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Evaluation of side effects of two *Providencia entomophila* strains associated with major olive tree insect pests on the parasitoid *Trichogramma oleae*

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Abstract

The control of olive tree insect pests has mostly been performed for several decades by chemical means. However, in an attempt to develop more natural treatments, two *Providencia entomophila* Ksentini *et al*. (Enterobacterales Morganellaceae) strains (IO-6 and IO-20) previously isolated from the olive tree insect pests *Prays oleae* Bernard (Lepidoptera Hyponomeutidae) and *Bactrocera oleae* (Rossi) (Diptera Tephritidae), respectively, were tested and were found to be effective against *B. oleae.* The side effects of both strains on beneficial insects were evaluated on the preimaginal stages and the adults of the egg parasitoid *Trichogramma oleae* Voegele et Pointel (Hymenoptera Trichogrammatidae) at concentrations ranging from 10^5 to 10^9 cfu/ml. The results showed that all IO-6 tested concentrations harmed *T. oleae* larvae, as well as the concentration 10^9 cfu/ml on prepupae. In contrast, different concentrations of strain IO-20 were found to be harmless for all developmental stages but slightly harmful at concentrations of 10^9 and 10^6 cfu/ml on larvae and prepupae, respectively. Nevertheless, none of the tested strains and concentrations were found to be persistent even 2 days after treatment application, as *T. oleae* adult survival was not negatively affected by any bacterial strain. However, when treatments were applied during developmental stages, some side effects on females external appearance, as brachypterous, two-male-antennae and single-male-antenna, were detected. Thus, taking into consideration all the aforementioned parameters, only *P. entomophila* IO-20 at a concentration of 10^5 cfu/ml guarantees perfect respect to all *T. oleae* developmental stages and offspring appearance.

Key words: *T. oleae*, preimaginal stages, adults, *P. entomophila*, mortality, persistence.

Introduction

With over 100 million grown olive trees (National Observatory of Agriculture, 2021), Tunisia, in which olive cultivation is strategic, is among the six top-ranking countries in terms of olive oil production in the world (International Olive Council, 2022). The Tunisian production of olive oil, which is mainly carried out in dry conditions and thus depends greatly on rainfall, is characterized by a strong annual variation (Chebbi *et al.*, 2019). Thence, the yearly exported olive oil quantity oscillated between 325 and 240 thousand tons in 2017/2018 and 2021/2022, respectively (International Olive Council, 2022). Hence, in parallel to the yearly variation of production that causes negative impacts on the economic level, this millennial sector faces many other problems such as the attack of various insect pests of economic importance. The most significant ones are the olive fly *Bactrocera oleae* (Rossi) (Diptera Tephritidae), the olive moth *Prays oleae* Bernard (Lepidoptera Hyponomeutidae), and the olive psyllid *Euphyllura olivina* (Costa) (Homoptera Psyllidae). In Tunisia, these pests are subject to control in treatment campaigns insured by the Tunisian state and are part of a national strategy undertaken since the independence of the country. However, apart from the organic farmers that should ensure their own treatments with very expensive and narrow spectrum products, chemical insecticides are exclusively used during national campaigns. The decision to use chemical insecticides is, unfortunately, driven by the organic treatments' high prices, hence the impossibility of change despite the concerns that repetitive chemical usage creates for many decades. In fact, chemical insecticides cause disastrous effects on the environment and consumers' health. These reasons pushed many Tunisian research teams to focus more on the development of biological control methods such as those based on microbial formulations. In that context, effective *Bacillus thuringiensis* Berliner (Bacillales Bacillaceae) bacteria strains on Lepidoptera (Boukedi *et al*., 2015) and Diptera larvae have been reported and patented (Zribi Zghal *et al*., 2017). These results follow a series of long research on this bacterium (Saadaoui *et al*., 2009; Elleuch *et al*., 2016; Zribi Zghal *et al*., 2019) and its effects on many agricultural pests, such as *Ephestia kuehniella* Zeller (Lepidoptera Pyralidae) (Saadaoui *et al*., 2010), *Spodoptera littoralis* (Boisduval) (Lepidoptera Noctuidae) (BenFarhat-Touzri *et al*., 2018; 2019) and *Ectomyelois ceratoniae* (Zeller) (Lepidoptera Pyralidae) (Boukedi *et al*., 2018). The same interest has been given to the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota Hypocreales) (Guesmi-Jouini *et al.*, 2014), with some isolates' performance being successfully tested on *Aphis gossypii* Glover (Hemiptera Aphididae) (Mseddi *et al.*, 2022).

Likewise, other bacterial (Gharsallah *et al.*, 2018) and fungal (Gharsallah *et al.*, 2020) isolates have been isolated from olive tree insect pests, with the bacterial species *Providencia entomophila* Ksentini *et al*. (Enterobacterales Morganellaceae) showing a promising performance when tested against the flour moth *E. kuehniella*

(Gharsallah *et al.*, 2018) and the olive fly, *B. oleae* (Ksentini *et al.*, 2019). To our knowledge, these studies are among the first to define *Providencia* species among olive tree insects' pathogens.

However, before considering *P. entomophila* isolates as future bio-insecticides against olive tree insect pests, their impact on olive oil quality, as well as their side effects on consumers, on auxiliary fauna, and the environment should be thoroughly assessed. With respect to this aspect, the egg parasitoid *Trichogramma oleae* Voegele et Pointel (Hymenoptera Trichogrammatidae) was used in this study as a test organism. *Trichogramma* species are distributed all over the world and play an important role in biological control against pests of several cultivated plants. Indeed, their success is mainly due to the fact that they cause the death of their host before it hatches, thus interrupting its biological cycle (Hassan, 1998). This genus, among other beneficial organisms, has been used for decades in order to detect the side effects of agricultural insecticides (Hassan *et al.*, 2000; Suh *et al.*, 2000; Ksentini *et al.*, 2010). Thus, the aim of the current study was to assess *T. oleae* preimaginal stages' and adults' sensibility toward two selected *P. entomophila* strains. The obtained results will help to categorize these bacterial strains' level of harmfulness and determine their suitability as potential organic treatments against agricultural pests.

