

# Changes in Monoterpene Emission Rates of *Quercus ilex* Infested by Aphids Tended by Native or Invasive *Lasius* Ant Species

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**Abstract** The emission of volatile organic compounds (VOCs) depends on temperature and light. Other factors such as insect herbivory also may modify VOC emission. In particular, aphid feeding promotes the release of new compounds and changes the composition of plant volatile blends. Given that some aphids are tended by ants, we investigated whether ants change the emission of VOCs indirectly through attendance on aphids. The effect of *Lachnus roboris* aphids and two different tending ant species on terpene emission rates of 4-year-old holm oak (*Quercus ilex*) saplings was investigated during a field experiment. There were five treatments: saplings alone (T1), saplings infested with *L. roboris* aphids (T2), saplings

infested with aphids tended by the local ant *Lasius grandis* (T3), those tended by small colonies of the invasive ant *Lasius neglectus* (T4), and those tended by large colonies of the same invasive ant species (T5). The infestation by *L. roboris* elicited the emission of  $\Delta^3$ -carene and increased the emission of myrcene and  $\gamma$ -terpinene. Terpene emissions were modified depending on the tending ant species. Attendance by the local ant *L. grandis* increased  $\alpha$  and  $\beta$ -pinene and sabinene. Attendance by the invasive ant *L. neglectus* only decreased significantly the emission of myrcene, one of the major compounds of the *Q. ilex* blend. Aphid abundance decreased with time for all treatments, but there was no difference in aphid abundance among treatments. Total terpene emission rates were not correlated with aphid abundance. These results highlight that aphids and tending ants may change terpene emission rates, depending on the ant species.

**Key Words** Aphid-ant interaction · Induced volatiles · Indirect effect · Invasive ant species · Terpene emissions · Biotic factors

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## Introduction

Plants emit a broad spectrum of volatile organic compounds (VOCs), mainly isoprene and monoterpenes, which have multiple protective and signaling functions (Peñuelas and Llusia 2004), including defensive and allelopathic roles. For example, they act as herbicides and may inhibit seed germination, cytochromic respiration, and growth of annual plants (Peñuelas et al. 1996). Under certain conditions, i.e., abiotic stress or herbivore attack, new volatiles may be produced *de novo* or released

from storage organs (Kesselmeier and Staudt 1999). These induced emissions have received increasing attention since the first studies that showed emission in response to herbivore attack (Dicke and Sabelis 1988). Some compounds of the emitted blend act as deterrents of the herbivore or as signals to attract predators or parasitoids that protect the plant against insects.

Holm oak (*Quercus ilex* L.) is a widespread tree of the Mediterranean basin, and it is a major source of VOCs (Staudt et al. 2001). In contrast to many deciduous oak species that emit large amounts of isoprene, *Q. ilex* emits huge amounts of monoterpenes, although it has no specific VOC storing organs (Staudt and Seufert 1995), and there is no storage pool of VOCs in the leaves or bark (Pasqua et al. 2001). The compositional fingerprint of the emissions is controlled mainly by genetics (Staudt et al. 2003). However, the synthesis and emission of these VOCs are modulated continuously by environmental factors such as light, temperature (Staudt and Bertin 1998; Llusia and Peñuelas 1999) and severe drought (Llusia and Peñuelas 1998), or by biological factors such as herbivore attacks (Staudt and Lhoutellier 2007). Identifying those biotic factors that promote changes of VOC emissions from holm oak is crucial for Mediterranean ecosystems because the monoterpenes released play a key role both in the biology of the community and in the formation of oxidants and secondary aerosols in the troposphere (Kesselmeier and Staudt 1999; Peñuelas and Staudt 2010). This has consequences for air quality and visibility, and even for the climate on a regional scale (Peñuelas and Llusia 2003).

In Spain there are many aphids that feed on *Q. ilex* (Nieto-Nafría and Mier-Durante 1998), in particular *Lachnus roboris* L., which is an obligate myrmecophilous aphid (Michel 1942; Sudd and Sudd 1985), and is tended by several ant species that collect honeydew as food (Paris and Espadaler 2009). Honeydew, a sugar solution excreted by aphids while feeding on sap plants, is the main food of several ant species (Carroll and Janzen 1973). Through aphid attendance, ants may exert indirect effects on plants, such as a decrease in seed production (Rico-Gray and Castro 1996), plant herbivory (Suzuki et al. 2004), and pollination (Lach 2007). The degree of these indirect effects varies among native ants, primarily as a result of differences among species in aggressiveness, territoriality, colony density, worker abundance, and the strength and persistence of the interactions between particular ant species (Styrsky and Eubanks 2007). In the case of invasive ants, it is assumed that the indirect effect on plants through aphid attendance may increase in magnitude because invasive ants frequently cause local hemipteran outbreaks (Styrsky and Eubanks 2007). The aggressive behavior and unicolonial structure of invasive ants may achieve higher colony densities and worker

abundance compared with those of native ants (Passera 1994). As a consequence, native ants are displaced, and honeydew sources are dominated by tending invasive ants (Holway et al. 2002). In response to ant attendance, aphids may increase sap feeding (Buckley 1987; but see Yao and Akimoto 2001) as well as the frequency of honeydew excretion, and they may change the sugar composition of honeydew (Fischer et al. 2002). Previous studies have shown that *L. roboris* excretes more drops of honeydew per minute when tended by the invasive ant *Lasius neglectus* than when tended by the native ant *Lasius grandis* (Paris and Espadaler 2009). This apparently is related to the higher intensity of attention shown by this invasive ant (Paris and Espadaler 2009).

