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Olive fruits infested with olive fly larvae respond with an ethylene burst and the emission of specific volatiles

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Abstract Olive fly (*Bactrocera oleae* R.) is the most harmful insect pest of olive (*Olea europaea* L.) which strongly affects fruits and oil production. Despite the expanding economic importance of olive cultivation, up to now, only limited information on plant responses to *B. oleae* is available. Here, we demonstrate that olive fruits respond to *B. oleae* attack by producing changes in an array of different defensive compounds including phytohormones, volatile organic compounds (VOCs), and defense proteins. *Bactrocera oleae-infested* fruits induced a strong ethylene burst and transcript levels of several putative ethylene-responsive transcription factors became significantly upregulated. Moreover, infested fruits induced significant changes in the levels of 12-oxo-phytodienoic acid and C_{12} derivatives of the hydroperoxide lyase. The emission of VOCs was also changed quantitatively and qualitatively in insect-damaged fruits, indicating that *B. oleae* larval feeding can specifically affect the volatile blend of fruits. Finally, we show that larval infestation maintained high levels of trypsin protease inhibitors in ripe fruits, probably by affecting post-transcriptional mechanisms. Our results provide novel and important information to understand the response of the olive fruit to *B. oleae* attack; information that can shed light onto potential new strategies to combat this pest.

Keywords: *Bactrocera oleae*; biotic stress; *Olea europaea*; phytohormones; plant defense; volatile organic compounds
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INTRODUCTION

The olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is the most dangerous fruit pest of olive trees in the Mediterranean basin. Damages appear during fruiting, when the insect females lay their eggs in the olive fruits pulp and, subsequently, larvae feed and grow in the fruit tissues inducing serious losses, both qualitative and quantitative, to the fruit and oil production. Despite the severe impact on yield, the expanding economic importance of olive cultivation and the need of reducing chemical control of the insect, up to now, only a few molecular studies aimed to study the olive defense mechanisms against the fruit fly have been conducted.

Plant defense mechanisms against insects are complex and involve an array of different and coordinated responses, modulated by the orchestration of different phytohormones (Wu and Baldwin 2010). Previous studies on olive tree demonstrated that ethylene treatments significantly decrease the olive moth *Prays oleae* (Bern) population, suggesting that this phytohormone has a role in the activation of olive defense responses against herbivores (Ramos et al. 2008). Ethylene signaling induces an immediate transcription response in plants and increases the transcript levels of defense-related genes encoding for toxins or antidigestives, including chitinase, β-1,3 glucanase (Wu and Bradford 2003), pathogenesis-related (PR) genes (Lawton et al. 1994), and protease inhibitors (PIs) (Cervantes et al. 1994; Jones et al. 1995; Harfouche et al. 2006). Similar defensive genes have been found to be induced in olive fruits under *B. oleae* herbivory attack. In this regard, chitinases, PR proteins, protease inhibitors, and β-1,3 glucanases have been found to be significantly induced at the mRNA level in *B. oleae*-infested fruits compared with controls (Corrado et al. 2012). In olive fruits, ethylene also plays a central role in regulating maturation, with the levels of this phytohormone peaking at the later phases of fruit development (Lavee and Martin 1981; Kitsaki et al. 1999; Tsantili and Pontikis 2004).

In addition to defensive proteins, defense compounds include a diverse array of secondary metabolites. For example, within the Oleaceae family, herbivore-attacked tissues of privet trees (*Ligustrum obtusifolium*) release enzymes from organelles that activate oleuropein - a secoiridoid compound present also in olive - into a very strong protein denaturant that has protein-cross-linking and lysine-alkylating activities. This defense mechanism has been proposed to exist also in olive (Kubo et al. 1998; Iannotta et al. 1999) and is thought to have adverse effects against herbivores by

decreasing the nutritive value of dietary protein (Konno et al. 1999).

Plants also protect themselves by producing indirect defenses such as volatile organic compounds (VOCs) and extrafloral nectars (Dicke et al. 1990; Turlings et al. 1990; Dicke and Baldwin 2010). Herbivore-induced plant volatiles have multiple functions, such as the attraction of predatory arthropods (Turlings et al. 1995; De Moraes et al. 1998; Schuman et al. 2009), the repellence of herbivores (De Moraes et al. 2001), the induction of systemic-induced resistance within an individual (Karban et al. 2006), and the activation of defenses in neighboring plants before being attacked (Baldwin et al. 2006).

Olive volatiles acting as attractive or repellent for the olive fly females have been identified. Attractive compounds include ethylbenzene, α-pinene, toluene, and *n*-octane, whereas (*E*)-2-hexenal is considered repellent (Lo Scalzo et al. 1994; Scarpati et al. 1996). α-Pinene acts as an oviposition stimulator, while (*E*)-2-hexenal, hexanal, and *o*-diphenolic derivatives of oleuropein act as oviposition deterrents (Lo Scalzo et al. 1994; Scarpati et al. 1996). It has also been shown that olive flies are also attracted by volatiles emitted by epiphytic bacteria, probably contributing to the location of plant hosts (Sacchetti et al. 2007, 2008). A wide range of parasitoids, as for example *Psyttalia concolor*, *Psyttalia lounsburyi*, *Eurytoma martellii*, and *Pnigalio agraules* live at the expenses of *B. oleae* third instar larvae, whereas *Fopius arisanus* is an egg-pupal parasitoid and *Eupelmus urozonus* attacks the third instar larvae and the pupae (Boccaccio and Petacchi 2009; Daane and Johnson 2010). The presence of a high number of olive fly parasitoids has led to the hypothesis that olive may have evolved indirect defense responses by modulating emitted volatiles to attract natural enemies of olive fly.

Olive fruit volatiles were previously characterized in processed table olives and in virgin olive oil. High amounts of C_6 compounds have been detected, indicating that lipoxygenases (LOXs) and hydroperoxide lyase (HPL) are active in the olive fruits. Minor volatile constituents include, among others, C_9 aldehydes, hydrocarbons, uncharacterized sesquiterpenes, and unknown compounds (Campeol et al. 2003; Baccouri et al. 2008; Sabatini and Marsilio 2008; Masi et al. 2014; Sansone-Land et al. 2014). To the best of our knowledge, an extensive study on fruit volatiles emitted in response to olive fly attack has not been reported so far.

The significance of the change in the fruit volatile blend induced by the olive fly attack can be diverse and may have consequences on the ecological interactions of olive fruits with the environment. For example, it may affect the behavior of the *B. oleae* population itself but also that of its natural enemies. Moreover, emitted volatiles may be involved in plant-plant communication, leading to elicitation of defense responses in non-damaged plants, or may also have an effect on the bacterial community living on this plant.

The aim of this study was to shed light on the induced defense responses of olive (*Olea europaea*) to the olive fly through the analysis of major players of the defense response system in plants, including phytohormones, emitted volatiles, activation of gene expression, and anti-herbivore defensive proteins. We report on several defense-related mechanisms that become activated in infested olive fruits and which start to unveil how olive plants may deploy counteracting responses to herbivory by the fruit fly.

