Repellence of Myzus persicae (Sulzer): evidence of two modes of action of volatiles from selected living aromatic plants.

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Repellence of *Myzus persicae* (Sulzer): evidence of two modes of action of volatiles from selected living aromatic plants

Running title: Repellent volatiles against *Myzus persicae*

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Abstract

BACKGROUND: Intercropping companion plants (CPs) with horticultural crops could be an eco-friendly strategy to optimize pest management. In this research, volatile organic compounds (VOCs) emitted by some CPs were investigated for their repellent properties towards the green peach aphid (*Myzus persicae* Sulzer). The aim of this study was to understand the modes of action involved: direct effects on the aphid and/or indirect effects *via* the host plant (pepper, *Capsicum annuum* L.).

RESULTS: We identified two promising repellent CPs species: the volatile blend from basil (*Ocimum basilicum*, direct repellent effect) and the mixture of (or previously intercropped) *C. annuum* plants with *Tagetes patula* cv. Nana (indirect effect). This effect was cultivar-dependent and linked to the volatile bouquet. For the 16 compounds present in the *O.*

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basilicum or *T. patula* bouquets tested individually, (*E*)-β-farnesene and eugenol reported good repellent properties against *M. persicae.* Other compounds were repellent at medium and/or at highest concentrations. Thus, the presence of repellent VOCs in a mixture does not mean that it had a repellent propriety.

CONCLUSION: We identified two promising repellent CPs species towards *M. persicae*, with a likely effect of one CPs' VOCs on the host plant repellency and highlighted the specific effectiveness of VOC blends.

Keywords: Olfactory behavior, Companion plant, *Myzus persicae* (Sulzer), Pepper (*Capsicum annuum* L.), Volatile Organic Compound (VOC), repellent,

Abbreviations: PPI, Pepper (*C. annuum*) Previously Intercropped; EBF, (*E*)-β-farnesene; CPs, Companion Plants; VOCs, volatile organic compounds.

1 Introduction

For many years, the intensification of agriculture and monocultures has caused various problems, in particular, the fast development of pest populations such as aphids (Hemiptera: Aphididae). Among these, the green peach aphid, *Myzus persicae* (Sulzer) is one of the main agricultural pests in temperate regions. *Myzus persicae* is responsible for considerable damage on crops, the transmission of various viruses, and major economic losses.[1] Mostly, the control of this polyphagous aphid is dependent on chemical measures. However, despite their high effectiveness in reducing pests, they have strong drawbacks as they favor the development of resistant pest populations and contribute to environmental contamination.[2]

Various alternative methods (e.g. using natural products, release of natural enemies, and push-pull strategy) have been investigated to reduce aphid infestations and limit their

damage to an acceptable level. Among these methods, intercropping with companion plants (CPs) is a promising eco-friendly approach.[3-5] These CPs can be suggested as natural diffusers of semiochemicals, primarily monoterpenes ($C_{10}H_{16}$) and sesquiterpenes ($C_{15}H_{24}$), which function as repellents or arrestants and can increase the protection of cultivated plants towards aphids.[6] The perception of potentially repellent plant volatile organic compounds (VOCs) through aphids sensilla (rhinaria – located in the insect antenna), can increase the deterrence effect, keeping populations away from crops, disrupting their settlement and most of all, reducing their performance and thus inhibiting the development of their population.[4]

Several authors state that some plants, belonging to the Lamiaceae and Asteraceae family, produce a number of VOCs with repelling properties against pests, including aphids.[5] However, most of the studies were carried out with essential oils or plant extracts.[7,8] Nonetheless, information available on living plants volatile effects is scarce. Various odor effects of aromatic plants with potential to influence aphid behavior were investigated in previous studies. Ben Issa *et al.* [9] reported that Marigolds (*Tagetes* spp.) produce a large number of VOCs which can affect aphid performance, an aspect also verified in the plant essential oils.[10] Basil (*Ocimum basilicum* L.) volatiles are well known for their repulsive effects on different aphid species.[5,9,11,12] VOCs from rosemary (*Rosmarinus officinalis* L.) plants are effective as a good aphid repellent.[9,13] Similar properties have been reported for volatiles from lavender (*Lavandula latifolia* L.) and peppermint (*Mentha piperita* L.).[9,14-16]

Scientists have put great effort into understanding CP volatile effects on pests. The properties of plant VOCs against aphids, such us repellency, have been frequently discussed in previous studies.[4,6,17-19] In contrast, as it is difficult to monitor whole-system experiments, few studies were focused on their mode of action[8] or on the underlying mechanisms.[20]

Nevertheless, in order to optimize the use of CP in pest management, we need not only the confirmation of the effectiveness of the plant VOCs tested but also a greater understanding of the mechanisms involved.[3] For this purpose, it is necessary to understand how CP acts and several hypotheses have been proposed for the mode of action of their volatiles towards aphids. By the emission of VOCs, CPs may disrupt aphid behavior directly through repellent activities.[4] They can also mask the attractant host plant odoriferous stimuli, thus preventing its recognition by the pest.[21] Therefore, chemical interaction between host plant VOCs and VOCs emitted from the surrounding environment and neighboring plants can also have a combined effect on herbivores. Another mechanism evocated is an indirect effect *via* the airborne communication between an emitter plant (CP) and a receiver plant (host plant).[22-24] Without physical contact, some plants can adsorb and re-release VOCs perceived from neighboring plants[25] and react to diverse signals. VOCs from neighboring plants can be perceived as biologically relevant information by the receiver plant i.e. aerial allelopathy, [26] and consequently modify its biochemical metabolism and/or its volatile emission.[22,23] Thus, VOCs as plant secondary metabolites can play an important role in plant-plant and plant-aphid chemical interactions and therefore be used as a tool to control aphids.

Furthermore, in order to better understand the action mechanisms of promising CPs, we need to know the effect of their compounds.[27] According to the information in literature, numerous single volatile compounds present in volatile mixtures of aromatic plants have been referenced to have a good repellent activity towards aphids and various pests, namely bisabolene, β-caryophyllene, camphor, (*E*)-β-farnesene (EBF), pinene, and linalol [10,13,16,17,19,28,29]. However, we lack information on the effects of many other compounds towards *M. persicae*. Also, there is a need to obtain more knowledge of the effect of individual compounds on *M. persicae,* in order to limit the field area dedicated to CPs and to find the best cultural practices to promote the emission of efficient VOCs.

The aims of the present study were to investigate the effects of six living aromatic plant species volatiles towards *M. persicae* orientation responses under laboratory conditions, and to define their mode of action. In order to identify the mechanisms triggered by plant repellent volatiles, we examined the aphids' orientation under two conditions: (1) when *M. persicae* were submitted to VOCs emitted by the CPs alone (direct repellency hypothesis); and (2) when they were submitted to interactions between the host plant and CP VOCs (indirect effect of CP VOCs on aphid via host plant). Furthermore, we characterized the VOCs emitted by CPs using GC-MS. For a better understanding of the mechanisms involved, we also tested the repellency of individual compounds to establish the link between their repellent properties and the emitted VOC profile.

2 Material and methods

2.1 Plant material

Pepper plants (six weeks old) (*C. annuum* L., cv. Yolo Wonder) were used as host plants and six CPs [basil (*O. basilicum* L.), lavender (*L. latifolia* L.), peppermint (*M. piperita* L.), rosemary (*R. officinalis* L.) (Lamiaceae) and two French marigold cultivars (*T. patula* L., cvs. Nana and Bonita Bolero) (Asteraceae)] were potted in 1 L pots except, for *L. latifolia* (3 L pots). The choice of these species was mainly based on the previous work of Ben Issa *et al.* (2016) [9]. For *T. patula*, we tested two cultivars to check another source of variability in volatile emissions so as to determine if genetic variability influences the CP effectiveness. Based on previous works on the effective phenological stage[30] against *M. persicae* (Dardouri *et al.*, not published), plants were used at non-flowering stage except for *L. latifolia* and *T. patula*, used at flowering stages. In order to prevent any interaction between their VOCs, CPs and host plants were placed in two separate greenhouses at the National Institute for Agricultural Research (INRA) of Avignon (France). Both greenhouses were maintained under

similar climatic conditions (temperature of 20 ± 5 °C; relative humidity (r. h.) of 60%-70%). All plants were cultivated without the use of chemical pesticides or fertilizers and were watered using a drip irrigation system.

Based on the results of the direct effect of plant VOCs on *M. persicae* (Y-tube olfactometer), we selected two species to test the indirect effects of their volatiles on aphids' orientation i.e. *O. basilicum* (direct repellent effect), *T. patula* cv. Nana (no direct repellent effect but a significant repellent effect in the presence of the host plant). CP (emitter) and *C. annuum* (receiver) plants were intercropped in a phytotron (22 ± 2°C, 60%–70% r. h. and L16:D8 photoperiod cycle) for 5 days before the experiment.[23] We used potted plants arranged in alternate rows of CPs and rows of *C. annuum* plants (20 cm between rows and pots in a row). Pots were placed in pot saucers so as to avoid any interaction between plant roots. Control *C. annuum* plants were grown simultaneously in another phytotron without CPs. Controls and treatment were used in olfactory bioassays. Pepper (*C. annuum*) Previously Intercropped will be further referred to as PPI.

