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RESEARCH

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Novel approaches to assess lethal and sublethal effects when evaluating risks of biopesticides toward beneficial arthropod

Ilaria Laterza[1†], Maria Luisa Vitale[2†], Maria Francesca Agostinacchio[1], Zineb Bennani[2], Enrico de Lillo[1], Giovanni Tamburini[1], Vincenzo Verrastro[2], Giuseppe Cavallo[2], Nicolas Desneux[3], Antonio Biondi[4], Elisa Santovito[5] and Daniele Cornara[1,2*]

Abstract

Background Biopesticides are defined as substances derived from naturally occurring materials (i.e., plants, microorganisms and minerals) characterized by low environmental effects, rapid degradation, and low toxicity for humans and beneficial insects. However, the assumption of safety for beneficials is generally solely based on the evaluation of acute mortality upon exposure, overlooking possible underhanded sublethal effects.

Methods We selected the parasitoid *Leptomastix dactylopii* Howard (Hymenoptera: Encyrtidae) attacking the mealybug pest *Planococcus citri* Risso (Hemiptera: Pseudococcidae) as case study to investigate whether the exposure to four biopesticides authorized in organic citrus orchards in the EU, in addition to a *Clitoria ternatea* (Fabales: Fabaceae) extract, causes significant alterations in the parasitoid physiology and behavior. Classical ecotoxicological tests aimed at evaluating survival rate, parasitization rate, emergence rate and sex-ratio, have been combined with olfactometry to assess deviation in the dynamics of the orientation toward the host, and with a novel optical oxygen respirometry technique to observe possible alterations in oxygen consumption.

Results All the tested compounds except spinosad were found to not harm *L. dactylopii*, causing neither lethal nor significant sublethal effects in the exposed parasitoids compared to the control.

Conclusions In conclusion, all the tested biopesticides showed compatibility within biological control strategies in a field scenario (i.e., organic orange orchards). Moreover, the screening protocols described in the present manuscript, integrating classical ecotoxicological tests and novel approaches targeting specific behavioral and physiological toxicant-induced alterations, allowed to shed light on the multifaceted impact of biopesticides on natural enemies.

Keywords Ecotoxicology, Biological control, *Planococcus citri*, *Citrus*, Olfactometry, Optical oxygen respirometry

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[†] Ilaria Laterza and Maria Luisa Vitale equally contributed to the paper.

*Correspondence:
Daniele Cornara
danielecornara@uniba.it
Full list of author information is available at the end of the article

Background

The ecological impact of synthetic pesticides has been a growing concern since the second half of the twentieth century, concomitantly with the growing body of evidence on their detrimental effects on human health and on key beneficial arthropods in agroecosystems (Desneux et al. 2007; Lee and Choi 2020; Serrão et al. 2022). In this context, biopesticides, biological control agents and semiophysicals have drawn attention as suitable tools for gradually replacing synthetic pesticides for more sustainable pest control strategies (Butu et al. 2020; Ribeiro et al. 2021; Nieri et al. 2022). Specifically, biopesticides are defined as substances derived from naturally occurring materials (e.g. plants, microorganisms, and minerals) (Copping and Menn 2000). These molecules are generally considered safe compared to synthetic insecticides since they are characterized by low environmental effects, rapid degradation, and low toxicity for humans and beneficial arthropods (Biondi et al. 2012a; Monsreal-Ceballos et al. 2018).

However, in the last decade several studies reported negative side effects of biopesticides on several group of insects including pollinators and natural enemies, particularly in terms of behavioral and physiological alterations rather than mortality (Biondi et al. 2012b, 2013; Cappa et al. 2024). The public perception of insecticidal toxicity is in fact generally based on the consideration of acute mortality as the main toxicological endpoint, overlooking pesticide-induced stress and sublethal effects (Biondi et al. 2013; Guedes et al. 2016; Ogburn and Walgenbach 2019; Ranjbar et al. 2021; Giunti et al. 2022). Exposure to a toxicant may indeed cause physiological and/or behavioral alterations in the arthropods, ranging from detrimental effects on reproduction, development, longevity and fecundity, sex ratio of the progeny, immune system, and communication (Simmonds et al. 2002; Desneux et al. 2007; González-Zamora et al. 2013), to hormesis effects as observed in the parasitoid *Trichogramma japonicum* Ashmead (Hymenoptera: Trichogrammatidae) (Wang et al. 2022). Therefore, these sublethal effects can be of particular concern for beneficial arthropods, potentially affecting their efficacy in pest control (Smith and Krischik 2000; Tunca et al. 2012; Lisi et al. 2023).

