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Life stage-dependent susceptibility of *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) to two pesticides commonly used in citrus orchards

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highlights

- Lethal and sublethal effects of two pesticides were studied on *Aphytis melinus* .
- Significant differences in parasitoid instars susceptibility were recorded.
- Pesticide risk assessment and choice should include life-stage susceptibility.
- The natural origin of the mineral oil is not guarantee of non-target safety.
- Careful integration of A. *melinus* and the tested insecticides is recommended.

ABSTRACT

The assessment of pesticides compatibility with natural enemies is recommended before including agrochemicals in integrated and organic pest management schemes. The lethal and sublethal effects of a mineral oil and a juvenile hormone mimic (pyriproxyfen), on adults and larvae of *Aphytis melinus* , a key ectoparasitoid of armored scale insect pests of citrus, such as Aonidiella *aurantii* , were evaluated. Mineral oil caused very high mortality on the adults, while a lower acute toxicity was recorded on young instars. No significant effects on their reproduction capacity and on the sex-ratio of the progeny were observed. Pyriproxyfen had neither lethal nor sublethal effects (in terms of survived female fertility) on A. *melinus* adults. However, parasitoid larvae exposed to this insecticide suffered strong acute toxicity and fertility reduction (progeny number and proportion of female progeny). When adults were offered the choice to parasitize treated and untreated scales they significantly preferred the control ones, and when they were exposed to only treated scaled the parasitism rate was significantly lower only with mineral oil-treated hosts. The significant differences in the susceptibility of the two parasitoid instars highlight the importance of including this aspect in pesticide risk assessment procedures and in the choice of the pesticide and of the treatment timing in the field. Overall, the results indicate potential for integrating A. *melinus* , both naturally present and artificially released, and these insecticides only by appropriate timing of insecticide spraying and parasitoid releases.

INTRODUCTION

The California red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), is an invasive pest of citrus and despite its common name it is believed to have Asiatic origin (Compere, 1961). Nowadays, this species is considered one of the most important pests of citrus in the Mediterranean basin and in several other citrus growing areas worldwide (Jacas et al., 2010; Zappalà, 2010). The ectoparasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) is a key natural enemy of A. *aurantii* (Compere, 1961; Siscaro et al., 1999; Sorribas and Garcia-Marí, 2011) and it is mass reared for inoculative and augmentative releases in citrus orchards (Moreno and Luck, 1992; Zappalà, 2010). The effectiveness of Aphytis spp. in the scale control could depend on many agro-ecological factors, such as the fitness of the released insects (Vasquez and Morse, 2012), their field dispersal capacity (Zappalà et al., 2012), the availability of the susceptible host instars and their size (Luck and Podoler, 1985), the interaction with other natural enemies (Heimpel et al., 1997a; Borer et al., 2003; Vanaclocha et al., 2013a), and the presence in the tree canopy of insecticide residues (Suma et al., 2009; Garcerá et al., 2013; Vanaclocha et al., 2013b). Although various environmentally friendly pest control tools, such as habitat management, cultural practices, mating disruption, and biological control, are currently adopted and implemented in citrus crops worldwide (Lim et al., 2006; Vacas et al., 2012; Aguilar-Fenollosa and Jacas, 2013; Chueca et al., 2013), chemical treatments are frequently applied in citrus groves (Rill et al., 2008; Garcerá et al., 2014; Monzò et al., 2014). The use of selective pesticides is therefore crucial for maintaining natural enemy populations and their ecosystem services (Prabhaker et al., 2007; Suma et al., 2009). Thus, the correct evaluation of potential side effects of pesticides on non-target organisms, such as the natural enemies employed in citrus IPM and organic packages, is particularly crucial (Garcerá et al., 2013; Planes et al., 2013). Pesticides could have both lethal (i.e. acute toxicity) and sublethal (i.e. influence on various behavioral and physiological traits) effects on the exposed natural enemies (Desneux et al., 2007; Biondi et al., 2012a). A comprehensive evaluation of undesired effects of agrochemicals should be therefore based not only on short-term acute toxicity tests, but also on the assessment of physiological and behavioral sublethal effects. These should include the potential long-term effects on natural enemy population dynamics and their ecosystem services (He et al., 2012; Biondi et al., 2013; Saber and Abedi, 2013; Bengochea et al., 2014). Moreover, most of the tests used to determine the side effects of pesticides on natural enemies are performed on only a single developmental stage, primarily the adults, which are considered to be the most exposed life stage. Immature developmental stages, however, may also be affected by pesticides, even though they are usually concealed within the host (Schneider et al., 2004). In this framework, studying the potential effects of pesticides on different developmental stages of the natural enemies could be relevant, also considering their variable susceptibility which has been often highlighted, particularly when testing Insect Growth Regulator (IGR) compounds (Ishaaya and Horowitz, 1995; Hoddle et al., 2001; Schneider et al., 2004). Mindful of this context, we aimed at evaluating the compatibility of a narrow range mineral oil and a juvenile hormone mimic with A. *melinus* when applied on adults and young instars of the parasitoid. Laboratory bioassays were conducted to assess the acute toxicity and the sublethal effects on reproduction on adults and young instars, as well as A. *melinus* parasitization activity on treated hosts.