2.2 *Myzus persicae* insects

Viviparous wingless females of *M. persicae* (clone Mp05), originally collected from a peach orchard of the INRA of Avignon, were reared on potted *C. annuum* plants in controlled conditions (temperature of 22 ± 1°C; r. h. of 60%-70% and L16:D8 photoperiod). Ten-day-old adult aphids (issued from the nymphs laid by females for 24 hours) were used for all olfactory tests.

2.3 Response of aphids to companion plant volatiles (Y-tube olfactometer bioassays)

In order to study the effect of VOCs from CPs on aphid olfactory orientation behavior under controlled conditions (22 ± 1 °C; 60–70% r. h.), we used a tubular Y-shaped

olfactometer (one 14 cm length arm and two 15 cm length branched arms, ID = 1.5 cm, angle between branched arms = 110°) (Figure 1A). A Y-shaped steel rod was placed in the center of the Y-tube glass and the olfactometer was positioned vertically.[31] Approximately four hours before beginning the bioassay, the plants used as an odor source were placed inside two airtight glass cages (Figure 1B) and the following choices were tested: (A), six *C. annuum* plants *versus* (*vs*) clean air and six CPs *vs* clean air; (B), six *C. annuum* plants *vs* six CPs; (C), three CPs and three *C. annuum* plants *vs* six *C. annuum* plants; and (D), six *C. annuum* plants *vs* six PPI with CPs. Two other tests (clean air *vs* clean air and six *C. annuum* plants *vs* six *C. annuum* plants) were carried out as controls to test for device bias.

During testing, 300 mL min^{-1} of air (monitored by flow meter) was channeled from each cage (potential VOCs source) into one of the two olfactometer arms. Using a fine paintbrush, a single aphid, starved at least for 2 h before the tests, was placed on the rod at the base of the olfactometer. The aphid could exhibit negative geotaxis and climb on the rod until the Y-junction where it made a choice between one of the two odor sources. The decision was recorded when the aphid reached one of the two olfactometer arms finishing lines, located 8 cm from the Y-junction. Aphids that did not make any choice within 5 min were scored as non-responders and were excluded from statistical analysis. In order to homogenize light in the experimental room and avoid any visual influence, the observations were performed in the dark with a red light lamp placed centrally above the olfactometer. In order to avoid device bias, after testing 10 individuals, the Y-tube was cleaned with ethanol at 70% (v/v) and distilled water and let dry for 5 min in the vacuum oven at 110 °C. Then, the positions of the olfactometer arms were reversed. After each experiment, the device was washed with soapy water (odorless detergent), ethanol (70%) and distilled water. For each modality, 120 responder aphids were individually tested. A permutation of odor sources was carried out

between the two cages after each 50% of observations (60 responder aphids). All the olfactory tests were conducted between 1 pm and 5 pm.

2.4 Collection and analysis of volatile compounds

VOCs emitted by CPs were collected in controlled conditions (22± 1 °C; 60–70% r. h.) using HS-SPME (headspace solid phase micro-extraction). Two hours before VOC sampling (at 11 am), six plants of each treatment were enclosed in one of the two airtight glass chambers (60 × 60 × 60 cm) (same device and same plants used for olfactory experiments). A dynamic system was used to sample VOCs (Figure 1C). An airflow (8 L min^{-1}, filtered through an active charcoal filter) was pumped by a compressor into each glass cage containing the odor sources. During sampling, an airflow regulated at a constant rate of 5 L min^{-1} was pumped from the ventilated glass chamber containing the VOCs source into a glass tube. Then, an SPME fiber (PDMS/DVB, 65 µm; Bellefonte, USA) was inserted into the glass tube through a septum and exposed to the airflow aspired for 30 min. VOC collections were replicated four times for each treatment. After the end of each VOC collection, the SPME fiber was injected directly into a gas chromatograph coupled to a mass spectrometer (GC-MS) (Trace-ISQ. single quadrupole (Thermo Scientific. Austin. TX. USA)) equipped with an apolar capillary column TR-5MS (Thermo) 20 m; 0.1 mm ID; 0.1 µm film thickness. The fiber was left in the injector for 2 min at 250 °C followed by a 30 min analysis. Helium was used as a carrier gas at a flow rate of 0.4 mL min^{-1}. The oven temperature was set at 40 °C for 2 min and then programmed from 20 °C min^{-1} to 300 °C. The ionization was by electron impact at 70 eV in the m/z 35-450 range. The integration of the peaks was achieved manually, obtained by using the Thermo Xcalibur software. The identification of peaks and compounds was performed by comparing the mass spectra and retention indices with those obtained from

commercial standards (for available compounds), and with those found in the NIST11 (National Institute of Standards and Technology, Gaithersburg, MD, USA) spectral library.

2.5 Chemical standards and reagents

Based on VOCs profile analyses, the following compounds, identified as major compounds in the volatile profiles of effective plants against *M. persicae* (i.e. *O. basilicum* and *T. patula* cv. Nana) and/or common compounds of these plants, were tested individually in this study (respective standard purity represented in brackets): EBF (93%), eucarvone (99%), eugenol (98%), geranyl acetone (97%), limonene (97%), linalol (97%), methyleugenol (98%), myrcene (90%), ocimene mixture (90%), p-cymene (97%), piperitone (98%), sabinene (75%), terpinolene (90%), verbenone (93%), α-terpineol (96%), and β-caryophyllene (80%). All these compounds were purchased from Sigma-Aldrich® (France). EBF, the main component of the aphid alarm pheromone, was used as a positive control and pure ethanol was used as a negative control. All the tested compounds were diluted at 0.01, 0.1 and 1.0% (v/v) in ethanol. Five compounds (i.e. α-bergamotene, δ-cadinene, β-elemene, germacrene-D, and γ-muurolene) were not tested as they were not available for purchase.

2.6 Aphids' response to individual volatile compounds (still-air olfactometer bioassays)

The *Myzus persicae* olfactory response to single synthetic compounds was tested using the same assay protocol used by Abtew *et al.* [7], with some modifications so as to adapt them to aphids' behavior. The linear tube still-air olfactometer consisted of a glass cylinder (L = 10 cm; ID = 2 cm), which was closed at the top with a screen mesh, a treated filter paper (Whatman® N°1. 4.5 cm²) and a rubber cap, in this order, and was closed at the bottom with a perforated transparent rubber box (L = 2 cm; ED = 2 cm) (Figure 2). The olfactometer was divided into three equal parts (3.8 cm per section). A transparent yellow PVC washer (thickness = 0.2 mm; width = 0.5 cm) placed at the end of the top section as a visual cue,

combined with the negative geotactic behavior of aphids, naturally pushes the majority of aphids into this section. However, aphids prefer to remain in the lower part when the tested compound has a repellent property. Individual compound solutions for each volatile were prepared in absolute ethanol (as a solvent) at 0.01, 0.1 and 1% (v/v), dosed at a volume of 3 µL on filter paper (odor source), and fixed in the rubber cap which allows closing the top end of the olfactometer. A rubber box containing 10 *M. persicae* females, previously starved for 3 h, were placed at the bottom end of each olfactometer. The number of aphids in each section was recorded 10 and 20 minutes after the test began. The repulsion index was calculated according to the following formula [7]:

$$Repulsion\ index\ (Ri) = \frac{((mdT \times t)+(mdM \times m)+(mdB \times b))}{n} = \frac{((1.9 \times t)+(5.7 \times m)+(9.5 \times b))}{(t+m+b)} \quad (1)$$

Where Ri represents the repulsion index; mdT is the mean distance for the top olfactometer part; mdM is the mean distance for the middle section; mdB is the mean distance for the bottom section; t, m, b and n are the number of aphids counted at the top, middle, bottom section and the total number of aphids per olfactometer, respectively.

Each concentration of each compound was repeated eight times simultaneously with eight controls (3 µLof ethanol). The three concentrations of the same compound were tested per day, from the lowest to the highest concentration. All tests were conducted in controlled conditions (22 ± 1 °C; 60-70% r. h.), between 8 am and 6 pm, under a fume hood (Geometra®) illumined from above by fluorescent tubing. The device was washed with odorless detergent, ethanol (70%) and distilled water after each test, and then air-dried for at least 16 h.

2.7. Statistical analysis

Statistical analyses were performed with R 3.1.0 software. Concerning Y-tube olfactometer experiments, the distribution of responders on each arm of the olfactometer

(odor sources) was analyzed for each treatment conducted by means of Chi-square (χ^2) tests with a 50% expected response (α=0.05). The non-parametric Kruskall-Wallis test was used to compare the number of non-responders between the different treatments. For still-air olfactometer bioassays, repellency of tested compounds was calculated using the above-mentioned formula. These data were first checked for normality through the Shapiro-Wilk test and for unequal variance using Levene's test. Given that normality and homoscedasticity were not achieved for all data, the non-parametric Wilcoxon test was applied to compare the olfactory responses of *M. persicae* between control (no compound) and treatment (individual compounds).

