LIÈGE université
Gembloux
Agro-Bio Tech

Essential oils as potential botanical insecticide against rosy apple aphid (*Dysaphis plantaginea* P.) by trunk injection

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Year: 2022

FRENCH COMMUNITY OF BELGIUM

UNIVERSITY OF LIÈGE – GEMBLOUX AGRO-BIO TECH

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Original essay for graduation as doctor in agricultural sciences and biological engineering

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Abstract

The extensive use of synthetic pesticides leads to risks for both human health and ecosystems due to the non-target toxicity and stability they present. Natural molecules are investigated to achieve more sustainable production methods and meet societal/consumer's expectations. The present thesis focuses on apple production and control of its most detrimental pests in orchards the Rosy Apple Aphid (RAA), *Dysaphis plantaginea* Passerini, using essential oils (EOs). This work was realised as part of a larger project (Tree-Injection) whose aim was to use EOs as a botanical insecticide combined with trunk injection. This project presents an innovative and original approach to implementing practical application of biopesticide.

Due to their large spectra of biological activities EOs have been actively investigated as an alternative to numerous synthetic biocide products. However, apart from herbicidal applications their phytotoxic properties are a major drawback to their applicability as biopesticides for sustainable agriculture. The first part of the present thesis synthesises the cellular and functional impacts of EOs leading to phytotoxicity. Physiological disturbances and their putative molecular targets are described. New opportunities regarding the development of biopesticides are discussed, including biostimulation and defence elicitation properties occurring below the phytotoxicity threshold.

The phytotoxic properties of *Cinnamomum cassia* EO (CEO) on apple tree (*Malus x domestica* Borkh) was studied in terms of oxidative burst (glutathione redox state) and oxidative damage on lipids (malondialdehyde). A rapid/strong oxidative burst occurred at high CEO concentrations, decreasing the reduced glutathione content in in the leaves. This burst is followed by apparition of oxidative damage, as suggested by an increase in malondialdehyde. Furthermore, at lower concentrations, induction of systemic defence was investigated by following the gene expression level of specific defence pathways (PR proteins, secondary metabolism, oxidative stress, parietal modifications). Hence, these findings help to draft innovative pest management strategies that consider both the risks (phytotoxicity) and benefits (defence activation combined with direct biocide properties) of biopesticides based on EOs.

Due to EO rapid degradation in the environment and their volatility, the potential to use alternative methods of application, such as trunk injection was explored in this thesis. Systemic translocation of EOs through the xylem following the injection of *Cinnamomum cassia* and *Mentha spicata* nanoemulsions in plant's vascular system was demonstrated by targeted volatile organic compounds (VOCs) analyses. Given systemic translocation, increased production and release of biogenic VOCs, and absence of phytotoxicity, this work can be seen as proof of concept for the use of EOs with trunk injection.

Finally, laboratory and field trials assayed the potential to control RAA based on CEO trunk injection. Laboratory trials demonstrated promising results and were scaled up during two-year field trials on trees of the 'Jonagold' cultivar. Considering the pest life cycle, injections were applied as curative (during the vegetative stage) and preventive treatments (at budburst). RAA population dynamics (number of colonies and aphids) were tracked in addition to other predator and pest populations. Given their importance, tree physiology and the emission of VOCs were followed. Total and commercial apple yield were estimated, in addition to the absence of *trans*-cinnamaldehyde residue (main compound in EO) in fruit. The final part of this thesis investigates the practical feasibility of laboratory-effective solutions in agronomic conditions and identifies challenges and limitations that need to be addressed.

Globally, this thesis furthers the potential of biopesticides based on EOs in fruit arboriculture and highlights a fruitful research perspective for pest control compatible with integrated pest management (IPM).

Keywords: Apple, Rosy Apple Aphid (RAA), essential oils (EOs), trunk injection, *trans*-cinnamaldehyde, DHS-TDU-GC-MS

Résumé

L'utilisation intensive de pesticides de synthèse entraîne des risques pour la santé humaine et les écosystèmes en raison de leur toxicité non ciblée et de leur stabilité. Les molécules naturelles sont étudiées afin de parvenir à des méthodes de production plus durables et de répondre aux attentes de la société et des consommateurs. La présente thèse se concentre sur la production de pommes et le contrôle de son ravageur le plus nuisible dans les vergers, le puceron cendré du pommier (RAA), *Dysaphis plantaginea* Passerini, en utilisant des huiles essentielles (HE). Ce travail a été réalisé dans le cadre d'un projet plus large (Tree-Injection) dont le but était d'utiliser les HE comme insecticide botanique combiné à l'injection dans le tronc. Ce projet présente une approche innovante et originale pour mettre en œuvre l'application pratique des biopesticides.

En raison de leur large spectre d'activités biologiques, les HE sont activement étudiées comme alternative à de nombreux produits biocides synthétiques. Cependant, en dehors de l'application herbicide, leurs propriétés phytotoxiques sont un inconvénient majeur à leur applicabilité comme biopesticides pour l'agriculture durable. La première partie de la présente thèse synthétise les impacts cellulaires et fonctionnels des HE conduisant à la phytotoxicité. Les perturbations physiologiques et leurs cibles moléculaires putatives sont décrites. De nouvelles opportunités concernant le développement de biopesticides sont discutées, y compris la biostimulation et les propriétés d'élicitation de défense se produisant en dessous du seuil de phytotoxicité.

Les propriétés phytotoxiques de l'HE de *Cinnamomum cassia* (CEO) sur le pommier (*Malus x domestica* Borkh) ont été étudiées en termes de stress oxydatif (état redox du glutathion) et de dommages oxydatifs sur les lipides (malondialdéhyde). Un stress oxydatif rapide et fort s'est produit à des concentrations élevées de CEO, diminuant la teneur en glutathion réduit dans les feuilles. Cette explosion est suivie par l'apparition de dommages oxydatifs comme le suggère l'augmentation du malondialdéhyde. De plus, à des concentrations plus faibles, l'induction de la défense systémique a été étudiée en suivant le niveau d'expression des gènes de voies de défense spécifiques (protéines PR, métabolisme secondaire, stress oxydatif, modification pariétale). Ces résultats aident donc à élaborer des stratégies innovantes de lutte contre les ravageurs en tenant compte à la fois des risques (phytotoxicité) et des avantages (activation de défense combinée aux propriétés biocides directes) des biopesticides à base d'HE.

En raison de la dégradation rapide des HE dans l'environnement et de leur volatilité, le potentiel d'utilisation de méthodes alternatives d'application, telles que l'endothérapie végétale ou injection dans le tronc, a été exploré dans cette thèse. La translocation systémique des HE à travers le xylème suite à l'injection de nanoémulsions de

Cinnamomum cassia et *Mentha spicata* dans le système vasculaire des plantes a été démontrée par des analyses ciblées des composés organiques volatils (COVs). Compte tenu de la translocation systémique, de la production et de la libération accrues de COVs et de l'absence de phytotoxicité, ce travail peut être considéré comme une preuve de concept pour l'utilisation des HE par injection dans le tronc.

Enfin, des essais en laboratoire et sur le terrain ont permis d'évaluer le potentiel de contrôle du RAA basé sur l'injection de CEO dans le tronc. Les essais en laboratoire ont montré des résultats prometteurs et ont été étendus par des essais « terrains » pendant deux ans sur des arbres du cultivar Jonagold. En tenant compte du cycle de vie du ravageur, les injections ont été appliquées comme traitements curatifs (pendant le stade végétatif) et préventifs (au débourrement). La dynamique des populations du RAA (nombre de colonies et de pucerons) a été suivie ainsi que celle de ces prédateurs et ravageurs. Compte tenu de leur importance, la physiologie des arbres et les émissions de COVs ont été suivies. Enfin, le rendement total et le rendement commercial en pommes ont été estimés ainsi que l'absence de résidus de *trans*-cinnamaldéhyde (composé principal de l'HE) dans les fruits. La dernière partie de cette thèse étudie le transfert de solutions efficaces en laboratoire à des conditions agronomiques réalistes et identifie les défis et les limitations de la technique.

Globalement, cette thèse met en avant le potentiel des biopesticides à base d'HE dans l'arboriculture fruitière et souligne les perspectives de recherche fructueuses pour le contrôle des ravageurs compatibles avec la lutte intégrée (IPM).

Mots-clés : Pomme, puceron cendré du pommier (RAA), huile essentielle (HE), injection dans le tronc, *trans*-cinnamaldéhyde, DHS-TDU-GC-MS.

The Apple Orchard

Borgeby-Gård (translated by Alan Tucker)

Come just after the sun has gone down, see

the deepening green of the new-mown ground;

it's nothing, no more than any of us can be

when we recollect an awareness we've found

stored in a heightened sense of memory,

new hopes, half-forgotten things we know

obscurely working from within, secretly

regaining thoughts scattered long ago

like windfalls under these Durer trees

that bring us through hundreds of working days

back into the abundant yield of their fruitfulness,

a patient reserve held in undeniable ways

whatever dimension we have over-stepped

they lift us superbly with renewed delight

when through a long life we come to accept

their willingness to grow in peace and quiet.

Remerciements

Au terme de cette thèse, j'aimerais remercier toutes les personnes sans qui la réalisation de ce travail n'aurait pas été possible.

Tout d'abord je tiens à remercier ma promotrice Marie-Laure Fauconnier ainsi que Thierry Hance pour m'avoir fait confiance dans le cadre du projet de recherche dans lequel s'est inscrit ce travail de thèse. Je tiens en outre à remercier mon encadrante pour sa disponibilité et pour les nombreuses discussions scientifiques que nous avons eu, et ce malgré ces très nombreuses autres responsabilités. Un très grand merci également aux membres de mon comité de thèse et aux membres de mon jury pour leur disponibilité et leurs nombreux conseils qui ont permis la réalisation et l'amélioration de cette thèse.

Je tiens également à exprimer ma plus sincère gratitude aux étudiants que j'ai eu la chance de pouvoir encadrer et suivre aux quotidiens dans la réalisation de leur travail de fin d'étude respectif à savoir Clément Burgeon, Laura Lheureux, Thomas Dhont et Jean Bouvry. En effet, ils ont tous montré une abnégation bien supérieure à la volonté d'acquisition de leur diplôme et ce fut un réel plaisir de travailler avec eux. Je tiens également à remercier les stagiaires que nous avons eu le plaisir d'accueillir Arnaud Duchene et Tatiana Lima.

Je remercie également tous mes collègues du Laboratoire de Chimie des Molécules Naturelles avec qui j'ai eu le plaisir de travailler et pour tous les bons moments passés ensemble. Merci à Danny, Manon, Thomas, Matthew, Chloé, Estelle, Clément… pour les courses à pied/vélo, pour les séances de dégustation de café. Merci à Fatimata et Évelyne pour leur bonne humeur à toute épreuve et pour les bons moments passés ensemble. Un énorme merci à Franck, Danny et Thomas pour leur grande aide technique. Merci également à Franck pour son aide et les dépannages GC. Enfin merci à Fabienne et Danny pour les nombreux dépannages administratifs.

Je tiens également à remercier Thierry Hance et toute l'équipe du Laboratoire d'écologie des interactions et contrôle biologique pour leur accueil. Un merci particulier à Guillaume Le Goff pour la grande synergie que nous avons eu tout au long de ce projet.

Je tiens également à remercier Marc Lateur et l'équipe du laboratoire de Ressources Génétiques Fruitières du Centre wallon de Recherches agronomiques. Un merci particulier à Alexis Jorion et Alain Rondia pour leur aide dans les vergers.

Et enfin, je souhaite dédier ce travail à Florence, à ma famille et à mes amis. Merci de m'avoir soutenu, motivé et encouragé tout haut long de la montagne russe émotionnelle parfois solitaire que constitue une thèse.

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List of Abbreviations

AChE	Acetylcholinesterase
ANOSIM	Analysis of similarity
AsA-GSH	Ascorbate-glutathione
A.S.	Active substance
ASM	Acibenzolar-S-methyl
ATP	Adenosine triphosphate
CAT	Catalase
Chl	Chlorophyll
CIS	Cooled Injection System
DAI	Days after injection
AsA-DHA	Ascorbate-dehydroascorbate
DHS	Dynamic headspace sampling
DMNT	(*E*)-4,8-dimethyl-nonatriene
DTT	Dithiothreitol
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact
EL	Electrolyte leakage
(C)EO(s)	(Cinnamon) essential oil(s)
EOC	Essential oil constituents
ET0/RC	Electron transport flux
F0	Basal fluorescence
FAR	(E,E)- α-farnesene synthase
FPPS	Farnesyl pyrophosphate synthase
Fv/Fm	Maximum yields of photosystem II
GABA	γ-Aminobutyric acid
GAs	Gibberellins
GC	Gas chromatography
GLMs	Generalised linear models
GLV	Green leaf volatiles
GRAS	Generally recognised as safe
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H_2O_2	Hydrogen peroxide

HPLC	High performance liquid chromatography
IPM	Integrated pest management
ISO	International organisation for standardisation
ISR	Induced systemic resistance
JA	Jasmonic acid
LOD	Limit of detection
LOQ	Limit of quantification
LOX	Lipoxygenase
MBB	Monobromobimane
MDA	Malondialdehyde
MEP	Methylerythritol phosphate
MOA	Mode of action
MS	Mass spectrometer
MVA	Mevalonate
PAL	Phenylalanine ammonia-lyase
PAR	Photosynthetically active radiation
PCA	Principal component analysis
PCD	Programed cell death
PECT	Pectin methyl esterase
PERMANOVA	Permutational multivariate analysis of variance
PI ABS	Performance index
POX	Peroxidase
PPP	Plant protection product
PR proteins	Pathogenesis-related proteins
PS	Photosystem
QTLs	Quantitative trait loci
RAA	Rosy apple aphid
R^2	Correlation coefficient
RH	Relative humidity
RIs	Retention indexes
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SOD	Superoxide dismutase
TAT	Tyrosine aminotransferase
TC	*Trans*-cinnamaldehyde
TDU	Thermal desorption unit
TFI	Treatment frequency index

TMTT	(E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene
TRX	Thioredoxin
(B)VOCs	(Biogenic) Volatile organic compounds

1

General Introduction

1. Apple origin, cultivation and orchard production system

Apple trees belong to the genus *Malus* of Maloideae subfamily and the Rosaceae family. Maloideae includes agronomic and ornamental species (apple, pear, quinces and hawthorns) distributed worldwide, mainly in temperate regions (Velasco et al., 2010). More than 30 species of apple exist and can be easily hybridised (Hancock et al., 2008; Korban, 1986).

The commonly cultivated apple is an interspecific hybrid named *Malus x domestica* Borkh. The cultivation origin is hard to determine but dates back at least 2,500 years ago. Domestication occurred in Central Asia from wild apple *Malus sieversii* (Ledeb.) M. Roem (Cornille et al., 2014). Hybridisation with other wild apple species such as *Malus sylvestris* L., *M. orientalis* Uglitzk., *M. baccata* (L) Borkh, and *M. prunifolia* Borkh occurred along it's spread to the west following trade road forming a complex of interspecific hybrids (Coart et al., 2006; Cornille et al., 2012, 2019; Harris et al., 2002).

Desirable traits were selected, and budding and grafting occurred as soon as 2,000 years ago (Janick & Moore, 1996). Today, more than 6,000 cultivars exist across the world. However, most fruit production relies on a few cultivars developed during the twentieth century in Europe, North America and Asia. Those cultivars include 'Delicious', 'Golden Delicious', 'Granny Smith', 'Fuji' and 'Gala' representing 60% of world production (O'Rourke, 2003). In Belgium, apple production of 'Jonagold' (a hybrid between 'Golden Delicious' x 'Jonathan') comprises 60% of the total apple production (APAQ-w).

In 2018, worldwide apple production reached 85.9 million tonnes from 4.6 million hectares (Figure 1). In Europe (EU28), total apple production accounted for 13.8 million tonnes. Belgium's production in the same year reached 231,300 tonnes of apples on 5990 Ha (*FAOSTAT*, 2020). Apart from its economic importance, an interesting trend can be observed from this figure regarding the intensification of production. Indeed, production continuously increases, whereas harvested area has decreased since the late 1990s, implying a drastic yield increase.

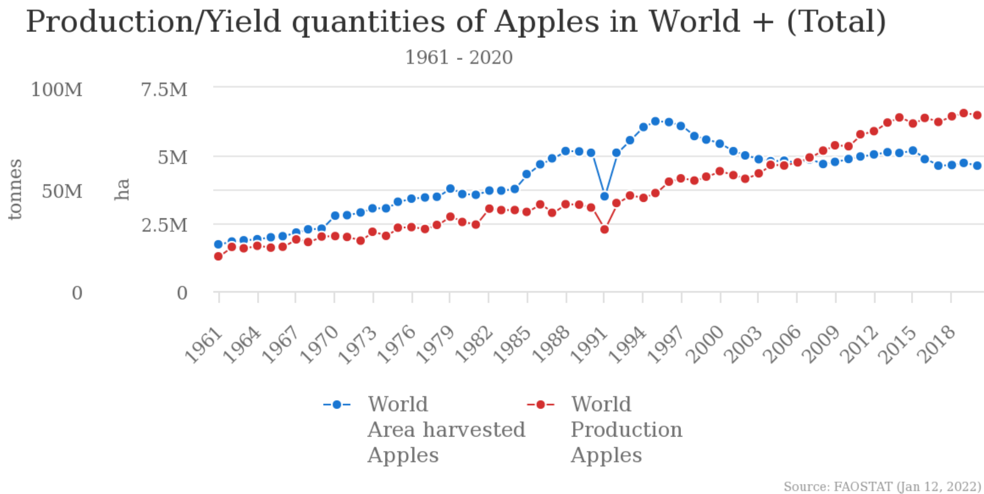


Figure 1. World production and area harvested of apples from 1961 to 2020 (*FAOSTAT*, 2020).

Commercial apple trees are grown as composite trees composed of a fruiting scion, presenting superior fruit tree genotypes, and rootstocks. The rootstock has significant effects on the scion, affecting tree size, performance, precociousness and flowering intensity (Marini & Fazio, 2018).

Eighty years ago, apple trees were propagated on seedling rootstocks. During the 20th century, because of the rapid turnover rate of commercial apple cultivars and for economic reasons, research focused on producing small, uniform and high yielding trees (Tukey & Brase, 1939; Zeiger & Tukey, 1960). Dwarf clonal rootstocks were investigated to meet these demands. Dwarf rootstocks were long known but confined to garden use due to precocity, which lead them to fall or break in an open environment.

To overcome this pattern while taking advantage of superior productivity, researchers designed new orchard systems using permanent tree support and specific training techniques. This drastic evolution led to high-density planting (from 2,000 to 4,000 trees/ha), doubling the yield over the last 25 years (Barritt et al., 1990; Weber, 2001). These growing methods greatly reduced production costs by maximising yields and increasing picking efficiency. Recently, research has focused on incorporating disease and pest resistance (Hancock et al., 2008).

2. Most common apple orchard pests and their management strategies

Similar to any type of production, apple yields are disrupted by pest insects and fungal and bacterial diseases, resulting in important quality and production loss, which leads to economic loss. The main fungal diseases are apple scab and mildew caused by *Venturia inequalis* and *Podosphaera leucotricha,* respectively. The main bacterial disease is fire blight caused by *Erwinia amylovora* (Jamar et al., 2010).

Arthropod species are referred to as pests when they compete on the same resources as humans. Many species of aphids, including rosy apple aphid (RAA), *Dysaphis plantaginea* Pass., the species complex of the leaf-curling aphids (*Dysaphis cf. devecta* Wlk.), the green apple aphid (*Aphis pomi* De Geer) and the woolly apple aphid (*Eriosoma lanigerum* Ham.), moths, such as the codling moth (*Cydia pomonella* L., winter moth (*Operophtera brumata* L.) and, the oriental fruit moth (*Grapholita molesta* Busck), and beetles, including Japanese beetle (*Popillia japonica* (Newman)) affect apple production (Schoonhoven et al., 2005). Pests targeting flowers or fruit have the strongest impact on yield due to product unmarketability occurring even at a slight infestation rate (Beers et al., 2009; Blommers, 1994).

Aphids quickly adapt to new environments and are very effective in colony build-up due to their life cycle, which is composed of sexual and asexual parts (Figure 2) and sometimes includes host-plant migration.

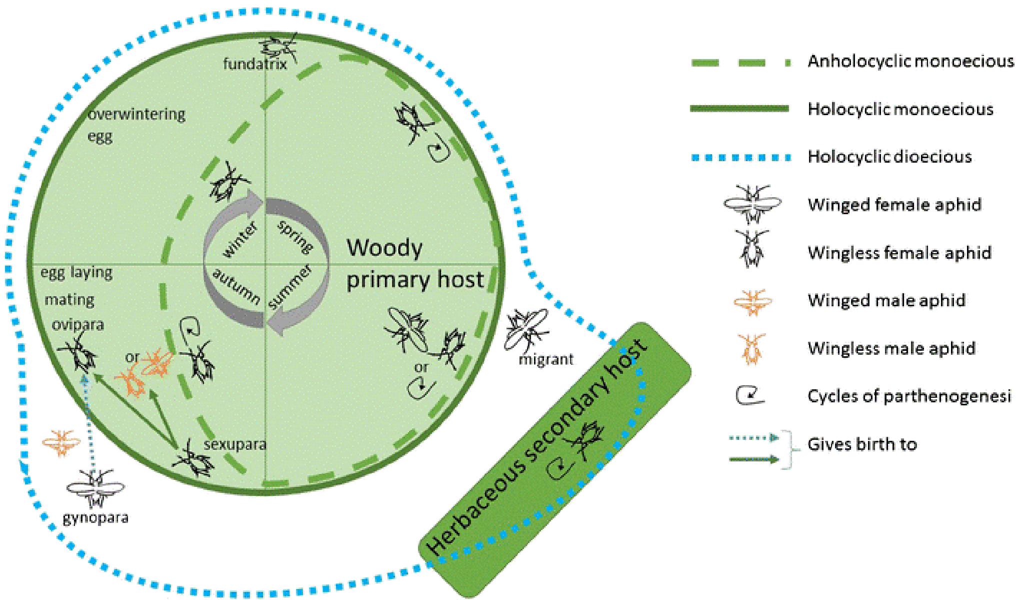


Figure 2. Life cycles of the different aphid species. The dashed blue cycle represents the *D. plantaginea's* (Rousselin et al., 2017).

In apple orchards, the rosy apple aphid *Dysaphis plantaginea* Passerini is considered among the most detrimental early-season pests in both conventional and organic production systems due to its very low economic damage threshold of two fundatrices per 100 leaves (Bürgel et al., 2005). RAA damages fruit through size reduction and shape deformation. Phloem sap-sucking activity also decreases tree vigour, deforms leaves and triggers premature chlorosis and leaf fall. It also indirectly enhances sooty mould development through the honeydew secretion.

They present a holocyclic dioecious life cycle on two different host plants, its primary host is the apple tree, cultivated or wild and its secondary host is the plantain (*Plantago* spp). Eggs spend the winter on apple and hatch early in spring at bud breaks into founder females. Multiple generations succeed by parthenogenesis (five to seven). In early summer winged individuals migrate to plantain. In autumn, winged individuals migrate back to apple where they mate and fertilised females lay winter eggs (Brown & Mathews, 2007; Bürgel et al., 2005; Nicolas et al., 2013; Rousselin et al., 2017).

The most popular approach to controlling pest arthropod populations is integrated pest management (IPM). This strategy relies on integrating multiple control levers, such as behavioural, microbial, cultural and biological control, as well as host resistance to stop populations from reaching economic injury levels (damage costs exceeding the control costs). Strategies can be divided into two categories: top-down or

bottom-up. The first implies a predator-prey relationship in which natural pest enemies reduce the population. These natural enemies, include entomopathogenic fungi (Bird et al., 2010), hymenopteran parasitoids (Peusens et al., 2006) and predators (Dib et al., 2010). Among those predators, the principals are *Episyrphus balteatus* De Geer (Diptera: Syrphidae), *Adalia bipunctata* L. (Coleoptera: Coccinellidae) and *Aphidoletes aphidimyza* Rondani (Diptera: Cecidomyiidae) (Wyss et al., 1999). Efficient control in orchards has not yet been achieved by predators, which occur too late in the aphid life cycle (Miñarro et al. 2005). Furthermore, other interactions such as mutualism occurring with the black garden ant (*Lasius niger* L.), mediate aphid performance (Pålsson et al., 2020). However, research on the management of functional biodiversity by interplanting herbaceous strips or extra-floral nectar-bearing trees to promote natural enemies, as a lure trap for aphids or to attract even more predators is still ongoing (Dib et al., 2010, 2016; Penvern et al., 2019).

During their co-evolution with insects, plants have developed diverse chemical and physical defence mechanisms, leading to cultivar resistance or tolerance of pests. This host plant resistance is considered a bottom-up management tool. Previous development of resistant cultivars has identified two RAA resistance candidate genes in apple germplasm, namely *Sm* from *M. robusta* Rehd. (Alston & Briggs, 1970) and locus *Dp-fl* from Florina cultivars (Miñarro & Dapena, 2007, 2008; Pagliarani et al., 2016). Quantitative trait loci (QTLs) for resistance were also acknowledged in 'Fiesta' (Stoeckli et al., 2008). However, until now, the bottom-up technique did not show sufficient fruit quality or yield from tolerant and resistant varieties (Alins et al., 2017). Nevertheless, as recently highlighted for 13 cultivars in north-eastern Belgium, cultivar selection drastically impacts bottom-up interactions with RAA and is of primary importance to develop eco-friendly RAA management strategies (Alhmedi et al., 2021).

Despite the previously described research efforts, methods to control *D. plantaginea* still rely on fundatrix elimination in early spring by chemical control, both in conventional and organic farming (Alins et al., 2017). A single treatment occurs pre-bloom at budburst, but in case of population outbreaks, another one can also be applied during the growing season or in overwintering eggs to prevent outbreaks the next year. These chemical control and other methods are represented in Supplementary Figure 1. In conventional farming systems, only a few carbamates and neonicotinoids such as pirimicarb, imidacloprid, thiacloprid and acetamiprid, have been authorised in Belgium (Peusens et al., 2006). In 2022, only 5 active substances (a. s.) were still authorised and used against RAA: flonicamid (neonicotinoids), acetamiprid (neonicotinoids), spirotetramate (tetramic acid), pirimicarb (carbamates), flupyradifurone (butenolide/ neonicotinoids superfamily) and azadirachtin (azadirachtins) (*Fytoweb*, 2022). These limitations can lead to pest resistance. A recent report from ANSES established that the first phenomenon of resistance to flonicamid was detected in *D. plantaginea*, in French orchards with a medium level of resistance

(Rapport Anses, 2021). Furthermore, these large spectra insecticides are not favoured for sustainable practices. Indeed, they may be responsible for many side effects, such as fauna disruption, health risks, and water, air and soil quality degradation (Krebs et al., 1999; Moss, 2008). In organic production, pest control currently rely solely on azadirachtin (Alins et al., 2017).

The level of pesticide use can be measured with the treatment frequency index (TFI), which is calculated by the theoretical number of pesticide treatments per hectare. Globally, in the current production system, the TFI varies between 30 and 40 (Simon et al., 2011). For susceptible cultivars, such as 'Golden Delicious', a mean of 35 TFI has been observed in orchards in South-eastern France, with a mean 26 TFI for scab-susceptible organic farming (Sauphanor et al., 2009).

To implement a sustainable agricultural system, a decrease in pesticide use is crucial and is the main option for limiting environmental contamination. Direct responses to this problem rely on IPM, as previously described, and research on alternative products (Campos et al., 2019).

In addition to organic farming (which come with technical specifications), agro-ecology is another trending concept that aims to reduce chemical input. Agro-ecology is defined by the OCDE as the study of agricultural crops and the environment. By their very nature, botanical insecticides are included in those movements as substitution practices, allowing the replacement of chemical pesticides by natural pesticides.

3. Essential oils as botanical insecticides

As a result of their secondary metabolism, plants synthesise a wide array of chemical compounds that are involved in multiple biotic and abiotic interactions, including those with arthropods (Rattan, 2010).

Essential oils (EOs) are defined as products resulting either by hydro-, steam- or dry distillation, or by a cold expression process (epicarp of citrus fruit) without heating from vegetable material organs, including buds, flowers, seeds, leaves, fruits, barks and roots (Rubiolo et al., 2010; Turek & Stintzing, 2013).

EOs are composed of a complex of lipophilic and volatile secondary metabolites called volatile organic compounds (VOCs). There are one or a few major compounds and many others for a complete composition of a few dozens to hundreds of different compounds. These chemicals usually belong to the terpenoid or phenylpropanoid classes of compounds. Terpenoid biosynthetic routes include the mevalonate (MVA) pathway in the cytosol and the methylerythritol phosphate (MEP) pathway in plastids. Phenylpropanoids originate through the shikimate pathway (Miguel, 2010; Pavela & Benelli, 2016). Due to their flavour and scent or their biological properties, such as

antioxidant and anti-inflammatory activities, over 300 EOs are commercially used in medicine, cosmetics, and the food industry.

Due to the acknowledged insecticidal, antimicrobial, antiviral, nematicidal and antifungal properties, EOs are actively investigated as an alternative to pesticides in the agricultural field (Bakkali et al., 2008). Regarding their insecticidal activities, they can act in many ways (contact toxicity, fumigation, repellence, etc.) due to a complex mixture of constituents enabling synergism. Their complex composition may also prevent resistance development compared to a single active ingredient, which typically constitutes conventional pesticides. Another advantage is their low mammalian toxicity and absence of persistence limiting residues in the environment and in food. Decreased risks of residual activity on non-target species (parasitoids, predators and pollinators) are compatible with IPM programmes (Isman & Machial, 2006; Machial, 2010). Due to these properties, some EOs have already been investigated for pre- or post-harvest crop protection (Bakkali et al., 2008; Pavela & Benelli, 2016; Regnault-Roger et al., 2012).

The insecticidal properties of EOs are often linked to their neurotoxic properties, affecting the insect's nervous system by blocking the gamma-aminobutyric acid (GABA) receptors, by binding to the octopamine receptors and by affecting acetylcholinesterase (AChE) enzymes through competitive and non-competitive inhibition (Mossa, 2016; Pavela & Benelli, 2016). Recent proteomic studies even suggest a larger impact on the development and functioning of the muscular and nervous systems, cellular respiration, protein synthesis, and detoxification (Renoz et al., 2021).

Indirect toxicity mechanisms have been acknowledged such as insect growth regulator (IGR), insect repellent and antifeedant activity mechanisms. Furthermore, the mode of application (direct contact, ingestion or fumigation) plays a major role in the intensity of EO's toxicity to insects.

Previous studies have demonstrated the toxicity of different EOs on aphids using the contact efficacy of EOs from 75 plant species and using the fumigant efficacy of EOs from 43 plant species (Ikbal & Pavela, 2019). Although idiosyncratic reactions are observed between species, *D. plantaginea* proved to be very sensitive to EO toxicity compared to *M. persicae* (Machial, 2010).

4. Mode of application of phytosanitary products

The most commonly used methods for pesticide application currently rely on spraying with an air blaster. However, reports suggest that only 29-56% reach the tree crown and as little as 0.1% could come into contact with the target pest (Wise et al., 2014). Indeed, leaf rolling, which occurs following aphid feeding behaviour, makes it harder to use direct contact insecticides once colonies are established. The remaining product drifts in the environment and other off-target end points are responsible for the previously listed hazards. To protect fruit crops, trunk or tree injection has been proposed as an alternative method that applies chemicals into plant vascular systems, which are then systemically translocated by xylem sap. The chemicals are injected by piercing the bark to access the xylem and are then systemically distributed via the xylem sap (Coslor et al., 2019; Doccola et al., 2012; Wise et al., 2014).

This method presents numerous advantages, such as efficient use of chemicals (especially to target sap-sucking pests), reduced load in agro-ecosystems, limited chemical degradation and usefulness when spray cannot be applied (urban area). Nevertheless, this technique is not routinely applied due to concerns about wounding of trunks or other limbs, which can impact the long-term tree fertility or longevity. The heterogeneous distribution of products leading to non-uniform supply in the canopy has also been demonstrated. Phytotoxicity may also occur at the wrong dosage, and this method requires much more trained labour representing an extra cost (Aćimović et al., 2014, 2015, 2016, 2020; Percival & Boyle, 2005).

This mode of application is not widespread; however, it has successfully protected various tree species from various fungi, bacteria and pests. Diverse compounds in trunk injection techniques have been reported, such as azadirachtin, imidacloprid, penthiopyrad, emamectin benzoate and other conventional chemicals specially designed for trunk injection, such as phosphites, imazalil, penconazole, pyrifenox, phosphonate and carbendazim (Percival & Boyle, 2005; Schulte et al., 2006; Mcmahon et al., 2010; Aćimović, 2014; VanWoerkom et al., 2014; Flower et al., 2015; Coslor et al., 2019).

5. Thesis outline

Today, apple production in conventional orchards relies on the high use of chemical inputs, such as fertilisers and pesticides. This production system is on the lookout for alternatives to enable sustainable production practices and meet public demand for residue-free fruit.

The main objective of this thesis is to contribute to botanical insecticide development based on EOs to control a major pest, RAA. This thesis was realised as part of

a larger project called Tree-Injection. This research was funded by the Department of Research and Technological Development of the Walloon regions of Belgium (DG03). This project was composed of a partnership between the Laboratory of Chemistry of Natural Molecules (ULiège) and the Biodiversity Research Center, Earth and Life Institute (UCL). In addition to RAA, *Cacopsylla pyri*, the main insect pest in pear, was also considered. In the framework of this project, the direct toxicity of EOs was measured through screening on an artificial diet after selection, following the literature review. More than 15 EOs were assayed, and the most active from *Cinnamomum cassia* and *Mentha spicata* L. were selected. Furthermore, the impact on other auxiliary insects, such as parasitoids and pollinators was determined. Finally, the impact of EO injection on insect-feeding behaviour was also considered. With its innovative approach, this project is a pioneer in combining EO application with trunk injection.

The second chapter is a bibliographic review investigating the putative mode of action of phytotoxicity arising following EO application. It systematically discusses the functional and cellular impacts and resulting physiological disturbances. It discusses rising opportunities, including the biostimulation and defence elicitation properties of EOs.

The third chapter investigates the potential phytotoxic properties of Cinnamon EO (*C. cassia*) on apple (*Malus x domestica*). Phytotoxicity is explored in terms of oxidative burst and damage. Plant defence induction was investigated via major defence pathway gene expression.

The fourth chapter investigates the translocation of essential oil constituents following trunk injection by targeted volatile organic compounds (VOCs) analyses, both contained and emitted by leaves. The impact on plant was determined using chlorophyll fluorescence and an untargeted analysis of VOCs.

The fifth chapter investigates efficiency against RAA, both at the laboratory and field scale. Biological activity was investigated following the population dynamics of RAA.

In the last chapter, all the results are discussed and perspectives are formulated regarding the development of EO biopesticides in apple orchards.

2

Phytotoxicity of essential oils: opportunities and constraints for the development of biopesticides.

Following the general introduction, the second chapter of this thesis presents a bibliographic review that was published in 2020 in Foods:

Werrie, P.-Y., Durenne, B., Delaplace, P., Fauconnier, M.-L (2020). Phytotoxicity of essential oils: opportunities and constraints for the development of biopesticides. A review. *Foods*, 9, 1291. doi: 10.3390/foods9091291

In the first part of this review, the diverse phytotoxic modes of action of EOs are discussed. The focus is on cellular functions, such as photosynthesis or respiration, as well as advances in molecular target identification. The mechanisms involved are classified according to water status alteration, membrane interaction, reactive species production, photosynthesis and mitochondrial respiration inhibition, microtubule disruption, enzymatic inhibition, and phytohormones status alteration. This mechanistic approach was selected due to the non-model nature of the ligneous plant considered in this work, as well as the various modes of EO application and the reported concentrations. Therefore, this review focuses specifically on biomarker selection, allowing to highlight potential phytotoxic phenomena rather than exhibiting precise thresholds.

The second part of this review exposes the potential mechanism of detoxification involving the metabolisation of phytotoxins or conjugation/sequestration followed by compartmentalisation or emissions.

The last part discusses the impact of this knowledge on potential agronomic applications. It summarises the observations of a large array of EOs according to their mode of action. Furthermore, it discusses the beneficial impact observed for mild stress or below the phytotoxicity threshold, leading to biostimulation, defence priming or direct plant defence induction.

Abstract

The extensive use of chemical pesticides leads to risks for both the environment and human health due to the toxicity and poor biodegradability that they may present. Farmers therefore need alternative agricultural practices including the use of natural molecules to achieve more sustainable production methods to meet consumer and societal expectations. Numerous studies have reported the potential of essential oils as biopesticides for integrated weed or pest management. However, their phytotoxic properties have long been a major drawback for their potential applicability (apart from herbicidal application). Therefore, deciphering the mode of action of essential oils exogenously applied in regards to their potential phytotoxicity will help in the development of biopesticides for sustainable agriculture. Nowadays, plant physiologists are attempting to understand the mechanisms underlying their phytotoxicity at both cellular and molecular levels using transcriptomic and metabolomic tools. This review systematically discusses the functional and cellular impacts of essential oils applied in the agronomic context. Putative molecular targets and resulting physiological disturbances are described. New opportunities regarding the development of biopesticides are discussed including biostimulation and defense elicitation or priming properties of essential oils.

1. Introduction

Essential oils (EOs) have been used historically in the food and perfume industries and are extracted from various plant organs (flowers, leaves, barks, wood, roots, rhizomes, fruits and seeds) through steam distillation, hydro-distillation and cold expression for citrus. These natural products are mainly composed of volatile organic compounds (VOCs), having a high vapour pressure at room temperature and belonging mainly to the phenylpropanoid and terpenoid families. Briefly, terpenes are classified according to the number of isoprene sub-units: 2 for monoterpenes ($C_{10}H_{16}$) and 3 for sesquiterpenes ($C_{15}H_{24}$). Oxygenated terpenes or terpenoids also contain additional functional groups such as alcohol, carboxylic acid, ester, etc. (Bakkali et al., 2008) and phenylpropanoids are produced from L-phenylalanine through deamination by phenylalanine ammonia-lyase (Dixon et al., 2002).

Many research studies have been undertaken on the use of EOs in more sustainable agronomic practices. In this regard numerous findings have described the strong biopesticidal potential of EOs thanks to their antibacterial (O'Bryan et al., 2015), antifungal (Cavanagh, 2007), insecticidal (Jankowska et al., 2018), acaricidal (Camilo et al., 2017), nematicidal (Andrés et al., 2012) and herbicidal activities (Tworkoski, 2002). Included under the Generally Recognised as Safe (GRAS) product categories of the US Food and Drug Administration, the impact of EOs on human health and ecosystems seems to be lower compared to synthetic plant protection products (PPP). Biocidal actions of EOs can be specific, therefore their use could be compatible with integrated pest management (IPM) (Koul et al., 2008).

The application of EOs is, however, subject to a major constraint. They may present phytotoxic properties to untargeted plants such as crops. The most effective EOs in pest control are phytotoxic as well and considerable precaution is required regarding product formulation (unless the objective is the formulation of a total herbicide) (Isman, 2000). Empirical tests for commercial EOs are commonly realised on major crops (Ibáñez & Blázquez, 2020). However these strategies have led to poor knowledge relating to other biological systems (Isman, 2016). Many parameters determine this impact such as the application mode (root watering, aerial spraying or injection in vascular system), the plant organs targeted, the phenological stage (seed, plantlet or mature plant), physiological state and product formulation. As illustrated by the opposing claims regarding the presence or absence of phytotoxicity of *Mentha pulegium* (pennyroyal) EOs towards *Cucumis sativus* (cucumber) and *Solanum lycopersicum* (tomato) it is necessary to gain insight into the molecular mechanism involved in order to design suitable biopesticides (Domingues & Santos, 2019; Rolli et al., 2014; Topuz et al., 2018).

Phytotoxicity can be defined as a negative impact on plant growth or plant fitness and can be linked to cellular dysfunctions. Physiological impairment can be observed through integrative measurement of stress, for example on the photosynthetic apparatus. However, determination of the primary site of action is much more challenging. Diverse phytochemical products have been demonstrated to influence several physiological processes of growth and development in plant cell division and root elongation (Yan et al., 2015). Blends of natural plant compounds often have numerous mechanisms of action making them very efficient at acting on a plant's primary metabolism. Therefore, it seems most important to gain insight into the physiological impact of EOs on plant crops to design proper bioassays and efficient biopesticides. Avoiding residual phytotoxicity which is currently an underestimated constraint in the field will allow the broader application of EOs (Singh & Pandey, 2018). However even if some processes seem to be inhibited in a dose-dependent manner, a concentration below the phytotoxic threshold could also stimulate the plant, a phenomenon referred to as biostimulation. New opportunities arising from this biostimulation and elicitation of defence will be discussed in this review.

All the mechanisms involved in the phytotoxicity of EOs cannot be easily interpreted individually (Grana et al., 2012). This review aims to discuss the latest putative molecular targets (mode of actions) involved in plant metabolism with a physiological approach including: water status alteration, membrane interaction/disruption, reactive oxygen/nitrogen species induction, genotoxicity and microtubule disruption, mitochondrial respiration, photosynthesis inhibition and enzymatic or phytohormones regulation. The different mechanisms presented throughout this review have been graphically summarised in Figure 3.

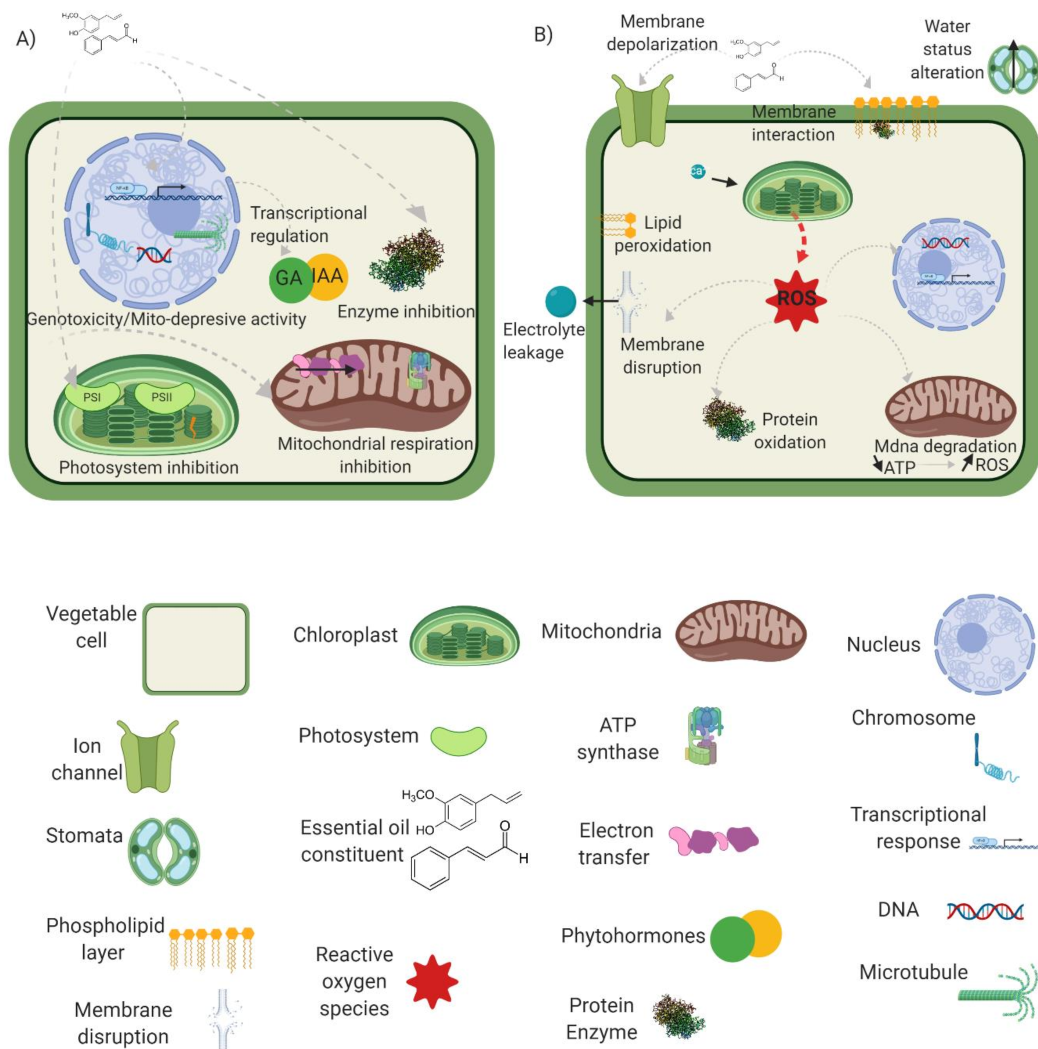


Figure 3. Mode of action of essential oil at the cellular level. (A) Photosynthesis and mitochondrial respiration inhibition, microtubule disruption and genotoxicity, enzymatic and phytohormone regulation. (B) Water status alteration, membrane properties and interactions, reactive oxygen species induction.

