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Unintentional effects of biopesticides on biocontrol agents

Méлина Cointe

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Méлина Cointe. Unintentional effects of biopesticides on biocontrol agents. Life Sciences [q-bio]. 2020. dumas-02986694

HAL Id: dumas-02986694

<https://dumas.ccsd.cnrs.fr/dumas-02986694v1>

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AGROCAMPUS
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INRAE



Année universitaire :2019-2020

Spécialité :

.....Agronomie.....

Spécialisation (et option éventuelle) :

.....Agroecology.....

Mémoire de Fin d'Études

- d'Ingénieur de l'Institut Supérieur des Sciences agronomiques, agroalimentaires, horticoles et du paysage
- de Master de l'Institut Supérieur des Sciences agronomiques, agroalimentaires, horticoles et du paysage
- d'un autre établissement (étudiant arrivé en M2)

Unintentional effects of biopesticides on biocontrol agents

Par : Mélina COINTE



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Soutenu à RENNES le* 09/09/2020

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Les analyses et les conclusions de ce travail d'étudiant n'engagent que la responsabilité de son auteur et non celle d'AGROCAMPUS OUEST

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ACKNOWLEDGMENTS

First, I would like to thank my two supervisors Louise Van Oudenhove and Vincent Calcagno for having me in their project and for having given me the opportunity to express freely my own ideas. I would also like to thank them for their passion, their advice, and their attention, especially during the period of the lockdown. It has represented a real motivation and helped me to keep going even in these unusual work conditions.

The second person I would like to thank is Manuel Plantegenest, my internship tutor. Even if we did not exchange a lot, he always demonstrated a great availability which has been really reassuring. Moreover, thanks to his quick answers at the beginning of my internship, I have been able to steer my report and my analysis to the right track quite quickly.

Then, I would like to thank my laboratory team within which I have been lucky to work. I would like to thank especially Louise, Guy, Lydia, Cécile, Hicham and Julie for always having been in such a good mood and for having demonstrated a lot of attention and care towards me. Special thanks to Guy and Lydia for their help during my laboratory experiments.

Finally, I would like to thank my best friend Margaux for her care and love. Thank you for having made the lockdown a joyful period, within which it was easy to work and to carry out my internship. Thank you also for your unfailing and long-distance support which helped me for six months and which will help me for the next coming years.

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LIST OF ABBREVIATIONS

AIC: Akaike Information Criterion

ACHE: Acetylcholinesterase

CT: Chemotype

EO: Essential Oil

FDR: False Discovery Rate

GLM: Generalized Linear Models

GlmmTMB: Generalized Linear Mixed Models using Template Model Builder

HR: Humidity Rate

IPM: Integrated Pest Management

PCA: Principal Component Analysis

T: Temperature

WSS: Within-Cluster Sum of Square

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1- Introduction

“Behind the discourse on sustainable development, it is actually a new social contract that is offered to farmers. And it cannot be excluded that sustainability represents, for the agriculture of the next decades, an "engine" as efficient as productivity was during the previous period” (Landais, 1998). Indeed, for several decades now, sustainability has been and still is the main focus for agronomy. This leads to the concept of agroecology which can be defined as the integrated study of the food supply as a whole, including its ecological, economic and social dimensions, or more simply as the “ecology of food systems” (Francis et al., 2003) . This sustainable approach deals with various issues such as the limitation of environmental pollution, by reducing the use of fertilizers and pesticides (Géo_rédaction, 2018). To achieve this objective, various strategies and methods can be implemented, including for instance, the use of biological control. Harry Scott Smith, an American entomologist, defined it as “the use of natural enemies (whether introduced or otherwise manipulated) to control insect pests (Smith, 1996). There are three types of biological control: by introduction (introduction of a natural enemy of exotic origin), by increase (increase in the density of natural enemies by recurrent releases), and by conservation (methods favouring the action of natural enemies already present in the system). The oophagous parasitoids *Trichogramma sp.* are commonly used as biocontrol agents. They are micro-hymenoptera which lay their eggs inside the eggs of their insect-hosts. They are commercialized by several companies and are the most used agents against some pests such as the corn borer (Naïbo, Druesne, 2008). Their ease of rearing on alternative hosts, and their wide geographic distribution make them interesting biological control agents (Souza et al., 2016).

However, the development of new agricultural policies may actually result in unforeseen problems for biological control and more especially in integrated pest management. For instance, in the context of the plan “Ecophyto II+” which aims to reduce by 50% the use of synthetic chemicals in agricultural practices from now until 2025 (agriculture.gouv, 2018), the use of essential oil seems to be an interesting approach. Essential oils can be defined as aromatic, volatile liquids obtained from plant material through steam distillation. These substances are chemically pure (non-diluted) compounds that are volatile under normal conditions (Ríos, 2016). Nowadays more and more studies state that essential oils are quite efficient substitutes for many synthetic insecticides. This efficiency comes from the fact that they have a widespread range of activities and most of the essential oils are lethal (table 1). Used as pesticides, the essential oils should not cause side effects on non-target insects (Regnault-Roger, 2013). But there are evidences that some essential oils utilized in integrated pest management can be harmful to these organisms. It has been demonstrated that several essential oils including tea tree, mint, Thyme, savory and clove, have a negative impact on *Typhlodromus pyri* (a mite, the natural enemy of the European mite (Deluche, 2019)) and honey bees by significantly increasing their mortality (Belleil, Chavassieux, 2014).

Table 1 : Summary table of essential oils effects on various pests. The oils indicated in bold are the ones which have been used in the experiments.

Essential Oil	Pest	Effect	Source
Clove, Savoury, Eucalyptus	Pear tree scab Potatoes downy mildew	Reduction of the disease progression	Vidal, 2016
Clove, Lavender	<i>Tribolium Castaneum</i>	Lethal effect	Mohamed et al., 2017
Eucalyptus, Coriander	Leafhopper <i>Amrasca devastans</i> (Homoptera: Cicadellidae)	Inhibition of oviposition	(Saxena, Basit, 1982)
Sweet flag	kelp fly <i>Coelopa frigida</i> (Diptera: Coelopidae)	Hatchability and fecundity reduced	(Ramos Ocampo, Hsia, 1986)
Basil , citronela	<i>Acanthoscelides obtectus</i> (Coleoptera: Chrysomelidae: Bruchinae)	Significant increase of the mortality Decrease of emergence	Rodríguez-González et al., 2019
Fennel	<i>Rhyzopertha dominica</i> (F.), <i>Sitophilus zeamais</i> Motschulsky and <i>Tribolium confusum</i>	Repellent	Bedini et al., 2016
Mugwort	<i>Tribolium castaneum</i> (Herbst), <i>Callosobruchus maculatus</i> (F.) and <i>Rhyzopertha dominica</i>	Lethal effect	Eljazi et al., 2020
Green Anise	Turnip aphids : <i>Lipaphis pseudobrassicae</i> (Davis) (Aphididae: Homoptera)	Lethal effect	Sampson et al., 2005
Oregano	<i>Sitophilus oryzae</i> (L.) (Coleoptera: Curculionidae) <i>Rhyzopertha dominica</i> (F.) (Coleoptera: Bostrychidae)	Lethal effect	Karan, Şimşek, 2018
Peppermint	Rice Weevil: <i>Sitophilus oryzae</i> L. Rice Moth: <i>Corcyra cephalonica</i> (St.)	Lethal effect	Khani, 2012
Sweet orange	Housefly : <i>Musca domestica</i> L. (Diptera: Muscidae)	Lethal effect (dehydration)	Kumar et al., 2012
Rosemary	Rice weevil: <i>Sitophylus oryzae</i>	Lethal effect	Sehari et al., 2018
Thyme	larvae of lesser mealworm, <i>Alphitobius diaperinus</i> Panzer (Coleoptera: Tenebrionidae)	Lethal effect Decrease of the young larvae growth	Szczepanik et al., 2012

According to other laboratory studies, these essential oils also make *Tryphlodromus pyri*'s fertility decrease , but do not affect the viability of the descendants (Vital et al., 2018). It is possible to add that the following essential oils: *Ocimum americanum*, *Hyptis suaveolens*, and *Lippia multiflora* significantly decrease the parasitism rate and increase the mortality of *Dinarmus basalis*, a parasitoid of *Callosobruchus maculatus* (cowpea seeds pest) (Ilboudo, 2009).

Until today, several non-intentional effects have already been assessed on different species of *Trichogramma sp.* (figure 1). For instance, 10 different essential oils (andiroba, black pepper, clove, garlic, ginger, neem, oregano, peppermint, sweet orange, and Thyme) have been tested and significantly reduce the female longevity and the parasitism rate of *Trichogramma pretiosum* (Parreira et al., 2018). Also, *T. evanescens* is repulsed by various essential oils and its survival is negatively impacted (Fillaud, 2019). The reaction could actually depend on the species. Indeed, the sensitivity of the sensory system varies between the different trichogram species (Voegelé et al., 1975). That is why it is interesting to assess the effect of various essential oils on different species of trichograms.

In this work, I will look for unintentional effects on different species of trichograms. In this context, it is important to mention that the fitness of female parasitoid wasps as *Trichogramma sp.* depends mainly on their ability to locate and parasitise hosts. The process of parasitisation can be divided into distinct steps: habitat finding, host finding and host acceptance, which can collectively be summarised as host selection (Nordlund, 1994). Host selection thus includes a set of behaviours, determined, and influenced by a variety of stimuli. However, insect eggs are largely odorless because they do not feed, nor release faeces. Moreover, many eggs are well protected physically and/or chemically to make up for their immobility and exposure to enemies and environmental influences (Fatouros et al., 2008). Thus, foraging females of egg parasitoids e.g. *Trichogramma* need to overcome the difficulty to locate host eggs by exploiting different cues (Noldus, 1989). Actually, there exist volatile cues corresponding to different types of semiochemical substances: the ones coming from the host: kairomones, and the ones coming from the plants: synomones (Klowden, 2013). Synomones can be constitutively produced: Volatile Organic Compounds (VOCs), or released as a defence mechanism when plants are attacked: Herbivore Induced Plant Volatiles (War et al., 2011). VOC such as the one released by prostrate mug bean varieties can significantly attract *Trichogramma ostrinae* (Bai et al., 2011). In that study, a preliminary control experiment showed that in the absence of an odour source, females of *T. ostrinae* distribute randomly over the four flow fields of a four-armed airflow olfactometer. Then, *T. ostrinae* were significantly attracted to the volatiles from whole plants of different prostrate mug bean varieties. Some effects of kairomones on *Trichogramma sp.* have also been assessed. For instance, *Trichogramma chilonis*, and its host insect, *Helicoverpa assulta*, were studied in laboratory experiments. *T. chilonis* was attracted to the sex pheromone of *H. assulta* (Boo, Yang, 2000).



Figure 1 : Picture of two trichograms females (Trichogramma Cordubensis, strain PUG029) laying their eggs on E.Khueniella's eggs

Similar results have been shown with sex pheromones of the lepidopteran hosts such as ZETA from *Ephestia spp.* on *T.evanescens* (Schöller, Prozell, 2002), as well as contact chemicals on the surface of the host eggs (Wilson, Woods, 2016) have also been identified or proposed as such cues. Females of *T.maïdis* are also attracted to synthetic sex pheromones and maize extracts (Kaiser, 1988).

This relies on processes of olfaction. That is the reason why it is interesting to focus on the way essential oils can impact *Trichogramma.sp* through olfactometry experiments. Two years ago, olfactometry experiments have already been initiated in the team that welcomed me, on three strains of *T.cacoeciae*: PMBIO1, ACJY144 and PMBIO1. They found out that Rosemary essential oil actually attracts the three strains and Mugwort essential oil attracts the strain ACJY144 (Cazier, 2018). Even if the Rosemary essential oil seems to attract *T.cacoeciae*, it also kills them after a certain amount of time. The chemotype used in the experiment is the cineole one (Cazier, 2018). Depending on the biotope (sun exposure, climate, soil composition, altitude, etc.), the same plant can secrete different essences. These variations in the biochemical composition of essential oils generate the concept of chemotype (CT). Two chemotypes of the same essential oil will show not only different activities but also very variable toxicities (ANON., 2016). Actually, it has been showed that some compounds of essential oils such as monoterpenes, representing a range of functional groups: citral (aldehyde), pulegone (ketone), linalool (alcohol), (-)-bornyl acetate (ester), and cineole (cyclic ether) can inhibit or inactivate the acetylcholinesterase (ACHE) which is one of the most important enzymes in neuro-neuronal and neuromuscular junctions in both insects and mammals (Jankowska et al., 2017). It causes acetylcholine to accumulate at the cholinergic site (figure 2). This produces continuous stimulation of cholinergic nerve fibres throughout the central and peripheral nervous system, followed by paralysis and death of the organism (Corbett et al., 1984). All of these terpenes kill the coleoptera *Tribolium castaneum*, and the mortality is dose responsive (Ryan, Byrne, 1988). Also, they all inhibit the ACHE.

It is possible to add that Cockroaches and house flies both exposed to linalool and terpineol, exhibit overt hyperactivity, loss of coordination, and tremors. In cat fleas, trembling and paralysis of legs can also be noted, followed by convulsions and death (Coats et al., 1991). Moreover, chemical analyses have showed that some volatiles produced by the plants such as cineole seems to attract the parasitoid *Cotesia glomerate* on cabbage which has been attacked by *Pieris brassicae* caterpillars (Scascighini et al., 2005). Also, the grape berry moth *Lobesia botrana* is significantly attracted by 1.8-cineole which has been extracted from Rosemary officinalis (Katerinopoulos et al., 2005). Based on these results, it is possible to suppose that the cineole contained in the Rosemary is responsible for the attraction but at the same time kills *T.cacoecie* by inhibiting the ACHE. In respect of Mugwort essential oil, 1.8 cineole is also one of the components but not the major one (Alizadehl et al., 2012; Juddzentiene, Buzelyte, 2006). This last statement reinforces the hypothesis that 1.8-cineole might be responsible for the attractive effect.

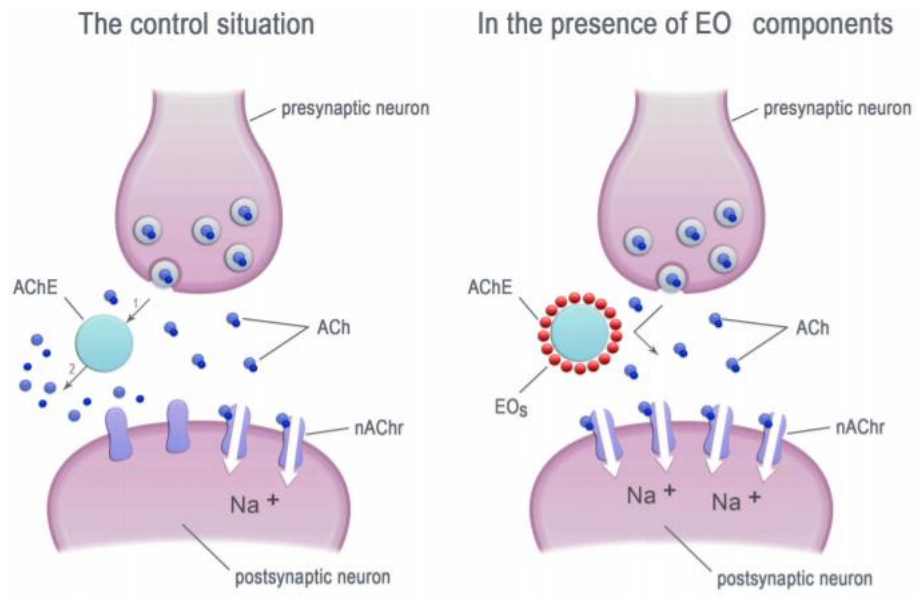


Figure 2: The essential oils' components inhibit the acetylcholinesterase (AChE) activity. Ach-acetylcholinesterase, nAChr-nicotinic acetylcholine receptors, Eos-essential oil components.

My project, in this internship, has been to follow up on the study of the unintentional effects of 10 essential oils (basil, coriander, Fennel, green anise, Mugwort, oregano, peppermint, Rosemary, sweet orange and Thyme) by completing the work that had been done (table 2) on one trichogram species of agricultural interest: *T.cacoeciae* (Ravaomanarivo Harivelo, 2014).

The hypothesis behind these unintentional effects is that the essential oils may be attractive, repulsive or neutral towards *T.cacoeciae*. Furthermore, I formulated and tested the hypothesis that 1.8-cineole was responsible for some observed attraction effects (Cazier 2018). This research seeks to optimize both the use of these biological auxiliaries and of essential oils in the context of integrated pest management. For this purpose, two types of laboratory experiment have been conducted: the first one consisting in olfactometry and the second one consisting in manual, and automatized olfactometry videos to better understand the effect of essential oils on trichograms' behaviour.

2- Material and methods

2.1 Biological materials for the experiments

2.1.1 Trichogram species and rearing conditions

Trichogram presentation

Trichograms are Chalcidian micro-Hymenoptera of the Trichogrammatidae family. There are currently 280 species known, all from the genus *Trichogramma* (Pintureau, 2012). Their size is often less than a millimetre. Trichograms are oophagous parasitoids: their larva develops inside the egg of the Insect-host, whose embryo is killed at an early stage during the larval life of the parasitoid. The parasitized eggs become black after a few days (the trichograms larva secretes a viscous substance which is applied against the vitelline membrane of the host egg and which hardens and darkens), which allows to distinguish them from healthy eggs. The disintegrated host tissues and yolk serve as food for the trichograms larva and ensure its development until metamorphosis, transformation into a nymph and then into an "imago" (adult) (figure 3). Then this imago leaves the egg and lives a free life (an average duration of 15 days at 18°C (Colombel et al., [sans date])), devoted to mating and for female, to host searching. Adult can feed on sweet substances (e.g aphid honeydew) or protein-rich substances (flower pollen) (Hawlitzky, 1982).

The range of hosts of trichograms is very wide since it includes many Lepidoptera, Diptera, Hemiptera and Coleoptera. Many studies aim to characterize this genus to improve its use in biological control (Smith, 1996). There exist two modes of reproduction in the genus *Trichogramma*, depending on the species or population considered (Consoli et al., 2010). The first mode of reproduction is by arrhenotokous parthenogenesis (for haplo-diploid species): males come from unfertilized eggs and are therefore haploid. As a consequence, unmated females only produce haploid males, and mated females strongly influence the sex ratio of their offspring by controlling the fertilization of oocytes (Suzuki et al., 1984), generally

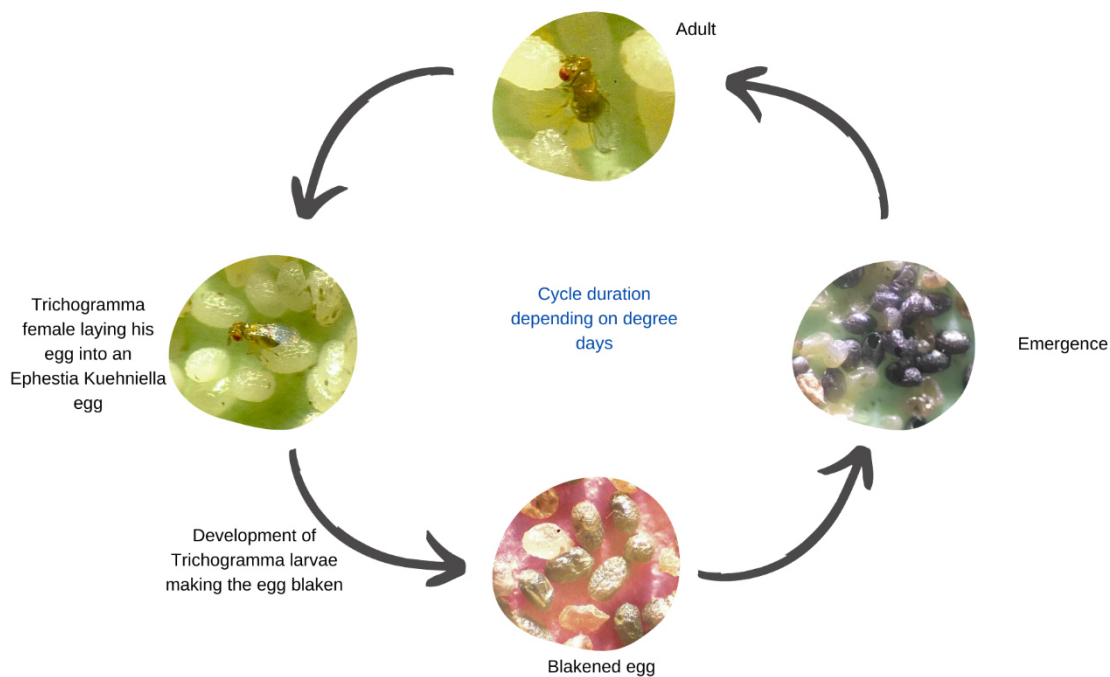


Figure 3 : Illustration of the development cycle of Trichogramma sp.