3 Results

3.1 Effect of companion plant volatiles on aphid olfactory response

The majority of the aphids assayed made a choice; only 3% had no response to the bioassays. The number of non-responders was not statistically different among the various tests (Kruskall–Wallis: K = 34.89; *P*-value = 0.07). For both tests setting similar content in both cages (either clean air *vs* clean air or *C. annuum vs C. annuum*), *M. persicae* responded equally to odors from the two Y-arms, confirming that there was no directional bias (Figure 3). No preference for *C. annuum* over the clean air was observed, indicating that the volatile blend from this host plant was not attractive to *M. persicae*. The results showed that of the six CPs tested, *M. persicae* were repelled only by *O. basilicum* volatiles when compared with clean control air or *C. annuum* volatiles (63 % and 66 % of repellency, respectively). *Rosmarinus officinalis, M. piperita, L. latifolia,* and both *T. patula* cultivars had no significant effect on *M. persicae* orientation responses. However, when the odors of CP and *C. annuum* plants were mixed against *C. annuum* odors, in addition to the significant effect observed with

O. basilicum-*C. annuum* mixture (61% repellency), the *T. patula* cv. Nana-*C. annuum* mixture affected the olfactory response of *M. persicae* (63% repellency). Also, when given the choice between *C. annuum* and PPI with CPs, aphids showed a significant preference for *C. annuum* (59 %) only against PPI with *T. patula* cv. Nana.

3.2 Analysis of the volatile profiles of companion plants

The GC-MS analyses of volatiles collected from six CPs are shown in Table 1. A total of 20, 12, 47, 25, 30, and 30 were identified in the respective volatile profiles *of R. officinalis, M. piperita. L. latifolia*. *O. basilicum. T. patula*. cv. Nana, and *Tagetes patula*. cv. Bonita Bolero. The main VOCs emitted by *R. officinalis* were borneol (19%), limonene (15%), o-cymene (12%), bornyl acetate (9%), camphor (8%), and α-pinene (7%). Few compounds were emitted by *M. piperita.* The major components were menthone (35%), menthofurane (24%), menthol (12%), pulegone (10%), limonene (8%), and menthol acetate (7%). *Lavandula latifolia* emitted important amounts of linalol (42%), camphor (17%), and borneol (18%). The *Ocimum basilicum* VOC blend was characterized by a high content of methyleugenol (76%) and a considerable percentage of EBF (7%), α-bergamotene (6%) and eugenol (5%). The main compound of *T. patula* cultivars Nana and Bonita Bolero was β-caryophyllene (32% and 36%, respectively). They also emitted an equal proportion of limonene (6%). Nevertheless, we observed that chromatographic profiles differed depending on the cultivar. The proportion of terpinolene was higher in Bonita Bolero (17%) than Nana (7%). An important proportion of piperitone (12%), (*E*)-β-ocimene (10%), verbenone (5%), eucarvone (4%), and p-cymen-8-ol (3%) were present in the Nana VOC profile. On the other hand, β-elemene (7%), neo-allo-ocimene (6%) and δ-cadinene (3%) were important volatiles in the Bonita Bolero profile. We verified the absence of EBF in the VOC blend from Nana plants and of α-terpineol in Bonita Bolero's. Finally, we observed that some compounds were equally present in different species. The most frequently detected compounds were eucalyptol, geranyl acetone,

limonene, linalol, β-myrcene, and terpinolene (identified in the volatile profiles of four different species). Globally, the compounds identified belong to two chemical groups of compounds of terpenic nature: monoterpenes and sesquiterpenes.

3.3 Effect of individual compounds on aphid olfactory response

Behavioral responses of *M. persicae* to individual compounds emitted in greater proportion (> 1%) by two promising CPs (*O. basilicum* and *T. patula* cv. Nana) were evaluated at three different concentrations and two assessing moments (Figure 4). Still-air olfactometer tests demonstrated that among the 16 compounds tested, only six (i.e. eucarvone, methyleugenol, limonene, p-cymene, terpinolene, and verbenone) did not have a significant repellent effect on *M. persicae,* whatever the concentration tested. The repellent activity depended on the concentration used. Only EBF and eugenol exhibited repellent action against *M. persicae* at the three concentrations. Aphids were significantly repelled by myrcene and α-terpineol at medium concentration (0.1%), and by myrcene, α-terpineol, linalol, geranyl acetone, ocimene, piperitone, sabinene, and β-caryophyllene at the highest concentration (1%). Globally, responses of *M. persicae* were similar at both exposure times except for α-terpineol, myrcene, sabinene, and linalol.

4 Discussion

The screening performed in the present study to evaluate the effectiveness of VOCs emitted by living aromatic plants against *M. persicae* confirmed recent works reporting that VOCs from aromatic plants could affect the olfactory behavior of aphids.[4,9,23] Among the six aromatic plant species tested, only two were repellent plants. The *Myzus persicae* olfactory response was directly affected by *O. basilicum* VOCs. In addition, the VOCs of *T. patula* cv. Nana only acted on their olfactory orientation in the presence or *via* the host plant (*C.*

annuum). Contrary to other studies, our findings demonstrated that none of the headspace volatiles from *L. latifolia, M. piperita* or *R. officinalis* plants exhibited significant negative effects against *M. persicae*.[9, 10, 13-16] Differences between results were probably related to a qualitative (presence/absence of specific VOCs) and quantitative variations among the chemical composition of volatiles perceived by aphids.[18] Variations in volatile profiles of the same plant species can be related to genetic differences and to various biotic and abiotic factors linked to plant nutritional status, stress conditions and phenological stages.[20,30,32,33] In addition, aphid olfactory responses to headspace volatiles released from living plants can be different from those of essential oils.[34] Aphid genotype could also account for the variability between studies as behavioral responses differ according to aphid species.[7,35]

One very interesting point in these findings is the specific response depending on the cultivar of *T. patula*: a significant effect was observed with the Nana cultivar while no significant effect was recorded with Bonita Bolero. This result may be related to a qualitative (e.g. absence of α-terpineol in Bonita Bolero) and/or a quantitative variability of VOCs emissions. The variation between the volatile profiles of the two cultivars is likely to have generated different interactions with the neighboring *C. annuum* plants[36] and consequently, a different aphid olfactory response[33]. These results support the idea that aphids are very sensitive to detect even a low chemical variability in the perceived volatile blend.[37]

From our observations, we showed that promising CP VOCs act by two different mechanisms. First, and in line with previous works [9,11], the findings of the present study confirmed that *O. basilicum* VOCs have a direct negative effect towards *M. persicae* olfactory behavior. These results are consistent with Digilio *et al.* [12] observation that vapors of basil essential oils have a repellent effect against two aphid species i.e. *M. persicae* and *Acyrthosiphon pisum.* According to Tiroesele and Matshela [5], *O. basilicum* repels *Brevicoryne brassicae* L. in kale cultivation. *Ocimum basilicum* is also known for its negative effects on

many pests.[38] For example, its essential oil volatiles have repellent activities against mosquitoes[39] and *Tribolium castaneum* (Coleoptera: Tenebrionidae).[40]

Probably, the repellent property of *O. basilicum* against *M. persicae* may result from the individual action of one or more of its volatile compounds.[27] In this sense, according to our results of the still-air olfactometer bioassay, it can be mainly related to their two predominant compounds demonstrated as repellent when tested individually i.e. EBF and eugenol. In this respect and as previously discussed, EBF, the main component of the aphid alarm pheromone, was repeatedly demonstrated in many studies as a good olfactory repellent against aphids.[41-44] However, other authors suggested that aphids could determine whether the EBF came from an aphid (pulsed emission) or a plant (continuous emission) and reported that the EBF emitted by a plant had no direct effect on their olfactory behavior.[42,43,45] The effectiveness of eugenol was consistent with previous studies reporting that eugenol has repellent activity against *M. persicae* [29] and other insects.[40,46,47] Despite the fact that the repellent effect of *O. basilicum* could be attributed to dominant VOCs [27,41], the role of other minor compounds cannot be disregarded[48] since they can be behaviorally active even in low doses.[49] As minor compounds emitted from *O. basilicum*, α-terpineol, myrcene, geranyl acetone, and sabinene were demonstrated as repellent at one or more concentrations tested. Our results corroborate previous reports on the repellency of these compounds against aphids and other insects.[10,13,17,27,28,44,48,49]

