Tree-injection: field-trial application of cinnamon essential oil as bio-insecticide in fruit arboriculture

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Abstract

Apple production is one of the biggest fruit business worldwide, estimated at 84.7 million tons in 2016. The Rosy Apple Aphid (*Dysaphis plantaginea*, Passerini) is amongst the most detrimental pest in apple orchard. Due to its rapid life-cycle and several generations of offspring per season, its resistance to insecticides is ever increasing and orchard sprayings have to be repeated several times to achieve limited impact on fruit production yield, causing damage to the environment and non-target organisms.

This project aims the development of a cinnamon (*Cinnamomum cassia*, J. Presl) essential oil-based biopesticide to deal with the Rosy Apple Aphid using tree-injection as the application method. It is part of the SPW "Tree-injection" project. This work evaluated the feasibility of implementing the technique in a 15 years old apple trees orchard in Gembloux (Belgique).

Product's formulation consisted in cinnamon essential oil with Tween80 as emulsifier and EDTA.

40 apples trees (*Malus domestica*, Borkh) of the Jonagold cultivar were followed during the experiment. The trees were divided into 8 blocks of 5 trees that were injected with 3mL of a different solution: one blank using only water [BSA], one blank with emulsifier [BEM], one cinnamon EO emulsion at 1% [CEO1], one cinnamon EO emulsion at 2% [CEO2] and one untreated blank tree [NEG]. Apple trees were injected on the 19th May 2020 and were followed until the 16th of June 2020. During this period, Aphids' and other insects' (pollinators, predators, parasitoids) populations were followed once a week. 3 days, 7 days, 14 days and 28 days after the injection time, apple trees' emissions of Volatile Organic Compounds were sampled on Tenax TA Cartridges. As a global health index, chlorophyll fluorescence (Fv/Fm) was measured at 5 different places on each tree.

Air samplings were analyzed through TDU-GC-MS for VOCs profile determination and cinnamon compounds detection. 55 compounds including terpenoïds and biological airborne signals were identified and analyzed using PCA and PermANOVA statistical analysis for detection of variation related to the treatment. Chlorophyll fluorescence measures were analyzed using repeated measures ANOVA, with Dunnett and Tukey tests for multiple comparison. Insects population monitoring were analyzed by Generalized Linear Model ANOVA and Fisher or Chi-square test.

Results showed actual emission of E-cinnamaldehyde from treated trees. No impact of the treatment on the trees' health index was detected. Significant differences were spotted in terms of VOCs emissions by the overall trees but could not be linked to a particular treatment. Aphids populations tended to increase in size on [NEG] trees but with a smaller number of colonies per tree where [CEO2], [CEO1], [BSA] and [BEM] tended to have smaller size colonies in higher number, indicating a response of the aphids to the injection.

Globally, the results are conclusive in terms of the efficiency of the treatment, no observed phytotoxicity on apple trees, cinnamon constituents' emissions and adaptability of the method in agronomic conditions.

Key-words: Tree-injection, essential oil, rosy apple aphid, *Malus domestica*, volatile organic compounds, orchard trial

Résumé

La production de pommes est une des plus importantes productions fruitières au monde, estimée à 84.7 millions de tonnes en 2016. Le puceron cendré du pommier (*Dysaphis plantaginea*, Passerini) est un des ravageurs les plus dommageables en verger de pommiers.

Ce travail fait partie du projet « Tree-injection » du SPW agriculture, visant la mise en place d'un traitement insecticide à base d'huile essentielle de cannelle (*Cinnamomum cassia*, J. Presl) appliquée par endothérapie pour lutter contre le puceron cendré du pommier. L'objectif est ici d'évaluer la faisabilité et l'efficacité de la technique dans un verger de pommiers de 15 ans situé à Gembloux (Belgique). La formulation du bio-pesticide est constituée d'huile essentielle de cannelle mise en solution avec du Tween80 et d'EDTA.

40 pommiers (*Malus domestica*, Borkh) de variété Jonagold ont été suivis durant l'expérience. Les arbres ont été divisés en 8 blocs de 5. Des ports d'injection ont été creusés dans 4 arbres sur 5 qui ont été injectés avec 3mL de différentes solutions : un « blanc » avec de l'eau [BSA], un « blanc » Tween 80 [BEM], un cannelle à 1% [CEO1], un cannelle à 2% [CEO2] et un « témoin » non traité. Les injections ont été réalisées le 19 mai 2020 et l'expérience a pris fin le 16 juin 2020. Pendant ce temps, les populations de pucerons et d'autres insectes (prédateurs, parasitoïdes, pollinisateurs) ont été suivies une fois par semaine. 3, 7, 14 et 28 jours après l'injection, des échantillonnages des émissions de Composés Organiques Volatiles par les pommiers ont été réalisés et la fluorescence chlorophyllienne (Fv/Fm) a été mesurée à 5 endroits sur chaque arbre en tant qu'indice de santé.

Les échantillons d'émissions ont été passés en TDU-GC-MS afin de déterminer le profil en COVs et de détecter des composés de cannelle. 55 composés ont été identifiés dont plusieurs terpènes et plusieurs signaux aériens d'importance biologique. Les profils ont été analysés via ACP et PermANOVA. Les mesures de fluorescence chlorophyllienne ont été analysées via ANOVA sur mesures répétées avec tests de Dunnet et de Tukey pour comparaisons multiples. Les suivis de populations d'insectes ont été analysés via ANOVA sur Modèle Linéaire Généralisé avec test de Fisher ou Chi-carré.

Les résultats ont montré une émission de composés de cannelle à partir des arbres traités. Aucun impact du traitement sur la santé des arbres n'a été détecté. Des différences significatives ont été observées en termes d'émissions de COVs par l'ensemble des arbres mais aucune n'a pu être liée à un traitement en particulier. Les populations de pucerons ont augmenté en nombre sur les arbres [NEG] avec un plus petit nombre de colonies par arbre alors que les colonies sur [CEO2], [CEO1], [BEM], [BSA] comptaient moins d'individus mais étaient plus nombreuses, ce qui indique une réponse des insectes au traitement.

Globalement, les résultats sont concluants en termes d'efficacité du traitement et celui-ci ne montre aucun signe de phytotoxicité. Des composés de cannelle sont émis par les feuilles, montrant l'adaptabilité de la méthode en conditions agronomiques.

Mots-clé : tree-injection, huile essentielle, puceron cendré du pommier, *Malus domestica*, composés organiques volatiles, essai verger

Table of contents

List of figures

List of tables

Abbreviations

(M)ANOVA: (Multivariate) Analysis of Variance

DAT: Day After Treatment

DF: Degree of Freedom

DHS: Dynamic Head Space

E-cinn: Trans-cinnamaldehyde or E-cinnamaldehyde

EO: Essential Oil

GC-MS: Gas Chromatography – Mass Spectrometry

GLM: Generalized Linear Model

GLV: Green Leaf Volatiles

HIPV: Herbivorous Induced Plant Volatiles

IPM: Integrated Pest Management

IT: Injection Time

LOD: Limit Of Detection

LOQ: Limit Of Quantification

PC(A): Principal Component (Analysis)

PSII: Photosystem II

Fv/Fm: Quantum efficiency of the PSII

p-value: probability value

RAA: Rosy Apple Aphid

RI: Retention Index

SAR: Systemic Acquired Resistance

SPW: Service Public de Wallonie

TD(U): Thermal Desorption (Unit)

(B)VOCs: (Biogenic) Volatile Organic Compounds

1 Introduction

The present work aims to assess the feasibility of insecticidal treatment on fruit trees using essential oils as active substance and tree-injection as application method. This work is part of the "Tree-Injection" project of the Walloon region (Belgium), funded by the SPW agriculture from the 01/09/2018 to the 31/08/2021. This Integrated Pest Management (IPM) technique lies on the known repulsive effects of some essential oils compounds towards insects and is part of a sustainable development strategy: reducing chemicals use, limiting the environmental hazard/health hazards of those chemicals and the creation of a range of high-quality fruits (apples).

1.1 Fruit production and pesticide use in arboriculture

In 2016, total apple production worldwide reached 84.7 million tons (FAOSTAT). This represents a gross production value of 45.8 billion US dollars (FAOSTAT). In 2018 in Europe (EU28), the total apple production accounted for 13.8 million tons. Belgium's production in the same year reached 231 300 tons of apples (EUROSTAT[1]). Unfortunately, the production yields of apple fruits are often disrupted by pest insects such as aphids, psyllas, beetles, moths (Jenser et al., 1999) or fungal or bacterial diseases (scab, fireblight, mildew,…) that result in important quality and production loss, thus leading to economical loss.

To deal with those numerous pests, the use of phytosanitary products has been widely spread since the 1950's (Isman, 2006; Valiuškaite et al., 2017). Nowadays, evidence suggests that the use of pesticides are responsible for a lot of side-effects: disrupting flora and fauna (Krebs et al., 1999), causing health risks (Lee et al., 2004), increasing resistance of target pests and affecting water, air and soil quality (Moss, 2008). It has been demonstrated that alternatives to pesticides have to be found. Development of organic agriculture and integrated pest management (IPM) are direct answers to this need of solutions (Sauphanor et al., 2009; Campos et al., 2019).

1.2 Apple orchards' insect pest: the Rosy Apple Aphid

In this thesis, one major pest in fruit production is considered: the rosy apple aphid (*Dysaphis plantaginea*) infesting apple trees (*Malus domestica*).

1.2.1 Rosy Apple Aphid's ecology and life cycle

Dysaphis plantaginea, Passerini, 1860 (Hemiptera: Aphidæ) is among the most detrimental pests in apple orchards in Europe (Albert et al., 2017). Its presence is problematic in both organic and conventional orchards due to its low abundance threshold for economic damage (Albert et al., 2017). Aphids cause damages on fruit, reducing their size and deforming their shape. They also reduce the overall tree vigor due to phloem sap sucking, organ deformation, chlorosis, leaf fall and they enhance the development of sooty mold on the honeydew they secrete (Rousselin et al., 2017). Rosy apple aphids are a specialized host-alternating (dioecious) species with apple (*Malus domestica* Borkh) as primary or winter host and plantain (*Plantago* spp) a secondary host plant (Blommers et al., 2004). Their development on apple trees alters fruiting and shoot development (Brown et al., 2007; Rousselin et al., 2017). Their feeding results in curving of leaves that renders the Rosy Apple Aphid (RAA) very difficult to monitor. Yet, for chemical control to be effective, it must be applied before the formation of curled leaves (Brown et al., 2007).

Aphids reproduce by parthenogenesis: the embryo develops from an unfertilized egg cell. For *D. plantaginea* however, the life-cycle is qualified as holocyclic (Figure 1): parthenogenetic generations are interrupted by a generation of sexual reproducers in autumn (Rousselin et al., 2017). This generation lays fertilized overwintering eggs on apple trees that hatch in early spring (Brown et al.,

[1] https://appsso.eurostat.ec.europa.eu/nui/submitViewTableAction.do accessed on 02/04/2020

2007) and give rise to several generations of wingless larviparous female aphids (fundatrigeniæ) which feed on the underside of young leaves, causing damages (Blommers et al., 2004). The portion of winged individuals increase within the successive generations and from late June, winged females move to their herbaceous secondary plants. There, females give birth to winged sexuparæ and males. Winged sexuparæ then go back to apple trees to lay winged female eggs. The larvæ spend the autumn on the apple tree, causing damages to leaves and altering fruiting. Before the cold season, the males mate with the adult sexual females which lay eggs that will overwinter on apple trees (Bonnemaison, 1959).

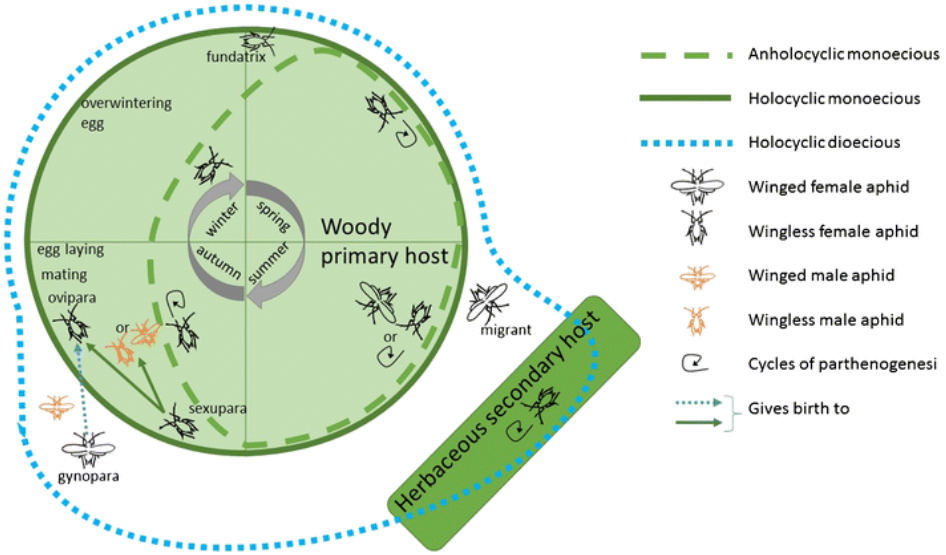


Figure 1: Variants of fruit tree aphid life cycle. The cycle of D. plantagniea is the blue dashed line. (Rousselin et al., 2017)

Nowadays, several 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 and (iv) development of aphid-resistant cultivars (Nicholas et al., 2005; Miñarro et al., 2008; Albert et al., 2017). Aphids' behavior is also very much influenced by the emission in the air of volatiles compounds 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' repellency, as a "run for your life" signal or as an attractant for aphids' natural predators (Francis et al., 2005; Vandermoten et al., 2012).

1.3 Use of essential oil as biopesticide

As said previously, concerns regarding environmental damages caused by pesticides have largely grown in recent years. Worldwide pesticide use has been estimated at 2.5 million tons each year and damage caused by this indiscriminate use of various products reaches $100 billion annually (Koul et al., 2008). Safe and eco-friendly biopesticides ("Green pesticides") are being developed and are expendably emerging on the market. These products are allowed as external inputs in organic production as "natural or naturally-derived substances" (EC No 834/2007[2]). This material can be of botanical origin such as pyrethrum, rotenone, neem, ryania, nicotine, sabadilla,… and can be used against a wide range of insects or fungi (Isman, 2006). It can also be constitued from microorganisms e.g. *Bacillus thuringiensis*-based products as general bioinsectide (Isman, 2000), *Streptomyces avermitilis*'s metabolites against plant parasitic insects, *Streptomyces aureus* that displays very low mammalian toxicity but highly effective insecticidal effect, *Metarrhizium anisopliæ* against

[2] ("EUR-Lex - 02007R0834-20130701 - EN - EUR-Lex," April-2-2020)).

lepidopterous insects, etc (Dev et al., 1997). The practice of using botanical insecticides dates back at least two millennia in ancient China, India, Egypt and Greece. The use of botanicals in Europe and North America was first recorded more than 150 years ago (Industrial Revolution). The development of the major classes of synthetic chemical insecticides is attributed to the documented use of plant derivatives as biopesticides (Isman, 2006). Among those botanical "Green pesticides", essential oils are well represented. Essential oils have traditionally been used as bug-repellent, in embalmment rituals and to protect stored commodities (grain, legumes) principally in Southern Asia and the Mediterranean region (Bakkali et al., 2008). Their use goes back to the Middle Ages but the analysis of their chemical constituents and the scientific investigation of their biological activities have only widely spread since the 1990's and their contact and fumigant activities against a wide range of pests (insects, fungi) have since greatly attracted attention as an alternative to synthetic pesticides (Isman, 2000; Koul et al., 2008). All those efforts to produce environmentally friendly crop protection products are in the global goal of reducing the ecological and health hazard[3] of currently used chemicals while ensuring food security and global food production for an ever growing world population (Isman et al., 2011). It is however to be noted that despite the prove of efficacy of botanical insecticides, their use and market development is still facing several issues e.g. (1) low availability of the natural resources, (2) standardization and quality control and (3) registration. Those issues still make botanical pesticides less attractive than synthetic chemicals where there is no scarcity of resources and where (2) and (3) are less of a problem as they have already been thought through since long (Isman, 1997; Isman, 2006; Isman et al., 2011; Campos et al., 2019).

1.3.1 Essential oil role and biosynthesis in plant

Essential oils are a complex mixture of volatile lipophilic compounds or volatiles organic compounds (VOCs). These VOCs are produced as secondary metabolites in plants and their extraction results in what is called the plant's "essential oil" (EO). The EO is not present as such within the plant, it is the extraction and the mix of the produced VOCs that formulates an EO. Some of these volatile compounds also do not exist as such within the plant as they may undergo structural modifications during their extraction. Essential oils are constituted of around 20 to 60 different substances of which 2 or 3 are major compounds found in high concentrations (Senatore, 1996; Russo et al., 1998; Burt, 2004) while the rest is typically present at trace levels (Campos et al., 2019). However, it seems that comprehensive GCxGC analysis allows to identify over a hundred compounds in some oils, up to 300 according (R Gomes da Silva et al., 2008). These oils are responsible for a plant's scent or taste and are thus widely used in the perfume and food industries (Isman, 2000). Nowadays, around 17500 aromatic species occurring in higher plants are recorded amongst 3000 known essential oils (Bakkali et al., 2008). However, the ability to elaborate VOCs is distributed to a limited number of higher plants families e.g. Myrtaceæ, Lauraceæ, Rutaceæ, Lamiaceæ, Asteraceæ, Apiaceæ, Cupressaceæ, Poaceæ, Zingiberaceæ, and Piperaceæ (Enan, 2001).

Essential oils play a capital role in plants' life cycle, in particular in the plant's interactions with its environment. The strong odor arising from plants are responsible (1) for attracting pollinators and seed disseminators (2) for the plant thermotolerance and (3) for the plant defense against pathogens and herbivores (Koul et al., 2008; Nagegowda, 2010; Pavela et al., 2016; Campos et al., 2019).

Essential oils constituents can be classified in two chemical groups according to their biosynthetical origin (Figure 2): (1) phenylpropanoïds and their aromatic derivatives (alcohols, ketones, aldehydes,…) and (2) terpenoïds divided into mono- and sesqui- terpenes (Bakkali et al., 2008). The various constituents can vary in concentration according the harvesting time of the plant and the region where it grows (Senatore, 1996).

[3] Attention, despite their low environmental impact, some biopesticides are no less dangerous. Further information will be given in 1.3.3 section.

Phenylpropanoïds are low molecular weight compounds and their functions are as diverse as their structural variations. They serve as antibiotics, pigments, UV protectants, insect repellents, signal molecules but are also present in more complex structures such as suberin, lignin or cell-wall components. They are largely distributed in dicot plants and in various individual plant organs (Hahlbrock et al., 1989). Aromatic compounds such as aldehyde (cinnamaldehyde), alcohol (cinnamic alcohol), phenols (eugenol) are derived from phenylpropane (Bakkali et al., 2008).

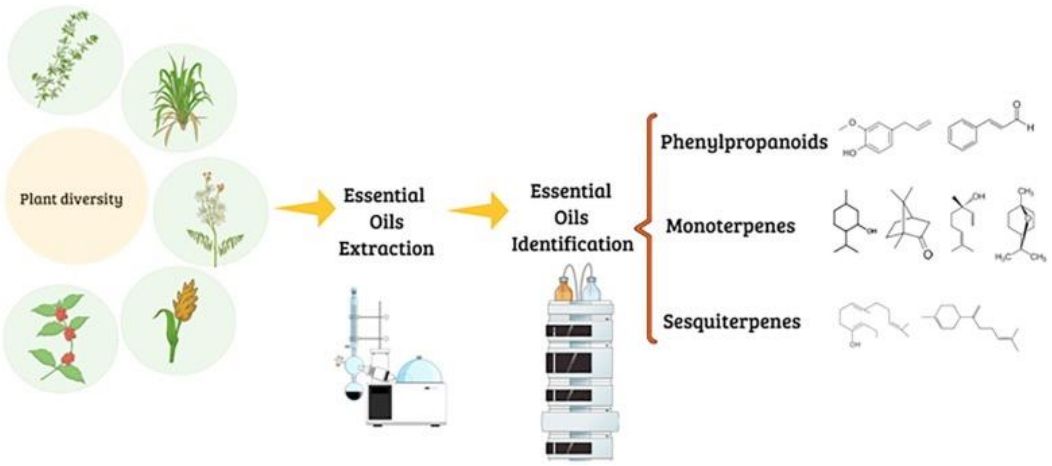


Figure 2: Global process of essential oils extraction and identification. (Campos et al., 2019)
NB: GC-MS apparatus are largely preferred over HPLC for EOs identification, unlike the scheme suggests.

Mono- and sesquiterpenoidal essential oil constituents are formed by the condensation of isopentenyl pyrophosphate units (Koul et al., 2008). Monoterpenes are synthesized via the methyerythritol phosphate pathway which takes place in the plastids. Sesquiterpenes synthesis takes place in the cytosol via the mevalonate pathway (Nagegowda, 2010; Campos et al., 2019). The terpenoïds' function in the essential oils will be further developed in section 1.3.3.

To identify these numerous compounds, Gas Chromatography (GC) using Flame Ionization Detector (FID) or Thermal Conductivity Detector (TCD) has been used for several years. Nowadays, Gas Chromatography coupled with a Mass Spectrometer (GC-MS) is the most common apparatus for EO analysis (Mossa, 2016). Analysis of EO will be further discussed.

1.3.2 Extraction and application methods

Essential oils are found in many plant organs e.g. flowers (bergamot), leaves (eucalyptus), barks (cinnamon), woods (rosewood), roots (vetiver), rhizomes (ginger), fruits (star anise) and seeds (nutmeg) (Enan, 2001; Bakkali et al., 2008). The most used technique for essential oils extraction is hydrodistillation or steam-distillation (Enan, 2001; Koul et al., 2008; Maes et al., 2019). Cold expression can also be employed for citrus fruits (Maes et al., 2019). It is to be noted that the extraction technique will interfere with the essential oil's chemical profile in both the number of molecules and the stereochemical types of the extracted molecules. The extraction method will thus also change according to the purpose of the use of a specific essential oil. Other innovative techniques include the use of liquid carbon dioxide, microwave extraction, low or high pressure distillation, solvent extraction (using lipophilic solvent) and even supercritical carbon dioxide or subcritical water extraction (Bakkali et al., 2008; Mossa, 2016). Although these techniques allow the obtaining of the same fraction of compounds, the products cannot be called essential oils as the international standard (ISO 9235[4]) reserves the term for products obtained via conventional methods (e.g. steam- or hydro-

[4] ISO9235 definition of an essential oil: product obtained from a natural raw material of plant origin, by steam distillation, by mechanical processes from the epicarp of citrus fruits, or by dry distillation, after separation of

distillation). Products obtained using other techniques should therefore be referred to as "plant extract" (Maes et al., 2019).

Application of essential oils as crop protection product is as varied as existing techniques either in IPM or conventional agriculture. Nevertheless, some problems still have to be resolved, such as optimizing plant growing conditions and extraction process in order to obtain homogeneous chemical composition, simplify the authorization requirements and stabilizing EO emulsions for optimized relief and efficiency. New technologies such as micro-encapsulation or nanotechnology might bring solutions to this last issue (Pavela et al., 2016). These techniques involves (i) emulsifications in droplets or capsules, (ii) coacervation (iii) spray drying, (iv) complexation, (v) ionic gelation, (vi) nanoprecipitation or (vii) film hydration. All these encapsulation methods allow an improvement of the products shelf-life and activities and allow slower compounds release (Maes et al., 2019). Spraying can be performed but given the volatility of essential oils compounds, the persistence of product is not always achieved and applications must be repeated. On the other hand, differences in constituents resulting in different physical properties of mixture of compounds may allow more deployment or longer persistence (Rattan, 2010). Moreover, the low persistence can be interesting as predators or parasitoids that would reinvade a treated crop would not be harmed (Isman, 2006). Some oils are used as traps or repellents, other as systemic insecticide (in tree-injection e.g.), some are used combined with other oils or even with synthetic pesticides. In a general manner, essential oils are use as contact or fumigants insecticides (Enan, 2001; Ikbal et al., 2019; Campos et al., 2019).

1.3.3 Biological activity of essential oils

Most of previous research regarding essential oils as potential plant protection products have identified the principal ecological activity of EO: (i) for virus and microorganisms: Antiviral, Antifungal and antimicrobial. (ii) to 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 (Isman, 2006; Koul et al., 2008; Rattan, 2010; Regnault-Roger et al., 2012; Mossa, 2016; Campos et al., 2019). However, despite the knowledge available on this subject, modes of action are in general not fully elucidated (Rattan, 2010; Campos et al., 2019).

First, it is interesting to note that a plant EO's ecological activity is not determined by its major compounds but rather by the complex mixture of all its constituents. Indeed, individual constituents rarely account for a major share of the EO's toxicity. Several studies suggest a synergistic effect among constituents: e.g. terpenes act as solvents to facilitate other toxins' passage through membranes (Rattan, 2010). In the same way, antagonistic effects are also observed that will be further explained. Oil chemical composition, molecular weight, entry zone of the toxin and functional groups also play a key role in their biological activity (Bakkali et al., 2008; Isman et al., 2011; Mossa, 2016). Successful plant species often show an EOs profile composed with either several moderately toxic compounds or a few highly toxic molecules (Rattan, 2010). In addition, EO's composition and therefore activity changes according the organs in which it is produced. Actually, some EO constituents (e.g. anthocyanins, monoterpenes) can act as attractants for pollinators when located in the flowers but may be insecticidal and antimicrobial when present in leaves. This allows the plants to have an impact on beneficial individuals and to be protected from herbivorous at the same time (Eisner et al., 2000; Rattan, 2010).

As far as insects are concerned, the identified mode of action mainly consists in the disruption of neurological pathways by monoterpenoïds (e.g. D-Limonene) which often leads to insect hyperactivity followed by rapid knock down and immobilization (Enan, 2001). Those pathways are very similar among the animal kingdom, allowing essential oils to apply as broad-spectrum insecticides. In

the aqueous phase — if any — by physical processes. ("ISO 9235:2013(en), Aromatic natural raw materials — Vocabulary," April-17-2020)

addition, insect resistance to those numerous compounds and complex effects is very rarely achieved or takes at least several dozen of generations (Isman, 2006; Rattan, 2010). Neurological toxicity is achieved by essential oil compounds disrupting (i) GABA-gated chlorine channel blocked by monoterpenes, leading to hyper excitation and death of insects; (ii) the Acetyl Choline Esterase (AChE) pathway where EO can act as a reversible competitive inhibitor, leading to ataxia; (iii) Octopamine (OA) a neurotransmitter (similar to norepinephrine in vertebrates) which is blocked by some EO compounds and (iv) in mitochondrial system by blocking enzyme (ATPase) or protein phosphorylation (H-ATP proton pump) (Isman, 2000; Isman, 2006; Rattan, 2010; Mossa, 2016).

In addition to those direct insecticidal effects and thanks to their non-specific effects on a wide range of molecular targets (Bakkali et al., 2008), EO and their constituents basically affect primary biochemical processes, disrupting insects' endocrinologic balance or behavior. Molecular targets can be proteins, enzymes, membrane, vital components (e.g. secondary metabolites) and nucleic acids. Those compounds act at sub-lethal concentration as deterrent (e.g. inhibiting food ingestion, egg laying), repellent or morphogenesis disruptors (Rattan, 2010; Isman et al., 2011; Mossa, 2016). Insecticidal effects of a wide range of EO's compounds (eugenol, camphor, linalool, cinnamaldehyde, carvone,…) on various insect families have been assessed by a great number of studies in the past years (Lindberg et al., 2000; Amer et al., 2006; Abramson et al., 2007; Regnault-Roger et al., 2012; Campos et al., 2019) and particularly on aphids (Isman, 2000; Ikbal et al., 2019), which is the main pest considered in this study. A review from (Nerio et al., 2010) displays a list of plant essential oils showing high repellency to arthropod species.

In bacteria and fungi, the activity of essential oils is associated with their ability to disrupt the cell walls and membranes, leading to cell lysis. EO can also coagulate the cytoplasm and damage lipids and proteins (Bakkali et al., 2008; Koul et al., 2008; Campos et al., 2019). Activity against DNA or RNA virus was also demonstrated (Bakkali et al., 2008). Some essential oils also display photoactive molecules that are cytotoxic and mutagenic under UV exposure (Bakkali et al., 2008). Some studies also highlighted the phytotoxicity of EO, suggesting their potential use as bioherbicides (Isman, 2000; Abd El-Gawad et al., 2016; Hazrati et al., 2017; Khare et al., 2019). EOs cause disturbance in the plants' membrane integrity, leading to oxidative stress of the plant. This stress is linked with electrolyte leakage (Hazrati et al., 2017) and an increase in proline and lipid oxidase content (Reactive Oxygen Species (ROS), molecules involved in plants defense). EOs were also proved to cause global plant growth inhibition as the alteration of the membrane influences all other physical and biochemical processes. In addition, sesquiterpene alcohols are strong inhibitors of protein synthesis (Khare et al., 2019). Seedling and germination were also demonstrated to be impacted by EOs application. The EOs allelopathic activity could thus be used as an ecofriendly herbicide (Abd El-Gawad et al., 2016). Cinnamaldehyde, which is intended to be injected into apple trees in this study, is a compound known for its phytotoxicity (Cloyd et al., 2002). It is noteworthy that the most efficacious EO against insects are often the most phytotoxic. This of course requires attention when using EO for plant protection product formulation. The plants' health can be monitored using the quantum efficiency of the photosystemII (PSII) ratio (Fv/fm) as a health index (Percival et al., 2005a; Dias, 2012). Indeed, damage to the PSII is often the first manifestation of stress in a leave (Maxwell et al., 2000). Assessing its efficiency is an easy, non-invasive measurement and a commonly used technique in plant physiology to understand the photosynthetic mechanisms but also to indicate a plant's response to environmental change (Murchie et al., 2013). This measure consists in evaluating the proper conduct of the photoexcitation of the chlorophyll. Basically, the sunlight turns chlorophyll molecules into an excited configuration. In normal conditions (healthy plant), the energy of this configuration is lost after a short period of time, mostly through the photochemical process (absorbed light energy in excess is lost through non-photochemical processes). In case of biotic or abiotic stress experienced by the plant, a greater part of this energy is dissipated in non-photochemical processes, often in the form of heat and re-emission of infra-red and red/far red radiations. This last form of energy dissipation is called chlorophyll fluorescence (Maxwell et al., 2000; Murchie et al., 2013). The ratio between the

variable fluorescence (Fv) and the maximal possible value for chlorophyll fluorescence (Fm) which are measured using specific apparatus such as the Handy PEA fluorimeter (Hansatech Instruments) have been demonstrated to be a robust indicator of the quantum efficiency of the PSII that can be related to the global health of the plant. Unstressed leaves display Fv/Fm values of ±0.83 and the ratio value decreases along with the stress undergone by the plant. Leaves showing a Fv/Fm value of 0.3 are considered dead (Murchie et al., 2013)

It is also interesting to note that EO, in spite of their known beneficial aspects for sustainable pesticide formulation, are not less dangerous than synthetic chemicals. To cite Paracelsus: *"The dose makes the poison"*. The growing interest of EO 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 (Stroh et al., 1998; Isman et al., 2011; Campos et al., 2019). Still, purified compounds such as some terpenoïds can be moderately toxic to humans with rat acute oral LD50 value of 0.150-3g/kg (e.g. carvacrol, pulegone) but the oil itself, containing this compounds at low concentrations and amongst lot of other constituents, is far less toxic (Isman, 2000; Isman, 2006; Mossa, 2016). Anyway, most EOs raise no concerns regarding their agricultural use in the commonly used concentrations or doses and still show lower impacts than synthetic insecticides or other botanical insecticides (Stroh et al., 1998; Isman, 2000; Pavela et al., 2016).

