Contribution to the study of the molecular physiological response of apple trees to an essential oil-emulsion biopesticide applied by trunk-injection

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Abstract

Apple production is among the most important ones in Belgium. Apple trees like any other plants are subject to abiotic and biotic stresses that may cause important economic losses. One example is its major pest, the rosy apple aphid (*Dysaphis plantaginea* (Passerini)). The common strategy for pest control relies on pesticides application. However, *D. plantaginea* becomes more and more resistant to these substances. Thus, the Walloon Region started a project to develop a biopesticide based on essential oils which take advantage of their insecticidal properties to come up with an environmentally friendly alternative to these chemical pesticides. This research study focuses on studying the physiological plant response at a molecular scale to this biopesticide directly injected into the trunk. Indeed, a good product for this context has to be efficient against the pest without damaging the plant nor the yield.

The study focused on the plant physiological response to a single essential oil at different concentrations than on comparing if the different EO emulsions already formulated induced the same physiological response. To this purpose, cinnamon essential oil (*Cinnamomum cassia* J.Presl) emulsions at 1% and 4% were injected in the trunk of two years old apple trees (*Malus domestica* Borkh). Leaves were sampled at 0h; 1h30; 3h; 4h30; 6h; 24h; 72h and 192h after injecting.

The efficiency of the apple tree redox system against the potential oxidative stress was assessed by measuring two antioxidants: glutathione and carotenoids by high performance liquid chromatography equipped with a FLD detector and by spectrophotometer, respectively. Glutathione play different roles in plants, especially in stress conditions. In this work, the evaluation of glutathione concentration in apple leaves was carried out by using monobromobimane labelling and HPLC-FLD. It presents a good linearity in the concentration range from 25 µM to 200 µM, a great repeatability and a GSH-mbb adduct stability for at least nine days. Furthermore, the leaf physiological status was investigated, on the one hand, with Fv/Fm ratio and F0 determined by a handy plant fluorimeter and, on the other hand, with chlorophylls (a and b) content also evaluated by spectrophotometer. Finally, oxidative damage was considered by the determination of conjugated dienes concentration, a marker of lipid peroxidation by spectrophotometer.

Results on apple leaves first show that it is not an easy task to study the oxidative stress in field conditions. A lot of other factors from the surrounding environment could influence the results including the researcher itself. In this kind of experiment, the challenge is to reflect the situation inside the plant at the time of sampling and not merely report the perturbation inflicted on the field. Moreover, the oxidative burst occurs in the few hours after the injection and it seems that the apple trees are able to manage this stress. Even if studies demonstrate the herbicidal activity of the cinnamon essential oil, it does not in this context. Finally, the plant antioxidant defences are composed by non-enzymatic and enzymatic scavengers. When these defences are not able to counterbalance the oxidative stress, different kinds of damage can be produced. This work was more concentrated on non-enzymatic antioxidant and the photosynthetic performances. Thus, the study has to be continued.

Key words: biopesticides, oxidative stress, trunk-injection, glutathione, photosynthetic apparatus, phytotoxicity

Résumé

La production de pommes est l'une des plus importantes en Belgique. Comme toutes les plantes, les pommiers sont sujets à des stress abiotiques et biotiques qui peuvent entraîner de lourdes pertes économiques. Un bon exemple est son ravageur principal, le puceron cendré du pommier (*Dysaphis plantaginea* (Passerini)). Une stratégie de lutte couramment utilisée contre les ravageurs est l'utilisation de pesticides. Cependant, *D. plantaginea* y devient de plus en plus résistant. Dans ce contexte, la Région wallonne a donc lancé un projet pour développer un biopesticide à base d'huiles essentielles possédant des propriétés insecticides pour proposer une alternative respectueuse de l'environnement à ces substances chimiques. Ce travail se focalise sur l'étude de la réponse physiologique d'arbres à une échelle moléculaire suite à l'injection du biopesticide dans le tronc. En effet, un bon produit pour ce genre d'application se doit d'être efficace contre le ravageur ciblé sans endommager la plante, ni le rendement.

Cette étude s'est concentrée sur la réponse physiologique de l'arbre par rapport à un seul biopesticide injecté à plusieurs concentrations plutôt que de comparer cette réponse à différents biopesticides formulés antérieurement. Par conséquent, des émulsions d'huile essentielle de cannelier de Chine (*Cinnamomum cassia* J.Presl) à des concentrations de 1% et 4% ont été injectées dans le tronc de pommiers âgés de deux ans (*Malus domestica* Borkh). Des feuilles ont été récoltées à différents temps après injection: 0h; 1h30; 3h; 4h30; 6h; 24h; 72h et 192h.

L'efficacité du système redox du pommier contre le stress oxydatif potentiel induit par l'émulsion et l'injection a été évaluée par la mesure de deux antioxydants à savoir, le glutathion par chromatographie en phase liquide à haute performance équipée d'un détecteur FLD et les caroténoïdes par spectrophotométrie. Le glutathion joue différents rôles au sein de la plante, notamment en conditions de stress. Dans ce travail, l'évaluation de la concentration en glutathion dans des feuilles de pommiers a été réalisée avec du monobromobinane par CLHP-DFL. Cette méthode présente une bonne linéarité dans la gamme de concentration comprise entre 25 µM et 200 µM, une très bonne répétabilité et un adduit mbb-GSH qui reste stable pendant au moins neuf jours. L'état physiologique des feuilles a aussi été étudié d'une part, par le ratio Fv/Fm and F0, déterminés à l'aide d'un fluorimètre portatif et d'autre part, par les teneurs en chlorophylles a et b, mesurées par spectrophotométrie. Finalement, les dégâts oxydatifs ont été évalués par la détermination de la concentration en dienes conjugués, un marqueur de la peroxydation des lipides, par spectrophotométrie.

Les résultats obtenus sur les feuilles de pommiers montrent tout d'abord qu'il n'est pas facile d'étudier le stress oxydatif sur le terrain. De nombreux autres facteurs liés à l'environnement immédiat peuvent altérer les résultats, le chercheur y compris. L'enjeu consiste à refléter ce qui se passe réellement à l'intérieur de la plante au moment de l'échantillonnage et non simplement les perturbations infligées sur le terrain. De plus, le sursaut oxydatif se produirait dans les quelques heures qui suivent l'injection et il semblerait que les pommiers soient capables de gérer ce stress. Même si des études scientifiques démontrent l'activité herbicide de l'huile essentielle de cannelle, ce n'est pas le cas dans ce contexte-ci. Enfin, les défenses antioxydantes de la plante sont composées d'antioxydants non enzymatiques et enzymatiques. Lorsque celles-ci ne sont pas capables de contrebalancer le stress oxydatif, différents types de dommages peuvent être occasionnés. Ce travail s'est davantage concentré sur les antioxydants non enzymatiques et les performances photosynthétiques. L'étude devra donc être poursuivie.

Mots-clés : biopesticides, stress oxydatif, injection dans le tronc, glutathion, appareil photosynthétique, phytotoxicité

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Abbreviations

γGC: γ-glutamylcysteine
γ-GCL: γ-glutamylcysteine synthetase or γ glutamylcysteine ligase
ϒ-Glu-Cys-Gly: γ-glutamyl-L-cysteinylglycine
ABA: Abscisic acid
ACC: 1-aminocyclopropane-1-carboxylic acid
AChE: Acetylcholinesterase
ANOVA: Analysis of variance
ANOVA1: One-way analysis of variance
ANOVA2: Two-way analysis of variance
APX: Ascorbate peroxidase
AsA-GSH: Ascorbate-glutathione pathway
BR: brassinosteroids
BTH: Benzothiadiazole
CAT: Catalase
CDs: Conjugated dienes
CEO: Cinnamon essential oil
CHES: 2-(Cyclohexylamino)ethanesulfonic acid
chl a: Chlorophyll a
chl b: Chlorophyll b
CK: cytokinins
CRA-W: Walloon Agricultural Research Centre
Cys: Cysteine
DAMPS: Damaged-associated molecular
DHA: Dehydroascorbate
DNA: Deoxyribonucleic acid
DTT: Dithiothreitol
EDTA: Ethylenediaminetetraacetic acid
EO(s): Essential oil(s)
EPA: the United States Environmental Protection Agency
ET: Ethylene
ETI: effector-triggered immunity
ETS: effector-triggered susceptibility
Fm: maximal chlorophyll fluorescence
F0: fluorescence origin
Fv: variable fluorescence
Fv/Fm: Maximum quantum efficiency of photosystem II
GA: Giberellins
GABA: ɤ-aminobutyric acid
GC-FID: Gas Chromatography using Flame Ionization
GC-MS: Gas Chromatography coupled with a Mass Spectrometer
GC-TCD: Gas Chromatography using Thermal Conductivity Detector
Glu: Glutamate
Gly: Glycine
GMOs: Genetically modified organisms
GR: Glutathione reductase
GSH: Glutathione
GS: Glutathione synthetase
GSSG: Glutathione disulphide
GST: Glutathione-S-transferases
GT: Glycosyltransferases

HPLC: High performance liquid chromatography
HR: Hypersensitive response
IAA: indole-3-acetic acid
ICS/SID2: isochorismate synthase
IPM: Integrated pest management
ISO: International organization for standardization
ISR: Induced systemic resistance
JA: Jasmonic acid
Kow: Water/octanol partition coefficient
LOD: Limit of detection
LOQ: Limit of quantification
MAMPS: Microbe-associated molecular
MAPKs: Mitogen-activated protein kinases
mbb: monobromobimane
mcb: monochlorobimane
MDA: Malondialdehyde
MeSA: Methylsalicylate
MPA: Metaphosphoric acid
$NADP^+$: Nicotinamide adenine dinucleotide phosphate
NADPH: reduced form of nicotinamide adenine dinucleotide phosphate
NB-LRRs: Nucleotide-binding leucine-rich repeat domain class
NEM: N-ethylmaleimide
NPR1: Non expressor of pathogenesis-related genes 1
OPA: O-phthalaldehyde
PAL: Phenylalanine ammonia lyase
PAMPS: Pathogen-associated molecular
PCD: Programmed cell death
PET: Photosynthetic electron transport
POX: Peroxidases
PPM: Plant plasma membrane
PPPs: Plant protection products
PR: pathogenesis-related genes
PR1: Pathogenesis-related 1 protein
PRRs: Pattern-recognition receptors
PSII: Photosystem II
PTI: Patter triggered immunity
R-proteins: Resistance proteins
RI: Retention time
PGPR/F: plant-growth-promoting rhizobacteria/fungi
RNA: Ribonucleic acid
ROS: Reactive oxygen species
SA: Salicylic acid
SAM: S-adenosyl-Met
SAR: Systemic acquired resistance
SOD: Superoxide dismutase
SPW: Public Service of Wallonia
STR: Strigolactones
TBARS: Thiobarbituric acid-reactive substances
UV-Vis: Ultraviolet-visible
VOCs: Volatile Organic Compounds

1. Foreword

Apple production is among the most important ones in Belgium. It can be impacted by pest damage that can induce significant economic losses. Pesticides have been used for ages but their noxious consequences on environment, humans and non-target animals are obvious. The Walloon Region thus started the "Tree-injection" project whose global objective is to efficiently protect trees in fruit growing by developing an environmentally friendly essential oil-based pesticide. This project falls within the integrated pest management[1] (IPM) techniques.

This study occurs in the second year of the project. During the first year, an essential oil-based biopesticide was formulated for trunk-injection purposes on *Malus domestica* Borkh (var. Jonagold) and some tests were run to assess its efficiency on its major pest, *Dysaphis plantaginea* (Passerini). At the same time, a study of phytotoxicity was initiated. A next step naturally seemed to be a further study of the physiological plant response.

There are clearly too many mechanisms and interactions at stake and too short a time to pretend to study the physiological response in its entirety. Therefore, this work will focus on the apple tree physiological response at a molecular scale where the oxidative stress is the main actor.

2. Introduction

Before bringing up the topic, it seems important to remember the general context that justifies the relevance of this study. This section will thus concern the apple production in Belgium, the "Tree-injection" project and essential oils in general.

It will be followed by the impacts of exogenous essential oil (EO) application and the plant physiological response assessment.

2.1. Apple production in Belgium

Fruit production is quite important in Belgium. The three mainly cultivated are pears, apples and strawberries in Wallonia. 1500 hectares are devoted to these productions of which the half for apples. Belgium is the chief European producer with an apple production of 273 950 tons in 2018 (FAOSTAT, 2018). There are many apple varieties on the market. The five most commercialized are Jonagold - Jonagored - Cox orange pippin - Elstar - Belle de Boskoop. Jonagold which represents 60% of the Belgian apples production will be given full consideration here (Apaq-W, no date).

Apple trees like any other plants are subject to abiotic and biotic stresses. Freezing events are the most important cause of economic losses in agriculture. For example, the Belgian apple production in 2017 corresponded to a third of the 2018 crop because of frost (FAOSTAT, 2018). However, this element is not as well studied as herbivory or drought. Injuries happen when trees are in dormancy period and may become visible only during the growing season. The

[1] Integrated Pest Management: "The careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human and animal health and/or the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms" (FAO and WHO, 2014).

highest-risk periods are autumn and spring which correspond to transition periods (Charrier et al., 2015). Apple trees may also suffer from several attacks from fungal, viral and bacterial diseases, pests, dust mites and nematodes (Belkair, 2018). All these factors can impair production or commercialization yields because apple fruits do not fulfil minimum quality criteria according to the Codex alimentarius commission (2010).

Several strategies have been implemented for pest control. The most frequently applied consists in using pesticides. However, they have some drawbacks. Indeed, their misuse causes adverse effects on non-target organisms and a reduction of biodiversity in the long term. They give rise to contamination problems in the environment and risks for human life. More and more active substances have therefore been removed from the market by various legislative authorities. Besides, pests can also develop resistance to these substances (Belkair, 2018; Hasanshahi et al., 2016; Pavela and Benelli, 2016). This combination of disadvantages suggests that plant protection products (PPPs) become an economic and ecological challenge not only in Belgium but around the world. This is why alternative solutions have been investigated such as biopesticides based on plant essential oils or hydrodystillates.

Nowadays, a lot of research focuses on those alternative products. A good example is the "Tree-injection" project presented in the next point. This current study is a part of it and will mainly centre on apple orchards and one of its main pests, *Dysaphis plantaginea* (Passerini).

2.2. Tree-injection project[2]

As explained above, the major objective of the "Tree-injection" project of the Walloon Region of Belgium is to develop a biopesticide to protect trees against pests. Essential oils and other plant hydrodistillates are well-known to have insecticidal properties and are therefore being considered for this project. The formulated biopesticide will be injected directly into the xylem of the fruit tree by a system called "trunk-injection".

The project is funded by the Public Service of Wallonia (SPW) agriculture[3] and run in collaboration with the Agro-Bio Tech Faculty Gembloux (Liège University), the Catholic University of Louvain and the Walloon Agricultural Research Centre (CRA-W).

It concentrates on apple and pear trees, more specifically on the control of their main pests, respectively the rosy apple aphid (*Dysaphis plantaginea* (Passerini)) and the pear psylla (*Carcopsylla pyri* (L.)) in order to study the effectiveness of the biopesticide. This project ultimately wants to find an environmentally friendly alternative to conventional chemical pesticides to efficiently control those two insects.

2.2.1. Apple tree biology

Malus domestica Borkh (var. Jonagold), called "apple tree" in English or "pommier commun" in French belongs to the *Rosaceae* family which includes agronomic and ornamental species distributed worldwide, mainly in temperate regions (Velasco et al., 2010).

[2] The information in this part is taken from the Annual Report No. 1. This document was communicated internally.

[3] The PSW's funding are granted from the 1rst September 2018 to 31srt Augustus 2021.

The Jonagold variety is the American hybrid cross between apple trees “Golden Delicious” x “Jonathan”. It is cultivated in Belgium since the early 1980s and as previously mentioned, Jonagold is nowadays a big part of the total Belgian apple production (60%). Jonagold apple is rather large. Its skin is smooth and greasy with colour shades. Its flesh is pale yellow, crisp and juicy. Apple harvest takes place between mid-September and October. The supply period goes from late September and July (Apaq-W, no date).

2.2.2. *Dysaphis plantaginea* biology and current control methods

One of the most common pests affecting apples is the rosy apple aphid or *Dysaphis plantaginea* (Passerini) (Hemiptera: Aphididae) in Europe and North America (Brown and Mathews, 2007). Aphids are biting-sucking, stylus-piercing insects (Belkair, 2018). The injuries they inflict are many and various. On the one hand, they feed by ingesting phloem sap directly from the cells of the sieve tubes. Their saliva contains toxic irritant substances triggering leaf curling. A high population density may thus lead to leaf rolling, shoot twisting, reduction of fruit size, deformation of fruit shape and premature fruit fall, in brief, to the reduction of tree vigour (Hasanshahi et al., 2016; Rousselin et al., 2017). In addition, repeated attacks will induce root deterioration and a reduction in the number of flowers and fruits. On the other hand, high aphid populations produce abundant honeydew, which favours the establishment of mould and fungus whose presence limits photosynthesis (Belkair, 2018; Peusens et al., 2006).

D. plantaginea is considered as a major early-season pest (De Berardinis et al., 1994). The holocyclic[4] dioecious[5] life cycle of the rosy apple aphid takes place on two different host plants. Its primary host is the apple tree, cultivated or wild, (*M. domestica*) and (*M. sylvestris*) (Alins et al., 2017). Its secondary host is the plantain (*Plantago spp*) (*Figure 1*).

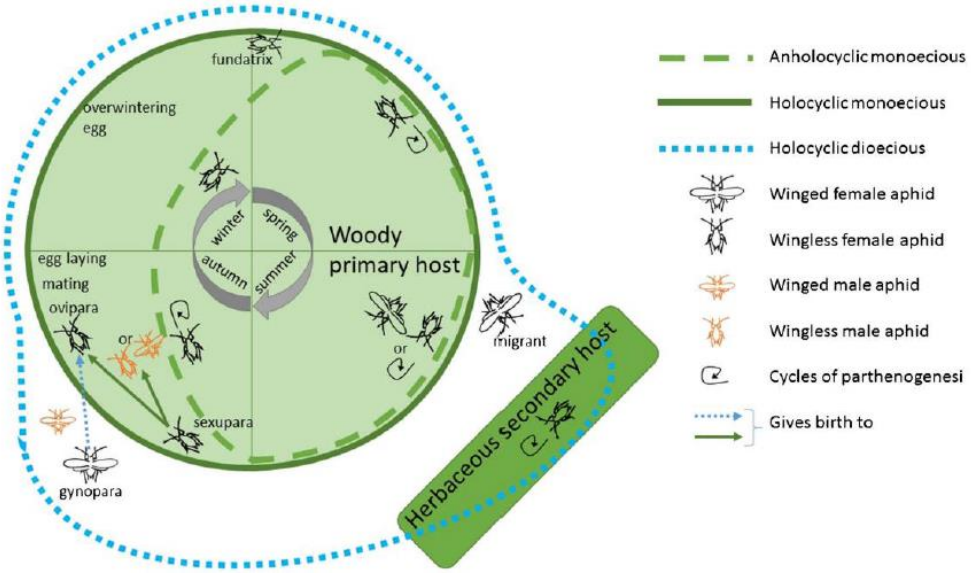

Figure 1. Sum up of life cycles of the different aphid species. The dashed blue cycle represents the D. plantaginea’s cycle (Rousselin et al., 2017).

[4] Holocyclic: “Parthenogenetic generations are interrupted by one generation of sexual reproduction” (Rousselin et al., 2017).
[5] Dioecious: “Having the male reproductive organs in one individual and the female organs in another; having separate sexes” (Collins dictionary online, consulted: 17/03/2020).

After spending the winter on their first host, eggs of rosy apple aphids hatch into founder females in early spring (April) which concurs with apple bud break. These females spawn other females by parthenogenesis[6] occurring during long-day conditions. Several generations of females will then succeed. Note that most of the females will be wingless in the first generations and then the proportion of winged females will gradually enlarge in the following generations. There are between five and seven generations between spring and early summer. It is during this period that aphid outbreaks are frequently recorded (Simon and Peccoud, 2018). The larvae are beige, then turn pink and greyish during their development (Nicolas et al., 2013). The nymphs move to bursting buds after hatching. They do not cause significant damage whereas adult fundatrices and spring generation do. Moreover, no deformation is caused by summer and autumn forms in both hosts (Bonnemaison, 1959).

In early summer (May-June), the winged females migrate to plantain (*Plantago lanceolata L.*) where they give birth to several more generations of females. In autumn, winged males and females appear because nights become longer and longer. They return to apple trees to mate. The fertilized females lay winter eggs. A new cycle will eventually begin in the next spring (Brown and Mathews, 2007; Nicolas et al., 2013; Wyss and Daniel, 2004).

The overwintering eggs have an elliptical shape. They are black, shiny and about half a millimetre long. They are usually laid in small groups in bark folds or in crevices around branches and buds. Aphid adults are about 2 to 3 mm long and are usually covered with waxy dust. They have a rounded body, and their antennae reach up to half of their body. Winged aphids are black with a dark brown spot on the abdomen and their wings are about twice as long as their body whereas the wingless form is purple (Nicolas et al., 2013).

D. plantaginea has developed a mutualistic relationship with ants (Hymenoptera: Formicidae). Ants feed on honeydew secreted by aphids. They provide some services in return: (i) ants have a negative impact on the population of aphid natural enemies such as Syrphidae and Chrysopidae populations, (ii) they maintain the colony hygiene in order to avoid fungi development (because of the honeydew secretions), (iii) they minimize competition with other aphid species and (iv) they structure aphid communities. This mutualism has thus harmful consequences for apple trees (Belkair, 2018, Miñarro et al., 2010).

Several methods for the control of *Dysaphis plantaginea* have been investigated on both conventional and organic apple productions. Nowadays, the elimination of fundatrices is the most widespread strategy (Alins et al., 2017). In classical apple production, insecticides can be sprayed in early spring. However, only a few groups of active substances, carbamates and neonicotenoids, are allowed for aphid control in Belgium (*Figure 2 (a) and (b)*) (Peusens et al., 2006). Unfortunately, this limitation leads to pest resistance. Moreover, these chemical substances are not specific to aphids and may be adverse on advantageous arthropods. This method can nevertheless be very efficient but only in early spring, before the expanding of aphid populations because when tree bloom begins, curled leaves provide protection to the remaining aphids (Brown and Mathews, 2007, Wyss and Daniel, 2004). In organic production, only azadirachtin controls the pest when it is sprinkled against *D. plantaginea* fundatrices (Alins et al., 2017).

[6] Parthenogenesis: "Type of reproduction, occurring in some insects and flowers, in which the unfertilized ovum develops directly into a new individual" (Collins dictionary online, consulted: 17/03/2020).

Some alternatives for both kinds of apple production would be using either insecticides or repellent in autumn in order to prevent the migration for the sexual reproduction. Wyss and Daniel (2004) showed that the use of pyrethrins or kaolin as autumn treatment could be a practicable substitute for spring treatment. Alins et al. (2017) and Cross et al. (2007) investigated garlic extracts, potassium soap, kaolin, pyrethrins and rotenone as permitted insecticides in organic productions during autumn. Only pyrethrins showed real effects to reduce damage but they expressed some variability as found by Wyss and Daniel (2004). Pyrethrins could therefore replace azadirachtin in aphids control in countries where it is not allowed and to decrease pest resistance (*Figure 2 (c) and (d)*) (Alins et al., 2017). However, Alins et al. (2017) showed that potassium soap also had an impact at the beginning of the expansion of the colonies of the *D. plantaginea.*

Moreover, they discuss the fact that the efficiency of a product to control aphid populations is not advisable when the infestation occurs at full bloom. With these studies, autumn is an even more fitting time. All these previously mentioned strategies worked by contact action.

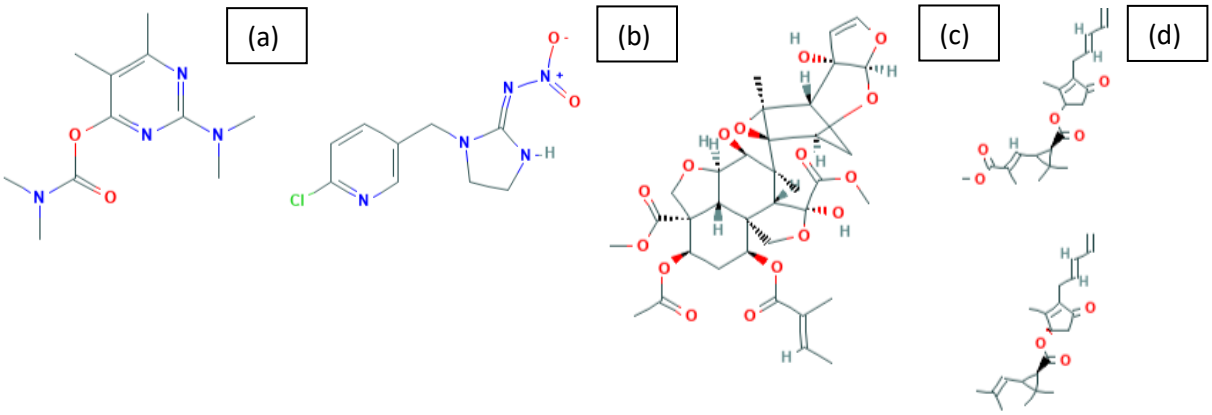

Figure 2. 2D structure of (a) pirimicarb (carbamate), (b) imidacloprid (neonicotenoid), (c) azadirachtin and (d) pyrethrins (PubChem).

Other strategies can be considered: either top-down or bottom-up processes that use aphid enemies or enhance the host plant resistance (genetically modified organisms (GMOs) or resistant hybrids) (Rousselin et al., 2017). However, on the one hand, the bottom-up technique did not show enough high yields nor a good fruit quality from these resistant and tolerant apple varieties (Iglesias et al. 2009). On the other hand, apple tree is not in the register of the authorised GMOs in the European Union for food and/or feed use including import and processing. This register is established and maintained by the European Commission and available online.[7]

Natural parasitoids and aphid predators helped by an increase of varied planting strips can play an additional control role. Wyss (1995) showed that this kind of habitat manipulation was productive. There is a non-exhaustive list of aphid enemies: Syrphidae, Coccinellidae, Braconidae, Chrysopidae, Anthocoridae, Forficulidae, Miridae, Nabidae, Cecidomyiidae and species of spiders (Alins et al., 2017; Peusens et al., 2006; Wyss, 1995). Weed strips are a source of food, habitat or egg-laying stimulation depending on considered insects (Wyss, 1995).

[7] https://webgate.ec.europa.eu/dyna/gm_register/index_en.cfm (Consulted: 10/08/2020).

