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Tobacco overexpressing β-ocimene induces direct and indirect responses against aphids in receiver tomato plants

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Abstract

In the last decade plant-to-plant communication has received an increasing attention, particularly for the role of Volatile Organic Compounds as possible elicitors of plant defense. The role of β-ocimene as an interspecific elicitor of plant defense has been recently assessed in multitrophic systems including different plant species (Solanaceae, Poaceae, legumes) and different pest species including chewer insects and phytophagous mites. Both chewer insects and phytophagous mites are known to elicit specific plant defensive pathways which are different (at least in part) from those elicited by sap feeders. The aim of this research was to fill this gap of knowledge and to assess the role of β-ocimene as an elicitor of plant defense against aphid pests, which are sap feeders.

For this purpose we used as transgenic tobacco plant releasing an odour plume enriched in this compound as emitter and a tomato plant as receiver. We selected the aphid \textit{Macrosiphum euphorbiae} and its natural enemy, the parasitoid \textit{Aphidius ervi}, as the targets of plant induced defense.

Tomato plant defense induced by β-ocimene was assessed by characterizing the aphid performance in terms of fixing behaviour, development and reproduction (direct plant defense) and the parasitoid performance in terms of attraction towards tomato plants (indirect plant defense). The characterization of tomato response to β-ocimene was completed by the identification of Volatile Organic Compounds as released by conditioned tomato plants.

Tomato plants that were exposed to the volatiles of transgenic tobacco enriched in β-ocimene resulted in less suitable for the aphids in respect to control ones (direct defense). On tomato plants “elicited” by β-ocimene we recorded: a significant lower number of aphids settled; a significant lower number newborn nymphs; a significant lower weight of aphids feeding.

In addition, tomato plants “elicited” by β-ocimene resulted became more attractive towards the parasitoid \textit{A. ervi} than control ones.

These results could be explained at least in part by examining the composition of the Volatile Organic Compounds released by tomato plants “elicited” by β-ocimene. Indeed, we found a significantly higher release of several compounds including methyl salicylate and cis-3-hexen-1-ol. These two compounds have been demonstrated to impair aphid development and reproduction and to be involved in the attraction of the aphid parasitoid \textit{A. ervi}.

By considering the ubiquity of β-ocimene and its ability to regulate the communication of plants belonging 30 to different species (if not families), we concluded that this compound is an ideal candidate for new 31 strategies of sustainable control of agricultural pests.
Keywords

- *Macrosyphum euphorbiae*;
- *Aphidius ervi*;
- Methyl salicylate;
- *cis*-3-Hexen-1-ol

**Introduction**

Plant volatiles play a crucial role in plant communication with the surrounding environment, performing a variety of functions from attraction of pollinators and seed dispersers to protection from biotic stresses such as phytopathogens and insect herbivore (Maffei et al., 2011). Among these functions, the ability to promote plant defence has received increasing attention in the last decades, leading to the characterization of Volatile Organic Compounds (VOC) that elicit a physiological response in the emitter and in neighbouring individuals (Arimura et al., 2000, Frost et al., 2007 and Glinwood et al., 2009). Heil and Karban (2010) reviewed papers retrace the main steps that strengthened the hypothesis of plant-to-plant communication. Field and laboratory studies have provided compelling evidence that receiver plants are able to respond to volatile cues from conspecific or interspecific emitter plants, by activating signalling pathways eventually leading to gene expression and the synthesis of defence metabolites. In most cases, the emitter plant was induced by mechanical or caterpillar damage, while the receiver one was tested for attractiveness towards predators and parasitoids and/or characterizing the “induced” VOC emission (Zebelo and Maffei, 2012 and references cited therein). Conversely, a few studies have focused on sap-sucking arthropods, demonstrating the existence of airborne plant-to-plant signalling that alters acceptance of the receiver plant by aphids, spider mites and attractiveness towards their natural enemies (Glinwood et al., 2004, Glinwood et al., 2009 and Muroi et al., 2011). Nevertheless, there is a scarcity of information about the identity of chemical compounds that regulate these complex interactions. One functional approach to characterize the effect of single volatile compounds is the use of transgenic or mutant plants that are genetically modified in their potential to emit or receive VOC signals. Some evidence has highlighted the role of monoterpenoids (e.g., ocimene and myrcene) that regulate gene transcription and prime indirect defences in response to herbivore attack (Godard et al., 2008 and Muroi et al., 2011). Such ubiquitously released VOCs are often present in the blends of volatiles emitted by damaged or induced plants. Broad bean plants that had been exposed to (Z)-jasmone in the vapor phase subsequently released significantly enhanced levels of β-ocimene compared with control plants, resulting more attractive for the aphid parasitoid *Aphidius ervi* (Birkett et al., 2000). The enhanced attractiveness could be partially explained by the high level of response of female parasitoids to purified β-ocimene in wind tunnel bioassay (Birkett et al., 2000).

