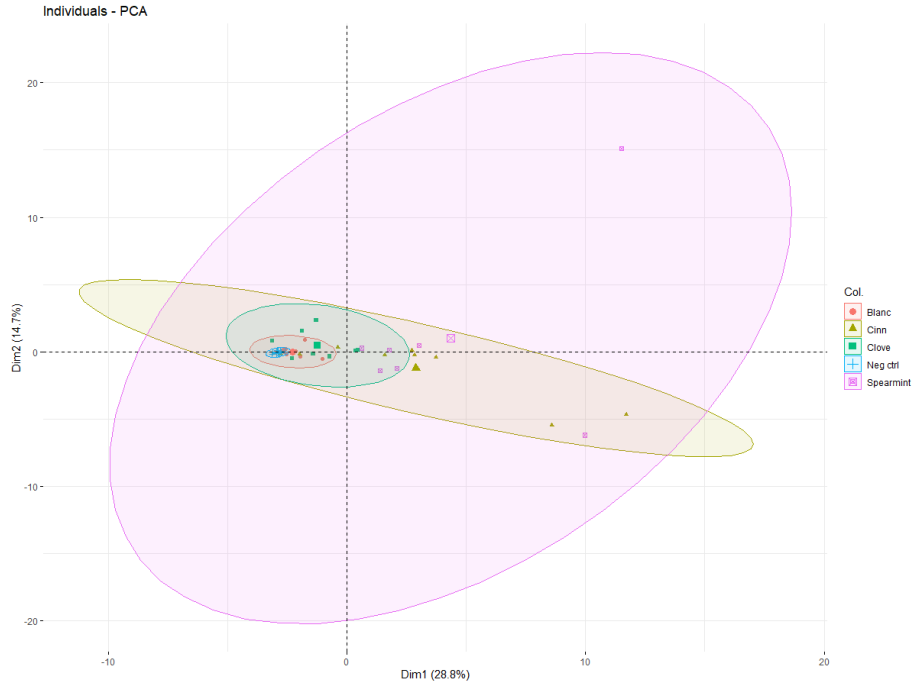


*Figure 31. Graphical representation of all **leaf content** samples from all treatments explained by the first and second dimension (43.5% of the total variance). Legend: Red = Blanc, Mustard = cinnamon treat. , Green = clove treat. , Pink = spearmint treat. and Blue = Negative control*

In Figure 31, it can be noticed that spearmint and cinnamon treated trees see most of their variance explained by dimension 1. On the other hand, clove treated trees are relatively centered compared to the two other treatments.

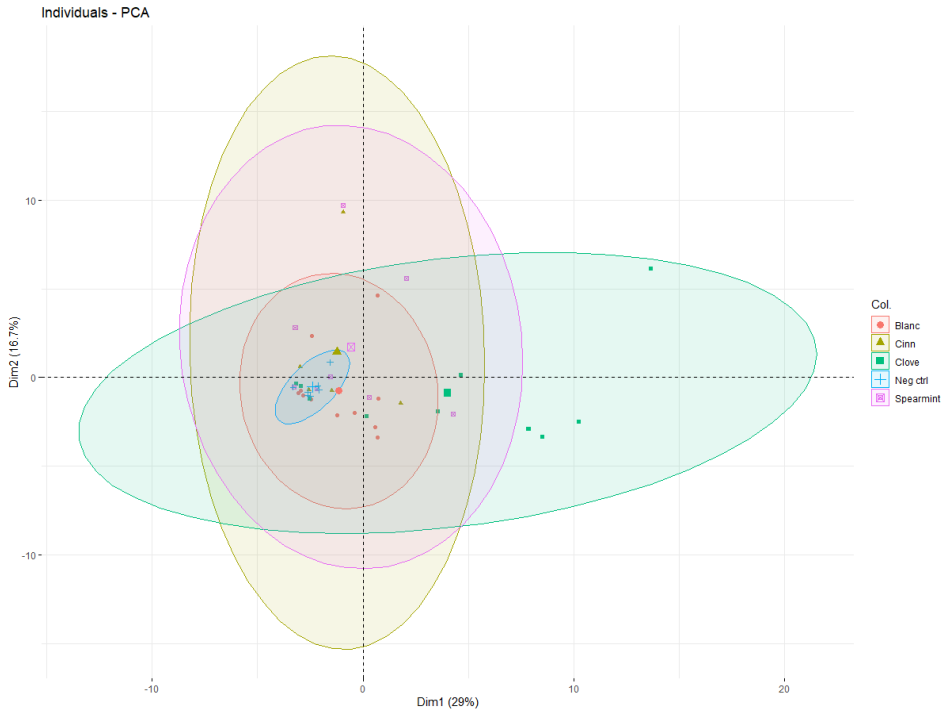


*Figure 32. Graphical representation of all **leaf emission** samples from all treatments explained by the first and second dimension (45.7% of the total variance). Legend: Red = Blanc, Mustard= cinnamon treat. , Green = clove treat. , Blue = spearmint treat. and Pink = Negative control*

Figure 32 representing the emission samples in the first two dimensions, two main trends can be observed. The clove (green ellipse) samples see most of their variance explained by dimension 1 as opposed to cinnamon and spearmint treatments which are more explained by dimension 2 than dimension 1.

This graphical representation is interesting as it allows to rapidly detect trends. Nevertheless, this representation explains in this case less than 50% of the total variance in both leaf content and emission cases (43.5% for the content and 45.7% for the emissions).

For both leaf content and emission datasets, the creation linear models taking into account mean coordinate values for each treatment (analyzed at each separate time) was considered. These models would later be tested through an ANOVA procedure to known if there is a significant difference between the treatments at a given time and when explained by one dimension at a time.

The number of samples being too small to create a correct linear model in the leaf content's case, its PCA was analyzed only in a qualitative manner. Several combinations of two dimensions out of the nine have been tested to detect major differences between each treatment. The ones which seemed the most pertinent are shown in Figure 33 and Figure 34.

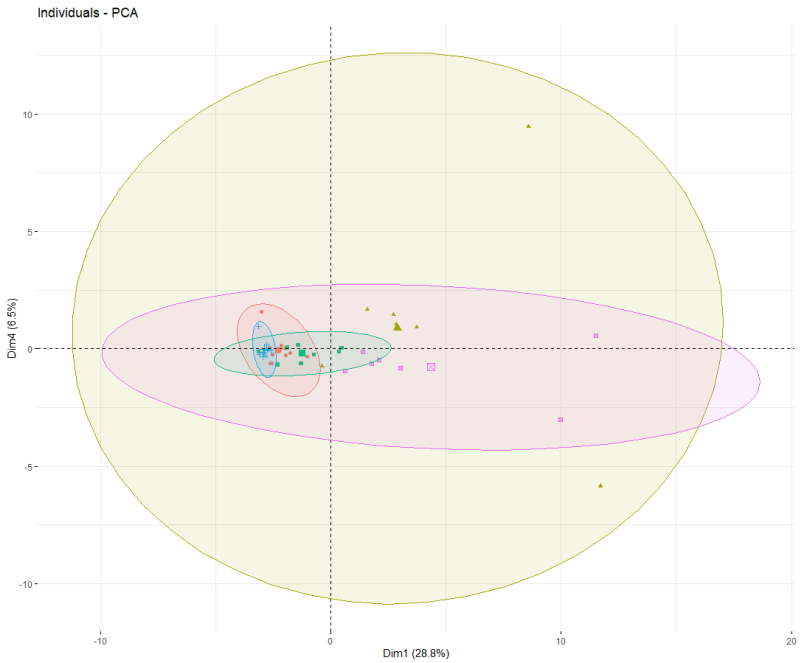


*Figure 33. Graphical representation of all **leaf content** samples from all treatments explained by dimensions 1 and 4. Legend: Red = Blanc, Mustard = cinnamon treat. , Green = clove treat. , Pink = spearmint treat. and Blue = Negative control*

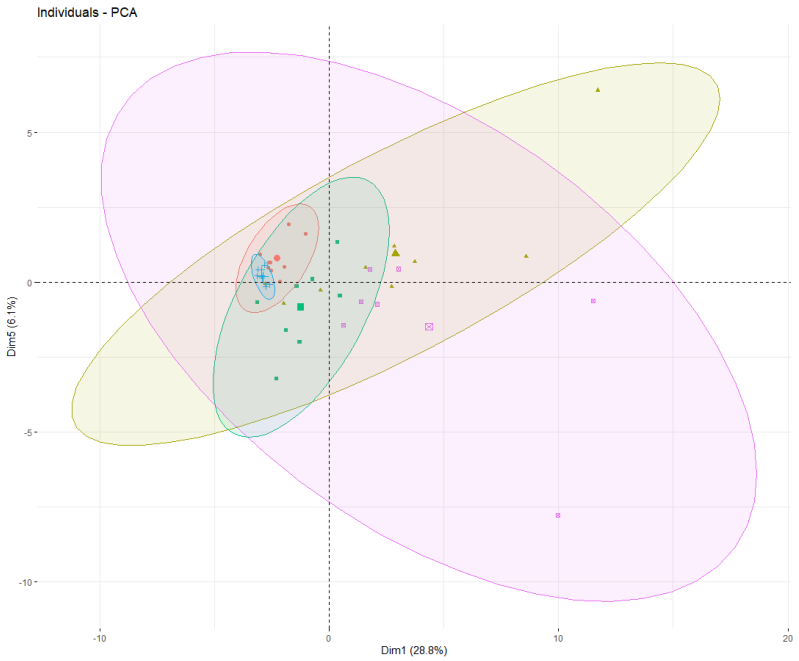


*Figure 34. Graphical representation of all **leaf content** samples from all treatments explained by dimensions 1 and 5. Legend: Red = Blanc, Mustard = cinnamon treat. , Green = clove treat. , Pink = spearmint treat. and Blue = Negative control*

It can be noticed that both Figure 33 and Figure 34 display as x-axis the first dimension created. It was thought that this would allow a better representation of the dimension presented on the y-axis given that the first dimension has already been discussed previously (Figure 31). Figure 33 indicates that little variance is explained by dimension 4 for every treatment, except for cinnamon oil treatment which presents two points on opposite sides. In Figure 34 on the other hand, the blanc and negative control treatment are relatively centered when compared to the three other dimensions. Additionally, a trend appears for the clove treatment. In fact, dots start from the origin of the axes and seem to spread towards negative values for dimension 5. Spearmint samples also show extreme values for this dimension.