It has to be noticed that not all steps of predators' development are implicated in aphid control and that these different environmentally friendly control methods require a good knowledge of the life cycle of the aphid.

Last but not least, essential oils have been studied for their promising properties to fight pests (Atanasova and Leather, 2018). This point is developed in the following section called "Essential oils".

2.3. Essential oils

2.3.1. Definition and general information

Essential oils (EOs) are "lipophilic and highly volatile secondary plant metabolites of molecular weight below 300" (Pavela and Benelli, 2016). International Organization for Standardization (ISO) defines essential oil as "product obtained from a natural raw material[8] of plant origin, by steam distillation, by mechanical processes from the epicarp of citrus fruits, or by dry distillation, after separation of the aqueous phase — if any — by physical processes" (ISO 9235, 2013).

They are produced by approximately 17 500 aromatic higher plants which are related to a few families counting the Asteraceae, Lamiaceae, Lauraceae and Myrtaceae (Regnault-Roger et al., 2012). EOs contain about 20-60 active substances but only two or three are present at high concentrations (20%-85%). They determine the biological properties of EOs in most cases. Others appear at trace levels but can modulate the activity of the big ones. They are also soluble in organic solvents. They are mainly constituted by two groups of compounds: terpenes/terpenoids (which are the most abundant group) and aromatic/aliphatic constituents. Compounds derived from glycosides or fatty acids are to a lesser extent part of EOs.

Chromatographic procedures are commonly used to characterize the chemical profile of EOs. The most common apparatus nowadays is gas chromatography coupled with a mass spectrometer (GC-MS). Other methods are gas chromatography using flame ionization (GC-FID) or thermal conductivity detector (GC-TCD) for example. Around 3000 EOs are known so far (Bakkali et al., 2008).

EOs are extracted from various plant organs such as roots (vetiver grass), barks (cinnamon), flowers (bergamot orange), woods (rosewood), fruits (star anise), rhizomes (ginger), leaves (lemon grass and eucalyptus) and seeds (nutmeg) usually by hydrodistillation or steam distillation and by cold expression for citrus fruits (Regnault-Roger et al., 2012). Modern extraction methods (e.g. microwave extraction and supercritical fluid extraction) also allow to obtain the same fraction of compounds. However, this fraction is called "plant extract" rather than "essential oil" reserved for the fraction obtained by conventional extraction methods (e.g. steam- and hydro-distillation) according to the ISO 9235 definition (ISO 9235, 2013). They are accumulated in secretory cells, canals, cavities, glandular trichomes, epidermic cells or resin ducts (Bakkali et al., 2008; Mossa, 2016; Regnault-Roger et al., 2012).

[8] Natural raw material: "natural raw material of vegetal, animal or microbiological origin, as such, obtained by physical, enzymatic or microbiological processes, or obtained by traditional preparation processes (e.g. extraction, distillation, heating, torrefaction, fermentation)" (ISO 9235, 2013).

The chemical profile of the EO varies in terms of the number of components and in stereochemistry depending on extraction parameters accordingly to the purpose of their use. Other variations are caused by climate, soil composition, impurities or contamination as well as organ, age and vegetative cycle stage of the plant (Bakkali et al., 2008).

EOs are also sensitive to chemical transformations (*Figure 3*). Indeed, components of a same chemical group can convert themselves through different reactions (oxidation, cyclization, isomerization or dehydrogenation reactions, generated either chemically or enzymatically) leading to a loss of quality, bioactive and organoleptic properties. These reactions can be furthered by light, temperature, water and oxygen availability. So, the conditions in which the EO is extracted, manipulated or stored must be precisely controlled (Turek and Stintzing, 2013).

Therefore, developing new modes of application or formulation will help conserve EO bioactivities. Trunk-injection (described later in 1.3.4.) is being considered here.

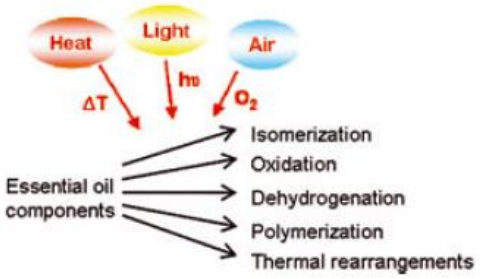

Figure 3. Possible reactions of EOs due to exposition to heat, light or air (Turek and Stintzing, 2013).

2.3.2. Biological properties

EOs have specific flavours and scents, bactericidal, virucidal, fungicidal and insecticidal properties, useful characters to repel unwanted insects and attract pollinators for examples. It makes thus sense to investigate an EO formulation for a biopesticide application which is the aim of the "tree injection" project. Besides, EOs also have anti-inflammatory and antioxidant activities that can explain why around 300 EOs are nowadays commercially important and commonly used in various fields such as medicine (natural remedies), cosmetics (perfumes, make-up products, massage oil), agriculture and food industries (additives and preservers). Some EOs have already been tested in pre- and post-crop harvest protection (Bakkali et al., 2008; Pavela and Benelli, 2016; Regnault-Roger et al., 2012).

2.3.3. Biopesticide

Biopesticide is "a generic term generally applied to a substance derived from nature, such as a microorganism or botanical or semiochemical, that may be formulated and applied in a manner similar to a conventional chemical pesticide and that is normally used for short-term pest control *[adapted from ISPM Pub. No. 3, 1996 (IPPC, 2005)]*" (FAO and WHO, 2017).

EOs are good candidates because of their insecticidal activities that can act in many ways (contact toxicity, fumigation, repellence, etc.) due to a complex mixture of constituents that also enables synergism. Moreover, they contain different compounds synthesized by many biological pathways and analogues of a same compound class. All these properties would eventually decrease or slow down the development of pest resistance which can come very fast

for *D. plantaginea* with chemical pesticides because of its short generation times, its rapid development and the single active substance of conventional pesticides.

EOs allow overcoming other current chemical pesticide problems. Indeed, they are highly volatile, it means they are not persistent in the environment. EO residues are hardly likely to be found on food products or soil and to pollute ground water, so minimizing risks for workers too. Moreover, they are compatible with IPM programs because non-target species like parasitoid, predator and pollinator insect populations will be more preserved due to the lesser risk of residual activity (Isman and Machial, 2006; Machial, 2010). Despite all these advantages, EOS, nevertheless, have to be correctly manipulated to prevent them from becoming toxic.

Directive 2009/128/EC, "Establishing a Framework for Community Action to Achieve the Sustainable Use of Pesticides," suggested promoting the use of integrated pest management and alternative agricultural approaches to pesticides in order to reduce risk and impact on the environment and human health (European Commission, 2009).

In plant protection (and by extension in the "Tree-injection" project), the aim is to prevent pests from causing plant damage. In this context, the examined EO bioactivities are either insecticidal or repellent. It means they will ultimately lead to the death of the insect or dissuade it from getting too close to the plant. Both biological effects have been assessed by a large number of studies in the past years.

EOs are lipophilic liquids able to penetrate the waxy cuticle of insects and impair biochemical dysfunctions (Mossa, 2016). Various factors influence the toxicity of EOs to insects: the chemical composition influenced by the part of the plant used to collect the oil, its phenological state, the time of year, the soil and climatic variations, the nature and position of functional groups, the point of entry of the toxin (inhalation, ingestion, skin absorption, etc.), molecular weight and the mechanisms of action. These last ones are reviewed in *Figure 4.* EO insecticidal modes of action often target the nervous system of insects by different means: (i) inhibition of insect cytochromes P450 which act in the metabolism of xenobiotics and endogenous compounds, (ii) bond with ɤ-aminobutyric acid (GABA) receptors to disrupt the function of GABA synapses to cause hyper-excitation of the insect central nervous system (*Figure 5 (a)*), (iii) inhibition of acetylcholinesterase (AChE) which engenders a general deficit of coordination in the neuromuscular system (*Figure 5 (b)*) and (iv) activation receptors for octopamine which is "a neurotransmitter analogous to the vertebrate noradrenaline" (Regnault-Roger et al., 2012) (*Figure 5 (c)*) (Isman, 2000; Isman and Machial, 2006; Mossa, 2016; Rattan, 2010; Regnault-Roger, 1997; Regnault-Roger et al., 2012).

Finally, Machial (2010) demonstrated that an EO-based biopesticide suitable for one insect species is probably not for another one depending on the composition of the EO and the variable responses between insect species.

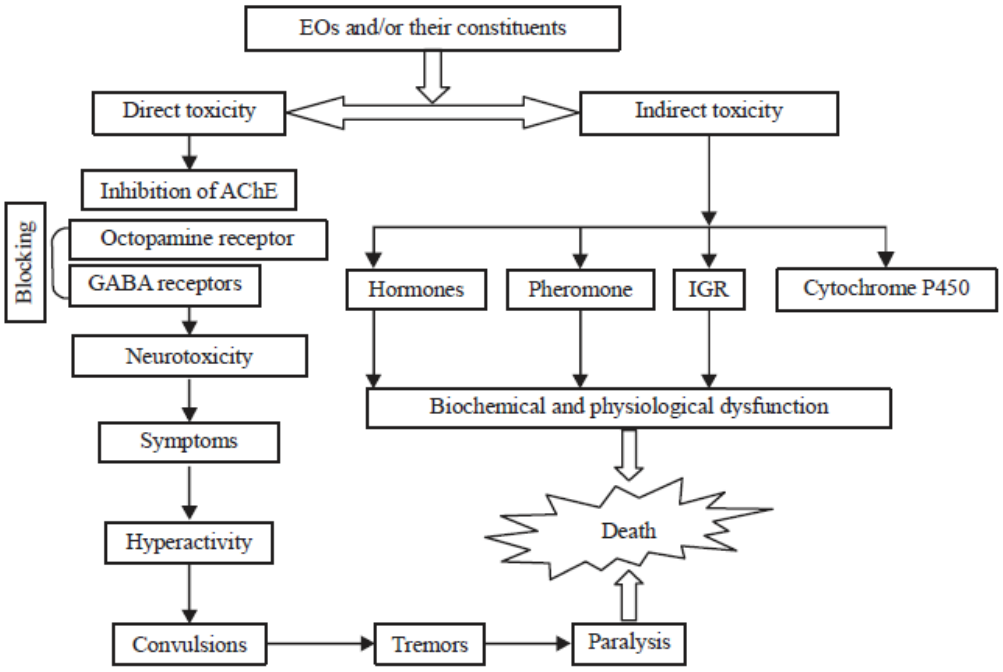

Figure 4. Overview of mechanisms induced by EOs toxicity that lead to the death of insects (Mossa, 2016).

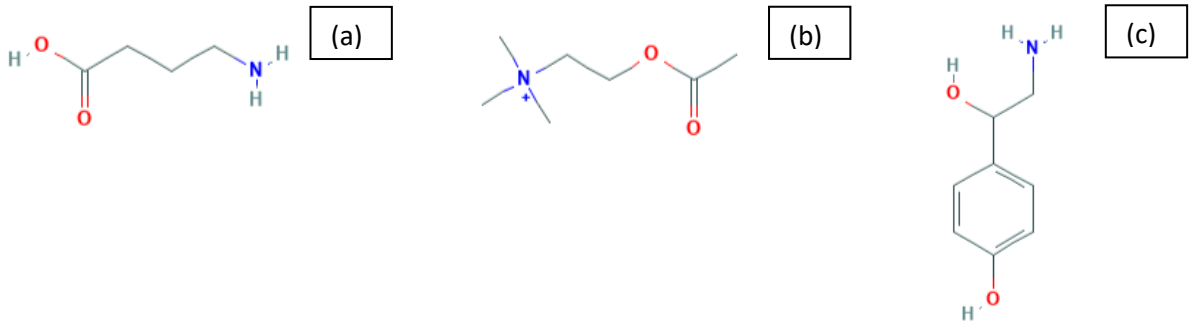

Figure 5. 2D structure of (a) ɤ-aminobutyric acid, (b) acetylcholine and (c) octopamine (PubChem).

Despite the efficient properties of the EOs against pests, the supply of commercial biopesticides is still low. Pavela and Benelli (2016) give four reasons: (1) few practical results are published, (2) European legislation is strict, (3 and 4) there is a low endurance of effects and a limited availability of these active substances for production.

Some essential oils are well known to have an undoubted insecticide bioactivity (Isman and Machial, 2006; Walia et al., 2015). Studies have already begun to investigate EOs against *D. plantaginea* (Gorski, et al., 2009, Machial, 2010). Clove oil (*Syzygium aromaticum* (L.) Merrill & Perry), spearmint oil (*Mentha spicata* L.) and cinnamon oil (*Cinnamomum cassia* J. Presl) were selected for the formulation of the biopesticide during the first year of the project based on studies at the UCLouvain. However, in the present study, it has been decided that only[9] cinnamon oil would be used to test the physiological response of the plant.

[9] This choice is explained in more details in the section 3.1 of "material and method" part.

The main component (85%) of *C. cassia* (*Lauraceae* family) is trans-cinnamaldehyde followed by o-methoxy-cinnamaldehyde (8,8%) (*Figure 6 (a) and (b)*) (Ooi et al., 2006). Cinnamaldehyde is already used as bioactive substance in commercialized pesticides. Examples are Cinnacure composed of 30% of cinnamaldehyde and 70% of inert ingredients and Cinnamite which also contains 30% of cinnamaldehyde. Both can be used, among other things, against aphids (Cloyd, 1999; Ooi et al., 2006; Proguard, 2002).

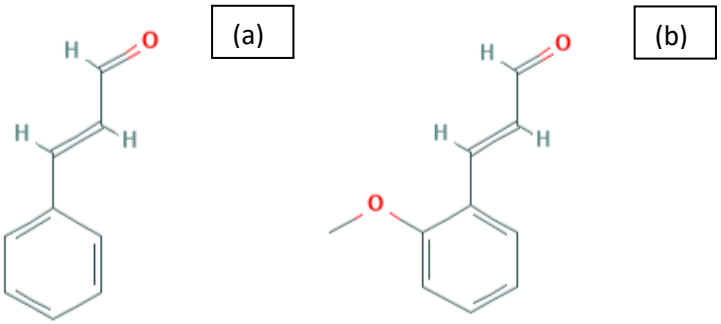

Figure 6. 2D structure of (a) trans-cinnamaldehyde and (b) o-methoxy-cinnamaldehyde (PubChem).

2.3.4. Application of EO-based pesticide by trunk-injection

As above stated, chemical or biological active substances are applied to trees for various purposes. They repel or kill insects, treat or prevent fungal/bacterial diseases. The most common way to apply these substances is by spraying. However, there are some potential inconveniences to this approach. For example, it cannot deliver the materials precisely where they are the most needed in the tree because a non-negligible part of the product is lost in the surrounding area and that may enhance the negative impact on non-target organisms (Kuhns, 2011). So other modes of injection have been investigated. Kuhns (2011) made an overview of systems (trunk implantation and injection, soil injection/drenching and trunk basal spray) to get chemicals into trees which are more targeted and efficient and stated their specificities.

In the context of the "Tree injection" project, trunk injection has been tested as an alternative system. As for any novel technique, laboratory bioassays, field studies and residue profile analysis must be performed to determine its effectiveness against apple insect pests and impacts on the tree plant and in the surrounding environment (Wise et al., 2014).

The principle of trunk injection consists in "placing water soluble chemicals at or inside the cambium" (Kuhns, 2011). The chemicals are injected by drilling a hole in the bark to reach the xylem. Thanks to transpiration by evaporation of water from the leaves, the water-soluble chemicals in the xylem sap are pulled up and can be distributed throughout the tree canopy. The challenge is to bring the chemical to the right portion of the tree, in the correct concentration and at the proper time of the year. The trunk is composed of five layers: from outside to inside, (1) the bark, (2) the phloem, (3) the cambium, (4) xylem and (5) the heartwood (*Figure 7*). The phloem, cambium and xylem make up the vascular system of trees (Kuhns, 2011, Wise et al., 2014).

Various devices are available on the market. In this work, an ENDOkit Manual PRO ©'s device (ENDOterapia ™ Vegetal) was used to inject the EO-emulsion pesticide in the trunk (*Figure 8*). It is a non-pressure system[10] which will be more detailed in the "Material and method" part.

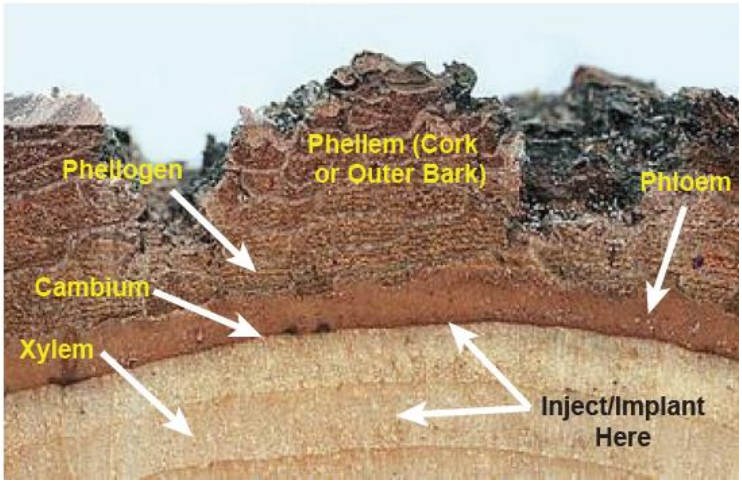

Figure 7. Cross section of trunk (Kuhns, 2011).

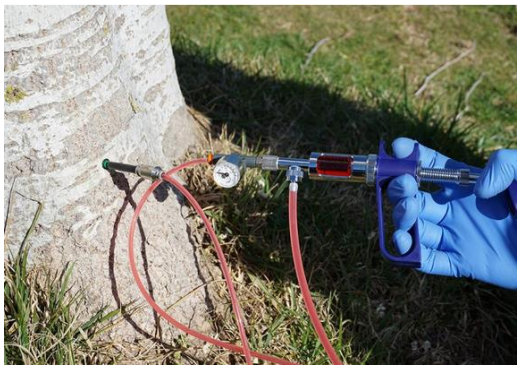

Figure 8. ENDOkit Manual PRO (https://endoterapiavegetal.com/en/equipment/endokit-manual-solution/).

VanWoerkom et al. (2014) and Wise et al. (2014) worked on insecticides formulated for trunk injection. They could conclude that (i) the activity by trunk injection lasted longer after application than by spraying; (ii) because of the transport of compounds inside the trunk, both environmental exposures and degradations were minimized; (iii) the "activity spectrum" of some tested insecticides expanded because the delivery of compounds was enhanced by trunk injection; (iv) there might be residues in trunk wood tissues which played the role of a "reservoir" for next year. Wise et al. (2014) also concluded that vascular delivery mainly ended up in foliage and that insecticidal residues were far below the United States Environmental Protection Agency (EPA) maximum residue limits in apple fruits. Finally, their preliminary work indicated that insecticide residues did not increase in flower nectar or pollen with trunk injection. However, further research should confirm this hypothesis. Indeed, despite some advantages presented above, the possible (long-term) impact on non-targeted organisms and apple trees as well as an economic analysis have to be pondered.

[10] The interested lecturer can find more information about pressured systems as well as other techniques (mainly using the soil) in the same perspective as tree-injection in Kuhns (2011)'s document.

Considering the advantages, trunk-injection seems to be a good method to treat aphids. It can be part of an IPM program. In addition, loaded pesticide contamination is reduced in the environment. It also eliminates the short- and long-term threats associated with pesticide previously developed in the introduction.

It is noteworthy that depending on the chosen device and to optimize the treatment, several parameters concerning the biological, ecological, chemical and technological aspects have to be taken into account. Some of them are unfortunately uncontrollable and may alter the efficiency (Aćimović, 2014). In any case, the challenge is to obtain an optimal compound distribution among the tree canopy and compound persistence to ensure a good pest management while ensuring limited wounding and plant health hazards due to the injection procedure and the nature of the active ingredient injected, respectively.

2.4. Impacts of exogenous EO application

Biological properties of EOs have already been approached in the previous point. Some research studies investigate the impacts of exogenous EO applications. EOs act either as a biostimulant or as a phytotoxic compound depending on the applied concentration. However, in most cases, EOs are more often phytotoxic than biostimulant. Thus, biostimulation will be still quick addressed.

2.4.1. Biostimulation

Biostimulation is characteristic of plant growth promotion. Many compounds have been studied as biostimulant such as humic acids[11], salicylic acid, amino acids and vitamins. The biostimulant property is helpful in ecological, commercial and agricultural fields. Indeed, it stimulates nutrient uptake efficiency, general plant growth and its traits such as root dry matter or root length in a lot of plant species but is also beneficial for symbiosis formation, proliferation of essential organisms for soil microbiota and protection against attacks of pathogens (Ben-Jabeur et al., 2019; Canellas et al., 2015; Souri and Bakhtiarizade, 2019). Recent studies demonstrate that EOs have influenced characteristics of plant growth as a biostimulant in a similar way to other organic compounds:

- Ben-Jabeur et al. (2019) brought to the fore that thyme oil (at 5 ppm) which coated durum wheat seeds (cultivar ‘Karim’) improved the biomass under water stress compared to well-watered control plants. They also showed that seedling development, seed germination, water and nutrient status of the developing plants under no stress conditions were enhanced by the oil.

- Souri and Bakhtiarizade, (2019) studied the effects of two concentrations (500 or 1000 ppm) of foliar and soil applications of rosemary essential oil on nutrient uptake and growth of tomato seedlings (under greenhouse conditions). The most marked effect was on seedling root system. All oil treatments (foliar and soil applications) improved root fresh weight.

- From their study, Barroso et al. (2019) concluded that besides root development of the plant (*Stylosanthes guianensis),* eucalyptus EO biostimulated photosynthesis.

[11] Canellas et al. (2015) reviewed humic substances in a horticultural context.

2.4.2. Phytotoxicity

EOs can also induce phytotoxicity against other plants. This property is already exploited as herbicide. It is the subject of more and more studies. For example, several scientists studied the impact of EOs on seed germination. They concluded that some EO compounds act as inhibitors of seed germination and suppressors of seedling growth. The best known to be phytotoxic are α-β pinene, 1,8-cineole, camphene, camphor, eucalyptol, geraniol and thymol. They are mainly monoterpenes. It is not surprising since the terpenes/terpenoids group is the most abundant in EOs. The phytotoxicity among the oils varies, of course, because of the difference in their major/minor constituents and their synergistic/antagonistic effects on the plant/membrane integrity (de Almeida et al., 2010; Gniazdowska et al., 2015; Khare et al., 2019; Poonpaiboonpipat et al., 2013; Sukegawa et al., 2018).

The lipophilic nature of EO lets them interact with the plant plasma membrane (PPM). As a result, the structure of the PPM which is made of layers of phospholipids, polysaccharides and fatty acids is disrupted. The plant membrane fluidity and permeability are changed. The damage can cause cell lysis. All other biochemical and physiological processes related to the membrane functioning could be impacted. The penetration depends on the water/octanol partition coefficient (Kow) of each molecule (Bakkali et al., 2008; Khare et al., 2019; Lins et al., 2019).

Thus, EOs can also be phytotoxic by attacking the photosynthetic metabolism in plant leaves as seen in several studies (Poonpaiboonpipat et al., (2013); Synowiec et al., 2015). EOs damage photosystem II (PSII) which is implicated in the photosynthetic electron transport (PET) system and located in chloroplast thylakoid membranes. Disturbing the electron transport causes over-energized chlorophyll molecules that provoke reactive oxygen species (ROS) formation and plant death because of a surplus of absorbed light energy compared to the photosynthesis capacity to use it through PET (Khare et al., 2019; Sewelam et al., 2016; Synowiec et al., 2015). ROS are described as "oxygen-containing molecules exhibiting higher chemical reactivity than O_2" (Waszczak et al., 2018). An overproduction of ROS can be lethal for plant cells. Indeed, they are highly reactive, toxic and must be minimized to protect the cell from oxidative damage (e.g. DNA and RNA damage, membrane lipid peroxidation, enzyme inhibition and protein oxidation). Their main production sites are where there is an excessive rate of electron flow such as chloroplasts and mitochondria occurs. The major ROS in plants are singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot -}$), stable hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$). $^1O_2$ is formed when excess energy is transferred from excited chlorophyll to molecular oxygen (reaction (1) and (2)) whereas the three others are produced by a transfer of one, two or three electron(s) to O_2 respectively (reactions (3), (4) and (5)) (Sewelam et al., 2016; Waszczak et al., 2018).

$$\text{Pigment} + \text{light} \rightarrow \text{excited pigment (P*)} \quad (1)$$

$$P^* + O_2 \rightarrow P + {}^1O_2 \quad (2)$$

$$O_2 + e^- \rightarrow O_2^{\cdot -} \quad (3)$$

$$O_2^{\cdot -} + e^- + 2\,H^+ \rightarrow H_2O_2 \quad (4)$$

$$H_2O_2 + e^- + H^+ \rightarrow H_2O_2 + \cdot OH \quad (5)$$

Some studies have demonstrated the phytotoxicity of cinnamon. It can cause electrolyte leakage resulting in cell death (Gniazdowska et al., 2015). Lins et al. (2019) showed the herbicidal activity of cinnamon EO on *A. thaliana* and its interactions with membranes as described in the previous paragraph.

Fortunately, plants have strategies to avoid or reduce phytotoxicity of xenobiotics. All of these mechanisms may have a price, of course. The mobilized energy and resources are not available any more for the primary metabolism, for example. A first mechanism is the compartmentalization of the xenobiotic away from its molecular target site(s). A second one is the metabolic detoxification coupled with sequestration. Several enzymatic steps are generally involved before obtaining a detoxified stable product depending on the nature of the foreign compound. It may react directly with conjugation partners or it may be first transformed into a substrate by oxidation, reduction ring hydroxylation of aromatics or hydrolysis of chemical bonds, for example. The detoxification process begins with an activation step which is mostly catalyzed by P-450 monooxygenases or peroxidases (POX) located in membrane fractions of the cells and in the cytosol, respectively. This step is then followed by the conjugation reactions. The xenobiotic reacts with sugars or amino acids depending on the active sites and the structure of the molecule. On the one hand, glycosyltransferases (GT, E.C. 2.4.1.x) mediate glycosyltransfer that is usually triggered by -NH_2, -OH, -COOH and -SH functions on a molecule. On the other hand, glutathione-S-transferases (GST, E.C.2.5.1.18) catalyze glutathione conjugation determined by nitro- or halogen functions and conjugated double bonds. Detoxification is totally dependent on the availability of these molecules and determines the susceptibility or tolerance toward exogenous toxic substances. At the end of the reaction chain, a detoxified and quite stable compound (a glycoside or a glutathione adduct) is in the cytosol (Inderjit and Duke, 2003; Schröder and Collins, 2002). These detoxification strategies have not been demonstrated yet in the case of essential oils.