2. Essential oils' cellular and physiological impacts

2.1. Essential oils' translocation

Essential oil constituents (EOC) must access specific targets in order to carry out the physiological impact previously listed within a plant. Numerous publications describe the VOCs released by plants (Dong et al., 2016; Maffei, 2010; Widhalm et al., 2015). However little is known about their cellular entrance and translocation in plant organisms in the case of a systemic effect.

When sprayed, the first interaction occurs with the cuticular wax components of the leaves. In fact, the cuticle is considered as the plant's first barrier to molecule penetration. The interaction between monoterpene with epicuticular waxes and stomata will be further described. Briefly, once it has entered through the stomata opening by gas exchange or diffusion through the waxy cuticle, each EOC is partitioned into the gas phase and liquid phase following a defined ratio determined by Henry's law. The liquid phase is materialised by the cell wall in which EOC accumulate. Compounds then diffuse to the cytosol following their oil/water partition coefficients (Sugimoto et al., 2016). Finally, active transport should also be considered as it has been demonstrated for emissions (Adebesin et al., 2017).

Regarding root uptake, a study with radio-labelled thymol demonstrates the translocation of monoterpenes in citrus trees. However, the determination of the mechanism was beyond the scope of the study although the authors suggest it could be similar to that for EDTA (Wong & Coats, 2018).

2.2. Water status alteration

Depending on the mode of application (aerial or root), two different phenomena have been suggested for disturbing the water status of plants after treatment with EOs.

The deleterious effect of monoterpenes (camphor and menthol) on cuticular wax and stomatal closure inhibition has been observed (Schulz et al., 2007). These two effects act synergistically on plant transpiration leading to guard cell disruption and desiccation. Interestingly, an opposite growth promoting effect is described for *Arabidopsis thaliana* during short vapour exposure to these terpenes. The molecular mechanism responsible for this prevention of stomatal closure is mediated through modification in the cytoskeleton and especially in the actin filament. Furthermore, stress symptoms appear together with a change in gene expression (Kriegs et al., 2010). The amount of leaf epicuticular waxes determines the sensitivity of crops seedlings and weed species (Bainard et al., 2006).

Water status alteration of plants was also observed after root watering application with citral, a mixture of two monoterpene isomers neral and geranial (Graña et al., 2016). In a similar study with the sesquiterpene *trans*-caryophyllene, the authors suggest that this alteration could be responsible for the oxidative burst and a strong proline accumulation due to its osmo-regulative function (Araniti et al., 2017).

2.3. Membrane properties and interactions

After entering the intercellular space and through the mesh of the cell wall, EOC directly solubilize within the plasma membrane depending on their physical properties, particularly the vapour pressure and the molecular mass. Their specific accumulation was demonstrated to modify the lipid packing density, membrane-bound enzymes and ion flux (Griffin et al., 2000).

This interaction can lead to a reversible depolarisation of the membrane potential (Vm) and to membrane disruption (Maffei et al., 2001). Furthermore, stronger membrane depolarisation occurs for more water soluble monoterpenes presenting a low octanol/water partition coefficient (Kow). A change in the polarisation state implies ion mobility through the membrane. A drastic entrance of Ca^{2+} in the cytosol is triggered by opening the calcium channel. Ca^{2+} is known to be largely involved in cellular signalling. It performs allosteric regulation of many enzymes and proteins. Moreover, Ca^{2+} is an intracellular second messenger of signal transduction pathways and gene expression. Finally, the increase of Ca^{2+} concentration can lead to an oxidative burst (Marcec et al., 2019).

Studies on artificial monolayer membranes of dipalmitoyl-phosphatildylcholine describe the penetration of monoterpenes such as camphor, cineole, thymol, menthol and geraniol which affect the vesicles topology (Turina et al., 2006). Similar work on model bilayer interactions with related monoterpenes, including limonene, perillyl alcohol and aldehyde demonstrates the diffusion across the membrane and an ordering effect on the lipid bilayer (Witzke et al., 2010). More recently, novel molecular techniques of dynamic interaction were applied to study the interaction between citronellal (monoterpene), citronellol (monoterpene) and cinnamaldehyde (phenylpropanoid) with biomimetic membrane (Lins et al., 2019). Briefly, the *in silico* insertion model predicted different behaviours between the two classes (monoterpenes and phenylpropanoids). These predictions were confirmed using *in vitro* biophysical assays. Citronellal and citronellol interaction with the model membranes was demonstrated without permeabilizing it, while cinnamaldehyde did not interact with the model membrane. This suggests two different mechanisms of action: (i) the modification of lipid bilayer organisation by monoterpenes and (ii) the interaction with membrane receptors for phenylpropanoid pathway metabolites.

Associated with the modification of membrane properties, a change in the membrane's composition also occurs. In fact, an increase in unsaturated fatty acids was demonstrated following application of monoterpenes such as 1,8 cineole, geraniol, thymol, menthol and camphor (Zunino & Zygadlo, 2004). Quantitative and qualitative changes in most abundant free and esterified sterols (sitosterol, stigmasterol, and campesterol) and phospholipid fatty acids (16:0, 16:1, 18:0, 18:1, 18:2, 18:3) were also highlighted in a study investigating the effect of the same monoterpenes (Zunino & Zygadlo, 2005). It results in an increase in the percentage of unsaturated (PLFAs) and stigmasterol. Interestingly, alcoholic monoterpenes seem to have a different mode of action affecting more unsaturated fatty acid and stigmasterol leading to seedling growth interferences.

2.4. Reactive oxygen and nitrogen species induction

Reactive oxygen species (ROS) are essential in cellular signalling. They can be produced in various locations in plant cells such as in the chloroplast, the peroxisome, the mitochondria and in the endoplasmic reticulum. ROS are very reactive compounds that in excess lead to the degradation of macromolecules such as lipids, carbohydrates, proteins and DNA (Gniazdowska et al., 2015).

Oxidative burst or generation of ROS has long been proposed as one of the main mechanisms of action of phytotoxins (Dayan et al., 2000). We know that the uncoupling of photosynthesis and respiration leads to the production of superoxide radicals ($O^{2.-}$) which are transformed into oxygen peroxide (H_2O_2) by the superoxide dismutase. Moreover, the reaction with transition metal triggers reduction of H_2O_2 to $OH^{.}$, another very reactive species (Mittler, 2002).

Oxidative stress was acknowledged after treatment with α-pinene through hydrogen peroxide, proline and the lipid peroxidation product malondialdehyde (MDA). Moreover, antioxidant enzyme activity assay (superoxide dismutase, catalase, ascorbate, peroxidase, guaiacol peroxidase and glutathione reductase) was also performed in the roots. The oxidative stress generated by these ROS leads to membrane lipid peroxidation and ultimately to membrane disruption launching the programmed cell death. These membrane disruptions are evidenced via electrolyte leakage (EL) and vital staining (Sunohara et al., 2015).

In a similar experiment determining germination and growth inhibition by β-pinene EL, lipid peroxidation and lipoxygenase activity were assessed. The result showed a strong increase in EL, dienes content and H_2O_2 content and the authors suggest that despite an increase in the activity of ROS scavenging enzymes, root membrane integrity was lost (Chowhan et al., 2013). Later on they studied the early ROS generation and activity of antioxidant defense system in root and shoot of hydroponic wheat. The damages being more severe in the root and a higher lipoxygenase (LOX) activity

was observed in parallel with accumulation of MDA (Chowhan et al., 2014). The up-regulation of LOX activity has been observed for citronellol as well and authors suggest its hydroperoxide derivatives destroy the membrane (Kaur et al., 2011).

EOs inhibiting growth of tested plant via ROS overproduction leading to oxidative stress and degradation of membrane integrity evidenced via increased level of MDA, REL and decreased levels of conjugated dienes were demonstrated for other EOs such as *Pogostemon benghalensis* (Dahiya et al., 2020), *Monarda didyma* (Ricci et al., 2017) and *Artemisia scoparia* (Kaur et al., 2012).

Secondary effect of ROS generation includes depigmentation of cotyledons in *A. thaliana* by *Heterothalamus psiadioides* EOs. The effects are here observed in a dose-dependent manner and very small amount. Authors also suggest alteration on auxin levels occurs as secondary effect. Exogenous addition of antioxidant did not reverse effects on adventitious rooting indicating that damages were too severe (Lazarotto et al., 2014).

The generation of ROS, one of the most prevalent plant responses to stress, is described in direct response to the application of EOs. However, it is unlikely to be the main mechanism of toxicity but rather an indirect consequence resulting from LOX activity, chloroplast or mitochondria alteration (Gniazdowska et al., 2015). The fundamental involvement of ROS in stress signalling as well as their interaction with other signalling components such as transcription factors, plant hormones, calcium, membrane, G-protein, mitogen-activated protein kinases need to be highlighted (Sewelam et al., 2016). These interactions may explain many of the numerous physiological impacts induced by EOs' application in plants. Moreover, after treatment with α-farnesene, they also observed the induction of nitric oxide production, a reactive nitrogen species, associated with an oxidative burst (Gniazdowska et al., 2015).

2.5. Photosynthesis inhibition

Photosynthesis inhibition has also been proposed as one of the putative modes of action of EOs. While the impact of certain allelochemicals on photosynthesis is well established, for instance quinone, this is not the case for EOs where numerous mechanisms have been proposed. Direct ROS-mediated disruption through oxidation of photosystem II protein has been suggested to inhibit photosynthesis as suggested by the increase in the proline content whose function is to accept electrons to protect the photosystem (Singh et al., 2006). The effect of β-pinene on the chloroplast membrane has long been demonstrated by the inhibition of the electron transport of photosystem II (PSII) (Klingler et al., 1991; Pauly et al., 1981).

Numerous studies report a decrease in the photosynthetic pigments chlorophylls (a and b) and carotenoids after treatments with EOs in a dose-dependent way (Chowhan

et al., 2011; Poonpaiboonpipat et al., 2013; Sharma et al., 2019). This can result from a direct pigment photo-degradation or from a decrease in *de novo* synthesis. Plants have developed a non-photochemical quenching (fluorescence) strategy to avoid the ROS production resulting from this photo-inhibition. The decrease in carotenoids content could explain a higher fluorescence emission and a decrease of the PSII performance due to some damage to the complex antenna via ROS production and lipid peroxidation (Araniti et al., 2016).

Artemisia fragrans EO impacts on the photosynthetic apparatus of perennial weed *Convolvulus arvensis* were studied using the most important chlorophyll fluorescence parameters F0, Fv/Fm, ΦPSII, qP and NPQ. Increase in minimal fluorescence level (F0) implies a restriction in the PSII transport chain. The decrease in maximum quantum yield of PSII (Fv/Fm) results from photosystem inactivation (photo-damage) and/or a blockade in electron transport. PSII electron transport chain state (ΦPSII) reduction in plants treated with EOs restricts the non-cyclic electron transport chain. The last two parameters represent energy used in photochemical quenching (qP) and non-photochemical quenching (NPQ). qP decreases following concentration of EOs whereas NQP increases. Taken altogether those results imply that the excited energy was not used in photosynthesis due to photosystem degradation by the EOs treatment (Pouresmaeil et al., 2020).

Two specific fluorescence parameters QYmax (a maximum quantum yield of PSII photochemistry) and Rfd (a fluorescence decrease ratio) have even been proposed as early predictors of broccoli plant response treatment to clove oil (Synowiec et al., 2015).

Moreover, in a study of photo respiratory pathway alteration by *Origanum vulgare* EOs in *A. thaliana*, Araniti et al. (2018) suggested that alteration of glutamate and aspartate metabolism leads to leaf chlorosis and necrosis. Glutamine synthetase is crucial to incorporate ammonia in organic compounds and may be a molecular target of *O. vulgare* EO. Finally, ammonia accretion has direct inhibiting properties on PSI and PSII due to its bonding with the oxygen-evolving complex. In addition, the decrease in pH gradient across membranes is able to uncouple photophosphorylation.

2.6. Mitochondrial respiration inhibition

Mitochondrial respiration inhibition is another putative target in the cellular mode of action of EOs. Monoterpenes treatment has long been reported to decrease respiratory oxygen consumption in whole plants, dissected organs and isolated mitochondria for 1,8-cineole (Muller et al., 1969) and juglone (Peñuelas et al., 1996).

The effect of monoterpenes has been well documented on isolated mitochondria, on germination and on primary root growth of maize (Abrahim et al., 2000). Briefly, the authors demonstrated that α-pinene triggers two different mechanisms which are the uncoupling of oxidative phosphorylation and the inhibition of electron transfer. This action drastically decreases ATP production and the authors suggest it occurs following unspecific disruption in the inner mitochondrial membrane (Abrahim et al., 2003; Mucciarelli et al., 2001). The mode of action of other monoterpenes such as camphor and limonene has been investigated. They respectively cause mitochondrial uncoupling and act on ATP synthase or on adenine nucleotide translocase complexes (Abrahim et al., 2003; Weir et al., 2004).

Accessibility to mitochondria in vivo can strongly affect phytotoxicity. A study performed using soy hypocotyl showed that the effect on mitochondria alone did not fully explain the resulting phytotoxic effect. Absence of correlation between respiratory inhibition in mitochondria and seed germination or root growth treated with α-pinene and limonene suggest that their inhibition property is probably dependent on their ability to permeate intracellular compartments (Weir et al., 2004).

Furthermore, the description of the cytochrome-oxidase pathway inhibition highlights the fact that this inhibition is likely to increase mitochondrial reactive oxygen species and membrane lipoperoxidation as demonstrated by increased concentrations of lipoperoxide products, activation of lipoxygenase and antioxidant enzymes (Ishii-Iwamoto et al., 2012).

Microscopic evaluation highlights the drastic reduction in the number of intact organelles among which mitochondria and membranes disruption of nuclei, mitochondria and dictyosomes (Lorber & Muller, 1976). This mitochondrial membrane deleterious effect leads to a decrease in energy production and ROS generation affecting numerous biochemical processes and cellular activities as observed for BY-2 treated with 1,8-cineole (Sakai & Yoshimura, 2012; Yoshimura et al., 2011).

2.7. Microtubule disruption and genotoxicity

Vapour exposure of citral at µmolar concentrations completely depolymerizes microtubules without any damage to the plasma membrane (Chaimovitsh et al., 2010). Results suggest an in vitro dose/time relationship for microtubule disruption whereas the actin filament remained intact. Finally, mitotic microtubules were more damaged than the cortical ones, leading to impairment in the mitosis process (Chaimovitsh et al., 2012).

To determine whether the microtubule impact results from direct depolymerisation or from indirect phytohormones balance modification, Graña et al. (2013) studied the short- and long-term effects of citral application in the plant model *A. thaliana*. Auxin (indole 3-acetic acid) polar transport is rapidly inhibited and ethylene content increases. These two hormones have numerous points of interaction and are essential for microtubule organisation, which leads to a long-term disorganisation of cell ultrastructure. Citral treated samples present a large number of Golgi complexes together with thickening of the cell wall. Those phenomena affect cell division and intracellular communication in the long-term.

More recently, Chaimovitsh et al. (2017) studied microtubule and membrane damages for a large number of terpenes and further demonstrated the difference in their mechanisms of action. In fact, they observed strong microtubule depolarisation for limonene and (+)-citronellal and moderate for citral, geraniol, (−)-menthone, (+)-carvone and (−)-citronellal. Moreover, many compounds lacked antitubular activity such as pulegone, (−)-carvone, carvacrol, nerol, geranic acid, (+)/ (−)-citronellol and citronellic acid. Furthermore, they demonstrated enantioselectivity of microtubule disruption for citronellal and carvone, the (+) enantiomers being more effective. They compare this antitubular activity with the membrane disrupting properties and found that citral did not cause membrane disruption. Carvacrol induced membrane leakage, and limonene both depolymerised microtubules and induced membrane leakage. Finally, through *in vivo* quantification of applied monoterpene they discover the biotransformation of citral (i) and limonene (ii) to (i) nerol and geraniol and (ii) carvacrol, respectively. This conversion explains the dual mode of action of limonene in both membrane and microtubule. Dual mode of action was recently highlighted for menthone in tobacco BY-2 plant cells and seedlings of *A. thaliana* (Sarheed et al., 2020).

Concerning direct genotoxicity, numerous chromosome abnormalities have been observed such as sticky chromosome, chromosome bridges, spindle disturbance, c-mitosis and bi-nucleated cells in root tip cells after treatments with EOs of *Schinus terebinthifolius*, *Citrus aurantiifolia*, *Lectranthus amboinicus*, *Mentha longifolia* and *Nepeta nuda*. The damaging reaction of EOs on the chromatin organisation could lead to chromosome bridges or sickness and ultimately to apoptosis. Interestingly,

different results for EOs with the same principal terpene suggest that there is synergic interaction between major and minor compounds (Bozari et al., 2013; Fagodia et al., 2017; Pawlowski et al., 2012; Pinheiro et al., 2015; Singh et al., 2020).

Another mito-depressive activity of EOs could be mediated by the inhibition of DNA synthesis. It was effectively demonstrated by Nishida et al. (2005) that monoterpenes are able to hinder organelle and nuclear DNA synthesis. Direct damage to DNA has been highlighted through the effect of EOs on head and tail DNA. Although the mechanism behind this are still vague, authors suggest that reactive oxygen species (ROS) following EOs treatments may be responsible for the genotoxic effect (Issa et al., 2020).

2.8. Enzymatic inhibition and regulation

Beside glutamine synthetase as a particular enzymatic target of EOs, studies suggest direct or indirect inhibition of specific enzymes as a putative mode of action. For example, a first case is related to the long known potato tuber bud dormancy inhibition using peppermint oil. A decrease in the activity of 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMGR; E.C. 1.1.1.34), a key-enzyme in the mevalonate pathway, was observed but without explanation at the transcriptional level (Oosterhaven et al., 1993, 1995).

Rentzsch et al. (2012) demonstrated a specific monoterpene interaction with gibberellins (GAs) signalling at dose-, tissue- and gene-level during dormancy release and sprout growth. They also described a typical case of biostimulation. At low concentration, peppermint essential oil and carvone promote bud sprouting and dormancy release, whereas at high concentration they completely inhibit it. They demonstrated that dormancy release is associated with tissue specific α- and β-amylase modulation and that EOs could affect this modulation. Indeed, at low concentration, amylase expressions were modulated by carvone through specific enhancement of a-AMY2 gene transcription by interacting with its transcription factor. This was not the case for peppermint EO for which they proposed interaction with specific components of the GA signalling pathway that enhanced the GA-mediated responses (Rentzsch et al., 2012).

These enzyme modulating activities have been reported for other compounds such as β-pinene reduction of hydrolyzing enzyme (protease, α- and β-amylase) in rice seedlings. At the same time, peroxidases and polyphenol oxidases activity increase suggesting their role in resistance against β-pinene-induced oxidative stress (Chowhan et al., 2011).

Strict inhibition phenomena have been proposed for cinmethylin which is a synthetic analogue of 1,4 and 1,8-cineole through asparagine synthetase inhibition. Authors suggested that benzyl ether moiety cleaved to generate toxophore that inhibits the enzyme. However due to an inability to reproduce these results *in vivo* afterwards, the authors decided to retract the paper. This illustrates well the difficulties in rigorously establishing a single molecular target (Romagni et al., 2000).

Later another target was proposed for the herbicide cinmethylin, the tyrosine aminotransferase (TAT) (EC 2.6.1.5). Indeed, TAT provides quinones for the prenylquinones pathway in the inner chloroplast membrane. Furthermore, plastoquinone is a cofactor in the carotenoid pathway. Therefore, the decrease in carotenoid resulting from this inhibition may trigger photo-oxidative degradation of chlorophyll and photosynthetic membrane disturbing chloroplast function (Grossmann et al., 2012).

More recently, Abdelgaleil et al. (2014) postulated that phytotoxicity of EOs could be mediated through carbonic anhydrase inhibition. Indeed, this enzyme plays a key role in the (de)carboxylation reaction involved in both respiration and photosynthesis and contributes to the movement of inorganic carbon to photosynthetic cells. Thus, CO_2 content in these cells would decrease leading to the formation of ROS by diverting a photosynthetic electron from CO_2 (Abdelgaleil et al., 2014).

2.9. Phytohormones and priming of plant defence

A first evidence of the interaction with phytohormones has already been developed previously concerning the gibberellin (GAs). Two other interconnected hormones have been suggested as main targets, auxin and ethylene. Indeed, citral impacts the polar auxin transport resulting in alteration of its content, cell division and ultrastructure of *A. thaliana* root meristem seedlings cell (Graña et al., 2013). Concentration balance between auxin and ethylene is responsible for root growth, radicle elongation and root hair formation. Citral was suggested as a promising herbicide with strong short term and long lasting toxicity. Similar results on polar auxin transportation were obtained with farnesene (Araniti et al., 2017) which affects specific PIN-FORMED protein. Furthermore, modification in PIN gene expression leads to a decrease in meristem size and a left-handed phenotype. Interestingly, a previous study reported an increase in the auxin content (Araniti et al., 2016). This loss of gravitropism was suggested to result from an alteration in the hormonal balance and stimulation of oxidative stress via ROS and RNS production interfering with cell division and cytokinesis through microtubule disruption altering root morphology.

Phytohormones balance is also involved in priming and plant defence induction mechanisms. Monoterpenoids are able to activate defence genes by signalling processes and Ca^{2+} influx causes by membrane depolarisation, protein phosphorylation/dephosphorylation and the action of ROS (Maffei, 2012). This gene expression

can lead to direct defence elicitation of specific pathway in the absence of stress or to defence priming. Priming is another phenomenon that improves the defensive capacity of plants by enabling plants to respond more rapidly and/or strongly following biotic or abiotic stress exposure.

Priming of plant defences has already been acknowledged in agricultural practices, as for example exposure to mint volatiles which enhanced transcripts levels of defence genes in soy through histone acetylation within the promoter regions (Sukegawa et al., 2018). This priming was stronger at mid distance implying a non-linear relationship to concentration. Recently, priming against bacteria was observed in apple using thyme oil. Indeed, the authors noted a much stronger expression of pathogenesis-related (PR) genes PR-8 following *Botrytis cinerea* application (Banani et al., 2018).

Regarding elicitation of plant defence, resistance can either be constitutive with the systemic acquired resistance (SAR) or induced with the induced systemic resistance (ISR). There is large cross-talk between the two systems which rely on salicylic acid (SA) and jasmonate (JA) hormones.

Transcriptomic study following exposure to volatile monoterpenes myrcene and ocimene demonstrated that plants develop a similar response to that induced by methyl jasmonate (MeJA) (Godard et al., 2008). Microarray profiling revealed the induction of several hundreds of transcripts annotated as stress or defence genes or transcription factor. Multiple stages of the octadecanoid pathway were present, and metabolite analysis demonstrates an increased level of MeJA in *A. thaliana* tissues.

The induction of SAR has also been acknowledged when using *Gaultheria procumbens* EO which is composed almost only of methyl salicylate. To demonstrate the effectiveness of the EO, they inoculated GFP-labelled fungal pathogen and showed a strong reduction of its development similar to commercial solution (Vergnes et al., 2014). Thyme EO also triggers constitutive defence in tomato against grey mould and *fusarium* as demonstrated by phenolic compounds and peroxidase activity measurement. Furthermore, root application is more effective than foliar. The authors also suggest that an increase in peroxidase activity resulting from oxidative burst (ROS) is a precursor of phenolic compounds accumulation. It seems that activation of plant defence gene and secondary metabolite production can be attributed to Peroxidase-Mediated Reactive Oxygen Species production (Ben-Jabeur et al., 2015). Moreover, induction of defence enzymes associated with SAR such as ß-l,3-glucanase, chitinase and peroxidase activity have been observed for different essential oil/constituents namely *Cinnamomum zeylanicum* oil/*trans*-cinnamaldehyde (Perina et al., 2019), Indian clove EO/eugenol (Lucas et al., 2012) and citronella EO/citronellal (Pereira et al., 2012).

3. Mechanism of detoxification

Plants have evolved pathways to decrease the toxicity of allelochemicals released from neighbours and xenobiotics. These mechanisms can be summarised as the metabolisation of phytotoxins or conjugation/sequestration followed by compartmentalisation or emissions.

Reduction and esterification of aldehydes to their alcohols have been demonstrated for green leaf volatiles such (GLV) as (Z)-3-hexenal (Matsui et al., 2012) but also as previously mentioned for monoterpenes such as citral to nerol and geraniol and limonene to carvacrol (Chaimovitsh et al., 2017). Similar reactions pathways were mentioned for citronellal by *Solanum aviculare* suspension cultures to menthane-3,8-diol, citronellol and isopulegol (Vaněk et al., 2003). Wheat seeds exposed to EOs were also able to oxidize and reduce different terpenes namely neral, geranial, citronellal, pulegone, carvacrol to the corresponding alcohol and acids using non-specific enzyme systems. The authors have suggested that the reduction activity was catalysed by non-specific dehydrogenase and oxidation by P-450 types enzymes (Dudai et al., 2000). Interestingly, part of the applied compound is degraded as demonstrated by the impossibility to account for all the compounds supplied to the germinated seeds. Moreover, derivates are less toxic compared to parent compounds (Dudai et al., 2000). *Anethum graveolens* hairy root cultures biotransform two oxygen-containing monoterpene substrates, menthol or geraniol in 48 h to menthyl acetate, linalool, α-terpineol, citronellol, neral, geranial, citronellyl, neryl, geranyl acetates and nerol oxides (Faria et al., 2009).

Other detoxifying mechanisms rely on conjugation with carbohydrates, or glycosylation, to sequestrate VOCs. Compared to the free aglycones they present a higher solubility in water and a smaller reactivity which facilitates their storage in the vacuoles and protects from the aglycones toxicity (Rivas et al., 2013). Numerous studies demonstrate this glycosylation by *Eucalyptus perriniana* culture cell which converts thymol, carvacrol, and eugenol into the corresponding β-glucosides and β-gentiobiosides (Shimoda et al., 2006). Biotransformation products were isolated following administration of 1,8-cineole as well. Following the administration of camphor seven new mono-glucosides products were isolated. Interestingly, the oxygen function was introduced before the glycosylation and ketone group reduction was not observed (Orihara & Furuya, 1994a). (−)-fenchone administration delivered six new biotransformation products with specific regio- and stereoselectivity for the hydroxylation reaction (Orihara & Furuya, 1994b). Similar results were obtained for sesamol (Shimoda et al., 2009) and vanillin (Sato et al., 2012).

Cell suspension of *Achillea millefolium* administrated with geraniol, borneol, menthol, thymol and farnesol converts these into several products and glycosylate both

the substrates and the biotransformation products. The decrease in glycosylated compounds afterwards implies that this glycolisation mechanism is both used as detoxification and to convert VOCs in readily usable forms to incorporate them in the metabolism (Figueiredo et al., 1996).

This mechanism was also acknowledged *in planta* as demonstrated for (*Z)*-3-hexenol produced by plants under insect attack (Sugimoto et al., 2015). This glycolised form acts as a defence molecule against herbivores, and is accumulated as prevention for the next attack. A large number of plant families use glycolisation as a common pathway of exogenous VOCs plant perception. Similar results are observed for other types of alcohols including aromatic, aliphatic and terpene compounds (Yamauchi et al., 2015).

Another sequestrating reaction consisted in the glutathionylation of GLV which has been demonstrated for methacrolein whose gluthation conjugates have been isolated from vapour exposed tomato (Muramoto et al., 2015). α, β-unsaturated aldehydes also react with gluthation (Galindo et al., 1999). Overall various processes have been developed by plants to detoxify and they are summarised in Figure 4.

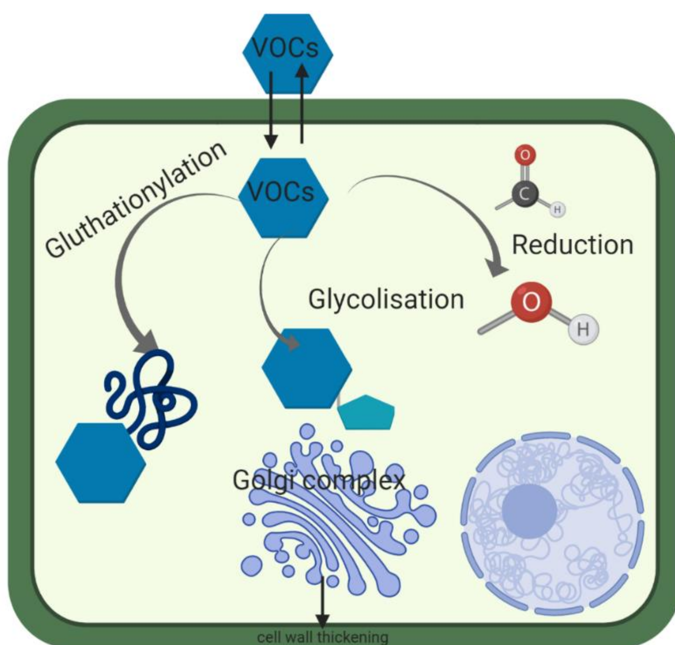


Figure 4. Sequestration and biotransformation of exogenous volatile organic compounds (VOCs) in plant.

4. Discussion

EOs physiological impacts have been and can be studied at the metabolomic (Synowiec et al., 2019), proteomic (Araniti et al., 2020) and transcriptomic (Rienth et al., 2019) levels and large amounts of untargeted data will emerge by grouping these techniques of research together. As phytotoxicity is either a goal (herbicide) or a constraint (other biopesticidal application or biostimulation), both parts will be discussed separately.

Regarding herbicidal application, cellular metabolism reactions are clearly involved in the phytotoxic properties of EOs. The scientific community is making progress in identifying the cellular functions affected, such as photosynthesis, respiration, etc. and research is advancing in molecular target identification. Nevertheless, due to the many interconnecting pathways that are involved simultaneously, no clear distinction has appeared between the diverse chemical classes of EOs compounds. Most of them are grouped within one EO which makes the unravelling of the specific mode of action a complex process. However, their effect can be distinguished between a general stress type response (ROS or osmotic related) compared to a more specific target (microtubule for example) leading to cellular impairment at much lower concentration.

To demonstrate persistence and efficiency in the targeted biological system, medium and long-term effects are most important. To answer these questions, it seems most interesting to deepen the study on the dynamics of the compounds and their fate in plant metabolism in regards to the capacity of the plant to metabolize, detoxify, sequestrate and compartmentalize. Phytotoxicity towards weeds without affecting the crop is essential to develop selective bio-herbicides. In this regard, the identification of other molecular mechanisms such as sugar and amino acid accumulation to prevent EO stress seems promising as demonstrated in maize (Synowiec et al., 2019).

A last point is the composition of the EOs. High complexity of EOC needs to be characterised properly as hundreds of compounds sometimes occur (Brokl et al., 2013). Moreover, variability within the same genus or plant has been frequently observed depending on many parameters such as chemotype, climate, soil, exposure, and from one year to the next (Benini et al., 2012; Tanoh et al., 2020), sometimes leading to fundamentally different composition (Nea et al., 2019). However, even if fundamental interaction cannot be studied properly for hundreds of compounds, their diverse mechanisms of action can constitute a strong opportunity for synergistic effect and prevent adaptation by the weed species. Interaction between different essential oil components can allow to reduce the application while still effectively preventing germination and weed growth (Mirmostafaee et al., 2020).

On the other hand, the phytotoxicity of EOs has long been considered as its main constraint regarding the development of other biopesticides (insecticides, fungicides, etc.). Phytotoxic consideration is currently often limited to the trade-offs of efficiency against the targeted pest versus visual innocuousness to the protected crop. As illustrated in Table 1, large variation occurs regarding the phytotoxic properties of EOs or their constituent's depending on the application systems and mode of action considered.

Table 1. Phytotoxic properties of essential oils or constituents solutions in diverse application mode/rate.

Mode of action	Essential oils or Constituents	Application mode (time)	Plant target	Observation	Ref
Water status alteration	Camphor (10 mg/L) menthol (5 mg/L)	Vapor exposure (for 24 to 96h)	*A. thaliana*	Scanning electron microscopy, Transpiration, PCR, western blot	(Schulz et al., 2007)
	Camphor (10 mg/L)	Vapor exposure (for 24 to 96h)	*A. thaliana*	Real time PCR, *in vivo* cytoskeleton visualisation	(Kriegs et al., 2010)
	Clove oil (2.5%) eugenol (1.5%)	Sprayed at 50 mL/m^2	Broccoli, lambsquarte, pigweed	Membrane integrity (EL), spray solution retention	(Bainard et al., 2006)
	Citral (1200 - 2400 µM)	Watered every 2 day (25 mL per pot)	*A. thaliana*	Water/osmotic potentials (Ψw/Ψs) pigment, protein, anthocyanin, stomata density	(Graña et al., 2016)
	Trans-caryophyllene (450 - 1800 µM)	Watering (25 mL/pot) or spraying (15 mL/pot)	*A. thaliana*	Chlorophyll a fluorescence, osmotic potential, MDA, pigment, proline, protein and element content	(Araniti, et al., 2017)
Membrane properties and interaction	*Mentha piperita* (5 - 900 ppm)	Perfusion	*Cucumis sativus*	Root segment membrane potential determination	(Maffei et al., 2001)
	C. zeylanicym *C. winterianus* (3%)	Sprayed (10 L/m^2)	*A. thaliana*	Herbicide Tests + in silico approach	(Lins et al., 2019)

	1,8-cineole, thymol, menthol, geraniol, camphor (21.7, 2.0, 1.9, 2.5, 7.4 mg/L)	Vapor exposure	*Zea mays*	Lipid, peroxide and lipid peroxidation	(Zunino & Zygadlo, 2004)
				Sterols and phospholipid fatty acid (PLFA) composition	(Zunino & Zygadlo, 2005)
Reactive oxygen and nitrogen species induction	*α*-pinene (1.36 - 136 mg/mL)	Vapor exposure in petri dish For 3to 7 days	*C.occidentalis, A. viridis, T. aestivum, P. sativum,*	EL, MDA, H_2O_2, proline, ROS scavenging enzymes (SOD, APX, GPX, CAT, GR)	(Singh et al., 2006)
	β-Pinene (0.02 - 0.80 mg/mL)				(Chowhan et al., 2013)
	β-pinene (1.36 - 13.6 μg/mL)	Vapor exposure for 4 to 24 h	Wheat seed	H_2O_2, O^{2-}, MDA, ROS scavenging enzymes, LOX	(Chowhan et al., 2014)
	Citronellol (50 - 250 μM)	Watered for 24, 48, and 72 h	Wheat seed	MDA, EL, CDs, LOX In situ histochemical analyses	(Kaur et al., 2011)
	P. benghalensis (0.25 - 2.5 mg/mL)	Vapor exposure	*Avena fatua Phalaris minor*	H_2O_2, O^{2-}, MDA, CDs, EL, ROS scavenging enzymes	(Dahiya et al., 2020)
	Monarda didyma (0.06 - 1.250 μg/mL)	Vapor exposure for 5 days	Weed seed	H_2O_2, MDA	(Ricci et al., 2017)
	Artemisia scoparia (0.14 - 0.70 mg/mL)	Vapor exposure for 5 days	wheat seed	O^{2-}, H_2O_2, proline, root oxidizability, cell death	(Kaur et al., 2012)
	Heterothalamus psiadioides (1 - 5 μL)	Vapor exposure for 7 days	*A. thaliana*	Histochemical detection of H_2O_2	(Lazarotto et al., 2014)

	β-pinene (135 μM)	Applied to Organelles suspension	Chloroplast (*Spinacia oleracea)*	O_2, protein, chlorophyll, electron microscopy	(Pauly et al., 1981)
	β-pinene (945 μM)	Applied to Organelles suspension	Chloroplast (*Cucurbita pepo)*	O_2, protein, chlorophyll Gel electrophoresis and immunoblotting	(Klingler et al., 1991)
	β-pinene (0.02 - 0.80 mg/mL)	Vapor exposure for 3, 5 and 7 days	*Oryza sativa*	Chlorophyll, protein, carbohydrate, proteases, *α*- and *β*-amylases, POD, PER	(Chowhan et al., 2011)
Photosynthesis inhibition	*Cymbopogon citratus* (1.25 - 10% (v/v))	Foliar sprayed at 1000 L ha^{-1}	Barnyardgrass	Chlorophyll a, b and carotenoid, EL, MDA	(Poonpaiboonpipat et al., 2013)
	Hyptis suaveolens (1% - 5% (v/ v))	Foliar sprayed (10 mL/plant)	*Oryza sativa* *E. crus-galli*	Total chlorophyll content, cell viability, Cytogenetic analysis	(Sharma et al., 2019)
	Farnesene (0 - 1200 μM)	Grown in medium for 14 days	*A. thaliana*	Root gravitropism, structural studies, electron microscopy, O^{2-}, H_2O_2, microtubule, ethylene, auxin	(Araniti et al., 2016)

	Artemisia fragrans (0.5, 1, 2 and 4%)	Spraying (100 mL/pot) for 5 days	*Convolvulus arvensis*	Chlorophyll a fluorescence, chlorophyll, ROS scavenging enzymes, H_2O_2, MDA	(Pouresmaeil et al., 2020)
	Clove oil (2.5%), eugenol (1.95%)	Covered by solutions	Broccoli	Chlorophyll a fluorescence imaging at 20, 40 and 60 min	(Synowiec et al., 2015)
	Origanum vulgare (0 - 500 μL/L)	Grown in medium for 10 days	*A. thaliana*	Chlorophyll a fluorescence, chlorophyll, protein, MDA, Ionomic, metabolomic	(Araniti et al., 2018)
Mitochondrial respiration inhibition	1,8-cineole (6 mM)	Apply to organelle	*A. fatua*	O_2 consumption	(Muller et al., 1969)
	Juglone (10 mM)	Bathed in dark for 30 min	Soybean cotyledons	O_2 consumption and isotope fractionation	(Peñuelas et al., 1996)
	α -pinene, camphor, eucalyptol and limonene (0.1 to 10mM)	Vapor exposure / apply to organelle	Maize	Protein, seed germination, growth test and oxygen uptake	(Abrahim et al., 2000)
	α –pinene (50 - 500 μM)	Grown in medium for 10 days	Coleoptiles and primary roots of maize	O_2 consumption, mitochondrial ATP production	(Abrahim et al., 2003)
	Pulegone, menthol, menthone (0 - 1500 ppm)	Foliar sprayed	Cucumber seeds (roots segments, mitochondria)	O_2 uptake, mitochondrial respiration	(Mucciarelli et al., 2001)
	Camphor,	Apply to organelle suspension	Corn and soybean	Mitochondrial respiration	(Ishii-Iwamoto et al., 2012)

	1,8-Cineole, Limonene, *α*–pinene (0 – 500 µM)				
	1,8-Cineole (0 - 2000 µM)	Vapor exposure	*N. tabacum* (seeds)	Growth, protoplasts proliferation, starch accumulation of BY-2	(Yoshimura et al., 2011)
Microtubule disruption and genotoxicity	Citral (0 - 1.0 µL)	Vapor exposure	*A. thaliana*	Microscopy, *in vitro* polymerisation of microtubules	(Chaimovitsh et al., 2010)
	Citral (0 - 1.200 µM)	Grown in medium 14 days	*A. thaliana*	Ultra-structural, pectin/callose, mitotic indices, ethylene, auxin	(Chaimovitsh et al., 2012)
	Limonene, citral, carvacrol, pulegone (4.6 - 9.2 µmol/20 mL)	Vapor exposure for 0, 15, 30 and 60 min	*A. thaliana*	Membrane, microtubules, F-actin, (confocal microscopy), *in Planta* monoterpene concentrations	(Chaimovitsh et al., 2017)
	Menthone	Vapor exposure	*Tobacco BY-2* *A. thaliana*	GFP-tagged markers for microtubules and actin filaments	(Sarheed et al., 2020)
	Schinus molle *Schinus terebinthifolius*	Vapor exposure 0.1 mL for 72 h	*Allium cepa* *Lactuca sativa*	Cytogenetic assay	(Pawlowski et al., 2012)
	Citrus aurantiifolia (0.10 - 1.50 mg/mL)	Vapor exposure (10 mL) for 3 h to 24 h	*Avena fatua*, *E. crus-galli*, *Phalaris minor*	Phytotoxicity: dose-response assay, cytotoxicity (*Allium cepa)*	(Fagodia et al., 2017)

	Plectrantus amboinicus (0 - 0.120% w/v)	Vapor exposure for 48 h	*Lactuca sativa Sorghum bicolor*	Germination speed index, percentage of germination	(Pinheiro et al., 2015)
	Mentha longifolia (10 - 250 µg/ml) (0.5 - 5%)	Vapor exposure Foliar sprayed (5 mL/pot)	*Cyperus rotundus E. crus-galli, Oryza sativa*	Germination, root length, coleoptile length, chlorophyll, cytotoxicity assay (*Allium cepa*)	(Singh et al., 2020)
	Nepeta nuda (0.1 - 0.8 µl/mL)	Vapor exposure (10 mL) for 7 days	*Zea mays*	Randomly amplified polymorphic DNA, quantitative analysis of proteins	(Bozari et al., 2013)
	Salvia leucophylla (0 - 1300 µM)	Vapor exposure for 4 days	*Brassica campestris*	DAPI-fluorescence microscopy, immunofluorescence microscopy, DNA Synthesis Activities	(Nishida et al., 2005)
	Vitex negundo (0.1 - 2.5 mg/mL)	Vapor exposure (12 mL)	*Avena Fatua, E. crus-galli Onion bulbs*	Phytotoxicity, cytoxicity	(Issa et al., 2020)
	S-carvone (125 µL)	Vapor exposure (several days)	*Solanum tuberosum*	Potato sprout growth, HMGR activity, membrane protein composition, transcription activity	(Oosterhaven et al., 1993)

	R/*S*-carvone (25 -125 µL)	Vapor exposure (several days)	*Solanum tuberosum*	Growth inhibition, carvone and conversion products in potato sprouts	(Oosterhaven et al., 1995)
	Peppermint oil (0.1% (v/v))	Vapor exposure	*Solanum tuberosum*	Potato sprout growth, protein extraction, enzyme activity, qRT-PCR for potato α –amylase	(Rentzsch et al., 2012)
Phyto-hormones	Ten monoterpenes (0.5 - 2 mM)	Vapor exposure (6 mL) for 9 days	*Silybum marianum*	carbonic anhydrase activity	(Abdelgaleil et al., 2014)
	Farnesene (250 µM)	Grown in medium for 14 days	*A. thaliana*	anatomy/meristem, mitotic indices, quantitative PCR, auxin gradient and polar transport	(Araniti et al., 2017)

Bioassays should ideally provide a range of toxic concentrations according to the mechanism involved in the toxicity process. Standardised methodologies/protocols to define the toxicity level of individual compounds as well as their blends are needed at macroscopic or remote level and specific scale to allow prediction. It is always a question of targeting an applied plant model and then defining the toxicity level in those specific application conditions. In this regard, *in vivo* redox and osmotic status sensor should be used as a specific marker for toxicity level. Indeed, wide differences in concentration assayed are reported in Table 1 depending on EO, model plant and mode of application considered which prevent to determine and/or predict precise phytotoxicity threshold. Hence, those concentrations aspects were not discussed in the framework of the mechanisms presented previously. Furthermore, all reported data concerned herbaceous species. Indeed, apart from blossom thinning experiment in apple using EO, very few data are available regarding phytotoxicity phenomena and threshold on woody crop species.

