Symbiosis disruption in the olive fruit fly, *Bactrocera oleae* (Rossi), as a potential tool for sustainable control

Martina Sinno[1], Annie Bézier[2], Francesco Vinale[3,4], David Giron[2], Stefania Laudonia[1], Antonio Pietro Garonna[1], Francesco Pennacchio[1]

[1] Department of Agricultural Sciences, University of Naples Federico II, 80055 Portici (NA), Italy;

[2] IRBI - UMR 7261 CNRS/Université de Tours, 37200 Tours, France;

[3] Department of Veterinary Medicine and Animal Production, University of Naples Federico II, 80137 Napoli, Italy;

[4]CNR Institute for Sustainable Plant Protection, 80055 Portici (NA), Italy.

Abstract

BACKGROUND: The olive fruit fly *Bactrocera oleae* (Rossi) (OLF) is a major agricultural pest, whose control primarily relies on the use of chemical insecticides. Therefore, the development of sustainable control strategies is highly desirable. The primary endosymbiotic bacterium of the OLF, "*Candidatus* Erwinia dacicola", is essential for successful larval development in unripe olive fruits. Then, targeting this endosymbiont with antimicrobial compounds may result in OLF fitness reduction and may exert a control action of its natural populations.

RESULTS: Here we evaluate the impact of compounds with antimicrobial activity on OLF endosymbiont. Copper Oxychloride (CO) and the fungal metabolite Viridiol (Vi), produced by *Trichoderma* spp., were used. Laboratory bioassays were carried out to assess the effect of the oral administration of these compounds on OLF fitness and molecular analyses (qPCR) were conducted to measure the load of OLF-associated microorganisms in treated flies.

CO and Vi were both able to disrupt the symbiotic association between OLF and its symbiotic bacteria, determining a significant reduction of the endosymbiont and gut microbiota load as well as an OLF fitness decrease. CO had a direct negative effect on OLF adults. Conversely, exposure to Vi significantly undermined the larval development of the treated females' progeny but did not show any toxicity in OLF adults.

CONCLUSIONS: These results provide new insights on the symbiotic control of the OLF and pave the way toward the development of more sustainable strategies of pest control based on the use of natural compounds with antimicrobial activity.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ps.5875

1 INTRODUCTION

The olive fruit fly *Bactrocera oleae* Rossi (OLF) causes severe yield losses and quality decay of olives and derived products,[1-3] with an estimated 15% reduction in world production per year.[4] During the last 40 years, the control strategies of *B. oleae* were based on the wide use of chemical insecticides, both as bait and cover sprays.[5-8] However, the misuse of insecticides promoted the development of resistance,[6, 7, 9-13] exerting a negative impact on non-target arthropods,[14] and olive oil quality.[15] Therefore, sustainable control strategies of OLF are highly desirable,[16, 17] and in line with the inspiring principles of the EU directive 128/2009 on sustainable use of pesticides.

The currently available alternatives to chemical pesticides are the attract-and-kill method, mass trapping devices, the use of Biological Control Agents (BCA) or Sterile Insect Techniques (SIT), with effects that are not always satisfactory on all instances.[16, 18-26] Unfortunately, olive cultivars showing resistance/tolerance against the OLF are not available,[27] even though this could be a promising area of research.[28-30]

An alternative new opportunity for pest control relies on the disruption of insect symbiosis also known as "symbiotic control".[31-34] Insect life and evolution are indeed strongly influenced by microorganisms associated with them.[35, 36] The insect is, therefore, a metaorganism (or holobiont) controlled by a complex network of interactions with the associated microbiota, which can be manipulated and/or suppressed by targeting either the insect or the microbial component.[31, 32, 35, 37-39] The symbiotic control of insect pests has been recently reviewed by Arora and Douglas.[33] Among the different options that can be pursued, the disruption of the vertical transmission (mother to offspring) of obligate endosymbiont required for nutrition, insect growth, reproduction or survival, seems to be an amenable target.

Implementation of symbiotic control strategies can be remarkably fostered by the discovery of novel natural molecules with antimicrobial activity. This appears to be a promising research area, as indicated by the successful use of antimicrobial peptides against the primary endosymbiont of *Sitophilus* sp. (Schoenherr) or *Buchnera*, the bacterial symbiont of the pea aphid (*Acyrthosiphon pisum* Harris) and the green peach aphid (*Myzus persicae* Sulzer).[40-42]

Since the beginning of the past century, *B. oleae* is known to harbor endosymbiotic bacteria.[43] A study published by Capuzzo *et al*. confirmed that a single, not culturable bacterial species, "*Candidatus* Erwinia dacicola" (Enterobacteriaceae: Gammaproteobacteria), represents nearly the entire symbiotic population associated with OLF.[44] The constant association of this symbiont with all life stages of different widespread OLF populations,[44-50] its vertical transmission to offspring,[46] and its specific localization, both within larvae and adults,[46,47] collectively indicate a highly specific and long-term symbiotic relationship.[47, 52, 53]

During mass-rearing, the standard artificial diet routinely used, contains antibiotics that block the natural transmission of the endosymbionts, which are replaced by environmental bacteria. In these conditions, both the adult and larval stages show a remarkable fitness decrease.[48, 54, 55] In order to alleviate this problem, the development of antibiotic-free and probiotic-supplemented diets, as well as the horizontal transfer of the endosymbiont from wild flies to lab reared strains, were successfully pursued.[56-59]

Recent studies suggested that "*Ca.* Erwinia dacicola" is able to use many different nitrogenous sources present in the diet of wild OLF populations to synthesize essential amino acids, which are then made available to the

host to complement its poor nutritional substrate, in order to support both development and reproduction.[60-62] Furthermore, the capacity to influence the oviposition behavior of its insect host was recently demonstrated.[51]

Unlike most fruit fly species, which lay eggs in ripe and nutritionally suitable fruits,[63-67] OLF larval stages can also develop in unripe olives, exploiting, then, a broader temporal window for multiple generations on a single host.[68, 69] Unripe fruits are generally resistant to herbivores and pathogens attack thanks to the high content of secondary metabolites, which have antimicrobial, anti-nutritive and toxic effects.[70, 71] The unripe fruit of *Olea europaea* (L.) is no exception; in fact, it contains several secondary metabolites, the most abundant of which is oleuropein, a bitter phenolic glycoside that can contribute up to 14% of the fruits dry weight.[29, 71, 72] Ben-Yosef *et al.* have lately shed light on the role of bacterial symbionts in overcoming the nutritional constraints imposed by phenolic compounds present into the unripe olive fruits.[54] In fact, while symbiotic larvae were able to develop in unripe olive fruits, their aposymbiotic counterparts could not reach the pupal stage, demonstrating that "*Ca.* Erwinia dacicola" is essential for the development of *B. oleae* larvae into unripe olive fruits.[54, 73]

Thus, "*Ca.* Erwinia dacicola" is thought to play a key-role for OLF larval stage survival,[54, 60, 61, 73] and the disruption of this symbiotic association can offer new opportunities for pest control. A recent study by Bigiotti *et al.*, showed a fitness reduction of adult flies with reduced symbiont loads in the oesophageal bulb as a consequence of exposure to antimicrobial compounds, such as copper and propolis.[74] However, no data are available on transgenerational effects of antimicrobial treatments, and, thus, on their potential to limit the larval development in unripe olive fruits, which should be the major goal of a symbiotic control strategy.