Table 2 : Summary table of the conducted experiments. In yellow, the experiments previously conducted in the laboratory. In green, the experiments conducted by myself

Trichogram Species	Strain	Rosemary	Sweet Orange	Oregano	Peppermint	Basil
<i>Cordubensis</i>	PUG029					
<i>Cacoeciae</i>	PJ					
<i>Cacoeciae</i>	PMbio1					
<i>Cacoeciae</i>	ACJY144					
Trichogram Species	Strain	Thyme	Mugwort	Fennel	Green Anise	Coriander
<i>Cordubensis</i>	PUG029					
<i>Cacoeciae</i>	PJ					
<i>Cacoeciae</i>	PMbio1					
<i>Cacoeciae</i>	ACJY144					
Trichogram Species	Strain	Cineole	Cineole 0,05µL / 0,5µL	Cineole 1µL/ 1,5µL		
<i>Cacoeciae</i>	ACJY144					

generally producing a majority of diploid females. The second mode is the thelytokous parthenogenesis: diploid females produce other females with the same genome (clones). This is the case, for example, of *T.cacoeciae*. Finally, some arrhenotokous populations are permanently infected with Wolbachia bacteria, resulting in thelytoky (Cook, Butcher, 1999).

All our experiments were done with three strains of *T.cacoeciae*: PMBIO1, ACJY144 and PJ.

Rearing conditions

The production of trichograms depends on the availability and quality of an insect host. A suitable host of substitution must be easy to produce and sustain trichogram reproduction with high fitness (Colombel et al., [sans date]). *Ephestia kuehniella* is one such. It allows a better fertility, longevity, and a higher capacity of flight compared to alternatives like *Sitotroga cerealella* (Lepidoptera: Tineidae) which was for long the most commonly used host. Eggs of *E.Kuehniella* are obtained from the private company Bioline Agrosiences. Batches of *E.Kuehniella* irradiated eggs were then stored at $T 2 \pm 1 \text{ } ^\circ \text{C}$, HR $75 \pm 5\%$ to ensure its quality. Irradiation blocks *E.Kuehniella*'s development and allows its conservation. This stock is renewed every Wednesday to work with fresh eggs.

Unparasitized eggs from *E. kuehniella* were attached (with $\frac{1}{3}$ of transparent Rubafix glue without solvent + $\frac{2}{3}$ of distilled water) on a cardboard strip (4.5 cm x 1 cm). This strip is then placed in a glass tube, in contact with a second strip covered with parasitized *E. kuehniella* eggs, containing trichograms ready to emerge. The two strips are maintained in the same tube for 24 hours in order to let the trichograms females lay their eggs in the non-parasitised host eggs. After that, the strip carrying the newly parasitised eggs is put in another tube and kept at 25 or 19 celsius degrees depending on the desired development time. Indeed, the development cycle of trichograms strongly depends on climatic temperature as it is typical insects. Temperature regulates metabolism speed (Colombel et al., [sans date]). Thus, the temperature used for rearing varied across species. It was determined in such a way to produce the desired development time, i.e to facilitate the organization of lab work. The temperatures were calculated based on the total number of degree-days required by a given species and by its minimal development temperature.

Example of calculation for *T. cacoeciae*:

- Degree days required: 165
- Minimal development temperature: 10°C
- Chosen rearing duration: 14 days
- Two rearing temperatures are used: 25°C and 19°C
- x: rearing duration at 25°C
- y: rearing duration at 19 °C

With all this information, it is only a matter of solving the following linear system with two equations, with two unknowns:

$$x + y = 14$$

$$x(25 - 10) + y(19 - 10) = 165$$

This leads to x equals to 6,5 and y equals to 7,5. Consequently, trichograms from cacoeciae species must spend 6,5 days at 25°C and then 7,5 days at 19°C to accumulate 165-degree days.

Based on this possible manipulation of the rearing duration, it was possible predict the date of adult emergence and organize the different experiments.

2.1.2 Essential oils

The olfactometry experiments as well as the survival tests have been conducted with 10 different essential oils: Basil, Coriander, Fennel, Green Anise, Mugwort, Oregano, Peppermint, Rosemary, Sweet Orange, and Thyme (see table 3). They were chosen based on their possible use as bio-pesticides. They were all purchased from the Italian company Esperis.

As an addition to these ten essential oils, the component which may actually be the one responsible for the attraction of the Rosemary essential oil, namely cineole, has also been tested on ACJY144 (Cazier, 2018). 1,8-cineole is a monoterpene cyclic ether found abundantly in nature. It is the major component of an essential oil isolated from *Eucalyptus globulus* leaves (up to 80%), but it can be also found in other species such as Rosemary (*Rosmarinus officinalis*, cineole chemotype), sage (*Salvia officinalis*), and mint (*Mentha* ssp.) (Aprotosoie et al., 2019). This component has been tested as pure but also diluted in paraffine oil in order to obtain different concentrations: 1%, 10%, 20% and 30%. Knowing that 5 μ l has been used in the odour zones for the pure cineole, these concentrations are respectively equivalent to the following volumes: 0.05 μ L, 0,5 μ L, 1 μ L, and 1,5 μ L.

2.2 Olfactometry

2.2.1 Experimental device

To study the way the different essential oils influenced the behaviour of trichograms, a four-way olfactometer has been used.

The experimental device (figure 4) is composed of a blower air pump (aquaOxy 2000) (1), from which two air outlets are connected to air filters (2) allowing to remove any impurities contained in the air. Then the system is divided into four connections. Each connection is connected to a flowmeter (DK 800 / PV) (3). The air then passes through a first glass bottle (4) containing distilled water (400mL) in order to be humidified. Then he goes through a second glass bottle (5) which, according to the experience, contains an odour of essential oil or nothing (no odour). Thanks to a tap system, it is possible to switch from the odorless bottle (control) to the bottle containing the odour during the experiment. Finally, the air is transmitted to the 4 arms of the olfactometer (6) and exits through the hole in the central area where the trichograms have been introduced. The air is then sucked out thanks to the vacuum system and expelled outside the room. The elements are connected by Teflon pipes, an inert material

Table 3 : Essential oils' presentation

Essential oil common name	Latin Name	Family
Basil	<i>Ocimum basilicum</i>	Lamiaceae
Coriander	<i>Coriandrum sativum</i>	Apiaceae
Fennel	<i>Foeniculum vulgare</i>	Apiaceae
Green anise	<i>Pimpinella anisum</i>	Apiaceae
Mugwort	<i>Artemisia vulgaris</i>	Asteraceae
Oregano	<i>Origanum vulgare</i>	Lamiaceae
Peppermint	<i>Mentha piperita</i>	Lamiaceae
Rosemary	<i>Rosmarinus officinalis</i>	Lamiaceae
Sweet orange	<i>Citrus aurantium</i>	Rutaceae
Thyme	<i>Thymus vulgaris</i>	Lamiaceae

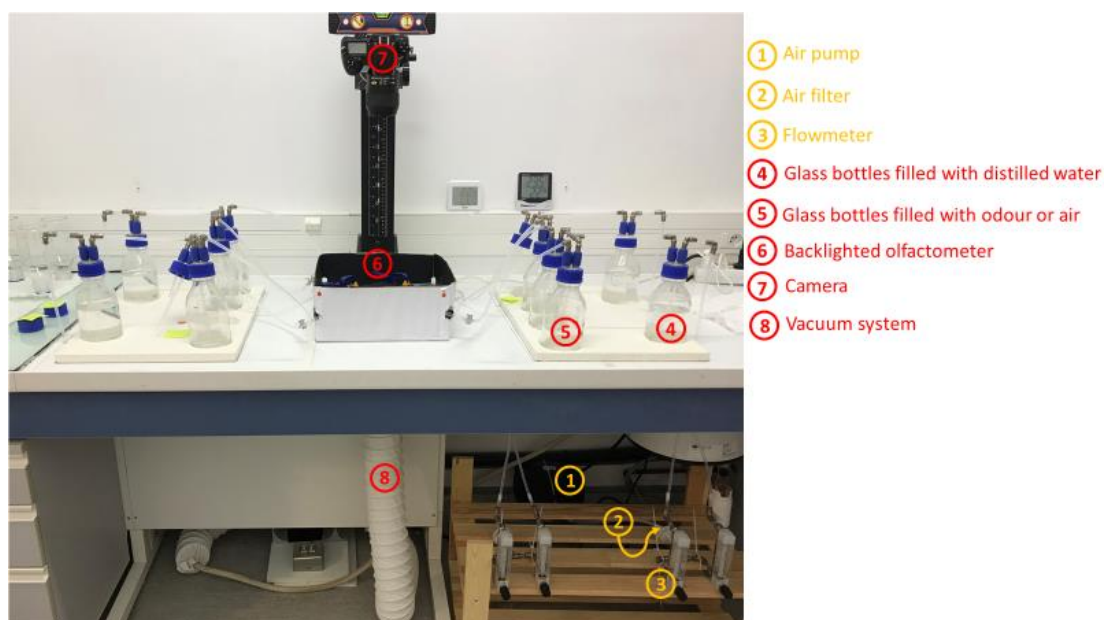


Figure 4 : Illustration of the experimental device

which does not emit or absorb any odour so as not to have an odorous background noise liable to have undesirable effects on the behaviour of the trichograms.

The olfactometer (6) is composed of a 4-arm star observation chamber (figure 5) and has dimensions adapted to the size and the moving capacities of the trichograms (14 x 14 x 0.8 mm). Each branch receives the same flow rate, that is adjusted in order to avoid the generation of turbulence that would mix the odours (Cazier, 2018). The four air flows enter the chamber, dividing it in four equal-sized odour fields, and together exit through the central hole. The parasitoids are introduced into the arena from below, through the central hole of the olfactometer, and can move freely in the 4 odour fields and through the boundary zones separating them. The olfactometer is placed on a light plate (LED) in order to obtain a uniform background light. Each observation is filmed using a digital camera (Nikon D810) (7) for further analysis.

The camera settings to obtain correct videos are as follows: Aperture: F8; Shutter speed: 1/30; Sensitivity: ISO 160. A resolution of 6.7 Megapixels is used. The framerate is of 25 pictures per second. Focus is done on the trichograms before starting the video recording.

2.2.2 Protocol

First, the trichograms are introduced into the olfactometer chamber from a tube containing 60 individuals and that is connected to the central hole of the arena. To make this transfer easier and quicker trichograms' phototaxis is used by turning the olfactometer light plate on so that the trichograms are attracted into the arena. Then, trichograms are three to move from the tube to the olfactometer arena for 10 minutes on average. When at least 15 trichograms have entered into the arena, the experiment is started. Trichograms are free to come and go during the experiment.

Before starting any video recording, the camera level is checked thanks to a spirit level. Then, the lens focus is made on the trichograms situated on the superior face of the olfactometer. The air flow of each inlet is checked to be equal to 1,6L/h.

The first video corresponds to a control. Trichograms are recorded for 5 minutes without being exposed to any odour. This is a way to check that their repartition is random and uniform across olfactometer. Then, trichograms are exposed to an odour: a zone with 5 μ l and another one with 10 μ l. Two other zones are odorless. A second video is started for 15 minutes. It allows to determine if insects are attracted, repulsed or unaffected by the presence of an essential oil odour. To test the diluted cineole, the controls corresponds to two odorless zones, and two zones filled with paraffine odour. During the experiment, the odours zones are facing paraffine zones.

This entire sequence is repeated 4 times, each time with new fresh individuals, yielding 4 different replicates (A, B, C and D). Each replicate also has a different (rotated) position of odour/odorless air flows (figure 6). This is a way to control for possible external biases.

At the end of an experiment, the olfactometer is cleaned with ethanol as well as the pipe making the connection between the olfactometer and the air flow arrival.

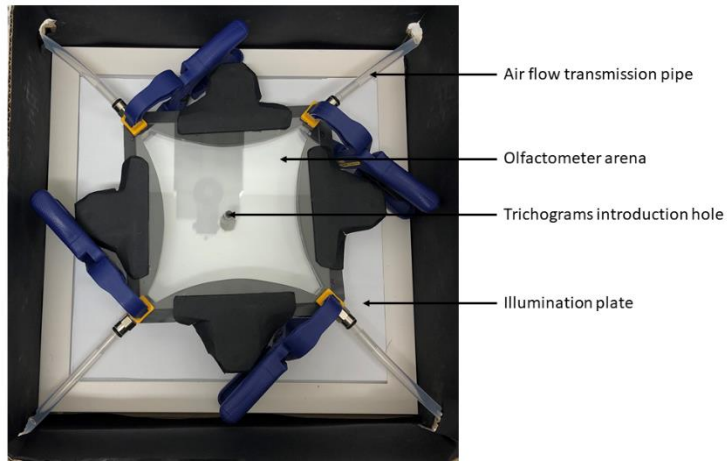


Figure 5 : Illustration of the olfactometer chamber

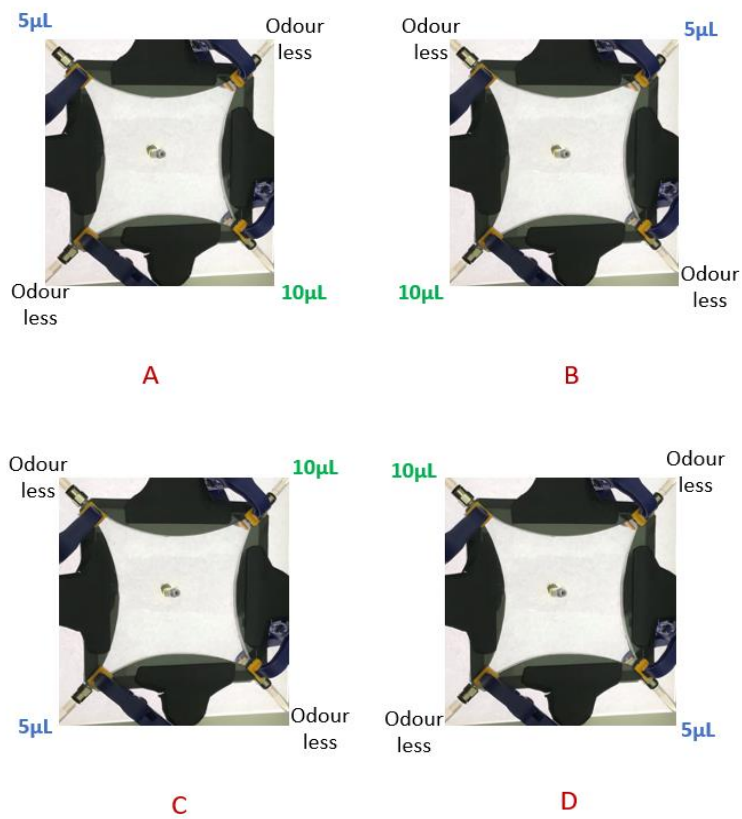


Figure 6 : Illustration of the different experimental replicates

The bottles in which odour has been introduced are cleaned with soap and water and then rinsed with ethanol. All the experiments are done in the same conditions, starting at 8 am and finishing at 12pm. Temperature and humidity can vary so they are recorded before each experimental session.

2.2.3 Analyses

Videos processing

Every two minutes, images are then extracted from the recorded videos using the FFmpeg software. This leads to three images for the controls and eight images for the tests, i.e. nine images per replicate.

These images are then processed with the ImageJ software: each trichogram is marked with a red point; the air inlets in the olfactometer as well as its centre are marked with a blue point. Afterwards, the individuals present in each zone are counted thanks to a macro.

This macro actually divides the olfactometer into sixteen zones and counts the individuals situated in each zone (figure 7). The zones which have been effectively used for the analysis are: 1, 4, 13, and 16. The individuals in all the other ones were not considered.

Statistical analysis

The output table given by ImageJ is then processed thanks to R software version 4.0, leading to a usable table.

The first step of the analysis is to study the repartition of the individuals in the olfactometer for the controls. The trichograms are expected to be distributed in a random way. If not, this will have to be considered for the results interpretation.

To verify the homogeneous distribution of the trichograms in the different areas of the olfactometer, a quasi-binomial GLM is constructed for the controls for each strain, and each time (0, 2 and 4min). For these tests, the two odour zones are not distinguished by their concentration. They are considered as one odour zone versus one odourless zone. A value of 0.5 is included as the intercept corresponding to a random distribution. The significance of this intercept is determined using a likelihood ratio test with a model without intercept (test F). The p-values extracted from these tests are then corrected using the FDR (False Discovery Rate) correction to consider the number of constructed GLMs.

The second step of the analysis is to determine the effect of the essential oil on the trichograms. If the essential oil is attractive, it is expected to have more than 50% of the individuals in the odour zone. On the contrary, if the essential oil is repulsive, it is expected to have more than 50% of the individuals in the odourless zone. An absence of one of the previous situations (50%/50%) is interpreted as a “neutral” effect of the essential oil.

Before starting, quasi-binomial GLMs are constructed for each strain, and each time, between the two odour zones to compare the distribution of the individuals between both zones. It allows to check if there is an effect of the different concentrations used: 5 and 10µl.

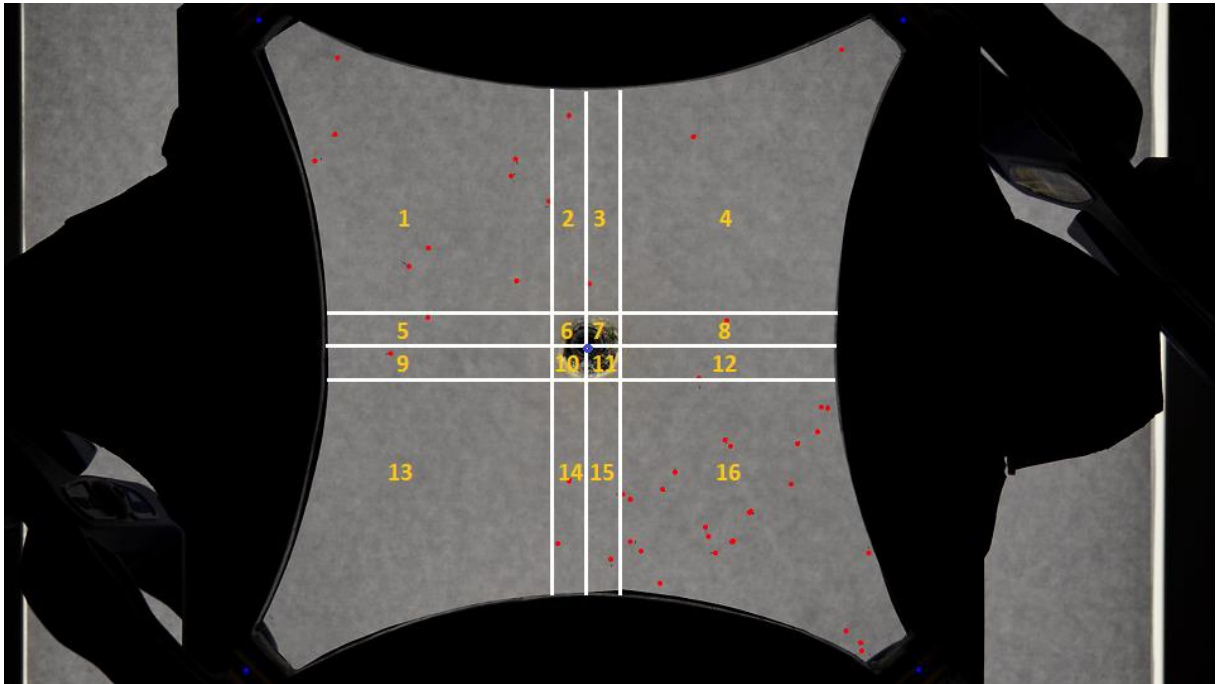


Figure 7 : Illustration of the ImageJ treatment

To determine if the proportion of individuals is significantly higher or lower than 50%, an exact binomial test is run on each replicate, for different times. The p-values obtained from these independent tests are then corrected with the False Discovery Rate method and pooled thanks to the fisher method, using the R package “survcomp” (Haibe-Kains et al., 2020). The False Discovery Rate method corrects the mistakes which could be made while the calculation of the p-values is run for a high number of tests.