In Table 12, the top five contributors explaining these dimensions were extracted.

*Table 12. List of the five greatest contributors to the various dimensions (for the **leaf content** analysis). From left to right: the variable name, the correlation between the variable and the dimension concerned (Dim. X), the coordinate of variable on this axis (coord), the quality of the representation (cos2) and the contribution of these molecules to the dimension (contrib).*

	Dim.4	Coord.	cos2	Contrib.
Unknown B	0.785	0.617	0.617	17.490
Methyl salicylate	0.738	0.545	0.545	15.450
Caryophyllene	0.650	0.423	0.423	11.999
β-Ionone	-0.520	0.270	0.270	7.672
(E)-4,8-Dimethylnona-1,3,7-triene	0.446	0.199	0.199	5.636

	Dim.5	Coord.	cos2	Contrib.
Heptadecane	-0.727	0.528	0.528	16.086
Hexadecane	-0.718	0.515	0.515	15.683
Ethyl heptanoate	0.574	0.330	0.330	10.051
3-Hexenal	0.545	0.297	0.297	9.035
α-Farnesene	-0.493	0.243	0.243	7.390

Out of the five treatments tested, samples of the three EO based treatments seem to vary the most on these dimensions (Figure 33 and Figure 34). Looking at Table 12, it can be noted that out of the ten top contributors for both dimensions, four are terpenic molecules (Caryophyllene, β-Ionone, (E)-4,8-Dimethylnona-1,3,7-triene, α-Farnesene) and four are typically produced under stressed conditions (Methyl salicylate, (E)-4,8-Dimethylnona-1,3,7-triene and α-Farnesene and 3-hexenal).

This could hence imply that injecting EOs in the tree causes a modification of the terpenic profile contained in the leaves and additionally stresses the plant. It must be noted that analyzing trends for molecules contributing maximum at 17.49% (e.g. Unknown B in Dimension 4) to a dimension that itself describes only a maximum of 6.6% of the total variance (e.g. dimension 4) must only be considered as potential effects and not as verified facts. These potential effects determined through the analysis of the leaf content can also be verified by analyzing the emissions.

In the case of emitted volatiles, a linear model was created for each dimension and time combination and then analyzed with an ANOVA test. This model created mean coordinate values, centered around the mean blanc coordinate, in each dimension for trees confronted to the same treatment. The results obtained are presented in Table 13.

*Table 13. Model designed for the leaf emission analysis, for each combination of dimensions, time and treatment a coordinate value is found when compared to the blanc treatment. The p-value issued from the ANOVA is also displayed. This one indicates whether there is a significant difference between the values obtained for each treatment at a specific given time. * significant difference, ** highly significant difference, *** very highly significant difference. Note: coordinate values are absent in the case of spearmint at t=72h, the p-values obtained in this case only consider the four other treatments.*

		24H						48H				
Dimensions	**P-Value**	**Cinn.**	**Clove**	**Spear.**	**Neg ctrl**	**Blanc**	**P-Value**	**Cinn.**	**Clove**	**Spear.**	**Neg ctrl**	**Blanc**
1	0.062	-0.818	14.075	-0.249	0.315	0.000	0.676	-3.326	4.072	-0.127	-1.991	0.000
2	0.493	5.121	4.190	2.638	-3.534	0.000	0.407	2.347	1.538	0.798	0.097	0.000
3	0.685	-0.395	-0.656	6.129	1.410	0.000	0.029*	1.550	0.307	4.961	-0.550	0.000
4	0.566	0.787	2.319	-2.847	-0.085	0.000	0.007**	0.938	0.556	6.109	0.936	0.000
5	0.504	2.307	1.326	-0.332	-0.754	0.000	0.283	-4.859	-4.592	-2.274	-3.557	0.000
6	0.183	8.270	-3.135	1.389	0.538	0.000	0.076	1.378	0.581	-0.800	0.123	0.000
7	0.177	3.362	-0.693	1.531	0.643	0.000	0.417	-1.795	-0.980	1.942	0.457	0.000
8	0.654	0.203	-0.081	-1.718	1.335	0.000	0.915	-0.369	-0.872	0.324	1.650	0.000
9	0.267	-2.604	-4.482	-2.633	-3.473	0.000	0.078	-1.592	-1.248	-1.885	-2.470	0.000
10	0.596	2.858	0.802	-1.010	0.679	0.000	0.730	0.554	1.067	1.150	1.330	0.000
11	0.273	0.400	3.128	2.326	1.377	0.000	0.901	0.846	-0.309	-0.382	0.145	0.000
12	0.655	-1.083	-1.397	-1.403	-0.253	0.000	0.933	-0.105	0.352	0.220	0.527	0.000
13	0.207	-0.559	-1.550	0.283	1.895	0.000	0.282	-1.357	0.903	0.546	1.316	0.000
14	0.783	-2.003	-1.520	-0.648	-1.512	0.000	0.317	0.645	0.034	1.534	1.276	0.000
15	0.966	0.825	0.199	-0.167	-0.027	0.000	0.063	2.358	1.142	0.096	0.852	0.000

		72H						96H				
Dimensions	**P-Value**	**Cinn.**	**Clove**	**Spear.**	**Neg ctrl**	**Blanc**	**P-Value**	**Cinn.**	**Clove**	**Spear.**	**Neg ctrl**	**Blanc**
1	0.057	1.471	5.784	NA	-1.378	0.000	0.935	-0.304	2.604	1.175	-0.536	0.000
2	0.104	0.104	-0.187	NA	0.882	0.000	0.757	1.274	0.556	0.814	0.564	0.000
3	0.031*	3.994	1.211	NA	0.058	0.000	0.018*	5.450	0.167	1.725	0.475	0.000
4	0.005**	1.676	-0.493	NA	0.723	0.000	0.001**	7.496	-0.132	1.121	0.748	0.000
5	0.050	-1.139	-2.897	NA	-2.568	0.000	0.239	-2.786	-3.914	-2.914	-2.471	0.000
6	0.020*	3.144	1.580	NA	0.491	0.000	0.281	1.026	1.067	0.894	0.691	0.000
7	0.026*	-3.971	0.197	NA	-0.783	0.000	0.782	-0.491	0.051	-0.310	-0.863	0.000
8	0.074	2.320	0.474	NA	0.807	0.000	0.017*	-1.669	-0.204	1.146	1.429	0.000
9	0.912	0.022	-0.453	NA	-0.644	0.000	0.094	2.666	0.760	-0.452	-0.561	0.000
10	0.617	-0.524	0.521	NA	0.165	0.000	0.298	0.790	-0.099	-1.685	0.439	0.000
11	0.996	0.151	0.211	NA	-0.021	0.000	0.725	1.277	0.275	-0.634	0.033	0.000
12	0.193	0.245	1.660	NA	-0.102	0.000	0.887	0.268	0.482	-0.482	-0.363	0.000
13	0.537	-0.195	0.384	NA	-0.010	0.000	0.234	-0.790	-0.878	-0.680	0.948	0.000
14	0.915	0.422	0.035	NA	0.315	0.000	0.482	-1.069	-0.041	-0.385	0.754	0.000
15	0.550	-0.343	-0.848	NA	-0.107	0.000	0.782	-0.393	-0.355	0.465	0.117	0.000

Note that the p-values indicated in the table concern each time only the coordinates obtained for one specific time. In fact, considering that the data collected from one time to another for each treatment is dependent (leaves were collected on the same tree for each time analyzed) a repeated ANOVA should have been performed to test the presence or absence of a significant difference between times.

The 15 dimensions extracted from the PCA have been created taking into account data from all the different times. The various ANOVA have hence always been performed using the same 15 dimensions. This therefore allows a certain comparison between times even in the absence of a repeated ANOVA.