As a consequence of aphids feeding on plants, VOC emissions may change and new compounds may appear (Du et al. 1998; Powell et al. 1998). Considering the direct effect of ants on aphid feeding and the indirect effect on plants, we hypothesized that the composition of the VOC blend of *Q. ilex* changes when the aphid *L. roboris* is tended by the invasive rather than the native *Lasius* ant species. Given that the effect of invasive ants on other components of the ecosystem is in part related to their abundance (Holway et al. 2002), we investigated whether the VOCs emitted by holm oak change according to the size of the *L. neglectus* colony.

We hypothesized that VOCs emitted by holm oaks would change qualitatively and quantitatively (i) according to the presence of the aphid *L. roboris*, (ii) depending on which ant species tended the aphids (invasive *L. neglectus* or native *L. grandis*), and (iii) depending on the size of the invasive ant colony. In this study, we aimed: a) to test these three hypotheses and to discover whether such changes occurred; b) to identify the compounds whose emission rates changed when holm oaks became infested with aphids; and c) to determine the compounds linked to ant attendance. We sampled volatiles emitted by holm oak saplings alone or infested with *Lachnus roboris* that were tended or not tended by *L. grandis* or *L. neglectus*.

## Methods and Materials

### Study Site

This experiment was performed at a field close to the Center of Ecological Research and Forestry Applications (CREAF) at the Autonomous University of Barcelona (41° 30' N, 2° 6' E). We used 4-yr-old saplings of *Quercus ilex* (mean±SE diameter, 13.08±0.62 mm) placed in 10-L pots the previous year that were filled with a mixture of commercial humus and a sandy soil (1:1). We used this mixture because previous work showed that in commercial humus alone, ant digging promotes rapid loss of water and consequent drying of the plant.

## Ant and Aphid Colonies

At the beginning of June, when new inseminated queens of *L. neglectus* were particularly easy to find under flat rocks and leaf litter, we sampled several queens and workers from an invaded area of the University campus. Fertilized queens are easy to identify because they are huge compared with workers and because they lack wings. Young queens are adopted by workers (Espadaler and Rey 2001). The queens began to lay eggs in the first few days after their capture (Paris, pers. observ.). Given that *L. neglectus* is a polygynic ant species (Boomsma et al. 1990), we placed three queens in each nest to ensure that the colony remained polygynic. In a preliminary experiment, we placed 30, 65, and 150 workers plus 3 queens in artificial nests. We realized that in small colonies of *L. neglectus* the queens were decapitated, but this did not occur in colonies with 150 workers. Therefore, we tried 30 workers per queen and found that with this proportion the colony grew. Finally, we decided to place three queens with 90 or 210 workers, to simulate small and large colonies, respectively. Newly inseminated queens of the native ant *L. grandis* were collected in June of the previous year during a nuptial flight in the campus. Recently landed queens were sampled immediately and brought to the laboratory to rear colonies for the planned experiment. One queen of the native ant *L. grandis* was placed in each nest because this is a monogynic ant species.

Until the experiment started, ants were kept in artificial nests consisting of 3.77 cm<sup>3</sup> (7.5×0.8 cm) nesting tubes, one-third filled with water, plugged with cotton wool, and covered with aluminium foil. The tubes were placed inside plastic boxes (17.7×11.2×3.5 cm) which were set in a growth chamber adjusted to a photoperiod and temperature regime that simulated the ongoing season. We fed the ants *ad libitum* with an artificial diet, according to Bhatkar and Whitcomb (1970), and with Tenebrionidae larvae that had been killed previously by freezing.

In the middle of June, we sampled *L. roboris* aphids, larvae and adults from holm oaks in invaded and control areas. The aphids were pooled and distributed evenly among saplings. We placed 10.5±1.1 (mean±SE aphids per sapling) aphids on saplings 2 d before transferring the artificial ant nests to the pots. Previous work to develop the general design of this experiment showed that after 4–9 days only between 12.5 and 20% of the aphid colonies (a colony = more than five individuals) survived without attention from ants. The ants were starved for 48 hr before transferring the artificial ant nests to the pots. The artificial nest was placed on the soil of the pot without the aluminium paper, to motivate the ants to move into the soil. At that moment, we checked how many queens were still inside the artificial nest. The colonies of the invasive ant began to move immediately, while the colonies of the

native ant began to move 3 to 5 hr after placing the tube on the soil. After one day, all the colonies had moved into the soil. During the experiment, Tenebrionidae larvae were provided to avoid aphid predation by the ants. We placed on the soil, close to the sapling trunk, a glass with a freshly killed larva on it. The ants tended the aphids for 1 wk prior to the first measurements of VOCs.

The number of aphids added to each sapling was chosen as a trade-off among several variables: mean aphid abundance per tree, the percentage of infested twigs and mean ant activity per tree that had been estimated under natural conditions on mature holm oaks (oaks that produce acorns), and the size and age of the saplings. Previous data showed that at the University campus, *L. roboris* were scarce on holm oaks colonized by the native or by the invasive ant species (mean aphid abundance per tree ±SE, *L. grandis*: 28.54±11.92, *L. neglectus*: 59.43±16.90; % of infested twigs ±SE, *L. grandis*: 1.37±0.63%, *L. neglectus*: 2.75±0.91%) (Paris and Espadaler 2009). If we had extrapolated these estimations to the saplings used for this experiment, which had a mean of 16 twigs per sapling, only three aphids should have been added to each sapling. This aphid abundance would not have provided enough honeydew for ant colonies and would probably not have exerted any effect on VOC emissions from the holm oak.

