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# Behavioural response of green peach aphid *Myzus persicae* (Sulzer) to volatiles from different rosemary (*Rosmarinus officinalis* L.) clones

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- Abstract**
- 1 Previous studies have shown that rosemary (*Rosmarinus officinalis* L.) can serve as a companion plant to control *Myzus persicae* (Sulzer) because of the repellent effect of certain volatile organic compounds (VOCs) that it emits. In the present study, we investigated which of five clones of rosemary may reveal a repellent effect on *M. persicae* and aimed to determine the possible VOCs involved.
  - 2 Analyses of the mixtures of VOCs released by the different clones revealed the presence of 15 main components. However, each clone was characterized by a specific volatile profile showing the existence of marked chemical variability.
  - 3 By testing the identified VOCs individually, using a dual-choice olfactometer, we observed that five volatiles had a significant repulsive effect on *M. persicae*: bornyl acetate, camphor,  $\alpha$ -terpineol, terpinene-4-ol and geranyl acetone. In addition, only one clone of rosemary elicited a significant repulsive action.
  - 4 Nevertheless, all of the tested clones released compounds that are repellent to the aphid when tested individually. Therefore, the emission of individual volatiles by a rosemary plant is not sufficient to elicit a repellent effect.
  - 5 The concentration, proportion and even the association/synergy of VOCs in the released olfactory bouquets can probably explain these contrasting results and are worthy of additional exploration in future studies.

**Keywords** Agroecology, aphid, companion plants, *Myzus persicae*, repulsion, rosemary, volatile organic compounds.

## Introduction

Aphids (Hemiptera, Aphididae) are piercing-sucking insects that feed on phloem sap, which results in severe damage to the host plants. *Myzus persicae* (Sulzer), the so-called green peach aphid, causes considerable damage to several crops not only by removing phloem sap from plants, but also by transmitting plant viruses (Blackman & Eastop, 2000). This polyphagous insect, for which the primary host is the peach tree, causes significant damage to many secondary hosts, such as pepper species in greenhouses (Sanchez *et al.*, 2011).

To limit the severity of the damage caused by this pest, several control methods have been carried out, among which chemical control methods are the most widespread. Despite being rapid and effective, the chemical control methods present

several disadvantages as a result of their harmful effects on the environment and the continuous development of resistant populations (Bass *et al.*, 2014).

To solve this problem and to ensure sustainable, economically competitive and ecologically intensive agriculture, the scientific community has adopted a strategy for the development of alternative control methods (Penvern *et al.*, 2010). One of these methods is based on the association of cultivated crops with companion plants aiming to provide services to the agro-ecosystem (Ben Issa *et al.*, 2017a). This agro-ecological strategy presents a promising alternative control method for maintaining phytophagous populations below their economic threshold, thus reducing the frequency of phytosanitary treatments without significantly reducing the level of production of the target crop (Uvah & Coaker, 1984; Mal ezieux *et al.*, 2009; Tang *et al.*, 2012). The different mechanisms explored with these companion plants are repulsion (plants with repellent properties) and attraction (trap plants). Furthermore, companion plants are able to mask the volatile

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emissions from host plants and can also attract natural enemies to control phytophagous populations (Ben Issa *et al.*, 2017a).

Regarding the repulsion mechanism (Deletre *et al.*, 2016), the choice of species associated with the crop to be protected remains paramount for the strategy to succeed (Shrivastava *et al.*, 2010) and is dependent on several factors. In addition to the ability to disturb the behaviour of aphids, criteria such as the phenology, hardiness and commercial exploitation of these plants need to be considered (Castro *et al.*, 2017).

Recent research has shown that the presence of rosemary (*Rosmarinus officinalis*, Lamiaceae) in the vicinity of sweet pepper plants hinders the development of *M. persicae* populations (Ben Issa *et al.*, 2016, 2017b). Rosemary is an aromatic plant that appears to meet the criteria required for a companion plant for the control of green peach aphid in Mediterranean horticultural crops. According to several studies (Hori, 1998, 1999a; Ben Issa *et al.*, 2016, 2017b), the remote action of rosemary plants, eliciting repulsive effects in *M. persicae* is related to the emission of volatile organic compounds (VOCs). These VOCs, which are not essential for cell growth and development, accumulate in specialized anatomical structures called trichomes (Marin *et al.*, 2006). The mixture of VOCs, which gives rosemary its characteristic odour, is mainly composed of monoterpenes (C<sub>10</sub>H<sub>16</sub> compounds). Chromatographic analyses of rosemary essential oils and plants show that the main VOCs are bornyl acetate, borneol, camphene, camphor, eucalyptol (1,8-cineole), linalol, verbenone and  $\alpha$ -pinene (Angioni *et al.*, 2004; Miresmailli *et al.*, 2006; Chopa & Descamps, 2012; Zhang *et al.*, 2014a; Sadeh *et al.*, 2017). It can be hypothesized that the efficacy of these plants is related to the emission level of one or more of these compounds, which would increase as their production increases. However, Ben Issa *et al.* (2017b) found that the range of action of the companion plants is quite limited and that effective protection requires such a density that it can be restrictive and cause a decline in the yield of the main crop. Therefore, it is imperative to optimize VOC production. In this sense, genetic selection can be an essential lever for optimizing the effectiveness of rosemary and promoting its use by producers. Indeed, VOC production varies quantitatively and qualitatively according to the cultivars (Satyal *et al.*, 2017), generating a variable olfactory response from aphids (da Costa *et al.*, 2010; Staudt *et al.*, 2010; Rajabaskar *et al.*, 2013; Schröder *et al.*, 2015). Still, by the same logic, understanding the action modes of companion plants and identifying the molecules involved should help us to select the most successful genotypes. However, there is little information available on this topic, especially with respect to the dualistic interaction between plants and insects via VOCs (Hori, 1999b).

The present study aimed to assess the effect of rosemary genetic variability on its ability to disrupt the behaviour of green peach aphids. The olfactory bouquets of five rosemary clones were analyzed by gas chromatography-mass spectrometry (GC-MS) and their VOC profiles were established. The repellent properties of the studied clones, as well as those of each of the VOCs identified in the olfactory bouquets, were assessed in dual-choice bioassays using olfactometers (Ameline *et al.*, 2007; Alvarez *et al.*, 2007). In the end, our ultimate goal was to establish a link between aphid repellence and the presence of certain VOCs from rosemary clones.