When analyzing the p-values in Table 13, it can be noticed that some of them are close to being significant at a given time (e.g. p-value for dimension 1 at t=24h) and then becomes far from being significant at the next time. The dimensions for which there is a significant difference between the treatments are indicated by one, two or three stars depending on the level of significance. As a summary:

- At t=48h: dimensions 3 and 4
- At t= 72h: dimensions 3, 4, 6, 7
- At t=96h: dimensions 3,4,8

The models display significant differences for dimension 3 and 4 from t= 48h to t= 96h. This implies that a trend probably exists for compounds majorly contributing to these dimensions. Hence, the top five major contributors for these two dimensions as well as for the other three dimensions have been extracted from the PCA (Table 14).

*Table 14. List of the five greatest contributors to the various dimensions (for the **leaf emission** analysis). From left to right: the variable name, the correlation between the variable and the dimension concerned (Dim. X), the coordinate of variable on this axis (coord), the quality of the representation (cos2) and the contribution of these molecules to the dimension (contrib).*

	Dim 3	Coord.	cos2	Contrib.
Caryophyllene	0.879	0.772	0.772	11.512
(3E,7E)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	0.796	0.633	0.633	9.438
Germacrene D	0.729	0.532	0.532	7.928
Methyl salicylate	0.599	0.359	0.359	5.346
β-Ocimene	0.593	0.352	0.352	5.244

	Dim 4	Coord.	cos2	Contrib.
γ-Muurolene	0.769	0.591	0.591	14.423
δ-Cadinene	0.767	0.589	0.589	14.376
α-muurrolene	0.766	0.587	0.587	14.331
Unknown 12	0.498	0.248	0.248	6.053
Methyl salicylate	-0.457	0.209	0.209	5.091

	Dim 6	Coord.	cos2	Contrib.
trans-Cinnamaldehyde	0.570	0.324	0.324	11.742
Dodecanal	0.510	0.260	0.260	9.406
Unknown 5	-0.484	0.235	0.235	8.495
1-Hexanol	0.471	0.222	0.222	8.042
α-Farnesene	0.464	0.215	0.215	7.782

	Dim 7	Coord.	cos2	Contrib.
7-Tetradecene	0.446	0.199	0.199	9.634
Germacrene D	-0.436	0.190	0.190	9.207
Terpinen-4-ol	-0.362	0.131	0.131	6.355
Unknown 8	-0.333	0.111	0.111	5.350
α-Farnesene	-0.323	0.104	0.104	5.047

	Dim 8	Coord.	cos2	Contrib.
Linalool	-0.425	0.181	0.181	11.275
D-Carvone	-0.336	0.113	0.113	7.053
Unknown 17	0.325	0.106	0.106	6.598
(3E,7E)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	0.311	0.097	0.097	6.047
Unknown 7	0.300	0.090	0.090	5.604

Overall, it can be noticed that the dimensions created are in this case also, affected by a large amount of terpenic molecules (e.g. β-ocimene, germacrene D, delta-cadinene, terpinene-4-ol, etc…). Thus explaining the source of variation found between the treatments. This finding is confirmed by the literature which states that the emission of terpenes is stimulated by abiotic stresses. These molecules improve plant resistance by direct quenching of reactive oxygen species or by stabilizing cell membranes (Brilli, Loreto and Baccelli, 2019)

The third dimension (upper left of the table) is composed of particularly interesting molecules. (β-) caryophyllene and β-ocimene (monoterpenes) are two terpenes emitted by the apple trees in normal conditions, but which has been found to greatly increase when subject to biotic stress like insect feeding and in some cases of biotic coupled to abiotic stresses.

Germacrene D and methyl salicylate are two other molecules which have not been found in emissions of unstressed apple trees but in which are related to the case of herbivory attacks (Holopainen and Gershenzon, 2010; Giacomuzzi *et al.*, 2016). As explained previously in the material and method section, the trees had been infected by a variety of pests, which could explain their presence. Nevertheless, it has been noticed when analyzing the PCA matrix, that methyl salicylate is only detected in the spearmint treatment after 96h compared to Germacrene D found in every sample. This could imply that its release is due to this oil's injection rather than herbivory attacks.

Similarly, (E,E)-4,8,12-trimethyl-1,3,7,11- tridecatetraene (TMTT) is a homoterpene which is not released by apples trees and other plants (e.g. lima beans) in normal conditions and which has been detected in the case of mites infestation and ozone exposure (Vuorinen, Nerg and Holopainen, 2004; Vallat and Dorn, 2005). In this project, TMTT has been found to be emitted by apple trees treated with cinnamon, spearmint but also in the negative control. A hypothesis could therefore be that mechanical wounding induced by the needle insertion, rather than EO-induced stress, could stress the plant leading to the emission of this molecule.

Some other very interesting molecules are present within the other dimensions. For example, α-farnesene is the fifth biggest contributor of dimension 6. This sesquiterpene emitted by all apples trees seems to explain much of the variance observed (Figure 35).

In fact, α-farnesene emitted varies greatly from one treatment to another. However, it can be noticed that overall, the amount is greater for trees that have been injected with EO compared to the blanc and negative control. Additionally, the α-farnesene emitted is in general greater when the trees are injected with cinnamon oil. This statement is only hypothetical given that there is only one repetition in most cases for this oil.

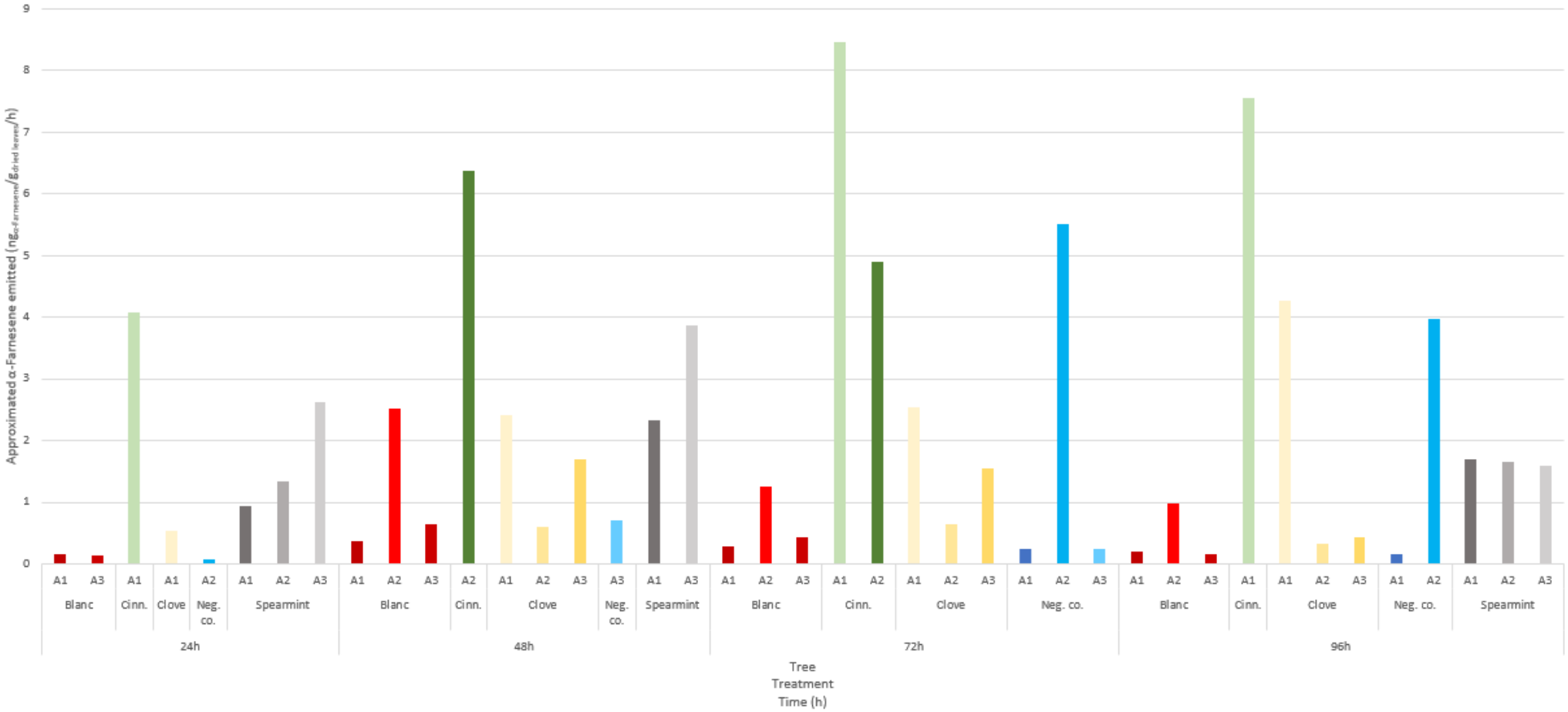


Figure 35. α-farnesene emitted ($ng_{\alpha\text{-farnesene}}/g_{dried\ leaves}/h$) over time for each treatment applied.

The first contributor to dimension 6 is trans-cinnamaldehyde. it was previously explained that trans-cinnamaldehyde was only found at t=24h in the emissions due to a contamination. In fact, when looking Table 14 it can be noticed that cinnamaldehyde at t=24h shows the highest coordinate value for dimension 6 out of all treatments and times. Nevertheless, given the absence of cinnamaldehyde at any other times, the significant different that exists at t=72h for dimension 6 is explained by the other top contributors (Dodecanal, Unknown 5, 1-Hexanol and α-Farnesene).