Materials and methods

Insects

The species *T. oleae* was used in all experiments. It was previously collected from a capsule baited with *E. kuehniella* eggs in the region of Sfax, Tunisia. Its phylogenetic identity was confirmed using a molecular approach. Sequences were published in the GenBank database under the accession numbers OQ450343 and OQ450319. This strain is known to be thelytokous following its infection with the endobacteria *Wolbachia* Hertig (Rickettsiales Ehrlichiaceae). *T. oleae* was maintained under laboratory conditions (25 ± 1 °C; 65 ± 5% RH; L/D: 16/8) on the eggs of the Mediterranean flour moth *E. kuehniella*. The latter species is being reared for many generations at the "Olive Tree Institute" on diets based on whole-wheat flour. Before their presentation to *Trichogramma* for parasitization, the host eggs were UV killed and then glued on thin carton cards with diluted Arabic gum. Egg parasitoids were kept under the same laboratory conditions mentioned above and were fed with a 50% honey solution throughout the rearing and also the following experimental process.

Bioassays

To conduct bioassays on development stages, approximately 300 *E. kuehniella* eggs were glued on 0.7 cm^2 of area on filter paper and then exposed to around 20 *T. oleae* females for 5 hours at 25 ± 1 °C, 65 ± 5% RH and L/D: 16/8. These females were then gently discarded with a camelhair brush, and the *E. kuehniella* eggs were transferred to new containers and put under the same climatic conditions mentioned above. When reaching the desired experimental stage either three, six, or nine days after the initial parasitization, the filter papers carrying parasitized eggs were dipped each for 10 seconds into the respective treatment solution (table 1). These days of treatments corresponded to the larval, prepupal, and pupal stages of *Trichogramma* wasps, respectively (Knutson, 1998). The insecticidal efficacy of two *P. entomophila* strains previously isolated from olive tree pests, namely, IO-6 (from *P. oleae*) and IO-20 (from *B. oleae*) (Ksentini *et al.*, 2019), were tested. These strains were incubated at 37 °C and 180 rpm in liquid LB for 48 hours and served for the preparation of the relative stock solution. Then, five successive concentrations of each bacterial suspension were carefully prepared, encompassing a range of 10^5 to 10^9 colony-forming units per millilitre (cfu/ml). These concentrations were carefully calculated and designed to guarantee a rise in bacterial density. The initial concentration of 5.10^9 cfu/ml and 3.10^9 cfu/ml for the bacterial strain IO-6 and IO-20 respectively, indicated a higher inoculum. In order to accurately and thoroughly prepare the serial concentrations. Following amounts of 10^8, 10^7, 10^6, and 10^5 cfu/ml obtained by 10-fold serial dilution supplied successively lower levels of bacterial cells, allowing a wide range of bacterial densities to be investigated in the following tests. In addition, the two commercial insecticides Delfin® (active ingredient: *B. thuringiensis*) and Biomat® (active ingredient: dimethoate) were used as positive controls. The concentration of each insecticide solution reflected the recommended field rates (table 1), and as for the bacterial suspensions, new solutions were made for each day of exposure. The negative control group was exposed to distilled water only. Following treatments with the corresponding solution and concentration in the relative day, areas of filter papers were kept at room temperature till the excess solution dried and then put together in corresponding clean Petri dishes (60 mm in diameter) sealed with parafilm® and kept under laboratory conditions (25 ± 1 °C; 65 ± 5% RH; L/D: 16/8). Each of the treatments was replicated 6 times, and eggs were considered successfully parasitized if they blackened (a shiny black appearance of egg chorion) due to *T. oleae* development.

Table 1. Used commercial insecticides (organic and chemical) and bacterial strains culture solutions on the egg parasitoid *T. oleae*. The different concentrations of the culture solutions and the recommended field rates (RFR) for commercial insecticides are shown. (EC = emulsifiable concentrate; WP = wettable powder; CS: culture solution).

Active ingredient	Trade name	Formulation	RFR	Solutions concentration
B. thuringiensis	Delfin®	WP	250 g hl^{-1}	-
Dimethoate	Biomat® 40 E.C	EC	100 cc hl^{-1}	-
Providencia entomophila strain IO-6	-	CS	-	$10^5/10^6/10^7/10^8/10^9$ cfu/ml
Providencia entomophila strain IO-20	-	CS	-	$10^5/10^6/10^7/10^8/10^9$ cfu/ml

Under the already mentioned environmental conditions, *Trichogramma* adults usually emerge after 10 days of initial parasitization. However, the final assessment was made 10 days after initial emergence and the mortality occurring during immature development was checked as well as partial emergence. Thus, parasitized eggs were visually checked under a stereomicroscope for emergence holes, and those with partially chewed exit holes with dead adults remaining inside were categorized as partially emerged. In parallel to these two parameters, emerged adults were also inspected post-mortem for wings and antennal aspects. Thus, females with only one or two vestigial wings were considered brachypterous, and those bearing two male antennae at once or only one male antenna were considered as two-male-antennae or single-male-antenna individuals throughout the study, respectively.

The LC50 for every bacterial formulation was calculated based on the mortality percentage for each of the five tested concentrations and every developmental stage (larva, prepupa, and pupa). Before calculation on Microsoft Excel, concentrations were log-transformed, and mortality percentages were transformed using Finney's table (Finney, 1952). In addition, the Program AAT Bioquest, available online via the link (https://www.aatbio.com/tools/lc50-calculator), was used for graphics drawing.

Persistence, which is the time required for a formulation residue to lose its effectiveness, was assessed on pomegranate leaves under field conditions. Thus, pomegranate branches carrying at minimum six leaves were sprayed with the equivalent concentration of every bacterial formulation and the equivalent insecticide following the recommended field dose (table 1), along with water control. Every solution was sprayed with its relative handgun sprayer, and the application was undertaken carefully to make sure that all leaf surfaces were covered. Branches were exposed to direct weather conditions, and the leaves were detached from plants at the desired period. Thus, either two, five, or eight days after application, six leaves were picked up from the relative branch. Square surfaces (1 by 1 cm) were cut from leaves and placed individually in vials containing approximately 30 *Trichogramma* wasps (<24 hours old) with a drop of diluted (50%) honey solution under the climatic conditions mentioned above. Six replications were used for every treatment. The leaf sections were placed in tops of vials with a light source adjacent to them to ensure that *Trichogramma* adults frequently touch them. Twenty-four hours later, the number of dead wasps was counted, and the percentage of mortality was established after 10 days based on the total number of individuals initially put in vials.