Other opportunities seem to arise at low concentration far below the toxicity threshold, such as biostimulation (Souri & Bakhtiarizade, 2019), priming and direct elicitation of defence mechanisms (Banani et al., 2018). This direct elicitation of the systemic defence mechanism can also result in broader abiotic pest protection and be a pertinent agronomical strategy. However, limitations arise in regard to the allocation of resources (growth-defence trade-off) and reduced efficiency compared to a synthetic product. The same essential oils/constituents are sometimes mentioned to be phytotoxic at high concentration and beneficial at a low one following a dose response concept with a concentration threshold effect. It has been proposed that these low doses simulate mild stress (Hara, 2020). However, such threshold models as hormesis are still debated in biology and very little is known about the underlying mechanisms (Calabrese, 2016).

An additional consideration concerns the kinetic release of EOs. Indeed, their persistence and application methods are limited due to low molecular weight, hydrophobicity and high volatility. To overcome these limitations, much work has been done regarding formulation technique to allow a control release profile. A recent promising domain is the formulation of nano-emulsion using bio-based surfactants (Prasad et al., 2019) as well as other encapsulation techniques (Maes et al., 2019).

A final constraint is the market approval by the different regulatory agencies throughout the world as well as economic considerations. Even if procedures are sometimes available for plant based products such as GRAS, list 25b of the EPA (Isman, 2016) or the European Pesticide Regulation (EC) No. 1107/2009 (Villaverde et al., 2014), few a. s. have been registered so far. Easier registration also leads to misevaluation regarding efficacy and safety for consumers. Indeed, in high concentration their use may be economically disadvantageous and exhibit undesirable phytotoxicity (Cloyd et al., 2009). In fact, the mammalian toxicity (LD50) is >1000 mg

kg^{-1} except for some EOs that are moderately to very toxic such as boldo, cedar and pennyroyal with LD50 values of 130, 830 and 400 mg kg^{-1} (Regnault-Roger et al., 2012). Reports of allergenic potential have been made regarding the use of cinnamon and citronella oil (Barceloux, 2008; Environmental Protection Agency, 1997).

Regarding economic considerations, areas of production are increasing every year decreasing the prohibitive cost of EOs. With controversial products being removed from the market, such as the sprout-preventing chemical chlorpropham (CIPC), alternative products such as EOs are expected to rise. Techno-economic assessments are still lacking regarding a large number of applications. These evaluations combining efficacy, plant safety, social and environmental impact should clarify many opportunities for the application of EOs (Mishra et al., 2015).

To conclude, the use of EOs for sustainable agricultural practices seems promising, and extensive research will probably clarify or deny their relevance in diverse applications. Due to their inherent characteristics, the pest control properties are usually very transitory and less effective than synthetic products. However, EOs can be an efficient alternative to conventional plant protection products when properly formulated and integrated with other pest management strategies.

3

Phytotoxicity and plant defence induction by *Cinnamomum cassia* essential oil

The third chapter of this thesis presents a research article published in 2022 in Agronomy:

Werrie, P.-Y., Juillard, A., Heintz, C., Brisset, M.-N., and Fauconnier, M.-L. (2022). Phytotoxicity and plant defence induction by *Cinnamomum cassia* essential oil application on *Malus domestica* Tree: a molecular approach. *Agronomy*, *12*(2), 512. doi:10.3390/AGRONOMY12020512

As detailed in the previous chapter, phytotoxicity to non-target crop organisms can hinder botanical insecticide development based on EOs. Therefore, these phenomena must be considered in the global attempt to control the RAA population in orchards. It must be stressed that due to the very transitory aspect of the mechanism considered, this investigation have been performed by foliar application and not by trunk injection. Furthermore, foliar application constitutes the conventional method for determining the mode of action. Although direct comparison with trunk injection could not be performed, this unravelled mechanism knowledge helps explain and design appropriate applications.

In the first part of this chapter, the phytotoxic reaction triggered by cinnamon EO is investigated by oxidative burst reaction (following glutathione redox state) and oxidative damage quantification (photosynthetic pigments and malondialdehyde).

The second part of this chapter evaluates the potential of cinnamon EO to modify the plant transcriptome, especially regarding the defence pathways. Thus, relative expression levels of 29 defence genes from PR proteins, secondary metabolism, oxidative stress, parietal modification and hormonal signalling were measured.

The last part discusses the potential of such reactions in an agronomic application context, as well as the research perspective.

Contribution: Conceptualisation, methodology, formal analysis (apart from RNA extraction and RT-PCR analysis), writing—original draft review and editing. Molecular biology RT-PCR analysis was performed by a specialised laboratory of the INRA which developed and patented a procedure to follow apple tree defence activation.

Abstract

Essential oils (EOs) are actively investigated as an alternative to numerous synthetic biocide products. Due to their large spectra of biological activities, the impact of EOs on non-target organisms should be characterised for biopesticide development purposes. In this study the potential phytotoxicity of *Cinnamomum cassia* EO (CEO) on apple trees (*Malus x domestica*) was investigated in terms of oxidative burst (glutathione redox state) and damage (malondialdehyde). At 2% CEO concentration, the reduced glutathione leaf content drops from 269.6±45.8 to 143.0±28.4 nmol g_{FW}^{-1}, after 30 min, illustrating a rapid and strong oxidative burst. Regarding oxidative damage, malondialdehyde increased significantly 24 h post application to 10.7±3.05 nmol g_{FW}^{-1}. Plant defence induction was previously suspected after *trans*-cinnamaldehyde (CEO main compound) application. Therefore, the elicitor potential was investigated by qRT-PCR, on the expression level of 29 genes related to major defence pathways (PR proteins, secondary metabolism, oxidative stress, parietal modification). Multivariate analysis and increased expression levels suggest induction of systemic resistance. Hence, the present research illustrates the dose–dependent phytotoxicity of CEO in terms of lipid peroxidation. Transcriptional data illustrates the elicitor properties of CEO. These findings can help to design pest management strategies considering both their risks (phytotoxicity) and benefits (defence activation combined with direct biocide properties).

1. Introduction

Owing to their antibacterial (O'Bryan et al., 2015), fungicidal (De Clerck et al., 2020), insecticidal (Jankowska et al., 2018), acaricidal (Peixoto et al., 2015), nematicidal (Andrés et al., 2012) and herbicidal (Raveau et al., 2020) properties, essential oils (EOs) are increasingly investigated to be included in agricultural practices as biopesticides. According to Dayan et al. (2009), the fungicidal mode of action (MOA) consists of the inhibition of synthesis of the fungal cell wall component chitin. In addition, EOs have some properties that make them suitable for insects management. EO physiological actions on insects suggest a neurotoxic MOA (Coats et al., 1991; Kostyukovsky et al., 2002), notably through octopamine synapses, γ-aminobutyric acid (GABA) and acetylcholinesterase (AChE) inhibition (Mossa, 2016; Pavela & Benelli, 2016). Recent studies reveal larger impacts on the development and functioning of the muscular and nervous systems, cellular respiration, protein synthesis, and detoxification (Renoz et al., 2021). The aforementioned biological properties make them a good alternative to synthetic pesticides. Moreover, they follow the European directive (2009) of a reduced risk for human health and to the environment. Their phytotoxic properties make them suitable for weed control, but are not desirable in other application contexts. In order to enable their large-scale use in the field, their potential phytotoxicity with non-target organisms especially crop plants from an agronomic perspective must be assayed.

Adverse physiological impact following EO application are disparate: water status alteration, inhibition of respiration and photosynthesis, membrane interaction/disruption, reactive oxygen/nitrogen species induction, microtubule disruption and enzymatic or phytohormones regulation (Werrie et al., 2020). From a mechanistic point of view, most of these alterations originate from, or lead to, reactive oxygen species (ROS) production. An oxidative burst following abiotic stresses is one of the largest shared responses in plants. The cell redox state modification, if not properly handled by the antioxidant system, can result in oxidative damage and lead to programmed cell death (PCD). Therefore, ROS were long considered as a toxic by-product of metabolism. Nevertheless, they also play a key role as regulators of growth and defence pathways (Mittler et al., 2004). Plant cells are well equipped to efficiently scavenge ROS and their reaction products by the coordinated action of non-enzymatic and enzymatic antioxidant components. Among the non-enzymatic ones, glutathione is a major component of the ascorbate-glutathione (AsA-GSH) pathway, playing a significant role in protecting cells against ROS-accrued potential anomalies (Gill et al., 2013). Most data suggest that enhanced ROS availability, especially hydrogen peroxide (H_2O_2), has less impact on the ascorbate-dehydroascorbate (DHA) ratio than on the redox status of the glutathione pool (Noctor et al., 2011). Various stress conditions drive characteristic changes in the intracellular amount and redox state of glutathione. Thus, modifications in the whole glutathione status can be taken as a reliable marker of the degree of intracellular oxidative stress (Hajdinák et al., 2019; Queval

et al., 2010). The main function of glutathione consists of a redox-homeostatic buffering, serving as a ROS scavenger, but it also plays a role in stress perception, signalling and defence reactions (Davey et al., 2003; Noctor et al., 2012).

In biological systems, oxygen-derived free radicals have repeatedly been demonstrated to play a role in cellular injury through chain reactions leading to the degradation of macromolecules such as lipids, carbohydrates, proteins and DNA (Gniazdowska et al., 2015). Indeed, much of the injury caused by exposure to biotic and abiotic stresses is associated with oxidative damage at the cellular level, particularly losses in bio-membrane integrity due to formation of lipid peroxides (Foyer et al., 1997). It should be noted that following a pathogen invasion or injury, this reaction may also originate from increased lipoxygenase activity (Morales & Munné-Bosch, 2019). Primary lipid hydroperoxides are highly unstable and reactive, quantification of lipid peroxidation is usually estimated by focusing on secondary oxidation products derived from them, such as malondialdehyde (MDA) (Davey et al., 2005). In studies related to oxidative stress, the measurement of MDA content has been demonstrated to be a reliable lipid peroxidation marker, representative of a rather late stage of oxidation (Miguel, 2010; Morales & Munné-Bosch, 2019). The accumulation of MDA following EO application is frequently observed with, for example, *Origanum vulgare* (Araniti et al., 2018), *Artemisia Fragrans* (Pouresmaeil et al., 2020), *Cymbopogon citratus* (Poonpaiboonpipat et al., 2013) or pure compounds including cinnamaldehyde (Gao et al., 2018).

After treatment with EOs, a decrease in the photosynthetic pigments namely chlorophylls and carotenoids in a dose-dependent way have also been reported, resulting from a direct pigment degradation or from an impairment in pigment biosynthetic pathways (Chowhan et al., 2011; Poonpaiboonpipat et al., 2013). Total leaf chlorophyll (Chl) content is a popular trait used to get an idea of the plant's photosynthetic capacity. Chl a and Chl b are the two forms of pigments that predominate in higher plants. Differently involved in light assimilation, Chl a is linked to the photosystems energy-processing centres whereas Chl b is an accessory pigment for harvesting light energy and transmitting it to Chl a (Bresson et al., 2018). Concerning carotenoids (Car), they act first as collectors of light energy driving photosynthetic processes. As antioxidants, their second role is the protection of the photosynthetic system against detrimental effects of light and O_2 (photo-oxidation), by scavenging ROS and the quenching of Chl excited states (Gitelson, 2004; Radhakrishnan et al., 2018).

Apart from phytotoxicity, glutathione and malondialdehyde play a role as regulators of plant defence pathways (Gomez et al., 2004; Han et al., 2013; Weber et al., 2004). Moreover, monoterpenoids are able to activate defence genes by signalling processes and Ca^{2+} influx causes by membrane depolarisation, protein phosphorylation/dephosphorylation and the action of ROS (Maffei, 2012). This gene expression can either lead to priming (an accelerated gene-response to biotic stress) or direct

defence elicitations. Priming properties have been observed in wheat seed with application of thyme EO (Ben-Jabeur et al., 2019) and also in apple against *Botrytis cinerea* with thyme and savory EO (Banani et al., 2018). Priming following exposure to mint volatiles resulted in enhanced transcript levels of defence genes in soy through histone acetylation within the promoter regions (Sukegawa et al., 2018). Regarding defence elicitation, systematic resistance induction is divided between systemic acquired resistance (SAR) and induced systemic resistance (ISR). A complex crosstalk exists between the two systems relying on salicylic acid (SA) and jasmonic acid (JA) hormones. Transcriptomic studies following exposure to volatile monoterpenes myrcene and ocimene demonstrated that plants develop a similar response to that induced by methyl jasmonate (MeJA) (Godard et al., 2008). The induction of SAR by EO has also been acknowledged in multiple pathosystems with nerodiol on tea plant (Chen et al., 2020), thyme (Ben-Jabeur et al., 2015) and clove EO (Jhonata et al., 2015) on tomato, *C. zeylanicum* and *trans*-cinnamaldehyde EO on tangerine (Perina et al., 2019), and citronellal on coffee plant (Pereira et al., 2012). Expression of defence-related genes is considered the hallmark to decipher the potential elicitor properties (Dugé De Bernonville et al., 2014).

For example, *Cinnamomum cassia* EO (CEO) has been previously commercialised by the Mycotech Corporation U.S. company as an aphicide/miticide/fungicide based on cinnamaldehyde (30% in the formulation) as the active ingredient (Koul et al., 2008). Cinnamaldehyde is also synthesised chemically for use as a fungicide in agriculture (e.g., Vertigo™, Cinnacure™) on a variety of crops. Depending on the biological activity targeted different concentrations of EO can be applied. Indeed, *in vivo* herbicidal activity of cinnamaldehyde has been observed at 3% (V/V) concentration on *A. thaliana* leaves (Lins et al., 2019). Field insecticidal activity against two spotted mites in cherry fruit was observed after five applications at 0.25% (V/V) concentration (Mezőfi et al., 2018). *C. zeylanicum* and cinnamaldehyde was applied against *Alternaria* brown spot in tangerines in the field at 0.1% (V/V) (Perina et al., 2019). Finally, EPA registration for Cinnacure™ has recommended an application rate at 0.4% (V/V) as a fungicide and insecticide in fruit trees (Washington & Mavian, 1999).

The objective of this study is to investigate the molecular mechanisms resulting from different concentrations of *Cinnamomum cassia* EO application (1-2%) on young *Malus x domestica* trees, especially the resulting oxidative burst and the potential oxidative damage. Moreover, the potential plant gene defence activation properties have been investigated by following 29 transcripts from major defence pathways.

2. Materials and methods

2.1 Plant material

Experiments on redox status, oxidative damage and photosynthetic pigments were conducted on mature leaves of two-year-old micropropagated *Malus x domestica* Borkh cv. Jonagold apple trees (height = 53±8 cm; diameter = 4.4±0.6 mm). They were kept in a climate chamber under the following conditions: 21±0.5°C, 60±10% RH, 16:8h light: dark periods and a photosynthetic active radiation (PAR) intensity equal to 50 µmol m^{-2} s^{-1}.

Experiments for transcriptional studies were conducted on open-pollinated apple seedlings (4-6 leaves) of cv. Golden Delicious, grown under greenhouse conditions (natural photoperiod supplemented with artificial light if needed, 17°C night and 20–23°C day according to the sun light).

2.2 Emulsion formulation and application

A cinnamon EO emulsion (1-2% (v/v) Pranarôm, batch number: CCB114) was obtained using Tween 80 (2%). The emulsion was stabilised using high speed homogenisation (HSH) at 9500 rpm for 6 min (Ultra-Turrax T25) followed by high pressure homogenisation (HPH) with 8 cycles at 5000 psi (FMC). Following a previously published protocol (Dugé De Bernonville et al., 2014; Le Mire et al., 2018, 2019; Warneys et al., 2018) approximately 30 mL of solution was applied on each plot to runoff.

2.3 Redox status: Determination of reduced (GSH) and oxidised glutathione (GSSG)

GSH can be derivated using monobromobimane (MBB), the amount of GSH-MBB adduct formed was then measured by high performance liquid chromatography with fluorescence detection (HPLC-FLD). As MBB only reacts with the reduced form GSH, the content of oxidised glutathione GSSG in the samples must be reduced by the addition of dithiothreitol (DTT) in order to obtain the total amount of glutathione (i.e. GSH + GSSG). In this way, the approximate redox state of glutathione can be estimated. The developed method is based on (Queval & Noctor, 2007), (Queval et al., 2010) and (Hajdinák et al., 2019). Briefly described, apple leaves were ground in liquid nitrogen. 100 mg of this powder were mixed with 1 mL of ice-cold acid extraction buffer (0.4 M HCl, 1 mM Na_2EDTA, 1% PVP). The samples were vortexed, centrifuged at 12000 rpm for 2 min and the supernatant was filtered through a 0.45 µm syringe filter. For GSH 100 µL of supernatant was mixed with 100 µL $NaHCO_3$, 20 µL H_2O, 200 µL CHES (0.5 M, pH 9) and 20 µL MBB (30 mM in acetonitrile)

and left to incubate for 15 min in the dark on ice. The reaction was then stopped by adding 660 µL of acetic acid (10%), followed by transfer to amber vials. For GSH+GSSG 100 µL of supernatant was neutralised and reduced in Eppendorfs with addition of 100 µL $NaHCO_3$ and 20 µL DTT and incubated for 30 min in the dark and on ice. Then 200 µL CHES and 20 µL MBB were added. The reaction medium was left to incubate for 15 min in the dark and on ice. The reaction was then stopped by adding 660 µL of acetic acid (10%), followed by transfer to amber vials. All analyses were performed on an Agilent 1260 Infinity HPLC system equipped with a FLD detector (λex: 395 nm, λem: 477 nm). The autosampler was thermostated at 6°C and 50 µL was injected onto the Eclipse XDB-C18 column (150 x 4.6 mm,5 µm). The GSH-bimane derivatives were separated from the other molecules using a linear gradient of 0.25% (v/v) acetic acid (pH 3.5) as solvent A, and 100% methanol as solvent B, at a flow rate of 0.8 mL min^{-1} and a column temperature of 40°C. The linear gradient started at 18% (v/v) solvent B until 17.5 min then increased to 100% (v/v) solvent B from 20 min to 27.5 min and returned to original condition 18% (v/v) solvent B at 28 min until the run ended at 32.5 min. GSH typical sample chromatogram, calibration curve, LOD and LOQ are available on Supplementary Figure 2.

2.4 Oxidative damage

2.4.1 Determination of malondialdehyde (MDA)

The thiobarbituric acid reactive substances (TBARS) content was determined according to the method of (Davey et al., 2005) with modifications mainly based on (Velikova et al., 2000) and (Bresson et al., 2018). Apple leaves were ground in liquid nitrogen with a mortar and pestle. 100 mg of this powder was mixed in an Eppendorf with 1 mL of ice-cold 5% (w/v) HCl. The samples were vortexed and centrifuged at 13,400 rpm for 10 min. 200 µL of supernatant was added to 40 µL of BHT (0.1% EtOH) and 760 µL of TBA (0.5% in MPA 20%), giving a final pH of approximately 1.0. The reaction mixture was heated for 30 minutes at 95°C and then quickly cooled on ice. Once the reaction had stopped the reaction mixture was centrifuged at 4000 g for 5 min and the supernatant is placed in a vial. All analyses were performed on an Agilent 1200 series HPLC system with MWD detector (RF-10AXL). Chromatograms were monitored at 532 nm and the injection volume was 10 µL. Samples were analysed on a Halo® C18 75 x 4.6 mm, 2.7 µm column thermostated at 40°C and eluted isocratically with 35% MeOH in 50 mM KPO_4 buffer (pH 6.8) at 1 mL min^{-1}. MDA typical sample chromatogram, calibration curve, LOD and LOQ are available on Supplementary Figure 2.

2.4.2 Determination of photosynthetic pigments

50 mg of leaf sample were ground in liquid nitrogen with a mortar and pestle. After 15 min extraction on ice in the dark in 10 mL of 96% (v/v) ethanol, the extract was

centrifuged at 4000 rpm for 10 min at 4°C. The absorbance of the supernatant was measured at 470, 649 and 665 nm using an Ultrospec 7000 spectrophotometer.

The concentrations of chlorophyll a and b and carotenoids were calculated as follows:

Ca (µg/g FW) = [(13.36 * A665) - (5.19 * A649)] / sample mass.

Cb (µg/g FW) = [(27.43 * A649) - (8.12 * A665)] / sample mass.

Ccarotenoids (µg/g FW) = [(1000*A470 - 2.13*Ca - 97.64*Cb)/209]/sample mass.

2.5 Induction of defences (RT-PCR)

The different treatments consisted of foliar application on apple seedlings (4-6 leaves, from open-pollinated M. domestica cv Golden Delicious) of: Bion® 50WG (salicylic acid analogue), Tween 80 aqueous solution (surfactant 2%), and emulsions of CEO at 1% (v/v) concentration (the 2% concentration proving to be phytotoxic). The sampling was performed after one, two or three days (corresponding to D1, D2 and D3). Four biological replicates of the same modalities (pooling of five apple seedlings each) were carried out. At each sampling time (24, 48 and 72 h), the five youngest expanded leaves per modality were collected, pooled, frozen in liquid nitrogen, and stored at -80°C until extraction. Each experiment was repeated four times. RNA extraction, reverse transcription and real-time quantitative PCR were performed as previously described (Vergnes et al., 2014) using the same proprietary primer set for the 29 defence genes and 3 reference genes (Bernonville et al., 2011). Relative changes in defence genes' expression (log2 ratio) were calculated using the $2^{-\Delta\Delta CT}$ method with 3 internal reference genes for normalisation, and against initial time (T0) from control plants.

2.6 Data analysis

All data were collected in Excel and processed using R studio software (version 4.1.2), with all results presented as a boxplot using the ggplot2 package. The main statistical procedure performed was a simple two-factor analysis of variance (ANOVA 2) the fixed factors were time and treatment. The samples came from a randomised design which guarantees their independence. Normality was assayed by the Shapiro-Wilk normality test. Homogeneity of variance was also demonstrated by Levene's test. In case of interaction, ANOVA tests were performed at each time point independently, followed by a pairwise analysis (t-test). A probability cut-off of α = 0.05, was used for tests of significance in all statistical analyses and adjusted with the Bonferroni correction. As qRT-PCR data were non-normally distributed, the nonparametric Kruskall-Wallis test was applied followed by the Conover post-hoc test with

holm correction. Multivariate visualisation was performed with heatmap and principal component analysis (PCA) using Complex Heatmap and FactoMiner packages.

3. Results

3.1. Redox status: determination of reduced (GSH) and oxidised glutathione (GSSG)

The soluble tripeptide GSH (L-γ-glutamyl-L-cysteinyl-glycine) is the principal low-molecular-weight thiol compound in plants (Foyer et al., 1997). Glutathione typically accumulates in plant tissues in the range of 200-600 nmol g_{FW}^{-1} (Noctor et al., 2011). GSH was derivated using monobromobimane (MBB), the amount of GSH-MBB adduct formed was then quantified by HPLC-FLD. As MBB only reacts with the reduced form GSH, the content of oxidised glutathione GSSG in the samples must be reduced by the addition of dithiothreitol (DTT) in order to obtain the total amount of glutathione (i.e. GSH+GSSG).

The results presented in Figure 5 display the reduced glutathione (GSH) leaf content, total content (GSH+GSSG) and GSH/(GSH+GSSG) ratio, over time, after CEO treatment at two concentrations, or tween. Those contents are between 100.7 nmol g_{FW}^{-1} and 486.6 nmol g_{FW}^{-1}. The relatively large standard deviation in the boxplot highlight the heterogeneity of glutathione content between and within *Malus x domestica* leaves. The two-way ANOVA displayed on the top of the graph present significant interactions between the treatment and time implying that the treatment effect is time dependent. This result is consistent with the transitory aspect of the oxidative burst and with the existence of circadian variation within the glutathione ascorbate cycle (Gallé et al., 2019). However pairwise t-test comparisons at each time shows that the GSH content, as well as its ratio, is significantly decreased after 30 minutes following 2% CEO applications. This result underlines indirectly, the production of ROS i.e., the oxidative burst occurring rapidly after CEO application. Under normal (unstressed) conditions, it is maintained mostly in its reduced form, resulting in a GSH/GSSG ratio of 10 to 1 (i.e. GSH/(GSH+GSSG) = 91%) (Pereira et al., 2012). In contrast, under oxidative conditions, two GSH molecules react together to form glutathione disulfide (GSSG). The specific enzyme glutathione reductase (GR), reduces GSSG back to GSH (Mittler et al., 2004). Therefore, GSH fluctuates in cells between two different forms: reduced GSH and oxidised GSSG, as a function of GR activity (with NADPH as an electron donor) (Koul et al., 2008). The proportion of GSSG increases substantially only in a strongly oxidizing environment (Mittler et al., 2004). Therefore, a decrease in GSH and the GSH/(GSH+GSSG) ratio is interpreted as evidence of redox imbalance. Indeed, it was previously established that detoxification of H_2O_2 through the glutathione–ascorbate cycle leads to a transient change in

the oxidation degree of the glutathione pool (Meyer, 2008). Such a transient change is also observed here and may therefore result from H_2O_2 detoxification.

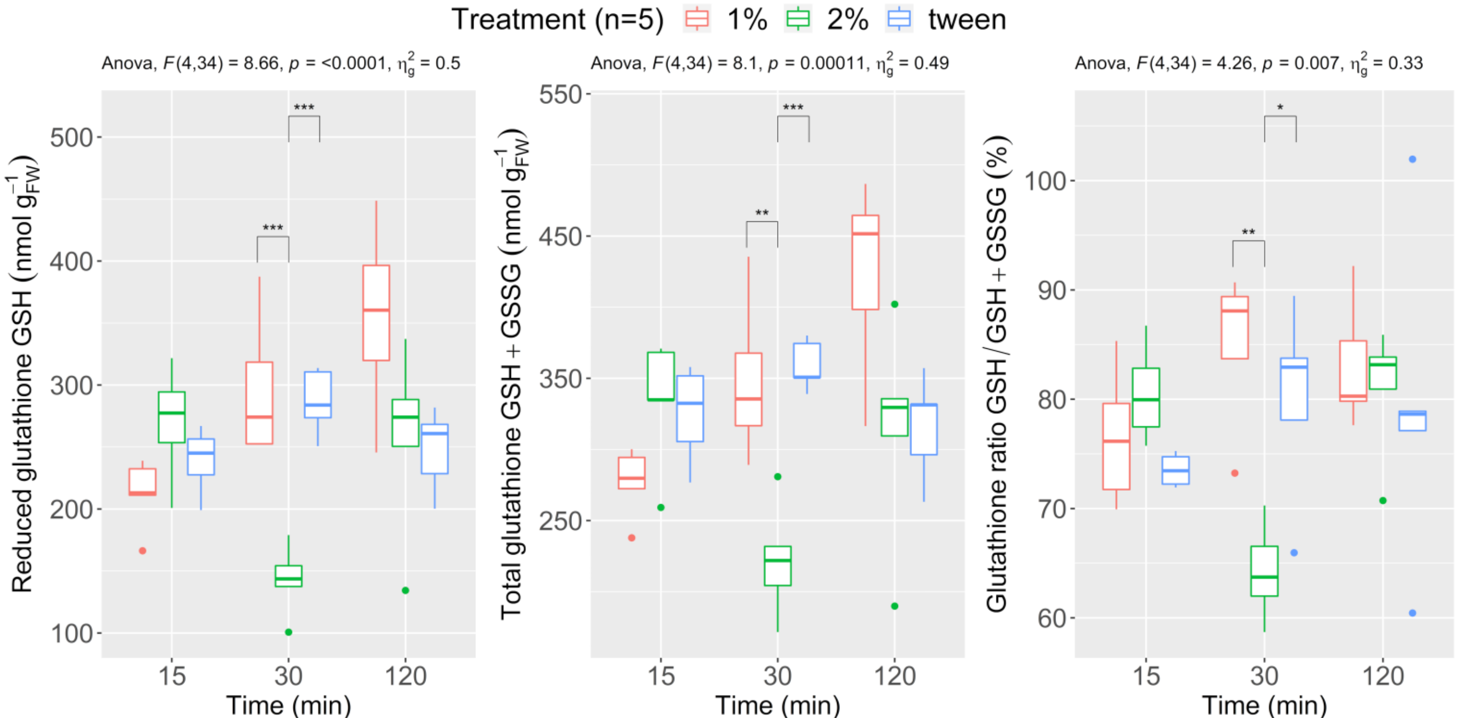


Figure 5. Effect of *C. cassia* EO (CEO) (1 and 2% v/v) and Tween 80 application on glutathione leaf content over time (n = 5): (left) Reduced glutathione GSH (nmol g_{FW}^{-1}); (center) Total glutathione GSH+GSSG (nmol g_{FW}^{-1}); (right) Glutathione ratio GSH/GSH+GSSG (%). Star on boxplot indicates significantly different distributions (*$p<0.05$, **$p<0.01$, ***$p<0.001$, pairwise t-test).

3.2. Oxidative damage

3.2.1 Malondialdehyde content (MDA)

The bulk of MDA in leaf tissue originates from the poly-unsaturated fatty acids' (PUFAs) peroxidation in response to oxidative stress. Its content was monitored by the TBARS assay, combined with a final HPLC-DAD separation step. The results following *C. cassia* EO or Tween 80 applications are shown in Figure 6. The main conclusion that can be drawn from these results is that, while the MDA concentration seems to fluctuate between 0 and 6 h of treatment, it increases drastically after 24h to reach 10.7±3.05 nmol g^{-1}_{FW} for the 2% concentration of CEO. In view of this trend, we can confirm that the peroxidation of membrane lipids causing MDA production would occur between 6 and 24 hours after treatment with 2% CEO. For the data that presented a positive skewness, a square-root transformation was applied for the statistical analysis. The two-way ANOVA displayed on the top of the graph represents significant interaction between the treatment and time implying that the treatment effect is also time dependent. Results of the pairwise t-test confirmed that from 24 h to 72 h, the 2% CEO treatment modality displays significantly higher values of MDA content compared to the other modalities. This result shows that the antioxidant capacities were not sufficient to inhibit the MDA accumulation in plant cells.

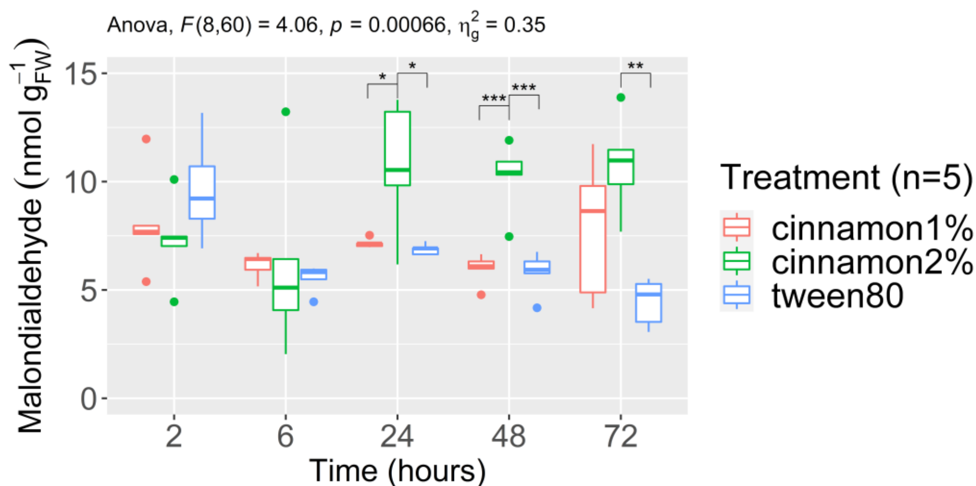


Figure 6. Effect of *C. cassia* EO (CEO) (1 and 2% v/v) and Tween 80 application on malondialdehyde (MDA) leaf content (ng g^{-1}) over time (n = 5). Star on boxplot indicates significantly different distributions (*p< 0.05, **p<0.01, ***p<0.001, pairwise t-test).

3.2.2 Photosynthetic pigment content (chlorophyll a, chlorophyll b and carotenoids)

To follow the potential photosynthetic pigment degradation resulting from CEO application, chlorophyll a, chlorophyll b and carotenoids were measured by spectroscopy in apple leaf ethanolic extract. Their respective contents following CEO application are presented in Figure 7. Chlorophyll a and b contents are in agreement with the literature, with on average, twice the amount of chlorophyll a than b (Tamburini et al., 2015). Moreover, Chl a and b content shows quite a similar tendency, with values sharply decreasing for the 2% concentration after 24h of treatment and increasing again after 48 hours to finally reach initial values. This could be a sign that plant stress management achieved after 48h. The two-way ANOVA displayed on the top of the graph demonstrate significant interaction between the treatment and time for Chl a and b. The previous hypothesis is confirmed by statistical analysis only for chlorophylls b, with the 24-h CEO 2% treated plants significantly different from all others. Concerning the carotenoids, the trend is quite different, with values remaining broadly stable from one-time step to the next. There is no significant observable difference over time apart for the significantly higher content of the 2% CEO treatment modality after 6 h.

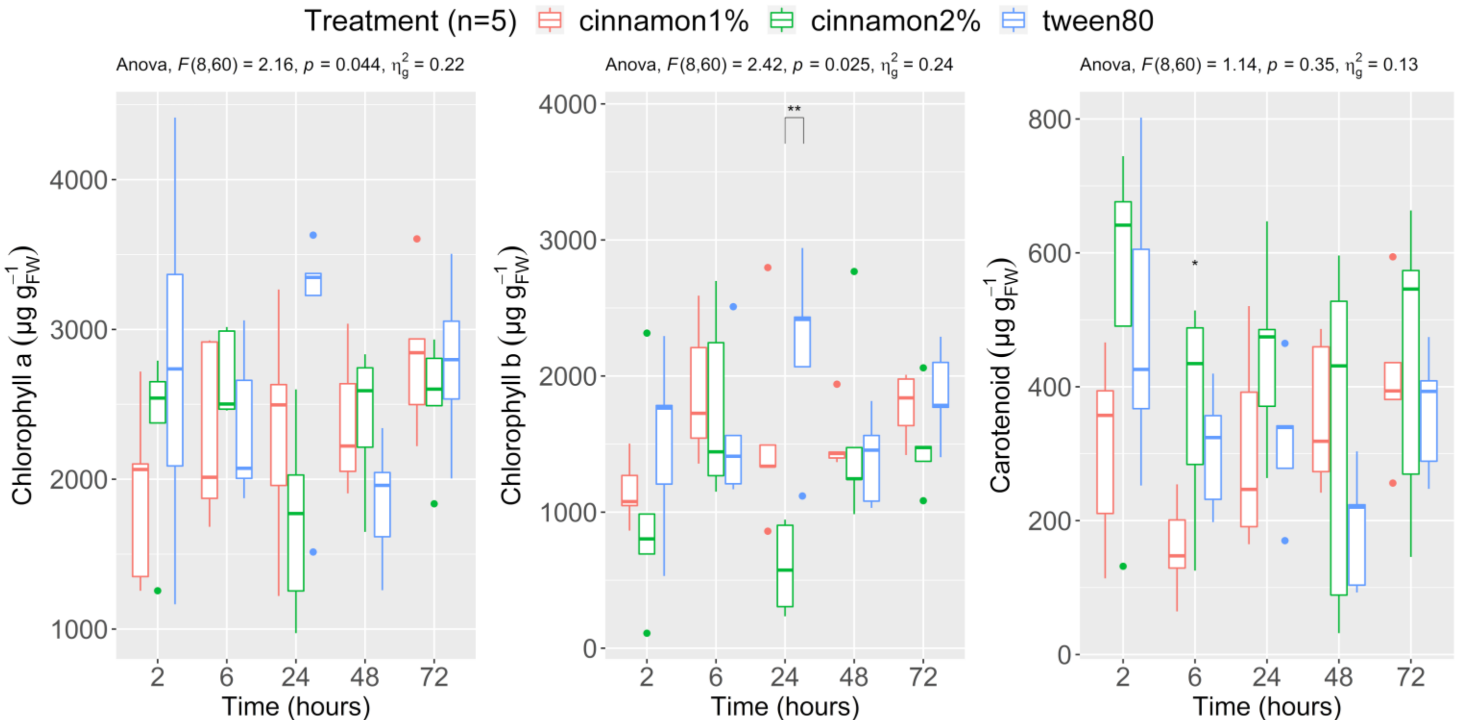


Figure 7. Effect of *C. cassia* EO (CEO) (1 and 2% v/v) and Tween 80 application on photosynthetic pigment leaf content over time (n = 5): (left) Chlorophyll a content (µg g^{-1}); (center) Chlorophyll b content (µg g^{-1}); (right) Carotenoid content (µg g^{-1}). Star on boxplot indicates significantly different distributions (*p<0.05, **p<0.01, ***p<0.001, pairwise t-test).

3.3. Induction of defences

Modification of cellular redox state as well as alteration in the previously mentioned metabolites can lead to reprogramming the expression of diverse genes. To investigate this transcriptional reprogramming, we have applied quantitative real time polymerase chain reaction (qRT-PCR) techniques on 29 transcripts of chemical and physical barriers (PR proteins, phenylpropanoids, isoprenoids, cysteines, oxidative stress, parietal modification and hormonal signalling (salicylic acid (SA), jasmonic acid (JA) and ethylene (ET)). Their detailed codes and names can be found in Supplementary Table 1. Principal component analysis (PCA) was performed to investigate the treatments' impact on the whole expression profile and representation of daily mean barycenters (with confidence intervals) are displayed in Figure 8 (left).

The first two dimensions accounted for 53.5% of the total variability. Initial variable contribution to those dimensions are represented on a variable factor map (right). Regarding barycenters, they separate remarkably following treatment as illustrated by the confidence intervals. Bion parts following the first dimension whose variable contributions are mostly SAR-related genes such as PR-proteins (PR-1, PR-10, PR-14), oxidative stress (GST, POX), isoprenoids (Far, HMGR) and SA signalling (WRKY, EDS1). Tween 80 is closer to the initial time before treatment (T0) and water. Those samples located left (negative value of first dimensions) imply an absence of up-regulation of the previously cited genes. Lastly, the impact of CEO 1% can be highlighted, especially at day 1 (D1). Indeed, it separates close to Bion following the first dimension with up-regulation of SAR-related genes. However, opposite to Bion, this up-regulation of defence genes diminishes drastically after 3 days. Regarding the second axis, no clear features can be underlined. In the PCA, FPPS and EIN3 are the strongest contributors to axis 2 (PC2). These defence genes can also respond strongly to environmental conditions. Therefore, PC2 represents above all a manipulative or sampling day effect, while axis 1 clearly represents the treatment effect.

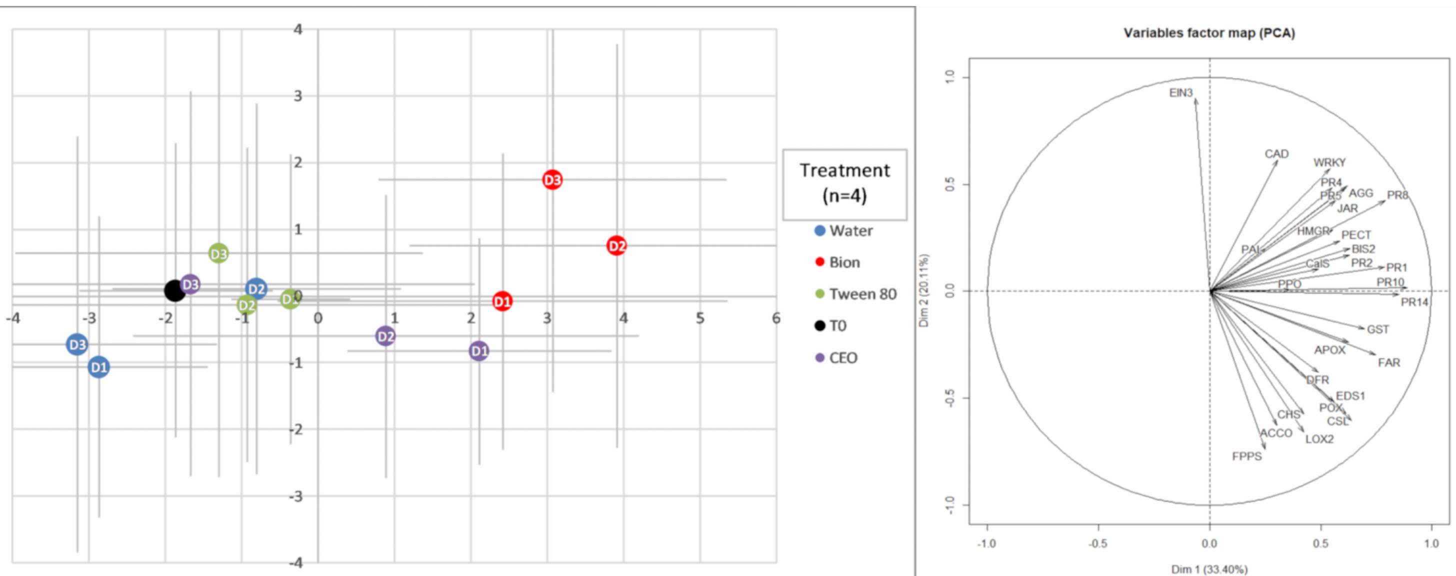


Figure 8. Effect of *C. cassia* EO (CEO 1% (v/v)), Bion, Tween 80 and water application on mean normalised log 2 expression level (n=4) by days of 29 mRNA transcripts of selected defence genes analysed by principal component analysis (PCA) with barycenter representation (left) and variable contribution to dimensions (right).

