



HAL
open science

Chemical Composition and Behavioral Effects of Five Plant Essential Oils on the Green Pea Aphid *Acyrtosiphon pisum* (Harris) (Homoptera: Aphididae)

Abir Kasmi, Majdi Hammami, Emmanuel G Raelison, Manef Abderrabba, Jalloul Bouajila, Christine Ducamp

► To cite this version:

Abir Kasmi, Majdi Hammami, Emmanuel G Raelison, Manef Abderrabba, Jalloul Bouajila, et al.. Chemical Composition and Behavioral Effects of Five Plant Essential Oils on the Green Pea Aphid *Acyrtosiphon pisum* (Harris) (Homoptera: Aphididae). *Chemistry and Biodiversity*, 2017, 14 (5), 10.1002/cbdv.201600464 . hal-01792236

HAL Id: hal-01792236

<https://hal.science/hal-01792236>

Submitted on 15 May 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Chemical Composition and Behavioral Effects of Five Plant Essential Oils on the Green Pea Aphid *Acyrtosiphon pisum* (Harris) (Homoptera: Aphididae)

Abir Kasmi^{1,2,3*}, Majdi Hammami⁴, Emmanuel G. Raelison⁵, Manef Abderrabba³, Jalloul Bouajila⁶, Christine Ducamp¹

¹Laboratory of Evolution and Biologic Diversity UMR 5174 CNRS / UPS / ENFA, Narbonne road PB 22687, 31326 Castanet Tolosan Cedex-France

²Department of Biology, Faculty of Sciences of Bizerte, Jarzouna Bizerte 7021, University of Carthage, Tunisia

³Laboratory of Materials, Molecules and Applications (LMMA), IPEST, PB 51, La Marsa 2070, Tunisia, University of Carthage

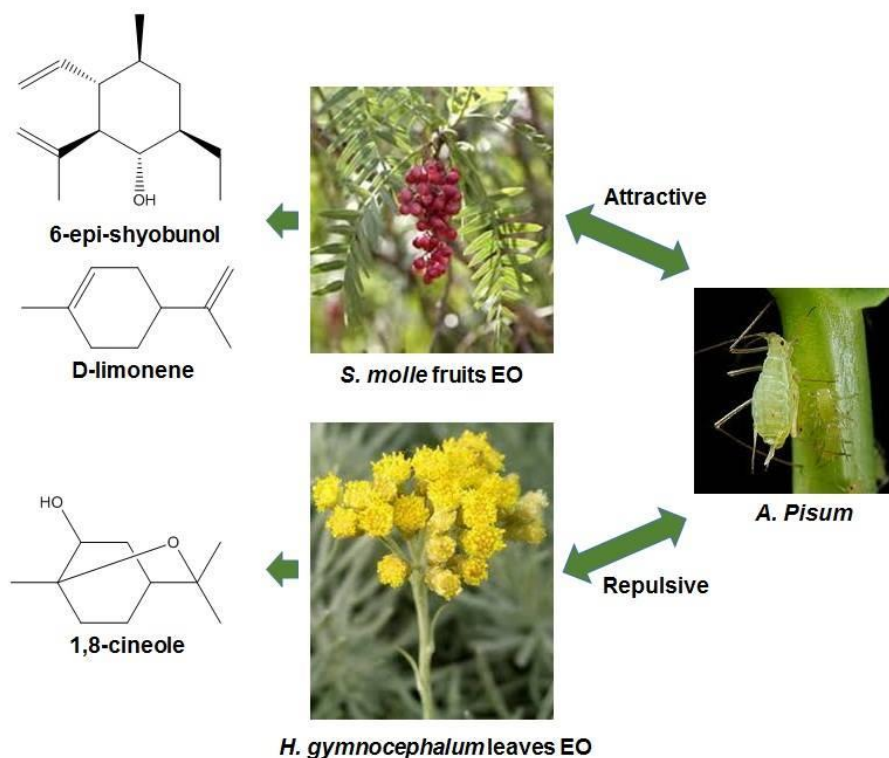
⁴Laboratory of Bioactive Substances, Biotechnology Center in Borj-Cedria Technopol, PB 901, 2050 Hammam-Lif, Tunisia

⁵Pharmacy Department, Faculty of Medicine, University of Antananarivo, PB 375, Antananarivo 101, Madagascar

⁶Laboratory of Molecular Interactions and Chemical and Photochemical Reactivity UMR CNRS 5623, Paul-Sabatier University, 118 Narbonne road, F-31062 Toulouse, France

*Corresponding author: Abir KASMI ; Regular address: Laboratory of Materials, Molecules and Applications (LMMA), IPEST, PB 51, La Marsa 2070, Tunisia, University of Carthage; E-mail address: kasmi_abir@yahoo.fr; Tel. : +216 53 683 841

Graphical abstract



Abstract

Essential oils (EOs) from *Schinus molle*, *Helichrysum gymnocephalum*, *Cedrelopsis grevei* and *Melaleuca viridiflora*, four aromatic and medicinal plants, are commonly used in folk medicine. EOs were characterized by Gas Chromatography-Mass Spectrometry (GC-MS) and quantified by Gas Chromatography-Flame Ionization Detection (GC-FID); then evaluated for their behavioral effects on adults of the green pea aphid *Acyrtosiphon pisum* (Harris) using a Perspex four-armed olfactometer in order to test the compatibility of their use as phytoinsecticides to control this insect pest. Our results showed that the EOs from leaves of *S. molle*, *M. viridiflora* and *C. grevei* did not change aphids' behavior. However, *S. molle* fruits EO seemed to be attractive while *H. gymnocephalum* leaves EO exhibited repellency towards

aphids at a dose of 10 μ l. The major compounds in *S. molle* fruits *EO* were 6-epi-shyobunol (16.22%) and d-limonene (15.35%). While, in *H. gymnocephalum* leaves *EO*, 1.8 cineole was the main compound (47.4%). The difference in aphids' responses to these two *EOs* could be attributed to the differences in their compositions. Our findings suggest that these two *EOs* have potential applications for the integrated pest management (*IPM*) of *A. pisum* (Harris).

Keywords: Natural products; Biological activity; *A. pisum* (Aphididae); *IPM*

1. Introduction

Aphids (Homoptera: Aphididae) are the most important agricultural pests of temperate regions [1] [2]. *Acyrtosiphon pisum* (Harris) is one of the key pests in pea crops. It is a phloem sap feeding insect with a very short life span of the order of 20 to 30 days average [3] [4]. Its success as plant pest is based on its high reproductive potential due to parthenogenesis during the spring and summer, transmitting viruses to plants and its polyphenism. This way of asexual multiplication explains its important demographic dynamics and damage to crops.