Material and methods

Insects

A parthenogenetic strain of the oleander scale, Aspidiotus nerii Bouché (Hemiptera: Diaspididae), was reared on organically grown squashes, Cucurbita maxima Duch. var. Butternut. Infested squashes were used for the parasitoid rearing, following A. lingnanensis rearing methodology as described by DeBach and White (1960), and modified by Raciti et al. (2003). All the insects used in this study were reared and kindly provided by the Sicilian Regional Insectary (Biofabbrica Insetti Utili, Ramacca, Catania, Italy). In particular A. nerii-infested and A. nerii-infested and parasitized squashes were promptly transferred to the laboratory, when needed for the toxicological bioassays.

Pesticides

We evaluated under laboratory conditions the effects of two agrochemicals. The first was the narrow range mineral oil Biolid E " (Emulsifiable Concentrate 80% a.s., Sipcam, Italy), generally used as adjuvant and/or alone to control mites and scale pests when at the first instars on many different crops under conventional, integrated and organic pest management programs (Biondi et al., 2012a). The other was pyriproxyfen, a juvenile hormone mimic [Admiral 10EC" (Emulsifiable Concentrate 10.86% a.s.), Sumitomo Chemical, Japan], which inhibits metamorphosis and embryogenesis in several insects (Ishaaya and Horowitz, 1995). Both chemicals were applied at their maximum label dose: 2 L hL"1 and 75 mL hL"1 respectively. Untreated controls were sprayed with tap water only. All the bioassays were conducted at 23 ± 1 #C, 60 ± 10% R.H. and a photoperiod of 16:8 (L:D).

Acute toxicity on A. *melinus* adults

The acute toxicity was assessed exposing newly emerged (24–48 h) adults to freshly dried pesticide residues on glass plates. Uniform deposits (1.3–1.8 mg/cm2) of pesticide solution or of water were obtained using a Potter Precision Spray Tower (Burkard Manufacturing Co. Ltd.) to spray 6 glass plates (9 # 9 cm). Subsequently, the plates were left into a chemical hood for about 2 h to complete drying and then assembled to put together a cube (Suma et al., 2009). To avoid fumigation effect, each set of experimental arenas was provided with a electric pump (Air fizz 100, Ferplast Spa, Italy) ensuring a constant 36 mL # min"1 air-flow (Suma et al., 2009). To mimic the field scenario where parasitoid adults use external nutrients (Beltrá et al., 2013), a solution of pure honey and water (1:1) was provided in the experimental arena through a cotton ball imbibed of honey solution. Ten parasitoids (five females and five males) were introduced in the arena and left in contact with the pesticide residues for 24 h and then their survival was recorded. Adults were considered dead if they did not react after being touched by a fine paint brush. Ten replicates per each treatment were carried out.