Statistical analysis

All statistical analyses were undertaken with SPSS for Mac OS Version 20.0, and all the data were angle transformed and compared by Tukey's test ($P \leq 0.05$) whenever differences were demonstrated. Further, mortality occurring during developmental stages was analysed according to the International Organization of Biological Control (IOBC), with class 1: harmless (E < 30%), class 2: slightly harmful (30 ≤ E ≤ 79%), class 3: moderately harmful (80 ≤ E ≤ 99%) and class 4: harmful (E > 99%) (IOBC, 1994).

Results

Development stages experiment

The impact of all treatments on mortality varied according to the developmental stage. The data in table 2 show that Delfin® (df = 2, F = 9.800, P = 0.002) as well as the concentrations 10^6 (df = 2, F = 58.973, P = 0.0001), 10^7 (df = 2, F = 174.806, P= 0.0001), and 10^8 (df = 2, F = 72.512, P = 0.0001) cfu/ml of strain IO-6 and the concentration 10^5 (df = 2, F = 4.689, P = 0.026) cfu/ml of strain IO-20 caused significantly higher mortality on larvae than on prepupae and pupae. The concentration of 10^9 (df = 2, F = 28.224, P= 0.0001) cfu/ml of strain IO-6 caused significantly equally high mortality on larvae and prepupae. However, Biomat® (df = 2, F = 492.201, P = 0.0001) was significantly more harmful to prepupae and pupae than to larvae (table 2). According to the harmfulness scale established by IOBC (IOBC, 1994), all IO-6 concentrations ranging from 10^5 to 10^8 cfu/ml were found to be slightly harmful only on *T. oleae* larvae, while the concentration 10^9 cfu/ml was found to be moderately harmful on larvae and prepupae as well. The same scale showed that concentrations of 10^6 and 10^9 cfu/ml IO-20 were slightly harmful to prepupae and pupae, respectively.

The impact of treatments on adult mortality during emergence is shown in table 3. In fact, in comparison to the two other development stages, the mortality during emergence was significantly higher when the treatment was applied to the pupae stage with Delfin® (df = 2, F = 11.317, P = 0.001), the concentrations 10^5 (df = 2, F = 7.067, P = 0.007), 10^8 (df = 2, F = 6.401, P = 0.010), 10^9 (df = 2, F = 9.503, P = 0.002) cfu/ml of IO-6 and the concentrations 10^5 (df = 2, F = 5.030, P = 0.021), 10^6 (df = 2, F = 81.154, P = 0.0001) and 10^9 (df = 2, F = 12.269, P = 0.001) cfu/ml of IO-20 (table 3). However, as an exception, partial emergence was significantly higher when Biomat® was applied to larvae than to prepupae or pupae (df = 2, F = 91.104, P= 0.0001 with 2.15 ± 0.76; 0.07 ± 0.16 and 0 ± 0.00; respectively). Also, no significant difference was observed between the three developmental stages when the other concentrations as well as the negative control were applied. Nonetheless, the recorded partial emergence percentages for both bacterial formulations and their concentrations were significantly lower on larvae (df = 12, F = 16.799, P = 0.0001) and prepupae (df = 12, F = 9.049, P = 0.0001) and not much different on pupae (df = 12, F = 9.154, P = 0.0001) than those of the negative control.

Treatment effects on emerged adults

Among the tested substances, Biomat® was found to cause 68.89% brachyptery in emerged adults when administered to larvae (figure 1). Although particularly important when this insecticide was used, this natural anomaly ranged from 4.9% to 10.81% when different IO-6 concentrations were applied and from 0.97% to 13.79%

Table 2. Mortality (%) of immature stages of *T. oleae* treated with commercial insecticides and different concentrations of two *P. entomophila* strains solutions under laboratory conditions (25 ± 1 °C; 65 ± 5% RH; L/D: 16/8).

Treatment	Immature developmental stage treated						
	larvae	class	prepupae	class	pupae	class	
Distilled water	9.15 ± 2.22 Aab	1	5.46 ± 1.73 ABa	1	10.67 ± 5.77 ABb	1	df = 2, F =3.976, P= 0.041
Delfin®	5.62 ± 2.31 Ab	1	3.58 ± 1.09 Aab	1	2.15 ± 0.69 Aa	1	df = 2, F = 9.800, P = 0.002
Biomat®	79.09 ± 3.59 Fa	3	99.93 ± 0.16 Db	3	100 ± 0.00 Cb	4	df = 2, F = 492.201, P = 0.0001
IO-6 10^5	30.07 ± 7.68 Ca	2	23.93 ± 5.77 Ba	1	20.80 ± 7.27 Ba	1	df = 2, F = 2.621, P = 0.106
IO-6 10^6	53.62 ± 12.51 Dc	2	19.28 ± 3.97 ABb	1	9.33 ± 2.66 ABa	1	df = 2, F = 58.973, P = 0.0001
IO-6 10^7	60.82 ± 7.08 DEb	2	15.33 ± 3.78 ABa	1	11.68 ± 2.34 ABa	1	df = 2, F = 174.806, P = 0.0001
IO-6 10^8	75.67 ± 9.09 EFb	2	20.07 ±7.73 ABa	1	17.64 ± 8.51 Ba	1	df = 2, F = 72.512, P = 0.0001
IO-6 10^9	79.99 ± 11.54 Fb	3	90.88 ± 12.34 Db	3	23.39 ± 20.09 Ba	1	df = 2, F = 28.224, P = 0.0001
IO-20 10^5	28.13 ± 13.75 Cb	1	14.37 ± 6.51 ABa	1	15.21 ± 3.06 Bab	1	df = 2, F = 4.689, P = 0.026
IO-20 10^6	25.98 ± 6.09 BCa	1	52.97 ± 38.35 Ca	2	24.87 ± 23.61 Ba	1	df = 2, F = 2.172, P = 0.148
IO-20 10^7	8.29 ± 2.89 Aa	1	18.69 ± 13.68 ABa	1	14.95 ± 9.54 ABa	1	df = 2, F = 1.586, P = 0.237
IO-20 10^8	11.78 ± 1.27 ABa	1	25.25 ± 11.79 Ba	1	15.16 ± 10.14 ABa	1	df = 2, F = 3.312, P = 0.064
IO-20 10^9	30.56 ± 5.79 Cb	2	22.78 ± 4.02 ABb	1	13.53 ± 8.08 ABa	1	df = 2, F = 11.193, P = 0.001
	df = 12 F = 70.612 P = 0.0001		df = 12 F =38.241 P= 0.0001		df = 12 F = 46.497 P= 0.0001		