Several control methods have been considered against aphids, among other regulation "Top-down"; which is the approach by natural enemies such as ladybirds and various parasitoids; and chemical control by the use of pesticides. The massive use of pesticides increases production costs, is toxic to mammals and beneficial insects, can reduce populations of natural enemies, leads to the development of pest resistance by selective pressure [5] and causes harmful effects on environment and human health [6]. In order to avoid these problems, alternative environmentally friendly and ecologically safe compounds are proposed, as the phytochemical insecticides based on *EOs* (phytoinsecticides) and the biological control.

Essential oils have been suggested as an attractive alternative for the control of insect pests because some are selective, biodegrade, provide efficient and safe repellents for humans and

the environment compared to synthetic insecticides [7] [8], have not or little impact on natural enemies and beneficial insects [9] [10] and can act on multiple sites of action thus the probability of developing a resistant population is very low [7] [8] [11]. Numerous *EOs* and their compounds have shown to have good repellent and insecticidal activity on insect pests of agricultural importance. Four biorational insecticides including Bugoil, a mixture of canola oil, thyme oil, tagetes oil and wintergreen oil were tested for their repellency. Bugoil provided a substantial repellency to *B. cockerelli* adults and deterred oviposition [12]. A significant repellent effect of the *EO* of rosemary (*Rosmarinus officinalis* L.) to onion thrips, *Thrips tabaci* Lindeman, adult females was observed in a glass Y-tube olfactometer [13]. *Mentha pulegium* *EO* showed no repellency to *Myzus persicae* (Sulzer), the green peach aphid, in olfactometer bioassays, but it inhibited feeding and settling [14]. The effect of limonene in carrot (*Daucus carota* L.) *EO* on carrot psyllids, *Trioza apicalis* Förster, was studied by [15]. It significantly increased the number of eggs laid by psyllids. However, in other studies, limonene was shown to have a repellent effect on carrot psyllids [16] [17] [18]. To our knowledge, no effects of *EOs* of *S. molle* (Anacardiaceae), *H. gymnocephalum* (Asteraceae), *C. grevei* (Rutaceae) and *M. viridiflora* (Myrtaceae) on *A. pisum* (Harris) were previously reported. Thus, this study aimed to characterize the composition of these *EOs* by *GC-MS* and *GC-FID* analysis, then to assess their behavioral effects on adults of the green pea aphid *A. pisum* using a four-armed olfactometer in order to test the compatibility of their use to control this insect pest.

2. Results

2.1. Chemical composition of EOs

The chemical compositions of the studied *EOs* were determined by *GC-FID* and *GC-MS* analysis and are listed in Table 1. A total of 36, 41, 23, 64, 37 compounds corresponding to 100%, 100%, 99.3%, 100% and 100% of the total *EO* content were respectively identified in

EOs from leaves and fruits of *S. molle* and leaves of *H. gymnocephalum*, *C. grevei* and *M. viridiflora*. The main compounds in the *EO* from leaves of *S. molle* were β -eudesmol (14.82%), elemol (13.71%) and α -eudesmol (12.76%); considerable amounts of d-limonene (9.25%), spathulenol (7.21%), γ -eudesmol (5.75%) and β -cadinene (5.03%) were also present in this oil. The *EO* from fruits of *S. molle* was characterized by 6-epi-shyobunol (16.22%) and d-limonene cubebol (15.35%). Important quantities of spathulenol, 4-epi-cubebol and l-phellandrene were also present in this oil (8.16%, 7.84% and 5.45% respectively). The major compounds in *C. grevei EO* were (*E*)- β -farnesene (27.67%) and δ -cadinene (14.52%). α -copaene and β -elemene were also present in considerable amounts in this oil (7.67% and 6.98% respectively). Finally, *M. viridiflora* and *H. gymnocephalum EOs* were characterized by the chemotype 1.8-cineole (47.4% and 55.54% respectively).

Table 1. Chemical compositions of *EOs* of *Schinus molle*, *Helichrysum gymnocephalum*, *Cedrelopsis grevei* and *Melaleuca viridiflora*.

N°	RI	Compound	<i>S. molle</i> Leaves	<i>S. molle</i> Fruits	<i>H. gymnocephalum</i> Leaves	<i>C. grevei</i> Leaves	<i>M. viridiflora</i> Leaves
			(%)	(%)	(%)	(%)	(%)
1	928	α -thujene	-	-	1	-	-
2	931	α -pinene	0.68	1.6	-	0.14	12.96
3	948	α -fenchene	-	-	-	-	0.12
4	967	sabinene	-	-	0.3	-	3.02
5	971	β -pinene	-	-	1.1	0.2	-
6	984	2,3-dihydro-1,8-cineole	-	-	2.1	-	-
7	986	(<i>Z</i>)-mentha-4,8-diene	-	-	-	-	0.23
8	988	β -myrcene	0.58	1.53	-	-	-
9	1003	pseudolimonene	-	-	-	-	0.05
10	1010	α -terpinene	-	-	1.3	-	-
11	1014	3-carene	-	-	-	-	0.06
12	1015	<i>o</i> -cymene	-	-	-	0.13	-
13	1020	sylvestrene	-	-	-	0.06	-
14	1022	limonene	-	-	0.5	-	-
15	1024	1,8-cineole	-	-	47.4	-	55.54
16	1026	1-phellandrene	2.32	5.45	-	-	-

17	1033	p-cymene	1.94	2.54	4.3	0.18	-
18	1036	d-limonene	9.25	15.35	-	-	-
19	1051	(<i>E</i>)- β -ocimene	-	-	-	-	0.24
20	1052	β -ocimene	-	-	2.4	-	-
21	1064	artemisia ketone	-	-	-	-	0.01
22	1079	kewda ether	-	-	-	-	0.11
23	1084	α -terpinolene	-	-	1.3	-	-
24	1088	artemisia alcohol	-	-	-	-	0.22
25	1105	isodihydrolavandulyl aldehyde	-	-	-	-	0.07
26	1133	3-terpinenol	-	-	-	-	0.17
27	1137	(<i>E</i>)-pinocarveol	-	-	-	0.11	-
28	1143	1-menthen-8-ol	-	-	-	-	0.03
29	1152	menthone	-	-	-	-	0.19
30	1162	(<i>Z</i>)-chrysanthemol	-	-	-	-	0.71
31	1164	α -phellandrene	-	-	0.2	-	-
32	1175	terpinen-4-ol	-	-	2.7	-	8.43
33	1177	isopinocampheol	-	-	-	0.03	-
34	1178	p-cymen-8-ol	-	-	-	0.03	-
35	1187	α -terpineol	-	-	1.8	0.08	-