On other hand, the aphid abundance should be related to the size of the ant colonies. Larger colonies than those used for this experiment may need a greater supply of honeydew, which would require more aphids. We considered that a 4-yr-old *Q. ilex* sapling would not be able to carry more than 20 aphids without becoming stressed by aphid feeding.

## Experimental Design

Five treatments with 4 saplings per treatment were used: sapling alone (T1); sapling + aphids (T2); sapling + aphids + *L. grandis* (native ant) colony (T3); sapling + aphids + *L. neglectus* (invasive ant) colony of 90 workers (small colony) (T4); and sapling + aphids + *L. neglectus* colony of 210 workers (large colony) (T5). T1 represented the situation of basal emissions of VOCs (without any biotic stress). T2 simulated an aphid infestation without ant attendance, to determine which VOCs are elicited by aphid feeding alone (first hypothesis). To investigate the effect of the attendance of different ant species (second hypothesis), we established T3, T4, and T5. The number of *L. neglectus* workers in T4 and T5 was altered to test the effect of colony size (third hypothesis). The morphological traits (height, trunk diameter, dry weight, number and area of leaves) of the saplings did not differ among treatments (Kruskall Wallis,  $P>0.324$ ).

Each pot was placed in a plastic box (49×29×25 cm) filled with the same soil mixture as that used for the pots. The plastic boxes were surrounded by two barriers of non-

toxic sticky resin (Tanglefoot®, Tanglefoot Company, MI, USA) to prevent ants escaping and other ant species entering. This device worked as a buffer area, which developed a gradient of humidity, from the pot, where we watered the plant, to the plastic box. This gradient of humidity was necessary to allow the ants to establish in an environment with sufficient humidity according to their preferences.

### VOC Sampling and Analysis

Before sampling VOCs, we counted the number of aphids. We sampled VOCs 1 wk after the ants had begun to tend the aphids. During the daily sampling protocol, we sampled VOCs from the empty chamber to test whether adsorption occurred on the enclosure walls of the chamber. Given that soil may act as a source or sink for VOCs (Asensio et al. 2007), we sampled VOCs from extra boxes filled with soil but without plants or roots (termed soil boxes). The values obtained from soil boxes represent the blank for the system, so they were subtracted from the measured plant VOC emissions. The plants and soil boxes were watered the evening before sampling of VOCs.

Five saplings, belonging to different treatments, were sampled for VOC emissions each day on 4 different days between 9:30 and 14.00 hr. VOCs were sampled from the soil boxes at 3 different times during the morning: at the beginning of the daily sampling, after 2 samplings of holm oak VOCs had been made, and at the end of the daily sampling.

Sampling of VOC emissions was conducted by enclosing the sapling in a cylindrical dynamic chamber made of Tedlar, which was mounted in a cylindrical aluminium frame (65 cm high, 35 cm diam) and had a volume of 62.5 L. After the cylindrical chamber had been installed on each tree, we waited 10 min before sampling the VOCs. Inside the chamber was a fan that homogenized the air. The air entering and leaving the chamber passed through respective glass tubes (11.5 cm long and 0.4 cm internal diam) filled with Carbotrap C (300 mg), Carbotrap B (200 mg) and Carbosieve S-III (125 mg) adsorbents from Supelco (Bellefonte, PA, USA), which were separated by plugs of quartz wool. These tubes were conditioned previously for 20 min at 350°C with a stream of purified helium.

In order to obtain comparable results, the emission rates were expressed on a leaf dry weight basis. The sampling time was 10 min, and the flow was adjusted to 500 ml min<sup>-1</sup>. The glass tubes (with trapped VOCs) were stored in a portable refrigerator at 4°C, and taken to the adjacent laboratory. At the laboratory, the glass tubes were stored at -30°C before analysis, for no longer than 48 hr.

For analysis of VOCs, a GC-MS (Hewlett Packard HP59822B, Palo Alto, CA, USA) was used. Tubes with trapped emitted monoterpenes were inserted in an OPTIC3 injector (ATAS GL International BV 5500 AA Veldhoven, The Netherlands) connected to a Hewlett Packard HP59822B GC-MS (Palo Alto, CA, USA), where they were desorbed at 250°C during 3 min. Terpenes were separated using a TRB-5 Fused Silica Capillary column, 30 m×0.25 mm×0.25 mm film thickness (Teknokroma, Barcelona, Spain). After sample injection, the initial temperature was increased from 46 to 70°C at 30°C min<sup>-1</sup>, and thereafter at 10°C min<sup>-1</sup> up to 150°C, held at 150°C for an additional 2 min, and thereafter increased at 30°C min<sup>-1</sup> up to 250°C, then held at 250°C for an additional 2 min. The helium flow was 1 mlmin<sup>-1</sup>. The identification of  $\alpha$ -pinene and  $\Delta^3$ -carene was conducted by comparing the retention times with standards from Fluka (Buchs, Switzerland). The other monoterpenes were identified tentatively by comparing the fractionation mass spectra with standards, literature spectra, GCD Chemstation G1074A HP and the mass spectra library Wiley.