Last but not least, it has been shown that the application of essential oils can impact the soil's microorganisms and enzymes either positively (stimulating effects, increased microbial population) or negatively (inhibitory effects) (Campos et al., 2019; Khare et al., 2019). Reduction in soil microbial activity was indeed observed after EO spraying, which is consistent with their antibacterial activity. On the other hand, this spraying enhanced the activity of β-glucosidase and alkaline phosphatase in soil, letting appear negligible effects on soil biological activities (Khare et al., 2019). Nevertheless, data is still lacking in this matter (Campos et al., 2019) and to mistakenly believe that since botanical pesticides are obtained from natural sources, they are therefore 100% safe and are always safer than synthetic pesticides has led to a lack of attention in this particular domain. Thus, some studies want to raise awareness about the lack of papers concerning toxicological data and the effects on non-targets organisms: selectivity among invertebrates is not well documented and beneficial arthropods such as natural predators of pests or pollinators are left apart (Pavela et al., 2016; Campos et al., 2019). In particular, honeybees are already endangered by the intensive use of plant protection products and they also appear somewhat susceptible to the application of essential oils (Isman, 2000; Lindberg et al., 2000; Abramson et al., 2007).

1.3.4 Cinnamon essential oil

The essential oil considered in this very project is extracted from the bark of the plant *Cinnamomum cassia* (J.Presl). This oil was selected earlier in the project amongst several others after a literature search and pre-test on aphids in artificial medium. Aphids showed mortality at concentration of 0.05%, 0.1% and 0.2% of cinnamon essential oil in the medium (Tree-injection report, 2019).

Apart from those results obtained in the project, cinnamon extracts have been widely used since ancient age as a spice but also for many medicinal properties such as anti-inflammatory, analgesic, gastritis and dyspepsia treatments or yet against blood circulation disturbance (Wang et al., 2009; Geng et al., 2011; Gunawardena et al., 2015). Similarly, cinnamon essential oils is used in a lot of fields (medicinal, food and cosmetic additives) due to its known antifungal, antioxidant and antibacterial properties (Geng et al., 2011; Jardim et al., 2018). Most recently, its use as a bio-pesticidal product gained more and more interests and most particularly the effect on insects, fungi and bacteria of its main compound: E-cinnamaldehyde (Burt, 2004; Lee et al., 2008; Wang et al., 2009; Chang et al., 2013; Ojaghian et al., 2016; Jardim et al., 2018). It was even investigated as acaricidal for house use against House Dust Mite (Oh, 2011). This feature is promising but must be carefully considered, especially as a house product, as cinnamon also displays allergenic properties (Tremblay,

2008; Ackermann et al., 2009; Isaac-Renton et al., 2015). Its use as a plant treatment product must also be cautiously thought as E-cinn displays phytotoxic effects (Cloyd et al., 2002).

1.4 Tree-Injection technique

Tree- (or trunk-) injection is a method to apply chemicals directly into the tree's vascular system. The chemicals are injected by piercing the bark to access the xylem and are then systemically distributed via the xylem sap. This allows optimal pests targeting while reducing pesticide inputs and environmental exposure (Doccola et al., 2012; Coslor et al., 2019).

1.4.1 Historical evidence

First records for the development of fruit tree injection in agriculture apparently goes back as early as the 12th century (writings from Hadj de Granade (1158) cited by Ibn al-Awwam in 1864). Leonardo da Vinci also worked on tree-injection in the 15th century: he studied the delivery of poisonous cyanide compounds from the trunk to the fruits. Tree-injection for plant protection and control of insects as we intend here was first reported in the London's "The Orchards and Garden" magazine of 1602 (Aćimović, 2014). However, complete study and development of tree-injection technique came a bit later, at the beginning of the 20th century, with the arising of invasive and new pathogens and insects that could not be dealt with using sprays. Among the pioneers in tree-injection for plant protection, we can cite the work of (Roach, 1939) for the cure of physiological disorders in fruit trees and the work of (Rumbold, 1920), who experimented injection of salicylic acid on chestnut trees. With the arrival of pesticides in the late 1950's, the technique evolved as a successful pest controlling method. (Harries, 1965) successfully controlled several pest insects using trunk-injection of pesticides and particularly the Pear psylla using Bidrin©. For the last 50 years, a lot of research has been carried out regarding tree-injection for insect or disease control in various tree species (Doccola et al., 2012; VanWoerkom et al., 2014; Aćimović, 2014).

1.4.2 Current use

Tree-injection is used as an alternative to conventional airblast sprayers for delivering crop protection products. Although it is not yet widespread, the technique has made its worth in successfully protecting various tree species from various pests and particularly in urban areas where sprays cannot be applied. The most used compounds in tree-injection techniques are biopesticides such as Azadirachtin® (Neem extract), compounds classed as "Reduced Risks"[5] such as Imidacloprid® (Imajet), Penthiopyrad® (Fontelis), compounds classed as "Organophosphate alternative" such as Emamectin Benzoate® (Tree-age) and other conventional chemicals specially designed for tree-injection such as phosphites, imazalil, penconazole, pyrifenox, phosphonate and carbendazim (Percival et al., 2005a; Schulte et al., 2006; Mcmahon et al., 2010; Aćimović, 2014; VanWoerkom et al., 2014; Flower et al., 2015; Coslor et al., 2019). Regarding apple pest management, tree-injection has been limited primarily to disease control and lacks extensive research on insect pest management (VanWoerkom et al., 2014).

The insecticide used for tree-injection have more or less the same effects on insects than the previously explained essential oils. Imidacloprid® is a neonicotenoïd that acts as a nerve poison after contact or ingestion by binding to the nicotinic receptor thus blocking the acetylcholine receptors resulting in paralysis and eventual death. Emamectin benzoate® is an avermectin effective by ingestion that also acts as a nerve poison by binding to GABA-receptors (VanWoerkom et al., 2014). Given this information, we can get a sense that EOs should work to effectively control insects in apple trees. There is however no previous study regarding the use of EOs for pest management using tree-injection techniques.

[5] By the United States Environmental Protection Agency (EPA) https://www.epa.gov/

The injection practice in itself varies a lot according to the device used, the compound injected and the tree species, age, health and vascular system. The Figure 3 shows various tree-injection devices.

Figure 3: Various tree-injection techniques. From left to right: (i) Drilled hole injector (Moore Tree Care), (ii) Injection system without drilled holes, (iii) No pressure system (Kuhns, 2011)

Most of the injection techniques require the delivery of a small amount of a given compound per injection called "micro-injections" or "micro-infusion". Micro technology allows to improve plant safety and reduce the application time but greater amount of substances can be injected as well without trouble. For effective protection, the injected compounds must be translocated from the injection site to the active site (insect feeding or disease infection site). Different devices are available according to the targeted trunk tissue e.g. xylem or cambium targeting trunk injection device. Most devices deliver active ingredients into the xylem. Once in the xylem, chemicals are either dependent on the tree's transpiration stream to move upward and to be distributed in the canopy ("infusion") or on the external pressure applied thanks to the injection device (compressed air, CO2). Devices are thus separated in two classes: active or passive, whether it requires external (high or low) pressure or not, respectively. Active pressure is usually about 6-110 psi (0.4-7.6 bar). Devices can also be classified based on the type of injection port: (i) needle insertion (lenticular injection) (ii) drill-based insertion. In this case, the port is sealed by the feeder tube or by a plug when the treatment is not applied. The plug technology allows to repeat treatments in the same injection site from year to year. (iii) Microcapsule trunk-injection which consists to drill a hole to place a microcapsule that will release the compound. In all cases, injection ports are perpendicular to the trunk's axis or (rarely) at a 45° angle (Percival et al., 2005a; Kuhns, 2011; Doccola et al., 2012; Aćimović, 2014; Wise et al., 2014; Huang et al., 2016). The last main parameter to assess is the compounds' concentration and the volume of the injection delivery. This is of course according to the device and product used in the operation. The most common parameter to determine the concentration of active ingredient to inject is based on the trunk-diameter e.g. 0.17g a.i. per centimeter trunk diameter for treatment of the litchi stink bug using azadirachtin (Schulte et al., 2006), 0.25g a.i. per 2.5 cm of girth in a micro-capsule fungal treatment (Percival et al., 2005a), 1 mL of compound per 2.54cm of DFH[6] at a rate of 0.2g of a.i. per 2.5cm of DFH for insecticide delivery in apple trees (VanWoerkom et al., 2014; Wise et al., 2014), 30mL on insecticidal solution for olive tree (Huang et al., 2016). The number of simultaneous injections, the hole dimension, the injection site and various abiotic or biotic factors are others key parameters for optimal treatment. Technical aspects regarding parameters that impact compounds translocation and distribution in the tree will be further investigated in the (1.4.5) section.

It is noteworthy that there are other techniques for getting chemicals into trees without spraying that can be used in the same perspective as tree-injection. Amongst those are (i) soil injection, which consists in directly injecting the chemicals into the soil for the roots to assimilate. (ii) soil drench, which is basically watering the soil around the tree with the chemical solution, again for the root to

[6] Diameter Foot High. Diameter of the trunk at one foot (30.48cm in non-retard units) above the ground.

uptake and (iii) trunk basal spray, where the chemicals are applied to the trunk base and is absorbed through the bark (Kuhns, 2011).

As far as insects are concerned, adults feeding on the leaves or in the bark are the primary targets. But studies suggest that tree-injection can also significantly reduce the viable eggs of *Ommatissus lybicus* laid on trees (Ghani et al., 2017).

Another very interesting aspect is tree-injection for the purpose of baited traps for pest insects. Indeed, some insect species typically colonize physiologically-stressed or deciduous hosts. Those sick trees release stress-related volatiles such as acetaldehyde, ethanol, acetone or methanol. Lure-traps baited with those compounds do not seem to be effective. However, injecting sacrificial trees with those stress-related compounds was proven successful in attracting insect pests in a tree nursery. This could be part of a push/pull strategy: injecting valuable trees with repelling compounds and injecting martyr/sacrificial trees with those attracting compounds. The biggest interest of this technique is that it does not matter to harm or poison the injected tree as it is not the one of interest (Ranger et al., 2010a).

1.4.3 Advantages

Reports suggest that with pesticide application by spraying, as little as 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. In addition, to cope with this poor efficiency, growers tend to apply more pesticide than needed (VanWoerkom et al., 2014; Coslor et al., 2019). Those drifts are responsible for most of the pesticides environmental damage and workers health hazards. Pesticides are actually estimated to be responsible for 4% deaths from all accidental poisonings. This rate increases among poor farm workers or in less developed countries where individual protection equipment is lacking (Wise et al., 2014)

Today, tree-injection is a very promising alternative technique for chemical application with certain advantages over conventional airblast sprayers by reducing the drifts and environmental or health hazards, in particular when working with biopesticides or reduced-risks pesticides. Those advantages are listed hereunder:

1. Efficient use of chemicals. Firstly because the ingestion of the active ingredient (a.i.) by the target pest is far more effective than contact or fumigant activity. Secondly because lower amounts of chemicals are required for effective protection and thirdly because annual or even bi-annual injection were proved efficient for pest or control disease where conventional pesticide spraying requires up to 8 applications per season for apples in the US (Doccola et al., 2012; Wise et al., 2014; VanWoerkom et al., 2014; Huang et al., 2016; Ghani et al., 2017)
2. Reduced potential environmental exposure (Doccola et al., 2012; Huang et al., 2016).
3. Useful when soil and foliar applications are ineffective or difficult to apply e.g. for treatments of tall trees, in urban areas or in tight orchards where access with a sprayer is not possible (Percival et al., 2005a; Doccola et al., 2012)
4. Less affected by weather, although under certain circumstances, weather is an important factor for tree-injection efficacy as described in (1.4.5) section. In any case, tree-injection is less impacted by wind or rain than spraying is. Wind does not impact injection efficacy and rain does not cause wash-off of the previously sprayed chemicals (VanWoerkom et al., 2014; Huang et al., 2016).
5. Can have curative effects as the efficient pest treatment allows trees to recover from previous infestations or as the treatment is simply beneficial to the tree (Percival et al., 2005a; Flower et al., 2015).
6. Economical perspective. Effectively, tree-injection does not represent a considerable investment, uses considerably lower levels of a.i, requires less fuel (tractor and machine consumption), provides superior seasonal control resulting in improved yields and benefits,

allows users to access high value markets (organic farming or improved quality products) and tools maintenance is ridiculous. This would allow small-holder farmers of horticultural crops to increase their margin while reducing non-economic issues such as soil compaction, pest resistance, non-target effects, environmental impacts and health hazards that also account for economic losses on the longer term (Mcmahon et al., 2010; Wise et al., 2014; VanWoerkom et al., 2014)

7. Simple apparatus and ease of use (Percival et al., 2005a; Mcmahon et al., 2010).
8. Low residues of compounds in fruits (Doccola et al., 2012; VanWoerkom et al., 2014)

In addition, tree-injection compared to soil injection or soil drenching is more efficient in some cases (Doccola et al., 2012; Ghani et al., 2017; Coslor et al., 2019) but in a study regarding imidacloprid® injection in hemlock, it exhibited poorer product uniform distribution and final concentrations (Dilling et al., 2010).

1.4.4 Constraints

Tree-injection also have some disadvantages that are often related to its complex technical aspects. Indeed, the efficacy of the treatment depends on a lot of factors, sometimes fairly difficult to fully master. Poor efficacy often results from one or several technical breaches. This mainly concerns (i) the tree species (ii) the tree's health, (iii) the attributes of the chemical applied, (iv) the frequency of application, (v) the timing of injection and (vi) the injection technique. All these factors, when incorrectly applied or evaluated, can lead to disease infection, embolism, rot, decay, compartmentalization in the tree tissues, growth ring disruption, girdling, necrosis and chlorosis, added to incomplete pest protection that also greatly impacts the tree (Aćimović, 2014; Doccola et al., 2012; VanWoerkom et al., 2014). Those aspects are discussed in (1.4.5) section.

In addition to complexity, the main concerns regarding tree-injection is the wounding of the tree's roots, trunks or other limbs that can impacts the tree's health or longevity and fertility on longer term (Doccola et al., 2012; Aćimović, 2014). The lack of experience or the need for enhancing the ingestion exposure to the pest and improving the material distribution and longevity into the crop is also to be considered (Aćimović, 2014; Wise et al., 2014). Phytotoxic reactions are also to be feared in case of product misuse or wrong dosage (VanWoerkom et al., 2014).

It was also reported that current technology is not able to provide slow, continuous (controlled) release of the compound over time. The non-uniformity of the distribution of the chemical in the tree, leading to oversupply or undersupply of the product inside the canopy or the trunk, is also criticized (Aćimović, 2014). Previously reported minimal residues levels in fruits also question the global efficacy of the method: how to protect the production from fruit infesting pests when it is known that a low amount of substance effectively reaches the fruits (for direct fruit pest of course)? (VanWoerkom et al., 2014). Flower residues also questioned the impact of tree-injection to pollinators like honeybees.

A final concern is the extra time and effort required for effective treatment using tree-injection: equipment preparation, trunk diameter measurements, rate calculations,… Basically "single tree monitoring" that 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 (VanWoerkom et al., 2014).

1.4.5 Technical aspects

Understanding the technical aspects of tree-injection is crucial to obtain good results, without endangering the tree's health. Most of the information in this chapter comes from (Doccola et al., 2012; Aćimović, 2014; VanWoerkom et al., 2014). Methodology relies on the following points considered one at a time but in practice, they overlap one another. The main goal of all these aspects is to obtain an optimal compound distribution among the tree canopy and compound persistence (= good

pest management) while ensuring limited wounding due to the injection hole or pressure and limited health hazards due to the nature of the compound.

1. Tree anatomy and physiology. The anatomy of a tree trunk is made up of five layers. From the exterior to the interior are the bark, the phloem, the cambium, the xylem and the heartwood. The vascular system is composed of the phloem, cambium and xylem. Different species have a different physiology and the technique used should be accorded to the tree species ("species specific injection"). Xylem (sapwood) is the conductive tissue of plant. It is made up of cellulose, lignin and other substances. It consists in straw-like vessels, varying in type and size. For example, apple trees are classified as diffuse porous angiosperm. This type of wood contains both tracheids and vessels which are scattered among each other in each annual growth. The water conductance within the xylem itself varies, the most active conducting sections being the current year growth ring.
   Parenchyma cells, another conductive cell type, are able to transfer water laterally throughout the xylem. This property can help homogeneous compound distribution but is practically rarely significant.
   The daily water uptake rate is also typical for a tree species. The compound concentration must thus be subsequently adapted to the tree daily transpiration. For apple trees that use around 50 to 200 liters of water per day (depending on the weather, the tree's age and wealth), it is recommended to amend the compound delivery with water to decrease its viscosity and facilitate its transport through the xylem. On the other hand, compound dilution is not necessary for forest trees that can absorb up to 1000+ liters of water per day.
2. Timing of injection. It has to correspond with the tree's phenology and the pest development. Tree physiology will rule the uptake and translocation rate of the sap and therefore of the product as far as passive injection is concerned. Injecting before bud break stages exhibits limited movement in the trunk and in the branches only at the end of the season. Injection in May provided a significant degree of apple scab protection in the next two seasons (Percival et al., 2005a). Pear trunk injection of thiabendazol ®shortly before leaf fall resulted in fast distribution of compounds into branches and leaves. Translocation was reported to continue during the next season in the new canopy growth. The same injection in winter gave poor primary translocation and secondary was limited. In the same research paper, spring injections exhibited considerable primary distribution but deposit in young leaves was delayed (Aćimović, 2014)
   The injection also must correspond with the beginning of the pest development. Injecting too soon exposes the product to a decrease in concentration or efficacy (if persistence is not achieved) and injecting too late will not optimally prevent the pest damages. One's also need the typical uptake rate of the compound for optimal injection time determination.
3. The distribution of injected compounds. The injection site is the main influencing factor. Concentrations of the products are higher in the leaves from branches in the plane of the injection point (0°) than in the branches from the opposite side (180°). Leaves from branches at a 90° angle from the plane of the injection site showed higher concentrations than 180° but lower than at a 0° angle (Tanis et al., 2012) The left or right side may have an impact in trees with spiral vascular system, causing injected compounds to follow a sectorial winding accent relocating to more locations in the canopy. Species with straight vascular system tend to distribute compounds in a straight line leading to heterogeneity. To overcome this issue, it is recommended to apply the solution via several injection sites. Indeed, spatial uniformity is achieved with more injection ports, four being a good compromise between optimal spatial distribution and fewer wounding of the tree. Soil drench and soil injection are reported to provide better concentration in imidacloprid® and better uniformity of distribution compared to tree-injection (Dilling et al., 2010). The pressure of injection is also very important. Relying on the sap flow does prevent damages but does not allow control. Pressure injection allows the

control of the uptake rate but high-pressure injection may cause several injuries e.g. blocked xylem, bark splitting or cambial damage. The translocation ability of the vascular system also represents a key factor.

4. Injection methodology. First of all, the hole's depth: diffuse porous apple tree xylem with vessels and tracheids scattered among the annual rings will benefit from deeper injection points. Piercing a clean-cut hole is also critical: higher drill speeds may cause friction to the conducting tissue causing damage and decrease in uptake. Slower drill speeds may tear apart some material, resulting in severe wounding of the tree at the injection site. Drill-based injection provides higher residue concentration compared to other injection method (Aćimović, 2014)
5. The tree's response. Parenchyma cells (cambium) are responsible for the callus tissue responding to cut or wounding (VanWoerkom et al., 2014). The tree does not really heal: the injured tissues are not replaced by living tissues. The tree actually isolates the wounded structure by sealing it and separating from the healthy tissue to prevent further damage. The process is called compartmentalization. To this day, the key traits of trunk injection method that would allow limited wounding and efficient compound distribution are still unknown.
6. The behaviors of injected chemistries. The uptake and translocation of pesticides inside the tree's vascular system will depend on numerous factors: (i) solubility: the correct compound formulation is crucial. Basically, the more water-soluble the compound is, the more efficient it will distribute. (ii) molecular size: some compounds are too large at the molecular level to fit through vascular tissues. In apple xylem, the average diameter of vessels lumen is between 17-48µm. Imidacloprid® (Bayer AG) has a volume of 199.15$Å^3$ (1.9915e^{-10} $µm^3$) (Aćimović, 2014). (iii) viscosity of the compound, resulting in poor translocation. (iv) pH (v) carbon adsorption coefficient (Koc) or organic Carbon-Water partitioning coefficient (mL/g) of the a.i. and the formulation expresses the level of adhesion of the injected chemicals with to the carbon rich compounds located in the xylem. High level of Koc implies strong binding to the organic molecules in the sap or xylem resulting in slow and poor compound distribution. (vi) time of degradation (persistence) of the compound, influenced by the plant metabolism.
   The compounds should, of course, not exhibit phytotoxic effects.
7. The uptake rate. The passive translocation of compounds is based on the tree transpiration potential (water potential): water transpires through the leaves' stomata, creating a negative pressure gradient that sucks the sap upwards from the roots to the leaves. Higher leaves density means more stomata, hence greater transpiration potential. Stomata open and close depending on the water pressure in the guard cell. The weather greatly influences the stomata opening and thus transpiration processes: radiation, vapor pressure deficit (VPD) and relative humidity are key parameters (Dragoni et al., 2005), but unfortunately impossible to monitor. On drought period, transpiration is limited as the tree tends to "save" water and on cold or humid periods, the low temperature or high relative humidity does not allow maximal transpiration. Optimal weather conditions would be medium to high temperatures, low relative air humidity (ensuring high VPD), sunny and windy weather, completed with substantial water supply in the soil. In the same manner, the cardinal orientation of the tree can impact the compound distribution. Water potential is also ruled by the gravitational potential, the pressure potential, the surface tension and the osmotic potential.
   In case of grafted trees, the cultivar/rootstocks combination is also said to impact compound distribution as fruit trees grafted on more vigorous rootstock tend to have somewhat higher and/or faster daily water uptake rate than less vigorous rootstocks.

1.4.6 Residues profile

To evaluate the product's distribution in the tree, the residues profile of chemicals in various organs must be measured. This aspect regarding essential oils will be further discussed in the (1.5) section. Previous reports suggested that vascular delivery was predominantly to foliage with limited residues in

fruits (far below the Maximal Residue Limits (MRLs) set by the EPA) and flowers. Fruit production is therefore not much impacted, which is good from a marketing perspective, but less interesting in case of pests feeding directly on fruits or flowers. In this particular experiment, the aphid species is detrimental to the flowers at their early development. The presence of aphids on the leaves are not as much disrupting the fruit production than their presence in flower buds. Plus, there is no data suggesting that the residues in fruit are sufficient for pest management. On the other hand, low residue in flowers are also good news as it should therefore not impact honeybees or other beneficial pollinators. A post-harvest treatment could be considered if fruits or flowers residues tend to exceed MRLs or have an impact on pollinators (Aćimović, 2014; VanWoerkom et al., 2014). In addition, a pre-flowering treatment must be investigated in the case where the vascular deliveries of compounds mainly to the leaves is inefficient for significant aphids monitoring, causing damages to the flowers and jeopardizing the production yield.

1.5 Profile of leaf-contained and emitted Volatile Organic Compounds (VOCs)

In this study, the distribution of the essential oils throughout the tree must be assessed. Other compounds such as the tree's defense VOCs in response to insect infestation or wounding from injection will also be inventoried. In order to do so, basic knowledge about the VOCs role in tree metabolism and their analysis methods are required.

1.5.1 Chemical ecology of biogenic VOCs

VOCs are plants' secondary metabolites. Those secondary metabolites mainly consists in isoprenoids (isoprene, monoterpenes, sesquiterpenes), phenolics and nitrogenous compounds (Fineschi et al., 2013). Oxygenated molecules (alcohols, aldehydes, ketones, acetates) also account for VOCs released by plants (Joó et al., 2010). Biogenic VOCs are key compounds in the communication between organisms and they also play an important role in the interaction between the biosphere and the atmosphere (Spinelli et al., 2010; Dudareva et al., 2013; Fineschi et al., 2013).

Two patterns are distinguished regarding how VOCs are stored. (i) Specifically stored compounds, happening in specific leaves organs such as resin ducts, secretory cavities or glandular trichomes at concentration of the order of µg-mg/g. The second pattern concerns (ii) directly emitted or temporary stored in small concentration (ng/g) compounds which storage happens in leaf aqueous or lipid phase and occurs for-water soluble volatiles (e.g. isoprene). Although a major fraction of these compounds is directly emitted, stress-induced stomatal closure may result in the build-up of these compounds inside the leaf (Ormeño et al., 2011). Stored VOCs are released in particular conditions and especially under mechanical stress. All plants organs (roots, trunk, stems, leaves, flowers) can emit volatile isoprenoids. These volatiles are responsible for the plant scent (flowers, pine needles,…) excepted for isoprene that cannot be detected by the human olfactory system (Fineschi et al., 2013).

The release of those stored compounds that count for up to 10% (and much under stress conditions) of the plants fixed carbon is at high metabolic costs and the purpose of this mechanism is still a debated issue. It is likely however that the plants invest this energy cost to protect vital organ from biotic and abiotic stresses (Fineschi et al., 2013). The composition and emission pattern of those VOCs are thus variable and depend on phenological and/or environmental changes (Rapparini et al., 2001; Fares et al., 2012). Temperature and photosynthetic active radiation (PAR) actually impact monoterpene emissions and must be taken into account when studying stress-released VOCs (Joó et al., 2010). Rainfall and relative humidity also influence the changes in VOCs profile tested on apple trees *in situ* (Vallat, Gu, et al., 2005).

Emission of stored or *de novo* VOCs comes massively after wounding of the plant or metabolic changes after infestation by pests (Herbivore-Induced Plant Volatiles, HIPVs). Their role is to act as deterrents (direct defense) to pathogens and herbivores, as attractants for parasitoids and predators of herbivores (indirect defense) and to contribute to healing (Scutareanu et al., 1997; Joó et al., 2010;

Jones et al., 2011; Fineschi et al., 2013). VOCs such as nonanal, (+)-Carvone, citral or eugenol also exhibited a strong activity in fungi growth inhibition (Brilli et al., 2019). In addition, Volatile isoprenoids were reported to mitigate the effects of oxidative stress by mediating the oxidative status of the plants (direct quenching of ROS) and by stabilizing cell membranes (Vickers et al., 2009; Brilli et al., 2019). Volatile differs according to plant-insect interactions, suggesting that defense mechanisms are species specific (Joó et al., 2010). Emissions from clean and mechanically wounded plants also differ from emissions of herbivore infested plants (Scutareanu et al., 1997). Brilli et al., (2019), also reported that VOCs emission can have allelopathic effects that can impair the growth of competitive plant species (inhibition of seed germination and root growth), revealing their potential use as weed control molecules. VOCs emitted by stressed plants can also enhance resistance of neighboring healthy plants when they are exposed to theses airborne signals (Girón-Calva et al., 2012).

Furthermore, VOCs are involved into the plant's resistance by priming and enhancing tolerance to stress, quenching reactive oxygen species (ROS), having potent antimicrobial activity and allelopathic effects. VOCs might also be important for plant growth regulation, development and senescence through interactions with plant hormones. They can act as airborne signals, allowing quick defense signaling between distant organs. Apart from their repellent or attractant activity, VOCs are regarded with great interests as an eco-sustainable strategy for enhanced plant protection and productivity. Other VOCs such as methyl salicylate and monoterpenes are actively involved in the mechanisms leading to systemic acquired resistance (SAR). Salicylic acid for example was reportedly a SAR elicitor in apple trees (Lateur, 2002). The treatment of lima beans with a synthetic analog to salicylic acid showed a priming of antibacterial defense thanks to the production of nonanal. VOCs do not only act as direct defense mechanisms but also trigger important defense patterns in the plant, enhancing global protection (Brilli et al., 2019). This global protection often involves more than one biochemical pathways and the rate of biosynthesis of VOCs is often controlled by the availability of substrate and precursors rather than by the activity of enzymes responsible for the VOCs formation. Among those precursors are the linoleic and linolenic acids that undergo the lipoxygenase pathway to form a variety of fatty acid-derived VOCs such as methyl jasmonate, 1-hexenal, nonanal, etc or also Green Leaf Volatiles (GLVs) that consists in saturated and unsaturated aldehydes and alcohols that are usually synthesized in green organs of plants in response to wounding (Dudareva et al., 2013). It has been shown that the phloem feeding of pests insects released specific LOX-derived volatiles that suppress or induce the synthesis of other VOCs or GLVs involved in defense mechanisms (Liu et al., 2010). The regulation of VOCs biosynthesis pathways (LOX, alcohol dehydrogenase,…) thus seems to be ruled by the activity of signal molecules such as methyl jasmonate, nonanal, methyl salicylate etc (Li et al., 2006). This indirect defense was reported less energetically costly than direct defense. Moreover, it was proposed that VOCs' combined actions with secondary metabolites can prevent cell degradation and death, thus prolonging the life span of leaves and flowers, improving the whole plant conditions (Brilli et al., 2019). However, it seems that the capacity of plants to perceive volatiles and to convert them into internal signals remains poorly understood (Girón-Calva et al., 2012).