36	1196	myrtenal	-	-	-	0.16	-
37	1216	(<i>E</i>)-(+)-carveol	0.28	-	-	-	-
38	1122	(<i>E</i>)-para-2,8-menthadien-1-ol	-	0.91	-	-	-
39	1128	(<i>Z</i>)-p-mentha-2,8-dien-1-ol	-	0.63	-	-	-
40	1182	1,8-menthadien-4-ol	-	0.36	-	-	-
41	1189	cryptone	-	0.64	-	-	-
42	1196	isopiperitenol	-	3.73	-	-	-
43	1216	(<i>E</i>)-(+)-carveol	-	0.64	-	-	-
44	1227	<i>cis</i> -p-mentha-1(7),8-dien-2-ol	-	0.48	-	-	-
45	1289	sabinyl acetate	-	0.36	-	-	-
46	1312	(<i>E,E</i>)-2,4-decadienal	-	-	-	-	0.29
47	1334	δ -elemene	-	-	-	0.96	0.29
48	1346	α -cubebene	-	-	-	0.72	-
49	1351	α -longipinene	-	-	-	0.09	-
50	1368	cyclosativene	-	-	-	-	0.13
51	1376	α -copaene	0.68	0.73	0.4	7.67	-
52	1378	β -patchoulene	-	-	-	-	1.65
53	1382	β -bourbonene	-	-	-	0.17	-
54	1391	β -elemene	0.19	0.66	-	6.98	-

55	1396	isoitalicene	-	-	-	0.15	-
56	1400	cyperene	-	-	-	0.27	0.24
57	1404	9,10-dehydro-isolongifolene*	-	-	-	0.21	-
58	1405	Υ -caryophyllene	-	-	-	-	0.32
59	1410	α -gurjunene	0.5	0.74	-	0.36	-
60	1413	α -cedrene	-	-	-	0.19	-
61	1415	isocaryophyllene	-	-	-	-	0.77
62	1419	α -caryophyllene	1.22	4.3	-	0.59	-
63	1428	β -gurjunene	-	-	-	0.34	-
64	1429	(<i>Z</i>)-thujopsene	-	-	-	-	0.11
65	1432	α -bergamotene	-	-	-	0.15	-
66	1438	aromadendrene	-	-	2	-	-
67	1443	(<i>Z</i>)- β -farnesene	-	-	-	2.31	-
68	1449	<i>epi</i> - β -santalene	-	-	-	-	2
69	1453	neoclovene	-	-	-	3.83	-
70	1454	α -humulene	0.46	0.9	-	-	-
71	1465	α -acoradiene	-	-	-	-	0.42
72	1469	(<i>E</i>)- β -farnesene	-	-	-	27.67	-
73	1470	bicyclosesquiphellandrene	-	-	5.6	-	-

74	1473	γ -curcumene	-	-	5.6	-	-
75	1475	β -chamigrene	-	-	-	-	0.21
77	1476	α -curcumene	-	-	-	1.69	-
78	1477	valencene	0.17	0.3	-	-	-
79	1479	α -amorphene	0.23	0.38	5.1	0.56	-
80	1481	germacrene D	0.24	2.02	-	-	-
81	1482	ar-curcumene	-	-	-	0.1	-
82	1485	β -selinene	0.36	0.23	3.3	0.77	-
83	1487	β -guaiene	-	-	-	0.17	-
84	1488	(Z)-6,11-eudesmadiene	-	-	-	-	0.03
85	1493	α -selinene	0.68	1.13	-	-	-
86	1494	bicyclogermacrene	-	-	5	-	-
87	1495	γ -muurolene	-	-	-	1.61	-
88	1496	viridiflorene	-	-	-	2.8	-
89	1498	α -muurolene	1.36	0.75	-	2.58	-
90	1502	germacrene A	-	0.27	-	-	-
91	1503	2,3-di-tert-butylphenol	-	-	0.5	-	-
92	1504	α -bulnesene	-	-	-	0.62	-
93	1505	2,6-di-t-butyl-4-methylphenol	-	-	-	-	0.07

94	1510	β -dihydroagarofuran*	-	-	-	0.27	-
95	1512	4-epi-cubebol	1.42	7.84	-	-	-
96	1513	γ -cadinene	-	-	-	0.87	-
97	1527	hedycaryol	-	-	-	-	0.15
98	1531	calamenene	-	-	1.8	-	-
99	-	ledol isomer	-	0.27	-	-	-
100	1519	β -cadinene	5.03	4.27	-	-	-
101	1521	δ -cadinene	-	-	3.6	14.52	-
102	1532	1,4-cadinadiene	-	-	-	0.31	-
103	1538	8,14-cedranoxide	-	-	-	-	0.4
104	1539	4,5,9,10-dihydro-isolongifolene	-	-	-	0.53	-
105	1540	elemol	13.71	1.24	-	0.79	9.89
106	1545	α -calacorene	0.19	-	-	0.99	-
107	1561	palustrol	1,13	0.44	-	-	-
108	1565	lauric acid	-	-	-	0.18	-
109	1568	caryophyllene alcohol	-	-	-	-	0.51
110	1571	germacrene-D-4-ol	-	-	-	0.57	0.23
111	1578	spathulenol	7.21	8.16	-	0,25	-
112	1580	caryophyllene oxide	2.85	1.08	-	-	-

113	1590	viridiflorol	2.46	1.34	-	1.23	-
114	1596	ledol	1.29	0.73	-	-	-
115	1598	humulene oxyde	0.73	-	-	-	-
116	1599	cedrol	-	-	-	-	0.11
117	1600	α -ylangene	-	0.41	-	-	-
118	1605	oplopenone	0.42	-	-	-	-
119	1610	epicedrol	-	-	-	2.5	-
120	1616	(<i>E</i>)-isolongifolanone	-	-	-	0.44	-
121	1622	dillapiol	-	-	-	-	0.19
122	1627	2-p-tolyl-6-methyl-5-hepten-2-ol*	-	-	-	0.25	-
123	1631	γ -eudesmol	5.75	0.38	-	-	-
124	-	7- α -H-eudesma-3,5-diene	3.97	4.25	-	-	-
125	1636	hinesol	-	-	-	1.26	-
126	1639	daucol	-	-	-	0,33	-
127	1651	β -eudesmol	14.82	0.99	-	0.27	-
128	1652	α -eudesmol	12.76	-	-	-	-
129	1654	α -cadinol	-	-	-	1.62	-
130	1664	bulnesol	-	-	-	0.14	-
131	1668	valeranone	-	-	-	0.18	-

132	1677	5-allyl-4,7-dimethoxy-1,3-benzodioxole*	-	-	-	0.15	-
133	1680	6-epi-shyobunol	3.03	16.22	-	-	-
134	1683	α -bisabolol	-	-	-	0.05	-
135	1688	γ -dodecalactone	-	-	-	2.05	-
136	1693	junper camphor	-	-	-	0.09	-
137	1709	(<i>Z</i>)- β -santalol	-	-	-	2.14	-
138	1723	isolekene	0.66	3.23	-	1.01	-
139	1734	oplopanone	0.18	-	-	-	-
140	1740	<i>cis-Z</i> - α -bisabolene epoxide	1.26	1.31	-	-	-
141	1874	alloeodionol*	-	-	-	1.32	-
142	1886	verticol*	-	-	-	0.03	-
Total identified compounds			100	100	99.3	100	100

RI: Retention Index relative to (C₆-C₂₂) n-alkanes on the *HP5-MS* column; - : not detected; * = Tentative identification supported by good match of MS spectra

2.2. Olfactory bioassays

Independently of the different treatments, 140 aphids were used. Among these, 20 were subjected to the odors of the whole bean plant (Reference manipulation) and 120 to odors emanating from the various *EOs* tested. Our results showed that the majority of aphids introduced in the olfactometer made a choice (Table 2). In fact, 86% of the total number of aphids headed one of the four arms of the olfactometer (Choice column) while a small minority of aphids (14% of the total number) remained on the center of the olfactometer without engaging in one of the four branches (No choice column).

