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The biological efficacy of pear ester on the activity of Granulosis virus for codling moth

Silvia Schmidt · Cristina Tomasi · Edison Pasqualini · Claudio Ioriatti

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Abstract Ethyl (*E*,*Z*)-2,4-decadienoate (pear ester) is an adult and larval kairomonal attractant for *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). The possibility of using a microencapsulated formulation of pear ester (DA-MEC, a.i. 5%, Trécé Inc.) to interfere with the host location behaviour was evaluated. Laboratory leaf disc bioassays and field efficacy trials were carried out on apple to determine the potential of improving the insecticidal performance of the granulovirus of *C. pomonella* (CpGV) by co-mixing with pear ester in a sprayable formulation. In laboratory bioassays, adding DA-MEC at low doses of CpGV insecticide increased the larval mortality as a function of time. The field tests performed revealed a significant effect of the blank DA-MEC formulation in reducing fruit injury compared to an unsprayed control.

Keywords Codling moth · Granulovirus · Host location disruption · Pest control · Pear ester · Spray adjuvant

S. Schmidt
SafeCrop Centre, IASMA, Via E. Mach 1,
38010 San Michele a/A (TN), Italy
e-mail: silvia.schmidt@iasma.it

C. Tomasi · C. Ioriatti (✉)
Plant Protection Department, IASMA Research Center,
Via E. Mach 1, 38010 San Michele a/A (TN), Italy
e-mail: claudio.ioriatti@iasma.it

E. Pasqualini
DiSTA (Dipartimento di Scienze e Tecnologie Agroambientali),
Facoltà di Agraria, Università di Bologna,
Viale G. Fanin 42, 40137 Bologna, Italy
e-mail: epasqualini@entom.agrsci.unibo.it

Introduction

A few plant and fruit volatiles are able to affect, as single compounds, the behaviour of adults and larvae of *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). (*E*,*E*)-α-farnesene attracts adults and neonate larvae over a short range (Wearing and Hutchins 1973; Hern and Dorn 1999). Hughes et al. (2003) evaluated the use of apple odour and (*E*,*E*)-α-farnesene on larvae and mated females to disrupt host location in laboratory trials and they obtained a specific disruptive effect; however, only with the apple odour. In olfactometer bioassays, the esters, hexyl hexanoate and butyl hexanoate, attract females (Hern and Dorn 2004). Moreover, the pear ester, ethyl (*E*,*Z*)-2,4-decadienoate (Et-*E*,*Z*-DD), elicits the attraction and capture of both male and female codling moths in the field (Light et al. 2001; Ioriatti et al. 2003; De Cristofaro et al. 2004). Laboratory bioassays showed that the pear ester also attracts neonate larvae (Knight and Light 2001). Pasqualini et al. (2005a, b) reported that the pear ester caused a disorienting effect on egg-laying females and suggested that the kairomone may confuse the neonate larvae in finding and recognizing the fruit.

Knight and Light (2001) speculated that the application of pear ester in sprayable formulation might increase the time neonate larvae spend walking on foliage prior to entry into fruit. As a consequence, the mortality of larvae could be enhanced because of the longer exposure time to biotic and abiotic factors. Ballard et al. (2000a, b) demonstrated that the longer the time codling moth larvae spend "browsing" on foliage, the greater probability they have of dying from *C. pomonella* granulovirus (CpGV) infection. Moreover, their results suggest that the addition of additives or adjuvants, like feeding stimulants or attractants such as (*E*,*E*)-α-farnesene, to the virus formulation can increase the uptake of CpGV from sprayed leaves. For that reason a

granulovirus based insecticide could be an appropriate candidate partner to investigate whether the disorienting effects of pear ester on the codling moth female and larval behaviour could affect insecticidal efficacy.

The aim of the present study was to demonstrate the possibility of improving CpGV insecticide performance by adding pear ester in a sprayable formulation. Because the kairomone acts as larval attractant, it might promote the uptake of virus. We tested the hypothesis that the time taken to acquire a lethal viral dose decreases as a consequence of the stimulation of the larval motility.