2.3 Observations of videos

2.3.1 Manual protocol

After having observed several videos and from the observations made during the experiments, I noticed that the individuals of the strain PMBIO1 from the cacoeciae species seemed to naturally jump more often than others. Knowing this, I focused on this type of movement through in the video's analyses. Therefore, I have seen that the jump frequency of the individuals in the odour zone seemed to be higher than the one of the individuals in the odourless zone, for the essential oils with an apparent repulsive effect. To test this hypothesis, and to identify which explanatory factors actually explain this behaviour modification (strain, species, essential oil, apparent effect of the essential oil on the trichograms) I have created an observatory protocol for that purpose.

For each replicate video (A, B, C, D), I randomly chose three individuals in an odour zone and three others in an odourless zone at 2min, 7min and 14min without distinguishing the two concentrations (5 or 10 μ L). Indeed, sometimes the number of trichograms in the experiment was very low making it hard to find individuals in each odour zone. For the same reason, the number of observed individuals was only 3 to make sure than even under the effect of an essential oil, it was possible to find some individuals in a non-preferred zone. After having randomly chosen the individuals, I counted the number of jumps each of them performed for one minute. I observed one trichogram at a time. For each control video, I randomly chose three individuals in the entire olfactometer and applied the same protocol.

These observations have been conducted on three strains of *T.cacoeciae*: PMBIO1 ACJY144 and PJ.

For those three strains, I also studied the activity rate individuals following a similar protocol as previously described. Instead of counting the number of jumps, I timed the periods of activity of the trichograms for one minute. I considered an individual as active when it was moving from a point A to a point B by walking or jumping. When trichograms were hovering, running in circles, or not moving anymore I considered them as non-active. This way I was able to quantify the behavioural modes I was observing on the videos. I then divided the time of activity by the length of the entire observation to get a percentage of activity.

This manual observations' protocol has also been applied to the olfactometry experiments conducted on ACJY144 with 1,8-cineole.

2.3.2 Manual observations' statistical analysis

First of all, a PCA was run (thanks to the “ade4” package) on the nine quantitative variables: jumps' frequency, proportion of individuals (obtained thanks to the counting table given by the ImageJ macro) and the activity rate, recorded in the odour zones, at the three different times (2, 7 and 14min). Previously, each variable was centred on the control. Moreover, The activity rate, and the proportion of individuals in the different zones were transformed using the following function $\log(p/(1-p))$, to take into account the fact that there are distributed on (0,1), and make them more normally distributed. The different essential oils were grouped using the k-means clustering method with 3 clusters. To determine the optimal number of clusters which should be used, the Elbow method was utilized. The aim of clustering is to define clusters such that the total intra-cluster variation (total within-cluster sum of square (WSS)) is minimized. The Elbow method looks at the total WSS as a function of the number of clusters. The smaller the value of WSS and the elbow graph decreases the better the cluster results (Syakur et al., 2018). For all the strains, the Elbow graph corresponded to the one in figure 8.

After this, a focus on the different variables is done by plotting the different variables according to the time, the zone, and the essential oil thanks to a facet grid from the package “ggplot2”. Errors bars are added to the different graphs in order to help the reading of the results. They correspond to twice the standard error, as an estimate of an interval confidence of 95%.

Secondly, different statistical models are fir to better identify which factors are actually influencing the three variables. For the jumps' frequency which is a counting data, glmmTMB models are used with a quasi-binomial family to consider the (visible) overdispersion while comparing the mean and the variance, and the inflation of the zero value. Regarding the activity rate and the proportion of individuals in the zones (odour and odourless) glm and glmer models were tested with a binomial family. In both cases, different combinations of explanatory variables were tested. To identify the best model among those tested, one metric is used: the AIC. AIC deals with the risk of overfitting and underfitting. It can be used as a tool to compare different models and find the best balance between model fit and generalizability. After this step, the best model retained.

2.3.3 Automatic video processing protocol

Ctrax is a computer vision program for estimating the positions and orientations of a range of insects through time. After starting Ctrax, the first step is to open a video to be processed. Then, the tracking parameters must be adjusted in order to match the video. I adjusted the parameters by using the Tracking Wizard. It allows to set the following parameters, in orders (ANON., 2011):

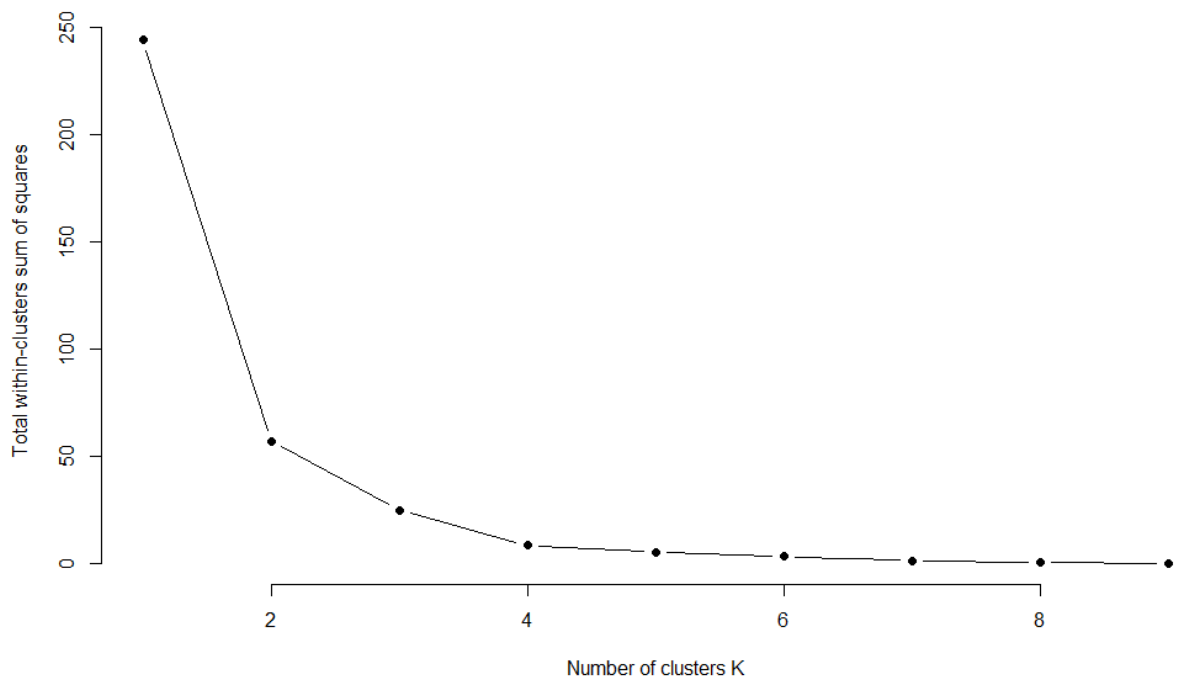


Figure 8 : Elbow graph for ACJY144 to determine the number of clusters that should be used

The exact same graph has been obtained for the two other strains.

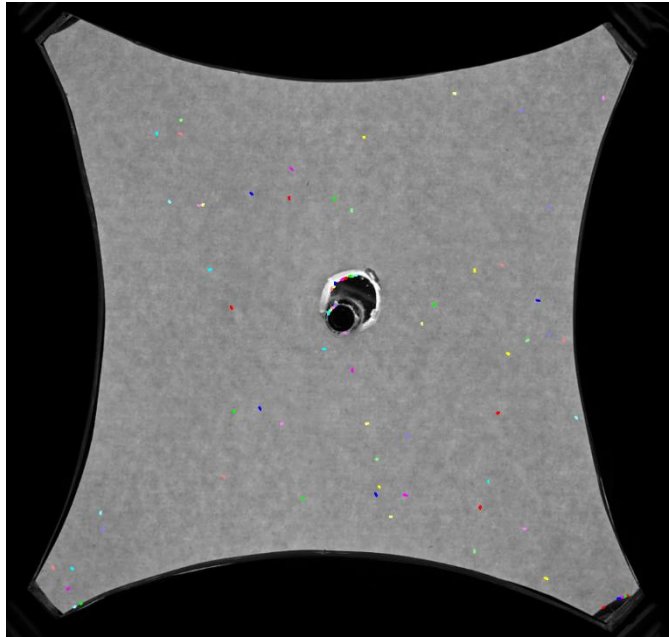
1. The tracking type: choose between dark individuals on a light background or light individuals on a dark background. In the case of olfactometry experiments, trichograms are dark individuals on light background.
2. The region of interest: it allows to precisely define the zone which must be studied in order not to waste tracking power. For the conducted experiments, it has been defined manually to match the arena's particular shape.
3. Fixing background: this parameter is used to make sure that the individuals which do not move during the movie are not becoming part of the background. It allows the identification of "ghosts", particles which appear on the image, but which are not trichograms.
4. High threshold: set the minimum required brightness (distance from the background) in order to discriminate foreground pixels (the individuals) from the background. (Used high threshold: 20).
5. Low threshold: this threshold determines the edges of each foreground component. The area surrounding each foreground pixel is also considered as foreground as long as it is above the low threshold. (Used low threshold: 7).
6. Shape computation: definition of the average and allowed variance in size.

After having set the parameters, the tracking can begin (figure 9). For a video of 15min, approximately two hours and a half are needed to achieve the tracking. At the end of tracking, the output data are saved in a .mat file.

Processing of the .mat file with R

The first step of the analysis, before using and interpreting the variables measured by Ctrax, is to know how accurate Ctrax actually is. To achieve this, the manual detections of individuals done with ImageJ for the three different times (2,7 and 14min) are compared to the detections of individuals done by Ctrax at the same times. The sensitivity (percentage of individuals which are not detected) and the specificity (percentage of particles which are not trichograms but are still detected) of Ctrax are therefore estimated. This gives an idea of how much filtering the data provided by Ctrax is needed.

The second step is now to define the filtering parameters which will be used to clean the data. To choose the minimum duration of a fragment, the distribution of the fragment's duration is analysed. Then, the same is done to identify the minimum speed i.e the speed to be declared active and the maximum length a particle can have to be considered as a trichogram. Different filtering parameters have been tested to plot at the different times used previously (2,7 and 14min) the particles detected by Ctrax i.e a portion of movement for which the duration was at least equals to the defined minimum duration. These plots have been compared to the manual detection of individuals realised with the ImageJ software. This allows the estimation of the sensitivity i.e the false negative rate and the estimation of the specificity. The aim is to adapt the filters in order to reduce as much as possible the number of particles which are actually not trichograms, whilst making sure that Ctrax still detects trichograms.



*Figure 9 : Screenshot of a tracking session by Ctrax
Each coloured point corresponds to a trichogram detected by Ctrax.*

The different parameters that have been tested are presented in table 4. The last line of the table corresponds to the parameters which have been taken to proceed with the analysis.

2.3.4 Computation of trajectory metrics

The behaviour of trichograms has been analysed by focusing on four different variables, computed in R with the package “trajr” (McLean, Volponi, 2018): first the proportion of individuals in the different zones. This was also a way to check that the observations done by Ctrax were closed to the one manually done (figure 10). Then, the activity, the average speed and the sinuosity of their movement have been calculated and analysed through time, in the different zones.

The activity rate corresponds to the proportion of time that individuals spend moving actively. It was first necessary to identify the minimum speed threshold to qualify a displacement as active, based on histograms on all velocities. The threshold has been estimated at 0.015 cm.s⁻¹. The sinuosity corresponds to the variation of angles averaged over all the changes in directions of a trajectory (Burte, 2019; Benhamou, 2004). To obtain the average movement's sinuosity in the odour and odorless zones, the fragments' sinuosity offset with their length were summed and then divided by the total fragments' length. Finally, the average individuals' speed has been obtained by summing the individuals speed offset with their duration and then by dividing it by the total duration. For the graphs of the speed, the activity rate and the sinuosity, the controls correspond to the four zones of the olfactometer without odours, knowing, thanks to the olfactometry experiments, that there is actually no “zone effect” inside the olfactometer.

This automatic protocol allows a more precise characterization of the effect of essential oils on trichograms but was only applied to the olfactometry videos with Rosemary and Thyme with the strain ACJY144, because of time constraints.

3- Results

3.1 Olfactometry

3.1.1 Check of the random repartition of the individuals in the olfactometer

For the controls, the individuals are expected to be randomly distributed in the olfactometer. The p-values obtained from the quasi-binomial GLM which have been constructed for the controls for each strain, each time, and each area of the olfactometer to verify this distribution are all superior to 0.05 after the FDR correction. Therefore, there is no effects which could have affected the distributions which have been observed during the experiments.

Table 4 : Summary table of the tested parameters in Ctrax

Tested parameters			
Min_Duration (in frames i.e seconds*25)	Max_length In pixels	MinSpeed Cm/s	MaxSpeed Cm/s
0	10000	0	10000
2,5*25	3	0,015	2
3,5*25	3	0,015	2
5*25	3	0,015	2

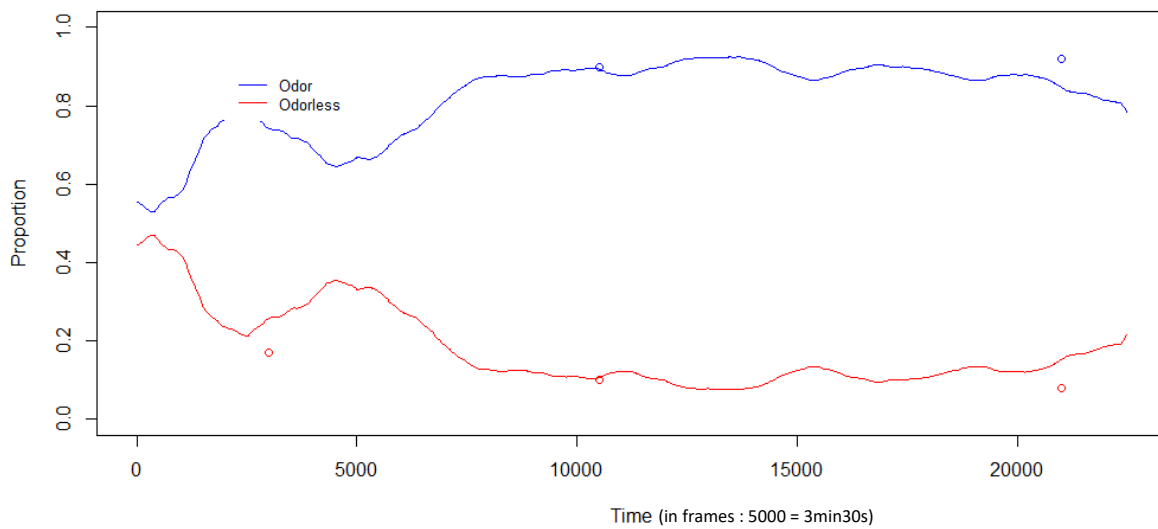


Figure 10 : Evolution of the proportions of individuals through time for the replicate C. The lines are the proportions obtained thanks to Ctrax. The points are the ones obtained from the manual count.

3.1.2 Effect of the different tested essential oils on the different strains of trichogramma

First of all, the p-values obtained from the quasi-binomial GLMs constructed for each strain, and each time to test the concentration effect, are all superior to 0.05 after correction. Therefore, there is no concentration effect. Thus, the two “odour” zones have not been distinguished in the different analysis.

Secondly, for each strain and each essential oil, graphs representing the evolution of the proportion of the individuals through time, in the odour zones have been generated. On each graph, the four replicates (A, B, C, D) are represented with different colours. The mean and the standard error of the four replicates are represented in black. The vertical bar at 0 represents the introduction of odour and the red dashed horizontal bar, 50% of individuals in the odour. The instantaneous average values showing a significant p-value after FDR correction are represented by red asterisks.

ACJY144

For this strain, it is possible to notice that depending on the essential oils, significant effects (repulsive or attractive) are recorded as of different times (2min ,4 min or 6min). It seems that Basil, Coriander, Green Anise, Peppermint, Oregano and Thyme are significantly repulsive (figure 11). For some essential oils, the effect seems to evolve through time. Indeed, Fennel and Mugwort are first responsible for a significant repulsive effect and then the trend reverses and

becomes attractive. The orange essential oil is neutral, and the Rosemary is the only one being significantly attractive. Regarding this attractiveness, the major component 1-8 cineole has been tested on its own to see if it is actually the one responsible for the trichograms' behaviour (appendix I). Without dilution (concentration of 5 and 10 μL as for essential oils), no concentration effect has been recorded. From 2 to 12 min, it seems to be significantly repulsive, and becomes significantly attractive after 12 minutes. It is important to note that the variability between the different replicates is quite high, as well as the one inside a same replicate (example of the replicate B at $t=-2$ and $t=8$ min). The effect which affects trichograms' behaviour seems to evolve quite quickly. For The lower concentrations of cineole which have been tested 0.05 μL , 0,5 μL , 1 μL , and 1,5 μL , there is still no concentration effect between 0.05 μL and 0,5 μL , neither between 1 μL , and 1,5 μL . Therefore, as for the other experiments, these different groups of odours have been treated as one odour zone. None of them show a significant attractivity (appendix II and appendix III), even if, for 20 and 30%, the proportions in the odour zone tend to be higher.

PMBIO1

As for ACJY144, Basil and Coriander essential oils are significantly repulsive (figure 12). Fennel Mugwort, Green anise, and Oregano show the same repulsive effect. Orange, Peppermint and Thyme seem to be quite neutral towards the tested individuals.