Table 2. The number of aphids, depending on the source of odor, who chose or not to leave the central zone and to head one of the four arms of the olfactometer.

	A	B	C	D	Choice	No choice	χ^2 test	<i>p</i>
Reference manipulation with the whole bean plant	6	5	6	3	20	0	0.1254 NS	0.7233
<i>EO</i> from <i>Schinus molle</i> fruits	11	6	2	1	20	8	3.75 NS ¹	0.0528
<i>EO</i> from <i>Schinus molle</i> leaves	6	5	4	5	20	3	0.1254 NS	0.7233
<i>EO</i> from <i>Melaleuca viridiflora</i> leaves	4	6	4	6	20	2	0.1434 NS	0.705
<i>EO</i> from <i>Helychrysum gymnocephalum</i> leaves	3	7	5	5	20	6	0.625 NS	0.4292
<i>EO</i> from <i>Cedrelopsis grevei</i> leaves	8	4	4	4	20	1	1.0256 NS	0.3112

A: treated area; B, C, D: control areas

NS : accepted null hypothesis ;¹ This value is significant at the probability level of 0.10.

Aphids had to make a choice between the three sources of pure air (B, C, D) and the source of air contaminated with the odor of the whole bean plant or the tested *EO* (A). The right section of the Table 2 showed that the proportion of aphids heading the source of air contaminated by the odor of the *EOs* are never significantly different of the expected proportions. These have been calculated taking into account the fact that aphids will choose the odor source with a probability of 0.25 and the three sources of pure air with a probability of 0.75. The results achieved by offering different odors to aphids showed in each case that the X^2 test is not significant which does not reject the null hypothesis (1 degree of freedom d.f.). The behavior of aphids depends on the tested *EO*. In fact, when we tested the *EO* from *S. molle* fruits, we noticed that aphids tended to prefer the air contaminated by the odor of this *EO* (X^2 : 3.75; 1 d.f.; p: 0.0528). The *EO* from *S. molle* fruits caused aphids to respond positively, suggesting it was attractive. When we presented to aphids the *EOs* from leaves of *S. molle*, *M. viridiflora*, *C. grevei* and especially the *EO* from leaves of *H. gymnocephalum* we noticed that aphids tended to prefer the sources of pure air and they turned away from air laden with the odors of these essential oils: *EO* from leaves of *S. molle* (X^2 : 0.1254; 1 d.f.; p: 0.7233) ; *EO* from leaves of *M. viridiflora* (X^2 : 0.1434; 1 d.f.; p: 0.705) ; *EO* from leaves of *C. grevei* (X^2 : 1.0256; 1 d.f.; p: 0.3112). It seems like the *EO* of *H. gymnocephalum* elicited a negative behavioral response, suggesting it was repellent towards aphids (X^2 : 0.625; 1 d.f.; p: 0.4292). Although the statistical test does not put in evidence a significant difference, it seems like the aphids tended to head the air contaminated by the odor of *EOs* (32 observations, expected number: 25).

3. Discussion

Insects, like other animals, respond to various signals in search of shelter, food, nestling site or mates. The signals emitted by plants, prey or congeners are detected by vision, olfaction or other sensory systems. Studies conducted on the mechanisms of olfaction have been driven by

growing interest into how behaviorally active volatiles (semiochemicals) might be incorporated into *IPM* strategies for the control of insect pests [19] [20]. Studies focused mainly on assessing the bioactivity of plant derivatives for pest control. However, few researches explored the lethal and sublethal effects on the beneficial fauna such as the natural enemies. Essential oils are generally known to have no or little impact on natural enemies and beneficial insects [9] [10]. In this context, ‘Chiasson *et al.* [21]’ reported that *EOs* were effective in controlling aphids, thrips, and whiteflies, but showed no effect on natural enemies of whitefly. However, ‘Bostanian *et al.* [22]’ showed that 4 days after topical spray, a *Chenopodium*-based insecticide can affect natural enemies including minute pirate bugs and a parasitoid wasp (*Aphidius colemani*) that are popularly used to control thrips and aphids.

The best we know, no effects of the *EOs* of *S. molle*, *H. gymnocephalum*, *C. grevei* and *M. viridiflora* on *A. pisum* (Harris) were previously reported. However, ‘Gonzalez *et al.* [23]’ analysed the effect of *EOs* from leaves of *S. molle* var. *areira* on the parasitoides *Trissolcus* ; which are biologic control agents against the green bug *Nezara viridula* (pest) ; using an X-shaped olfactometer. In the same context, ‘Leyva *et al.* [24]’ indicated also that the *EO* from *Melaleuca quinquenervia* is a promising natural alternative of mosquito control.

Several *EOs* have been studied to assess their repellent and insecticidal effects on insect pests of agricultural importance. The repellent effects of *EOs* from thyme, *Thymus vulgaris*, patchouli, *Pogostemon cablin*, and lemon-scent gum, *Corymbia citriodora*, on *Bemisia tabaci* biotype B were assessed in choice test experiments and the three *EOs* had a significant effect on *B. tabaci* [25]. ‘Koschier and Sedy [13]’ reported that rosemary oil (*Rosmarinus officinalis* L.) exhibited a significant repellent effect on onion thrips, *Thrips tabaci* Lindeman, adult females in a glass Y-tube olfactometer. The effect of garlic chive *EO* (*Allium tuberosum* Rottler); compared with pure air and citrus leaves; was examined in a T-olfactometer and it exhibited repellency on Asian citrus psyllid, *Diaphorina citri* Kuwayama, adults [26].

Furthermore, several studies reported the repellent effects of *EOs* on aphids. In this context, wormwood oils are very effective against some aphids: the corn leaf (*Rhopalosiphum maidis*) [27], the bird cherry oat (*Rhopalosiphum padi*) and the peach potato (*Myzus persicae*) [28]. It was reported by ‘Hori and Komatsu^[29]’ that rosemary oil interrupt the host selection behavior of aphids such as the green peach aphid *Myzus persicae* (Sulzer) and the onion aphid *Neotoxoptera formosana* (Takahashi). Two years later, ‘Hori^[14]’ reported also that *Mentha pulegium EO* showed no repellency on *M. persicae* in olfactometer bioassays, yet it inhibited feeding and settling. Then he found that rosemary oil equally affects *M. persicae* alighting and settling behavior [30].