Materials and methods

Insects

C. pomonella pupae were obtained from a mass-reared laboratory culture (Andermatt, Biocontrol, CH). Insects were kept at 25 ± 2°C under L17:D7. Neonate larvae were collected from waxed-paper oviposition sheets previously placed in adult mating cages. Only vigorous larvae were chosen for the experiments.

Laboratory bioassay

A leaf disc bioassay technique, as described by Ballard et al. (2000a), was used to assess whether the DA-MEC formulation enhances the biological efficacy of CpGV insecticide (Madex®, Intrachem, Italy). Fifteen mm diameter apple leaf discs were cut from fresh apple leaves (cultivar *Golden Delicious*) collected from a field control plot untreated with insecticides. Groups of 21 discs were fixed, lower surface uppermost, onto the outside of 9-cm petri dish lids, previously smeared with sticky-trap glue (Tangletrap, Tangle-Foot Co., MI, USA). Tangletrap applied to the leaf margin was used to immobilize and separate neonate larvae that moved away from the leaf discs. Leaf disc surfaces were then sprayed with the various treatments using a Potter Tower (Burkhard Manufacturing Co. Ltd, Rickmansworth, UK) calibrated before each assay to achieve a foliar deposit of approximately 3 µl/cm^2, in order to simulate similar application rates as used in the field trials. Once the leaf discs had dried, two neonate larvae were transferred to each disc using a fine-hair paintbrush. The petri dishes were transferred to a dark 25°C incubator. Larvae were left to walk around the leaf discs for set exposure times of 3, 7, 15, 30 and 60 min. Then, the larvae remaining on leaf discs were transferred individually to separate wells (2.5 ml) of a 128-well culture plate (Bio-Assay Tray, Bio-BA-128) half filled with a soybean-wheat artificial diet (Stonefly Inc., Bryan, TX, USA). Virus induced mortality was recorded after rearing at 25°C for 14 days in the dark. CpGV-infected larvae were identified by the milky-white colour. In case of uncertainty, larvae were prodded with a needle in order to provoke the characteristic disintegration of the larval tissues. Forty-two larvae were used for each exposure time for each treatment, and non-virus deaths or missing larvae were subtracted from the total. For each assay, two controls were set up: "diet only"—with 32 larvae placed directly on diet; and "leaf control"—with 32 larvae exposed to unsprayed leaf discs for 60 min prior to transferring to a clean diet.

Exposure time mortality data for each treatment were analysed using the probit analysis program, POLO-PC (LeOra Software 1987). We used the program to estimate the lethal time expected to cause 50% mortality (LET_{50}) of each treatment tested and the 95% confidence intervals for these exposure times. The slope (+ standard error) of the probit line was also estimated. POLO-PC was used to test equality and parallelism of the slopes of the probit lines.

In the first experiment, LET_{50} responses were compared for Madex at the dosage 160 ppm (0.16 ml/l), the lower recommended field dose that corresponds to a virus granule content of about 2.4×10^6 /ml, with and without pear ester in microencapsulated formulation, DA-MEC (a.i. Et-*E*,*Z*-DD 5%, Trécé Inc.), tested at the dosage of 10 ppm (0.01 ml/l) per solution. In assay 2, a higher dosage of 667 ppm of Madex (10^7 granules /ml; 0.67 ml/l solution) was tested alone and mixed with pear ester at 10 ppm per solution. Compared to the lowest dose of *E*,*E*-α-farnesene tested by Ballard et al. (2000b), the pear ester content applied here at 10 ppm corresponds to a 200-fold lower dosage. This dosage for the pear ester was chosen based upon the results of Knight and Light (2001) that reported a minimum threshold dose for larval attraction between 10 and 1,000-fold lower for the pear ester than for (*E*,*E*)-α-farnesene, depending on the type of bioassay performed. In assay 3, "control treatments" were carried out to evaluate the biological activity of the pear ester putative active ingredient by comparing the DA-MEC formulation with both the un-encapsulated pear ester ingredient alone ("DA-Neat") and the microcapsule coformulants ("Blank-MEC") present in the commercial product. DA-Neat consisted of the pure compound ethyl (*E*,*Z*)-2,4-decadienoate at ≥97.0% purity. This was undertaken due to the possibility that the coformulants could interact with the insect's behaviour. Both DA-Neat and Blank-MEC formulations were supplied by Trècè Inc.