Frequent calibration (once every 3 analyses) with external standards ( $\alpha$ -pinene,  $\Delta^3$ -carene, *p*-cymene, limonene) was used during the quantification. The detection limit was about 0.6 ng. The calibration curves were always highly significant ( $r^2 > 0.99$ ). At the end of the experiment, the leaves were harvested from all the saplings to determine the dry weight after drying at 70°C in a ventilated oven until the weight was constant. Monoterpene emission rates were calculated as the difference between the amount of each compound emitted in samples from plants and the amount emitted from soil boxes without the plant (blank). The VOC emissions were expressed as  $\mu\text{g}$  per g of dry weight of leaves per hour.

### Statistical Analysis

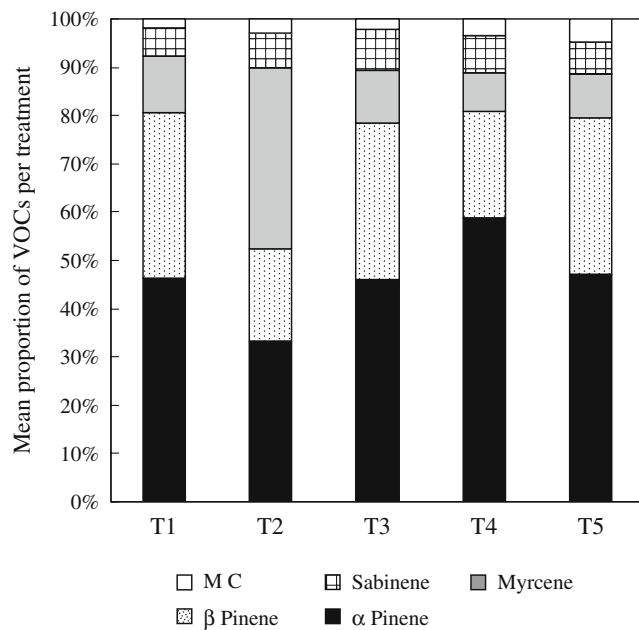
We applied Mann-Whitney and Kruskal-Wallis procedures because the data did not fit a normal distribution. To test the first hypothesis, we compared the VOC emissions of holm oaks alone (T1) against the emissions of saplings whose aphids were not tended (T2) by using the Mann-Whitney procedure. Given that the colony size of the invasive ant *L. neglectus* (T4 vs. T5, third hypothesis) did not produce differences in any emitted VOCs (Mann-Whitney,  $P > 0.2$ ), these treatments were combined. To test the effect of different tending ant species on VOC emissions (T2 vs. T3 vs. T4+T5, second hypothesis) we used the Kruskal-Wallis test. Two tailed exact *P* values are reported. When significant values arose, we performed the nonparametric Dunn's test for multiple comparisons, according to the process proposed by Zar (1996). Finally, aphid abundance was compared among treatments and by day using the

Kruskall-Wallis procedure and correlated with the total emission of VOCs using the Spearman procedure. All analyses were conducted using STATISTICA 6.0 for Windows (StatSoft, Inc. Tulsa, OK, USA, 1996).