S. molle is characterized by the pungent odor of its *EO* which is mainly concentrated in fruits and leaves ; while the *EOs* of *C. grevei*, *H. gymnocephalum* and *M. viridiflora* are mainly concentrated in leaves ; which explains why we choosed to study both leaves and fruits of *S. molle* and only leaves of the other three plants. The major compounds of our *EOs* (Table 1) were 6-epi-shyobunol, d-limonene in *S. molle* fruits *EO*; β -eudesmol, elemol and α -eudesmol in *S. molle* leaves *EO*; (*E*)- β -farnesene and δ -cadinene in *C. grevei* leaves *EO*; while *M. viridiflora* and *H. gymnocephalum* leaves *EOs* were characterized by the chemotype 1,8-cineole. Our aphids were attracted only by the *EO* from *S. molle* fruits. For the remaining *EOs*, it could be that blends which characterize them do not contain the preferred volatiles by aphids. Thus, aphids did not respond to these *EOs* and preferred to head the pure air sources.

It’s well known that aphids could detect odors emitted by plants through their antennas [31]. Furthermore, volatiles substances emitted by plants could influence the acceptance process of host plants by aphids [32]. Studies focused on the search behavior of host plants deployed by the bean black aphid, *Aphis fabae* Scopoli, showed that winged forms of the aphid respond positively to a mixture of 15 molecules [33] [34]. These molecules were (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 1-hexanol, benzaldehyde, 6-methyl-5-hepten-2-one, octanal, (*Z*)-3-hexen-1-yl

acetate, (*R*)-linalool, methyl salicylate, decanal, undecanal, (*E*)-caryophyllene, (*E*)- β -farnesene, (*S*)-germacrene D and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). (*E*)- β -farnesene is the aphid alarm pheromone and caused them to disperse [32] [35]. Our study supports this as (*E*)- β -farnesene is a major compound (27.67%) in *C. gevei* leaves *EO*. The odor of this oil elicited a negative behavioral response from aphids suggesting it was repellent. Another research on the aphid *Phopalosiphon padi* Linnaeus, exhibited that the aphid headed oat or wheat plants in response to a mixture of volatile compounds. In fact, the wingless aphids responded to 11 molecules while winged individuals responded only to 4 molecules. This difference have been explained by the specific lifestyle of these two morphs of aphids.

On the other hand, several pentatomids influence on the mixture of volatiles emitted by their host plants. For instance, the main volatiles released by maize plants injured by *N. viridula* are linalool, β -caryophyllene, (*E*)- α -bergamotene, and β -farnesene [36]. Volatile release in bean plants (*Phaseolus vulgaris* L. and *Vicia faba* L.) is also affected by *N. viridula*. In fact, feeding and oviposition raise 4,8,12-trimethyl-1,3,7,11-tridecatetraene linalool and β -caryophyllene [37]. These authors also reported that mixtures with large quantities of β -caryophyllene attract *T. basalis* females. Our study support this since we found that α -caryophyllene was abundant in our *S. molle* fruits *EO*. This oil elicited a positive behavioral response from aphids suggesting it was attractive.

Generally, *EOs* tend to be more effective on soft-bodied insects such as aphids, thrips and whiteflies, than hard-bodied insects [10]. Moreover, *EOs* could be mixed with conventional pesticides as synergists to achieve higher activity [38]. 'Lichtenstein et al. [39]' reported that carvone greatly increases the insecticidal activity of carbofuran, carbaryl, parathion, and dichlorodiphenyltrichloroethane.

Two strategies, namely natural plant products and the biologic control constitute the essential components of *IPM*. In biological agriculture, the biological control is the preferential option

to regulate the abundance of herbivores. The natural enemies such as ladybirds and different parasitoids are perceived as able to regulate the abundance of aphids. But the analysis of life strategies of these organisms indicated that they are rarely able.

4. Conclusions

According to our laboratory results, the *EO* of *S. molle* fruits caused aphids to respond positively suggesting it was attractive so it could be used as a trap for aphids. While the *EO* of *H. gymnocephalum* exhibited repellency towards aphids. These oils are potential candidates for the control of the green pea aphid *A. pisum* (Harris). They should be used in combination of the natural enemies of aphids such as ladybirds and various parasitoids as biological control agents. Furthermore, Additional studies should be conducted in the field to assess the efficacy of these *EOs* and their compatibility with natural enemies. Finally, to confirm our observations, it would be interesting to characterize the key compounds of *EOs* that deliver useful information to aphids. The only method that allows us to identify with certitude the molecules perceived by the nervous system of aphids would be antenno-electrography coupled to *GC-MS*.

5. Experimental Section

5.1. Insects

This manuscript is containing animal particularly insect experiments. No permission is in place in France where the experiments were performed.

We used adults of the green pea aphid *A. pisum* (Harris) previously reared on bean plants *Vicia faba* L. (more resistant than green pea plants) for many generations to provide for breeding populations of ladybirds. For the purposes of this experiment, adult individuals aged from 2 to 3 weeks were randomly selected and isolated in a Petri dish; where we put some leaves of bean plants to keep them fed; for 24 h. Aphids were then left in the bioassay room for at least 2 h to acclimatize prior to experiments.

5.2. Essential oils

Leaves and fruits of *Schinus molle* were collected in Lac II Region, Tunisia (March 2012). Samples were identified by Mr. Ridha El Mokni and voucher specimens were deposited in the herbarium of the Department of Biology of the Faculty of Sciences of Bizerte -Tunisia. Leaves of *Melaleuca viridiflora*, *Cedrelopsis grevei* and *Helichrysum gymnocephalum* were collected in Antananarivo, Madagascar (June-July 2008). After collection, plant material were dried at room temperature and subjected to hydrodistillation in a Clevenger apparatus for 2h. The *EOs* obtained were dried over anhydrous sodium sulfate and stored in airtight containers, wrapped in aluminum foil and stored in a refrigerator at 4°C until use.