Field studies

During the 2004 and 2005 seasons, efficacy trials were carried out in a 10-year-old, experimental apple orchard at

the IASMA Research Center, in S. Michele all'Adige (Trento, Italy). Double rows of *Golden Delicious* apples on M9 rootstocks are interspersed with single rows of *Florina* that act as "buffers". The planting is 3.5 × 1.2 m, with a tree height of approximately 3 m, and the training system "Spindel". The treatments were distributed in a randomized complete block design, with four blocks and conducted in plots comprised of 40 trees over two rows. The trials were carried out during the first generational flight of codling moth. The efficacy was measured by the extent to which fruit damage attributable to *C. pomonella* was reduced by each treatment, compared with the unsprayed control. The applications were performed using an experimental sprayer (Diethart Waibl, Merano, Italy), designed with separate small tanks for each treatment mix. All treatments were applied at an equivalent water volume of 15 hl/ha. Madex was applied at the lowest recommended field dosage (7 ml/hl containing 3×10^{13} granules/l in 2004 and 16 ml/hl containing 1.5×10^{13} granules/l in 2005). The pear ester dosage of 12 ml/hl used in 2004 trials was chosen according to the results obtained in semi-field trial carried out by Pasqualini et al. (2005b), while both 12 and 1 ml/hl doses were used in the 2005 trials. Gusathion 20 SC (18.4% a.i. azinphos-methyl) was used as an insecticide standard.

In 2004 trial, the first spray was timed at the beginning of egg hatch on May 25. Following applications were performed at 8-day intervals (every other week for azinphos-methyl) with the last spray being carried out on July 2. Six applications were required to cover the first generation flight. On July 8, 1,000 fruits/treatment were examined for codling moth damage. Damage caused by either active still living larva (with presence of wet, brown frass) or by larva that had stopped their feeding activity (superficial stings), was distinguished.

In 2005, two treatments were added to the experimental design in order to evaluate the activity of a lower dosage of the kairomone both alone and mixed with CpGV. The first spray was conducted on May 10 at the beginning of oviposition, in order to exploit fully the period of time in which the reported "disorienting effect" on egg laying females (Pasqualini et al. 2005a) could act. Additional applications were made at intervals of approximately 8 days (every other week for azinphos-methyl) with the last one being carried out on June 28. Seven sprays with kairomone and CpGV were necessary to cover the first generation flight. Fruit damage was checked on July 6 by inspecting 1,320 fruits/treatment.

Fruit damage data was processed by analysis of variance. Data obtained were subjected to one-way ANOVA after Bliss angular transformation. Levene's test was used to verify homogeneity of variances. The Duncan test was used to compare unsprayed control vs. mean of all other treatments (Statistica® 7.1 for Windows®, Statsoft Inc., Tulsa, OK, USA).

Results

Laboratory bioassay

The regression line parallelism test (student *t*), performed for the same treatments throughout the three assays, did not reveal significant differences (at $P = 0.05$) and therefore data were pooled.

At the Madex concentration of 160 ppm, corresponding to 2.4×10^6 CpGV granules/ml, virus-induced mortality was negligible. Mixing Madex at this concentration with 10 ppm of DA-MEC increased the neonate mortality and established a time-mortality response (Table 1). Even if the differences are not statistically significant, higher mortality levels were recorded after 15 min exposure time and the trend continued until the maximum exposure time of 60 min (Table 2). At the higher Madex concentration of 667 ppm, corresponding to 10^7 CpGV granules/ml, the addition of DA-MEC did not decrease the LET_{50}, indicating no improvement of the virus efficacy for this rate. Regarding the separated components of the commercial product DA-MEC, when added to the lower rate of virus (160 ppm), the LET_{50} determination was lowest for the virus mixed with DA-MEC. The next lowest value was obtained with the DA-Neat treatment and a significantly longer though weakly positive effect on virus efficacy with the Blank-MEC formulation (Table 1).