It is interesting to note that repellent and non-repellent plants share some VOCs that have shown such activity. For example, EBF was present in the odor bouquet of *L. latifolia, O. basilicum,* and *T. patula* cv. Bonita Bolero. Odor blends from *L. latifolia*, *O. basilicum, R. officinalis,* and *T. patula* contain α-terpineol, which was associated with *M. persicae* repellent effects.[13] Our results confirmed that aphid responses to some individual compounds were not the same when these compounds were mixed with others.[20] The repellent activity of some

compounds could be modulated by other compounds which can either inhibit or mask their effects.[50] Thus, the absence of repellent activity of a blend containing active compounds can be linked to synergic and antagonistic interactions between the VOCs perceived by aphids.[13,27] For example, Webster *et al.* [49] demonstrated that the volatile blend of *Vicia faba* (*Aphis fabae* attractive host plant) contains 10 compounds that repelled the aphid when individually tested. Recently, we showed that five rosemary clones all emitted a number of repellent compounds against aphids, whereas only one clone presented a repellent activity on *M. persicae* (Dardouri *et al.*, not published). The presence of repellent or attractive compounds in the plant volatile headspace does not necessarily mean that this plant is repulsive or attractive to the insect.[28] The function of volatile compounds varies according to volatile combinations.[49] For example the β-caryophyllene was reported as an effective compound against *M. persicae* [10] and as an inhibitor of EBF repellent activity.[28,45] Also, using a Y-tube olfactometer and different *Medicago* species, Mostafavi *et al.* [51] showed that *A. pisum* Harris and *A. kondoi* Shinji were only repelled by volatile blends released by the species with high ratios of EBF relative to β-caryophyllene. These hypotheses could probably explain why volatile blends of the *T. patula* cultivar cv. Bonita Bolero and *L. latifolia* had no repellent effect on *M. persicae* despite containing EBF. Thus, aphids have more sensitivity to specific ratios than to single compounds.[37,48] Our findings also show that some individual compounds were behaviorally active only when their concentration exceeded a detection threshold (high concentration) *i.e.* ocimene, piperitone, β-caryophyllene, linalol, sabinene, and geranyl acetone.[49] Repellency response to volatile compounds depends on their concentration in the blend perceived by the insect.[27] The same compounds can be attractive at a concentration and repulsive at another and can function as either a kairomone or an allomone. Obviously, most of the plant repellent properties were related to the mixture of VOCs from these plants and were not only generated by one compound alone.[49] Further testing should be

conducted in order to investigate the interaction between different combinations of VOCs and to establish a dose-response relationship that would identify profiles that may have a direct repellent effect on *M. persicae*.[50]

In general, effective repellent CPs directly influence aphids' behavior through their VOCs. Nevertheless, in line with previous studies[20,22,23,36,52,53], our work showed that some plants can act by means of a second mechanism that requires the presence of the host plant. We demonstrated that *C. annuum* and *T. patula* volatiles alone did not exhibit a significant effect on *M. persicae* aphids, nevertheless, the mixture of these two plants acts significantly on *M. persicae* olfactory behavior. Since the odors of the host plant were not attractive to *M. persicae*, the hypothesis that *T. patula* VOCs can mask the *C. annuum* odor[21] can be ruled out. *Tagetes patula* cv. Nana seems to have a repellent effect on aphids' behavior that requires the presence of *C. annuum*. Two possible mechanisms may be involved: i) the volatile combination of *C. annuum* and *T. patula* cv. Nana plants has repellent effects on *M. persicae*, ii) *T. patula* cv. Nana volatiles changed the *C. annuum* plant emission from a neutral volatile profile into a repulsive blend.[23,52]

In order to answer this question, we compared the choice of *M. persicae* between *C. annuum* (control) and PPI with CPs during five days. Olfactory test results show a significant preference of *M. persicae* for control *C. annuum* only when given the choice between *C. annuum* and PPI with *T. patula* cv. Nana. Our results show that the volatile profile of PPI with *T. patula* cv. Nana apparently becomes repellent to *M. persicae*. Although the information concerning an indirect effect of neighboring plants on pests *via* their host plants is rare, our results are consistent with previous works. Results obtained by Dahlin *et al.* [22] confirmed that chemical changes can be induced on host plants by an interaction with neighboring plant VOC profiles, thus becoming repellent to *M. persicae*. The same observations were shown in the study by Ninkovic *et al.* [23], who demonstrated that attractive potato plants have turned into

repulsive to *M. persicae* after their exposure to volatiles from onion plants. In addition, these authors showed that a synthetic mixture simulating the volatile bouquet emitted from the potato exposed to the onion plant VOCs was more repellent to *M. persicae* than a synthetic blend that mimicked the headspace from unexposed potato plants. Our results corroborate those of Amarawardana *et al.* [53], who demonstrated that *C. annuum* plants previously exposed to chive volatiles became repellent to *M. persicae*. This mode of action was also observed with other aphids and other pests. For example, exposing barley plants to volatiles from various thistle species may change their volatile profile and reduce their attractiveness to *Rhopalosiphum padi*[52]. Bean fly infestations were reduced when bean plants were exposed to volatiles from leek plants.[54]

Probably, the repellent effect of PPI with *T. patula* cv. Nana compared to unexposed plants is a change in its volatile profile.[36] Unfortunately, the headspace sampling using SPME and the analysis of volatiles emitted by *C. annuum* compared to PPI with *T. patula* did not allow us to identify qualitative differences (data not shown). It seems that the difficulty could be linked to the small quantities of volatiles collected from the *C. annuum* plant headspace. Any changes in emitted VOCs should be investigated in future studies with other volatile extraction techniques.[55] On the other hand, electroantennograpic assays (EAG) can be used to investigate aphid olfactory responses to very small peaks and to distinguish active *C. annuum* volatiles modulated by the presence of PPI with *T. patula* cv. Nana.[35,56]

Nevertheless, we can propose two conceivable hypotheses that can explain our results: (1) either the *T. patula* cv. Nana volatiles can adhere by adsorption to the surface of *C. annuum* plants and be re-released (passive mechanism)[25], or (2) the *T. patula* cv. Nana volatiles induced a chemical change of the *C. annuum* plants *via* changes in their physiology (active mechanism).[36,54] Since we observed a significant effect on aphids' orientation just after a short time of exposure of *C. annuum* to *T. patula* cv. Nana volatiles (i.e. tests started 4

hours after placing the mixture of CP and *C. annuum* plants inside the glass cage), the first hypothesis seems closer than the second one. However, all assumptions remain valid. The exposure of a receiver plant to particular VOCs (for example β-ocimene and (*E*)-β-ocimene present in *T. patula* volatile profiles), can cause a change in its defense mechanisms, and can generate definite aphid responses.[57] In addition, receiver plants can modify their metabolism[24] and their biomass allocation.[58,59] For example, Godard *et al.* [26] showed that the methyl jasmonate accumulation of intact *Arabidopsis thaliana* changed when they were exposed to ocimene or myrcene blends. Indeed, more research is needed to understand the function of chemical communication signals between *C. annuum* and *T. patula* cv. Nana plants.[26,36]

From an ecologic point of view, this laboratory olfactory study has allowed us to select two companion plants that can be introduced into the culture system in order to reduce the nefarious effects of *M. persicae*. Mixing crops with *O. basilicum* or *T. patula* would contribute to reduce *M. persicae* infestations. The choice of the cultivar together with the adequate species is a key condition. Likewise, we have identified an indirect effect of the *T. patula* cv. Nana odors *via* host plants, which can be an important mode of action. It can have a great potential to reduce the appeal of *C. annuum* and could be used to minimize aphid incidences. However, the sensitivity of aphids to small differences between VOC blends with the complexity of CP-hostplant-aphid interactions requires deeper investigations which take into consideration the major sources of variability (e.g. genetic variability, cultural practices, edaphoclimatic conditions, among others). Furthermore, a combination of repellent plants and diffusers containing repellent compounds, such as eugenol, EBF, α-terpineol or a blend of synthetic compounds could represent an interesting method in an Integrated Pest Management (IPM) program and those could be used as 'push' elements for applying the "Push-pull" strategy. Finally, before offering this pest management system to farmers, we need to determine their effectiveness in natural conditions.

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Figures Captions

Figure 1. Schematic representation of the device used for (A), dynamic sampling of VOCs from living companion plants using the headspace solid phase micro-extraction (HS-SPME) technique and for (B), to study the effect of plant volatile compounds on *Myzus persicae* olfactory behavior using the Y-tube olfactometer. (C), Odor source placed in two airtight glass cages. All connections were made using PTFE (Teflon®) tubing (8 and 10 mm diameter).

Figure 2. Schematic representation of the vertical tube still-air olfactometer used to study the effect of individual volatile compounds on *Myzus persicae* olfactory behavior.

Figure 3. Olfactory behavioral responses of *Myzus persicae* to living plant odors on a Y-tube olfactometer: (A), companion plant (CP) volatiles (dark gray bars) or *C. annuum* plant volatiles (light gray bars) *vs.* clean air (control; white bars); (B), volatiles from CPs *vs. C. annuum* plant volatiles; (C), mixture of CP and *C. annuum* plant volatiles (gray hatched bars) *vs. C. annuum* plant volatiles; and (D), *Capsicum annuum* previously intercropped with CP volatiles (dark gray bars) and *vs. C. annuum* plant volatiles (light gray bars). *Capsicum annuum* and clean air were used as controls. "N"= 120 responding aphids per trial. *P*-value determined with Chi-square (χ^2) (α=0.05) tests with a 50 % expected response (*$P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$; NS. not significant).