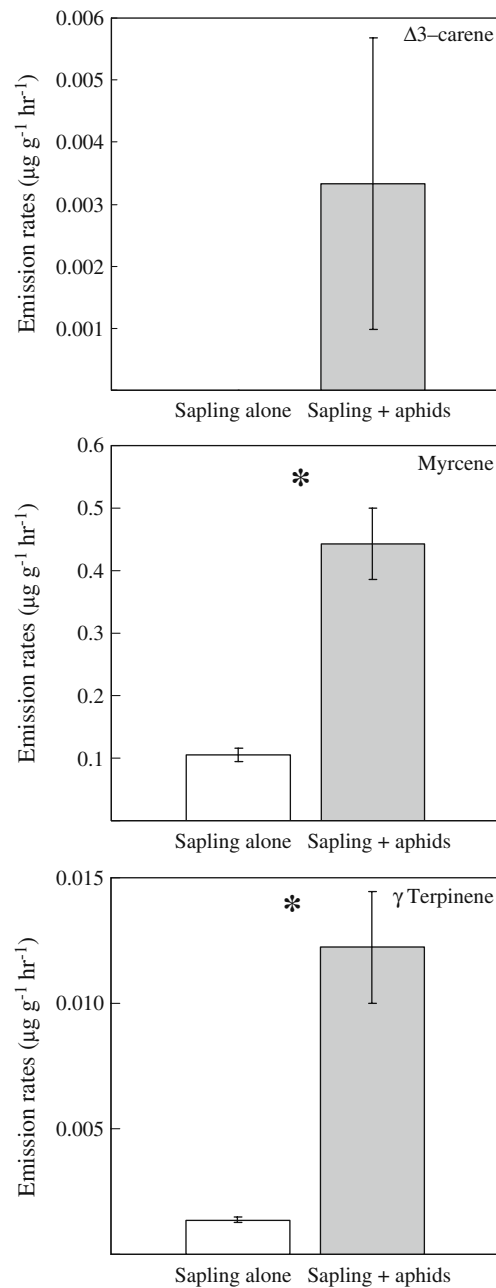
**Results**

One day after placing the artificial nests on soil pots, all ant colonies had moved into the soil and begun to tend aphids. Total emissions from the holm oak increased 1.31-fold when aphids were present (range 0.62–2.13  $\mu\text{g}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ ) while the attendance by *L. grandis* and *L. neglectus* increased the total emissions 5- and 1.19-fold, respectively (*L. grandis* range: 3.60–5.06  $\mu\text{g}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ ; *L. neglectus* range: 0.27–2.19  $\mu\text{g}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ ). In all treatments,  $\alpha$ - and  $\beta$ -pinene, myrcene, and sabinene comprised the main compounds of the *Q. ilex* blend (Fig. 1).  $\Delta^3$ -Carene, a minor compound, was not emitted by uninfested saplings and was produced only by infested plants, independent of whether the aphids were tended or not (Fig. 2). The emission of myrcene and  $\gamma$ -terpinene increased significantly when holm oak saplings were infested with *L. roboris* (Mann-Whitney, T1 vs. T2,  $U_{4,4}=0$ ,  $P<0.03$  for both components) (Fig. 2).

The terpene emissions of holm oak saplings changed according to the tending ant species (Table 1). In particular,  $\alpha$ - and  $\beta$ -pinene, camphene, sabinene and  $\Delta^3$ -carene increased when aphids were tended by the native ant *L.*



**Fig. 1** Proportion of terpenes emitted by *Quercus ilex* saplings alone (T1), from saplings infested with *Lachnus roboris* aphids (T2), saplings with aphids tended by the native ant *Lasius grandis* (T3) or tended by the invasive ant *Lasius neglectus* from small colonies (T4) or from larger colonies (T5). MC: minor compounds (less than 1.5 % of total emission). They were  $\gamma$ -terpinene, terpilone,  $\alpha$ -thujene,  $\Delta^3$ -carene, Camphene and  $\alpha$ -terpine



**Fig. 2** Mean and standard error of volatile organic compounds emitted at different rates by *Quercus ilex* saplings alone (T1) or infested with *Lachnus roboris* aphids (T2). Asterisk shows statistical significance between treatments according to Mann Whitney’s test ( $P<0.05$ )

*grandis* compared with unattended aphids or those tended by the invasive ant *L. neglectus* (except camphene and  $\Delta^3$ -carene) (Kruskall-Wallis,  $\alpha$ - and  $\beta$ -pinene:  $H_{2,16}=8.74$ ,  $P<0.01$ ; sabinene:  $H_{2,16}=8.49$ ,  $P<0.01$ , camphene:  $H_{2,16}=7.65$ ,  $P<0.02$ ;  $\Delta^3$ -carene:  $H_{2,16}=7.50$ ,  $P<0.02$ ; Dunn’s test  $Q_{0.05,3}=2.39$ ) (Table 1). The attention of the invasive ant decreased the emission of myrcene (Kruskall-Wallis,  $H_{2,16}=11.31$ ,  $P<0.003$ ) but no changes were detected for the other compounds (Table 1).



**Table 1** Means and standard error ( $\mu\text{g. g}^{-1}\cdot\text{h}^{-1}$ ) of main compounds emitted by *Quercus ilex* saplings ( $N=4$  for T2 and T3 and  $N=8$  for T4+T5) infested with not tended aphids (T2) or infested with aphids tended by the native ant *Lasius grandis* (T3) or by the invasive ant *Lasius neglectus* (T4 and T5 coupled). Different letters indicate statistical differences of non parametrical comparisons treatments (Dunn's test,  $P<0.05$ )

Compound	T2	T3	T4+T5
$\alpha$ -Thujene	0.007 $\pm$ 0.004 a	0.015 $\pm$ 0.005 a	0.005 $\pm$ 0.001 a
$\alpha$ -Pinene	0.391 $\pm$ 0.182 a	2.072 $\pm$ 0.033 b	0.551 $\pm$ 0.105 a
Camphene	0.007 $\pm$ 0.003 a	0.047 $\pm$ 0.014 b	0.012 $\pm$ 0.004 ab
Sabinene	0.084 $\pm$ 0.042 a	0.387 $\pm$ 0.045 b	0.075 $\pm$ 0.017 a
$\beta$ -Pinene	0.227 $\pm$ 0.105 a	1.454 $\pm$ 0.269 b	0.306 $\pm$ 0.075 a
Myrcene	0.443 $\pm$ 0.057 a	0.482 $\pm$ 0.044 a	0.093 $\pm$ 0.020 b
$\Delta^3$ -Carene	0.003 $\pm$ 0.002 a	0.018 $\pm$ 0.001 b	0.010 $\pm$ 0.003 ab
$\alpha$ -Terpine	0.004 $\pm$ 0.001 a	0.003 $\pm$ 0.001 a	0.001 $\pm$ 0.000 a
$\gamma$ -Terpinene	0.012 $\pm$ 0.002 a	0.011 $\pm$ 0.004 a	0.013 $\pm$ 0.005 a
Terpinolene	0.001 $\pm$ 0.000 a	0.002 $\pm$ 0.001 a	0.005 $\pm$ 0.002 a

The abundance of aphids decreased with time for all infested saplings (mean aphids per day  $\pm$ SE, day 1: 10.5 $\pm$ 1.1, day 2: 17.8 $\pm$ 0.9, day 3: 6.5 $\pm$ 1.0, day 4: 3.3 $\pm$ 0.7) ( $H_{2, 16}=11.59$ ,  $P<0.01$ ), but aphid abundance did not differ among treatments (mean aphids per treatment  $\pm$ SE, T2: 13 $\pm$ 4.2; T3: 9.4 $\pm$ 1.6; T4+T5: 11.9 $\pm$ 1.4;  $H_{2, 16}=0.02$ ,  $P<0.99$ ). No correlation was found between aphid abundance and total emitted VOCs ( $r_s=-0.01$ ,  $P<0.9$ ,  $N=12$ ).