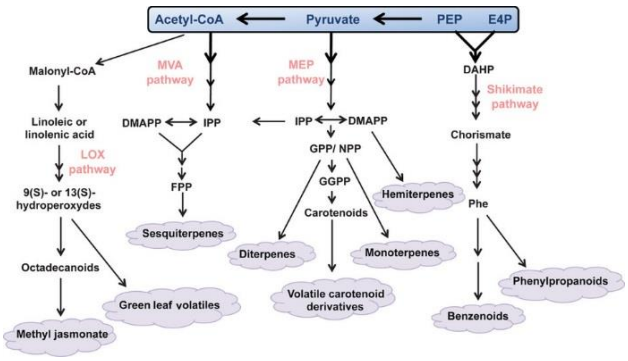


Figure 4: Overview of biosynthetic pathways that lead to the synthesis and emission of plant VOCs (Dudareva et al., 2013)

VOCs emission also exhibits variations according to the season and phenological stage of the plant: e.g. at full bloom, apple flowers emits about 366 ng of monoterpenes hydrocarbons per gram of dry weight per hour. This emission decreases at fruit-set to reach its lowest value of 3-9 ng per g DW per hour at harvest time. Indeed, fruit trees tend to be strong monoterpene emitters only at blooming for pollinators attraction (Baraldi et al., 1999; Rapparini et al., 2001)

In apple, linalool is the main contributor of monoterpene emission at blooming (94% of the emitted carbon in apple). α-pinene, camphene and limonene were for their part identified in the foliage emission. (Rapparini et al., 2001). Linalool was found to be emitted exclusively by apple and pear flowers (Baraldi et al., 1999). The changes in the VOCs' profile of agricultural crops (e.g. apples and pears) can have a significant influence on the flavor (Vallat, Gu, et al., 2005).

In previous studies on pear and apple trees, the main VOCs that have been encountered under natural or stress conditions are: linalool, α-farnesene, camphor, methyl salicylate, limonene, nonanal, squalene, benzaldehyde, cis-3 hexenyl acetate, (*E*)-2-hexenal and (*Z*)-3-hexen-1-ol (Scutareanu et al., 1997; Joó et al., 2010; Jones et al., 2011; Vallat et al., 2005).

In spite of the positive aspects of VOCs previously put forward, some VOCs display phytotoxicity (Brilli et al., 2019) and some VOCs, in a very sneaky way, are reported to be induced by infestation of females rosy apple aphids and serve as attractants for males RAA when in combination with aphid sex pheromones. It was indeed proven that host-specific pests use their host-plant VOCs as attractants for their conspecifics. It is noteworthy that those VOCs are induced by the presence of the pests (Van Tol et al., 2009).

1.5.2 Sampling and analysis of VOCs

In this study, treated trees will exhibit (i) HIPVs caused by the rosy apple aphid, (ii) stress-related VOCs caused by the injection and (iii) by the injected essential oil VOCs or at least the main compounds. In order to qualitatively and quantitatively analyse the VOCs contained and emitted by the leaves and flowers, several approaches have to be considered for sampling and analysis.

Two approaches must be distinguished: (i) stored compounds (mono- and sesqui- terpenes) analysis and (ii) emitted compounds analysis. As it is not known whether the EOs compounds will be stored or emitted and at which concentration, both analyses require precision and sensitivity. A comprehensive understanding of the treated plant volatolome is also of highest importance in order to know whether the identified compounds come from the plant, from the treatment or from the reaction of the plant to the treatment.

Stored compounds analysis can be performed directly through traditional extraction technique (e.g. hydrodistillation, soxhlet solvent extraction, simple solvent extraction), novel techniques (e.g. microwave assisted extraction, ultra-sound extraction, supercritical fluid extraction) or indirectly by trapping and collecting within adsorbent materials such as Dynamic HeadSpace (DHS), Solid-Phase Micro Extraction (SPME) or Stir Bar Sorptive Extraction (SBSE) (Bicchi et al., 2008; Ormeño et al., 2011; Li et al., 2019). These methods can be applied on harvested leaves or flowers, cryo-freezed in liquid nitrogen after sampling and kept at -80°C until extraction to avoid post-harvest VOCs emissions.

The analysis of non-specifically stored compounds in leaves or flowers requires the use of trapping techniques, more sensitive to trace amounts. Unlike for stored BVOCs, their analysis cannot provide information regarding the emission potential of a plant (Ormeño et al., 2011). That is why the analysis of those compounds must be performed in the atmosphere as well. Air sampling can be performed using various apparatus (e.g. Radiello ™ sampler, electric nose EOS835) that allow rapid and reliable screening of plant material (Vallat et al., 2005; Spinelli et al., 2010). However, those methods are often less sensitive than chromatography analysis or only allow comparative rather than quantitative analysis. However, the Proton-Transfer Reaction "Time-of-Flight" mass spectrometer (PTR-FT-MS),

a new analytical technology, seems to offer high-resolution and highly sensitive detection of the whole spectra of VOCs in vivo and in real time with high resolving power (Brilli et al., 2019). Air analyses are often realized by flowing the air from a dynamic flow-through Teflon growth chamber containing the tested plants through adsorbents that are further analyzed by Thermal Desorption – Gas Chromatography and Mass Spectrometry (TD-GC-MS) (Baraldi et al., 1999; Rapparini et al., 2001). This is practical for small plants in lab conditions but impossible to apply in situ. Field measurements can be performed using cuvettes or Nalophan® ™ bags that are placed on the tree branches. Pumps can be used to flow purified (thanks to active carbon filters) air inside the bag and to trap VOCs on adsorbent cartridges that are further desorbed using TD-GC-MS (Joó et al., 2010; Rodriguez et al., 2017). Another rising technique consist in the use of Direct Contact Sorptive Tape Extraction (DC-STE) coupled with GC-MS. This consists in placing non adhesive polydimethysiloxane tapes in direct contact (DC) with a biologically active surface such as a leaf or in the headspace in equilibrium with the matrix (in this case, no direct contact, only STE). This technique can be used for qualitative and quantitative analysis of plant volatiles and the tape placing allows to target specific regions of the matrix, for instance part of leaves that have undergone herbivorous attack or other damages to identify specific stress-related emissions (Boggia et al., 2015).

Trapping techniques and particularly HeadSpace – Gas Chromatography (HS-GC) are often preferred over extraction methods as they do not require solvent or specific apparatus, are versatile, rapid and are often more sensitive. Matrix effects are also more easily overcome (no contamination from non-volatile constituents) (Bicchi et al., 2008; Ochiai et al., 2014; Soria et al., 2015).

Multi-Volatile Method (MVM), consisting in three different DHS samplings from one vial was reported to allow more uniform recovery of a variety of aroma compounds in aqueous samples (Ochiai et al., 2014). This technique seems interesting as it allows to adsorb compounds from the same vial in 3 different cartridges adsorbent and therefore to obtain better sensitivity as one single adsorbent type is not necessarily efficient for all the compounds present in the sample. Choosing the optimal adsorbent type is crucial. In regard to this particular study, Tenax TA, Porapak Q, Chromosorb 106 or Porapak Rxn RP cartridges seem the most suited based on their characteristics (Rodriguez et al., 2017). Using several cartridges to ensure best sensitivity and selectivity can also be interesting.

The extracts or trapped compounds can afterwards be analyzed using GC coupled to a mass spectrometer.

Fruits analysis is not considered here. However, the use of Solvent Assisted Flavor Evaporation (SAFE) seems the most suited to extract compounds from complex food matrices such as fruits. Low temperature used during SAFE allow gentle extraction of VOCs and prevent the formation of artifact of the modification of these compounds (Li et al., 2019). This last point is crucial, regardless of the sample type, the alteration of compounds or the formation of artifacts must at all cost be avoided. That is why the extraction method very often depends on the sample type (Li et al., 2019).

1.6 Field trials

In this part, a state of the art of the previous field trials regarding insect monitoring will be presented. The goal is to identify the insect population monitoring method, the type of experimental design, the sampling and injecting methods and the statistical analysis that would best suit the experiment proposed in this study.

The insect infestation can be artificial or natural. In case of artificial infestation, the insects can come from a lab-maintained population or can be field-collected in situ to be breed in laboratory and placed on selected trees afterwards.

1.6.1 Studies not related with tree-injection

A study assessing the tolerance of scab-resistant cultivars to the RAA field collected the aphids. The collection on trees from different orchards allow a large genetic variability. In the first phase of this study (young trees in pots), six adult apterous females (each one coming from a different population) were placed on the first expanded leaf of the selected trees (placed in a completely randomized design with 8 replicates). Aphid movement from one tree to another was avoided by putting the pots in dishes with water. The formation of alates was not an issue as the Rosy Apple Aphid is a dioecious species and the alates thus do not feed or reproduce on apple trees. Three weeks are reportedly enough to see differences in trees' susceptibility: leaf rolling appearing in 24h in some cases. The shoot deformation was recorded using a scale from 0 to 5. Aphids were counted and their abundance was evaluated on a scale of 0 to 6. The experiment was then repeated in the field using the same counting techniques. A Spearman test was applied to evaluate the relationship between the damage level and the abundance of aphids (Miñarro et al., 2008).

Another study aiming the autumn control of the Rosy Apple Aphid disposed the trees in a randomized complete block design with 4 replicates. Here, the number of RRA colonies was evaluated and an analysis of variance was performed (Cross et al., 2007).

A 2017 large scale experiment evaluated the RAA population dynamics by counting the total number of aphids on each monitored tree and characterize the population dynamics using 3 variables: (i) presence/absence (ii) the log of the area under the curve of the tree population abundance, indicating in situ RRA population dynamics (iii) the presence duration (in days) indicating the aphids emigration process. All those data was analyzed using generalized linear mixed model to assess the influence of each considered factor (agroecological infrastructures and natural predators) (Albert et al., 2017).

1.6.2 Studies considering tree-injection for pest monitoring

In this part, previous publications regarding tree-injection and its various aspects are briefly presented. Those studies were investigated principally for the methodology of the injection, the sampling of plants organs for compound uptake monitoring and the effect on the controlled pests.

In a 2014 study, apple trees were injected at the end of May for protections against various insects and fungi. 4 injections were administrated at each tree, each under the 4 main scaffold branches to allow optimal delivery. Leaves and flower sampling schedule followed a geometric sequence: samplings were executed 2 days after treatment (DAT), 7 DAT, 14 DAT, 30 DAT, 60 DAT and 90 DAT. Sampling of minimum 10g of leaves and flowers was collected from high and low positions of the tree crown and from the 4 cardinal direction (N,S,W,E). Evaluations of the efficacy of the treatment took place when the first symptoms were expected to be visible according to predictors. No artificial infestation was performed. Counting (insects) was realized according the species during a 2 minutes observation per tree or by counting the number of lymphs present on 20 randomly selected shoots. Scores were attributed for each tree according the level of damage and the number of pests. Insecticide bioassays in laboratory were also performed using collected leaves that were placed in a dish with insect larvæ. Mortality and area of leaf consumed was assessed for each treatment. ANOVA were performed on the data sets (VanWoerkom et al., 2014).

In a study using micro-capsule trunk injection, fungicide injections were performed every 15 cm of trunk girth. Trees injected with water served as control. Disease severity was visually assessed and rated on a 0-5 scale. Trees vitality was assessed by chlorophyll fluorescence measurement: 10 randomly selected leaves per tree were adapted to darkness for 30min by attaching light exclusion clips to the leaf surface. Measurements were recorded up to 1s using a HandyPEA portable fluorescence spectrometer (Hansatech Instruments Ltd, UK). The wound closure was also monitored and rated on a 0-5 scale. For statistical analysis, normality was checked and homogeneity of variance was verified (Anderson-Darling test). An ANOVA was then performed (randomized complete block design) (Percival et al., 2005b)

A 2010 Australian field study injected 100 cocoa trees (50 with phosphonate and 50 with water as a control) for stem canker and black pod incidence (Mcmahon et al., 2010).

Another study controlled insect pests using tree-injection in an apple orchard. Leaves for insecticide residues were collected 4 and 8 weeks after treatment. Repetitions for whole tree sampling where realized for that purpose only. Those trees were fully dismantled and analyzed. The insect abundance was evaluated by checking the underside of leaves for 30s per tree. Damage was evaluated by counting the number of chlorotic and curled leaves reported on the total number of leaves per shoot for 20 shoots per tree (Coslor et al., 2019).

One last study of interest consisted in injecting sacrificial tree with pests attracting volatiles (ethanol, methanol, acetone, acetaldehyde). Volatile sampling was achieved by solid phase micro extraction (SPME) by first positioning a tree branch within a polyacetate bag. Volatiles were thermos-desorbed and analyzed via GC. Peak identifications were made by comparing mass spectra and retention times with those of authentic standards. One-way ANOVA and repeated measures ANOVA were performed for collected data analysis (Ranger et al., 2010b).

2 Objectives

The goal of the present study is to evaluate the efficiency of a tree-injection treatment considering cinnamon (*Cinnamomum cassia,* J. Presl)*)* essential oils as active ingredient of a biopesticide formulation against the Rosy Apple Aphid. This oil was selected based on previous studies of the UCLouvain (partner of this project) regarding response of the Rosy Apple Aphid to the essential oil extracted from cinnamon.

Experiments will take place on 15 years old Jonagold cultivars apple trees in an orchard located in Gembloux (Belgium). The rest of the experiments will be performed on young apple and pear trees kept in semi-controlled conditions using cinnamon, clove and spearmint essential oils. The two experiments have two different objectives: the field trial aims to demonstrate the biopesticidal potential of the cinnamon EO for aphids monitoring in agronomic conditions. The effects of the treatment on the aphid population will be controlled and the presence or absence of specific VOCs or cinnamon essential oil compounds or derivatives in the leaves or emitted by the apple trees will be put in relation with the aphids' population dynamics. The experiment on younger trees is essentially to investigate the aphids' response due to the treatment only (artificial infestation) in the tree and the reaction of the tree to the treatment. The first aspect is mainly qualitative and the second experiment is both qualitative and quantitative.

The whole experiment aims to:

- Demonstrate the biopesticidal potential of the cinnamon essential oil against the Rosy Apple Aphids in agronomic conditions.
- Demonstrate translocation of the injected cinnamon essential oil main constituent via emitted and contained VOCs analysis.
- Demonstrate the potential incidence of the treatment on the trees' health using chlorophyll fluorescence measurements.
- Demonstrate the trees' reaction to the treatment by major emitted VOCs analysis and comparison between different treatments.

Some technical constraints and challenges are to be expected all along the experiments, especially in field trials. Some "on-the-go" modifications may thus interfere with the original plan. Edits of the original plans will be notified in the Material and Methods section.

3 Material and methods

This section is divided in the main aspects of the work:

- Experimental design
- Tree-injection methodology
- Characterization of the EO
- Calibration curve of cinnamon EO major compound
- Contained and emitted VOCs sampling and analysis
- Evaluation of the treatment's phytotoxicity
- Insect population dynamics
- Data analysis

3.1 Experimental design

In this section, the experimental set up and the sampling of vegetal matter or VOC schedules are described. The section 3.5 explains how the sampling is executed and the subsequent analysis method.

3.1.1 Field trial on adult apple trees

Part of this whole experiment took place in an apple orchard containing various cultivars (Figure 5). The experiment concerned the Jonagold cultivars. This orchard is the property of the CRA-W[7] and is located rue bois Godeaux in Grand-Manil (Gembloux, Belgium), coordinates: 50.555103, 4.661554. The trees were planted in March 2005.

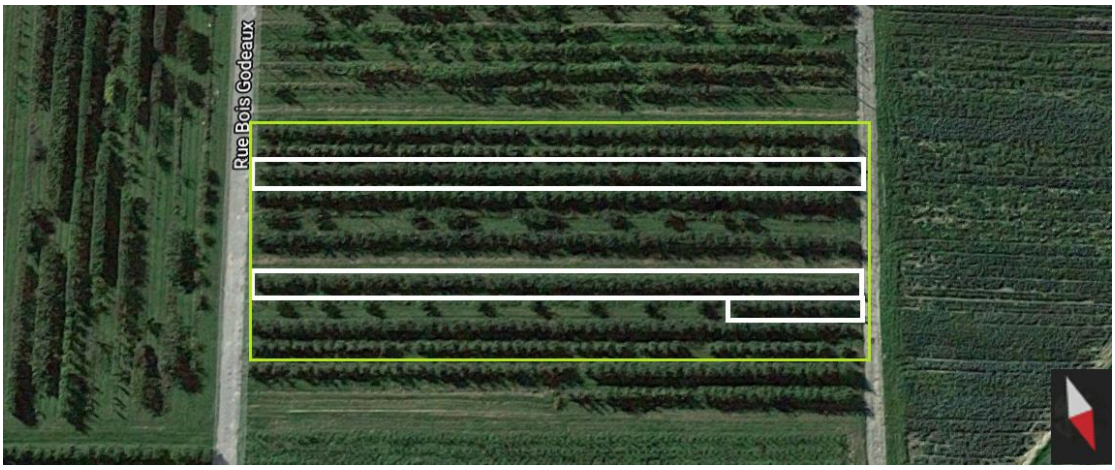


Figure 5: Apple orchard (green rectangle) located in Grand-Manil (Gembloux, Belgium). The white rectangles are the Jonagold cultivars.

As a total, 119 Jonagold apple trees were available for experiment, distributed among 2 lines of 54 trees each and an additional 11 trees at the end of another row. The first white box is later referred to as the "D" line and the second one as the "I" line. The 11 spare Jonagold trees are referred to as "J".

NB: The orchard rows are roughly orientated on a South-North axis and the trees are on an East-West axis. The rows are listed from B to K on the S-N axis (Up to bottom on the figure) and the trees are numerated from 1 to 52 following the E-W axis (left to right on the figure).

For the orchard experiment, five tree-injection treatments were considered, applied on 8 repetitions disposed in 8 blocks of 5 trees each, so 40 trees. The five treatments are as follows:

[7] Centre de Recherche Agronomique Wallon (Walloon Research Center in Agronomics)

a. No injection [NEG]. This negative "blank" treatment will allow to determine the normal profile of VOCs in the apple trees and to identify the potential changes due to aphids' infestation or to the treatment.
b. Injection blank with water [BSA]. This "blank" treatment will inform on the changes in the VOCs profile due to the stress caused by the injection only.
c. Injection blank with the emulsifier [BEM]. Previous work showed that the emulsifier used in compound's formulation may be phytotoxic or toxic towards insects. This "blank" treatment will show the impact of the emulsifier on the trees and the insects, regardless of the cinnamon essential oil.
d. Injection with cinnamon essential oil emulsion at 2% [CEO2].
e. Injection with cinnamon essential oil emulsion at 1% [CEO1].

The injection consists in 3mL of the treatments solution. The quantity of compound to deliver was determined according several considerations: As no previous studies that considered the injection of essential oils as biopesticide were found, data on this specific parameter was missing. The volume of injection treatment was set to 3mL following the previous results obtained in this project and after the EndoKit user's manual. The cinnamon essential oil concentration in the injection volume was set to 1% (V:V) and 2% (V:V) so 0.03g and 0.06g of active substance per tree, respectively. This was similar to the quantity used for other pesticide injected by tree-injection (Coslor et al., 2018; Coslor et al., 2019).

Each treatment was applied once in each block (8 repetitions) and at least one untreated tree was left between each relevant tree inside the blocks. To determine which trees were to be treated, an evaluation prior the experiment beginning was performed in which the trees' health was evaluated in order to discard the trees that were considered in poor health conditions on a global visual assessment. An inventory of the aphid colonies was also made and each tree was given a score based on the number of colony that counted more than 10 individuals. The method of inventory is later described in this chapter. The treatment modalities were then assigned to the trees considering their infestation scores and so that the medium infestation was similar for all the treatments whilst ensuring no pattern was repeated in term of treatment order in the blocks. Following this approach, 8 blocks were constituted (Figure 6)

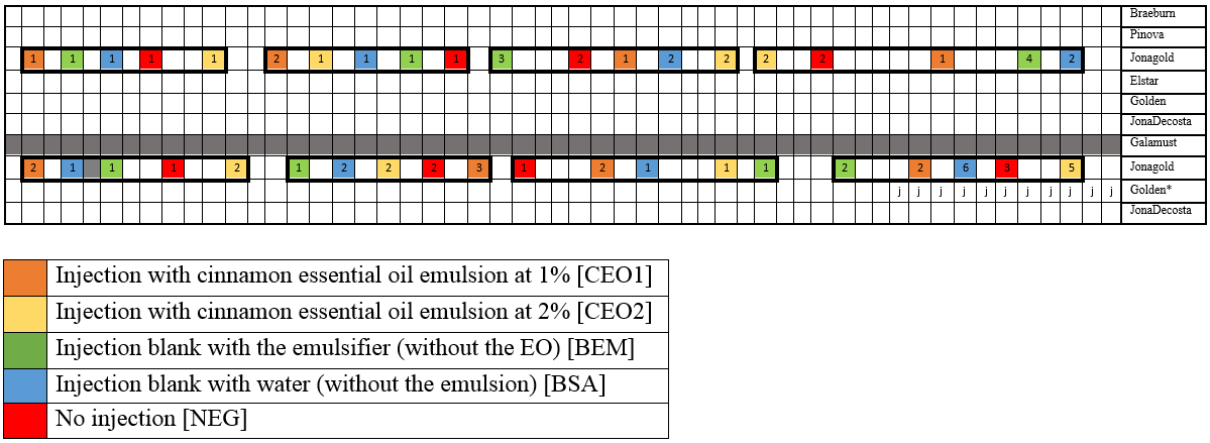


Figure 6: Orchard experimental design

For the reader sake's, the (Figure **Erreur ! Source du renvoi introuvable.**6) scheme is further explained hereafter:

- Each square represents one tree in the orchard.
- The "j" letter in the Golden cultivars row represents the 11 Jonagold cultivars located at the end of the row.

- The grey line (Galamust cultivar) is no longer existent.

The health evaluation and the aphid population inventory took place on the 11th of May 2020. On the same day, 6 trees from the 11 spare Jonagold cultivars in the Golden row were injected (3 with 3mL of water and 3 with 3mL of cinnamon essential oil emulsion at 2%). The purpose was to get used to handling the injection kit and to keep on hand plugged trees for the case where some other test were to be performed without compromising the experimental design. The global health of those 6 trees using fluorimeter was assessed 3 days later, on the 14th of May 2020. The fluorimeter measurement will be further detailed.

The injection of the 32 trees considered in the experimental design took place on the 19th of May 2020.

In order to evaluate the product uptake and dispersion in the trees of cinnamon essential oils components, leaves-retained compounds and compounds emitted by living leaves must be sampled. The sampling of contained and emitted VOCs was performed 72h (3days), 168h (7 days), 336h (14 days) and 672h (28 days) after IT (injection time). The VOCs sampling and analysis will be detailed in (section 3.5). The experimental phase thus ended on the 16th June 2020.

NB: The sampling must always be performed in the same order between the sampling sessions in order to avoid bias according the time of the day when the sampling occurs. As a matter of fact, the sampling sessions always began with tree D2 at around 9.30 AM.

Edit: On the first sampling session that took place on the 22sd of May 2020, the team members were unable to sample the last 8 trees. For the rest of the experiment, the trees I2, I4, I6, I9, I12, I15, I17 and I21 were not further considered for the VOCs part. As a consequence, the [CEO1] and [CEO2] lost one repetition and [BSA], [BEM] and [NEG] lost two repetitions which makes respectively 7 and 6 repetitions instead of 8 as originally planned. The aphids monitoring and chlorophyll fluorescence still took place on those 8 trees.

3.1.2 Semi-controlled trial with young apple trees

For this part of the experiment, 60 apple trees (Early Jonagold cultivar - Milenga® M9 2 years "knip") were purchased from Nicolaï Fruit and Vegetables (St-Truiden, Belgium).

The trees were 2 years old and had been kept in cold conditions before repotting and were thus still in their winter phase. They were repotted on the 15th May 2020 in 15L plastic jar. The jar were first filled with approximately 5cm of compacted soil in order to increase the jar weight and ensure its stability (Figure 7). Universal potting soil (HumuForte MycoPlus) was then added up to half of the jars. It is composed of black peat, peat litter, wood fibre, calcium and magnesium fertilizers, organic compound fertilizer, mixture of bacteria and mycorrhizae. The dry matter represents 25% of the total weight. Its pH zone is from 5 to 6.5 with an electrical conductivity of 550µS/cm.

Figure 7: On the left row sits 3 jars with soil covered with potting soil. On the right row are 3 jars prior to the potting soil addition. On the extreme left is the right arm's shadow of a handsome young man.

The trees were then placed in the pots and held straight while more potting soil was added and lightly compacted in order to maintain the tree in place (Figure 8). The trees were then watered until the pot reached field capacity.

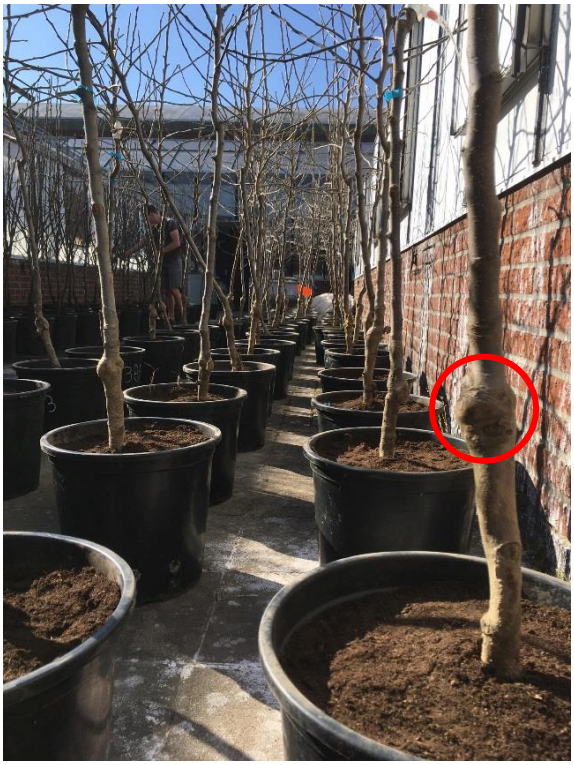


Figure 8: Apple trees after repotting. The red circle indicates the injection site (thicker part of the trunk where the graft is inserted in the rootstock).

The injection device in the younger trees is the same as the one used for the field trial. The hole was however drilled less deeply (1.5cm) to avoid consequent damages (Figure 8).

For this pilot scale trial, the experiment will take place on 32 trees. After repotting, 3 branches on each tree were encapsulated with a net hold in place with a string. This allowed the branches to remain free of any insect or any egg deposit. Artificial infestation with D. plantaginea on the apple trees later took place in the net, to keep track of the development of each colony and to avoid any migration or external factor influence (predation, wind,…).

For this setup, 4 modalities are considered:

- 8 trees treated with a cinnamon essential oil emulsion at 1% [CEO1] in the presence of insects
- 8 trees treated with a cinnamon essential oil emulsion at 2% [CEO2] in the presence of insects
- 8 trees treated with a cinnamon essential oil emulsion at 4% [CEO4] in the presence of insects
- 8 trees left untreated [NEG] in the presence of insects

The injection of 3mL of the emulsion was performed in the same manner as described previously, excepted that the plug was inserted in the thicker part of the trunk were the graft was inserted in the rootstock (Figure 8). The injection and aphid infestation took place on the 24th of June 2020.

However, due to rescheduling of the overall work due to the Covid pandemia, the results will not be treated in this particular work but are still a go for the overall project.

3.2 Tree-Injection methodology

This part is dedicated to the formulation of the emulsion that was delivered to the trees and to the tree-injection devices that was used to do so.

3.2.1 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 the xylem sap's physical properties (VanWoerkom et al., 2014; Wheeler, 2020). The sap being an aqueous medium, the essential oils must be part of an aqueous solution. The formulation of an essential oil/water (O/W) emulsion is necessary.

For this purpose, 15mL of water are placed under 1250 rpm agitation. The emulsifier (Tween80) is added, in the meantime with cinnamon essential oil (Pranarôm International SA, Belgium) at a ratio of 4:1 (V:V) Tween:EO. For a 100mL emulsion, here are the respective volume of Tween and essential oil to use according the desired essential oil concentration (Table 1):

Table 1: Volume of Tween and EO to use following the desired EO concentration for a 100mL emulsion.

EO concentration	Tween volume (mL)	EO volume (mL)
0.5%	2	0.5
1%	4	1
2%	8	2

Then, 20mL of EDTA 100mM are added to the solution that is afterwards completed to the final volume with water (EDTA is thus at 20mM in the final solution). The solution is kept under constant agitation for 5min and is then stabilized using high-speed homogenization (HSH) at 9500rpm for 6min (Ultra-Turrax™ T25) and high-pressure homogenization (HPH) with 8 cycles at 5000psi (FMC). This whole process is realized as much as possible in a dark container to avoid EO degradation by light. The EO emulsions are then stored at 4°C and away from light. The emulsion's stability was not evaluated for this experiment but earlier emulsions were evaluated through EO particle size using a

particle sizer (Beckman Coulter – Delsa™ Nano C Particle Analyzer). A nano-emulsion was obtained for all the previous emulsions.

Blank injections were performed during the experiment. They consist in a water solution (blank saline using tap water) and an aqueous solution using Tween 80 at 6% and EDTA 20mM (blank emulsifier).