Figure 4. Olfactory behavioral responses (mean ± standard deviation) of *Myzus persicae* to individual compounds at three concentrations (0.01, 0.1, and 1%) and two different times (10 and 20 min) in a still-air olfactometer. Black bars represent the control (no compound) and

gray bars represent compounds at a defined concentration and time. a: *O. basilicum* major compound, b: *T. patula* cv. Nana major compound and c: compound shared between *O. basilicum* and *T. patula* cv. Nana; error bars represent standard deviation, *P*-value determined with Wilcoxon's test (α=0.05): *$P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$; NS. not significant. (ocimene mixture: composed of 69% of β-ocimene and 31% of neo-allo-ocimene).

Table 1. Relative percentages (mean ± standard deviation) of VOCs emitted by companion plant species. The relative area of each compound was calculated by dividing the peak area of this compound by the total peak area (n = 4). VOCs are listed according to their retention time (RT).

No.	Compounds	RT	RI	Companion plant species					
				Lavandula latifolia	*Mentha piperita*	*Ocimum basilicum*	*Rosmarinus officinalis*	*Tagetes patula* cv. Nana	*Tagetes patula* cv. Bonita Bolero
1†	α-Pinene	4.51	929	0.02 ± 0.02	-	0.10 ± 0.08	**7.23 ± 0.54**	-	-
2†	Camphene	4.7	945	0.01 ± 0.01	-	-	**3.12 ± 0.14**	-	-
3†	Sabinene	5.01	968	-	-	0.18 ± 0.16	-	0.39 ± 0.11	0.63 ± 0.53
4†	β-Pinene	5.05	971	-	-	0.06 ± 0.05	**1.50 ± 0.11**	-	-
5†	β-Myrcene	5.2	984	**1.08 ± 0.67**	-	0.18 ± 0.24	**1.10 ± 0.23**	0.43 ± 0.12	0.21 ± 0.17
6†	α-Phellandrene	5.38	998	-	-	-	**1.93 ± 1**	-	-
7§	(*Z*)-3-hexenyl acetate	5.43	1002	-	-	-	-	**3.28 ± 0.61**	**1.42 ± 1.21**
8†	*o*-Cymene	5.64	1017	-	-	-	**12.15 ± 2.37**	0.69 ± 0.48	0.09 ± 0.08
9†	Limonene	5.69	1022	0.24 ± 0.22	**7.64 ± 1.02**	-	**15.38 ± 1.82**	**5.68 ± 0.89**	**5.94 ± 3.54**
10†	Eucalyptol	5.73	1025	**3.12 ± 1.11**	0.26 ± 0.12	0.40 ± 0.21	**2.67 ± 0.38**	-	-
11†	(*E*)-β-Ocimene	5.78	1028	-	0.19 ± 0.05	-	-	**9.94 ± 0.71**	**3.51 ± 1.86**
12§	1-Hepten-4-ol	5.84	1032	-	0.60 ± 0.19	-	-	-	-
13†	β-Ocimene	5.95	1042	0.87 ± 0.59	-	-	-	**2.11 ± 0.04**	**3.40 ± 2.94**
14†	γ-Terpinene	6.08	1051	0.07 ± 0.02	-	-	**2.84 ± 0.22**	-	-
15†	(*Z*)-β-Terpineol	6.2	1062	0.03 ± 0.03	0.71 ± 0.20	-	**1.60 ± 0.24**	-	-
16†	Linalol oxide	6.35	1072	0.20 ± 0.09	-	-	-	-	-
17†	Terpinolene	6.49	1083	0.31 ± 0.26	-	0.64 ± 0.33	0.81 ± 0.05	**7.39 ± 0.98**	**17.52 ± 7.05**
18†	Linalol	6.62	1094	**42.14 ± 3.33**	-	0.30 ± 0.13	**1.15 ± 0.16**	**1.88 ± 0.71**	**1.73 ± 1.51**
19†	Neo-allo-ocimene	7.06	1122	**1.02 ± 0.29**	-	-	-	**1.61 ± 0.31**	**6.21 ± 3.52**
20†	(*Z*)-Tagetone	7.31	1138	-	-	-	-	0.87 ± 0.21	0.55 ± 0.23

21†	Camphor	7.34	1141	**16.97 ± 2.47**	-	0.11 ± 0.12	**8.40 ± 0.62**	-	-
22†	Menthone	7.47	1148	-	**34.88 ± 4.02**	-	-	-	-
23†	Menthofurane	7.63	1155	-	**24.35 ± 1.93**	-	-	-	-
24†	Borneol	7.69	1162	**16.77 ± 4.23**	-	-	**18.88 ± 0.79**	-	-
25†	Menthol	7.77	1168	-	**12.49 ± 2.20**	-	-	-	-
26†	Terpinen-4-ol	7.85	1173	0.15 ± 0.07	-	-	**4.44 ± 0.52**	-	-
27†	*p*-Cymen-8-ol	7.99	1182	-	-	-	-	**3.35 ± 0.65**	0.09 ± 0.07
28§	Crypton	8.07	1187	0.6 ± 0.28	-	-	-	-	-
29†	α-Terpineol	8.08	1188	0.56 ± 0.30	-	0.26 ± 0.24	**2.93 ± 0.35**	0.83 ± 0.47	-
30§	Myrtenol	8.24	1197	0.17 ± 0.10	-	-	-	-	-
31†	Verbenone	8.41	1207	0.11 ± 0.06	-	-	0.74 ± 0.13	**4.77 ± 2.64**	0.05 ± 0.05
32§	(*Z*)-Carveol	8.6	1217	0.19 ± 0.08	-	-	-	-	-
33†	2-Hydroxycineole	8.68	1221	0.12 ± 0.09	-	-	-	-	-
34†	Isobornyl formate	8.78	1226	**1.77 ± 0.43**	-	-	-	-	-
35†	Pulegone	8.92	1235	-	**10.08 ± 0.47**	-	-	-	-
36§	Cuminaldehyde	8.98	1237	0.34 ± 0.19	-	-	-	-	-
37†	Carvone	9.04	1241	0.28 ± 0.07	-	-	-	-	-
38†	Linalyl anthranilate	9.18	1248	**4.56 ± 0.74**	-	-	-	-	-
39†	Piperitone	9.23	1251	0.15 ± 0.06	-	-	-	**11.53 ± 3.81**	**3.29 ± 2.42**
40†	(+)-*p*-Mentha-1.8-dien-3-one	9.54	1268	-	-	-	-	0.35 ± 0.23	0.19 ± 0.11
41†	Bornyl acetate	9.78	1281	**1.98 ± 0.46**	-	0.39 ± 0.29	**9.33 ± 1.83**	-	-
42†	Menthol acetate	9.9	1287	-	**7.39 ± 0.62**	-	-	-	-
43†	Cuminol	9.91	1288	0.12 ± 0.09	-	-	-	-	-
44‡	γ-Elemene	10.82	1333	-	-	-	-	0.43 ± 0.14	0.47 ± 0.38
45†	Eucarvone	10.91	1338	-	-	-	-	**4.38 ± 2.02**	0.72 ± 0.63
46§	Eugenol	11.24	1353	-	-	**4.72 ± 2.57**	-	-	-
47§	Neryl acetate	11.29	1356	0.73 ± 0.34	-	-	-	-	-
48‡	Copaene	11.62	1372	-	-	0.05 ± 0.02	-	-	-