RESULTS

Bactrocera oleae induces ethylene accumulation in olive fruits

To investigate the potential role of major defense-related phytohormones in olive responses to *B. oleae* larvae, the level of ethylene, jasmonic acid (JA), JA-isoleucine (JA-Ile), 12-oxo-phytodienoic acid (OPDA), and salicylic acid (SA) were quantified in infested drupes and control undamaged fruits at 100-110 d after flowering (DAF). Infested drupes of the medium-susceptible cultivar Leccino showing oviposition puncture were collected in the field and immediately frozen in liquid nitrogen. Subsequently, the fruits were dissected in order to check the olive fly attack stage and only those containing II-III instar larvae were selected for phytohomone analysis (Figure 1A). Additionally, to investigate the potential activation of the HPL pathway by larval feeding, C_{12} products of this pathway were also quantified (e.g. 12-oxo-dodecenoic acid (traumatin), 12-hydroxy-dodecenoic acid, and 9-hydroxy-12-oxo-dodecenoic acid (9-hydroxy-traumatin)) (Kallenbach et al. 2011).

Comparison of ethylene levels in larval infested and control drupes showed a strong significant increase in the level (~23 fold) of this phytohormone in infested fruits of the cultivar Leccino. This result was also confirmed in fruits of the cultivar Coratina, where ethylene increased approximately 10 fold in infested fruits (Figure 1B). Coratina is considered a more susceptible variety to olive fly attack compared with Leccino (Iannotta and Scalercio 2012), and based on these results, increased susceptibility may be associated with differences in the ethylene induction levels (see Discussion).

Small but significant increased levels of OPDA (~2 fold) were also detected in larval infested drupes compared with control (Figure 1C), suggesting a weak activation of the allene oxide synthase (AOS) pathway. Non-significant changes in JA levels and undetectable levels of JA-Ile (Figure 1C) in both infested and control drupes suggested that activation of the AOS pathway was largely limited to the plastidial steps. In contrast to the AOS pathway, the HPL pathway was strongly activated in infested fruits. Increased accumulation of 12-oxo-dodecenoic acid (traumatin), 12-hydroxy-dodecenoic acid, and 9-hydroxy-traumatin (1.8, 5.8, and 1.6 fold, respectively) was detected in infested drupes (Figure 1C). In particular, 12-OH-dodecenoic acid, as a mixture of the two isomers 12-OH-(9Z)-dodecenoic acid and 12-OH-(10E)-dodecenoic acid, was the compound showing the highest increase. Finally, SA levels were decreased by 2.7 fold in infested fruits (Figure 1C).

Analysis of ethylene induced genes in *B. oleae*-infested olive fruits

To investigate if the ethylene burst detected in infested fruits was associated with the induction of mRNA levels of genes involved in ethylene biosynthesis and signaling pathways, 41 candidate genes from the olive fruit transcriptome database (OLEA ESTdb: http://140.164.45.140/oleaestdb/; Alagna et al. 2009) were identified according to their similarity to 1-aminocyclopropane-1-carboxylate synthase (ACS),

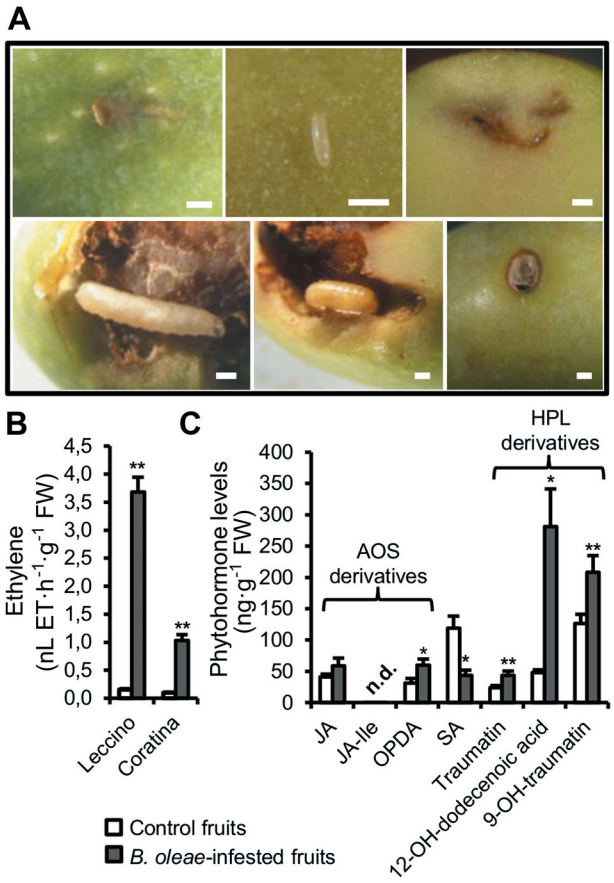

Figure 1. *Bactrocera oleae* induces changes in phytohormones levels of olive (*Oleae europaea*) fruits

(A) Stages of *B. oleae* attack in olive fruits at 100-110 d after flowering (DAF). Triangular oviposition puncture, egg, feeding tunnels, III-instar larva, pupa, exit hole. Scale bar = 1 mm. **(B)** Ethylene emissions collected from control undamaged fruits (white bars) and from fruits infested by *B. oleae* larvae (gray bars) of Leccino and Coratina cultivars at 100-110 DAF. Values are means of three biological replicates with standard error (SE) indicated. Statistically significant differences were determined by Student's *t*-test (**$P < 0.01$). **(C)** Levels of jasmonic acid (JA), JA-Isoleucin (JA-Ile), cis-12-oxo-phytodienoic-acid (OPDA), salicylic acid (SA), traumatin (mixture of 12-oxo-(10E)-dodecenoic acid and 12-oxo-(9Z)-dodecenoic acid), 12-OH-dodecenoic acid (mixture of 12-hydroxy-(9Z)-dodecenoic acid and 12-hydroxy-(10E)-dodecenoic acid), and 9-OH-traumatin (9-hydroxy-12-oxo-(10E)-dodecenoic acid) were measured in *B. oleae*-infested fruits (white bars) and control undamaged fruits (gray bars) of cultivar Leccino. Values are means of 10 biological replicates with SE indicated. Statistically significant differences were determined by Student's *t*-test (*$P < 0.05$, **$P < 0.01$). n.d. = not detected.

1-aminocyclopropane-1-carboxylate oxidase (ACO), ethylene receptor 1 (ETR1), ethylene receptor 2 (ETR2), ethylene-insensitive protein 2 (EIN2), ethylene-insensitive protein 3 (EIN3), and APETALA2/ethylene-responsive factor (AP2/ERF) transcription factors. The expression level of these genes was analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in control undamaged and *B. oleae*-infested fruits at 100-110 DAF (Tables 1, S1). Consistent with the increased ethylene levels in infested fruits, the mRNA level of three transcripts putatively involved in ethylene biosynthesis (*OeACS1*, *OeACO1*, and *OeACO2*) (Table 1) were 71, 34, and 8 fold higher in infested fruits compared with the control, respectively (Figure 2). Additionally, three ERF transcripts (*OeERF1*, *OeERF2*, and *OeERF3*) (Table 1), putatively involved in ethylene signaling were 7, 4, and 13 fold higher than in the control fruits, respectively (Figure 2). Ethylene is known to be the main phytohormone modulating the olive fruit ripening process; therefore, to assess if the increased expression of genes putatively involved in ethylene biosynthesis, observed in the infested fruit, was a consequence of a ripening-independent response, their relative mRNA levels were also analyzed in ripe fruits and compared with green undamaged and *B. oleae*-infested fruits (Figure 2). The results indicated that the induction of *OeACS1*, *OeACO1*, *OeERF1*, and *OeERF3* transcripts in infested fruits is not an effect of a ripening-controlled program, but more likely it is related to the activation of plant response to *B. oleae*. In fact, *OeACS1* and *OeERF3* do not change significantly along fruit maturation, *OeACO1* and *OeERF1* transcript levels decrease with the ripening. In contrast, *OeERF2* mRNA levels slightly increase in ripe fruits but they reach values lower than in *B. oleae*-infested fruits, while *OeACO2* mRNA levels increase in ripe fruits reaching values not significantly different from those observed in infested fruits.