Figure 9 is a gene expression heatmap representing the mean deviations in the water controls at each sampling date for the 29 defence genes considered, with all values normalised to the initial treatment (represented on Supplementary Table 2). Among the interesting information that can be derived from this figure, the first is that, the control Tween 80 alone produces effects compared to water treatment, that are quite marked for PR proteins and agglutinin. However, Tween 80 has been assessed to be a nontoxic and biocompatible surfactant (Prieto & Calvo, 2013). Concerning CEO, the activation effects are visible especially at day 1 as evidenced previously in the PCA. Prolonged activation effects until day 3 are observed for some genes, notably for hormonal signalling (ACCO), for pathogenesis-related protein (PR8, PR10 and PR14), for parietal modification (Pect) and phenylpropanoids (BIS2). Bion (Acibenzolar-S-methyl) the positive control, clearly triggers SAR-related genes. Due to their non-normal distribution, impact of treatment was analysed by the Kruskal-Wallis test and post hoc Conover test for pairwise comparison between treatments. We can see significant impact at each day and between treatment. Indeed, Bion upregulated the following genes (expression level) on day 1, PR-2 (4.24±0.47), PR-5 (3.7±0.68), PR-8 (1.44±0.86), AGG (7.95±1.28), Far (3.47±0.27), CSL (1.72±0.27) and EDS1 (2.61±0.28). At day 2 this increase is significant solely for PR-5(3.19±0.41). Finally, this increase is prolonged until day 3 for PR1(2.77±0.31), PR2(4.9±0.33), PR5(3.48±0.8), PR8(2.46±0.62), AGG (7.29±1.43) and FAR (2.48±0.54). Tween 80 produced a significant increase on day 1 for PR-14 (3.44±0.27) and on day 3 for AGG (4.59±0.2). CEO upregulated PR-8 (1.67±0.53), PR-14 (4.14±0.92), PAL (1.56±0.18), CSL (1.95±0.73), GST (0.51±0.17) and ACCO (0.69±0.21) on day 1. Increases are prolonged on day 3 for Pect expression levels (3.43±0.43). Taken individually CEO specifically up-regulated transcripts from different pathways compared to Bion such as ethylene from hormonal signalling (ACCO), oxidative stress (GST) and especially phenylpropanoids (PAL) and parietal modifications (PECT).

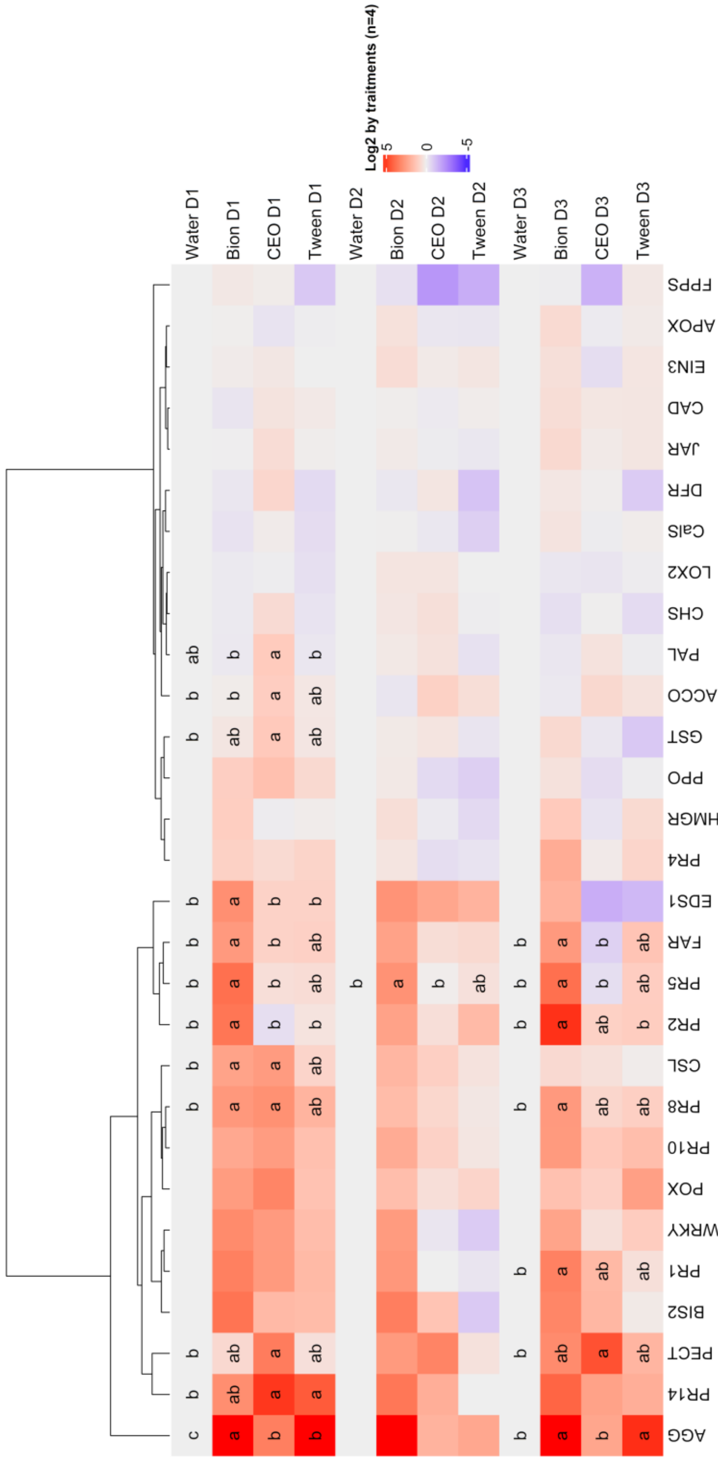


Figure 9. Effect of *C. cassia* EO (CEO 1% (v/v)), Bion, Tween 80 and water application on mean normalised log 2 expression levels (n=4) by days of 29 mRNA transcripts of selected defence genes analysed by heatmap (deviations to the water controls) and the pair-wise Conover test with compact letter displays (different letter representing significantly different means).

4. Discussion

As for many other EOs it seems that foliar application of CEO triggers an oxidative burst as suggested by the drastic decrease in reduced GSH and ratios observed. Whether CEO directly triggers the production of ROS or results from metabolic alteration cannot be deduced from the present study. The pro-oxidative character of CEO depends on its concentration. From a broader perspective, this pro-oxidative character may differ depending on plant sensitivity, tissue type, physiological and/or phenological state. Plants are equipped with numerous soluble antioxidants and many ROS scavenging enzymes (superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), thioredoxin (TRX)), and the enzymes of the Asada–Halliwell–Foyer pathway (Foyer & Noctor, 2005). Their simultaneous measurement gives a better insight of the cellular redox state, but is labour and cost intensive (Queval & Noctor, 2007). Glutathione is at the heart of the antioxidant systems. Therefore, GSH redox couple measurement has been proposed as a preferred marker for H_2O_2 availability in plant cells (Foyer et al., 1997). Since it has been used to monitor many abiotic stresses, including in apple trees, such as heavy metal (He et al., 2020), drought (Ma et al., 2011) and temperature (Ma et al., 2008). In this framework we propose to include the glutathione redox state measurements as early markers of oxidative burst following EOs or VOCs applications. Another function of glutathione is to detoxify xenobiotics in plants through conjugation reactions. These reactions were observed *in planta* for hexenal (Davoine et al., 2006), methacrolein (Muramoto et al., 2015) and are suggested as a conversion method for volatile organic compounds (VOCs) in plant–plant communication (Sugimoto et al., 2016). This reaction could also take place and explain part of the GSH consumption.

If not handled by the previously described antioxidant systems, oxidative burst can lead to the appearance of oxidative damage in many macromolecules and in cell membranes, leading to MDA production. MDA leaf content reported in apple leaf ranges from pmol g_{FW}^{-1} to µmol g_{FW}^{-1} depending on the protocol applied. However, a two-fold increase in content following different types of stress has been acknowledged in heavy metal (He et al., 2020), drought (Ma et al., 2011) and extreme temperature (Ma et al., 2008) exposure known to trigger ROS production. MDA originates from PUFA and it is well known that in *Arabidopsis thaliana* leaves for example, mostly linoleic acid and other tri-unsaturated fatty acids are the source of up to 75% of MDA produced (Weber et al. 2004). This specificity makes it a limited marker of oxidative damage. Reactive carbonyl species (RCS) production is considered a ubiquitous reaction to oxidative burst. RCS can inactivate chloroplasts and mitochondrial enzyme accelerating oxidative stress and consuming GSH. Therefore, besides MDA, other compounds should be considered, such as acrolein and 4-hydroxy-2-nonenal (Mano, 2012). Finally, oxidative damage can occur for other biomolecules beside lipids. Protein inhibition (Araniti et al., 2018), microtubule depo-

larisation (Chaimovitsh et al., 2017) or DNA damaging (Bozari et al., 2013) properties have been demonstrate for other VOCs. Those reactions lead to long-term phytotoxicity and should also be considered. Indeed, CEO application can be considered phytotoxic in the short-term only at a 2% concentration, but we can't rule out other mechanisms that lead to long-term phytotoxicity at lower concentrations.

Pathogenesis-Related (PR) proteins have been defined as plant host proteins that are produced only in response to attack by pathogens or a related event (van Loon et al., 1994). Demonstrating the expression of PR genes has been widely accepted as a hallmark of plant defensive systemic acquired resistance (SAR) induction (Bonasera et al., 2006; Oliveira et al., 2016). The SAR, which is a form of systemic resistance in plants with a specific defence signalling pathway, can also occur after spraying with a synthetic or natural compound, commonly known as an inducer such as the Bion used in this study (Dugé De Bernonville et al., 2014). From our results (PCA) it would seem that defence induction pathways following CEO application is similar to SAR. The most commonly screened PR genes expressed in apples and other plant-pathogen systems are PR-1 (antifungal activity), PR-2 (β-1,3-glucanase), and PR-8 (class III chitinase) (Saboki et al., 2011). Thyme EO has been suggested to increase PR-8 expression in apple (Banani et al., 2018). Our results also showed significant increases in expression levels of PR-8 and PR-14 compared to water. Tween 80 alone also impacted PR-related protein agglutinin and PR-14. Similar results on defence related genes have been previously highlighted in wheat after Tween 20 treatment (Le Mire, 2018). Therefore, the formulation needs to be investigated to determine if part of defences induction is actually due to the elicitor compounds themselves. Formulation also drastically impact apparition of phytotoxicity phenomena by controlling the release of active substance. Therefore, further investigations of its impact on this particular model will help to precise specific threshold of activity.

Apart from those coding for PR proteins, other genes represent the wide diversity of known plant defence mechanisms. The metabolic pathways to which these genes are related include secondary metabolic pathways (phenylpropanoids and isoprenoids), oxidative stress, parietal modifications and hormonal signalling pathways of salicylic acid, jasmonic acid and ethylene. In our study phenylalanine ammonia-lyase (PAL) expression levels from the phenylpropanoids pathway was significantly (but transiently) increased. This is coherent with previous results obtained regarding this enzyme activity in citrus following *trans*-cinnamaldehyde application (Perina et al., 2019). Changes in PAL activity have been shown to precede the increases in BD and BIS activities in Asian pear *Pyrus pyrifolia* leading to production of phytoalexins (Saini et al., 2017). This result was not verified here, but only BIS2 was followed out of the 9 genes detected in the genome sequence of the apple 'Golden Delicious' (Chizzali et al., 2012). Alternatively, phenylpropanoid pathway activation could be investigate through production of biphenyls and dibenzofurans. Indeed, production of aucuparin and noraucuparin have been demonstrate in apple following elicitor

treatment (Sarkate et al., 2018). Cell wall modification may also occur, as suggested by the increase in pectin methyl esterase (PECT) expression levels. Regarding hormonal signalling, ACCO up-regulation suggest an impact on ethylene. Hormonal signalling is known to be a very transient signal; therefore, the balance of phytohormones should be consider when investigating signal perception following CEO application. Concerning oxidative stress, glutathion S-transferase (GST) is specifically up-regulated after CEO application which is consistent with the GSH results presented, regarding redox status. Finally, regarding isoprenoids, α-farnesene production was acknowledged in response to SAR induction by Bion application. The same result was obtained following CEO injection into apple trees with a modification of VOC emission (Werrie et al., 2021). However Farnesyl pyrophosphate synthase (FPPS) and (E,E)-α-farnesene synthase (FAR) do not seem to be upregulated following CEO application. However, deep transcriptome analyses such as RNA-seq should provide a more complete and less biased picture on all the genes modulated following CEO treatment.

Regarding signal transduction, it has been suggested that while the monoterpenes could disturb the lipid organisation and/or domain formation, the phenylpropanoid cinnamaldehyde could rather interact with membrane receptors (Lins et al., 2019). Recent evidence suggests that cinnamaldehyde regulates endogenous Ca^{2+} in the root of *Brassica rapa* (Cheng et al., 2021). Furthermore, precedent investigation observed generation of endogenous hydrogen sulphide (H_2S) in roots treated with cinnamaldehyde, supposedly by increasing the activity of L-cysteine desulfhydrase (Xue et al., 2016). They proposed that cinnamaldehyde could regulate Ca^{2+} directly by targeting transient receptor potential A1 (TRPA1) as observed in mammals but also that Ca^{2+} regulation by H_2S may operate downstream of cinnamaldehyde through a linear signalling pathway during the induction of lateral root formation. The transcriptional reprogramming that follows CEO application could be explained by modification of those prominent signalling molecules.

To conclude, this work highlights modification of oxidative stress related metabolites; namely glutathione and malondialdehyde following CEO application in a dose-response relationship. Furthermore, it investigates transcriptional reprogramming of major defence pathway. Increases in expression levels of specific genes belonging to PR-proteins (PR-8, PR-14), hormonal signalling (ACCO), oxidative stress (GST), phenylpropanoids (PAL) and parietal modification (PECT) pathways were observed following CEO application. Multivariate analysis of the 29 transcripts acknowledged similar but more transient modification of expression levels than the SAR inducer Bion.

In a broader scope, the defence induction occurring below the phytotoxicity threshold represents an engaging research path for EO application in agronomy to design appropriate and sustainable agricultural pest management strategies.

4

Proof of concept for the systemic movement of mint and cinnamon essential oils

The fourth chapter of this thesis presents a research article published in 2021 in Frontiers in Plant Science:

Werrie, P.-Y., Burgeon, C., Le Goff, G.J., Hance, T., and Fauconnier M.-L. (2021) Biopesticide trunk injection into apple trees: a proof of concept for the systemic movement of mint and cinnamon essential oils. *Front. Plant Sci.* 12:650132. doi: 10.3389/fpls.2021.650132

As presented in the general introduction, an alternative mode of application of EOs was considered in the framework of the project: trunk injection.

The first part of this chapter therefore seeks to investigate EO translocation following passive injection into plant stems. Indeed, with this type of application it is important to demonstrate the systemicity of the applied molecules through the xylem sap. Thus, major compounds of two EOs carvone and cinnamaldehyde (of mint and cinnamon EO, respectively), contents in leave where monitored. Furthermore, emissions of those VOCs from leaves after injection were also considered. These two EOs were assayed because they had strong insecticidal activity on an artificial diet against RAA in the framework of the Tree-Injection project. Furthermore, their major compounds belong to two different classes of compounds, namely terpenoids and phenylpropanoids whose specific physico-chemical properties may alter the systemicity profile.

The second part investigates potential phytotoxicity on the tree using non-targeted analysis of the VOCs and ecophysiological measurement (chlorophyll fluorescence).

The last part of this chapter discusses the relevance of the application method and its results in the framework of botanical insecticide development based on EOs.

Contribution: Conceptualisation, methodology, formal analysis, writing—original draft review and editing.

Abstract

The use of conventional pesticides is debated because of their multiple potential adverse effects on non-target organisms, human health, pest resistance development and environmental contaminations. In this setting, this study focused on developing alternatives, such as trunk-injected essential oil (EO)-based biopesticides. We analysed the ecophysiology of apple trees (*Malus x domestica*) following the injection of *Cinnamomum cassia* and *Mentha spicata* nanoemulsions in the tree's vascular system. Targeted and untargeted volatile organic compounds (VOCs) analyses were performed on leaf-contained and leaf-emitted VOCs and analysed through DHS-GC-MS and TDU-GC-MS. Our results showed that carvone, as a major constituent of the *Mentha spicata* EO, was contained in the leaves (mean concentrations ranging from 3.39 to 19.7 ng g_{DW}^{-1}) and emitted at a constant rate of approximately 0.2 ng g_{DW}^{-1} h^{-1}. *Trans*-cinnamaldehyde, *Cinnamomum cassia*'s major component, accumulated in the leaves (mean concentrations of 83.46 and 350.54 ng g_{DW}^{-1}) without being emitted. Furthermore, our results highlighted the increase in various VOCs following EO injection, both in terms of leaf-contained VOCs, such as methyl salicylate, and in terms of leaf-emitted VOCs, such as caryophyllene. Principal component analysis (PCA) highlighted differences in terms of VOC profiles. In addition, an analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) revealed that the VOC profiles were significantly impacted by the treatment. Maximum yields of photosystem II (Fv/Fm) were within the range of 0.80-0.85, indicating that the trees remained healthy throughout the experiment. Our targeted analysis demonstrated the systemic translocation of EOs through the plant's vascular system. The untargeted analysis, on the other hand, highlighted the potential systemic acquired resistance (SAR) induction by these EOs. Lastly, *Cinnamomum cassia* and *Mentha spicata* EOs did not appear phytotoxic to the treated trees, as demonstrated through chlorophyll fluorescence measurements. Hence, this work can be seen as proof of concept for the use of trunk-injected EOs given systemic translocation, increased production and release of biogenic VOCs (BVOCs) and absence of phytotoxicity. Further works should focus on the ecological impact of such treatments in orchards, as well as apple quality and production yields.

1. Introduction

Apple *Malus x domestica* Borkh is the most cultivated fruit crop worldwide, reaching a production of 84.7 million tonnes in 2016 and representing a gross product value of US $ 45.8 billion (FAOSTAT). As any other plant, apple trees are subject to abiotic and biotic stresses that cause important economic losses. Apple trees suffer from fungal, viral, and bacterial diseases; insects; mites; and nematodes (Kellerhals et al., 2012). The rosy apple aphid, *Dysaphis plantaginea*, and the codling moth, *Cydia pomonella*, are among the most serious apple pests (Rousselin et al., 2017), while the main diseases are apple scab, powdery mildew, and fire blight caused by the fungi *Venturia inequalis* and *Podosphaera leucotricha* and by the bacteria *Erwinia amylovora*, respectively (Jamar et al., 2010). All these factors can impair production or marketable yields because apples do not fulfil the minimum quality criteria. Currently, the most applied delivery method for pest control is air-blast spray application of pesticides to the tree canopy (Damos et al., 2015). However, pesticide off-target drift can lead to adverse effects on non-target organisms. Over the last 50 years, biodiversity has been reduced by up to 50% in European bird species and by 20–30% in British and German flora (Geiger et al., 2010). Pesticides can cause environmental contamination and risks for human health through excessive residues on the fruit (Damalas & Eleftherohorinos, 2011). Additionally, pests can develop resistance to these pesticides, which usually contain a single active molecule (Alins et al., 2017). Altogether, this suggests that the plant protection product (PPP) mode of application selection is an economic and ecological challenge around the world. As a result of the negative perception of synthetic pesticides, causing negative effects on human health during and after application, and fears of their excessive residues in or on fruit, consumer demand for agricultural products without synthetic pesticide residues from excessive phytosanitary treatments has increased. This is why alternative solutions have been investigated, such as biological pesticides or biopesticides. An abundant body of literature is published each year concerning the prospect of plant essential oils (EOs) as active ingredients in the production of biopesticides (Campos et al., 2019).

The international organisation for standardisation (ISO) defines an EO as a “product obtained from vegetable raw material, either by distillation with water or steam, or from the epicarp of citrus fruits by a mechanical process, or by dry distillations.” Due to their biological activity, they have long been applied in cosmetics, therapeutics, and food applications (Bașer & Buchbauer, 2015). The composition of EOs is highly variable and comprises a tremendous diversity of compounds. However, most of them belong to the terpenoids (mono- or sesqui-) or phenylpropanoids class of compounds, both of which have high lipophilicity and volatility, especially at room temperature. The secondary metabolites of EOs originate from methylerythritol phosphate and phenylalanine pathways (Rehman et al., 2016).

Some of the volatile organic compounds (VOCs) contained in EOs play a major role in plant defence mechanisms against bacteria, fungi, viruses, and herbivores (Bakkali et al., 2008). Therefore, much research has been performed to integrate these antibacterial, fungicidal, and insecticidal EOs as alternatives for sustainable agronomic practices, limiting environmental and health hazards. Indeed, due to their rapid degradation and since they are generally recognised as safe (GRAS), they represent an interesting alternative application of most synthetic conventional pesticides (Koul et al., 2008). Two EOs were used in this study: cinnamon EO (*Cinnamomum cassia* J. Presl) and mint EO (*Mentha spicata* L.). They both present well-documented biopesticidal activity (De Clerck et al., 2020; Singh & Pandey, 2018) due to their insecticidal and fungicidal (Lee et al., 2020; Muchembled et al., 2018) properties, which have already led to commercial product development (Isman, 2020; Isman et al., 2011). For example, mint EO has presented an inhibition concentration between 24 and 83 mg L^{-1} on apple scab, depending on the strain (Muchembled et al., 2018). *Cinnamomum cassia*, on the other hand, possesses a lethal dose fifty of 17.41 µL mL^{-1} on aphid *Myzus persicae* (Ikbal & Pavela, 2019).

Nevertheless, particular attention must be paid to the formulation of EO-based pesticides (Aćimović et al., 2020). A well-studied formulation could, on one hand, counter the high volatility of EOs and ensure the prolonged release of the active substance (a. s.) and, on the other hand, attenuate potential phytotoxic effects (Maes et al., 2019; Moretti et al., 2002). EOs can impact many plant physiological processes (water status alteration, membrane integrity, respiration, and photosynthesis inhibition) through diverse modes of action, such as reactive oxygen species (ROS) induction and enzymatic or phytohormone regulation (Werrie et al., 2020). In this regards, chlorophyll fluorescence has been proven useful to evaluate plant vitality and response to abiotic stress (Kalaji et al., 2016). The application of EOs in apple tree may lead to phytotoxicity depending on the application method, concentration, and adaptive duration. For example, 7% of flowers were injured for clove oil in a thinning experiment for concentrations as low as 2% (Miller & Tworkoski, 2010). Fruit damages were also reported in post-harvest treatment with savory, oregano, and thyme EOs at concentrations of 1–10% for the purpose of controlling *Botrytis cinerea* and *Penicillium expansum* (Lopez-Reyes et al., 2010). Nevertheless, fruit damage was not observed with thermal fogging treatment of lemongrass and citrus EOs at a concentration of 0.125% to control *B. cinerea* (Mbili et al., 2017). Therefore, the mode of EO application, the formulation, and the selection of the a. s. must be adapted for specific purposes and carefully evaluated.

Trunk injection is a method of applying chemicals directly to the vascular system of the tree after bark piercing, and the chemicals are then distributed systemically through the xylem tissue. This application method directly targets pests while reducing environmental exposure to pesticides and input quantities (Doccola and Wild, 2012; Wise et al., 2014). It has recently been experimented to fight fungi, such as

apple scab, *Venturia inaequalis*, and powdery mildew, *Podosphaera leucotricha*, with disease severity reductions of 22% to 55% and 41.8% to 73.5% depending on the season and the product considered (potassium phosphites and synthetic fungicides) (Aćimović et al., 2016; Percival & Boyle, 2005). A similar experiment on insect species [codling moth *Cydia pomonella* (L.), rosy apple aphid *Dysaphis plantaginea* (Passerini), green apple aphid *Aphis pomi* (Passerini)] reported up to two seasons of control after a single injection of imidacloprid or emamectin benzoate (Wise et al., 2014). Spatial and temporal distributions of imidacloprid in leaves have been investigated (Aćimović et al., 2014), as well as residues to nectar and pollen, which were below the EPA (Environmental Protection Agency) threshold of 25 ng g^{-1} for imidacloprid (Coslor et al., 2019). Although management of the injection timings may help to keep residue under the toxic limit, systemic resistance inducers have also been explored with the injection of acibenzolar-S-methyl (ASM) to induce systemic acquired resistance (SAR) and to control fire blight (Aćimović et al., 2015).

In the present study, we aimed to determine the distribution of trunk-injected EOs in young apple trees, thus proving their systemic movement by quantifying target VOCs, both within leaves and by aerial emissions. We also determined the impact of injected EOs on tree physiology by monitoring chlorophyll fluorescence and untargeted VOCs.

2. Materials and methods

2.1. *Essential oils*

The cinnamon EO (*Cinnamomum cassia* J. Presl) and mint EO (*Mentha spicata* L.) used in this study were purchased from Pranarôm (Pranarôm & Herbalgem, Ghislenghien, Belgium). Before formulation of the EOs, the oil composition was analysed by gas chromatography associated with mass spectrometry (GC-MS). These analyses were carried out on a 7890A-5975C GC-MS equipped with an HP-5MS 30 m × 0.25 mm × 0.25 µm capillary silica column (Agilent Technologies Inc., Santa Clara, USA). The operating conditions were as the following: helium flow of 1.0 mL min^{-1}; the oven temperature was programmed at 40°C for 2 min, increased to 100°C at a rate of 5°C min^{-1}, increased to 120°C at a rate of 3°C min^{-1}, held for 3 min, increased to 220°C at a rate of 5°C min^{-1}, and finally increased to 310°C at a rate of 15°C min^{-1}. One microlitre of a 1 mg mL^{-1} EO solution in hexane (HPLC grade, Merck KGaA, Darmstadt, Germany) was injected in splitless mode. The injector, quadrupole, and MS temperatures were 250°C, 150°C, and 230°C, respectively. The mass spectrometer (MS) ran in electron impact (EI) mode at an electron energy of 70 eV. Mass spectra were acquired in the range of 30 to 400 atomic mass units (amu).

2.2. *Emulsion formulations*

To facilitate injection and diffusion of EOs in the tree vascular tissue, a water soluble, stable, and homogenous EO emulsion was prepared. To prepare 100 mL of the 0.5% (v/v) EO/water emulsion, 2 mL of Tween 80 (CAS 9005-65-6, Merck KGaA, Darmstadt, Germany) and 20 mL of 100 mM Ethylenediaminetetraacetic acid (EDTA) (Titriplex III, Merck KGaA, Darmstadt, Germany) solution were added to 15 mL of water under constant agitation at 1,250 rpm. Water was then added to bring the final volume to 100 mL. After 5 min under constant agitation, the solution was then stabilised by high-speed homogenisation for 6 min at 9500 rpm (Ultra-Turrax T25, IKA WERKE, Staufen im Breisgau, Germany) and by high-pressure homogenisation with 8 cycles at 5000 psi (FMC, Philadelphia, USA). The emulsion stability was checked by analysing the EO particle sizes and distribution in solution with a particle sizer (Beckman Coulter DelsaTM Nano C Particle Analyser, California, USA).

2.3. *Biological material*

Experiments were performed on 2-year-old apple trees (*Malus x domestica* Borkh, cv 'Jonagold' grafted on M26 rootstock) obtained locally (Serres de Sauvenières, Gembloux, Belgium). The trees were 155±15 cm high and presented a trunk diameter of 2±0.2 cm above the graft union. During the experimental phase, the plants were placed in an environmental chamber with controlled environmental conditions (21±0.5°C, 62±10% relative air humidity, and 16:8 h light: dark periods and photosynthetically active radiation (PAR) of 50 µmol m^{-2} s^{-1}). Plants were watered every day with 500 mL of water. They developed fully expanded leaves but were free of flowers or fruit.

2.4. *Trunk injection system*

The trees used in the experiment were drilled right above the grafting union with holes that were 1 mm wide and 1 cm deep. Three trunk injection ports per tree trunk were created and were positioned at an equal distance from each other (each 120° of trunk radius). Each injection port was slanted upward at a 60° angle in relation to the trunk axis (Figure 10). Needles (BD vacutainer® safety lock 23G, Becton Dickinson, New Jersey, USA) were inserted into the ports and connected on the other side to drip bags (Baxter®, Baxter International Inc, Deerfield, USA) filled with the solution injected (Figure 10). Four different treatments were tested using three biological replicates over a period of 96 h. The first two modalities were treated with EO emulsions (one with cinnamon oil and the other with mint oil), the third was a negative control (emulsion exempt of EOs), and the fourth was a blank treatment (no injection). To avoid cross-contamination, the treatments were delivered separately from each other

at different times. Treatments were applied on different trees each time with a chamber ventilating period of two days.

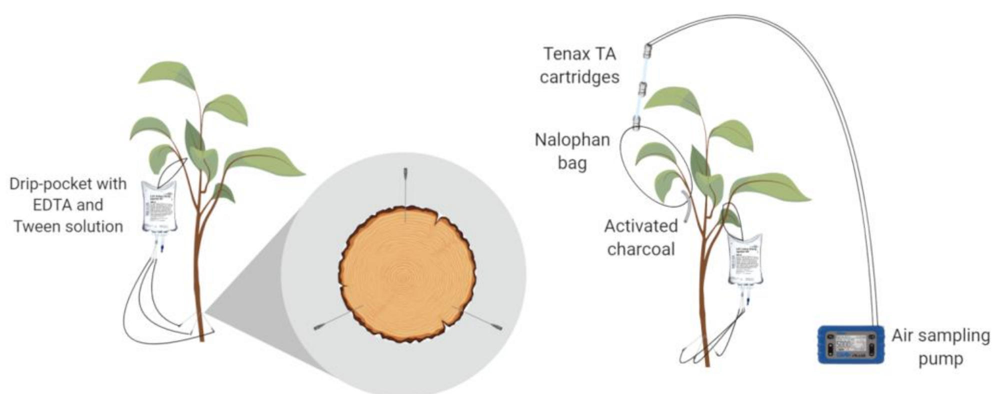


Figure 10. Laboratory trunk injection device (left) and sampling of leaf-emitted VOCs (right).

2.5. Volatile organic compound (VOC) sampling by headspace techniques

2.5.1. Leaf-contained VOCs

Ten leaves were homogeneously sampled at t=0 h, 24 h, 48 h, 72 h, and 96 h on each replicate tree. Sampling was performed by cutting the leaves at their base and dipping them into liquid nitrogen, before storage at –80°C prior to DHS-GC-MS analysis. A dry weight (DW) measurement was performed at the end of the experiment at 60°C until constant weight to obtain content results in ng g_{DW}^{-1}.

2.5.2. Leaf-emitted VOCs

The headspace was sampled following the protocol for the volatile collection of aphid-infested leaves from an apple tree (Stewart-Jones & Poppy, 2006). Briefly, two Tenax TA® 60/80 cartridges (Camsco©, Houston, USA) were attached to an inert polyethylene terephthalate (PET) bag (Nalophan®, Odometrics, Arlon, Belgium) enclosing a single branch. The trapping of emitted VOCs was performed by constant air sampling of 50 mL min^{-1} using a Gilian air sampling pump (Sensidyne©, St. Petersburg, USA) attached to the other side of the cartridges (Figure 10). Briefly, air enters the bag through the activated charcoal tube, loads in the VOCs, and exits the bag through the Tenax TA cartridges, which capture VOCs. The bag and its connected

cartridges were set up on each tree (n=3) at t=0 h. The cartridges were then replaced at t=24 h, 48 h, 72 h, and 96 h and stored at –80°C prior to the GC-MS analysis. At the end of the experiment, all leaves enclosed in the bag were sampled and weighed. A DW measurement was also performed on these leaves at 60°C until constant weight to obtain results in ng g_{DW}^{-1} h^{-1}.

2.6. VOCs analysis: sample preparation and GC-MS analysis

Leaf-contained VOCs were analysed by DHS-GC-MS. Before dynamic headspace sampling (DHS), the leaves were ground (A11 basic grinder, IKA WERKE, Staufen im Breisgau, Germany) with liquid nitrogen. Then, 1 g of freeze-grinded leaves was put in a 20 mL screw cap vial (Gerstel©, Mülheim an der Ruhr, Germany) and 2 mL of a 20% (w/v) NaCl solution was added to create a salting out effect (Liberto et al., 2020). Afterwards, the sealed vial was incubated in the dynamic headspace system at 35°C for 20 min (Automated dynamic headspace DHS, Gerstel©, Mülheim an der Ruhr, Germany). The headspace was then dynamically transferred to a Tenax TA cartridge by applying 1200 mL of nitrogen at a flow of 30 mL min^{-1}. The cartridge was then dry purged at 50 mL min^{-1} for 4 min. The cartridge was then sent to the thermal desorption unit (Thermal Desorption Unit TDU 2, Gerstel©, Mülheim an der Ruhr, Germany) for GC-MS analysis. The thermal desorption parameters used were the same as those described below for leaf-emitted VOCs. Tenax TA® porous polymers, based on 2,6-diphenyl-p-phenylene oxide, are widely used as an adsorbent in purge trap applications and plant headspace analysis due to their high versatility.

Leaf-emitted VOCs were analysed by TDU-GC-MS. Before thermal desorption, 1 µL of 0.4 mg mL^{-1} 1-phenyloctane (CAS 2189-60-8, Merck KGaA, Darmstadt, Germany) in hexane was added to the cartridge by a multipurpose sampler (MultiPurpose Sampler MPS, Gerstel©, Mülheim an der Ruhr, Germany). The addition of 1-phenyloctane as an internal standard (IS) allowed for semi-quantification of the VOCs present on the cartridge. The VOCs were then thermally desorbed in the TDU and cryofocused in the Cooled Injection System (CIS) (Gerstel©, Mülheim an der Ruhr, Germany). The TDU temperature program was 40°C for 1 min, and was increased to 280°C at a rate of 100°C min^{-1} and held for 5 min. CIS was mounted with a baffled glass liner and operated in solvent vent mode, and the temperature program was –60°C for 0.10 min, which was increased to 250°C at a rate of 12°C s^{-1} and held for 2 min following existing protocols (Delory et al., 2016; Durenne et al., 2018).

The analyses were carried out on a 7890B-5975C GC-MS equipped with an HP-5MS 30 m × 0.25 mm × 0.25 µm capillary silica column (Agilent Technologies Inc., Santa Clara, USA). The operating conditions were the following: helium flow of 1 mL min^{-1} and oven temperature 40°C for 2 min, which was increased to 220°C at a rate of 5°C min^{-1} and finally increased to 310°C at a rate of 15°C min^{-1} and held for

3 min. The quadrupole and MS temperatures were 150°C and 230°C, respectively. The MS ran in EI mode at an electron energy of 70 eV. Mass spectra were acquired in the range of 30 to 400 amu.

For untargeted analysis, identification was based on comparison of the obtained spectra with the reference mass spectra from the NIST 17, Wiley 275, and pal 600 databases. Moreover, experimental retention indexes (RIs) were calculated using C_7–C_{30} solutions and compared to literature RIs. Technical grade standards were injected to ensure identification (Nea et al., 2019; Tanoh et al., 2020). Semi-quantification was performed using the following formula:

$$\textbf{compound A concentration} = \frac{\textbf{compound A area}}{\textbf{IS area}} * \textbf{IS concentration}$$

Detection and quantification of the major compounds of EOs were performed in single-ion monitoring (SIM) mode. Based on the characterisation of the selected EOs, calibration curves in TDU-GC-MS using pure standards were established for each major component of the EO: (+)-carvone (CAS 2244-16-8, 99.9% purity, Supelco©, Missouri, USA) for mint and *trans*-cinnamaldehyde (CAS 14371-10-9, ≥99% purity, Merck KGaA, Darmstadt, Germany) for cinnamon oil. The 6-point calibration curves were established by injecting 1 µL of the standard solution in hexane (Merck KGaA, Darmstadt, Germany). For (+)-carvone, ions 108 and 93 were selected as qualifiers and ion 82 was selected as the quantifier. A calibration curve ($y=0.527x+0.020$, $R^2=0.985$) was established in triplicates between 1.50 and 861.05 µg mL^{-1}. For *trans*-cinnamaldehyde, ions 132 and 103 were used as the qualifier and ion 131 was used as the quantifier. A calibration curve ($y=0.628x+0.018$, $R^2=0.989$) was established in triplicates between 0.623 and 954.50 µg mL^{-1}. The IS 1-phenyloctane was also used at a concentration of 400 µg mL^{-1}.

2.7. Chlorophyll fluorescence measurements

The potential phytotoxic effect of EOs on the photosynthetic efficiency of plants was evaluated by estimating the maximum quantum efficiency of photosystem II (Fv/Fm) with a fluorimeter (Handy PEA+, Hansatech Instruments Ltd, Norfolk, United Kingdom). For a healthy sample, this ratio is around 0.83 and lowers as plant stress increases, reaching 0.3 at the end of senescence (Bresson et al., 2018). Moreover, the maximum quantum yield of photosystem II has been used to evaluate foliar response after EO application (Synowiec et al., 2015, 2019). Measurements were performed at the same time of day for each time considered (t=0 h, 24 h, 48 h, 72 h, and 96 h for each modality tested). Fv/Fm was assessed on three leaves randomly selected on each tree at different height (low, middle, high). Before the measurement, the leaves were dark-adapted for 20 min using leaf clips. Fv/Fm measurements were then performed by exposing the leaves to light intensity of 3000 µmol m^{-2} s^{-1}.

2.8. *Statistical analysis*

The results from the targeted VOCs were visualised, and detailed non-parametric statistical analysis (Kruskall Wallis test and Dunn's test) was generated in Rstudio with ggstatplots (Patil, 2018). The untargeted VOC profiles, either contained or emitted, underwent several statistical analyses to understand the impact of the treatments performed on the apple trees. Firstly, one-way analysis of variance (ANOVA) was performed for each VOC present in the profiles to understand which of them were significantly different between treatments using Tukey's post hoc test. Descriptive statistics were coupled with principal component analyses (PCA) and heatmaps to visualise treatment effects and which VOCs they impact. All of these analyses were performed with metaboanalyst (www.metaboanalyst.ca) (Pang et al., 2020). Analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were performed between the different treatments. PERMANOVA tests the simultaneous response of one or more variables to one or more factors based on a similarity/distance matrix with permutation methods (Anderson, 2017). The ANOSIM and PERMANOVA were calculated in R studio (R 3.5.2 software, R-Development-CoreTeam, Boston USA) using the VEGAN package. ANOSIM and PERMANOVA were performed to establish if the contained and emitted VOC profiles were significantly impacted by the treatment. For fluorescence measurements, two-way repeated measures ANOVA was performed on the Fv/Fm dataset with treatments and time as a factor, followed by the pairwise t-test. A probability cut-off of $\alpha=0.05$ was applied for tests of significance in all statistical analyses and adjusted with the Bonferroni correction.

3. Results

3.1. *Essential oil compositions and formulations*

GC-MS analysis of the EOs demonstrated that *Cinnamomum cassia* oil was composed of 91.22% *trans*-cinnamaldehyde, and *Mentha spicata* was mainly composed of carvone (57.78%) and limonene (25.28%). A detailed composition can be found in the supplementary material (Supplementary Table 3 and 4). The EO compositions are similar to those reported before (Snoussi et al., 2015; Zhang et al., 2019). A stable nanoemulsion had a mean particle size diameter below 200 nm and a polydispersion index <0.2.

3.2. VOCs spectra analysis

3.2.1. Targeted essential oil compounds

Regarding mint EO, the main compound, carvone, was found in both the emission and in the leaves, as displayed in Figure 11. The emission rate into the air was constant throughout the experiment at around 0.2 ng g_{DW}^{-1} h^{-1}. The leaf content, however, was more variable within and between 24 h and 48 h. Indeed, the carvone content varied between 3.39 and 19.70 ng g_{DW}^{-1}, with a maximum two days after injection. However, as this compound was not found in the other treatments of the experiment, it demonstrates the systemic translocation of the trunk-injected mint EO.

Trans-cinnamaldehyde, the main compound of cinnamon EO, was only recovered in the content of the leaf (and not in the air emission). However, this content was much higher in comparison to carvone, i.e., mint EO content, as observed in Figure 12, reaching 350.54 ng g_{DW}^{-1} 72 h after injection.

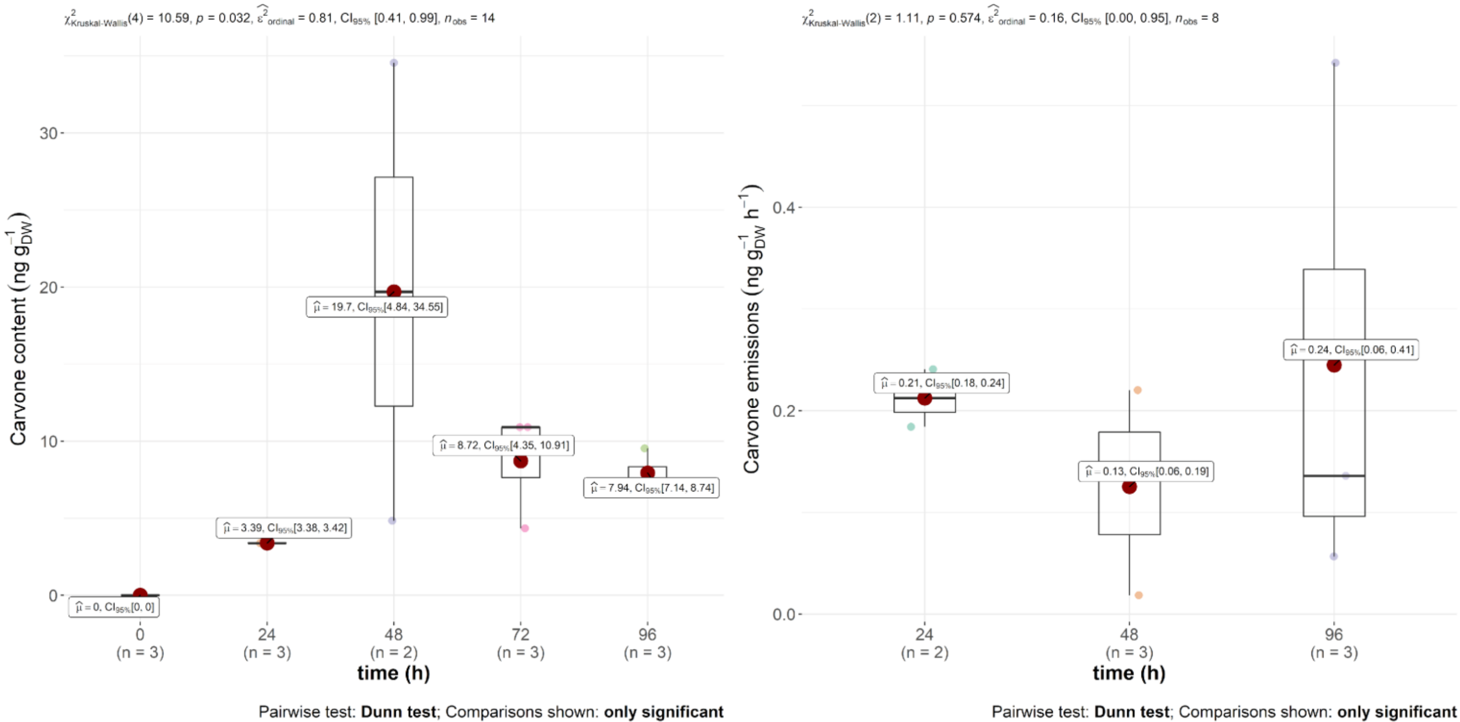


Figure 11. Boxplot of D-carvone contained (in ng g_{DW}^{-1}) in the leaves (left) and in the emissions (ng g_{DW}^{-1} h^{-1}) (right) over time after injection.