49§	Geranyl acetate	11.69	1376	**1.54 ± 0.83**	-	-	-	-	-
50‡	Zingiberene	11.89	1385	0.06 ± 0.04	-	-	-	-	-
51‡	β-elemene	11.94	1387	-	-	0.38 ± 0.22	-	**2.45 ± 0.94**	**6.80 ± 6.73**
52‡	α-Guaiene	12.16	1398	-	-	-	-	0.32 ± 0.32	**1.27 ± 1.23**
53‡	(*Z*,*E*)-α-Farnesene	12.21	1401	0.13 ± 0.04	-	-	-	-	-
54§	Methyleugenol	12.23	1402	-	-	**76.11 ± 4.73**	-	-	-
55‡	(*E*)-α-Bergamotene	12.43	1411	0.11 ± 0.03	-	-	-	-	-
56‡	β-Caryophyllene	12.55	1417	**1.09 ± 0.32**	0.60 ± 0.22	-	**3.49 ± 0.98**	**31.95 ± 4.07**	**35.80 ± 6.45**
57‡	Aromandendrene	12.55	1418	-	-	0.22 ± 0.03	-	-	-
58‡	β-ylangene	12.75	1426	0.09 ± 0.04	-	-	-	**2.41 ± 1.62**	**2.07 ± 1.44**
59‡	α-Bergamotene	12.85	1431	0.14 ± 0.05	-	**5.73 ± 0.58**	-	-	-
60§	Geranyl acetone	13.14	1445	-	0.81 ± 0.16	0.36 ± 0.04	0.30 ± 0.10	0.09 ± 0.07	0.45 ± 0.20
61‡	(*E*)-β-Farnesene	13.25	1449	0.35 ± 0.20	-	**7.04 ± 1.28**	-	-	**1.88 ± 2.13**
62‡	Humulene	13.25	1450	-	-	-	-	0.41 ± 0.32	0.38± 0.38
63‡	Linalyl isobutyrate	13.41	1457	0.08 ± 0.03	-	-	-	-	-
64‡	β-Cuvebene	13.47	1459	0.03 ± 0.02	-	-	-	0.06 ± 0.02	0.15 ± 0.15
65‡	β-Copaene	13.49	1460	0.04 ± 0.02	-	-	-	-	-
66‡	Germacrene-D	13.87	1478	0.03 ± 0.01	-	0.24 ± 0.14	-	0.97 ± 0.53	**1.37 ± 1.01**
67‡	(*Z*)-β-Farnesene	13.9	1480	-	-	0.08 ± 0.01	-	-	-
68§	Isomethyleugenol	14.12	1489	-	-	**1.07 ± 0.17**	-	-	-
69‡	α-Selinene	14.26	1497	0.03 ± 0.02	-	-	-	-	-
70‡	α-Farnesene	14.35	1500	-	-	-	-	0.20 ± 0.08	0.44 ± 0.44
71‡	α-Bulnesene	14.37	1501	-	-	0.39 ± 0.18	-	-	-
72‡	β-Bisabolene	14.4	1503	-	-	-	-	0.14 ± 0.08	0.32 ± 0.32
73‡	γ-Muurolene	14.54	1509	0.81 ± 0.39	-	0.52 ± 0.40	-	0.51 ± 0.39	0.55 ± 0.56
74‡	δ-Cadinene	14.72	1517	0.11 ± 0.04	-	0.13 ± 0.03	-	0.56 ± 0.35	**2.51 ± 2.39**
75‡	Caryophyllene oxide	16.06	1579	0.19 ± 0.10	-	-	-	-	-
76§	Cubenol	16.72	1611	0.06 ± 0.04	-	-	-	-	-
77§	α-epi-Cadinol	17.24	1638	0.43 ± 0.32	-	0.34 ± 0.18	-	-	-

Σ of VOCs areas	55.6E+08 ± 40.9E+08	3.33E+08 ± 1.48E+08	16.5E+08 ± 11.5E+08	9.79E+08 ± 0.80E+08	2.86E+08 ± 1.90E+08	2.93E+08 ± 1.27E+08

RI, retention indices relative to (C8–C20) n-alkanes series on a TR-5MS column;
Compounds with relative percentages higher than 1% are represented in bold;
"-"not detected;
† Monoterpene;
‡ Sesquiterpene;
§ Others.

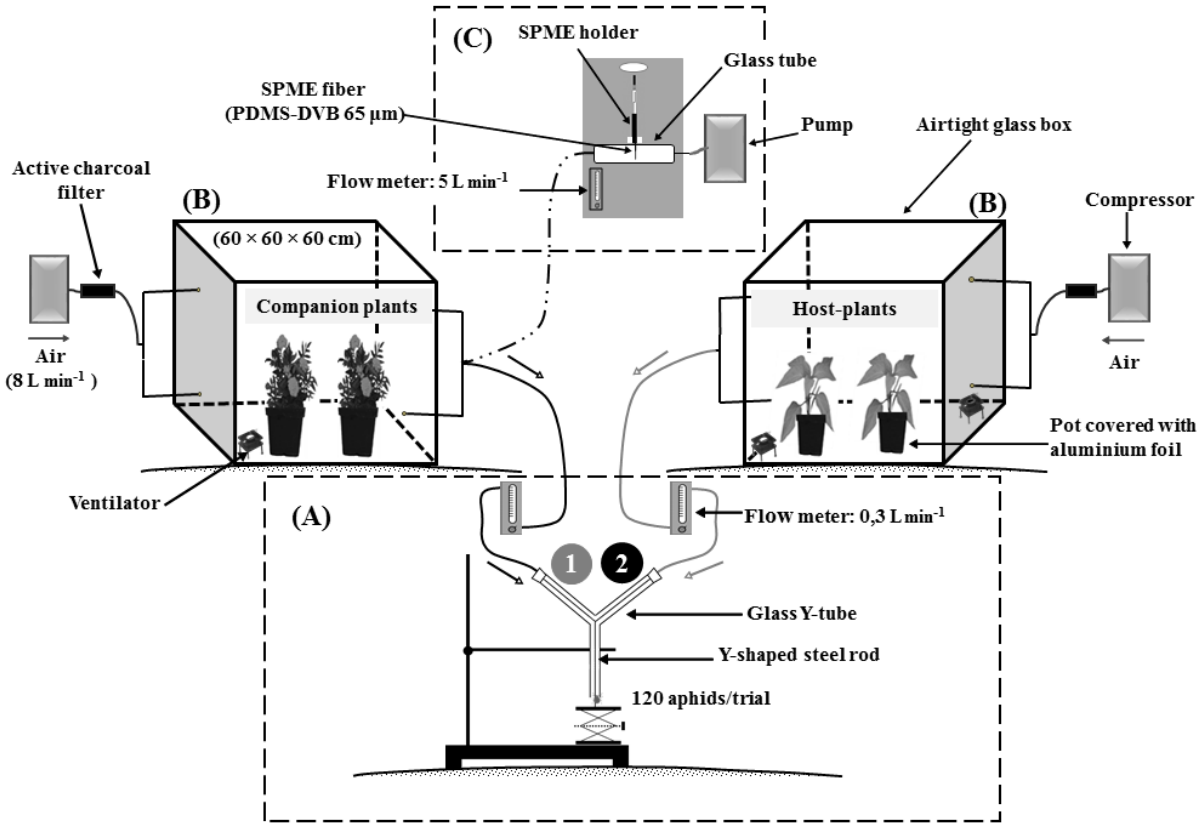


Figure 1.