The aim of our study was to assess the role of β-ocimene as a trans-specific elicitor of plant defences against aphids. For this purpose we used transgenic tobacco plants releasing a plume enriched by β-ocimene as emitters (Muroi et al., 2011), and tomato plants cv San Marzano as receivers. We aimed for the biological, chemical and behavioural characterization of direct and indirect defensive responses against aphids in tomato elicited by transgenic tobacco volatiles.
Materials and methods

Plant material

The emitter plants used in all assays were wild-type tobacco, *Nicotiana tabacum* L. (Solanaceae) cv SR1 and the relative transgenic plants overexpressing a lima bean $\beta$-ocimene synthase that emits an odour plume enriched by $\beta$-ocimene (*Muroi et al., 2011*). The receiver plants used in all assays were tomato plants *Solanum lycopersicum* L. (Solanaceae) cv San Marzano (*Digilio et al., 2012*). All plants were grown in sterilized soil in three different greenhouses (one to rear the plants and the others for each treatment, see below) under the following conditions: 24 ± 2 °C and 70 ± 10% RH, and a 16:8 light/dark photoperiod.

Plant treatments

Three week-old tomato plants were conditioned by placing them downwind (flow rate 30 cm s$^{-1}$) of transgenic (tomato transgenic tobacco system = TTT) and wild-type genotypes of tobacco (tomato wild-type tobacco system = TWT) for 72 h as described in *Muroi et al. (2011)*.

Insects

The tomato and potato aphid *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae) is permanently reared in an environmental cabinet at the Institute for Sustainable Plant Protection, National Research Council, on tomato plants, cv San Marzano under the same conditions as described above. The original strain was field-collected in 2003 in Scafati (Campania, Italy) from tomato cv San Marzano. The parasitoid wasp *Aphidius ervi* (Haliday) (Hymenoptera: Braconidae) is continuously reared in an environmental chamber at 20 ± 1 °C, with a photoperiod of 18/6 h of light/darkness and 60 ± 5% RH, on its natural host, the pea aphid *Acythosiphon pisum* (Harris), maintained on potted broad bean plants (*Vicia faba* L. cv Aquadulce), as previously described (*Guerrieri et al., 2002*).

Induction of direct defence

The induction of direct defence against aphids was assessed in terms of behaviour and performance of *M. euphorbiae* on conditioned tomato plants (TWT and TTT). Immediately after conditioning, receiver plants were sealed in a Perspex cage (4.7 dm$^3$) together with a 2 ml Eppendorf tube, containing twenty aphids (apterous fourth instars or newly born adults), weighed as a pool. The tube was placed with its base in the soil, and its opening next to the tomato stem, to facilitate the aphids in walking/climbing on the plant to choose a feeding site. Each experiment was repeated 6 times.

The Perspex cage was opened after 48 h and aphids, dead or alive, were counted indicating the number remaining in the Eppendorf tube, wandering on cage walls, pot or tube, or feeding/standing on the plant. For living aphids, the pooled weights, the nymphs produced and the aphids settled after 48 h on TTT and TWT were compared by ANOVA.

Induction of indirect defence (*A. ervi* attraction)

Attraction assay for the aphid parasitoid *A. ervi* was conducted in a single-choice wind tunnel bioassay. Behavioural observations were carried out as described previously (*Sasso et al., 2009*). In brief, ninety parasitoid females were tested for each target by releasing them individually in the odour plume 35 cm downwind from the target; each female was only used once and observed for a maximum time of 5 min. Experimental conditions were: 20 ± 1 °C, 65 ± 5% RH, 70 μmol m$^{-2}$ s$^{-1}$
PPF, 30 cm s\(^{-1}\) wind speed. Flight behaviour data were recorded by calculating the percentage of oriented flights towards the target plant and landings on the target plant. Behavioural experiments were conducted on several days and the target plants offered in a random order to reduce any daily or hourly bias. For each target (TTT or TWT), five conditioned plants were tested. The percentage of response (oriented flights and landings on the target) to each target was evaluated. The number of parasitoids responding to each target in all experiments was compared by a \(G\)-test for independence with William's correction (Sokal and Rohlf, 1995). The resulting values of \(G\) were compared with the critical values of \(\chi^2\) (Sokal and Rohlf, 1995).