However, EO phytotoxicity may activate the plant immune defences. Indeed, EO (because of its lipophilic nature) can interact with the PPM that provokes membrane depolarisation by changing the membrane potential and an electrolyte leakage. As a consequence, the influx of Ca^{2+} increases and leads to the induction of an oxidative burst and ROS accumulation. These two events participate in the very early responses of the pattern-triggered immunity (PTI) of the plant and signal the intermediate and late defence responses such as the activation of defence genes, hypersensitive response (HR) and induced resistance (i.e. systemic acquired resistance (SAR) and induced systemic resistance (ISR)) (Boller and Felix, 2009; Maffei, 2012; Meng and Zhang, 2013).

2.4.3. ROS

2.4.3.1. Roles of ROS in plant physiology

As briefly explained in the previous points, ROS play an important part in various plant mechanisms. They can be found at different concentration levels. In any case, they act as signalling molecules. In low concentrations, they are by-products of usual metabolic pathways during the electron transfer reactions that take place in the mitochondria and chloroplasts or during photorespiration in peroxisomes whereas in excessive levels, ROS are responsible for oxidative damage and are implicated in plant immunity through biphasic oxidative burst. The first step is unspecific because ROS production can be due to non-biotic stresses (e.g. wounding). The second phase is specific because it corresponds to prolonged ROS accumulation which can be associated with effector-triggered immunity (ETI) and HR. Indeed, elevated ROS cause oxidative damage to biomolecules (lipids, proteins, carbohydrates and nucleic acids). They disrupt the cell functioning and lead to cell death. So the overproduction of ROS has cytotoxic effect beside serving as signalling molecules (Stael et al., 2015).

2.4.3.2. Evaluation of plant physiology response

Plants must develop self-defence mechanisms to detoxify the excess of ROS levels and keep cellular redox homeostasis in normal conditions (without any stress). This "machinery" is constituted by enzymatic and non-enzymatic antioxidant components such as ascorbate, ascorbic acid, glutathione, peroxidases, superoxide dismutases and catalases (Gill et al., 2013; Torres et al., 2006). As other components in plant, antioxidants are also dependent upon a number of variables: plant development stage, age, environmental conditions and other components as phytohormones (Majer et al., 2016). So the evaluation of ROS and antioxidant ability are relevant in plant stress physiology studies. Moreover, some indicators (e.g. chlorophyll fluorescence, chlorophylls and carotenoids contents) are commonly measured to assess the plant physiological status. This work will evaluate the plant physiology response by measuring some antioxidants, indicators of the plant physiological status and oxidative damage.

2.4.3.2.1. Plant physiological status by photosynthetic systems assessment

First of all, the potential pesticide impact on the photosynthetic efficiency can easily be evaluated by estimating the maximum quantum efficiency of PSII with a fluorimeter. In a few words, the maximum efficiency of photosystem II (the variable fluorescence (Fv) /the maximal chlorophyll fluorescence (Fm)) is the ability of photon energy absorbed by PSII to be used in photochemistry under dark-adapted conditions (Ekmekci and Terzioglu, 2005; Hansatech, no date). This non-destructive and rapid method will be further explained in the "Material and method part".

This Fv/Fm ratio can be used as a sensitive indicator of plant physiological status. This ratio will be around 0,83 for a healthy sample, a lower value for stressed samples and lower than 0,3 for a dead sample (Bresson et al., 2018).

Additionally to chlorophyll fluorescence, the content of chlorophyll a and b can also be evaluated. Chlorophyll a (chl a) and b (chl b) pigments are essential to convert light energy into stored chemical one in photosynthesis process (*Figure 9*). They are differently involved in this process. Chl a is a primary electron donor in the electron transport chain whereas chl b, an accessory pigment is linked to the transfer of light energy to chl a. The chl a/b ratio and the total chlorophyll (a+b) can give an indication of the chloroplast degradation/disturbance and of photosynthetic capacity during plant senescence or when the plant is exposed to stresses (Bresson and al., 2018; Sytykiewicz and al., 2013).

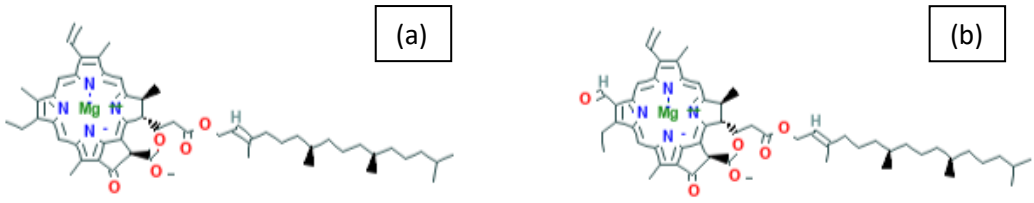

Figure 9. 2D structure of (a) chlorophyll a and (b) chlorophyll b (PubChem).

Finally, carotenoids play a crucial role in photosynthetic systems protection from photo-oxidation and from ROS thanks to their long-unsaturated chain (*Figure 10*). Their content decreases when plants are exposed to oxidative events (Gitelson et al., 2006). They basically have two main functions in photosynthesis in high plants: photoprotector and light collector involving either interactions with chlorophyll by channelling energy away from it or by passing energy on to it (Demmig-Adams et al., 1996).

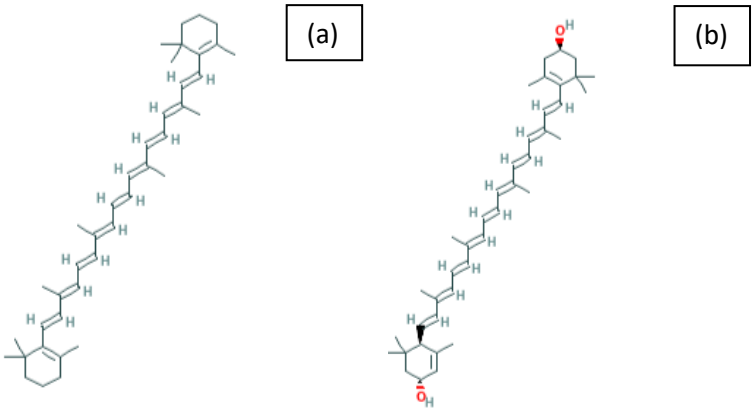

Figure 10. 2D structure of examples of the two classes of carotenoids (a) β-carotene (carotene class) and (b) lutein (xanthophyll class) (PubChem).

So the changes of leaf carotenoids and chlorophyll content are widely used to get information about the plant physiological status (Gitelson and Merzlyak, 2004). Their concentrations can be easily determined by spectrophotometric measurements.

2.4.3.2.2. Glutathione

Glutathione (GSH) is a ubiquitous low-molecular-weight simple water-soluble thiol tripeptide. It is composed of the amino acids glutamate (Glu), cysteine (Cys), and glycine (Gly) and called γ-glutamyl-L-cysteinylglycine (ϒ-Glu-Cys-Gly or $C_{10}H_{17}O_6N_3S$) (*Figure 11 (a)*) (Noctor et al., 2011).

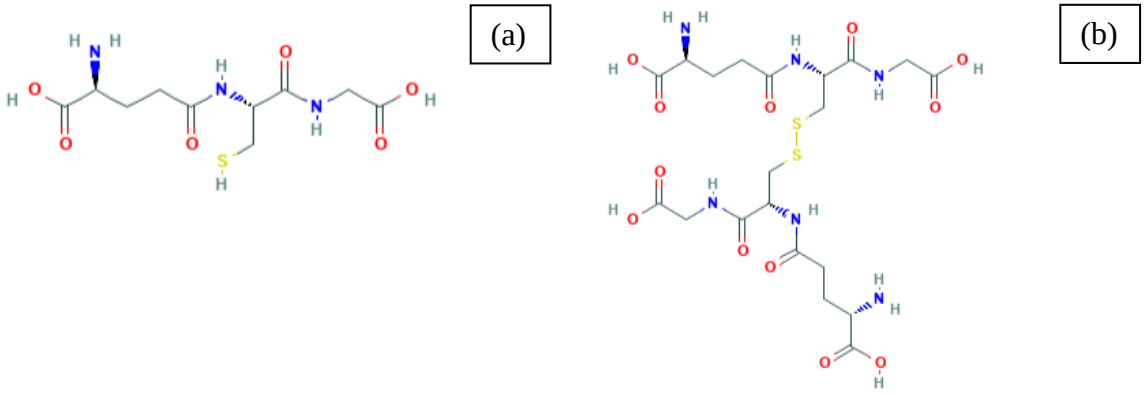

Figure 11. 2D structure of (a) glutathione (GSH) and (b) glutathione disulphide (GSSG) (PubChem).

It is found in most cells, including bacteria, animals and plants in millimolar concentrations. However, the GSH quantity in leaves is determined by plant species, state of development, environmental conditions, etc. It plays multiple roles such as plant development, modification/ transport of some hormones, ROS scavenging and signalling/defence reactions (*Figure 12*). Many of them are associated to reversible redox reactions of its cysteine sulphide or thiol group. Genetic modifications of GSH are currently made in order to study its mechanisms and its importance in plant physiology (Cereser et al., 2001; Foyer and Halliwell, 1976; Gullner and Komives, 2001; Gullner and Komives, 2006; Hajdinák et al., 2018; Majer et al., 2016).

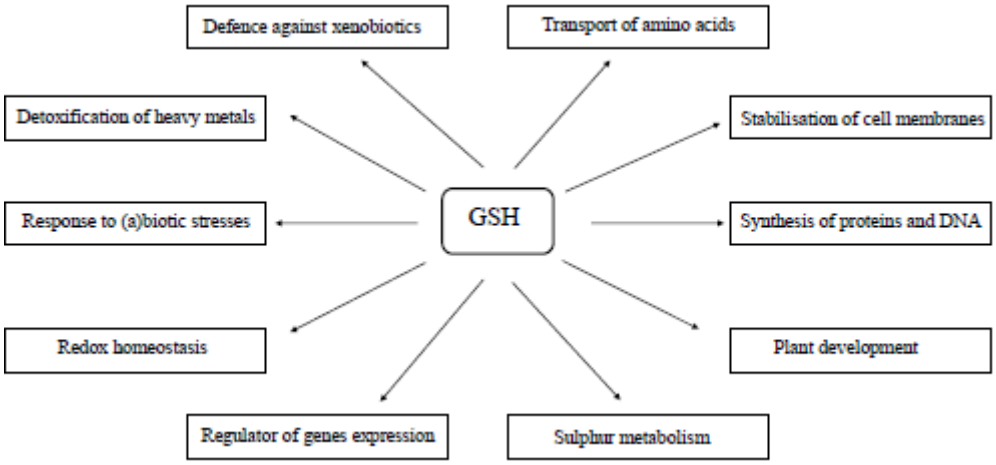

Figure 12. Overview of GSH roles in plants.

GSH biosynthesis is in a two-step ATP-dependent process and occurs in the cytosol and the plastid: γ-glutamylcysteine (γGC) is first formed from glutamate (Glu) and cysteine (Cys). This reaction is catalyzed by γ-glutamylcysteine synthetase or γ glutamylcysteine ligase (γ-GCL, GSH1; EC 6.3.2.2). Glycine (Gly) is then added to the C-terminal end of γGC to produce GSH under glutathione synthetase (GS or GSH2; EC 6.3.2.3) (*Figure 13*) (Frendo et al., 2013; Gill et al., 2013; Noctor et al., 1998).

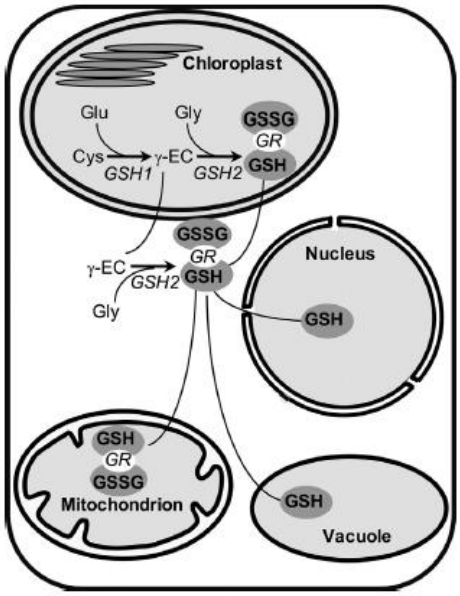

Figure 13. Biosynthesis pathway and transport of glutathione inside a plant cell (Frendo et al., 2013).

Its oxidized form is represented by glutathione disulphide (GSSG): the sulphur atoms of two GSH molecules give both one electron and convert into GSSG by the formation of a disulphide bridge (*Figure 11 (b)*). GSSG can be reconverted back to GSH thanks to glutathione reductase (GR; EC 1.6.4.2) which can be found in mitochondria, plastids, peroxisomes and cytosol. GR uses NADPH to do so (1) provided by the electron transport chain or by glucose-6-phosphate dehydrogenase in the light and in the dark respectively (Foyer and Halliwell, 1976; Gill et al., 2013).

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \quad (1)$$

The ratio of GSH/(GSH+GSSG) can be used as an indicator of oxidative stress. In unstressed plants, reduced GSH represent more than 90% of the total glutathione pool (Noctor et al., 1998; Hajdinák et al., 2018). GSH can be oxidized into GSSG by many oxidants (reactive oxygen/nitrogen species) and free radicals. Glutathione is also a redox buffer and acts in redox homeostasis which has a function in modulating the induction of defence processes such as the oligomer-monomer transition of the NPR1 protein occurring in infected plant cells. NPR1 is a main plant regulator of SAR. (Gullner and Komives, 2006).

GR and GSH are also important ascorbate-glutathione (AsA-GSH) pathway. Indeed, GSH helps to re-reduce ascorbate by reducing dehydroascorbate (DHA) chemically or DHA reductases, a class of glutathione transferases (GSTs) (2) (Noctor et al., 2011). Ascorbate and glutathione are both antioxidants and important cofactors in the enzymatic antioxidant plant defence (Majer et al., 2016).

$$dehydroascorbate + 2GSH \xrightarrow{\text{DHA reductase}} GSSG + ascorbate \quad (2)$$

Plant glutathione S-transferases (GSTs; EC 2.5.1.18) are "homo- or heterodimeric combinations of different subunits" (Gullner and Komives, 2001). They are multifunctional enzymes which participate in toxic substance detoxification, oxidative stress attenuation and hormone transport. Their genes are highly induced in a broad-spectrum of stress conditions (Gullner et al., 2018).

Nowadays, there is a range of methods for glutathione determination in biological samples in vitro and in situ such as enzymatic quantification (Ellman's reagent) and liquid chromatography-based assays that use different detectors as well as various fluorescent labelling in high performance liquid chromatography (HPLC). However, each quantification technique has its own limitations (inadequate detection limits, low reproducibility, etc.). This part will be detailed in the "material and method" part.

2.4.3.2.3. Lipid peroxidation

As previously stated, high ROS levels can cause the oxidation of lipids among other things. The primary target of oxidative reaction are the unsaturated lipids because of the sensitivity of hydrogen atoms adjacent to unsaturated olefinic bonds (Davey et al., 2005).

Dulf et al. (2010) determined the major fatty acid in *Malus domestica* Bokh leaves: α-linolenic acid, palmitic acid, linoleic acid and oleic acid. Three of them are unsaturated (*Figure 12*).

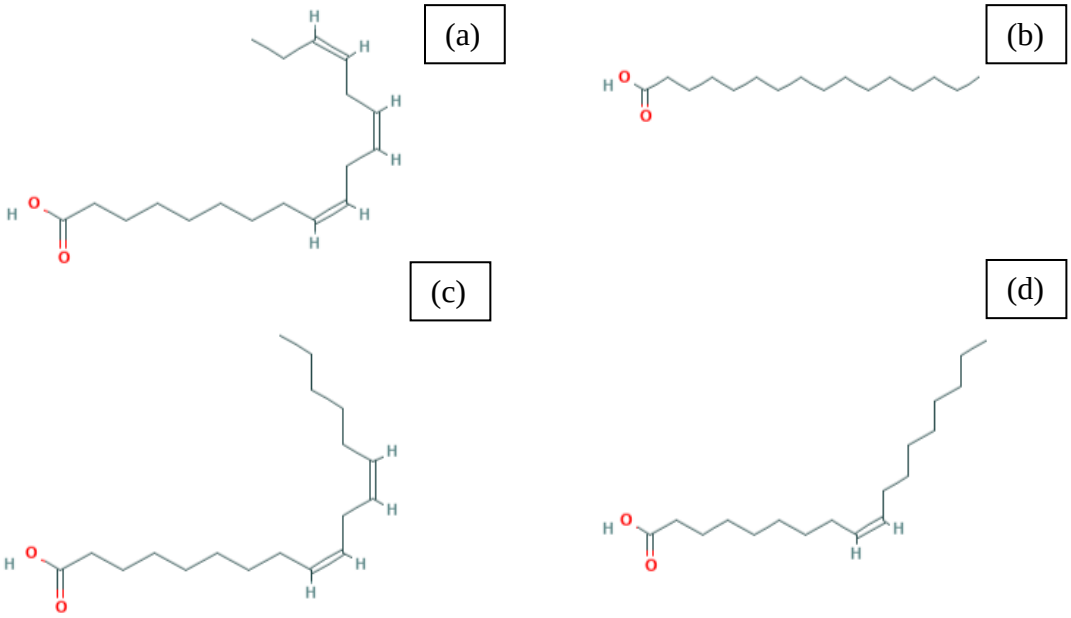

Figure 14.The four major fatty acids in leaves of Malus domestica Bokh: (a) α-linolenic acid, (b) palmitic acid, (c) linoleic acid and (d) oleic acid (PubChem).

Two markers of lipid peroxidation commonly used in literature are malondialdehyde (MDA) and conjugated dienes (CDs) in studies concerning plant responses to (a)biotic stresses. Depending on the stress origin, elevated lipoxygenase activity due to plant response to wounding or pathogen invasion can also cause of lipid peroxidation. So the formation of MDA can be the result of (non)enzymatic lipid peroxidation (Davey et al., 2005). Moreover, lipid peroxidation naturally occur in aerobic plant life (Morales and Munné-Bosch, 2019).

MDA is one of the secondary oxidation products derived from hydroperoxides. MDA is more easily measurable than other lipid peroxidation products because of the instability and reactivity of primary lipid hydroperoxides. They appear due to oxidized (poly)unsaturated fatty acids. However, the MDA measurement is only based on a single aldehyde which make it less precise. It is commonly quantified by the thiobarbituric acid reactive substances (TBARS) assay (Davey et al., 2005; Morales and Munné-Bosch, 2019). However, its concentration in apple leaves will not be determined here. Only conjugated dienes will be.

CDs are related to first products of lipid oxidation: double bonds are moved from the malonic position to the conjugated position during polyunsaturated fatty acid oxidation (Roman, 2012). They can be measured by spectrophotometry.

2.4.4. Defence induction by EOs

2.4.4.1. General reminder of plant local and systemic immune defences

Plants are sessile organisms. They are subject to abiotic (drought, heavy metal, salinity, high temperature, ultraviolet radiation, pollution, nutrient deficiency or toxicity) and biotic stresses (bacteria, viruses, fungi, nematodes, herbivores and insects) (Hakim et al., 2018). They have to face these stresses because they cannot escape. So they have developed structural barriers/traits and chemical/physiological defence mechanisms. Plants and their enemies are in a perpetual co-evolutionary battle for dominance. The final outcome of the fight depending on the side is the capability of the pathogen to overcome the plant immune system or the ability/reactivity of the plant to recognize the pathogen and to set up a defence strategy. So, in order to protect themselves, plants have evolved highly effective and complex immune systems[12] (*Figure 15*) (Pajerowska-Mukhtar et al., 2013; Pieterse et al., 2009).

The first implemented strategy is physical barriers including rigid cell walls, waxy cuticular layers and bark to avoid enemy attachment, infection or invasion. It is known as the plant constitutive defences. They also have inducible ones which consist of preformed toxic chemicals, antimicrobial components (phytoanticipins) and restructuring cell walls (cell suicide) that help to detect pathogen invasion and respond it. This first defence line can inhibit most of the micro-organisms invasion (Freeman and Beattie, 2008; Sels et al., 2008).

The basal resistance (also called innate immunity) corresponds to the first line of inducible defences. The plant is able to recognize diverse non-self-molecules (pathogen/microbe/damaged-associated molecular patterns or P/M/DAMPS) by transmembrane pattern-recognition receptors (PRRs) resulting in pattern-triggered immunity (PTI). It is effective against non-adapted pathogens. This recognition is the beginning of a cascade of reactions that will initiate the attacked plant immunity. The cascade of reaction includes ions fluxes that lead to plasma membrane depolarization, production of ROS and activation of Mitogen-Activated Protein Kinases (MAPKs) (Boller and Felix, 2009; Burketova et al., 2015; Meng and Zhang, 2013; Rasmussen et al., 2012; Rienth et al., 2019).

Unfortunately, adapted pathogens can circumvent PTI by employing effectors which are virulence protein factors secreted into plant cells to promote pathogenesis and induce susceptibility. This kind of effector is called effector-triggered susceptibility (ETS). Another pathogen approach is to affect phytohormone homeostasis in order to foil the host immune response. Pathogens interfere in biosynthesis and/or signalling pathways of phytohormones. A last plan is to mimic the phytohormone to have the advantage over the plant, exploiting jasmonic and salicylic acids (JA-SA) antagonism (which will be explained later) for example (Kazan and Lyons, 2014).

The effectors used by pathogens can (in)directly be recognized thanks to intracellular plant receptors of the nucleotide-binding leucine-rich repeat (NB-LRRs) domain class which activate the second plant immunity called effector-triggered immunity (ETI) (Boller and Yang He, 2009; Pieterse et al., 2009; Stael et al., 2015). ETI is based on highly polymorphic resistance proteins (R-proteins) and is usually longer and stronger-lasting than PTI. Moreover, ETI is often associated with a hypersensitive response (HR) which lead to programmed cell death (PCD) around the infection site to avoid nutrients and water supply for pathogens. Rapid appearance

[12] Warning: plants do not possess an immune system comparable to animals. However, plant strategies are very efficient against pathogen attacks.

of necrotic spots corresponding to sites of attempted pathogen penetration is usually referred to as HR. The process of HR is associated with mitogen-activated protein kinases (MAPKs) activation which encourages ROS generation in chloroplasts, salicylic acid (SA) accumulation and metabolic reprogramming. Mechanisms of ETI are similar to PTI ones including regulatory proteins, phytohormones biosynthesis, ROS production, cell wall reinforcement through the synthesis of lignin and callose, the production of antimicrobial secondary metabolites (e.g. phytoalexins) and the accumulation of pathogenesis-related (PR) proteins (e.g. chitinases and β-1,3-glucanases) (Burketova et al., 2015; Glazebrook, 2005; Gullner and Komives, 2001; Pieterse et al., 2009; Rienth et al., 2019; Stael et al., 2015).

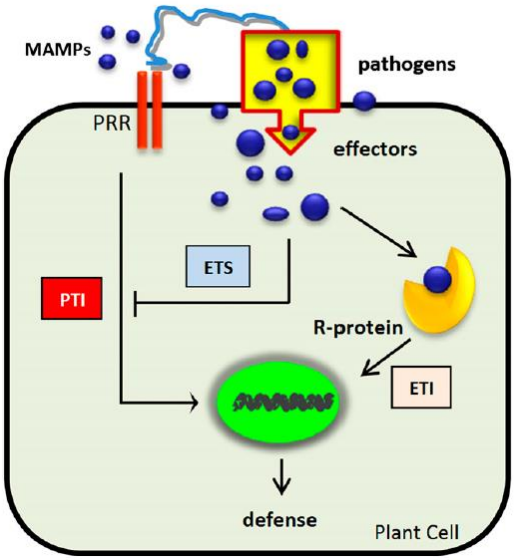

Figure 15. Simplified diagram of plant immunity against pathogens. Forward arrows correspond to positive interactions whereas blunt arrows represent negative interactions (Kazan and Lyons, 2014).

In addition to the plant immunity of infested parts, distal and uninfected tissues usually develop systemic acquired resistance (SAR), an enhanced resistance to a subsequent attack by pathogens. Compared to vertebrates, plants exposed to microbes do not acquire adaptive immunity. However, they can generate "a working memory" called systemic acquired resistance which provides a wide range and long-lasting resistance to various pathogens in not directly exposed tissues. SAR establishment begins with the perception of the pathogen, foliar treatment, chemical activators or plant phytohormones. Then, the signal is relayed by a complex network of signalling molecules (e.g. salicylic acid (SA)) to induce the transcription of defence proteins and production of required molecules that mediate the activation of pathogenesis-related (PR) genes. The increase of the SA level and of the expression of pathogenesis-related (PR) genes in both local and systemic tissues characterize SAR. It can be brought by priming with SA or active analogues for example (Burketova et al., 2015; Conrath et al., 2006; Durrant and Dong, 2004; Kazan and Lyons, 2014; Meng and Zhang, 2013).

Nevertheless, other phytohormones (i.e. jasmonic acid (JA) and ethylene (ET)) regulate the induced systemic resistance (ISR) which is also a defence response in distal parts of the plant to a first infection. In contrast to SAR, ISR can be started by root colonization with plant-growth-promoting rhizobacteria/fungi (PGPR/F). ISR is SA-independent but NPR1-dependent. Even if it is has begun in roots, ISR leads to resistance priming in both roots and aerial parts of plant. There are similarities between SAR and ISR: the two of them act against different pathogens, require a functioning NPR1 and can be associated with an accumulation of phytoalexins/PR-proteins and alterations in the cell wall composition. Briefly, induced resistance can be divided in two parts: SAR and ISR that prime accelerated and enhanced defences upon subsequent (a)biotic stresses *(Figure 16)* (Burketova et al., 2015; Walters and Fountaine, 2009; Walters et al., 2013).

This systematic induced resistance can be triggered by a pre-treatment with elicitors. This "priming" phenomenon is here only stated but will be developed later in this introduction.