Here we contribute to fill this research gap by investigating the insecticide activity on OLF adult and larval stages, and the concurrent impact on its microbiota, exerted by antimicrobial compounds of different origin. We focused our attention on Viridiol (Vi), a secondary metabolite biosynthesized by beneficial fungi belonging to *Trichoderma* genus (Hendrik).[75-78] Vi is a steroid-like molecule with broad antimicrobial activity both *in vivo* and *in vitro*, produced by *T. virens* (Mill), *T. viride* (Pers.), *T. hamatum* (Bonorden), *Hypocrea virens* (Chaverri), and certain *Gliocladium* (Corda) species.[79-81] *Trichoderma* is one of the most studied fungal biocontrol agents and its activity is also related to the variety of metabolites they produce. These metabolites have been found to directly inhibit the pathogens, increase disease resistance and enhance plant growth also in a field experiment.[82]

We also focused on Copper Oxychloride (CO), which is widely used in olive groves for disease control. Field observations of reduced OLF infestations associated with the use of CO led to the hypothesis that this compound may act as a symbioticide,[83-91] which was corroborated by a recent laboratory study.[74] A remarkable negative effect of copper treatments on larval growth has been reported,[90] along with a deterrent effect on the OLF oviposition.[91] Furthermore, CO was found to be moderately toxic when orally administered both to adults and larval stages of several insect species including Diptera.[92-95] Here we try to assess if these negative effects of CO on the OLF can be partly due to its impact on associated microbiota.

Laboratory bioassays were carried out on wild populations to assess the suitability of these compounds for the OLF symbiotic control. We recorded the mortality and fecundity of adult flies and the larval development of their progeny in unripe olive fruits. To measure any concurrent change in the associated microbiota, we also estimated the endosymbiont load in the oesophageal bulb and the midgut, as well as the overall microbiota resident in the midgut using a quantitative PCR approach.

2 MATERIALS AND METHODS

2.1 Sampling, origin, and maintenance of *B. oleae* wild population

The olives were collected from trees in a pesticide-free experimental field of *Olea europaea* at the Department of Agricultural Sciences, University of Naples Federico II (Portici, NA, Italy). Infested olives were weekly collected, from September to December, in 2015 and 2016, placed in plastic trays, bottom lined with paper, and incubated in a controlled rearing room (24 ± 2°C, 60 ± 10% RH, and 12:12 light/dark photoperiod). Mature larvae and puparia were daily collected and caged; the emerged adults were sexed and separately maintained in groups of 30 flies, using cylindrical cages (20 cm diameter, 15 cm height).

2.2 Viridiol production and purification

For Viridiol production, *T. virens* strain GV41, obtained from actively growing margins of potato dextrose agar (PDA – Hi Media India) cultures, was used. The fungal strain was maintained on PDA slants at room temperature, and sub-cultured every two months. Two 7 mm diameter plugs of the above-mentioned strain were inoculated into 5 L conical flasks containing 1 L of sterile potato dextrose broth (PDB - Hi Media India). The stationary cultures were incubated for 31 days at 25°C and then filtered under vacuum through filter paper (Whatman No. 4). Subsequently, the filtrates were stored at 2°C for 24 h. Two liters of the filtered culture broth of strain GV41 were extracted with ethyl acetate (EtOAc). The combined organic fractions were dried (Na_2SO_4) and evaporated under reduced pressure at 35 °C. Then, the recovered yellow residue was subjected to column chromatography (Si gel; 50 g), eluted with a gradient of EtOAc:petroleum ether (from 8:2 to 10:0). Similar Thin Layer Chromatography (TLC) profiles fractions were combined and subsequently purified with preparative TLC separation (Si gel; EtOAc:petroleum ether; 6:4) or silica gel flash chromatography (EtOAc/petroleum ether, 8:2 to 10:0). Preparative silica gel TLC of fraction 2, 3 and 4 yielded 6 mg of Viridiol. Analysis of the Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectra finally revealed the expected signals previously described for such compound, confirming the purity of the obtained product.[96]

2.3 Bioassays

2.3.1 Adult survival

The basic diet for OLF adults was made of sucrose (0.5 g) solubilized in water (200 µl), in which were dissolved the following experimental compounds: CO (0.5%, 0.1%, and 0.02% *w/w* sucrose) and Vi (0.5% and 0.1% *w/w* sucrose). As negative control, this sucrose diet, completed with the antibiotic Piperacillin (Sigma, 100 µg/mL), which we will abbreviate as "Antibiotic" in this study, was used to clear the endosymbiotic bacteria from the fly's gut,[60, 61] while the plain diet (only sucrose) acted as positive control. In all cases, the diet was dried in the oven at 50°C for 3 h and offered in a solid form to the experimental insects.

Newly emerged adults were sexed then kept in groups of 30 individuals for each sex, with *ad libitum* access to one of the different experimental diets; for each diet/treatment 3 groups of females and 3 groups of males where used, for a total amount of 1,260 experimental flies. From the onset of the experiment to sexual maturity, which was attained after 14 days,[97] the percentage of surviving flies was daily recorded along with the diet

consumption *per* fly/day. This was obtained by calculating the difference between the weight of the diet on day 1 and that recorded on day 14, which was divided by the average number of flies found alive every day.

2.3.2 Adult reproduction

On the 14th day, mature adults (at least 15 females and 15 males) were allowed to mate. Un-infested and unripe olive fruits were offered to mated females for the oviposition (1 olive/female) The olives in the cage were replaced after three days to assess any time-related change in the reproductive activity. Half of the olives of these two groups was sectioned to assess the number of laid eggs *per* female, while the second half was incubated for 3-4 weeks to allow larval development.

At the end of the oviposition tests, all treated adults were stored at -80°C, until further processing.

2.3.3 Larval development

The number of eggs laid by reared females that completed the development and gave rise to adults was daily scored. The number of offspring/female was used to assess the successful development in unripe olives, which requires the presence of primary endosymbionts.[54]

2.4 DNA extraction

The frozen flies were surface-sterilized by vortexing for 15 s in a 1% sodium hypochlorite – 0.1% Triton X solution, rinsed twice with distilled water and then dissected under sterile conditions in a laminar flow hood to isolate the head and the abdomen, where the oesophageal bulb and the midgut reside, respectively.

After dissection, pooled samples of four heads and four abdomens were separately used for DNA extraction to assess the level of the bacterial load within the oesophageal bulb and the midgut. The DNA was extracted using the PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific), following the manufacturer's instructions. The extracted DNA quality and concentration were evaluated using the Varioskan (Thermo Fisher Scientific) and samples with 260/280nm ratio <1.6 and 230/260nm ratio <1.5 were discarded. Then all the remaining DNA samples were stored at -80 °C until further processing.

2.5 Quantification of the endosymbiotic bacterial titer by qPCR

The amplification of "*Ca*. Erwinia dacicola" 16S rDNA region was obtained with primers EdEnRev and EdF1.[47, 48] These primers were previously validated in a study on the relative abundance of "*Ca*. Erwinia dacicola" across life stages of *B. oleae*.[48] Even though the specificity of these primers was recently questioned,[59] this did not significantly affect the present study since the oesophageal bulb is colonized nearly exclusively by the primary endosymbiont,[44,62] and the gut environment of the experimental population was also predominantly colonized by the primary endosymbiont (see supporting information, Fig. S1).

Moreover, only for midgut samples, the total bacterial load was assessed using a couple of primers, designed with Primer Express Software (Thermo Fisher Scientific), within the 16S rDNA region shared by all bacteria.[98]

Out of the three tested couples of candidate primers, only the most efficient one, 16SuniF and 16SuniR, was used for further analyses.