To conclude this section, it can be said that the trees are to be affected by the injection of essential oils. In fact, not only most of major components of essential oils are found in the trees and in their emission but the overall volatile profiles seem to differ largely from one treatment to the other.

It is therefore primordial to now analyze the phytotoxic effects of such treatments on the plant, i.e to have a look at the maximum quantum efficiency of PSII (through Fv/Fm measures) and the leave's gas exchanges with the environment (through net CO_2 assimilation rates).

4.3. Phytotoxicity

Phytotoxins have different sites and modes of action depending on their chemical structures. These include for example photosystem II, photosystem I, protoporphyrinogen oxidase, glutamine synthetase and asparagine synthetase. One of the most studied remains the effect of phytotoxins of PSII. Phytotoxins can act in a multitude of ways. An example of such action for terpenes is that of odoratol (isolated from *Cedrela odorata*). This molecule has been found to inhibit PSII by competing with the binding site of plastoquinone (PQ) making it impossible to receive the electrons and the H^+ it needs to move across the thylakoid membrane and hence interrupting the photosynthetic electron transport (Duke and Dayan, 2006).

Regardless of the exact mode of action on PSII, a good way to evaluate the effect of these molecules on PSII is to analyze the maximum quantum efficiency of PSII, i.e. the Fv/Fm ratio. These have been measured for all five treatments and are described in Figure 36[32]. Injecting an EO emulsion in the tree does not seem to stress the latter significantly. In fact, the Fv/Fm values remain close to 0.83 (considered optimum) for almost every treatment at every given time(Maxwell and Johnson, 2000).

Only one value deviates from the average, that is the Fv/Fm obtained for negative controls at t=0h. In fact, the Fv/Fm values obtained for the negative controls in the next times follow the same trend as the other treatments. The fact that this outlier value is obtained at t=0h only implies that the plants photochemical efficiency is not affected by the treatment but has been affected by events taking place in the previous days.

The temperature the week before this part of the experiment (started on 09/07/19) has been controlled as extreme temperatures could have explained this abnormal value. Yet, the minimum temperature was 9°C on the night of Thursday 04/07/19 and maximum 28°C on Saturday 06/07/19. These temperatures do not seem particularly extreme given than temperature up to 33°C were recorded the previous week (Saturday 29/06/19) and had no impact on the plant's fitness from the previous treatment (Blanc treatment).

[32] Note that the results are expressed here as mean ± std. This was possible given that three measurements were done on three separate trees.

An explanation could therefore simply be that the plants chosen for this experiment were not easily attainable in the greenhouse and therefore maybe not watered as well as others. For the Fv/Fm recorded at other times, no treatment particularly stands out from the rest at first sight. This will be confirmed later through an ANOVA.

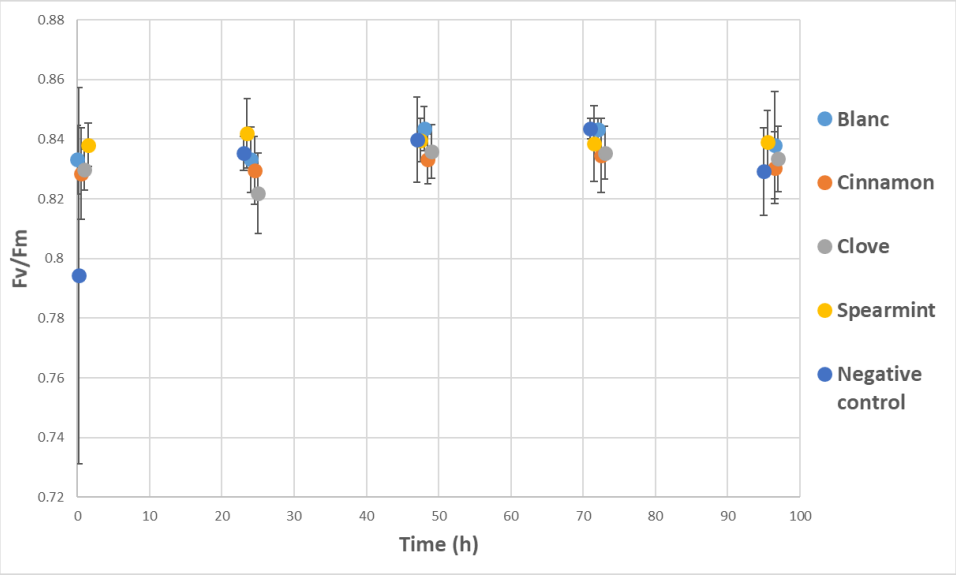


Figure 36. Maximum quantum efficiency of photosystem II (Fv/Fm) for each treatment over time. Note that the points are dispersed around their corresponding times to allow a better visualization of all points (i.e. to prevent stacking of points for a given time).

Another parameter measured during the experiments was the net photosynthetic rate/net CO_2 assimilation rate – A. Figure 37 describes the A values obtained for each treatment over time. Several observations can be made when looking at this graph.

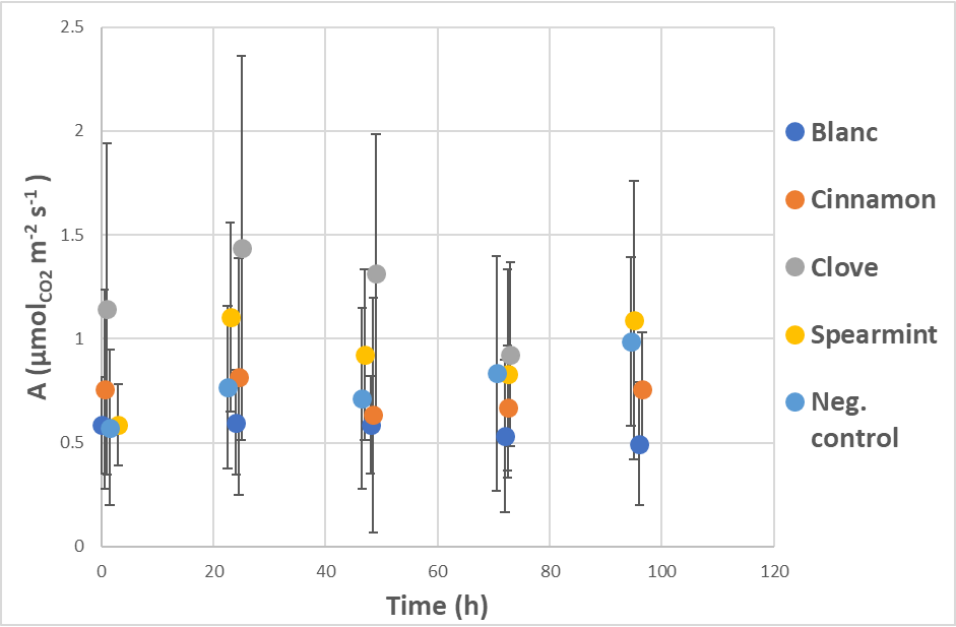


Figure 37. Net CO_2 assimilation rate/ net photosynthetic rate (A) for each treatment over time. Note that the points are dispersed around their corresponding times to allow a better visualization of all points (i.e. to prevent stacking of points for a given time).

Firstly, the values obtained ranging from 0.5 to 1.5 $\mu mol_{CO2}\ m^{-2}s^{-1}$ are quite low when compared to values found in the literature. In fact, Glenn et al. (2003) found carbon assimilation rates ranging from 5.2 to 7.4 $\mu mol_{CO2}\ m^{-2}\ s^{-1}$ for "Empire" apple trees (Malus ×sylvestris (L.) Mill var. domestica (Borkh Mansf.)) depending on the treatment applied. Several reasons could explain these differences: the differences in variety (Jonagold in this project vs. Empire), the potential difference in leaf-age and most importantly an incorrect calibration of the IRGA used here. In fact, the IRGA used in this project was an old model which was not calibrated. This makes the results' comparison to literature difficult, but these still make a relative comparison for the data within the project possible.

Secondly, no abnormal value stands out from all the rest. In fact, what has been said for the negative control at t=0h is not represented here. The A value obtained here overlaps the value found for the spearmint and blanc treatments.

Thirdly, the clove treatment shows the highest A values from t=0h to t= 72h. Unfortunately, no values were recorded for clove at t=96h due to an IRGA malfunctioning error. This data point would have been very interesting as it would have confirmed the fact that clove showed the highest photosynthetic rates.

It can also be noticed that the values obtained for the blanc treatments are always the lowest, which could imply that injecting a solution in the tree has a positive impact on its photosynthetic efficiency. This is unexpected as it has been found earlier when analyzing the volatile profiles that molecules typically released under stressed circumstances were found in the leaf content and emissions of the treated-leaves. This trend though has not been noticed in Figure 36, reminding the importance of analyzing both kind of measurements before drawing any conclusion.

Finally, the general trend for all treatments (except for the blanc) seems to be an increase of the photosynthetic rate from t=0h to t=24h followed by a decrease from t=24h to t=48h to then rise from t=48h to t=96h.