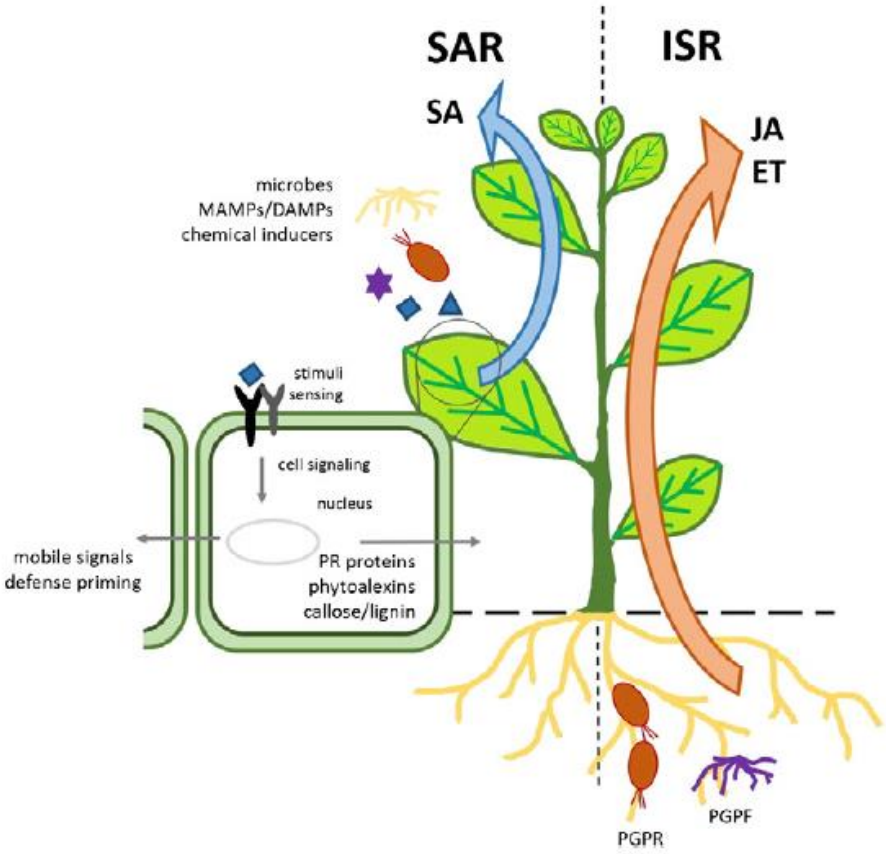

Figure 16. The two induced resistances in plants: SAR and ISR (Burketova et al., 2015).

2.4.4.2. Phytohormonal interactions linked to the plant immunity in general

The phytohormones take part in various plant mechanisms. Here are some examples with a more specific focus on plant defences. They are induced by MAPKs in plant immunity. Phytohormones such as ET, JA and SA act as important secondary signalling molecules and trigger the activation of a complex range of defence-related genes in plant defence responses (Meng and Zhang, 2013). JA, ET and SA finely interplay with other phytohormones including abscisic acid (ABA), auxins (indole-3-acetic acid, (IAA)), brassinosteroids (BR), cytokinins (CK), giberellins (GA) and strigolactones (STR) to coordinate plant immune defences because

several defence responses strictly depend on them (*Figure 17*) (Burketova et al., 2015; Kazan and Lyons, 2014). These last small molecules are also indispensable for the regulation of plant development, growth, survival, and reproduction even in low concentration. As a result, the plant can adapt its defences against the invaders thanks to the (synergistic or antagonistic) crosstalk between hormonal signalling pathways.

One mostly studied example is the antagonistic interaction between the ET/JA and SA response pathways in plant immunity (Pieterse et al., 2009). Broadly speaking, JA and ET signalling mediates resistance against wounds, necrotrophs and herbivorous pests whereas SA signalling provokes resistance to (hemi-)biotrophic pathogens[13]. The activation of one pathway often inhibits or increases the susceptibility of the other. The positive point is that plants can at once invest more resources in the most appropriate pathway depending on the kind of pathogen. Unfortunately, pathogens may turn it into their own advantage (Denancé et al, 2013; Kazan and Lyons, 2014; Rienth et al., 2019; Robert- Seilaniantz et al., 2011).

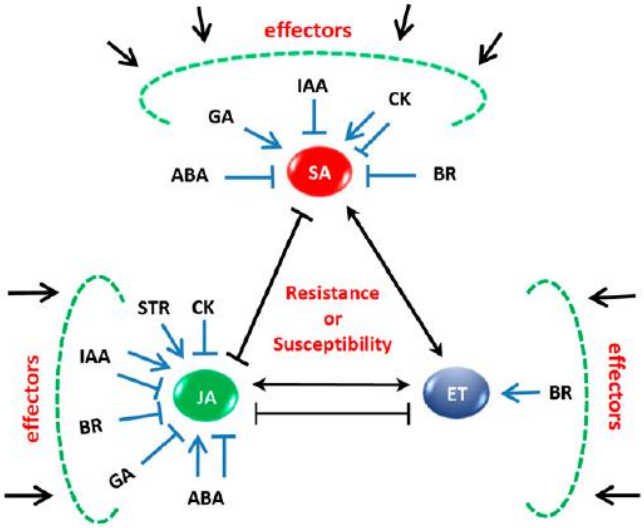

Figure 17. Summary of the complexity in signalling phytohormonal interactions. Forward arrows correspond to positive interactions whereas blunt arrows represent negative interactions (Kazan and Lyons, 2014).

SA is synthesized from chorismate which is a primary metabolite by two distinct enzymatic pathways isochorismate synthase (ICS/SID2) and phenylalanine ammonia lyase (PAL). Let's remember that its biosynthesis is activated during immune defence response throughout PAMPs or effectors pathogen recognition. **JA** biosynthesis begins with the hydrolysis of galacto- and phospholipids by a phospholipase activity. α-linolenic acid is then released and undergoes enzymatic reactions that lead to JA production. **ET** is synthesized from the conversion of S-adenosyl-Met (SAM) into methylthioadenosine and 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is then converted into ET through enzymatic conversion (*Figure 18*) (Rienth et al., 2019).

[13] They need living host tissues for their proliferation.

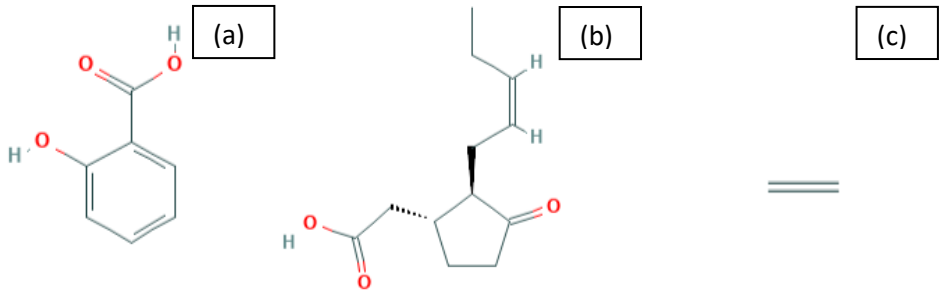

Figure 18. 2D structure of (a) salicylic acid, (b) jasmonic acid and (c) ethylene (PubChem).

It has to be kept in mind that the hormone crosstalk and its induced impacts on plants are very complex and still far from being completely elucidated.

2.4.4.3. Volatile Organic Compounds (VOCs)

When a plant is attacked, Volatile Organic Compounds (VOCs) can be released to warn surrounding plants and induce their emission of volatiles or expression of defence genes, so that they respond faster.

VOCs are "lipophilic liquids with a low molecular weight and high vapour pressure at ambient temperatures" (Dudareva et al., 2013). They represent about 1% of plant secondary metabolites. They are able to freely cross cellular membranes and be delivered into the environment (the atmosphere by leaves, flowers and fruits and the soil by roots) thanks to physical properties. They allow the plant to communicate and interact with the immediate environment (Dudareva et al., 2006). There are four major classes of VOCs: terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives and amino acid derivatives.

Their roles are various and species-specific. First of all, they allow plants to reproduce and disperse seeds thanks to blends of volatile molecules from the flowers, fruits and seeds that attract pollinators and dispersers. They can be active at short- and long-distances. For example, VOCs released from flowers act as olfactory cues or mimicry of the insect pheromones and their profiles vary according to seasonal or day-light patterns. Indeed, they can chase pollinators away from pollinated flowers and conduct them to unpollinated ones (Dudareva et al., 2006).

In a defence point of view, VOCs act as above- and below-ground plant defences. First of all, some VOCs have antifungal, antimicrobial and repellent activities against insect pests. For example, flower VOCs protect reproductive plant organs from florivores. They can also attract natural predators of their enemies as carnivores or parasitoids of herbivores. It is called tritrophic interactions. The more the information sent to the predator is complete (location, activity of the attacking animal, etc.), the more it can be helpful to the plant. As mentioned in the previous paragraphs, VOCs are thus a communicating facility between plants. VOCs released from damaged vegetative tissues may induce the expression of defence genes and emission of VOCs in healthy tissues on the same plant or surrounding unattacked ones. Finally, the last role of VOCs is to protect plants against abiotic stresses (Dudareva et al., 2006; Dudareva et al., 2013; Rienth et al., 2019).

2.4.4.4. Defences induced by EO application

As developed earlier, EOs can induce phytotoxicity against (higher) plants. As in any other attacks, plants are able to react thanks to different strategies. More and more studies reveal that EOs may also act as elicitors to prepare the host defences. This priming effect differs among plant species. The efficiency of EOs depends on the application method, timing and the molecular interactions between the EO, the pathogen and the host (Rienth et al., 2019).

Priming corresponds to "the physiological condition in which plants are able to better or more rapidly mount defence responses, or both, to biotic or abiotic stress" (Conrath et al., 2006). Priming is encouraged through various mechanisms by several molecules. Frost et al. (2008) highlighted the fact that priming does not force up the metabolic and energetic costs associated with the complete establishment of an induced defence response as one of the advantages. Indeed, in such "potentiated" cells, the defence compounds are only expressed on the pathogen attack. It has been known for a long time, but much progress has been made toward a better understanding of priming over the past few years.

In a general context, resistance can be stimulated by treatment with a variety of (a)biotic inducers. It works exactly as explained before: the plant recognizes elicitor molecules that leads to the activation of signalling pathways and chain reactions to induce plant defences (Walters and Fountaine, 2009). Elicitor molecules can come from various origins. Treatment with (purified) non-self-molecules, plant-beneficial micro-organism interactions, natural and synthetic chemical inducers as benzothiadiazole (BTH) or its commercialized forms Bion or Actigard (Syngenta) and selected strains of PGPR are some examples that were shown to trigger local and systemic resistance to the pathogen attack (Burketova et al., 2015; Conrath, 2011; Conrath et al., 2006; Rienth et al., 2019).

First of all, the composition of EOs can provoke SAR thanks to compounds that are close to SA phytohormone as some synthetic functional and structural elicitors similar to SA already do (e.g. BTH). Indeed, SA is very important in defence signalling (Bektas and Eulgem, 2015). Molecules which cause SAR is an interesting strategy since it can provide an efficient protection against a wide range of pathogens. Vergnes et al., (2014) noticed a high induction of the expression of defence biomarkers after treating *Arabidopsis* plants with *Gaultheria procumbens* essential oil. This induction is due to methylsalicylate (MeSA) which represents more than 96% of this EO. It can be metabolized to SA in plant tissues. SA is known to influence the transcriptional regulator NPR1 that controls the expression of a broad spectrum of SAR-related genes like the pathogenesis-related 1 protein (PR1). Godard et al., (2008) also demonstrated that an accumulation of MeJA and changes in the transcriptome of Arabidopsis thaliana plants had appeared after the exposure of plants to monoterpene volatiles.

EOs can induce resistance through targeting gene expression as well. Banani et al. (2018) studied the effectiveness of thyme and savoury essential oils against *Botrytis cinerea* on apple fruit by characterizing the expression of PR-8 (a class III chitinase) and PR-5 (a thaumatin-like protein) in apple tissues. Indeed, these two pathogenesis-related (PR) genes were identified as being induced in response to different resistance inducers (Bonasera et al., 2006). They observed that thyme essential oil promoted the accumulation of protein PR-8 and as a result, generated resistance in apple fruit. The efficacy of thyme essential oil (1% concentration) was more visibly shown with the lesser disease incidence and the smaller lesion diameter provoked by grey mould. In addition, thymol, carvacrol, and p-cymene are volatile phenolic compounds of thyme and savory oils are well known for their high effectiveness against fungi, especially in synergism.

Bill et al. (2016) also worked on thyme oil. They found that applied by fumigation it induced a higher expression of β-1,3-glucanase (a PR-2 protein) and chitinase genes that can both damage the fungal cell wall. The induced defence response was initiated at transcript and enzymatic level. Avocados used in this study were less vulnerable to anthracnose[14] development. The treatment with thyme oil was as successful as prochloraz (an imidazole fungicide). Cindi et al, (2016) obtained similar results with peaches. Sellamuthu et al. (2013) and Bill et al. (2014) also observed an increase of peroxidase, ammonia-lyase and antioxidant enzymes in avocado fruits treated with thyme essential oil.

Finally, Perina et al. (2019) reported the mutual activity of *C. zeylanicum* essential oil against the fungus *A. alternate* on citrus plants. It could work by a direct fungal activity and by a capacity to stimulate plant defence enzymes in citrus leaves. They compared commercial tangerines treated by this EO with a copper fungicide and with a commercial plant activator. *C. zeylanicum* essential oil was able to reduce more the incidence and the progression of diseases at comparable levels. A dual effect of citronella (*Cymbopogon nardus*) essential oil in coffee plants was confirmed by Pereira et al. (2012b).

It is, of course, a non-exhaustive list of studies that deal with priming effect of EOs or defences generated by EO applications. There are still unexplored effects of essential oils that could act on plant immunity.

Since scientists have illustrated a double mode of action on plants against a pathogen that characterize some EOs (i.e. a direct activity against the pathogen and an indirect effect by priming the plant defences), it may also be the case for the cinnamon essential oil (CEO) applied by trunk-injection on apple trees (*Malus domestica* Borkh (var. Jonagold)).

[14] Anthracnose: "any of several fungus diseases of plants and trees, such as vines and beans, characterized by oval dark depressed spots on the fruit and elsewhere" (Collins dictionary online, consulted: 25/03/2020).

3. Objectives and strategy adopted

This work is the continuation of what has already been done in the first year. This master thesis is more devoted to the possible EO-emulsion impact on apple trees after its injection in the trunk. The oxidative stress will be prioritized here because ROS have a signalling role to play in the plant response initiation by their accumulation causing an oxidative burst. In this context, this work will be more focused on a molecular scale.

Thus, the specific goals targeted in this work are:

1. Determining if the applied treatments induce phytotoxicity on apple trees. If so, which one is the most phytotoxic.

2. Evaluating the efficiency of the apple tree redox system against the potential oxidative stress induced by the EO-emulsion.

3. Measuring the produced oxidative damage, especially on the photosynthetic apparatus.

In order to achieve these objectives at a molecular scale, the experiment will be about the measurement of glutathione and carotenoids to assess the plant redox system. Chlorophyll fluorescence, chlorophyll a and b pigments and conjugated dienes will give an idea of the global plant vitality and ROS damage.

It has to be kept in mind that choices had to be made from the beginning. In addition, as the plant response can come very fast, other kinds of damage may not have been taken into account or the most relevant plant reaction time may have been missed.

4. Material and method

This third part will be divided depending on the main goals presented above:

- Characterization of the EOs
- Experimental implementation
- Assessment of plant physiological response at a molecular scale
- Statistical analysis

4.1. Characterization of the essential oils

In this work context, the emphasis will be more on studying the plant physiological response to a single essential oil at different concentrations than on comparing if the different EO emulsions formulated last year induce the same physiological apple tree response. As a consequence, only Cinnamon oil - *Cinnamomum cassia* J. Presl (Pranarôm, lot number: CCB114) was considered here. Its molecular profile was established (*Annex 1*).

A 10 milligrams of EO dissolved in 10 millilitres of n-hexane was prepared following its analysis by GC-MS system (7890B-5975C, Agilent Technologies Inc.) equipped with a HP-5 MS capillary column (30m x 250 µm x 0.25 µm, Agilent Technologies Inc.).

One microliter of the EO solution was injected in splitless mode at 250°C with helium as carrier gas at a flow rate of 1 mL/min. The gas chromatography oven program was as set out: (i) 40°C for 2 min, (ii) an increase to 100°C at a rate of 5°C/min, (iii) an increase to 120°C at a rate of 3°C/min, (iv) a continuance at 120°C for 3 min, (v) an increase to 220°C at a rate of 5°C/min, (vi) a final increase to 310°C at a rate of 15°C/min. The quadrupole and MS source temperatures were fixed at 150°C and 230°C, respectively. The mass spectra were recorded in Electron Ionisation (EI) mode at 70 eV with a scanned mass ranged from 30 to 400 m/z. The component identification was carried out by a comparison of the recorded spectra with computed reference databases (NIST14, NIST17, PAL600K and WILEY275) and by a comparison of the retention indices (RI) with RIs from the literature. RI values were calculated by analysing a mixture of homologues n-alkanes (C7–C30) under the same conditions. The chromatogram was processed with Mass hunter software.

Unfortunately, the obtained molecular profile did not correspond to the one present in the analysis sheet supplied by Pranarôm (*Annex 1*). Their analysis characteristics are presented in *Table 1*.

Table 1. Analysis characteristics performed by Pranarôm control quality department.

GC-MS system HEWLETT PACKARD / GC-FID
Capillary column : HP INNOWAX 60-0.5-0.25
Set-ups of the gas chromatography oven program: 6 min at 50°C -2°C/min→250°C-20min at 250°C
carrier gas He : 22 psis

4.2. Experimental implementation

4.2.1. Preparation of EO-based pesticide

In order to optimize the EO transport from the xylem sap by transpiration, an emulsion of an essential oil/water has to be first prepared.

To get a 100 mL EO-emulsion, 15 mL of water were put under 1 250 rpm agitation with a magnetic stirrer. Tween 80 (the emulsifier) was then added to respect a ratio of 1:4 (v/v) EO:Tween 80 in view of the different EO concentrations tested (*Table 2*).

Table 2. Volumes of EO and Tween necessary depending on the EO concentration for a 100 mL EO-emulsion.

EO concentration	EO volume (mL)	Tween volume (mL)
1%	1	4
4%	4	16

After that, 20 mL of Ethylene Diamine Tetra Acetic acid (EDTA, 100 mM) were added to the mixture which was finally filled with water to reach the final volume of 100 mL and concentration of 20 mM. This solution was kept under a constant agitation at 1 250 rpm during 5 min and then was stabilized using the high-speed homogenization (HSH) at 9 500 rpm for 6 min (Ultra-Turrax T25) followed by a high-pressure homogenization (HPH) with eight cycles at 5 000 psi (FMC). The EO-emulsion was stored at 4°C and in aluminium foil to avoid EO degradation.

An emulsion without EO corresponding to an aqueous solution using Tween 80 at 8% and EDTA (20 mM) was also prepared. This solution will be referred to as “negative control” afterwards.

4.2.2. Trunk-injection settings

In this work, an ENDOkit Manual PRO ©’s device (ENDOterapia TM Vegetal) was used to inject the EO-emulsion pesticide in the trunk (*Figure 19*).

Figure 19. ENDOkit Manual PRO (https://endoterapiavegetal.com/en/equipment/endokit-manual-solution/).

The use of this device requires three steps (*Figure 20*):

1. The drilling of one hole in the trunk
2. The insertion of a plug into the hole
3. The introduction of a syringe into the plug septum to inject the EO-emulsion

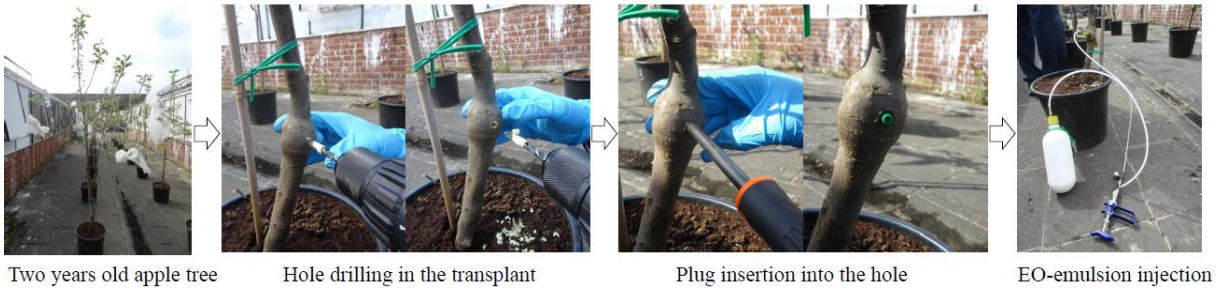

Figure 20. Overview of the pesticide injection process on apple trees (Photos Lheureux L.).

Here are more details about the injection procedure and the parameters. These were determined according to the device user's guide and previous studies on the subject.

- Hole drilling parameters: The hole was drilled at the height of the transplant thanks to an 8 mm diameter drill bit with a battery-operated drill. First of all, the external bark layer was gently pierced at a low speed. The bark residues were then cleaned off in order to avoid contamination of the bark layer during the rest of the drilling. The hole had a depth of 1,6 cm, the length of the black part of a plug, helped with a white ribbon as a depth indicator. Before the plug insertion, the hole was cleaned off once more.

- Plug insertion: The plug was put into the hole by means of the EndoKit's insertion device. It is correctly inserted when its green part only is visible. Some smooth hammer blows may help to position it.

- Injection: An injector needle linked to the pesticide container was introduced into the plug septum. 3 mL of the pesticide solution was injected. Only a hand pressure on the syringe plunger is required. The needle was then withdrawn.

- Injection volume*:* The volume of the compound to deliver was only determined by the EndoKit user's guide and according to previous results obtained in this project because no studies dealing with the injection of essential oils as biopesticide were found in literature.

4.2.3. Experimental design

The two factors studied in this experiment are:

1. Four types of tree-injection treatment:
    1) The cinnamon EO emulsion at 1% [CEO 1%].

    2) The cinnamon EO emulsion at 4% [CEO 4%]. Pre-tests done in UCLouvain showed that a CEO emulsion at 2% becomes toxic for the plant. As a consequence, CEO 4% should represent an overdose and induce a physiological apple tree response.

    3) An emulsion without EO injection also called negative control [NC]. This modality would allow to determine the impact of injection on apple trees.

    4) No injection also termed “blanc” [B]. It would inform on the normal apple tree response due to the environment.

2. Time to assess a kinetic of the apple tree physiological response. Apple leaves were sampled the week of the injection. More leaves were collected during the first day: 0h, 1h30, 3h, 4h30 and 6h according to preliminary results. Then, one sample was taken successively at 24h, 72h and 192h.

The experimentation was made on two year old apple trees, *Malus domestica* Borkh (cov. Early Jonagold) purchased from the nursery “Johan Nicolaï s.a” (St-Truiden, Belgium). The trees had been grafted on EARLY JONAGOLD Milenga® M9 rootstocks.

The whole experiment took place outdoors next to the greenhouses belonging to CRA-W and located rue de Liroux, building “Emile Marchal” (Gembloux, Belgium; 50°33’58” Nord, 4°43’32’’ Est) (See orange rectangle, *Figure 23*). All trees are in identical environmental conditions.

Figure 21. Experiment localisation (google maps).

In total, 16 Jonagold apple trees were required for the experiment, four treatments were applied in four repetitions. They were completely randomly distributed on two lines of eight trees each. Each tree was wearing a colour ribbon corresponding to a specific treatment for greater clarity during the samplings. The experiment took place from the 22nd of June to 29th of June 2020 in meteorological conditions presented in *Table 3*.

Table 3. Mean ± standard deviation of the meteorological conditions during the field experimentation that took place from the 22nd of June to 29th of June 2020. "/" means data no available.

	Temperatures (°C)	Relative humidity (%)
Average	19,7 ± 2,79	73,6 ± 6,25
Maximum	27,4 ± 4,01	/
Minimum	11,6 ± 2,99	/

It was very likely that the whole of the vascular bundles would not receive the same amount of biopesticide. That was why one sampling consisted in randomly collecting two leaves at three different heights (low, medium and high) that were randomly chosen. The leaves were mixed together in order to have an average of leaves per tree. The six leaves were immediately cryo-frozen in liquid nitrogen after sampling and were stored at -80°C before analysis in the lab.

The two years old apple trees were kept in cold conditions in order to maintain them in their winter phase before the experimentation. The experimentation should have been carried out in April 2020 corresponding to the apparition of rosy aphid founder females on apple trees but it had to be postponed because of the Covid-19 confinement. However, the physiological development of the tree corresponded thanks to this artificial extension of the dormant period.

They were repotted on the 15th of May 2020 in plastic jars (15 L capacity). The universal potting soil (HumuForte MycoPlus) used was composed of black peat, peat litter, wood fibre, calcium and magnesium fertilizers, organic compound fertilizer, mixture of bacteria and mycorrhizae. The dry matter represented 25% of the total weight. Its pH zone was from 5 to 6.5 with an electrical conductivity of 550µS/cm. When the trees were repotted, they were watered until reaching the field capacity. Stakes were placed on the 11th of June 2020.

4.3. Assessment of plant physiological response

As mentioned before, studying apple tree response to the biopesticide in its entirety was here hardly conceivable. Choices had to be made. The focus would be on experiments that were relevant to assess the tree redox system, the oxidative burst and the potential ROS damage produced. Each method has drawbacks and advantages that will be discussed all the way along.

4.3.1. Plant physiological status

4.3.1.1. Maximum quantum efficiency of PSII

Theoretical aspects

A first indicator of plant physiological status is the maximum quantum efficiency of the photosystem II (PSII) measured with a fluorimeter.

When plant leaves receive light, the absorbed energy is transferred as excitation energy and is used in photochemistry. The excess of absorbed light energy is dissipated thanks to non-photochemical processes including re-emission at the wavelengths of infrared and red/far-red light energy. This process is known as chlorophyll fluorescence. In stress conditions, the photosynthetic performances decline, so more energy is dissipated by chlorophyll fluorescence (Hansatech Instruments, no date).

In a few words, the maximum efficiency of photosystem II (Fv/Fm) is the ability of photon energy absorbed by PSII to be used in photochemistry under dark-adapted conditions.

PSII is the first photosystem that is activated in the photosynthesis process after a light stimulus. Thus, the measurement mainly concerns its fluorescence. The leaf sample is first "dark" adapted using a leaf-clip (*Figure 22*) system designed for this purpose. It allows the PSII electron acceptors (chlorophyll a) to be re-oxidized to react when a rapid illumination of the sample follows the dark-adaptation step. When illumination happens, a rapid polyphasic rise in chlorophyll fluorescence occurs followed by a decline in intensity. This is called the Kautsky induction that can be graphically represented (*Figure 23*).

Figure 22. Leaf-clip used in the experimentation (Photos Lheureux L.).