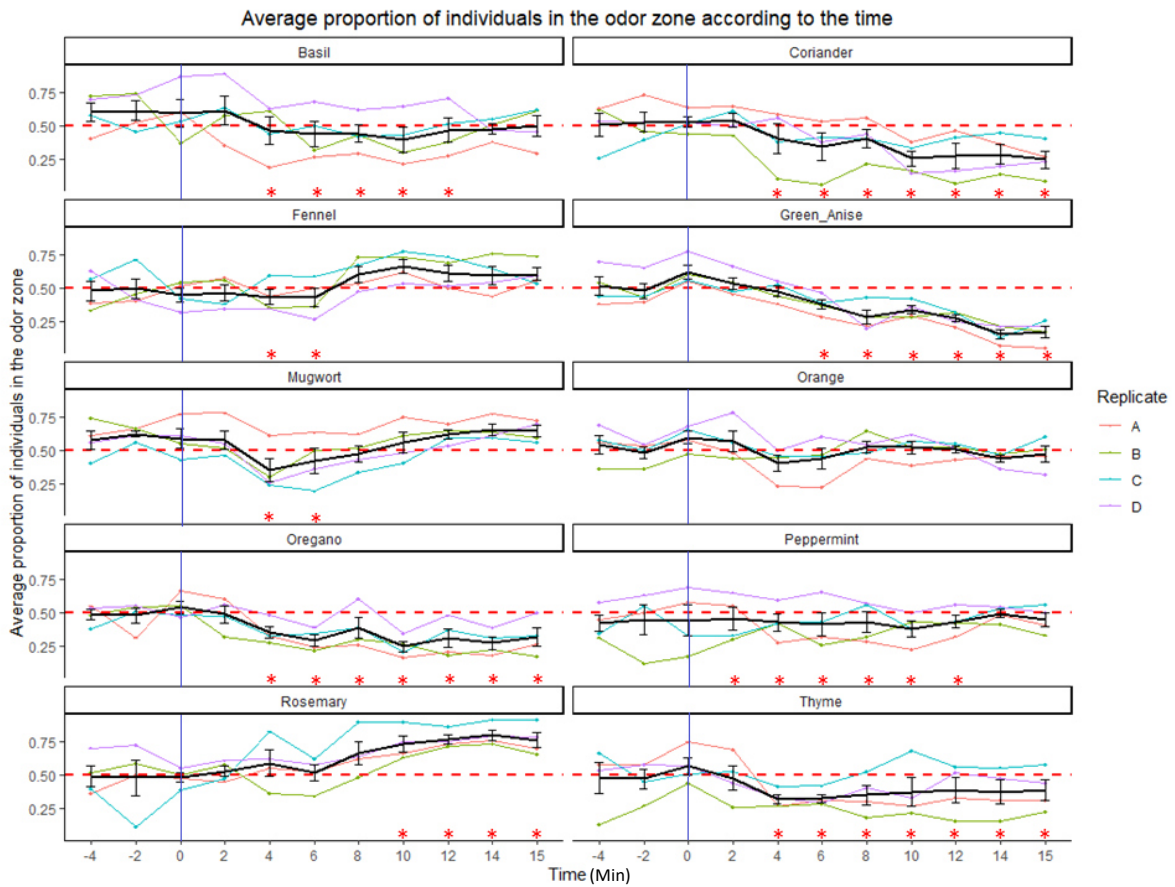


Figure 12 : Graphs representing the different evolutions of the proportions of individuals in the odour zones for the ten essentials on *T.cacoeciae*, strain ACJY144

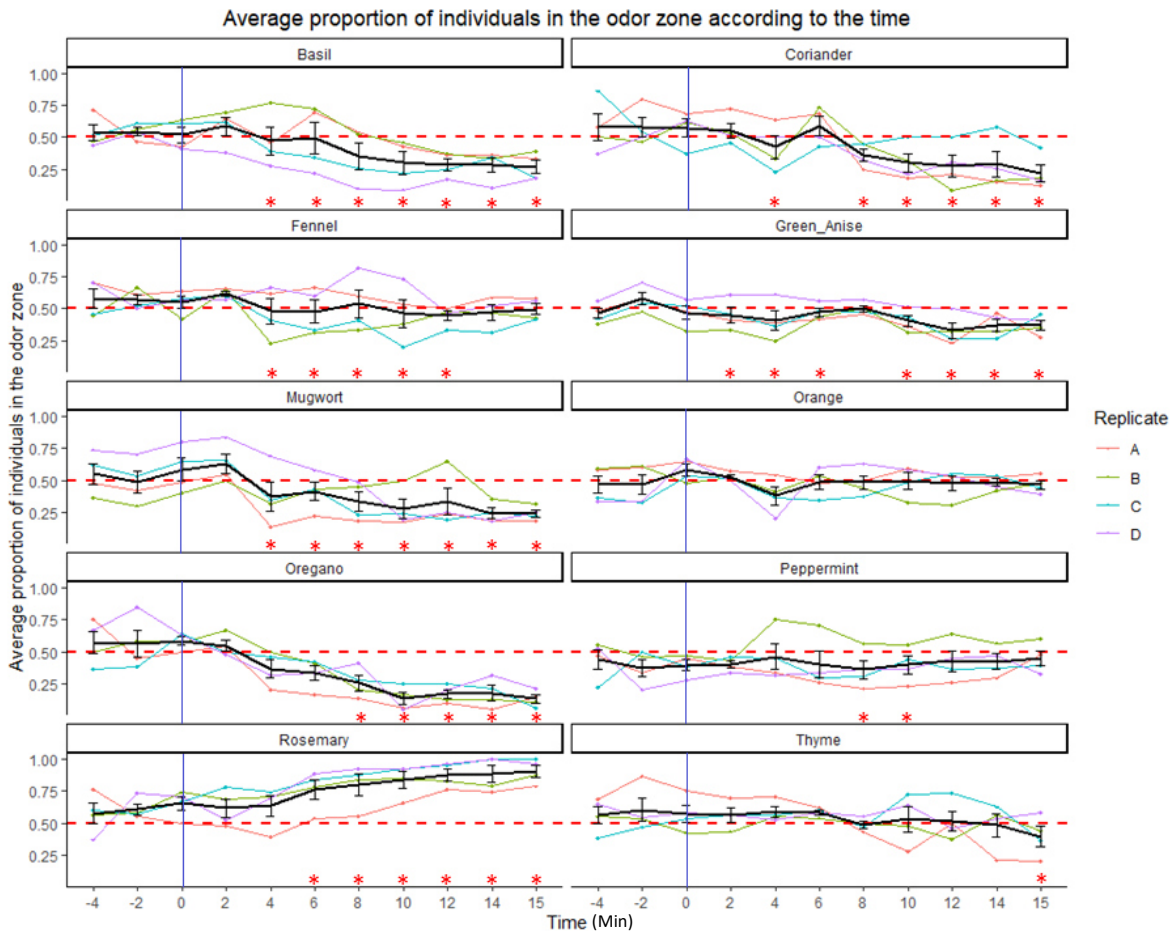


Figure 11 : Graphs representing the different evolutions of the proportions of individuals in the odour zones for the ten essentials on *T.cacoeciae*, strain PMBIO1

Rosemary is also significantly attractive for this strain, but the effect appears earlier. For this strain, there is no change in trichograms' reaction towards the odour.

PJ

Regarding this strain, seven of the ten essential oils have a significant repulsive effect as of different moments: Oregano, Peppermint, Thyme, Green Anise, Coriander and Fennel. Unlike the other strains, Rosemary essential oil is neutral toward this strain. The same result can be observed for Orange and Mugwort. Nevertheless, even if it is not significant, Mugwort essential oil shows an attractive trend (Appendix IV).

The different essential oils' effects for each strain are summarized in appendix V.

3.2 Observations of videos

3.2.1 Manual observations

To have a better understanding of the way the essential oils could be classified and influence the trichograms, the proportion of individuals in the odour zone as well as the activity rate and the jump's frequency have been measured at three different times (2,7 and 14 min) for the three strains. First the summary statistics, in this case PCAs centred and scaled with the control (no odour at all) completed with a K-means clustering, show that it is possible to group together several essential oils. The groups differ from one strain to another.

ACJY144

Indeed, for ACJY144 it is possible to distinguish three groups of essential oils. The first one is constituted of Mugwort, Rosemary, pure Cineole, diluted Cineole (between 1 and 10%), and diluted Cineole (between 20 and 30%). The second one is represented by Orange, Coriander, Oregano, Green Anise and Basil. The third one is composed with Thyme and Peppermint (figure 13). Thanks to the variable projection which is quite good except ($\cos^2 > 0.75$), except for different proportions (at 2min especially) and the activity rate at 2 min (Appendix VI), it is possible to say that actually the first axis opposes the essential oils for which most individuals stand in the odour zone from the ones for which there are less individuals in the odour zone but they jump more frequently. In this case, Thyme and Peppermint appear to be the most repulsive ones. The second axis opposes the essential for which at 2min the activity rate at two minutes is quite high and then strongly decreases at 7 and 14min. With the graphs of the average activity rate according to the time and zone it is effectively possible to see that the essential oils which actually make the trichograms' activity rate significantly decrease through time in the odour zone (figure 13) are Mugwort and Rosemary. This is also true for Fennel. A look at the average jump's frequency (Appendix VII) and the proportion of individual in the different zones state that Fennel, Mugwort, and Rosemary essential oils lead to the same kind of reaction towards trichograms. Indeed, for the three of them, the jump's frequency tends to be equivalent as during the control.

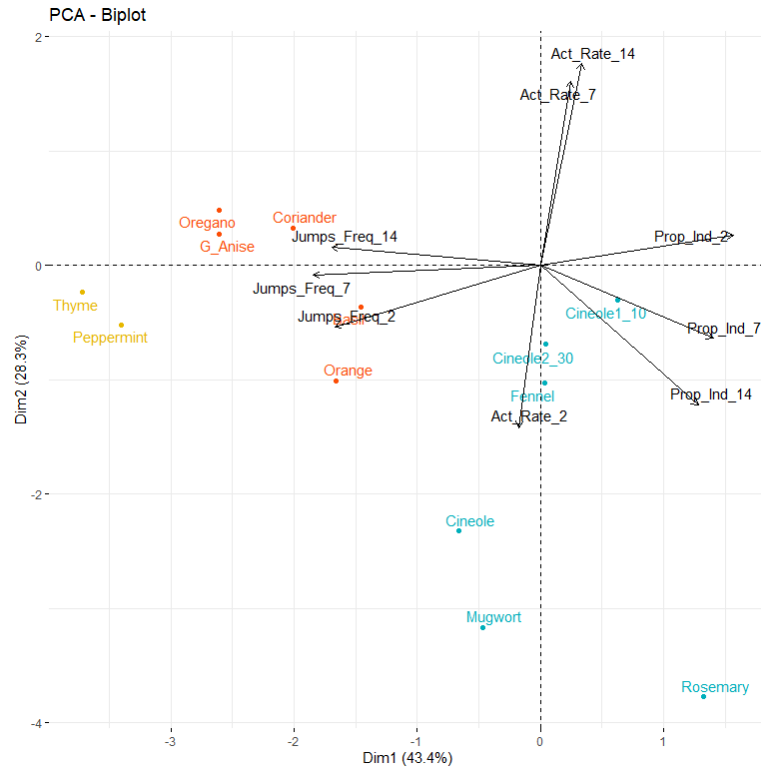


Figure 13 : PCA representing the ten essential oils, plus the three tested modalities for cineole for *T.cacoeciae* ACJY144, according to the three studied variables at three different times: Proportion of individuals at 2, 7 and 14 min (Prop_Ind_2, Prop_Ind_7), Activity rate at 2, 7 and 14 min (Act_Rate_2, Act_Rate_7, Act_Rate_14), and Jump's frequency 2, 7 and 14 min (Jumps_Freq_2, Jumps_Freq_7, Jumps_Freq_14)

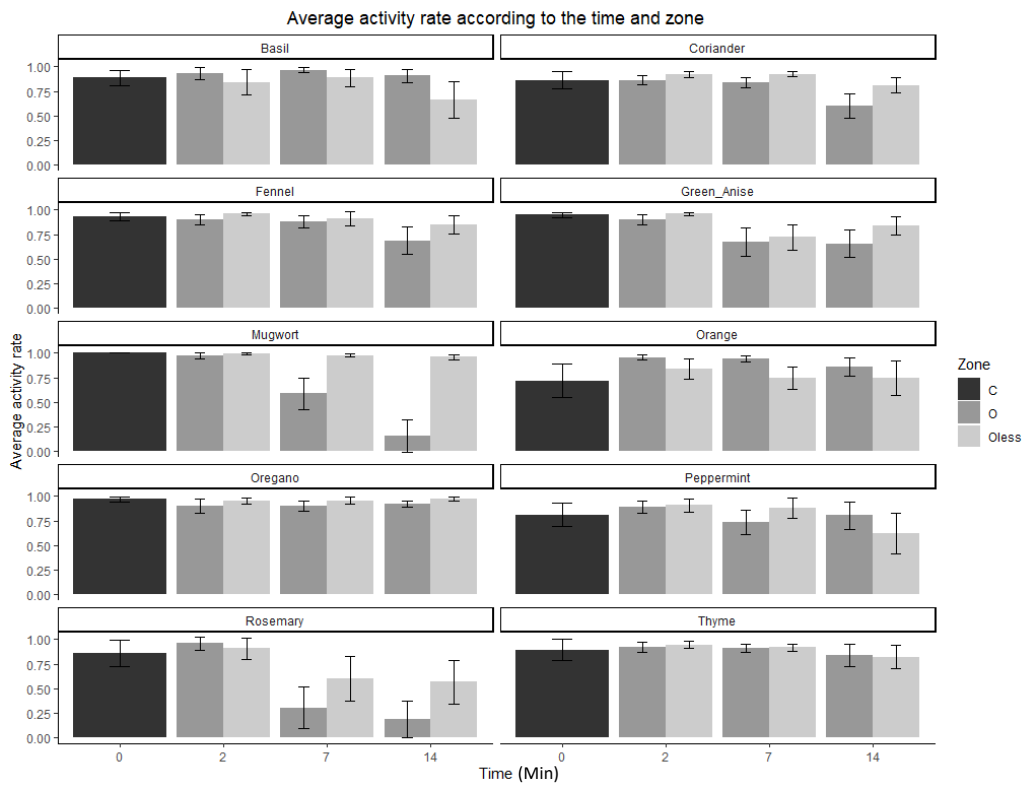


Figure 14 : Average activity rate observed through time for the ten essential oils tested on *T.cacoeciae* ACJY144. C: the controls, O: Odour Zone, Oless: Odourless Zone. The error bars correspond to twice the standard error i.e an estimation of the confidence interval at 95%.

With the PCA, the different modalities of cineole tested are classified in the same group than Mugwort, Rosemary and Fennel. This is mainly because the activity rate strongly decreases after 2min with pure cineole (Appendix VIII). The diluted cineole is closer to the controls because the change in the activity rate is not marked, neither it is for the jump's frequency (Appendix IX). For this group, the proportion of individuals in the odour zone does not appear as a good indicator to determine their nature towards trichograms. However, for the repulsive oils, especially for Thyme and Peppermint, the jumps frequency increases quite a lot compared to the controls whereas there is no noticeable change in the activity rate.

The groups identified thanks to the different variables seem to match with the ones identified thanks to the olfactometry graphs. Even if the cineole appears closer to the Rosemary with the PCA than with the results shown by the previous graphs.

PMBIO1

Regarding PMBIO1, it is also possible to identify three groups. The first one being composed with: Rosemary, Fennel, Green Anise, Thyme, Coriander, Mugwort and Oregano. The second one is constituted of Orange and Peppermint. The last one only counts Basil (figure 15). In this case, the variables are not correlated to one dimension more than another. Nevertheless, because of their quite good projection ($\cos^2 > 0.75$, except for the proportion of individuals at 2min and the activity rate at 14min, appendix X), it is possible to say that the first dimension is more explained by the proportion of individuals and the activity rate whereas the second is more about the jump's frequency.

Actually, Rosemary is grouped with Fennel, Green Anise, Thyme, Coriander, Mugwort and Oregano because of its activity rate and jump's frequency. Indeed, these oils do not significantly impact the trichograms jump's frequency (Appendix XI) but create a decreasing trend for the activity rate (Appendix XII). Rosemary appears quite isolated from the others because the decrease in the activity rate is much more intense. Then the jump's frequency explains the other groups. Indeed, Basil, Peppermint and Orange are the only ones for which the jump's frequency is significantly higher than the one in the odorless zone, Peppermint and Basil being repulsive. Orange is closer to the control because the repulsive effect does not appear. Nevertheless, Basil does not appear in the same group than Peppermint and Orange because the increase in the jump's frequency is higher and maintains quite high through time compared to the others.

PJ

As for PMBIO1, it is possible to create three groups of essential oils (figure 16). The dimensions oppose the groups based essentially on the jump's frequency and the activity rate because the proportion of individuals are not well projected except for 14min (Appendix XIII).

It is possible to notice that Rosemary, Fennel, Green Anise and Basil are part of the same group because of their activity rate decreasing quite a lot after 7min (Appendix XIV).



Figure 15 : PCA representing the ten essential for *T.cacoeciae* PMBIO1, according to the three studied variables at three different times: Proportion of individuals at 2, 7 and 14 min (Prop_Ind_2, Prop_Ind_7), Activity rate at 2, 7 and 14 min (Act_Rate_2, Act_Rate_7, Act_Rate_14), and Jump's frequency 2, 7 and 14 min (Jumps_Freq_2, Jumps_Freq_7, Jumps_Freq_14)

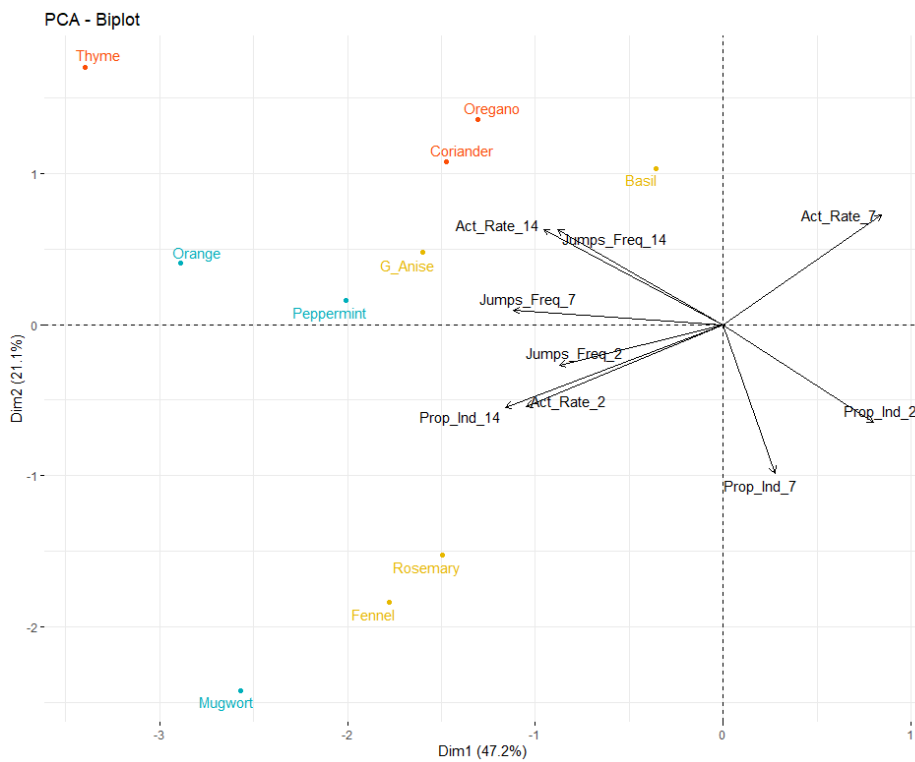


Figure 16 : PCA representing the ten essential for *T.cacoeciae* PJ, according to the three studied variables at three different times: Proportion of individuals at 2, 7 and 14 min (Prop_Ind_2, Prop_Ind_7), Activity rate at 2, 7 and 14 min (Act_Rate_2, Act_Rate_7, Act_Rate_14), and Jump's frequency 2, 7 and 14 min (Jumps_Freq_2, Jumps_Freq_7, Jumps_Freq_14)

Nevertheless, Rosemary and Fennel are far from Green Anise and Basil because they make the proportion of individuals increase through time unlike Basil and Green Anise which make it decrease. The two other groups are: Oregano, Coriander, Thyme and Orange, Peppermint, and Mugwort. All of them appear to be quite repulsive to trichograms. Nevertheless, only Mugwort, Orange and Peppermint make their jump frequency increase after 2min (Appendix XV).

Statistical models

First of all, simple glm models have been run for the three variables with “Strain” as a fixed effect. It shows that intraspecific variability must be considered. Then, different explanatory variables have been added step by step, and kept or not based on the way they affect the AIC. All the tested models for the jump’s frequency are listed in table 5. For both the activity rate and the jump’s frequency, the best model is the one built with Strain, the interaction between the essential oil and time, plus the zone (odour or odourless) as fixed effects; and the identity of each individual as a random effect. Regarding the proportion of individuals in the different zones, the best model is built with the same parameters, except that the random effect is not used (the proportion is not measured on an individual scale). This modelling confirms the fact that the evolution of the observed variables effectively depends on the time which is closely related to the essential oil. Also, it demonstrates that the observations must always be related to the strain because of the intraspecific variability.

3.2.2 Automatic video analyses

Example of an attractive essential oil: Rosemary

First of all, the automatic analyses confirmed the proportions’ evolution through time in the different zones. Through time, the proportion of individuals in the odour zones becomes effectively higher than 50% (figure 17).

Then, it shows that actually in the odour zones, the individuals speed decreases for two replicates (A and D) and stays lower than the one in the odorless zone. But it is not the case for the replicates B and C for which the opposite is observed (figure 18). Regarding the activity rate, it is lower in the odour zones for all the replicates except for replicate C (Appendix XVI). Moreover, in the odour zone the individuals’ movement seem to be more winding (Appendix XVII). Indeed, for three of the replicates, the sinuosity observed in the zones with odour is 4 times higher than in the zones without. Nevertheless, for the replicate B, the sinuosity is higher in the odorless zone.