Acute toxicity on A. *melinus* larvae

Bioassays on A. *melinus* juveniles were conducted using squashes infested with 1000 ± 100 oleander scale third instar nymphs, i.e. the parasitoid preferred host instar (Rosen and DeBach, 1979). Infested squashes were exposed to 10 females and 10 males of the parasitoid for 48 h in ventilated plastic boxes (35 # 25 # 45 cm) (l # w # h), provided with honey droplets. Two similar squashes infested with 500 third instars A. nerii each, were simultaneously exposed to A. *melinus* adults per each replicate and treatment. In order to expose A. *melinus* third instars to the tested insecticides or to the control solution, only one squash was sprayed until run-off [using a 2 L aerosol hand sprayer (Matabi" , Antzuola, Guipuzcoa, Spain)] 5 d after oviposition initiation (Raciti et al., 2003). While, the other infested squash was moved to a different box kept under the same environmental conditions. This second set of squashes provided a specific control data on parasitoid emergence per each sprayed squash. The number and the sex-ratio of the emerged progeny from all the squashes were scored daily for one week after the juvenile development completed, i.e. 12 d at these environmental conditions (Raciti et al., 2003). The experiment was replicated ten times. The percentage of young instars mortality was estimated as: ½ðNcx " NtxÞ=Ncx' (100 where Ncx is the number of A. *melinus* adults emerged in the control squash cx and Ntx is the number of emerged adults from the corresponding treated squash tx.

Sublethal effects on reproduction

The adults that survived the 24-h exposure to pesticide dry residue within the glass cubes, were placed for one week with new hosts susceptible to be parasitized (third instar nymphs), at the rate of 100 scales per parasitoid female. Survived parasitoids were released in couples (one male and one female (Pan et al., 2014)) inside plastic cylindrical isolators (45 cm3) fixed on infested squashes using a strip of modeling paste (Jovi" , Barcelona, Spain). The isolators were ventilated and supplied with honey droplets. The reproductive capacity of the adults developed from topically exposed larvae (those coming from the ‘‘Acute toxicity on A. *melinus* larvae’’ experiment) was assessed by placing a newly emerged couple for one week with new susceptible hosts (at the density of 100/female), inside isolators similar to those described above for the adults survived to the residual exposure to the pesticides. The number and the sex-ratio of the emerged progeny were scored daily for one week starting 12 d after the oviposition beginning. All the trials were replicated 10 times.

Parasitism on treated hosts: choice and no-choice tests

In order to assess whether or not A. *melinus* is able to parasitize treated hosts on a treated substrate, the parasitization activity of adult wasps on hosts previously treated with mineral oil, pyriproxyfen or water was assessed. The squashes were left in a chemical hood for about 2 h to complete drying. Then, newly emerged (624 h) A. *melinus* adults were released, in groups of 20 couples, on 2000 A. nerii third instars (100 hosts/female), and left for 48 h in a dual-choice and in a no-choice test. For both kinds of test, two squashes infested with 1000 scales were exposed to the parasitoids for 48 h into the plastic boxes previously described for the acute toxicity test on young instars. In the choice test one treated and one untreated infested squash were placed in the same plastic box and exposed to A. *melinus* adults, while in the no-choice test, two treated and two untreated infested squashes were placed in two different boxes and exposed to 20 A. *melinus* adults per each box. Forty-eight hour after the end of the parasitization, all the scales were observed under the binocular to assess the parasitism rate, i.e. the portion of parasitized scales (with at least one A. *melinus* egg or larva) on the total exposed scales, was calculated. Each test and combination was replicated five times