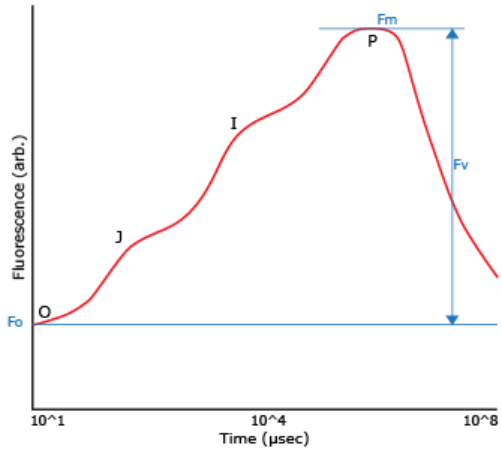

Figure 23. The Kautsky fluorescence induction curve (Hansatech instruments'web site).

Where:

- O, J, I and P represent the maximum in each phase
- F0 is the fluorescence origin
- Fm is the fluorescence maximum
- Fv is the variable fluorescence, i.e. Fm-F0

The Fv/Fm ratio can then be calculated and will be used as a rapid and sensitive indicator of plant physiological status. This ratio will be around 0,83 for a healthy sample, a lower value for stressed samples and lower than 0,3 for a dead sample (Bresson et al., 2018).

This measurement is made on non-frozen leaves and is non-destructive compared to other analyses performed in this work.

Manipulation achieved

Sample preparation

Three leaves[15] randomly sampled were placed in the dark for 20 minutes using a leaf-clip prior the measurement.

The parameters set for the fluorescence measurement are:

- Pre-light: none
- Duration pre-illumination: 0,0s
- Light intensity: 3000µmol.$m^{-2}s^{-1}$
- Gain: 1
- Duration illumination: 1,0 s (every 10 µs)
- Flash(es): 1

This measurement was taken at each sampling time (0h; 1h30; 3h; 4h30; 6h; 24; 72h and 192h).

[15] Each at various heights (low, medium and high)

Analysis method

Measurements were done using a handy Plant Efficiency Analyser (PEA) fluorimeter (Hansatech Instruments)

4.3.1.2. Chlorophyll a and b pigments

Theoretical aspects

Chlorophyll a and chlorophyll b content as well as the chl a/b ratio and the total chlorophyll (a+b) serve as indicators of the chloroplast degradation/disturbance and of photosynthetic capacity during plant senescence or when the plant is exposed to stresses (Bresson and al., 2018; Sytykiewicz and al., 2013).

Leaf Chlorophyll a and b concentrations are determined by spectrophotometric measurements.

Manipulation achieved[16]

This protocol was inspired by Mrs Blondiaux from the plant biology unit of F.U.S.A.Gx.

Sample preparation

Cryo-frozen apple tree leaves were crushed in liquid nitrogen with a mortar and pestle during about ten seconds to obtain a thin homogeneous powder. 10 ± 4 mg of this powder was mixed with 2 mL of 96% (v/v) cold ethanol. The solution was first put in the dark and on ice for 15 min before being centrifuged at 12 000 rpm during 5 min at 4°C.

The absorbance of the supernatant was measured at 649 nm and 665 nm. The concentration of chlorophyll a and b were calculated as follows:

Chlorophyll a: C_a (µg/mL)= (13,36*A_{665})-(5,19*A_{649})
C_{wa} (µg/g)= [(13,36*A_{665})-(5,19*A_{649})]/sample weight in mg *1000

Chlorophyll b: C_b (µg/mL)= (27,43*A_{649})-(8,12*A_{665})
C_{wb} (µg/g)= [(27,43*A_{649})-(8,12*A_{665})]/sample weight in mg *1000

Analysis method

Absorbances were measured with an Ultrospec 7000 spectrophotometer at room temperature.

[16] This protocol is the same used for measuring the absorbance of carotenoids and conjugated dienes. However, they were split here to present the work more clearly.

4.3.2. Plant redox system and oxidative boost

4.3.2.1. Carotenoids

Theoretical aspects

Carotenoids play the roles of photoprotector and light collector in higher plants thanks to their long-unsaturated chain. The photosynthetic systems are thus protected from photo-oxidation and from ROS (Gitelson et al., 2006).

A simple technique to dose leaf carotenoids content is recording absorbance by UV-VIS spectroscopy.

Manipulation achieved

This protocol was inspired by "Dosage des pigments foliaires d'*Arabidopsis* au spectrophotometer" protocol by Mrs Blondiaux.

Sample preparation

The sample preparation is the same as for the measurements of chlorophylls pigments and conjugated dienes.

Cryo-frozen apple tree leaves were crushed in liquid nitrogen with a mortar and pestle during about ten seconds to obtain a thin homogeneous powder. 10 ± 4 mg of this powder was mixed with 2 mL of 96% (v/v) cold ethanol. The solution was first put in the dark and on ice for 15 min before being centrifuged at 12 000 rpm during 5 min at 4°C.

The absorbance of the supernatant was measured at 470 nm. The concentration of carotenoids was calculated as follows:

$$C_{carotenoids}\ (\mu g/mL) = (1000*A_{470} - 2{,}13*C_a - 97{,}64*C_b)/209$$

$$C_{carotenoids}\ (\mu g/g) = [(1000*A_{470} - 2{,}13*C_a - 97{,}64*C_b)/209]/\text{sample weight} *1000$$

Analysis method

Absorbances were measured with an Ultrospec 7000 spectrophotometer at room temperature.

4.3.2.2. Glutathione

Theoretical aspects

Glutathione is a ubiquitous low-molecular-weight simple water-soluble thiol tripeptide found in most cells. Only plant glutathione will be considered in this part. The GSH/(GSSG+GSH) ratio can be used as an indicator of oxidative stress (Noctor et al., 1998; Hajdinák et al., 2018). Nowadays, there is a range of sensitive methods for glutathione determination in biological samples both in vitro and in situ by spectrophotometric and HPLC assays. No absolute technique gives exhaustive information about glutathione measurement. Each quantification technique has its own limitations and has to be replaced in the experiment context.

The most common spectrophotometric technique is the recycling method, DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) or Ellman's reagent. GSSG is converted into GSH by glutathione reductase (GR) in the presence of NADPH. The addition of the GR and NADPH increases the specificity of this quantitative glutathione technique. It is rather easy, quick and cheap. The measurement of the total glutathione (GSH+2GSSG) can be done by the addition of thiol masking reagent (e.g. vinylpyridine or N-ethylmaleimide (NEM)) even at low concentration (Giustarini et al., 2013; Lőrincz and Szarka, 2017; Noctor et al., 2011).

The most employed sophisticated high performance liquid chromatography (HPLC) method is based on fluorescent labelling by O-phthalaldehyde (OPA) or bimanes that offer high specificity and sensitivity. To quantify the low molecular glutathione molecule, it is first derivatized in order to form a conjugate with a fluorescent derivatization reagent to improve the detection limit. The OPA is very toxic for the environment. That is one of the reasons why monobromobimane (mbb) was preferred. There are two bimanes that can be utilized, the monochlorobimane (mcb) and the monobromobimane (mbb). The first one is more specific to glutathione than mbb because mbb can react with any thiol functional group that may lead to an overestimation of glutathione in samples. However, it is more reactive than mcb that needs to be catalyzed by glutathione S-transferases (GSTs) to accelerate the reaction (Noctor et al., 2011; Queval et al., 2011). Moreover, bimanes can react with glutathione inside intact cells. This advantage can be helpful in microscopy and flow cytometry. Furthermore, bimanes are suitable for routine assessment of GSH in (intact) cells because of their rapidity, specificity and sensitivity (Čapek et al., 2017). The measurement of the glutathione disulphide content can be done by the addition of the dithiothreitol (DTT) or Cleland's reagent that will reduce it. The total glutathione pool is then determined and the GSSG content can be calculated (Hajdinák et al., 2018).

Manipulation achieved

The developed method was inspired by (Hajdinák et al., 2018; Queval and Noctor, 2007; Queval et al., 2011).

Sample preparation

Cryo-frozen apple tree leaves were crushed in liquid nitrogen with a mortar and pestle during about ten seconds to obtain a thin homogeneous powder. 100 ± 5 mg of this powder was mixed with 1 mL of HCl (0,2 N). The extract was then centrifuged at 13 400 rpm during 15 min at 4°C in Eppendorf tubes (centrifuge MiniSpin®). 0,5 mL of supernatant was neutralised with 0,4 mL of sodium hydroxide (1 M), 50 µL of sodium phosphate monobasic (1M, pH 5,6). The final pH of neutralized supernatants was around 6 or 7.

Then two tests were performed on the supernatant to measure GSH and total GSH (one part of GSH is converted into GSSG) respectively.

For GSH, 0,2 mL of the supernatant was incubated with 0,2 mL of 2-(Cyclohexylamino)ethanesulfonic acid (CHES, 0,5 M, pH 9), 20 µL of distilled water and 20 µL of monobromobimane (mbb) (30 mM) during 15 min in the dark. The reaction was stopped with 0,66 mL of acetic acid (10% v/v).

For total GSH, 0,2 mL of the supernatant was incubated with 0,2 mL of CHES (0,5 M, pH 9) and 20 µL of 1,4-dithiothreitol (DTT) (10 mM) during 30 min in the dark. 20 µL of mbb (30 mM) was then added. The reaction was let during 15 min in the dark. The reaction was stopped with 0,66 mL of acetic acid (10% v/v).

Once the reactions were stopped, Eppendorf tubes were put on ice. Each of them was then centrifuged at 10 000g during 10 min at 4°C. The supernatant was filtered (0,45 µm) in vials. Vials were put on ice before their injection (50 µL).

Calibration

The stock solutions have to be freshly prepared on the very day of the analysis.

The calibration curve of GSH was established in triplicates at concentrations of 25 µM; 50 µM; 100 µM; 150 µM and 200 µM from a 10 mM GSH stock solution (CAS Number: 70-18-8, 98% µM; Acros Organics).

The dilutions were performed in 1 mL or 2 mL balloons with 800 µL of metaphosphoric acid (10 % v/v). Chloridric acid (0,2 N) was used to complete the final volume.

Each calibration point followed the exactly same steps as samples (neutralization, derivatization depending on GSH or total GSH and filtration).

Analysis method

The apparatus was an Agilent 1260 Infinity equipped with a FLD detector (λ_{EX}: 395 nm, λ_{EM}: 477 nm). The column used was a Zorbax 300 SB from Agilent (C18; 150 mm x 4,6 x 3,5 µm dimensions) at 40 °C. The autosampler was at 4°C. The run lasted for 32,5 min with 0,8 ml/min for the eluent flow rate per analysis. A linear gradient of aqueous acetic acid (0,25% (v/v)) and NaOH as solvent A (final pH at 3,5) and methanol as solvent B (*Table 4*).

Table 4. Details of the linear gradient set for the measurement of glutathione by HPLC-FLD.

Time (min)	**Solvent A - Aqueous acetic acid (0,25% (v/v) and NaOH (final pH at 3,5)** (%)	**Solvent B – Methanol** (%)
0	82	18
17,5	82	18
20	0	100
27,5	0	100
28	82	18
32,5	82	18

Based on standards, the retention time of glutathione was 4.6 min.

The GSSG concentration and the redox state of apple tree leaves were calculated by the Hajdinák et al. (2018) formula:

[GSSG] = ([Total glutathione]-[GSH])/2 expressed in µM

The redox state = ([GSH]/[GSSG+GSH])*100 expressed in %

Where

[GSSG] = glutathione disulphide concentration in apple tree leaves expressed in µM

[Total glutathione] = total GSH concentration in apple tree leaves expressed in µM

[GSH] = glutathione concentration in apple tree leaves expressed in µM

4.3.3. Lipid peroxidation

4.3.3.1. Conjugated dienes

Theoretical aspects

Conjugated dienes (CDs) are related to first products of lipid oxidation.

They can be measured by spectrophotometry.

Manipulation achieved

This protocol was inspired by Singh et al. (2007).

Sample preparation

The sample preparation is the same as for the measurements of carotenoids and conjugated dienes.

Cryo-frozen apple tree leaves were crushed in liquid nitrogen with a mortar and pestle during about ten seconds to obtain a thin homogeneous powder. 10 ± 4 mg of this powder was mixed with 2 mL of 96% (v/v) cold ethanol. The solution was first put in the dark and on ice for 15 min before being centrifuged at 12 000 rpm during 5 min at 4°C.

The absorbance was measured at 234 nm and the concentration of conjugated dienes was calculated thanks to the extinction coefficient ($\varepsilon = 26.5\ mM^{-1}cm^{-1}$) and was expressed as µmol g^{-1} fresh weight (FW).

Analysis method

Absorbances were measured with an Ultrospec 7000 spectrophotometer at room temperature.

4.4. Statistical analysis

An analysis of variance at two factors (two-way ANOVA[17] or ANOVA2) was achieved for each test carried out in the laboratory and the Fv/Fm ratio accomplished on the experimental field (at each time independently). The factors here are the applied treatment and the experimental blocks.

In order to use this procedure, it was necessary to check the application conditions which were the normality and the variance equality of the 16 populations (4 treatments*4 blocks) from an infinity of populations. The samples were randomly and independently chosen. The number of observations per population varied depending on the analysis. Thus, the normal distribution was supposed because the number of repetitions has to be higher than ten ($n>10$) to correctly verify the normal distribution. The variance equality was demonstrated by using Levene's test with a P-value lower than 0,05 ($P < 0.05$).

The null hypotheses implied that:

1. There was no interaction between the two factors.
2. The mean of all treatments applied was equal meaning that there was no significant difference between the four treatments.
3. There was no variability within the blocks.

If the P-value was lower than 0,05 for a null hypothesis, it was rejected.

- If there was a significant difference between treatments Dunnett's test was performed to allow comparing the pesticide formulated (with and without CEO) to the "blanc".

- If there was variability within the blocks, no further analysis of the variability was performed because the blocks set up to control heterogeneity.

The data collected were processed on excel and R studio.

[17] Despite the fact that an online random number generator was used to assign the different treatments to apple trees, blocks have been created accidentally. In order to do a relevant statistical analysis, it has to be taken into account.

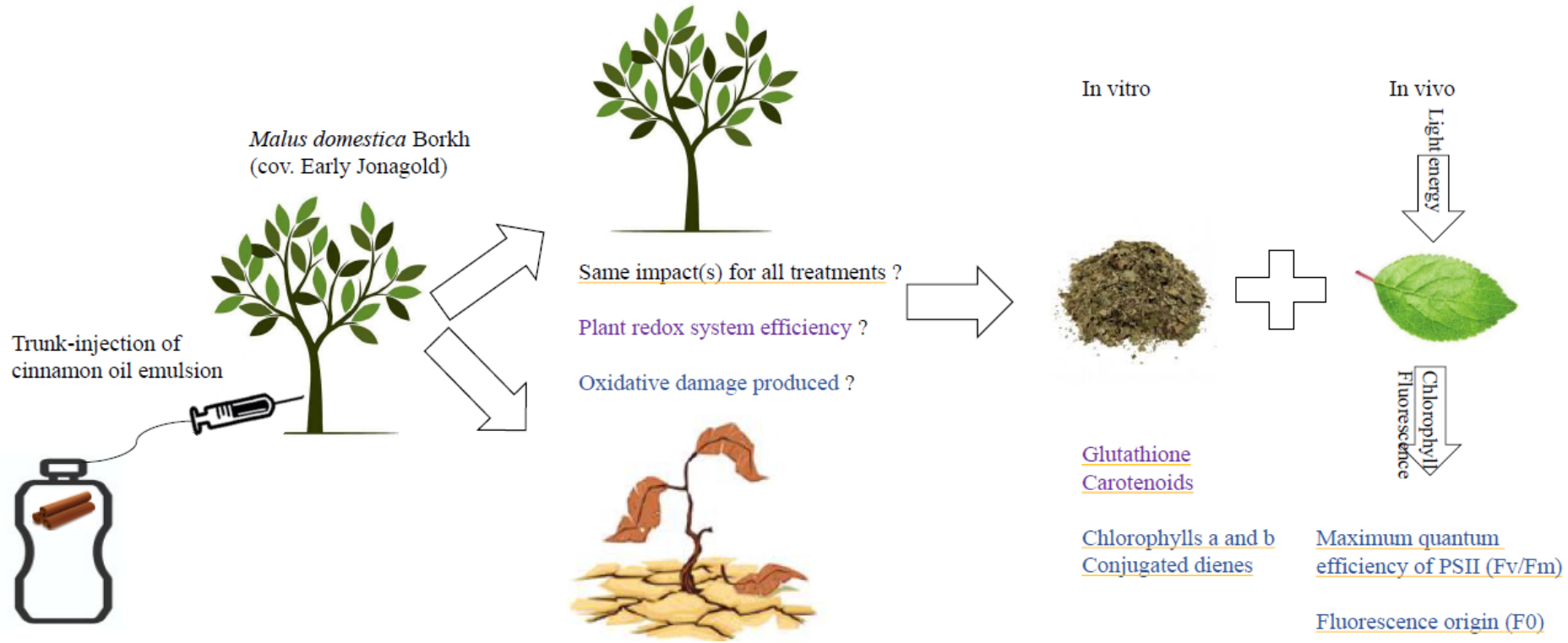

Figure 24. Schematic representation of the approach taken to assess the molecular physiological response of apple trees to the EO-emulsion biopesticide.

5. Results and discussion

This section will be divided into two main parts: first, the protocol development to measure the physiological response of apple trees to the injection of the biopesticide into the trunk, then the field experimentation. The aim of this work is to prove the biopesticide safety for plants in this kind of application. If it is, a second advantage will be added at the tree-injection project. Indeed, the biopesticide formulation showed promising results against the rosy apple aphid last year. Thus, it will be well on track to become an environmentally friendly alternative to the conventional pesticides.

The quantification of glutathione will be the only protocol developed. Further thoughts needed to be given to it due to the thiol group oxidative sensitivity and the specific chemical conditions for the fluorescent conjugate between GSH and mbb. This molecule has a pivotal role in plant antioxidant defence. It is a ROS scavenger, a substrate of antioxidant enzymes and an actor in the regeneration of other antioxidants (Noctor et al., 2011). Thus, it was chosen to be the cornerstone of the assessment of the plant antioxidant defences. Furthermore, glutathione was never measured in a similar context to this work. Finally, the bimane labelling will offer nice perspectives in situ in fluorescence imaging.

In order to evaluate the efficiency of the apple tree redox system against the oxidative burst, a preliminary test helped to adapt the sampling times for the field experimentation. The molecules assessed on apple tree leaves during this main field test were antioxidants (i.e. carotenoids and glutathione), different indicators of the plant physiological status and damage (i.e. chlorophyll fluorescence, chl a, chl b and conjugated dienes). Indeed, in stress studies, different aspects have to be considered.

5.1. Protocol development

5.1.1. Measurement of glutathione by HPLC-FLD in apple tree leaves

5.1.1.1. Advantages and pitfalls of glutathione determination in biological samples

Glutathione is commonly determined in studies because of its involvement in various mechanisms such as biosynthetic pathways, antioxidant biochemistry, redox homeostasis and detoxification (Noctor et al., 2012). As a consequence, the protocol of glutathione determination can give different information depending on the context of the study. In this work, the redox glutathione ratio is considered as a good indicator to evaluate the oxidative stress and eco-toxicological injury in plant cells (Hajdinák et al., 2018). The ratio decreases in oxidative stress conditions because of the oxidation of GSH and so the rise of GSSG concentration. Additionally, the fluorescent labelling between GSH and bimanes allow the observation of glutathione in situ in different cell organelles by fluorescence imaging. Indeed, GSH scavenges ROS whose concentration and lifetime vary between different cellular compartments (Majer et al., 2016).

In the literature, scientists usually agree that the protocol implementation of glutathione can be delicate in biological matrices. The cause is mainly due to the oxidation of the thiol group of GSH. The pitfalls of GSH quantification using bimane as fluorophore will be investigated here. So far, no unanimously recognized technique has been defined to measure glutathione in biological samples (animal or plant) causing a lot of variability between laboratories. The most criticized issues are the sample processing (GSH auto-oxidation and sample acidification) and the specific chemical conditions for the formation of the GSH-fluorescent agent conjugate.

Sample preparation

First of all, GSH is recognized as unstable in aqueous solution and it can thus auto-oxidize leading to an overestimation of GSSG concentration. This auto-oxidation can be prevented by using a masking agent such as bimane or N-ethylmaleimide (NEM) that must be added before the sample acidification (Cereser et al., 2001; Giustarini et al., 2016; Hajdinák et al., 2018; Rossi et al., 2002).

Next, the acidification step does not guarantee a complete protection from the oxidation of the -SH group (Rossi et al., 2002). In this study, the possible oxidation of glutathione in acetic acid and metaphosphoric acid (MPA) was investigated. The GSH standard was initially dissolved in a mix of chloridric acid (0,2 N) and acetic acid (10%, v/v). Then, the acetic acid was replaced by metaphosphoric acid (MPA) (10%, v/v). The chloridric acid was not substituted by another one because it was used as extraction solvent in leaf samples. In *Figure 25*, it appears that MPA induces less oxidation of GSH than acetic acid at the beginning. Then, if the glutathione dissolved in acetic acid is put on ice and in the dark, there is no further oxidation of glutathione during at least one hour. It is quite different with MPA whose peak area considerably falls between 45 min and 60 min. It could thus be more interesting to use MPA to minimize the GSH oxidation but the calibration concentrations must not last too long in the acidification step.

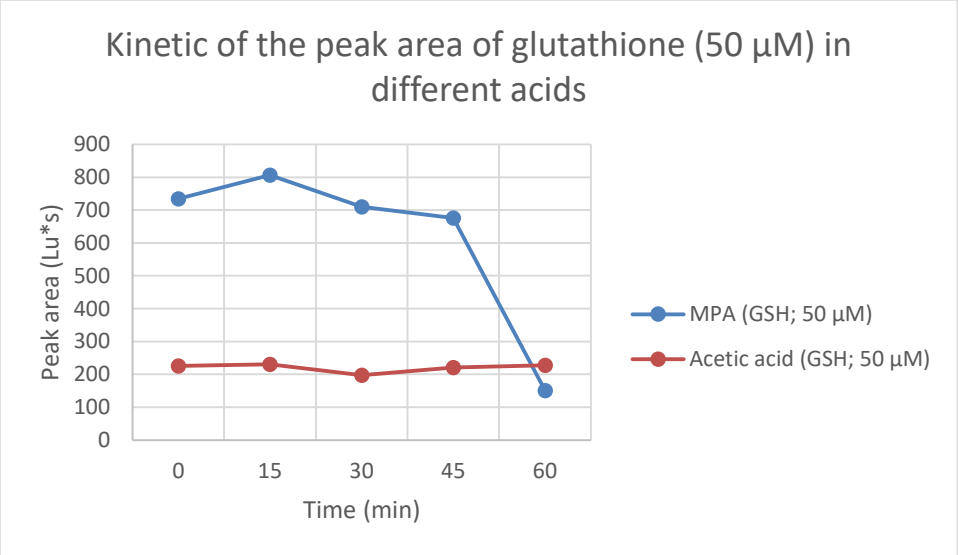

Figure 25. kinetic of the peak area of glutathione at the same concentration (50µM) during the acidification step in (a) acetic acid (10%, v/v)+HCl (0,2N) and (b) metaphosphoric acid (10%, v/v)+HCl (0,2N) (n=1).

The most suitable conditions for the formation of mbb-GSH fluorescent derivate

Before derivatizing the acidified GSH solution, the neutral-alkaline pH must be restored (Rossi et al., 2002). Indeed, the formation of mbb-GSH fluorescent derivate is pH-dependent and best occurs at a slightly alkaline pH (around 7,5-8,0) (Hajdinák et al., 2018). Though this step is essential, it can cause oxidation after restoring the neutral-alkaline pH. For example, Rossi et al. (2002) discovered that 50% of GSH content was converted into GSSG within a few minutes at pH 8,0-8,5.

Additionally to the protocol itself, conditions of sample collection, transport and storage are also very important factors that may influence the glutathione determination in biological samples. Cereser et al. (2001) noticed a rapid decrease in the quantity of GSH in red blood cells and fibroblast pellets and an increase in the GSSG amount stored in -80°C. Despite strict precautions, the leaf samplings were unfortunately negatively impacted (See further in this present section).

5.1.1.2. Validation of the glutathione analytical method

Chromatographic analysis

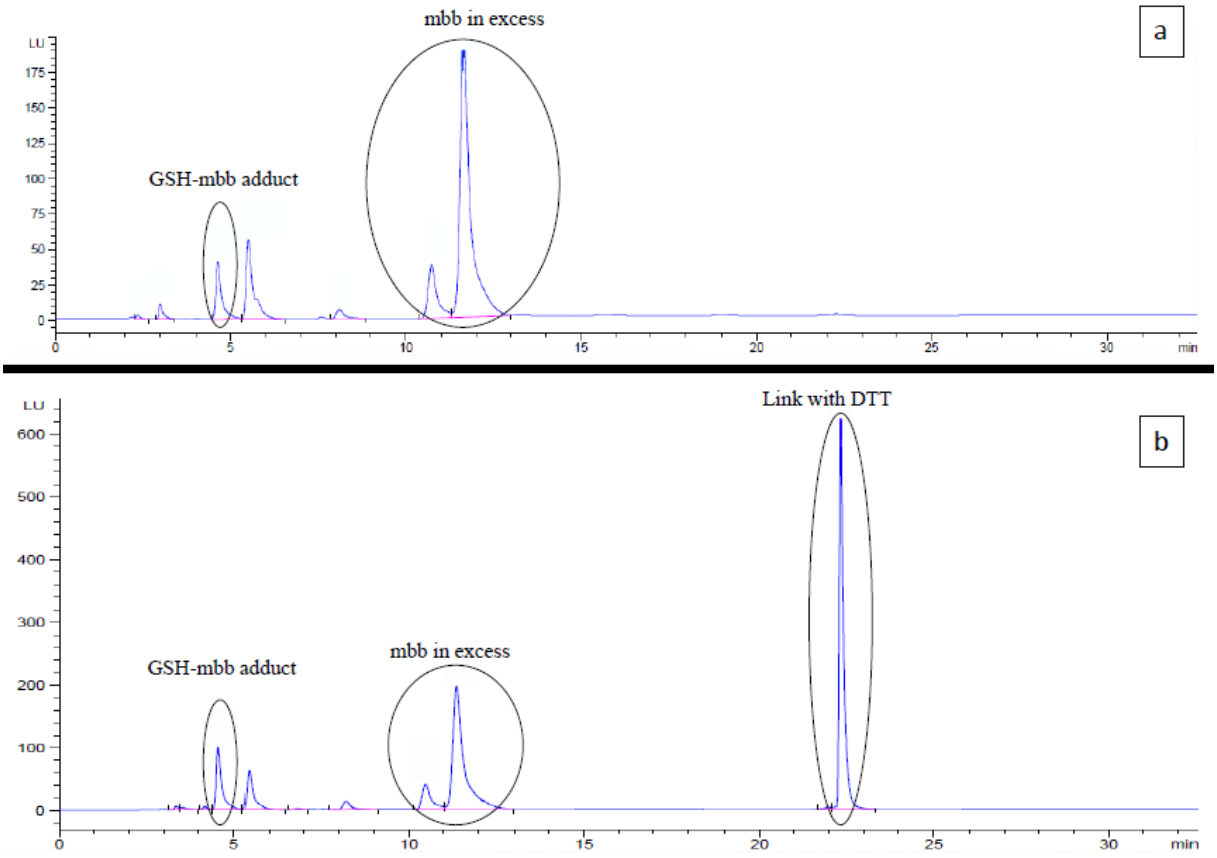

Figure 26. Peak identification of (a) GSH standard at 50µM and (b) GSH in apple leaves non-treated with a biopesticide.