Example of a repulsive essential oil: Thyme

As for Rosemary, the automatized observations confirm the proportions’ evolution through time in the different zones. Indeed, through time, the proportion of individuals in the odour zones becomes effectively lower than 50% (Appendix XVIII).

Then, unlike the attractive essential oil, with Thyme, the individuals speed is a bit higher in the odour zones than in the odourless zones (Appendix XIX).

Table 5.: Summary table of the tested models for the jump's frequency

Models for the jump's Frequency			
Model	Fixed effects	Random effects	AIC
m1	EO :Time+Zone+Strain	EXP_IND	5137.7
m2	EO :Time+Zone+Strain	None	5255.8
m3	EO :Time+Zone	None	5512.6
m4	EO :Time	None	5801.5
m5	EO	None	5878

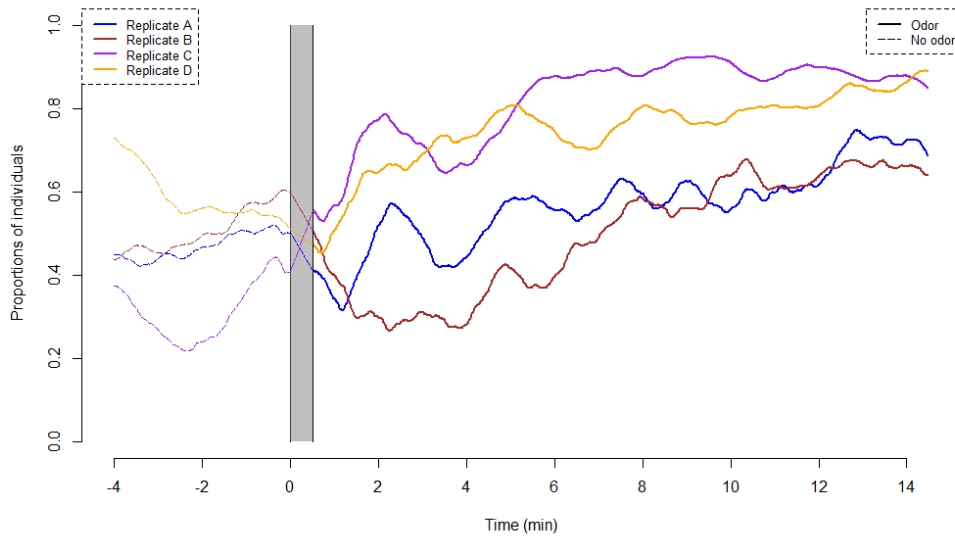


Figure 17: Evolution of the proportions of individuals for the different zones through time for the Rosemary essential oil. The grey bar corresponds to the transition between the controls and the introduction of the odour.

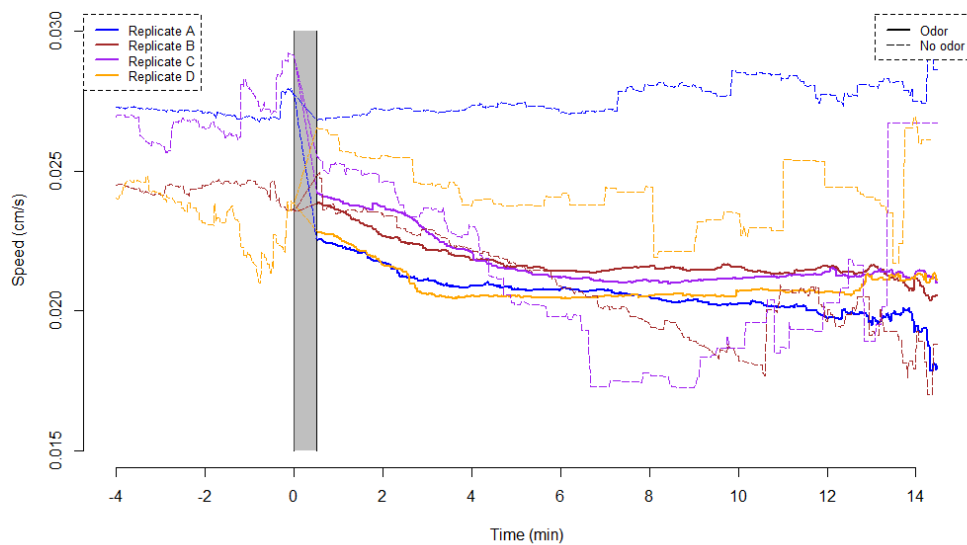


Figure 18: Evolution of the individuals' speed for the different zones through time for the Rosemary essential oil. The grey bar corresponds to the transition between the controls and the introduction of the odour.

The activity rate is quite similar in both zones (figure 19). The sinuosity increases in the odour zones but the difference of sinuosity between the odourless and the odour zones is lower than the one observed for Rosemary (figure 20).

4- Discussions

The results from the conducted experiments and analysis have demonstrated that essential oils effectively have an effect on trichograms' behaviour. Indeed, the different strains of *Trichogramma cacoeciae* respond to the various tested essential oils' odour by being repulsed or attracted. Regarding the responses' nature, an intraspecific variability has been spotlighted. Indeed, the attraction and repulsion to an essential oil is different from one strain to another, the reaction depending also on the time exposure. Some strains react more quickly than others. The time effect, as the strain, have been identified through statistical modelling, to interact with the identity of essential oils as explanatory variables for the activity rate, the jump's frequency, and the proportion of individuals in the odour zone. These last variables appear as indicators for the essential oils' nature i.e attractive, repulsive, or neutral (no effect identified), which must always be brought back to the strain.

The first behaviour which has been identified is the escape, as a response to a repulsive essential oil. Escaping is actually characterized by an increase in the jump's frequency. Therefore, jumping would actually be a runaway behaviour which allows the trichograms to escape more quickly the repulsive odour and join the odorless zones. Indeed, in the case of the repulsive odours, there was no more individuals in the odour zones. Moreover, the trichograms' activity rate, as well as their movement sinuosity and their speed tend to stay similar to the control one. Being repelled is the most common behaviour towards essential oil odours shown by *Trichogramma cacoeciae* similarly to other species such as *T.evanescens* (Fillaud, 2019) or for *T.cordubensis* (strain PUG029) (appendix XX).

The second behaviour which has been revealed is attractivity, mainly expressed when the individuals are exposed to Rosemary odour. Attractivity is characterized by a decrease in the individuals' activity and in their speed in the odour zone. On the contrary their movement's sinuosity increases. The jump's frequency is not modified. However, this effect raises a question. Does this attractivity result from a choice or is it the result of a stricken toxicity? Indeed, cineole which has been identified as the component responsible, at least partially, for the attractivity of Rosemary, is also responsible for the accumulation of acetylcholine in the cholinergic sites which leads to paralysis and death of the organism (Corbett et al., 1984). Thus, the individuals would actually stay in the odour zone only because they are not able to flee anymore. As a partial answer, it is possible to use the fact that these movement characteristics have also been identified on *T.pretosium* while facing the attractive sex pheromone of its host, yet not demonstrated as toxic for trichograms. It increases its walking times and decreases its flight times and its walking velocity (Noldus et al., 1990). Therefore, the observed changes in their movements would be more about a "real attractivity"

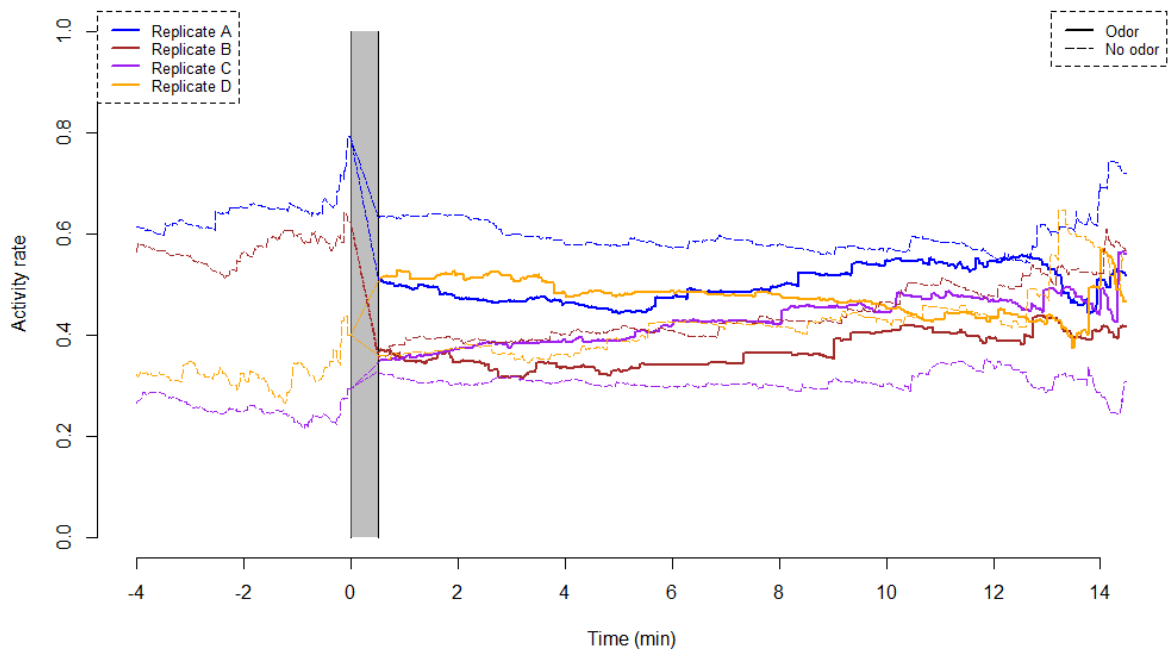


Figure 17 : Evolution of the individuals' activity rate for the different zones through time for the Thyme essential oil. The grey bar corresponds to the transition between the controls and the introduction of the odour

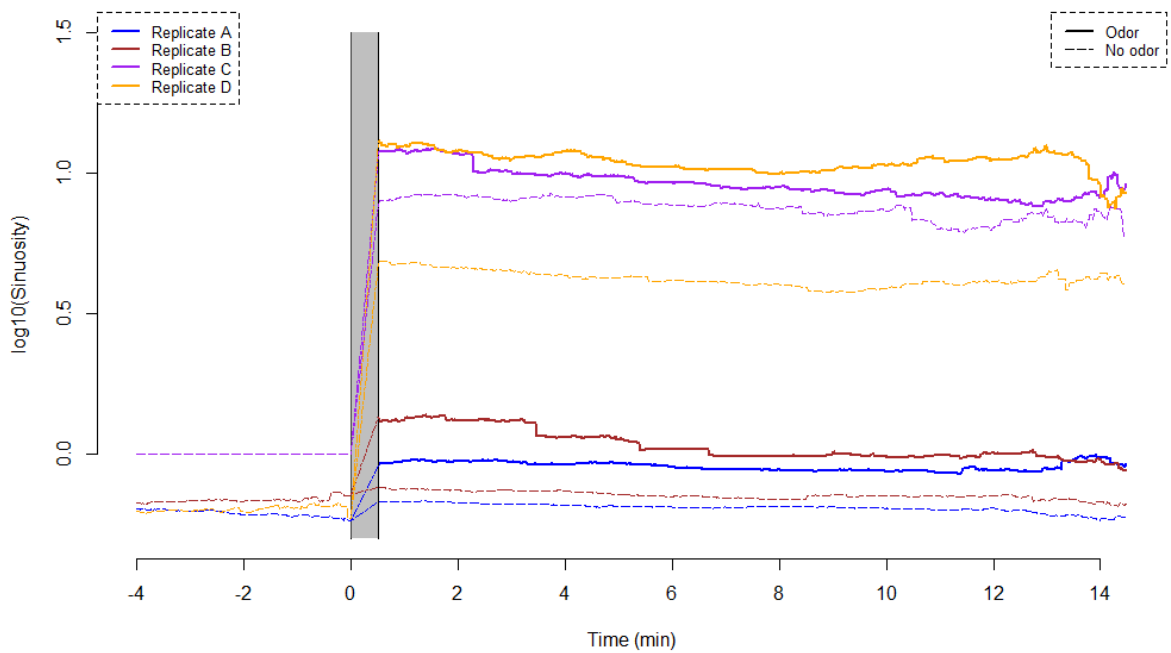


Figure 18 : Evolution of the individuals' movement sinuosity for the different zones through time for the Thyme essential oil. The grey bar corresponds to the transition between the controls and the introduction of the odour

than a toxicity. Nevertheless, there is no guarantee that it would not in fact be a combination of the two.

The last behaviour which has been spotlighted is characterized by flight at first, then by a period of habituation leading to attractivity. This apparent repulsive effect as a first reaction is put forward by the significant increase of the proportion of individuals in the odorless zones. However, at the same time, the jump's frequency does not show an increase such as in the case of totally repulsive oils. It suggests that fleeing during the first minutes is more a behaviour which expresses the fact that the trichograms are startled by this new odour than the expression of a real repulsion. Indeed, the trichograms which have been used in the experiments have never been exposed to an odour. Moreover, it has already been shown that trichograms' behaviour towards an odour can be different if they have or have not previously experienced the odour (Kaiser et al., 1989). Effectively, the hedonic value of odours is something highly plastic (Midroit, 2018). For these reasons, the change in the trichograms' behaviour may result from a habituation phenomenon which can be defined as the progressive decrease of the amplitude or frequency of a motor response to repeated sensory stimulation that is not caused by sensory receptor adaptation or motor fatigue (Schmid et al., 2014). Moreover, the fact that the first repulsive effect reverses to become attractive is also supported by the trends of the activity rate and the jump's frequency which are closer to the ones characterizing the attractive essential oils.

In the experimental conditions which have been used, there are other essential oils which are not responsible for any kind of particular trichograms' behaviour. Therefore, they are considered as neutral. They may effectively appear as neutral considering the studied variables and the duration of the experiment. The notion of neutral must be considered with precautions. Indeed, there may in fact be an effect but too slight or more variable than the others to be revealed considering the statistical power of the analysis. Another possible explanation would be that these odours, apparently "neutral", are not perceived by the trichograms. Or, they might be perceived but without having any effect on the trichograms. In this case, they would be truly neutral. One last possibility would be that to notice the effects of these essential oils longer experiments would be needed.

Based on all the indicators, Fennel, Mugwort, and Rosemary appear to be part of the same group. Knowing it is quite tempting to attribute this similarity to a common component. The monoterpene 1,8-cineole has been proposed as the one responsible for the attractiveness of Rosemary and has been tested on ACJY144. Actually, as it has been seen before, Mugwort and Rosemary contain 1,8-cineole which might be responsible for the attractiveness of these oils. It is interesting to notice that Fennel essential oil also contains 1,8-cineole but in a lower quantity than Mugwort and Rosemary (Qiu et al., 2012 ; Saharkhiz, Tarakemeh, 2013). The attractive effect may be directly linked to the concentration of cineole. Indeed, the less the oil has this component, the lower are the consequences on the chosen indicators. This is actually matching with the way the different cineole concentrations are located on the PCA graph. The

less the cineole has been diluted, the closer it is to Rosemary. However, according to the olfactometry experiments, the attractivity is not as intense as the one observed with Rosemary. The concentrations 1 μ L and 1,5 μ L show an attractivity in tendency, which disappears with the concentrations 0,05 μ L and 0,5 μ L. These surprising results might be linked to the fact that there is an important variability between the replicates: some of them having opposed trends, which make the interpretation of the mean quite difficult. Moreover, cineole might not be the only one responsible for the attractivity. Actually the attractivity of Rosemary and also of Mugwort and Fennel, to a lesser extent, could be due to the perception of an “aromatic harmony” which is defined as mixtures whose fragrant notes merge to form a new one, different from those of its components (Sinding, 2012)

Material and methods

The different protocols used for the olfactometry experiments and the videos' observations were sufficient to get a first idea of what is happening to the trichograms in the presence of essential oils by focusing on different behavioural aspects. However, it presents some limits which could be overpassed by changing the olfactory experiments. Indeed, the individuals effectively in an odourless zone might have already spent time in the odour zone. Therefore, the observed behaviour may be influenced. This could be avoided by doing two control tests: one without any odour and one with odour in the four arms of the olfactometer. This would allow to evaluate the effect of the odour on individuals which would have spent the whole time being exposed to it. However, this would suppress the notion of choice and attractivity/repulsion. Another important limit is that the identity of the observed individuals, manually or automatically, is not conserved through time. Actually, it would be interesting to observe one group of individuals not exposed to an odour and the exact same one exposed to odour with the possibility to keep the identity of each of them so that the individual variability would be properly assessed. Moreover, all the effects which have been assessed depends on the time exposure. For some essential oils, nothing in particular happens for 15 minutes. For them, a longer time duration might be more appropriate in order to tell with more confidence if the absence of effect is rather a question of time exposure or just a fact, specific to the studied essential oil. Nevertheless, by combining manual and automatic observations, it has been possible to assess the evolution of trichograms' behaviour when they are exposed to odours. Indeed, thanks to the automatic observations, it has been possible to add the speed and the sinuosity as a descriptor to characterize the trichograms' movements (Yong et al., 2007). Moreover, Ctrax allows to follow a big number of individuals and obtain an important quantity of information. This makes the method much more powerful than the manual observations. The comparison of the proportions observed by Ctrax with the ones manually calculated have shown that Ctrax's detection and tracking of the individuals is quite accurate. This verification made it possible to go further in the trichograms movements assessment.

Also, it appeared that there is actually no concentration effect, whether with 5µl or 10µl. This might be caused by air saturation. Indeed, the bottles which have been filled with 10µl of essential oil will be more quickly saturated in odour than the ones with 5µl.

But after a few minutes, both of them will be equally saturated. This does not actually produce two different air concentrations. To manipulate concentrations more precisely, one saturated airflow could be mixed with a neutral airflow in a certain proportion, before entering the olfactometer chamber, in order to produce the desired concentration.

5- Conclusion and perspectives

The conducted experiments have shown that non-intentional effects of essential oils, on trichograms, must be considered while using them against pests because they effectively cause significant side effects. Indeed, most of them are repulsive to *Trichogramma sp.* For *Trichogramma cacoeciae*, three essential oils have been identified as attractive: The Rosemary essential oil as significantly attractive, Mugwort and Fennel as attractive in tendency but no significance has been identified with the methods which have been used. This particular group of essential oils is also associated to the modification of trichograms' movements. They decrease their speed and activity rate and increase their movement sinuosity which can explain their accumulation in odour-containing areas. Considering that essential oils have toxic (lethal and sublethal effects) on the longer term, a repulsive effect mitigates their potential impact on trichograms, but attractive effects would make the impact even worse: trichograms would actually be driven to deadly components.

The sequence processing and the complete automation of observations are possible with the computer vision program Ctrax. There would no longer be any need to carry out manual image pointing, and a large amount of information would be accessible in a reasonable amount of time. This makes it possible to consider high-throughput phenotyping which would thus make the study of all these interactions more efficient and faster decision-making. In addition, this high throughput phenotyping could also improve the choice of the species and strains of potentially marketable biological control agents.

Identifying the side effects is essential in the development of biological control strategies, especially for the one relying on integrated pest management. Indeed, Integrated Pest Management (IPM) is an effective and environmentally sensitive approach that relies on a combination of common-sense practices. IPM is based on the comprehension of the life cycles of pests and auxiliaries and their interaction with the environment. This information, in combination with available pest control methods, is used to manage pest damage by the most economical means, and with the least possible hazard to people, property, and the environment (US EPA, 2015). Using essential oils as a replacement for chemicals is an interesting initiative towards the reduction of the chemicals use, and it matches the idea of integrated pest management. Indeed, the first possible solution is to use both essential oils and trichograms as two complementary methods to manage pests. For instance, it has been showed that the essential oils of *Thymus capitatus* and *Tetraclinis articulata* are quite efficient

against *Tuta absoluta* (Bouayad Alam et al., 2017), a devastating pest of tomato originating from South America (Cabello et al., 2009). But, *Trichogramma cacoeciae* is also used against *Tuta absoluta* (Cherif, Grissa, 2015). In this case, both essential oils and biocontrol could be used as complementary methods against *Tuta absoluta*, constituting two phases of treatment. The second possible solution is to use the essential oils to make the trichograms more efficient by developing a push-pull strategy. Therefore, essential oils would be used to push the beneficial insects out of the surrounding area and to pull them to where they are required for biological control (Cook et al., 2007). To illustrate this second solution, an attractive essential oil such as Rosemary could be used as a pull strategy to attract the trichograms on the considered crops. But before creating any kind of integrated pest management by combining both methods, a study should be done on the effects of the envisaged essential oils on the biocontrol agents to identify and understand all the interactions which could happen. Moreover, even if using both methods is not considered, thinking of the side effects that essential oils can have on trichograms must still be done. Indeed, trichograms are pest enemies naturally present in the fields. So, using one control method such as essential oils, that actually impacts the natural regulations which already exist becomes quite questionable.