To further explore the regulation of gene expression induced by ethylene in infested olive fruits, we searched for regulatory motifs involved in ethylene responses in the promoter and 5′- and 3′-untranslated regions (UTRs) of defensive genes (*OeChit*, *OeTPI2*, and *OePRp27*) - previously reported to be strongly induced in *B. oleae*-infested fruits of cultivar Leccino (Corrado et al. 2012) and in known ethylene-responsive genes (e.g. *OeACO1*, *OeACO2*, *OeERF1*, *OeERF2*, and *OeERF3*). We found the 8 bp motif, ATTTCAAA, previously recognized as an ethylene-responsive element (ERE) (Itzhaki et al. 1994) in *OeChit* and *OeACO1* promoters, positioned at −1,455 bp and −1,390 bp before their respective start codons, supporting the hypothesis that these genes may be regulated by ethylene in olive fly-attacked fruits. In contrast, we could not find canonical ethylene-responsive motifs in the promoter regions of *OeTPI2* and *PRp27* and in the analyzed ERFs, which are also induced in *B. oleae*-infested fruits. In this case, however, we cannot exclude the possibility that these genes are regulated by uncharacterized ethylene-responsive motifs or transcription factors in olive fruits.

Constitutive and induced levels of TPI proteins in green and ripe fruits

Trypsin protease inhibitors (TPIs) represent an important component of defense responses against herbivorous insects in several plant species. The mRNA levels of TPI-encoding genes have been previously shown to increase in olive fruits infested by *B. oleae* (Corrado et al. 2012), however data on TPI protein amount have never been reported in olive tissues. Olive fruits are rich in phenols and secoiridoids (Alagna et al. 2012) that interfere with TPI assays; therefore, by using the original protocol developed by Jongsma and co-workers

Table 1. Transcripts of ethylene biosynthesis and signaling network differentially expressed in olive (*Olea europaea*) infested by *Bactrocera oleae*

Putative function	Transcript	Olea DB ID[a]	Length (bp)	Blast X Best hit	E-value	Score	Similarity (%)
1-Aminocyclopropane-1-Carboxylate synthase	*OeACS1*	OLEEUCl009961:Contig1	275	NP_001236141.1 (*Glycine max*)	3E-41	150	90
1-Aminocyclopropane-1-Carboxylate oxidase	*OeACO1*	OLEEUCl056880:Contig1	294	AEM62885.1 (*Actinidia chinensis*)	3E-26	107	90
		E8NTSAO03G8TB3:singleton	258	EYU33867.1 (*Mimulus guttatus*)	2E-43	154	97
	OeACO2	OLEEUCl054285:Contig2	223	AFJ75398.1 (*Salvia miltiorrhiza*)	1E-46	160	99
		OLEEUCl041676:Contig1	364	ABK59094.1 (*Manihot esculenta*)	2E-48	168	95
AP2/ERF transcription factor	*OeERF1*	OLEEUCl002278:Contig1	271	AEK82608.1 (*Hevea brasiliensis*)	5E-16	75	80
	OeERF2	OLEEUCl089730:Contig1	266	XP_002276572.2 (*Vitis vinifera*)	4E-22	95	80
	OeERF3	E8NTSAO04IDOK9:singleton	260	BAF75651.1 (*Daucus carota*)	2E-28	112	83

[a]The accession numbers of the OLEA EST database (http://140.164.45.140/oleaestdb/) (Alagna et al. 2009) are provided.

(Jongsma et al. 1993; Jongsma et al. 1994), it was impossible to obtain good protein isolates from fruit tissues. For this reason, we modified and optimized a protocol for the isolation of proteins from olive fruits suitable for the analysis of TPI proteins by radial diffusion assay. In particular, after the extraction, total soluble proteins were passed twice in a polyvinylpolypyrrolidone (PVPP) column of approximately 3 cm in order to eliminate the polyphenolic compounds. Afterwards, they were concentrated by trichloroacetic acid (TCA) precipitation or lyophilization and used for the radial diffusion assay.

Initially, in contrast to what we observed at the mRNA level and was reported by Corrado and co-workers (Corrado et al. 2012), the analysis of TPI protein amount in green fruits (100 DAF) revealed no significant differences between *B. oleae*-infested and undamaged control fruits. We found that in green fruits, TPI represented approximately 0.6% of the total soluble proteins (Figure 3A, B). To examine the eventual dependence of TPI content to the ripening process, we also analyzed the TPI protein content of ripe fruits (150 DAF). We observed a variation in TPI constitutive amount (of undamaged control fruits), which decreased up to 0.05% of the total soluble proteins, whereas in ripe fruits under *B. oleae* attack it remained at high levels, similar to those observed in green fruits (Figure 3A, B). In contrast to the differences observed in TPI protein content between green and ripe undamaged fruits, no significant changes were observed in the mRNA levels of *OeTPI2* (Figure 3C), the *TPI* gene considered the major driver of TPI activity of *B. oleae*-infested fruits (Corrado et al. 2012). These results could be explained by a post-translational regulation of TPI, probably controlled by ethylene.

Bactrocera oleae affects the VOC profile of olive fruits

Given the strong activation of the HPL pathway in infested fruits as detected by accumulation of C_{12} products, we analyzed VOCs emitted by fruits infested with *B. oleae* larvae. For this purpose, *B. oleae*-infested (BI) and undamaged control fruits (C) at 150 DAF were collected from plants of the cultivar Leccino growing in the field and the headspace of fruits were collected for 22 h in controlled conditions. As an additional treatment known to induce VOC production in different plant tissues, artificial wounding (W) of the fruit was also conducted. Analysis of the VOC profiles was conducted by high-resolution two dimensional gas chromatography time-of-flight mass spectrometry analysis (Gaquerel et al. 2009) and comparisons were also performed with VOC profiles of control undamaged fruits. The analysis detected 334 and 346 peaks in *B. oleae*-infested and artificial wounded fruits, respectively. The intensity and statistical significance of the changes elicited by *B. oleae* infestation or wounding were examined using Volcano plots and anova (BI vs C, and W vs C) was performed on normalized $\log_2$-transformed ion intensities to screen for differentially regulated volatiles.