Table 1 Effect of the DA-MEC formulation on the median lethal exposure time (LET_{50}) and different concentrations of Madex tested upon neonate codling moth larvae

Treatment	Rates (ppm)	LET_{50} (95% CL)	Slope (±SE)	χ^2
Madex	160	0.2×10^7 –	0.15 (±0.33)	4.39
Madex + DA-MEC	160 + 10	54.1 (31.5–150.0)	1.0 (±0.26)	1.50
Madex + Blank-MEC	160 + 10	1253.1 (0.1–>10^6)	0.28 (±0.3)	2.75
Madex + DA-Neat	160 + 0.5	92.2 (36.6–256.9)	2.32 (±1.61)	1.50
Madex	667	9.1 (4.8–16.2)	0.97 (±0.27)	5.7
Madex + DA-MEC	667 + 10	14.6 (8.0–25.2)	0.93 (±0.27)	4.18

Table 2 Original mortality values of neonate codling moth larvae recorded during the leaf disc bioassay

Time on leaf discs (min)	% Mortality		$\chi_c^{2\,a}$	P
	Madex 160 ppm	Madex 160 ppm + DA-MEC		
3	31.6	23.4	0.33	0.41
7	31.6	31.3	0.05	0.81
15	34.4	37.7	0.01	0.93
30	18.8	41.9	4.07	0.04
60	43.8	62.5	2.33	0.13

[a] Yates corrected

The likelihood ratio (LR) test of parallelism did not reveal significant differences within the treatments ($\chi^2 = 9.36$, $df = 5$) assuming parallel lines.

Field studies

The addition of DA-MEC, at the higher dosage of 12 ml/hl, to the CpGV improved the control activity of the granulovirus on *C. pomonella* (Table 3), but the difference was not significant. Unexpectedly, in both the years, 2004 and 2005, the field efficacy trials performed revealed a significant effect of the blank DA-MEC formulation in reducing fruit injury compared to an unsprayed control. No significant differences in the percentage of total damaged fruit were found between DA-MEC + Madex, DA-MEC alone, and Madex alone treatments applied at the recommended field dosage, indicating similar control efficacy for the experimental conditions applied.

Discussion

In the laboratory bioassays performed, it was possible to increase the larval virus infection by adding pear ester to a sublethal virus dose. Ballard et al. (2000b) obtained a similar result by mixing virus, at a granules content of 2×10^6 ml^{-1}, with molasses, which is reported to be one of the most effective feeding stimulants for lepidopteran larvae (Burges and Jones 1998). Pear ester did not act as feeding stimulant for codling moth neonate larvae when applied to apple fruits (Pasqualini et al. 2005b). Increased mortality after identical exposure times can be explained if larval motility is induced by the kairomone odour stimulus. The Blank-MEC formulation, without kairomone, showed some weak effect in increasing larval mortality. Possibly, the microcapsules are ingested accidentally by the larvae and favour in that way, contact with the virus granules.

The dosage of Madex that we demonstrated as a laboratory sublethal dose, is actually the recommended dose used in the field trials. In the leaf disc bioassays, the larval exposure to surface viruses was designed to be limited in time and space, while in the field, larvae can ingest, accidentally or through active feeding, a higher amount of virus granules by moving about, sensing and feeding more frequently, over larger surface areas until they penetrate into the fruit. As a consequence, a virus content corresponding to the recommended field dosage did not enable a time mortality response to be determined and established in the laboratory bioassays.

In the field trials the efficacy of Madex, applied at the recommended field dosage, was not improved when mixed with DA-MEC. Furthermore, no positive effect was observed during the laboratory trial when DA-MEC was added to a high dose of Madex, containing about 10^7 virus granules/ml of product. However, in the laboratory bioassay, increased mortality, or simply lethal effects, became evident when the kairomone was mixed with the virus at a sublethal dose. These results of dose-dependent enhanced virus efficacy suggest that the failure to resolve improved efficacy of the tank mixed DA-MEC in the field could be ascribed to the high, perhaps overwhelming, virus dosage applied. The dosage used in the field corresponds to

Table 3 Percentages of damaged fruits obtained in the field trials carried out in 2004 and 2005 in S. Michele all'Adige (TN)