In this context, an intimate knowledge of the impact of biopesticides on natural enemies is crucial for limiting the impact of the agricultural practices on the ecosystem and for a safe and sustainable crop production. Nevertheless, the evaluation of insecticidal side effect on highly specialized organisms as parasitoids requires experimental efforts beyond the simple "spray and count" and should consider holistically how the toxicant may interfere on the antagonist-host interaction through a multidisciplinary approach. For instance, olfactometry, traditionally used for evaluating insect response to volatile organic compounds (VOCs), can be employed to investigate pesticide-induced effects on antagonist orientation toward the host (Desneux et al. 2004, 2006a, 2006b). Respirometry may on the other side unveil crucial information on how exposure to a pesticide could affect the metabolism of the antagonist and, in the long term, the whole ecosystem services they provide. However, the equipment used for respirometry presents several limitations (i.e., high costs, size constraints of the tested specimens, relatively short time of observation permitted, sophisticated tools) that possibly restrained its application in ecotoxicological studies.

In the present study, we combined risk assessment of physiological (survival rate, parasitization rate, emergence rate and sex-ratio) and behavioral (olfactory orientation) sublethal effects together with a new optical oxygen respirometry (Santovito et al. 2021) to shed light on eco-toxicological profile of biopesticides. Specifically, the parasitoid *Leptomastix dactylopii* Howard (Hymenoptera: Encyrtidae), the mealybug *Planococcus citri* Risso (Hemiptera: Pseudococcidae), and the sweet orange plant *Citrus sinensis* L. (Rutaceae), were investigated as a model system to explore the biopesticides impact on the beneficial-pest-plant tri-trophic interaction. We hence tested four active substances (azadirachtin, pyrethrin, mineral oil and sweet orange essential oil) authorized in organic citrus orchards in the EU derived, and a new molecule obtained from an extract of *Clitoria ternatea* L. (Fabales: Fabaceae), that has recently gained attention for its insecticidal property (Mokrane et al. 2020). The results on the eco-toxicological profile of the tested biopesticides will be useful for enabling their effective use in integrated biological control programs.

Methods

Insects and plants rearing

A colony of *P. citri* was established from individuals collected on sweet orange plants in the premises of CIHEAM-Bari (Valenzano, Bari, Italy). The colony was maintained for 15 generations on butternut squashes, *Cucurbita moschata* Duchesne (Cucurbitales: Cucurbitaceae), under controlled conditions (24 ± 1 °C, 60 ± 5% RH, 14L/10D).

A colony of the parasitoid *L. dactylopii* was set using individuals provided by Biofabbrica di Ramacca (Ramacca, Italy). The parasitoids were reared on *P. citri*, maintained on butternut squash in Plexiglas and mesh boxes at 24 ± 1 °C, 60 ± 5% RH, 14L/10D, and supplemented with water and drops of honey. Virgin *L. dactylopii* individuals were obtained from cocooned pupae of

P. citri individually isolated into 15 mL plastic tubes supplemented with honey drops under the same environmental conditions described above.

Plant material consisted of 6-month old sweet orange plants 20–30 cm in height (var. Madam Vinous) grafted on potted sour orange plants, maintained in a greenhouse under controlled conditions (25 ± 2C°, 60 ± 5% RH, 16L/8D). Plants were grown in 1.4 L pots (20 cm in height and 9 cm in diameter), using Brill® 3 Special soil (Brill, Germany), and watered daily with tap water.

Tested biopesticides

The following active substances were tested: Azadirachtin (AZA), Pyrethrin (PYR), Mineral oil (MO), sweet Orange Essential Oil (OEO), *C. ternatea* (CT), a combination of *C. ternatea* and sweet Orange Essential Oil (CT + OEO) (see Table 1 for commercial names, doses and mode of actions of the pesticide tested). The selected products were applied at the maximum doses reported on the labels; tap water and spinosad were used as negative and positive control, respectively (see Table 1 for commercial names, doses and mode of actions of the biopesticide tested).

Lethal effect of biopesticides residues on parasitoid survival

In the current study we aimed to recreate a field scenario, thus the parasitoids were exposed to the dried biopesticide residues directly on the plants to firstly evaluate the toxicant impact on parasitoid survival. Specifically, the exposure was performed adapting the method described by Biondi et al. (2012a): sweet orange plants were sprayed until run-off (7.5 ± 0.5 ml approx. volume per plant) with the tested biopesticides at the doses indicated in Table 1 using a hand-sprayer. After 3 h of drying into a fume hood (25 ± 3C°, RH 60 ± 5%), each plant was inserted into plastic and 0.5 mm-mesh isolators (hereafter, the arena), one plant per arena, keeping the pot outside the arena (See Additional file 1: Fig. S1). Five females and five males (virgin adults, 24–72 h old) were then introduced into each arena for 72 h to expose the parasitoids to the residues on the plant. Honey drops were supplied as source of food, while water was placed in 1.5 ml tube plugged with cotton. A total of 50 individuals (25 males and 25 females; 5 arenas) were tested per treatment. Parasitoids' survival was hence recorded at 1, 3, 12, 24, 36, 48, 60, and 72 h after their introduction into the arena. The tests were conducted in a growth chamber under controlled conditions (24 ± 1 °C, RH 60 ± 5%, 14L/10D).