Developing the integrated pest management by using natural auxiliaries or by replacing chemicals by natural products such as essential oils is a way to start the agroecological transition towards sustainable agricultural systems. Effectively, agroecology is based on the fact that agroecosystems are ecosystems which have been modified by the human activity, but which cannot escape the general mechanisms of biology (Calame, 2016). Thus, its point is to implement new and sustainable agricultural systems by considering all the existing and natural interactions but also the ones, humanely implemented. That is the reason why studying nonintentional effects of essential oils on auxiliaries is a key point for the development of such systems.

Nevertheless, from an agroecological point of view, envisaging the use of the kind of biological control presenting is only a starting point for the transition and it is not the aim. Indeed, the final point of improving the systems by implementing new pest control strategies is to become independent towards the use of products even greener such as the essential in order to emphasize and enhance beneficial biological interactions and synergisms among agrobiodiversity components thus resulting in the promotion of key ecological processes and services (Altieri, 1995).

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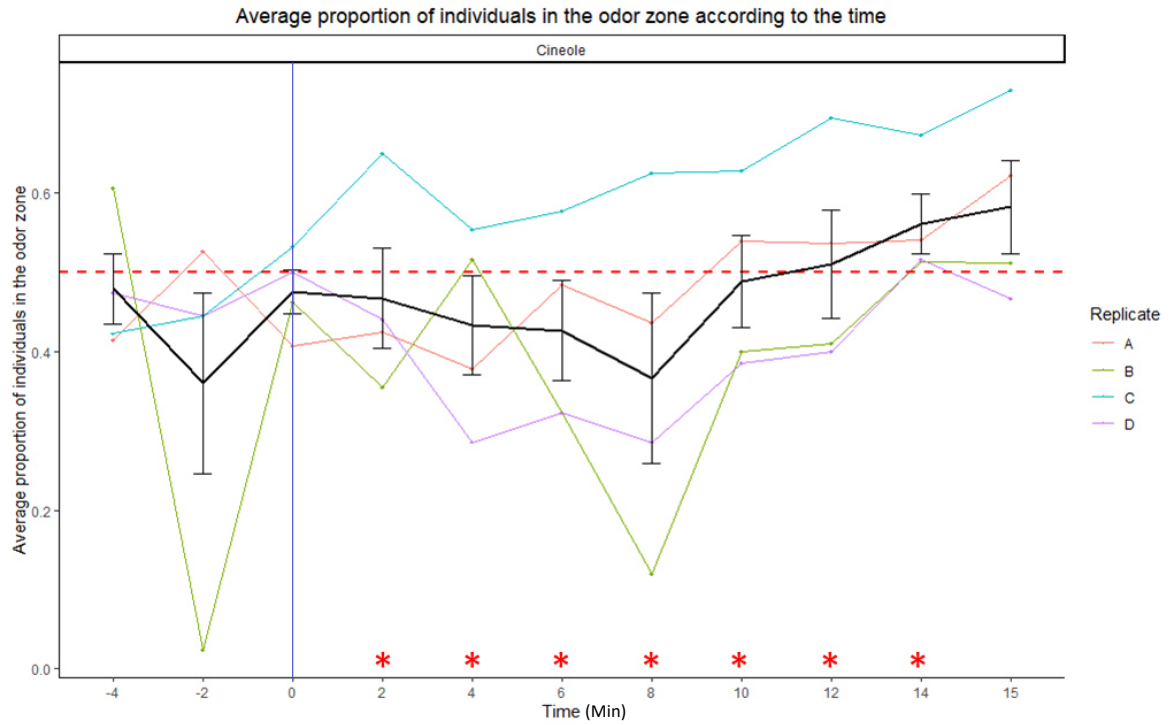
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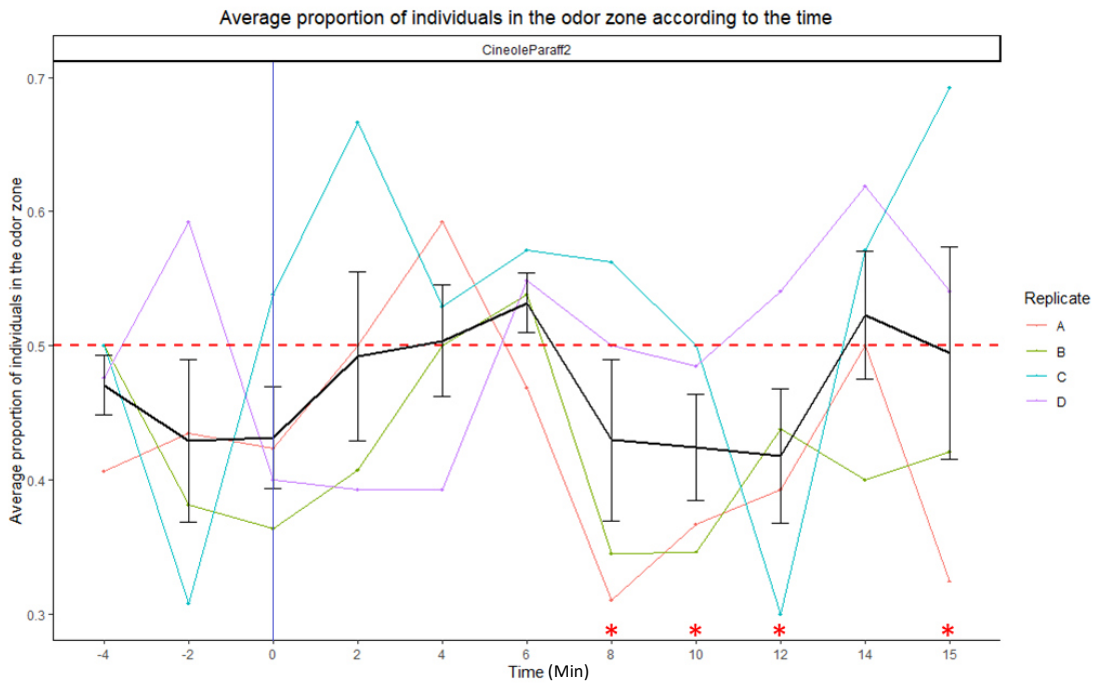
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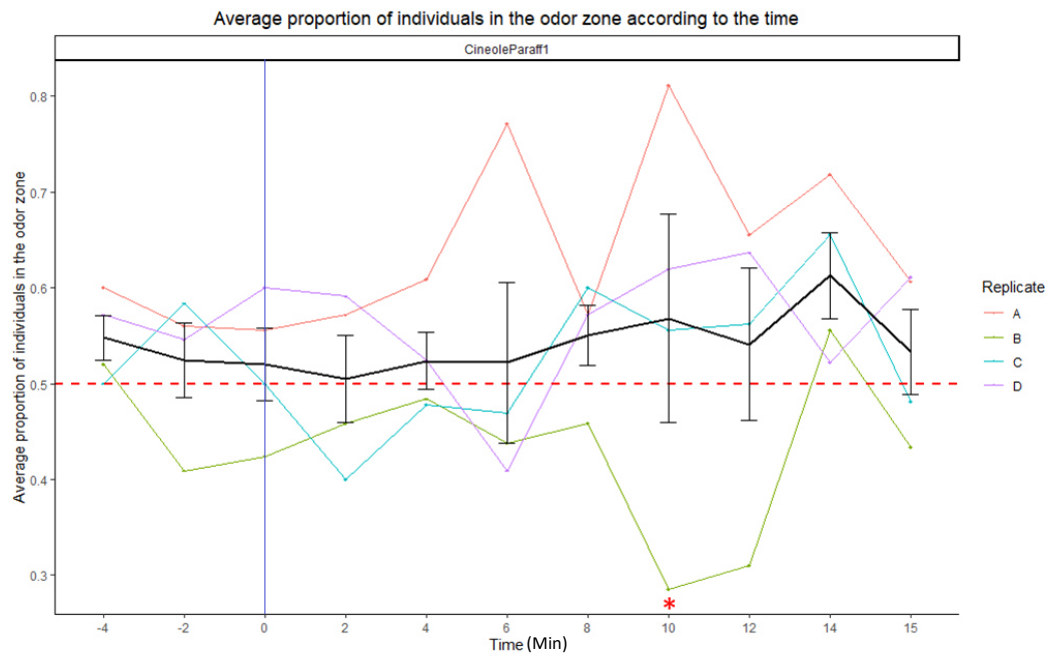
Appendix I: Graphs representing the different evolution of the proportions of individuals in the odour zones for pure Cineole on *T.cacoeciae*, strain ACJY144



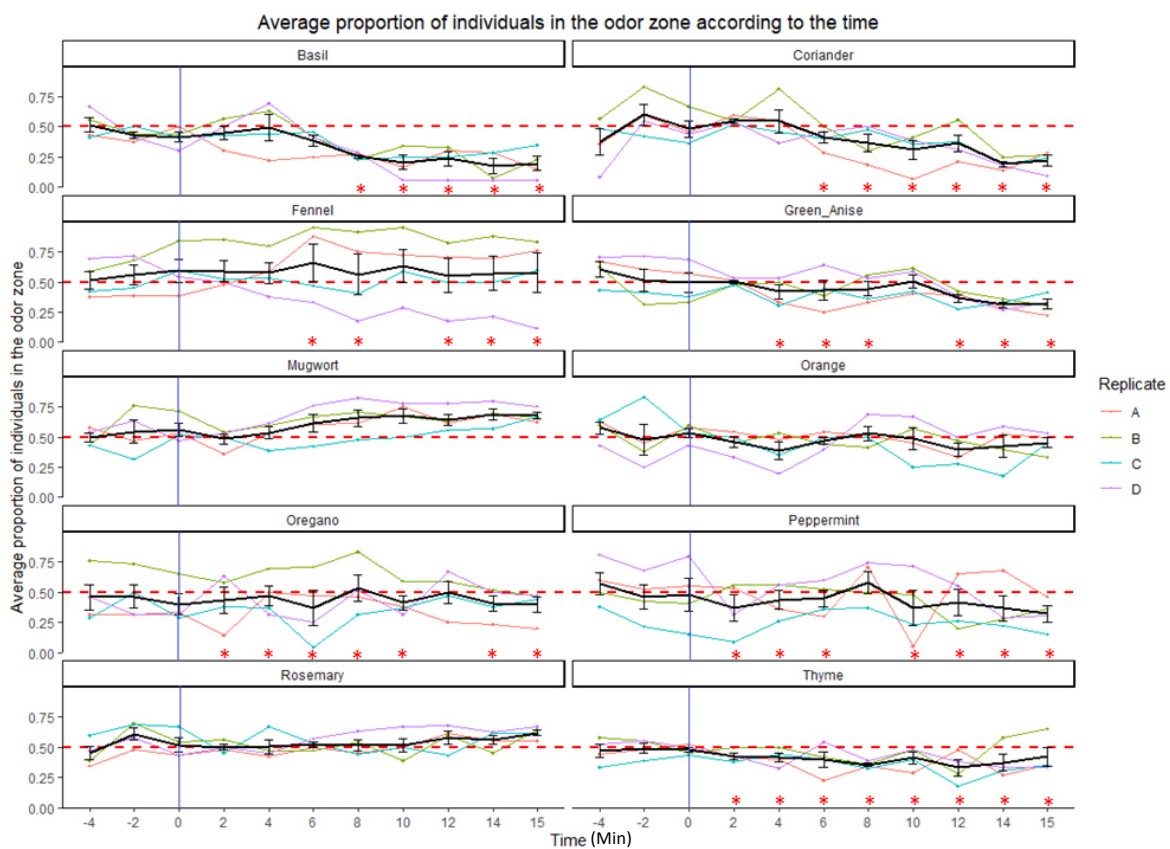
Appendix II : Graphs representing the different evolution of the proportions of individuals in the odour zones for Cineole 1/10% i.e 0.05 and 0.5 μL on T.cacoeciae, strain ACJY144



Appendix III : Graphs representing the different evolution of the proportions of individuals in the odour zones for Cineole 20/30% i.e 1 and 1,5μL on T.cacoeciae, strain ACJY144



Appendix IV : Graphs representing the different evolutions of the proportions of individuals in the odour zones for the ten essentials on T.cacoeciae, strain PJ

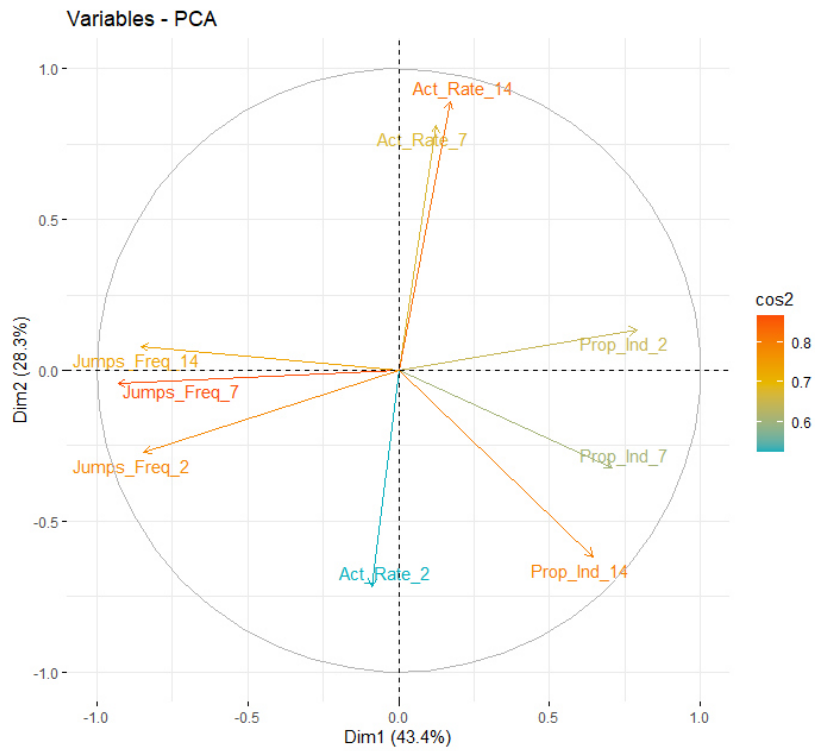


Appendix V : Summary table of the different effects of essential oils on *T.cacoeciae*.

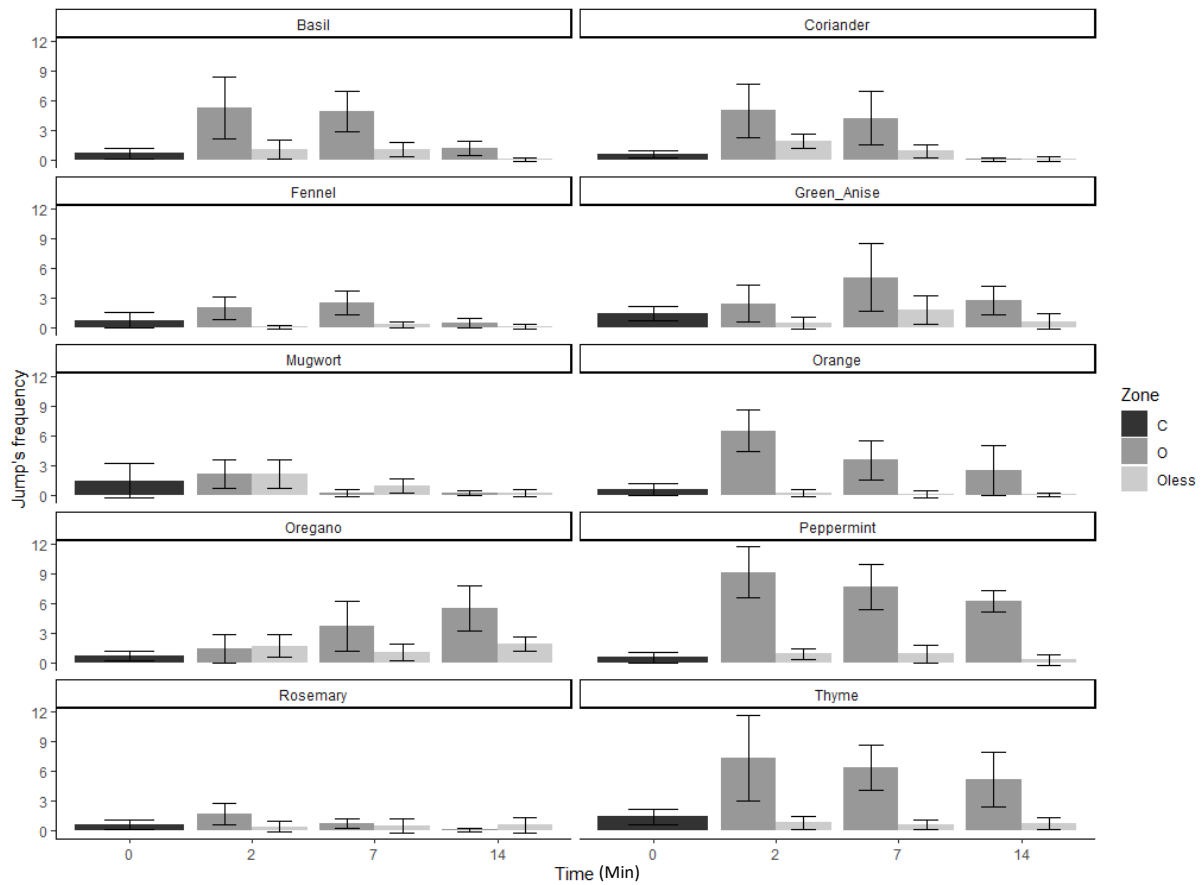
R: Repulsive, R-A: Repulsive then attractive, A: Attractive, N: Neutral

Essential oil	ACJY144	PMBIO1	PJ
Basil	R	R	R
Coriander	R	R	R
Fennel	R-A	R	A
Green Anise	R	R	R
Mugwort	R-A	R	A
Oregano	R	R	R
Peppermint	R	R	R
Rosemary	A	A	N
Sweet Orange	N	N	N
Thyme	R	N	R