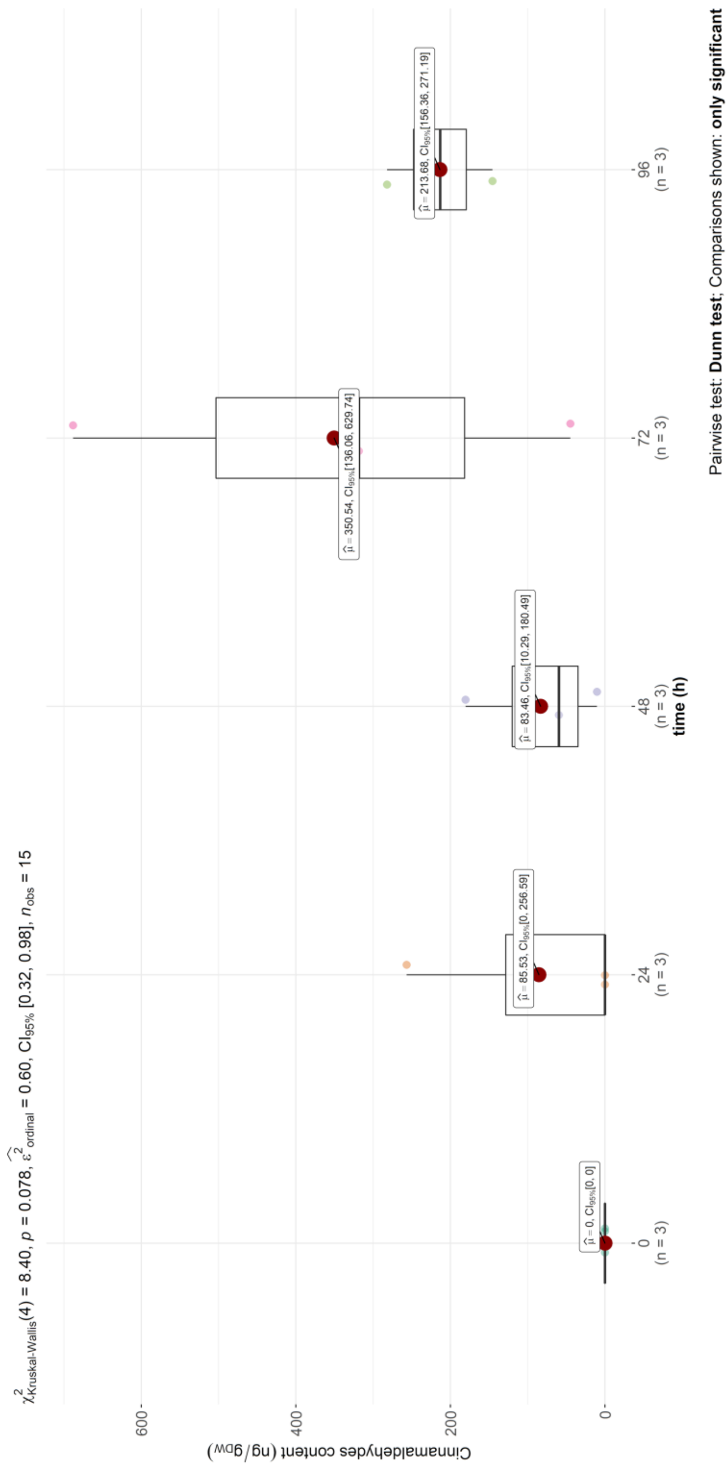


Figure 12. Boxplot of *trans*-cinnamaldehyde contained (in ng g_{DW}^{-1}) in the leaves over time after injection.

3.2.2. Untargeted VOCs emitted (TDU-GC-MS)

A total of 56 compounds were detected in the headspace emissions profiles of *Malus x domestica* trees belonging to the alkanes, alkenes, alcohols, aldehydes, aliphatic and aromatic esters, furanes, homoterpenes, ketones, monoterpenes, sesquiterpenes, and terpenoids (Supplementary Table 5). A selection of biogenic VOCs (BVOCs) that have a major biological role in the environment, such as pest attractant, attraction of pest-killing parasitic wasps, antennal response elicitor, or herbivory-induced plant volatile (Gershenzon & Dudareva, 2007; Hare, 2011; Souza et al., 2017), is presented in Figure 13. The apple trees we injected with both EOs emitting the largest amounts of caryophyllene, linalool, and germacrene D and significantly larger amounts of α-farnesene and (E)-4,8-dimethyl-nonatriene (DMNT).

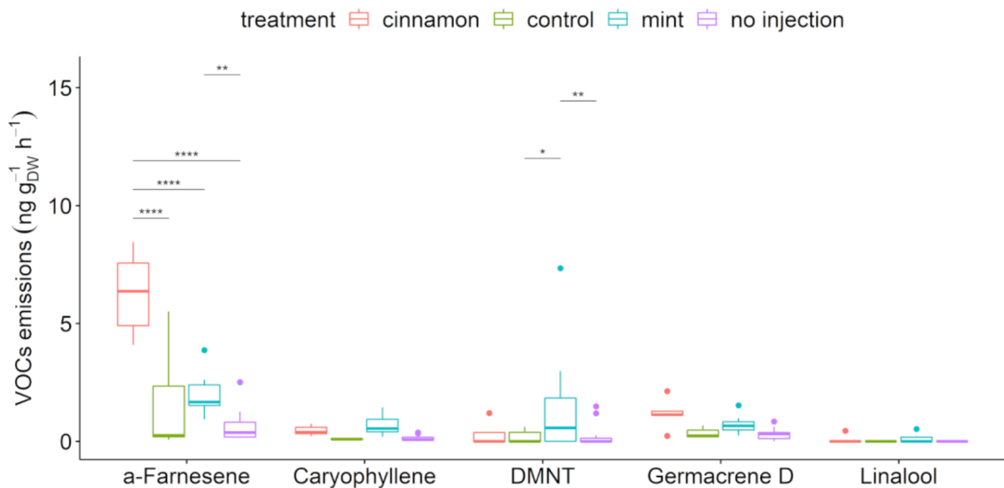


Figure 13. Boxplot of a selection of *Malus x domestica* VOCs emitted (ng g_{DW}^{-1} h^{-1}) from plants injected with EOs and the control. The star symbols above the bars indicate a significant difference between the means ($p<0.05$).

Multivariate analysis of the emitted VOC profiles performed by PCA captured 83.3% of variance in the first two dimensions (Figure 14). VOC profiles of EO-injected trees separated well from the control and no injection treatment. On the other hand, as can be observed in the heatmap (Figure 14), some compounds are produced for both oils, such as caryophyllene, germacrene D, bergamotene, (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), and linalool, whereas some of them are specific to a particular oil. Indeed, cinnamon oil injected trees emitted more terpinen-4-ol, α-farnesene, and trees injected with mint oil emitted more DMNT and β-ocimene. Among the compounds previously mentioned, linalool, germacrene D and terpinen-4-ol are found in mint EO and caryophyllene is found in cinnamon EO, but as minor compounds at concentrations below 1%.

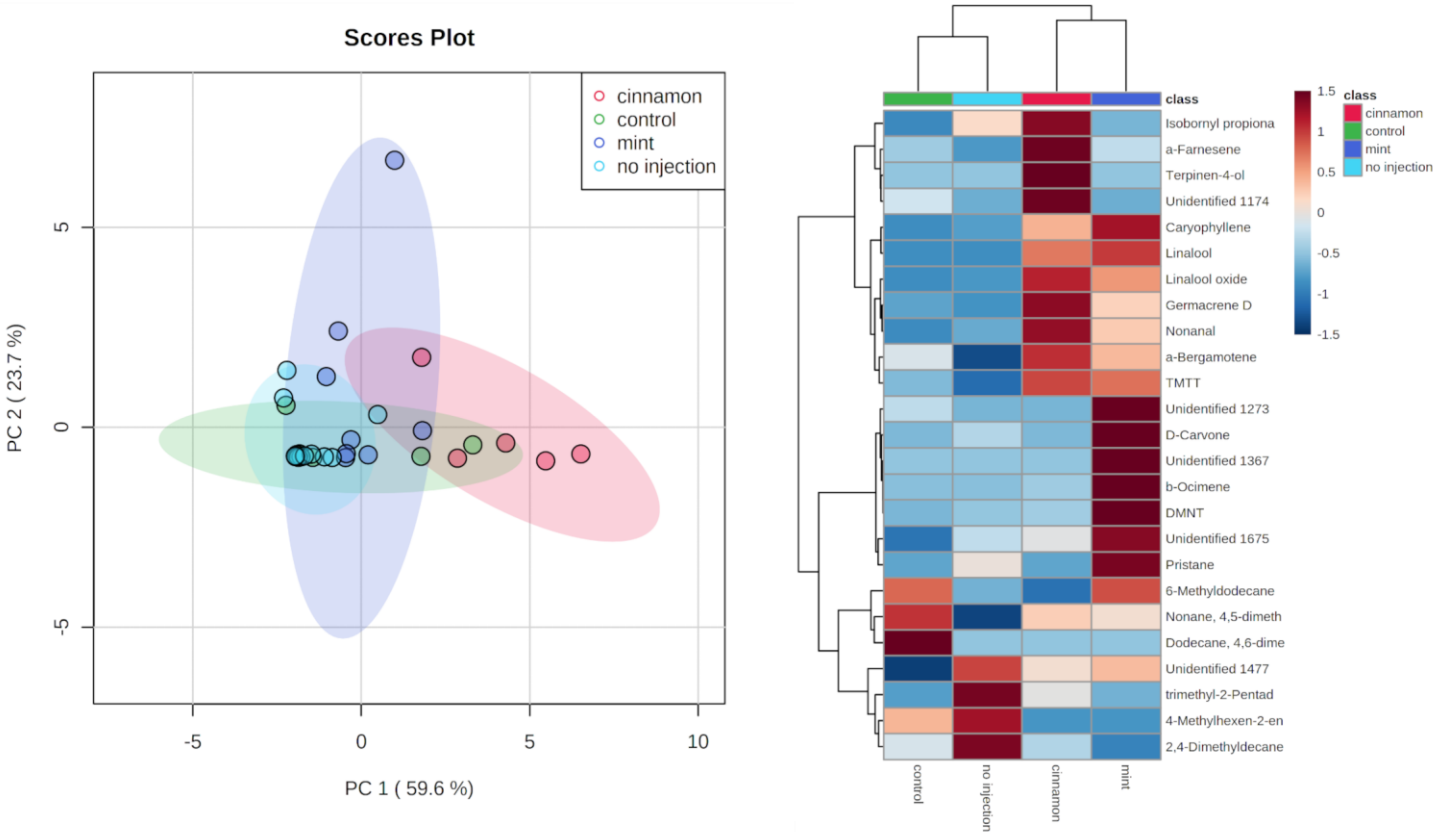


Figure 14. Principal component analysis (PCA) (left) and heatmap of the top 25 contributors merged by group (right) of *Malus x domestica* VOC emissions generated on metaboanalyst after data processing.

The VOC emission profiles were significantly impacted by the treatment. ANOSIM revealed significant structural differences for VOC profiles between treatments, with some overlapping ($R=0.281$, $p=0.002$). On the other hand, PERMANOVA performed on the same data set revealed similar outcomes for comparisons between treatment ($F=3.95$, $p=0.001$). Pairwise PERMANOVA yielded significant differences for multiple comparisons in all cases, except for the no injection-control and mint-control, as shown in Table 2.

Table 2. Pairwise PERMANOVA comparisons for VOCs emissions between treatment. The asterisks indicate significant differences. * $p \leq 0.05$, ** $p \leq 0.01$.

	No injection	**Cinnamon**	**Control**
Cinnamon	0.006**	–	–
Control	0.400	0.013*	–
Mint	0.006**	0.006**	0.253

3.2.3. Untargeted VOCs contained (DHS-GC-MS)

A total of 67 compounds were detected in the VOCs contained within leaves. These compounds belong to the alcohols, aldehydes, alkadienes, alkanes, aromatic and aliphatic esters, fatty acid esters, homoterpenes, and ketones (Supplementary Table 6). Injection of EOs significantly increased methyl salicylate, benzaldehyde, benzeneacetaldehyde, β-ionone, and nonanal (Figure 15). Among those compounds only benzaldehyde was found in the cinnamon EO, but also as a minor compound below 1%.

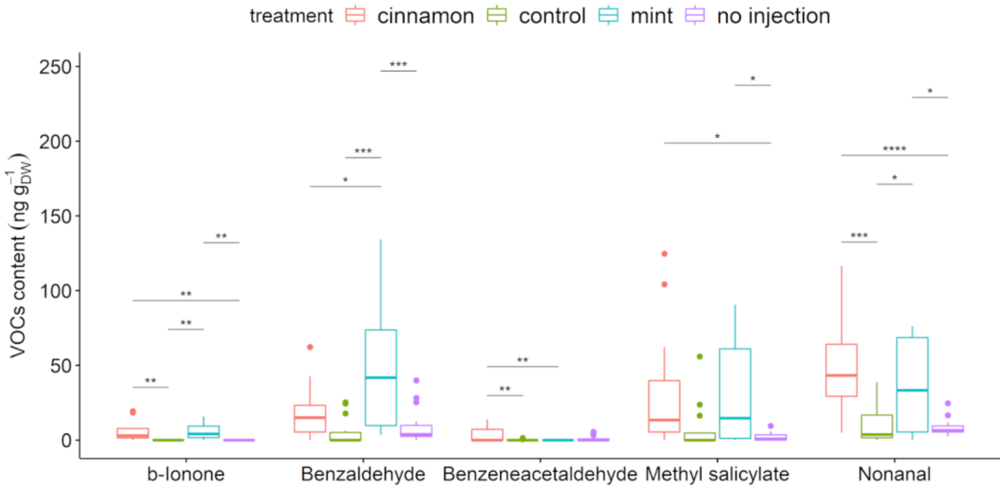
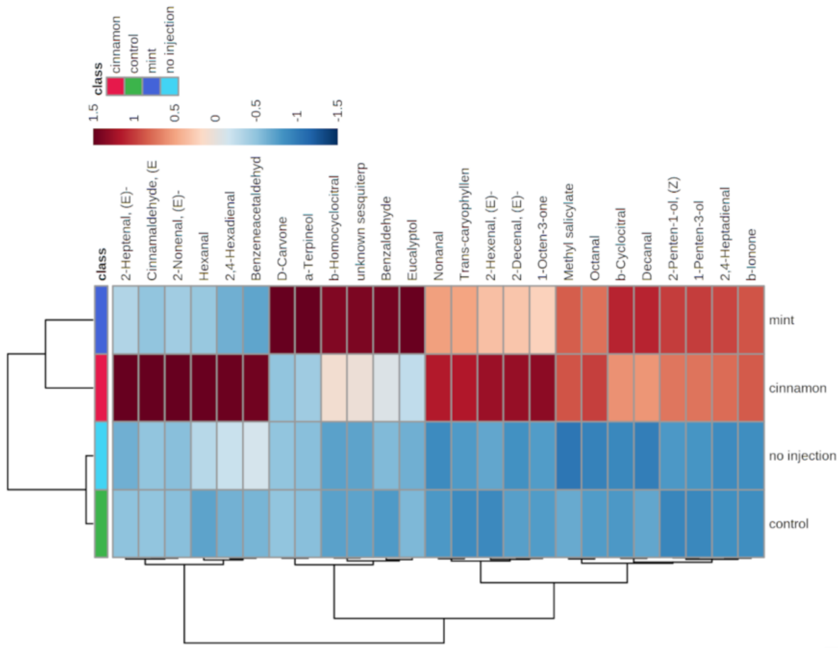


Figure 15. Boxplot of a selection of *Malus x domestica* VOCs contained (ng g_{DW}^{-1}) from plants injected with mint and cinnamon EOs and the control. The asterisk symbols above the bars indicate a significant difference between the means ($p<0.05$).

VOC profiles for EO-treated trees were much more dispersed in comparison to the control and no injection treatments (Figure 16). As for the emitted VOCs, it seems from the heatmap that some compounds increased for both oil treatments, such as decanal, caryophyllene, and 1-penten-3-ol. Some increases were specific, such as numerous aldehydes for cinnamon oil (2-heptenal, 2-nonenal, 2,4-hexadienal) and terpenes for mint oil (α-terpineol, eucalyptol, β-homocyclocitral). In regard to the EO composition, only α-terpineol was found in trace amounts within the mint EO at 0.25%.



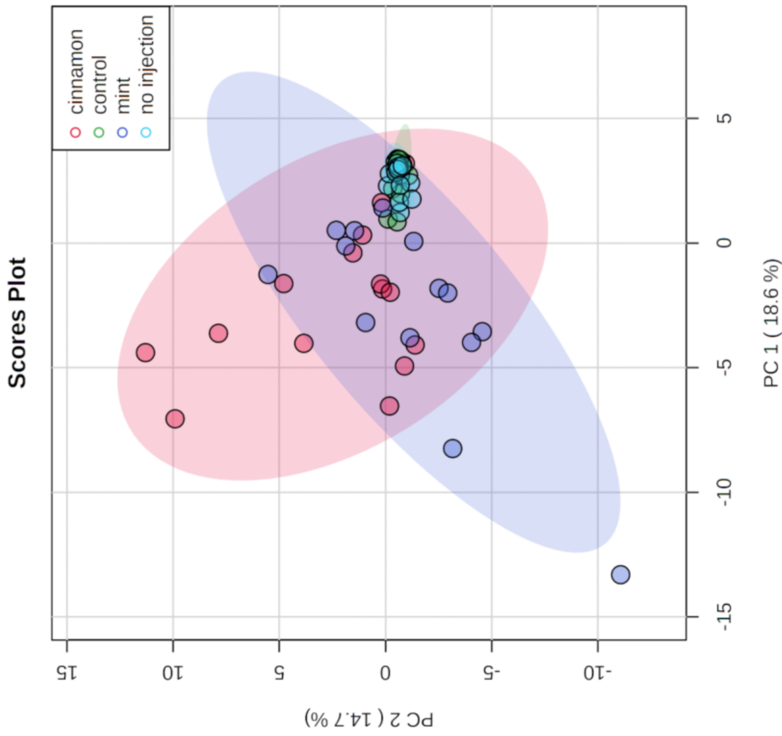


Figure 16. PCA (left) and heatmap of top 25 contributors merged by group (right) of *Malus x domestica* VOCs contained generated on metaboanalyst software after data centring processing.

ANOSIM revealed significant differences for VOC profiles between treatments, with some overlapping between group (R=0.271, p=0.001). PERMANOVA analysis of the same dataset revealed similar outcomes for comparisons between treatments (F=7.37, p=0.001). Finally, pairwise PERMANOVA revealed significant pairwise differences between all treatments, except for the control-no injection and cinnamon-mint, as shown on Table 3.

Table 3. Pairwise PERMANOVA comparisons for VOCs contained between treatments. The asterisks indicate significant differences. * $p \leq 0.05$, **$p \leq 0.01$.

	No injection	Cinnamon	Control
Cinnamon	0.006**	–	–
Control	0.585	0.006**	–
Mint	0.016*	0.151	0.022*

3.3. Chlorophyll fluorescence

Maximum yields of photosystem II (Fv/Fm) over time are presented in Figure 17. Chlorophyll fluorescence showed that most values were located between 0.80 and 0.85, implying that the trees maintained good ecophysiological performances throughout the experiment (Figure 17). Two-way repeated measure ANOVA revealed a significant impact of factors (treatment: F=4.76, p=0.003, ges=0.082; day: F=4.78, p=0.001, ges=0.107) without interaction (F=1.59, p=0.099, ges=0.107). However, pairwise comparison between treatments at each day demonstrated significant differences only at the start of the experiment (day 1) with a lower yield for the control.

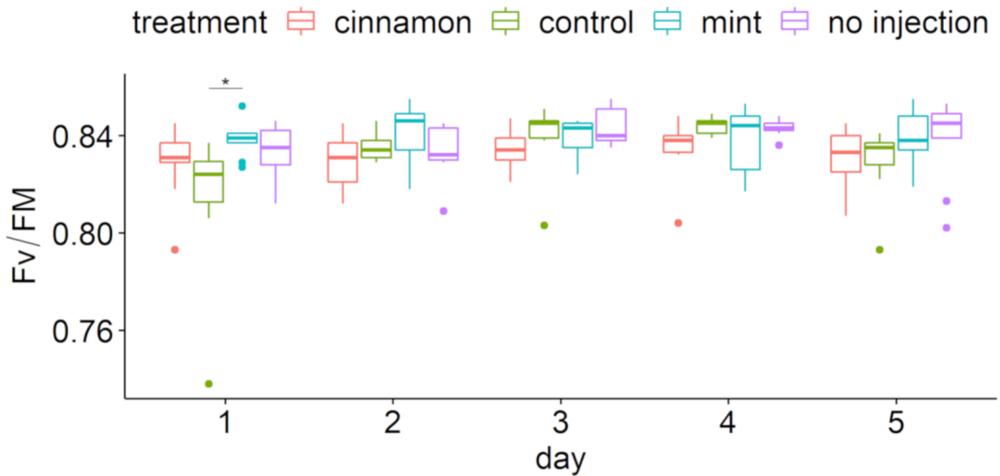


Figure 17. Maximum quantum yield of photosystem II (Fv/Fm) boxplot per treatment during time after injection. The star symbols above the bars indicate a significant difference between the means ($p<0.05$).

4. Discussion

Taken altogether, our results demonstrate, for the first time, the systemic translocation of trunk-injected EOs in apple plants. Carvone increased in the leaf content and was emitted at a constant rate, and *trans*-cinnamaldehyde content increased in the leaves but was not found in detectable amounts in the air emissions. The strong spatial heterogeneity combined with the relatively small sampling may also contribute to the variability of the results. However, it appears that the EO translocation within apple tree tissues and its diffusion in ambient air must be conditioned by its own physico-chemical properties. Among those properties, vapour pressure, organic carbon–water partitioning coefficient (Ko/c), and the octanol water partition coefficient (Ko/w) may explain the differences observed between carvone and *trans*-cinnamaldehyde (Doccola and Wild, 2012; Montecchio, 2013; Aćimović, 2014). Out of these two, *trans*-cinnamaldehyde was the molecule with the smallest vapour pressure of 15.3 Pa and 3.853 Pa at 25°C for carvone and *trans*-cinnamaldehyde, respectively (European Chemical Agency). This molecule, following Henry's law, has a smaller tendency to volatilise and hence accumulates in the leaves. Moreover, from a histological point of view, they diffuse slowly through aqueous phases in the mesophyll, lipid bilayer membranes, and internal airspace (in the substomatal cavity) before release through the stomata (Calfapietra et al., 2013). This diffusion is conditioned following the compounds' octanol water partition coefficients (Ko/w), which are 2.7 for carvone and of 2.1 for *trans*-cinnamaldehyde. It is worth mentioning that other phenomena could concurrently take place, such as the potential transformation or degradation of

these xenobiotic compounds by the apple plants. Diverse mechanisms such as reduction/oxidation, esterification or conjugation with carbohydrates (glycosylation), or glutathione (glutathionylation) were demonstrated *in planta* for numerous GLVs and terpenes (Matsui et al., 2012; Rivas et al., 2013) and by diverse microorganisms (Asakawa et al., 2018). This was specifically demonstrated for *Arabidopsis* aldehyde oxidase 4 (AAO4) extracted from *Arabidopsis thaliana* developing seeds that could convert *trans*-cinnamaldehyde *in vitro* (Ibdah et al., 2009).

In addition to the established systemic circulation of carvone and *trans*-cinnamaldehyde, it is most interesting to look at the modification of other VOCs in the emission profiles that can strongly impact trophic interaction within ecosystems. BVOC emissions can mediate herbivore interactions (Trowbridge & Stoy, 2013). Within the framework of this discussion, one should bear in mind that numerous factors can influence apple tree VOC emissions, including meteorological (Vallat et al., 2005), circadian (Giacomuzzi et al., 2017), physiological (Zeng et al., 2017) and phenological (Casado et al., 2006), as well as interactions with herbivores (Suckling et al., 2012) or fungi (Souleyre et al., 2019). However, systemic release of induced volatiles also occurs in plants in the case of insect feeding to recruit natural enemies. The homoterpenes DMNT and TMTT, the monoterpenes ocimene and linalool, and the sesquiterpenes farnesene and caryophyllene are a shared response to herbivores in diverse plant systems (Holopainen & Gershenzon, 2010; Paré & Tumlinson, 1999). Therefore, modification of emitted VOCs, such as those observed in our work, may alter trophic interactions in regard to chemical ecology. Moreover, germacrene-D, α-farnesene, and methyl salicylate may have resulted from SAR activation by the injected EOs since SAR has been detected after trunk injection of SAR activators (Aćimović et al., 2015). Indeed, monoterpenes have been acknowledged to support SAR among different plants (Riedlmeier et al., 2017). The elicitation of resistance in young apple trees by acibenzolar-S-methyl was observed to specifically increase the production of the compounds that were effective against rosy apple aphids and *Erwinia amylovora* (Aćimović et al., 2015; Warneys et al., 2018). Moreover, *Cinnamomum zeylanicum* oil and *trans*-cinnamaldehyde were proven to be efficient against *Alternaria* brown spot in tangerine by direct effects and resistance induction (Perina et al., 2019). A prospective molecular tool such as quantitative real-time PCR to detect changes in expression levels of genes involved in plant defense mechanisms may prove useful to challenge this hypothesis (Aćimović et al., 2015; Dugé De Bernonville et al., 2014). The plant defence responses include other mechanisms, such as cell wall fortification, antimicrobial compounds such as pathogenesis related (PR) protein productions, phytoalexins, or ROS (Marolleau et al., 2017). Phytoalexins include diverse plant secondary metabolites biosynthesised in response to pathogens and certain abiotic stresses. In the subtribe Malinae of the Rosaceae family, the phytoalexins biphenyl and dibenzofuran are produced upon pathogen attack (Chizzali & Beerhues, 2012) and after elicitor-treated cell cultures (Saini et al., 2019; Teotia et al., 2019). The production of phytoalexins following treatment with EOs could also

be an interesting prospect in order to determine and clarify the defence induction potential of these compounds as well as their potential impact on pathogens.

The results presented in this work clearly exposed the possibility that EO application could trigger different physiological processes within plants, leading to other BVOC emissions. Some compound production seems to be shared for both EOs, whereas some seem to be specifically induced by each EO. These results support the hypothesis of different modes of action for each EO and further demonstrated the plant's reaction to these EO injections. The differences between the two EO profiles may result from their specific interactions with the plant and, more precisely, with the plasma membrane. Recently, molecular techniques of dynamic interaction were applied to study the interaction between a biomimetic membrane with monoterpene (citronellal and citronellol) and with cinnamaldehyde (phenylpropanoids). Briefly, the *in silico* insertion model predicted different behaviours between the two classes (monoterpenes and phenylpropanoids), which are the stable interactions with plant lipids for monoterpene, while *trans*-cinnamaldehydes had no stable interaction with the membrane. These predictions were confirmed using *in vitro* biophysical assays (Lins et al., 2019).

Regarding the contained VOC profiles, green leaf volatiles (GLV) generated by the lipoxygenase (LOX) pathway such as 2-hexenal constitutes the major compounds. Due to the extraction protocol, this profile may not be interpreted as a potential pool for VOC emissions in the environment as *de novo* synthesis could have occurred during incubation and trapping after grinding, especially for GLV. DHS is the most widely used sampling approach in the plant field because of its flexibility (sampled volume, trapping approaches, and materials) (Bicchi et al., 2008). A high concentration factor was applied for the trace components under study. However, the analysis of these contained profiles may prove useful to further establish the metabolomic impact of EOs injection into apple trees. The presence of greater amounts of other aldehydes, such as nonanal, and the plant volatile hormone methyl salicylate reinforces the previously formulated hypothesis of resistance induction (Wenig et al., 2019). Other compounds emerged from the degradation of carotenoids, namely β-ionone and homocyclocitral (Dudareva et al., 2013).

Our work did not express foliar phytotoxicity. Indeed, maximum yields of photosystem II demonstrated significant differences only at the start of the experiment. Chlorophyll fluorescence is a non-destructive and sensitive method that is widely used in eco-physiological studies to assess abiotic stress in plants. Indeed, perturbation in plant metabolism may decrease photosystem II (PSII) performance. However, local toxicity at the injection site cannot be excluded, as well as the mechanical damage that occurs due to the injection procedure (Aćimović et al., 2016; Doccola et al., 2012). Furthermore, the specific mode of action of carvone can lead to microtubule depolarisation within cells. Lastly, unspecific generation of ROS has been frequently

observed after EO application (Dahiya et al., 2020; Kaur et al., 2010; Sunohara et al., 2015). Carotenoids are among the first non-enzymatic antioxidants acting to protect photosystem II from photo-inhibition and ROS (Pospíšil, 2012). Therefore, the higher content of its degradation product in the leaf may be explained by such a phenomenon. Physiological disorder in phytohormones or ROS balances that may result in chronic and long-term toxicity from EO applications should be addressed before concluding a lack of harmful effects of the treatments.

In terms of agricultural application, trunk injection and EO applications are rarely used (Aćimović et al., 2020). This work was established as proof of concept that the combination of both may be a suitable strategy to develop the biopesticidal potential of EOs while avoiding most of their drawbacks. However, we must highlight that more works in terms of reproducibility of results over different years, with other apple varieties, rootstock and efficiency on diverse pests are needed to establish the agronomic potential of such treatment. The absence of impact on apple quality or yield and on tree growth through long-term phytotoxicity should be established as well. Field trials should be performed to establish efficacy as a biopesticide and the lack of harmful effect to beneficial insects.

Plant VOCs are a promising tool as they have numerous applications in agriculture, such as parasitoids attractant or through defence induction or priming, growth regulators, and abiotic stress protectants (Brilli et al., 2019). Moreover, use of natural substances that elicit systemic resistance has been proven to be a suitable strategy for pathogen management in orchards (Lateur, 2002). The possibility of combining EOs due to their biopesticidal properties with a new mode of application—trunk injection—was hereby demonstrated. Furthermore, the variations in the emitted and contained VOCs clearly demonstrate that young apple trees react to EO injection and that this reaction may be explored to design sustainable agricultural practices.

5

Laboratory and field-trial application of cinnamon essential oil as botanical insecticide in fruit arboriculture

The fifth chapter of this thesis combines two different experiments. The first synthesises the preliminary work of laboratory trials investigating the population control of RAA using the injection of cinnamon EO. The second part presents a research article that will soon be submitted soon, synthesising field trials:

Werrie, P. Y., Le Goff, G. J., Kumps, V., Dhont, T., Lateur, M., Fauconnier, M.-L., Hance, T. Field-trial application of *Cinnamomum cassia* essential oil by trunk injection as a bio-insecticide in fruit arboriculture.

In this second part, the methodology of field trials for 2020 and 2021 and their results are presented. Those trials were performed in an apple orchard belonging to and managed by the Walloon Agricultural Research Center (CRA-W) in Belgium. Indeed, these injections were applied as curative and preventive treatments during the vegetative stage and at bud burst. Insect population dynamics were monitored for RAA, as well as its predator and other pest populations following treatments. To identify the potential impact on biological airborne signals, tree VOCs emissions of were sampled. Tree physiology was followed by visual assessment, growth and chlorophyll fluorescence measurement. The total and commercial apple yields were estimated, as well as the *trans*-cinnamaldehyde residue (main EO compound) in fruit.

The chapter ends by discussing the drawbacks of the actual methodology and the perspectives to improve practical implementation.

Contribution: Conceptualisation, methodology, formal analysis (apart from insect population dynamics), writing—original draft review and editing. The entomologic evaluations were performed by trained scientists specialised in apple orchard insect fauna from the laboratory of "Ecology of interactions and biological control" (UCLouvain).

Abstract

Apple production is one of the biggest fruit businesses worldwide. The Rosy Apple Aphid (RAA), *Dysaphis plantaginea* Passerini, is amongst the most detrimental pests in apple orchards. This study synthesises laboratory and two years' field trials aiming to develop a biopesticide based on cinnamon (*Cinnamomum cassia*, J. Presl) essential oil to deal with the RAA using tree-injection as the application method. In the frame of the laboratory design RAA population development was evaluated during 4 weeks (3 generations) on (non)-injected trees. In the same time tree physiology was evaluated by chlorophyll fluorescence and photosynthetic activity. As part of field trials 40 and 45 apple trees (*Malus x domestica*, Borkh) of the Jonagold cultivar were followed respectively during the 2020 and 2021 experiment. Injections were applied as preventive and curative treatments during vegetative stage and at budburst. Insect population dynamics (number of colonies and aphids) was monitored as well as other pest and predator populations following treatments. Moreover, tree emissions of volatile organic compounds (VOCs) were sampled to identify potential impact on biological airborne signals. Due to EO potential phytotoxicity tree physiology was followed by visual assessment, growth and chlorophyll fluorescence measurement. Lastly total and commercial yield were estimated as well as *trans*-cinnamaldehyde residue (EO main compound) in fruit by stir bar sorptive extraction (SBSE) methods. Globally treatment impacted aphid population dynamics but did not lead to a complete *D. plantaginea* control. Significant differences were spotted in terms of VOCs emissions by the overall trees but were associated with seasonal variation and not with treatments. Increased concentration of active substance (a. s.) leads to visual phytotoxicity, reduced performance index and growth on apple trees allowing to establish maximal application rate. The treatment presented no residue in fruit or impact on yield. This study investigates the practical feasibility of laboratory effective solutions in agronomic conditions and identifies challenges and limitations needing to be addressed.

1. Introduction

In 2016, total apple production worldwide reached 84.7 million tonnes (FAOSTAT). This represents a gross production value of 45.8 billion US dollars (FAOSTAT). Unfortunately, the production yields of apple fruits are often disrupted by pest insects such as aphids, psyllids, beetles, moths (Jenser et al., 1999) or fungal/bacterial diseases (scab, fire blight, mildew, etc.) that results in important quality, production and economic loss. *Dysaphis plantaginea* Passerini (Hemiptera: Aphidæ) is among the most detrimental pests in apple orchards (Albert et al., 2017). Its presence is problematic in both organic and conventional orchards due to its low abundance threshold for economic damage (two fundatrix per 100 leaf) (Bürgel et al., 2005). Aphids damage fruits by reducing their size and deforming their shape. They also reduce the overall tree vigour due to phloem sap sucking, organ deformation, chlorosis, premature leaf fall and they enhance the development of sooty mold on the honeydew they secrete (Rousselin et al., 2017).

To deal with those numerous pests, phytosanitary products have been widely spread since the 1950's (Isman, 2006; Valiuškaitė et al., 2017). Nowadays, evidence suggests that pesticide misuse may be responsible for flora and fauna disruption (Krebs et al., 1999), causing health risks (Alavanja et al., 2004), increasing resistance of target pests and affecting water, air and soil quality (Moss, 2008). Worldwide use has been estimated at 2.5 million tons each year and damages caused by this indiscriminate use of various products reaches $100 billion annually (Koul et al., 2008).

Reports suggest that with pesticide application by spraying, only 0.1% of the chemical comes into contact with the target pest. Moreover, airblast sprayers are reported as inefficient means of applying pesticides to their target as only 29-56% of the solution actually reaches the tree crown while remaining product drifts to the ground, water, air or other off-target end points. Those drifts are responsible for most of the pesticides environmental damage and workers health hazards (Coslor et al., 2019; Wise et al., 2014). Tree (or trunk) injection is an alternative method to conventional airblast sprayers to apply crop protection products directly into the tree's vascular system. The chemicals are injected by piercing the bark to access the xylem and are then systematically distributed via the xylem sap. This allows optimal pests targeting while reducing pesticide inputs and environmental exposure (Coslor et al., 2019; Doccola et al., 2012). Although it is not yet widespread, trunk injection has made its worth in successfully protecting various tree species and particularly in urban areas where sprays cannot be applied (Aćimović, 2014).

Nowadays, several integrated pest management (IPM) techniques are available to deal with aphids in tree orchards: e.g. (i) promoting natural predators or parasitoids such as ladybirds, syrphidæ, spiders (Miñarro et al., 2005), (ii) the systematic removal of the secondary host plant (iii) interplanting herbaceous strips or extra-floral nectar

bearing trees to promote natural enemies or as a lure trap for aphids or to attract even more predators (iv) parasitoid release and (v) development of aphid-resistant cultivars (Albert et al., 2017; Miñarro & Dapena, 2008; Nicholas et al., 2005). Aphid behaviour is also very much influenced by the emission in the air of volatiles organic compounds (VOCs) such as plants' stress volatiles that have a repellent or toxic effect (Rousselin et al., 2017) or by the detection of aphids alarm pheromones, such as E-β-farnesene, that also triggers aphids' repellence, as a "run for your life" signal or as an attractant for aphids' natural predators (Francis et al., 2005; Vandermoten et al., 2012). However, several studies concluded that natural enemy populations are unable and insufficient to control and regulate aphid populations (Brown & Mathews, 2007; Dib et al., 2010; Miñarro et al., 2005; Wyss, 1995; Wyss et al., 1999). Therefore, new strategies to manage aphids in tree orchards are needed.

Safe and eco-friendly biopesticides ("green pesticides") are being developed and are emerging on the market. These products are allowed as external inputs in organic production as "natural or naturally-derived substances" (EC No 834/20072). They can be of botanical origin such as pyrethrum, rotenone, neem, ryania, nicotine, sabadilla, … and may be used against a wide range of insects or fungi (Isman, 2006). Among those botanical "green pesticides", essential oils (EOs) are well represented. EOs are a complex mixture of volatile lipophilic compounds or volatile organic compounds (VOCs). These VOCs are produced as secondary metabolites in plants and their extraction by distillation or pressing results in what is called "essential oil" (EO). EOs are constituted of a wide array of substances of which 1 to 3 are major compounds found in high concentrations (Russo et al., 1998; Senatore, 1996) while the rest is typically present at trace levels (Campos et al., 2019). The growing interest of EOs in IPM or sustainable agriculture lies in their rapid breakdown in the environment, their low accumulation in soil and water and their non-significant toxicity to non- target organisms such as fish, birds and mammals (Campos et al., 2019; Isman et al., 2011; Maes et al., 2021; Stroh et al., 1998).

Most of the previous research regarding EOs as potential plant protection products have identified their principal biological activity: (i) for virus and microorganisms: antiviral, antifungal and antimicrobial. (ii) for insects: attractants (both for pests or natural predators), antifeedant (feed deterrent), ovicide/oviposition inhibitors, repellent, insecticidal (to both larvæ and adults), fumigants, insects growth regulator (IGR) and (iii) nematicide (Campos et al., 2019; De Clerck et al., 2020; Koul et al., 2008; Miresmailli et al., 2006; Mossa, 2016; Rattan, 2010; Regnault-Roger et al., 2012). Regarding insecticidal activity, despite the knowledge available on this subject, modes of action are in general not fully elucidated (Campos et al., 2019; Rattan, 2010). Neurotoxic mode of action is suspected through inhibition of neurotransmitters such as GABA, ACEh. Recent proteomic studies even suggest a larger impact on the development and functioning of the muscular and nervous systems, cellular respiration, protein synthesis, and detoxification (Renoz et al., 2021). The EO used in

our study was obtained from *Cinnamomum cassia*. Cinnamon essential oils (CEO) is used in a lot of fields (medicinal, food and cosmetics) due to its known antifungal, antibacterial, antioxidant and flavour properties (Geng et al., 2011; Jardim et al., 2018). Most recently, its use as a biopesticide gained interests and most particularly for the effect on insects, fungi and bacteria of its main compound: *trans*-cinnamaldehyde (Burt, 2004; Chang et al., 2013; Lee et al., 2008; Ojaghian et al., 2016; Wang et al., 2009). It was even investigated as acaricidal for house use against House Dust Mite (Oh, 2011). Commercial solutions are already on the market such as Vertigo™ and Cinnacure™.

A previous study by Werrie et al. (2021) showed that following the injection of an emulsion of cinnamon essential oil (CEO) into the trunk of an apple tree, the main compound was able to diffuse into its vascular system and reaches the leaves. Subsequent study show that CEO application could trigger plant defence but could also be phytotoxic to the tree (Werrie et al., 2022). The present work aims to assess the feasibility of insecticidal treatment on fruit trees using CEO as a. s. and trunk injection as application methods in laboratory and orchard conditions. This IPM technique lies on the known repulsive effects of some EO compounds towards insects and is part of a sustainable development strategy: reducing chemical use, limiting the environmental hazard/health hazards of those chemicals and the creation of a range of high-quality fruits. First, a preliminary laboratory design studied influence of injection on survival of *D. plantaginea* while monitoring plant physiological status. Then field trials experiment synthesised a two years' trial aiming to: 1) Determine the botanical insecticide potential of the CEO against the RAA in agronomic conditions population dynamics, 2) Determine the translocation of the injected CEO main constituent via VOCs analysis, 3) Determine the potential incidence of the treatment on the trees' health and production through ecophysiological measure of chlorophyll fluorescence and non-targeted VOCs emissions.

2. Materials and methods

2.1 Laboratory trials

2.1.1 experimental design

The experiment was carried on two-year-old *Malus x domestica* Borkh (var. Jonagold) micropropagated apples trees, having developed mature and fully expanded leaves (plant length = 53±8 cm; stem diameter = 4.4±0.6 mm). They were conserved in an environmental chamber operating under the following conditions: 21±0.5°C, 60±10% relative humidity (RH), 16:8h light: dark periods and a PAR intensity equals to 50 µmol m^{-2} s^{-1}. Apple trees were watered every three days with 50 mL of water.

Four modalities in the experimental design were considered: A) non-injected trees, as controls B) non-injected trees with aphids deposited on leaves, C) trunk-injected trees during around 2 days (44 h) with CEO 2% (v/v) emulsion, D) injected trees as C) with aphids. Each treatment was tested on seven apple tree replicates (n = 7).

2.1.2 Insect population dynamics

For modalities B and D, 15 second instar larvae of *D. plantaginea* were deposited at the end of the injection, just after the drip pockets have been disconnected from the trees. Aphid survival monitoring, the number of living individuals was recorded during 4 weeks (3 generations).

2.1.3 Tree physiological performance

One leaf located at mid-height of the tree was selected for measurements of chlorophyll fluorescence and another one for photosynthetic capacity. All the measures were performed on the same leaves and at the same moment of the day between the sampling sessions, every two days during the first week, then every week during one month.

Measurements were done using a fluorescence monitoring system (FMS2, Hansatech Instruments, Kings Lynn, UK). Before measurements, leaves were dark adapted for 30 min with leafclips. The baseline fluorescence is then measured (F0). Then, a flash of saturating light is sent (“SP”: 18 000 µmol $m^{-2}s^{-1}$) and the maximum fluorescence is measured (Fm). A constant light is sent for 2 minutes (“Actinic light”: 660 µmol $m^{-2}s^{-1}$), after which a new saturating flash is used to measure the maximum fluorescence of the photosystem (Fm') as well as the basic fluorescence in the presence of constant light (F' or Fs). Other commonly used fluorescence parameters are directly calculated by the device, such as quantum yield of PSII in light conditions (ΦPSII), proportion of open PSII (qP), maximum quantum yield of PSII (Fv/Fm) and non-photochemical quenching (NPQ).

Water vapour and CO_2 exchange were measured with a portable infrared gas analyser (ADC LCi-SD, ADC BioScientific Ltd., Hoddesdon, Herts., UK). The CO_2 assimilation rate (A, in µmol CO_2 m^{-2} s^{-1}), sub-stomatal CO_2 mole fraction (Ci, in µmol mol^{-1}), stomatal conductance (gs, in mol H_2O m^{-2} s^{-1}) and transpiration rate (E, in mol H_2O m^{-2} s^{-1}) values were collected. The measurement conditions were as follows: leaf temperature, 21°C; leaf chamber area, 6.25 cm^2; relative air humidity, 60%; photosynthetic photon flux density (PPFD), 50 µmol m^{-2} s^{-1}; leaf to air vapour pressure deficit, 1.5±0.5 kPa; and ambient CO_2 concentration, 400±5 µmol mol^{-1} (or volumes per million vpm).

2.1.4 Data analysis

All the data were gathered on Excel and processed using Minitab 19 and R studio (v 4.1.2) softwares. The main statistical procedure performed was a simple one-way analysis of variance (ANOVA 1). In the case where the null hypothesis of ANOVA was rejected, a post-hoc Dunnett's test was performed to compare each modality with the control. In this case, ANOVA were performed for each parameter at each time independently. For aphids counting, a Generalised Linear Model (GLM) with a Fisher test (family = quasipoisson (link= log)) was performed. For all significance tests, $\alpha = 0.05$ was applied as probability cut-off.