The chromatograms obtained after HPLC analysis are shown in *Figure 26 (a) and (b)*. The GSH-mbb adduct peak was determined by GSH standard at different concentrations. Its retention time is around 4,6 min. The peaks between 10 and 11 min correspond to the excess of mbb because it is self-fluorescent and has to be in excess to ensure the good determination of GSH concentrations (Lavigne et al., 2007).

The peak with a retention time at 22 min is related to the addition of DTT when the total glutathione is measured. This peak does not appear neither in the standard curve nor in the reduced glutathione in leaf sample chromatograms. It could be an excess of DTT because of its fluorescence properties at these excitation and emission wavelengths (λ_{EX}:395 nm, λ_{EM}:477 nm) (Sun et al., 2018). Another possibility is that it reduces other disulphides than GSSG that would react with mbb because this fluorophore is not specific to glutathione and can react with any thiol group (Noctor et al., 2011). This second explanation is unlikely because the peak area for the mbb in excess does not lessen when the DTT is added. Even if the mbb is not selective for glutathione, no other peak interferes with the peak of interest on the chromatogram.

It is noteworthy that even if the mbb is not selective for glutathione, no other peak interferes in GSH determination in both GSH standard (*Figure 26 (a)*) and the plant matrix (*Figure 26 (b)*) if they are compared.

Calibration curve and linearity

The linearity was assessed for concentrations between 25 µM and 200 µM which corresponded to the most suitable concentration range to determine glutathione in leaf samples (See the preliminary test part). The equation of the regression line is y= 22,241x-434,77 with a coefficient of determination (R^2) equal to 0,9954 showing a good linear correlation in the concentration ranges considered (*Annexe 2*).

The calibration curve was built from reduced glutathione standard. Despite the fact that the diverse GSH solutions were kept in the dark, on ice and the restricted loss of time during manipulations, GSH may have been (auto)-oxidized during the acidification step as previously mentioned at the beginning of this section. To play it safer, adding a reducing agent (e.g. DTT) may be contemplated.

The limit of detection (LOD) and the limit of quantification (LOQ) are 20,77 µM and 23,64 µM, respectively.

Once the glutathione protocol was correctly developed, it was tested on biological samples and different parameters were evaluated.

Repeatability

The repeatability was assessed by injecting the same solutions of the GSH and total glutathione from a single apple leaf sample five times consecutively (*Figure 27 (a) and (b)*). Only the peak of the GSH-mbb adduct is shown.

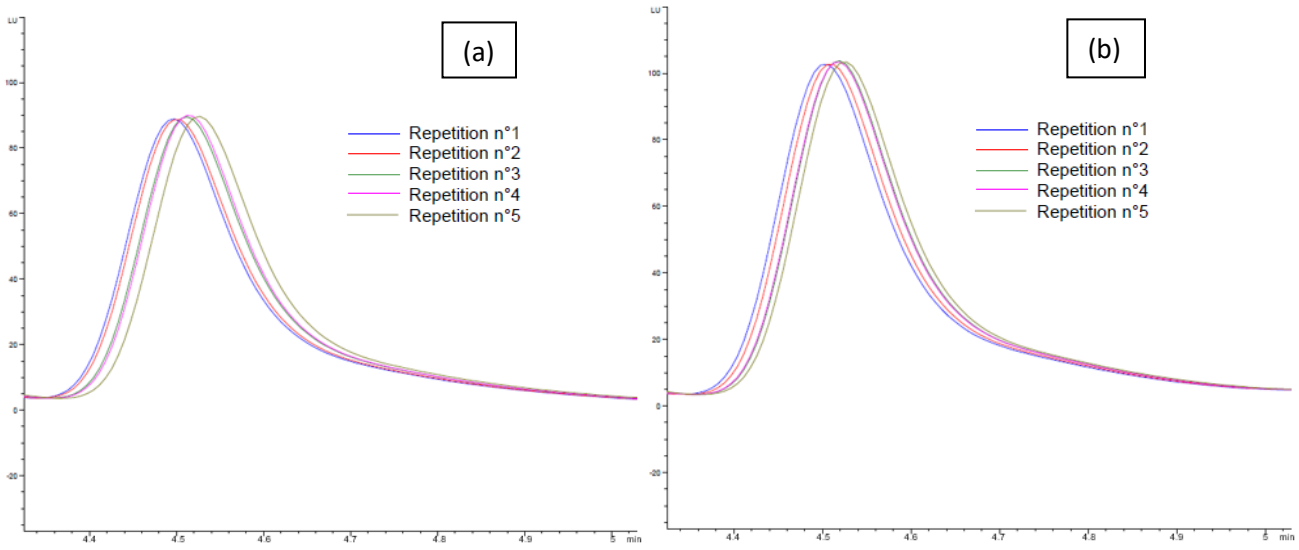

Figure 27. Repeatability of (a) glutathione in its reduced form and (b) the total glutathione from a single apple leaf sample (n=5).

The mean ± standard deviation for the five repetitions of reduced glutathione is 62,07±0,43 µM or 583,95±4,03 µM/g fresh weight and the one for the five repetitions of total glutathione is 69,66±1,0 µM or 655,3±9,52 µM/g fresh weight.

After that the repeatability was determined on independent leaf weighing (*Figure 28*). Five independent analyses from a large initial ground apple leaf pool were performed according to the established glutathione protocol. The leaves were sampled the day of the analysis. They were stored at -80°C.

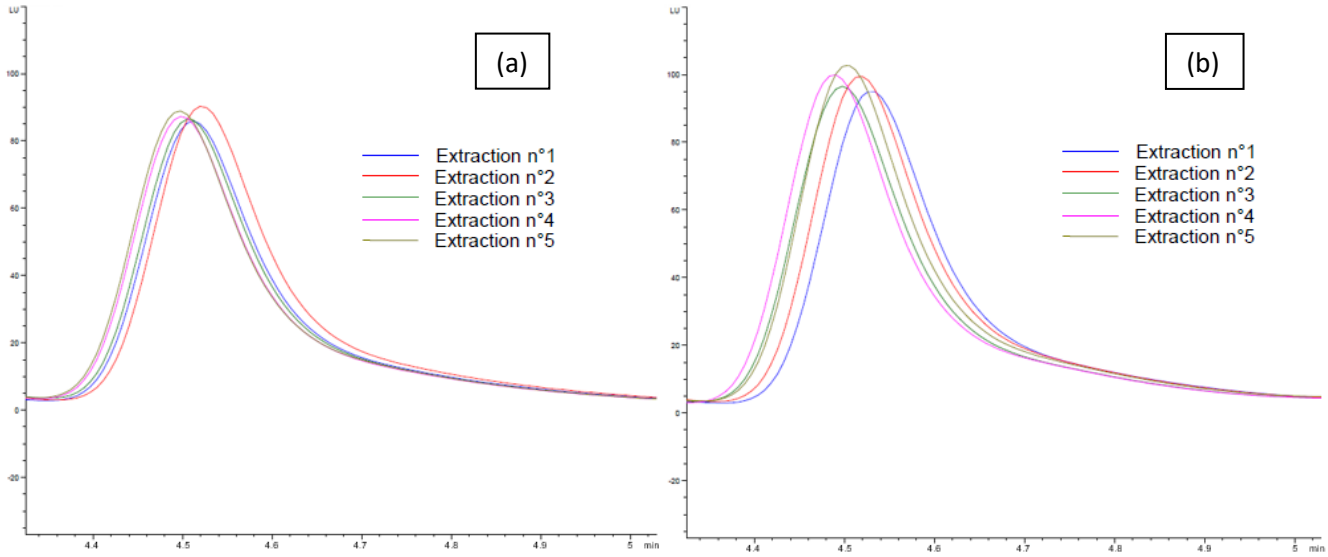

Figure 28. Determination of (a) reduced glutathione and (b) total glutathione on apple leaves (n=5).

The mean ± standard deviation for the five reduced glutathione extractions is 598,37±9,94 µM/g fresh weight and the one for the five total glutathione extractions is 655,4±8,43 µM/ g fresh weight.

Then, the GSSG concentration could be calculated from the two others: 28,85±5,19 µM/g fresh weight.

Finally, the ratio could be estimated: 95,45±0,84 %. It corresponds to the ratio generally found in unstressed plants (Noctor et al., 1998).

Stability of GSH-mbb derivatives

The stability of the GSH-mbb adduct was finally estimated by injecting the same solutions of GSH and total glutathione from a single apple leaf sample for a few days (i.e. D, D+1, D+2, D+3, D+5, D+6, D+7 and D+8) (*Figure 29 (a) and (b)*). The vials containing the solutions were stored on the HPLC rack at 4°C in the dark.

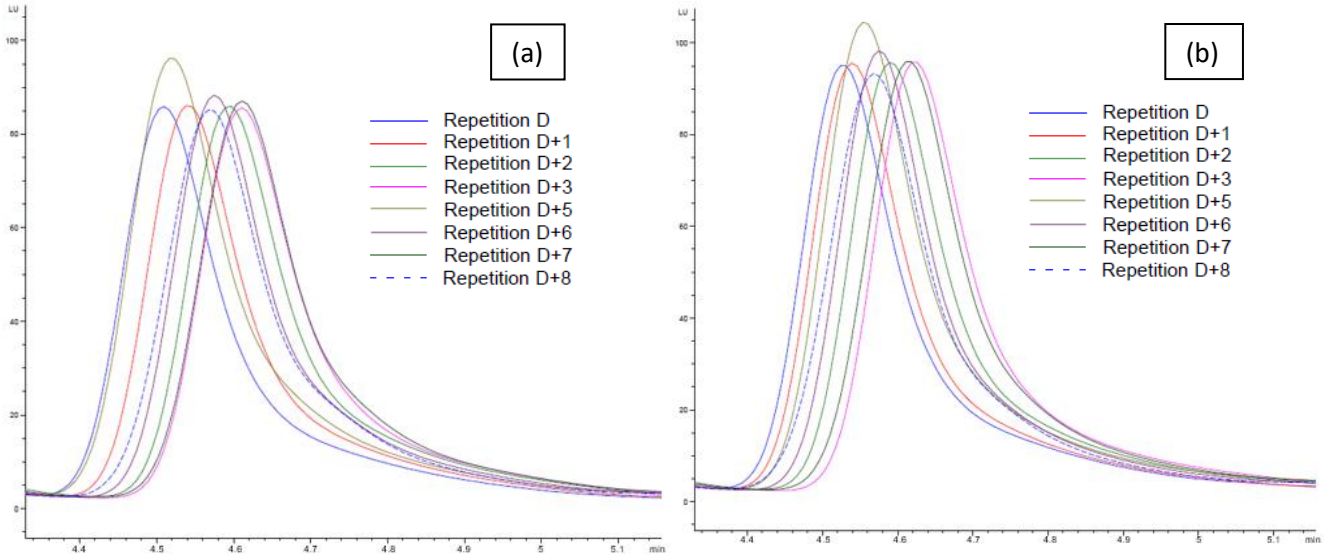

Figure 29. Stability of (a) GSH-mbb adduct and (b) total glutathione-mbb adduct from a single apple leaf sample (n=1).

The GSH-mbb adduct is stable for 9 days at least in the storage conditions tested. The mean ± standard deviation for the five reduced glutathione repetitions is 63,55±2,34 µM or 627,36±23,1 µM/g fresh weight and for the total glutathione is 66,89±1,80 µM or 660,39±17,81 µM/g fresh weight.

To sum up this protocol development part, the results revealed a good linearity in the concentration range of the biological samples, a great repeatability and an adduct stability for at least nine days.

5.2. Evaluation of the molecular physiological response of apple trees

5.2.1. Preliminary tests

In order to get a better chance to observe the oxidative burst, a preliminary test was achieved. Two treatments were therefore studied: one constituted by a cinnamon EO emulsion at 1% [CEO 1%] and the second by tap water. Both solutions were injected into two trees, so four trees in all.

Apple leaves were sampled for two days after injecting at 0h, 3h, 6h, 27h and 30h. Sampling times close to the injection were favoured because the oxidative burst is a very early physiological response to abiotic stress in plant (Boller and Felix, 2009). Only glutathione was considered for the pre-testing because it is the most precise protocol to adjust better the sampling time of the oxidative burst (*Figure 30*).

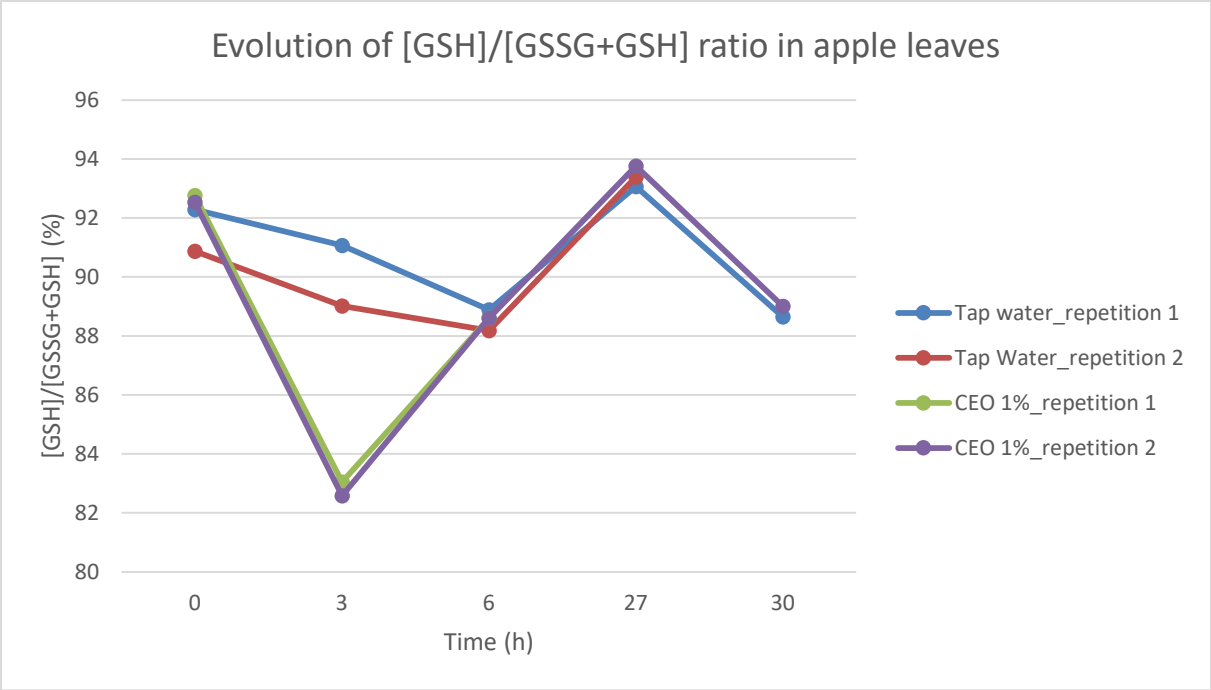

Figure 30. Evolution of the [GSH]/[GSSG+GSH] ratio in apple leaves (n=2).

The repetitions of a same treatment show a shape similarity of the two curves. Broadly speaking, all curves reach a low point between zero and six hours before increasing and then slightly decreasing again. The plunge is more important with CEO 1% treatment than with tap water.

At this point in the work, some remarks need to be made. First of all, using mbb overstates the concentration of glutathione because mbb can form a fluorescent conjugate with any thiol function in biological samples. It is not the case with standard glutathione, of course. Then, some repetition points are missing because their concentrations were under the LOQ which leads to the question of the variability within the biological sample. It will be further discussed in this part.

In the light of these results, it seems more relevant to favour sampling between zero and six hours after injection to assess the oxidation burst. That is why sampling times chosen for the experimental fields were at 0h; 1h30; 3h; 4h30; 6h; 24h; 72h and 192h.

5.2.2. Experimental results

5.2.2.1. Tree-injection device

Before moving on to the results of leaf samples, it is important to note that the injection step could also influence the plant response as it can be seen on *Figures 31 and 32*. Injuries were noticed on the preliminary test after about one and a half week of injection. There were not any on the experimental field after the same period of time.

A leak appeared for some injection sites during the experimental trial implying that the absorbed volume was not identical for all repetitions. This did not occur during the preliminary test. Nevertheless, the biopesticide not directly injected could still be absorbed by the trunk. Each

technique has advantages and drawbacks. Two disadvantages were clearly perceived on the field. Even so, a tree injection reduces potential environmental exposures, allows working with lower active substance which reduces the pesticide inputs and is a useful alternative when foliar and soil applications are either difficult to apply or ineffective (Doccola and Wild, 2012).

Figure 31. Physical injuries due to the plug insertion after one and a half week of preliminary test performed on two years old apple trees.

Figure 32. Example where not all the three mL of the biopesticide was injected into the trunk (fourth CEO1% modality).

5.2.2.2. The chlorophyll fluorescence

The chlorophyll fluorescence was recorded each time. The other analyses were performed at 0h; 1h30; 3h; 4h30; 6h and 72h because the chlorophyll fluorescence was directly measured in vivo on the experimental field.

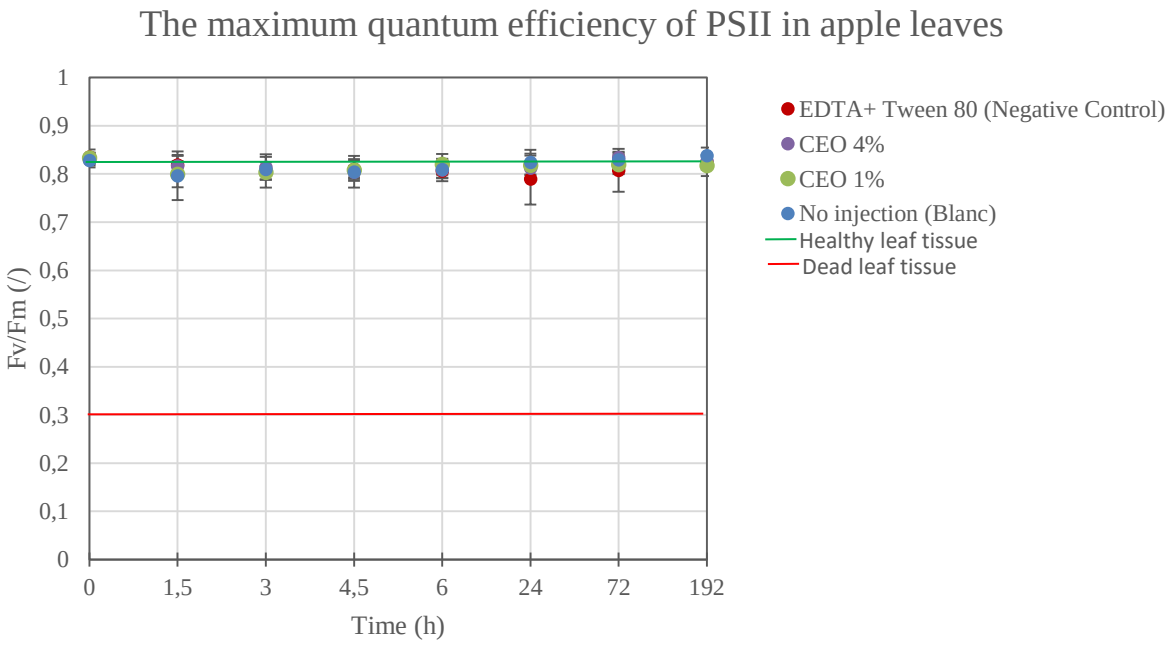

Figure 33. Maximum quantum efficiency of PSII of apple leaves (n=12).

Different chlorophyll fluorescence parameters were evaluated: Fv/Fm ratio and F0. They give information about the maximum quantum efficiency of PSII and the distribution of energy to PSII, respectively (Bresson et al., 2018; Ekmekci and Terzioglu, 2005).

Each (Fv/Fm) ratio was around 0,83 at all sampling times (*Figure 33*) meaning that the leaf tissues assessed were healthy and there was no damage on the photosynthetic apparatus (Bresson et al., 2018). It seems that, a priori, no treatment stands out from the others on the Fv/Fm ratio recorded at different times. The two-way ANOVA will confirm or invalidate this first observation. Each time was tested independently. P-value was equal to 0.05 for all statistical tests executed in this work. They were divided into three parts: (1) verification of the homogeneity of variances by Levene's test; (2) achievement of the analysis of variance and (3) performance of the Dunnett's test (i.e. post-hoc test) when the null hypothesis of ANOVA was rejected.

The homogeneity of variances of the maximum quantum efficiency of PSII was verified. The p-values can be consulted in *Annexe 3*.

The ANOVA2 results presented in *Table 5* shows that the interaction between the two factors are significantly different at time t=3h, t=72h and t=192h. In addition, for these two last times, blocks and treatments are also (highly) significant. It indicates that, on the one hand, there is a variability taken care of by these blocks and on the other hand, the treatment has an impact on the Fv/Fm ratio because the means of different treatments were significantly different, thus rejecting the null hypothesis.

The Dunnett's test was then performed on p-values lower than 0.05. It compared each treatment with a control. In this case, the control consisted of non-injected trees (i.e. No injection treatment or blanc). When there was no significant difference between the means of a treatment and the control, this test grouped them, meaning that the tested treatment induced the same response as the control. These results are presented in *Table 6*. It reveals that the EDTA-Tween 80 or the negative control causes a significantly different response compared to the three others at t=24h and at t=192h so does CEO 1% at t=192h. However, this response does not impact the health of leaf tissues because the Fv/Fm ratio remains around 0,83.

*Table 5. P-values obtained for each of the two-way analysis of variance performed on Fv/Fm ratio (p-value = 0.05). The significant p-values are in bold: *significant (0.05); ** highly significant (0.01) and *** very highly significant difference (0.001).*

	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
Treatment	0.48	0.28	0.76	0.86	0.373	**0.014** *	0.10	**0.0029** **
Block	0.91	0.68	0.40	0.92	0.390	**0.020** *	0.072	**0.0036** **
Treatment *Block	0.28	0.36	**0.0071** **	0.14	0.830	**0.0033** **	0.58	**0.0011** **

Table 6. Dunnett's test performed on the Fv/Fm ratio at (a) t=24h and (b) t= 192h. The same letter indicates that the treatments belong to the same group as the control.

Treatment	Mean (a)	Treatment	Mean (b)
No injection (control)	0,82417A	No injection (control)	0,8360A
CEO 1%	0,81842A	CEO 4%	0,82675A
CEO 4%	0,81233	EDTA+Tween 80	0,82375
EDTA+Tween 80	0,79225	CEO 1%	0,81767

When there was no interaction between the two factors, they were regrouped and a one-way analysis of variance (one-way ANOVA or ANOVA1) was performed on the treatment factor (*Table 7*). In brief, the type of treatment has no influence on the Fv/Fm ratio.

Table 7. P-values obtained for one-way ANOVA performed on Fv/Fm ratio (p-value = 0.05).

	t=0h	t=1h30	t=4h30	t=6h	t=72h
Treatment	0.46	0.27	0.87	0.33	0.10

To sum up, even if variations were generated by the different treatments at some times, the Fv/Fm ratio stayed constant. The plant photosynthetic performances were optimal (i.e. around 0,83) over the tested time lapse. However, some leaf tissues were visually oxidized from t=72h *(Figure 34)*. They were related to trees which were treated with an emulsion of CEO 4% and a negative control (i.e. EDTA-Tween 80 only). This visual observation did not appear in *Figure 33* where the Fv/Fm ratio should go down and reach less than 0,3 and was partially shown in the Dunnett's test at t=192h in *Table 6*. It was not observed on the preliminary test for the same period of time. The treatments injected were CEO1% and tap water.

At this stage, the sampling can be discussed. The oxidized leaves were located at different heights but not uniformly. It can be deduced that the biopesticide was not distributed in the whole tree throughout the vascular bundles in a uniform way. Three leaf-clips (one at different heights: low, medium and high) randomly put per tree and per time to evaluate Fv/Fm ratio may not be enough to reflect the whole organism tissue health.

Figure 34. Apple leaves oxidized during the experiment at (a) t=72h and (b) t=192h after injection.

F0 represents the basal fluorescence or the fluorescence origin. It is the first point on the Kautsky fluorescence induction curve when the leaf is light-stimulating after the dark-adaptation step. An increase of F0 is associated with a decline in PSII reaction centres and oxidative damage (Ekmekci and Terzioglu, 2005).

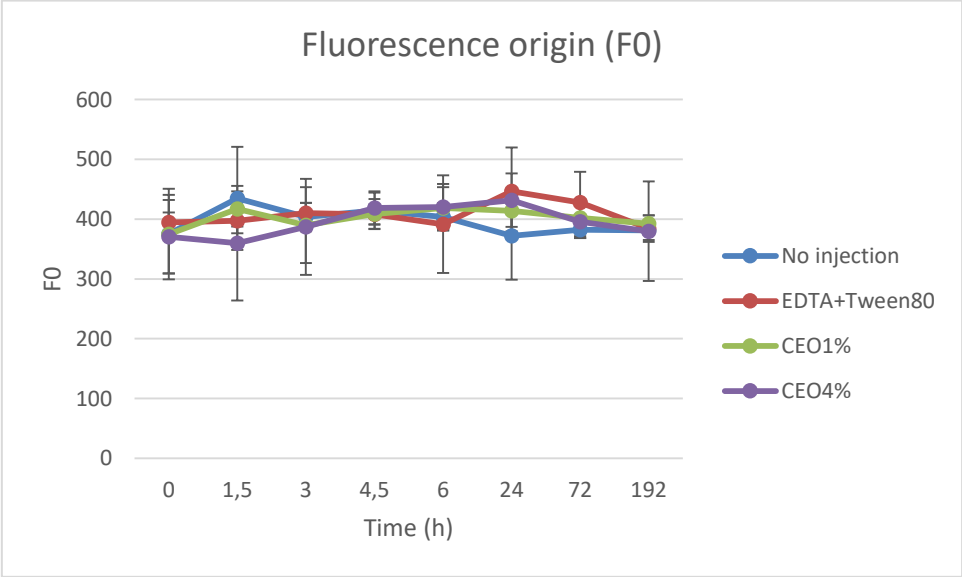

Figure 35. Fluorescence origin of apple leaves (n=12).