Identification of plant volatiles

Plant volatiles were collected from all target plants (TTT, TWT) immediately after the wind tunnel bioassay. All glassware, silicon, and Teflon connections were scrupulously cleaned and heated at 100 °C overnight before use. Plant volatiles were collected by an airtight entrainment system immediately after the wind-tunnel bioassay. A single plant in a pot was placed into a bell jar (20 L) sealed with parafilm and connected to a circulation pump with a 200 ml/min flow rate. Before returning to the pump, the air was passed through an adsorbent trap made of Tenax TA\textsuperscript{®} (Cat. no. 226-336, SKC, Eighty Four, PA, USA) connected to the system by a Teflon-capped glass plug. In order to reduce any stress to the plant in the system, each collection lasted 3 h. Collected volatiles were analyzed by an integrated system including thermal desorber (Tekmar TD-800) mounted on a gas chromatograph (column: RTX-200, 60 m, 0.25 mm ID, 0.25 μm, carrier gas: He) coupled to a mass spectrometer detector as described in Digilio et al. (2010). The resulting peaks were compared with 36 available authentic standards (Appendix 1) and a compound database library (National Institute of Standards and Technology). The relative quantity of each compound was calculated by comparing the relative area peak with that of a calibration curve created for each identified compound. The volatiles are indicated as micrograms per plant fresh weight. The volatile patterns of treated plants were analyzed using principal component analysis (PCA) and multivariate analysis of variance (MANOVA) using SPSS. Fourteen major compounds were used to each analysis. The volatiles collected from TTT, TWT plants were compared with ANOVA test for each single volatile compound separately.

Results and discussion

The role of VOCs in direct and indirect defences of tomato plants against aphids has been recently characterized (Digilio et al., 2010, Digilio et al., 2012, Sasso et al., 2007 and Sasso et al., 2009). In this study we demonstrate the interaction between interspecific emitter and receiver plants in both direct and indirect defences. Based on the nature of \(\beta\)-ocimene, ubiquitously emitted form a suite of plant species when damaged, and from specific organs such as floral tissues (Shimoda et al., 2012), we used the different plant species (tomato) for plant-to-plant communication assays, and observed their responses.

Emission of \(\beta\)-ocimene by transgenic tobacco induces VOCs in receiver tomato plants

Muroi et al. (2011) showed the ability of maize plants to behave as a \(\beta\)-ocimene receiver, inferring that a wide range of plant taxa, including not only Solanaceae but also Poaceae and legumes, respond to \(\beta\)-ocimene. Exposure of tomato plants to the volatiles of either TTT or TWT induced the release of 14 VOCs, including green leaf volatiles (i.e., trans-3-exen-1-ol and cis-3-exen-1-ol), terpenoids (e.g., limonene) and benzenoids (e.g., methyl salicylate) (Table 1). PCA showed that the volatile blend composition of TTT and TWT are separated in two groups (Fig. 1). Among volatiles,
**trans**-3-exen-1-ol, **cis**-3-exen-1-ol and methyl salicylate were released from TTT in higher amounts than from TWT (Table 1). Overall, TTT released a significantly higher total amount of VOCs than TWT (Wilks’ lambda = <0.001, *F* = 1036.147, *N* = 6, *P* = <0.001). The significant increase in methyl salicylate is noteworthy since this compound is active in the attraction of the aphid parasitoid *A. ervi*, being detected at antennal level at concentration as low as 100 μg/ml (Sasso et al., 2009). Nonetheless, this compound is commonly related to aphid infestation (Blande et al., 2010, Sasso et al., 2007 and Zhu and Park, 2005). In the field, this compound was particularly attractive for syrphid flies (Diptera: Syrphidae) (James, 2005) and the lacewing *Chrysopa nigricornis* Burmeister (Neuroptera: Chrysopidae) (James and Price, 2004), which are among the more effective natural enemies of aphids. A similar consideration holds for the green leaf volatile **cis**-3-hexen-1-ol. This compound was found to alter the settling of *M. euphorbiae* on tomato plant in both aerial and contact bioassay, resulting in a significantly lower number of aphids settled after 24 h (Digilio et al., 2012). Moreover, **cis**-3-hexen-1-ol plays a key role in the attraction of *A. ervi*, being detected at concentrations as low as 0.1 mg/ml (Sasso et al., 2009). Therefore, higher emission of these compounds by TTT plants than by TWT plants are likely to exert a potential effect on both aphid and its parasitoid.