Values followed by the same letter are not statistically different using mean comparisons (Tukey test, $P < 0.05$) angular transformed data. Capital letters following the values represent comparisons within a column and lower-case letters represent comparisons within a line. Mortality estimation: class 1 = harmless ($E < 30\%$), class 2 = slightly harmful ($30 \leq E \leq 79\%$), class 3 = moderately harmful ($80 \leq E \leq 99\%$) and class 4 = harmful ($E > 99\%$).

Table 3. Adult mortality during emergence (%) of *T. oleae* treated with different insecticides and concentrations of bacterial solutions during immature life phases.

Treatment	Immature developmental stage treated			
	larvae	prepupae	pupae	
Distilled water	1.44 ± 1.11 CDa	1.15 ± 0.63 Ba	1.05 ± 0.49 BCDEa	df = 2, F = 0.047, P= 0.954
Delfin®	0.77 ± 0.43 BCa	1.54 ± 1.09 Ba	3.45 ± 1.12 Eb	df = 2, F = 11.317, P= 0.001
Biomat®	2.15 ± 0.76 Db	0.07 ± 0.16 Aa	0 ± 0.00 Aa	df = 2, F = 91.104, P= 0.0001
IO-6 10^5	0 ± 0.00 Aa	0.05 ± 0.13 Aa	0.78 ± 1.12 ABCb	df = 2, F = 7.067, P= 0.007
IO-6 10^6	0 ± 0.00 Aa	0.1 ± 0.15 Aa	0.11 ± 0.17 ABa	df = 2, F = 1.250, P= 0.315
IO-6 10^7	0 ± 0.00 Aa	0.31 ± 0.53 Aa	0.11 ± 0.3 ABa	df = 2, F = 1.258, P= 0.313
IO-6 10^8	0 ± 0.00 Aa	0.1 ± 0.15 Aab	0.79 ± 0.64 ABCb	df = 2, F = 6.401, P= 0.010
IO-6 10^9	0 ± 0.00 Aa	0 ± 0.00 Aa	0.98 ± 0.83 ABCDb	df = 2, F = 9.503, P= 0.002
IO-20 10^5	0.03 ± 0.08 Aa	0.07 ± 0.11 Aab	0.78 ± 0.7 ABCb	df = 2, F = 5.030, P= 0.021
IO-20 10^6	0.04 ± 0.11 Aa	0.04 ± 0.10 Aa	2.81 ± 1.06 DEb	df = 2, F = 81.154, P= 0.0001
IO-20 10^7	0.38 ± 0.43 ABa	0.23 ± 0.26 Aa	0.63 ± 0.52 ABCa	df = 2, F = 0.712, P= 0.507
IO-20 10^8	0.3 ± 0.4 ABa	0.06 ± 0.14 Aa	0.16 ± 0.28 ABCa	df = 2, F = 0.868, P= 0.440
IO-20 10^9	0 ± 0.00 Aa	0.12 ± 0.18 Aa	1.59 ± 1.27 CDEb	df = 2, F = 12.269, P= 0.001
	df = 12 F = 16.799 P= 0.0001	df = 12 F = 9.049 P= 0.0001	df = 12 F = 9.154 P= 0.0001	

Values followed by the same letter are not statistically different using mean comparisons (Tukey test, $P< 0.05$) angular transformed data. Capital letters following the values represent comparisons within a column and lower-case letters represent comparisons within a line.

when IO-20 concentrations were tested on larvae. All these percentages were within the norms as 13% of adults were brachypterous following water treatment on larvae. Nonetheless, when water was applied to prepupae and pupae, 20.34% and 31.75% of adults, respectively, were brachypterous. For both aforementioned developmental stages, all IO-6 and IO-20 concentrations caused brachyptery percentages lower than those obtained with the negative control.

The percentages of females carrying two male antennae, in the population of adults that emerged following treatments with different solutions on the three developmental stages, are reported in figure 2. As indicated with the negative control, the natural percentages of two-male-

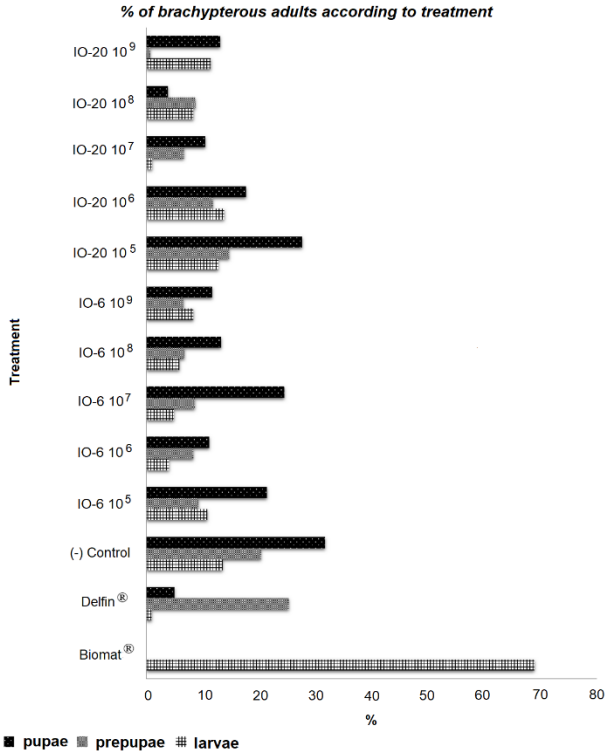

Figure 1. Percentage of *T. oleae* brachypterous females emerging following treatment of preimaginal stages with *P. entomophila* strains IO-6 and IO-20.