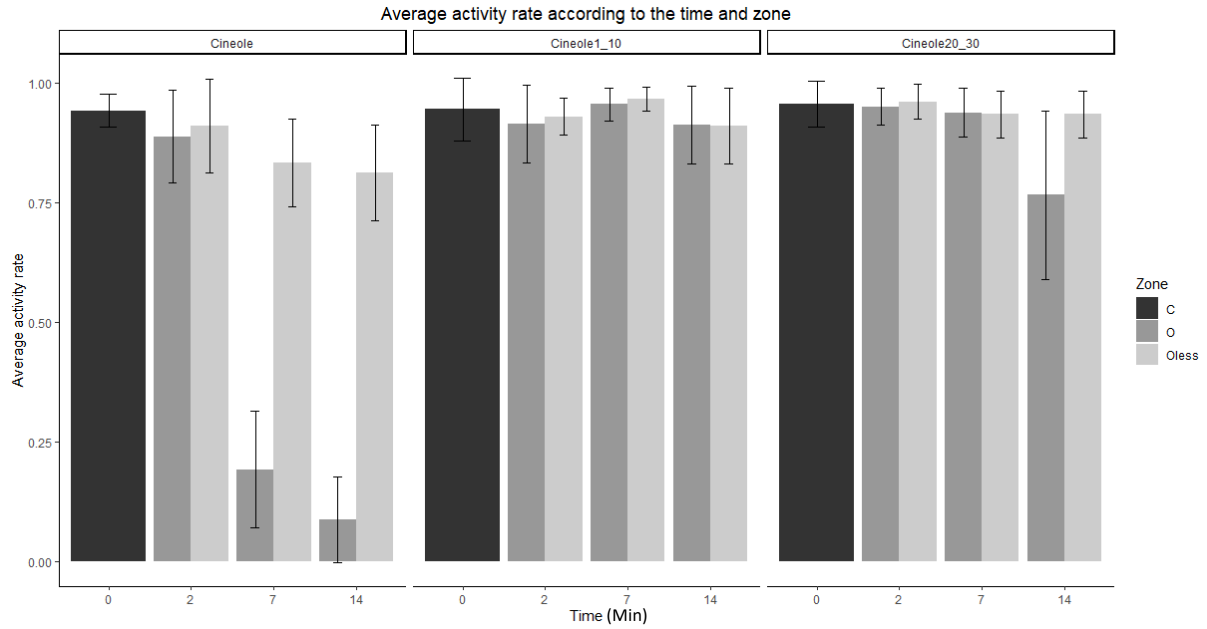
Appendix VI : PCA representing the three studied variables at three different times for the strain ACJY144: Proportion of individuals at 2, 7 and 14 min (Prop_Ind_2, Prop_Ind_7), Activity rate at 2, 7 and 14 min (Act_Rate_2, Act_Rate_7, Act_Rate_14), and Jump's frequency 2, 7 and 14 min (Jumps_Freq_2, Jumps_Freq_7, Jumps_Freq_14)



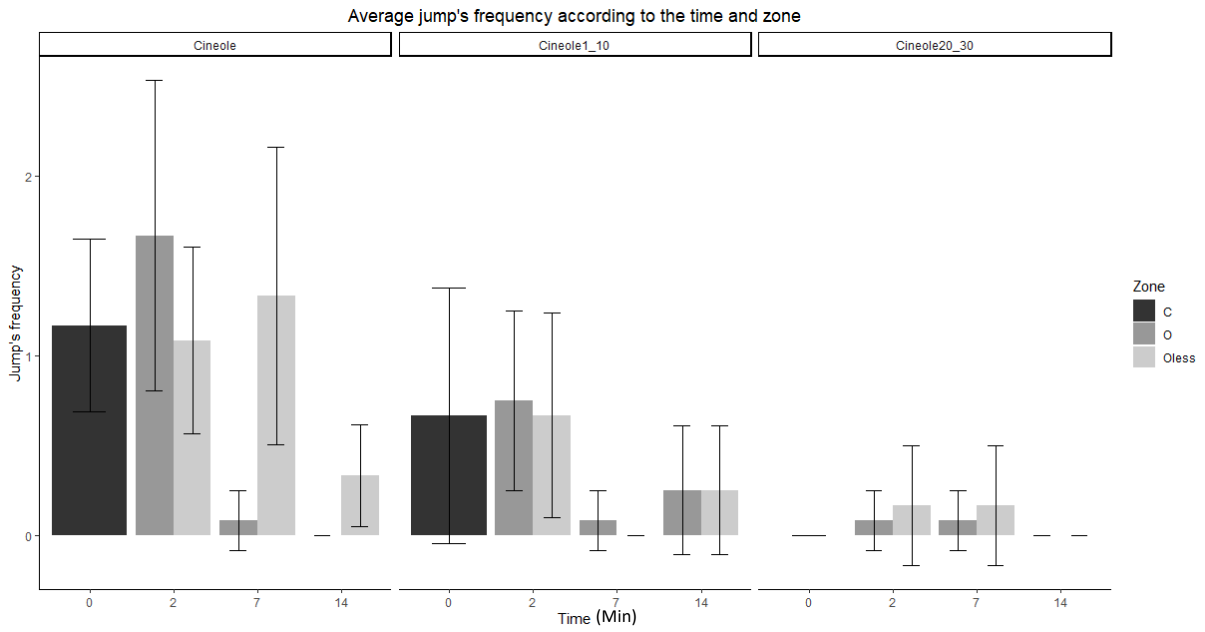
Appendix VII : Average jump's frequency observed through time for the ten essential oils tested on T.cacoeciae ACJY144. C: the controls, O: Odour Zone, Oless: Odourless Zone. The error bars correspond to twice the standard error i.e an estimation of the confidence interval at 95%



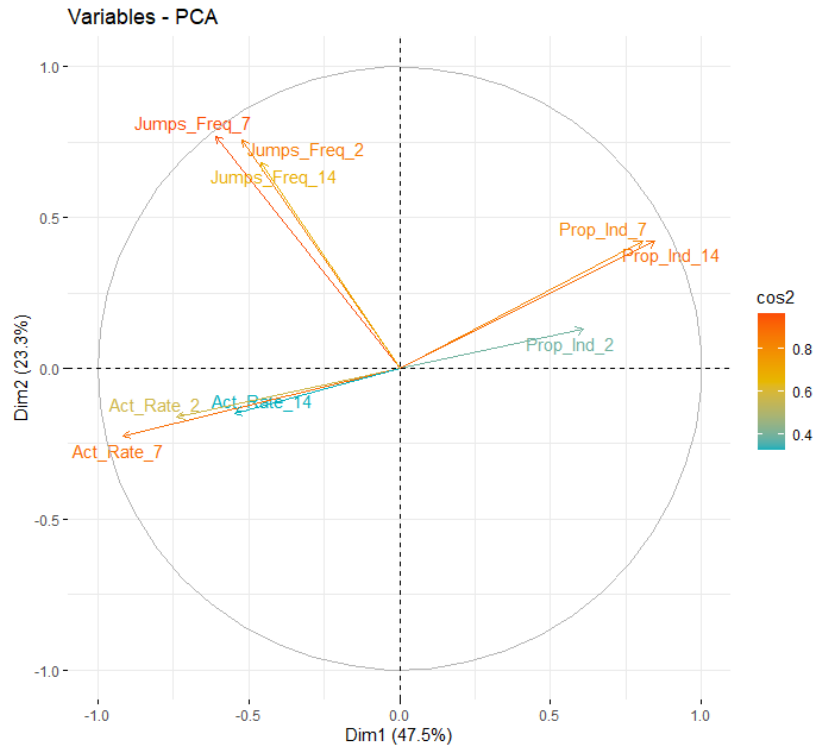
Appendix VIII : Average activity rate observed through time for the three modalities of cineole tested on T.cacoeciae ACJY144. C: the controls, O: Odour Zone, Oless: Odourless Zone. The error bars correspond to twice the standard error i.e an estimation of the confidence interval at 95%. Cineole i.e Pure Cineole, Cineole1_10 i.e 0.05µL and 0.5µL, Cineole20_30 i.e 1 and 1,5µL



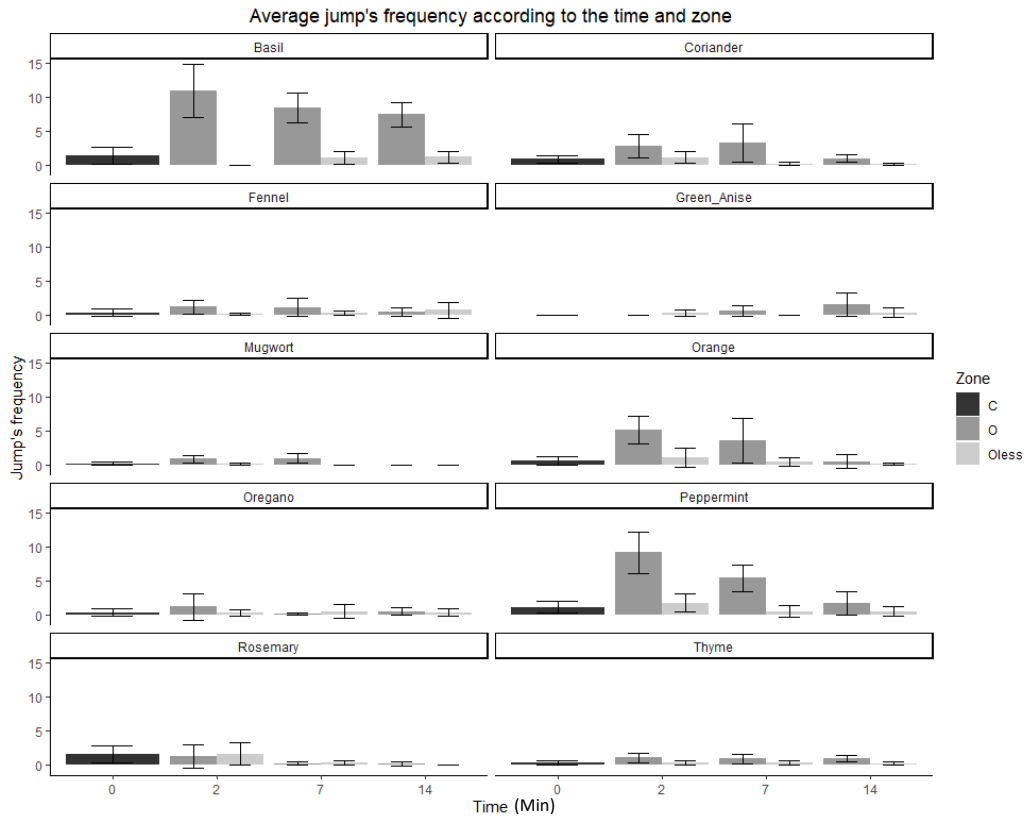
Appendix IX : Average jump's frequency observed through time for the three modalities of cineole tested on T.cacoeciae ACJY144. C: the controls, O: Odour Zone, Oless: Odourless Zone. The error bars correspond to twice the standard error i.e an estimation of the confidence interval at 95%. Cineole i.e Pure Cineole, Cineole1_10 i.e 0.05 μ L and 0.5 μ L, Cineole20_30 i.e 1 and 1,5 μ L



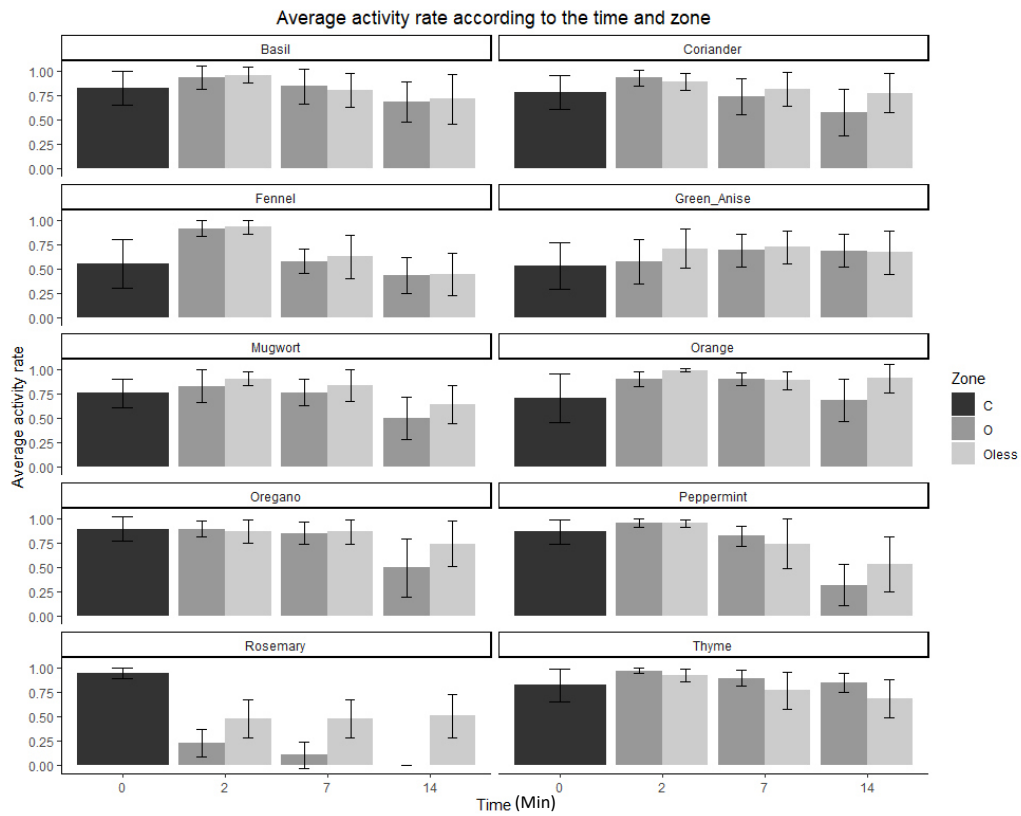
Appendix X : PCA representing the three studied variables at three different times for *T.cacoeciae* PMBIO1: Proportion of individuals at 2, 7 and 14 min (Prop_Ind_2, Prop_Ind_7), Activity rate at 2, 7 and 14 min (Act_Rate_2, Act_Rate_7, Act_Rate_14), and Jump's frequency 2, 7 and 14 min (Jumps_Freq_2, Jumps_Freq_7, Jumps_Freq_14)



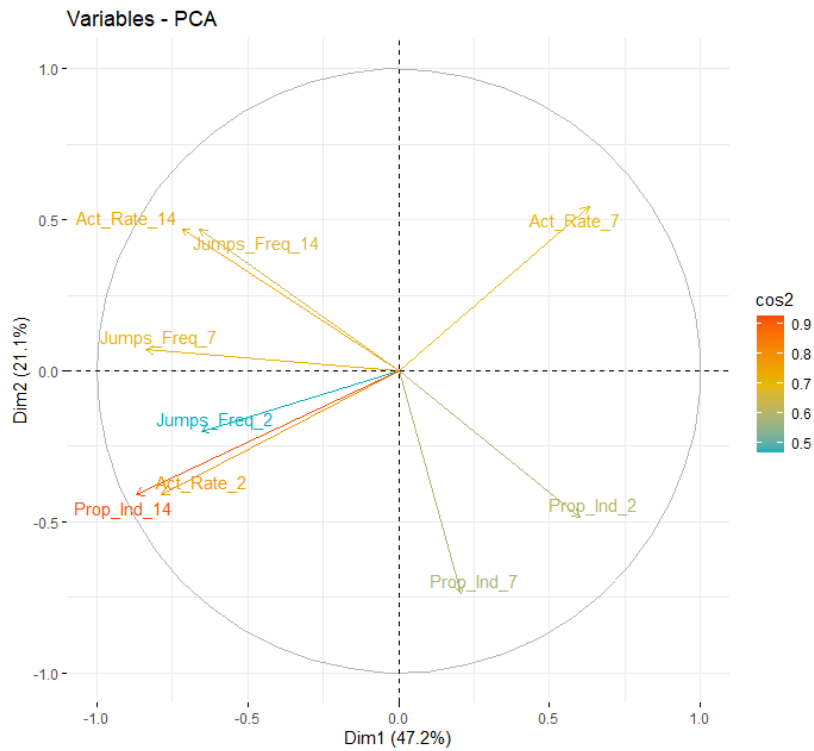
Appendix XI : Average jump's frequency observed through time for the ten essential oils tested on T.cacoeciae PMBIO1. C: the controls, O: Odour Zone, Oless: Odourless Zone. The error bars correspond to twice the standard error i.e an estimation of the confidence interval at 95%



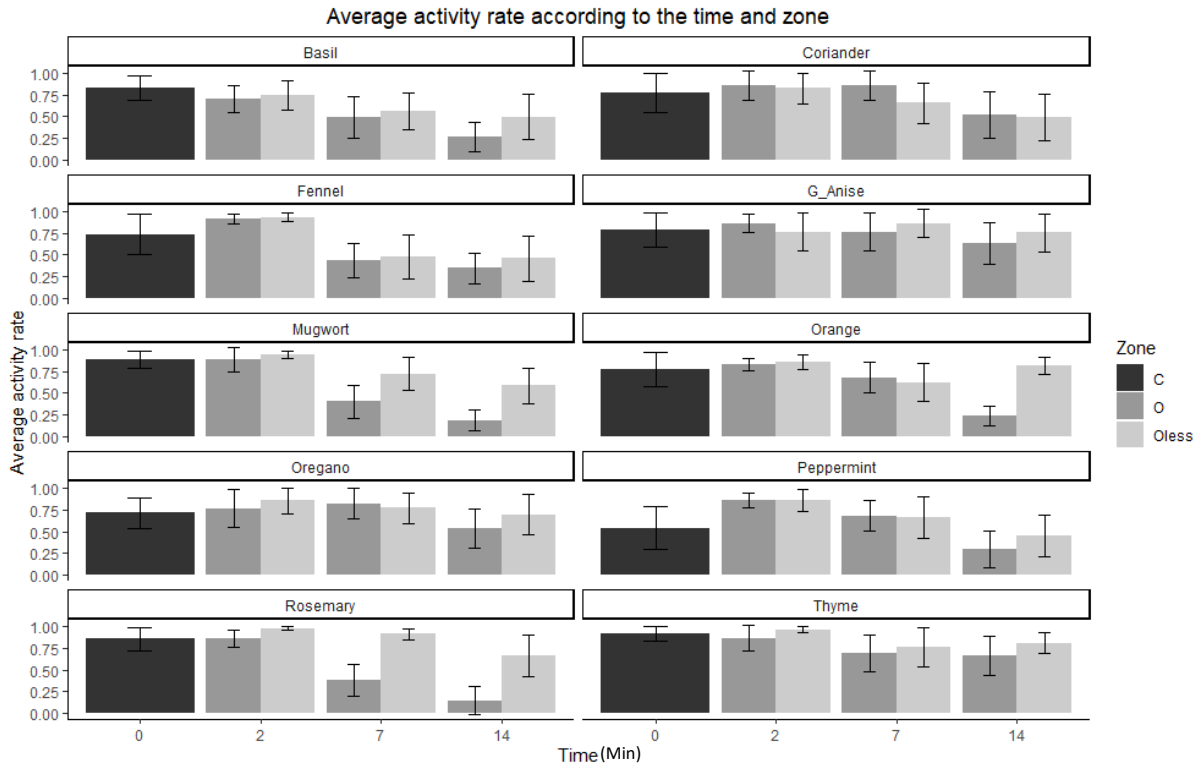
Appendix XII : Average activity rate observed through time for the ten essential oils tested on *T.cacoeciae* PMBIO1. C: the controls, O: Odour Zone, Oless: Odourless Zone. The error bars correspond to twice the standard error i.e an estimation of the confidence interval at 95%.