Table 1.

Volatiles emitted by tomato plants exposed to transgenic tobacco (TTT) and wild-type tobacco plants (TWT). Compounds are listed in order of retention time. Values are expressed as mean values (μg g⁻¹ fr wt) ± standard error; see footnote for significance levels *n* = 5.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>TTT</th>
<th></th>
<th>TWT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td><strong>trans</strong>-3-Exen-1-ol</td>
<td>4.490</td>
<td>0.223</td>
<td>0.951</td>
<td>0.061</td>
</tr>
<tr>
<td><strong>cis</strong>-3-Hexen-1-ol</td>
<td>1.355</td>
<td>0.211</td>
<td>0.315</td>
<td>0.023</td>
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<tr>
<td>α-Pinene</td>
<td>3.398</td>
<td>0.417</td>
<td>4.611</td>
<td>2.079</td>
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<tr>
<td>β-Pinene</td>
<td>0.239</td>
<td>0.042</td>
<td>0.064</td>
<td>0.010</td>
</tr>
<tr>
<td>Limonene</td>
<td>0.939</td>
<td>0.146</td>
<td>0.249</td>
<td>0.037</td>
</tr>
<tr>
<td><strong>cis-β-Ocimene</strong></td>
<td>1.156</td>
<td>0.206</td>
<td>0.265</td>
<td>0.034</td>
</tr>
<tr>
<td><strong>trans-β-Ocimene</strong></td>
<td>1.150</td>
<td>0.284</td>
<td>0.337</td>
<td>0.032</td>
</tr>
<tr>
<td>γ-Terpinene</td>
<td>0.103</td>
<td>0.018</td>
<td>0.025</td>
<td>0.002</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>0.066</td>
<td>0.014</td>
<td>0.015</td>
<td>0.002</td>
</tr>
<tr>
<td>Methyl salicylate</td>
<td>46.722</td>
<td>7.757</td>
<td>11.992</td>
<td>1.164</td>
</tr>
<tr>
<td><strong>cis-Caryophyllene</strong></td>
<td>7.826</td>
<td>1.526</td>
<td>1.816</td>
<td>0.181</td>
</tr>
<tr>
<td><strong>trans-β-Caryophyllene</strong></td>
<td>0.095</td>
<td>0.050</td>
<td>0.113</td>
<td>0.055</td>
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<tr>
<td>γ-Elemene</td>
<td>3.505</td>
<td>0.680</td>
<td>1.597</td>
<td>0.495</td>
</tr>
<tr>
<td><strong>cis-β-Farnesene</strong></td>
<td>14.480</td>
<td>2.929</td>
<td>3.622</td>
<td>0.324</td>
</tr>
<tr>
<td>Total</td>
<td>85.523</td>
<td>13.994</td>
<td>25.973</td>
<td>3.564</td>
</tr>
</tbody>
</table>

** Significant difference between tomato plants exposed to TTT and TWT volatiles. ANOVA, <0.01.
**Significant difference between tomato plants exposed to TTT and TWT volatiles. ANOVA, <0.05.***

**Significant difference between tomato plants exposed to TTT and TWT volatiles. ANOVA, <0.001.***

Fig. 1.

Principal component analysis (PCA) of volatile pattern of tomato plants exposed to volatiles from transgenic tobacco (TTT) or wild-type tobacco (TWT) plants. First and second principal component plotted against each other. Percentage variation explained between brackets. TWT variables are indicated by squares (■) and TTT by circles (●).

**Emission of β-ocimene by transgenic tobacco induces direct defence in receiver tomato plants**

Along with the induction of VOCs, the emission of β-ocimene by transgenic tobacco plants caused (TTT) a significant alteration in the behaviour and performance of *M. euphorbiae* on receiver tomato plants, compared to tomato plants exposed to the volatiles of TWT. In particular, a significant alteration in the aphid settling was recorded, with only a half of tested aphids fixed on
TTT after 48 h (Fig. 2). In addition, both development and reproduction were impaired on TTT plants. The overall weight of aphids feeding on TTT was significantly lower than that recorded on TWT plants. Similarly, the number of nymphs produced on these plants was half that recorded on TWT (Fig. 2). The higher amount of methyl salicylate released by TTT plants compared with TWT can account, at least in part, for the better defensive performance recorded. Indeed, tomato plants treated aerially with methyl salicylate became unfavourable for the settlement and reproduction of *M. euphorbiae* (Digilio et al., 2012). However, the defensive response elicited by TTT was characterized not only by a reduced number of aphids settled on the treated plant but also by slower development as exemplified by a lower total weight and lower fecundity in terms of nymphs produced by settled aphids (Fig. 2). The total number of newly developed nymphs on TTT was about one-third of that on TWT. These results confirm findings with bird cherry oat aphid *Rhopalosiphum padi*, whose population growth in the field is reduced by treating plants with a pellet formulation releasing methyl salicylate (Ninkovic et al., 2003).