To normalize data, different *B. oleae* housekeeping genes were used for the two body parts. *β-actin* gene was used for the data normalization in the oesophageal bulb samples and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used for data normalization in midgut samples, according to the study of Sagri *et al*.[91] The amplification of a portion of the *β-actin* gene was obtained with a couple of primers designed with Primer Express Software (Thermo Fisher Scientific). Three couples of primers were designed and their efficiency was tested. The most efficient couple, Act2F and Act2R, was chosen. The amplification of a portion of the *gapdh* gene was obtained with the couple of primers GapF and GapR.[99] All primers used are reported in Table 1.

Quantitative PCR experiments were carried out with SYBR® Green PCR Master Mix (Thermo Fisher Scientific) in a 13 µl total reaction volume, containing 3 µl of diluted genomic DNA, 6.5 µl of Master Mix and a solution of primers with a final concentration of 300 nM. The amplification procedure used by Estes *et al.* was tested and adapted.[48] The experiments were performed with a StepOnePlus Real-Time PCR System (Applied Biosystems) as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. Reactions were followed by a quality control melting curve and terminated by a cooling.

For quantification, standard curves were generated using purified amplicons of each selected target, produced with the above-mentioned primers, as a template. First, a classic PCR was performed with DreamTaq PCR Master Mix (Thermo Fisher Scientific) in a total volume of 50 µl, using 3 µl of genomic DNA extracted from the control samples and 2 µl of primers (10 mM each). The following condition was used for the PCR reactions: initial denaturation at 95°C, 2 min; 35 cycles of denaturation at 95°C for 30 s; annealing at 60°C for 30 s and extension at 72°C for 60 s; final extension at 72°C for 10 min. Then, PCR products were separated by electrophoresis on a 1.2% agarose gel and the expected bands were successively excised from the gel and purified with QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. The concentration of purified amplicons was determined using the Qubit 2.0 Fluorometer (Life Technologies).

The following equation was used to calculate the numbers of amplicon copies into the purified template with a predetermined concentration: weight of PCR fragment (g/µl) / (660 g/mol × pair bases number of the PCR fragment) × (6.023×10^{23}) = number of genomic copies per microliter.[100]Taken the number of amplicon copies of the purified templates per microliter, five 10-fold serial dilutions were made, ranging from 1,00E + 07 = 10^7 to 1,00E + 03 = 10^3 number of amplicon copies. These serial dilutions were used to generate the standard curves. All the standard curves had very similar efficiency and slope allowing the use of $2^{-(\Delta\Delta Ct)}$ method for the relative quantification.[101]

In the oesophageal bulb samples, the abundance of "*Ca*. Erwinia dacicola" 16S rDNA was calculated relative to the *B. oleae β-actin* reference, while in the midgut samples, the abundance of "*Ca.* Erwinia dacicola" 16S rDNA and the abundance of the universal bacterial segment of 16S rDNA were calculated relative to the *B. oleae gapdh* reference gene.

The ΔCt between the reference gene (*B. oleae β-actin* and *gapdh*) and the target gene ("*Ca*. Erwinia dacicola" 16S rDNA and 16S rDNA universal bacterial segment) was calculated for each sample using the following equation: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$. Then, the ΔΔCt between the ΔCt of the treated samples and the average ΔCt of the control groups was calculated using the following equation: $\Delta\Delta Ct = \Delta Ct_{\text{treatment}}$ – mean

$\Delta Ct_{control}$. Finally, the fold change $2^{-(\Delta\Delta Ct)}$ was calculated and averaged. Obtained data were log-transformed and the logarithm of the fold change $2^{-(\Delta\Delta Ct)}$ was used as an index of the relative abundance of the bacterial loads in comparison with the control.

2.6 Statistical analysis

The analyzed parameters were: adult mortality rate, daily diet consumption, fecundity, number of offspring/female, relative bacterial load of "*Ca*. Erwinia dacicola" and of total bacteria.

For both fitness and molecular data, the distributions were analyzed and the summary statistic calculated. Boxplots and bar-charts of each distribution were generated.

For each parameter One-way analysis of variance (One-way ANOVA) was carried out. Levene's test for homogeneity of variance from means and medians was performed to assess if the assumption of homoscedasticity, required for ANOVA, was verified and the H_0 was accepted with p-value $\geq$ 0.05%. The normality distribution of the residual was verified with the Shapiro-Wilk test with a $W \geq 0.05$ and a p normal ≥ 0.05. Tukey's pairwise *post-hoc* test was used to identify the treatments that were statistically different and Dunnett's simultaneous test for level mean versus control mean revealed the treatments that statistically differ from the control. When the data were not normally distributed, these were analyzed through the non-parametric Kruskal–Wallis test. Where significant differences were observed, *post-hoc* comparisons were performed using Mann–Whitney *post-hoc* tests.

Analysis of covariance (ANCOVA) was performed to investigate if the variability of the analyzed parameters was linked with some variables. Sex and treatment concentration were used as covariates for bioassay data.

Statistical analyses of the results were carried out with Minitab 18 Statistical Software®, GraphPad Prism 8®, PAST 3 ® and Microsoft Excel®.

3 RESULTS

3.1 Impact of experimental treatments on OLF fitness

Taking into accounts all the recorded parameters, the overall outcomes of the bioassays clearly indicates that both CO and Vi negatively affect OLF fitness.

The highest experimental concentration of CO (0.5%) determined a significant increase of the **adult mortality rate** for both sexes (ANOVA, $F_{6, 28} = 7.621$; Dunnett's pairwise post-hoc, P = 0.0224, P = 0.0001 – for females and males respectively) (Fig. 1). Moreover, a dose-dependent mortality trend was observed for CO (ANCOVA, $F_{2, 16} = 16.00$, P = 0.00011) (Fig. 1), which further corroborates the occurrence of direct toxicity of this compound on adult flies. None of the other experimental treatments had a significant impact on adult mortality (Fig. 1).

CO and Vi treatments had an overall negative impact on **daily diet consumption** with an inverse correlation between the treatment concentration and the diet intake (ANCOVA: treatment concentration: $F_{1, 10} > 6.64$, P < 0.030). A reduced daily diet consumption, compared to controls, was observed for both sexes at higher CO

doses (ANOVA, $F_{6, 14} = 12.67$; Dunnett's pairwise post-hoc P = 0.00015 and P = 0.00028 for females and males, respectively), and only for females for CO 0.1% and Vi 0.5% (P = 0.0029 and P = 0.0040, respectively) (Fig. 2). Moreover, the diet intake for each treatment was sex-related, with a higher level of diet consumption in females (ANCOVA: sex: $F_{1, 10} > 7.10$, $P < 0.026$).

The overall **fecundity (=** sum of the mean number of laid eggs/female recorded in the two oviposition tests**)** was significantly reduced only by the two higher CO concentrations (ANOVA: $F_{6, 21} = 10.44$; Dunnett's pairwise: P = 0.00017 and P = 0.00134 – for 0.5% and 0.1%, respectively) (Fig. 3A).

The **offspring *per* female** was statistically lower for all compounds tested (ANOVA: $F_{6, 21} = 45.21$; Dunnett's pairwise: $P < 0.05$) (Fig. 3B).

The results reported above indicate the occurrence of a direct negative impact of the higher concentrations of the experimental compounds tested on OLF. However, their negative impact at lower concentrations only on progeny development, mirroring the antibiotic response, indicates that part of their overall effect on fitness is likely mediated by the negative impact they may have on OLF associated microbiota, in particular the symbiont, which is essential for larval development.