## Discussion

The infestation by *L. roboris* elicited the emission of  $\Delta^3$ -carene and increased significantly the emissions of myrcene and  $\gamma$ -terpinene. The most important biogenic volatile organic compounds (BVOCs) produced by terrestrial plants are isoprene and monoterpenes, such as those mentioned above (Karl et al. 2009). In the Mediterranean basin there are several plants that emit isoprenoids, particularly those of the *Quercus* genus (Owen et al. 2002). Among them is the holm oak (*Quercus ilex*), which despite lacking storage organs is a strong emitter of monoterpenes in comparison with other *Quercus* spp. that emit mainly isoprene (Kesselmeier and Staudt 1999). The blend produced by holm oak has been studied mainly in relation to its modulation by abiotic factors (temperature, light, drought, different CO<sub>2</sub> concentrations) (Staudt and Bertin 1998; Llusia and Peñuelas 1999; Rapparini et al. 2004; Peñuelas and Staudt 2010). In accordance with previous reports of holm oak terpene emissions, we found that the blend produced by holm oak saplings was composed mainly of  $\alpha$ - and  $\beta$ -pinene, sabinene and myrcene (Street et al. 1997;

Staudt and Bertin 1998). Recently, Staudt and Lhoutellier (2007) showed that massive outbreaks of the gypsy moth (*Limantria dispar*) raise by 16% the total emitted VOCs, including new compounds, in *Q. ilex* after a delay of several hours from the start of the infestation. The presence of this moth promotes the release of DMNT, germacrene D,  $\beta$ -caryophyllene, and several other sesquiterpenes that were not emitted or emitted only in trace amounts from non-infested leaves. To our knowledge, no study on the effect that aphids have on oak trees, and in particular the indirect effect that the ant, through aphid attention, has on holm oak VOCs, has been performed previously. Changes in the composition of emitted VOCs associated with the presence of aphids have been reported in studies of tea and willow trees (Han and Chen 2002; Inui et al. 2003) and of other plants with agricultural value (Heil 2007). The aphid-induced plant volatiles act as synomones for foraging aphid parasitoids and predators (Du et al. 1998; Powell et al. 1998). On our saplings, we have never seen predators or any evidence of parasitoid oviposition (mummies). In addition, during a 2-year study of the abundance of *L. roboris* on mature holm oaks in the same study area and with the same ant species, parasitized aphids or predators have never been found.

The volatiles induced by insect damage depend on the feeding habits (Delphia et al. 2007) and on the density of herbivores (Dicke et al. 1993; Tumlinson et al. 1993). In general, phloem feeders induce lower emissions of plant volatiles compared with chewing insects, even under high levels of plant infestation (Staudt and Lhoutellier 2007). Aphids are all phloem feeders, and their feeding behavior comprises two phases. The first is the probing phase, during which the aphid inserts its stylet and tests sap quality, and the second is the ingestion phase, during which the aphid regulates sap intake using its cybarial pump (Miles 1987). During probing, the stylet of the aphid transiently punctures epidermal, mesophyll, and parenchyma cells. For the most part, however, aphids penetrate plant tissues via a primarily intercellular route, and their impact on plants is thought to be largely due to removal of photosynthesis products and injection of saliva (Goggin 2007). After identifying a suitable plant and locating on the plant, aphids begin to ingest sap from the sieve tube (Miles 1987). The ability to prevent sieve tube plugging is an important adaptation that allows aphids to remain at a single feeding site for hours at a time (Goggin 2007). Despite the low level of cell damage that aphids produce when inserting their stylets between cells (Goggin 2007), some aphid species may trigger large responses in plant volatiles shortly before starting to feed. The plant response induced by aphids usually appears with a delay of several hours after infestation (El-Aouni et al. 2007) and may cause systemic production of volatiles for up to 24 hrs after the aphids have been removed from the

plant (Guerrieri et al. 1996). Du et al. (1998) found that 6-methyl-5-hepten-2-one, linalool, geranic acid, and (E)- $\beta$ -farnesene appeared during the first day after *Acyrtosiphon pisum* infestation of broad bean plants and increased in concentration with increasing duration of aphid feeding. These facts highlight an immediate plant response that is amplified with time and does not stop immediately.

In our study, we began to sample VOCs 1 week after aphids began to feed on *Q. ilex* saplings. This suggests that we probably sampled VOCs when the response of the saplings was amplified. Moreover, the response of the saplings may have continued, because aphid abundance decreased with time in all treatments, but no correlation between aphid abundance and total emitted VOCs was found. The decrease in the number of aphids could be due to ant predation, despite the fact that ants were provided with freshly killed Tenebrionidae to prevent aphid predation. On holm oak, the main prey of *L. grandis* and *L. neglectus* are Psocoptera (33.8% and 31.1% of total predated insects, respectively) and the untended aphid *Hoplocallis picta* (35.4% and 37.8% of total predated insects, respectively) (Paris and Espadaler 2009). *Lachnus roboris* represents only 1.5% and 2.5% of total predated insects for *L. grandis* and *L. neglectus*, respectively (Paris, unpublished data). We have never seen ants carrying aphids to the nest but, as mentioned, both ant species prey on *L. roboris* so we cannot completely discount aphid predation.