## Materials and methods

### Aphid rearing

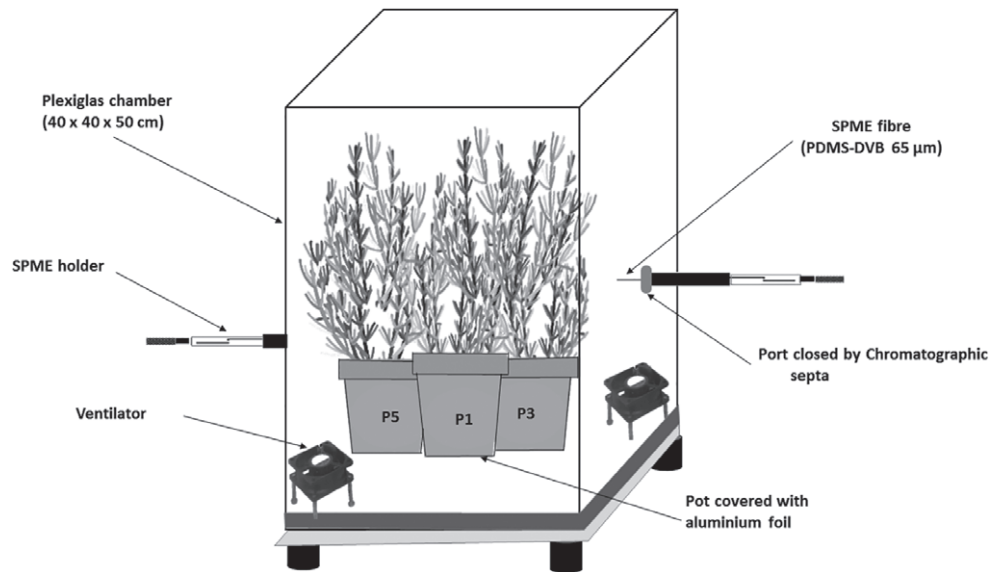
*Myzus persicae* were obtained by mass rearing (clone Mp05) from a fundatrix collected from a peach orchard of the French National Institute for Agricultural Research (INRA) of Avignon (France) (Sauge *et al.*, 2010). The insect culture was maintained on young pepper plants under controlled rearing conditions, comprising an LD 16 : 8 h photocycle at  $22 \pm 1$  °C and 60–70% relative humidity. For each experiment conducted, the aphids used were all of the same age. For breeding synchronization, apterous females were deposited per plant. Twenty-four hours later, these females were removed, whereas the larvae generated remained on the pepper plant. After 10 days, this cohort of 24 h gave the necessary adult individuals to be used in the experiments.

### Plant material

Five clones of rosemary (*Rosmarinus officinalis* L.) were propagated by cuttings from specimens collected from plots from CRIEPPAM (Centre Régionalisé Interprofessionnel d'Expérimentation en Plantes à Parfum, Aromatiques et Médicinales, France). The clones considered in the present study were: Esselte (E), Nonza (N), Pigette (P), Sudbery Blue (SB) and Voltz Splindler (VS). These clones were selected based on preliminary results that reported the existence of variability in the chemical profile of their essential oils (L. Gomez *et al.*, unpublished data). The five clones were propagated at the same moment and cultivated in a greenhouse of INRA Avignon under controlled conditions (temperature of  $20 \pm 5$  °C and 60–70% relative humidity). The cultivated clones did not receive any fertilization or phytosanitary treatments. The irrigation was carried out with a drip system to homogenize the production conditions. For all of the experiments carried out, the plants were used at the same vegetative stage (6–8 months old). The pepper plants (*Capsicum annuum* L., var. Yolo Wonder) used for the *M. persicae* mass rearing were 6 weeks old (with 5–6 developed leaves) and were obtained by sowing in another glass greenhouse to avoid any interaction with the rosemary clones. All of the plants were cultivated in 1-L pots filled with peat.

### Volatile profile characterization

The profile of the VOCs from each clone was analyzed by HS-headspace solid phase micro-extraction (SPME) and GC-MS. For the analyses, 15 plants were used per clone, divided into three groups of five plants each. On the day before sampling, the plants were placed under the experimental conditions: temperature of  $22 \pm 1$  °C and  $60 \pm 5\%$  relative humidity. The plants were watered and the pots were wrapped in aluminium foil to prevent sample pollution by VOCs from the culture substrate and plastic pot. For each analysis, five plants were carefully introduced without touching the foliage inside a parallelepiped-shaped Plexiglas chamber (width 40 cm, length 40 cm, height 50 cm) (Fig. 1). To homogenize the atmosphere inside the Plexiglas chamber during sampling and to promote



**Figure 1** Device for static sampling of volatile organic compounds (VOCs) emitted from rosemary clones using the headspace solid phase micro-extraction technique.

the adsorption of the VOCs on the SPME fibre, two ventilator fans were installed at the base.

After 10 min of stabilization, two polydimethylsiloxane/divinylbenzene SPME fibres (PDMS-DVB, 65 µm; Supelco, Bellefonte, Pennsylvania) were introduced simultaneously through two septa positioned on the walls. After an exposure time of 5 min, the SPME fibres were removed together with the sampled plants. This experiment was conducted at 10.00 h, and the other two groups of plants were analyzed at 13.00 h and 16.00 h, with a total of six samples per clone (2 fibres × 3 sampling moments), reporting an average profile throughout the day. Before each SPME analysis, the air inside the chamber was renewed with a vacuum cleaner and the cage was cleaned with 70% ethanol and distilled water to avoid contamination. The SPME analyses were carried out at a rate of one clone per day.

The VOC samples were rapidly analyzed by GC-MS (Trace-ISQ, single quadrupole; Thermo Scientific, Austin, Texas) equipped with an apolar capillary column TR-5MS (Thermo Scientific) (20 m; 0.1 mm inner diameter; 0.1 µm film thickness). The carrier gas was helium at a constant flow of 0.4 mL min<sup>-1</sup>. The desorption of the SPME fibre was performed during 2 min in the injector at 250 °C in splitless mode. The oven temperature was set at 40 °C for 2 min after desorption, followed by an increase of 20 °C min<sup>-1</sup> up to 100 °C, then 5 °C min<sup>-1</sup> up to 160 °C and, finally, 30 °C min<sup>-1</sup> up to 300 °C. The mass spectra were recorded in electron impact mode with an ionization energy of 70 eV in the range *m/z* 35–450.