3.2 Alteration of associated bacterial community

The results obtained from qPCR showed a widespread negative impact exerted by several experimental treatments on the load of the main bacterial endosymbiont of *B. oleae*, "*Ca.* Erwinia dacicola", both in the oesophageal bulb (Fig. 4A) and the midgut (Fig. 4B). The **endosymbiotic bacterial** load in **oesophageal bulb samples** was significantly lower (Kruskal-Wallis: $P < 0.036$) in all experimental treatments, compared to controls, except for the lowest CO concentration (0.02%) (Fig. 4A). The endosymbiotic bacterial titer in the **midgut** was significantly lower in all treated insects, except for the lowest concentration of CO and Vi (0.02% and 0.1%, respectively) (Kruskal-Wallis: $P < 0.036$) (Fig. 4B). These results are in tune with the reduced fitness recorded in the bioassay, which is, then, associated with a widespread decrease of the endosymbiont titer.

The **total bacterial community** resident in the midgut was significantly lower in all experimental treatments (Kruskal-Wallis: $P < 0.0121$), except for the antibiotic and the lowest concentration of CO (0.02%) (Fig. 5). These results suggest that CO and Vi have a broad and more intense effect on the gut microbial community, while the high specificity of Piperacillin on Enterobacteriaceae may likely allow the development of resistant bacteria harbored in the gut.

4 DISCUSSION AND CONCLUSION

In order to impair the fitness of insect pests, several authors suggested the use of symbioticide compounds to interrupt the vertical transmission of primary insect endosymbionts.[31-33] Here we contribute to test the potential of symbiosis disruption for control of *Bactrocera oleae*, one of the major agricultural pests of olive orchards.

Bioassays performed in this study, across two subsequent generations, have allowed teasing apart (1) the direct negative impact (i.e. mortality, reduced food intake, reduced fecundity) of the experimental compounds and (2) the indirect effects mediated by the alteration of symbiotic load and the gut-associated microbial

community (i.e. larval progeny development, number of offspring). The reduced survival and fecundity of treated flies are due to the direct negative impact of the administered compound. In fact, it is known that the presence of "*Ca*. Erwinia dacicola" is irrelevant for *B. oleae* females' fecundity when the flies are reared on a nitrogen-free diet, such as in our experimental bioassays.[60-61] This was also confirmed by our results since the survival and fecundity of aposymbiotic females, treated with antibiotics, were not statistically different from controls. On the contrary, the survival and fecundity of CO-treated flies were highly reduced in a dose-dependent manner, as expected based on previous studies demonstrating Copper toxicity on insects.[92-95] In contrast, Vi treatment did not affect the females' survival and fecundity displaying no significant toxic effect.

The presence of "*Ca*. Erwinia dacicola" is essential for *B. oleae* larval survival in unripe olive fruit.[54] Our results demonstrate that the fitness reduction induced by part of the experimental treatments can be exclusively due to their negative impact on insect-associated microorganisms. Indeed, the number of offspring *per* female clearly decreases in the treated insects, suggesting an anti-symbiotic effect of the administered compounds. Antibiotic treatment led to a drastic reduction in the number of offspring up to 97%, compared with the control cohort, in agreement with previous results by Ben-Yosef *et al*.[54] Additionally, all the administered compounds led to a significant reduction of the offspring, with a decrease ranging from 99% to approximately 50%, for CO 0.5% and Vi 0.1%, respectively. It is worth noting in the case of Vi these results are associated with a total absence of direct negative effects on OLF. This is an interesting issue when considering the direct toxic effect this compound may have on non-target arthropods.

In the case of CO, the higher experimental concentrations used (0.5% and 0.1%) exerted a direct toxic effect, which reduced the number of eggs laid by females, further reinforcing the negative impact on insect fitness mediated by a reduction of symbionts. Our study provides additional evidence for an anti-symbiotic effect of CO on the OLF and a possible explanation for the high larval mortality reported in CO sprayed olive fruits.[83-90] In the present study, strong evidence supports that the oral administration of CO can affect both OLF adult and larval fitness, as a result of both a toxic and a symbioticide effect. The symbioticide effect persists at the lowest concentration used, while a strong toxic effect occurs when higher doses are used supporting the toxicity of Copper for insects in a dose-dependent manner.[84-87] The double effect of CO could make this compound particularly useful for the OLF control, as suggested by previous field observations.[92-95] A recent study published by Bigiotti *et al*. demonstrated the symbioticide effect on the OLF of two different copper products after oral administration.[74]

The quantification of microbial loads in the OLF exposed to the experimental treatments considered clearly indicates that the capacity of larvae to develop in unripe olives is highly dependent on the presence of the primary symbionts, which are affected to a various extent by the compounds tested. Changes in progeny development can be linked to substantial changes in "*Ca.* Erwinia dacicola*"* load but treatments also strongly affected the entire microbiome composition. If and how alterations of the whole bacterial community compared to "*Ca.* Erwinia dacicola" are partially responsible for the observed fitness reduction remains to be studied.

Our results shed light on the possible use of natural products for symbiotic control of the OLF. Indeed, Vi has a strong negative impact on endosymbiont load and on the overall load of gut microbiota, which is associated with a reduced OLF larval survival. Vi has shown remarkable antibiotic activities against some bacteria and phytopathogenic fungi,[76-82,102] but, to our knowledge, this is the first time that a fungal metabolite is used for

symbiotic control of the OLF, *via* the disruption of its endosymbiotic bacterial load. Interestingly, the fungal metabolite 3-O-methylfunicone from a beneficial endophytic strain of *Talaromyces pinophilus* displayed toxic effects against the pea aphid *Acyrthosiphon pisum* (Homoptera, Aphidiidae).[76]

In conclusion, our results pave the way towards the use of this natural compound to disrupt *B. oleae* endosymbiosis, fostering the development of more sustainable strategies of OLF control. However, even though the absence of direct negative effects on OLF adults exerted by the lower concentration of CO and Vi appears to be promising in terms of possible toxic effects on non-target arthropods, further studies are necessary to assess the risk associated with the use of these compounds.

AUTHOR CONTRIBUTIONS

FP, APG, SL, DG, FV, and MS conceived and designed the study. MS conducted experiments. AB assisted in the qPCR set-up. FV provided the fungal metabolites. FP and MS wrote the manuscript, and APG, SL, AB, FV, and DG revised and improved the manuscript. All authors reviewed and approved the final version of the manuscript.

ACKNOWLEDGMENTS

This work was supported by the following projects:
MIURPON [grant number Linfa 03PE_00026_1; grant number Marea 03PE_00106]; POR FESR CAMPANIA 2014/2020- O.S. 1.1 [grant number Bioagro 559]; MISE CRESO [grant number Protection n. F/050421/01-03/X32]; PSR Veneto 16.1.1 [grant number Divine n. 3589659]. COST Action FA1405 [grant number: COST-STSM-ECOST-STSM-FA1405-270217-083727].
The Région Centre Project Insect Effect to DG [grant number: 2014-00094521].
Thanks are due to Paola Varricchio, Rosalba Ferrara and Ilaria Di Lelio (Department of Agricultural Sciences, University of Naples, Federico II) for their help in the set-up of molecular experiments, to Francesco Nugnes (CNR Institute for Sustainable Plant Protection, 80055 Portici (NA), Italy) for his help with the DGGE analysis, and to Prof. Antonio Belcari and Prof. Patrizia Sacchetti (Department of Agrifood Production and Environmental Sciences - DISPAA, University of Florence, Florence, Italy) for sharing their know-how about the *B. oleae* rearing and bioassay set up.