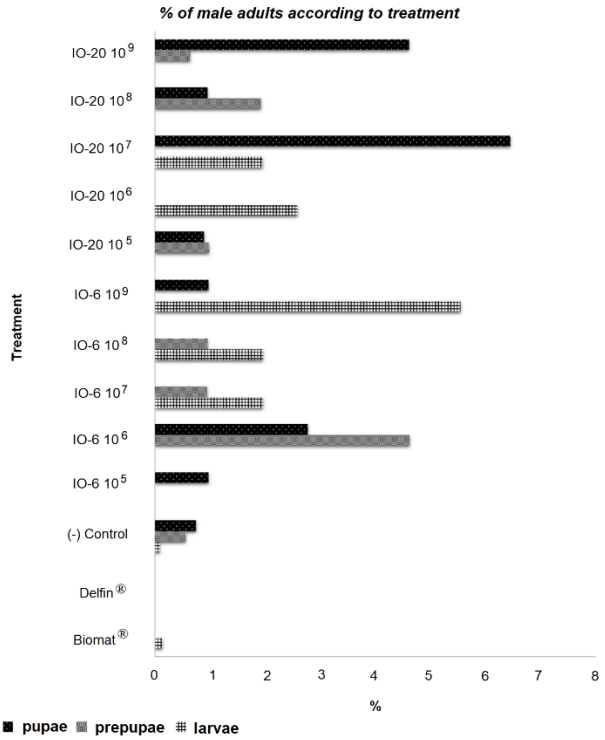

Figure 2. Percentage of *T. oleae* two-male-antennae females emerging following treatment of preimaginal stages with *P. entomophila* strains IO-6 and IO-20.

antennae females in *T. oleae* range from around 0.01% and 0.75%, for larvae until pupae, respectively. However, up to 2.59% and up to 5.55% of *T. oleae* was composed of two-male-antennae females when larvae were dipped into IO-20 and IO-6, respectively. On prepupae, 1.93% and 4.63% was composed of two-male-antennae females when the suspensions IO-20 (10^8 cfu/ml) and IO-6 (10^6 cfu/ml) were used, respectively. On pupae, the latter concentration had also an effect as 2.78% of two-male-antennae females was detected, whereas up to 6.46% of two-male-antennae females were noticed when the bacterial solution IO-20 was used (figure 2).

According to the data reported in figure 3, single-male-antenna females were quite rare but never appeared when eggs, whatever their developmental stage, were dipped into water (negative control). Individuals carrying at once a male and a female antenna appeared when prepupae and pupae were dipped into IO-20 (10^9 cfu/ml) and when prepupae were dipped into Delfin®. Their percentages in the total population were below 0.2% for Delfin® and ranged from 1.5% to 1.91% with IO-20 (10^9 cfu/ml) (figure 3).

The LC50 calculation

The LC50 values calculated for both tested bacterial suspensions are reported in table 4. However, despite the increasing concentrations of both tested bacterial suspensions, the mortality rates of almost all *T. oleae* developmental stages showed non-linear evolution. Thus, the calculated LC50 values were considered valid only for *T. oleae* larval stages treated with IO-6 strain (table 4, figure 4). Therefore, the concentration of IO-6 that causes 50% mortality in larvae of *T. oleae* is equal to $10^{6.21}$ cfu/ml.

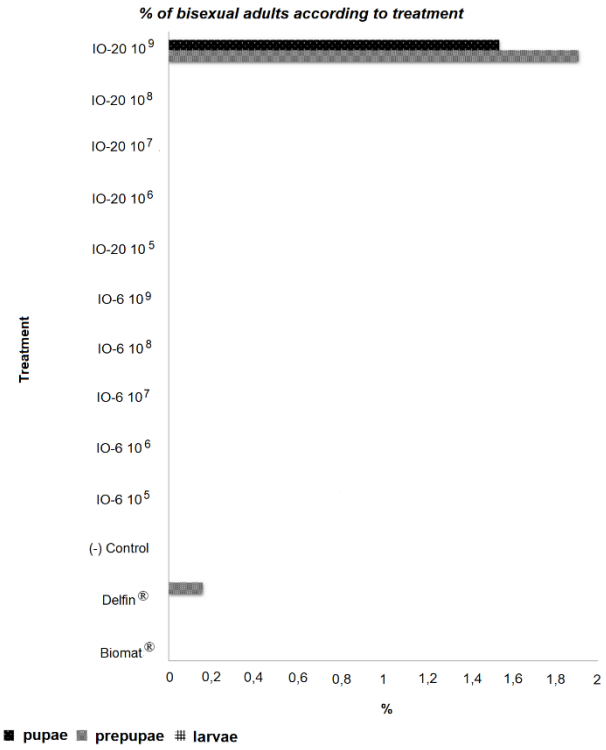

Figure 3. Percentage of *T. oleae* single-male-antenna females emerging following treatment of preimaginal stages with *P. entomophila* strains IO-6 and IO-20.

Table 4. Lethal concentration rates of strains IO-6 and IO-20 of *P. entomophila*, causing 50% of death on *T. oleae* preimaginal stages.

Tested formulation	Development stage	Coefficients		LC50
		Intercept	Variable 1	
IO-6	larvae	2.951	0.333	$10^{6.21}$
IO-6	prepupae	1.604	0.42	* ($10^{8.086}$)
IO-6	pupae	3.61	0.056	* ($10^{24.82}$)
IO-20	larvae	4.404	−0.038	* ($10^{-15.68}$)
IO-20	prepupae	4.391	−0.007	* (10^{-87})
IO-20	pupae	3.645	0.071	* ($10^{19.08}$)

The equation $Y = ax + b$; with "a" corresponding to variable 1; "b" corresponding to intercept; and "Y" corresponding to 50% of mortality after transformation using Finney's table (Finney, 1952), used to calculate the amount of LC50.
* although calculated, the obtained values are considered invalid due to large fluctuations in the corresponding mortality rates.