**Fig. 2.** Performance of *Macrosiphum euphorbiae* on tomato plants exposed to transgenic tobacco (TTT) volatiles or wild-type tobacco plants (TWT) volatiles. Values are means ± standard error recorded after 48 h, n = 6, each replicate was made of 20 aphids. Asterisks indicate significant difference within the same raw (*t*-test, *P* < 0.05, ***P* < 0.001).

**Emission of β-ocimene by transgenic tobacco induces attraction of aphid parasitoids in receiver tomato plants**

Having assessed the ability of tomato to alter aphid behaviour on exposure to transgenic tobacco volatiles, we evaluated tomato potential to attract parasitoids in wind tunnel bioassays. TTT plants were significantly more attractive to *A. ervi* than were TWT plants (Fig. 3). Significant differences were recorded for oriented flights (63% TTT vs 24% TWT; $G = 24,234$, df = 1, $\chi^2 = 10,827$) and for landings on the target (42% TTT vs 16% TWT; $G = 6930$, df = 1, $\chi^2 = 6635$) (Fig. 3). It has previously been reported that tomato plants infested by *M. euphorbiae* release significantly higher amounts of β-ocimene compared with uninfested plants (Sasso et al., 2007), leading to high attraction of *A. ervi* when tested in wind-tunnel bioassay (Birkett et al., 2000). In our bioassay, TTT plants emitted a significantly higher amount of β-ocimene, four times higher than control plants (TWT) (Table 1). We cannot exclude a possible “buffer” effect mediated by adhesion of β-ocimene to the superficial waxy layer of the receiver plants that could have influenced the flight behaviour of *A. ervi*. However, in TTT plants we found a significantly higher release of methyl salicylate and cis-3-hexen-1-ol that were both implicated as key compounds in the foraging behaviour of *A. ervi* (Sasso et al., 2009). In addition, methyl salicylate was found to alter the performance of the aphid.
(settling and/or reproduction) (Digilio et al., 2012) and this may account for the reduced performance of this aphid on TTT plants (see above).

![Graph showing flight and landing behavior of Aphidius ervi](image)

**Fig. 3.** Flight behaviour of the aphid parasitoid *Aphidius ervi* behaviour towards on tomato plants conditioned by wild-type tobacco (TWT) volatiles and transgenic tobacco (TTT) volatiles. Values indicate the percentage of female showing oriented flights and landings on tomato plants: each assay was conducted using 90 females tested against 6 plants. Asterisks indicate significant differences (*G*-test, **P** < 0.01, ***P** < 0.001).

### Conclusions

Volatile compounds play a crucial role in plant–insect interactions. They are used by phytophagous insects to identify a suitable plant as well as by natural enemies of phytophagous insects to identify an infested plant where to forage. The more an insects is able to detect key volatile compound(s), the more ecological advantage at all levels of the multitrophic chain it receives. The degree of species specificity is highly variable and in this paper we stressed the effect of a compound, β-ocimene, whose release by different plant species is associated to the attack of different pest species.

Tomato plants exposed to transgenic tobacco releasing a VOC blend including β-ocimene as a predominant product (TTT) resulted in greater defense against *M. euphorbiae* and greater attraction of the aphid parasitoid *A. ervi*, in comparison with tomato plants exposed to the volatiles of untransformed tobacco plants (TWT). The current study confirms the role of β-ocimene in eliciting a diverse defense response in tomato plants, in addition to results reported for lima bean and maize (Muroi et al., 2011). Since VOCs are increasingly tested in the field for their possible use in biological control systems (James, 2005 and Ninkovic et al., 2003), we suggest that β-ocimene might represent an ideal compound for this application.

### Appendix 1. Standards used for the identification of volatiles collected by air-entrainment of head space from tomato plants

(+)-longifolene, (Z)-3-hexen-1-ol, 3-carene, 6-methyl-5-hepten-2-one, anisole-p-allyl, camphor, chlorobenzene, cis-nerolidol, decane, dodecene, eucalyptol, eugenol, hexanal, humulene (=α-caryophyllene), linalool, methyl salicilate, menthol, ocimene, p-cymene, p-dichlorobenzene (IS),

References