Analysis of the VOC profiles showed that the amounts of 11 volatile compounds were increased in the headspace of *B. oleae*-infested fruits compared with undamaged control fruits whereas the amounts of three volatiles significantly decreased (Figure 4A). The 11 volatiles included C_9 aldehyde (i.e. nonanal), unknown sesquiterpene, aromatic compounds, and hydrocarbons (Figures 4B, S1, Table S2). Increased amounts of emitted volatiles ranged 1.3-2.8 fold while decreased amounts ranged 1.6-10 fold (Figure 4D). Seventeen compounds were differentially emitted in artificial wounded fruits compared with control fruits, and included C_6 aldehydes, unknown aromatic compounds, and hydrocarbons (Figures 4A, B, S1, Table S3). In this case, increased amounts of these volatiles ranged 1.6-5.4 fold while decreased amounts ranged 1.7-4 fold. The unknown compound 1 was exclusively emitted in wounded olives, and it was below detection limits in infested or control fruits (Figure 4D).

Despite a partial overlapping between wounding and *B. oleae* feeding responses, most of the differentially emitted volatiles were specific of the two set of samples (Figure 4C),

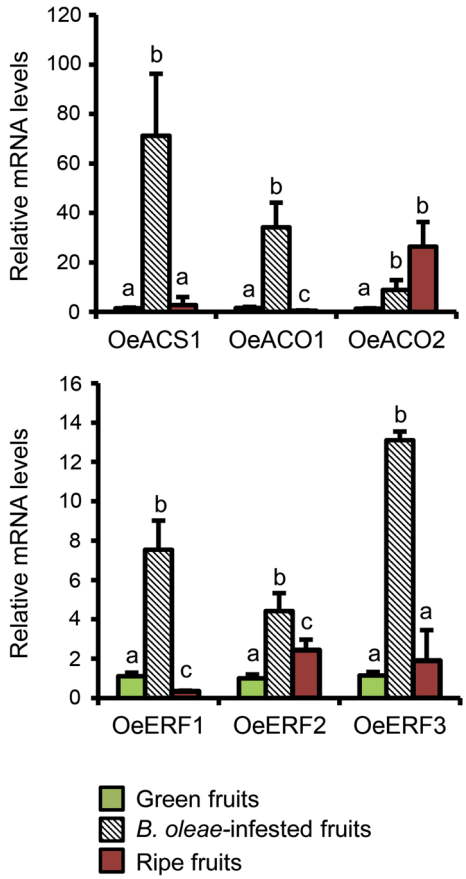

Figure 2. Relative mRNA levels of genes involved in ethylene biosynthesis and signaling network in olive fruits
Relative mRNA levels were measured in green undamaged fruits collected at 100 d after flowering (DAF) (green bars), in *Bactrocera oleae*-infested green fruits collected at 100 DAF (gray bars) and in ripe fruits collected at 150 DAF (purple bars) from cultivar Leccino. *OeACS1*, 1-aminocyclopropane-1-carboxylate synthase; *OeACO1-2*, 1-aminocyclopropane-1-carboxylate oxidase; *OeERF1-2-3*, ethylene-responsive transcription factor. Values are means of three biological replicates with standard error indicated. Different letters indicate significant differences between samples, as determined using anova followed by Student-Newman-Keuls post-hoc test ($P < 0.05$).

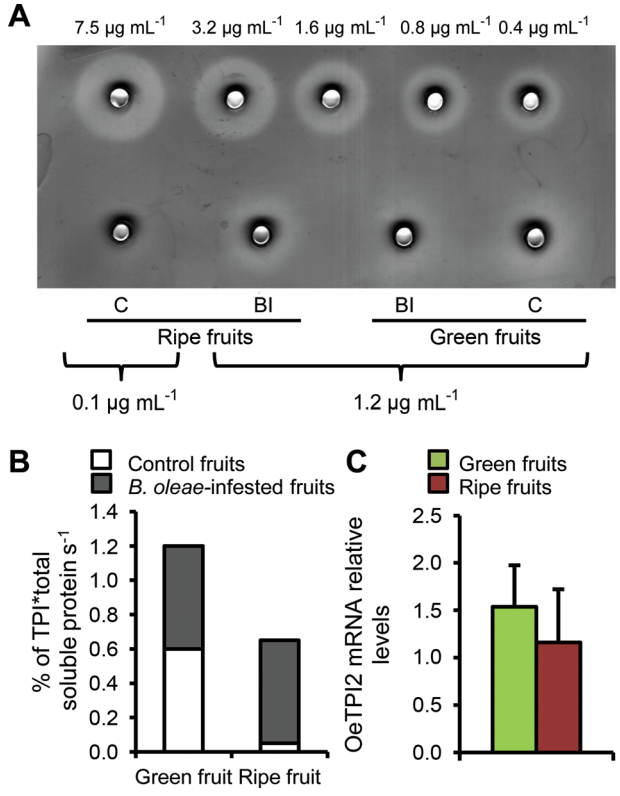

Figure 3. Trypsin protease inhibitors (TPIs) content of green (100 d after flowering (DAF)) and ripe (150 DAF) olive fruits
(A, B) TPI content was determined by radial diffusion assay using TPI from soybean to build a standard curve. TPI levels for control undamaged fruits (C, light pink bars) and *Bactrocera oleae*-infested fruits (BI, magenta bars) were reported as µg/mL or as percent on total soluble proteins. **(C)** The relative mRNA levels of *OeTPI2* (Corrado et al. 2012) were measured in undamaged fruits collected at 100 DAF (green) and at 150 DAF (ripe). Values are means of three biological replicates with standard error indicated.

suggesting that mechanical damage inflicted by the feeding larvae is only a component of the fruit response to olive fly attack, and therefore *B. oleae* larval feeding can specifically affect the volatile blend of fruits. VOCs specifically up- or downregulated in *B. oleae*-infested fruits and not in artificial wounded olives include sesquiterpenes, aromatics, and unknown compounds.

DISCUSSION

In this study, we show that olive fruits respond to *B. oleae* attack by producing changes in an array of different defensive compounds. A hypothetical scheme of the mechanisms involved in responses to *B. oleae* infestation is depicted in Figure 5. Our results suggest that the increase of ethylene could be responsible for the transcriptional induction of defense and signaling genes such as *OeChit* and *OeERFs* and that the accumulation of defense proteins such as TPI could be regulated at the post-translational level.