5.3. Gas Chromatography and Gas Chromatography-Mass Spectrometry

Quantitative and qualitative analysis of the *EOs* were carried out by *GC-FID* and *GC-MS*. Gas Chromatography analyses were performed on an Agilent 7890A *GC* system, coupled to an Agilent 5975C mass spectroscopy detector with electron impact ionization (70 eV). An apolar *HP5-MS* capillary column (5% phenyl methyl silicone, 95% dimethylpolysiloxane 30 m × 0.25 mm, film thickness 0.25 μm) was used. Injector and detector temperatures were set, respectively, at 250 and 300°C, respectively. Temperature was programmed to rise from 60 to 260 °C with a 5° C/min rate. A second gradient was applied up to 340 °C at a gradient of 40 °C/min. For analysis purposes, the *EOs* were dissolved in petroleum ether. 1μl of sample was injected in the split mode ratio of 60:1. The carrier gas was helium with a flow rate of 1.2 mL/min. Scan time and mass range were 1s and 40–300 m/z, respectively. Volatile components were identified by comparison of their Kovats index (KI) obtained on a non-polar *HP5-MS* column relative to (C₆- C₂₂) *n*-alkanes, with those provided in the literature or with those of authentic compounds available in literature and the authors' laboratory. Further identification was made by comparison of their mass spectra with those recorded in the Wiley 09 NIST 2011

(National Institute of Standards and Technology) and other published mass spectra [40] and by-coinjection of available reference compounds (>99% purity) provided from commercial suppliers (Sigma–Aldrich, Darmstadt, Germany) and dissolved in dimethyl sulfoxide (DMSO) Sigma–Aldrich (USA). The samples were analysed in duplicate. The percentage composition of the *EOs* was computed by the normalization method from the *GC* peak areas, assuming identical mass response factor for all compounds. Results were calculated as mean values of two injections from *EOs*, without using correction factors. All determinations were performed in triplicate and averaged.

5.4. Olfactory bioassays

A Perspex four-armed olfactometer as described by ‘*Vet et al.* ^[41]’ was used to assess the behavioral responses of aphids to the odors of the tested *EOs* (Fig 1). The olfactometer’s arena had a star-shape consisting of four arms between which an aphid could move freely. Each region had an area of 6.2 cm². The distance from one end to the opposite end of the olfactometer was 12 cm. The olfactometer was lined with a white filter paper 90 mm in diameter (Whatman International Ltd Maidstone England) to allow the movement of aphids. It was replaced after each experiment to avoid memory effect. The center of the chamber of the olfactometer was pierced with a hole of 2 mm of diameter. A piece of glass was inserted into this hole and a plastic pipe of the same diameter was fitted to this fitting. This hole was connected to a pump which thus sucked air through the four branches of the olfactometer. The air first passed through a device which enables it to saturate with water and then through a charcoal filter for purification. The charcoal filter was used to remove volatile compounds which could contaminate the air flow. A flowmeter set at 100 ml/min allowed to control the air flow in each pipe and between the inputs of the olfactometer [42]. If the four air flows were equal, they determine four odor fields of the same surface, centered on the orifice through which air is

sucked. Thus, when aphid will be introduced at the center of the olfactometer, he will analyse the odors carried by each of the airflow. We regularly checked the setting of the flowmeters and the equality of odor fields by sucking NH_4Cl vapors in the olfactometer. Prior to each experiment all glassware was washed with Teepol detergent and then with 70% ethanol and subsequently with distilled water [32] and baked in an oven overnight at 160 °C.

The experiment was carried out in a room at an ambient temperature of $20 \pm 1^\circ\text{C}$ with a relative humidity of 70%. To avoid heterogeneity in lighting, the olfactometer was placed in a cylindrical dark room (10.5 cm in diameter, 22cm in height) and was illuminated from below by a lamp.

The reference handling consists on placing the whole bean plant inside a bell and leaving it for 24h to allow the condensation of odors. After 24 hours, treatment was started. One branch of the olfactometer was randomly attributed to the odor source which is the bean plant, the three other branches served as control (air). We let odors impregnate for five minutes then we introduced the aphid and followed its movements using a camera. To test the behavioral effects of the *EOs* on the aphids, a piece of filter paper impregnated with 10 μl of the tested pure *EO* was placed randomly in one of the four arms of the olfactometer. The three remaining arms served as control and each contained an untreated piece of filter paper. Air was drawn through the central hole at a rate of 100 ml/min. The flowmeter was started 5 minutes before the introduction of the aphid to allow impregnation of the odor in the olfactometer. A fine paintbrush was used to introduce a single aphid into the central chamber through a hole in the top of the olfactometer. When the aphid entered in the olfactometer, he met the four fields of odor and he could eventually make a choice. The experiment was then run for 20 min. Aphids were allowed to move freely in the olfactometer during the experience. A video camera (CCD Sony SPT M324CE fitted with a 4–50 mm/F1:1.6 zoom lens with an infrared filter) coupled to SACAM software [43] was used to monitor and record the aphid's behavioral parameters. If an

aphid remained motionless for 5 min we replaced it by a more active one and the replicate was rejected. No aphid was tested more than once to avoid “the memory effect”. For each tested *EO*, twenty replicates where the aphid was moving were recognized. After every four replicates, the olfactometer was cleaned and the positions of the branches were inverted, between controls and treatments to eradicate any effects of directional bias. We considered that the first choice was made when the aphid entered in more than a half of the branch length and remained there for at least 30 s. The *EOs* elicited a behavioral response from the aphids if they caused them to spend significantly more or less time in the treated arm compared to control arms.

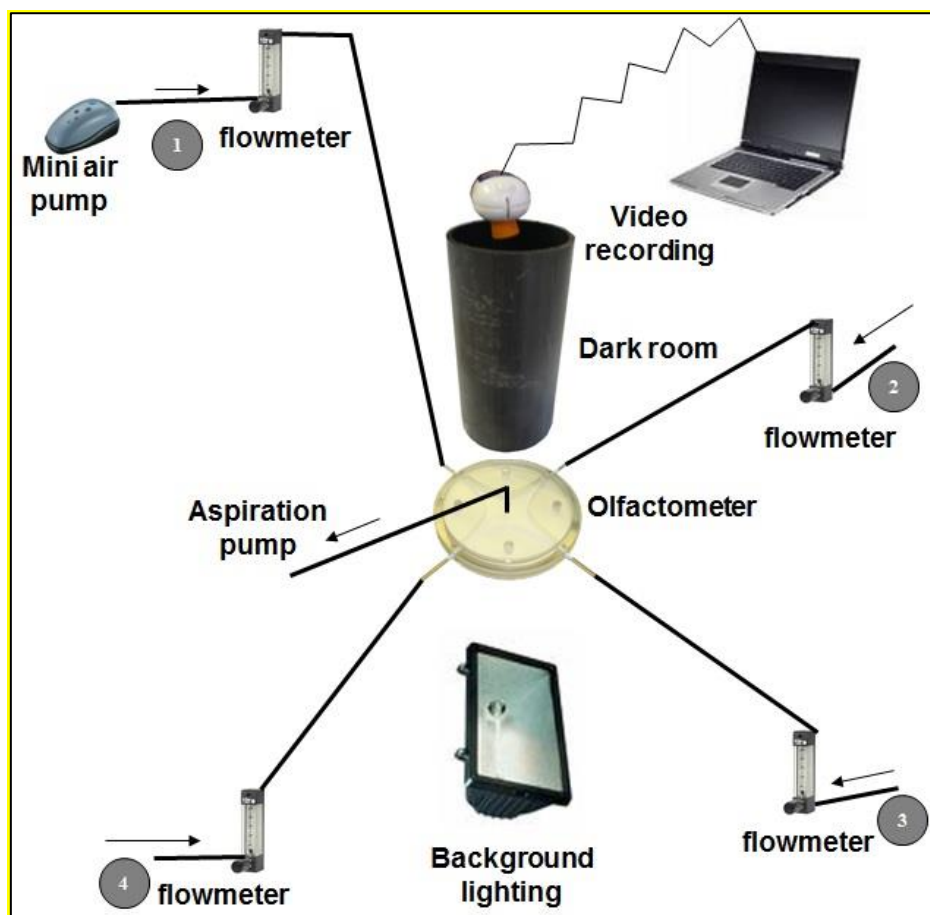


Fig 1. Four armed olfactometer

5.5. Statistical analysis

For each variant of the experiment, the number of aphids moving towards the air source containing the odor (of the bean plant or the tested *EO*) was compared to the number of aphids who were heading for the three pure air source using a X^2 test for a sample [44]. The null hypothesis was that aphids choose the pure air sources with a probability of 0.75 and the air contaminated by the odor of the plant or the *EO* with a probability of 0.25. The results were analysed using the software R [45].