As stated above, our results showed that feeding by *L. roboris* induced the emission of  $\Delta^3$ -carene and increased the emission of myrcene and  $\gamma$ -terpinene. It has already been reported that mature holm oaks release  $\Delta^3$ -carene in very low quantities (1.9–8.3% of total emissions depending on the month) (Street et al. 1997), but previous studies did not report whether or not the sampled holm oaks were infested by aphids because this trait was not related to the aims of those studies. The reported temporal variations of  $\Delta^3$ -carene emissions in these studies coincided with the population dynamics of *Lachnus roboris* on holm oaks (Street et al. 1997), which shows a main peak abundance at the end of spring or beginning of summer and a secondary peak abundance at the beginning of autumn (Paris and Espadaler 2009). This leads us to think that the sampled mature holm oaks could have been infested by aphids.

#### Why Different Species of Tending Ants Resulted in Different Emissions

Our results also showed that the attendance of ants may change the VOC emissions of plants, and that their effect is dependent on the ant species. When the local ant *L. grandis* was present,  $\alpha$ - and  $\beta$ -pinene, sabinene, camphene, and  $\Delta^3$ -carene increased significantly compared with the emissions of saplings with untended aphids. On the other

hand, attention from the invasive ant *L. neglectus* decreased only the emission of myrcene, one of the main VOCs emitted by holm oak. However, when the effect of *L. neglectus* colony size was tested, no difference was found in BVOC emissions. We suspect that this finding could be explained by the adjustment that *L. roboris* can make to its rates of feeding and excretion when tended by different ant species. According to electrical penetration graphs obtained during feeding, aphids are able to regulate the ingestion of sap (Tjallingii 1995). Once a plant is accepted as a suitable source, the total time of penetration of sieve elements is the same with or without the presence of tending ants (Rauch et al. 2002). The total duration of penetration is composed of two waveforms: E1 (salivation) and E2 (ingestion of sap). Rauch et al. (2002) suggested that it is the duration of ingestion of sap that aphids change when they are tended. However, these authors were unable to distinguish between E1 and E2 waveforms but suggested that an increase in sap ingestion may occur as honeydew excretion increases when ants tend aphids. Therefore, *L. roboris* probably adjusted its feeding rate, not necessarily by increasing it but by modifying its frequency and the opening time for the cybarial pumping of sap. In addition, aphids are able to regulate the number of drops excreted per minute and the mean volume of honeydew drops, depending on whether or not they are tended. It has been demonstrated that when aphids are tended by ants, they increase the number of drops per minute but decrease the volume of drops (Yao and Akimoto 2001). This evidence shows that aphids apparently maintain a constant excretion rate (drops\*hour<sup>-1</sup>\*aphid<sup>-1</sup> per drop volume) regardless of whether tended or not. The excretion frequency (drops\*hour<sup>-1</sup>\*aphid<sup>-1</sup>) was significantly higher when *L. roboris* was tended by the invasive ant (Paris and Espadaler 2009). However, we cannot determine whether or not the volume of the drops changes when aphids become tended by the invasive or by the native ant. Therefore, we cannot be totally sure whether the rate of honeydew excretion changes. However, some kind of regulation of excretion rate should have occurred because the frequency (drops per minute) changed. This change in the frequency of excretion of *L. roboris* could reflect an adjustment of aphid feeding when tended by *L. neglectus* instead of *L. grandis*, which may affect the VOC emissions of the plant.

#### Ants and Aphids as Sources of VOCs

Volatiles are involved in ant social behaviors such as recruitment, foraging, alarm, caste and nestmate recognition (Hölldobler and Wilson 1990), as well as in their interactions with other species. On the other hand, aphids emit volatiles in association with crowding, dispersion, alarm, and sexual attraction (Pickett et al. 1992). As a

result, in our experiment both insects may have been sources of BVOCs. We searched each compound detected in this study in the literature and in Pherobase, a web-based library of BVOCs elicited from plants and insects (<http://www.pherobase.com/>). Terpenes can be emitted in large amounts by aphids as a component of the alarm blend (Francis et al. 2005). In a study of the volatile compounds emitted by 23 different aphid species, myrcene,  $\alpha$ - and  $\beta$ -pinene, camphene, and  $\gamma$ -terpinene were emitted by several aphid species after crushing but not during their feeding on plants (Francis et al. 2005). Total emission of the main component of the alarm pheromone in the pea aphid (*Acyrtosiphon pisum*), when attacked by predators, averages  $16.33 \pm 1.54$  ng per aphid and ranges from 1.18 to 48.85 ng per aphid (Schwartzberg et al. 2008).