REFERENCES

1. Pereira JA, Alves MR, Casal S and Oliveira MB, Effect of olive fruit fly infestation on the quality of olive oil from cultivars Cobrancosa, Madural and Verdeal Transmontana. *Ital J Food Sci* **16**:355–365 (2004).

2. Malheiro R, Casal S, Baptista P and Pereira JA, Physico-chemical characteristics of olive leaves and fruits and their relation with *Bactrocera oleae* (Rossi) cultivar oviposition preference. *Sci Hort* **194**:208–214 (2015).

3. Medjkouh L, Tamendjari A, Keciri S, Santos J, Nunes MA and Oliveira MB, The effect of the olive fruit fly (*Bactrocera oleae*) on quality parameters, and antioxidant and antibacterial activities of olive oil. *Food Funct* **7**(6):2780–2788 (2016).

4. Montiel Bueno A and Jones O, Alternative methods for controlling the olive fly, *Bactrocera oleae*, involving semiochemicals. *IOBC/WPRS Bull* **25**:1–11 (2002).

5. Thomas DB and Mangan RL, Nontarget impact of spinosad GF-120 bait sprays for control of the Mexican fruit fly (Diptera: Tephritidae) in Texas citrus. *J Econ Entomol* **98**:1950–1956 (2005).

6. Skouras PJ, Margaritopoulos JT, Seraphides NA, Ioannides IM and Kakani EG, Organophosphate resistance in olive fruit fly, *Bactrocera oleae*, populations in Greece and Cyprus. *Pest Manag Sci* **63**:42–48 (2007).

7. Kakani EG and Mathiopoulos KD, Organophosphate resistance-related mutations in the acetyl-cholinesterase gene of Tephritidae. *J Appl Entomol* **132**:762–771 (2008).

8. Margaritopoulos JT, Skavdis G, Kalogiannis N, Nikou D and Morou E, Efficacy of the pyrethroid alpha-cypermethrin against *Bactrocera oleae* populations from Greece, and improved diagnostic for an iAChE mutation. *Pest Manag Sci* **64**:900–908 (2008).

9. Stasinakis P, Katsares V and Mavragani-Tsipidou P, Organophosphate resistance and allelic frequencies of esterases in the olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae). *J Agric Urban Entomol* **18**:157–168 (2001).

10. Kakani EG, Zygouridis NE, Tsoumani KT, Seraphides N, Zalom FG and Mathiopoulos KD, Spinosad resistance development in wild olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae) populations in California. *Pest Manag Sci* **66**(4):447–453 (2010).

11. Kampouraki A, Stavrakaki M, Karataraki A, Katsikogiannis G, Pitika E, Varikou K, Vlachaki A, Chrysargyris A, Malandraki E, Sidiropoulos N, Paraskevopoulos A, Gkilpathi D, Roditakis E and Vontas J, Recent evolution and operational impact of insecticide resistance in olive fruit fly *Bactrocera oleae* populations from Greece. *J Pest Sci* **91**:1429–1439 (2018).

12. Vontas J, Hernandez-Crespo P, Margaritopoulos JT, Ortego F, Feng HT, Mathiopoulos KD and Hsu JC, Insecticide resistance in Tephritid flies. *Pest Biochem Physiol* **100**:199–205 (2011).

13. Pavlidi N, Kampouraki A, Tseliou V, Wybouw N, Dermauw W, Roditakis E, Naueng, Van Leeuwenbe T, Vontas J, Molecular characterization of pyrethroid resistance in the olive fruit fly *Bactrocera oleae*. *Pestic Biochem Phys* **148**:1–7 (2018).

14. Spanedda AF and Terrosi A, A field method for assessing the harmfulness to olive tree entomofauna of pesticides used for olive fly control. In: Vitagliano C, Martelli GP (eds) Proceedings of the 4th International Symposium on olive growing, Valenzano, Italy, 25- 30 September 2000. *Acta Hortic* **586**(2):849–852 (2002).

15. Spanedda AF and Terrosi A, Toxic residue patterns in olive fruit, oil, and waste water of the most common in- secticides used for controlling olive fly in Central Italy. In: Proceedings of the 4th International Symposium on olive growing, Valenzano, Italy, 25- 30 September 2000. *Acta Hortic* **586**(2**)**:853–856 (2002).

16. Daane KM and Johnson MW, Olive fruit fly: managing an ancient pest in modern times. *Annu Rev Entomol* **55**:151–169 (2010).

17. Hladnik M, A review of plant protection against the olive fly (*Bactrocera oleae* (Rossi, 1790) Gmelin) and molecular methods to monitor the insecticide resistance alleles. *Acta Agr Slov* **109**(1):135 (2017).

18. Dias NP, Zotti MJ, Montoya P, Carvalho IR and Nava DE, Fruit fly management research: A systematic review of monitoring and control tactics in the world. *Crop Prot* **112**:187–200 (2018).

19. Raspi A and Loni A, Alcune note sull'allevamento di *Opius concolor* (Szèpl.) (Hymenoptera: Braconidae) e su recenti tentativi d'introduzione della specie in Toscana ed in Liguria. *Frustula Entomol* **17**:135–145 (1994).

20. Wharton RA Classical biological control of fruit-infesting Tephritidae, in World Crop Pests: Fruit Flies—Their Biology, Natural Enemies and Control, 3B, ed. by Robinson AS, Hooper G, Elsevier, Amsterdam, pp. 303–313 (1989).

21. Miranda MA, Miquel M, Terrassa J, Melis N and Monerris M, Parasitism of *Bactrocera oleae* (Diptera, Tephritidae) by *Psyttalia concolor* (Hymenoptera, Braconidae) in the Balearic Islands (Spain). *J Appl Entomol* **132**:798–805 (2008).

22. Yokoyama VY, Rendon PA and Sivinsk J, *Psyttalia* cf. *concolor* (Hymenoptera: Braconidae) for biological control of olive fruit fly (Diptera: Tephritidae) in California. *Environ Entomol* **37**:764–773 (2008).

23. Noce ME, Belfiore T, Scalercio S, Vizzarri V and Iannotta N, Efficacy of new mass-trapping devices against *Bactrocera oleae* (Diptera tephritidae) for minimizing pesticide input in agroecosystems. *J Environ Sci Health B* **44**(5**)**: 442–448 (2009).

24. Estes AM, Nestel D, Belcari A, Jessup A, Rempoulakis P and Economopoulos AP, A basis for the renewal of sterile insect technique for the olive fly, *Bactrocera oleae* (Rossi). *J Appl Entomol* **136**:1–16 (2011).

25. Canale A and Benelli G, Impact of mass-rearing on the host seeking behavior and parasitism by the fruit fly parasitoid *Psyttalia concolor* (Szépligeti) (Hymenoptera: Braconidae). *J Pest Sci* **85**(1):65–74 (2012).

26. Sagri E, Reczko M, Tsoumani KT, Gregoriou ME, Harokopos V, Mavridou AM, Tastsoglou S, Athanasiadis K, Ragoussis J and Mathiopoulos KD, The molecular biology of the olive fly comes of age. *BMC Genet* **15**(2):S8 (2014).

27. Fabbri A, Lambardi M and Ozden-Tokatli Y, Olive Breeding. In Breeding Plantation Tree Crops: Tropical Species, ed. by Jain SM, Priyadarshan PM, Springer, New York, NY, pp 423–465 (2009).