The VOCs were identified by comparing their mass spectra with the spectra of the NIST11 library (National Institute of Standards and Technology, Gaithersburg, Maryland). The retention times of the compounds to be identified were compared with those obtained from commercial standards. The quantification was carried out by manual integration of the peaks obtained using XCALIBUR (Thermo Scientific).

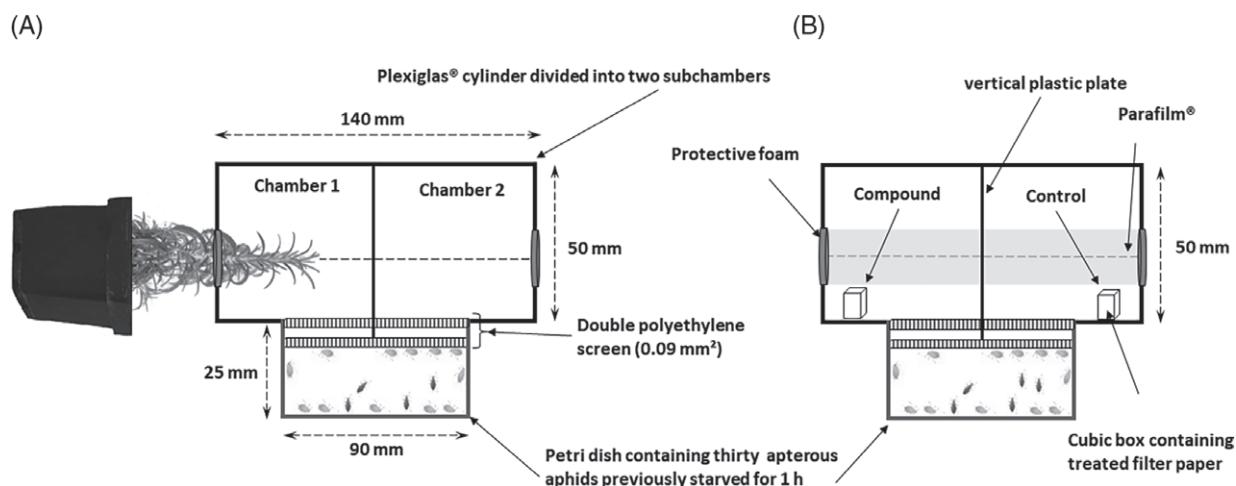
#### Standards and reagents

The VOCs identified in the obtained olfactory mixtures produced by the rosemary clones were supplied by Sigma-Aldrich (France). The standards were purchased to individually test their effect on green peach aphids. The standards obtained were (with the respective standard purity represented in brackets): bornyl acetate (95%), borneol (99%), camphene (95%), camphor (96%), eucalyptol (99%), geranyl acetone (97%), limonene (97%), linalol (97%), terpinene-4-ol (95%), terpinolene (90%), verbenone (93%),  $\alpha$ -pinene (98%),  $\alpha$ -terpineol (96%) and  $\gamma$ -terpinene (97%). (*E*)- $\beta$ -farnesene (93%) was used as a positive control. Only  $\beta$ -terpineol was not available for purchase. Each compound was tested at a concentration of 1% diluted in ethanol (v/v; m/v in the case of camphor). The concentration used makes it possible to obtain a peak area similar to the sum of the peak areas emitted by a whole plant to avoid saturation of the olfactometer.

#### Olfactometer bioassays with *M. persicae*

The dual-choice olfactometer used (Eigenbrode *et al.*, 2002; Ameline *et al.*, 2007; Alvarez *et al.*, 2007) is a device with a static atmosphere, consisting of two overlapping containers of different sizes, with facing openings provided with a polyethylene grid (0.09 mm<sup>2</sup> mesh) (Fig. 2). The upper container is a cylindrical Plexiglas arena (diameter 14 cm, height 5 cm) hermetically subdivided into two equal chambers. Each side of this container is pierced to allow the introduction of a leaf or plant part through a 5-mm opening. To prevent mechanical injuries, each opening is protected with foam. The lower container (diameter 9 cm, height 2.5 cm), where aphids are placed, is positioned below the arena polyethylene grid.

All of the bioassays were performed in a ventilated room, maintained at 22 ± 1 °C and relative humidity of 60 ± 5%. The



**Figure 2** Dual-choice olfactometer used for the olfactometry bioassays conducted with *Myzus persicae*: (A) rosemary versus control (without plant); (B) 10  $\mu\text{L}$  of volatile compound (1%) versus control (10  $\mu\text{L}$  of ethanol). An aphid is considered as a responder if it is positioned on the grid under one of the two chambers.

bioassays were conducted in darkness to avoid any visual cue that could influence the orientation of aphids. One hour before starting the bioassays, the plants were placed in the room. Thirty apterous females from a cohort of 10 days were placed in the lower container with a brush soaked in demineralized water. A fasting period of 1 h in the dark allowed aphids to equalize their physiological state. A fragment of rosemary plant was introduced into one upper chamber versus control (empty chamber). To test the VOCs, 10  $\mu\text{L}$  of the VOC (1% solution in ethanol) was deposited on filter paper (2  $\times$  2 cm) in a cubic box (2.1  $\times$  2.1 cm), which was then fixed in a chamber from the upper arena. A second paper with 10  $\mu\text{L}$  of ethanol was placed in the second chamber, serving as a control. The papers were left to dry for 5 min. The upper arena was then closed and sealed with Parafilm® to seal the olfactometer. The lower container, with the aphids homogeneously distributed, was immediately placed under the upper container. Aphids underwent negative geotaxis and climbed onto the upper grid (Eigenbrode *et al.*, 2002) where they positioned themselves in accordance with their olfactory orientation under one of the two upper chambers. Each bioassay lasted for 1 h. After this period, the upper container was removed and the lower container was photographed to record the distribution of the aphids. An aphid was considered as a 'responder' when it was placed on the grid and as a 'nonresponder' when it was placed in the edges of the container or when it was not in the grid. In each bioassay, only the responders were counted considering the side of the upper chamber where they were positioned. Each choice hypothesis was tested eight times in eight olfactometers used in parallel. Per treatment and hypothesis to be tested, 240 aphids were studied (8 olfactometers  $\times$  30 apterous females). To avoid the risk of contamination, a single clone or VOC was tested per day and the entire structure was cleaned with 70% ethanol and distilled water.