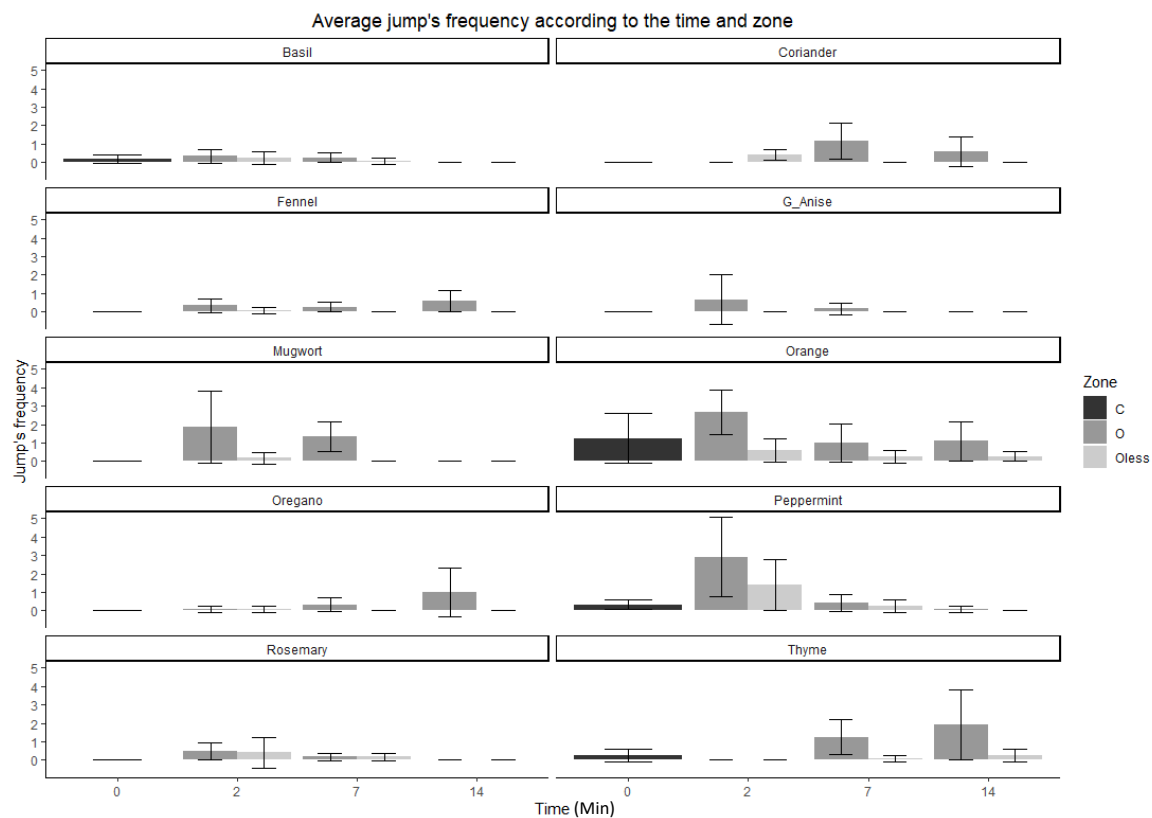
Appendix XIII : PCA representing the three studied variables at three different times for the strain PJ: Proportion of individuals at 2, 7 and 14 min (Prop_Ind_2, Prop_Ind_7), Activity rate at 2, 7 and 14 min (Act_Rate_2, Act_Rate_7, Act_Rate_14), and Jump's frequency 2, 7 and 14 min (Jumps_Freq_2, Jumps_Freq_7, Jumps_Freq_14)



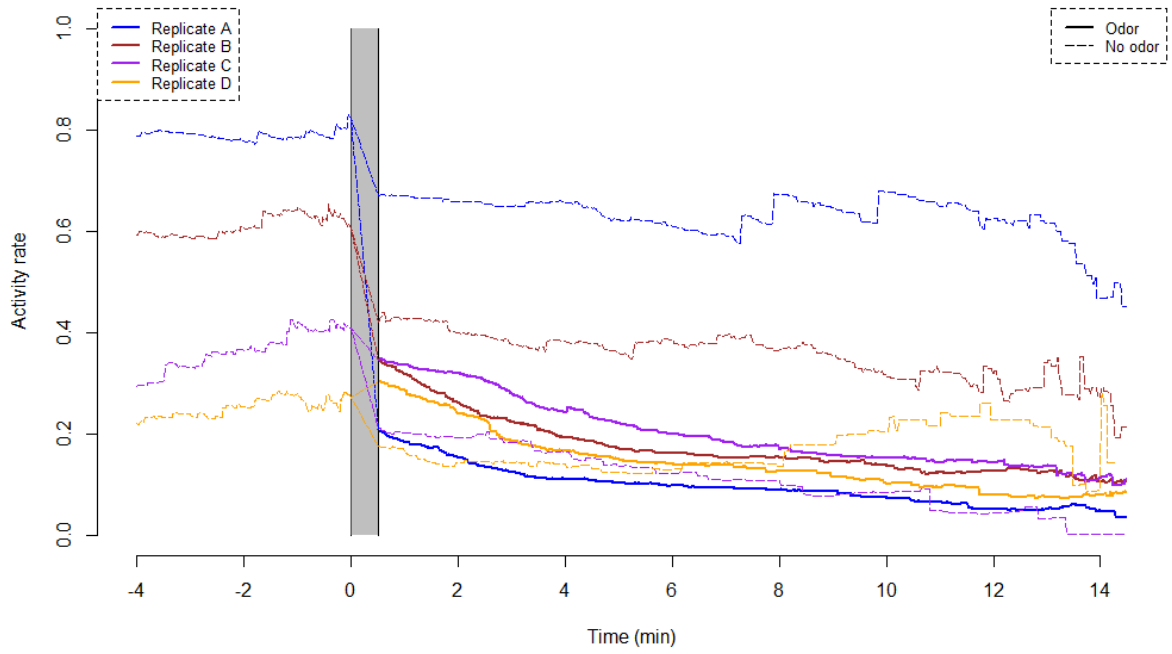
Appendix XIV : Average activity rate observed through time for the ten essential oils tested on *T.cacoeciae* P.J. C: the controls, O: Odour Zone, Oless: Odourless Zone. The error bars correspond to twice the standard error i.e an estimation of the confidence interval at 95%.



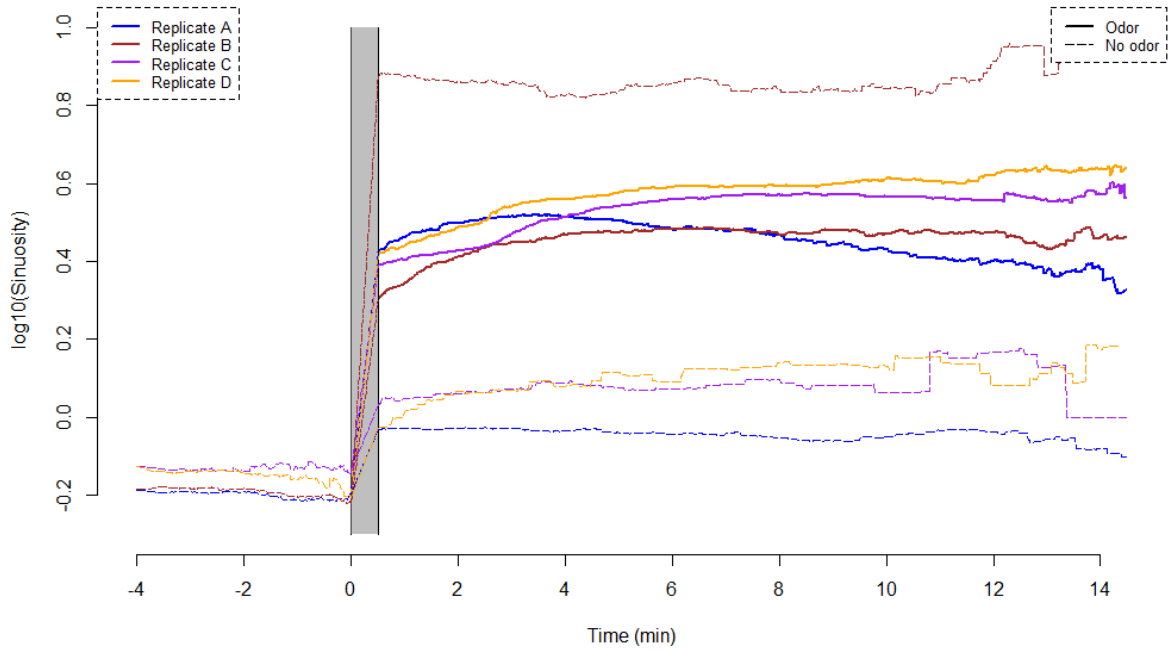
Appendix XV : Average jump's frequency observed through time for the ten essential oils tested on *T.cacoeciae* P.J. C: the controls, O: Odour Zone, Oless: Odourless Zone. The error bars correspond to twice the standard error i.e an estimation of the confidence interval at 95%.



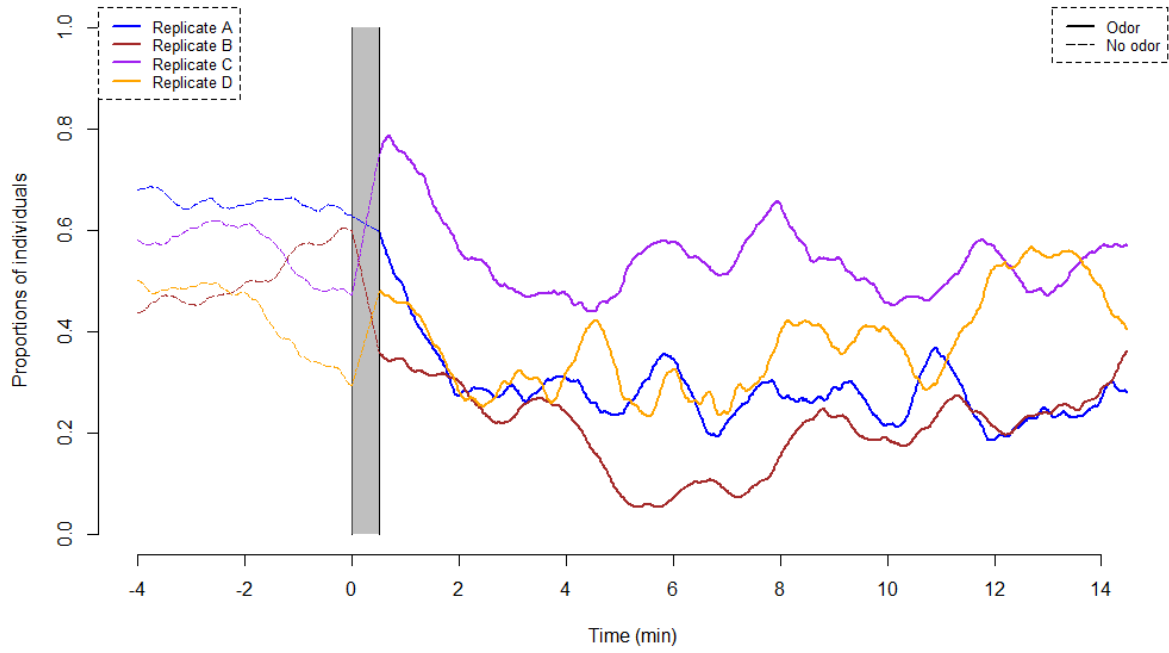
Appendix XVI : Evolution of the individuals' activity rate for the different zones through time for the Rosemary essential oil. The grey bar corresponds to the transition between the controls and the introduction of the odour.



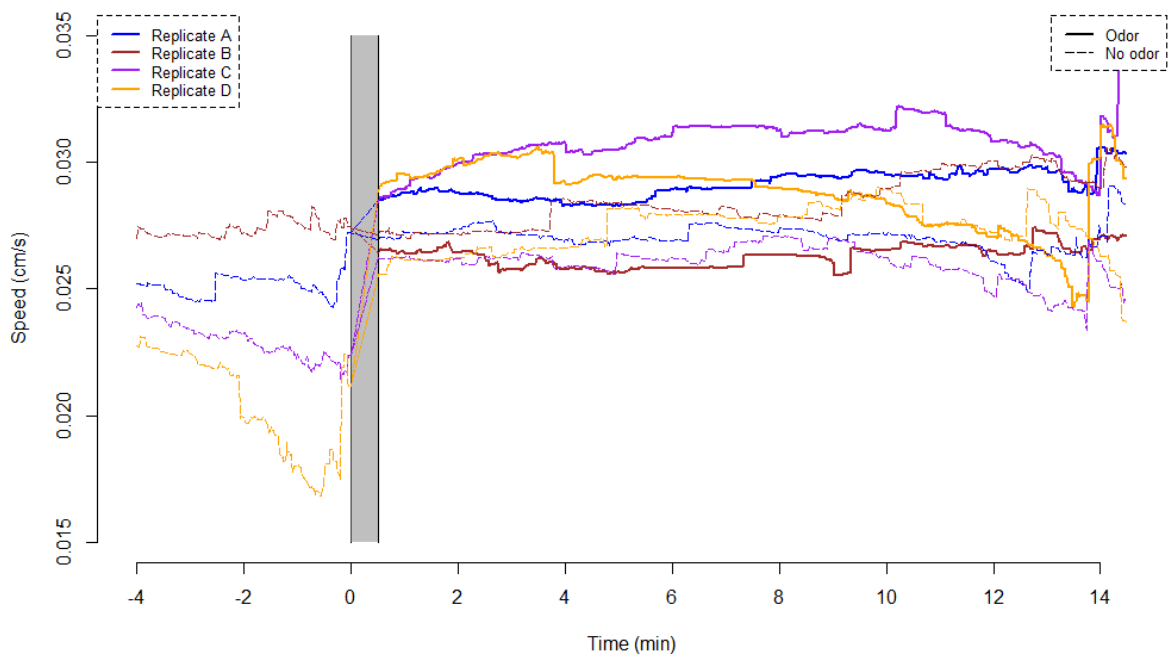
Appendix XVII : Evolution of the individuals' movement sinuosity for the different zones through time for the Rosemary essential oil. The grey bar corresponds to the transition between the controls and the introduction of the odour.



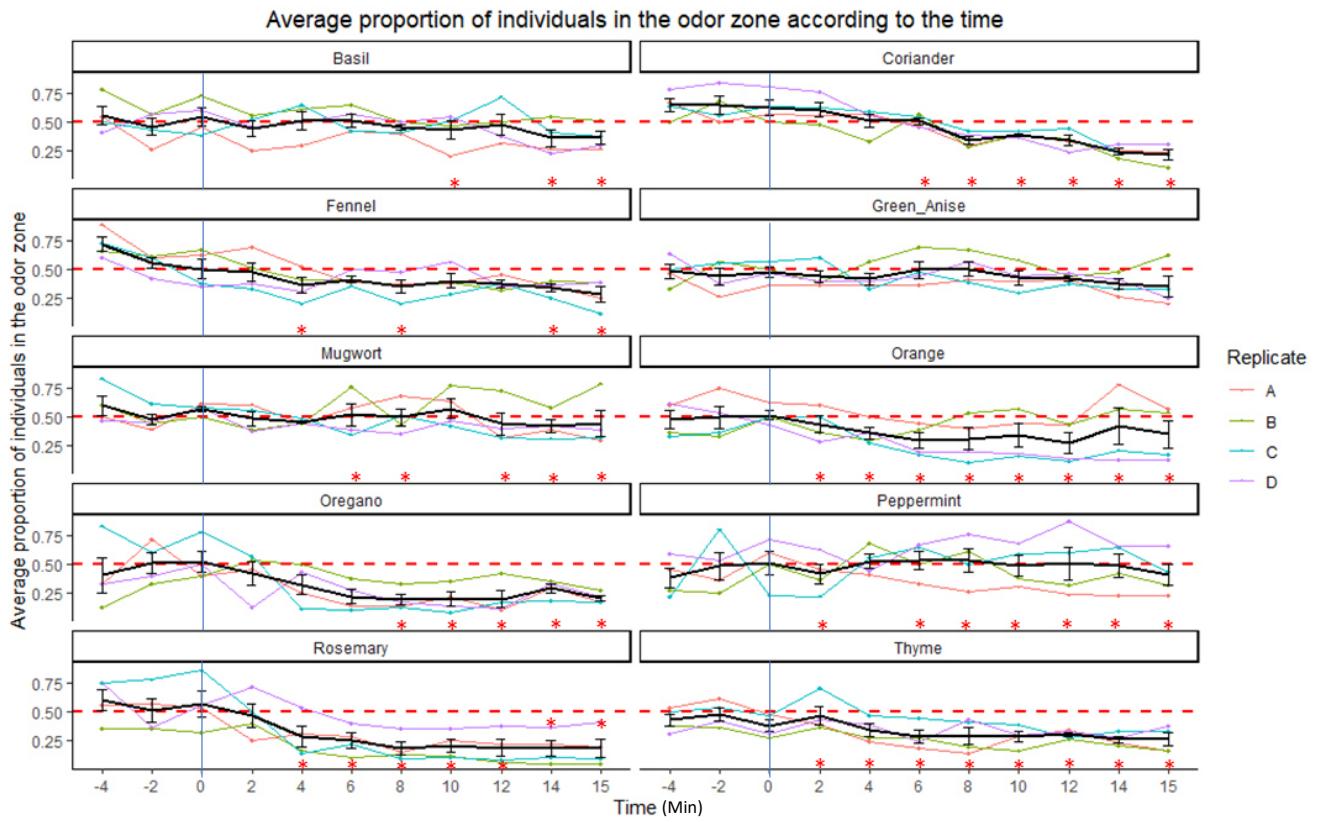
Appendix XVIII : Evolution of the proportions of individuals for the different zones through time for the Thyme essential oil. The grey bar corresponds to the transition between the controls and the introduction of the odour.




Appendix XIX : Evolution of the individuals' speed for the different zones through time for the Thyme essential oil. The grey bar corresponds to the transition between the controls and the introduction of the odour.



Appendix XX: Graphs representing the different evolutions of the proportions of individuals in the odour zones for the ten essentials on *T.cordubensis*, strain PUG029



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Nb pages :21 (76 au total) Annexe(s) : 20	
Année de soutenance :2020	Maîtres de stage : Louise VAN OUDENHOVE et Vincent CALCAGNO
Titre français : Effets non-intentionnels des biopesticides sur les auxiliaires de lutte biologique	
Titre anglais : Unintentional effects of biopesticides on biocontrol agents	
<p>Résumé (1600 caractères maximum) : L'agroécologie traite de diverses problématiques telles que la réduction de l'utilisation d'engrais et de pesticides. La lutte biologique ou l'utilisation de produits « naturels » comme les huiles essentielles peuvent être envisagées. Cependant, pour une mise en œuvre simultanée, ces produits ne doivent pas causer d'effets secondaires sur les insectes auxiliaires. Le but de cette étude est d'identifier les effets non intentionnels de dix huiles essentielles (basilic, coriandre, fenouil, anis vert, armoise, origan, menthe poivrée, romarin, orange douce et thym), et d'un composé contenu dans certaines d'entre elles : 1,8 cinéole ; sur le comportement de trois souches de l'auxiliaire <i>Trichogramma Cacoeciae</i> (micro-hyménoptère). Des expériences d'olfactométrie, des observations de vidéos, manuelles et automatisées ont été réalisées. Les réactions des trichogrammes vis-à-vis des odeurs sont dépendantes de la souche et du temps d'exposition. Pour toutes les souches, trois groupes d'huiles essentielles ont été identifiés. Les huiles essentielles attractives (Romarin, Armoise et Fenouil) sont responsables d'une diminution du taux d'activité et de la vitesse des parasitoïdes. La sinuosité de leur mouvement a tendance à augmenter. Les huiles répulsives augmentent leur vitesse ainsi que la fréquence de leurs sauts (possible comportement de fuite). Ces résultats suggèrent que l'utilisation de la lutte intégrée contre les ravageurs, basée à la fois sur les huiles essentielles et les agents de la lutte biologique doit faire l'objet d'une réflexion approfondie avant d'être mise en œuvre.</p>	
<p>Abstract (1600 caractères maximum): Agroecology deals with various issues such as the limitation of environmental pollution, by reducing the use of fertilizers and pesticides. The use of biological control or the use of "natural" products such as essential oils can be considered as solutions. But, if implemented together, they should not cause side effects on non-target insects. That is why the identification of natural products' unintentional effects on biocontrol agents is a key point in integrated pest management. The aim of this study is to identify the unintentional effects of ten essential oils (Basil, Coriander, Fennel, Green anise, Mugwort, Oregano, Peppermint, Rosemary, Sweet orange and Thyme), and one of their compound: 1.8 cineole; on the behaviour of three strains of the biocontrol agent <i>Trichogramma Cacoeciae</i> (micro-hymenoptera). Olfactometry experiments, manual, and automatic olfactometry observations of videos have been conducted. The trichograms' reactions towards essential oils odours were found to depend on the strain and the time of exposure. For all of the strains, three groups of essential oils have been identified. The attractive essential oils (Rosemary, Mugwort, and Fennel) are responsible for a decrease of their activity rate and their speed. Their movement sinuosity tends to increase. Repulsive oils set themselves apart by making their jump's frequency increase as as their speed, as a possible escape behaviour. These results suggest that the use of an integrated pest management based on both essential oils and biocontrol agents must be deeply thought before implementation.</p>	
<p>Mots-clés : Biocontrôle, Lutte biologique, huiles essentielles, effets non intentionnels, olfactométrie, analyse comportementale, <i>Trichogramma cacoeciae</i>. Key Words: Biological control, essential oils, unintentional effects, olfactometry, behavioural analysis, <i>Trichogramma cacoeciae</i>.</p>	