Role of ethylene as potential modulator of olive defense responses to herbivores

Enhanced ethylene production is an active response of plants to biotic stress (Wang et al. 2002). It synergizes volatile emission (Ruther and Kleier 2005) and modulates many other defense responses to herbivorous insects (Baldwin et al. 2002; Lorenzo et al. 2003; Harfouche et al. 2006; Maffei et al. 2007). We reported a strong increase in ethylene production in olive fruits infested by *B. oleae*, accompanied by a significant increase in the mRNA level of genes involved in ethylene

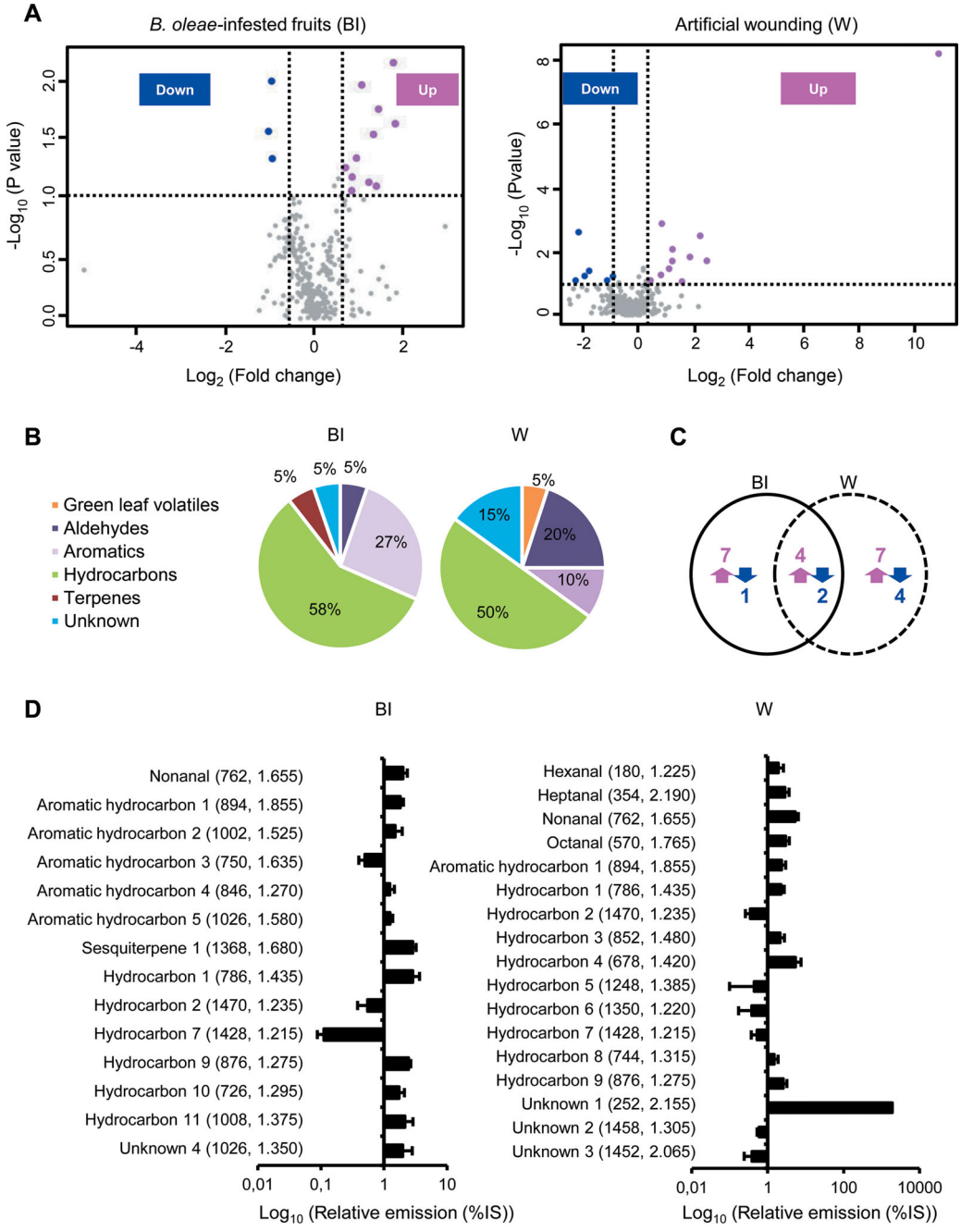

Figure 4. *Bactrocera oleae* feeding and artificial wounding affect volatile organic compounds (VOCs) of olive (*Olea europaea*) fruits

(A) Volcano plot analysis of the intensity, and statistical significance of the changes in chromatographic peak areas between volatile samples collected from *B. oleae*-infested fruits compared with the control undamaged fruits (left) and between volatile samples collected from artificial wounded fruits compared with the control (right). The $\log_2$ ratio of mean intensities is shown on the x axis, and the negative $\log_{10}$-transformed *P*-value, from anova on normalized log-transformed peak intensities, is shown on the y axis. Each dot represents an analyte from the common set of 344 normalized peaks. The vertical dashed lines represent 1.5 fold change ratios. The colored circles represent analytes above the threshold (fold change threshold, 1.5; *t*-test threshold, 0.1). Plant volatiles found to be upregulated and downregulated in *B. oleae*-infested fruits or in artificial wounded fruits are highlighted in pink and blue (see Tables S2, S3, Figure S1). The further its position away from the (0,0), the more significant the feature is. **(B)** Class of plant volatiles significantly regulated in *B. oleae*-infested (BI) and artificial wounded (W) fruits compared with control. **(C)** Venn diagrams showing the number of overlapping and non-overlapping volatiles, induced (pink arrows) or repressed (blue arrows) between the two sets of comparisons (Tables S2, S3). **(D)** Bar chart showing the VOCs differentially emitted after *B. oleae*-infestation (BI) or artificial wounding (W). Numbers in brackets indicate retention times on the first and second dimension.