Sublethal effects induced by biopesticide residues

To evaluate the sublethal effects of biopesticides on *L. dactylopii,* the first bioassay was conducted immediately after the parasitoid's survival assay (Bioassay 1: Sublethal effect on the parasitization efficiency). Parasitoids were exposed to biopesticides residues following the methodology described above for the survival test (Bioassay 2: Sublethal effect on the olfactory orientation; Bioassay 3: Sublethal effect on the oxygen consumption).

Bioassay 1: Sublethal effect on the parasitization efficiency

After the exposure to residues, the surviving females were used to assess whether the biopesticides affected the parasitization rate and the emergence rate, time of emergence, and sex ratio of their offspring. A non-treated sweet orange plant was hence caged inside a new arena and infested with 20 third-instar *P. citri* nymphs for 24 h, to give the mealybugs the time to settle on the plant. A single exposed female parasitoid was then released into the arena for 24 h, then removed and discarded (Fig. 1A). For each treatment, a total of 25 females were tested in 25 different arenas. After ten days, all *P. citri* nymphs were singly isolated in clean vials provided with honey drops and kept in a growth chamber (24 ± 1°C, RH 60 ± 5%, 14L/10D) until adult wasps' emergence. The number of mummies, the number and sex of emerged wasps,

Table 1 Details of the commercial name, mode of action and dose of the tested biopesticides

Active ingredient	Commercial name	Dose (ml/L)	Mode of action	Reference
Azadirachtin (AZA)	Oikos®	1.5	Anti-feedant, repellent, molting disruptor	(Sparks and Nauen 2015)
Pyrethrin (PYR)	Pyganic 1.4	1.5	Sodium channel modulator, disrupts insects' nervous system	(Sparks and Nauen 2015)
Paraffinic mineral oil (MO)	UFO	20	Asphyxiant	(Sparks and Nauen 2015)
Sweet orange essential oil (OEO)	Prev-am®	6	Interfering with basic metabolic, biochemical, and physiological functions; histological modifications	(Campolo et al. 2018)
Clitoria ternatea extract (CT)	Sero-x®	20	Cell membrane disruption (predicted)	(Oguis et al. 2019)
Clitoria ternatea extract + Sweet orange essential oil (CT + OEO)	Prev-am® + Sero-x®	20 + 6	–	
Spinosad (SP)	Laser®	0.50	Acetylcholine receptor allosteric	(Sparks and Nauen 2015)

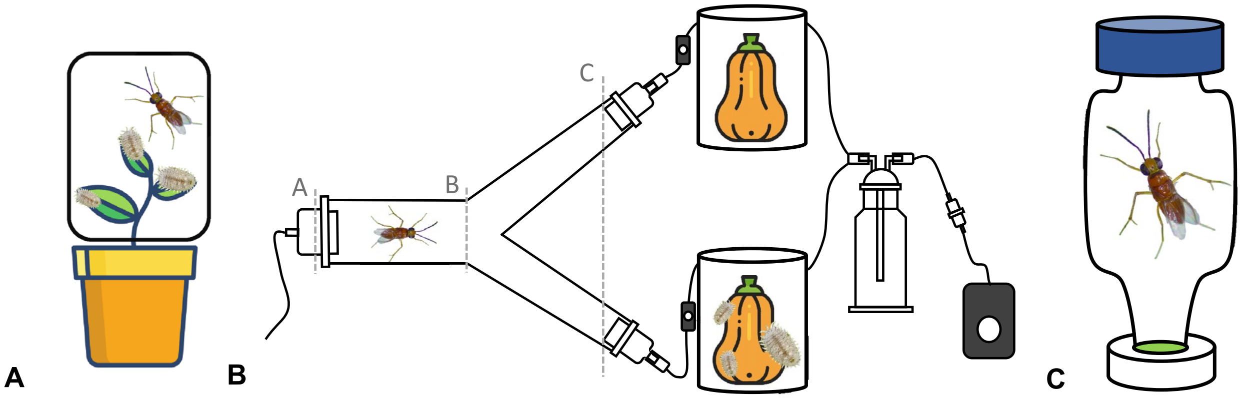

Fig. 1 Experimental set-up used for the evaluation of parasitization efficiency (**A**), orientation toward the host (**B**) and oxygen consumption (**C**) after exposure to biopesticides residuals

and the time needed for emergence (calculated from the day of nymph isolation) were recorded. Finally, the parasitization (number of parasitized nymphs over the total recovered individuals) and emergence (number of emerged parasitoid wasps over the total number of parasitized individuals) rate, the mean time of emergence, and the sex ratio (expressed as proportion of the number of males and the total number of insects emerged) were calculated.