2.2 Orchards trials

The whole experiment took place in an apple orchard containing various cultivars and concerned solely the Jonagold cultivars. This orchard is located in Belgium (coordinates: 50.555103, 4.661554). The trees were planted in March 2005 on M9 rootstock. No other phytosanitary treatment was applied during the experiment. The experiment was realized during two successive years (2020 and 2021). The orchards plantation design and cultivar repartition is presented on Supplementary Figure 3. The weather conditions were also recorded for the entire duration of the experiment (Supplementary Table 7).

2.2.1 Trunk injection method

The device used was an ENDOkit Manual PRO © purchased from ENDOterapia Vegetal (Spain). This device requires the drilling of a hole in the tree bark prior to injection. The hole was drilled at 60 cm height using an 8 mm drill bit. Bark outer layer was removed using low drilling speed and residues were cleaned. Hole was then completed at a higher speed to plug length (2 cm). The plug was hammered to correct positioning to ensure optimal healing. The presented methodology is illustrated on Supplementary Figure 4.

2.2.2 Formulation of the essential oil emulsion

To ensure optimal compound delivery, the physical properties of the solution delivered to the trees must be as close as possible of the xylem sap's physical properties. CEO was therefore formulated as an oil/water emulsion using Tween 80 biocompatible surfactant and EDTA following previously described procedure (Werrie et al., 2021).

2.2.3 Experimental design

The first year (spring 2020) consisted in the evaluation of injection impact on the tree health, and in a curative treatment of aphids' infestations. The second year (spring 2021) consisted in a preventive treatment with the injection of different volumes of the CEO.

For the first year, the injection took place on the 19th of May 2020. Therefore, aphid colonies were already in place. An inventory of aphid colonies (more than 10 individuals) was made to ensure that trees started with the same number of aphids. The treatment modalities were assigned considering infestation scores (number of colonies) so that the medium infestation was similar. Height random blocks of 5 trees were constituted with each treatment by block (n=8). The five treatments consisted in no injection [NEG] (control to determine the potential changes due to the treatment), injection with water [BSA] (to measure stress caused by the injection only), injection of emulsifier [BEM] (to show impact of the emulsifier), injection with CEO emulsion at 1% [CEO1] (0.03 g of active substance (a.s.)) and injection with CEO emulsion at 2% [CEO2] (0.06 g of a. s.).

For the second year, following the results of the first year, a second experiment took place in the same orchard with a gradient of the injected volume of CEO. Injection took place on the 1st of April 2021, 9 blocks were constituted following the previously described procedure. Five treatments were considered as follows: no injection [NEG], Injection of CEO at 2% with 3 mL, 10 mL, 20 mL and 50 mL (0.06 g, 0.2 g, 0.4 g, 1 g of a. s.). For this last treatment the volume of emulsion was injected through 2 plugs (25 mL of emulsion in each plug) on each side of the tree.

2.2.4 Insect population dynamics

2.2.4.1 Dysaphis plantaginea populations

During the experiment, aphid colonies development was monitored once per week for 6 weeks since the injection of the EO emulsion for the first year, and for 7 weeks since the appearance of aphids for the second year. The total number of colonies per tree was registered one per week, and the total number of aphids was evaluated for one colony (always the same) on each tree at each monitoring session.

2.2.4.2 Predator and pest populations

Using a stick, tree branches (high, medium and low on the tree) were beaten 3 times each. As a result, the insects or other living organisms fell onto a white board placed under the beaten branch and the number of predators (spiders, predatory bugs, ladybirds, and earwigs) and pests (caterpillars, other aphids than *D. plantaginea* (Green apple aphid/*Aphis pomi*, and Woolly aphids/*Eriosoma lanigerum*), weevils, and psyllids) were counted. The operation was repeated two times per tree (on each side of the row).

2.2.5 Volatile organic compounds (VOCs) sampling and analysis

2.2.5.1 Emission sampling

For the first year A 60 L PET Nalophan® bag (odometric©) was placed over a branch with an activated charcoal filter. At the other extremity, an PTFE tube was enclosed

inside the bag and screwed inside a stainless steel tube connector (Swagelock™). One cartridges (Tenax TA 60/80, Camsco™) was relied to a Gilian™ "GilAir Plus" air sampling pump (Sensidyne). Prior to sampling, sorbent tubes were conditioned for 11h at a 300°C program in the conditioner (TC2 conditioner, Gerstel©). Air pumping is executed for 90 min at a 100 mL min^{-1} rate. For the second year, sampling device was miniaturised to a 12*12 cm PET Nalophan® bag to enclose a single flower bud. Identical cartridges and sampling pumps were used. Air pumping was executed for 30 min at 200 mL min^{-1} rate. Cartridges were then stored at -80°C prior to analysis. Each cartridge received 1µL of 1-phenyloctane solution at 40 µg mL^{-1} as internal standard (IS) prior thermal desorption using a multipurpose sampler 2 (MPS2, Gerstel©).

2.2.5.2 CEO residue analysis (apple) sampling

After yield estimation apples were stored at -80°C prior analysis. CEO main compound, *E*-cinnamaldehyde content was analysed by stir bar sorptive extraction (SBSE) using stir bars of 10 mm in length and 0.5 mm in film thickness coated with polydimethylsiloxane (PDMS) from Gerstel. For analysis, each sample was freeze-grinded. Then 4g of apple and 1.2g of NaCl was weighed in a 20 mL headspace vial. Then, 1µL of methyl trans-cinnamate solution at (50 µg mL^{-1} in heptane) is added as IS in the vial. Stir bars were twisted at 700rpm during 2h at room temperature. *E*-cinnamaldehyde quantification was performed using single ion monitoring mode with m/z=131 as quantifier ion and m/z=103,132 as qualifier ions. Calibration curve between 2.5 and 140.5 ng g^{-1} was performed in triplicates using the relative response.

2.2.5.3 Thermal desorption and GC-MS analysis

Analyses were performed on HP-5 MS capillary column (30 m × 250 µm × 0.25 µm, Agilent Technologies©) by GC-MS (7890B-5975C, Agilent Technologies©). Thermal desorption conditions were as follows for Tenax, 40°C to 280°C at 100°C min^{-1} with 5 min hold, and as follow for SBSE, 35° to 250°C at 60°C min^{-1} with 3 min hold. COV were Cry Focused at -60°C and injected at 12°C sec^{-1} to 250°C with 2 min hold. GC runs with a helium flow rate of 1.2 mL min^{-1}. The oven temperature program was: 40°C with 2 min hold; increase of 5 °C min^{-1} up to 170°C then 20°C min^{-1} up to 310°C with a hold for 3 min. The mass spectrometer was set to have a temperature of 230°C at the ion source and 150°C at the quadrupole. The mass spectrometer was programmed with a SCAN acquisition mode. Mass spectra were scanned from 35 to 500 amu. Then, component identification was performed by comparison of the obtained spectra with mass spectra in a reference database (NIST17, pal600, whiley275). Additionally, experimental retention indices (RI) were calculated following the injection of a mixture of n-alkanes C8-C30 (Sigma Aldrich©) under the same chromatographic conditions.

2.2.6 Tree ecophysiology: Chlorophyll fluorescence

The fluorescence measurements were taken using the Hansatech fluorometer (Handy PEA). with following conditions, no Pre-illumination, Illumination of 3000 µmol m^{-2} s^{-1} during 1.0 s with gain of 1. Leaf was "dark" adapted for 30 min before measurement. For each tree, 5 measurements were performed at various heights (low, medium and high) on both sides. Four parameters were considered in the analysis namely: the maximum potential quantum efficiency of Photosystem II (Fv/Fm), the basal fluorescence (F0), the performance index (PI ABS) and the electron transport flux (ET0/RC).

2.2.7 Analysis of tree growth and apple production

For each condition of both years, trees growth was estimated at the end of the season (09/09/2020 and 15/09/2021) by measuring on each side of each tree, three randomly selected year shoots at various heights (low, medium and high). On the same dates as the growth measurement, all apples from each tree were harvested, separated into two categories (with and without symptoms of *D. plantaginea* attack), counted and weighed.

2.2.8 Data analysis

Generalised linear models (GLMs) were fitted to the data to test the potential influence of our treatments and time on the dynamics of population of aphids (number of colonies and number of aphids per colony), predators, and other pests than *Dysaphis plantaginea* dynamics of population (family = quasipoisson(log)). VOCs were compared between sampling sessions and between treatments using principal component analysis (PCA) and permutational multivariate analysis of variance (permanova) followed by pairwise permanova. Permanova was computed using the "Adonis" function with a Euclidean method and a Fisher test. Permanova were done using a distance matrix generated with the R "dist" function (Euclidean method) and a Wilks test. Chlorophyll fluorescence data was analysed by GLM (family= gaussian). Yield and growth parameters were analysed by one-way analysis of variance. The normality of the data set was determined using a levene test. Homogeneity of variance was assessed by shapiro test and data were square root transformed for 2021 yield. Pairwise comparison between the treatments was performed using the Tukey's test. Visual phytotoxicity burned bud count number was analysed by GLMs (family= poisson).

3. Results

3.1 Laboratory trials

3.1.1 Insect population dynamics

The primary objective of the laboratory experiment was to investigate impact of CEO injection on RAA population development. To do so number of living aphids counted in each colony and evolved as shown in Figure 18. If we still consider the date of injection as day 0, aphids are therefore deposited on leaves on day 2. Already 6 days after injection (DAI), the mean number of survivors on treated plants dropped sharply by approximatively 60%. From Day 11 until the end, the number of aphids start to increase on both types of apple trees, but more rapidly on untreated ones. Five weeks after the beginning of the monitoring, the number of aphids on control plants is in average 4,6 times higher than on injected trees (172 compared to 37). GLM analysis confirm differences in population development, with effects of time and injection significant, with p-values are below 0.001, and no interaction between the two factors ($p>0.05$). Although encouraging, controls taking into consideration the effects of the injection and the formulation alone should be taken into account to investigate the agronomic potential and confirm the effects obtained.

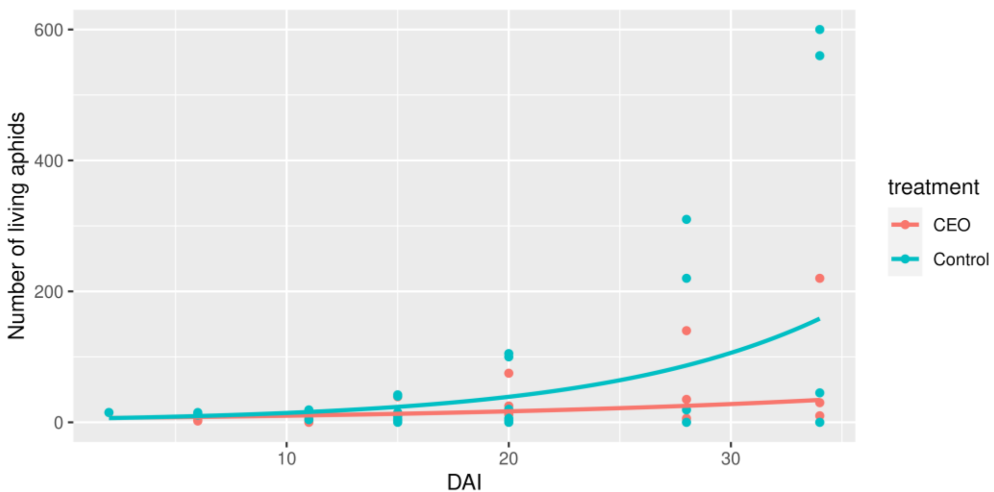


Figure 18. Overview of the number of living aphid on injected trees (red) and control (blue) and the GLM models.

3.1.2 Tree ecophysiology

ANOVA 1 have been performed for each of the five fluorimeter parameters Fv/Fm, F0, ΦPSII, qP, NPQ, to know the impact of the treatment, with each time tested independently. P-values obtained are summarised in Table 4. The Dunnett's test was performed for significant one comparing each treatment with the control (modality A). The only tests which display a significant difference between injected (C & D) and non-injected apple trees (A & B) are on day 1 for F0 and on day 8 for ΦPSII with both higher values for injected trees. A higher F0 value could support the assumption that these plants would be more stressed. However, the significant difference observed for F0 on day 1 is not visible in the Fv/Fm ratio, while it depends on F0. Finally, it seems surprising that the injected plants also have a higher quantum yield of PSII in light condition.

Table 4. P-values obtained for ANOVA-1 performed on chlorophyll fluorescence parameters over time.

	D0	D1	D5	D8	D11	D18
Fv/Fm	0.491	0.753	0.695	0.099	0.861	0.730
F0	0.409	**0.003****	0.725	0.013*	0.271	0.107
ΦPSII	0.813	0.281	0.769	**0.014***	0.390	0.902
qP	0.287	0.447	0.244	0.297	0.214	0.216
NPQ	0.063	0.094	0.038*	0.002**	0.312	0.164

p-value<0.05: significant * / < 0.01: highly significant ** / < 0.001: very highly significant ***

Similar ANOVA 1 were performed (4 parameters at 6 days of measurement) for photosynthetic activity and the corresponding p-values are summarised in Table 5. A surprising thing to point out is that for the carbon assimilation rate A at day 0 (i.e. the beginning of the experiment, before any special treatment applied), there is a significant difference occurring, which continues to be observed thereafter until the end of the experiment. It could imply that carbon assimilation rate is affected by events taking place previous of the experiment, in addition to being affected by the treatment. We can therefore conclude that in our case, this parameter does not seem to be very reliable to highlight the presence of stress due to the treatment. Measurements under standardised artificial light could increase reliability of these data. Finally, for the E and gs parameters, the only distinction between injected and non-injected apple trees occurs on day 1 for transpiration rate E. The lower E values for the injected plants are further evidence of a photosynthetic yield negatively impacted 24 h after the beginning of the injection.

Table 5. P-values obtained for ANOVA-1 performed on IRGA parameters over time.

	D0	**D1**	**D5**	**D8**	**D11**	**D18**
A	0.017	0.002**	0.011*	0.003**	0.005*	0.000**
C	0.182	0.529	0.000**	0.000**	0.004*	0.004**
E	0.280	0.003**	0.583	0.335	0.028*	0.132
gs	0.431	0.000**	0.027*	0.978	0.04*	0.290

p-value<0.05: significant * / < 0.01: highly significant ** / < 0.001: very highly significant ***

To sum up, we can affirm that neither injection of CEO emulsion nor aphid deposition does seem to impact significantly on the long-term the photosystem II and photosynthetic apparatus efficiency. It is consistent with the fact that visually, leaf tissues were and remain fully green, with no chlorophyll bleaching brown traces on apple leaves on which the fluorescence measurements were repeated. Those results combine with previous biological activity on RAA allow the transfer of those preliminary results to similar experiences at the field scale.

3.2 Field trial experiments

3.2.1 Insect population dynamics

3.2.1.1 Dysaphis plantaginea populations

For the first year of experiment (2020), the GLM test found a significant effect of the treatments on the number of aphids per colony ($F= 2.81$, $df=4$, $p < 0.05$). Indeed, at the third week, the colony of the control condition contains about 800 aphids while for the colonies of the other conditions it is 400 in average. A highly significant effect of the date of sampling was also observed ($F= 46.50$, $df= 5$, $p < 0.01$) with an increase of the number of individuals in the colony from the first week to the third week (from 100 to 500 aphids) followed by a decrease until the end of the season. In order to better visualise population dynamics, cumulative number of aphids was represented on Figure 19.

The number of colonies increased since the beginning of the experiment ($F=10.45$, $df= 1$, $p < 0.01$) to reach a peak at the fifth week with around 30 colonies per tree that fall back at the 6th week with only 2 colonies per tree on average. This dynamic observed for the number of colonies per tree was the same for all treatments ($F=0.94$, $df= 4$, $p > 0.05$). In order to better visualise colony build up, cumulative number of colonies was represented on Figure 20.

For the second year of experiment (2021), regarding the number of aphids per colony, it was in the same range as the previous year and did not differ between treatments ($F=0.44$, $df=4$, $p=0.780$) with about 400 aphids per colony at the time of the population peak (mid-June) that then collapse at the end of the month ($F=14.90$, $df=1$, $p<0.001$) (Figure 19).

According to Figure 20, the average number of colonies found per tree in 2021 globally increases until June ($F=87.58$, $df=1$, $P<0.001$) to reach a peak of 5 colonies that is 6 times less than in 2020. The GLM performed indicates that the treatment had a significant effect with more colonies found in the 3 mL condition ($F=7.73$, $df=4$, $p<0.001$) with 8 aphid colonies found in this condition against 3 on average for the other conditions (Figure 20).

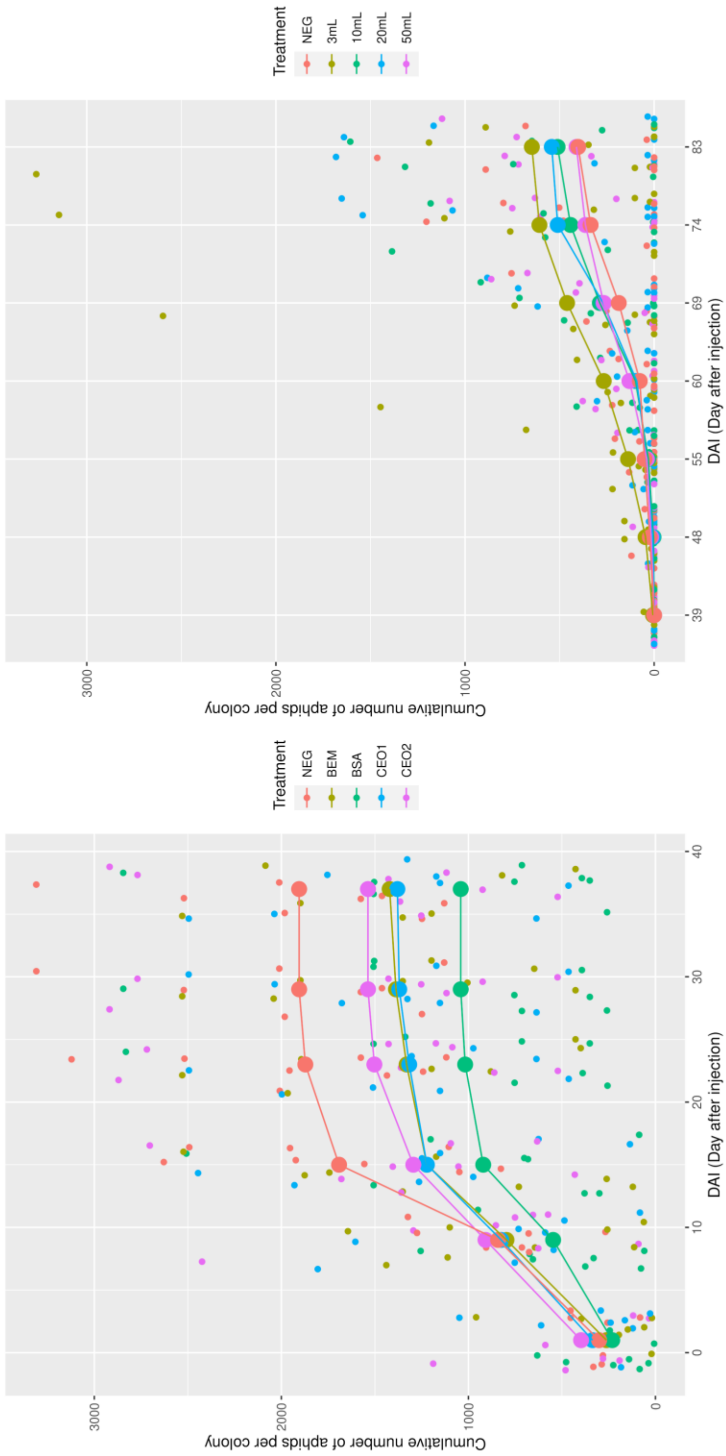


Figure 19. Cumulative number of aphids per colony according to the DAI (Day After Injection) for all the treatments and for both years (2020-left; 2021-right).

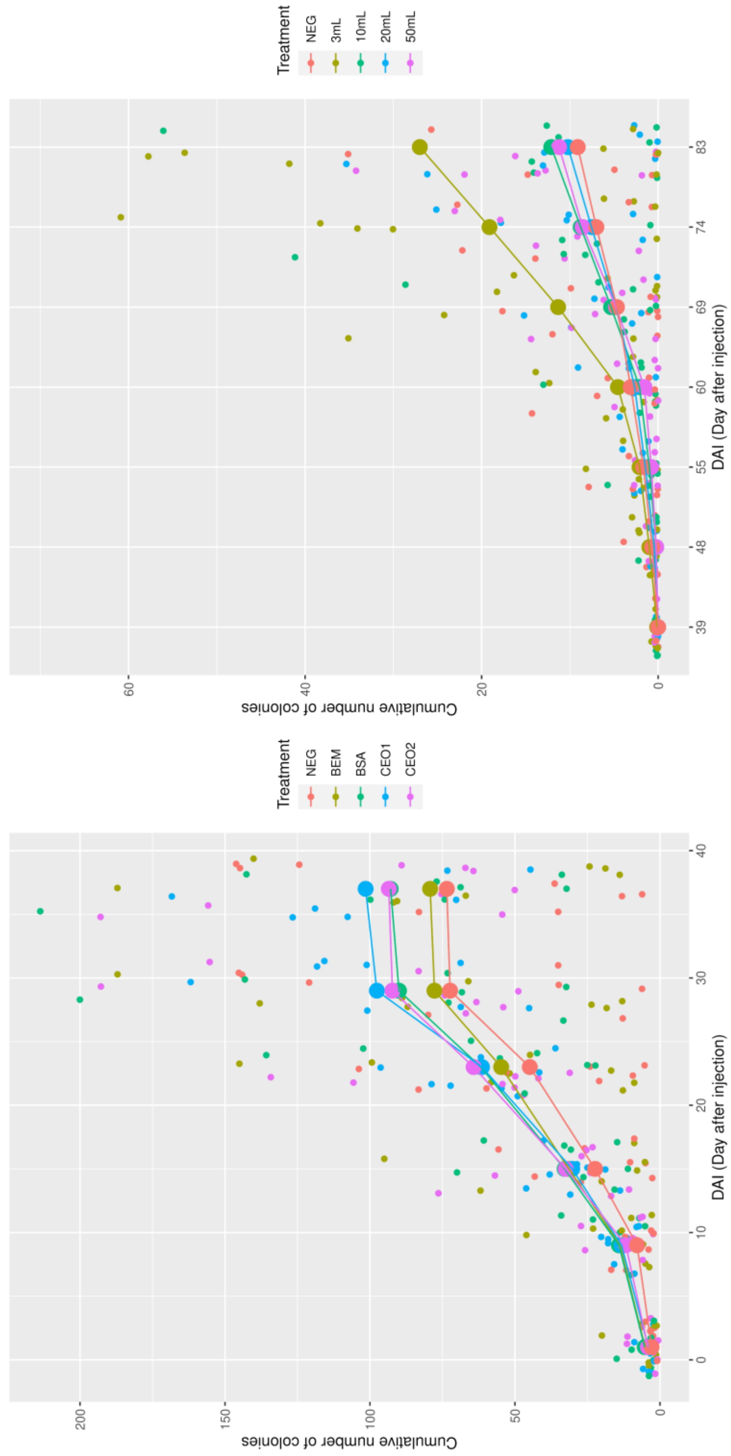


Figure 20. Cumulative number of aphid colonies according to the DAI (Day After Injection) for all the treatments and for both years (2020-left; 2021-right).

3.2.1.2 Predators population

The first year, 565 individuals were collected with the beating tray techniques. The main predators are the spiders (46%), followed by the bugs (28%), ladybugs (20%) and the earwigs (6%). The GLM analysis showed a significant effect of time on the mean number of predators ($F=20.46$; $df= 1$; $p<0.001$). Indeed, the number of predators increased from the third week (average of 1.9 predators) to the 6th week (average of 4.2 predators). No significant difference was found between the treatments ($F=1.22$, $df= 4$, $p= 0.300$) (Figure 21).

In 2021, a similar result was observed regarding the predators found (1307 harvested predators, 73% spiders, 12% earwings, 11% ladybirds, and 4% bugs) with a progressive increase in the number of predators during the season ($F=110.24$; $df= 1$; $p < 0.001$), and no significant difference between the treatments ($F=0.76$, $df= 4$, $p=0.550$) (Figure 21).

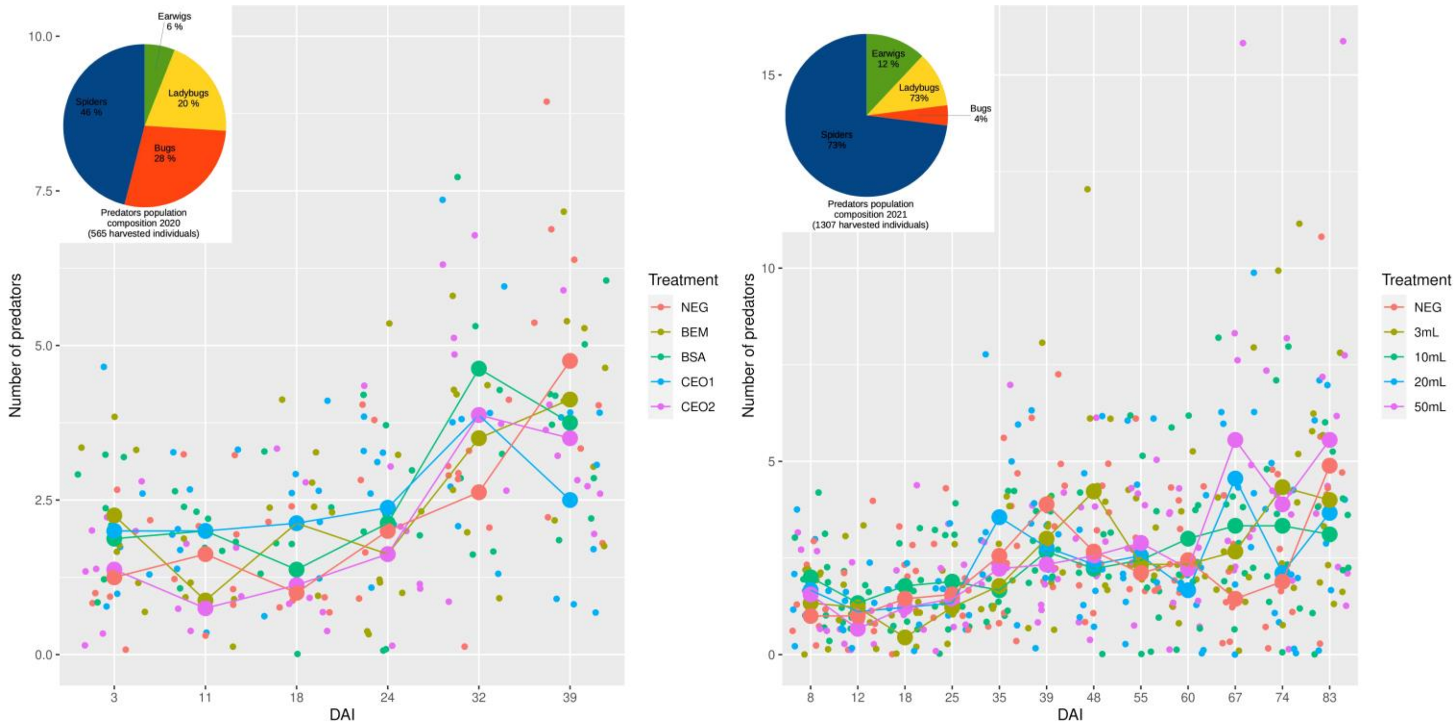


Figure 21. Number of harvested predators according to the DAI (Day After Injection) for all the treatments and for both years (2020-left; 2021-right).

3.2.1.3 Pests populations

In 2020, 169 pest individuals were harvested with the beating tray techniques. The different organisms identified were weevils (31%), psyllids (30%), caterpillars (27%), green apple aphid/*Aphis pomi* (8%) and Woolly aphids/*Eriosoma lanigerum* (4%). Significant results were found for the date of sampling ($F=9.46$; $df= 1$; $p<0.001$) with a decrease from the week 1 (average of 1.35 other pests' species) to the week 2 (average of 0.38 other pest species), and a stable value for the following weeks. No difference was observed between the treatments ($F=1.38$; $df= 4$; $p= 0.240$).

In 2021, for the other pests found on the trees (1147 harvested individuals, 74% caterpillars, 20% weevils and 5% psyllids) a similar impact of time than the previous year, was observed with around 4 individuals harvested per tree at the beginning of the experiment and less than 1 in average at the last sampling date ($F=55.07$; $df= 1$; $p < 0.001$). No impact of our treatments was observed ($F=0.28$, $df= 4$, $p=0.890$) (Figure 22).

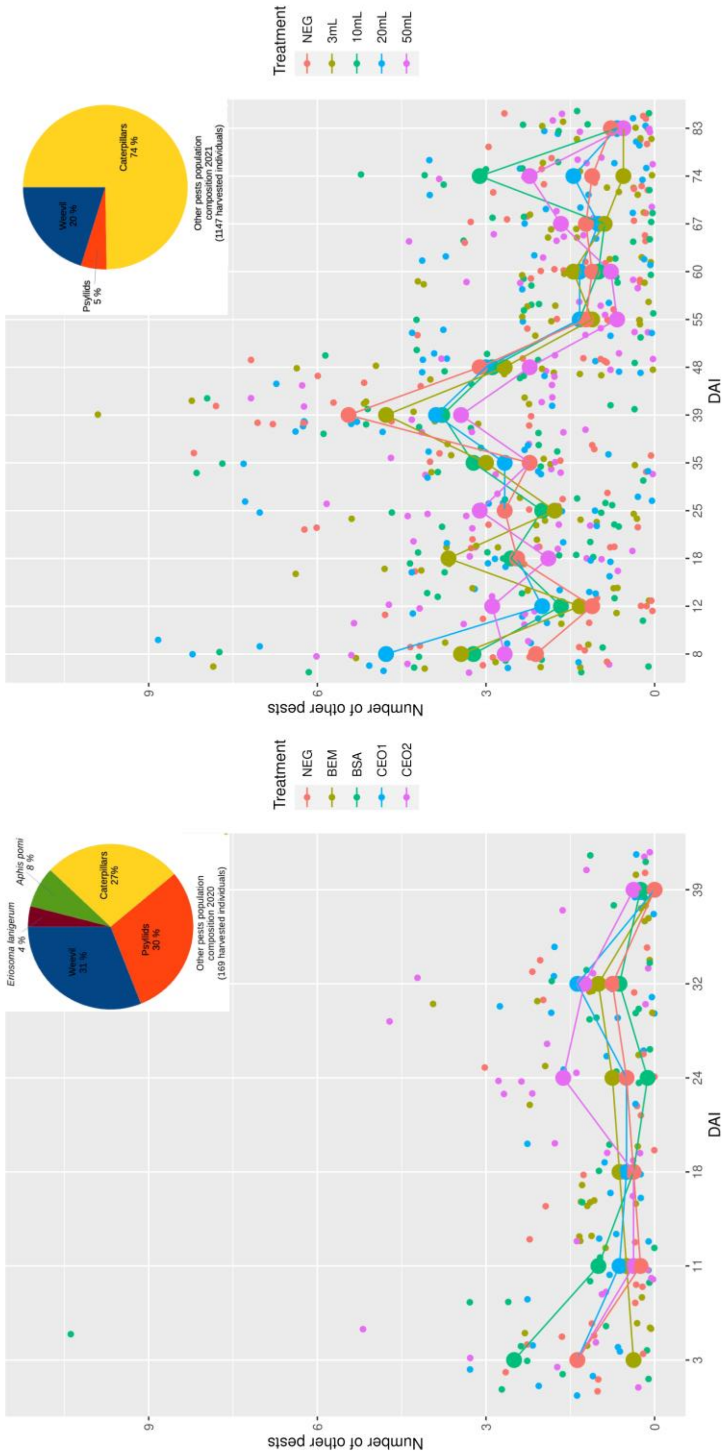


Figure 22. Number of harvested pests other than *Dysaphis plantaginea* according to the DAI (Day After Injection) for all the treatments and for both years (2020-left; 2021-right).

3.2.2 Volatile organic compounds (VOCs)

3.2.2.1 M. domestica VOC emissions

In 2020 samples were taken 3,7,14 and 28 days after injection (DAI) from the 22nd of May to 19th of June. Therefore, during the vegetative stage of trees. Fifty-five volatile species were detected in *M. domestica* emissions. Twenty-eight compounds were detected at a rate superior to 0.5 ng g^{-1} h^{-1} and were kept for the following analysis. Data can be found in Supplementary Table 8. They belonged to the alkanes, alcohols, aldehydes, esters ketone, carboxylic acid, sesquiterpenes and monoterpenoids family. Major compounds of the profile consisted in benzoic acid, 3-hexen-1-ol acetate, α-farnesene, DMNT, nonanal and caryophyllene. Two types of multivariate analysis were performed on the dataset, namely principal component analysis (PCA) to visualise potential impact of treatments or sampling date and permutational multivariate analysis of variance (permanova) to investigate significance of the impact. First 2 PC's plots representing 48.9% of dataset variability are displayed on Figure 23 upper left (treatment) and right (DAI) panels. Groups centroid and confidence ellipse clearly overlapped for treatment whereas they tend to separate for DAI. This analysis is strengthened by permanova results being non-significant for treatment (F=1.11, df= 4, p= 0.313) and highly significant for DAI (F=9.83, df= 3, p= 0.001). Pairwise analysis is displayed on Table 6. VOC emissions were not impacted by 2020 treatments and are highly dependent on the sampling date climatic conditions.

In 2021 samples were taken 21, 29 and 41 DAI from 22nd of April to 12th of May corresponding to the beginning (10% open flower), middle (50% open flower) and end (petal falling) of flowering. Thirty-nine compound contents were superior to 0.5 ng h^{-1} and were kept for the following analysis. Their summary can be found in Supplementary Table 9. They belonged to the alkenes, alcohols, aldehydes, alkanes, aromatic, carboxylic acid, esters, ketones, sesquiterpenes and monoterpenoids family of compounds. Major compounds consisted of benzyl alcohol, 3-hexen-1-ol (acetate), benzaldehyde and 1-hexanol. Similar PCA and permanova analysis was performed according to treatment and DAI. First 2 PC's plots representing 36.3% of dataset variability are displayed on Figure 23 lower left (treatment) and right (DAI) panels. Again confidence ellipses overlapped for treatment whereas they separate for DAI. This analysis is also strengthened by permanova results being non-significant for treatments (F=1.12, df=4, p= 0.300) and highly significant for DAI (F=13.29, df=2, p= 0.001). Pairwise analysis is displayed on Table 6. As for 2020, 2021 flower VOC emissions were not impacted treatments which is coherent with the absence of impact on insect population dynamics.

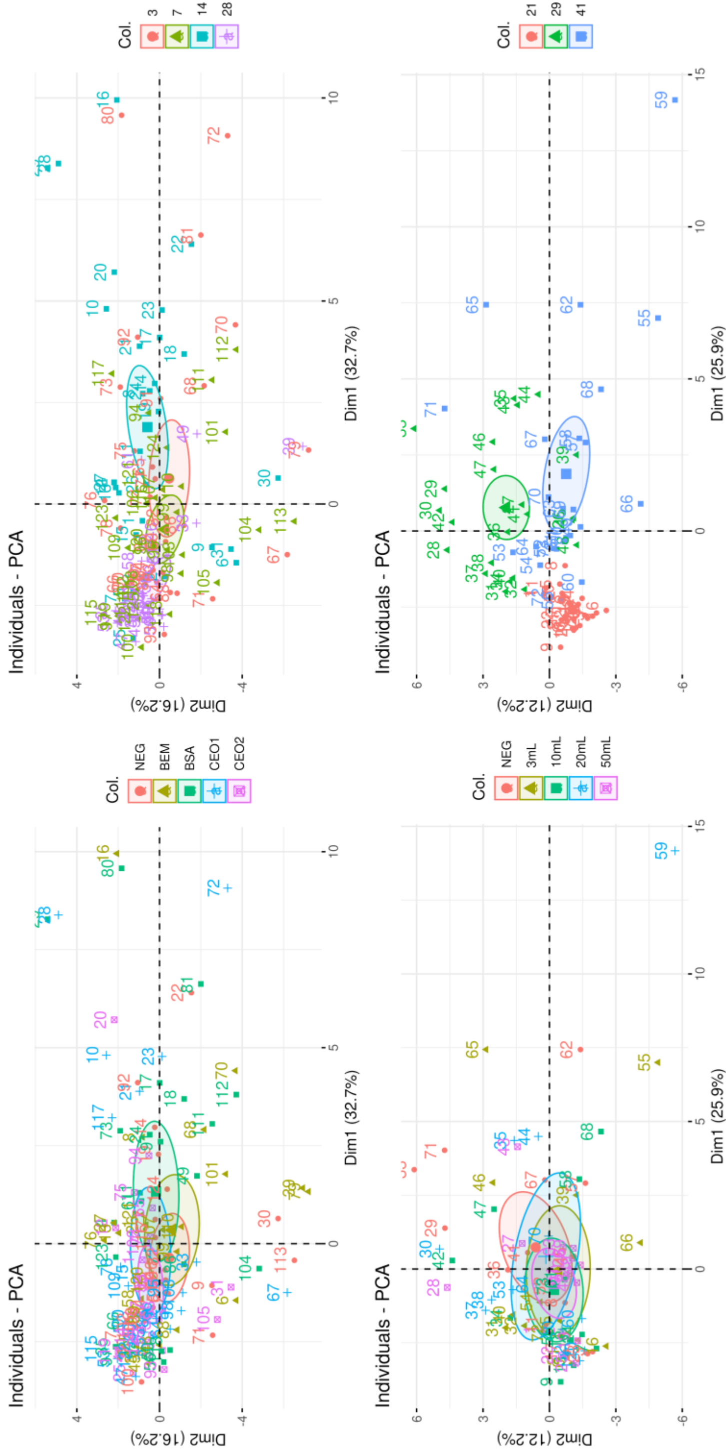


Figure 23. Principal component analysis (PCA) of VOC headspace emissions of *M. domestica* tree according to the DAI (Day After Injection) for all the treatments and for both years (2020-left;2021-right).

Table 6. Pairwise permanova of VOC headspace emissions for 2020 (left) and 2021 (right) trials.

2020				**2021**		
DAI	3	7	14	**DAI**	21	29
7	0.005**	-	-	**29**	0.002**	-
14	0.002**	0.002**	-	**41**	0.002**	0.034*
28	0.002**	0.002**	0.002**			

3.2.2.2 CEO residue analysis

Trans-cinnamaldehyde (TC) presents low toxicity properties to humans. However, residue in the fruit may have an allergic potential and may result in taste alteration due to its low odour threshold (50-750 ppb). Therefore, though treatments were applied early in the season we have investigated potential TC residue in fruit using SBSE-TDU-GC-MS methods. Linearity range was observed between 2.5 ng g^{-1} to 140.5 ng g^{-1} (R^2= 0.993). Limit of detection (LOD) and limit of quantification (LOQ) were respectively 0.80 and 2.24 ng g^{-1}. Recoveries were calculated by direct TDU liquid injections for 12.5 ng g^{-1} and 140.5 ng g^{-1} and were respectively 51.1±2.54% and 47.63±0.7%. No TC residue was detected in the 2020 samples. In 2021, TC was detected for three samples, two of them belonging to 10 mL and one to the 50 mL modality. However, content was below the LOQ established for the methods.

3.2.3 Evaluation of the treatment's ecophysiological impact

Chlorophyll fluorescence was measured throughout the all experiment in order to evaluate the potential impact of the treatment on tree physiology. Fv/Fm results are displayed in Figure 24 following day after injection (DAI). At every time and condition considered Fv/Fm was superior to 0.7 and no distinction seems to arise in between treatments. Interestingly it seems that values were more impacted depending on the day considered than on treatment.

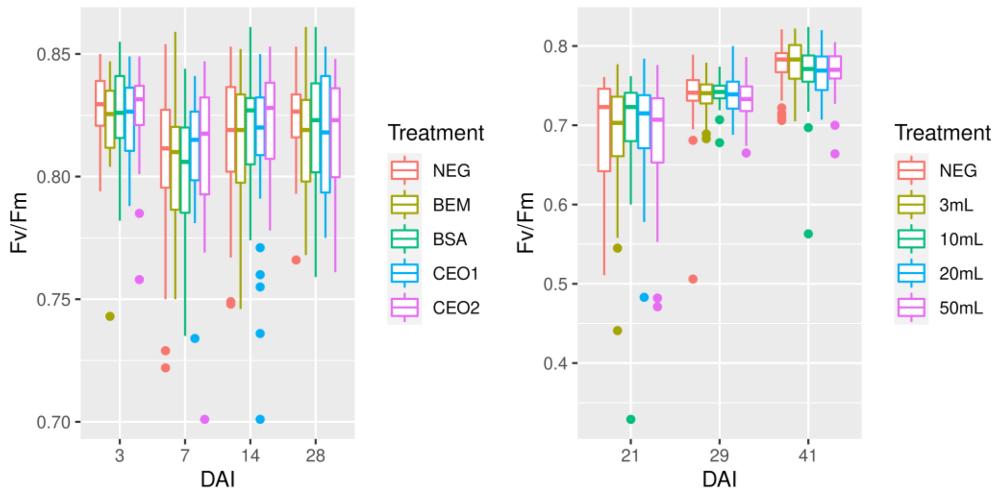


Figure 24. Maximum potential yield of photosystem II (Fv/Fm) according to the DAI (Day After Injection) following treatment in (2020-left; 2021-right).

GLM statistical analysis was performed to investigate the potential impact of day and treatment factors on the four parameters considered. The results are presented in Table 7. From those results it appears that the day has a significant impact on all parameters in 2020 and on two in 2021 (Fv/Fm and PI ABS). It also highlights that the treatment had a significant effect only on the performance index (PI ABS) during the 2021 trial. This index is a multiparametric parameter describing alterations within and between F0 and FM.

Table 7. Data summary (mean± standard deviation) by treatment and GLM analysis of 4 chlorophyll fluorescence parameters.