3.2.2 Tree-injection device and parameters

Various devices are available on the market, especially in the US (TreeCareScience ©, Arborjet ©, ArboSystems ©) where the technology is spreading. In this study, the device used is a ENDOkit Manual PRO © (Figure 9) purchased from ENDOterapia Vegetal (Spain).

Figure 9: ENDOkit Manual PRO (https://endoterapiavegetal.com/en/equipment/endokit-manual-solution/)

This device requires the drilling of one hole prior to the injection. The parameters to set before injecting were determined using previous studies on the subject and according the device user's manual. Here are listed the injection volume and settings:

- Depth and diameter of the hole: The hole was drilled at 60cm height on the trunk using an 8mm diameter drill bit with a battery operated drill. First, the outer layer of the bark was gently scratched off using minimal pressure and low drilling speed. The bark residues were then cleaned, allowing the rest of the hole to be carved without contamination from the outer bark layer (Figure 10).

Figure 10: Scratching of the outer bark layer. The green ribbon on the drill bit is a depth indicator, placed at 2 cm from the tip of the bit.

The hole was then completed at higher speed at the depth of 2 cm, the length of a plug (Figure 11). The shards of wood were pulled out of the hole as much as possible prior the plug insertion.

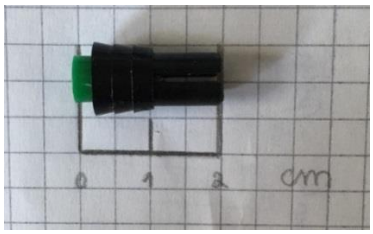


Figure 11: EndoPlug from the EndoKit Manual Pro.

- Plug insertion: the plug was pushed in the hole using the insertion device supplied with the EndoKit. To ensure that the plug was fully pushed into the hole, the back of the plug-inserter was gently hammered until correct positioning of the plug (Figure 12). The plug must be fully inserted and the green part must make flush with the tree bark to ensure optimal healing. Correct positioning of the plug allows the tree to form callus on the black part of the plug, accelerating and facilitating the healing process through compatmentalization (Aćimović et al., 2016a).

Figure 12: Plug insertion.

- Injection: the solution to inject was poured into the container. The injector needle was inserted into the plug as shown in Figure 13. After correct positioning of the needle, the solution was injected and the needle was withdrawn from the plug after the plunger returned to its original position. The injection requires the pressure of the hand on the syringe plunger, no additional pressure is required.

Figure 13: Insertion of the needle in the plug

3.3 Volatile Organic Compounds (VOCs)

3.3.1 Characterization of the Essential Oils

In order to identify the essential oils residues in the vegetal matter (endogenous VOCs) or in the VOCs samplings (emitted VOCs), it is necessary to previously investigate the cinnamon essential oil in the percentage of its main constituents (characterization and molecular profile establishment).

A 10mg EO in 10mL n-hexane (CAS 110-54-3, Chem-Lab, 99+% HPLC Grade) solution was prepared for each essential oil. The GC-MS system (7890b-5975C, Agilent Technologies) used for the analysis was equipped with a HP-5 MS capillary column (30m x 250µm x 0.25µm, Agilent Technologies Inc.).

1µL of the hexane containing EO solution was injected in splitless mode at 250°C with Helium as the carrier gas (1mL/min). The oven temperature program was as follows: (i) 40°C for 2min, (ii) increase to 100°C at a rate of 5°C/min, (iii) increase to 120°C at 3°C/min, (iv) a plate at 120°C for 3 min, (v) an increase to 220°C at a 5°C/min rate, (vi) an increase to 310°C (final temperature) at 15°C/min.

The quadrupole temperature was fixed at 150°C and the MS source at 230°C. The mass spectra was recorded in EI mode at 70eV with scanned mass range from 30 to 400 m/z.

Chromatograms were treated using Masshunter (Agilent) software and compounds spectra identification was performed based on a comparison with the NIST17 spectra reference database and a comparison of the retention index (RI) of each compound with the RI found in the literature.

3.3.2 Calibration curve of trans-cinnamaldehyde

Based on the characterization of the EO used for the formulation of the biopesticide, a calibration curve of its major compound (Trans-cinnamaldehyde) was established using commercial pure standards.

- Trans-cinnamaldehyde (CAS 14371-10-9, Sigma-Aldrich, ≥99%)

A 200 µg/mL trans-cinnamaldehyde solution in hexane (CAS 110-54-3, Chem-Lab, 99+% HPLC Grade) was prepared (stock solution). This solution was kept refrigerated and away from light.

Two curves were established: one for the quantification of E-cinnamaldehyde in the leaves emissions (emitted VOCs) using 1-phenyloctane (CAS 2189-60-8) as internal standard and the other for the quantification of E-cinnamaldehyde in the leave content (endogenous VOCs) using methyl trans-cinnamate (CAS 1754-62-7, Sigma-Aldrich, 99%) as internal standard.

The two curves followed a different path of dilution, best fitted with the expected amount of trans-cinnamaldehyde in the different samples (emissions and contents) (Table 2).

Table 2: Calibration curves set up

	Calibration curve (Emissions)		Calibration curve (Content)	
Level	E-cinnamaldehyde (ng/mL)	*1-phenyloctane (ng/mL)*	E-cinnamaldehyde (ng/mL)	*Methyl trans-cinnamate (ng/mL)*
1	80000	25000	160000	50000
2	60000	25000	130000	50000
3	40000	25000	100000	50000
4	30000	25000	80000	50000
5	20000	25000	60000	50000
6	10000	25000	40000	50000
7	5000	25000	20000	50000
8	1000	25000	10000	50000
9	-	-	1000	50000

The vials were then injected in GC-MS for establishment of the calibration curve. Several curve were set up:

- DHS[8]-GC-MS on Tenax TA cartridge. This curve aims to assess the recovery of the tool: the amount of E-cinnamaldehyde detected compared to the real amount in the sample.
- DHS-GC-MS on Tenax TA cartridge, with spiked ground up leaves. This curve aims to determine the recovery of the E-cinnamaldehyde amongst other VOCs contained in apple tree leaves.
- TDU[9]-GC-MS on Tenax TA cartridge.

Each curve was repeated 3 times in order to compute the LOD and LOQ of the method and to evaluate the reproducibility of the results. The comparison of the TDU-GC-MS on Tenax curve with the matrix effect curve allowed to evaluate the method recovery.

For each curve, 1µL of solution from the vials was injected, either in a DHS vial that was further sampled (advial) or either directly on a cartridge placed in the thermal desorption unit (TDU).

The curve with ground up leaves was established as follows:

- Leaves were randomly collected in the orchard and were immediately frozen by placing them into a plastic bag filled with liquid nitrogen and pouring some over it afterwards.
- For each vial, a handful of leaves was placed in an IKA™ grinder and was kept frozen by adding some more liquid nitrogen. Once the liquid nitrogen was almost vaporized, the leaves were grinded and 1g was weighted and placed in a 20mL vial.
- As the calibration solution could not be performed in "advial" by the GC-MS apparatus for the equilibration time between the headspace and the matrix would have been too short, the solution of the content calibration curve were diluted 10 times and 10µL of this diluted solution was added to the vial along with 2mL 20% NaCl solution for salting out. The samples were homogenized, were kept refrigerated for 1 hour and were then injected in DHS-GC-MS after a second homogenization. The 80000ng/mL and the 20000ng/mL points were performed 5 times for repeatability assessment.

The technical characteristics of the apparatus and the parameters were set as follows: a TDU/CIS coupled to a GC-MS system (7890B-5975C, Agilent Technologies Inc.) equipped with a HP-5 MS capillary column (30m x 250µm x 0.25µm, Agilent Technologies Inc.). For a sharper analysis, calibration curve were acquired using both SCAN and Single Ion Monitoring (SIM). The selected ions for each compound were as follows: (**Quantifier**, qualifier 1, qualifier 2)

- Trans-cinnamaldehyde: **131**, 132, 103
- 1-Phenyloctane: **91**, 92, 190
- Methyl trans-cinnamate: **131**, 103, 162

From the NIST Mass Spectrometry Data Center[10].

The parameters of the TDU-GC (Table 3) and DHS (Table 4) are explained herafter:

[8] Dynamic Head Space
[9] Thermal Desoprtion Unit
[10] https://www.nist.gov/srd/nist-standard-reference-database-1a

Table 3: TDU-GC parameters

Step	GC	TDU Tenax	TDU Chromosorb 106
Run time	38 min	-	-
Initial Temp	40°C	40°C	40°C
Hold time	2 min	-	-
Post run temp	70°C	-	-
#1 Rate	5°C/min	100°C/min	100°C/min
#1 Value	170°C	280°C	225°C
#1 Hold time	0 min	5 min	10 min
#2 Rate	20°C/min	-	-
#2 Value	310°C	-	-
#2 Hold time	3 min	-	-
Flow	1.2 mL/min	-	-
Cool Down time	7 min	-	-
Cryo timeout	15 min	-	-
MS Source Temperature	230°C	-	-
MS Quad Temperature	150°C	-	-

DHS parameters:

Table 4: DHS parameters

Step	DHS
Incubation temperature	35°C
Incubation time	20 min
Agitation speed	300 rpm
Transfer heater temperature	50°C
Trapping volume	1200 mL
Trapping flow	30 mL/min
Trap temperature	35°C
Incubation temperature (trapping phase)	35°C
Drying volume	200 mL
Drying flow	50 mL/min
Trap temperature (drying phase)	25°C

3.4 Emitted and contained VOCs sampling and analysis

Leaves will be sampled on treated and [NEG] trees for VOCs analysis by DHS-GC-MS and VOCs released in the atmosphere will be sampled by entrapping emissions on adsorbent cartridges using an encapsulation technique: wrapping one random branch into a Nalophan® bag. Emissions will then be analyzed using TD-GC-MS.

All the data collected will be analyzed using MassHunter software (Agilent Technologies Inc) and compound identification will be based on the comparison of spectrum obtained from the analysis with spectrum from several database and on the comparison of the compounds' retention time compared with the kovats index and the retention index found in the literature.

3.4.1 Leaves sampling and analysis

For each sampling, approximately 10g of fresh matter must be sampled.

In the orchard, for each tree, sampling will take place at 3 different height (low, medium and high). 2 leaves per height will be randomly sampled on each side of the tree[11].

Leaves will immediately be cryo-freezed in liquid nitrogen after sampling and will be stored at -80°C prior to analysis.

[11] Each side of the row.

For analysis, each sample of vegetal matter is placed in a grinder and liquid nitrogen is poured onto it to keep it frozen prior to grinding using an IKA grinder. One gram of the obtained powder is placed in a DHS vial. Two mL of NaCl solution at 20% (w/v) are then added for salting out and the sample can finally be injected in DHS-GC-MS (Figure 14).

The injection was executed as follows: First, 1µL of 1 Methyl trans-cinnamate solution at 50µg/mL is added as internal standard in the vial ("advial" technique). Each vial is inserted in the DH sampler and incubates for 20min at 35°C. Then, 1200mL of He carrier gas are passed onto the sample's headspace at a 30mL/min flow rate.

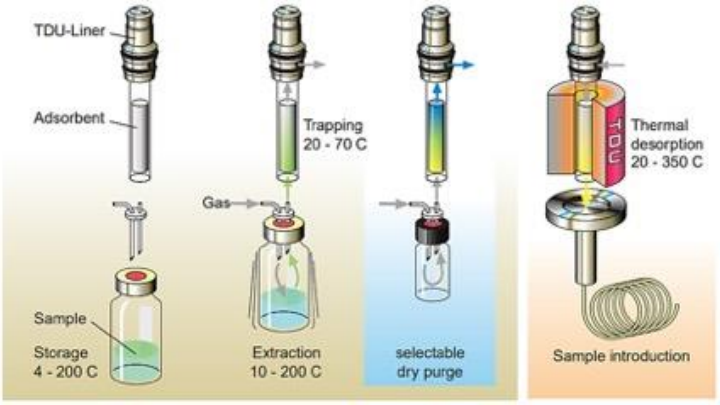


Figure 14: DHS sampling and TD scheme (https://www.gerstel.com/en/dhs-scheme.htm)

The VOCs contained in the headspace can therefore be absorbed on an adsorbent Tenax TA cartridge. To prevent an ice block forming in the CIS, a dry purge is performed by blowing 200mL of nitrogen through the cartridge at 50mL/min. The cartridge is then placed in the TDU for desorption and the rest of the analysis follows the same path as previously explained in the 3.3.2 section.

All the handling of the samples, cartridges and internal standard is fully automated using a multipurpose sampler 2 (MPS2, Gerstel, Germany).

3.4.2 Volatiles sampling

For each sampled tree, a 60L PET Nalophan® (odometric) bag was placed over a branch (encapsulation). This branch was randomly selected but must be approximatively at the same height and location between each tree and must remain the same all along the experiment. The bag is sealed at one extremity over the branch using a zip tie. The tie also helps to hold in place an activated charcoal filter tube that purifies the air entering the bag. At the other extremity, an inox or PTFE tube is enclosed inside the bag and screwed inside a stainless steel tube connector (Swagelock™). Two cartridges (Tenax TA 60/80, Camsco™) are placed in series onto the connector for the trees treated with cinnamon essential oil (in order to be certain to obtain complete trapping of the compounds of interest, in this case trans-cinnamaldehyde) and relied to a Gilian™ "GilAir Plus" air sampling pump (Sensidyne). Other treatments only required one cartridge. Prior to sampling, sorbent tube were conditioned for 11h at a 300°C program in the conditioner (TC2 conditioner, Gerstel). The air is pumped from the bag and passes through the cartridges allowing VOCs to be adsorbed. Air pumping is executed for 90 minutes at a 100mL/min rate. So, as a total, 9 liters of air are pumped (Figure 15).

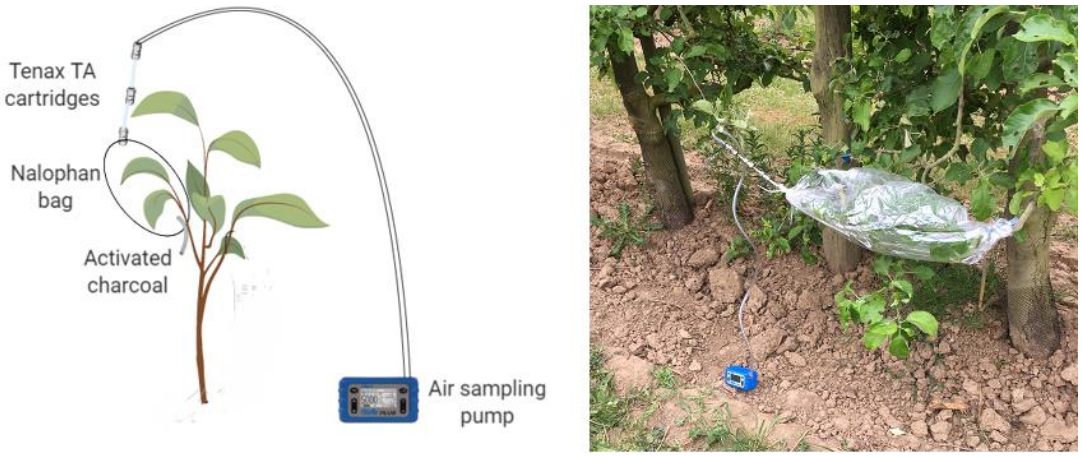


Figure 15: Scheme (Burgeon et al., 2019) *and picture of the exogenous VOCs sampling technique using Tenax TA cartridges (Camsco).*

Cartridges were then stored at -80°C prior to analysis. Each cartridge received 1µL of 1-phenyloctane solution at 400µg/mL as internal standard prior desorption. The thermal desorption and the GC-MS parameters are identical to those described in 3.3.2 section. After desorption, the cartridges were conditioned in a shorter cycle (30min at 300°C) prior the following sampling.

To be able to compare each branch emissions with the others, the detected and quantified compound in the GC analysis were brought up to a nanogram of compound per gram of fresh leaves or gram of dried leaves, per hour scale. To do so, all the leaves (and apples if there happens to be one or several on the branch) of the enclosed branch were withdrawn from the branch on the last sampling session. The total weight of fresh matter (leaves + apples) was weighted and triplicates of approximatively 2 gram of fresh matter were dried at 50°C and weighted every 24h until constant weight (considered achieved after 48h). To ensure that the apples had reached constant weight, they were dried 2 hours longer at a higher temperature (100°C). Although the literature on the subject worked at temperature of 60-70°C for apples drying (McGlone et al., 2003; Palmer et al., 2010), it was decided nonetheless to pursue the drying of the apples at a higher temperature but for a shorter period.

3.5 Insect population dynamics

During the experiment, the insect populations will be monitored, both in the case of an artificial infestation and of a natural presence.

This part was supervised by the UCLouvain partners on this project, from the biology faculty (Louvain-la-Neuve). Results and statistical analysis were computed and designed by them. The first aphids counting involved all the project's member and other insects counting on the field were under their supervision.

In the orchard, the Dysaphis plantyaginea colonies were counted on the 11th of May 2020. A colony was defined as "more than 10 individuals present on the same leave". Each colony was marked with a colored string tied around the branch supporting the leave of interest. Each tree was given a score based on the number of colony spotted.

During the experiment, the colonies development was monitored once per week for 6 weeks and each new colony was marked using a string of a different color for each census. The total number of colony per tree was registered at each monitoring session. The total number of aphids was evaluated for one colony (always the same) on each tree at each census.

The natural predators, pollinators and other class of insects or other arthropods (e.g. spiders) were also monitored. This was performed using a stick and a white board. Using the stick, tree branches (high, medium and low on the tree) were beaten 3 times each. As a result, the insects or other living

creatures fell onto the white board and a census of the biodiversity was executed for 30 seconds. The operation was repeated two time per tree (on each side of the row) and for each census. The number of different species or genus was determined and the number of individuals per species or genus was counted (EcoOrchard, 2017).

3.6 Evaluation of the treatment's ecophysiological impact

Phytotoxicity will be assessed using a fluorimeter to measure the maximum quantum efficiency of the photosystem II (PSII).

The measured leaf is "dark" adapted using a clip that does not allow the light to reach its surface. This allows the PSII electron acceptors (chlorophyll *a*) to be re-oxidized and to react when a rapid illumination of the sample happens after dark adaptation. When this illumination occurs, the leave displays a rapid rise in emitted fluorescence that fades quickly after. This is called the Kautski induction that is graphically represented at (Figure 16).

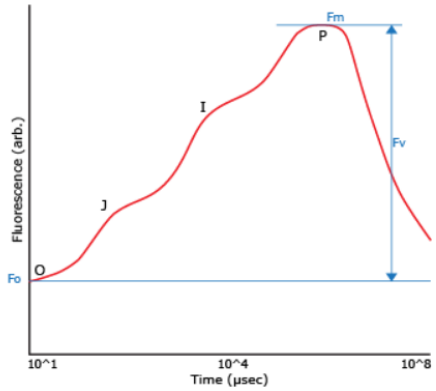


Figure 16: Kautsky fluorescence induction curve (Hansatech instruments: http://www.hansatech-instruments.com/continuous-excitation-chlorophyll-fluorescence/ 07-07-2020)

Where:

- F0 is the fluorescence origin
- Fm is the fluorescence maximum
- Fv is the variable fluorescence (= Fm – F0)

The fluorescence measurements will be taken every 10µs during one second using the fluorimeter (Hansatech – Handy PEA).

The illuminating conditions are as follows:

- Flashes: 1
- Pre-illumination: none
- Duration pre-illumination: 0.0s
- Illumination: 3000µmol.$m^{-2}s^{-1}$
- Duration illumination: 1.0s
- Gain: 1

The Fv/Fm ratio was taken as a global health index for the plants (Pavlović et al., 2014). For each tree, 5 clips were placed as follows: 3 at various height (low, medium and high) on the north exposed side of the tree and 2 at intermediate height (between low and medium and between medium and high) on the other (south exposed) side. The clips were held in place for at least 20min before the measure was taken. For each tree, the measure was taken at the same moment of the day between the sampling sessions.

3.7 Data analysis

The data obtained after the various GC-MS analysis were dealt with using MassHunter Workstation Software (Agilent Technologies, Inc. 2016). Various spectra database were used for spectra analysis and compounds identification: NIST17, NIST14, PAL600K and WILEY275.

The data treatment was executed using the R Project for Statistical Computing software.

3.7.1 Fluorescence analysis

As the Fv/Fm measure consists in a repeated measure over time on the same individuals, the data were treated using repeated measure ANOVAs. The homogeneity of variances and co-variances were assessed using the Mauchly's test (H0= sphericity of variance and co-variance). The normality of the data set was determined using Shapiro-Wilk's normality test (H0= the population is normally distributed). The data acquired on the treated trees was compared to the untreated trees [NEG] using the Dunnett's test and a pairwise comparison between the treatments was performed using the Tukey's test.

3.7.2 Aphids population analysis

This part was supervised by the UCLouvain partners on this project, from the biology faculty (Louvain-la-Neuve). Results and statistical analysis were computed and designed by them.

The number of aphids of one colony per tree and the number of colony per tree were analyzed using a Generalized Linear Model (GLM) with a Fisher test (family = quasipoisson(log)). The other insects monitored (pollinators, predators, others pests, ants and parasitoïds) were analyzed using a GLM with a Chi-square Test.

3.7.3 Emitted and contained VOCs analysis

Using MassHunter Qualitative analysis Workflow, the chromatograms were extracted using Total ion Chromatogram and compound mining was performed by integration. The obtained results were exported as Microsoft Excel file.

Each compound's area was then divided by the total chromatogram area and by the area of the internal standard. The compounds that accounted for less than 0.01% of the total chromatogram area and non-biogenic compounds (e.g. –siloxane compounds coming from the GC-MS column) were discarded. The relative amount of each compound was then computed using the internal standard area (semi-quantification). The relative amount was then expressed in ng per gram of dried leaves per hour to have a mean of comparison between the samples. The [CEO2] and the [NEG] results of the two first samplings were deeply analyzed and the compounds that were redundant in a majority of the total 26 samples were considered for the analysis.

The relative amount of the compounds were then compared between sampling sessions and between treatments using Principal Component Analysis (PCA). A PermANOVA and pairwise PermANOVA were then applied to the complete set of data and on the first two dimensions obtained with the PCA analysis. PermANOVA was computed using the "Adonis" function with a Euclidean method and a Fisher test. Pairwise permutations MANOVAs were done using a distance matrix generated with the R "dist" function (Euclidian method) and a Wilks test.

4 Results and discussion

4.1 Cinnamon essential oil characterization

The characterization of the cinnamon essential oil was given by Pranarôm International SA from whom the oil was purchased. The chromatogram is available at (Figure 17).

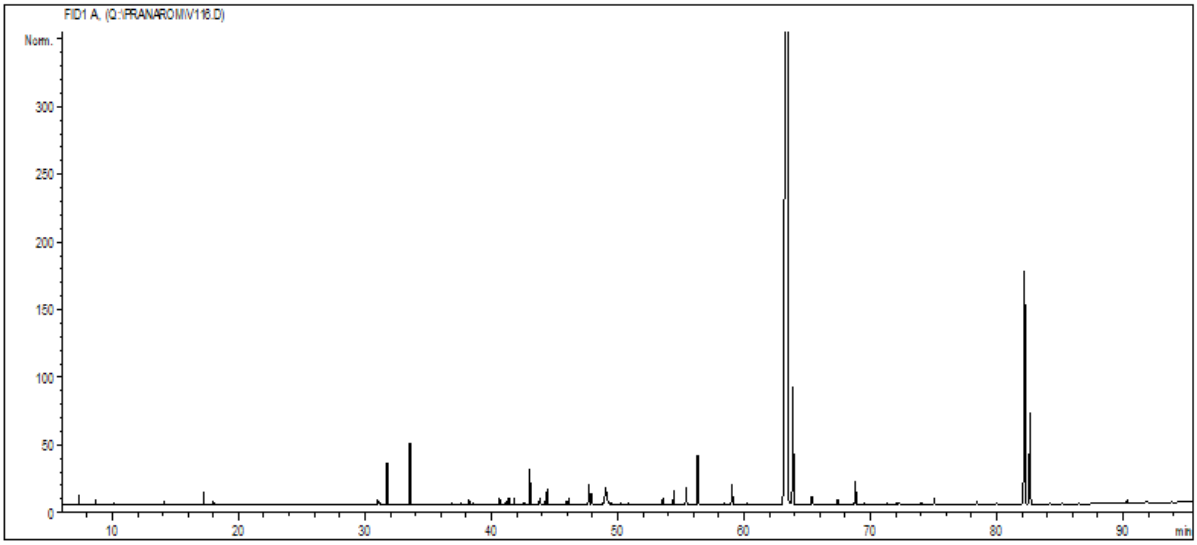


Figure 17: Cinnamon essential oil chromatogram (Pranarôm)

E-cinnamaldehyde accounted for 79.5% of the cinnamon essential oil. This is consistent with the values found in the literature, ranging from 30% (Wang et al., 2009) to values as high as 83% (Jardim et al., 2018) and everything in between (Geng et al., 2011; Chang et al., 2013; Ojaghian et al., 2016). The rest of the profile was however slightly different in our extract than in the literature found on the matter. The second most present compound in our extract is Trans-o-Methoxy cinnamaldehyde, representing 5.3% of the total cinnamon EO. This compound was also reported as bioactive in (Gunawardena et al., 2015). The obtained profiles of cinnamon essential oil extracts from *Cinnamomum cassia* were very different from one article to another, depending on the cinnamon age, growth region, type of bark used, etc. which can explain high variability between extracts.

As a result, each tree treated with [CEO2] and [CEO1] were injected with 0.046g and 0.024g of E-cinnamaldehyde, respectively. The complete characterization table is available in the Appendix section (Appendix 1).

4.2 E-cinnamaldehyde's calibration curve

4.2.1 TDU-GC-MS - Emissions

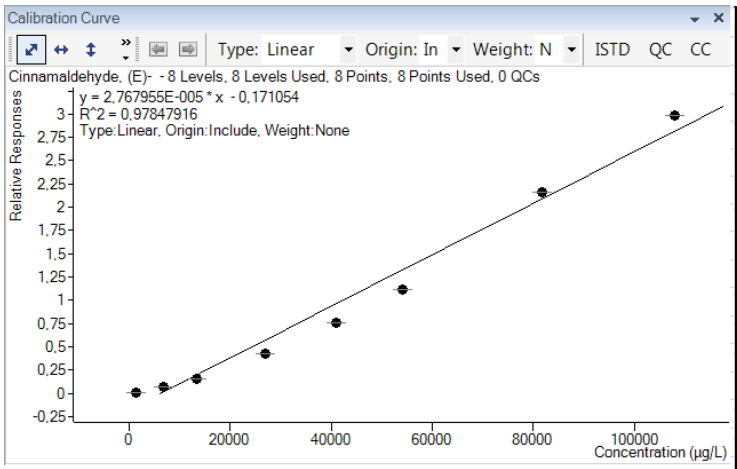
The following curve (Figure 18) was passed in TDU-GC-MS on Tenax TA cartridge. Chromatograms were analyzed using MassHunter Quantitative Analysis (Agilent).
In order to quantify the E-cinnamaldehyde content in the VOCs emissions, the LOD and LOQ were predicted using the method described in (Wenzl et al., 2016). The X and Y value (relative concentration and relative response, respectively) given by Masshunter were used to obtain a linear regression using Microsoft Excel Data Analysis. The LOD and LOQ were then predicted as follows (Wenzl et al., 2016):

$$LOD = 3.8 * \frac{SD}{b} * \sqrt{1.1 + \frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}}$$

$$LOQ = 3.3 * LOD$$

Where:

SD: standard deviation of the residuals of the linear regression
b: slope of the curve (y = 0.5565x – 0.2221)
$\bar{x}$*:* mean calibration level
x_i*:* analyte concentration at calibration level i
3,8 and 3,3: constants



Trans-cinnamaldehyde			
R^2 = 0,9809			
y = 0,557x -0,222			
[C] µg/L	S/N	X	Y
113931,7	15919,23	5,5	3,0
84429,4	24815,12	4,2	2,2
46358	5045,93	2,8	1,1
33837,9	2880,9	2,1	0,8
21490,4	1575,17	1,4	0,4
11775,8	36,36	0,7	0,2
8592,6	26,6	0,3	0,1
6731,5	13,09	0,1	0,0
SE of Intercept		0,1	
SD of Intercept		0,4	
LOD		0,7	
LOQ		2,2	

Figure 18: MassHunter Quantitative Analysis curve (left) and LOD/LOQ table value (right)

The LOD and LOQ given in (Figure 18) are expressed in relative response (X unit). When comparing to the calculated concentration ([C] column), the LOD is close to 11775µg/L and the LOQ is located between 33837 and 46358µg/L. Those are huge values, way above the lowest calibration concentration of 1000µg/L of E-cinnamaldehyde. To address this issue, another calculation was made using the actual response as Y value and the calculated concentration as X (Table5).

Table 5: LOD and LOQ computed using the calculated concentration and the analyte's response.

Trans-cinnamaldehyde			
R^2 = 0,999			
y = 1133,9x - 6E+06			
[C] µg/L	S/N	X	Y
113931,7	15919,23	113931,7	122046039
84429,4	24815,12	84429,4	90541824
46358	5045,93	46358	47817971
33837,9	2880,9	33837,9	33688735
21490,4	1575,17	21490,4	18839011
11775,8	36,36	11775,8	7126290
8592,6	26,6	8592,6	3080877
6731,5	13,09	6731,5	713801
SE of Intercept		560635,2	
SD of Intercept		2387245,0	
LOD		2105,3	
LOQ		6947,6	

The LOD and LOQ are about 2105µg/L and 6947.6µg/L, respectively. This was a lot better than the previous results, even too good: when using the calibration curve with the LOD, the corresponding response is negative, which is practically impossible. This method of statistically defining the LOD and LOQ is not suited to this analysis and should therefore not be taken into account. Furthermore, LOD and LOQ are more valuable when calibration samples are realized in triplicates. In this case,

only one repetition was available due to several malfunction and unavailability of the apparatus. The LOQ value was thus kept as the reference value for later E-cinnamaldehyde detection in the VOCs emissions. The values presented here are therefore to be considered cautiously: not as a genuine representation of the reality but rather as an idea of the range of concentration at which E-cinn can be at least detected (C = 6000µg/L) in trees' emissions using this TDU-GC-MS method. Improvement with assessment of the matrix effect and repetitions of the method should be made to obtain validated analytical LOD and LOQ.