28. Corrado G, Alagna F, Rocco M, Renzone G, Varricchio P, Coppola V and Rao R, Molecular interactions between the olive and the fruit fly *Bactrocera oleae*. *BMC Plant Biol* **12**(1):86 (2012).

29. Alagna F, Kallenbach M, Pompa A, De Marchis F, Rao R, Baldwin IT, Bonaventure G and Baldoni L, Olive fruits infested with olive fly larvae respond with an ethylene burst and the emission of specific volatiles. *J Integr Plant Biol* **58**(4):413–425 (2016).

30. Grasso F, Coppola M, Carbone F, Baldoni L, Alagna F, Perrotta G, Pérez-Pulido AJ, Garonna A, Facella P, Daddiego L, Lopez L, Vitiello A, Rao R and Corrado G, The transcriptional response to the olive fruit fly (*Bactrocera oleae*) reveals extended differences between tolerant and susceptible olive (*Olea europaea* L.) varieties. *PLoS ONE* **12**(8):e0183050 (2017).

31. Darby AC, Symbiosis research as a novel strategy for insect pest control. In: Biorational Control of Arthropod Pests: Application and Resistance Management, ed. by Ishaaya I, Horowitz, AR, Springer, Netherlands, pp 207–231 (2009).

32. Klepzig KD, Adams AS, Handelsman J and Raffa KF, Symbioses: a key driver of insect physiological processes, ecological interactions, evolutionary diversification, and impacts on humans. *Environ Entomol* **38**(1):67–77 (2009).

33. Arora AK and Douglas AE, Hype or opportunity? Using microbial symbionts in novel strategies for insect pest control. *J Insect Physiol* **103**:10–17 (2017).

34. Giron D, Dedeine F, Dubreuil G, Huguet E and Mouton L, Influence of microbial symbionts on plant-insect interactions. In: Insect-Plant Interactions in a Crop Protection Perspective, vol 81, ed. by Sauvion N, Thiéry D, Calatayud P, Elsevier, GBR, pp 225-257 (2017).

35. Douglas AE, Microbial brokers of insect-plant interactions revisited. *J Chem Ecol* **39**:952-961 (2013).

36. Macke E, Tasiemski A, Massol F, Callens M and Decaestecker E, Life history and eco-evolutionary dynamics in light of the gut microbiota. *Oikos* **126**(4):508–531 (2017).

37. Relman DA, "Til death do us part": coming to terms with symbiotic relationships. *Nat Rev* **6**:721–724 (2008).

38. Vasanthakumar A, Handelsman IJ, Schloss P, Bauer L and Raffa KF, Gut microbiota of an invasive wood boring beetle, the emerald ash borer: community composition and structure across different life stages. *Environ Entomol* **37**:1344–1353 (2008).

39. Giron D, Dubreuil G, Bennett A, Dedeine F, Dicke M, Dyer LA, Erb M, Harris MO, Huguet E, Kaloshian I, Kawakita A, Lopez-Vaamonde C, Palmer TD, Petanidou T, Poulsen M, Sallé A, Simon JC, Terblanche JS, Thiéry D, Whiteman NK, Woods HA and Pincebourde S, Promises and challenges in insect–plant interactions. *Entomol Exp Appl* **166**(5):319–343 (2018).

40. Login FH, Balmand S, Vallier A, Vincent-Monegat C, Vigneron A, Weiss-Gayet M, Rochat D and Heddi A, Antimicrobial peptides keep insect endosymbionts under control. *Science* **334**:362–365 (2011).

41. Le-Feuvre RR, Ramírez CC, Olea N and Meza-Basso L, Effect of the antimicrobial peptide indolicidin on the green peach aphid *Myzus persicae* (Sulzer). *J Appl Entomol* **131**(2):71–75 (2007).

42. Luna-Ramirez K, Skaljac M, Grotmann J, Kirfel P, Vilcinskas A, Orally delivered scorpion antimicrobial peptides exhibit activity against pea aphid (*Acyrthosiphon pisum*) and its bacterial symbionts. *Toxins* **9**(9):261 (2017).

43. Petri L, Ricerche sopra i batteri intestinali della mosca olearia. Roma: Memorie della Regia Stazione di Patologia Vegetale di Roma (1909).

44. Capuzzo C, Firrao G, Mazzon L, Squartini A and Girolami V, "*Candidatus* Erwinia dacicola", a co-evolved symbiotic bacterium of the olive fly *Bactrocera oleae* (Gmelin). *Int J Syst Evol Microbiol* **55**:1641–1647(2005).

45. Belcari A, Sacchetti P, Marchi G and Surico G, La mosca delle olive e la simbiosi batterica. *Inf Fitopatol* **9**:55–59 (2003).

46. Sacchetti P, Granchietti A, Landini S, Viti C, Giovannetti L and Belcari A, Relationships between the olive fly and bacteria. *J Appl Entomol* **132**:682–689 (2008).

47. Estes AM, Life in a fly: the ecology and evolution of the olive fly endosymbiont, *Candidatus* Erwinia dacicola. Ph.D. thesis, University of Arizona, Tucson, AZ, USA (2009).

48. Estes AM, Hearn DJ, Burrack HJ, Rempoulakis P and Pierson EA, Prevalence of *Candidatus* Erwinia dacicola in wild and laboratory olive fruit fly populations and across developmental stages. *Environ Entomol* **41**(2):265–274 (2012).

49. Kounatidis I, Crotti E, Sapountzis P, Sacchi L, Rizzi A, Chouaia B, Bandi C, Alma A, Daffonchio D, Mavragani-Tsipidou P and Bourtzis K, *Acetobacter tropicalis* is a major symbiont of the olive fruit fly (*Bactrocera oleae*). *Appl Environ Microbiol* **75**:3281–3288 (2009).

50. Savio C, Mazzon L, Martinez-Sanudo I, Simonato M, Squartini A and Girolami V, Evidence of two lineages of the symbiont "*Candidatus* Erwinia dacicola" in Italian populations of *Bactrocera oleae* (Rossi) based on 16S rRNA gene sequences. *Int J Syst Evol Micrbiol* **62**:179–187 (2012).

51. Polpass AJ, Ben-Yosef M, Jurkevitch E and Yuval B, Symbiotic bacteria affect oviposition behavior in the olive fruit fly Bactrocera oleae. *J Insect Physiol* **117**:103917 (2019).

52. Paracer S and Ahmadjian V, Symbiosis: an introduction to biological associations, 2nd ed. Oxford University Press, Oxford, United Kingdom (2000).

53. Ishikawa H, Insect symbiosis: an introduction. In Insect symbiosis, ed. by K. Bourtzis and T. Miller, CRC Press, Boca Raton, pp. 1–16 (2003).

54. Ben-Yosef M, Pasternak Z, Jurkevitch E and Yuval B, Symbiotic bacteria enable olive fly larvae to overcome host defences. *Roy Soc Open Sci* **2**(7):150–170 (2015).

55. Ras E, Beukeboom LW, Càceres C and Bourtzis K, Review of the role of gut microbiota in mass rearing of the olive fruit fly, *Bactrocera oleae*, and its parasitoids. *Entomol Exp Appl* **164**:237–256 (2017).

56. Dimou I, Rempoulakis P and Economopoulos AP, Olive fruit fly [Bactrocera (Dacus) oleae (Rossi) (Diptera: Tephritidae)] adult rearing diet without antibiotic. *J Appl Entomol* **134**:72–9 (2010).

57. Sacchetti P, Ghiardi B, Granchietti A, Stefanini FM and Belcari, A, Development of probiotic diets for the olive fly: evaluation of their effects on fly longevity and fecundity. *Ann Appl Biol* **164**:138–150 (2014).