Finally, two complementary assays were carried out (blank versus blank and solvent versus solvent) to ensure a lack of experimental bias. An assay with pepper versus blank allowed the characterization of the behaviour of *M. persicae* insects in the presence of the host plant (pepper).

### Statistical analysis

Statistical analyses were performed using XLSTAT, version 2016.02 (Addinsoft, France). The number of nonresponders between the different treatments (insects that did not orient to any chamber of the olfactometer were designated as nonresponders) was compared using the nonparametric Kruskal–Wallis test. In the case of significant differences, the number of nonresponders was compared using Tukey's test ( $\alpha=0.05$ ). For each treatment conducted, the distribution of responders on each chamber of the olfactometer was analyzed using a Wilcoxon test for paired samples. The significance level was  $\alpha=0.05$ . To compare the differences in emissions of VOCs of different rosemary clones, Tukey's test with a 95% confidence interval was performed. A principal component analysis (PCA) was also carried out to show the variability of the VOCs between the different clones.

## Results

### Analysis of the VOC profiles from the rosemary clones

The VOC profiles of the five different clones of rosemary (Esselte, Nonza, Pigette, Sudbery Blue and Voltz Splindler) allowed the identification of 15 compounds. From these 15 compounds, 11 were present in the five clones, whereas linalol,  $\alpha$ -terpineol and verbenone were absent from the Voltz Splindler clone. Other VOCs, namely (*E*)- $\beta$ -terpineol and terpinolene, were also absent from the volatile profile of Esselte (Table 1). Analysis of VOC profiles revealed that VOC emission was highly dependent on the clone. From these, there were five major components (Table 1) in the different clones:  $\alpha$ -pinene, eucalyptol (except for Nonza), camphor, borneol and bornyl acetate. Bornyl acetate was the main VOC in Nonza, Sudbery Blue and Voltz Splindler, and camphor was the main VOC in Pigette and Esselte (Table 1). The proportion of  $\alpha$ -pinene was homogeneous between the clones that were analyzed, varying on average between 16% and 26% (Pigette and Esselte,

**Table 1** Relative percentages (mean  $\pm$  SD) of the components of the volatile organic compound (VOC) mixtures released by the rosemary clones studied, sampled by solid phase micro-extraction and identified by gas chromatography-mass spectrometry ( $n = 6$ )

Code	VOCs	Clones						
		RT	RI	Esselte	Nonza	Pigette	Sudbery Blue	Voltz Splindler
1	$\alpha$ -pinene	4.65	938	<b>26.20 <math>\pm</math> 2.46b</b>	<b>18.72 <math>\pm</math> 1.75 a</b>	<b>16.50 <math>\pm</math> 1.58 a</b>	<b>19.91 <math>\pm</math> 2.79 a</b>	<b>19.66 <math>\pm</math> 2.09 a</b>
2	Camphene	4.88	957	0.96 $\pm$ 0.5a	2.01 $\pm$ 1.35b	0.90 $\pm$ 0.50a	1.26 $\pm$ 0.37ab	0.91 $\pm$ 0.39a
3	Limonene	5.9	1034	1.20 $\pm$ 0.71a	3.71 $\pm$ 2.16b	1.44 $\pm$ 0.63a	1.65 $\pm$ 0.71a	1.60 $\pm$ 0.93a
4	Eucalyptol	5.97	1039	<b>9.33 <math>\pm</math> 0.80c</b>	0.64 $\pm$ 0.12a	<b>8.09 <math>\pm</math> 1.05bc</b>	<b>7.55 <math>\pm</math> 1.07b</b>	<b>7.37 <math>\pm</math> 0.35b</b>
5	$\gamma$ -terpinene	6.28	1064	0.93 $\pm$ 0.41a	<b>5.07 <math>\pm</math> 1.70c</b>	0.85 $\pm$ 0.45a	3.30 $\pm$ 0.56b	4.57 $\pm$ 1.26c
6	( <i>E</i> )- $\beta$ -Terpineol	6.53	1079	ND a	1.61 $\pm$ 0.44bc	1.38 $\pm$ 0.55b	2.04 $\pm$ 0.35c	3.18 $\pm$ 0.37d
7	Terpinolene	6.69	1092	ND a	1.00 $\pm$ 0.45c	0.39 $\pm$ 0.14b	0.59 $\pm$ 0.22b	1.14 $\pm$ 0.34c
8	Linalol	6.91	1107	4.98 $\pm$ 0.61d	1.42 $\pm$ 0.58b	4.67 $\pm$ 1.50d	2.25 $\pm$ 0.28c	ND a
9	Camphor	7.84	1167	<b>26.26 <math>\pm</math> 1.45d</b>	<b>5.01 <math>\pm</math> 1.90a</b>	<b>25.15 <math>\pm</math> 5.11d</b>	<b>6.89 <math>\pm</math> 2.31b</b>	<b>12.81 <math>\pm</math> 1.90c</b>
10	Borneol	8.26	1191	<b>10.92 <math>\pm</math> 0.72b</b>	<b>6.26 <math>\pm</math> 1.58a</b>	<b>14.02 <math>\pm</math> 2.03d</b>	<b>10.84 <math>\pm</math> 0.51b</b>	<b>12.23 <math>\pm</math> 2.13c</b>
11	Terpinen-4-ol	8.34	1196	1.05 $\pm$ 0.08a	2.20 $\pm$ 0.79b	1.80 $\pm$ 0.39 b	2.37 $\pm$ 0.45b	0.94 $\pm$ 0.54a
12	$\alpha$ -terpineol	8.62	1211	0.34 $\pm$ 0.34ab	0.78 $\pm$ 0.48bc	1.13 $\pm$ 0.49c	1.10 $\pm$ 0.51c	ND a
13	Verbenone	8.94	1229	1.46 $\pm$ 0.39a	<b>8.12 <math>\pm</math> 1.98c</b>	4.58 $\pm$ 1.86b	<b>18.17 <math>\pm</math> 2.53d</b>	ND a
14	Bornylacetate	10.22	1295	<b>12.92 <math>\pm</math> 0.88a</b>	<b>40.43 <math>\pm</math> 1.23 e</b>	<b>17.09 <math>\pm</math> 2.30b</b>	<b>20.26 <math>\pm</math> 2.49c</b>	<b>31.16 <math>\pm</math> 3.39d</b>
15	Geranylacetone	13.66	1456	3.45 $\pm$ 0.51bc	3.05 $\pm$ 2.15abc	2.02 $\pm$ 1.18ab	1.82 $\pm$ 0.59a	4.44 $\pm$ 1.54c
<b>Total peak area</b>				<b>5.06 <math>\times 10^7 \pm 1.90 \times 10^7</math> ab</b>	<b>12.3 <math>\times 10^7 \pm 4.27 \times 10^7</math> c</b>	<b>11.6 <math>\times 10^7 \pm 2.67 \times 10^7</math> c</b>	<b>9.49 <math>\times 10^7 \pm 3.08 \times 10^7</math> bc</b>	<b>3.79 <math>\times 10^7 \pm 1.32 \times 10^7</math> a</b>