Treatment	2004		2005	
	% damaged apples	% active larvae	% damaged apples	% active larvae
Untreated	51.4 a	41.9 a	24.3 a	19.5 a
DA-MEC 12 ml/hl	18.2 b	6.5 b	12.0 b	6.5 b
Madex + DA-MEC 12 ml/hl	16.0 b	1.9 c	10.4 b	2.7 c
Madex	17.9 b	4.3 bc	12.1 b	3.4 c
DA-MEC 1 ml/hl			11.6 b	5.0 bc
Madex + DA-MEC 1 ml/hl			15.8 b	4.3 bc
Gusathion SC	0.8 c	0.0 c	0.8 c	0.2 cd
ANOVA, $df_{2004} = 4, 15$	$F = 23.43$	$F = 59.24$	$F = 20.00$	$F = 37.85$
$df_{2005} = 6, 21$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Different letters within the same column indicate significant differences (Duncan's test, $P < 0.05$)

3.15×10^{14} granules/ha and coincides with the recommended label rate. Field tests by Ballard et al. (2000b) showed that spraying at 10^{14} granules/ha did not significantly improve control, compared to 10^{13} granules/ha. Our results indicate that with high population densities, as we found in the orchard studied, CpGV works already at its maximum control potential at the rate applied. Ballard et al. (2000b) demonstrated that improved efficacy of the virus could be obtained by adding (*E,E*)-α-farnesene or feeding stimulants to a 10^{12} granules/ha CpGV dose. Thus, the utility of mixing DA-MEC with CpGV might be the enhanced ability to reach acceptable control efficacy levels with lower rates of insecticide, rather than improvement of its overall potential efficacy.

The use of codling moth granulovirus as a selective and biological alternative to conventional insecticides has attracted considerable interest in the last 25 years (Cross et al. 1999). In mating disrupted treated orchards, CpGV is often applied as a complementary control method in order to reduce the population density (Kienzle et al. 2001). The chief disadvantage of using CpGV insecticide is its low persistence in field conditions (Glen and Payne 1984; Arthurs and Lacey 2004) and subsequently the higher cost due to repeated applications (up to weekly). Spraying the virus at lower dosages and exploiting an enhancement effect of DA-MEC to obtain a satisfactory fruit damage control could result in a more cost-effective solution. For that reason further efficacy trials are also needed to prove the enhancement effect of DA-MEC in the field, by testing lower virus dosages.

In the field efficacy trials performed, the kairomone treatment without insecticide showed significant activity in reducing fruit damage compared to an untreated control. The studies carried out by Pasqualini et al. (2005a), showed a more diffuse pattern and distribution of egg-laying by mated females which was attributed to the foliar spraying of the kairomone microencapsulated formulation. Additionally, previous trials, which they performed on neonate larvae, indicated apparent "difficulties" for the larvae in entering kairomone treated fruit, although they did not explain the mechanism by which the pear ester may act upon the larvae (Pasqualini et al. 2005b). In the field efficacy trials reported in the present study the increased larval mortality recorded in DA-MEC treated plots (without any insecticide) could be ascribed to an increased exposure to natural enemies or abiotic stresses due to the longer time period larvae are enticed to spend on the skin surface before they enter into the fruit. The DA-MEC formulation is not known to have toxic effects itself on codling moth (Douglas Light, USDA Agricultural Research Service, and Bill Lingren, Trécé Inc., Adair, OK, USA; personal communications). The damage recorded in all DA-MEC treated plots, with and without the virus, consisted mainly of superficial stings produced by larvae that had been stopped in their feeding activity. This quantified result might be explained either by a certain fatigue of the larvae, which had realised energy (perhaps additional) in searching for the fruit, or because the larvae failed to recognise the target fruit even after tasting it. The latter would suggest a possible influence of the kairomone on the gustatory receptors.

In conclusion, the present study showed that adding DA-MEC to low doses of Madex promoted an increase in virus-induced mortality as a function of exposure time in the laboratory bioassays, and that in field applications, DA-MEC was able to reduce codling moth fruit damage. Further studies are needed to define the optimal field application dosages of DA-MEC and Madex tank-mixed together. Prospects about the use of pear ester in combination with other conventional insecticides in order to lower the recommended field dosages should be taken into account (Light 2007). The biggest advantage in the use of this kairomonal spray-adjuvant lies in the development of new or modified control strategies, which would be more sustainable for both the environment and farm worker safety. Hence, specific investigations are needed to clarify the interaction between DA-MEC and the evoked larval behaviour.

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