Supplementary Material

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/MS-number>.

Acknowledgements

We would like to give our gratitude to Félipé Ramon Portugal and Gilles Espinasse for helping us with the olfactometer experiments, without your help and knowledge this study would not have been possible to accomplish.

Author Contribution Statement

Abir Kasmi: redaction of the paper and olfactory bioassays; Majdi Hammemi: *GC-MS/GC-FID* analysis; Emmanuel G. Roaellisson: providing us with the Malagasy plants; Manef Abderrabba: Abir's Professor; Jalloul Bouagila: *GC-MS/GC-FID* analysis; Christine Ducamp: Helping in olfactory bioassays

Conflict of Interest

The authors declare that there are no conflicts of interest.

References

- [1] D.S. Hill, 'Agricultural insect pests of temperate regions and their control', Cambridge University Press, UK, 1987.
- [2] A. K. Minks, P. Harrewijn, 'Aphids and their biology, natural enemies and control'. Vol C. Elsevier, Amsterdam, 1989.
- [3] A. F. G. Dixon, 'Aphid Ecology An Optimization Approach', Chapman & Hall, London, 1992.
- [4] A. F. G. Dixon, 'Patch quality and fitness in predatory ladybirds', In Dettner et al. (eds): Vertical food web interactions. *Ecological Studies* **1997**, *130*, 205 – 223.
- [5] D. G. Liu, J. T. Trumble, 'Comparative fitness of invasive and native populations of the potato psyllid *Bactericerca cockerelli*', *Entomol. Exp. Appl.* **2007**, *123*, 35 – 42.
- [6] W. A. A. Peres , B. S. Corrêa-Ferreira, ' Methodology of mass multiplication of *Telenomus podisi* Ash. and *Trissolcus basalus* (Woll.) (Hymenoptera: Sacelionidae) on eggs of *Euchistus heros* (Fab.) (Hemiptera: Pentatomidae) ', *Neotrop. Entomol.* **2004**, *33*, 457 – 462.
- [7] M. B. Isman, 'Plant essential oils for pest and disease management', *Crop Prot.* **2000**, *19*, 603 – 608.
- [8] M. B. Isman, 'Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world', *Ann. Rev. Entomol.* **2006**, *51*, 45 – 66.
- [9] W. de Souza Tavares, I. Cruz, F. Petaci, S. L. de Assis Junior, S. Sousa Freitas, J. C. Zanuncio, J. E. Serrao, 'Potential use of Asteraceae to control *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and selectivity to their parasitoids *Trichogramma pretiosum* (Hymenoptera; Trichogrammatidae) and *Telenomus remus* (Hymenoptera: Scelionidae)', *Ind. Crop Prod.* **2009**, *30*, 384 – 388.

- [10] M. C. Egigu, M. A. Ibrahim, A. Yahya, J. K. Holopainen, 'Holopainen Yeheb (*Cordeauxia edulis*) extract deters feeding and oviposition of *Plutella xylostella* and attracts its natural enemy', *Biocontrol*. **2010**, *55*, 613 – 624.
- [11] C. Regnault-Roger, C. Vincent, J. T. Arnason, 'Essential oils in insect control: low risk products in a high-stakes world *Ann. Rev. Entomol.* **2012**, *57*, 405 – 424.
- [12] X. B. Yang, Y. M. Zhang, L. Hua, L. N. Peng, J. E. Munyaneza, J. T. Trumble, T. X. Liu, 'Repellency of selected biorational insecticides to potato psyllid, *Bactericera cockerelli* (Hemiptera: Psyllidae)', *Crop Prot.* **2010b**, *29*, 1320 – 1324.
- [13] E. H. Koschier, K. A. Sedy, ' Labiate essential oils affecting host selection and acceptance of *Thrips tabaci* Lindeman', *Crop Prot.* **2003**, *22*, 929 – 934.
- [14] M. Hori, 'Antifeeding, settling inhibitory and toxic activities of labiate essential oils against the green peach aphid, *Myzus persicae* (Sulzer) (Homoptera: Aphididae)', *Appl. Entomol. Zool.* **1999a**, *34*, 113 – 118.
- [15] A. Nissinen, M. Ibrahim, P. Kainulainen, K. Tiilikkala, J. K. Holopainen, 'Influence of carrot psyllid (*Trioza apicalis*) feeding or exogenous limonene or methyl jasmonate treatment on composition of carrot (*Daucus carota*) leaf essential oil and headspace volatiles', *J. Agric. Food Chem.* **2005**, *53*, 8631– 8638.
- [16] G. Nehlin, I. Valterová, A. K. Borg-Karlson, 'Use of conifer volatiles to reduce injury caused by carrot psyllid, *Trioza apicalis*, Förster (Homoptera, Psylloidea)', *J. Chem. Ecol.* **1994**, *20*, 771 – 783.
- [17] I. Valterová, G. Nehlin, A. K. Borg-Karlson, 'Host plant chemistry and preferences in egg-laying *Trioza apicalis* (Homoptera, Psylloidea)', *Biochem. Syst. Ecol.* **1997**, *25*, 477 – 491.