Data analyses

Each dataset (adults and young instars) and the parasitism rates obtained in the no-choice test, were first tested for homoscedasticity (Levene test) and normality (Shapiro–Wilk test) of their distribution and transformed when necessary. Then, they were subjected to one-way ANOVA and Fisher's Least Significant Difference (LSD) test was used to determine which insecticidal treatments were significantly different at the $P < 0.05$ level. The parasitism rates obtained in the choice test were analyzed as follows: the null hypothesis that A. *melinus* parasitization choice was not affected by treatments (a response equal to 50:50 of parasitized scales) was subjected to a chi-square goodness of fit test. All statistical analyses were conducted with STATISTICA 7.0 (StatSoft Inc., OK, USA). Lethal (mortality) and sublethal effects (fertility output and proportion of female progeny) of the tested pesticides were summarized in a reduction coefficient (Ex), calculated for adults and young instarsseparately, according to the formula described by Biondi et al. (2012a). These values were then compared to the standards for laboratory ecotoxicological tests of the International Organization for Biological Control (IOBC) which include four categories: (1) harmless: $E < 30\%$, (2) slightly harmful: $30\% < E < 80\%$, (3) moderately harmful: $80\% < E < 99\%$, and (4) harmful: $E > 99\%$ (Sterk et al., 1999).

Results

Acute toxicity. The acute toxicity trials showed a significant effect of the treatment both on the adults ($F = 69.14$; df = 2, 23; $P < 0.001$) and on the young instars ($F = 11.24$; df = 2, 48; $P < 0.001$). Mineral oil killed most of the tested adult wasps (97.2 ± 1.9%), and all the females, making impossible the effects on the progeny evaluation. Pyriproxyfen resulted less toxic towards adults (38.4 ± 5.6%), although the mortality was significantly higher than that registered for the control (Table 1). The highest percentage of estimated young instars mortality was obtained from the parasitized scales sprayed with pyriproxyfen (37.0 ± 6.1%), which resulted significantly higher than the mortality of both scales treated with mineral oil (13.2 ± 3.6%) and that of the control (Table 1). No significant differences ($F = 0.45$; df = 2, 48; $P = 0.637$) were found in the sex-ratio of the adults emerged from the treated parasitized scales (Table 2).

Sublethal effects on reproduction

Pyriproxyfen did not significantly affect either the fertility of the adult females that survived the exposure to its residues (42.8 ± 11.1 offspring/female; $F = 3.67$; df = 1, 18; $P = 0.092$) (Table 1) or the sex ratio of their progeny ($F = 0.35$; df = 1, 18; $P = 0.57$) (Table 2). While, the trials conducted on A. *melinus* young instars showed a significant effect of the treatment in terms of progeny production of the developed individuals ($F = 3.75$; df = 2, 28; $P < 0.001$). Indeed, the mean fertility of the adults developed from the young instars treated with pyriproxyfen was 14.2 ± 4.1 progeny/female and it significantly differed from mineral oil (30.9 ± 5.4) and the control (31.1 ± 5.9) (Table 1). Besides, pyriproxyfen significantly disrupted the sex ratio of the progeny produced by the specimens treated as larvae (F = 6.55; df = 2, 28; P < 0.001), causing a reduction in the female proportion (Table 2).

Parasitism on treated hosts

In the dual-choice tests a significantly lower parasitism rate on the hosts treated with both pesticides (mineral oil: 19.2 ± 4.5%; pyriproxyfen: 29.6 ± 5.3%) compared to the control (61.6 ± 2.6% and 43.2 ± 1.62%, respectively) was recorded (Fig. 1). In the nochoice test the effect of the treatment was also significant (F = 7.09; df = 2, 14; P < 0.01). However, the parasitism rate was significantly lower (LSD test P < 0.05) only on hosts treated with mineral oil (26.5 ± 9.9%), compared with pyriproxyfen (63.2 ± 9.9%) and with the control (82.7 ± 9.1%). Treated scales were observed and no significant difference in mortality between treated and control scales was found.

Reduction coefficient (Ex) and IOBC toxicity classes

The calculated reduction coefficient was 96.81% for the mineral oil and 42.22% for pyriproxyfen in the trials on lethal and sublethal effects on adults. According, pesticides were classified as moderately harmful (class 3) and slightly harmful (class 2) to adults, respectively. By contrast, the reduction coefficient estimated forthe impact of the two pesticides on the wasp young instars was 16.55% for mineral oil, which was classified as harmless, and 81.03% for pyriproxyfen, then classified as moderately harmful.