58. Sacchetti, P., Pastorelli, R., Bigiotti, G. *et al.* Olive fruit fly rearing procedures affect the vertical transmission of the bacterial symbiont *Candidatus* Erwinia dacicola. *BMC Biotechnol* **19**:91 (2019).

59. Bigiotti G, Pastorelli R, Guidi R, Belcari A and Sacchetti P, Horizontal transfer and finalization of a reliable detection method for the olive fruit fly endosymbiont, *Candidatus* Erwinia dacicola. *BMC Biotechnol* **19**(Suppl 2):1–12 (2019).

60. Ben-Yosef M, Aharon Y, Jurkevitch E and Yuval B, Give us the tools and we will do the job: symbiotic bacteria affect olive fly fitness in a diet-dependent fashion. *P Roy Soc B* **277**:1545–1552 (2010).

61. Ben-Yosef M, Pasternak Z, Jurkevitch E and Yuval B, Symbiotic bacteria enable olive flies (*Bactrocera oleae*) to exploit intractable sources of nitrogen. *J Evol Biol* **27**:2695–2705 (2014).

62. Blow F, Gioti A, Goodhead IB, Kalyva M, Kampouraki A, Vontas J, *et al.*, Functional Genomics of a Symbiotic Community: Shared Traits in the Olive Fruit Fly Gut Microbiota. *Genome Biol Evol* **12**:3778–3791 (2020).

63. Fletcher BS, The biology of dacine fruit flies. *Annu. Rev. Entomol.* **32**:115–144 (1987).

64. Greany PD, Host plant resistance to tephritids: an under-exploited control strategy. In Fruit flies: their biology, natural enemies and control, ed. by Robinson AS and Hooper G, Elsevier Science, Amsterdam pp. 353–362 (1989).

65. Messina FJ and Jones VP, Relationship between fruit phenology and infestation by the apple maggot (Diptera: Tephritidae) in Utah. *Ann Entomol Soc Am* **83**:742–752 (1990).

66. Joachim-Bravo IS, Fernandes OA, Bortoli SRA and Zucoloto FS, Oviposition behavior of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae): association between oviposition preference and larval performance in individual females. *Neotrop Entomol* **30**:559–564 (2001).

67. Rattanapun W, Amornsak W and Clarke AR, *Bactrocera dorsalis* preference for and performance on two mango varieties at three stages of ripeness. *Entomol Exp Appl* **131**:243–253 (2009).

68. Kapatos ET and Fletcher BS, The phenology of the olive fly, *Dacus oleae* (Gmel.) (Diptera, Tephritidae), in Corfu. *Z Angew Entomol* **97**:360–370 (1984).

69. Neuenschwander P, Michelakis S, Holloway P and Berchtol W, Factors affecting the susceptibility of fruits of different olive varieties to attack by *Dacus oleae* (Gmel.) (Dipt., Tephritidae). *Z Angew Entomol* **100**:174–188 (1985).

70. Whitehead SR and Bowers MD, Evidence for the adaptive significance of secondary compounds in vertebrate-dispersed fruits. *Am Nat* **182**:563–577 (2013).

71. Gutierrez-Rosales F, Romero MP, Casanovas M, Motilva MJ and Minguez-Mosquera MI, β-Glucosidase involvement in the formation and transformation of oleuropein during the growth and development of olive fruits (*Olea europaea* L. cv. Arbequina) grown under different farming practices. *J Agric Food Chem* **60**:4348–4358 (2012).

72. Amiot MJ, Fleuriet A and Macheix JJ, Importance and evolution of phenolic compounds in olive during growth and maturation. *J Agric Food Chem* **34**:823–826 (1986).

73. Pavlidi N, Gioti A, Wybouw N, Dermauw W, Ben-Yosef M, Yuval B, Jurkevich E, Kampouraki A, Van Leeuwen T and Vontas J, Transcriptomic responses of the olive fruit fly *Bactrocera oleae* and its symbiont *Candidatus* Erwinia dacicola to olive feeding. *Nature Sci Rep* **7**:42633 (2017).

74. Bigiotti G, Pastorelli R, Belcari A and Sacchetti P, Symbiosis interruption in the olive fly: Effect of copper and propolis on *Candidatus* Erwinia dacicola. *J Appl Entomol* **143**(4):357–364 (2019).

75. Rubio MB, Hermosa R, Reino JL, Collado IG and Monte E, Thctf1 transcription factor of *Trichoderma harzianum* is involved in 6-pentyl-2H-pyran-2-one production and antifungal activity. *Fungal Genet Biol* **46**(1):17–27 (2009).

76. Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Barbetti MJ, Li H, Woo SL and Lorito M, A novel role for Trichoderma secondary metabolites in the interactions with plants. *Physiol Mol Plant Pathol* **72**:1–3 (2008).

77. Vinale F, Sivasithamparam K, Ghisalberti EL, Ruocco M, Woo SL and Lorito M, *Trichoderma* secondary metabolites that affect plant metabolism. *Nat Prod Commun* **7**(11):1545–1550 (2012).

78. Keswani C, Singh HB, Hermosa R, García-Estrada C, Caradus J, He Y, Mezaache-Aichour S, Glare TR, Borriss R, Vinale F and Sansinenea E, Antimicrobial secondary metabolites from agriculturally important fungi as next biocontrol agent. *Appl Microbiol Biotechnol* **103**: 9287-9303 (2019).

79. JS, Bu'Lock JD and Yuen TH, Viridiol, a steroid-like product from *Trichoderma viride*. *J Chem Soc Chem Commun* **14**:839 (1969).

80. Howell CR and Stipanovic RD, Effect of sterol biosynthesis inhibitors on phytotoxin (viridiol) production by *Gliocladium virens* in culture. *Phytopathology* **84**:969-972 (1994).

81. de Souza Sebastianes FL, Montes Peral Valente AM, Boffo EF, Ferreira AG, Soares Melo I, Beraldo Moraes LA, Azevedo JL, Pizzirani-Kleiner AA and Teixeira Lacava P, Isolation of the antibacterial agent Viridiol from the Mangrove endophytic fungus *Hypocrea virens*, as monitored by a biologic assay against *Escherichia coli* and NMR Spectroscopy. *Current Biotec* **6**:325 (2017).

82. Vinale F, Nicoletti R, Lacatena F, Marra R, Sacco A, Lombardi N, d'Errico G, Digilio MC, Lorito M and Woo SL, Secondary metabolites from the endophytic fungus *Talaromyces pinophilus*. *Nat Prod Res* **31**(15):1778-1785 (2017).

83. Tzanakakis ME, Considerations on the possible usefulness of olive fruit fly symbionticides in integrated control in olive groves. In: Integrated Pest Control in Olive-Groves, ed. by Cavalloro R and Crovetti A, *Proc CEC/FAO/IOBC*, International Joint Meeting, Pisa, 3-6 April **1984**:386-393 (1985).

84. Belcari A and Bobbio E, L'impiego del rame nel controllo della mosca delle olive, *Bactrocera oleae*. *Inf Fitopatol* **12**:52–55 (1999).

85. Sacchetti P, Belcari A and Del Pianta R, Utilizzo di prodotti ad azione antibatterica per il controllo della mosca delle olive. In: La difesa dai fitofagi in condizioni di olivicoltura biologica, Atti Accademia nazionale dell'Olivo e dell'Olio, Spoleto, 29-30 October **2002**:23-33 (2004).