	Treatments	**Quantum efficiency of PSII (Fv/Fm)**	**Basal fluorescence (F0)**	**Performance index (PI ABS)**	**Electron transport flux (ET0/RC)**
2020 (n=40)	BEM	0.81±0.02	406.78±44.1	3.13±1.7	0.67±0.09
	BSA	0.82±0.02	394.99±66.8	3.47±1.8	0.67±0.08
	CEO1	0.82±0.02	402.5±61.16	3.2±1.87	0.67±0.08
	CEO2	0.82±0.02	405.14±46.8	3.42±1.6	0.67±0.07
	NEG	0.82±0.02	399.77±58.6	3.47±1.7	0.66±0.08
GLM	Treatments	0.324	0.334	0.212	0.794
	DAI	<0.001	<0.001	<0.001	<0.001
	Interactions	0.455	<0.05	0.431	0.29
2021 (n=45)	3 mL	0.74±0.06	432.87±52.9	0.97±0.5	0.55±0.09
	10 mL	0.73±0.05	438.69±56.3	0.93±0.4	0.54±0.08
	20 mL	0.73±0.06	425.17±65.7	1.06±0.6	0.55±0.07
	50 mL	0.73±0.06	423.31±52.0	0.92±0.4	0.55±0.08
	NEG	0.73±0.06	428.91±62.6	1.06±0.6	0.55±0.07
GLM	Treatments	0.752	0.195	<0.05	0.696
	DAI	<0.001	0.246	<0.001	0.378
	Interactions	0.858	0.865	0.348	0.813

Moreover, in 2021 clear signs of phytotoxicity appeared following treatments. Indeed, injections were performed at BBCH 54 at the beginning of budburst (compared to BBCH 69 in 2020). Visual signs of phytotoxicity appeared two weeks after injection for high volume modalities (20 mL and 50 mL). Due to their brown colour and absence of development some of the flowering buds were identified as chemically

"burned buds" (Supplementary Figure 5). The number of burned buds per modality after injection are represented on Figure 25. From this graph we can clearly deduce the phytotoxicity of high application rates with up to 144.55±42.43 burned buds. This is confirmed by GLM analysis that the treatment significantly impacts ($F=147.62$, $df=4$, $p<0.001$) as well as the date ($F=41.02$, $df=5$, $p<0.001$) and their interactions ($F=10.53$, $df=20$, $p<0.001$). Interestingly, after 40 days of injection, the number of burned buds progressively decreased to reach control level at the end of the experiment (DAI= 68) except for 50 mL modality. Finally, clear sign of wounding at the injection site has appeared at the end of the season as observed on Supplementary Figure 6 with different degree of bark-cracking.

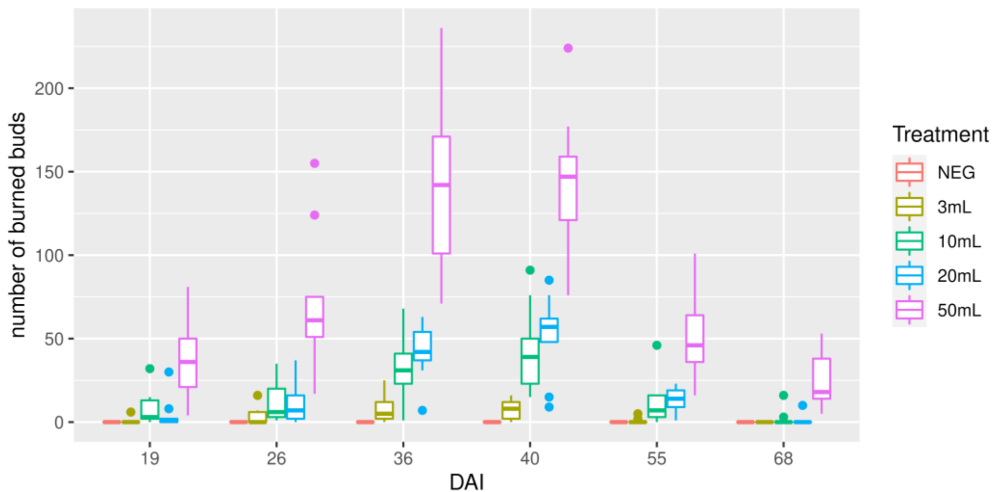


Figure 25. Number of burned buds according to the DAI (Day After Injection) following treatment in 2021 trials.

3.2.4 Analysis of tree growth and apple production

To estimate potential long-term impact on the tree physiology, growth of year shoot was considered. The total yield was estimated in terms of mass and number of apples. Finally, apples presenting *D. plantaginea* sign of attack were used to estimate commercial impact. Summary of these observations are displayed on Table 8. Regarding the year shoot growth, it was stable for the two year considered. More interestingly it was significantly reduced ($F=3.37$, $df=4$, $p=0.010$) from 29.1±17.69 cm to 22.54±18.31 cm for 50 mL and 22.96±16.22 cm for 20 mL injection in 2021 illustrating the long-term adverse impact of phytotoxicity previously highlighted. In terms of yields (total and commercial) it appears that the treatment did not impact them (nor positively or negatively) for both years. However, all modalities in 2021 present a drastically reduced total yield of less than 1 kg compared to 14 kg the previous year due to adverse climatic conditions.

Table 8. Data summary (mean± standard deviation) by treatment and ANOVA analysis of tree growth and apple production.

	Treat-ments	**Year shoot growth (cm)**	**Total apple yield (kg)**	**Total apple num-ber**	**% attacked by *D. plantaginea* (kg)**	**% attacked by *D. plantaginea* (num)**
2020 (n=8)	BEM	28.71±19.83	14.83±7.93	112.88±74.07	21.67±16.85	32.33±24.05
	BSA	28.9±17.35	15.08±5.66	107.5±32.5	17.98±12.51	30.15±12.75
	CEO1	27.75±22.59	14.89±6.67	110.75±54.72	28.39±18.45	42.21±19.82
	CEO2	27.65±16.51	12.34±5.64	86.38±37.46	19.54±9.77	36.86±19.1
	NEG	29.27±17.13	12.68±7.69	85.88±48.4	20.42±17.32	31.88±21.65
2021 (n=9)	3 mL	30.52±17.75	0.77±0.65	8.57±10.37	29.66±35.37	34.06±34.67
	10 mL	28.52±15.41	0.4±0.22	4.29±1.89	16.14±14.76	31.19±24.24
	20 mL	22.96±16.22	0.43±0.32	3.57±2.51	19.56±36.01	22.86±35.46
	50 mL	22.54±18.31[a]	0.48±0.28	4.38±3.29	14.68±20.78	22.14±28.4
	NEG	29.15±17.69	0.7±0.89	6.5±7.53	7.5±8.29	19.84±26.67

4. Discussion

The results gathered in a control laboratory experiment on small trees determine the potential of such injections of CEO to control aphid's population without presenting a drastic impact on plant physiology. However, scaling up to agronomic apple orchards furnishes much more ambiguous results that will be discussed. Indeed, the results obtained in the trial experiments suggest that our treatments have a moderate impact on aphid population dynamics. In 2020, the results showed a higher peak of aphids in the control treatment without injection compared to the other conditions in our experiment. This decrease in the aphid population dynamic may have two non-exclusive origins: 1) the essential oil of *Cinnamomum cassia* used in this study is known for its antifeedant and insecticidal effect against insect pests (Huang & Ho, 1998; Kim et al., 2003; Lee et al., 2008) caused by its major compound, *trans*-cinnamaldehyde (Park et al., 2017). Therefore, its presence in the vascular system of the trees may cause its emission at the leaf level and/or its ingestion by aphids, and thereby causing their death. However, Werrie et al. (2021), who also injected CEO into apple trees, showed that *trans*-cinnamaldehyde was not found in the emissions of the injected apple trees. Moreover, the fact that there was an aphid population decrease not only in the EO treatments but also in the control conditions with injection (BEM and BSA) indicates that the ingestion of *trans*-cinnamaldehyde did not cause the death of the aphids. 2) The second possibility for explaining this phenomenon is that the injections themselves with or without EO triggered physiological responses from the trees (Perina et al., 2019). This would mean that the treatment indeed has an influence on aphids' development but rather in terms of injection-linked stress' emissions of repellent VOCs than the presence of cinnamon compound. Concerning the 2021 trial, the meteorological conditions jeopardised the entomological survey, particularly concerning the aphid populations, with six times fewer individuals harvested during this trial than in the previous one. However, the results obtained during this experiment showed a higher number of aphids in the condition with the lowest volume of CEO emulsion injected (3 mL), while higher doses presented smaller aphid colonies. This result indicates that a toxic effect could occur. However, it is difficult to draw conclusions as the aphids also developed less in the control conditions without injection.

Although the results of our study have shown the impact of our treatment on the populations of *D. plantaginea*, we failed to manage them. The main reason for this is that the CEO emulsion we injected was not homogeneously distributed in the crown of the tree (Doccola et al., 2007; Percival & Boyle, 2005). The non-uniformity of the distribution of the chemical in the tree, led to oversupply or undersupply of the product inside the canopy or trunk (Aćimović, 2014). Indeed, the fact that some aphid colonies developed well in our strongest treatment (50 mL), despite a high number of burned buds, shows that some branches were given a high dose of emulsion while others were not. Some colonies may have then developed on branches that received

little or no emulsion. Injection timing is a paramount factor in achieving active concentration content before pest outbreaks. Therefore, optimisation considering *D. plantaginea*'s life cycle may allow a better control of its population. Other main parameters to assess include the compounds' concentration and injection. The amount of active substance (a. s.) to inject is conventionally based on trunk diameter. A recent experiment on 'Red Delicious' apple trees presenting a comparable diameter (17.8–20.2 cm) reported a range of a. s. from 0.04 to 0.8 g a. s. per tree for emamectin benzoate and from 0.1 to 0.2 g for imidacloprid (Coslor, 2019b).

We did not observe any impact of our treatment on predator populations. Indeed, during the two years of treatment, we observed that pest populations decreased throughout the season, while predator populations increased. Therefore, it seems that our treatment does not impact natural biological control in the orchard. The same conclusion was reached in a study on the cotton mealy bug *Phenacoccus solenopsis* (Ghada & Naglaa, 2020), where the CEO was used to control the pest while its predator *Chrysoperla carnea* was not impacted.

Volatile organic compound (VOC) emissions are of paramount importance, as they can impact aphid behaviour and trophic interactions. However, multiple factors can alter those emissions, such as seasonal, meteorological (Vallat et al., 2005), physiological (Zeng et al., 2017) and, phenological factors (Casado et al., 2006), as well as interactions with fungi (Souleyre et al., 2019) and herbivores (Suckling et al., 2012). Herbivore-induced plant volatiles (HIPV) play a key role in indirect defence responses, such as predator and parasitoid attraction to attacked plants (Turlings & Erb, 2018). Headspace emissions were consistent with previously cited study, and among the detected compounds, many have an acknowledged biological interest such as methyl salicylate, 3-hexen-1-ol, (*E*)-4,8-dimethylnona-1,3,7-triene (DMNT), δ-3-carene, linalool, α-bergamotene and β-bourbonene. Some of the identified compounds have also been shown to be produced in direct defence response to *D. plantaginea*, such as α-farnesene, caryophyllene and germacrene-D (Warneys et al., 2018). The absence of modification in a single compound and the total profile of VOC emissions triggered by this curative treatment is consistent with the previously discussed entomological results. In contrast, seasonal variation is highly significant, indicating that environmental and physiological/phenological parameters impact the tree more than the treatment. In 2021, VOC emissions increased due to flower blooming. Less information has been published on the topic; however, profiles are similar to the literature (Cellini et al., 2019; Rachersberger et al., 2019). Interestingly, terpenoids, alcohols and esters, such as 3-Hexen-1-ol, benzyl alcohol, 3-hexen-1-ol acetate, β-ocimene, DMNT and linalool were the main constituents. Emission profiles were not impacted by treatment regardless of the quantity and were highly dependent on the flowering stage. These results confirm the absence of impact of our treatment on chemical ecology, predator populations and probably on pollinators and parasitoids populations.

Regarding tree physiological impact, the analysis of chlorophyll fluorescence alone would suggest the absence of impact for injection during the vegetative stage and the limited impact for preventive treatment in 2021 trials, as only the performance index (PI) decreased at a high injection volume. However, the empirical observations highlighted a bias in this measure. Indeed, a higher volume of injections leads to the absence of bud development and only the less impacted buds were measured. Therefore, preventive treatment to prevent colony establishment may not be optimal in terms of tree physiology. Indeed, xylem sap flow is limited at bud break (Dragoni et al., 2005). Short-term phytotoxicity seems to depend on the physiological stage of the tree. Moreover, the growth alteration of young shoots at 20 mL and 50 mL suggests long-term effects.

During our two field trials, we did not observe any effect of our treatments on apple production, either in the proportion of fruit attacked by *D. plantaginea* or in the quantity of harvested apples. These results are in line with the lack of significant effect of our treatments on *D. plantaginea* and the other pest populations. Our treatments did not have a sufficient impact on pest populations and thus did not affect apple production. In our second field trial, we harvested very few apples (less than 1 kg per tree, on average). This can be explained by the poor weather conditions during the flowering period in 2021 (colder and windier than in the previous trial) (Supplementary Table 7), which did not favour the activity of pollinating insects. Concerning the residues of TC contained in the fruit, this molecule presented low toxicity properties to humans (Zhang et al., 2019), but it may have an allergic potential and may result in taste alteration due to its low odour threshold (50-750 ppb). Although treatments were early in the season, we investigated the potential TC residue in the fruit. The residues recovered in apple fruits were lower than the limit of quantification established for the methods (2.24 ng g^{-1}). The very low amount of residues presents in the apples following our treatment would not affect their taste. However, because of the low presence of TC in the fruits, the method presented in this paper with an early season injection, cannot be used to control fruit pests, such as *Cydia pomonella* larvae; later injections could therefore be considered to increase the level of TC in the fruit and manage this type of pest.

One of the main concerns regarding trunk injection is the wounding of the tree's roots, trunks or other limbs that can impact the tree's health or longevity and fertility and the distribution heterogeneity (Aćimović et al., 2014; Doccola et al., 2012). The lack of experience or the need for enhancing ingestion by the pest and improving the material distribution and longevity into the crop should also to be considered (Aćimović, 2014; Wise et al., 2014). Phytotoxic reactions are also feared in case of product misuse or incorrect dosage (Wise et al., 2014). It has also been reported that current technology is unable to provide slow, continuous (controlled) release of the compound over time. (Aćimović et al., 2014) showed that increasing the number of trunk injection ports allows a homogeneous distribution of injected imidacloprid in

the apple tree canopy. Injecting the CEO emulsion through 4 injection ports instead of 1 or 2 would allow a more homogeneous distribution within the tree and therefore would probably have a greater impact on aphid populations with lower stress for the trees. Developing and testing new methodologies of injection of EO emulsions could be an interesting next step in this study. A final concern is the extra time and effort required for effective treatment using trunk injection: equipment preparation, trunk diameter measurements, rate calculations, ect that are basically "single tree monitoring", which renders the technique less appealing. The feasibility of integrating it into current fruit production systems and convincing farmers of the economic viability of the system is also a great challenge (Wise et al., 2014).

In conclusion, this study synthesises two-years of preliminary trials aiming to develop biopesticides based on trunk injection of EO emulsions. The first year showed that a CEO treatment by trunk injection could impact aphid population dynamics, while in the second year, when the weather conditions were worse for the insect populations, we were able to assess the stress caused by our treatment and thus determine the amount of emulsion not to exceed 10 mL, to not cause damage to the plant and still manage the pest populations. CEO presents strong antifungal and bactericidal properties therefore potential impacts on other orchards, such as fire blight (*Erwinia amylovora*), apple scab (*Venturia inaequalis*) or oidium (*Podosphaera leucotricha*), may strengthen interest in its techniques. In the same way, the potential activation of plant systemic defence could be a suitable strategy for previously cited pests. Moreover, only one EO was investigated in this trial. Therefore, screening of other EOs or their blends (for synergism) presenting desirable fungicidal and/or insecticidal properties is an attractive perspective to consider for future field trials and to control other pest pathogens. Botanical pesticides, such as CEO, have shown promising results concerning crop pest management but are not directly comparable to synthetic pesticides (Miresmailli & Isman, 2014). This study identified practical challenges and limitations that need to be addressed.

6

General discussion, conclusion and perspectives

The general aim of the present thesis was to formulate and potentially inject a botanical insecticide based on EOs to control the RAA population in apple orchards. Considering the results presented in the previous chapters, different aspects will be considered in the present discussion. First, the physiological response of apple trees was considered to meet this challenge. Second, the mechanisms governing the biological activities observed in the systemic translocation of EO major compounds and the parameters governing these mechanisms are examined. Third, aspects regarding the mode of action of the target insect pest (RAA) are hypothesised. Finally, the relevance of such treatments and modes of application in apple orchard production systems are discussed.

1. General consideration of cinnamon EO phytotoxicity

Following the literature review the primary research hypothesis was to determine how long after and at which concentration of CEO foliar application oxidative stress is observed. Indeed, some studies have already shown the herbicidal activity of CEO (Lins et al., 2019; Muñoz et al., 2020; Saad et al., 2019). Oxidative stress occurs when ROS over-accumulate, changing the redox status of the cells. The antioxidant system of plants is complex and composed of plenty of (non)-enzymatic molecules operating in interconnected pathways (Noctor et al., 1998). ROS are considered hallmark generic biomarkers in phytotoxicity studies related to abiotic stress and EO application. To assess potential phytotoxicity, the efficiency of the apple tree redox system against potential oxidative stress was assessed through the glutathione ratio and oxidative stress-related gene transcript analysis. Drastic changes following application at 2% CEO explained why plant physiological status has been considered in each step of this thesis.

However, it must be stressed that the molecules investigated here (even if they are pertinent) are not sufficient to assess the whole plant's physiological response. No universal oxidative stress indicator exists. Therefore, to evaluate it correctly, other relevant factors must be considered, such as ROS, antioxidants, metabolite markers, protein oxidation and modifications at a transcript level. Each measurement providing specific useful information, and each approach has limitations (Noctor et al., 2016). ROS accumulation, and probably EO treatment is not uniform across the cell (Noctor et al., 2016). Therefore, ROS detection by histochemical staining techniques, such as superoxide anions with nitroblue tetrazolium (NBT), hydrogen peroxide by diaminobenzidine tetrahydrochloride (DAB) staining or total ROS with 2',7'-dichlorofluorescein (H_2DCF), could be considered (Jambunathan, 2010). *In vivo* observation of glutathione in different cell organelles by fluorescence imaging using the same bimane probes could be investigated as a non-destructive marker of oxidative stress (Majer et al., 2016).

When the plant is subject to the slow development of oxidative stress, its antioxidant content increases with the intensity of the stress and the plant acclimates (Šircelj et al., 2005). When the antioxidant plant defence cannot manage stress, oxidative damage appears. The second objective was to detect the extent of the cellular damage that occurs because of oxidative stress. In the framework of this study, photosynthetic pigment and malondialdehyde were considered. Instead of MDA to assess membrane lipid peroxidation, other reactive carbonyl species derived from lipid peroxidation could be quantified, such as like n-hexanal, 4-hydroxy-2-nonenal and 4- hydroxyl-2-hexenal (Esterbauer et al., 1991; Shulaev & Oliver, 2006). The previously described mechanisms are quick and highly dynamic; therefore, in addition to considering dose-dependent activity, the time range must also be considered. Although larger considerations should be made regarding this oxidative approach, it has been shown to be suitable to help identify dose-dependent phytotoxicity of CEO. Indeed, phytotoxicity seems to appear after exceeding a certain concentration threshold, whose determination may help to identify its suitable use as a botanical insecticide. However, although they are largely recognised as reliable markers, other markers can be considered, such as chlorophyll fluorescence and VOCs emission.

Indeed, chlorophyll fluorescence is commonly measured in plant stress studies. It is a simple and quick way to observe abiotic stress. Indeed, a decrease in PSII quantum efficiency (Fv/Fm) is considered an oxidative stress sign and is therefore considered in practical trunk injection applications. Apple trees being perennial crops treatment should not lead to long-term phytotoxicity. Long-term studies of photosynthetic efficiency and damage to photosystems have reported that stressed plants either modify their metabolic pathways, reducing Fv/Fm, or that ROS directly degrade their photosystem by interrupting the electron transport chain (Schöttler & Tóth, 2014). In our case, no significant differences between injected and non-injected trees were highlighted for chlorophyll fluorescence parameters, except in the high ratio of a. s. in trials. This suggests the potential of this non-invasive technique to monitor phytotoxicity occurring by EO application (Pouresmaeil et al., 2020; Synowiec et al., 2019). However, the limitations of these results may also come from spatial heterogeneity. Therefore, methodologies allowing larger and precise quantification, such as pulse-amplitude-modulated imaging chlorophyll fluorometers is an interesting approach (Gog et al., 2005).

Untargeted VOC emissions were also considered to highlight physiological modifications. A large array of stress factors is known to affect the emission of BVOCs (Possell & Loreto, 2013). In this regard, the modification of different terpenoids and green leaf volatiles observed in a controlled environment implies the larger impact of EOs on apple physiology. However, the origins of these biogenic VOCs may result from other environmental factors and/or from the micro-organism of the phyllosphere. Indeed, similar results were not observed in field trials. Multiple factors are also able to alter these emissions, such as seasonal, meteorological (Vallat et al.,

2005) and phenological factors (Casado et al., 2006) and, biotic interactions (Souleyre et al., 2019; Suckling et al., 2012). It can therefore be concluded that such a methodology is not appropriate to consider physiological modifications in orchards.

The previously discussed mechanisms do not allow for the identification of the primary molecular target of *trans*-cinnamaldehyde in plant cells. Previous works suggest an interaction with specific sites of the plant plasma membrane (Lins et al., 2019), endogenous H_2S and Ca^{2+} regulation (Cheng et al., 2021). Transient receptor potential A1 (TRPA1) targeting could regulate Ca^{2+} as observed in mammals, but regulation could also be performed by H_2S operating downstream of cinnamaldehyde through a linear signalling pathway (Xue et al., 2016). It is pertinent to investigate these fundamental mechanisms in non-model plant species, such as apple.

As they are involved in the signal transduction pathway, H_2O_2, GSH and MDA play a role in systemic defence induction and act as regulators of gene expression (Davey et al., 2003; Noctor et al., 2012; Stone & Yang, 2006; Velikova et al., 2000). The results regarding the induction of the defence pathway highlighted an increased level similar to the SAR-elicitor, specifically in phenylalanine ammonia-lyase, PR-proteins and pectin methyl esterase. However, this unbiased molecular biology approach is hard to implement in non-model plants. The main inconvenience of the direct induction of plant defences is the great resource cost resulting from its activation, potentially leading to yield decline. The priming phenomenon, which allows stronger and/or faster defence activation only when stress occurs could also be investigated using these type of secondary metabolites.

A final perspective concerns phytohormone measurement. Indeed, it may be useful to further investigate systemic defence induction and regulation. The GSH redox state influences the cross talk of JA/SA and the content and redox state of GSH may modulate JA-associated genes (Frendo et al., 2013). Therefore, as phytohormones take part in the establishment of plant defence mechanisms and interact with antioxidants, they should also be investigated.

In conclusion, this work has considered multiple aspects of physiological impact, allowing us to gain insight into potential EO applicability and humbly suggests new research perspectives. This work has been performed on non-model woody species, for which very little data are available regarding phytotoxicity phenomena and thresholds. Therefore, it addresses this gap and contributes to botanical insecticide development in perennial ligneous species.

2. Systemicity of trunk injection methods

The purpose of this project was to move forward with the development of a trunk-injected biopesticide using EOs as active substances (a.s.).

One of the objectives of this work was to demonstrate the systemicity of EO in injected trees by first analysing the kinetics of the EOs specific compounds contained and emitted by leaves. This was made possible due to the development of green and headspace analytical methods that were sensitive enough to allow the quantification of EO major components through (DHS)-TDU-GC-MS. Regarding the content, rigorous analytical validation of the method should be performed to consider the matrix effect and source of variation to allow exact quantification and to decrease the linearity range as much as possible to help in the quantification of components found at very low concentrations. DHS allows a tailored method for any type of vegetable matrix and for the specific physico-chemical properties of targeted analytes (Ko/w, vapour pressure, ect.) (Liberto et al., 2020). However, other methods with increased recoveries developed for apple, such as SBSE-TDU-GC-MS, could increase detection performance in other tissues and, of course, so will the use of more sensitive apparatus such as triple quadrupole mass spectrometer or time-of-flight mass analysers. Increased sensitivity will allow to better characterise spatial and temporal distribution within trees after injection.

Great differences in both emission and storage patterns in the leaves were observed. The results indicated that *trans*-cinnamaldehyde accumulated in the leaf over time, but it was not emitted by cinnamon oil-treated trees. However, carvone content increased and was emitted at a constant rate in the spearmint-treated trees. Molecules presenting higher lipophilicity can adsorb to the lignin of the xylem wall and cell plasma membrane (Berger & Laurent, 2019). However, many other parameters control uptake and translocation, such as factors related to the tree (anatomy of the vascular system and leaves) and weather conditions (vapour pressure) (Berger & Laurent, 2019). This work first focused only on the leaves. Active constituent residues in apple were later considered in field trials. However, it could be interesting to extend this study to the other parts of the tree (e.g., roots and trunk).

Pharmacokinetic study considering absorption distribution, metabolism and expression could explain previously developed impacts on plants. Modification of the kinetic properties through appropriate formulation to control the release of a. s. could mitigate phytotoxicity and improve bio-activity. Indeed, through the presented work, stable and non-phytotoxic emulsions based on an EO emulsified with Tween 80 (and EDTA) have been developed, successfully injected and taken up by apple trees using a relatively simple and affordable device. However, although this emulsion is biocompatible, an effort to create a bio-based emulsion should be considered. Indeed, bio-based substitutes for both EDTA and Tween 80 can be found. As an alternative

to Tween 80, sucrose esters that are completely biodegradable and possess much lower critical micelle concentrations should be considered (Polat & Linhardt, 2001). A bio-based chelator should also be considered to replace EDTA in its role to chelate Ca^{2+} ions and reduce the occlusion of sieve plate pores. Addition of metal chelator was performed following preliminary test with methylene blue dyes (data not show) and whose role was suggested by (Wong & Coats, 2018). Several groups have used EDTA for sampling *Arabidopsis* Phloem Exudate and observed no adverse effect on cell ultrastructure for EDTA concentrations from 10 mM to 20 mM (selected concentration) (Tetyuk, 2013). Direct agro-infiltration in apple leaf however suggest phytotoxicity potential reinforcing the need for adequate formulation as biopesticide for injection (Belhassen, 2021). Other encapsulation methods may also prove suitable to improve the formulation of the selected a. s. (Maes et al., 2019).

A final aspect to consider regarding this systemic translocation is the precise localisation within the tissue. Indeed, to better understand the physiological impact and the expected insecticidal effect, both temporal and spatial aspects must be investigated. Deuterated compounds that are radiolabelled or label free could be investigated using methods such as Raman spectroscopy histological analysis.

It can therefore be said that this work was a real steppingstone in the “Tree-injection” project and proved that EO-based pesticides could be injected into trees, an emerging concept to the authors’ knowledge.

3. Biocide properties of cinnamon essential oil

RAA population dynamics were greatly impacted in laboratory trials, whereas EO injection failed to control the population in field trials. Therefore, it may be relevant to characterise the potential of the CEO applied by trunk injection by investigating its mode of action resulting either by ingestion or repulsion occurring following VOCs exposition.

Antifeeding and insecticidal properties against insect pests caused by cinnamon’s major compound, *trans*-cinnamaldehyde, have already been acknowledged (Huang & Ho, 1998; Kim et al., 2003; Lee et al., 2008). As indicated by EO systemicity, the impact of fumigation and ingestion should be considered in this model.

Indeed, the *trans*-cinnamaldehyde found in the leaves could be interesting against insects by ingestion toxicity. Aphids, by piercing the leaf to reach the phloem, often pass through the xylem and would hence be in contact with the oil droplets. However, it is unclear whether the small amounts of CEO emulsion injected are sufficient to cause their death due to the ingestion of toxic compounds in the xylem. Indeed, the concentration measured in the leaves was below $\mu g\ g^{-1}$. In contact toxicity, it has been

reported that *C. cassia* possesses a lethal dose 50 equivalent to 17.41 µg cm^{-2} on the aphid *Myzus persicae* (Ikbal & Pavela, 2019).

To clarify this mode of action a fitness study considering feeding behaviour modification by electropenetrography (EPG) and life history traits (performance) was recently performed in the frame of the Tree-Injection project by a laboratory specialised in the technique (UMR CNRS EDYSAN (Écologie et Dynamique des Systèmes Anthropisés) of Jules Verne University of Picardie. This implies that the injection of an EO emulsion into trees can impact hemipteran host-plant colonisation, as for both species (*Cacopsylla pyri* and *Dysaphis plantaginea*), a modification of their preference and of their performance was observed. EO injection altered the feeding behaviour of *Dysaphis plantaginea*, as a significantly lower proportion of aphids ingested phloem sap on injected trees. Concerning stylet probing, the injection of CEO emulsions shortened their duration and increased their latency time, while their number remained the same regardless of the modality. Short probing duration indicates the presence of negative factors in the epidermis and/or mesophyll, which cause the stylets to withdraw (Crompton & Ode, 2010; Dancewicz et al., 2016; Kordan et al., 2012; Marchetti et al., 2009; Slesak et al., 2001). The longer latency time seemed to indicate that the aphids were reluctant to probe the leaf. The fact that the aphids ingested more xylem from the injected trees, combined with the toxic effects observed, would therefore reinforce the idea that the mortality observed in our experiments was not due to the presence of the CEO itself, but rather to a reaction of the latter.

Indeed, plant defence elicitation by synthetic SA analogue Bion was efficient in controlling RAA and modifying apple tree VOC emissions (Warneys et al., 2018). In the present thesis, a clear activation similar to Bion was observed by the CEO. Interestingly, the phenylpropanoid pathway was specifically increased by CEO application. Metabolites from this pathway, hydroxycinnamic acids, particularly 4-caffeoylquinic acid (4-CQA) and 4-p-coumaroylquinic acid (4-pCoQA), were identified as the major players in RAA-resistant apple cultivars (Berrueta et al., 2018). Secondary metabolites accumulation leads to similar effects on RAA feeding behaviour (increased latency time). Therefore, the studies on the activation of the plant defence mechanism by the CEO, as well as evaluation of the timing of this effect and specific secondary metabolites production, such as VOCs, hydroxycinnamic acids or dibenzofurans could clarify the physiological impact leading to RAA mortality.

Moreover, control of the pathogen considered in this thesis, direct biocidal properties of EO and indirect elicitor properties could impact other fungi and bacterial pests, such as fire blight and apple scab.

In this regard, large screening of EOs presenting biocide or elicitor properties could be considered as well as synergism potentially occurring in their blend (Bedini et al., 2016).

4. Relevance of EO and trunk injection methods to manage rosy apple aphids in orchards

Replaced in the global SPW "Tree-Injection" project, the objectives were to evaluate the feasibility of CEO trunk injection into apple trees to control the RAA population in orchards. These experiments allowed us to ease some concerns and highlight perspectives for similar trials. Although their potential was established in the laboratory, much work is still needed to improve trunk injection performance in field applications.

A first aspect concerns the timing of injection to match pest occurrence. Finding the exact moment of application between preventive measures or once the adult founders have colonised the leaves (curative measure) is critical. Indeed, synchronisation between bud burst and egg hatching determine the success of RAA infestation, and economic damage is produced by founders (Miñarro & Dapena, 2007). Both aspects are considered in this work. However, previous research revealed that movement in the trunk after injection before bud burst was limited in apples (Clifford et al., 1987). Therefore, an optimum between those considerations may be hard to reach for the RAA-apple interaction.

It is important to determine the number of applications for guaranteed and prolonged efficiency to consider this method as a viable alternative. In this regard, the distribution heterogeneity observed for trunk injections is a major drawback that has been acknowledged on multiple occasions. An identical phenomenon has been observed in this work and constitutes a major obstacle to efficiency due to the mobility of the aphid. Furthermore, oversupply for some branches has led to phytotoxicity. A compromise concerning the injected dose must also be found to ensure that it is not too high and that the product acts well.

Due to its invasive nature, damage occurs to the plant vessels. This damage could lead to poorer sap circulation and, in the long-term, could impact plant growth and production yield. Recent analysis of wound evolution in apple at cellular and tissue levels showed that, after three seasons, bark and wood had recovered their normal structure and xylem had recovered sap transport capacity (Berger, 2019). Moreover, innovations using small ports or alternatives to drilling have been developed (Aćimović et al., 2016; Montecchio, 2013).

The previous remarks are relevant regardless of the production system considered. Nevertheless, this technology should be of economic interest to fruit producers. Both the cost of production and the cost of implementation should be investigated. Previous estimation in different pathosystems regarding economic costs results in an increase (Berger, 2019) or decrease in protection costs compared to spraying (Li et al.,

2021). Currently, it requires 2 to 5 min per tree to deliver crop protection materials. Economic viability in conventional agriculture may depend on reducing the time and labour required to cover the acres of crops (Wheeler et al., 2020). However, a broader impact is expected for smallholder agriculture (Vandervoort, 2014). The technique requiring no heavy mechanisation is hardly accessible in developing countries.

From a wider point of view, the profitability of such techniques will also depend on its potential compatibility in integrated pest management (IPM) program for improved regulation of this pest. In this regard preliminary data from field trial regarding impact on pest and predators are favourable.

Another aspect that could increase technique attractiveness is the multi-use of the product. Indeed, the alternative investigated in this work would solely replace 1 to 3 of the current 30 to 40 treatments per year. Economic interest may therefore be greater in multi-use conditions. Finally, such application methods have been investigated for other biological pest control agents, such as endophytic bacteria (Bahadou et al., 2017; Berger et al., 2015; Rabiey et al., 2019) and RNA interference (Dalakouras et al., 2018). Investigation of botanical insecticides or biopesticides applied with a precise mode of application, limiting drift potential and considering environmental interactions to limit systematic application, could integrate perfectly with biological or agroecological modes of production.

As part of the Tree-Injection project, this thesis led to major findings. Following CEO application on apple trees, oxidative stress seems to be managed by the plant and enables systemic defence induction. Furthermore, after injection into cambium, *trans*-cinnamaldehyde diffuses to the leaf and can restrict the development of the targeted pest (RAA).

Overall, the use of cinnamon EO as a botanical insecticide combined with a trunk injection method seems a very promising alternative to conventional plant protection products to treat RAA and could be part of a larger IPM programme. Finally, preliminary field trials highlighted that further research is still necessary.

The main hypothesis resulting from this work is that in combination with direct activity, plant defence triggering, especially phenylpropanoid pathway induction occurring after CEO application can affect RAA feeding behaviour by secondary metabolite production, regardless of the application method considered.

7

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8

Appendix

Scientific communications and publications

First author articles:

Werrie, P.-Y., Durenne, B., Delaplace, P., Fauconnier, M.-L. Phytotoxicity of Essential Oils: Opportunities and Constraints for the Development of Biopesticides. A Review. Foods, 2020, 9, 1291. doi:10.3390/foods909129

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Werrie, P.-Y., Le Goff, G. J., Kumps V., Dhont T., Fauconnier, M.-L., Hance, T. Tree-injection: field-trial application of cinnamon essential oil as bio-insecticide in fruit arboriculture. (to be submitted)

Articles as co-author:

Denoirjean T., Belhassen D., Doury G., Ameline A., Werrie P.-Y., Fauconnier M.-L., Hance T., Le Goff, G. J. Essential oil trunk injection into orchard trees: consequences on the performance and preference of Hemipteran pests. (submitted in journal of economic entomology)

Dorland J., Werrie P.-Y., Couty A., Fauconnier M.-L., Lateur M., Doury G., Ameline A. *Geranium macrorrhizum*, a potential novel companion plant disturbing aphid host plant colonization. (submitted in journal of pest science)

Congress oral/written communications:

Werrie P.-Y., Le Goff, G. J., Hance T., Fauconnier M.-L. (2018) Development of an essential oil-based new tool for pest-management in orchards: research methodology, NSABS, Gand (poster)

Werrie P.-Y., Fauconnier M.-L. (2019) Biopesticide based on essential oils by tree-injection application in fruit orchards, map expo global market place for medicinal & aromatic plant, Eindhoven (communication orale hors académique)

Werrie P.-Y., Le Goff, G. J., Hance T., Fauconnier M.-L. (2019) Development of a biopesticide based on essential oils by tree-injection application in fruit orchards ISEO 50th International symposium on essential oils, Vienne (poster)

Werrie P.-Y., Le Goff, G. J., Hance T., Fauconnier M.-L. (2020) Injection of essential oils in xylem of apple tree for management of sap-sucking pest in orchards NSABS, Gembloux (poster)

Werrie P.-Y., Fauconnier M.-L. (2021) Development of a new tool for pest management in fruit arboriculture using essential oils applied by trunk injection ISEO 51th International symposium on essential oils (communication orale)

Supplementary Figures and Tables

Supplementary Table 1. List of 29 defence genes followed by qRT-PCR.

Defence Classes and Sub-classes		Defence Genes	
		Gene Codes	**Complete Names**
Chemical and/or physical barriers	PR proteins	PR-1	Pathogenesis-related protein 1
		PR-2	Pathogenesis-related protein 2 (glucanases)
		PR-4	Pathogenesis-related protein 4 (hevein-like)
		PR-5	Pathogenesis-related protein 5
		PR-8	Pathogenesis-related protein 8 (class III chi-
		PR-10	Pathogenesis-related protein 10
		PR-14	Pathogenesis-related protein 14 (lipid trans-
	Agglutinin	AGG	Agglutinin synthetase
	Phenylpropanoids	PAL	Phenylalanine ammonia-lyase
		CHS	Chalcone synthase
		DFR	Dihydroflavonol reductase
		BIS2	Biphenyl synthase
		PPO	Polyphenol oxidase
	Isoprenoids	HMGR	Hydroxymethyl glutarate-CoA reductase
		FPPS	Farnesyl pyrophosphate synthase
		Far	(E,E)-alpha-farnesene synthase
	Cysteines	CSL	Cystein-S-lyase
	Oxidative stress	APOX	Ascorbate peroxidase
		GST	Glutathion S-transferase
		POX	Peroxidase
	Parietal modification	CalS	Callose synthase
		Pect	Pectin methyl esterase
		CAD	Cinnamyl alcohol dehydrogenase
Hormonal signalling	Salicylic acid (SA)	EDS1	Disease resistance protein EDS 1
		WRKY	WRKY transcription factor 30
	Jasmonic acid (JA)	LOX2	Lipoxygenase AtLOX2
		JAR	Jasmonate resistant 1
	Ethylene (ET)	ACCO	1-aminocyclopropene-1-carboxylate oxi-
		EIN3	EIN3-BINDING F BOX PROTEIN 1

Supplementary Table 2. Log 2 expression level of 29 defence genes followed by qRT-PCR.

Treat-ment	Bion			CEO			Tween 80			Water		
Day	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
PR1	1.54± 0.56	3.72± 0.6	2.77± 0.31	0.78± 1.21	0.98± 1.05	1.1± 0.8	0.19± 0.54	0.72± 0.52	0.13± 0.56	1.87± 0.75	0.98± 0.29	0.61± 0.16
PR2	4.24± 0.47	2.68± 0.97	4.9± 0.33	0.14± 0.96	0.76± 1.14	0.69± 0.19	0.92± 0.51	1.94± 0.24	0.81± 0.69	0.55± 0.25	0.26± 0.51	0.26± 0.59
PR4	1.28± 0.94	1.28± 0.32	1.64± 0.66	0.98± 0.32	0.51± 0.44	0.31± 0.22	1.19± 0.53	0.66± 0.44	0.34± 0.44	0.34± 0.92	0.95± 0.19	0.46± 1.06
PR5	3.7±0 .68	3.19± 0.41	3.48± 0.8	0.37± 0.35	0.38± 0.48	0.8± 0.5	0.44± 0.57	0.74± 0.55	0.68± 0.34	0.14± 0.54	0.3± 0.52	0.38± 0.83
PR8	1.44± 0.86	1.9± 0.74	2.46± 0.62	1.67± 0.53	1.05± 0.45	0.56± 0.2	0.58± 0.46	0.54± 0.49	0.77± 0.53	1.27± 0.49	0.29± 0.34	0.22± 0.44
PR10	2.75± 1.08	4.02± 1.12	3.21± 0.94	3.13± 0.36	2.83± 0.77	1.74± 0.83	2.01± 0.39	2.19± 0.71	2.1± 0.77	0.53± 0.26	1.89± 0.44	0.55± 0.5
PR14	2.08± 0.96	4.84± 1.46	3.42± 0.91	4.14± 0.92	3.22± 1.08	1.72± 1.78	3.44± 0.27	1.19± 1	1.33± 0.95	0.94± 0.29	1.18± 0.89	0.7± 0.4
AGG	7.95± 1.28	7.73± 0.78	7.29± 1.43	1.44± 1.07	3.75± 1.09	1.68± 0.84	3.92± 0.39	4.12± 0.7	4.59± 0.2	1.99± 0.67	1.87± 1.97	0.62± 0.99
PAL	0.28± 0.33	0.27± 0.21	0.24± 0.17	1.56± 0.18	0.48± 0.29	0.83± 0.22	0.22± 0.41	0.27± 0.32	0.36± 0.07	0.42± 0.24	0.06± 0.22	0.44± 0.3

Treatment	Bion			CEO			Tween 80			Water		
Day	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
CHS	0.33± 0.41	0.4± 0.39	0± 0.38	1.09± 0.21	0.61± 0.42	0.41± 0.56	0.18± 0.22	0.06± 0.42	0.11± 0.66	0.45± 0.18	0.12± 0.4	0.39± 0.17
DFR	0.77± 0.15	1.05± 0.22	1.39± 0.38	1.76± 0.5	1.53± 0.18	1.18± 0.67	0.48± 0.39	0.12± 0.82	0.23± 0.83	0.99± 0.37	1.22± 0.55	1.14± 0.42
BIS2	3.11± 2.61	2.52± 1.42	1.62± 1.54	1.07± 0.68	0.41± 1.21	0.14± 0.64	0.98± 0.89	1.9± 1.77	1.45± 0.41	0.63± 0.58	0.94± 0.35	1.63± 0.55
PPO	0.65± 0.46	0.64± 0.6	0.45± 0.59	0.2± 0.86	0.08± 0.41	0.41± 0.7	0.99± 1.04	0.35± 0.57	0.01± 0.44	1.68± 0.06	0.44± 0.6	0.04± 0.85
HMGR	0.16± 0.38	0.18± 0.36	0.22± 0.55	0.95± 0.19	0.48± 0.27	1.23± 0.37	0.8± 0.54	0.89± 0.74	0.27± 0.34	0.89± 0.56	0.34± 0.41	0.94± 0.4
FPPS	1.38± 0.22	1.87± 2.72	1.72± 2.64	1.25± 0.45	3.8± 3.86	3.26± 3.2	0.15± 0.65	3.19± 3.75	1.45± 2.05	1.14± 0.47	1.5± 2.57	1.67± 2.62
FAR	3.47± 0.27	3.24± 0.35	2.48± 0.54	1.7± 0.43	1.36± 0.58	0.96± 0.82	1.74± 0.34	1.51± 0.5	1.14± 0.65	0.79± 0.12	0.81± 0.54	0.2± 0.89
CSL	1.72± 0.27	1.34± 0.33	0.55± 0.59	1.95± 0.73	0.55± 0.63	0.33± 0.85	0.18± 0.75	0.07± 0.53	0.02± 0.8	0.67± 0.18	0.45± 0.62	0.12± 0.87
APOX	0.26± 0.14	0.46± 0.1	0.51± 0.12	0.06± 0.15	0.19± 0.34	0.24± 0.32	0.16± 0.24	0.21± 0.37	0.04± 0.17	0.22± 0.16	0.02± 0.46	0.13± 0.27

Treat-ment	Bion			CEO			Tween 80			Water		
Day	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
GST	0.37±0.28	0.02±0.31	0.08±0.3	0.51±0.17	0.12±0.41	0.97±0.58	0.39±0.2	0.45±0.33	1.74±0.42	0.68±0.2	0.19±0.27	0.76±0.21
POX	2.57±0.37	3.05±0.4	1.36±1.01	3.23±1.03	2±1.81	0.87±1.8	1.38±0.8	2.34±0.73	2.42±0.69	0.04±0.82	1.5±0.92	0.09±1.11
CalS	0.11±0.13	0.44±0.09	0±0.17	0.32±0.04	0.23±0.24	0.43±0.25	0.26±0.21	0.37±0.65	0.27±0.28	0.19±0.27	0.42±0.26	0.35±0.08
PECT	0.66±1.11	2.22±1.08	1.93±1.13	3.48±0.33	2.85±0.85	3.43±0.43	0.44±0.68	0.03±1.12	0.65±0.82	0.03±0.55	0.44±0.72	1.16±0.15
CAD	0.46±0.24	0.12±0.32	0.23±0.23	0.17±0.29	0.3±0.14	0.05±0.23	0±0.28	0.1±0.29	0.02±0.24	0.19±0.37	0.19±0.26	0.32±0.2
EDS1	2.61±0.28	1.62±0.73	2.24±0.94	0.53±0.36	1.06±0.5	1.34±0.98	0.54±0.47	0.65±0.84	1.06±1.59	0.36±0.54	1.23±0.96	0.33±0.48
WRKY	0.83±1.16	3.34±2	0.99±0.93	0.37±0.69	0.48±0.47	0.88±1.1	0.71±0.48	0.21±0.48	0.28±0.37	2.28±0.67	0.7±1.79	1.37±0.8
LOX2	0.09±0.12	0.31±0.15	0.28±0.33	0.18±0.28	0.3±0.27	0.33±0.44	0.16±0.28	0.02±0.27	0.13±0.44	0.22±0.1	0.02±0.19	0.07±0.32
JAR	0.38±0.24	0.07±0.27	0.12±0.33	0.23±0.29	0.31±0.22	0.4±0.29	0.29±0.2	0.42±0.35	0.27±0.02	0.36±0.36	0.24±0.17	0.56±0.21

Treat-ment	Bion			CEO			Tween 80			Water		
Day	1	2	3	1	2	3	1	2	3	1	2	3
ACCO	0.25± 0.15	0.43± 0.5	0.93± 0.76	0.69± 0.21	0.76± 0.57	0.07± 0.64	0.11± 0.23	0.35± 0.28	0.39± 0.47	0.37± 0.16	0.18± 0.6	0.78± 0.55
EIN3	0.37± 1.55	0.28± 1.42	0.07± 1.44	0.25± 1.45	0.71± 1.01	0.86± 0.99	0.48± 1.54	0.57± 1.44	0.1±1 .34	0.49± 1.56	0.87± 1.47	0.41± 1.3

Supplementary Table 3. *Cinnamomum cassia* essential oil composition analysis.