Bioassay 2: Sublethal effect on the olfactory orientation

An olfactometer bioassay was carried out to assess potential biopesticide-induced alterations on the parasitoid olfactory response, adapting the Tunca et al. (2014) protocol. Butternut squashes (*C. moschata*) infested or not infested with *P. citri* (adults and third instars nymphs) were used as volatile source. We chose butternut squashes instead of sweet orange plants since preliminary olfactometer assays did not show a consistent response of the parasitoid to citrus odors, irrespective of the infestation status (data not shown).

Female parasitoids were tested after exposure to residues (as described in the "Lethal effect of biopesticides residues on parasitoid survival" section) using an Y-shaped olfactometer (inner diameter of 1 cm) (Fig. 1B). Specifically, the olfactometer consisted of a central arm, 7 cm long, and two lateral arms 9 cm long, each at 45° angle to the central arm. Each lateral arm was connected through a silicone tube to a squash (infested/not infested) located inside plastic bags (oven bags, 25 × 38 cm, Cuki®) and used as a source of olfactory stimuli. A continuous airflow (0.2 L/min) was set into the olfactometer lateral arms using an air pump placed at the end of the system. The air produced, cleaned by passing through bubbler and charcoal filters, finally inflated the plastic bags containing the squashes. The olfactometer was located horizontally on a plane surrounded by cardboard walls 60 cm high and illuminated by a white, fluorescent light tube (700 lx), mounted ca. 1 m above the olfactometer and aligned with the olfactometer central arm.

The olfactometer was conceptually divided into three main sections (Fig. 1B) with each female introduced into the zone A and observed for ten minutes. Females that appeared to be not active during the testing time (i.e., scarce antennal movement, no locomotory behavior) were discarded. At least 20 replicates (active females) per treatment were carried out. After each replicate, the air inside the olfactometer was cleaned with clean air for five minutes, while the position of the squashes (infested and non-infested) was inverted every five observations to avoid position bias.

We measured the following parameters: reactivity (i.e., female entering the zone B); number of insects that made a choice (i.e., females entering one of the two lateral arms of the zone C); number of insects changing their first choice; latency period (i.e., time required to enter the zone B from the insect introduction in the olfactometer). Every five tested insects, the olfactometer arms were washed with ethanol (90%) and rinsed with distilled water.

Bioassay 3: Sublethal effect on the oxygen consumption

An innovative technology based on optical oxygen sensing (Santovito et al. 2021, 2022) was tested for the first time here on insects, to investigate the oxygen consumption by *L. dactylopii* after exposure to biopesticides' residues (as described in the "Lethal effect of biopesticides residues on parasitoid survival" section). The experimental set-up consisted of a solid-state coating, prepared according to the protocol of Santovito et al. (2021), and dispensed onto the bottom of sterile 1 mL glass vials (Agilent Technologies Italia, Cernusco sul Naviglio, Milan, Italy) in a sterile environment (Fig. 1C). The vials were then left to air dry in a laminar flow hood for 1 h before being used. The exposed parasitoids, both females and males, (22 to 38 for each treatment) were singly introduced into the vials containing the sensor coating, provided with a drop of honey as food source. The vials were hermetically closed and left at room temperature (24 ± 1 °C). The phosphorescence emitted by the sensor at the bottom of the vial was hence measured two times a day for three days.

As described by Santovito et al. (2021), the sensor is quenched by the presence of O_2: as the insect in the vial consumed O_2, the sensor signal increased. The sensor signal was recorded using the hand-held sensor reader FirestingGO_2 Pyrosciences (Aachen, Germany) as phase shift signals, measured in dphi° (degrees angle units). Then, the signal was then converted in percentage of oxygen available in the vial using the calibration equation provided by Santovito et al. (2021). The decrease of oxygen concentration inside the vial was ascribable to the insect respiration.