A statistical analysis should help in the interpretation of these results. ANOVA have therefore been performed to compare the Fv/Fm values and A values obtained for each treatment within each time (10 ANOVA in total: Fv/Fm and A at 0,24,48,72 and 96h).

The first step was to verify the homogeneity of variances through Levene's test. The carbon assimilation rates (A) at t=96h were the only set of values which did not fulfill this homogeneity of variances condition (p-value = 0.031, i.e < 0.05). It was still decided to go on with the test for this set of data but it is important to remind this unsatisfactory condition for application during its interpretation.

The p-value obtained for the various ANOVA performed have been summarized in Table 15.

*Table 15. P-values obtained for each of the ANOVA performed on Fv/Fm and A data. Level of significance: p-value = 0.05, *significant; **0.01, highly significant; ***0.001, very highly significant difference.*

	0h	**24h**	**48h**	**72h**	**96h**
Fv/Fm	0.027*	0.007**	0.227	0.129	0.444
A	0.062	0.027*	0.018*	0.392	0.035*

Dunnett's test has therefore been performed when the p-values were lower than 0.05, indicating that there is a significant difference between the means of different treatments. This test allows the comparison of each treatment with a control treatment (i.e. the Blanc treatment in this case).

This procedure groups the treatments with the control one (blanc treatment) if there is no significant difference between their means.

Table 16. Dunnett's performed for the Fv/Fm dataset at (a) t= 0h, (b) t= 24h. Treatments belonging to the same group are indicated by the same letter.

Treat	N	Mean	Grouping
Blanc (control)	9	0.83322	A
Spearmint	9	0.83811	A
Clove	9	0.82967	A
Cinnamon	9	0.82844	A
Neg. Control	9	0.7943	

Treat	N	Mean	Grouping
Blanc (control)	9	0.83311	A
Spearmint	9	0.84189	A
Neg. Control	9	0.83522	A
Cinnamon	9	0.82956	A
Clove	9	0.82189	A

Concerning Fv/Fm results, Dunnett's test helped in confirming that the negative control was significantly different from the Blanc at t=0h. For t=24h on the other hand, the p-value being lower than 0.05 indicated that there was a significant difference between the treatments but Dunnett's test showed that this one was not found between each treatment and the blanc treatment. Looking back at Figure 36, one can understand that the significant difference probably stands between the clove and spearmint treatment.

Table 17. Dunnett's test performed for the A ($\mu mol_{co2}\ m^{-2}\ s^{-1}$) dataset at (a) t=24h, (b) t= 48h and (c) t=96h. Treatments belonging to the same group are indicated by the same letter

Treat	N	Mean	Grouping
Blanc (control)	9	0.5977	A
Clove	9	1.439	
Spearmint	9	1.106	A
Cinnamon	9	0.817	A
Neg. control	9	0.767	A

Treat	N	Mean	Grouping
Blanc (control)	9	0.5851	A
Clove	9	1.313	
Spearmint	9	0.923	A
Neg. control	9	0.715	A
Cinnamon	9	0.634	A

Treat	N	Mean	Grouping
Blanc (control)	9	0.4953	A
Spearmint	9	1.091	
Neg. control	9	0.987	A
Cinnamon	9	0.7546	A

Dunnett's test performed on the carbon assimilation rate (A) data confirms what has been described earlier in Figure 37, the clove treatment is significantly different compared to the rest at t=24h and 48h. At t=96h, the spearmint treatment is said to stand out compared to the other treatments. Although, Levene's test being unsatisfied for A data at t= 96h, this result cannot be considered significant but only considered as a possible representation of reality.

To conclude this section, it can be said that even if variations exists between the treatments, no dramatic drop in Fv/Fm or A has been observed. The plant's fitness therefore does not seem affected by the injection of EOs over this time lapse.

5. General discussion

The purpose of this project was to move forward on the development of a trunk-injected biopesticide using essential oils as active substances. To achieve this, three main objectives were set out.

Firstly, developing stable and bio-compatible emulsions was necessary. This objective can be considered met given the good results obtained not only in the preliminary tests[33] but also in later tests whereby the solution was correctly taken up by the plant. However, although this emulsion is bio-compatible, an effort to create a bio-based[34] emulsion should be looked into. Indeed, bio-based substitutes for both EDTA and Tween 80 can be found. Sucrose esters which are completely biodegradable and possess much lower critical micelle concentration[35] should be considered as an alternative to Tween 80 (Polat and Linhardt, 2001). A bio-based chelator that could replace EDTA in its role to chelate Ca^{2+} ions and hence reduce the occlusion of sieve plate pores is citric acid. Although no research has been found to prove the effect of citric acid on callose deposition in plants, many have been performed where the use of citric acid was compared to that of EDTA in its capacity to chelate Ca^{2+} ions in dentistry and other metals in depollution experiments (Scelza, Teixeira and Scelza, 2003; Turgut, Katie Pepe and Cutright, 2004; Di Palma and Mecozzi, 2007).

The second objective of this work was to analyze the kinetics of EOs contained and emitted by trunk-injected trees. This was possible due to methods which were sensitive enough to allow the quantification of most of the EO major components. Great difference in both emission and storage patterns in the leaves were observed. Cinnamaldehyde was found in the leaves but missing in the emissions, carvone accumulated in the leaves and was emitted always at a constant rate and finally limonene presented constant concentrations over time in both content and emissions.

The global content and emission profiles were also analyzed through PCA and ANOVA which allowed to better understand the different impacts of treating a tree with an EO emulsion. The exact impact of each oil on the volatile profile must yet still be established. Several ways methodologies could be looked into to achieve this. For instance, performing an ANOVA to analyze whether there is a significant difference between the concentrations of each dimension's top contributors (e.g. performing an ANOVA on caryophyllene concentrations, top contributors of the 3rd dimension). This second task however revealed that the greatest variance seems to exist between terpenic molecules, found to be increasingly released in stress conditions.

The last objective of the present study was to analyze which EO induced phytotoxicity. This was determined through maximum quantum efficiency of PSII and net carbon assimilation rates analysis. It was found that none of the tested oil negatively affected the plant's fitness in a significant way over this time period and at this concentration (0.5%). Indeed, looking at the carbon assimilation rates, one could even think that clove positively affected the plant (Table 17. Dunnett's tests). These results would not have been obtained with many other essential oils well-known for their herbicidal activity given their high phytotoxicity.

[33] i.e. Fv/Fm of plants injected with an EDTA and Tween 80 solution, as well as average particle size and polydispersity index of a cinnamon oil in Tween 80 emulsion.
[34] i.e. "wholly or partly derived from biomass, such as plants, trees or animals" (European Committee for Standardization)
[35] i.e. the concentration needed to solubilize hydrophobic molecules in water.

Over all, it is difficult to determine a “fits for all” essential oil to use as active substance in a pesticide as both cinnamon and spearmint, give interesting results. Indeed, trans-cinnamaldehyde (cinnamon oil’s major component) found in high concentrations in the leaves could be interesting against insects by ingestion toxicity. Indeed, aphids and psyllids, by piercing the leaf to reach the phloem, often pass through the xylem and would hence be in contact with the oil droplets. Secondly, there seems to be an additional release of α-farnesene by the plant when treated with this oil and because α-farnesene is known to repel the aphids (Warneys *et al.*, 2018), using this EO is all the more interesting for induced repellent activity. Similarly, spearmint oil also has its advantages. Indeed, carvone is found to be accumulated and emitted with time. Hence, spearmint could potentially have both ingestion toxicity and repellency activities at the same time. The effects and mode of actions of these EOs should be analyzed in greater depth to help in selecting the oil for further tests. Repellency effects could, for example, be assessed through a Y-tube olfactometer test (Mann *et al.*, 2012). These tests should be performed with pure standards of the major components as well as the oil itself in order to better understand synergistic or antagonistic effects taking place between the molecules in the oil.

6. Conclusion

Through the work hereby presented, stable and non-phytotoxic emulsions based on an EO emulsified with Tween 80 (and EDTA) have been developed. As part of the project these were successfully injected and uptaken by apple trees using a relatively simple and affordable device. The success of this work was proven by the detection and quantification of the injected EO’s major molecules present in the leaves and emitted by these through the use of analytical procedures allowing to be sensitive enough for the purpose of this study.

Results indicate that trans-cinnamaldehyde accumulated in the leaf with time with time but that it was not emitted by cinnamon oil treated trees. Limonene’s content and its emission rate remained constant for spearmint and all other treated trees. Carvone on the other hand increased in the content and was emitted at a constant rate for spearmint treated trees. Finally, concerning clove oil treated trees, eugenol has not been found in neither the content nor the emissions. This work therefore shows that, out of three EOs tested, cinnamon and spearmint oils revealed good potential insecticidal and repulsive effects. Further lab and field works are necessary to confirm these promising results

To conclude it can therefore be said that this work was a real stepping stone in the “Treeinjection” project and proved that EO based pesticides could be injected in trees, an emerging concept to the authors’ knowledge. Furthermore, these EO emulsions proved to have potential ingestion toxicity and repulsive effects at a relevant biological concentration. This work therefore opens the road to the development of promising new types of biopesticides.