Name	Score (Lib)	CAS	Relative area (%)
Cinnamaldehyde, (E)-	98	104-55-2	91.22
o-Methoxycinnamaldehyde	97.06	1504-74-1	1.98
Benzaldehyde	90.59	100-52-7	0.75
Benzenepropanal	93.23	104-53-0	0.60
Copaene	91.68	3856-25-5	0.55
Benzaldehyde, 2-methoxy-	93.18	135-02-4	0.39
Butylated Hydroxytoluene	89.6	128-37-0	0.39
Acetic acid, cinnamyl ester	95.36	103-54-8	2.30
Benzaldehyde, 2-hydroxy-	85.01	90-02-8	0.24
Caryophyllene	89.78	87-44-5	0.23
Acetic acid, 2-phenylethyl ester	93.29	103-45-7	0.21
Benzofuran, 2-methyl-	87.02	4265-25-2	0.20
Naphthalene-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	84.6	483-76-1	0.19
Phenylethyl Alcohol	85.89	60-12-8	0.18
(1S,4aR,8aS)-1-Isopropyl-7-methyl-4-methylene-octahydronaphthalene	82.39	6980-46-7	0.13
Total			99.56

Supplementary Table 4. *Mentha spicata* essential oil composition analysis.

Name	**Score (Lib)**	**CAS**	**Relative area (%)**
Carvone	93.77	99-49-0	57.78
D-Limonene	95.74	138-86-3	25.28
Dihydrocarvone	93.55	5524-05-0	2.06
(-)-β-Bourbonene	94.35	5208-59-3	1.72
Caryophyllene	96.81	87-44-5	1.57
β-Pinene	92.87	127-91-3	1.34
β-Myrcene	94.27	123-35-3	1.33
Unknown terpene			0.86
Terpinen-4-ol	93.68	562-74-3	0.72
Germacrene D	95.26	23986-74-5	0.71
Piperitone	85.22	89-81-6	0.6
Levomenthol	97.34	2216-51-5	0.52
(E)-β-Farnesene	94.57	18794-84-8	0.41
Butylated Hydroxytoluene	90.77	128-37-0	0.31
Dihydrocarvyl acetate	95.79	20777-49-5	0.28
4-Thujanol	88.97	546-79-2	0.27
γ-Terpinene	92.26	99-85-4	0.25
α-Terpineol	93.61	98-55-5	0.25
unidentified			0.25
cis-sabinene	92.58	3387-41-5	0.23

o-Cymene	91.21	527-84-4	0.23
Carvyl acetate (Z)	94.47	1205-42-1	0.22
Acetic acid, hexyl ester	89.91	142-92-7	0.21
trans-Carveol	89.07	1197-07-5	0.21
α-Bisabolene	88.31	17627-44-0	0.2
Isogermacrene D	90.5	317819-80-0	0.19
Naphthalene, -hexahydro-4,7-dimethyl-1-(1-methylethyl)	90.89	483-76-1	0.16
α-Terpinolene	81.96	586-62-9	0.15
Dihydroedulan	81.8	41678-32-4	0.15
(E)-β-Elemene	91.48	515-13-9	0.14
Unidentified sesquiterpene			0.14
Isomenthone	90.73	491-07-6	0.13
Caryophyllene oxide	83.74	1139-30-6	0.13
(+)-4-Carene	83.46	29050-33-7	0.12
3-Octanol, acetate	88.16	4864-61-3	0.12
δ-Terpineol	84.64	98-55-5	0.1
Limonene oxide, trans-	82.77	4959-35-7	0.08
γ-Muurolene	84.54	30021-74-0	0.05
1,3,6-Heptatriene, 2,5,6-trimethyl-	81.89	42123-66-0	0.04
Linalool	82.17	78-70-6	0.02
Total			99.53

Supplementary Table 5. Untargeted VOC emissions profiles (TDU-GC-MS): detailed composition of headspace emissions profiles of *Malus x domestica* tree belonging to the alkanes, alkenes, alcohol, aldehydes, aliphatic and aromatic esters, furanes, homoterpenes, ketones, monoterpenes, sesquiterpenes and terpenoids. Compounds with Asterisks indicate significant differences after one-way ANOVA and Different letters indicate significant differences based on post hoc Tukey's HSD test.

Family	Name	Calculated RI	Literature RI	Blank (n=11)	Cinnamon (n=5)	Mint (n=8)	Control (n=7)
Alcohol/ Phenol	1-Octen-3-ol	963.3	962	0.33±0.14	0.13±0.08	0.21±0.14	0.11±0.06
	2-ethyl-1-Hexanol	1772.8	1790	0.15±0.09	0.16±0.11	0.16±0.11	0.11±0.06
	3-Hexen-1-ol, (Z)-	839.3	856.6	0.67±0.3	0.51±0.23	0.11±0.04	0.36±0.14
	l-Menthol	1153.7	1150.0	0.12±0.06	n.d.	n.d.	0.05±0.02
	1-Hexanol	853.2	869.7	0.21±0.06	0.74±0.33	0.12±0.04	n.d.
	1-Octanol	1219.1	1218.3	0.22±0.07	0.24±0.11	0.18±0.09	n.d.
Aldehydes	2-Decenal, (E)-	1243.2	1263.4	0.11±0.03	0.07±0.03	0.07±0.02	n.d.
	4-Methylhexen-2-enal*	1013.1	1011.5	0.1±0.07[a]	n.d.	n.d.	.07±0.04[ab]
	Decanal	1186	1205.4	0.18±0.14	0.43±0.34	0.37±0.33	0.15±0.11
	Dodecanal	1385.8	1408.1	n.d.	0.05±0.02	0.1±0.04	n.d.
	Nonanal	1086.1	1103.3	0.29±0.33	0.93±1.23	0.74±0.68	0.19±0.22
	Undecanal	1285.7	1306.5	0.06±0.02	0.08±0.04	0.1±0.05	0.05±0.02

Aliphatic esters	3-Hexen-1-ol, acetate, (Z)-	993.3	1011.5	3.27±1.34	1.44±1.22	0.48±0.25	2.33±0.88
Alkanes	6-Methyldodecane	1196.1	1250	0.06±0.02	n.d.	0.06±0.03	0.05±0.03
	Decane, 2,3,5,8-tetramethyl-	656.7	720	0.08±0.04	0.09±0.04	0.08±0.05	0.06±0.03
	Dodecane, 2,6,11-trimethyl-	686.7	776	0.14±0.08	0.16±0.1	0.13±0.08	0.11±0.03
	Nonane, 4,5-dimethyl-*	1205.7	1205.4	n.d.	0.08±0.04^{ab}	0.06±0.03^{ab}	0.06±0.02^{a}
	Dodecane	1180.3	1200	0.16±0.1	0.14±0.09	0.19±0.1	0.13±0.04
	Phytane	1782.9	1790	0.06±0.02	n.d.	0.06±0.03	n.d.
	Tetradecane	1377	1400	0.21±0.13	0.14±0.06	0.19±0.12	0.16±0.03
	Tridecane	1279	1300	0.25±0.15	0.2±0.12	0.29±0.16	0.22±0.05
	Undecane	1082	1100	0.17±0.13	0.17±0.13	0.16±0.09	0.08±0.05
	Unidentified alkane RI 1477*	1477	-	0.12±0.07^{a}	0.1±0.06^{ab}	0.12±0.07^{ab}	n.d
	Unidentified RI 1174	1174	-	n.d.	0.11±0.05	0±0	0.04±0.01
	Unidentified alkane RI 1675	1675	-	0.08±0.03	0.09±0.04	0.07±0.04	n.d.
	3-Ethyl-2,6,10-Trimethylundecane	1441.6	-	0.09±0.05	0.13±0.08	0.09±0.05	n.d.
	2,4-Dimethyldecane	1040.2	1106	0.12±0.09	0.1±0.05	0.06±0.04	0.08±0.04
	Dodecane, 4,6-dimethyl-	1360	-	n.d.	n.d.	n.d.	0.05±0.03

	Pristane	1681.2	1684	0.06±0.02	n.d.	0.07±0.04	n.d.
Alkenes	7-Tetradecene	1371.3	1374	0.07±0.04	n.d.	0.08±0.05	n.d.
Aromatic esters	3-Hexen-1-ol, benzoate, (Z)-	1550.1	1550	n.d.	0.13±0.06	0.31±0.11	n.d.
	Methyl salicylate	1177.4	1192.9	n.d.	n.d.	0.66±0.41	n.d.
Esters	Isobornyl propionate	1169.9	1171.3	0.11±0.07	0.2±0.13	0.13±0.05	n.d.
Furanes	Trans-Linalool oxide	1070.6	1071	0.1±0.06	0.21±0.16	0.18±0.1	0.06±0.03
Homoterpenes	TMTT*	1557.7	1566	n.d.	0.21±0.12^{a}	0.2±0.16 ^{a}	0.16±0.08^{ab}
	DMNT	1098.3	1105	0.98±0.54	0.79±0.52	2.58±2.53	0.46±0.28
Ketones	2-Undecanone	1276.5	1294	0.06±0.03	0.1±0.04	0.07±0.04	n.d.
	trimethyl-2-Pentadecanone	1833	1842	0.09±0.06	0.12±0.05	0.1±0.04	0.04±0.02
Monoterpenes	Geranylacetone	961.9	NA	0.07±0.02	0.18±0.08	0.14±0.06	n.d.
	l-Menthone	1136.3	1136	0.04±0.02	n.d.	0.07±0.03	n.d.
	Dihydroactinidiolide	1046.4	1011.3	0.08±0.02	n.d.	0.11±0.04	n.d.
	α-Bergamotene*	987.9	977.7	n.d.	0.13±0.07^{a}	0.14±0.08^{a}	0.08±0.04^{ab}
	β-Ocimene	1033.7	1037.8	n.d.	0.07±0.03	0.36±0.33	n.d

Sesquiter-penes	α-Farnesene*	1484.2	1490.9	0.65 ± 0.72^{b}	6.27 ± 1.81^{a}	2 ± 0.92^{b}	1.56 ± 2.23^{b}
	α-Muurrolene	1494.9	1498.3	n.d.	0.3±0.13	0.2±0.09	n.d.
	Caryophyllene*	1398	1406.5	0.16 ± 0.13^{b}	0.45 ± 0.21^{ab}	0.67 ± 0.44^{a}	0.1 ± 0.03^{b}
	D-Cadinene	1503.7	1523.2	n.d.	0.31±0.14	0.26±0.13	n.d.
	Germacrene D*	1459.3	1480.6	0.33 ± 0.26^{b}	1.18 ± 0.67^{a}	0.73 ± 0.39^{ab}	0.35 ± 0.21^{b}
	γ-Muurolene	1457.9	1476.2	n.d.	0.1±0.04	0.08±0.04	n.d.
Terpenoids	D-Carvone*	1178.7	1242	n.d.	n.d.	0.08 ± 0.05^{a}	n.d
	Linalool	1084.6	1099	n.d.	0.45±0.2	0.3±0.19	n.d
	Terpinen-4-ol	1159.6	1177.1	n.d.	0.14±0.06	n.d.	n.d
	Dihydrocarvone	1179.2	1201.4	n.d.	n.d.	0.17±0.06	n.d.
Unknown	Unidentified RI 1367	1367	-	n.d.	n.d.	0.07±0.03	0±0
	Unidentified RI 1273	1273	-	n.d.	n.d.	0.08±0.05	0.04±0.01
	Unidentified RI 1686	1686	-	n.d.	n.d.	0.08±0.03	n.d.

Supplementary Table 6. Untargetted VOCs Contained (DHS-GC-MS): Detailed composition of VOCs contained in leaves of *Malus x domestica*. These compounds belong to the alcohols, aldehydes, alkadienes, alkanes, aromatic and aliphatic esters, fatty acid esters, homoterpenes, ketones.

Family	Name	Calculated RI	Literature RI	Blank (n=15)	Cinnamon (n=15)	Mint (n=14)	Control (n=14)
Alcohol/ Phenol	Terphenyl-2-ol *	2213.9	2275	1.94±0.5[b]	0.91±0.43[b]	8.71±6.48[a]	n.d.
	1-Octanol*	1071.9	1272.1	1.66±0.43[b]	17.83±8.47[a]	9.14±3.97[b]	n.d.
	1-Penten-3-ol*	679	675	31.48±25.9[b]	333.67± 284.47[a]	277.31± 220.6[a]	19.38±8.17[b]
	2,4-Hexadien-1-ol	817.4	882	n.d.	n.d.	54.96±0	41.19±14.87
	2-Octen-1-ol, (Z)-	1052.6	1039	n.d.	3.26±1.11	n.d.	n.d.
	2-Penten-1-ol, (Z)-*	669.5	771.2	68.14± 66.76[b]	333.22± 274.02[a]	354±187.52[a]	47.82±67.9[b]
Aldehydes	2,4-Heptadienal*	1013.1	1011.5	15.02± 19.43[b]	67.57±24.21[a]	68.58± 23.82[a]	34.63±19.95[b]
	2,4-Hexadienal*	913.6	913.2	73.37± 99.68[b]	212.39±198.4[a]	47.29±44.9[b]	48.26±56.87[b]
	2-Decenal, (E)-*	1261.9	1263.4	n.d.	13.23±13.19[a]	9.3±7.01[b]	4.04±4.52[b]
	2-Heptenal, (E)-*	958.4	960.5	8.33±7.61[b]	42.5±29.31[a]	14.19±8.42[b]	18.5±7.86[b]

	2-Hexenal, (E)-*	796.3	853	1350.58±833.5[b]	3816.23±1634.35[a]	2384.14±1393.71[ab]	1145.79±1223.41[b]
	2-Nonenal, (E)-*	1160.4	1162.2	n.d.	2.4±1.77[a]	0.85±0[b]	n.d.
	2-Octenal, (E)-	1058.9	1060.2	n.d.	4.96±2.38	3.05±0.53	n.d.
	2-Pentenal, (E)-	656.7	720	1.47±0	60.03±82.45	6.62±2.29	n.d.
	Benzaldehyde*	961.9	949	10.93±12.26[b]	22.69±17.12[b]	46,22±42,69[a]	12.64±11.23[b]
	Benzene-acetal-dehyde*	1044	1039	3.7±1.67[b]	7.89±4.73[a]	n.d.	0.9±0.47[b]
	Decanal*	1205.7	1205.4	1.21±0.41[b]	4.45±2.95[ab]	5.78±7.72[a]	2.42±1.47[ab]
	Heptanal	903.5	902	3.94±2.95	25.64±20.81	14.81±11.42	5.32±9.12
	Hexanal*	734.2	799.9	278.56±348.69[b]	889.75±450.14[a]	299.17±205.76[b]	163.69±372.1[b]
	Nonanal*	1104.8	1103.3	8.4±5.82[b]	51.19±34.08[a]	40.83±31.12[a]	11.17±13.04[b]
	Octanal*	1004.1	1002.8	2.93±2.17[b]	13.66±10.98[a]	17.94±7.86[ab]	3.75±3.43[ab]
Alkadiene	3-Ethyl-1,5-octa-diene*	941.2	939	n.d.	2.53±1.19[ab]	8.65±9.13[a]	n.d.
Alkanes	Decane, 2,3,6-trimethyl-	1069.3	1466	n.d.	18.8±13.23	1.93±0	n.d.

	Dodecane	1199.9	1200	1.03±0.45	1.33±0.69	2.63±2.11	1.02±0.66
	Heptadecane	1699.1	1700	n.d.	1.56±0.45	1.55±0.95	n.d.
	Heptane, pentamethyl-2,2,4,6,6-	1010.1	1003	n.d.	8.7±14.54	2.96±1.94	n.d.
	Hexadecane	1599	1600	n.d.	1.41±0.52	2.3±1.61	n.d.
	Nonane, 3-methyl-	1051.3	970	n.d.	6.48±6.05	3.05±1.02	n.d.
	Octadecane	1760.5	1800	n.d.	1.28±0.24	1.45±1.11	n.d.
	Tetradecane	1399.5	1400	0.84±0	1.44±0.28	4.03±2.12	n.d.
Aromatic esters	1-ethyl-cyclohexene	998.5	1011.5	n.d.	4.59±1.51	10.85±18.18	n.d.
	Methyl salicylate*	1191.4	1192.9	3.82±2.78[b]	32.8±39.31[a]	41.82±32.97[a]	20.93±21.29[ab]
	1,3,5-Trimethylbenzene	990.7	996	n.d.	n.d.	9.76±9.5	n.d.
	3-Hexen-1-ol benzoate	1569.2	1569.5	1.6±0	15.28±21.95	5.29±5.1	n.d.
	Benzoic acid, ethyl ester	1169.9	1171.3	n.d.	3.28±0	11.73±13.32	n.d.

Aliphatic esters	3-Hexen-1-ol, acetate, (Z)-	1006.1	1004	17.3±9.98	59.25±33.98	4.43±3.2	4.84±0
	Glutaric acid, butyl isobutyl ester	1659	1647	20.82±3.76	43.17±30.76	32.51±11.59	15.8±6.23
	Heptanoic acid, ethyl ester	1097.9	1096	0.94±0.23	1.67±1.13	n.d.	0.83±0.3
Fatty acid esters	Hexadecanoic acid	1957.9	1978	n.d.	272.26± 534.77	7.16±0	n.d.
	Octadecanoic acid	2158.8	2177	n.d.	179.85±273.49	n.d.	n.d.
Homoterpenes	DMNT	1113.3	1107.5	7.05±4.76	7±7.77	4.93±1.52	3.8±3.79
Ketones	1-Octen-3-one*	978.2	978	n.d.	7.46±3.37[a]	5.13±1.16[ab]	n.d.
	Cyclohexanone, 2,2,6-trimethyl-	1035.4	1013	n.d.	3.23±1.13	3.57±2.47	n.d.
	α-Ionone*	142.9	1425.6	0.4±0	14.52±9.71	6.27±6.18	n.d.
	α-Pinene	925.5	936	n.d.	3.15±1.56	3.56±2.76	n.d.
	β-Cyclocitral*	1219.1	1218.3	1.97±0[b]	6.1±3.92[a]	7.58±4.73[a]	1.97±0.87[b]
	β-Ionone*	1478.2	1485.9	n.d.	7.6±5.92[a]	7.28±4.82[a]	n.d.
	δ-Carene	1046.4	1011.3	2.94±2.07	19.89±32.2	21.54±29.14	0.65±0

Monoterpenes	β-Homocy-clocitral*	1252.2	1236	n.d.	0.93±0.67[b]	1.38±0.78[a]	n.d.
	β-Pinene	987.9	977.7	n.d.	13.27±0	6.64±4.55	n.d.
	Limonene	1027.7	1029.5	1.26±0.85	15.33±10.05	15±21.01	3.12±2.13
	Eucalyptol*	1028.6	1032	7.2±2.43[b]	30.01±21.47[ab]	100.88±114.12[a]	12.16±9.94[b]
	p-Cymene	1023.7	1024	0.4±0	3.42±1.31	8.17±7.49	1±0
Phenyl-pro-panoids	Cinnamaldehyde, (E)-*	1272.3	1271.3	n.d.	21.81±15.65[a]	n.d.	n.d.
Sesquiterpenes	α-Farnesene	1495.6	1504.1	n.d.	115.84±189.68	22.05±14.22	n.d.
	δ-Cadinene	1505	1533	n.d.	0.77±0.17	1.35±1.38	n.d.
	Unknown ses-quiterpene*	1477.5	-	n.d.	5.55±2.35[ab]	10.9±4.78[a]	n.d.
	γ-Gurjunene	1589.6	1472	3.94±4.15	3.72±1.21	19.03±25.55	1.63±0
	α-Longipinene	1485.9	1352	2.55±2.54	3.19±0.98	5.77±5.59	1.09±0.87
	δ-Elemene	1332	1337	n.d.	n.d.	0.58±0.43	n.d.

	Germacrene-D*	1481.3	1480	n.d.	6.23±2.76[a]	n.d.	n.d.
	Trans-caryo-phyllene*	1416.2	1465	1.34±1.44[b]	7.68±6.86[a]	5.55±2.71[ab]	0.37±0.26[b]
Terpenoids	Camphor	1147.2	1143	n.d.	7.04±5.85	12.46±0	n.d.
	D-Carvone*	1240.8	1242	n.d.	n.d.	1.17±0.92[a]	n.d.
	α-Terpineol*	1192.1	1190	n.d.	n.d.	17.27±17.47[a]	n.d.
	Linalool	1099.7	1084	1.54±0.08	37.79±0	4.07±3.88	n.d.
	p-Menthone	1155.9	1150	n.d.	17.67±15.1	23.34±0	n.d.
	Terpinen-4-ol	1178.2	1177	n.d.	n.d.	3.72±2.36	n.d.

Supplementary Table 7. Weather conditions for the entire duration of the experiment and the duration of flowering, for the 2020 and 2021 trials.

	Experiment duration		**Flowering period**	
	2020	**2021**	**2020**	**2021**
Temperature (°C)	13.55±4.02[a]	11.45±5.70[b]	10.50±2.81[a]	9.64±4.42[b]
Relative humidity (%)	73.35±13.06[a]	82.43±10.53[b]	74.47±11.79[a]	80.31±6.05[a]
Pluviometry (mm)	1.13±2.76[a]	3.32±9.72[b]	1.02±2.16[a]	1.19±0.99[a]
Wind speed (m/sec)	0.35±0.21[a]	0.54±0.81[b]	0.39±0.20[a]	0.61±0.52[b]

Supplementary Table 8. Volatiles organic compounds emission (ng h^{-1}) summary by treatment (mean+/- standard deviation) of 2020 trial.

Family	Name	BEM	BSA	CEO1	CEO2	NEG	Kovats library	Kovats experi-mental
Acids	Acetic acid	2.24±2.4	1.77±1.34	1.65±1.46	1.51±1.18	1.43±1.02	625	622.3
	Benzoic acid	10.1±6.76	9.44±6.91	9.03±6.49	8.16±5.1	9.32±6.29	1180	1178.7
	Nonanoic acid	0.68±0.61	0.88±0.85	0.42±0.36	0.52±0.49	0.57±0.54	1275.3	1271.9
	Octanoic acid	0.87±0.6	0.77±0.5	0.54±0.48	0.56±0.43	0.63±0.42	1182	1183
Alcans	Heptadecane	0.63±0.81	0.39±0.38	0.65±0.89	0.36±0.38	0.63±0.65	1700	1700
	Pentadecane	1.25±1.62	0.91±0.85	1.21±1.55	0.82±0.82	1.3±1.39	1500	1505.2
Alcohols	1-Dodecanol	0.72±0.57	0.71±0.48	0.56±0.5	0.59±0.38	0.64±0.31	1472.8	1478.3
	1-Octanol	0.6±0.36	1.01±0.84	0.59±0.45	0.42±0.32	0.57±0.32	1071.5	1074
	1-Octen-3-ol	0.65±0.61	0.93±1.18	0.53±0.52	0.62±0.76	0.64±0.53	980	978.8
	1-Tetra-decanol	0.55±0.83	0.84±1.35	0.43±0.58	0.5±0.53	0.52±0.61	1676.3	1678.4
	2-Propyl-1-pentanol	0.83±1.45	0.43±0.84	0.43±1.27	0.39±0.7	0.86±1.64	1052.8	1031.1
	3-Hexen-1-ol, (Z)-	2.7±4.07	2.61±2.79	2.1±3.2	1.23±1.33	2.25±2.79	856.6	848.7
	Phenol	1.62±1.97	1.31±1.33	0.86±1.07	1.03±1.11	1.53±1.87	983.3	982
Aldehydes	Benzaldehyde	1.17±0.56	1.29±0.99	1.17±0.7	1.02±0.51	1.07±0.55	962.7	958.4
	Decanal	2.66±2.25	2.17±1.38	1.95±1.48	1.57±1.01	2.42±1.65	1205.4	1206.9
	Nonanal	4.08±3.6	3.9±2.5	3.22±2.96	2.44±1.78	3.95±3.1	1103.3	1105.3

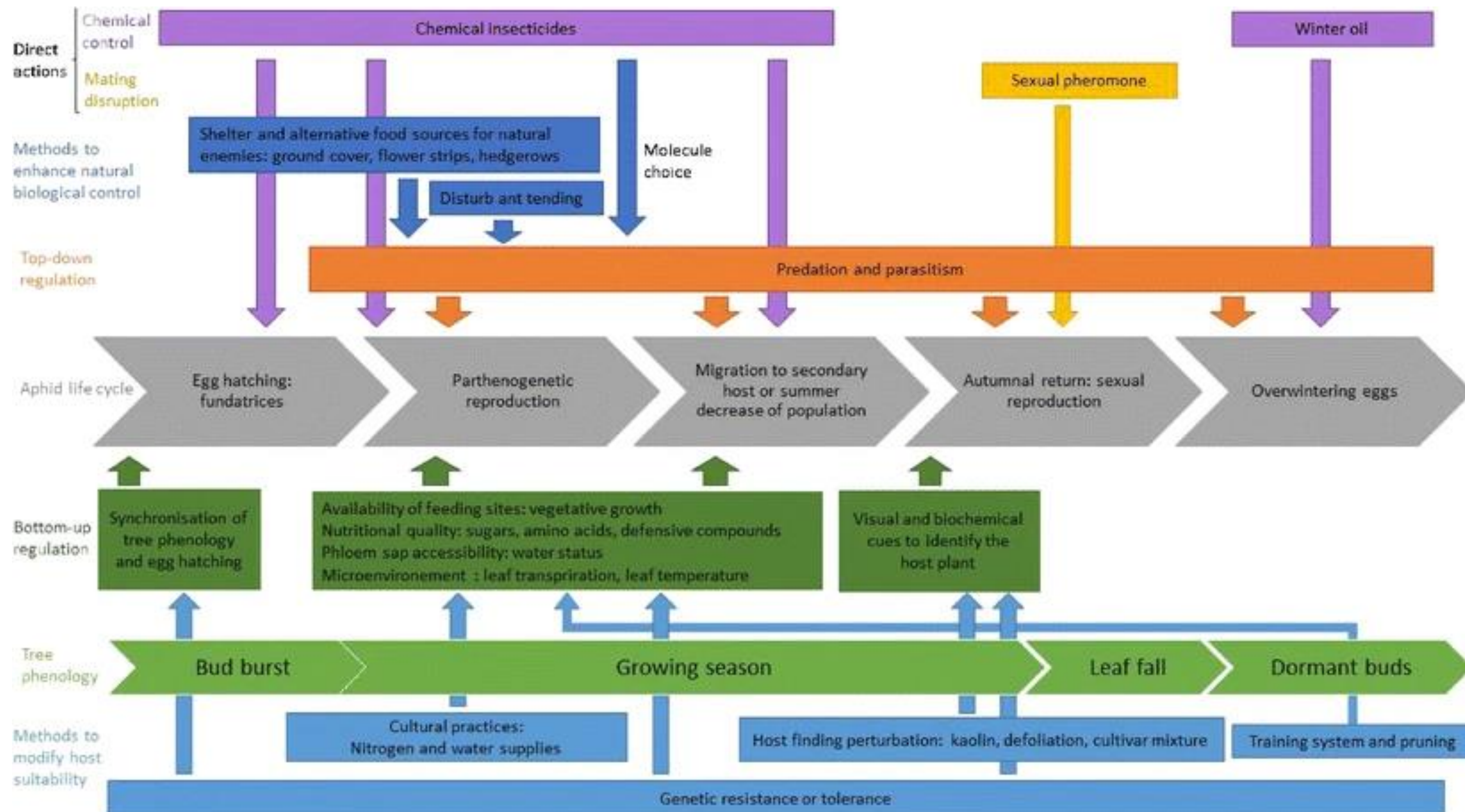
Esters	3-Hexen-1-ol, acetate,	6.71±5.51	11.1±13.62	5.86±5.89	4.83±5.17	5.87±3.96	1005	1008.2
	Methyl salicylate	1.51±2.12	1.8±2.96	1.6±2.62	0.84±1.13	1.39±1.88	1192.9	1200.9
Ketone	Acetophenone	2.06±1.17	2.14±1.28	1.75±1.06	1.73±0.74	1.97±0.87	1067.4	1068
Monoterpenoids	(E)-4,8-Dimethylnona-1,3,7-triene	4.15±4.4	5.02±3.92	3.81±4.67	2.43±2.45	3.28±3.11	1116	1118.5
	3-Carene	0.9±1.55	0.84±1.34	1.05±2.14	0.35±0.42	0.37±0.42	1011.3	1050.7
	Linalool	0.5±0.48	0.69±0.57	0.33±0.4	0.31±0.22	0.48±0.52	1100	1100.5
Sesquiterpenes	α-Bergamotene	1.86±3.08	2.43±2.89	1.72±2.56	1.3±2.09	1.06±1.3	1441	1501.8
	α-Farnesene	5.03±6.7	6.6±8.04	5.98±8.63	3.78±5.91	3.23±4.08	1504.1	1510.5
	β-Bourbonene	2.44±2.85	4.46±3.74	2.49±2.51	2.49±2.48	2.25±2.33	1384.2	1393.6
	Caryophyllene	2.66±2.59	5.93±11.72	3.19±4.54	2.4±2.64	1.95±1.86	1420.1	1425.1
	Germacrene-D	1.4±1.69	2.06±2.15	1.75±2.66	1.24±1.72	1.05±1.28	1480	1490.5

Supplementary Table 9. Volatiles organic compounds emission (ng h^{-1}) summary by treatment (mean+/- standard deviation) of 2021 trial.

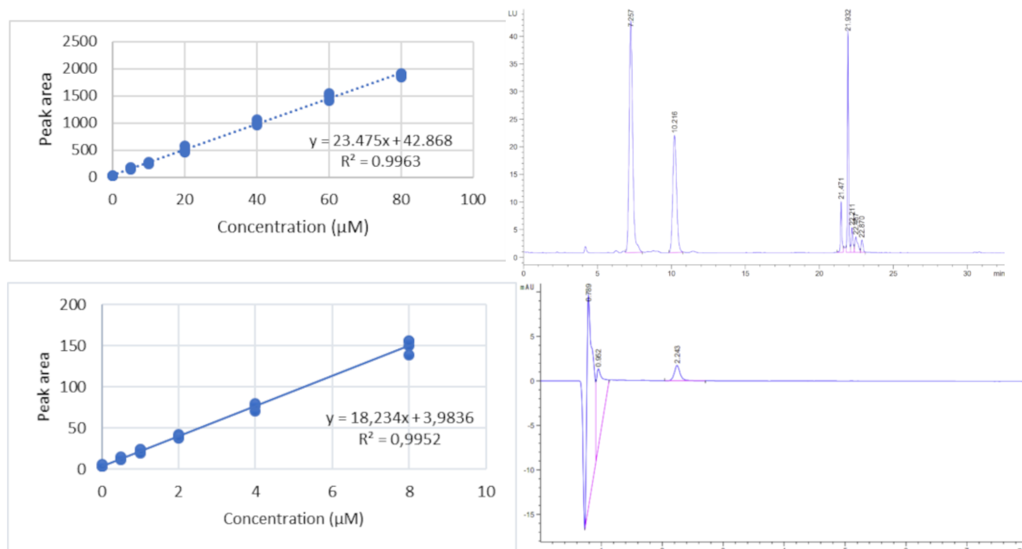
family	Name	3 mL	10 mL	20 mL	50 mL	NEG	Library RI	Hit RI
Alcenes	4-Cyanocyclohexene	1.39±2.18	0.77±2.27	1.31±2.74	1.11±2.79	1.74±3.98	1008	1018.78
Alcohols	1-Hexanol	20.23±27.59	11.48±8.74	21.05±24.29	18.3±9.19	22.94±19.81	860	869.58
	1-Pentanol	1.59±2.63	1.24±1.7	1.78±1.66	1.81±2.24	3.19±3.21	761	766.57
	3-Hexen-1-ol	127.72±197.75	106.73±113.85	153.09±153.17	139.64±99.23	217.75±174.75	856	855.45
	Benzyl alcohol	176.29±153.91	229.88±222.21	222.92±220.56	167.48±245.04	257.34±225.83	1036	1035.34
	Phenol	2.23±1.64	3.19±2.66	3.33±2.53	2.17±2.25	3.31±3.14	981	983.978
	Terphenyl-2-ol	3.32±3.72	4.07±4.03	9.13±14.48	6.03±5.54	7.57±7.43	2275	2261.94
Aldehydes	2-Nonenal	3.48±3.98	3.24±4.26	3.59±2.71	3.04±3.59	3.48±3.83	1112	1095.16
	Benzaldehyde	10.16±7.11	9±3.83	10.64±7.02	7.71±5.69	15.56±15.36	961	959.55
	Benzeneacetaldehyde	4.92±6.97	2.24±4.01	3.58±6.91	3.17±4.72	3.14±4.54	1044	1044.13
	Decanal	15.77±10.24	23.68±15.22	25.63±25.21	17.28±10.06	17.47±13.62	1204	1206.56
	Nonanal	34.87±34.22	37.33±35.47	33.64±18.63	24.3±9.54	27.13±12.63	1104	1105.20
	Octanal	3.16±3.68	5.34±3.54	5.11±7.45	2.19±3.4	4.27±4.54	1005	1003.77
Alkanes	4,5-dimethyl-Nonane	1.5±3.36	2.08±4.17	1.84±3.82	0.79±1.87	1.5±2.96	1035	1058.35
	Dodecane	0.72±1.44	0.23±0.59	1.24±2.38	0.46±1.21	1.06±2.61	1200	1200.33
Aromatic heterocyclic	Indole	2.17±2.83	2.71±4.71	2.28±2.93	1.16±1.92	2.82±2.87	1288	1294.27

Benzenes	Benzyl nitrile	4.66±12.22	26.2±84.42	8.03±15.16	26.21±56.06	6.57±9.63	1138	1140.42
Carboxylic acid	Hexadecanoic acid	18.97±19.22	30.12±11.95	62.26±77.51	46.92±32.5	22.35±17.43	1968	1964.64
	Octadecanoic acid	4.51±7.59	9.28±5.08	30.28±47.22	24.88±30.05	3.24±6.48	2167	2165.14
Esters	2-Hexen-1-ol, acetate	14.65±52.71	1.15±4.13	0.95±3.69	0.77±1.63	111.25±180.26	1011	1018.04
	3-Hexen-1-ol, acetate	106.73±174.43	119.41±150.81	146.16±169.31	141.7±134.88	83.41±133.04	1009	1008.28
	Acetic acid, hexyl ester	3.56±6.66	2.83±4.6	3.08±5.53	2.64±3.7	4.22±4.27	984	1015.03
	Acetic acid, phenylmethyl ester	11.17±16.77	13.28±17.46	13.74±21.68	13±19.96	15.76±20.18	1160	1165.88
	Benzoic acid, ethyl ester	6.71±10.75	13.87±24.27	17.56±32.34	8.92±16.79	30.4±80.92	1160	1164.85
	Butanoic acid, 3-hexenyl ester	1.65±3.97	1.62±2.52	2.34±3.71	2.07±2.82	4.4±7.42	1191	1187.58
	cis-3-Hexenyl-methylbutyrate	2.8±4.39	1.83±2.89	3.78±4.71	1.94±2.61	4.68±4.56	1226	1234.30
	Methyl salicylate	5.18±4.52	6.63±5.52	13.79±17.65	5.98±7.21	8.68±6.78	1190	1195.88
Ketones	6-methyl-5-Hepten-2-one	6.47±3.94	8.96±8.12	13.49±22.82	7.65±6.44	11.16±13.56	988	988.19
	Acetophenone	14.14±7.14	14.51±5.79	19.61±8.26	13.3±6.05	16.03±5.43	1058	1066.73
	Geranylacetone	0.5±1.33	0.08±0.3	1.94±5.74	0.59±1.51	2.15±3.86	1451	1455.04
	Isophorone	2.82±6.12	1.92±4.74	2.5±5.69	3±9.09	2.2±5.97	1117	1121.69
Monoterpenoids	β-Ocimene	10.32±23.93	4.6±7.08	3.9±9.19	5.89±5.98	5.42±9.26	1041	1049.62
	DMNT	25.82±61.31	16.86±27.29	31.25±61.82	27.88±29	15.84±15.68	1105	1117.71
	Linalool	23.5±21.37	21.15±23.83	21.22±13.74	18.06±9.54	31.23±33.96	1098	1100.65

Sesquiter-penes	α-Farnesene	9.88±20.88	7.54±11.07	15.13±33.71	8.67±11.37	8.24±12.05	1509	1509.70
	β-Bourbonene	9.38±16.85	5.12±8.72	8.97±11.2	5.26±4.19	11.47±11.67	1381	1388.72
	Caryophyllene	5.27±13.46	2.88±6.73	2±3.04	1.62±2.18	3.19±5.14	1419	1423.87
	Germacrene D	5.9±13.08	2.57±6.52	2.44±4.79	2.09±4.82	5.79±9.67	1480	1480.74
	trans-α-Bergamo-tene	4.26±6.65	1.82±3.24	2.81±7.18	3.21±4.69	3.58±5.24	1430	1496.33



Supplementary Figure 1. Possible methods to control aphid populations in apple and peach orchards by mobilizing bottom-up and top-down processes (Rousselin, 2017).



Supplementary Figure 2. Typical HPLC-FLD sample chromatogram (RT GSH-MBB= 7.2 min) and calibration curve for GSH (upper case); Limit of detection (LOD) = 1.16 µM; Limit of quantification (LOQ) = 1.41 µM. Typical HPLC-DAD sample chromatogram (RT MDA(TBA)2=2.1 min) chromatogram and calibration curve for MDA (lower case); Limit of detection (LOD) = 0.19 µM; Limit of quantification (LOQ) = 0.25 µM.

North west

54	B	P	J	E		JD		J	J	JD	P	P
53	B	P	J	E		JD		J	J	JD	P	P
52	B	P	J	E		JD		J	J	JD	P	P
51	B	P	J	E		JD		J	J	JD	P	P
50	B	P	J	E		JD		J	J	JD	P	P
49	B	P	J	E		JD		J	J	JD	P	P
48	B	P	J	E		JD		J	G	JD	P	P
47	B	P	J	E		JD		J	G	JD	P	P
46	B	P	J	E		JD		J	G	JD	P	P
45	B	P	J	E		JD		J	G	JD	P	P
44	B	P	J	E	G	JD		J	G	JD	P	P
43	B	P	J	E		JD		J		JD	P	P
42	B	P	J	E	G	JD		J		JD	P	P
41	B	P	J	E		JD		J	G	JD	P	P
40	B	P	J	E		JD		J		JD	P	P
39	B	P	J	E		JD		J		JD	P	P
38	B	P	J	E		JD		J		JD	P	P
37	B	P	J	E	G	JD		J		JD	P	P
36	B	P	J	E		JD		J	G	JD	P	P
35	B	P	J	E		JD		J		JD	P	P
34	B	P	J	E		JD		J		JD	P	P
33	B	P	J	E		JD		J		JD	P	P
32	B	P	J	E	G	JD		J		JD	P	P
31	B	P	J	E		JD		J	G	JD	P	P
30	B	P	J	E		JD		J		JD	P	P
29	B	P	J	E		JD		J		JD	P	P
28	B	P	J	E		JD		J		JD	P	P
27	B	P	J	E	G	JD		J		JD	P	P
26	B	P	J	E	G	JD		J	G	JD	P	P
25	B	P	J	E	G	JD		J		JD	P	P
24	B	P	J	E		JD		J		JD	P	P
23	B	P	J	E		JD		J		JD	P	P
22	B	P	J	E		JD		J		JD	P	P
21	B	P	J	E		JD		J	G	JD	P	P
20	B	P	J	E		JD		J		JD	P	P
19	B	P	J	E	G	JD		J		JD	P	P
18	B	P	J	E	G	JD		J		JD	P	P
17	B	P	J	E		JD		J		JD	P	P
16	B	P	J	E		JD		J	G	JD	P	P
15	B	P	J	E	G	JD		J		JD	P	P
14	B	P	J	E		JD		J		JD	P	P
13	B	P	J	E		JD		J		JD	P	P

Size
L=81m
l=42m
S=3402 m^2
Interline= 3.5m
Interplant = 1.5m

Variety
Braeburn = B
Pinova = P
Jonagold = J
Elstar = E
Golden = G
Jona Decosta = JD

Design
injected in 2020
injected in 2021

12	B	P	J	E		JD		J		JD	P	P
11	B	P	J	E		JD		J	G	JD	P	P
10	B	P	J	E		JD		J		JD	P	P
9	B	P	J	E		JD		J		JD	P	P
8	B	P	J	E		JD		J		JD	P	P
7	B	P	J	E		JD		J	G	JD	P	P
6	B	P	J	E	G	JD		J		JD	P	P
5	B	P	J	E		JD		J		JD	P	P
4	B	P	J	E		JD		J		JD	P	P
3	B	P	J	E	G	JD		J	G	JD	P	P
2	B	P	J	E	G	JD		J		JD	P	P
1	B	P	J	E		JD		J		JD	P	P

South east

Supplementary Figure 3. Apple orchards characteristic and design of experiment for two years (2020 and 2021).

Supplementary Figure 4. Trunk injection modalities: ENDOkit Manual PRO (upper left), removal of the outer bark layer (upper right), plug insertion (bottom left), insertion of the needle in the plug and injection (bottom right).

Supplementary Figure 5. Chemically “burned buds” appearing on high volume modalities.

Supplementary Figure 6. Degree of bark-cracking on different trees. 1) Little to no bark-cracking 2) Mild bark-cracking 3) Severe bark-cracking.