VOCs are listed according to their retention time (RT) in minutes. The retention index (RI) is relative to (C8-C20) *n*-alkanes on the TR-5MS column. 100% =  $\Sigma$  of the 15 volatiles identified. Mean values followed by different lowercase letters differ significantly (Tukey's test at  $\alpha = 0.05$ ). Compounds with a relative percentage higher than 5% are indicated in bold. ND, not detected.

respectively). The same observation was made for eucalyptol with a relative percentage below 10%, whereas borneol represented between 6% (Nonza) and 14% (Pigette) of the total VOCs emitted. Two other VOCs showed a higher variability among rosemary clones: camphor ranged from 5% (Nonza) to 25% (Pigette), whereas bornyl acetate varied from 13% (Esselte) to 40% (Nonza). It was also confirmed that verbenone is especially important in Sudbery Blue, at 18% (Table 1).

The PCA obtained using the VOCs of the five rosemary clones (Fig. 3) showed that clones formed distinct groups on the first plane of the PCA. Each clone was characterized by a specific profile, which allowed observation of the existence of marked chemical variability, thus allowing separation of the clone on the PCA. The first two dimensions (F1 and F2) of the PCA explained 61.5% of the total variability of the data (41.84% for the F1 axis and 19.74% for the F2 axis). The first axis, F1, allowed separation of clones richer in camphor (9), eucalyptol (4) and linalol (8), such as Esselte and Pigette, located on the left side of the PCA, from clones richer in  $\gamma$ -terpinene (5), bornyl acetate (14) and terpinolene (7), namely Nonza, Sudbery Blue and Voltz Splindler.

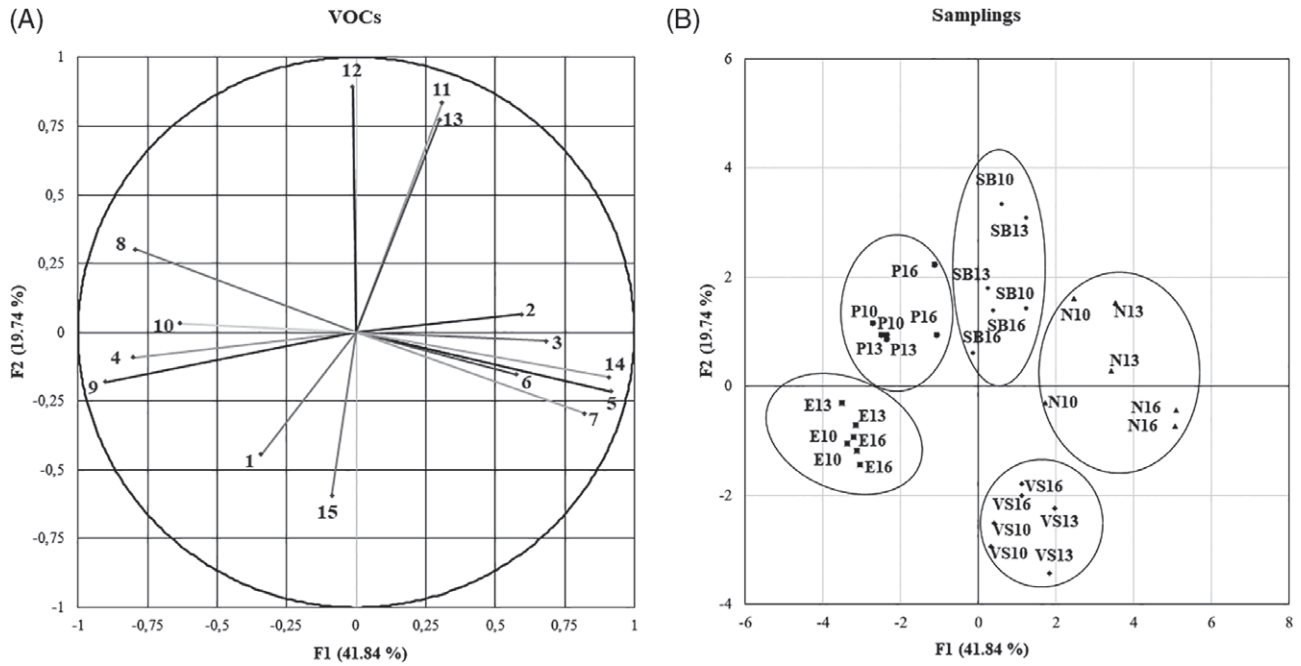
The second dimension of the PCA, F2, allowed distinction of the clones richer in  $\alpha$ -terpineol (12), terpinene-4-ol (11) and verbenone (13), namely Pigette, Sudbery Blue and Nonza, as represented in the positive region of F2, from those clones represented in the negative region of F2, characterized for being richer in geranyl acetone (15), namely Esselte and Voltz Splindler (Fig. 3).