Concerning ants,  $\alpha$ - and  $\beta$ -pinene, sabinene and camphene have been detected as components of the alarm blend in the ants *Crematogaster laboriosa*, *Pristomyrmex pungens* (except myrcene), *Lasius niger* (except camphene), *Technomyrmex albipes* (except camphene and  $\beta$ -pinene), and *Tetramorium caespitum* (except camphene and  $\beta$ -pinene) (Hayashi and Komae 1980). However, these authors gave information about the proportion of each compound represented in the blend and not the absolute values. Janssen et al. (1997) found that  $\beta$ -pinene occurs only in picogram quantities at foraging trails of *P. pungens* and is a major component of monoterpene secretions from the poison gland of this species. In leaf cutting ants, when a foraging trail is made by dribbling volatile trail pheromone, only 40 pg/ $\mu$ l–0.4 ng/ $\mu$ l is necessary to trigger trail-following activity (Riley et al. 1974). At high concentrations (4  $\mu$ g/ $\mu$ l), the ants are repelled from the trail (Riley et al. 1974). In ants of *Tetramorium* sp., the Dufour's gland (a gland that produces several volatiles) may contain between 30 and 70 ng of a chemical blend per worker (Hölldobler and Wilson 1990). In workers of *Pogonomyrmex badius*, the main alarm pheromone is stored in quantities of 0.2–34  $\mu$ g in the mandibular gland reservoir (Hölldobler and Wilson 1990). These examples highlight that the emission of VOCs by aphids and ants occurs at a level three or four orders of magnitude below those detected in this study, although ants can store high quantity of VOCs. Concerning  $\Delta^3$ -carene, there is no evidence that this component has been secreted by ants or by aphids.

Should Aphids and Tending Ants be Considered as a New Driving Factor for Monoterpene Emissions in BVOC Inventories of the Mediterranean Region?

To answer this question we should consider the seasonality of monoterpene emissions by *Q. ilex*, the abundance of trees infested with aphids and the amount of honeydew collection by ants, whether these factors overlap in time,

and the intensity of ant-aphid mutualism. In Europe, forests cover 40.4% of the vegetated surface and are the major source of monoterpene emissions (Karl et al. 2009). Monoterpene emissions arise in summer, and the main contribution comes from forests of the Boreal and Mediterranean regions where *Pinus* spp. and *Q. ilex* are the main emitters as a result of the area they cover (Karl et al. 2009). According to Köble and Seufert (2002), *Q. ilex* contributes 2.1% to the forested area and is the first tree species of newly synthesized monoterpene emissions (standard emissions factor of  $43 \mu\text{g g}^{-1}\text{h}^{-1}$ ). Monoterpenes are emitted mainly by *Q. ilex* during the summer, especially in July and August, owing to the light and temperature dependence of their production (Staudt and Bertin 1998) and the seasonality shown in the monoterpene synthase activity of *Q. ilex* (Fischbach et al. 2002). At fragments of mixed forest colonized by *L. neglectus* or by local ants, the number of trees colonized by ants peaks in June or July depending on the year (Paris 2007). Honeydew collection per holm oak, by *L. neglectus* and *L. grandis*, follows the same temporal variation (Paris and Espadaler 2009). This evidence suggests that the period of maximum monoterpene emission from forests, the number of trees infested with aphids, and honeydew collection by ants overlap in time. At forest stand level, it is the intensity of aphid infestation, i.e., the area covered by infested trees and the abundance of different tending ant species that could change the rate of monoterpene emission for the purposes of the BVOC inventories. In Spain, *L. roboris* seems to be a rare aphid (Paris and Espadaler 2009) that does not exceed a level of 10% of infested trees at stand level (Melia et al. 1993), in contrast to the situation in temperate forests, where this aphid species is more frequent (Sudd and Sudd 1985). However, *Lasius* spp. and other tending ant species tend many aphid species on different tree species, especially *Pinus pinaster* and *P. halepensis*, two monoterpene emitters that are broadly distributed in the Mediterranean region (Karl et al. 2009). Thus, monoterpene emission by other tree species apart from *Q. ilex* could be modified also by tending ants.

Monoterpenes play different roles in the troposphere and for plants. In the troposphere, monoterpenes can contribute to the formation of  $\text{O}_3$  from a series of reactions with  $\text{NO}_x$  emitted from anthropogenic and natural sources, and to the formation of secondary organic aerosols as a result of the gas/particle partitioning of their reaction products (Karl et al. 2009). At the plant surfaces, as well as in the mesophyll monoterpenes quench  $\text{O}_3$  that protects the plant against oxidative stress (Holopainen 2004), and protects the plant against higher temperatures (Copolovici et al. 2005). Taking into account the facts that the invasive ant *L. neglectus* decreased total monoterpene emissions by 76% in comparison with *L. grandis*, that this invasive ant is



expanding its range in Europe (Espadaler et al. 2007), and that it is displacing native ants we suggest that massive aphid outbreaks in areas where this invasive ant occurs should be considered when modelling the emission of VOCs. A rapid way to include this information in inventories will be to estimate the proportion of trees colonized only by *L. neglectus*. Moreover, it will be necessary to know how monoterpene emission changes in other tree species colonized by *L. neglectus*, in particular other *Quercus* and *Pinus* spp., which are the main monoterpene emitters in the Mediterranean region (Karl et al. 2009).

In summary, the results of this study highlight that the amount of various terpenes emitted by holm oaks can increase or decrease (depending on the compound), or new compounds can be released, according to the presence of aphids and the identity of the tending ant species. However, the range of ant colony sizes tested here had no effect on terpene emission rates. We discard the possibility that changes in  $\alpha$ - and  $\beta$ -pinene, sabinene, and camphene emissions were caused by emissions from ants or aphids because of the scale at which these insects emit VOCs. Therefore, from an ecological perspective, the altered monoterpene composition of the *Q. ilex* blend associated with the feeding of aphids and tending ants may affect the protection that monoterpenes exert against episodes of high O<sub>3</sub> and temperatures during the Mediterranean summer (Pinto et al. 2007).

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