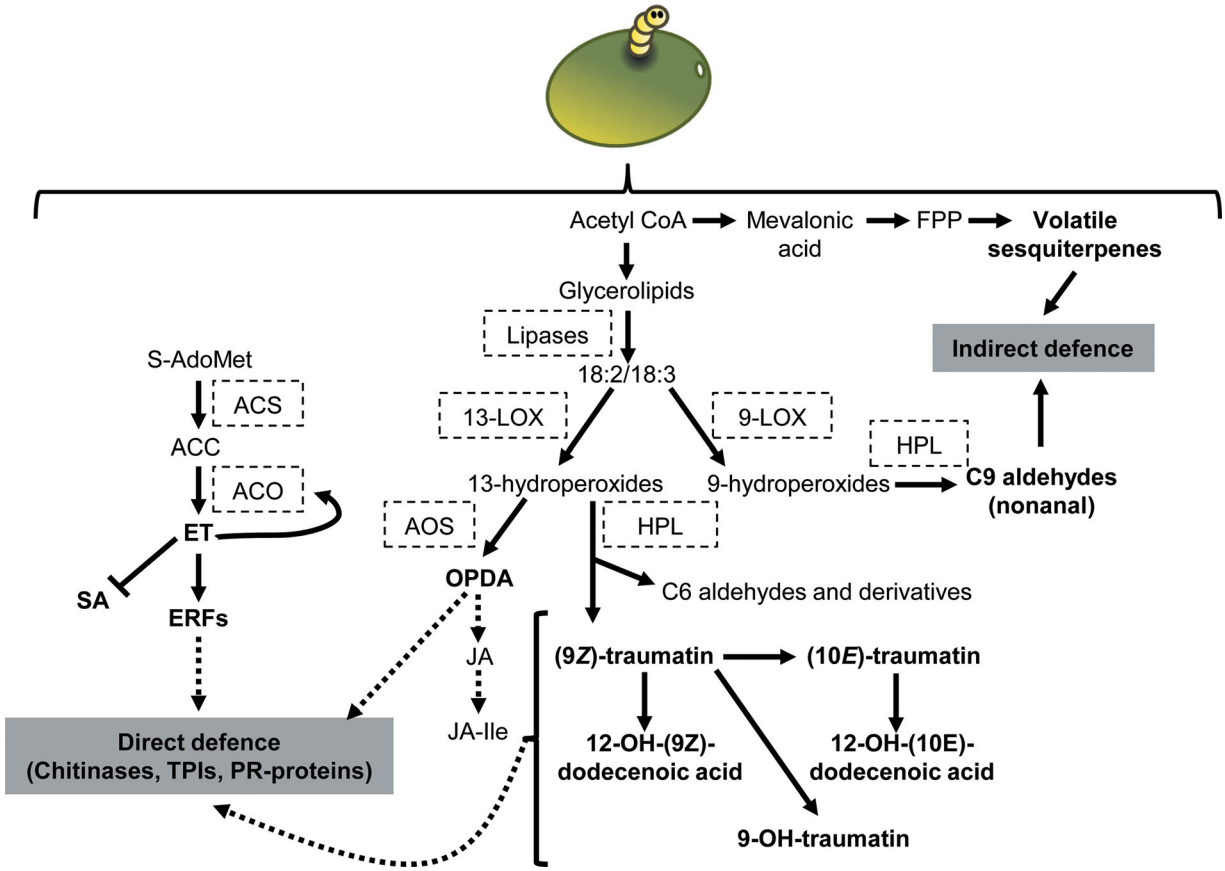

Figure 5. Schematic representation of olive responses to *Bactrocera oleae* feeding
A schematic representation of ethylene (ET), allene oxide synthase (AOS), lipoxygenase (LOX), hydroperoxide lyase (HPL) pathways activated during *B. oleae* infestation. Dotted arrows indicate hypothetic connections. Compounds induced or repressed by *B. oleae* infestation are indicated in bold. ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, 1-aminocyclopropane-1-carboxylate oxidase; ACS, 1-aminocyclopropane-1-carboxylate synthase; AldoMet, S-adenyl-methionine; ERFs, ethylene-responsive factors; FA, fatty acids; FPP, farnesyl pyrophosphate; JA, jasmonic acid; JA-Ile, JA-Isoleucin; OPDA, 12-oxo-phytodienoic acid; SA, salicylic acid. (9Z)-traumatin can undergo several enzymatic and non-enzymatic modifications to be converted into (10E)-traumatin, 12-OH-(9Z)-dodecenoic acid, 12-OH-(10E)-dodecenoic acid and 9-hydroxy-12-oxo-(10E)-dodecenoic acid (9-OH-traumatin) (Kallenbach et al. 2011; Mukhtarova et al. 2011).

biosynthesis (*OeACS*, *OeACO1*, *OeACO2*) and in ethylene-responsive transcription factors (*OeERF1*, *OeERF2*, *OeERF3*). We also demonstrated that the *OeACO1* promoter contains an ERE, indicating a positive feedback in the regulation of the ethylene pathway, as also reported in other species (Nakatsuka et al. 1998). In contrast, we did not find significant differences in the mRNA levels of other genes belonging to the ethylene pathway; however, we cannot exclude that there were some biases when analyzing these loci. Only deep transcriptome analyses will provide a complete unbiased picture on all the genes modulated by *B. oleae* attack.

OeACS1, *OeACO1*, *OeERF1*, and *OeERF3* mRNA levels, and TPI mRNA and protein content, did not increase in ripe fruits. These results suggest that the increase of ethylene elicited by the insect is not related to the ripening process and it is, most likely, related to the activation of the plant defense responses against fruit fly herbivory. On the other hand, the increase of *OeACO2* and *OeERF2* may indicate that, probably, a parallel pathway stimulating the increase of ethylene related to the ripening process is also active in *B. oleae*-infested fruits.

In agreement with the role of ethylene in olive defense against insect pests, we identified an ERE in the promoter of the defense-related gene *OeChit*, putatively encoding for a chitinase, previously showed by Corrado and co-authors (Corrado et al. 2012) to be significantly induced in *B. oleae*-infested fruits. Moreover, previous studies demonstrated that ethylene affects the olive moth (*Prays oleae*) egg population (Ramos et al. 2008), strengthening its potential role as modulator of olive defenses against herbivores. In the signaling events which translate the ethylene increase in a downstream-specific response, ethylene-responsive transcription factors play a key role. Elevated ethylene production leads to the increased activity of ethylene-responsive transcription factors, such as ERFs. These events are gradually translated into the accumulation of metabolites that function

as defensive compounds (Erb et al. 2012). Ethylene-responsive transcription factors can bind the promoters of defense-resistance proteins, either directly or through interaction with other transcription factors (Chakravarthy et al. 2003; Son et al. 2012). Interestingly, we identified three *OeERF* genes potentially involved in the response to *B. oleae*. Future analyses, aimed at identifying their gene target and to study their regulation, will help to complete the picture of their role in olive responses to herbivores. The discovery of transcription factors able to regulate defense genes is of fundamental importance to develop strategies to potentiate plant protection against insect pests. The plasticity and the specificity of members of the AP2/ERF transcription factor family make them valuable targets for genetic engineering and breeding of crops (Licausi et al. 2013).

Role of other phytohormones in olive responses to *B. oleae*

Our observation that levels of OPDA, SA, traumatin, and its derivatives (12-OH-dodecenoic acid and 9-OH-traumatin) varied in *B. oleae*-infested olive fruits, even though at a lower extent compared with ethylene, indicated that these compounds may contribute to the modulation of olive responses to the insect pest.

12-Oxo-phytodienoic acid is an intermediate of the JA pathway and JA is considered one of the most important modulators of plant defense responses to insect pests. We found a weak but significant increase of OPDA levels in infested fruits compared with control that was not coupled with changes in JA levels, and JA-Ile was not even detected, suggesting that a weak activation of the AOS pathway is probably limited to the plastidial steps. It is possible that OPDA contributes to the regulation of a certain subset of defense responses in a JA-independent manner. In *Arabidopsis*, it has been demonstrated that OPDA is not only a precursor for JA but it can also act as a JA-independent signal that may activate certain wounding- or herbivory-induced responses (Stintzi et al. 2001). In addition, higher levels of OPDA stimulate rice resistance to piercing-sucking insects (Yu et al. 2008).

In our study, we also observed a significant increase in the C_{12} derivatives of the HPL pathway in *B. oleae*-infested fruits. Our results are in accordance with the activation of HPL enzyme activity in olive fruit mesocarp reported in response to wounding (Padilla et al. 2014). In plants, 18:2 and 18:3 fatty acids released from membranes are dioxygenated by 13-LOXs to generate 13S-hydroperoxides, which are substrates of HPL and are cleaved to produce C_6 aldehydes (green leaf volatiles), traumatin, and other C_{12} derivatives (Kallenbach et al. 2011). Currently, the role of C_{12} derivatives of the HPL pathway in plant defense to herbivores has not yet been well clarified, however, their rapid increase after herbivore attack and their ability to elicit substantial changes in the expression of defense-associated genes were consistent with their possible role in the regulation of plant stress responses (Kallenbach et al. 2011).

Our studies also showed a decrease of SA levels in the infested fruits compared with controls. This may be an effect of the induction of the ethylene pathway. In fact, antagonistic interactions between ethylene and SA pathways have been reported in other plant species (van Loon et al. 2006; Zander et al. 2010; An and Mou 2011).