#### Orientation of *Myzus persicae* under the influence of olfactory bouquets from rosemary clones

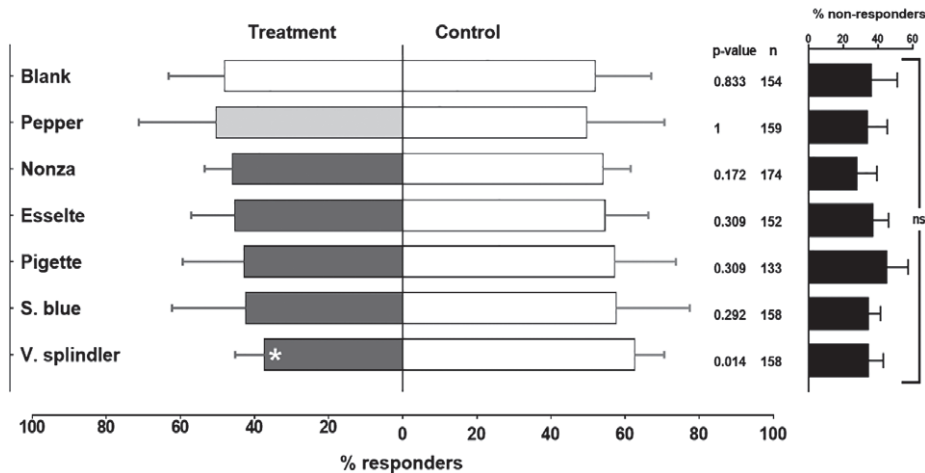
The percentage of aphids classified as nonresponders ranged from 27.5% to 44.5%. Nevertheless, these results were not statistically different among the different clones analyzed (Kruskall-Wallis:  $K = 8.43$ ;  $P = 0.208$ ). Figure 4 shows the distribution of aphid responders in the presence of a rosemary clone in one chamber of the olfactometer against control in the other chamber (empty chamber). The results of the blank versus blank (Wilcoxon:  $P = 0.83$ ) and the pepper versus blank (Wilcoxon:  $P = 1$ ) assays showed no significant differences in the choice of aphids between the two olfactometer chambers. In the tests carried out with rosemary clones, aphids tended to choose the blank compared with those with companion plants. However, only the Voltz Splindler clone reported a statistically significant effect on the orientation of aphids, at 37.34% ( $P = 0.014$ ). The remaining clones did not result in a significant change in the orientation of aphids: Nonza 45.98% (Wilcoxon:  $P = 0.17$ ); Esselte 45.39% (Wilcoxon:  $P = 0.30$ ); Sudbery Blue 42.41% (Wilcoxon:  $P = 0.29$ ); and Pigette 42.86% (Wilcoxon:  $P = 0.30$ ).

#### Effect of individual VOCs on the orientation of *M. persicae*

In the assays of individual VOCs, the percentage of nonresponders ranged from 24.2% to 46.7%. Although there was a significant difference in the number of nonresponders according to the VOC (Kruskall-Wallis:  $K = 41.19$ ;  $P = 0.0002$ ), none differed significantly from the negative and positive controls: blank and (*E*)- $\beta$ -farnesene, respectively (Fig. 5). Nevertheless,



**Figure 3** Principal component analysis obtained from the relative percentages of volatile organic compounds (VOCs) from rosemary clones: (A) correlations circle of the VOCs variables on the two axes F1 and F2; (B) projection of the 30 samplings of VOCs from the five rosemary clones during three periods (10.00 h, 13.00 h and 16.00 h) with two solid phase micro-extraction fibres on the two axes F1 and F2. The identity of the VOCs is shown in Table 1. E, Esselte; N, Nonza; P, Pigette; SB, Sudbery Blue; VS, Voltz Splinder.

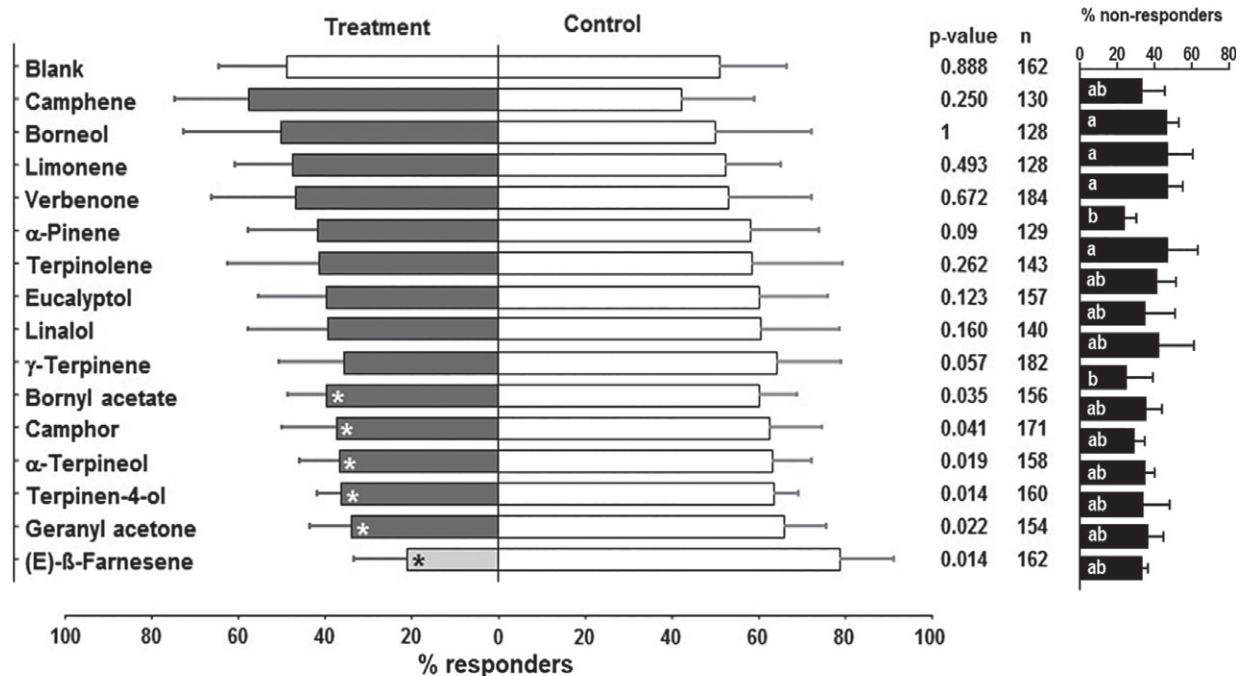


**Figure 4** *Myzus persicae* responder orientation in the presence of a rosemary clone in one chamber of the olfactometer (grey bars) and control in the other one (empty chamber; white bars). Light grey bars represent pepper. The asterisk (\*) indicates a significant difference (Wilcoxon test) with  $P < 0.05$ ;  $n$  represents the total number of responders and black bars in the histogram on the right represent the percentages of nonresponders for 240 aphids tested in each treatment; ns, no significant differences (Kruskal–Wallis test at  $\alpha = 0.05$ ); error bars indicate the SD.