Discussion

We used the squash/A. *nerii*/A. *melinus* laboratory system, to study how two agrochemicals, commonly used in citrus agro-ecosystems, can affect the population dynamics and the biocontrol activity of a parasitoid of a citrus key pest. The obtained results showed differences in the susceptibility of the two parasitoid developmental stages to the two pesticides. In particular, the narrow range mineral oil was lethal to A. *melinus* adults through residual contact on inert substrate, and it reduced the parasitization activity of the females when treated hosts were offered. Meanwhile, mineral oil did not show any toxicity on the parasitoid young instars when applied on parasitized scales. Interestingly, we found a divergent toxicological behavior of pyriproxyfen towards the two parasitoid life stages. Pyriproxyfen slightly affected A. *melinus* adult survival and did not reduce the reproduction of the adults survived to that exposure, but it strongly impacted the juvenile survival and especially the reproduction capacity (both progeny size and sex-ratio) of the adults that developed from the treated parasitized hosts. Moreover, when treated and untreated hosts were exposed simultaneously, parasitoidsalways preferred parasitizing the control hosts. While, when A. *melinus* was not given any choice, this trend was consistent only for the mineral oil-treated hosts on which the parasitization rate was lower than on untreated hosts. Although the parasitization reductions caused by both insecticides were not very high numerically, this decrease could be ecologically important for its effects on the parasitoid biocontrol services, including the recolonization capacity in a treated environment. The obtained results could be explained by the different mode of action of the two pesticides. In particular, the mineral oil was toxic to A. *melinus* adults as contacticide, most likely blocking the breathing pores and/or the insect cuticle, as in other minute and soft-bodied arthropods after residual or topical contact (NajarRodriguez et al., 2007). Similarly, a detrimental effect was noted on the parasitization activity of A. *melinus* on sprayed hosts both in the choice and no-choice experiment, which could decrease the recolonization ability of the parasitoid in treated environments (Desneux et al., 2006; Biondi et al., 2013). These data confirm those obtained in previous trials where the same parasitoid was highly susceptible to mineral oil fresh residue on glass surfaces (Suma et al., 2009) and on freshly sprayed citrus leaves for 24 h (Campbell, 1975). While, Vanaclocha et al. (2013b) found a lower (27%) A. *melinus* mortality after exposing parasitoid adults to petroleum oil in the laboratory. These divergent results may be due to the fact that Vanaclocha et al. (2013b) used another commercial product, tested at lower concentration (1.5 vs 2.0 L hL"1), and gaveto the parasitoids the opportunity to avoid the insecticide exposure and/or to recover in untreated areas of the experimental arena. This latter point, although it should be further investigated through behavioral observations, could have influenced the parasitoid survival significantly. Meanwhile, in the present study we provide for the first time evidences on the mineral oil safety towards both the parasitoid larvae feeding under the scale waxy cover, and the developed adults that did not suffer any potential exposure to the oil residues. In contrast, the injuriousness towards immature stages of pyriproxyfen was shown by the low emergence rates that we recorded from parasitized hosts sprayed with this IGR. To explain this important pyriproxyfen impact on young instars, we suggest three hypotheses: (i) the insecticide was able to penetrate into the scale cover and contaminated, directly by contact and/or indirectly by ingestion of contaminated hosts, the parasitoid young instars causing molt and/or metamorphosis disorders that affected their survival (Ishaaya and Horowitz, 1995; Planes et al., 2013); (ii) pyriproxyfen did not penetrate the scale cover, but newly formed adults ingested insecticide residues when chewing their way through the scale cover during emergence and caused behavioral impairment that prevented the successful parasitoid emergence, as already recorded for moth larvae emerging from pyriproxyfen-treated eggs (Tomé et al., 2012); and (iii) a combination of these two phenomena was responsible of the low emergence rates. Nevertheless, the results obtained by Rill et al. (2008), clearly showed that pyriproxyfen and another IGR insecticide, buprofezin, were not toxic towards A. *melinus* eggs and larvae, when California red scale infested lemons were dipped into the insecticide solutions. These divergent results could be explained by (i) the differential permeability of the cover of the two scale species employed in the two experiments, both intrinsic of the scales or induced by the two rearing vegetal hosts, i.e. lemons and squashes, as showed in the case of spinosad susceptibility of egg-parasitoid pupae into the eggs of various insect species (Biondi et al., 2012b) and (ii) or to the intraspecific variation in insecticide resistance that has been previously reported in A. *melinus* (Rosenheim and Hoy, 1986, 1988). The intraspecific variation in pyriproxyfen susceptibility of this parasitoid species may also explain the discrepancy between our results and those of Gonzalez-Zamora et al. (2013) that obtained 100% of mortality when exposing for 24 h adults to pyriproxyfen residues on inert substrate. However, in Vanaclocha et al. (2013b) the mortality of A. *melinus* adults was not significant when parasitoids were exposed to dry residues on leaves in the laboratory. Besides, this compound caused a reduction in the fertility of the adults that survived the juvenile exposure and emerged from the treated young instars. This could be due to the fact that juvenile hormone analogs, such as pyriproxyfen, are transported to the ovary or they penetrate into the eggs where they block embryogenesis at blastokinesis (Staal, 1975; Retnakaran et al., 1985). This would explain why the reduction in fertility did not occur when the biological instars exposed to the IGR residue is the adult one. Indeed, although A. *melinus* is a synovigenic parasitoid with an ovigeny index of 0 to)0.07 (Heimpel et al., 1997b), it may have already developed eggs before the pesticide exposure, i.e. 1–2 d after emergence, while pyriproxyfen may have blocked the ovigeny process of young adults that developed from treated young instars. Sex ratio was affected (higher male proportion) only in the progeny of adults exposed to pyriproxyfen as immatures. Considering that arrenotokous parthenogenesis (i.e. unfertilized females produce only males) occurs in A. *melinus* (Rosen and DeBach, 1979), we can argue that higher male progeny may be due to male sterility or mating behavior alterations.