- [18] P. Kainulainen, A. Nissinen, A. Piirainen, K. Tiilikkala, J. K. Holopainen, 'Essential oil composition in leaves of carrot varieties and preference of specialist and generalist sucking insect herbivores', *Agr. Forest Entomol.* **2002**, *4*, 211 – 216.
- [19] N. G. Agelopoulos, M. A. Birkett, A. J. Hick, A. M. Hooper, J. A. Pickett, E. M. Pow, L. E. Smart, D. W. M. Smiley, L. J. Wadhams, C. M. Woodcock, 'Exploiting semiochemicals in insect control', *Pesticide Sci.* **1999a**, *55*, 225 – 235.
- [20] C. R. Rodriguez-Saona, L. L., 'Stelinski Behavior-modifying strategies in IPM: theory and practice. In: Integrated Pest Management: Innovation-Development Process (Ed. by R. Peshin & A. K. Dhawan)', 2009, pp. 263–315. Dordrecht: Springer.
- [21] H. Chiasson, N. J. Bostanian, C. Vincent, 'Acaricidal properties of a *Chenopodium*-based botanical', *J. Econ. Entomol.* **2004**, *97*, 1373 – 1377.
- [22] N. J. Bostanian, M. Akalach, H. Chiasson, 'Effects of a *Chenopodium*-based botanical insecticide/acaricide on *Orius insidiosus* (Hemiptera: Anthocoridae) and *Aphidius colemani* (Hymenoptera: Braconidae)', *Pest Manag. Sci.* **2005**, *61*, 979 – 984.
- [23] J. O. W. González , R. A. Laumann, S. da Silveira, M. C. Blassioli Moraes , M. Borges, A. A. Ferrero, 'Lethal and sublethal effects of four essential oils on the egg parasitoids *Trissolcus basalis*', *Chemosphere.* **2013**, *92*, 608 – 615.
- [24] M. Leyva, L. French-Pacheco, F. Quintana, D. Montada, M. Castex, A. Hernandez, M. D. C. Marquetti, '*Melaleuca quinquenervia* (Cav.) S.T. Blake (Myrtales: Myrtaceae): Natural alternative for mosquito control', *Asian Pacific Journal of Tropical Medicine*, **2016**, *9*(10), 979 – 984.
- [25] N. W. Yang, A. L. Li , F. H. Wan, W. X. Liu , D. Johnson, 'Effects of plant essential oils on immature and adult sweetpotato whitefly, *Bemisia tabaci* biotype B', *Crop Prot.* **2010a**, *29*, 1200 – 1207.

- [26] R. S. Mann, R. L. Rouseff, J. M. Smoot, W. S. Castle, L. L. Stelinski, 'Sulfur volatiles from *Allium* spp. Affect Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), response to citrus volatiles', *Bull. Ent. Res.* **2011**, *101*, 89 – 97.
- [27] S. E. Halbert, D. Corsini, M. Wiebe, S. F. Vaughn, 'Plant-derived compounds and extracts with potential as aphid repellents', *Ann. Appl. Biol.* **2009**, *154*, 303 – 307.
- [28] M. Bailena, L. F. Julio, C. E. Diaz, J. Sanz, R. A. Martínez-Díaz, R. Cabrera, J. Burillo, A. Gonzalez-Coloma, 'Chemical composition and biological effects of essential oils from *Artemisia absinthium* L. cultivated under different environmental conditions', *Crop Prot.* **2013**, *49*, 102 – 107.
- [29] M. Hori, H. Komatsu, 'Repellency of rosemary oil and its components against the onion aphid, *Neotoxoptera formosana* (Takahashi) (Homoptera: Aphididae)', *Appl. Entomol. Zool.* **1997**, *32*, 303 – 310.
- [30] M. Hori, 'The effects of rosemary and ginger oils on the alighting behaviour of *Myzus persicae* (Sulzer) (Homoptera: Aphididae) and on the incidence of yellow spotted streak', *Appl. Entomol. Zool.* **1999b**, *34*, 351 – 358.
- [31] J. R. Storer, G. Powell, J. Hardie, 'Settling responses of aphids in air permeated with non-host plant volatiles', *Entomologia Experimentalis & Applicata.* **1996**, *80*, 76 – 78.
- [32] J. A. Pickett, L. J. Wadhams, C. M. Woodcock, J. Hardie, 'The chemical ecology of aphids', *Annual Review of Entomology*, **1992**, *37*, 67 – 90.
- [33] B. Webster, T. Bruce, S. Dufour, C. Birkemeyer, M. Birkett, J. Hardie, J. Pickett, 'Identification of volatile compounds used in host location by the black bean aphid *Aphis fabae*', *Journal of Chemical Ecology*, **2008a**, *34*, 1153 – 1161.

- [34] B. Webster, T. J. A. Bruce, J. Hardie, 'Olfactory recognition of host plants in the absence of host-specific volatile compounds', *Communicative and Integrative Biology*, **2008b**, *1*, 167 – 169.
- [35] M. E. Montgomery, L. R. Nault, 'Comparative response of aphids to the alarm pheromone, (E)- β -farnesene', *Entomologia Experimentalis Et Applicata*, **1977**, *22*, 236 – 242.
- [36] L. Williams III, C. Rodriguez-Saona, P. W. Paré, S. J. Crafts-Brandner, 'The piercing-sucking herbivores *Lygus hesperus* and *Nezara viridula* induce volatile emissions in plants', *Arch. Insect Biochem. Physiol.* **2005**, *58*, 84 – 96.
- [37] S. Colazza, A. Fucarino, E. Peri, G. Salerno, E. Conti, F. Bin, 'Insect oviposition induces volatile emission in herbaceous plants that attracts egg parasitoids', *J. Exp. Biol.* **2004**, *207*, 47 – 53.
- [38] M. B. Isman, S. Miresmailli, C. Machial, 'Commercial opportunities for pesticides based on plant essential oils in agriculture, industry and consumer products', *Phytochem. Rev.* **2011**, *10*, 197 – 204.
- [39] E. P. Lichtenstein, T. T. Liang, K. R. Schulz, H. K. Schnoes, G. T. Carter, 'Insecticidal and synergistic components isolated from dill plants(2007) *J. Agr. Food Chem.* 1974, *22*(4), 658 – 664.
- [40] R. P. Adams, 'Identification of essential oil components by gas chromatography /quadrupole mass spectroscopy', Allured Publishing Corporation, Carol Stream, III, USA, 2007.
- [41] L. E. M. Vet, J. C. Lenteren Van, M. Heymans, E. Meelis, 'An airflow olfactometer for measuring olfactory responses of hymenopterous parasitoids and other small insects', *Physiological Entomology*. 1983, *8*: 97 – 106.

- [42] M. H. Pham-Délégue, P. Etievant, R. Guichard, P. Marilleau, J. Douault, J. Chauffaille, C. Masson, ‘Chemicals involved in honey-bee-sunflower relationship’, *Journal of Chemical Ecology*. 1990, 16, 3053 – 3065.
- [43] L. C. Jorge, R. A. Laumann, M. Borges, M. C. B. Moraes, R. A. Cruz, L. Palhares, ‘Software para avaliação do comportamento de insetos. Circular Técnica, 30’, Embrapa Instrumentação Agropecuária, Brazil, 2005.
- [44] S. Siegel, ‘Nonparametric statistics for the behavioural sciences (Statistiques non paramétriques pour les sciences du comportement)’, Edition New York: McGraw-Hill, 1956.
- [45] R Development Core Team, 2010. R: A language and environment for statistical computing. R Foundation for statistical Computing, Vienna. ISBN 3-900051-07-0, URL: <http://www.R-project.org>, accessed the 22nd of September 2011.