Statistical analysis

Statistical analyses were carried out in RStudio (v. 2023.03.0, R Core Team 2022). General and generalized linear mixed-effects' models were used to explore the sublethal effects of the tested biopesticides on *L. dactylopii,* using the treatment as a predictor. For all the models tap water (i.e., control treatment) was set as baseline. Four general linear mixed-effects models were hence run to test the parasitoid response in the first bioassay, including parasitization rate, time of emergence, and sex ratio as response variables. Since all the *L. dactylopii* individuals completed their development by parasitizing their hosts, resulting in eventual homogeneous adult emergence, no model was run to assess the effect of treatment on this response variable. Data obtained in the olfactory orientation bioassay were analyzed running four generalized linear mixed-effected models considering a binomial family. We consequently included reactivity, number of insects making a choice, number of insects changing their first choice, and latency period as response variables. Regarding the analysis of the oxygen consumption bioassay, a general linear mixed-effected model was run including the oxygen consumption as response variable. Treatment (categorical; seven levels), time (categorical; three levels), sex (categorical; two levels), and the interaction between treatment and time were used as predictors. Tukey multiple comparison test was applied to determine the significance among treatments. Analyses were performed using the "glmmTMB" package (Brooks et al. 2017) implemented in R (R Core Team 2022). We checked the models for overdispersion and residual distribution using the "DHARMa" package (Hartig 2017).

Results

Lethal effect of biopesticides residues on parasitoid survival

All tested *L. dactylopii* individuals survived during the 72 h exposure to biopesticides residuals. Exposure to spinosad (used as positive control) caused 100% mortality of the parasitoids within 12 h (See Additional file 1: Table S1).

The surviving parasitoids were used in the sublethal effects bioassays (spinosad was consequently excluded from the assessment of sublethal effects).

Bioassay 1: Sublethal effect on the parasitization efficiency

The parasitization rate was significantly different among treatments CT (38.1% ± 4.4 SE) and OEO (53.0% ± 6.0 SE) (Fig. 2, Table 2), but not between treatments and the control (See Additional file 1: Table S2).

Conversely, no statistical differences were observed for all the other investigated variables (Table 2), with the emergence rate ranging between 96.1 to 99.00%, the progeny sex ratio varying from 0.6 to 0.7 (See Additional file 1: Table S2) and the mean time of emergence ranging between 20.9 (days) and 23.1 (days) (See Additional file 1: Table S2).

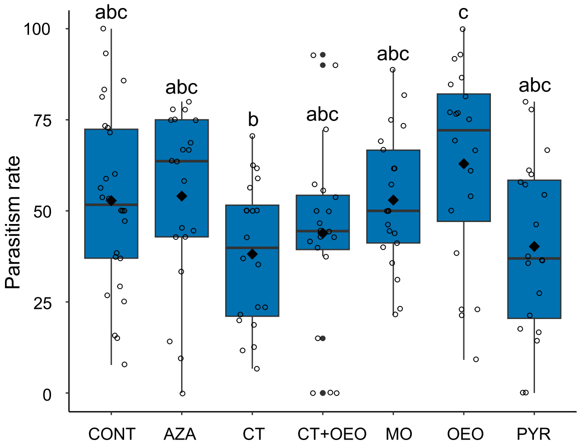

Fig. 2 Parasitization rate of *Planococcus citri* nymphs by *Leptomastix dactylopii* after biopesticides' residual exposure. Letters on box plots indicate the results of Tukey pairwise comparison test ($p < 0.05$); different letters indicate statistically significant differences between treatments. Treatment abbrevation: *CONT*: Control, *AZA*: Azadirachtin, *CT: Clitoria ternatea* extract, *CT + OEO: Clitoria ternatea* extract + Sweet orange essential oil, *MO:* Paraffinic mineral oil, *OEO*: Sweet orange essential oil, *PYR:* Pyrethrin

Table 2 Results of the generalized linear mixed models testing the effect of biopesticides on *Leptomastix dactylopii*

Bioassay	Variable	Chi-square	P-value
Parasitoid efficiency	*Parasitization rate*		
	Treatment	**17.841**	**0.007**
	Emergence rate		
	Treatment	2.507	0.868
	Time of emergence		
	Treatment	3.003	0.809
	Sex ratio		
	Treatment	4.199	0.650
Olfactory orientation	*Reactivity*		
	Treatment	4.938	0.552
	Individuals making a choice		
	Treatment	6.649	0.355
	Individuals changing the first choice		
	Treatment	1.586	0.954
	Latency period		
	Treatment	6.031	0.420
Respirometry	*Oxygen consumption (dphi°)*		
	Treatment	11.684	0.069
	Day	**211.196**	**0.000**
	Sex	0.127	0.722
	Treatment:Day	7.717	0.807

Bold variables and values represent the significant variable obtained from the ANOVA test