Conclusion

Overall, this research brings useful findings for optimizing integrated and organic management programs against insect scale pests of citrus. The results suggest a careful timing of the applications of these two insecticides when thought to be integrated with A. *melinus* field activity. In order to combine mineral oil applications and A. *melinus* augmentative releases, the latter should be performed before the treatments or when the mineral oil residues are degraded below a toxicity threshold for A. *melinus* in citrus orchards, i.e. one week after the treatment, as suggested by Campbell (1975). In contrast, pyriproxyfen applications should be strictly avoided when parasitoids are developing into their hosts. Then, all these findings stress the importance of pest and natural enemy monitoring for the optimization of pest control programs in citrus groves (Zappalà, 2010; Campolo et al., 2014; Monzò et al., 2014). The detrimental results obtained exposing the parasitoid to the narrow range mineral oil residues stress the idea that naturally derived pesticides, thus authorized in organic farming, are not necessarily safe for non-target organisms (Biondi et al., 2012a, 2012b, 2013; de Castro et al., 2013; Martinou et al., 2014), and that the origin of a given compound could not really relate to its toxicological properties in cropping conditions. Moreover, from the risk assessment perspective all these results stress how important is the accurate toxicity evaluation on both lethal and sublethal effects toward multiple developmental stages. Besides, the data on the fertility of emerged adults would suggest continuing the evaluation of potential transgenerational effects of pyriproxyfen, being these crucial on the demography of survived individuals (Ali et al., 2012; Liang et al., 2012).

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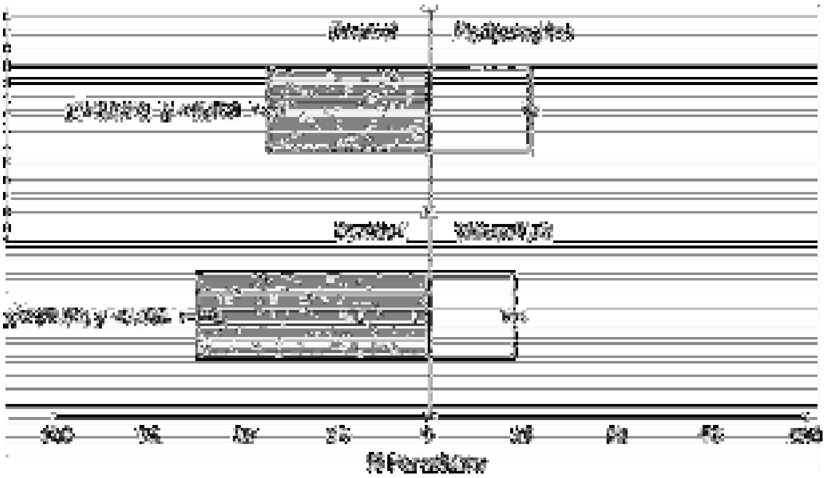