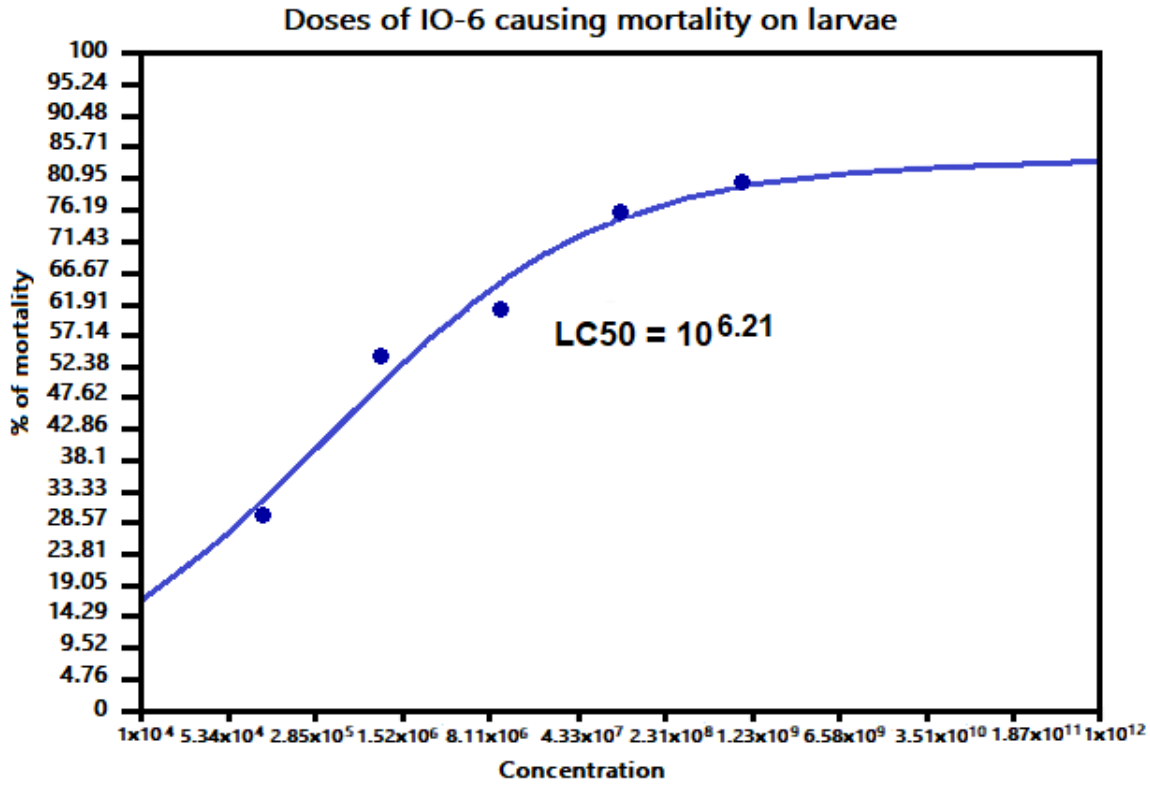

Figure 4. Percentages of mortality of *T. oleae* larval stages following treatment with different doses of *P. entomophila* strain IO-6.

The persistence experiment

The persistence of each solution was assessed through the survival percentages of adult *T. oleae* put into contact with treated leaves after two, five, and eight days of their spraying. Relative data are shown in table 5. Regardless of the day of exposure, the survival percentages of wasps exposed to leaves treated with both *P. entomophila* solutions and their concentrations were significantly similar. In fact, both bacterial suspensions and their different concentrations have significantly similar effects on wasps' survival than the negative control, eight days after leaf spraying (df = 12, F = 2.080, P = 0.031). On day five after sprinkling, only the concentration of 10^6 cfu/ml IO-6 among all the others caused, although the difference was inconsequential, a significant decrease in adult survival in comparison to the negative control (df = 12, F = 3.362, P = 0.0001). However, *Trichogramma* wasps being in contact with leaves sprayed two days ago with *P. entomophila* suspensions, and their different concentrations, show survival percentages significantly slightly higher than those of the negative control, but similar to those obtained with Delfin® (df = 12, F = 96.149, P = 0.0001). Nonetheless, as expected, Biomat® residues on leaves allowed only the survival of 5.62 ± 12.31% of adult wasps on day 2. This negative effect significantly decreased through the days and became similar to the negative control effect on day 8 (table 5).

Table 5. Mean ± SE percent survival of *T. oleae* adults 24 hours following exposure to insecticides and bacterial residues on pomegranate leaves at various days after application of field indicated rates.

Treatment		Treatment persistence after			
		2 days	5 days	8 days	
Distilled water		95.31 ± 2.13 Ba	99.47 ± 0.88 Bb	97.35 ± 4.17 Aab	df = 2, F = 4.890, P = 0.023
Delfin®		99.24 ± 1.17 BCa	96.66 ± 3.15 Ba	96.62 ± 3.05 Aa	df = 2, F = 1.632, P = 0.228
Biomat®		5.62 ± 12.31 Aa	75.12 ± 35.01 Ab	98.92 ± 2.63 Ab	df = 2, F = 34.155, P = 0.0001
IO-6	10^5	99.09 ± 1.26 BCa	98.95 ± 1.44 Ba	98.49 ± 2.39 Aa	df = 2, F = 0.054, P = 0.948
IO-6	10^6	97.64 ± 2.56 BCa	96.85 ± 3.11 ABa	97.87 ± 2.99 Aa	df = 2, F = 0.285, P = 0.756
IO-6	10^7	99.29 ± 0.78 BCa	98.89 ± 1.76 Ba	97.57 ± 2.42 Aa	df = 2, F = 0.983, P = 0.397
IO-6	10^8	99.38 ± 1.51 BCa	98.31 ± 1.96 Ba	97.96 ± 1.51 Aa	df = 2, F = 1.913, P = 0.182
IO-6	10^9	98.96 ± 1.7 BCa	98.31 ± 1.61 Ba	98.26 ± 2.04 Aa	df = 2, F = 0.376, P = 0.693
IO-20	10^5	99.71 ± 0.72 BCa	98.85 ± 2.82 Ba	100 ± 0 Aa	df = 2, F = 0.600, P = 0.561
IO-20	10^6	99.54 ± 1.13 BCa	99.53 ± 1.15 Ba	99.19 ± 1.26 Aa	df = 2, F = 0.222, P = 0.804
IO-20	10^7	100 ± 0 Ca	98.76 ± 1.40 Ba	99.72 ± 0.68 Aa	df = 2, F = 2.884, P = 0.087
IO-20	10^8	99.02 ± 1.59 BCa	99.82 ± 0.44 Ba	100 ± 0 Aa	df = 2, F = 1.567, P = 0.241
IO-20	10^9	99.44 ± 1.36 BCa	99.33 ± 1.63 Ba	100 ± 0 Aa	df = 2, F = 0.502, P = 0.615
		df =12 F =96.149 P=0.0001	df = 12 F = 3.362 P=0.0001	df =12 F = 2.080 P=0.031	

Values followed by the same letter are not statistically different using mean comparisons (Tukey test, $P < 0.05$) angular transformed data. Capital letters following the values represent comparisons within a column and lower-case letters represent comparisons within a line.