4.2.2 DHS-GC-MS – Leaf Contents

The leave content of E-cinnamaldehyde could not be determined during the time of this master's thesis. This is however still a go for the overall SPW project but it will not be treated here.

4.3 E-Cinnamaldehyde detection in VOCs emissions

In some samples, E-cinn was detected in SCAN mode by the MassHunter QualitativeWorkflow (Agilent). For samples where E-cinn was not identified but that still displayed a peak at the E-cinn acquisition time range (18.476 – 18.509), the peak's signal in Single Ion Monitoring (SIM) or via Extracted Ion Chromatogram (EIC) was further investigated (Figure 19). Peaks displaying E-cinn's typical ion fragments m/z = 131, 132 and 103 were manually identified and so for all the samples (even from non-cinnamon treated trees).

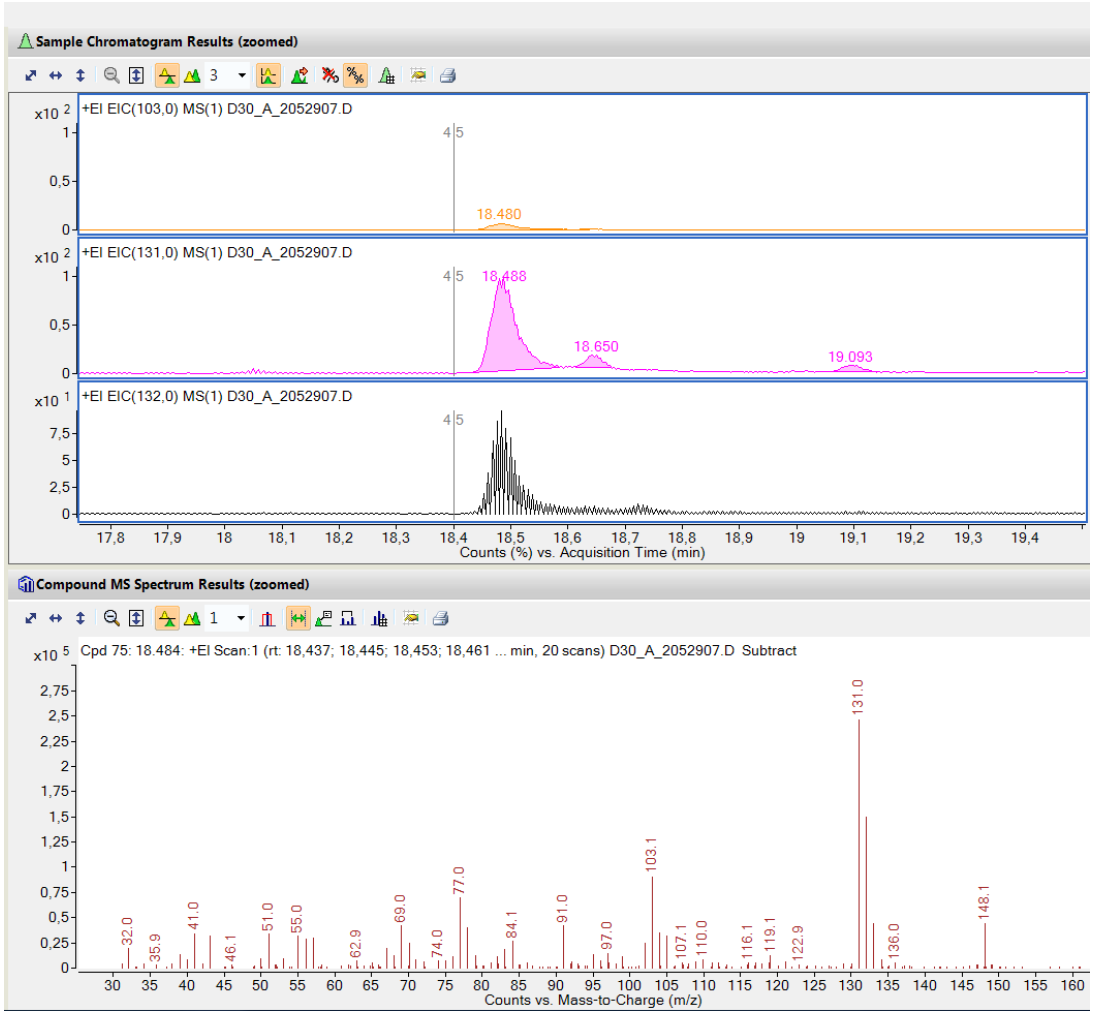


Figure 19: D30 tree's chromatogram at sampling 2. The chromatogram response for ion fragment m/z = 103, 131 and 132 are zoomed in and the mass spectrum of the peak detected at 18.484 min clearly displays all 3 fragments.

As a total, 16 trees displayed an E-cinnamaldehyde peak at a least one sampling session. The (Table 6) lists the trees where E-cinnamaldehyde was identified, the calculated amount of analyte (using the previously designed calibration curve) and the sampling session where it was present. As the considered LOD for the method is of 6000µg/mL, the calculated amount cannot be considered precise for an evaluation of the E-cinnamaldehyde emission rate or transfer rate in the tree. The values presented here are purely informative and should be considered as a proof of cinnamon essential oil transfer and emission in and by the tree rather than an actual measure of how much analyte was emitted.

Table 6: E-cinnamaldehyde calculated amount in trees VOCs emissions

Calculated amount of E-cinnamaldehyde (µg/L)

Tree	Sampling 1	2	3	4
D11 - CEO2	7304			
D14 - CEO1	5732		6350	
D16 - CEO1		6725	6361	
D2 - CEO1	14854		6265	
D22 - NEG		6718		
D24 - BEM				11358
D30 - CEO1		7689		
D32 - BSA		18448		
D37 - CEO2		7106		
D8 - NEG		16909		
I19 - CEO2		6121		6413
I31 - BSA		6177		
I49 - NEG			16451	
D28 - NEG	13692	5950	14556	6287
D35 - CEO2	5739	13562	6462	9360
I52 - CEO2		5954	8753	6352

Quite surprisingly, E-cinnamaldehyde was detected in several [NEG] trees, with D28 showing E-cinn peak in all four sampling sessions. Some E-cinn was also detected in [BSA] and [BEM] trees. There are several hypothesis to explain these unexpected results:

- During sampling sessions, only 8 Nalophan bags were available. When sampling was done on one tree in the orchard, the bag was almost immediately replaced on another tree. Condensation on the inner surface of the bag was formed during almost every sampling and particularly the sampling that occurred around noon where temperature in the orchard and sunlight was around its maxima on the sampling day. When bags were too wet, they were summarily dried using paper tissues or a cloth but this did not happen between all bags replacement. Nalophan bag are expected to be chemically inert but it is likely that the water remaining between samplings had retained VOCs, among which E-cinn could have been present as it presents a solubility in water of about 1.42mg/mL (Human Metabolome Database, HMDB). In the same manner, leaf residues might have been trapped in the foils of the bag, leading to contamination if the residues happened to come from a cinnamon-injected tree.
- It is not impossible that E-cinn found in non-cinnamon trees came from nearby trees treated with cinnamon. However, the coal filter placed at the fixation point of the bag should have prevented that from happening and the concentration of E-cinn in the air around cinnamon-injected tree would hardly be sufficient although it is emitted by all the tree.
- Cartridge were injected in the same order than the trees in the orchard. Therefore, the chromatogram from a “non-cinnamon” cartridge placed after a CEO1 or CEO2 cartridge in the analysis sequence could display an E-cinn peak in the case where E-cinn was retained in the column.

- Peak's identification is incorrect, despite corresponding the retention time frame and having the 3 ion fragments (m/z=131, 132, 103). Even if some peaks were manually identified, this is still very unlikely as the combination of the retention time and the three ion fragments leaves almost no doubt on the compound's identification. Moreover, some E-cinn was still identified in Scan mode from non-cinnamon trees, attesting the presence of the compound where there should not be.
- E-cinnamaldehyde was produced by apple trees. This is of course by far the most unlikely.

Anyway, this could explain a few aberrant results. Here, there happens to be 7 contaminations or false-positive, including one where E-cinn is detected at each sampling (D28). Further verifications of the chromatograms with computing of the ion ratios and using one sampling bag per tree may solve this problem in the future.

Again, the calculated concentration should not be taken into account as a genius value but rather as a proof of the presence of E-cinn in the VOCs emitted by the leaves. This is still a very promising result as it is now certain that the injected essential oil is successfully carried by the tree's sap, from the injection point to the leaves and is emitted by them. As it was (to the author's knowledge) the first time that cinnamon essential oil was used as an insecticide using application by tree-injection in an agronomics condition trial, the substantial proof that the delivery of the compounds through the tree works is positive. In this experiment design, the concentration of the active ingredient (cinnamon EO) injected in the trees was similar to the concentration used for traditional products in tree-injection, such as Imidacloprid. This will allow to easily compare the effectiveness of the cinnamon treatment to standard treatments. Furthermore, when comparing E-cinn's Koc (carbon to water partition coefficient, c.f. 1.4.5 section) of about 37 ("Cinnamaldehyde | C9H8O - PubChem," March-26-2020) to Imidacloprid's Koc ranging from 156 to 800 ("Imidacloprid | C9H10ClN5O2 - PubChem," March-26-2020), high mobility could have been expected from E-cinn compared to proven effective Imidacloprid. However, this high mobility would also be synonym of a short persistence of the product in the tree and thus a short-time effectiveness, excepted if it happens to be retained in leaves as stocked VOCs. In this matter, an analysis of the leaves will be necessary to ascertain the previous circulation hypothesis.

Now that the treatment was proved effective for cinnamon mobility and E-cinnamaldehyde emission, it is still to be assessed if it is effective against aphids as direct defense or by systemic acquired resistance (indirect defense elicitor) and how the trees respond to it.

4.4 VOCs emissions analysis

The VOCs profile analysis can inform on the treatment's impact on the tree, particularly if stress-related volatiles are emitted by (a) injected trees and (b) cinnamon injected trees that potentially suffers the toxicity of the oil added to the injection wounding. Emissions analysis can also inform on the insects response, as aphids or other insects (predators, pollinators) are sensitive to specific VOCs' concentration in the air and that a change in the VOCs' profile can induce a behavioral change in aphids (repellent, anti-feedant, attractant) or in other insects.

4.4.1 VOCs species

From the comparison of the [CEO2]'s and [NEG]'s global VOCs content of the two first samplings, a list of 55 volatile species was constituted. This list (Table 7) served as a basis for the comparison between treatments in terms of VOCs contents. Based on the comparison in terms of VOCs species, it appears that no new compounds were produced by injected trees as a response to the injection. The variations in profile are thus rather quantitative than qualitative. Subsequent analysis will thus be based on the relative amount of each VOC emitted by the trees, expressed in ng/g dried leaves/hour.

Table 7: VOCs species global list (to be continued on the next page)

CAS	RT	Name	Formula	Kovats (reference)	Kovats (computed)	Ratio Kovats ref/Kovats comp
Internal standard						
2189-60-8	23,591	Benzene, octyl-	C14H22	1461	1472,071	0,75%
Terpenoïds						
13466-78-9	12,026	3-Carene*	C10H16	1011,3	1050,713	3,75%
78-70-6	13,566	Linalool	C10H18O	1100	1100,574	0,05%
19945-61-0	14,065	(E)-4,8-Dimethylnona-1,3,7-triene	C11H18	1116	1118,505	0,22%
3856-25-5	21,333	Copaene	C15H24	1376,2	1383,152	0,50%
5208-59-3	21,595	.BETA. BOURBONENE	C15H24	1384,2	1393,662	0,68%
87-44-5	22,486	Caryophyllene	C15H24	1420,1	1425,169	0,36%
24268-39-1	23,108	.gamma-Muurolene	C15H24	1474	1451,57	-1,55%
23986-74-5	24,025	Germacrene-D	C15H24	1480	1490,492	0,7%
13474-59-4	24,291	trans-.alpha.-Bergamotene*	C15H24	1441	1501,782	4,05%
502-61-4	24,613	.alpha.-Farnesene	C15H24	1504,1	1510,479	0,42%
Acids						
64-19-7	2,638	Acetic acid	C2H4O2	625	622,3	-0,43%
79-09-4	3,503	Propanoic acid	C3H6O2	710	712,823	0,40%
65-85-0	15,74	Benzoic acid	C7H6O2	1180	1178,692	-0,11%
124-07-2	15,862	Octanoic acid	C8H16O2	1182,03	1183,075	0,09%
112-05-0	18,378	Nonanoic acid	C9H18O2	1275,3	1271,904	-0,27%
334-48-5	20,97	Decanoic acid	C10H20O2	1375,5	1368,592	-0,50%
Alcools/Phenols						
928-96-1	6,406	3-Hexen-1-ol, (Z)-	C6H12O	856,6	848,735	-0,93%
111-27-3	6,783	1-Hexanol	C6H14O	869,7	861,277	-0,98%
3391-86-4	9,942	1-Octen-3-ol	C8H16O	980	978,796	-0,12%
108-95-2	10,042	Phenol	C6H6O	983,3	982,084	-0,12%
58175-57-8	11,449	2-Propyl-1-pentanol	C8H18O	1052,8	1031,114	-2,10%
100-51-6	11,594	Benzyl alcohol	C7H8O	1036,9	1036,039	-0,08%
111-87-5	12,713	1-Octanol	C8H18O	1071,5	1074,048	0,24%
111-14-8	12,846	Heptanoic acid	C7H14O2	1080,1	1078,566	-0,14%
121959-70-4	18,648	Cyclopropanemethanol, .alpha.,2-dimethyl-2-(4-methyl-3-pentenyl)-, [1.alpha.(R*),2.alpha.]-	C12H22O	1299	1282,127	-1,32%
112-53-8	23,738	1-Dodecanol	C12H26O	1472,8	1478,31	0,37%
14035-34-8	27,662	2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol	C17H26O2	1644	1643,112	-0,05%
112-72-1	28,413	1-Tetradecanol	C14H30O	1676,3	1678,42	0,13%
32214-89-4	28,677	Caryophylla-3(15),7-dienol (6) II	C15H24O	1682	1690,832	0,52%
Aldehydes						
100-52-7	9,321	Benzaldehyde	C7H6O	962,7	958,382	-0,45%
122-78-1	11,87	Benzeneacetaldehyde	C8H8O	1045,9	1045,414	-0,05%
124-19-6	13,699	Nonanal	C9H18O	1103,3	1105,353	0,19%
112-31-2	16,662	Decanal	C10H20O	1205,4	1206,929	0,13%
3913-81-3	18,228	2-Decenal, (E)-	C10H18O	1263,4	1266,224	0,22%
14371-10-9	18,476	Cinnamaldehyde, (E)-	C9H8O	1271,3	1275,615	0,34%
112-44-7	19,473	Undecanal	C11H22O	1306,5	1308,543	0,16%
112-54-9	22,136	Dodecanal	C12H24O	1408,1	1410,314	0,16%
41610-68-8	27,681	Ylangenal	C15H22O	1674	1644,005	-1,82%
2765-11-9	29,048	Pentadecanal-	C15H30O	1714,4	1711,473	-0,17%

CAS	RT	Name	Formula	Kovats (reference)	Kovats (computed)	Ratio Kovats ref/Kovats comp
Ketons						
110-93-0	10,164	6-Methyl-5-hepten-2-one	C8H14O	985,9	986,094	0,02%
98-86-2	12,535	Acetophenone	C8H8O	1067,4	1068,002	0,06%
3796-70-1	23,253	5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	C13H22O	1451,8	1457,724	0,41%
119-61-9	27,43	Methanone, diphenyl-	C13H10O	1628	1632,204	0,26%
Alcans/Alcens						
112-40-3	16,489	Dodecane	C12H26	1200	1200,378	0,03%
629-50-5	19,265	Tridecane	C13H28	1299	1300,2	0,09%
629-59-4	21,889	Tetradecane	C14H30	1400	1405,455	0,39%
629-62-9	24,371	Pentadecane	C15H32	1500	1505,178	0,34%
629-78-7	28,816	Heptadecane	C17H36	1700	1700	0,00%
Esters						
142-92-7	10,984	Acetic acid, hexyl ester	C8H16O2	1010,4	1015,319	0,48%
119-36-8	16,359	Methyl salicylate	C8H8O3	1192,9	1200,934	0,67%
Alcool esters						
3681-82-1	10,774	3-Hexen-1-ol, acétate	C8H14O2	1005	1008,186	0,32%
Aromatics						
107983-42-6	15,026	exo-7-(trans-1-Propenyl)bicyclo[4.2.0]oct-1(2)-ene	C11H16	1140	1153,036	1,13%
58437-71-1	17,37	Ethanol, 2-(3,3-dimethylbicyclo[2.2.1]hept-2-ylidene)-*	C11H18O	1303	1233,737	-5,61%

In (Table 7), compounds marked with an "*" are the one for which the Kovats ratio is superior to 3% which means that their identification is uncertain. The Kovats (reference) index were taken from the NIST Mass Spectrometry Data Center or from the ChemSpider[12] website in last resource. When analyzing chromatograms, non-biogenic compounds were automatically discarded. Some compounds were found as biogenic but were not found referenced in apple trees emissions. Such compounds are: **(A)** exo-7-(*trans*-1-Propenyl)- bicycle[4.2.0]oct-1(2)-ene, reported in the composition of *Ligusticum chuanxiong (*Hort) essential oil (Yang et al., 2015), **(B)** Ethanol, 2-(3,3-dimethylbicyclo[2.2.1]hept-2-ylidene)-, reported in the profile of *Viola odorata* (Jasim et al., 2018) and **(C)** 2,6-bis(1,1-dimethylethyl)-4-(1-oxopropyl) phenol, reported in soybeans emissions (Cantúa Ayala, 2020). Anyway, neither of these 3 compounds seems to be of ecological importance in this particular experiment although some were reported to influence oviposition in limabeans or elicited antennal response, which means that it may have an impact on some insects' species.

The rest of the profile are compounds that are typical plant metabolites emitted by apple trees. Among them are compounds of biological importance, those compounds variation in the different VOCs profile may explain a marked difference in the insects dynamics or in the plants ecophysiological reaction to the treatment. Those compounds were listed in the (Table 8) and their ecological role was briefly explained.

The profile is qualitatively similar to referenced apple emissions' profile. The level of emissions are however difficult to compare as the method of sampling often differs, leading to different scale. Literature research gave apple emission rate of VOCs from the order of pmol/dm/h, nL/h, mg/L or often simply expressed in mean peak area (Vallat & Dorn, 2005; Vallat, Gu, et al., 2005; Soukoulis et al., 2013; Giacomuzzi et al., 2016).

[12] http://www.chemspider.com/

Table 8: Principal compounds of biological interest. (Rapparini et al., 2001; Bäckman et al., 2001; Vallat et al., 2005; Casado et al., 2006; Hare, 2011; Dudareva et al., 2013; Becker et al., 2015; Salamanca et al., 2017)

Compound name	Biological role	Reference
Linalool	Pollinators attraction	*Rapparini et al, 2001*
	HIPV	*Hare et al, 2011*
3-carene	HILV, toxic properties	*Dudareva et al, 2013*
Benzenoid compounds	HIPV, L-phenylalanine pathway	*Dudareva et al, 2013*
	HIPV	*Becker et al, 2015*
3-hexen-1-ol (Z)	GLVs from the LOX pathway	*Dudareva et al, 2013,*
	Antennal response elicitor	*Backamnn et al, 2001*
Nonanal	GLVs from the LOX pathway	*Dudareva et al, 2013*
beta-caryophyllene	Attraction of pest-killing parasitic wasps	*Dudareva et al, 2013*
	Pests attraction	*Vallat, 2005*
Methyl salycilate	Bees attraction	*Dudareva et al, 2013*
	Repellent	*Vallat et al, 2005*
	Predators attraction	*Salamanca et al, 2017*
	HIPV	*Becker et al, 2015*
(E)-4,8,dimethyl-1,3,7-nonatriene (DMNT)	HIPV	*Hare et al, 2011; Casado et al, 2006*
	Antennal response elicitor	*Backman et al, 2001*
α-farnesene	HIPV, pest repellent at high concentration	*Hare et al, 2011; Backman et al, 2001*
	Pest attractant at low concentration	*Casado et al, 2006*
α-bergamotene	HIPV	*Hare et al, 2011*
Germacrene-D	HIPV	*Casado et al, 2006*
	Antennal response elicitor	*Backman et al, 2001*

NB: HIPV = Herbivory Induced Plant Volatile, GLV = Green Leaf Volatile, LOX = Lypoxygenase.

The emission of HIPV is the major form of induced indirect defense for plants. This can be done either by the emissions of novel compounds or the emission of qualitatively similar but quantitavely different VOCs species or blends, following herbivory infestation or mechanical wounding. VOCs or blends emissions can even be species-specific (Wadhams et al., 1999). Direct defense aims the production of toxic or repellent/deterrent compounds by the plant itself. Successful strategies in plant management aims the eliciting of SAR (Systemic Acquired Resistance) that is going to produce direct defenses's compounds or blends, in addition with the triggering of indirect defense by predators/parasitoïds attraction.

By treating the trees with cinnamon essential oil, both a direct effect on aphids and in the trees' emission patterns was expected. Direct effect on aphids was expected through E-cinnamaldehyde toxicity or SAR triggering and indirect effects were expected to come from the stress induced by the treatments, resulting in HIPV release.

4.4.2 VOCs species concentration: comparison between treatments

Now the actual purpose of this experiment was to determine if there was one or several significant differences in the VOCs profile and concentration between the five different treatments factor. To do so, one could investigate one compound at a time and perform repeated measure ANOVA on each compound results. This would allow to identify the potential significant differences between treatment, sampling time, and tree for one compound. However, this would generate a lot of analysis and a lot of data that would be hardly put in perspective to the treatment's effect on the aphids or on the tree's condition. Indeed, 125 samples each containing 55 variables is a huge dataset and comparing each identified significant difference to aphids monitoring results or to chlorophyll fluorescence measure would be a laborious activity and would not even be pertinent. To deal with this matter, the use of PCA can help ease the analysis by extracting the important information contained in one dataset. It creates Principal Components (PCs), or dimensions, from the original dataset, each PC being a linear combination (vector) of the original variables (Gorban et al., 2008). PCs can be compared to one another to identify significant variations in the data. However, it shall be kept in mind that PCA only

gives visual (qualitative) appreciation of the treated data, unlike ANOVA which is a descriptive statistical analysis. To go further than the PCA visual aid, a PermANOVA (Permutational Multivariate Analysis Of Variance) can be performed. The interest of PermANOVA is that it is a semiparametric method: it allows to perform classical partitioning (interaction terms, various tests, hierarchical structure,…) while also being a robust multivariate method that can treat non-normal, ordinal, qualitative or zero-inflated data (Anderson, 2017). Its use is widely spread in ecological, agriculture, chemistry or genetics field where a lot of data is often generated from a complex experimental design.

First, a histogram of the eigenvalues of the dimensions created by the PCA can be drawn (Figure 20):

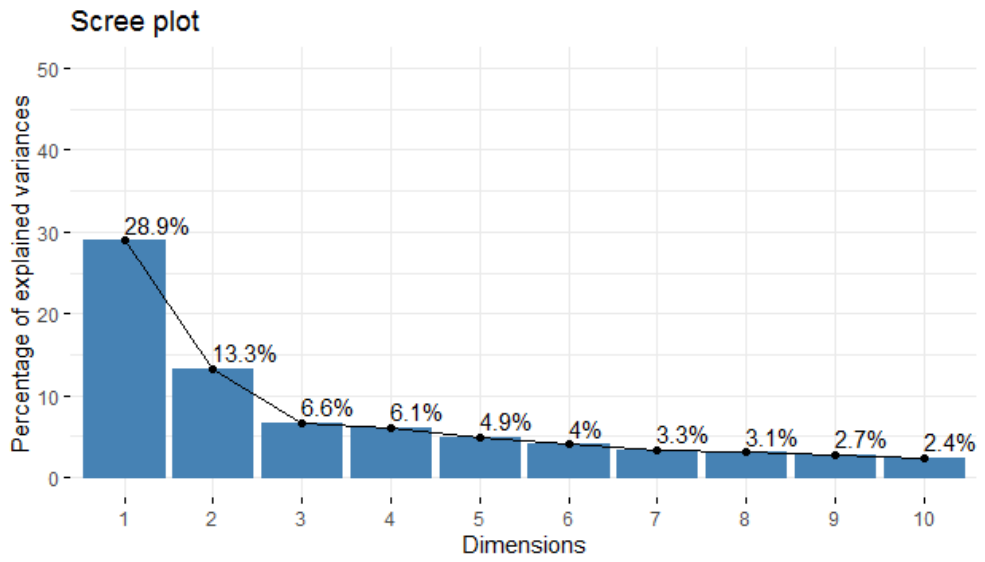


Figure 20: Histogram representing the eigenvalues of the 10 first dimensions created from the leaf emissions profile.

The eigenvalue informs on the percentage of the total variance explained by each dimension. Here, the two first dimensions (or PCs) explains 42.2% of the total variance. In other words, the variables (compounds) constituting the two first PCs are the one for which the relative amount (in $ng/g_{dried\ leaves}/hour$) changes the most from the sampling day and/or the treatment to another. The first 10 dimensions explains 75.3% of the total variance and the last four values are very similar to one another, which means that the next dimensions are not much representative of the variance and are therefore not worth further analyzing.

Then, by generating graphs where the axis are chosen from two dimensions out of the 10 and by drawing ellipses, it can be observed whether the individuals (treatment, sampling day or treatment+sampling day) separate or not from one another. In the case where individuals are well separated on the graphs, significant differences can be expected in the relative amount of compounds constituting the dimensions chosen for the axes. On the other hand, if individuals are not well separated, then no significant difference are to be expected. The first series of graphs were drawn using the PCs 1 and 2 as axis (figure 21, 22 and 23) and the representation of ellipses on other dimensions (1, 3), (2, 3), (1, 4), (2, 4) did not gave more interesting information, it was thus chosen to work with the first two dimensions as explicative variables for further analysis.

The compounds constituting the first two dimensions are listed in the (Table 9):

Table 9: Compounds constituting the first two dimensions of the PCA. Columns represent (from left to right): (A) Compound: the variable's name (B) Dim (X): the correlation between the variable and the dimension X. (C) Coord: the variable's coordinate on the axis. (D) Cos2: the quality of the representation. (E) Contrib: the contribution of the variable to the dimension.

Compound	Dim.1	coord	cos2	contrib	Compound	Dim.2	Coord	cos2	Contrib
Phenol	0,882	0,777	0,777	39.563	**Caryophyllene**	0,836	0,699	0,699	67.811
Nonanal	0,878	0,771	0,771	39.229	**Ethanol, 2-(3,3-dimethylbicyclo[2.2.1]hept-2-ylidene)-**	0,818	0,669	0,669	64.893
Decanal	0,861	0,742	0,742	37.761	**Beta-Bourbonene**	0,786	0,618	0,618	59.995
Methanone, diphenyl-	0,861	0,742	0,742	37.735	**Germacrene-D**	0,779	0,607	0,607	58.895
Undecanal	0,831	0,691	0,691	35.157	**.alpha.-Farnesene**	0,776	0,603	0,603	58.477
Heptanoic acid	0,822	0,676	0,676	34.404	**trans-.alpha.-Bergamotene**	0,712	0,508	0,508	49.244
2-Decenal, (E)-	0,810	0,656	0,656	33.384	**1-Tetradecanol**	0,702	0,493	0,493	47.863
Dodecane	0,803	0,645	0,645	32.821	**10,10-Dimethyl-2,6-dimethylenebicyclo[7.2.0]undecan-5.beta.-ol**	0,685	0,469	0,469	45.541
Benzyl alcohol	0,795	0,633	0,633	32.194	**3-Carene**	0,649	0,421	0,421	40.819
Dodecanal	0,789	0,622	0,622	31.672	**(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol**	0,602	0,362	0,362	35.128

It is interesting to note that in the case of PC1 and PC2, the variable segregated based on their chemical families. The first dimension is mainly explained by aldehydes and the second is primarily constituted of terpenoïds, which are compounds of high biological interest.

To identify the primary source of variance amongst our main expected source of variation (i.e. the treatment and the sampling conditions), 3 graphs were plotted using dimension 1 and 2 as axis: Ellipses of the factor "Treatment" (Figure 21), ellipses of the factor "sampling day" (Figure 22) and ellipses of the combined factor "treatment + sampling" (Figure 24). The "Tree" individuals are expected to be a major source of variation as well but such a plotting would require 32 ellipses and would be arduous to interpret and was therefore not considered.