the olfactory behaviour of *M. persicae* differed according to the VOC assayed. The positive control, (*E*)- $\beta$ -farnesene, clearly induced the orientation of aphids towards blank: (*E*)- $\beta$ -farnesene 20.99% ( $P = 0.014$ ). Only five VOCs elicited a significant repellent effect compared with blank: bornyl acetate 39.74% (Wilcoxon:  $P = 0.035$ ); camphor 37.43% (Wilcoxon:  $P = 0.041$ );  $\alpha$ -terpineol 36.71% (Wilcoxon:  $P = 0.019$ ); terpinene-4-ol 36.25% (Wilcoxon:  $P = 0.014$ ); and geranyl acetone 33.77% (Wilcoxon:  $P = 0.022$ ).

### Discussion

The volatile profile of our five rosemary clones has almost the same composition in terms of VOCs, although in different concentrations and proportions. All were previously identified in studies with whole plants (Zhang *et al.*, 2014a; Ben Issa *et al.*, 2016) or in rosemary essential oil (Hori & Komatsu, 1997; Hori, 1998; Isman *et al.*, 2008; Pausas *et al.*, 2015). These results confirm that genetic variability influences VOC production in a



**Figure 5** *Myzus persicae* responders' orientation in the presence of a volatile compound (10  $\mu$ L at 1%) in one chamber of the olfactometer (grey bars) and control in the other one (10  $\mu$ L of ethanol; white bars). Light grey bars represent positive control ((*E*)- $\beta$ -farnesene). The asterisk (\*) indicates a significant difference (Wilcoxon test) with  $P < 0.05$ ;  $n$  represents the total number of responders and black bars in the histogram on the right represent the percentages of nonresponders for 240 aphids tested for each treatment; values presented with the same lowercase letter are not significantly different (Tukey's test at  $\alpha = 0.05$ ); error bars indicate the SD.

quantitative and qualitative way (Tholl & Lee, 2011; Schröder *et al.*, 2015). Although VOC production varies throughout the day, overall, their proportion in the olfactory bouquet is relatively stable. This gives the olfactory bouquet, considered as a whole, a chemical signature that separates genotypes (see PCA in Fig. 3). Nevertheless, environmental factors, phenology and plant cultivation also influence the characteristics of the VOC production (Pichersky & Gershenzon, 2002).

One reason for interest in rosemary is its presumed potential repellency for pests (Koschier & Sedy, 2003; Miresmailli *et al.*, 2006; Zhang & Chen, 2015; Zhang *et al.*, 2014a), particularly towards the green aphid (Hori, 1998; Ben Issa *et al.*, 2016, 2017b). The arena olfactometer, similar to most of the olfactometers described previously (Hori, 1998; Wang *et al.*, 2015), allows for testing of choice or preference bioassays, which are used to estimate the repellent power. The fact that aphids were distributed homogeneously during the blank tests showed the absence of a preference for a chamber and thus of bias within the arena device. Similarly, bioassays carried out with pepper showed that this host plant does not emit an attractive olfactory volatile mixture for *M. persicae*. (*E*)- $\beta$ -farnesene (the aphid alarm pheromone) (Francis *et al.*, 2004), which was used as a positive control and is highly repulsive, confirmed the functionality of the olfactometer. Thus, when the aphids avoid the side with a volatile, it is repulsive. In this case, in the bioassays performed with five rosemary clones, all of the clones reported repulsive capacities towards *M. persicae*, although only the effect of Voltz Splindler was statistically significant ( $P < 0.05\%$ ). The same repellent effect has already been reported for the Pyramidal

clone (Ben Issa *et al.*, 2016). Therefore, the rosemary genetics may explain the observed differences in aphid behaviour. This is consistent with several studies reporting that the repulsion (or attraction) of the aphid differs according to the VOC profile produced by different cultivars (Storer & van Emden, 1995; Wang *et al.*, 2009; da Costa *et al.*, 2010; Schröder *et al.*, 2015). This evidence is also valid for other pests sensitive to the olfactory environment, such as thrips *Frankliniella occidentalis* (Pergande), (Thysanoptera: Thripidae) in rosebushes (Gaum *et al.*, 1994) and the whitefly *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) in tomatoes or eggplants (Darshane *et al.*, 2017). In this sense, genetic variability should be taken into consideration when choosing companion plants and can comprise a potential lever for improving companion plant effectiveness. It should be emphasized that the absence of a significant repellent effect does not prevent a clone from having an impact on the fecundity of the aphid negatively, as reported by Ben Issa *et al.* (2017b). The results of the present study support the ability of rosemary terpenes to affect the performance of *M. persicae* (Hori, 1998, 1999a; Ben Issa *et al.*, 2016, 2017b).

The olfactometer bioassays conducted with individual VOCs emitted from rosemary were performed to identify their potential effect with respect to the behavioural choice of the aphids, aiming to better understand the mode of action of the olfactory bouquet. The efficacy of the olfactory bouquet may result from the individual effect of certain VOCs on the behaviour of the aphid or from an effect resulting from the relative proportion in the blend of certain VOCs via an interactive and synergetic action (Zhang *et al.*, 2014a). Regarding the rosemary VOCs