Discussion

Under normal conditions and at the optimum development temperature of 25 °C, Harrison *et al.* (1985), Pizzol *et al.* (2010) and Ksentini *et al.* (2011), had shown that a normal percentage of mortality often occurs during *Trichogramma* sp. development stages, making that adults' emergence percentages could normally range between 84.4-96.88% (Carvalho *et al.*, 2017; Taha *et al.*, 2022). In this study, mortality values obtained, when eggs were dipped into distilled water or Delfin®, were within the normal range, whatever the development stage. Concerning both tested bacterial suspensions (IO-6 and IO-20), our results had shown significant and large variability in mortality percentages in comparison with the negative control. Thus, maximum mortality percentages of 90.88% and 52.97%, both on prepupae, were obtained with IO-6 10^9 cfu/ml and IO-20 10^6 cfu/ml, respectively. However, although significantly higher than the negative control, some tested concentrations were considered harmless, according to IOBC guidelines, as their mortality percentages were inferior to 30% (IOBC, 1994). This was particularly noticed with the majority of IO-20 concentrations in all developmental stages and almost all IO-6 concentrations in prepupae and pupae. This difference in developmental stages' susceptibility toward an insecticide was reported in previous research (Carvalho *et al.*, 2010; Braga Maia *et al.*, 2013; Souza *et al.*, 2014). In this context, when spraying the chemical insecticide Fipronil® on different immature stages of *Trichogramma brassicae* Bezdenko (Hymenoptera Trichogrammatidae), Ghorbani *et al.* (2016) noticed the accented susceptibility of larvae in comparison to prepupae and pupae. On the contrary, the application of the bio-insecticide Bactospeine® on three *Trichogramma* species showed that developmental stages' different sensitivities depended more on the species and the used bio-insecticide dose, with prepupae and pupae being generally more susceptible than larvae (Ksentini *et al.*, 2010). In parallel, in the current study, the thorough monitoring of the mortality rates showed that almost all IO-6 and IO-20 treated preimaginal stages followed a non-linear shape. In fact, we noticed an increase/decrease of mortality rates as a response for lower/higher concentrations, respectively. Indeed, in an attempt to determine *Oryzaephilus surinamensis* (L.) (Coleoptera Silvanidae), *Tribolium castaneum* (Herbst) (Coleoptera Tenebrionidae), *Sitophilus oryzae* (L.) (Coleoptera Curculionidae) and *Rhyzopertha dominica* (F.) (Coleoptera Bostrichidae) response to specific exposure intervals to phosphine, a "sweet spot", i.e., decrease of mortality with the increase of the insecticide concentration was noticed (Lampiri *et al*., 2021). The latter authors defined this sweet spot as a "global" phenomenon, that is expressed almost equally vigorously regardless of the resistance level of the specific examined population. According to Guedes and Cutler (2014), this phenomenon is called "hormesis" and describes a biphasic dose-response relationship that is characterized by a reversal of response between low and high doses of a stressor (e.g. insecticides). Still, conforming to Cutler (2012), the hormetic dose-response model is now widely recognized, while the study of dose-response relationships has traditionally been guided by the threshold and/or linear non-threshold models. Nonetheless, in the current study, only IO-6 treated larvae had the exception to be characterized by an increasing mortality rate following the concentrations increase.

For all preimaginal stages treated with distilled water, partial emergence was significantly similar and did not exceed 1.44%. However, in comparison to previous work (Ksentini *et al.*, 2010), this recorded rate appears quite important for the negative control treatment. Nonetheless, in the same previous work, authors detected differences

within the tested species and their developmental stages regarding the treatments, with *Trichogramma evanescens* Westwood (Hymenoptera Trichogrammatidae) being more sensitive to distilled water treatment, and *Trichogramma cacoeciae* Marchal (Hymenoptera Trichogrammatidae) pupae more sensitive to Bactospeine®. In the current study, both tested bacterial suspensions caused *T. oleae* partial emergence, with pupae being significantly more sensitive than larvae and prepupae. Delfin® as well caused an increased susceptibility of pupae in comparison to larvae and prepupae. In this context, and although the egg chorion offers protection to preimaginal stages from insecticides in most cases (Campbell *et al.*, 2016), the ingestion of the treated solution/insecticide might occur during the opening of the emergence hole (Cônsoli *et al.*, 2001). In fact, according to the latter authors, while the parasitoid is cutting a small area of the host chorion to prepare for its emergence, a small quantity of the chorion surface could be swallowed and with it the product covering the host surface. This explains better pupae increased sensitivity towards Delfin®, as well as all tested bacterial suspensions, except the harmful Biomat® that caused 100% of offspring mortality when applied on pupae.

Upon their emergence from eggs, *Trichogramma* adults immediately extend their wings. However, adults with stubby or fold wings are qualified as brachypterous (Knutson, 1998). Thus, following Keller and Lewis's (1985) field trials and releases, brachyptery rates of up to 34% for both sexes were noticed. In fact, according to Orr and Suh (1999), there was approximately a 14-fold increase in the brachyptery of females in insectary-reared *Trichogramma* when compared to field-collected specimens. Nonetheless, as reported by Schmidt *et al.* (2003), brachyptery rates can reach up to 78.5% for commercially reared *Trichogramma*. This may be the reason why brachyptery rates were closer to 32% for *Trichogramma* adults emerging from pupae dipped into distilled water. Still, for both bacterial strains (IO-6, IO-20), adults' brachyptery was lesser than those of the negative control, with generally increased rates for pupae in comparison with prepupae and larvae. Thereby, although an approximate percentage of 69% was recorded after larvae treatment with Biomat®, we fail to define whether the highly recorded brachyptery percentages among adults were due to repetitive laboratory rearing or to the treatments' nature.