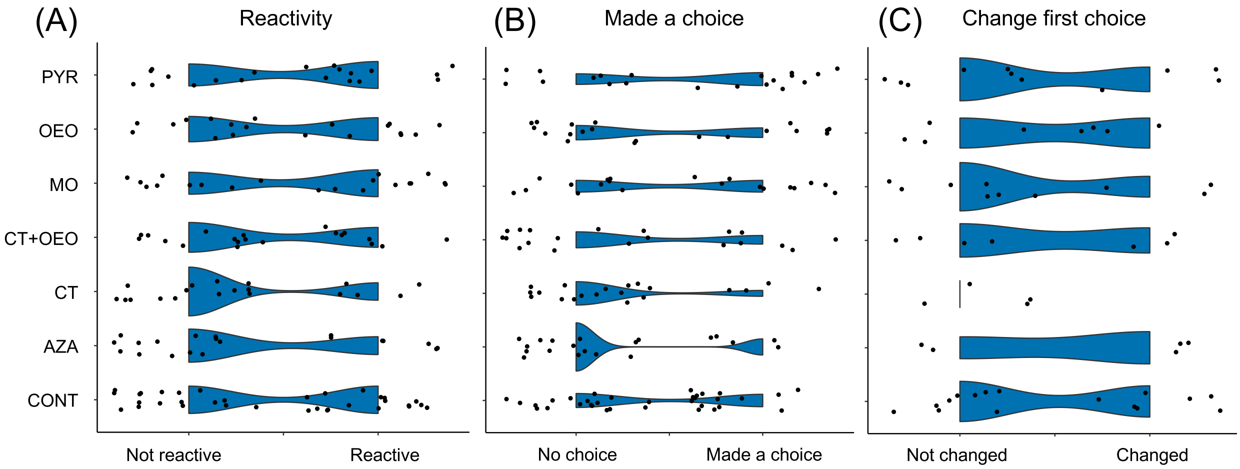

Fig. 3 Response of *Leptomastix dactylopii* in the olfactory orientation after residues exposure. Violin graphs show the parasitoid reactivity (**A**), the proportion of individuals making a choice (**B**) and changing their first choice (**C**). Tukey pairwise comparison test showed no significant differences between treatment ($p > 0.05$)

Bioassay 2: Sublethal effect on the olfactory orientation

No difference with the control in *L. dactylopii* response to host-substrate odor cues (i.e., reactivity, individuals making a choice, individuals changing the first choice, and latency period) was found after the exposure to the tested bioinsecticides (Fig. 3, Table 2). Considering all the treatments and individuals tested, we observed an average of 44.15% *L. dactylopii* females entering the zone B (See Additional file 1: Table S3), with 39.61% individuals making a choice, and 17.53% changing their first choice (See Additional file 1: Table S3). The latency period ranged between 2.53 and 5.06 min. The time required for the first choice ranged from 3.03 to 4.58 min (See Additional file 1: Table S3).

Bioassay 3: Sublethal effect on the oxygen consumption

In all the treatments including the control, we observed a general decrease of oxygen concentration over time, corresponding to an increase of oxygen consumption. Mean oxygen concentration inside the vials ranged from 20.5% O_2 (day 1), to 19% O_2 (day 2), and 18.2% O_2 (day 3), with statistically significant differences between days (Table 2, Fig. 4A). In contrast, no significant differences were observed among the treatments or between treatments and control (Table 2, Fig. 4B).

Discussion

Biopesticides are generally considered as environmental-friendly alternative to pesticides, with limited impact on non-target organisms and beneficial arthropods (Czaja et al. 2015; Benelli et al. 2018; Essiedu et al. 2020; Samada and Tambunan 2020). Nevertheless, several studies have reported either lethal or sublethal effects following biopesticides exposure in beneficial arthropods as pollinators and natural enemies (Suma et al. 2009; Biondi et al. 2012a, 2012b; Passos et al. 2022; Uzun Yigit et al. 2023). The general uncertainty on biopesticides toxicological profile calls for a more comprehensive risk assessment for both regulatory and integration in biological control programs. In this context, we investigated the lethal effect (i.e., acute mortality) and the risk of physiological (i.e., survival rate, parasitization rate, emergence rate, sex-ratio and respirometry) and behavioral (i.e., olfactory orientation) sublethal effects to shed light on eco-toxicological profile of biopesticides.

Since we aimed to simulate a post-exposure field scenario, we exposed the parasitoids to biopesticides sprayed directly on the plants. This exposure methodology offers the insect a choice: besides being exposed to the toxicant while walking or resting on the plant, the insect has the possibility to avoid the chemical remaining on surfaces that have not been reached by the pesticide (as for example the arena). Additionally, compared to the toxicant application on inert surfaces, the interaction of biopesticides residues with plant chemicals (enzymes and wax layer) may significantly affect their toxicity for the exposed organism (Schuler 1996; Desneux et al. 2005). Therefore, the exposure to a pesticide through the sprayed plant gives a more realistic representation of what can be the outcome of a treatment with a certain chemical on an insect under field conditions.