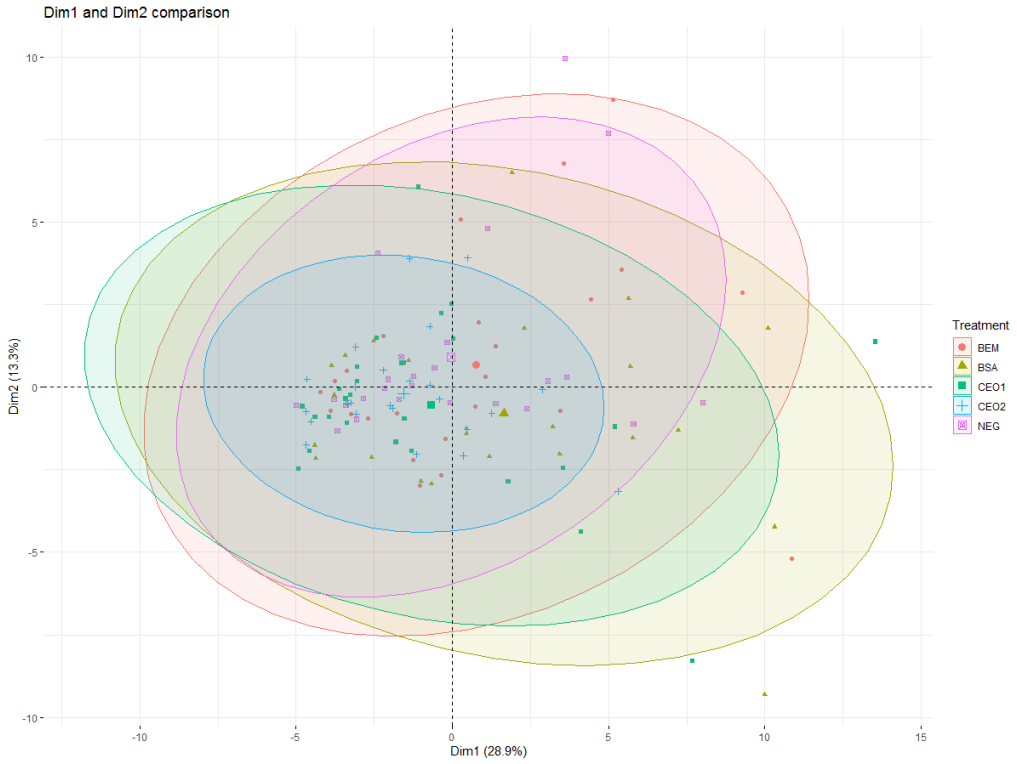


Figure 21: PCA representation of the 5 treatments on dimension 1 and dimension 2

A quick glance at this first graph easily allows to see the poor separation between treatments. Indeed, the centroids (i.e. center of the ellipses) are very close to the center of the axis. Although the ellipses vary in size and orientation, their location on the axis remains very close to one another. This globally means that although there are variations in the relative amount of emitted compounds from the first 2 PCs between treatments, they are not quite marked. It is noteworthy that the CEO2 treatment seems to have the smallest variability amongst all the treatments, as far as the first 2 dimensions are concerned. It is also interesting to take a closer look at the centroids distribution on the second axis. Indeed, the second PC contains stress-induced volatiles for which relative amount should evolve along with the stress induced by the injection or the aphids presence. The CEO1, CEO2 and BSA centroids are located under the axis where NEG and BEM are located over the axis. This may explain the observation made in the further (4.6.1) section. Still, the differences are not outstanding and should not lead to significant differences.

To verify the hypothesis, a PermANOVA was computed, still with the PC1 and PC2 as variables and with the Treatment as tested factor. The obtained p-value = 0.255 with a degree of freedom (df) of 4. As expected by the visual interpretation of the graph, the factor "treatment" is not significant in terms of variation in relative amount of the VOCs species constituting the first 2 dimensions.
This compared the "treatment" factor at once. A pairwise permutated MANOVA (Multivariate Analysis Of Variance) was then executed in order to perhaps spot differences between treatments compared one to another (Table 10).

Table 10: Pairwise comparisons using permutation MANOVAs on a distance matrix. Impact of treatment on PC 1 and 2.

Treatment's effect on the fisrt 2 PCs				
	NEG	**BEM**	**BSA**	**CEO1**
BEM	0.48	-	-	-
BSA	0.28	0.48	-	-
CEO1	0.48	0.75	0.48	-
CEO2	0.48	0.48	0.29	0.48

Again, no significant difference spotted. But this analysis only concerned the first 2 dimensions: such analysis extended to the whole dataset could vary. Plus, it shall be considered that p-value using 95% confidence level is very tight for a field trial like this one where a lot of parameters are not controlled. Here, a p-value under 0.1 can be considered significant (Frömke et al., 2004). The PermANOVA on the whole dataset gave a p-value = 0.053 (DF = 4) which can be considered significant as just stated. The treatment has thus an impact on the whole VOCs profile. This impact is however difficult to identify precisely as there are 55 variables, varying on a low scale among 125 samples. It cannot be said whether this change will have a significant influence on the insect's behavior/death rate or not or whether the tree will be physiologically damaged or not. The pairwise analysis on the whole dataset gave the following results (Table 11):

Table 11: Pairwise comparisons using permutation MANOVAs on a distance matrix. Impact of treatment on all variables.

Treatment's effect on all the variables				
	NEG	**BEM**	**BSA**	**CEO1**
BEM	0.83	-	-	-
BSA	0.12	0.67	-	-
CEO1	0.73	0.96	0.62	-
CEO2	0.11	0.11	0.07 .	0.12

The CEO2 treatment seems to separate the most from the other treatments in terms of global VOCs relative amount emitted by the leaves. Surprisingly, the BSA treatment seems to separate very well from the CEO2 treatment, as well as from the NEG treatment. When going back to the previous table, it can be observed that the two lowest value are also concerning CEO2 vs BSA and BSA vs NEG. This

also reinforces the observations made on the first ellipse regarding the centroids position of those treatments.

Knowing this, the ellipses regarding the sampling day (i.e. different weather conditions) were drawn (Figure 22).

Figure 22: PCA representation of the 4 sampling days on dimension 1 and dimension 2

As expected, the separation here is better than on the treatment's ellipses. The variation in size and orientation is more marked and the centroids are further separated although there is still no outstanding separation. Based on the previous results, it can be predicted that Day14 vs Day28 should show the lowest permANOVA p-value when compared to one another, with Day3 and Day7 as intermediate p-value. As the "Sampling day" seems to better separate the ellipses, the global p-value for PCs 1 and 2 should be lower than the one obtained with "treatment" as factor and idem for the whole set of data. Indeed, both p-value (for PCs 1,2 and for the whole variables set) showed high significance (p-value = 0.001, df = 3). Each tree's physiology and most importantly the sampling weather, changing between sessions, would therefore be responsible for the most variability in the relative amount of VOCs emitted by the leaves, which is a documented fact (Vallat, Gu, et al., 2005). As expected, the pairwise comparison gave the smallest p-value for Day14 vs Day 28 (Table 12). The comparison of the weather data on those two days would be very instructive.

Table 12: Pairwise comparisons using permutation MANOVAs on distance matrix. Impact of sampling day on PC 1 and 2.[13]

Sampling day's effect on the first 2 PCs			
	Day 14	**Day 28**	**Day 3**
Day 28	0.002 **	-	-
Day 3	0.050 .	0.002 **	-
Day 7	0.002 **	0.012 *	0.048 *

[13] Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

By adding more variables to the analysis, the pairwise comparison using all compounds tends to separate even more (Table 13).

Table 13 : Pairwise comparisons using permutation MANOVAs on distance matrix. Impact of sampling day on all variables.

Sampling day's effect on all the variables			
	Day 14	**Day 28**	**Day 3**
Day 28	0.002 **	-	-
Day 3	0.005 **	0.002 **	-
Day 7	0.002 **	0.008 **	0.025 *

When taking a look at the weather records obtained from a nearby meteorological station in the orchard, there are obvious differences between the 4 samples (Figure 23).

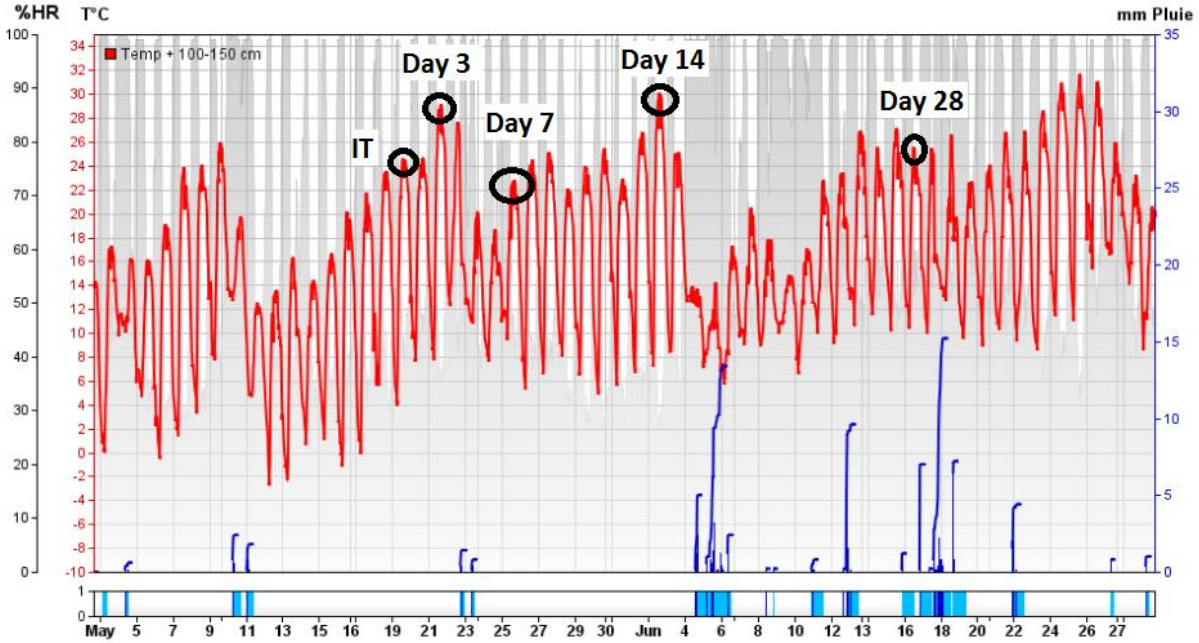


Figure 23: Weather records from a meteorological station located next to the orchard. IT = injection time (Obtained from the CRA-W).

This explains the major differences spotted between treatments and the higher rates observed during day 3 and 14. Besides, Day 3 and Day 14 shows high temperatures coming after small drought periods, which is an additional stress factor. The raw data is available in (Annex 4).

To compare the variance brought by each factor, a combined factor “treatment+day” was created and resulting ellipses were drawn (Figure 24).

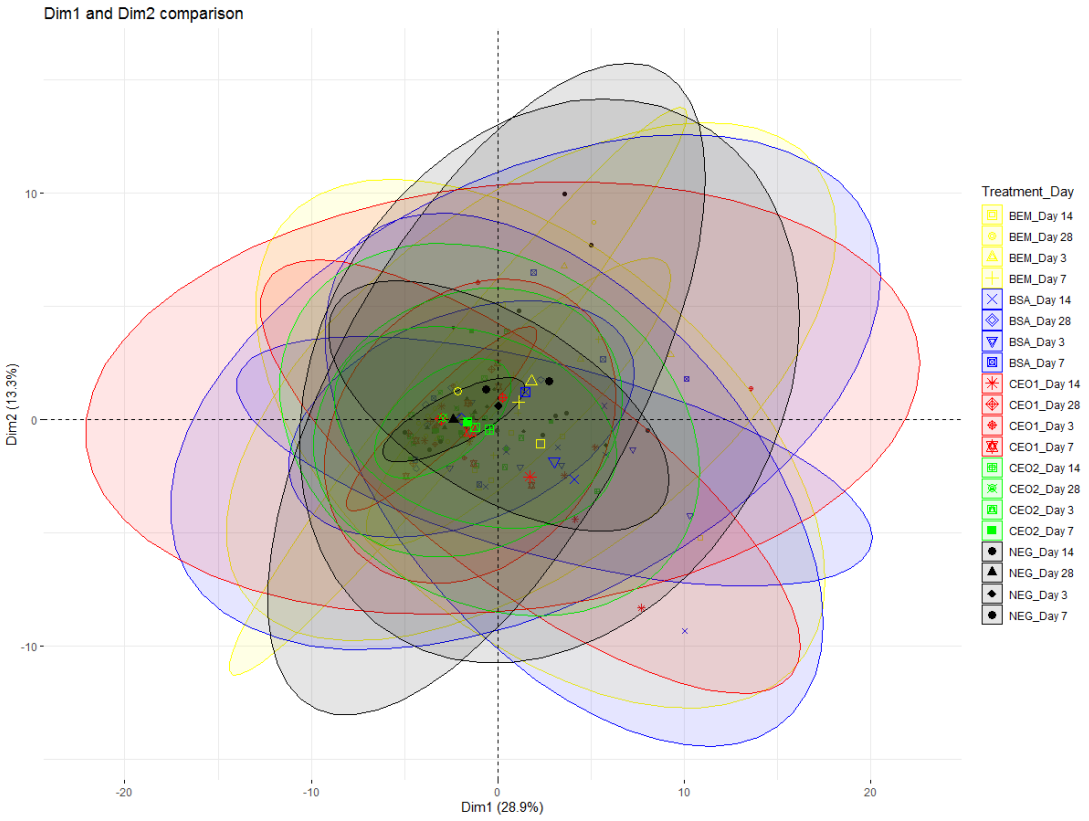


Figure 24: PCA representation of the sampling day mixed with the treatment factor on dimensions 1 and 2.

With 20 ellipses, the graphs became a little crowded. The centroids are however still visible and it can be observed that no particular group of treatment or day really stands apart from one another. It can nevertheless be noted that the CEO treatments (red and green) tends to be more grouped in the lower left part of the graph when the other treatments are more disparate and occupy mainly the 3 other quartiles. This, although maybe not leading to a significant degree of variation, indicates that the treatment is likely to influence the leaves emissions. The CEO2 (green) individuals are the one that show the less dispersion, meaning that neither the day nor the treatment seems to affect the relative amount of leave-emitted VOCs by the trees injected with 2% cinnamon essential oil. PermANOVA gave a p-value = 0.001 (DF = 19) for both PCs 1,2 and whole data analysis. The pairwise comparison being 19x19 matrix, they will not be shown here but are available in the Appendix (Appendix 2 and 3). Little to no significance difference were spotted in this analysis which means that the variation is highly present when comparing all the data at once but tends to be more discreet when comparing subject of different treatments and time to one another.

This being said, no real concentration data were shown for this whole analysis which tends to be quite abstract. So, to help the reader visualizing the variation in relative amount of some compounds, several graphs were plotted, first using the first 3 compounds from PC1, that should explains the most variance (Figure 25).

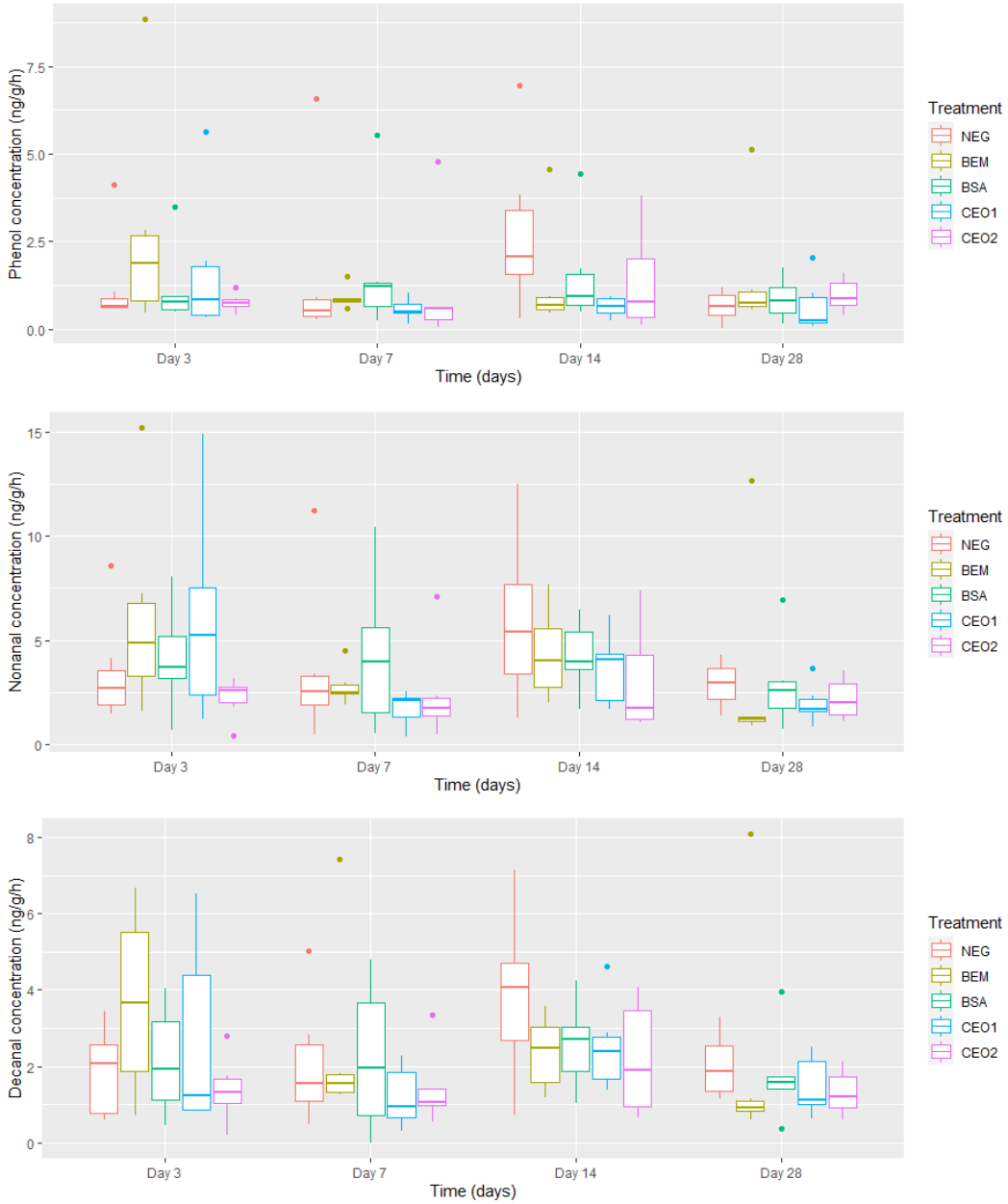


Figure 25: Plot of Phenol, Nonanal and Decanal

This seems to confirm that the variation does not much come from the treatment type but rather from the sampling day, hence from the sampling weather condition. As previously said, the weather plays a key role in a tree's evapotranspiration process (Vallat, Gu, et al., 2005). On hotter day, more water travels from the root to be emitted by the leaves. The profile in VOCs should therefore not change much qualitatively but as a consequence of an increased transpiration, higher levels of VOCs are to be expected. The treatment's impact could be better explained by a significant increase in the concentration of the tree's stress-related volatiles or volatiles of ecological importance (Wadhams et al., 1999). As said previously, numerous of those compounds constitute the second PC: Alpha-Farnesene, Caryophyllene, Germacrene-D and 3-Carene. Methyl salicylate is also a referenced stress-induced volatile that, despite not intervening in the first two dimensions, is worth considering. Significant changes in these compounds' concentration over time or due to the treatment could explain a change in the insect's behavior and in the tree's stress level (detected through chlorophyll fluorescence). A closer look on those VOCs species thus seems interesting (Figure 26).

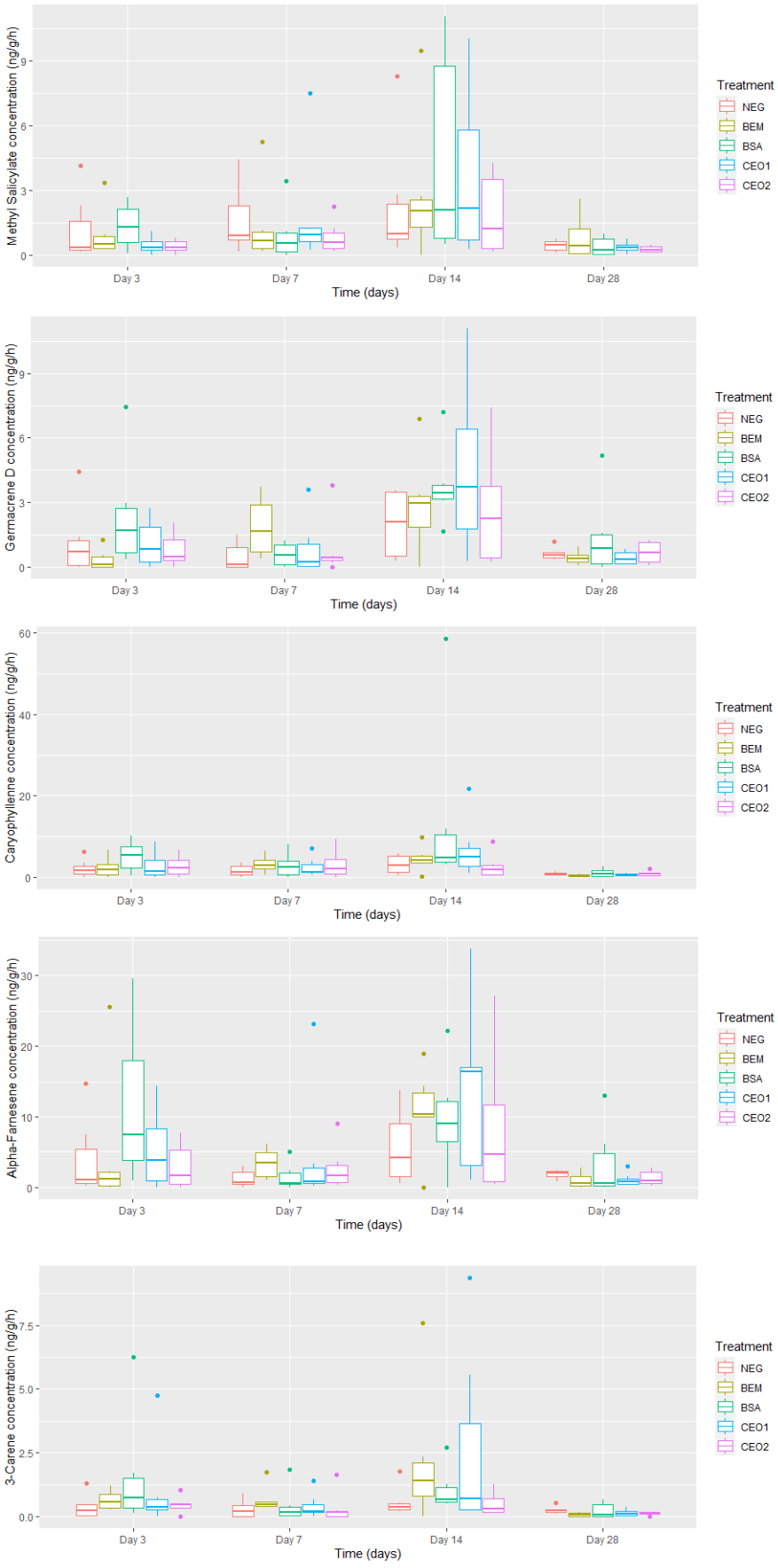


Figure 26: Graphs of several compounds of biological interest (Methyl Salicylate, Germacrène-D, Caryophyllene, Alpha-Farnesene and 3-Carene).

At first glance, the “Day 14” sampling seems to be the biggest source of variation, indicating a potential stressful trauma near this sampling, confirmed by the weather record (Figure 23). Again, to add some descriptive statistics to this visual approach, a repeated measure ANOVA was performed on three compounds that was the most relevant in this particular study: alpha-Farnesene and Germacrene-D that plays a key role in the apple tree-aphids interaction and methyl salicylate that is a major compound when it comes to mechanical damages (Vallat, Gu, et al., 2005 ; Vallat & Dorn, 2005). The repeated measures ANOVA were performed with the same parameters as the chlorophyll fluorescence measurements’ analysis. The normal distribution condition was never met for any compound but the qqplot allowed to perform the analysis anyway, as no value really stood out of the global distribution for the analysis to be a nonsense (Figure 27, 28 and 29).

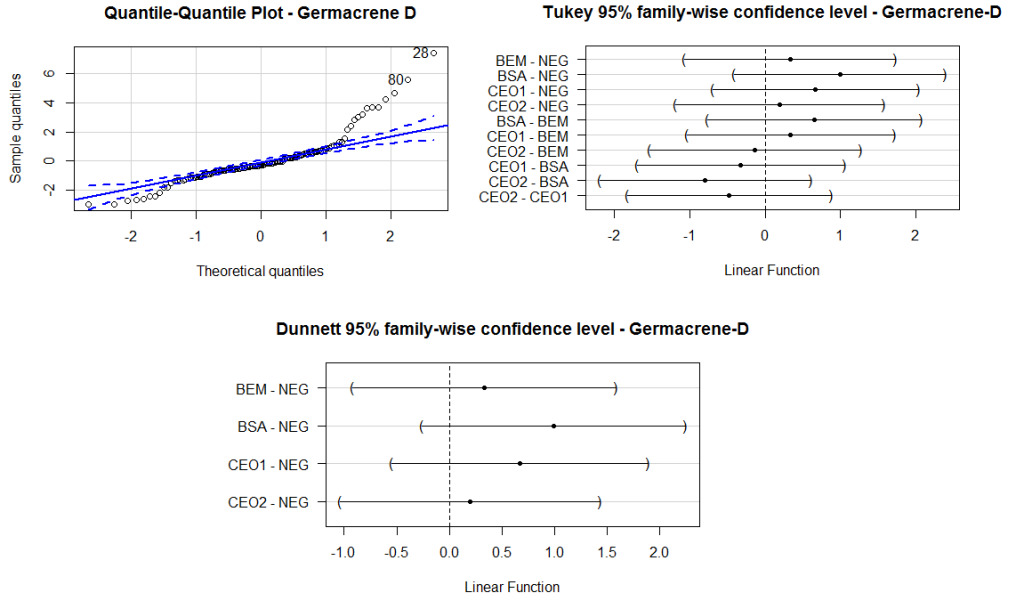


Figure 27: analysis of Germacrène-D

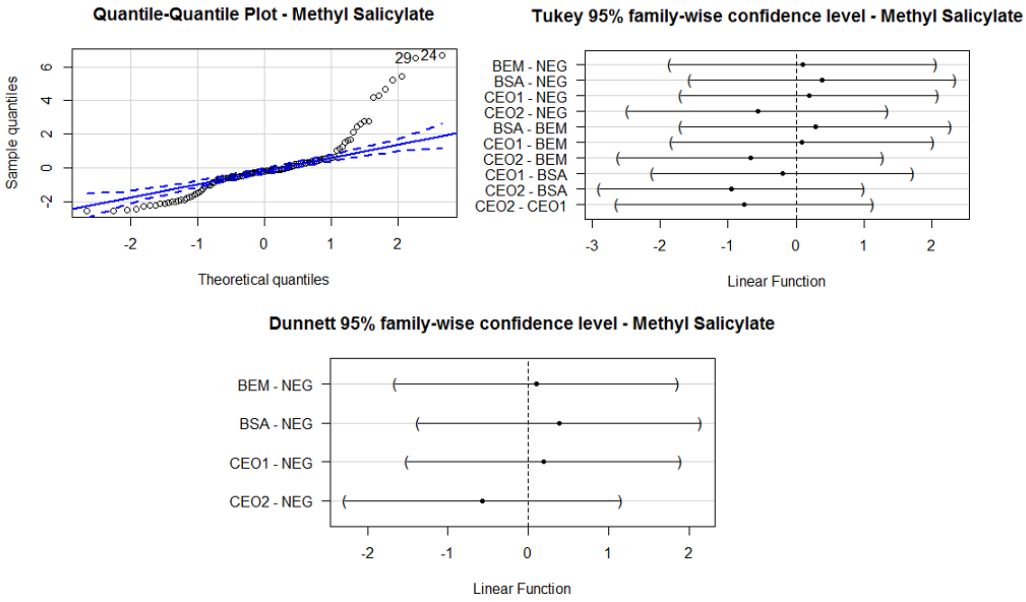


Figure 28: analysis of methyl salicylate

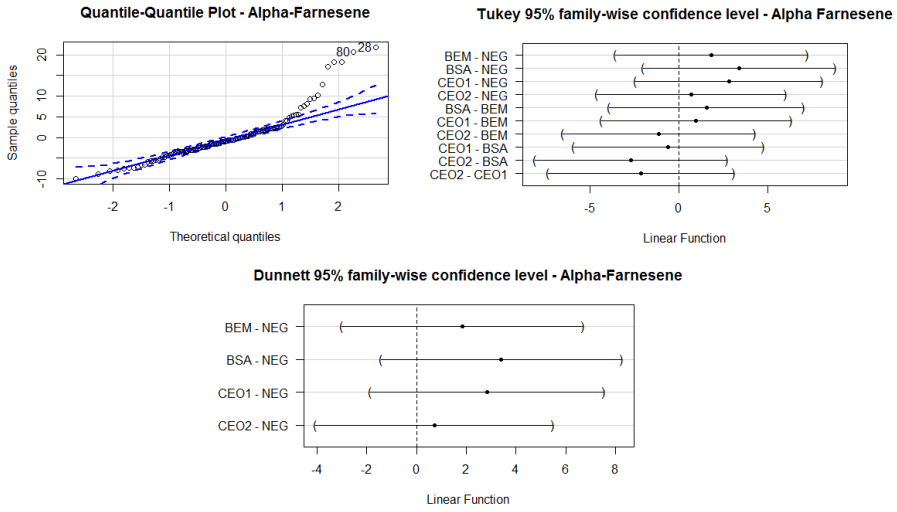


Figure 29: Analysis of alpha-farnesene

No significant difference was found for neither of these 3 compounds.

As a global comment for this VOCs analysis part, the treatment is considered significant on the whole dataset but little to no difference can be spotted between treatments. This means that variation is happening at the trees scale and conclusions are therefore hard to draw. It shall be considered that this whole experiment is a field-trial and that the analysis of the variation of compounds' relative amount at this such small scale (ng/g dried leaves/hour) is a laborious activity. Plus, each branch is different from one another and the variation sources are legion. In addition, as said previously, insects' (pest or predators) behavior is often impacted by a blend of several compounds at a specific concentration. To not spot these blends in this analysis does not mean that there will be no effect on the aphids' population or on the predators' presence in the orchard and particularly on the treated trees. Furthermore, as the potential change in the VOCs profile caused by the injection is supposed to come from HIPV/Stress volatiles emissions, it would seem logical to observe an effect between treated and NEG trees. But if this actually happens and that the aphids tends to decrease their presence/activity on those trees, that will not be the case for untreated trees that will stay with an ever-growing population of aphids and will therefore also emits HIPV. This is indeed a complex problem with numerous potential outcome.