In parallel to brachypterous adults, two-male-antennae individuals were detected among *T. oleae* populations following different treatments. However, although our laboratory-reared strain is considered a thelytokous one with a population of 100% females, a negligible percentage (less than 0.8%) of two-male-antennae individuals were detected following eggs dipping into distilled water. In fact, *T. oleae* is a thelytokous species (Pintureau *et al.*, 1999; Fricaux Warot, 2018), with asexuality being due to infection with parthenogenesis-inducing *Wolbachia* symbiont (Pintureau *et al.*, 2000; Lindsey and Stouthamer, 2017). Thus, in this case, female offspring results from unfertilized eggs, rather than from fertilization (Lindsey *et al.*, 2018). Nonetheless, completely parthenogenetic *Trichogramma* wasps can be rendered permanently bisexual by treatment with antibiotics or high temperatures (Stouthamer *et al.*, 1990). In this context, Grenier *et al.* (2002) noticed that endosymbionts of the genus *Wolbachia* were efficiently cured from *Trichogramma* species for up to 14 generations, by incorporating 0.02% tetracycline into the artificial diet used to rear larvae. This suggests that for the current study, an interaction might have occurred between *Wolbachia* and both tested *P. entomophila* strains, increasing the appearance of two-male-antennae individuals among the population. In fact, following their study on the impact of *Wolbachia* on the microbiota of the cabbage root fly *Delia radicum* (L.) (Diptera Anthomyiidae), Ourry *et al.* (2021) noticed that *Wolbachia* infection positively increased *Providencia* abundance. However, further studies and attention should be addressed in order to apprehend what caused the increase in two-male-antennae individuals' numbers after *T. oleae* treatment with *P. entomphila* different suspensions. Nevertheless, we should still keep in mind that according to Lindsey *et al.* (2018), *Wolbachia* infection is not only responsible for diploid females' production from unfertilized eggs, but also for diploid males and intersex individuals' appearance in appreciable quantities within the population. This explains better why some single-male-antenna individuals were noticed during the current study.

Parsaeyan *et al.* (2018) found out that while the recommended field concentrations of emamectin benzoate and cypermethrin caused 72.0 and 80.7% mortality of *T. brassicae* preimaginal stages, respectively, obtained LC50 values for both insecticides were respectively 2.7 and 0.5 µg of active ingredient/ml. This parameter is used as well for the assessment of natural formulations. In this way, the LC50 value of the commercially based *B. thuringiensis* bio-insecticide (Dipel®) was 84.2 (9.5-288.5) µg/µL, when *Trichogramma chilonis* Ishii (Hymenoptera Trichogrammatidae) adults were allowed to feed on honey supplemented with spores (Amichot *et al.*, 2016). However, according to the latter authors, the LC50 could not be calculated when *Trichogramma* adults were exposed to a *B. thuringiensis* formulation devoid of Cry toxins. In the same context, we noticed in the current study that LC50 values for almost all *T. oleae* preimaginal stages exposed to IO-6 and IO-20 different concentrations were categorized as invalid. This was the consequence of a non- linear evolution of mortality rates despite the tested suspensions' increasing concentrations. Nonetheless, only larvae exposed to IO-6 described a linear evolution of mortality rates following concentrations increase. This permitted the determination of an LC50 value equal to $10^{6.21}$ cfu/ml, and stressed by the way on IO-6 formulation's detrimental impact on larvae. In parallel to the LC50 measure, insecticide persistence is being considered an important tool in order to assess the insecticide/formulation impact on living organisms, days after treatment. In fact, insecticide persistence is being greatly examined in Integrated Pest Management (IPM) programs as it helps to calculate the necessary time in order to avoid the overlap of different kinds of treatments; e.g. chemical insecticide use and beneficials' mass releases. In this context, Ashtari (2019) found out that Confidor® persisted less than Avaunt® and Spintor®, and as a

consequence, *T. brassicae* and *T. evanescens* could be released 5 days after their field application. In the same context, Ksentini *et al.* (2010) previously noticed that Tracer® persisted far more than 6 days and could cause more than 94% of mortality on *T. evanescens* in comparison to Bactospeine® which was statistically comparable to the negative control. In the current study, although the concentration 10^6 cfu/ml of IO-6 showed a slight difference in comparison to the negative control, both *P. entomophila* formulations and their different concentrations showed no detrimental effects on *T. oleae* adults on days two, five, and eight after treatment. Thus, both tested *P. entomophila's* formulations could be considered safe on *T. oleae* adults.

Conclusions

To our knowledge, this research is the first to assess the side effect of *P. entomophila* strains on the *Trichogramma* genus. Therefore, none of these tested strains or their different concentrations caused significant mortality in adults, in comparison to the negative control. However, although IO-6 caused detrimental effects only on larvae, and only its concentration of 10^9 cfu/ml on prepupae - with no negative effects being noticed on pupae -, this strain should not be considered in control programs as it could cause harmful effects on some developmental stages, i.e. *T. oleae* larvae. Nonetheless, even though some IO-20 concentrations (10^6 and 10^9 cfu/ml) caused increased mortality in prepupae and larvae, respectively, this *P. entomophila* strain could be used. Yet, in order to avoid possible deleterious effects on different developing stages, only a concentration of 10^5 cfu/ml is advisable on *Trichogramma* and potentially on other auxiliary fauna, although IO-20 effectiveness on olive tree insect pests at this very exact concentration has still to be assessed. Besides, with the latter IO-20 concentration, percentages of single-male-antenna, two-male-antennae, and brachypterous organisms within the population were similar to the negative control. Last but not least, although the latter formulation was proved to be harmless to the beneficial insects i.e. *Trichogramma* genus, further studies are necessary in order to assess its exact impact on agroecosystem biocoenosis before considering it as a potential organic treatment against olive tree insect pests.

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