Considering our results, it is possible that ethylene may cooperate with other phytohormones for the induction of specific responses to *B. oleae*; however, further experiments are necessary to clarify the role of OPDA and HPL pathways and to evaluate their potential cross-talk with ethylene. In fact, ethylene regulates many plant biological processes and it is clear that the diversity of ethylene functions is achieved, at least in part, by its interactions with other hormone signaling pathways.

Modulation of TPIs in olive fruits

Protease inhibitors are widely distributed throughout the plant kingdom and are considered to be an essential part of the plant defense system against herbivorous insects (Ryan 1990; Zavala et al. 2004).

We reported for the first time a modulation of the TPI amount in olive, a member of the Oleaceae family. We found a high level of TPI in olive fruits which indicate their prominent role in the constitutive defense responses of olive. It was previously reported that the mRNA levels of TPI genes increase in olive fruits under *B. oleae* attack compared with the undamaged control fruits (Corrado et al. 2012). We observed that this response strongly depends on the fruit ripening stage. In the first stages of fruit development, TPIs are part of the constitutive defense responses, and their high levels in the fruits are probably sufficient to protect the plant from herbivores and this can explain why they are not induced by *B. oleae* attack. Besides, at ripening stage, when the constitutive TPIs decrease, they are induced by insect attack. It is possible that at the completion of fruit developmental and ripening processes, the plant reduces the constitutive defense responses being at the end of its reproductive cycle and saving energy that can be assigned to the complex physiological changes taking place during ripening. On the contrary, the plant maintains high levels of TPIs in the fruits only in the case of a herbivore attack. It is known in other species that the direction and magnitude of the defense responses to larval feeding depend on the age of the tissues that are damaged and that plant ontogeny constrains both constitutive and inducible PI production (van Dam et al. 2001; Diezel et al. 2011).

It is known that PIs are subjected to different levels of regulation and both transcriptional and post-translational mechanisms affect their amount and activity in plants (Horn et al. 2005; Mishra et al. 2012). The discrepancy that we observed between the *OeTPI2* mRNA levels and the TPI proteins in green and ripe olive fruits can be explained with a post-translational control of TPI levels.

***Bactrocera oleae* affects the volatile blend of olive fruits**

Our data showed that *B. oleae* feeding induced significant differences in the volatile profile of olive fruits and indicated the activation of lipoxygenases (9-LOX and 13-LOX) and the HPL pathway in response to olive fly infestation. These pathways have been previously shown to be active in olive fruits (Salas and Sanchez 1999; Muzzalupo et al. 2012) and their end-products as C_6 compounds and C_9 aldehydes (i.e. nonanal) have been detected (Sabatini and Marsilio 2008; Sansone-Land et al. 2014). Nonanal has been repeatedly reported in the context of plant biotic stress (Birkett et al. 2003; Yi et al. 2009; Yi et al. 2010) with proven *in vitro*

antifungal and bactericidal activities (Nakamura and Hatanaka 2002; Fernando et al. 2005). It has also been identified as an active volatile that primes the expression of defense genes, which become fully activated only when plants are subsequently challenged by a virulent pathogen (Yi et al. 2010). In particular, in Lima bean, it strongly induces the expression of LOX and PR protein 2 (PR-2) priming the plant defenses against bacterial pathogens (Yi et al. 2009).

Sesquiterpenes were upregulated exclusively in *B. oleae*-infested fruits but not in wounded fruits. They have been frequently reported as signaling molecules for attracting natural enemies of plant herbivores (Weissbecker et al. 2000; Ruther and Kleier 2005; Schnee et al. 2006). Moreover, Schmelz and co-authors (2003) showed that ethylene synergizes the emission of maize sesquiterpene compounds upon treatment with high doses of (*Z*)-3-hexen-1-ol, evidencing that ethylene appears to be an important component in the stimulation of insect-induced volatile emissions (Schmelz et al. 2003).

Our data demonstrate that *B. oleae*-infested fruits possess a unique blend of volatile compounds. The significance of the change in the fruit volatile blend induced by the olive fly attack can be diverse but it probably has consequences for the ecological interactions of olive fruits with the environment. Further analyses to assess the biological relevance of the change of volatile blend induced by the insect may help to identify new strategies for the control of this pest. In this context, it may be interesting to study the effect that the volatile blend produced by olive fly-infested fruits can have on the insect populations as well as on the epiphytic bacteria of the olive tree.

MATERIALS AND METHODS

Plant material and treatments

Control and infested fruits of olive (*Olea europaea* L.) cultivars Leccino and Coratina were harvested at 100-110 (green fruits) and 150 (ripe fruits) DAF from field plants growing in Perugia (Italy), under the same environmental and agronomical conditions. Fruit tissues surrounding the feeding tunnels of II-III instar larvae were used for phytohormones and qRT-PCR analyses and for trypsin protease inhibitor assays. For the analysis of volatile compounds, undamaged, damaged, and artificially wounded fruits of the olive cultivar Leccino collected at 150 DAF were used. For wound treatment, drupes were wounded with a sterile scalpel blade without damaging the stone. Olive fruits for high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS), quantitative qRT-PCR, and TPI assay were frozen in liquid nitrogen and stored at −80 °C, immediately after harvesting.

Phytohormone analyses

For phytohormone analysis, we extracted tissues from infested and control fruits using ethyl acetate spiked with 200 ng of 9,10-dideutero-9,10-dihydrojasmonic acid (JA-D2), 40 ng of each jasmonoyl isoleucine* (^{13}C6-labeled JA-Ile), 3,4,5,6-tetradeutero salicylic acid (SA-D4), and hexadeutero abscisic acid (ABA-D6) and 400 μL of royal jelly acid as internal standards (ISs). To avoid high amounts of oil, phytohormones were extracted from ethyl acetate with 0.1 mol KOH. The aqueous solution was adjusted to pH 4 with HCl and phytohormones were extracted with ethyl acetate. The extracts were dried and resuspended in 70% methanol and the phytohormone content analyzed by HPLC-MS/MS as described by Wang and co-authors (Wang et al. 2008). Phytohormones were quantified as ng IS/g fresh mass. Values were calculated as means of 10 biological replicates. Ethylene emissions were measured on three biological replicates per sample. The headspace was flashed into a photo-acoustic laser spectrometer with hydrocarbon-free clean air (INVIVO; https://www.invivo-gmbh.de), and the ethylene concentration was quantified by comparing ethylene peak areas with peak areas generated by a standard ethylene gas. Statistically significant differences were determined by Student's *t*-test (*$P < 0.05$, **$P < 0.01$, $n = 10$).