4.5 Trees' response

In order to explain variation (or not) in the VOCs profile and to prove the treatment's inocuity, the trees' response had to be investigated.

4.5.1 Ecophysiological impact of the treatment

The ecophysiological impact of the treatment was evaluated through the measure of the chlorophyll fluorescence and particularly the Fv/Fm ratio. As a quick reminder: the chlorophyll fluorescence measurement and thus the evaluation of the photosystem II quantum efficiency is a widely used, robust and easy method to measure a plant's stress level and global health. Indeed, a stressed plant tends to modify its metabolic pathways, which reduces the PSII quantum efficiency. The absorbed light is re-emitted at a different wavelength (fluorescence), reducing the efficiency of photosynthesis (Pavlović et al., 2014). Basically, the F0 (fluorescence origin) is higher in stressed plants than in healthy plants. As Fv (fluorescence variation) = Fm (fluorescence maxima) – F0; if F0 increases, then Fv tends to be smaller and therefore the global Fv/Fm measure is smaller as well. A healthy plant (low F0) will display a Fv/Fm value of about 0.80 where a stressed plant will display lower values. A plant displaying Fv/Fm = 0.3 value is considered dead (Murchie et al., 2013). The ratios of the different wavelength of the emitted fluorescence in red/far-red: F690/F740, blue/far red: F440/F740 and

blue/green: F440/F520 are also very good indicators of a plant's stress caused by environmental factors. It does not only measure the fluorescence of chlorophyll (F690/F740) but also the fluorescence of polyphenols (F440/F740 and F440/F520) which are indicators of cell-wall conditions (Pavlović et al., 2014). This is called "high resolution fluorescence imaging system" and although it is more representative, it was beyond the need covered in this experiment where the F0, Fv, Fm measurement was considered sufficient. The F0 value alone could be used for measuring the plant's stress level as it is the impacting factor in the Fv/Fm measure but it was chosen to work using the Fv/Fm measure as it is commonly used and was therefore comparable to other studies. F0 and Fm values alone might change between apparatus or conditions, but not the Fv/Fm ratio which makes it more practical.

The Fv/Fm measures comparison between treatments for the different sampling sessions are displayed in (Figure 30).

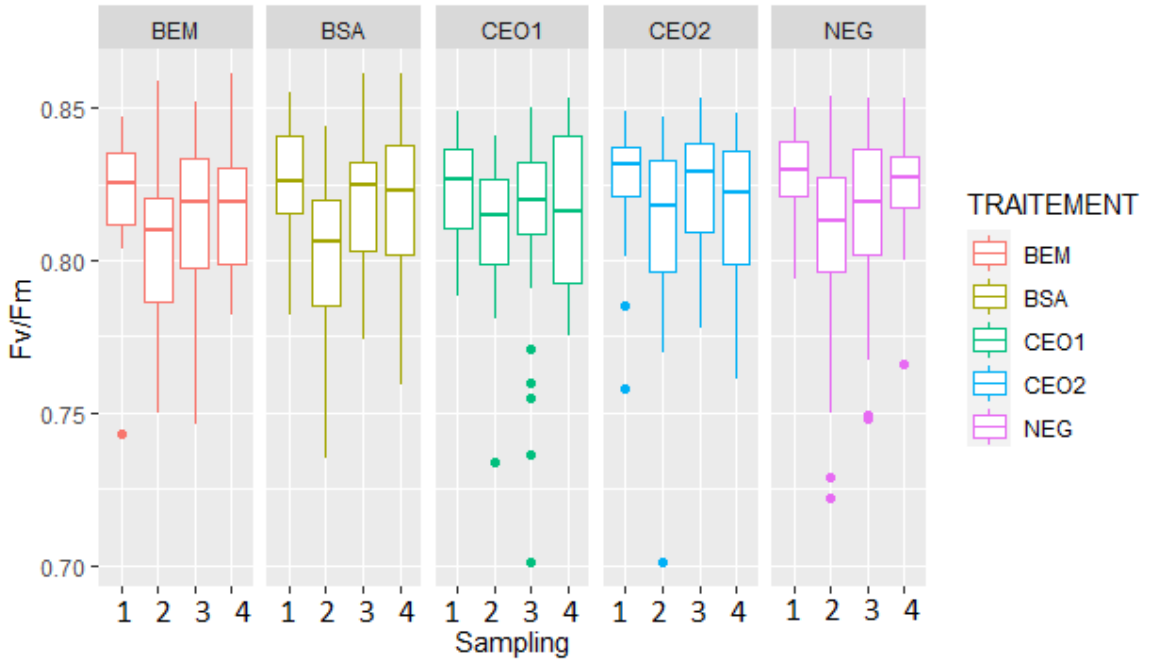


Figure 30: Fv/Fm measure over time

Most values are located between 0.75 and 0.85 of Fv/Fm ratio, which indicates a global good health of the sampled trees. As the VOCs analysis, the Fv/Fm ratio seems to be impacted by the sampling day (and conditions) rather than by the treatment provided to the trees. The major decrease observed at sampling 2 (Day7) in all treatments tends to correlate with this statement. This is interesting as well as the Day7 sampling displayed the mildest weather conditions. The stress occasioned by the harsh weather conditions of the previous days seems to interfere with the PSII efficiency with a slight delay. The repeated measure ANOVA will help to identify differences. The repeated measure allows to include the factor "time" as a parameter of the analysis and it was performed with the factor "tree" as well to take into account individuals' variation.

At first, measures did not meet the sphericity hypothesis (Mauchly's Test) and the normality (Shapiro-Wilk's test) hypothesis. The p-value for the Mauchly's test was 0.026 ($p<0.05$) and the null hypothesis was thus rejected. The p-value for the Shapiro-Wilk normality test was 1.284e-15 ($p<0.05$) and the null hypothesis was also rejected in this case.

To deal with the Mauchly's test value, a Greenhouse-Geisser correction was applied ("Test de Sphéricité de Mauchly dans R: Excellente Référence - Datanovia," August-4-2020). The p-value was

compared with the p-value obtained from the ANOVA test and were found identical, the analysis could be further performed.

The distribution of the data was further investigated in order to identify the proper correction to apply. A Quantile-Quantile plot and a distribution plot were drawn, along with a representation of the distribution with a normal distribution curb (Figure 31):

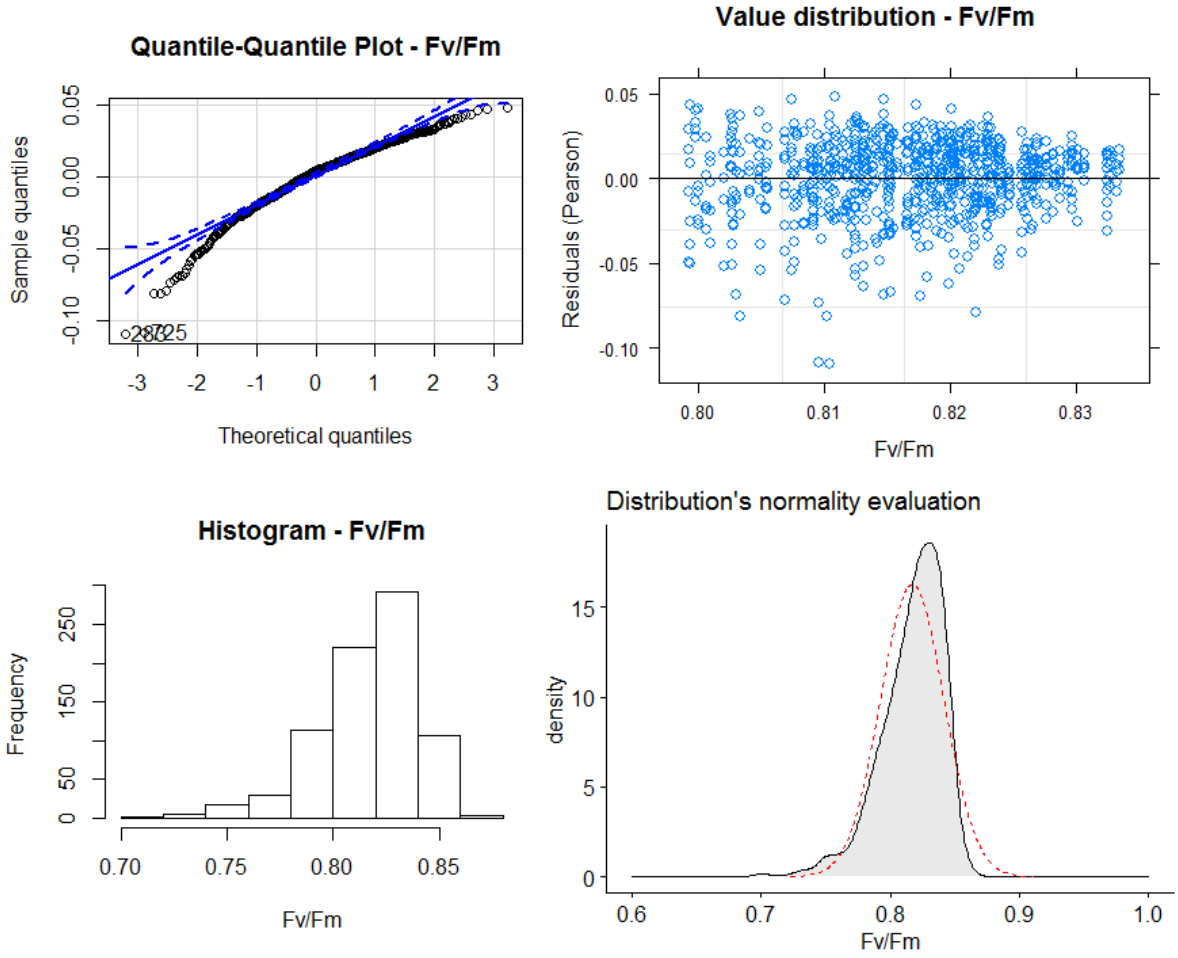


Figure 31: QQplot and representation of the values distribution (top) and histogram and distribution evaluation (bottom)

The data is distributed with a slight negative asymmetry (skewness = -1.13). Several corrections are available to deal with this type of asymmetry ("Skewed Distribution: Definition, Examples - Statistics How To," August-4-2020). After several trial with various functions (log, 1/x, sqrt), it was chosen to work with the 2*asin(sqrt) transformation function as it was the one giving the best skewness result (skewness = -0.96) (Figure 32).

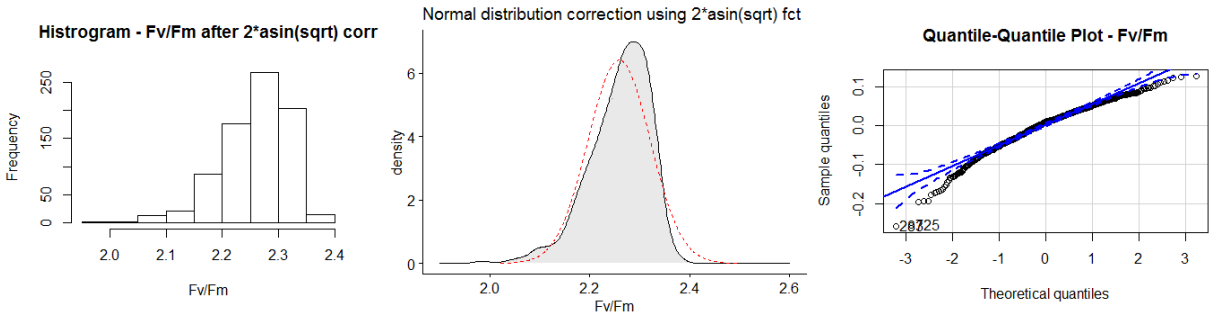


Figure 32: Histogram, Distribution and QQlot after transformation

The Shapiro-Wilk's test p-value after correction was better (p-value = 3.8e-13) but was still highly significant. Anyway, it was decided to perform the Tukey and Dunnett tests, as the qqplot displayed a regular curve with no value jumps near its center.

The Tukey's test compares each treatment with one another and the Dunnett's test compares each treatment to the chosen reference factor, here the [NEG] treatment. No significant difference was reported whatsoever (Figure 33).

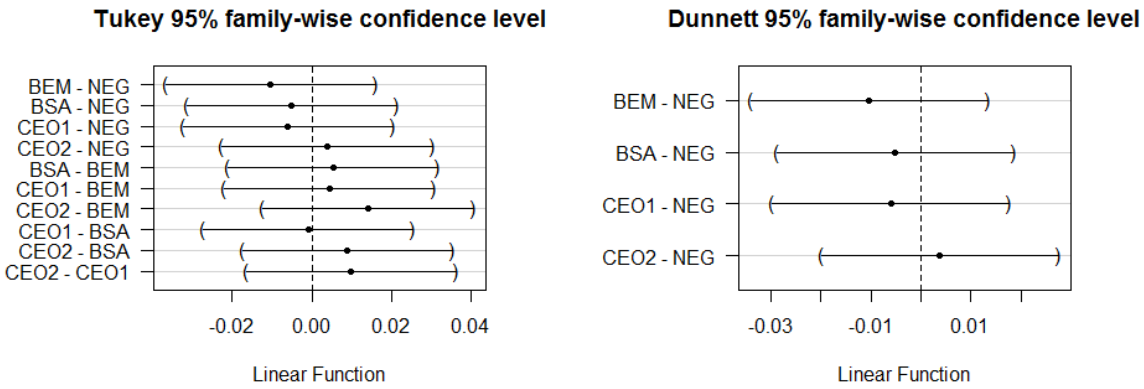


Figure 33: Tukey's and Dunnett's test results

So, as a global conclusion for this part and as the (Figure 34) showed, the treatment had no impact on the trees' health or stress level, as regard of the photosynthetic apparatus efficiency. This comfort the VOCs results in the following matter: as little to no additional stress seems to have been added by the treatment, it is quite logical to observe little to no difference in the stress-related volatiles' relative amount in the VOCs analysis. The difference in VOCs emissions thus seems to come from the weather conditions and from individuals' variations.

4.5.2 Outer bark layer cracking

Due to the drilling and the plug insertion, several trees suffered from longitudinal bark cracking (Figure 34). The cracks' length were measured on the last sampling day.

Figure 34: Degree of bark-cracking on different trees. 1) Little to no bark-cracking 2) Mild bark-cracking 3) Severe bark-cracking

Although some cracks were quite impressive (some reaching more than 30cm on each side of the injection port), it appears not to have had any consequence on the trees' health. Cracks were longer

over the injection port than under. (Aćimović et al., 2016a) conducted a study to evaluate healing processes of different tree-injection technique. Although they reported longitudinal cracks as well using 4.4mm and 9.5mm drill bit, the cracks were not as long as the cracks observed in the orchard. Bark cracking seems to be a direct response of the disrupting of the vertical sap flow: cambium and phloem tends to dehydrate due to lack of water from the xylem, leading to outer bark layer cracking vertically from the injection port. This could explain the length variation from the cracks above and under the injection port level. The fact that May 2020 was reportedly an extremely dry month sure is also not helping in the matter (IRM[14] + Annex 4). Wounds this type takes about 2 years to fully recover (Aćimović et al., 2016a). Some trees in the orchard were already marked by scars of bark cracking and some even showed spontaneous cracks during the experiment (Figure 35), which means that it is not uncommon for the bark to crack in its outer layers due to an impact/wound or maybe a drought period.

Figure 35: Spontaneous bark-cracking on an apple tree that was not part of the experiment.

This however raise questions about the safety of the technique for the tree. Even if it appears not to be impacted, the damages are serious and considering that the technique sometimes requires several injection ports on the same tree, this can become harmful over time (if the treatment is repeated each year) and in case of multi-ports injection. Plus, it is an open door for fungal or bacterial infestation. Less damaging technique could be considered for further experimentation such as needle-based injection, lenticular blade port or unsealed drill port. Indeed, callus forming around the plug tends to increase pressure around the port and therefore generates cracking. Plugged trees also heals slower than trees with unplugged ports. However, it seems that the plugs also helps protecting the injection port from infections and stimulates the healing process by compartmentalization. The plug also ensures better delivery of the injected substance in the sap (Aćimović et al., 2016a). A balance must thus be found in this matter.

[14] https://www.meteo.be/fr/climat/bilans-climatologiques/2020/mai

4.6 Insects monitoring

As said in the "Material and Method" part, data on insects in the orchard were mostly collected by members of the biology faculty of UCLouvain, partners on this project. The counting methods, parameters, insects' identification and statistical analysis was designed by their good care. The on-filed insects monitoring and counting were under their supervision. The conclusion hereby stated are however the author's.

4.6.1 Aphids ecology

The first target of this experiment was the Rosy Apple Aphid. As said previously, observed changes in the aphids' population density could come from several factors:

- Acute toxicity of the cinnamon essential oil for aphids.
- Repellent/anti-feedant effect of the cinnamon essential oil on aphids.
- Repellant/anti-feedant/attractant effect of the modification of the VOCs profile on aphids, linked with the injection's stress or the cinnamon compounds.
- Attractant/repellant effect of the modification of the VOCs profile on aphids' natural predators, linked with the injection's stress or the cinnamon compounds.

Consequently, this analysis will be put in perspective with the previous results and requires the monitoring of aphids' colony and of aphids natural predators (predators + parasitoïds).

The number of aphids counted in one colony per tree evolved as follows (Figure 36)

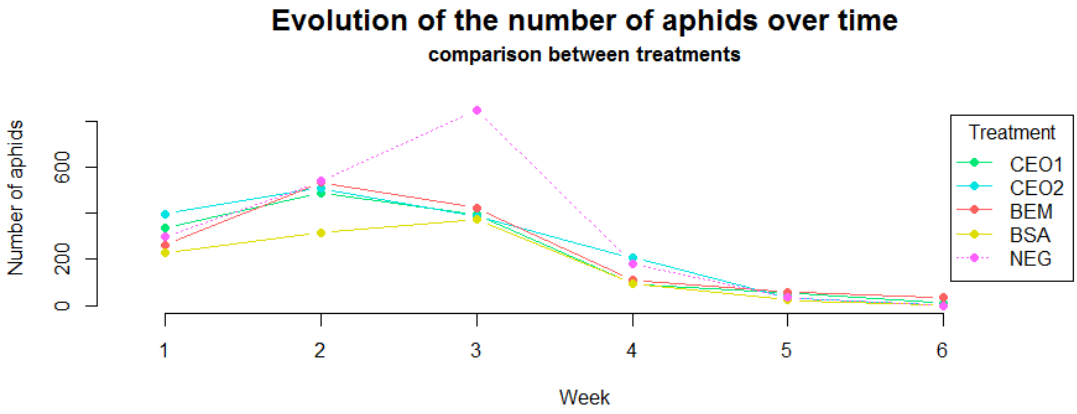


Figure 36: Graph of the evolution of the number of aphids in one colony per tree

The first monitoring (week 1) started on the 11th of May, then one counting per week was performed until the 16th of June (week 6). The injections happened on week 2. From this graphic, an effect of the treatment on the aphids' development seems pretty obvious. The decrease observed from week 4 to 6 can mainly be attributed to the aphids' development stage: the winged females move from the apple tree (primary host) to the *plantago spp.* (secondary host). This phenomenon is reported to happen in mid-late June (Blommers et al., 2004; Rousselin et al., 2017) and matches with the observation from the orchard.

For this analysis, the block factor was found significant, with a p-value = 0.002 (df = 7). The treatment factor was also reported significant over the number of aphids in one colony per tree: p-value= 0.027 (df =4). Let's now see if the same trend is observed for the evolution of the number of colony per tree (Figure 37):

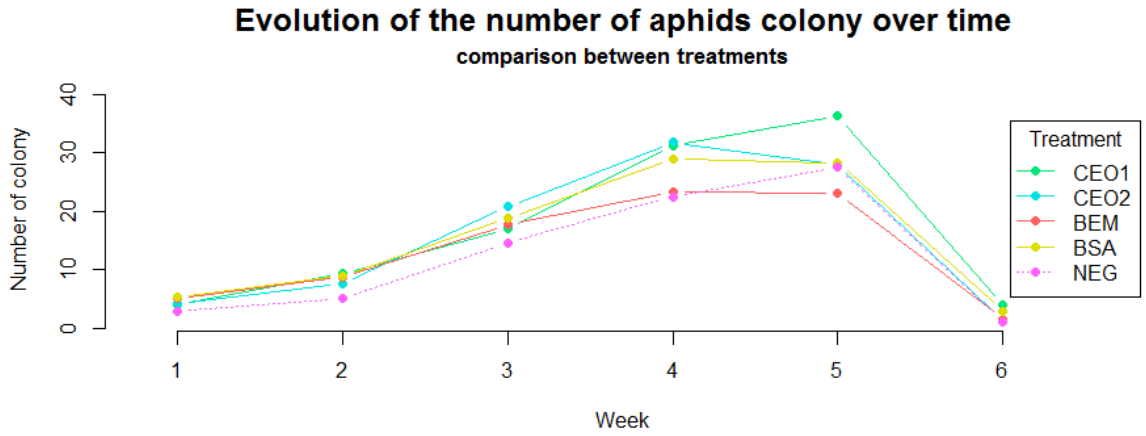


Figure 37: Graph of the evolution of the number of aphids' colony over time.

The treatment's impact seems less obvious but a trend can however be visualized concerning the saline blank and both cinnamon treatments where the number of colony seems to increase faster and sensibly more than the negative or blank emulsion treatment. Again, the block factor was found highly significant (p-value = 2E-16, df = 7) and the treatment was also reported significant, although less than in the previous result, with a p-value = 0.02 (df = 4)

When compared to previous results, this would mean that the NEG trees' aphids' population grows inside colonies but the number of colonies does not increase much where, on the contrary, aphids on treated trees tend to disperse in more colonies of smaller populations, excepted for the BEM treatment where the aphids colony tends to stay in small number with less individuals. The week 4 results seems to coincide with the observations made in point 4.4.2: the CEOs and BSA treatment are separated from the NEG and BEM treatments. When taking into account the two graphs, it seems that the BEM treatment is the more effective. This would mean that the repulsion effect or the stress brought to the trees come from the Tween80 rather than from the essential oil. But as the CEOs treatment also contains Tween80, the same effect should be observed. Unless there happens to be a retroactive effect of the oil on the Tween80's potential toxicity, which is very unlikely given the cinnamon essential oil's properties.

4.6.2 Aphids' predators and parasitoids monitoring

The determination of the treatment's impact on aphids alone would have been misevaluated if the aphids' natural predators/parasitoids were not taken into account. The predators' (*Coccinellidae, Syrphidae, Chrysopidae…)* or parasitoids' (*Aphidiinae* mostly) response to volatiles and especially Herbivore-Induced Plant Volatiles (HIPV) or alarm-pheromone from aphids is well documented (Wadhams et al., 1999; Becker et al., 2015) and as the VOCs emissions of the tested apple trees were modified, modification in the behavior of predators and parasitoids was to be expected (Figure 38).

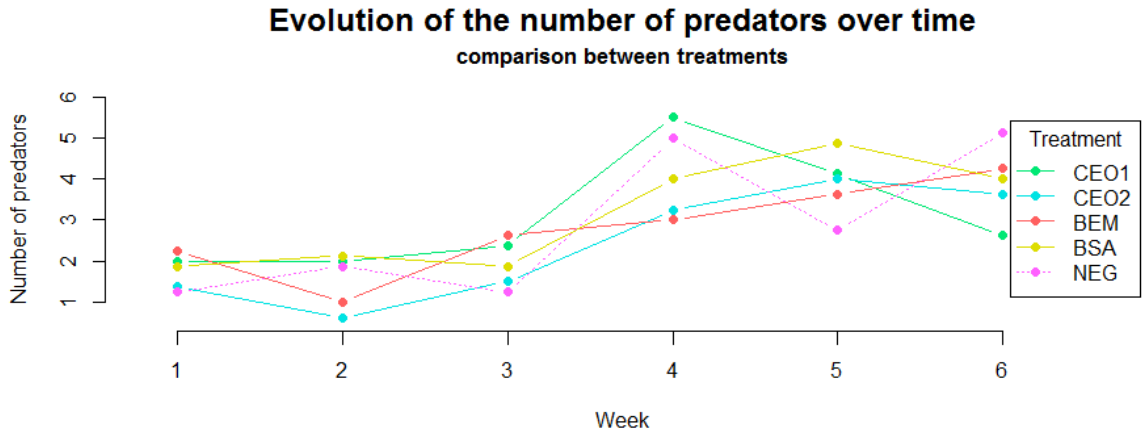


Figure 38: Graph of the evolution of the predators' population in the orchard.

The scale for the number of predators is notably small. No specific trend can be observed in this graph, plus, an increase of predators seems legit in most cases: if the tree emits HIPV, it can be due to both the treatment and the higher number of aphids on NEG trees.

Anyway, with a p-value of 0.197 (df =4), the number of predators does not seem to be much impacted by the treatment. The parasitoids monitoring showed a slight trend in favor of the CEO1 treatment (Figure 39):

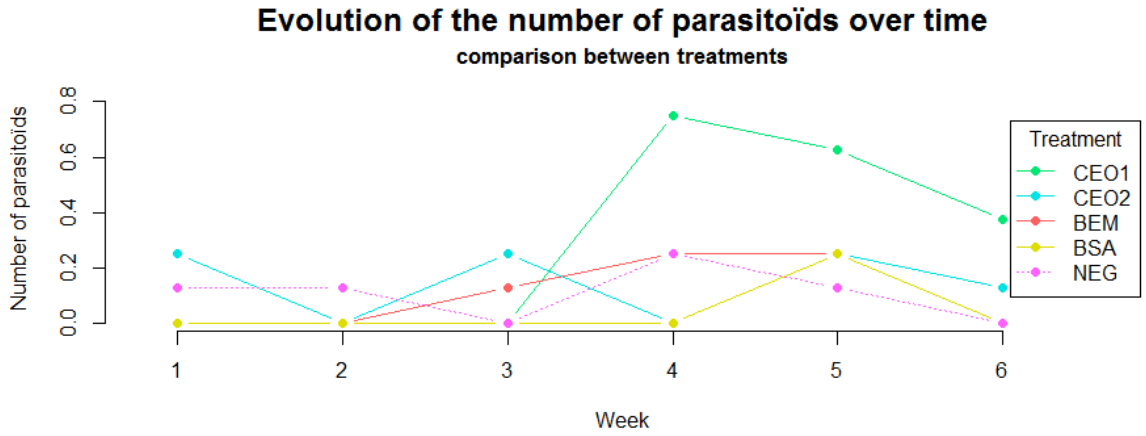


Figure 39: Evolution of the parasitoids in the orchard

However, the scale is even smaller than for the predators and it seems that relying the presence of a parasitoid insect on a tree during a 1 minute sampling once a week to a treatment that might influence its presence is a bit light to draw conclusions. So although the treatment impact seemed significant (p-value = 0.021, df=4), the parasitoids evolution will not be commented. For further investigations, trapping system or longer and more frequent observation sessions should be considered.

4.6.3 Others

Ants, pollinators and other pests were also monitored. Ants maintain a well-known symbiotic relationship with aphids and the presence of ants on trees can often be linked with the presence of aphids (Frömke et al., 2004).

Concerning pollinators, the experiment fell already quite late in the blooming season, few to no flowers were still present on the trees. Therefore, the presence of pollinators should not be linked to the treatment but rather to the absence of flowers. This feature should however be considered for later

experiment as the flowers content from treated trees could not have been tested here and as it is likely to interfere with the pollinators activity in the orchard. The graphs show no particular trend and as the two previous graphs, their scale are very limited (Figure 39).

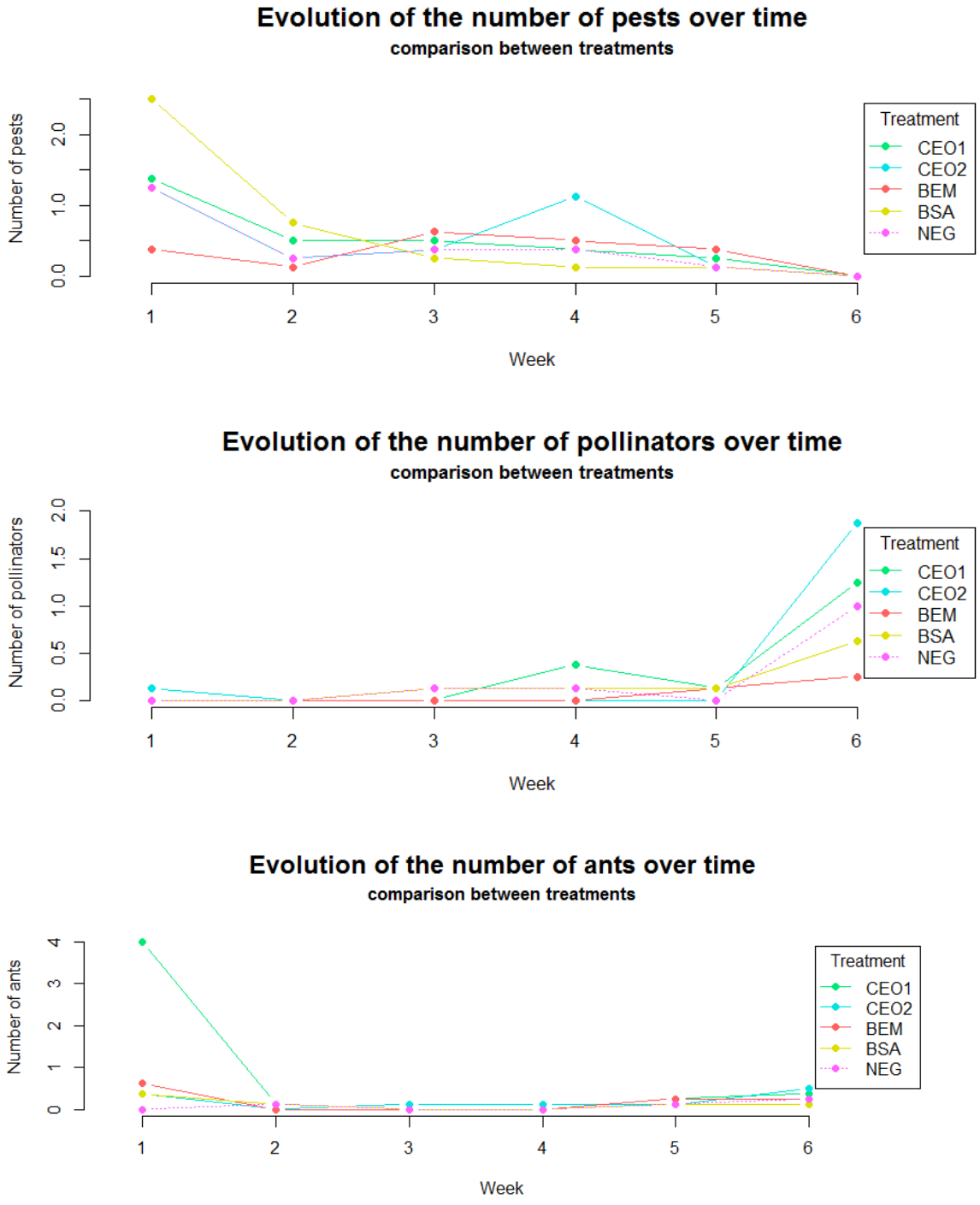


Figure 40: Evolution of the number of (1) pests (2) pollinators and (3) ants in the orchard

Again, really few observations were made. The higher value of the CEO1 treatment for ants monitoring is sufficient to state the change as significant. As for parasitoids, longer and more frequent observations should be performed to obtain more data. A binary approach could also be investigated with a presence/absence form of data. Of course, observations of mobile living "variables" is tough and is not easy to perform, neither to interpret. Yet, where aphids or predators larvae are quite easy to monitor over time, monitoring flying or highly mobile insects is far less simple.