7. Perspectives

The first short-term perspective is a more-in-depth characterization of the emulsion used through characterizing the viscosity, the thermodynamic stability (e.g. heating-cooling cycle) and kinetic stability (e.g. keeping the emulsion at room temperature and measuring at different time interval the particle sizes) (Ghosh *et al.*, 2013a). Secondly, determining the exact limits of quantification and detection in order to decrease the linearity range as much as possible would help in the quantification of components found at very low concentrations (e.g. cinnamaldehyde leaf-content at t=24h). These LOQs and LODs could also be lowered, for example by working in single-ion monitoring or with more

sensitive instruments such as a gas chromatography triple quadrupole tandem mass spectrometer (GC-QQQ MS/MS).

An important aspect needed from future studies is to increase the number of replicas (to reach a minimum of five repetitions) for the analysis of content and emission. This would allow to perform further statistical analysis and to increase the robustness of the trends observed in this work. Similarly, a repeated ANOVA model should be performed on the phytotoxicity measurements and VOC profiles. This would allow not only to detect differences between treatments but also between times. Additional sampling times could also be added to the experimental design to reduce the time interval between each sampling. This would enable to establish a more thorough vision of the oil's behavior in terms of storage in the leaves and emissions but also in terms of phytotoxicity. Other concentrations should be tested in order to establish the ideal concentration, i.e. the injected concentration which allows the same efficiency than the concentrations tested in biological tests by the UCLouvain partner. Finally, it is important to remind that the final purpose of this research is to develop a product against both the rosy apple aphid and the pear psylla. These experiments should be repeated on pear trees to examine and compare their VOC content and emissions to that of apple trees.

Before the commercialization of this biopesticide the following key aspects must also be analyzed.

First of all, tests in a controlled environment, where aphids are placed on apple trees (and psyllas on pear trees) should be performed. This would allow to test more realistically the hypothetical efficiency of such products. Furthermore, the injection apparatus should be revised to adapt to larger trees than the ones used in this work to scale up the system.

Following this work, field trials should be performed. These would allow to better understand the products real efficiency and should help in:

- Understanding the effect of abiotic factors on the product's efficiency (effect of wind on repellency, extreme temperatures on emission rates, etc…).
- Understanding how the tree's age could impact the EO's storage and emission.
- Establishing ideal application modalities:
    - Finding the exact moment of application, i.e preventive measure (e.g. before the imaginal molt) or once the adult founders have already colonized the leaves.
    - Finding the number of applications for a guaranteed efficiency.
- Assessing the effect of such product on other species living in orchards (e.g. bee pollinators or even syrphids, aphid's natural predators).

The product could also be tested as part of an integrated pest management program for improved regulation of this pest.

Finally, the product's administration should be of economic interest for the fruit producer. Both cost of production and cost of implementation of this emulsified product should be investigated. The impact of such administration on fruit production should be analyzed (e.g. yield modifications and traces in fruits).

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9. Annex

Annex 1. Inhibitory activities observed (or not) for several EOs (Hori, 2002)

Essential oils	Items of investigation[a]				
	AFA	REP	SIC	SIN	TOX
(Group 1)					
Thyme oil	○	○	○	○	○
Spearmint oil	○	○	○	○	×
Peppermint oil	○	○	○	×	×
Lavender oil	○	○	○	×	×
(Group 2)					
Pennyroyal oil	○	×	○	○	○
Mint oil	○	×	○	×	×
Marjoram oil	○	×	○	×	×
Basil oil	○	×	○	×	×
(Group 3)					
Rosemary oil	×	○	○	×	×
(Group 4)					
Sage oil	×	×	○	×	×

[a] AFA: antifeeding activity in the EMIF; REP: repellency in the olfactometer test; SIC: settling inhibition in the choice test; SIN: settling inhibition in the no-choice test; TOX: toxicity in the toxicity test.
○: the activity was clearly observed in the test; ×: the activity was not clearly observed in the test.

Annex 2. Standard specifications of AkseBio2 (Erler, Yegen and Zeller, 2007)

Formulation type	EC 7 (liquid)
Ingredient (70 ml [AI]/liter)	Aromatic (thyme, *Thymbra spicata* var. *spicata* L.; oregano, *Origanum syriacum* var. *bevanii* (Holmes); and anise, *Pimpinella anisum* L. essential oils)[b] and edible (sesame oil and maize oil) plant extracts; essential oil components (carvacrol, thymol and anethole); a natural emulsifier; and a bacterium, TR 2000
Appearance	Yellow to light brown
Smell	Thyme-like odor
Suspensibility	Completely suspensible in water
Toxicity to nontarget organisms	Low to moderate, depending on species and life stage (Erler 2004a)
Shelf life	Two years, when stored under proper conditions and without opening the lid
Recommended storage conditions	Store in a cool, dark, and dry place

[a] Some of the specifications were determined after a 4-yr study.
[b] The plant materials used as the source of essential oils originated in Antalya.

Annex 3. LD_{50} of the clove EO and its main components compared to the conventional pesticide abamectin when test in laboratory against C. chinesis (Tian *et al.*, 2015)

Table 2. Probit analysis of the insecticidal activity of the clove essential oil and its constituents against the summer adults of *Cacopsylla chinensis*

Treatment	LD_{50} (μg/adult)	95% Fiducial limit	Slope ± SE	χ^2	*P* value
Clove essential oil	0.730	0.676–0.755	1.742 ± 0.101	3.191	1.000
Eugenol	0.673	0.657–0.705	1.641 ± 0.183	4.313	1.000
Eugenol acetate	9.266	8.340–10.295	1.135 ± 0.252	15.465	0.894
β-Caryophyllene	0.708	0.641–0.799	1.186 ± 0.166	7.966	0.999
Abamectin	0.017	0.015–0.018	1.770 ± 0.172	9.923	0.992

Table 3. Probit analysis of the insecticidal activity of the clove essential oil and its constituents against the nymphs of *C. chinensis*

Treatment	LD_{50} (μg/nymph)	95% Fiducial limit	Slope ± SE	χ^2	*P* value
Clove essential oil	1.795	1.782–1.819	1.092 ± 0.141	5.983	1.000
Eugenol	1.668	1.410–1.842	0.253 ± 0.133	7.233	0.999
Eugenol acetate	9.942	8.911–10.987	0.932 ± 0.230	16.914	0.892
B-Caryophyllene	1.770	1.595–1.941	1.241 ± 0.133	6.432	1.000
Abamectin	0.021	0.019–0.023	1.363 ± 0.166	9.475	0.994

Annex 4. Numerical phytotoxicity rating scale (Cloyd and Cycholl, 2002)

Score	Description
0	No visible injury
1	Light injury, no influence on marketability
2	Moderate injury, 50% foliar injury, reduced market quality
3	Complete foliar injury, >75% foliar injury, not marketable

*Annex 5. Characterization of cinnamon (*Cinnamomum cassia *J.Presl*) EO. The name, identification score, CAS number and relative area (%) are presented.

Name	Score (Lib)	CAS	Relative area (%)
Benzaldehyde	90.59	100-52-7	0.75
Benzaldehyde, 2-hydroxy-	85.01	90-02-8	0.24
Phenylethyl Alcohol	85.89	60-12-8	0.18
Benzenepropanal	93.23	104-53-0	0.60
Benzofuran, 2-methyl-	87.02	4265-25-2	0.20
Cinnamaldehyde, (E)-	95.47	14371-10-9	0.44
Benzaldehyde, 2-methoxy-	93.18	135-02-4	0.39
Acetic acid, 2-phenylethyl ester	93.29	103-45-7	0.21
Cinnamaldehyde, (E)-	98	14371-10-9	91.22
Copaene	91.68	3856-25-5	0.55
2-Propenal, 3-phenyl-	82.98	104-55-2	0.01
2-Propenal, 3-phenyl-	80.65	104-55-2	0.00
Caryophyllene	89.78	87-44-5	0.23
Acetic acid, cinnamyl ester	95.36	103-54-8	2.29
Acetic acid, cinnamyl ester	81.01	103-54-8	0.01
(1S,4aR,8aS)-1-Isopropyl-7-methyl-4-methylene-octahydronaphthalene	82.39	6980-46-7	0.13
Butylated Hydroxytoluene	89.6	128-37-0	0.39
Naphthalene-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	84.6	483-76-1	0.19
2-Propenal, 3-(2-methoxyphenyl)-	97.06	1504-74-1	1.68
2-Propenal, 3-(2-methoxyphenyl)-	88.09	1504-74-1	0.02
2-Propenal, 3-(2-methoxyphenyl)-	82.31	1504-74-1	0.12
2-Propenal, 3-(2-methoxyphenyl)-	83.49	1504-74-1	0.07
2-Propenal, 3-(2-methoxyphenyl)-	84.81	1504-74-1	0.08
2-Propenal, 3-(2-methoxyphenyl)-	80.22	1504-74-1	0.01
Total			**100.00**

*Annex 6. Characterization of spearmint (*Mentha spicata *L.)* EO. The name, identification score, CAS number and relative area (%) are presented.