We found all the investigated compounds, excluding spinosad, to be safe for the parasitoid, with no significant lethal or sublethal effects observed on the parasitoids, showing compatibility within biological control strategies in the field.

The lethal effect observed for the spinosad is consistent with previous results which found the molecule to affect the nervous system of Hymenoptera parasitoids when ingested or through contact, resulting in spinosad-induced muscle contractions which ultimately lead to the insect's death (Biondi et al. 2012b).

Regarding mineral oil, in contrast with our data, Suma et al. (2009) reported 53.2% mortality rate of *L. dactylopii* exposed to the pesticide, with additionally significant effects on the progeny sex ratio. However, in their work, the authors used a narrow range mineral oil sprayed on

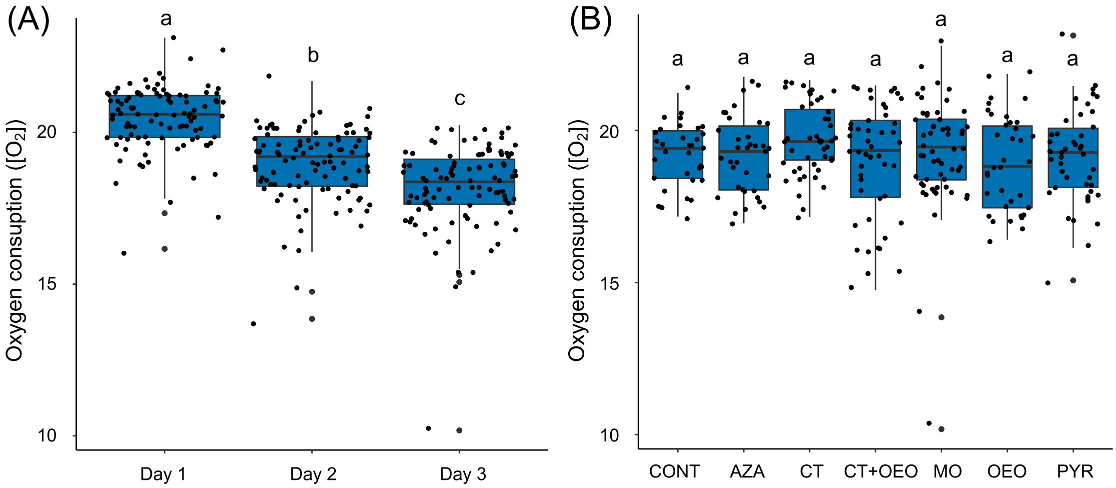

Fig. 4 Oxygen consumption of *Leptomastix dactylopii* after residual exposure in the respirometry bioassay. Letters on box plots indicate the results of Tukey pairwise comparison test ($p < 0.05$); different letters indicate statistically significant differences between days/treatments

an inert surface (e.g., glass cage) rather the mineral oil applied directly on the plants, as in our bioassay. Toxicological tests conducted by spraying the toxicant on inert surfaces rather than plants may indeed give a biased prediction of the toxicant impact on the target insect (Desneux et al. 2006a, 2006b).

In addition to the absence of evident lethal effects, the biopesticide residues did not alter the parasitization efficiency (i.e., parasitization rate, sex ratio, time of emergence), in contrast with data reported on other parasitoid species (Saber et al. 2004; Beloti et al. 2015; Ribeiro et al. 2021; Giunti et al. 2022).

The relative safety for *L. dactylopii* of the tested biopesticides residues was further confirmed by the olfactometry assay and the optical oxygen respirometry, with no significant changes observed in the parasitoid orientation and in oxygen consumption between the treatments and the control.

Olfactometry has been used by several authors to evaluate insects' response to inert materials or plant tissues treated with different molecules including pesticides (Staley et al. 2011; Pope et al. 2012; Tunca et al. 2014; Djukić et al. 2016). For example, insecticides such as azadirachtin and pyrethrins were reported as capable of interfering with the parasitoid–host interaction based on the observed repellent effect of filter papers sprayed with the compounds (Tunca et al. 2014). However, such fundamental investigations do not furnish a complete picture of the biopesticide–parasitoid interaction, given the behavior of the parasitoid and the ability to spot the host within the agroecosystem after the exposure to pesticide residuals is overlooked. In our work, we focused on this post-exposure scenario, investigating whether the exposure to biopesticides' residuals might impair parasitoid ability to locate the host. The results obtained suggest that in a field scenario (i.e., organic orange orchards) the use of *L. dactylopii* can be compatible with the tested active substances since no significant changes in the insect behavior (i.e., reactivity, latency period, number of wasps making a choice, or changing their first choice) or orientation toward the host were observed. *Clitoria ternatea* extract did also show no negative effects on the parasitoid, consistently with the study performed by Mensah et al. (2015) on natural enemies of *Helicoverpa* spp. (Lepidoptera: Noctuidae). This molecule is currently authorized on few crops (i.e., tomato, brassicas, cucurbits, cotton), but considering its insecticidal properties on pests of agricultural interest (Poth et al. 2011; Mensah et al. 2015; Zanotelli et al. 2017) and the unharmful effect showed in our work, further studies on citrus are highly encouraged.