Fig. 1. Mean parasitism rates (±SE) in the dual-choice test between treated and untreated hosts by *Aphytis melinus* .

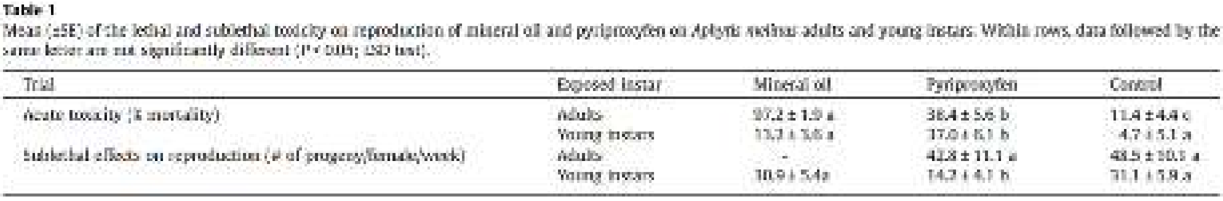

Table 1

Mean (±SE) of the lethal and sublethal toxicity on reproduction of mineral oil and pyriproxyfen on *Aphytis melinus* adults and young instars. Within rows, data followed by the same letter are not significantly different ($P < 0.05$; LSD test).

Trial	Exposed instar	Mineral oil	Pyriproxyfen	Control
Acute toxicity (% mortality)	Adults	97.2 ± 1.9 a	38.4 ± 5.6 b	11.4 ± 4.4 c
	Young instars	13.3 ± 5.6 a	37.0 ± 6.1 b	4.7 ± 5.1 a
Sublethal effects on reproduction (# of progeny/female/week)	Adults	-	42.8 ± 11.1 a	48.5 ± 10.1 a
	Young instars	10.9 ± 5.4a	14.2 ± 4.1 b	31.1 ± 5.8 a

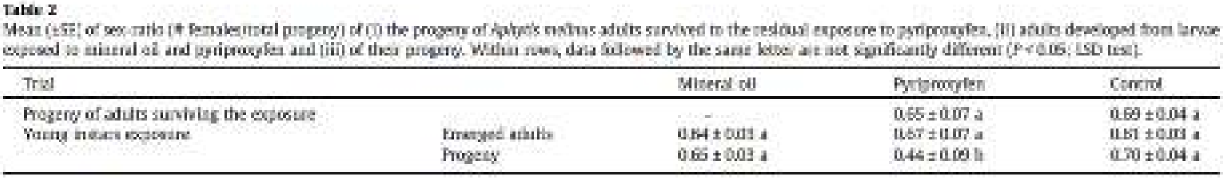

Table 2

Mean (±SE) of sex-ratio (# females/total progeny) of (i) the progeny of *Aphytis melinus* adults survived to the residual exposure to pyriproxyfen, (ii) adults developed from larvae exposed to mineral oil and pyriproxyfen and (iii) of their progeny. Within rows, data followed by the same letter are not significantly different ($P < 0.05$; LSD test).

Trial		Mineral oil	Pyriproxyfen	Control
Progeny of adults surviving the exposure		-	0.65 ± 0.07 a	0.69 ± 0.04 a
Young instars exposure	Emerged adults	0.64 ± 0.03 a	0.67 ± 0.07 a	0.61 ± 0.03 a
	Progeny	0.65 ± 0.03 a	0.44 ± 0.09 b	0.70 ± 0.04 a