86. Belcari A, Sacchetti P, Rosi MC and Del Pianta R, Control of the olive fly (*Bactrocera oleae*) through the use of copper products in Central Italy. *IOBC/WPRS Bull* **28**:45–48 (2005).

87. Tsolakis H, Ragusa E, and Tarantino P, Control of *Bactrocera oleae* by low environmental impact methods: NPC methodology to evaluate the efficacy of lure-and- kill method and copper hydroxide treatments. *Bull Insectology* **64**:1–8 (2011).

88. Caleca V, Lo Verde G, Lo Verde V, Piccionello MP, and Rizzo R, Control of *Bactrocera oleae* and *Ceratitis capitata* in organic orchards: Use of clays and copper products. *Acta Hortic* **873**:227–234 (2010).

89. Gonçalves F and Torres L, Effect of copper Oxychloride on the olive infestation by *Bactrocera oleae* in northeastern Portugal. *Acta Hortic* **949**:333–340 (2012).

90. Rosi MC, Sacchetti P, Librandi M and Belcari A, Effectiveness of different copper products against the olive fly in organic olive groves. *IOBC/WPRS Bull* **30**(9):277–281 (2007).

91. Prophetou-Athanasiadou DA, Tzanakakis ME, Myroyannis D and Sakas G, Deterrence of oviposition in *Dacus oleae* by copper hydroxide. *Entomol Exp Appl* **61**:1–5 (1991).

92. Jensen P and Trumble JT, Ecological consequences of bioavailability of metals and metalloids. *Recent Res Dev Entomol* **4**:1–17 (2003).

93. Cheruiyot DJ, Investigation of elemental defense and trophic transfer of metals using beet armyworm, *Spodoptera exigua*, Hübner. Ph.D. dissertation. Auburn University, Alabama, USA (2012).

94. Majumdar TN and Gupta A, Acute and chronic toxicity of copper on aquatic insect *Chironomus ramosus* from Assam, India. *J Environ Biol* **33**(1):139–142 (2012).

95. Cheruiyot DJ, Boyd RS, Coudron TA and Cobine PA, Biotransfer, bioaccumulation, and effects of herbivore dietary Co, Cu, Ni, and Zn on growth and development of the insect predator. *Podisus maculiventris* (Say). *J Chem Ecol* **39**(6):764–772 (2013).

96. Moffatt JS, Bu'Lock JD and Yuen TH, Viridiol, a steroid-like product from Trichoderma viride. *J Chem Soc Chem Commun* **14**:839 (1969).

97. Zervas GA, Reproductive physiology of *Dacus oleae* (Gmel.) (Diptera: Trypetidae). Comparison of a wild and artificially reared flies. *Geoponika* **282**:10–14 (1982).

98. Nadkarni M, Martin FE, Jacques NA, Hunter N, Determination of bacterial load by real-time PCR using a broad range (universal) probe and primer set. *Microbiology* **148**:257–266 (2002).

99. Sagri E, Koskinioti P, Gregoriou ME, Tsoumani KT, Bassiakos YC and Mathiopoulos KD, Housekeeping in Tephritid insects: The best gene choice for expression analyses in the medfly and the olive fly. *Sci Rep-UK* **7**:1–9 (2017).

100. Malorny B, Hoorfar J, Bunge C and Helmuth R, Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Appl Environ Microbiol* **69**(1):290-296 (2003).

101. Livak KJ and Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**(4):402–408 (2001).

102. Sawa R, Mori Y, Iinuma H, Naganawa H, Hamada M, Yoshida S, Furutani H, Kajimura Y, Fuwa T and Takeuchi T, Harzianic acid, a new antimicrobial antibiotic from a fungus. *J Antibiotics* **47**:731–732 (1994).

TABLES

Table 1: List of the primers used in molecular analyses

Target genes	Primers	Amplicon length (bp)
"*Ca*. Erwinia dacicola" 16S rDNA	EdF1: 5'-CTAATACCGCATAACGTCTTCG-3' EdEnRev: 5'-CCACCTACTAGCTAATCCC-3'	90
Bacteria universal segment 16S rDNA	16SuniF: 5'-GACGTTACCCGCAGAAGAA-3' 16SuniR: 5'-CGCCCAGTAATTCCGATTAA-3'	198
Housekeeping genes		
β-actin	Act2F: 5'-GCAGAGCAAACGTGGTAT-3' Act2R: 5'-TGTGATGCCACACTTTCT-3'	91
gapdh	GapF: 5'-GGTGTCTTTACAACAATCG-3' GapR: 5'-TAGATACGACCTTCATGTCAG-3'	148

FIGURES

Figure 1: Mean (± SE) adult mortality rate recorded on the 14th day of treatment. Bars marked with an asterisk were significantly different from the mean registered for controls of the same sex (Dunnett's pairwise post-hoc test, $P < 0.02$). Control flies were fed on a compound-free diet. Antibiotics cohorts were fed on a diet containing Piperacillin. CO-treated flies were fed on a diet containing Copper Oxychloride at different concentrations (0.5%, 0.1%, 0.02% w/w). Vi-treated flies were fed on a diet containing Viridiol at different concentrations (0.5%, 0.1% w/w).

Figure 2: Mean (± SE) daily diet consumption. Bars marked with an asterisk were significantly different from the mean registered for controls of the same sex (Dunnett's pairwise post-hoc test, $P < 0.004$). Control flies were fed on a compound-free diet. Antibiotics cohorts were fed on a diet containing Piperacillin. CO-treated flies were fed on a diet containing Copper Oxychloride at different concentrations (0.5%, 0.1%, 0.02% w/w). Vi-treated flies were fed on a diet containing Viridiol at different concentrations (0.5%, 0.1% w/w).

Figure 3: Mean (±SE) females fecundity (A) and mean number of offspring/female (B). Bars marked with an asterisk were significantly different from the control mean (Dunnett's pairwise post-hoc test, $P < 0.03$). Control flies were fed on a compound-free diet. Antibiotics cohorts were fed on a diet containing Piperacillin. CO-treated flies were fed on a diet containing Copper Oxychloride at different concentrations (0.5%, 0.1%, 0.02% w/w). Vi-treated flies were fed on a diet containing Viridiol at different concentrations (0.5%, 0.1% w/w).

Figure 3: Mean (±SE) endosymbiotic bacterial load resulted from qPCR and expressed as log ($2^{-(\Delta\Delta Ct)}$) in oesophageal bulb (A) and midgut (B) samples of *B. oleae* females. The zero-level corresponds to the mean control load. Bars marked with an asterisk were significantly different from the control mean in the Mann-Whitney's pairwise post-hoc test. Control flies were fed on a compound-free diet. Antibiotics cohorts were fed on a diet containing Piperacillin. CO-treated flies were fed on a diet containing Copper Oxychloride at different concentrations (0.5%, 0.1%, 0.02% w/w). Vi-treated flies were fed on a diet containing Viridiol at different concentrations (0.5%, 0.1% w/w).

Figure 5: Mean (±SE) total bacterial load resulted by qPCR and expressed as log ($2^{-(\Delta\Delta Ct)}$) in midgut samples of *B. oleae* females. The zero-level corresponds to the mean control load. Bars marked with an asterisk were significantly different from the control mean in the Mann-Whitney's pairwise post-hoc test. Control flies were fed on a compound-free diet. Antibiotics cohorts were fed on a diet containing Piperacillin. CO-treated flies were fed on a diet containing Copper Oxychloride at different concentrations (0.5%, 0.1%, 0.02% w/w). Vi-treated flies were fed on a diet containing Viridiol at different concentrations (0.5%, 0.1% w/w).