



# *Geranium macrorrhizum*, a potential novel companion plant affecting preference and performance of *Myzus persicae* on sweet pepper

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## Abstract

The combination of a companion plant with a cultivated plant is considered an interesting strategy to reduce pest pressure and, hence, the use of pesticides. Although several plants from the Alliaceae and Lamiaceae families are known to be efficient companion plants against aphid pests, only a few plants of the Geraniaceae family have been studied so far. The aim of this work was to investigate the potential effects of *Geranium macrorrhizum* as a companion plant on the colonization of sweet pepper (*Capsicum annuum*, Solanaceae) by the green peach aphid (*Myzus persicae*). Aphid's orientation behavior, probing behavior and life history traits were assessed on sweet pepper using a host choice preference setup, Electrical Penetration Graph technique and clip-cage laboratory bioassays, respectively. The potential disturbance through mechanical stimulation of geranium leaves was also assessed. The composition of VOCs from *G. macrorrhizum* leaves was analyzed using SPME technic followed by GC–MS. This study revealed that *G. macrorrhizum* as a companion plant was intrinsically repellent but not enough to completely mask the attractive odor of the sweet pepper host plant. Moreover, *G. macrorrhizum* negatively impacted the probing behavior, fecundity and survival rate of *M. persicae* on sweet pepper. The effects were exacerbated when *G. macrorrhizum* leaves were mechanically stimulated. This could be due to the greater amount of the main VOCs germacrone and  $\beta$ -elemenone emitted by *G. macrorrhizum* following mechanical stimulation. Our results bring new insights into the use of novel companion plants to regulate aphid pest populations.

**Keywords** Volatile compounds · Chemical ecology · Electrical penetration graph · Repellent · Geraniaceae

## Key message

- *Geranium macrorrhizum* as a companion plant can disturb aphid host plant colonization.

- Geranium VOCs were repellent but not enough to completely mask the host plant attractive odor.
- Geranium VOCs reduced aphid fecundity on the host plant without affecting its probing behavior.
- Mechanically stimulated geranium VOCs reduced aphid phloem sap ingestion, survival and fecundity.
- These latter effects could be due to the increase in  $\beta$ -elemenone and germacrone emission.

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## Introduction

A common eco-friendly method for reducing insect pest pressures in crops is to make the method of cultivation more diverse by introducing ‘companion plants’ (CPs) (Parker et al. 2013). Polyculture involving ‘companion planting’ relies on two plant species cultivated together that are known, or believed, to synergistically improve one another’s growth (Franck, 1983). It is expected that, planted together,

they will directly mask the specific chemical cues that one another's pests depend on to locate their respective host, or they will host particularly effective natural enemies of one another's pest. In a context of crop protection against insect pests, the effectiveness of this method can be assigned to the putative role of volatile organic compounds (VOCs) released by CPs (Basedow et al. 2006). A possible hypothesis based essentially on laboratory experiments to explain such effects is that VOCs emitted by CPs have a negative effect on insect pests, acting as behavioral repellents and deterrents or functioning via chemical alteration of the olfactory profile of the host target plant (Ben-Issa et al. 2017a).

Although field experiments on the effects of companion plants still need to be performed on target crops colonization by aphids, several works have already demonstrated their efficiency in regulating aphid pest populations through repellent activity (Ben-Issa et al. 2017b). The plant species most frequently and successfully used belong to the Lamiaceae (Basedow et al. 2006; Beizhou et al. 2011), Asteraceae (Glinwood et al. 2004; Jankowska, 2010), Alliaceae (Zhou et al. 2013) and Apiaceae (de Lima et al. 2014) botanical families. The use of these CPs can represent an effective strategy to disturb the aphid host plant colonization process at some of the steps described by Niemeyer (1990) and Powell et al. (2006). The habitat and host location, which are governed by visual and olfactory cues, could be altered by the emission of VOCs by CPs that could repel aphids from their host plant or mask the odor of the host plant (Mansion-Vaquié et al. 2020; Nottingham et al. 1991). Host acceptance, characterized by sustained phloem sap ingestion, can be impacted by CPs: airborne repellent VOCs can directly disturb aphids feeding behavior by interfering with their olfactory system (Baudry et al. 2021; Dardouri et al. 2021). These authors also hypothesized that CP VOCs could impregnate the host plant cuticle, making the plant less palatable. Finally, after aphids have settled on a plant, CPs can also impact their life history traits and significantly reduce their fecundity due to an alteration of the host plant acceptance process or to an intoxication following the inhalation of CP VOCs (Ben-Issa et al. 2017a).

With about 320 species distributed throughout the temperate regions, the genus *Geranium* L. (Geraniaceae) is chemically characterized by the presence of tannins, flavonoids, anthocyanins and VOCs (Aedo et al. 1998). Agronomical studies have also shown that Geraniaceae plants have interesting properties when used as companion plants, to improve the productivity of the focal plant (Verma et al. 2013), to repel coleopteran (Held et al. 2003) or lepidopteran pests (Yacoub et al. 2011), or to attract insect pollinators regarding their floral resource (Masierowska et al. 2018). *Geranium macrorrhizum* L., commonly known as 'big-root geranium,' was proposed by CRA-W (Centre Wallon de Recherches Agronomiques, Gembloux, Belgium) to be

investigated as a promising insect pest repellent cover-crop CP, due to the abundance of trichomes on its leaf surface (Kremer et al. 2013) known to be involved in plant defense mechanisms. Indeed, glandular trichomes form a protective layer around the leaves (Werker, 2000) and produce substances, especially when they are mechanically stimulated (Zhou et al. 2017), that can be toxic or repellent to herbivorous organisms. Ilić et al. (2020) emphasized the singularity of *G. macrorrhizum* VOCs chemical composition by comparing it with that of other species belonging to the *Geranium* genus. Their study revealed that volatile fractions of *G. macrorrhizum* were separated from all other samples due to a high sesquiterpene content (>70%), while the volatile fractions of other samples (*G. phaeum*, *G. sanguineum*, *G. robertianum*, *G. palustre*, *G. pyrenaicum*, *G. columbinum* and *G. lucidum*) were composed of sesquiterpenes, diterpenes and fatty acids derivatives.

Here, we aimed to investigate, through laboratory experiments, whether *G. macrorrhizum* as a CP could disturb the aphid host plant colonization. We hypothesized that this disturbance would be increased through mechanical stimulation of geranium leaves. The VOCs produced by *G. macrorrhizum* were sampled using solid-phase micro-extraction (SPME) and were measured by GC-MS. The green peach aphid (*Myzus persicae* Sulzer) was used as the aphid pest model and sweet pepper (*Capsicum annuum* L.) as the target host plant. The potential alteration of the aphid colonization process by *G. macrorrhizum* VOCs was investigated on (1) aphid orientation behavior using a dual choice set-up, (2) aphid probing behavior using the Electrical Penetration Graph (EPG) technique and (3) aphid survival and fecundity using clip-cages.

## Materials and methods

### Plant and insect material

To avoid any competition between sweet pepper and geranium and also to limit the impregnation of sweet pepper by geranium VOCs, sweet peppers and geraniums were grown in individual pots placed in separate rooms. Experiments were performed using sweet pepper (*C. annuum* L. cv. 'Sprinter') as the target plant and *G. macrorrhizum* as the companion plant (CP). Sweet pepper plants were provided as seeds by the "Provinciaal Proefcentrum voor de Groenteteelt Oost-Vlaanderen" (PCG, Belgium). Geraniums, provided as plants by the CRA-W, were obtained from offshoots of mother plants through vegetative propagation of their rhizomes. Plants were grown in 7 × 7 × 6 cm (sweet pepper) and 9 × 9 × 10 cm (geranium) pots containing potting soil (NPK 18-10-20, 0.5 kg/m<sup>3</sup>, FLORAGARD), under controlled conditions (24 ± 1 °C, 60 ± 5% relative humidity

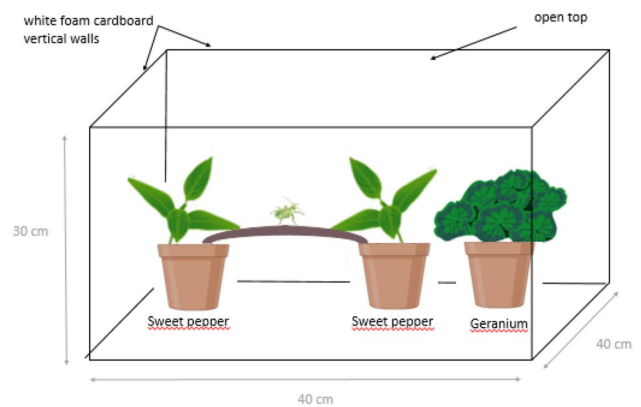
(RH), and 16L:8D photoperiod at 2,5 klux.). For all the experiments, sweet peppers were used five weeks after sowing, at a stage of 8 to 10 fully developed leaves and height of 15 to 18 cm, and geraniums at a stage of 8 to 12 fully developed leaves and height of 15 to 18 cm (mean mass of aerial parts  $6 \pm 1$  g). None of the plants used had flowered at the time of experiments and sweet pepper plants had never been in the presence of geranium plants until their use for the experiments.

The *M. persicae* colony was established from one parthenogenetic female collected in 1999 in a potato field near Loos-en-Gohelle (France) and maintained on rapeseed (*Brassica napus* L. cv. “Adriana”) (Brassicaceae). Pots (9×9×10 cm) containing each 3–4 rapeseed plants were placed in ventilated plastic cages (24×11×36 cm) and maintained under the same conditions as described above. All the experiments were performed using young apterous adults ( $9 \pm 2$  day-old, corresponding to their pre-reproductive period) synchronized on sweet pepper leaves embedded in 1.5% agar in Petri dishes (Ø 9 cm). All tested aphids were naive, i.e., they had never been submitted to geranium VOCs.

In order to assess the influence of geranium used as a companion plant, behavioral and physiological parameters of aphids on the target sweet pepper plant alone “P” were compared to those on the target plant associated to Geranium “P-G” or brushed Geranium “P-BG.” The plant association consisted in placing a pot with a sweet pepper plant contiguously to a pot with a geranium plant taking care that their respective foliage were 15 cm apart. Placing a companion plant beside a sweet pepper was performed at the precise moment a test started and their placement together lasted only for the duration of the test. For the “BG” procedure, in order to stimulate the emission of VOCs, all the leaves of the geranium were gently brushed with a paintbrush (MANET paintbrush—Petit Gris—series 408, N°10) by carrying out five successive passages in the direction of the central vein on the whole abaxial surface of each leaf.

### Aphid plant preference

The experimental setup adapted from Baudry et al. (2021) allowed contact-based, odor-based and visual cues (Fig. 1). This open system setup was used to evaluate the putative repulsive properties of geranium (G and BG) and the putative attractivity of sweet pepper (P). An apterous aphid was placed in the middle of an 8 cm×2 cm plastic bridge linking a pot containing a sweet pepper plant (P) to another pot containing a geranium (G or BG). Each individual aphid was then observed for three minutes after being released. The plant receiving the aphid first contact (sweet pepper or geranium) was recorded. The experiment was repeated on 46 different aphids for the P vs. G modality and 39 different



**Fig. 1** Schematic representation of the aphid preference setup using an arena for dual choice bio-assays. The example shown here is the one involving a choice between a sweet pepper plant (P) alone and a sweet pepper plant associated with a geranium plant (P-G) (C modality)

aphids for the P vs. BG modality. The setup was also used to evaluate the aphid preference when submitted to a choice between the sweet pepper associated with geranium (P-G or P-BG) and the sweet pepper alone (P), the two sweet pepper plants being linked by the bridge. The sweet pepper plant receiving the aphid first contact was recorded. The experiment was repeated on 40 different aphids for the P vs. P-G modality and 39 different aphids for the P vs. P-BG modality.

The whole setup was placed in an aerated room (devoid of any directional wind) under controlled conditions ( $24 \pm 1$  °C,  $60 \pm 5\%$  RH and 16L:8D photoperiod, 2,5 klux). Plants locations were alternated to avoid any environment variation between each modality. To minimize external stimuli, the setup was positioned between four white foam cardboard walls (30 cm×40 cm each).

### Aphid probing behavior

The probing behavior of *M. persicae* was studied using the Electrical Penetration Graph (EPG) technique (Tjallingii 1978, 1988) by comparing the probing behavior of aphids placed onto a sweet pepper alone (P) with that of aphids placed onto a sweet pepper associated with a geranium (P-G) or a brushed geranium (P-BG). Recordings were carried out for eight hours in the middle of the 16 h of photophase under controlled conditions ( $24 \pm 1$  °C,  $60 \pm 5\%$  RH and 16L:8D photoperiod, 2,5 klux). The records were obtained according to a setup consisting of sticking a thin gold wire (Ø 18 µm and 2 cm long) on the dorsal part of the aphid abdomen with a conductive water-based silver glue (EPG systems, Wageningen, the Netherlands). The wire was connected to an electrical closed circuit comprising the aphid and its sweet pepper host plant. Probing behavior was recorded with a direct current (DC) EPG device, model Giga-8 (EPG-systems,

Wageningen, The Netherlands) at 1 Giga Ohm input resistance. Output from the EPG at 100× gain was digitized at a rate of 100 samples per second per channel. Acquisition and analysis of the EPG waveforms were carried out with EPG Stylet+ software (EPG Systems, [www.epgsystems.eu](http://www.epgsystems.eu)). Relevant aphid behavior EPG parameters were calculated with EPG-Calc 6.1.7 software (Giordanengo, 2014). The probing behavior of 35 aphids for the P and P-G modalities, and 34 aphids for the P-BG modality, was recorded. Aphids were placed on the abaxial part of two fully expanded sweet pepper apical leaves.

Seventeen different EPG parameters were used to assess the probing behavior of *M. persicae* on sweet pepper alone (P) or associated with geranium (P-G or P-BG). Waveform "Pr" represents general probing behavior within plant tissues (parameters studied: the total duration of probing (s\_Pr), the number of probes (n\_Pr), the number of brief probes < 3 min. and the time before the first insertion of stylets within plant ( $t > 1Pr$ )). Waveform "C" represents the intercellular apoplastic stylet pathway where the insects show a cyclic activity of mechanical stylet penetration and secretion of saliva (parameters studied: the total number of pathway phases (n\_C) and the total duration of pathway phase (s\_C)). Waveform "Pd" (*i.e.*, Potential drops) represents intracellular stylet puncture occurring during stylet pathway (parameters studied: the total number of potential drops (n\_Pd) and the total duration of potential drops (s\_Pd)). Two waveforms related to phloem activity were recorded: waveform "E1," which represents salivation into phloem sieve elements at the beginning of the phloem phase (parameters studied: the total duration of salivation (s\_E1), the number of salivations (n\_E1)), and waveform "E2," which is correlated with passive phloem sap uptake from the sieve element (parameters studied: the total duration of phloem sap ingestion (s\_E2), the number of phloem sap ingestions (n\_E2), the total duration of sustained phloem sap ingestion, *i.e.*, > 10 min (s\_s E2), the number of sustained phloem sap ingestions, *i.e.*, > 10 min (n\_sE2)). Furthermore, waveform "G" represents active intake of xylem sap (parameters studied: the total duration of ingestion of xylem sap (s\_G) and the number of ingestions of xylem sap (n\_G)), and waveform "F" represents derailed stylet mechanics (parameters studied: the total duration of derailed stylets phase (s\_F), the number of derailed stylets phases (n\_F)).

## Aphid physiology

Several physiological parameters were assessed using "clip-cages" adapted from MacGillivray and Anderson (1957). Each clip-cage was composed of a plier supporting an acrylic transparent cylinder (Ø 1.5 cm, 0.8 cm long) closed by a nylon gauze, while the airtightness on the leaf was ensured using felt. Clip-cages were placed on the

abaxial part of two fully expanded top leaves of the non-associated sweet pepper (P) or sweet pepper associated with geranium (P-G or P-BG). Two to three clip-cages were installed per sweet pepper plant (10–15 for each modality), on each new growing leaf, each enclosing a single synchronized aphid adult. Survival and daily fecundity (number of newly larviposited nymphs) were assessed for each adult every 24 h for a duration equivalent to that of the pre-reproductive period duration (nine days). All these experiments were carried under controlled conditions ( $24 \pm 1$  °C,  $60 \pm 5\%$  RH and 16L:8D photoperiod, 2,5 klux). A total of 41 (P), 39 (P-G) and 38 (P-BG) individuals were, respectively, used to evaluate the survival rate and the total fecundity.

## Analysis of volatile compounds by gas chromatography coupled with mass spectrometry (GC–MS)

Before proceeding to the headspace solid phase microextraction GC–MS analysis (HS-SPME-GC–MS), geranium plantlets (G or BG) were individually enclosed for 24 h at room temperature in 35L Nalophan® bag. Then, sampling of VOCs was achieved with a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 µm) SPME fiber (Supelco, Darmstadt, Germany) through a 120 min exposition in the headspace. Injection was performed in splitless mode at 280 °C. The fiber was conditioned for 20 min at injection temperature. Analyses were performed by GC–MS (7890B-5977B, Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5 MS capillary column (30 m×250 µm×0.25 µm, Agilent Technologies, Santa Clara, CA, USA). Helium was used as a carrier gas at a flow rate of 1.2 mL/min. The oven temperature program was as follows: starting at 40 °C with a hold for 2 min; then, an increase of 4 °C/min up to 200 °C then 20 °C/min up to 300 °C with a hold for 5 min. The mass spectrometer was set to have a temperature of 230 °C at the ion source and 150 °C at the quadrupole and programmed with a SCAN acquisition mode. Mass spectra were scanned from 35 to 500 amu. Then, component identification was performed by comparison of the obtained spectra with reference databases (NIST17, pal600, whiley275). Additionally, experimental retention indices (RI) were calculated following the injection of a mixture of n-alkanes C8-C30 (Sigma Aldrich, Darmstadt, Germany) under the same chromatographic conditions as those previously mentioned. This allowed the comparison of these experimental RI to literature RI (Babushok et al. 2011). After the analysis, geranium plantlets leaves were sampled and dried at 60 °C until constant mass to normalize according to dry weight. The experiment was repeated on seven different plants for each of the G and BG modalities.

## Major compounds quantification

The two major compounds germacrone and  $\beta$ -elemenone were quantified by external calibration using essential oil (EO) of *G. maccorrhizum* (HERBORISTERIE Bardou™, France) due to the absence of commercial standard for the latter. EO was diluted in hexane (1 mg/mL) and 0.2  $\mu$ L was injected in GC–MS in the same chromatographic conditions. Germacrone and  $\beta$ -elemenone represent, respectively, 44.32% and 30.02% of the total area. Standards were prepared by diluting EO in hexane (representing 30–375  $\mu$ g/mL of  $\beta$ -elemenone and 44–554  $\mu$ g/mL of germacrone), and 1  $\mu$ L was injected by a multipurpose sampler (Multi-Purpose Sampler MPS2, Gerstel©, Mülheim der Ruhr, Germany) in 20 mL headspace vial. SPME fiber was then exposed to the headspace during 120 min and analyzed following the same conditions. Calibration curves were analyzed in triplicate between 1.5 and 18  $\mu$ g/L for  $\beta$ -elemenone and 2–27  $\mu$ g/L for germacrone with respective coefficients of determination of  $R^2 = 0.989$  and  $R^2 = 0.986$ .

## Statistical analysis

Data obtained on aphids did not follow a normal distribution (Shapiro test), and homoscedasticity (Fisher test) was not respected. Choice tests data evaluating aphid preferences were analyzed using conformity chi-squared tests. The effect of treatment on the percentage of non-responding aphids was tested using a chi-squared test of independence. Total fecundity data were analyzed using a Generalized Linear Model (GLM) for data following a Poisson distribution. When a significant effect was detected, a pairwise comparison using least squares means (package R: “lsmeans”) ( $p$  value adjustment with Tukey method) at the 0.05 significance level was used to test for differences between treatments. Mortality data and probing behavior data reporting the time before first probing ( $t > 1Pr$ ) and the time before first phloem sap ingestion ( $t > 1E2$ ) were analyzed using a Cox regression thanks to the “coxph” function (Package R: “survival”). This model is adapted to treat time-dependent parameters. Absence of an EPG reading was taken into account and treated as a « zero value». The assumption of validity of proportional hazards was validated using the “coxph” function. All the other probing behavior data were analyzed using a Kruskal–Wallis one-way analysis of variance (H), followed by multiple comparison Dunn tests using the R package “FSA” ( $p$  values adjustment method: ‘Holm’).

Concerning the chemical analysis, the dataset was condensed prior to analysis by removing trace compounds and compounds that accounted for less than 0.5% of the total peak area. Principal component analysis (PCA) was generated using FactoMiner and factoextra packages. Variable

contributions to component were visualized as vectors on the biplot.

Permutational multivariate analysis of variance (Permanova) and analysis of similarities (ANOSIM) were performed by the vegan package (with 999 permutations) based on Bray–Curtis distances of the proportions of each compound following Permutation test for homogeneity of multivariate dispersions. Major compound’s concentration in emissions was analyzed by a paired T test before and after mechanical stimulations.

R software version 3.6.2 (R Core Team, 2014) was used for all statistical analyses.

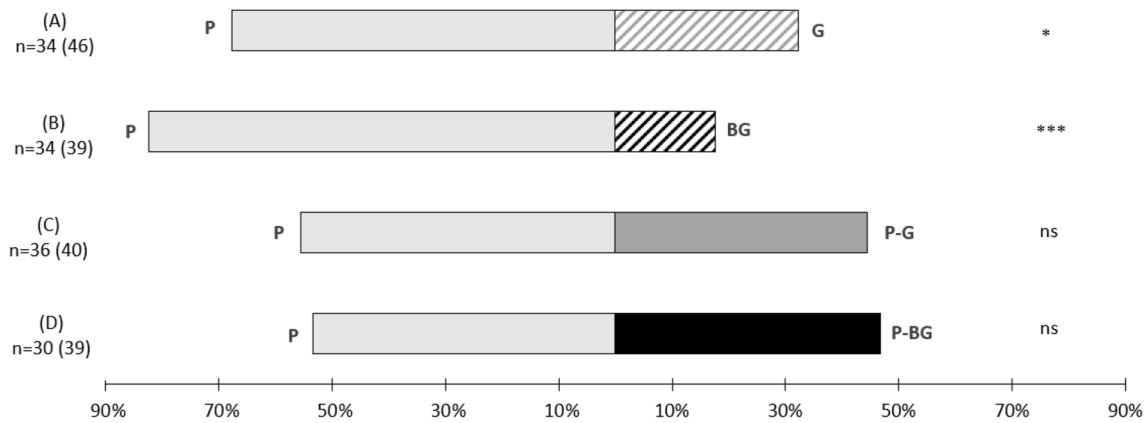
## Results

### Aphid plant preference

Aphids significantly chose sweet pepper over geranium whether brushed (Chi-squared test,  $\chi^2 = 14.24$ ,  $df = 3$ ,  $p < 0.001$ ) or not (Chi-squared test,  $\chi^2 = 4.24$ ,  $p = 0.039$ ) (Fig. 2). However, geranium companioning had no significant effect on aphid choice as no preference was observed between the sweet pepper plant (P) and the sweet pepper plant associated with geranium (P-G) (Chi-squared test,  $\chi^2 = 0.44$ ,  $df = 3$ ,  $p = 0.505$ ), or between the sweet pepper plant (P) and the sweet pepper plant associated with brushed geranium (P-BG) (Chi-squared test,  $\chi^2 = 0.13$ ,  $df = 3$ ,  $p = 0.715$ ). The percentages of non-responding aphids were not significantly different between the experimental situations (Chi-squared test,  $\chi^2 = 4.58$ ,  $df = 3$ ,  $p = 0.213$ ).

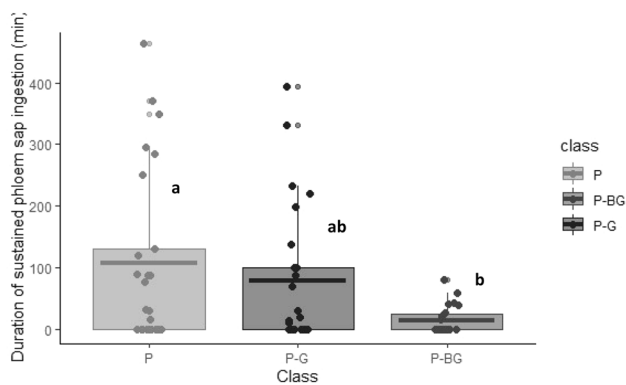
### Aphid probing behavior

Among the nineteen parameters associated with the probing behavior, there were only four major significant effects of treatment on the total number of brief probes (Kruskal–Wallis test  $\chi^2 = 7.58$ ,  $df = 2$ ,  $p = 0.023$ ), the total duration of sustained phloem sap ingestion (Kruskal–Wallis test  $\chi^2 = 6.34$ ,  $df = 2$ ,  $p = 0.042$ ), the total number of xylem phases (Kruskal–Wallis test  $\chi^2 = 13.25$ ,  $df = 2$ ,  $p = 0.001$ ) and the total duration of xylem sap ingestion (Kruskal–Wallis test  $\chi^2 = 19.30$ ,  $df = 2$ ,  $p < 0.001$ ). Indeed, the total number of brief probes was inferior for the (P-BG) modality compared to the (P-G) modality ( $p = 0.027$ ). The duration of sustained phloem sap ingestion was shorter for the (P-BG) modality compared to the (P) modality ( $p = 0.045$ ) (Fig. 3). The total number of xylem phases was inferior for the (P-G) modality compared to the (P) modality ( $p = 0.041$ ) and the (P-BG) modality ( $p = 0.001$ ). Finally, the total duration of xylem sap ingestion was shorter for the (P-G) modality compared to the (P) modality ( $p < 0.001$ ) and the (P-BG) modality ( $p = 0.003$ ). None of the other EPG data linked to the general



**Fig. 2** Percentage of aphids making a choice between a sweet pepper (P) and **A** a geranium (G); **B** a brushed geranium (BG); **C** a sweet pepper associated with a geranium (P-G); **D** a sweet pepper associated with a brushed geranium (P-BG). *n*: total number of responding

aphids (in brackets, the total number of tested aphids). \* indicates a significant difference ( $*p \leq 0.05$ ;  $***p \leq 0.001$ ; Chi-squared test). ns: non-significant



**Fig. 3** Total duration of phloem sap sustained ingestion by *Myzus persicae* on sweet pepper alone (P) or associated with geranium (P-G) or P-BG). Different letters indicate significant differences

probing phase, the pathway phase, or the derailed stylets phase showed any significant effect of treatment on the aphid stylet activity.

### Aphid physiology

Aphids survival rate on sweet pepper was significantly influenced by treatment (Cox model,  $\chi^2 = 8.80$ ,  $df = 2$ ,  $p = 0.01$ ) (Table 1). This rate was significantly smaller when aphids were reared on sweet pepper associated with brushed geranium (P-BG) (62%) compared to sweet pepper alone (P) (90%) (em-means,  $p = 0.029$ ). There was no other significant difference in the two other pairwise comparisons between modalities (P vs. P-G: em-means,  $p = 0.733$ ; P-G vs. P-BG: em-means,  $p = 0.120$ ). There was a significant effect of treatment on aphid fecundity (GLM,  $\chi^2 = 16.50$ ,  $df = 2$ ,  $p < 0.001$ ). Compared to aphids in the (P) modality, the total fecundity was significantly smaller in the (P-G) modality (ls-means,  $p < 0.001$ ) and in the (P-BG) modality (ls-means,  $p < 0.001$ ). There was no significant difference between the two modalities involving geranium companioning (P-G vs. P-BG: em-means,  $p = 0.779$ ).

**Table 1** Comparisons of fitness parameters (means  $\pm$  SEM) of *Myzus persicae* reared on sweet pepper alone (P) and sweet pepper associated with a geranium (P-G) or with a brushed geranium (P-BG)

	Aphids on sweet pepper alone (P)		Aphids on sweet pepper associated with geranium (P-G)		Aphids on sweet pepper associated with brushed geranium (P-G)	
	n		n		n	
Survival rate (%)	41	<b>90a</b>	38	<b>85ab</b>	38	<b>62b</b>
Total fecundity	41	<b>13.98 <math>\pm</math> 1.04a</b>	38	<b>9.85 <math>\pm</math> 0.62b</b>	38	<b>9.06 <math>\pm</math> 0.56b</b>

Within row, different letters indicate significance difference between treatments (GLM or COX test followed by multiple comparison)  $p$  values  $< 0.05$  (Cox model and GLM) are written in bold;  $n$  = sample size

## Analysis of volatile organic compounds (VOC) emission by gas chromatography coupled with mass spectrometry (GC–MS)

The headspace SPME analysis of *G. macrorrhizum* plantlets presented a total of 62 VOCs belonging to the monoterpenoids, sesquiterpenoids, esters, cetones and aldehydes families. The detailed composition is available in the supplementary materials (Table S2). The main compounds whose relative areas were superior to 0.5% are listed in Table 2. Those compounds belonged solely to the monoterpenoids, sesquiterpenoids families and represented 91.05% and 92.38% of total emissions for geranium (G) and brushed geranium (BG), respectively. As for the essential oil composition, germacrone and  $\beta$ -elemenone were the most abundant compounds in geranium emissions.

Multivariate analysis was performed to investigate the impact of mechanical stimulation on VOCs profile emission leading to aphid behavioral changes. The principal component analysis (PCA) allowed to visualize similarities between individuals (point) taking into account relative abundance of VOCs (Fig. 4). The first two components took into account 87.5% of total variability allowed to distinguish in between G (in blue) and BG (in red). ANOSIM analysis demonstrated that the VOCs profile distinction was statistically significant ( $R=0.3392$ ,  $p=0.015$ ). Permanova

also yielded significant profile distinction between the two groups ( $F=7.3339$ ,  $p=0.011$ ). The arrows represent the original variables (VOCs) contribution to the first and second dimension (PCA1&2). At first glance, differences between the samples followed PCA1 mainly composed of monoterpenoids (on the right) and sesquiterpenoids (on the left) (Fig. 4). The mechanical stimulation of geranium therefore led to an increase in sesquiterpenoids emission and a relative decrease in monoterpenoids emission.

To evaluate this impact for the two main sesquiterpenoids germacrone and  $\beta$ -elemenone, external calibration allowed to quantify their concentration after 24 h. Emissions boxplot are presented in Fig. 5. Emission of  $\beta$ -elemenone rised from  $7.14 \pm 3.86$  to  $18.65 \pm 8.62 \mu\text{g L}^{-1} \text{g}_{\text{DW}}^{-1}$  and germacrone emission from  $10.65 \pm 4.4$  to  $30.84 \pm 12.99 \mu\text{g L}^{-1} \text{g}_{\text{DW}}^{-1}$ . Paired T test demonstrated that those increases were significant ( $p < 0.01$ ) both for germacrone ( $t=5.4754$ ,  $df=6$ ,  $p$  value = 0.00155) and  $\beta$ -elemone ( $t=5.7929$ ,  $df=6$ ,  $p$  value = 0.001159).

## Discussion

The repellent properties of plants and essential oils from Geraniaceae have been highlighted in insects, and more particularly in aphids. *M. persicae* is repelled by VOCs emitted

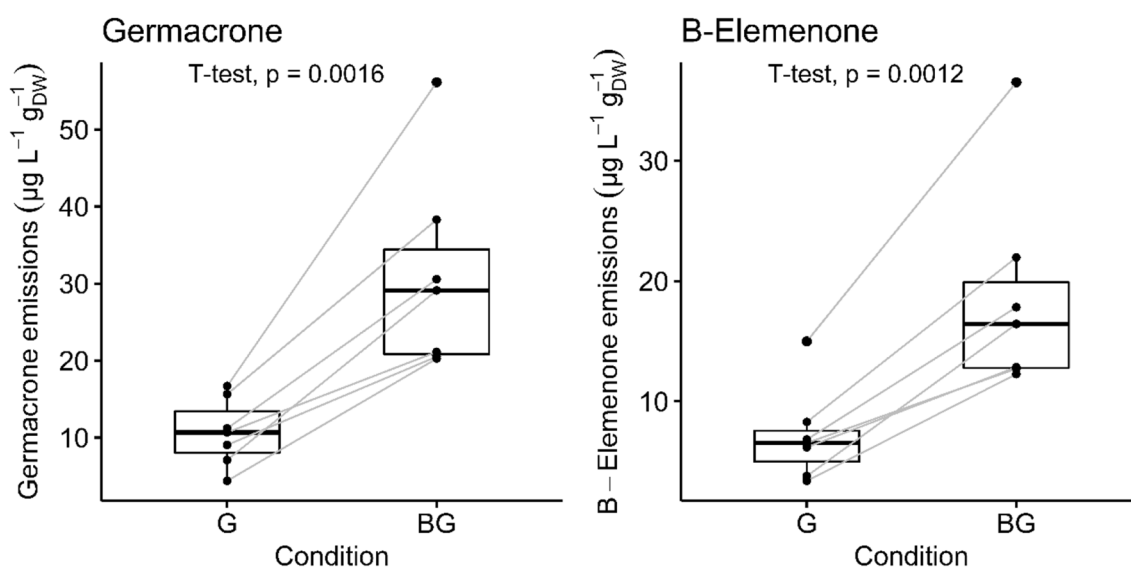
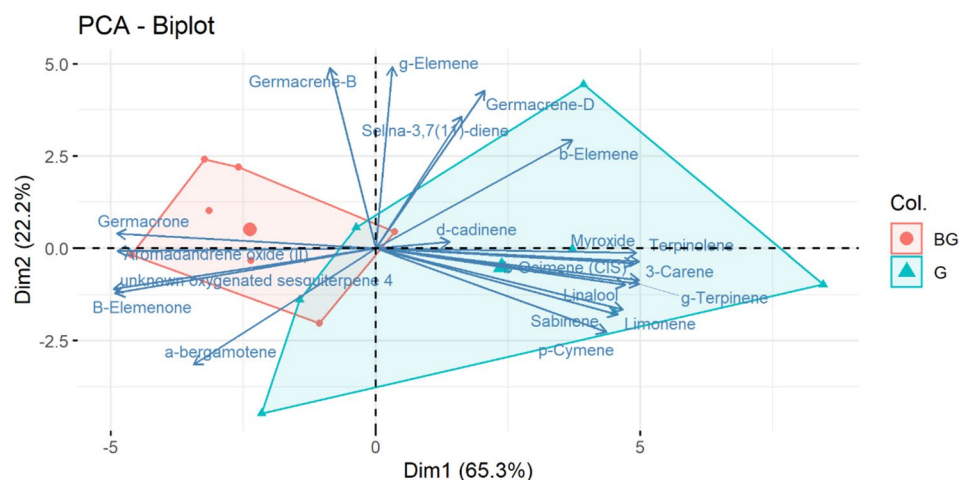
**Table 2** Volatile organic compounds emissions of *Geranium macrorrhizum* before (G) and after mechanical stimulation (brushed geranium BG)

Family	Compound	Formula	Exp RI*	Library RI	G (n=7)	BG (n=7)	Fold change
Monoterpenes	3-Carene	C10H16	1014	1011	$0.94 \pm 0.45$	$0.32 \pm 0.21$	0.33
	Limonene	C10H16	1026	1030	$1.3 \pm 0.75$	$0.45 \pm 0.34$	0.34
	p-Cymene	C10H14	1022	1024	$3.44 \pm 1.72$	$1.2 \pm 0.76$	0.35
	Sabinene	C10H16	970	973	$0.74 \pm 0.44$	$0.24 \pm 0.21$	0.33
	Terpinolene	C10H16	1086	1087	$2.32 \pm 1.09$	$0.85 \pm 0.47$	0.37
	$\beta$ -Ocimene (cis)	C10H16	1047	1048	$7.73 \pm 2.42$	$3.07 \pm 1.72$	0.40
	$\gamma$ -Terpinene	C10H16	1057	1060	$6.37 \pm 2.62$	$2.43 \pm 1.5$	0.38
Oxygenated monoterpenes	Linalool	C10H18O	1098	1099	$0.52 \pm 0.19$	$0.21 \pm 0.09$	0.40
	Myroxide	C10H16O	1141	1138	$1.18 \pm 0.44$	$0.39 \pm 0.2$	0.33
Oxygenated sesquiterpenes	Aromadandrene oxide (II)	C15H24O	1656	1658	$1.01 \pm 0.26$	$1.32 \pm 0.13$	1.31
	Germacrone	C15H22O	1697	1705	$14.22 \pm 4.61$	$20.64 \pm 2.11$	1.45
	unknown Oxygenated sesquiterpene 4	C15H22O	1600	0	$2.05 \pm 0.58$	$2.74 \pm 0.22$	1.33
	$\beta$ -Elemenone	C15H22O	1607	1602	$27.45 \pm 7.9$	$35.29 \pm 2.9$	1.29
Sesquiterpenes	Germacrene-B	C15H24	1557	1551	$2.47 \pm 0.78$	$2.95 \pm 0.49$	1.19
	Germacrene-D	C15H24	1481	1481	$1.11 \pm 0.44$	$1 \pm 0.16$	0.91
	Selina-3,7(11)-diene	C15H24	1542	1541	$0.66 \pm 0.13$	$0.7 \pm 0.14$	1.06
	$\alpha$ -Bergamotene	C15H24	1429	1429	$1.03 \pm 0.45$	$1.29 \pm 0.13$	1.25
	$\beta$ -Elemene	C15H24	1392	1398	$2.94 \pm 0.95$	$2.38 \pm 0.41$	0.81
	$\gamma$ -Elemene	C15H24	1434	1436	$12.92 \pm 2.99$	$14.25 \pm 2.29$	1.10
	$\delta$ -Cadinene	C15H24	1524	1523	$0.65 \pm 0.05$	$0.66 \pm 0.05$	1.01

Relative abundance was expressed as the mean value  $\pm$  SD of seven replicates

\*Exp RI = Experimental retention index

**Fig. 4** Principal component analysis (PCA) of geranium VOCs emissions following mechanical stimulation ( $n=7$ )



**Fig. 5** Germacrone and  $\beta$ -elemenone emissions ( $\mu\text{g L}^{-1} \text{g}_{\text{DW}}^{-1}$ ) ( $n=7$ ). The gray line links each individual before and after treatment (paired T test)

by *Pelargonium zonal* (Ben-Issa et al. 2016) and *Brevicoryne brassicae* by VOCs emitted by *Geranium maculatum* essential oils (Chopa 2019). In our choice experiment, aphids tested in a setup allowing contact-based, odor-based and visual cues, exhibited a preference for their host plant over *G. macrorrhizum*. This preference, being more marked when brushed geranium (BG) was used as a CP, suggests that volatile compounds play a determining role in the aphid orientation observed. In our study, GC-MS shed light on the main VOCs components emitted by the aerial parts of *G. macrorrhizum* leaves, i.e.,  $\beta$ -elemenone (27.45–35.29%), germacrone (14.22–20.64%),  $\gamma$ -elemene (12.92–14.25%),  $\beta$ -ocimene (cis) (7.73–3.07%),  $\gamma$ -terpinene (6.37–2.43%), p-cymene (3.44–1.2%),  $\beta$ -elemene (2.94–2.38%), germacrene-B (2.47–2.95%), terpinolene (2.32–0.85%) and limonene, myroxide, germacrene-D,  $\alpha$ -bergamotene,

aromadandrene oxide (II), 3-carene, sabinene, selina-3,7(11)-diene,  $\delta$ -cadinene, linalool (<2% each). Our results are in accordance with Chalchat et al. (2002) who analyzed the quantity and chemical composition of oils from the aerial parts of *G. macrorrhizum* and showed that  $\beta$ -elemenone and germacrone were the two main components. Our study also revealed that stimulation of trichomes via geranium leaf brushing significantly altered the VOCs profile with a relative increase in oxygenated sesquiterpenes emissions and a decrease in monoterpenoids as can be observed from the principal component analysis. The quantitative study of the two major oxygenated sesquiterpenes germacrone and  $\beta$ -elemenone revealed that their emitted amounts were almost multiplied by three. This could explain the increased repellency observed, as extracts of *Rhododendron* oil containing 20.8% germacrone, 35%  $\beta$ -elemenone and 11%



$\gamma$ -elemene had a repulsive action toward the Psocoptera *Liposcelis bostrychophila* and the Coleoptera (Liang et al. 2016). However, in our study, the repellent property of geranium seemed limited as geranium companioning had no significant effect on aphid choice. Indeed, no preference was observed between the sweet pepper plant (P) and the sweet pepper plant associated with geranium (P-G and P-BG). A possible masking or disrupting mechanism on the host plant attractivity by non-host VOCs, as described by (Nottingham et al. 1991; Schröder and Hilker 2008), can not be ruled out.

The probing behavior study confirmed the hypothesis of a disruptive effect of *G. macrorrhizum* VOCs. When aphids were submitted to volatiles of a geranium that had not been brushed, only parameters related to xylem phases were impacted. Their decrease could be due to the effect of geranium COV's on the water balance of the insect. When a brushed geranium was present, the observed perturbation of *M. persicae* sustained phloem sap ingestion could globally reflect their host plant acceptance disturbance through a decrease in sweet pepper palatability. However, the probing behavior was not drastically modified by the CP volatiles: among the nineteen probing parameters selected, only four were altered. Regarding the work of Dardouri et al. (2021) where the repellent effect of the two companion plants (*Tagetes patula*, marigold, and *Ocimum basilicum*, basil) translated into greater numbers of probes (n\_Pr) and a shorter total duration of probing (s\_Pr) performed by *M. persicae* on sweet pepper, the hypothesis of a probing behavior disruption as a result of geranium volatils repellency can be rejected in our study. Unlike (Navarro-Rocha et al. 2018), who demonstrated the antifeedant properties of germacrone and  $\beta$ -elemenone against *M. persicae* and *Rhopalosiphum padi* by adding them to their artificial diet, we can not conclude that geranium volatiles deposited on sweet pepper made it deterrent. Indeed, geranium as a CP (G and BG) did not seem to impact any of the behavioral phases occurring before the initiation of sap ingestion. Not only the time to first probe ( $t > 1Pr$ ), but also the parameters of the pathway phase (n\_C, S\_C, n\_Pd and S\_Pd) were unchanged, suggesting that the modifications of *M. persicae* probing behavior in the presence of geranium were probably not due to physical or chemical changes in epiderm and/or mesophyll tissues. Moreover, the potential drops revealing the mechanism used by aphids to discriminate and accept the host plant, far before reaching the phloem, were not impacted. The lower occurrence of brief probes in the P-BG condition could even reflect a decrease in the putative VOC-related disturbance. Consequently, the hypothesis that geranium VOCs could be adsorbed in the sweet pepper leaf cuticle up to phloem tissues can be ruled out here, although such phenomena have already been shown in other studies (Camacho-Coronel et al. 2020; Himanen et al. 2010). Thus, the altered acceptance of the host plant by brushed geranium VOCs is likely to be

the consequence of aphid intoxication by inhalation by germacrone and  $\beta$ -elemenone. Finally, certain VOCs have also been shown to stimulate neighboring plants to adjust their defenses at the right time and subsequently reduce herbivore feeding damages as demonstrated in several studies (Karban et al. 2014; Ninkovic et al. 2019; Tolosa et al. 2019).

As revealed by aphid performance on the host plant, volatiles of geranium (G and BG) did alter aphid reproduction by negatively impacting their total fecundity. Mechanical stimulation of the leaves and the subsequent increase in germacrone and/or  $\beta$ -elemenone VOCs emission seem to have amplified these negative effects through a decrease in aphid survival which could be attributed to a shorter duration of phloem sap intake. Our results thus confirm the aphicidal properties of *G. macrorrhizum* previously shown on *M. persicae* through the use of plant extracts (Velcheva et al. 2001). It should also be noted that *P. zonal* (Geraniaceae) used as a companion plant also affected the survival and reproduction of *M. persicae* but through other repellent compounds such as geranyl acetone and  $\beta$ -farnesene (Ben-Issa et al. 2016). Finally, it can be hypothesized that the *G. macrorrhizum* VOCs, particularly the germacrone and/or  $\beta$ -elemenone, negatively altered the preference and the performance of *M. persicae* toward its host plant, and that the process involved would probably be an intoxication via fumigation. However, to validate the putative repellency and toxicity of these two compounds, it would be worth confirming their effects by using synthetic compounds. The neurotoxic action via inhalation of non-host plant volatiles and essential oil volatiles has been demonstrated in different studies (Petrakis et al. 2014; Poorjavad et al. 2014; Regnault-Roger et al. 2012; Sousa et al. 2015), and more particularly with *Pelargonium graveolens* oils (Baldin et al. 2015; Jayakumar et al. 2017). Essential oils rich in oxygenated monoterpenes were more toxic compared to those rich in monoterpene hydrocarbons, whereas essential oils rich in sesquiterpenes were less active (Koutsaviti et al. 2018). Furthermore, germacrone and  $\beta$ -elemenone are terpene presenting a ketones function and this group of compounds have already been acknowledged for their strong insecticidal activity via the inhibition activity of ketones on acetylcholinesterase (AChE).

Finally, another plant species, *Rhododendron thymifolium*, exhibits a chemical profile (Liang et al. 2016) close to the one of *G. macrorrhizum*. It would therefore be interesting to compare their efficiency as CPs in protecting the focal plant against insect pests. Plant protection using chemical or plant extracts can have different effects on aphid behavior and physiology depending on whether they are applied topically, ingested, or inhaled (Martoub et al. 2011). It would be relevant to study the effect of germacrone and  $\beta$ -elemenone on the behavior and physiology of *M. persicae* when these two molecules are applied via the three main routes of exposure.

To conclude, this study demonstrated a disturbance induced by VOCs from *G. macrorrhizum* as a CP on *M. persicae* host plant colonization process. Regarding host plant location, *G. macrorrhizum* was shown to be intrinsically repellent but not enough to completely mask the attractive odor of the sweet pepper host plant. *G. macrorrhizum* as a CP reduced aphid fecundity without affecting plant acceptance. In a field context, using intact *G. macrorrhizum* is not likely to offer a suitable protection against aphids.

However, when *G. macrorrhizum* leaves were stimulated, the effects were more pronounced: sustained phloem sap ingestion, survival and fecundity were reduced. VOCs analysis revealed that these effects could be due to the increase in  $\beta$ -elemenone and germacrone which represent compounds that are rarely emitted by plants belonging to Geraniaceae. It would be interesting to test if the emission of  $\beta$ -elemenone and germacrone by the companion plant *G. macrorrhizum* could protect sweet peppers in field conditions and reduce the survival and reproduction of *M. persicae*. In this context, a comparison could be made with the work of (Ben-Issa et al. 2016) which showed that another Geraniaceae, *P. zonal*, affects the dynamics of *M. persicae* by emitting geranyl acetone and  $\beta$ -farnesene. Altogether, this suggests these volatile compounds may have a neurotoxic effect rather than a repellent or deterrent effect. In a context of applicability, it would be interesting to test under field conditions whether a mechanical stimulation of *G. macrorrhizum*, for example by watering the plants, could be sufficient to induce a boost in the emission of VOCs. In this situation, it would also be important to test to what extent such emissions might negatively affect other crop pests. Indeed, if companion plants repel aphids, they might also promote their attraction to crops and induce damages.

## Author contribution statement

PYW AC GD AA conceived and designed the experiments. JD PYW performed the experiments. JD PYW GD AC AA analyzed the data. JD PYW AC GD MLF ML AA wrote the paper.

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**Data availability** All data generated or analyzed during this study are included in this published article [and its supplementary information files].

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

**Ethical approval** The article does not contain any studies with human participants or vertebrate animals. No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

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