The relative safety of the biopesticides tested for *L. dactylopii* was further confirmed by our oxygen consumption bioassay. This innovative optical oxygen respirometry is based on a phosphorescent dye sensitive to the oxygen level, which can be real-time measured and registered by means of a hand-held reader. This technology has been devised to measure the metabolic activity of bacterial colonies. Here, for the first time, it has been applied to toxicological studies on insects. Previous studies have investigated the metabolic pattern of insects using other devices (i.e., Tartes et al. 1999; Malison et al. 2022; Abbas et al. 2023). Nevertheless, those tools showed limitations as the need for highly specialized and expensive equipment and reagents, possibility to observe the insect response only for short period, and size constraints for the specimens to be tested (Lighton and Turner 2004; Marais et al. 2005; Huang et al. 2014). In our experiment, using the sensor vials as respiration chambers, we recorded the oxygen consumption of a small insect (3 mm) via an accurate, simple (sample-and-measure), portable, low-cost (< $5 k) device (Santovito et al. 2021). This novel technology can be easily applied in ecotoxicological studies on pests and beneficial organisms.

In our trial, the oxygen consumption trends by parasitoids exposed to biopesticides' residuals did not diverge from the control. Previous studies showed how the exposure to insecticides can deeply affect the respiration of living beings in different ways, depending on the active ingredient: tebufenozide, permethrin and thiamethoxam caused a decrease of the respiration rate (Silva et al. 2020), while sub-lethal concentrations of beta-cypermethrin have led to an increase of the respiratory rate compared to the control in certain insect species (Xiao et al. 2017). Nevertheless, the exposure methodologies applied in those experiments were different from the one adopted in the present study, including submersion of the insect for 5 s in the insecticide solutions (Silva et al. 2020), or exposure to pesticide residues in glass tubes (Xiao et al. 2017), potentially affecting the respiratory response recorded by the authors.

Conclusion

A detailed knowledge of the multifaceted impact that both synthetic pesticides and biopesticides might have on natural enemies is crucial for limiting the ecosystem impacts of the agricultural activity and pursuing the final goal of a safe and sustainable production. In this context, the experimental approach used in the present study, integrating classical entomo-toxicology studies with modern tools, including the use of a novel optical oxygen respirometry, can be extended to others beneficial-pest-plant system to gather more insights on the potential of chemicals to interfere with biological control programs. The results obtained showed all the tested biopesticides to not harm *L. dactylopii* either in terms of lethal or sublethal effects, showing compatibility within biological control strategies in sweet orange orchards.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43170-024-00249-8.

Additional file 1: Table S1. Details of the number of dead Leptomastix. dactylopii at 1, 3, 12, 24, 36, 48, 60, and 72 hours after the release in the arena for the residues' exposure. **Table S2.** Details of the parasitism dynamic of L. dactylopii after residues' exposure. Means±SE Means followed by the same letter within a column are not significantly different (p<0.05). **Table S3.** Details of the L. dactylopii response after residues' exposure in the olfactometer bioassay. **Figure S1.** Details of the arena used for the exposure to residues of the tested molecules.

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Author contributions

DC conceived and designed the study; MV, IL, MA, ZB, GC and ES collected the data; IL and GT performed the statistical analysis; IL, MV and DC wrote the first draft of the paper; AB and ND substantively revised the paper. EdL, GT, VV and DC provided fundings. All authors read and approved the final manuscript.

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Availability of data and materials

Raw data and additional information are available from the corresponding author (daniele.cornara@uniba.it) upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Soil, Plant and Food Science, University of Bari, Via Amendola 165/A, 70126 Bari, Italy. [2]International Centre for Advanced Mediterranean Agronomic Studies-Institute of Bari (CIHEAM-Bari), 70010 Valenzano, BA, Italy. [3]INRA, University of Côte d'Azur, CNRS, UMR 1355-7254, 06903 Sophia Antipolis, France. [4]Department of Agriculture Food and Environment, University of Catania, Via S. Sofia 100, 95123 Catania, Italy. [5]Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Via Amendola 122, 70125 Bari, Italy.

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