5 Global discussion

If this experiment was to be looked from a wider point of view, the primary objective was to treat apple trees with cinnamon essential oil injected directly in the trunk for the control of the Rosy Apple Aphid in field trial. The injection was expected to have an impact on aphids by acute toxicity of the cinnamon or by triggering SAR and defense mechanisms in the trees that would thus emit airborne signals known to repel aphids (direct defense) or attract aphids predators (indirect defense) (Vickers et al., 2009; Joó et al., 2010; Jones et al., 2011). This would have also caused some stress that would have been perceived by measuring the foliar quantum efficiency of photosystemII.

The airborne signals are indeed the key of this whole experiment. As long as emitted cinnamon compounds are not in lethal or at least repulsive concentration for aphids, the role of the injection is to trigger the trees natural defense mechanisms and to act as an elicitor of HIPV/stress volatiles emissions.

The presence of E-cinnamaldehyde in the emissions profiles is an interesting and very promising result. Although the detected levels are imprecise and anyway should not be high enough to directly impact aphids development (as previous results in the project showed mortality in medium concentrated at 0.05% E-cinn), the emission of E-cinn from 15 years old trees injected with as few as 0.046g and 0.024g E-cinnamaldehyde is impressive and opens the way for further development of the technique: the method is successful for the delivery of an essential oil from the trunk to the tree canopy, hopefully leading to systemic defense when at the suited concentration (VanWoerkom et al., 2014). Quantification of E-cinn in the collected leaves will also give a better understanding of the compound retention/emission rate. The results suggested here that E-cinn did not directly impacted the aphids' development but was rather active at the tree level, for emissions of airborne signals.

The emitted VOCs profile, supposed to explain much of the tree's physiological state and the aphids response did not explained much in terms of changes in HIPV emissions. This is neither a bad nor a good result: global variations were still identified overall the VOCs profile and as insects are rather blends sensitive (Wadhams et al., 1999; Brown et al., 2007), it is normal to detect changes in the insects development but not especially in specific VOCs emissions. Few changes in HIPV emissions is however consistent with the chlorophyll fluorescence results that does not suggest a stressful incidence of the treatment (neither from the injection nor from the cinnamon compounds). The VOCs relative amount were rather influenced by the weather as could have been expected (Vallat, Gu, et al., 2005). Still, some differences were spotted when looking at the PCA analysis and comparing each treatment's ellipse's centroid. The CEOs treatment appeared to be separated from the other treatments and the BSA treatment also displayed separation from the NEG and BEM as well as from the two CEOs. The analysis of leaves VOCs is likely to explain a lot of observations regarding the insects' development and behavior. Besides, the tree VOCs response is often species-specific: wounds caused by aphids will triggers the emissions of aphids-specific compounds (alpha-farnesene, Germacrene-D, beta-caryphyllene) (Warneys et al., 2018) while the injection triggers the emissions of mechanical wounding VOCs (terpenoïds, methyl salicylate, nonanal) (Bertin et al., 1997; Rapparini et al., 2001; Vickers et al., 2009). The terpenoïds, including aphids-specific terpenoïds were responsible for a great share of the total variance, which means that both the aphids and the treatment had an impact: the tree responded to the treatment and auto-defended against aphids, with a better results on treated than untreated trees.

Regarding the method used for VOCs sampling, it is practical and the pumping rate and time seemed to be appropriate. It is however a bit more touchy when weather conditions (rainfall, extreme temperature) are not optimal: it may interfere with the sampling and with the apparatus (humidity on cartridges,…) thus jeopardizing the measure. The statistical approach, although thoughtful, can nevertheless be modified, allowing to maybe achieve other results. Here, it was chosen to work with

unsupervised PCA followed by PermANOVA analysis. Several other multivariate analysis models are available such as supervised PCA, PLSDA (Partial Least Squared Discriminant Analysis), Partial Least Squared Regression, MANOVA (Multivariate Analysis of Variance), K-means Cluster Analysis, ICA (Independent Component Analysis),… Statistical analysis is a tool of scientific research, in the same way as apparatus or facilities: no two different apparatus will give the exact same results.

The trees' health evaluation concluded that no discernible damage was induced by the treatment (at least regarding the chlorophyll fluorescence). This is positive as well as the injection method is quite invasive and that cinnamon essential oil displays phytotoxicity properties. However, the bark cracking is quite concerning and the injection method might be questioned: a needle-based injection would not cause any problem given the high dilution rate of the emulsion and would strongly reduce bark cracking due to injection port drilling (Aćimović et al., 2016b). Problem would be that needle-based injection would maybe not allow as efficient compound's mobility. This is a dilemma between product dispersal's efficiency vs preservation of the tree's integrity.

Regarding the insect's response, no particular trend could be observed regarding predators, pollinators, parasitoïds, others pests or ants. On the contrary, aphids' number per one population per tree clearly showed a decrease for all 4 injection treatments, the untreated trees being more severely infested. The number of aphids' colony per tree however seemed to increase in blank saline, cinnamon 1 and cinnamon 2 treatments while negative and blank emulsion treatments showed no specific increase. This would mean that the treatment indeed as an influence on aphids' development but rather in terms of injection-linked stress' emissions of repellent VOCs than in cinnamon compounds presence.

As a final global comment to this discussion, it seems meaningful to remind that all the (in)significant data obtained earlier, although statistically significant in terms for example of VOCs profile, may not be biologically significant i.e. the difference spotted in the chromatogram analysis might not have an impact in the field. The same way around: no significant difference does not mean that there was no biological impact and that the insect's behavior in the orchard was not modified (McKillup, 2012) as insect's sensitivity is expected to be far more precise and selective than the sensitivity of used apparatus.

6 Conclusion

Replaced in the global SPW "Tree-injection" project, the objectives of this work were to evaluate the feasibility of cinnamon essential oil injection into apple trees using tree-injection method to fight against the Rosy Apple Aphid.

Although the method is not yet in its final form, this first "trial-and-error" experimentation allowed to brush off some concerns and to better prepare the next series of tests.

The delivery of cinnamon essential oil to apple trees by tree-injection showed no evidence of serious damage to the tree's physiological state. Treated trees successfully emitted cinnamon compounds, and insects' response suggested that the treatment is working, although the insects' reaction is rather due to the injection itself than to the cinnamon essential oil repellent or toxic effect.

As a whole, the use of essential oils as biopesticide seems a very promising ecological alternative to conventional plant protection products that could be part of Integrated Pest Management program or integrated into organic production. Tree-injection technique was also proven efficient for compound delivery and seems a far better alternative than spraying to obtain systemic and long-term protection.

Though, several questions are still to be answered, notably in terms of (1) long term effects on the trees and on apples production yield, (2) products effectiveness duration and frequency of treatment's repetition, (3) impacts on blooming and pollinators, (4) impacts on fruit quality, (5) feasibility of large scale application and last but not least, (6) product's coherence.

This last point deserves a bit of development. Indeed, the formulation is using Tween80 as an emulsifier. Tween80 is not a biological compounds and still this formulation would be authorized in organic farming under annex II of CE 889/2008 regarding authorized active substances in organic farming. To the author's humble mind, a bioemulsifier would be preferable to be consistent in the biological approach of the product. Plus, massive development of such biopesticidal products often tends to jeopardize the original market niche of the product and also leads to competition of use. The development of such products should never lead to an industrial production for a large scale use as it would just become a repetition of the misuses of plant protection products up till now. On the contrary, development of this kind of biopesticide is interesting when combined with other IPM techniques and not used as a routine treatment but rather as a last resource solution.

7 Perspectives

Numerous aspects still have to be further investigated for this product to become the desired optimized, efficient, stable, ease of use and environmentally friendly plant protection product targeted by the SPW's "Tree-injection" project.

First, higher concentration of cinnamon essential oil have to be tested. This for two aspects: (1) determining the on-field aphids' response threshold for E-cinnamaldehyde (active concentration) and (2) determining the trees threshold for marked toxicity (toxic concentration). Hopefully, active concentration for aphids will be lower than the toxic concentration for the trees. The evaluation of the ecophysiological impact of the treatment on the trees' PSII quantum efficiency gave results on the direct acute toxicity potentially perceived by the trees. However, such a treatment could present long-term effects on the tree health or productivity. An evaluation on several years would allow to evaluate the long-term effect and other tests should be performed with other organs than the leaves sampled.

Then, injection should be performed earlier in the season. This would allow to target colony foundress, disrupting the development of colony during the season and avoiding damages to flower, which are the most detrimental. This would in the same time allow the assessment of the treatment's impact on flower emissions and on pollinators' behavior. Also, the injection earlier in the season would most certainly disrupts the trees' emissions, making it unattractive or better unrecognizable for aphids. In late season, apples should also be analyzed for possible cinnamon of Tween80 residues.

Also, to obtain more controlled results, aphids' development should be monitored both in natural conditions and in artificial infestation using sleeve-bags placed over branches in order to obtain repeatable results and allowing to draw conclusive evidence on the treatments effect on the aphids' development.

As far as the sampling technique is concerned, replicates of calibration curve and better LOD and LOQ should be achieved for a more precise analysis and one Nalophan bag per tree is also to consider to avoid contaminations of non-cinnamon trees. Two sampling per tree would be even better as it would allow to have some sort of repetitions and to have an idea of the product migration rate in the tree. Besides, as the potential compounds of interests are identified (HIPV, stress volatiles), a calibration curve of these compounds using pure standard can also be performed. This would allow greater precision than semi-direct quantitation using one internal standard and would allow to better understand the treatment's impact and the subsequent aphids or pests reaction. Designing an experimental design with injected trees kept free from any insect or abiotic stress would also help identify the part played by the injection in the stress production and the following change in VOCs emissions pattern. Also, to be able to assess the effect on aphids of the Tween80 emulsifier, tests should be performed by placing aphids on artificial Tween80 medium.

Given the cinnamon essential oil properties, an evaluation of the development of fungal diseases such as apple scab or bacterial diseases such as the fire blight could also be considered for the potential multi-use of the product on various pathogens and mainly as an elicitor of SAR (Lateur, 2002).

In addition, there are several other essential oils already considered for their anti-insecticidal properties. Other essential oils emulsions could be developed and tested. Moreover, a synergic effect of essential oil is often reported in the corresponding literature. In particular, (Burt, 2004) reported a synergic effect of E-cinnamaldehyde and Eugenol, one of the main constituent of Clove (*Syzygium aromaticum*) essential oil, another oil considered in this particular project.

Finally, an estimation of the cost of the treatment for a yearly application in a 1ha orchard could be an interesting fact to assess the economic viability of the technique and an overview of the legislative constraints that have to be overcame before the product could enter the market as a biological plant protection product would also be a key point in this biopesticide development.

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9 Appendix

Appendix 1: Characterization of cinnamon essential oil (Pranarôm)

Peak	RT	Compound	%	Peak	RT	Compound	%
1	7,3	alpha-PINENE	0,08	41	47,7	2-METHYL BENZOFURANE	0,4
2	7,4	alpha-THUYENE	0,01	42	47,9	delta-CADINENE + gamma-CADINENE	0,29
3	8,7	CAMPHENE	0,05	43	48,3	SALICYLATE DE METHYLE	0,09
4	9,1	HEXANAL	0,01	44	49	BENZENEPROPANAL	0,78
5	10,1	beta-PINENE	0,03	45	49,2	alpha-CURCUMENE	0,09
6	14,1	LIMONENE	0,04	46	49,5	Trans-alpha-BISABOLENE	0,05
7	14,5	1,8-CINEOLE	0,02	47	50,2	COMPOSÉ AROMATIQUE	0,04
8	17,2	STYRENE	0,17	48	50,8	COMPOSÉ AROMATIQUE	0,03
9	18	p-CYMENE	0,06	49	51,1	ACETATE DE 2-PHENYLETHYLE	0,03
10	18,7	TERPINOLENE	0,01	50	51,8	Trans-ANETHOL	0,02
11	21,9	6-METHYL-5-HEPTENE-2-ONE	0,01	51	52,1	CALAMENENE	0,03
12	25,5	NONANAL	0,02	52	52,9	ACIDE HEXANOIQUE	0,03
13	26,2	COMPOSÉ BENZYLIQUE	0,02	53	53,5	COMPOSÉ PHÉNOLIQUE	0,12
14	29,8	SESQUITERPENE	0,01	54	54,4	ALCOOL BENZYLIQUE	0,27
15	30,2	SESQUITERPENE	0,02	55	55,4	Z-CINNAMALDEHYDE	0,34
16	30,3	delta-ELEMENE	0,01	56	56,3	ALCOOL PHENYLETHYLIQUE	0,98
17	31	CYCLOSATIVENE + ISOLEDENE	0,12	57	58,5	COMPOSÉ AROMATIQUE	0,05
18	31,2	YLANGENE	0,06	58	59,1	2-METHOXY BENZALDEHYDE	0,42
19	31,8	alpha-COPAENE	0,8	59	60,2	OXYDE DE CARYOPHYLLENE	0,05
20	32,9	COMPOSÉ AROMATIQUE	0,04	**60**	**63,5**	**E-CINNAMALDEHYDE**	**79,49**
21	33,3	CAMPHRE	0,04	61	63,8	COMPOSÉ AROMATIQUE Mw=206	2,46
22	33,6	BENZALDEHYDE	1,14	62	65,4	2-METHOXYPHENYLACETONE	0,24
23	33,8	beta-BOURBONENE	0,02	63	67,4	SPATHULENOL	0,1
24	36,8	LINALOL	0,04	64	68,8	ACETATE DE CINNAMYLE	0,46
25	37,6	SESQUITERPENE	0,05	65	69,5	EUGENOL	0,04
26	37,9	alpha-trans-BERGAMOTENE	0,04	66	69,8	TRIMETHYL PENTADECANONE	0,02
27	38,2	beta-CARYOPHYLLENE	0,12	67	70,5	T-CADINOL	0,02
28	38,5	TERPINENE-4-OL	0,04	68	71,3	SANDARACOPIMARADIENE ISOMERE	0,03
29	38,8	AROMADENDRENE	0,03	69	72,1	alpha-BISABOLOL	0,05
30	40,6	COMPOSÉ AROMATIQUE	0,14	70	72,3	SESQUITERPENOL	0,03
31	41,1	ACETOPHENONE	0,08	71	72,6	alpha-CADINOL	0,02
32	41,3	COMPOSÉ AROMATIQUE Mw=132	0,14	72	74,4	2-METHOXY-CINNAMALDEHYDE	0,04
33	41,8	ALLO-AROMADENDRENE	0,12	73	75,1	ALCOOL CINNAMIQUE	0,13
34	42,6	alpha-HUMULENE + ESTRAGOLE	0,04	74	75,7	CARYOPHYLLA-3,7-DIEN-6-OL	0,02
35	43	ALDEHYDE SALICYLIQUE	0,69	75	78,4	EPOXYDE SESQUITERPENIQUE	0,07
36	43,8	gamma-MUUROLENE	0,14	76	82,2	Trans-o-METHOXY-CINNAMALDEHYDE	5,26
37	44,2	BORNEOL	0,3	77	82,6	COUMARINE Mw=146	2,1
38	44,4	alpha-TERPINEOL + LEDENE	0,08	78	90,3	BENZOATE DE BENZYLE	0,07
39	46	alpha-MUUROLENE	0,08	79	93,8	COMPOSÉ AROMATIQUE	0,04
40	46,2	beta-BISABOLENE	0,13	80	99,5	COMPOSÉ AROMATIQUE	0,14
						TOTAL	99,99

Appendix 2: P-Value of pairwise permutation MANOVA (df= 19) with "treatment+time" factor and PC 1,2 as explicative variables.

	BEM_Day 14	BEM_Day 28	BEM_Day 3	BEM_Day 7	BSA_Day 14	BSA_Day 28	BSA_Day 3	BSA_Day 7
BEM_Day 28	0.129	-	-	-	-	-	-	-
BEM_Day 3	0.499	0.499	-	-	-	-	-	-
BEM_Day 7	0.205	0.166	0.829	-	-	-	-	-
BSA_Day 14	0.645	0.063	0.332	0.127	-	-	-	-
BSA_Day 28	0.205	0.756	0.734	0.727	0.127	-	-	-
BSA_Day 3	0.959	0.127	0.499	0.332	0.749	0.271	-	-
BSA_Day 7	0.153	0.517	0.834	0.737	0.129	0.570	0.181	-
CEO1_Day 14	0.829	0.160	0.387	0.271	0.727	0.237	0.918	0.153
CEO1_Day 28	0.054	0.937	0.129	0.054	0.054	0.570	0.054	0.129
CEO1_Day 3	0.484	0.421	0.981	0.723	0.303	0.628	0.478	0.628
CEO1_Day 7	0.337	0.499	0.681	0.937	0.153	0.829	0.357	0.681
CEO2_Day 14	0.772	0.368	0.737	0.643	0.485	0.594	0.726	0.472
CEO2_Day 28	0.127	0.981	0.250	0.129	0.127	0.741	0.127	0.347
CEO2_Day 3	0.205	0.340	0.723	0.914	0.153	0.737	0.337	0.737
CEO2_Day 7	0.153	0.499	0.628	0.891	0.127	0.749	0.224	0.749
NEG_Day 14	0.499	0.203	0.980	0.499	0.346	0.409	0.499	0.499
NEG_Day 28	0.127	0.937	0.495	0.131	0.063	0.829	0.127	0.471
NEG_Day 3	0.301	0.591	0.853	0.937	0.146	0.918	0.337	0.737
NEG_Day 7	0.129	0.741	0.715	0.499	0.054	0.826	0.129	0.801

	CEO1_Day 14	CEO1_Day 28	CEO1_Day 3	CEO1_Day 7	CEO2_Day 14	CEO2_Day 28	CEO2_Day 3
BEM_Day 28	-	-	-	-	-	-	-
BEM_Day 3	-	-	-	-	-	-	-
BEM_Day 7	-	-	-	-	-	-	-
BSA_Day 14	-	-	-	-	-	-	-
BSA_Day 28	-	-	-	-	-	-	-
BSA_Day 3	-	-	-	-	-	-	-
BSA_Day 7	-	-	-	-	-	-	-
CEO1_Day 14	-	-	-	-	-	-	-
CEO1_Day 28	0.127	-	-	-	-	-	-
CEO1_Day 3	0.387	0.054	-	-	-	-	-
CEO1_Day 7	0.321	0.387	0.627	-	-	-	-
CEO2_Day 14	0.627	0.203	0.754	0.645	-	-	-
CEO2_Day 28	0.160	0.900	0.147	0.715	0.365	-	-
CEO2_Day 3	0.250	0.205	0.552	0.941	0.548	0.368	-
CEO2_Day 7	0.166	0.205	0.499	0.937	0.499	0.499	0.987
NEG_Day 14	0.421	0.054	0.937	0.494	0.749	0.127	0.387
NEG_Day 28	0.203	0.347	0.348	0.756	0.407	0.619	0.365
NEG_Day 3	0.277	0.307	0.800	0.854	0.645	0.499	0.900
NEG_Day 7	0.181	0.484	0.546	0.737	0.382	0.766	0.627

	CEO2_Day 7	NEG_Day 14	NEG_Day 28	NEG_Day 3
BEM_Day 28	-	-	-	-
BEM_Day 3	-	-	-	-
BEM_Day 7	-	-	-	-
BSA_Day 14	-	-	-	-
BSA_Day 28	-	-	-	-
BSA_Day 3	-	-	-	-
BSA_Day 7	-	-	-	-
CEO1_Day 14	-	-	-	-
CEO1_Day 28	-	-	-	-
CEO1_Day 3	-	-	-	-
CEO1_Day 7	-	-	-	-
CEO2_Day 14	-	-	-	-
CEO2_Day 28	-	-	-	-
CEO2_Day 3	-	-	-	-
CEO2_Day 7	-	-	-	-
NEG_Day 14	0.367	-	-	-
NEG_Day 28	0.478	0.127	-	-
NEG_Day 3	0.900	0.568	0.627	-
NEG_Day 7	0.737	0.357	0.894	0.756

Appendix 3: P-Value of pairwise permutation MANOVA (df = 19) with "treatment+time" factor and all compounds as explicative variables

	BEM_Day 14	BEM_Day 28	BEM_Day 3	BEM_Day 7	BSA_Day 14	BSA_Day 28	BSA_Day 3	BSA_Day 7
BEM_Day 28	0.46	-	-	-	-	-	-	-
BEM_Day 3	0.60	0.54	-	-	-	-	-	-
BEM_Day 7	0.49	0.54	0.91	-	-	-	-	-
BSA_Day 14	0.91	0.24	0.43	0.40	-	-	-	-
BSA_Day 28	0.50	0.95	0.50	0.51	0.30	-	-	-
BSA_Day 3	0.43	0.24	0.54	0.39	0.43	0.26	-	-
BSA_Day 7	0.43	0.54	0.95	0.97	0.40	0.50	0.41	-
CEO1_Day 14	0.99	0.39	0.54	0.43	0.91	0.41	0.43	0.41
CEO1_Day 28	0.41	0.95	0.43	0.43	0.24	0.93	0.24	0.46
CEO1_Day 3	0.66	0.54	0.99	0.95	0.43	0.52	0.43	0.95
CEO1_Day 7	0.56	0.81	0.80	0.95	0.41	0.88	0.30	0.91
CEO2_Day 14	0.68	0.51	0.51	0.45	0.43	0.66	0.30	0.43
CEO2_Day 28	0.24	0.54	0.30	0.28	0.24	0.53	0.24	0.40
CEO2_Day 3	0.30	0.43	0.66	0.56	0.24	0.41	0.40	0.75
CEO2_Day 7	0.41	0.56	0.50	0.47	0.24	0.55	0.24	0.53
NEG_Day 14	0.89	0.50	0.91	0.54	0.53	0.53	0.40	0.60
NEG_Day 28	0.40	0.94	0.44	0.41	0.24	0.95	0.24	0.44
NEG_Day 3	0.48	0.53	0.95	0.77	0.26	0.48	0.38	0.91
NEG_Day 7	0.30	0.69	0.66	0.95	0.24	0.53	0.30	0.95

	CEO1_Day 14	CEO1_Day 28	CEO1_Day 3	CEO1_Day 7	CEO2_Day 14	CEO2_Day 28	CEO2_Day 3
BEM_Day 28	-	-	-	-	-	-	-
BEM_Day 3	-	-	-	-	-	-	-
BEM_Day 7	-	-	-	-	-	-	-
BSA_Day 14	-	-	-	-	-	-	-
BSA_Day 28	-	-	-	-	-	-	-
BSA_Day 3	-	-	-	-	-	-	-
BSA_Day 7	-	-	-	-	-	-	-
CEO1_Day 14	-	-	-	-	-	-	-
CEO1_Day 28	0.28	-	-	-	-	-	-
CEO1_Day 3	0.53	0.43	-	-	-	-	-
CEO1_Day 7	0.51	0.65	0.91	-	-	-	-
CEO2_Day 14	0.51	0.47	0.45	0.75	-	-	-
CEO2_Day 28	0.24	0.60	0.24	0.50	0.43	-	-
CEO2_Day 3	0.32	0.40	0.54	0.85	0.45	0.27	-
CEO2_Day 7	0.28	0.54	0.43	0.91	0.60	0.47	0.68
NEG_Day 14	0.62	0.41	0.82	0.76	0.66	0.24	0.42
NEG_Day 28	0.30	0.97	0.40	0.68	0.43	0.41	0.24
NEG_Day 3	0.41	0.43	0.95	0.95	0.43	0.24	0.72
NEG_Day 7	0.28	0.47	0.87	0.97	0.41	0.24	0.54

	CEO2_Day 7	NEG_Day 14	NEG_Day 28	NEG_Day 3
BEM_Day 28	-	-	-	-
BEM_Day 3	-	-	-	-
BEM_Day 7	-	-	-	-
BSA_Day 14	-	-	-	-
BSA_Day 28	-	-	-	-
BSA_Day 3	-	-	-	-
BSA_Day 7	-	-	-	-
CEO1_Day 14	-	-	-	-
CEO1_Day 28	-	-	-	-
CEO1_Day 3	-	-	-	-
CEO1_Day 7	-	-	-	-
CEO2_Day 14	-	-	-	-
CEO2_Day 28	-	-	-	-
CEO2_Day 3	-	-	-	-
CEO2_Day 7	-	-	-	-
NEG_Day 14	0.43	-	-	-
NEG_Day 28	0.44	0.34	-	-
NEG_Day 3	0.60	0.66	0.30	-
NEG_Day 7	0.48	0.43	0.39	0.63

Appendix 4: Raw meteorological data. The blue line are the sampling sessions and the injection day (1st line). (CRA-W)

Date	T max°C	T min°C	T avg°C	RH% avg	Rain mm	Rain hours	Rain hours>=	Cropwet hou	ET0 mm
10/05/2020	19.7	9.6	14.9	98	2.4	2.0	2.0	10.8333	0.0
11/05/2020	12.5	4.7	8.2	67	1.8	2.5	2.5	7.8333	0.0
12/05/2020	13.6	-2.7	6.4	70	0.0	0.0	0.0	0.0	0.0
13/05/2020	16.4	-2.3	6.9	71	0.0	0.0	0.0	0.0	0.0
14/05/2020	14.5	0.7	8.1	62	0.0	0.0	0.0	0.0	0.0
15/05/2020	16.7	1.1	9.3	64	0.0	0.0	0.0	0.0	0.0
16/05/2020	20.2	-1.1	10.3	66	0.0	0.0	0.0	0.0	0.0
17/05/2020	21.8	0.0	11.5	67	0.0	0.0	0.0	0.0	0.0
18/05/2020	23.6	5.7	15.2	63	0.0	0.0	0.0	0.0	0.0
19/05/2020	**24.6**	**4.0**	**15.7**	**62**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
20/05/2020	24.6	7.7	17.0	73	0.0	0.0	0.0	0.0	0.0
21/05/2020	29.2	7.8	19.1	70	0.0	0.0	0.0	0.0	0.0
22/05/2020	**27.7**	**12.3**	**17.8**	**86**	**1.4**	**2.0**	**2.0**	**5.3333**	**0.0**
23/05/2020	20.2	10.7	14.1	68	0.8	1.5	1.5	2.4167	0.0
24/05/2020	18.7	7.7	12.9	84	0.0	0.0	0.0	0.0	0.0
25/05/2020	22.8	9.5	16.5	72	0.0	0.0	0.0	0.0	0.0
26/05/2020	**24.5**	**5.4**	**16.3**	**68**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
27/05/2020	25.2	6.6	16.6	66	0.0	0.0	0.0	0.0	0.0
28/05/2020	22.1	8.1	15.1	62	0.0	0.0	0.0	0.0	0.0
29/05/2020	24.0	6.5	15.9	53	0.0	0.0	0.0	0.0	0.0
30/05/2020	25.5	4.9	16.2	56	0.0	0.0	0.0	0.0	0.0
31/05/2020	22.9	5.7	16.2	53	0.0	0.0	0.0	0.0	0.0
01/06/2020	26.8	6.7	18.1	58	0.0	0.0	0.0	0.0	0.0
02/06/2020	**30.1**	**7.3**	**19.5**	**55**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
03/06/2020	25.2	8.4	17.1	65	0.0	0.0	0.0	0.0	0.0
04/06/2020	13.9	7.9	12.5	98	5.0	3.5	3.5	9.8333	0.0
05/06/2020	14.2	6.8	9.5	98	13.0	9.0	9.0	24.0	0.0
06/06/2020	17.3	5.8	11.4	76	2.8	2.0	2.0	12.9167	0.0
07/06/2020	20.5	9.1	13.8	72	0.0	0.0	0.0	0.0	0.0
08/06/2020	17.9	8.9	12.8	95	0.4	1.0	1.0	1.4167	0.0
09/06/2020	14.9	10.0	12.4	96	0.0	0.0	0.0	0.0	0.0
10/06/2020	17.1	6.6	12.3	97	0.4	1.0	1.0	1.75	0.0
11/06/2020	22.8	10.0	16.0	84	0.4	1.0	1.0	13.5	0.0
12/06/2020	23.5	9.2	16.1	87	9.2	3.0	3.0	4.25	0.0
13/06/2020	27.0	10.6	19.0	78	0.4	1.0	1.0	11.4167	0.0
14/06/2020	25.6	11.6	18.1	80	0.0	0.0	0.0	0.0	0.0
15/06/2020	27.2	10.2	19.1	76	1.2	1.0	1.0	2.6667	0.0
16/06/2020	25.6	10.4	18.0	81	7.0	1.0	1.0	15.0	0.0