Name	Score (Lib)	CAS	Relative area (%)
Ethyl ether	81.67	60-29-7	
Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-	90.35	4889-83-2	0.86
Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	92.58	3387-41-5	0.23
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	92.87	18172-67-3	1.34
.beta.-Myrcene	94.27	123-35-3	1.33
Acetic acid, hexyl ester	89.91	142-92-7	0.21
(+)-4-Carene	83.46	29050-33-7	0.12
o-Cymene	91.21	527-84-4	0.23
D-Limonene	95.74	5989-27-5	25.28
.gamma.-Terpinene	92.26	99-85-4	0.25
Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-,	88.97	15537-55-0	0.27
Cyclohexene, 1-methyl-4-(1-methylethylidene)-	81.96	586-62-9	0.15
Linalool	82.17	78-70-6	0.02
3-Octanol, acetate	88.16	4864-61-3	0.12
Limonene oxide, trans-	82.77	4959-35-7	0.08
Cyclohexanone, 5-methyl-2-(1-methylethyl)-, cis-	90.73	491-07-6	0.13
Cyclohexanemethanol, .alpha.,.alpha.-dimethyl-4-methylene-	84.64	7299-42-5	0.10
Levomenthol	97.34	2216-51-5	0.52
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	93.68	20126-76-5	0.72
.alpha.-Terpineol	93.61	98-55-5	0.25
Cyclohexanone, 2-methyl-5-(1-methylethenyl)-, trans-	93.55	5948-04-9	1.86
Cyclohexanone, 2-methyl-5-(1-methylethenyl)-, trans-	92.84	5948-04-9	0.23
trans-Carveol	89.07	1197-07-5	0.15
Carveol	86.82	99-48-9	0.06
Carvone	93.77	99-49-0	57.78
2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl)-	85.22	89-81-6	0.60
(-)-Carvone	87.19	6485-40-1	0.05
Carvone	89.51	99-49-0	0.02
Carvone	87.73	99-49-0	0.01
3-hydroxy-2-methyl-5-(prop-1-en-2-yl)cyclohexanone	85.73	1000365-66-6	0.08
(-)-Carvone	83.14	6485-40-1	0.00
2H-1-Benzopyran, 3,4,4a,5,6,8a-hexahydro-2,5,5,8a-tetramethyl-	81.80	41678-32-4	0.15
Dihydrocarvyl acetate	95.79	20777-49-5	0.28
1,3,6-Heptatriene, 2,5,6-trimethyl-	81.89	42123-66-0	0.04
2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, acetate, cis-	94.47	1205-42-1	0.22
(-)-.beta.-Bourbonene	94.35	5208-59-3	1.72
Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-,	91.48	515-13-9	0.14
Caryophyllene	96.81	87-44-5	1.57
NA	92.17	18252-44-3	0.25
(S,1Z,6Z)-8-Isopropyl-1-methyl-5-methylenecyclodeca-1,6-diene	93.13	317819-80-0	0.18
Cyclohexene, 4-(1,5-dimethyl-1,4-hexadienyl)-1-methyl-	88.31	17627-44-0	0.20
(E)-.beta.-Famesene	94.57	18794-84-8	0.41
Germacrene D	89.84	23986-74-5	0.14
.gamma.-Muurolene	84.54	30021-74-0	0.05
Germacrene D	95.26	23986-74-5	0.71
(S,1Z,6Z)-8-Isopropyl-1-methyl-5-methylenecyclodeca-1,6-diene	90.50	317819-80-0	0.19
Butylated Hydroxytoluene	90.77	128-37-0	0.31
Naphthalene, -hexahydro-4,7-dimethyl-1-(1-methylethyl)	90.89	483-76-1	0.16
Caryophyllene oxide	83.74	1139-30-6	0.13
1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-,	89.96	552-02-3	0.08
Total			100.00

*Annex 7. Characterization of clove (*Syzygium aromaticum *(L.) Merrill & Perry)* EO. The name, identification score, CAS number and relative area (%) are presented.

Name	Score (Lib)	CAS	Relative area (%)
cis-Muurola-4(15),5-diene	81.54	157477-72-0	0.029
Eugenol	96.1	97-53-0	77.691
Eugenol	86.5	97-53-0	0.110
Eugenol	81.77	97-53-0	0.101
Eugenol	87.84	97-53-0	0.016
Caryophyllene	97.73	87-44-5	10.841
Phenol, 2-methoxy-4-(1-propenyl)-, (Z)-	81.64	5912-86-7	0.022
Eugenol	89.08	97-53-0	0.005
Phenol, 2-methoxy-4-(1-propenyl)-	84.36	97-54-1	0.001
Humulene	93.09	6753-98-6	0.382
Phenol, 2-methoxy-4-(1-propenyl)-	81.06	97-54-1	0.003
Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl-, methylcarbamate	89.55	1918-11-2	0.189
Naphthalene,hexahydro-4,7-dimethyl-1-(1-methylethyl)-,	81.83	483-76-1	0.148
Phenol, 2-methoxy-4-(1-propenyl)-, acetate	94.62	93-29-8	10.069
Phenol, 2-methoxy-4-(1-propenyl)-, (Z)-	90.13	5912-86-7	0.016
Phenol, 2-methoxy-4-(1-propenyl)-, (Z)-	82.49	5912-86-7	0.012
Caryophyllene oxide	90.88	1139-30-6	0.362
Phenol, 2-methoxy-4-(1-propenyl)-	82.01	97-54-1	0.003
			100.000

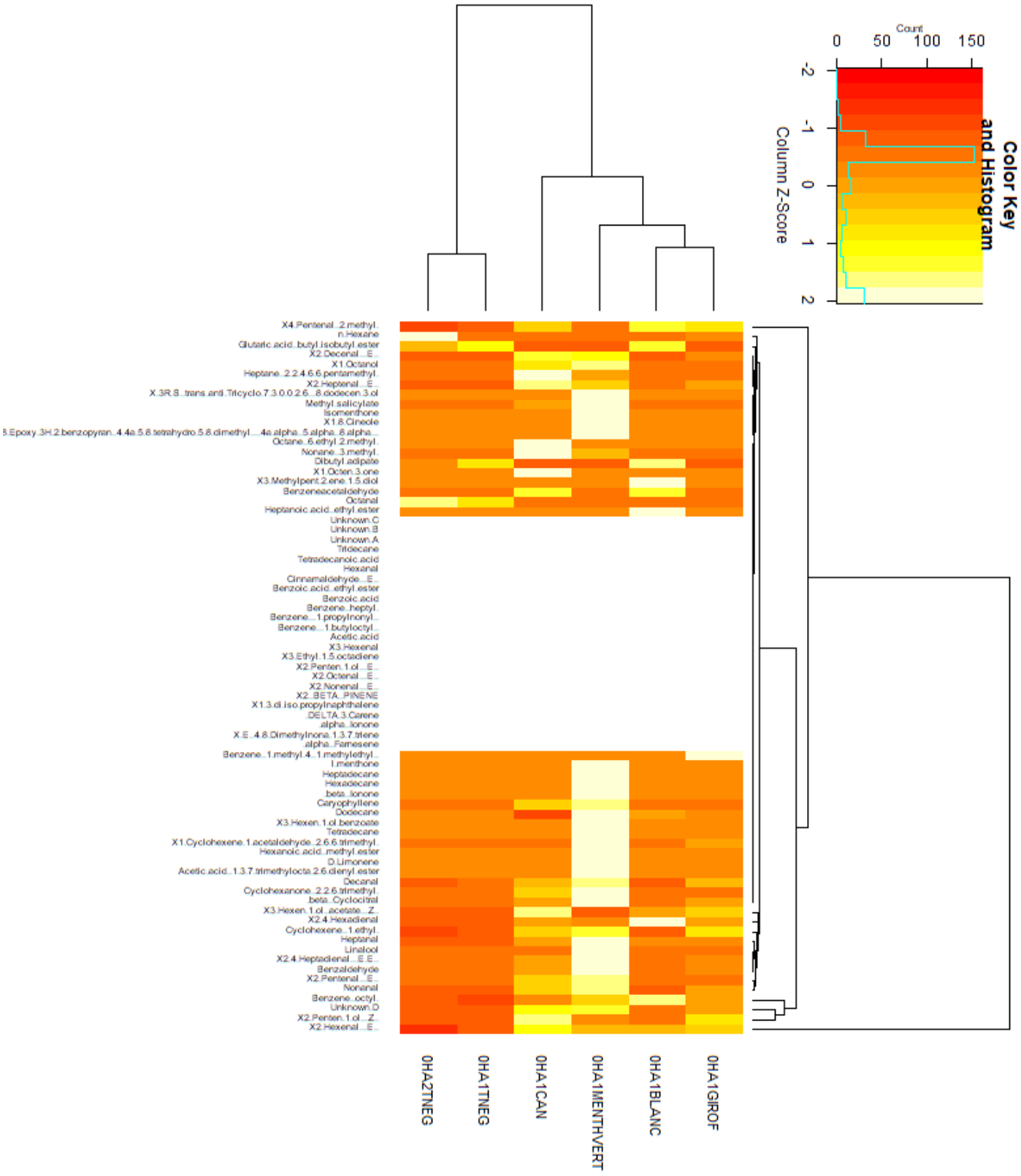
Annex 8. List of phytosanitary products used on one-year-old apple trees and the pests aimed by the product