As it can be seen in *Figure 35*, the value of F0 fluctuates slightly around 390. The profiles of the curves are more variable than with the Fv/Fm ratio (*Figure 33*). The negative control is significantly different at t=24h and t=72h as well as CEO 4% at this first time (*Tables 8 and 9*).

When no interaction between the two factors occurred, they were brought together and an ANOVA1 was performed on the treatment factor (*Table 10*). The type of treatment does no act upon the FO either.

*Table 8.P-values obtained for each of the two-way analysis of variance performed on F0 (p-value = 0.05). The significant p-values are in bold: *significant (0.05); ** highly significant (0.01) and *** very highly significant difference (0.001).*

	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
Treatment	0.73	0.059	0.66	0.79	0.45	**0.0024 ** **	**0.0078 ** **	0.92
Block	0.64	0.42	0.21	0.78	0.21	**0.0024 ** **	0.029	0.46
Treatment *Block	0.21	0.35	0.78	0.54	0.76	**0.012 * **	0.58	0.90

Table 9. Dunnett's test performed on the F0 at (a) t=24h and (b) t= 72h. The same letter indicates that the treatments belong to the same group as the control.

Treatment	Mean (a)	treatment	Mean (b)
No injection (Control)	371,917 A	No injection (Control)	382,167 A
EDTA-Tween 80	442,458	EDTA-Tween 80	427,750
CEO 4%	431,583	CEO 4%	396,667 A
CEO 1%	412,917 A	CEO 1%	396,625 A

Table 10.P-values obtained for one-way ANOVA performed on F0 (p-value = 0.05).

	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=192h
Treatment	0.76	0.059	0.73	0.78	0.42	0.90

5.2.2.3. Leaf pigments

In parallel to Fv/Fm ratio, further information about plant physiological status is brought by leaf pigments composition and content. Their changes are commonly used to evaluate this status during development, senescence, adaptation and acclimatization to different stresses. For example, chlorophylls absorb solar light energy and allow its utilization in photosynthetic reactions whereas carotenoids are major antioxidant metabolites that quench singlet oxygen to avoid its excessive accumulation (Gitelson and Merzlyak, 2004; Gitelson et al., 2006; Noctor, Mhamdi and Foyer, 2016; Ramel et al., 2012a).

Chlorophylls

In *Figure 36*, the non-injected trees have the lowest chl a leaf content, the highest is reached with the CEO4% treatment at t=0h. But *Table 11* informs that there is no significant difference between treatments at this time. This result is consistent because the sampling was done just before the injection. The curve of each treatment has a different profile but the means of each treatment per time are not significantly different.

The curves for chl b have a pattern of a "boom-bust" pattern apart from CEO 4% where the content maintains the same level from t=1h30 (*Figure 37*). EDTA+Tween 80 and CEO 4% are significantly different at t=3h compared to the control (*Table 12*).

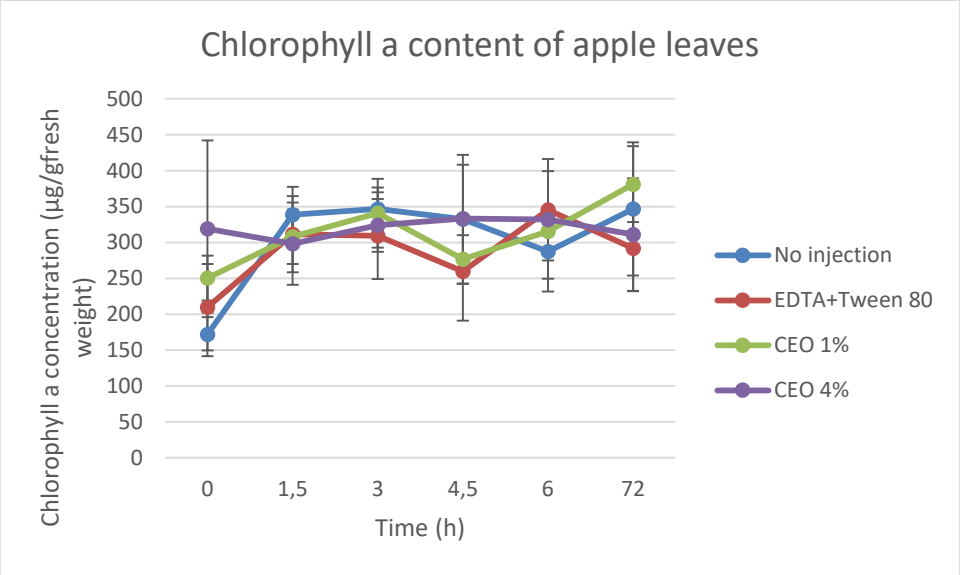

*Figure 36. Chlorophyll a content (µg*g fresh weight^{-1}) of apple leaves (n=4).*

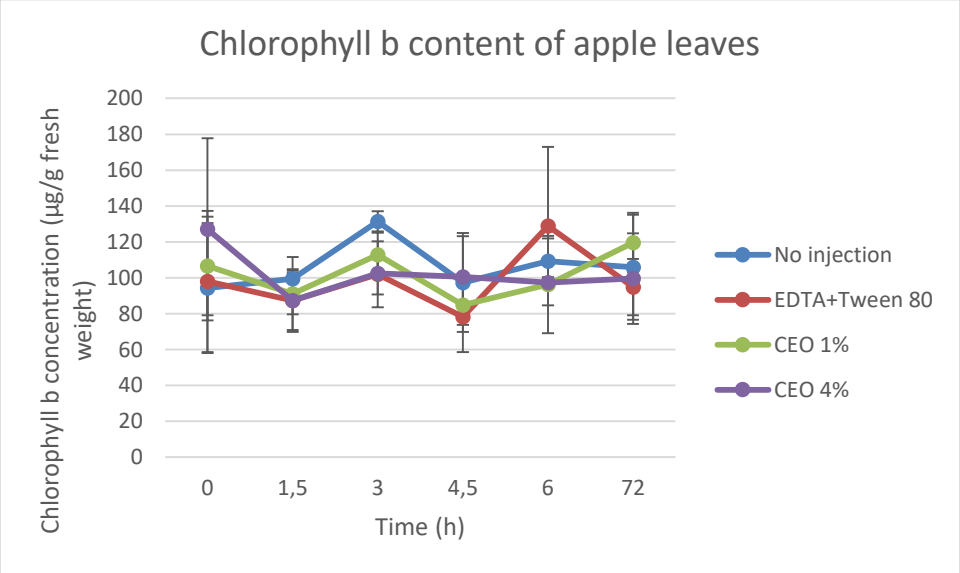

*Figure 37. Chlorophyll b content (µg*g fresh weight^{-1}) of apple leaves (n=4).*

The ANOVA2 could not be executed here because the number of repetitions per treatment and block was equal to one (n=1) each time. As a consequence, neither the homogeneity of variance test nor the ANOVA2 could be performed.

To increase the number of repetitions and be able to do a statistical analysis, different options can be explored:

1) The ANOVA2 is performed without taking time intervals into account.

2) The blocks are not taken into account. The statistical analysis then becomes a one-way ANOVA on the factor "treatment" each time. For the Fv/Fm ratio (*Table 5*), the interaction between the treatments and the blocks is only significant at t=3h. Even at this time, the p-value is higher than 0.05 meaning that there is no significant difference between the means for each factor taken independently. For this reason, this case will be favoured compared to the first option. This choice will also apply for carotenoids, conjugated dienes contents and glutathione ratio.

The homogeneity of variances for chlorophylls content was checked and the Levene's test can be consulted in *Annexe 3*.

*Table 11. P-values obtained for each of the one-way analysis of variance performed on chlorophyll a and b content (p-value = 0.05). The significant p-values are in bold: *significant (0.05); ** highly significant (0.01) and *** very highly significant difference (0.001).*

	Chlorophyll a content					
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=72h
Treatment	0.069	0.67	0.65	0.36	0.55	0.35
	Chlorophyll b content					
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=72h
Treatment	0.65	0.62	**0.023***	0.43	0.32	0.46

Table 12. Dunnett's test performed on chlorophyll b content at t= 3h. The same letter indicates that the treatments belong to the same group as the control.

Treatment	Mean
No injection (control)	131,43 A
CEO 1%	112,77 A
CEO 4%	102,42
EDTA+Tween 80	101,93

Changes in the chl a/b ratio and total chlorophyll (Chl a+b) content are also indicators of the physiological status in photosynthetically active tissues for plants exposed to a wide spectrum of (a)biotic stresses (Sytykiewicz et al., 2013). They are presented in *Figures 38 and 39*.

Regarding the results of the Levene's test in *Annexe 3*, the homogeneity of variances for the set of values of chl a/b ratio at t=3h was not met. The one-way ANOVA was performed all the same. This unsatisfactory condition of application must be kept in mind during its result interpretation.

More significant differences can be seen with the chl a/ chl b ratio than with the total chlorophyll or chl a and chl b content separately assessed. The chl a/b ratio increases from t=0h to t=1h30. The negative treatment is then significantly different from the control (*Tables 13 and 14*). At t=3h, all the treatments are significantly different from the control and at t=6h, only CEO treatments are. The CEO curves have the same shape. It is simpler with the total chlorophyll (a+b) content because all p-values are higher than 0.05.

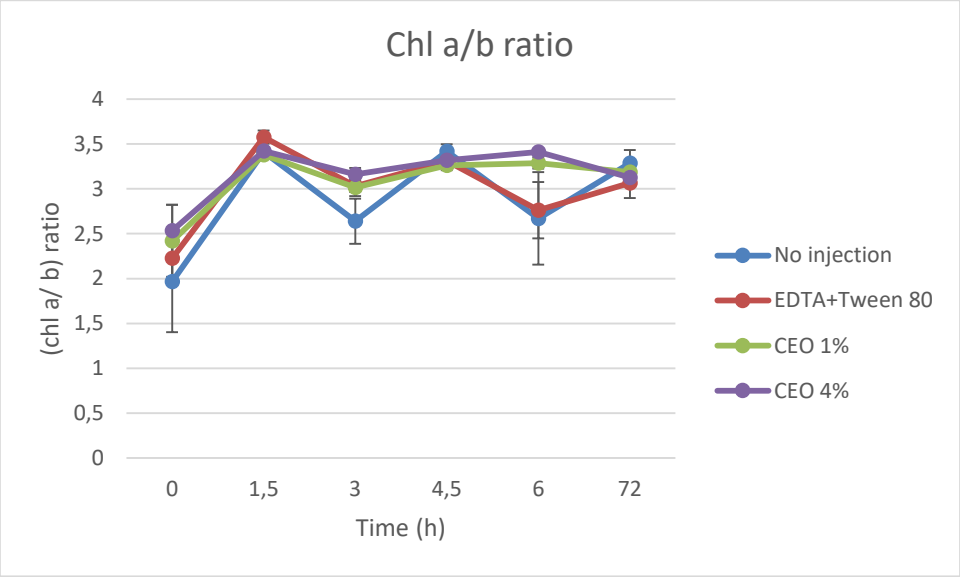

Figure 38. Chl a/b ratio of apple leaves (n=4).

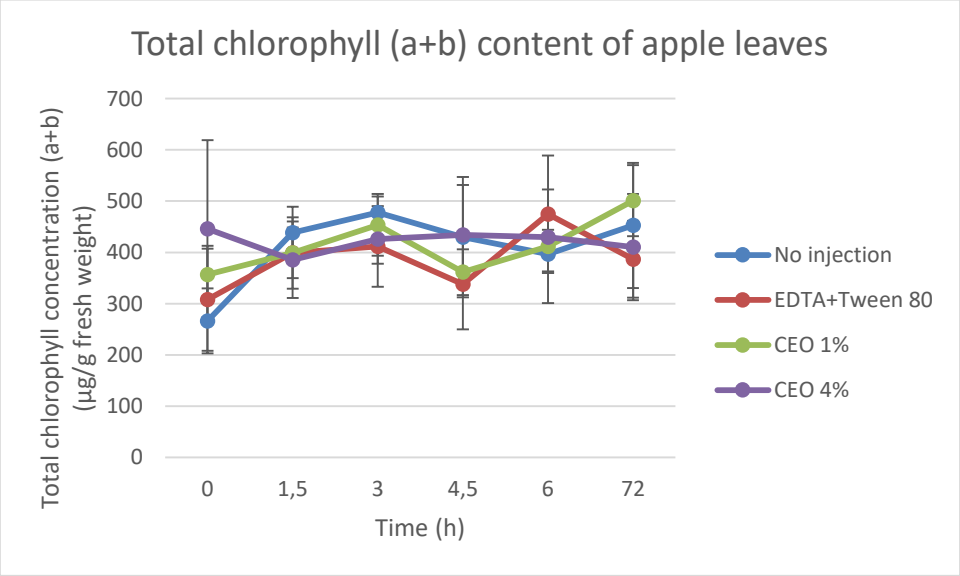

*Figure 39. Total chlorophyll (a+b) content (µg*g fresh weight^{-1}) of apple leaves (n=4).*

*Table 13. P-values obtained for each of the one-way analysis of variance performed on chl a/b ratio and total chlorophyll (a+b) content (p-value = 0.05). The significant p-values are in bold: *significant (0.05); ** highly significant (0.01) and *** very highly significant difference (0.001).*

	Chl a/b ratio					
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=72h
Treatment	0.26	**0.0011 ****	**0.0015 ****	0.060	**0.0098 ****	0.12
	Total chlorophyll (a+b) content					
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=72h
Treatment	0.16	0.67	0.40	0.38	0.58	0.38

Table 14. Dunnett's test performed on chl a/b ratio (a) at t=1h30h and (b) at t= 3h and (c) at t=6h. The same letter indicates that the treatments belong to the same group as the control.

Treatment	Mean (a)	Treatment	Mean (b)	Treatment	Mean (c)
No injection (control)	3,4105 A	No injection (control)	2,640 A	No injection (control)	2,670 A
EDTA +Tween 80	3,5765	CEO4	3,1616	CEO 4%	3,4117
CEO 4%	3,4232 A	EDTA +Tween 80	3,0304	CEO 1%	3,2876
CEO 1%	3,3809 A	CEO1	3,0137	EDTA +Tween 80	2,762 A

Carotenoids

The calculation of the carotenoids concentration is dependent on the chlorophylls a and b ones.

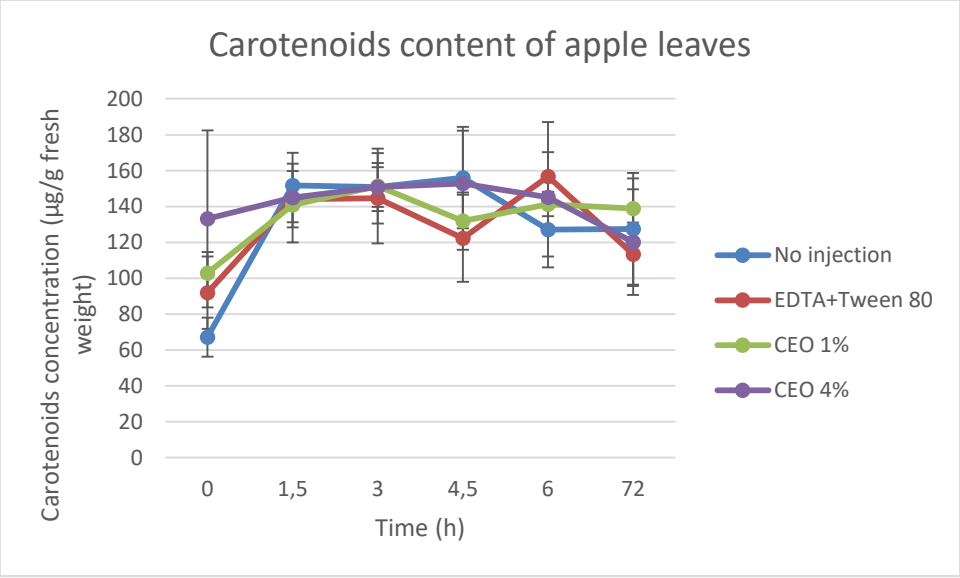

*Figure 40. Carotenoids content (µg*g fresh weight^{-1}) of apple leaves (n=4).*

From *Figure 40*, the curves for the carotenoids and the chl a contents globally look alike. The statistical analysis shows that CEO 4% is significantly different from the other treatments

(*Tables 15 and 16*) but the sampling was done just before the injection. Thus, this variation can only have a biological origin. This implies that in the next statistical analyses, the treatment may not be the single cause when the results are significantly different from the control.

At t=1h30, the carotenoids content increases for all treatments and stays globally higher than at the beginning. No big difference appears between the treatments.

The homogeneity of variances for carotenoids content was verified and the Levene's test can be consulted in *Annexe 3*.

*Table 15. P-values obtained for each of the one-way analysis of variance performed on carotenoids content (p-value = 0.05). The significant p-values are in bold: *significant (0.05); ** highly significant (0.01) and *** very highly significant difference (0.001).*

	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=72h
Treatment	**0.038 ***	0.83	0.95	0.22	0.42	0.53

Table 16. Dunnett's test performed on the carotenoids content at t=0h. The same letter indicates that the treatments belong to the same group as the control.

Treatment	Mean
No injection (control)	67,11 A
CEO 4%	133,1
CEO 1%	102,76 A
EDTA+Tween 80	92,0 A

Sircelj et al. (2005) studied the biochemical response to progressing drought on one year old apple trees (M. domestica) "Elstar" and "Jonagold Wilmuta". Measuring carotenoids, chl a and chl b was a part of the study. The conditions were different: Their samplings lasted longer (i.e. 6, 10, 15, 20 and 23 days) and they extracted the pigments with ice-cold acetone before analysing them by HPLC. They concluded that the total carotenoid concentration was not significantly different between water-stressed leaf plant and the control which also occurs in this study.

In 'Jonagold Wilmuta', they noticed that chlorophyll concentrations declined significantly upon severe drought stress at the end of the experiment which is not the case here. *Table 11* informs that the mean of all treatments is equal for chlorophyll implying that the different injected biopesticides have no impact on chlorophyll a content compared to not injected trees. Concerning chlorophyll b concentration, CEO 4% and the negative control are significantly different from the control at t=3h (*Table 12*).

Table 17 sums up the results collected at day six by these authors for carotenoids and chlorophylls content in order to give an idea of the range of concentrations they found in apple leaves. If they are compared with these obtained in this work, the pigment content is lower (µg/g fresh weight against mg/g fresh weight). It could be explained by the different protocols. HPLC is more precise than a spectrophotometer and acetone extraction is maybe more efficient than ethanol. The chl a concentration is higher than the chl b one at each time in both works. However, in this work, chl a is not twice as high as chl b.

Table 17. Sircelj et al. (2005)'results for carotenoids and chlorophylls content for control only for (M. domestica) "Jonagold Wilmuta" at day=6 (3⩽n⩽4).

	Carotenoids content (mg/g fresh weight[18])	Chlorophyll a content (mg/g fresh weight)	Chlorophyll b content (mg/g fresh weight)
M. domestica"Jonagold Wilmuta" control	3,4±0,17	13,8±1,23	5,91±0,31

5.2.2.4. Glutathione

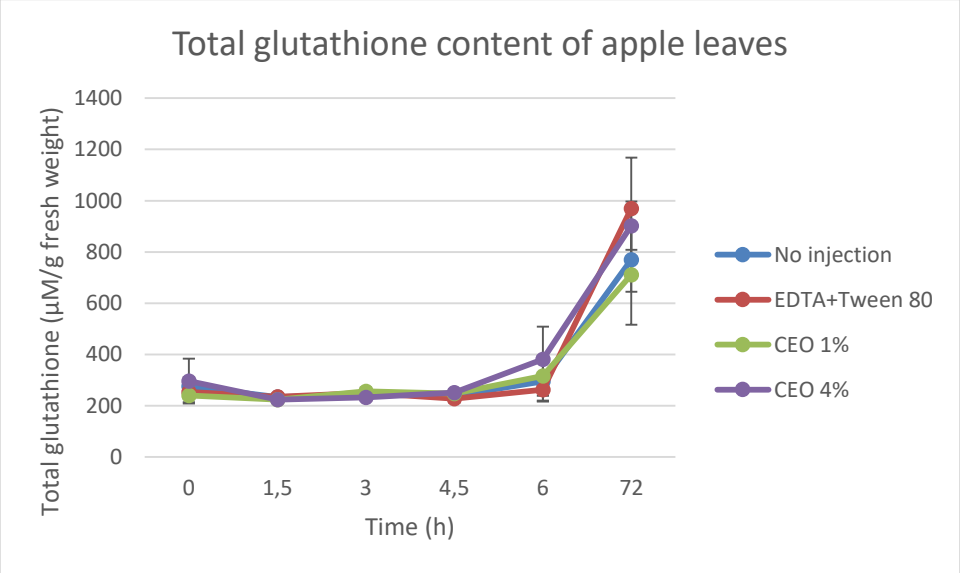

*Figure 41. Total glutathione (µM*g fresh weight^{-1}) of apple leaves (n=4).*

The standard deviation as well as the concentration difference between treatments increased with the hours passing as seen in *Figure 41*. Non-oxidized leaves were noticed at 72h which is correlated with a rise of the total glutathione content. It proves that something affected glutathione content before the analysis. The expected total glutathione concentration for t=0h should be at least equal to 800 µM/g fresh weight.

[18] Concentrations were given per mg/g dry weight. The values were then divided by 0,35 to approximate it in fresh weight and make a comparison with the results obtained in this work.

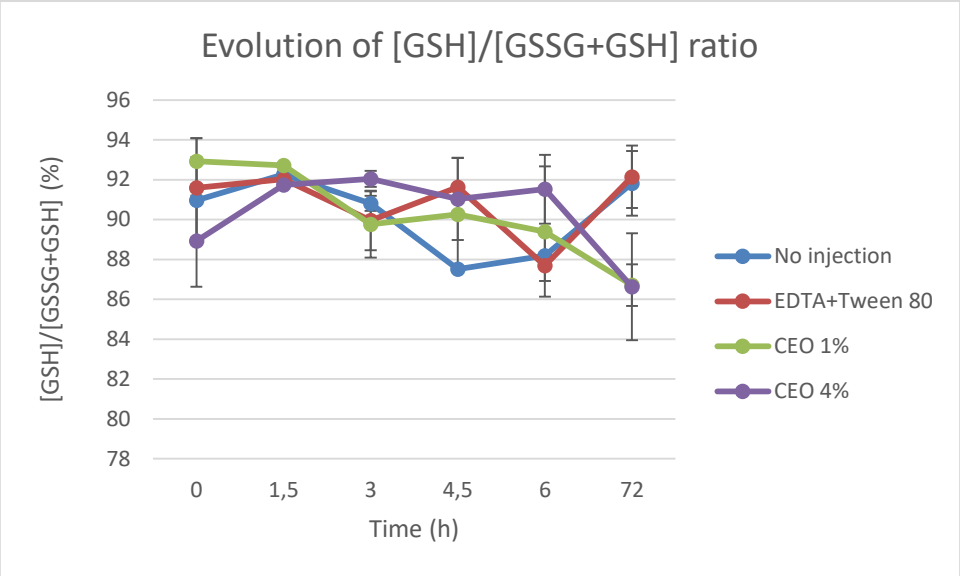

Figure 42. Glutathione ratio in apple leaves (1 ≤n ≤4).

Thanks to *Figure 42*, the biological variability is proved at t=0h once again. Regrettably, the oxidative burst that hypothetically happened between t=0h and t=6h in the preliminary test cannot be further studied through statistics due to a non-sufficient number of repetitions to make an accurate statistical analysis (homogeneity of variance and ANOVA1) within the other sets of values. Graphically, the glutathione ratio for the no-injection treatment only fell in this interval of time. It is the opposite for CEO 4%.

It seems that the treatment impacted the glutathione ratio at t=72h which is confirmed by the Dunnett's test: CEO treatments are significantly different from the negative control and the blanc at this time (*Tables 18 and 19*). The homogeneity of variances was checked for t=6h and t=72h. The Levene's test can be consulted in *Annexe 3*.

In a nutshell, the slight decrease in the ratio (93% to 87%) means that the plant could manage any stress as already noted in the preliminary test

*Table 18. P-values obtained for each of the one-way analysis of variance performed on total glutathione content and glutathione ratio (p-value = 0.05). "–"means that there is no result for this time. The significant p-values are in bold: *significant (0.05); ** highly significant (0.01) and *** very high (0.001).*

	Total glutathione					
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=72h
Treatment	0.31	-	0.27	0.29	0.68	0.14
	Glutathione ratio					
Treatment					t=6h	t=72h
	-	-	-	-	0.12	**0.00082 *** **

Table 19. Dunnett's test performed on glutathione ratio at t= 72h. The same letter indicates that the treatments belong to the same group as the control.

Treatment	Mean
No injection (control)	91,816 A
EDTA+Tween 80	92,145 A
CEO 1%	86,707
CEO 4%	86,63

Some standard deviations are missing in *Figure 42* because the reduced glutathione concentration was just under the LOD. Some of them were between the LOD and LOQ but were considered in the figure anyway to give an idea of the ratio evolution. As the concentration cannot be quantitatively calculated, they will not be taken into account for the statistical analysis. These samples oxidized when they were taken out from the -80°C freezer despite careful handling. The causal factors can be multiple. This is a review of all the tricky stages during this period.

First of all, a good sampling is essential. Indeed, the challenge consists in altering the plant system as little as possible, especially when the study is dealing with the plant antioxidant processes in order to get a real picture of the situation inside the plant at the time of the sampling and not merely report the perturbation inflicted by the researcher as underlined by Majer et al. (2016). Leaves should be frozen as fast as possible (30 seconds maximum) in liquid nitrogen after picking (Majer et al., 2016). The leaves were immediately plunged into freezer bags containing liquid nitrogen. The bags were kept in polystyrene boxes with liquid nitrogen at the bottom before the storage at -80°C.

Secondly, while removing the liquid nitrogen in excess (because of the small size of the sampling, i.e. six leaves each time), the mass might have warmed up quickly. A few seconds can be enough. Some molecules as H_2O_2, for example, can be degraded in a few seconds at ambient temperature (Noctor, Mhamdi and Foyer, 2016). Compared to the sampling used to assess the repeatability of the glutathione method, the collected leaf mass was much lower (six leaves for the experiment against a half-full freeze bag for the protocol development). Moreover, the storage time between the sampling and the analysis was not the same. The timing of the field experiment and the glutathione protocol did not allow a direct analysis. The results at t=72h (leaves that spent the shortest time in the freezer) are the best. So, another explanation for the leaf oxidation may be the non-inactivation of some molecules at -80°C. Their activity or degradation could influence the glutathione analysis.