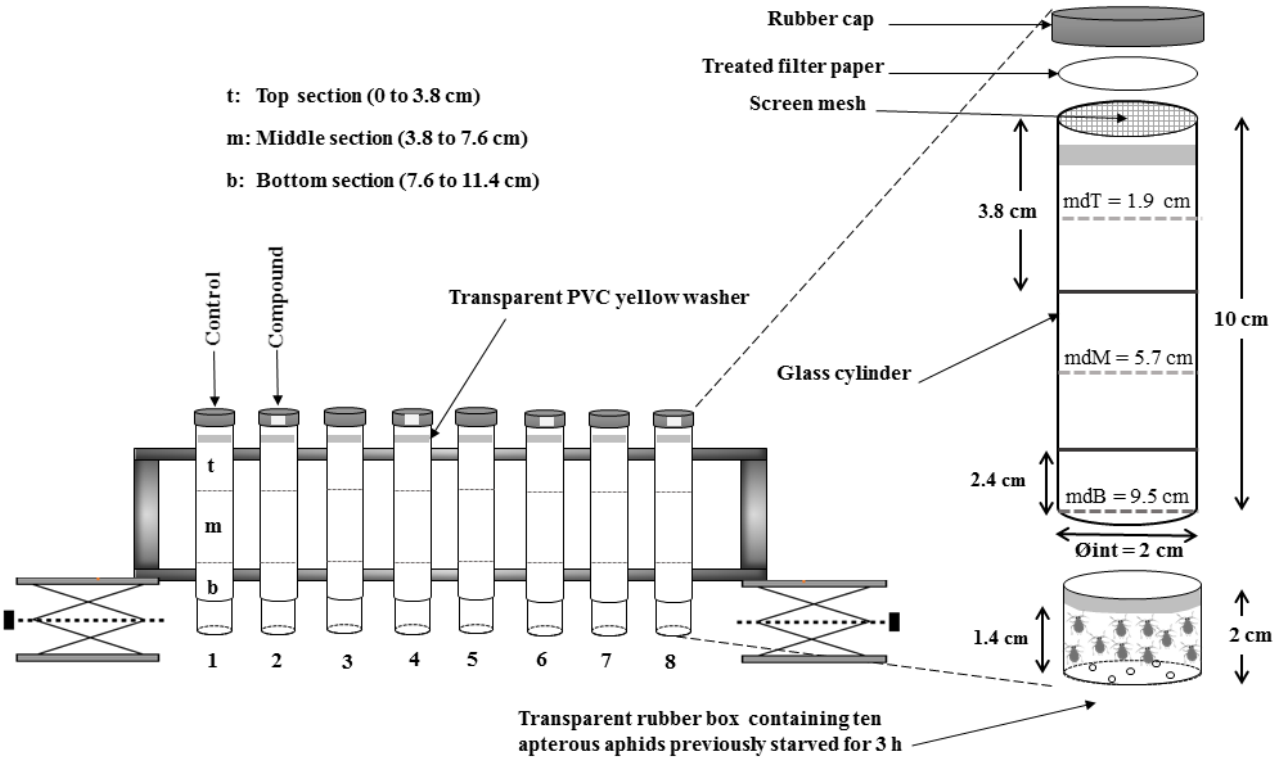
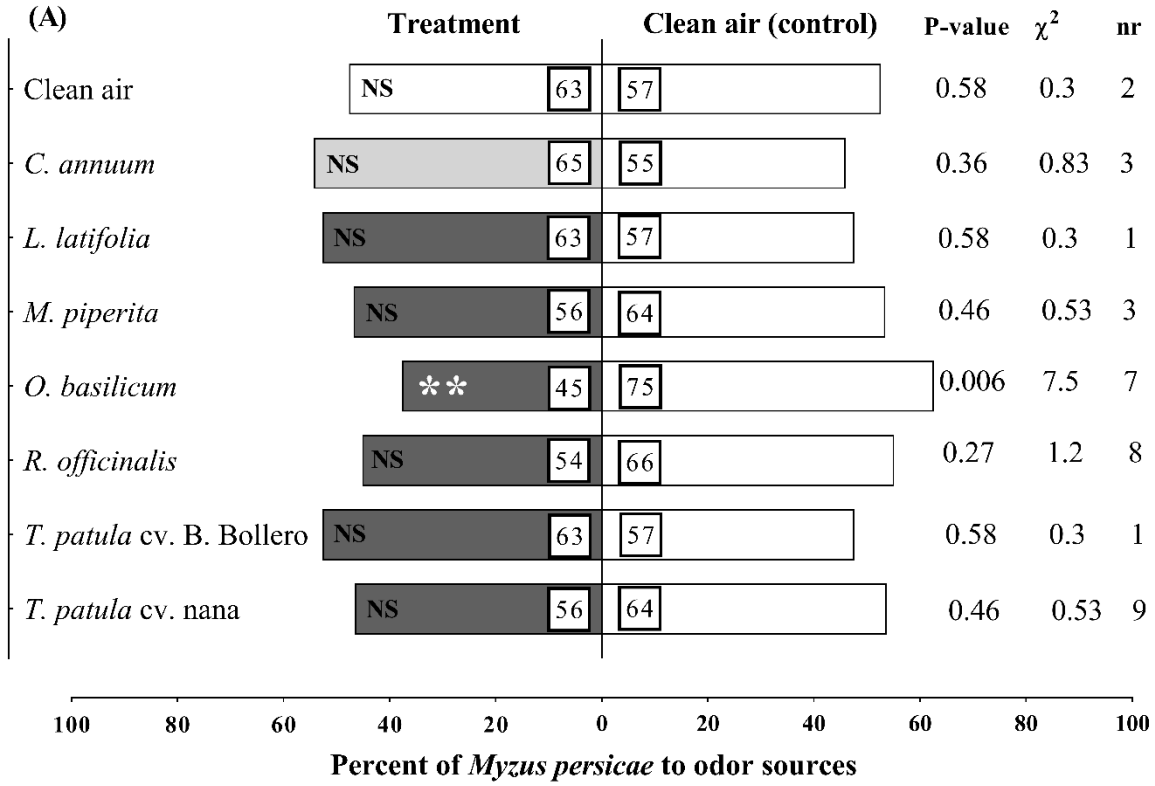
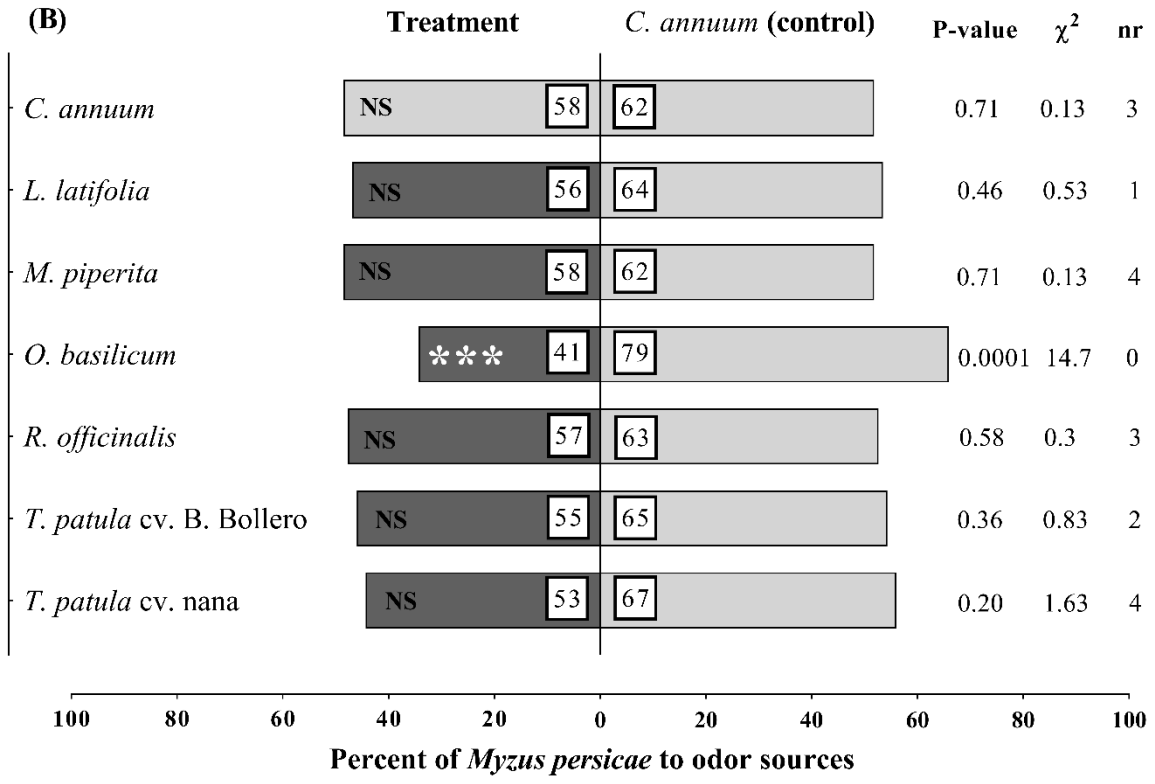


Figure 2.





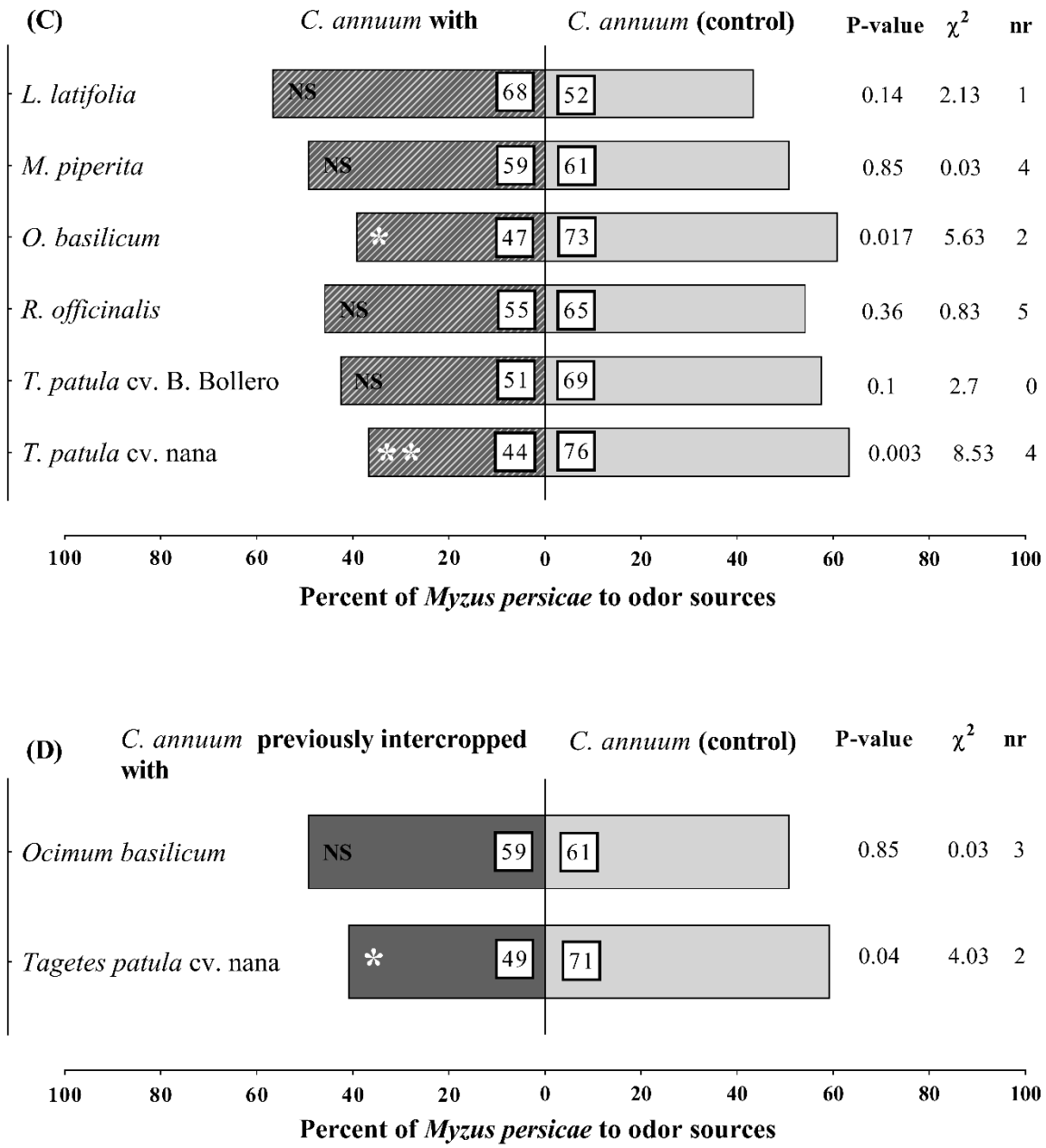
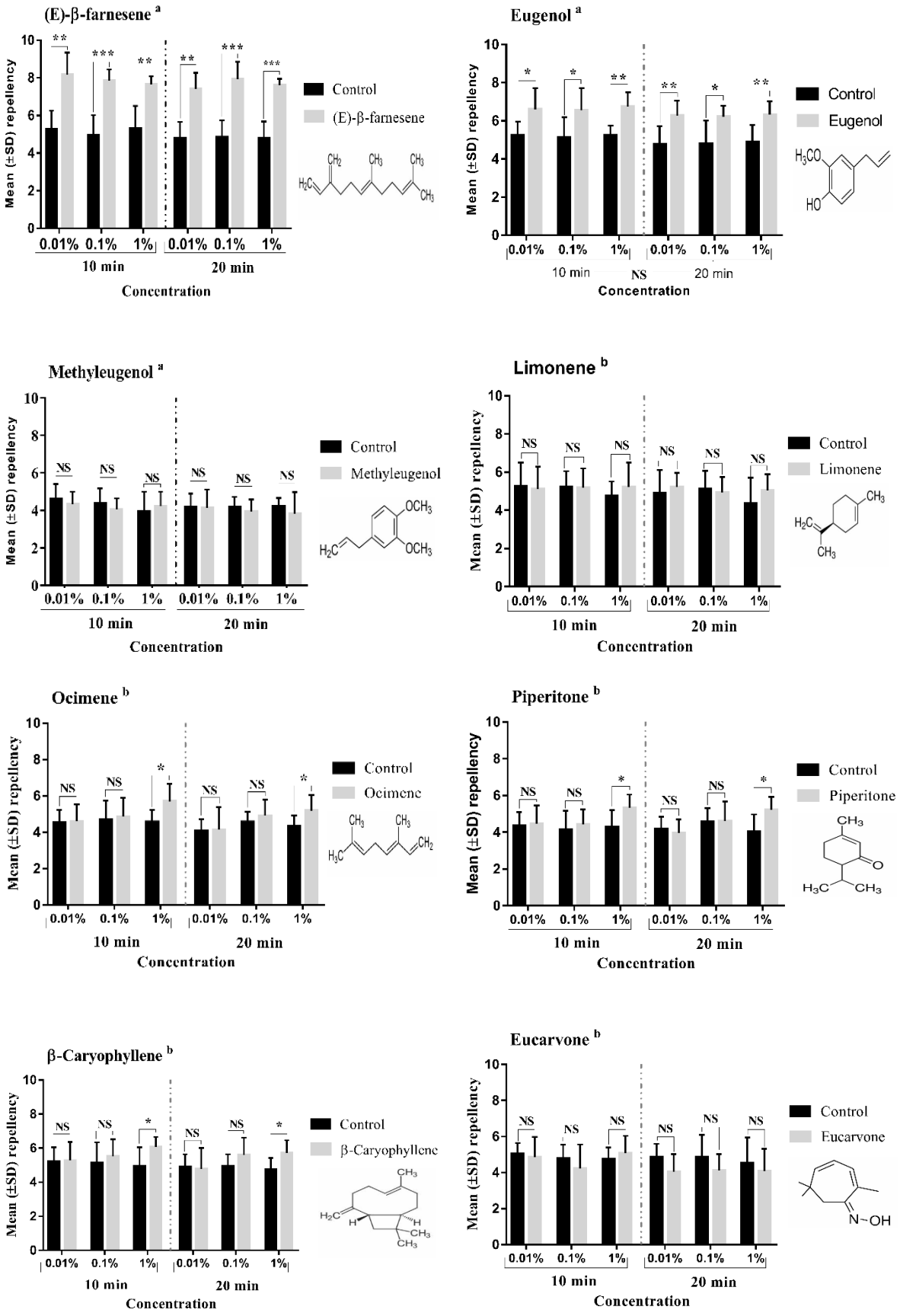


Figure 3.



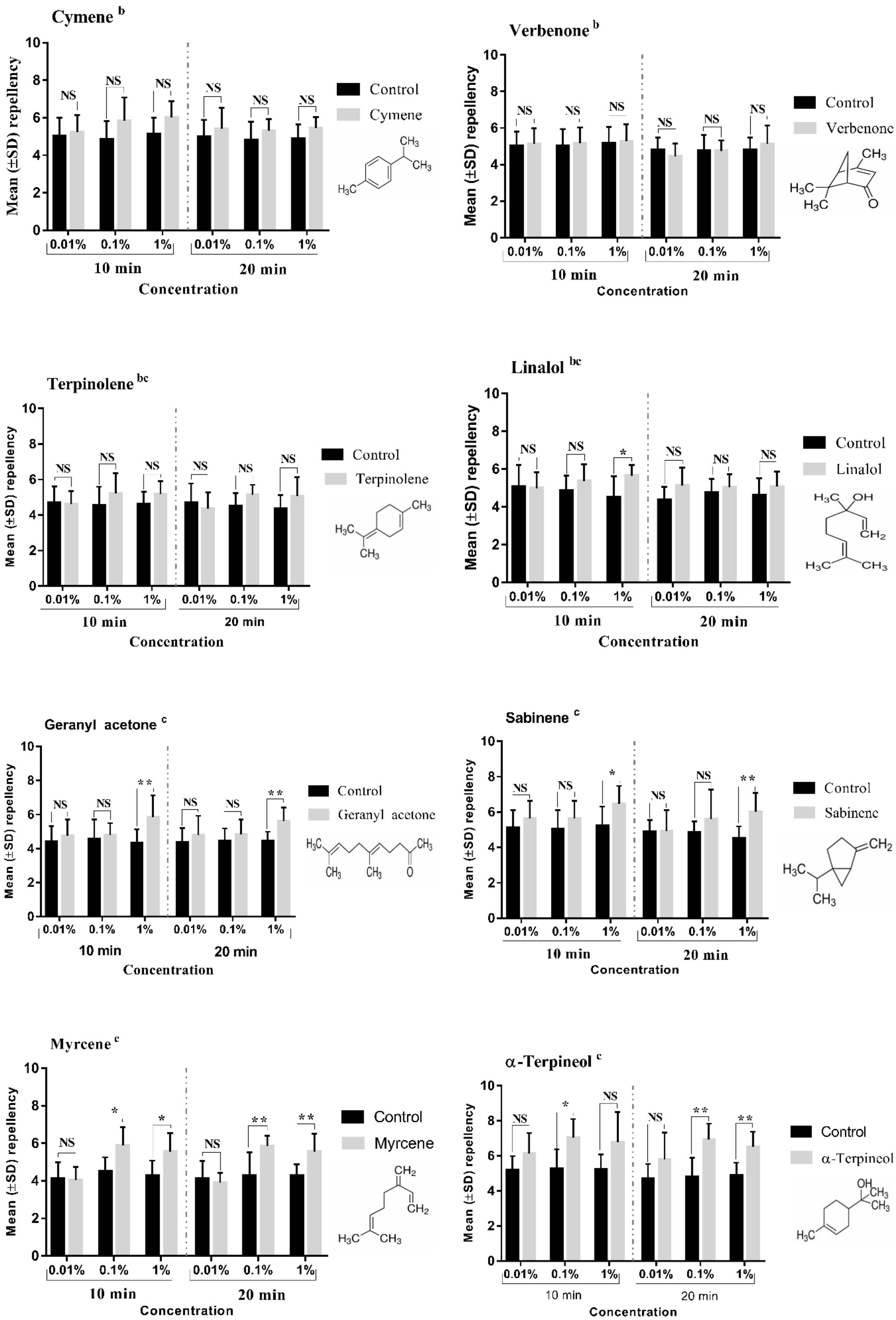


Figure 4.