tested at the same concentration, not all elicited a significant effect on the behavioural response and choice of aphids. From the VOCs emitted from rosemary, only five showed a significant repulsive effect on aphids, namely bornyl acetate, camphor,  $\alpha$ -terpineol, terpinene and geranyl acetone. These terpenic compounds were already reported to be involved in the ability of rosemary plants to reduce the performance of *M. persicae* (Hori, 1998, 1999a) and the behaviour of other insects. For example, camphor is a proven repellent not only for *M. persicae* (Hori, 1998), but also for the onion aphid *Neotoxoptera formosana* (Takahashi) (Homoptera: Aphididae) (Hori & Komatsu, 1997) and mosquitoes (Hwang *et al.*, 1985). Camphor also induces an anti-appetizing effect on *M. persicae* (González-Coloma *et al.*, 2011) and a toxic effect on the lesser grain borer *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae) (Rozman *et al.*, 2007) and also on the rice weevil *Sitophilus oryzae* (Linnaeus) (Coleoptera: Curculionidae) (Abdelgaleil *et al.*, 2009). Bornyl acetate has a repellent effect on mosquitoes and Coleoptera species (Hwang *et al.*, 1985; Park *et al.*, 2003; Rozman *et al.*, 2007) and an anti-appetising effect against the bird cherry-oat aphid *Rhopalosiphum padi* (Linnaeus) (Hemiptera: Aphididae) (González-Coloma *et al.*, 2011). Terpinene-4-ol is a toxic VOC and has insecticidal activity against several insects (Isman, 2004; Abbassy *et al.*, 2009). The  $\alpha$ -terpineol has a repellent activity towards *M. persicae* (Hori, 1998). Geranyl acetone demonstrated a highly repellent activity against the green pea aphid *Acyrtosiphon pisum* (Harris) (Hemiptera: Aphididae) (Zhang *et al.*, 2017) and *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Deletre *et al.*, 2015).

According to the results of the present study, the remaining VOCs emitted from rosemary ( $\alpha$ -pinene, camphene, limonene, eucalyptol,  $\gamma$ -terpinene, terpinolene, linalol, borneol and verbenone) do not cause a significant repellent effect on *M. persicae*. The lack of a repellent effect on *M. persicae* was also observed by Bruce *et al.* (2005) for linalol and by Hori (1998) for  $\alpha$ -pinene, camphene, eucalyptol and borneol. Nevertheless, several studies demonstrated the effect of these VOCs on the behaviour of aphids and other insects (Chapman *et al.*, 1981; Hori & Komatsu, 1997; Hori, 1998; Tomova *et al.*, 2005; Rozman *et al.*, 2007; Abdelgaleil *et al.*, 2009; Halbert *et al.*, 2009; Webster *et al.*, 2010; Zhang *et al.*, 2014b; Lamy *et al.*, 2016; Jiang *et al.*, 2016). Such contradictions are not necessarily surprising and testify the difficulty of making comparisons without considering the experimental conditions. The first cause of variability is undoubtedly the concentration of the VOCs tested (Foster *et al.*, 2007). It is also possible that these compounds are more repulsive in combination and in a synergetic action with other VOCs (Deletre *et al.*, 2016). Among the five repellent VOCs, bornyl acetate is the major component (35%) in the olfactory bouquet of the rosemary Voltz Splindler clone. Nevertheless, the same VOC was present at a relative proportion of 40% in the least repellent clone, Nonza. Therefore, this major VOC cannot alone explain the repellent action of Voltz Splindler on *M. persicae*. Consequently, we can hypothesize that the repellency level does not result from the accumulation of individual repulsive effects of the VOCs. For example, the Nonza clone accumulates 50% of repellent VOCs versus 48% in Voltz Splindler. These results suggest that the repulsion of a companion plant is more related to a blend of VOCs (with a significant synergetic effect), as noted by Bruce

and Pickett (2011), than the sole action of a specific repellent VOC. The results obtained in the PCA (Fig. 3) point toward the same direction, with it being difficult to establish a link between the repulsive efficacy of Voltz Splindler and the repellency of each VOC emitted. Repulsion may also be associated with the presence of minor components that are sometimes difficult to detect (McCormick *et al.*, 2014). Bruce *et al.* (2005) reported that the sesquiterpene (+)-bicyclogermacrene inhibited the effect of (*E*)- $\beta$ -farnesene on *M. persicae* and also that (–)-germacrene D resulted in the same effect in the pea aphid *A. pisum*. Dawson *et al.* (1984) showed that, depending on the concentration of VOCs in the olfactory bouquet,  $\beta$ -caryophyllene may inhibit the aphid response to the aphid alarm pheromone (*E*)- $\beta$ -farnesene. Also, the relative proportion of each VOC in the volatile profile perceived by the aphid plays an important role in its olfactory response. Some specific ratios may elicit a maximum repelling action (Zhang *et al.*, 2014a).

In this first comparative study, we noted the limitations of testing the VOCs individually at the same concentration (1%), which does not correspond to the relative proportion of each VOC in the olfactory bouquet of rosemary. A slight change in the concentration or absence of a designated VOC in the olfactory bouquet can disrupt the repellent action reported previously (Rajabaskar *et al.*, 2013; Zhang *et al.*, 2014a). Additional studies that test a range of concentrations and establish a dose–response curve for each VOC would be relevant. The relative proportion of VOCs perceived by the aphid also appears to affect its olfactory response (Zhang *et al.*, 2014a). A complementary approach might consist of determining whether VOC associations retrieve higher or lesser repellent properties, as reported by Webster *et al.* (2010). Finally, we could attempt to simulate the volatile bouquet emitted from rosemary, then create variations by removing one or more VOCs from the bouquet with the aim of isolating the most effective odour combination (Bruce & Pickett, 2011).

## Conclusions

In conclusion, the present study demonstrates the importance of the genetic variability of rosemary clones with respect to their level of repulsion for *M. persicae*. The five clones studied emit, with few exceptions, the same VOCs but in different concentrations and relative proportions. Most of the VOCs identified are terpenic compounds and, among them, five significantly repel aphids. The Voltz Splindler clone, which is the only to demonstrate real repellent properties, has four of the five VOCs that are themselves repellent in the individual assays. Nonza, the least repellent clone, emits these five VOCs in higher quantities and proportions compared with Voltz Splindler. Such results confirm that the effectiveness of an olfactory bouquet is related more to a blend of compounds and the synergetic actions between than to the sum of the effects of individual VOCs with repellent properties. Thus, the production of a specific repulsive VOC cannot be used as a reliable marker of the effectiveness of a companion plant and therefore promoting its emission by genetic selection or culture is not sufficient. On the basis of the results obtained in the present study, we must therefore assess and integrate the beneficial or harmful interactions within the olfactory bouquet emitted from the rosemary clones. Such an aspect should guide our approach with respect to optimizing the repulsive efficiency

of companion plants of interest, namely Voltz Splindler, by testing, for example, artificial combinations of VOCs.

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