The extraction could also explain the decline in the results. The cells burst in acid and compounds from different cell compartments mix. It increases the risk of changing the redox status of the chemical compounds of interest (Majer et al., 2016).

The grinding is also important. It should be reduced to the minimum to avoid possible generation of prooxidative surroundings in the crusher or the mortar (Majer et al., 2016). An electric crusher was first used but the leaf mass was too small to get a thin and uniform powder. Even if the powder had a better aspect with the mortar, it was not quite satisfactory either.

A last possible explanation for concentrations under or between LOD and LOQ is the drawbacks of mbb. Despite careful manipulation, it could play a negative role because of its self-fluorescent and photodegradation which would interfere with thiol determination (Lavigne et al., 2006). However, this is the less convincing hypothesis having regards to the results.

The small mass had a likely negative impact on the leaf storage here. The glutathione method was the most impacted probably because it is a more sensitive technique.

5.2.2.5. Conjugated dienes

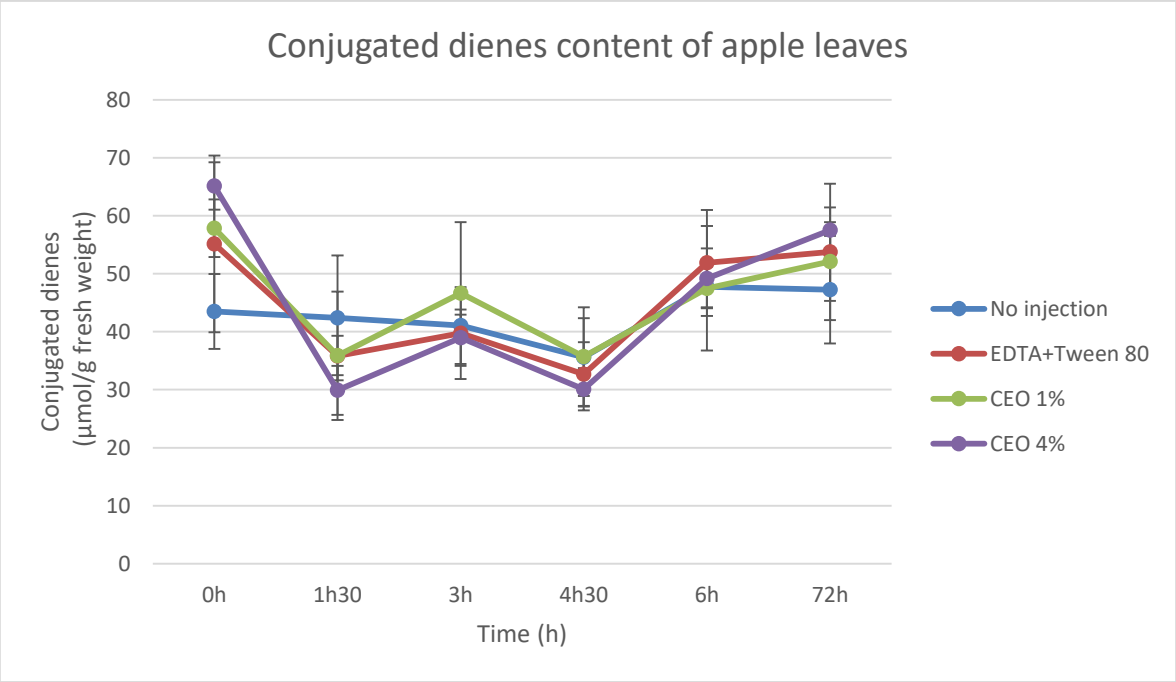

*Figure 43. Conjugated dienes content (µmol*g fresh weight^{-1}) of apple leaves (n=4).*

The blanc curve (i.e. No injection treatment) for the conjugated dienes (CDs) is relatively stable compared to the CDs content for the injected treatment where the CDs contents gently decrease before going up a little bit, then decrease once more and finally rise again in *Figure 43*.

However, during the protocol test, the non-stressed leaves were around 17,96 µmol/g fresh weight which represents about a third of the CDs content obtained here.

Regarding the results of the Levene's test in *Annexe 3*, the homogeneity of variances for the set of values at t=0h and t=6h was not met, unfortunately. The one-way ANOVA was performed all the same. This unsatisfactory condition of application must be kept in mind during its result interpretation. CEO 4% differs from the others at t=0h. Just as the carotenoid content does at t=0h, it illustrates the biological variation between leaves but does not implicate the treatment in *Tables 20 and 21*.

*Table 20. P-values obtained for each of the one-way analysis of variance performed on conjugated dienes content (p-value = 0.05). The significant p-values are in bold: *significant (0.05); ** highly significant (0.01) and *** very high.*

	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=72h
Treatment	**0.032 ***	0.25	0.51	0.56	0.84	0.42

Table 21. Dunnett's test performed on conjugated dienes content at t= 0h. The same letter indicates that the treatments belong to the same group as the control.

Treatment	Mean
No injection (control)	43,50 A
CEO 4%	65,14
CEO 1%	57,83 A
EDTA+Tween 80	55,14 A

To sum up the interpretations of the results obtained for all manipulations done in the lab, most of the time, the treatments were not significantly different from one another for whatever analysis. When they were, the EDTA+Tween 80 treatment in particular differed compared to the control followed by the CEO treatments as it can be seen in *Table 22*. The oxidative stress is managed by the plant antioxidant defences.

The observed leaf oxidation could distort the results. The difference between chl a and chl b content had to be higher. Chl a concentration is commonly equal to the double of the chl b in non-stressed plants (Sircelj et al., 2005). In addition, the CDs content was higher than in the healthy leaves taken to test the protocol. Finally, the major part of the glutathione results was unusable for the statistical analysis.

Moreover, the biological variability was demonstrated several times at t=0h. Thus, the significant difference between the results can also be explained by the biological variability in addition to the kinds of treatment. Unfortunately, they cannot be separated.

Table 22. Summary of the treatment(s) which is (are) significantly different from the control per time and per analysis. "–"means that there is no result for this time and "/" mentioned that all treatments are equal.

Plant physiological status and antioxidants								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
Fv/Fm ratio	/	/	/	/	/	EDTA+Tween 80	/	EDTA+Tween 80 CEO 1%
F0	/	/	/	/	/	EDTA+Tween 80 CEO 4%	EDTA+Tween 80	/
Carotenoids	/	/	/	/	/	-	/	-
Chl a	/	/	/	/	/	-	/	-
Chl b	/	/	EDTA+Tween 80 CEO 4%	/	/	-	/	-
Chl a/b ratio	/	EDTA+Tween 80	CEO 4% EDTA+Tween 80 CEO 1%	/	CEO 4 % CEO 1%	-	/	-
Total chl (a+b)	/	/	/	/	/	-	/	-
Glutathione ratio	/	/	/	/	/	-	CEO 1% CEO 4%	-
Lipid peroxidation								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
CDs	/	/	/	/	/	-	/	-

6. General discussion

The aim of this work was to study the physiological response of apple trees to an essential oil-emulsion pesticide at a molecular scale. One of the big challenges is to formulate and inject a biopesticide that shows great efficiency against the rosy aphid population without being phytotoxic.

Some molecules were targeted to initiate the assessment of the apple tree response. Gathering the results together is not an easy task not only because of the interconnecting pathways but also because the experiment was done as close as possible to agronomic conditions. Furthermore, a lot of other (a)biotic factors could influence the plant physiological response to oxidative stress. ROS production is one of the most prevalent responses to plant stress. ROS are also by-products of usual metabolic pathways in aerobic organisms during the electron transfer reactions that take place in the mitochondria and chloroplasts or during photorespiration in peroxisomes. As a consequence, they are naturally present in low concentrations in plant cells. The oxidative stress occurs when ROS over accumulate in the cell. They affect the plant metabolism in multiple ways and cause cellular damage. Their accumulation is not uniform across the cell (Noctor et al., 2016).

The main objective was to bring to light and evaluate the potential phytotoxicity induced by the emulsion with and without CEO compared to non-injected trees. To assess the phytotoxicity, the efficiency of the apple tree redox system against the potential oxidative stress was assessed thanks to the glutathione ratio and the carotenoids content determination. Carotenoids as well as the maximum quantum efficiency of PSII and chlorophylls (a and b) content also helped to investigate the physiological status that could decrease because of oxidative damage produced. Conjugated dienes were measured in this part too.

Only apple leaves were considered here. So, focusing on the photosynthesis apparatus was relevant. Chlorophyll a and b are abundant in green leaves and are responsible for light energy absorption and the transduction of this energy into a chemical one by photosynthesis. Carotenoids also absorb light energy and transfer it to chlorophyll. To protect the photosystems against a surplus of absorbed light energy compared to the photosynthesis capacity and ROS overaccumulation, lines of defence have to be efficient: (1) dissipation of excess excitation light as heat and re-emission of photons by fluorescence. These mechanisms that remove the trapped energy before it is transmitted along the electron transport chain are known as non-photochemical quenching (NPQ) and take place in the external antennae of PSII and (2) (non)-enzymatic antioxidant molecules that scavenge the ROS. In this study, the two defence lines were considered (Guidi et al., 2017).

The chlorophyll fluorescence is commonly measured in plant stress studies. It is a simple and quick way to address the stress. Fv/Fm ratio and F0 were investigated here. Indeed, in most study only Fv/Fm is taken into account. Its decrease is considered as an oxidative stress sign. This ratio is dependent on F0 and Fm. In oxidative stress case, F0 increases whereas Fm decreases (Ekmekci and Terzioglu, 2005). All in all, both Fv and Fm decrease (Fv=Fm-F0). However, it is possible that the ratio of the two does not decrease in the same way. That is why, F0 was also analysed in this study. Results showed that on the one hand, the Fv/Fm was around 0,83 and, on the other hand, that F0 was around 390. In conclusion, no inconsistency was observed for Fv/Fm ratio.

Nevertheless, remaining singlet-excited chl a can form a triplet-excited chl that can reduce molecular oxygen easily leading to ROS synthesis. ROS could also be produced by a deficiency of PSII caused by a stress factor. They have to be scavenged by enzymes and antioxidant molecules including carotenoids and glutathione. So, carotenoids can also be a photoprotective agent (Guidi et al., 2017).

In stress conditions, the carotenoids content diminishes to play the antioxidant role. No treatment led to a lower carotenoids concentration. Then, results showed that the oxidized leaves negatively impacted the total glutathione determination. Its concentration should be at least at 800µM. Thus, it also affected the ratio determination. Some total and reduced glutathione concentrations were under the LOQ. It was the only molecule impacted this way. As a consequence, the hypothesis of the oxidative burst between zero and six formulated during the preliminary test could not be verified on the field by a statistical analysis. The ratio decreases in oxidative stress conditions because the GSSG rises. Indeed, reduced glutathione scavenges ROS, serves as enzymes substrate to reduce H_2O_2 to water for example and takes a part in ascorbate regeneration which is another main antioxidant in plants. The oxidized form can be recycled to the reduced form by GRs found in the mitochondria, plastids, peroxisomes and cytosol (Noctor et al., 2011). It is impossible to say at which stage of the oxidative stress the rise of GSSG corresponds because of the recycling and the non-measurement of the other molecules that may interact with glutathione. When the plant is subject to a slow development of the oxidative stress, its antioxidant content increases with the intensity of the stress. The plant acclimates (Sircelj et al., 2005).

When the antioxidant plant defence cannot manage the oxidative stress, damage occurs. In this case, chl a and chl b should reduce whereas conjugated dienes should go up. In this study, the chl a/ chl b ratio was the most influenced by the treatments between t=1h30 and t=6h. The CDs content was higher than in non-stressed apple leaves analyzed during the protocol test.

On the whole, the plant seemed to manage the oxidative stress by itself. The negative control (i.e. EDTA+ Tween 80) already affected the apple trees. Sometimes it was the only treatment which was significantly different from the control. From this point, the formulation of the biopesticide can be discussed and revised. Some studies have already shown the herbicidal activity of CEO (Gniazdowska et al., 2015; Lins et al., 2019). However, it appeared here that the CEO-emulsion pesticide, even at 4% (chosen as an overdose) did not induce an oxidative stress than could not be managed by the plant antioxidant system in this kind of application. In the case where the significantly different response was only induced by the negative control, CEO might act as an elicitor to prepare the host defences or antioxidant system. Perina et al. (2019) proved that *C. zeylanicum* essential oil was able to stimulate scavenging ROS enzyme activity in citrus leaves.

As already said, only leaves were taken into account. It may well happen that a bigger oxidative stress occurs in another part of the plant that would then play a role of priming in leaves. Glutathione is known to have several other roles including regulator of genes expression. Thus, it could maximize the defences before the oxidative stress becomes too important. Some studies have already investigated the elicitor role of glutathione. For example, Wingate et al. (1988) proved that exogenous GSH applied to a suspension of cultured bean cells (*Phaseolus vulgaris* L.) stimulated the transcription of defence genes. The influence of GSH redox state in the cross talk of JA/SA was also explored. Frendo et al. (2013) reviewed some articles dealing with this subject and suggested that GSH content as well as its redox state may modulate JA-associated genes. In conclusion, GSH may seem to play an additional role in oxidative stress by interacting with phytohormones that regulate the induced resistances.

Last but not least, the molecules investigated here (even if they are pertinent for this study) are not sufficient to assess the plant physiological response in its entirety. There is no universal oxidative stress indicator. The whole antioxidant system of the plant is complex and composed by plenty of (non)-enzymatic molecules that work in synchrony (Noctor and Foyer, 1998). To evaluate it correctly some relevant factors must be considered such as ROS, antioxidants, metabolite markers, protein modifications at a transcript level and oxidative damage. Each measurement gives specific useful information and each approach has limitations that the scientist has to be aware of and it begins with sampling and extraction as it could be illustrated in this work (Noctor et al., 2016). The approach chosen for the analysis of oxidative stress depends on the aims of the work. So the oxidative stress may act more precisely than what the molecule tested in this work would be able to detect. This remark also concerns the damage that is possibly produced. This work only focused on the beginning of the injection. Cracks in the trunk were only noticed after two weeks. The sampling targeted the plant physiological response at an early stage of oxidative stress at the expense of damage that usually appears at a later stage. For example, damage on the vessels could lead to a poorer circulation of sap or can occur in long-term, impact the plant growth and in fine, the yield of fruit production.

It is noteworthy that the results could have been influenced by the oxidation occurred during the leaf storage, a non-optimal sampling/storage, (an)other (a)biotic factor(s) and/or the biological variability. For example, the Fv/Fm ratio revealed that the leaf tissues were healthy whereas unhealthy tissues were visible from t=72h. Thus, the question of what would be the best injection in time and space can therefore be asked.

7. Conclusion

This initiation study of the apple tree physiological response first allows the protocol development related to the quantification of glutathione by fluorescent labelling with monobromobimane by HPLC. This is a more sophisticated method though more complicated to implement correctly but it offers nice prospects in situ.

Then, this work set the oxidative burst between 0 and 6 hours after the injection. Results indicated that the plant could manage the oxidative stress by itself and so, no great damage was inflicted, especially on the photosynthetic apparatus. As a consequence, the herbicidal activity of CEO would have no effect in this kind of application. The results also demonstrated that studying the physiological plant response to oxidative stress is complex. The molecules investigated in this work were not enough to give a complete picture of what happens inside the plant. In addition, a lot of factors could influence the experiment carried out in conditions as close as possible of agronomic ones. Each result has to be replaced in its context.

To conclude, this study started to prove that the CEO-emulsion pesticide injected in the apple tree trunk is not phytotoxic for the plant. Its antioxidant system can manage the oxidative stress and no big oxidative damage is produced. Thus, this work adds a second advantage to the development of this kind of biopesticides which already got good results against the rosy aphid last year. Despite this promising conclusion, this study is not sufficient in itself. The work has to be continued.

8. Perspectives

Because each work has a limited time (even more this year because of the Covid-19 confinement), priorities had to be established. This work allowed two main things: the protocol development of glutathione and the initiation of the study of the physiological apple tree response to an EO-emulsion pesticide injected in the trunk.

Concerning the glutathione protocol, the validation of this protocol using mbb as fluorophore should be continued and some parameters must still be tested or improved such as the matrix effect and other acids that would limit thiol group oxidation or the use of a masking agent. To go further, this protocol should be modified to allow the observation of glutathione in situ in different cell organelles by fluorescence imaging. Indeed, the lifetime of ROS varies among different cellular compartments, their accumulation is not uniform across the cell and the plant plasma membrane is permeable to monobromo- and monochloro-bimanes (Majer et al., 2016).

The experiment showed that the kinds of treatments do not induce big changes inside the plant. The tree could manage the oxidative stress itself. However, it also revealed that the conditions of the sampling and of its storage must to be improved according to the kind of analysis. Some analyses will require fresh biological samples to not be altered. Moreover, the number of repetitions has to be generally increased to enhance the robustness of the results and their interpretations.

Then, the whole antioxidant system of the plant is composed by plenty of (non)-enzymatic molecules. This work investigated a small part of it and thus does not allow to correctly picture what occurs in the plant. The oxidative stress may act more precisely than the molecule tested in this work would be able to detect. There is no absolute molecule to assess the oxidative stress and the plant response. It is a whole. So, first of all, ROS which are at the heart of the oxidative stress must be measured (as fast as possible after the sampling because they can be very unstable). For example, H_2O_2 can be assessed with a fluorimetric kit purchased in the market. Then, there are the (non)-enzymatic molecules that could be investigated such as ascorbic acid and scavenging enzymes (e.g. superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX)). Finally, oxidative damage could be measured such as lipid peroxidation, oxidation of proteins and electrolyte leakage to assess the membrane integrity. Lipid peroxides can supply some information such as the location and source of ROS (Noctor et al., 2016). Damage could also occur on the plant vessels which could be evaluated by plant histological sections. Depending on the results obtained with these first perspectives, proteomic and transcriptomic studies could be interesting to consider.

The efficiency of each treatment should be tested on the rosy apple aphid population in parallel to VOCs analysis contained and emitted by apple leaves. It would allow correlating the impacts of the biopesticide according to its concentration and its detection in the leaves on the plant and the insect populations.

In addition, as phytohormones take part in the establishment of plant defence mechanisms and interact with antioxidants as well, they should be investigated.

Ultimately, this work was focused only on leaves. It could be interesting to extend the study to the other parts of the tree (e.g. roots and trunk) to be complete.

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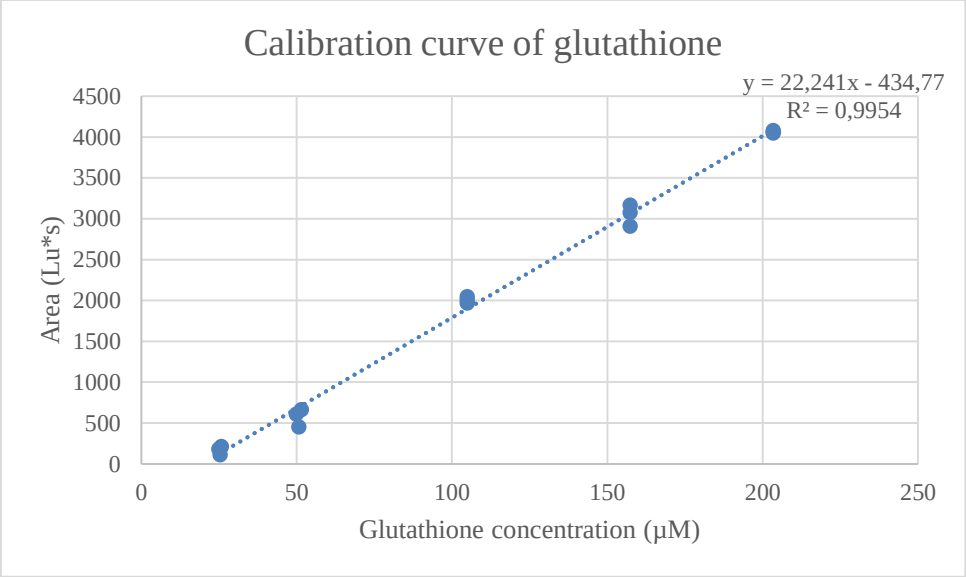
10. Annexes

Annex 1: Characterization of cinnamon (Cinnamomum cassia J.Presl) EO. The Retention Time (RT) (min), name and relative area (%) are presented (molecular profile provided by Pranarôm).

Retention time (min)	Name	relative area (%)
7,3	alpha-PINENE	0,08
7,4	alpha-THUYENE	0,01
8,7	CAMPHENE	0,05
9,1	HEXANAL	0,01
10,1	beta-PINENE	0,03
14,1	LIMONENE	0,04
14,5	1,8-CINEOLE	0,02
17,2	STYRENE	0,17
18	p-CYMENE	0,06
18,7	TERPINOLENE	0,01
21,9	6-METHYL-5-HEPTENE-2-ONE	0,01
25,5	NONANAL	0,02
26,2	COMPOSÉ BENZYLIQUE	0,02
29,8	SESQUITERPENE	0,01
30,2	SESQUITERPENE	0,02
30,3	delta-ELEMENE	0,01
31	CYCLOSATIVENE + ISOLEDENE	0,12
31,2	YLANGENE	0,06
31,8	alpha-COPAENE	0,8
32,9	COMPOSÉ AROMATIQUE	0,04
33,3	CAMPHRE	0,04
33,6	BENZALDEHYDE	1,14
33,8	beta-BOURBONENE	0,02
36,8	LINALOL	0,04
37,6	SESQUITERPENE	0,05
37,9	alpha-trans-BERGAMOTENE	0,04
38,2	beta-CARYOPHYLLENE	0,12
38,5	TERPINENE-4-OL	0,04
38,8	AROMADENDRENE	0,03
40,6	COMPOSÉ AROMATIQUE	0,14
41,1	ACETOPHENONE	0,08
41,3	COMPOSÉ AROMATIQUE Mw=132	0,14
41,8	ALLO-AROMADENDRENE	0,12
42,6	alpha-HUMULENE + ESTRAGOLE	0,04
43	ALDEHYDE SALICYLIQUE	0,69
43,8	gamma-MUUROLENE	0,14
44,2	BORNEOL	0,3
44,4	alpha-TERPINEOL + LEDENE	0,08
46	alpha-MUUROLENE	0,08

46,2	beta-BISABOLENE	0,13
47,7	2-METHYL BENZOFURANE	0,4
47,9	delta-CADINENE + gamma-CADINENE	0,29
48,3	SALICYLATE DE METHYLE	0,09
49	BENZENEPROPANAL	0,78
49,2	alpha-CURCUMENE	0,09
49,5	Trans-alpha-BISABOLENE	0,05
50,2	COMPOSÉ AROMATIQUE	0,04
50,8	COMPOSÉ AROMATIQUE	0,03
51,1	ACETATE DE 2-PHENYLETHYLE	0,03
51,8	Trans-ANETHOL	0,02
52,1	CALAMENENE	0,03
52,9	ACIDE HEXANOIQUE	0,03
53,5	COMPOSÉ PHÉNOLIQUE	0,12
54,4	ALCOOL BENZYLIQUE	0,27
55,4	Z-CINNAMALDEHYDE	0,34
56,3	ALCOOL PHENYLETHYLIQUE	0,98
58,5	COMPOSÉ AROMATIQUE	0,05
59,1	2-METHOXY BENZALDEHYDE	0,42
60,2	OXYDE DE CARYOPHYLLENE	0,05
63,5	**E-CINNAMALDEHYDE**	**79,49**
63,8	COMPOSÉ AROMATIQUE Mw=206	2,46
65,4	2-METHOXYPHENYLACETONE	0,24
67,4	SPATHULENOL	0,1
68,8	ACETATE DE CINNAMYLE	0,46
69,5	EUGENOL	0,04
69,8	TRIMETHYL PENTADECANONE	0,02
70,5	T-CADINOL	0,02
71,3	SANDARACOPIMARADIENE ISOMERE	0,03
72,1	alpha-BISABOLOL	0,05
72,3	SESQUITERPENOL	0,03
72,6	alpha-CADINOL	0,02
74,4	2-METHOXY-CINNAMALDEHYDE	0,04
75,1	ALCOOL CINNAMIQUE	0,13
75,7	CARYOPHYLLA-3,7-DIEN-6-OL	0,02
78,4	EPOXYDE SESQUITERPENIQUE	0,07
82,2	**Trans-o-METHOXY CINNAMALDEHYDE**	**5,26**
82,6	**COUMARINE Mw=146**	**2,1**
90,3	BENZOATE DE BENZYLE	0,07
93,8	COMPOSÉ AROMATIQUE	0,04
99,5	COMPOSÉ AROMATIQUE	0,14
	TOTAL	**99,99**

Annex 2: Calibration curve of glutathione.



*Annex 3: Summary of P-values obtained for each Levene's test on all molecules measured (p-value = 0.05). The significant p-values are in bold: *significant (0.05); ** highly significant (0.01) and *** very highly significant difference (0.001). "–"means that there is no result for this time.*

The maximum quantum efficiency of PSII (Fv/Fm ratio)								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
	0.92	0.79	0.72	0.73	0.97	0.87	0.38	0.93
F0								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
	0.45	0.72	0.23	0.83	0.91	0.89	0.60	0.61
Chlorophyll a content								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
	0.27	0.60	0.73	0.40	0.54	-	0.58	-
Chlorophyll b content								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
	0.54	0.58	0.52	0.55	0.39	-	0.53	-
Chl a/b ratio								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
	0.36	0.67	**0.00028 *** **	0.95	0.44	-	0.79	-
Logarithmic transformation	-	-	**4.8e-05 *** **	-	-	-	-	-
Square root transformation	-	-	**0.00012 *** **	-	-	-	-	-
Total chlorophyll (a+b) content								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
	0.34	0.59	0.52	0.44	0.44	-	0.57	-
Carotenoid content								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
	0.38	0.32	0.72	0.69	0.52	-	0.38	-

Total glutathione								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
	0.49	-	0.83	0.7053	0.60	-	0.87	-
Glutathione ratio								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
	-	-	-	-	0.67	-	0.33	-
Conjugated dienes								
	t=0h	t=1h30	t=3h	t=4h30	t=6h	t=24h	t=72h	t=192h
	0.012 *	0.25	0.56	0.35	**0.035 ***	-	0.78	-
Logarithmic transformation	**0.024 ***	0.30	0.60	0.49	**0.023 ***	-	0.79	-
Square root transformation	**0.016 ***	0.27	0.58	0.41	**0.028 ***	-	0.78	-