Phytosanitary product	Supplier	Aimed pest
Tracer	Corteva	Thrips
Mesurol	Bayer	Thrips
Corbel	BASF	Oïdium
Difcor	Globachem	Oïdium
Dimilin	Floraservis	Groundflies
Curater	Bayer	Groundflies

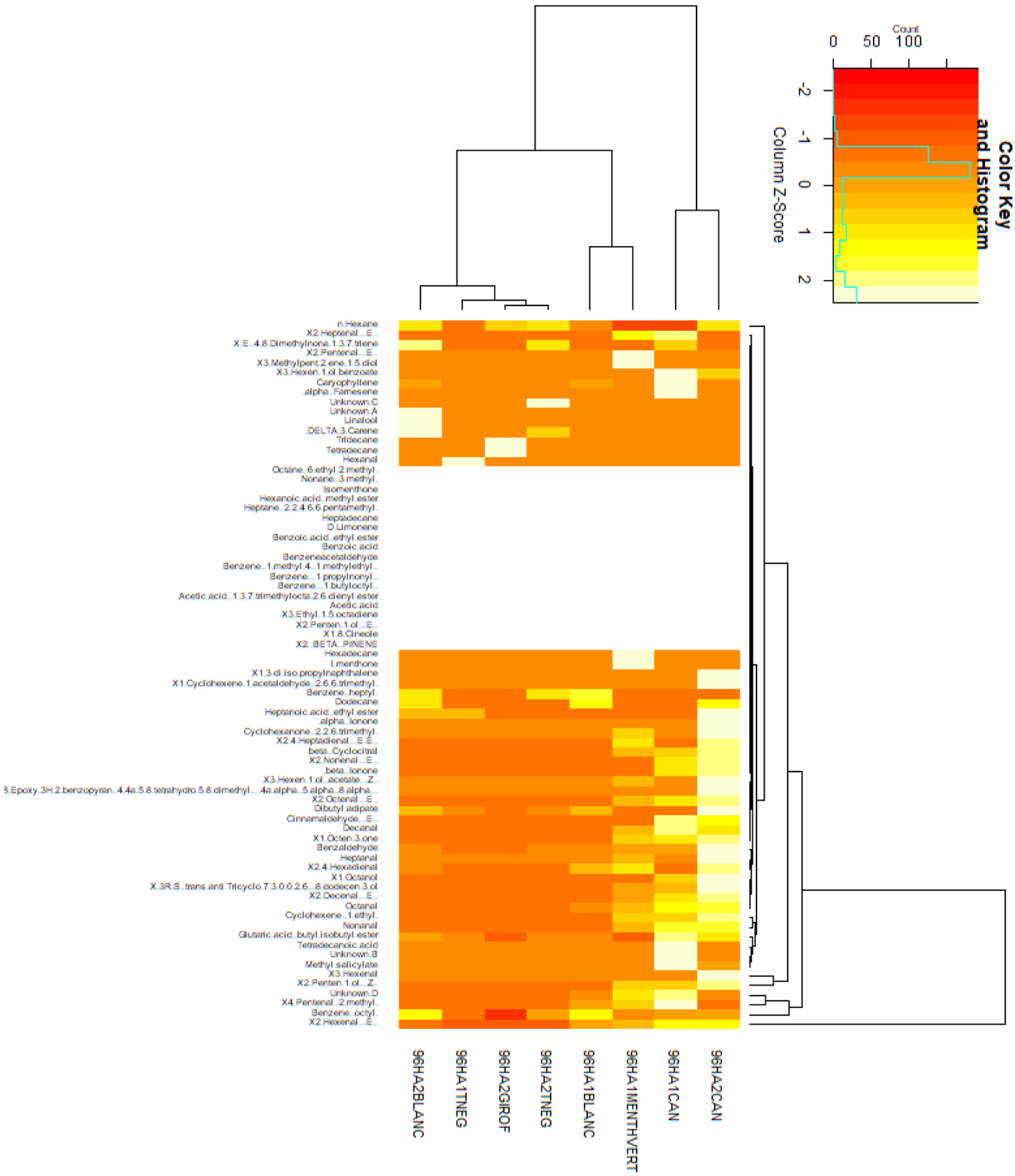
Annex 9. List of phytosanitary products used on two years old apple trees and the pests aimed by the product

Phytosanitary product	Supplier	Aimed pest
Confidor	Bayer	Mites
Spruzit	Neudorff	Mites
Admiral	Sumitomo Chemical	Cochineal
Calypso	Bayer	Aphids

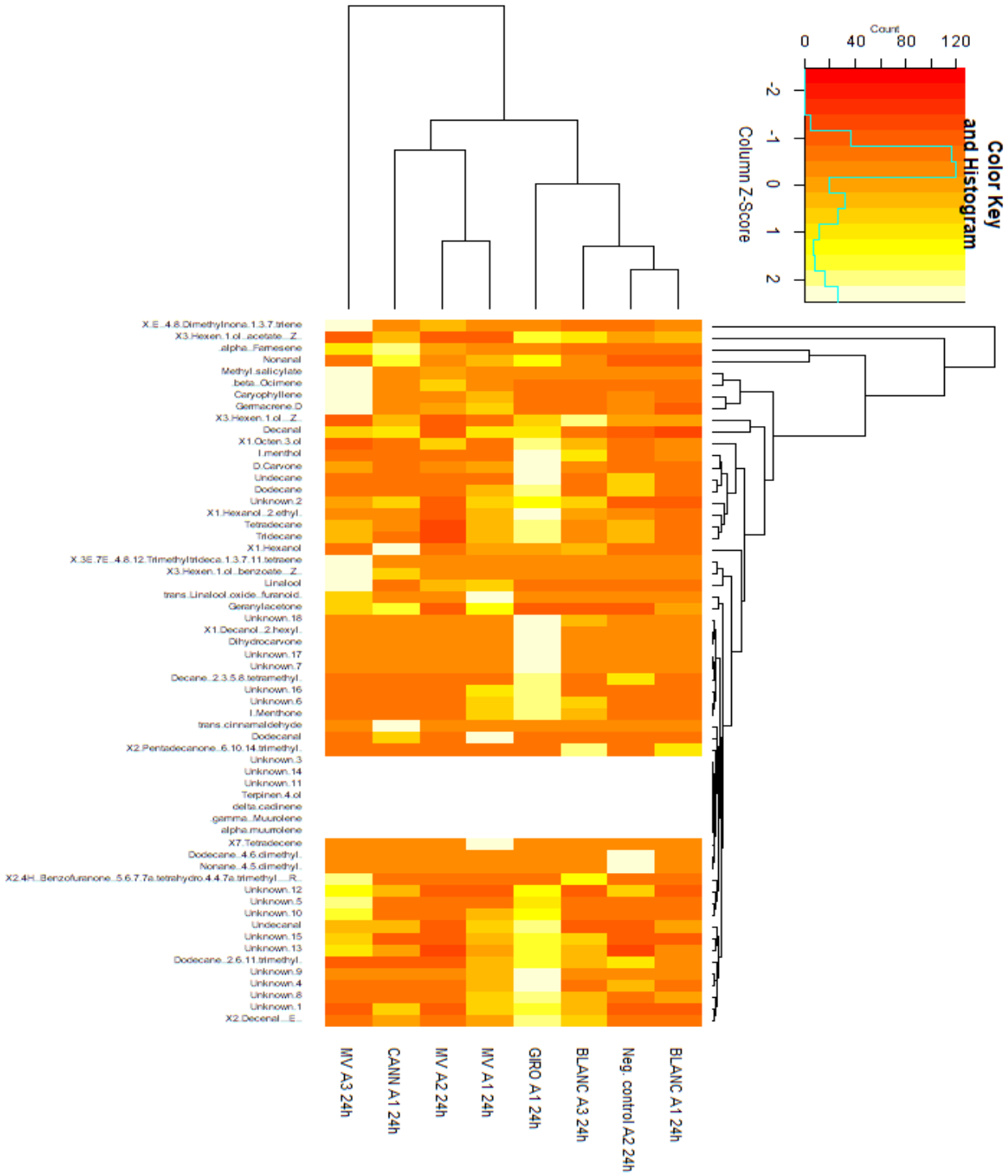
Annex 10. Normalised heatmap for the leaf content samples at t= 0h. Legend: "Girof" = Clove treat., "Blanc" = Blanc treat., "Menthevert"= Spearmint treat., "Can" = Cinnamon treat., "TNEG"= Negative control.



Annex 11. Normalised heatmap for the leaf content samples at t= 96h. Legend: "Girof" = Clove treat., "Blanc" = Blanc treat., "Menthevert"= Spearmint treat., "Can" = Cinnamon treat., "TNEG"= Negative control.



Annex 12. Normalised heatmap for the emission samples at t= 24h. Legend: "Giro" = Clove treat., "Blanc" = Blanc treat., "MV" = Spearmint treat., "Cann" = Cinnamon treat., "Neg. control" = Negative control.



Annex 13. Normalised heatmap for the emission samples at t= 96h. Legend: "Giro" = Clove treat., "Blanc" = Blanc treat., "MV" = Spearmint treat., "Cann" = Cinnamon treat., "Neg. control" = Negative control.