Quantitative RT-PCR

Total RNA was extracted from 0.2 g of fruit mesocarp and exocarp with the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and treated with DNase I (Ambion, Austin, TX, USA). Reverse transcription of 2 μg of RNA was performed using oligo(dT)$_{18}$ and the SuperScript III Reverse Transcriptase kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Genes putatively involved in ethylene biosynthesis and signaling network and TPI genes were analyzed by qRT-PCR). The analysis was performed on a PCR Real Time 7300 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol and using the Reagent kit for SYBR Green analysis (Applied Biosystems) and gene-specific primers (Table S4). Primer efficiency was, initially, verified by the presence of single PCR product bands on agarose gel electrophoresis. The thermal cycling program started with a step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Three technical and three biological replicates were performed per sample. After each assay, dissociation kinetics analysis was performed to verify the specificity of the amplification products. Moreover, PCR efficiency was assessed by producing a standard curve for each locus with five dilution points, each one replicated three times. Only primer pairs that produced the expected amplicons and showed a PCR efficiency ranging 1.8-2.1 were selected for use. Relative amounts of all mRNAs were calculated using the 2-△△CT method (Livak and Schmittgen 2001), where $\Delta CT = CT_{target\ gene} - CT_{reference\ gene}$. The housekeeping elongation factor 1-α gene was used as an endogenous reference gene for cDNA normalization. Data for three biological replicates were submitted to anova followed by Student-Newman-Keuls post-hoc test ($P < 0.05$).

Collection and analysis of plant volatiles

Infested, wounded, and control fruits were enclosed in 50 mL plastic containers. Considering the difficulties in selecting uniform material for volatile collection, we performed the fruit sampling based on trend of olive fly infestation in the field and selected a period characterized by a high infestation rate (corresponding to 150 DAF fruits), in order to obtain the

highest percentage of fruits infested by II-III instar larvae. Fruits with oviposition punctures (infested) and undamaged fruits (control) were collected. For wound treatment, drupes were wounded with a sterile scalpel blade without damaging the stone. Nine olives were placed in each container. Volatiles were collected on charcoal tubes ORBOTM-32 (Supelco-Sigma-Aldrich, Bellafonte, PA, USA). Ambient air flowing into the cage was filtered through charcoal tubes inserted both incoming and outgoing the container. Air flow was created by a manifold vacuum pump. Fruit volatiles were collected during a 22 h period at 18 °C. Background contaminants present in ambient air were collected using empty trapping containers. After volatile collection, olives were dissected and only containers carrying at least 80% of the fruits infested by II-III instar larvae and pupae were selected as infested samples and considered for the analyses, whereas containers with 100% of fruits without any insect attack were selected as control samples. Eight replicates per treatment were considered. Immediately after collection, traps were spiked each with 400 ng of tetralin as an internal standard and eluted with 500 μL of dichloromethane into a GC vial containing a glass insert.

Samples were run on an Agilent 6890N gas chromatograph equipped with an Agilent 7683 auto-injector (Agilent Technologies, Santa Clara, CA, USA) coupled to a LECO Pegasus III time-of-flight mass spectrometer with a 4D thermal modulator upgrade (LECO, St Joseph, MI, USA). Injected samples were separated first on a non-polar column (C1 RTX-5MS, 20 m × 250 μm i.d. × 0.5 μm; Restek, Bellefonte, PA, USA) and every 6 s (modulation time) transferred to a mid-polar column (DB-17, 0.890 m × 100 μm i.d. × 0.1 μm; Agilent Technologies) for the second separation. Chromatography and analysis conditions as well as deconvolution, alignment, and integration of VOC analyte peaks were performed as described by Gaquerel and co-authors (Gaquerel et al. 2009). The released amounts of volatiles were calculated from peak areas normalized to the peak area of tetraline as the internal standard (RT1, 846; RT2, 3.76). During peak table alignment using the comparison feature imbedded in the ChromaToF software (LECO), mass spectra alignment was accepted at a similarity threshold of 500/1,000. Peak lists were then checked prior to further processing for known artifact peaks and contaminants (e.g. column bleeding and plasticizers) identified from the analysis of ambient air samples trapped in the glasshouse.

The intensity and statistical significance of the changes elicited by *B. oleae* infestation or wounding were examined using Volcano plots (Cui and Churchill 2003). The variations in intensity across treatments were analyzed by univariate anova on $\log_2$-transformed normalized peak areas using MetaboAnalyst 2.0 (Xia et al. 2009; Xia et al. 2012). We selected a *P*-value of 0.1 for the threshold of statistical significance and a 1.5 fold change ratio as cut-off threshold to discriminate between significantly up- and downregulated peaks.

Identification of regulatory motifs

The cDNA sequences of *OeChit1* (GenBank accession no. JN696113.1), *OeTPI2* (A.N. JQ429796.1), *OePRp27* (A.N. JN696114.1), and those identified in the olive fruit transcriptome database (OLEA ESTdb: http://140.164.45.140/oleaestdb/) (Alagna et al. 2009), respectively, *OeACO1* (OLEEUCl056880:Contig1, E8NTSAO03G8TB3:singleton), *OeACO2* (OLEEUCl054285:Contig2, OLEEUCl041676:Contig1), *OeERF1* (OLEEUCl002278:Contig1), *OeERF2*, OLEEUCl089730:Contig1), and *OeERF3* (E8NTSAO04IDOK9:singleton) were used as queries to search by BLAST the genome sequences of cv. Leccino (http://oleagenome.org/). Promoters and coding regions were predicted by using Softberry tools (http://www.softberry.com/). Approximately 1,500 bp of the promoter region immediately preceding the start codon was analyzed for all the genes except *OeERF2*, for which a shorter region of 1,000 bp was analyzed. Regulatory motifs were identified in promoter and UTR sequences by PlantCARE software (Lescot et al. 2002).

Trypsin protease inhibitor assay

Immediately after harvesting, the olive fruits of cv. Leccino (infested and undamaged green fruits at 100 DAF; infested and undamaged ripe fruits at 150 DAF) were frozen in liquid nitrogen and stored at −80 °C until further analysis. Five grams of olive fruit mesocarp and exocarp were homogenized in a mortar, with 20 mL extraction buffer obtained by dissolving in 1 L of Tris-HCl 0.1 mol pH 7.6 the following compounds: 50 g PVPP, 110 mm particle size (Fluka-Sigma-Aldrich, Steinheim, Germany), 2 g phenylthiocarbamide, 5 g sodium diethyldithiocarbamate, and 18.6 g Na_2 ethylenediaminetetraacetic acid. Homogenates were centrifuged at 15,000 *g* for 15 min at 4 °C. Supernatants, recovered via a syringe, carefully avoiding drawing the oily residue, were passed twice on a PVPP column of approximately 3 cm made within a 10 mL syringe, to eliminate polyphenolic compounds. In the case that proteins were too diluted, they were concentrated by trichloroacetic acid precipitation (Sanchez 2001) or lyophilization. After quantification by Bradford assay, an equal amount of total proteins was loaded for the radial diffusion assay that was performed according to Jongsma and co-authors (Jongsma et al. 1993). The same protein amount was loaded per each sample. The experiment was repeated three times.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Mass spectra of volatile compounds differentially emitted from *Bactrocera oleae* attacked and wounded fruits compared to undamaged fruits

Table S1. Transcripts tested by quantitative reverse transcription polymerase chain reaction (qRT-PCR) which resulted not differentially expressed in *Bactrocera oleae* attacked fruits compared to control

Table S2. Compounds differentially emitted in Bactrocera oleae-infested fruits selected by Volcano plot

Table S3. Compounds differentially emitted in Bactrocera oleae-infested fruits selected by Volcano plot

Table S4. Primers used for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses