Journal für Kulturpflanzen, 62 (8). S. 299–304, 2010, ISSN 0027-7479 Verlag Eugen Ulmer KG, Stuttgart

Originalarbeit

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Bioassay suitable for determining the susceptibility of *Meligethes aeneus* to organophosphates

Ein Biotest für die Bestimmung der Empfindlichkeit von *Meligethes aeneus* gegen Organophosphate

Abstract

Bioassay procedures were developed for determining the susceptibility to organophosphates of oilseed rape pollen beetles. Technical-grade and formulated chlorpyrifos-ethyl were tested using single applications of a series of different dosages. The inner wall of glass tubes were treated with these test substances and dried by rotating them on a roller mixer. Larger containers (polypropylene 'Rotilabo' cups) were compared with glass tubes in a separate experiment. The use of technical-grade substances in glass tubes with lids, which allow an exchange of air, is recommended for use in future studies on pollen beetles because they are cheaper, needs less space, are faster to prepare because less time is required for evaporating the solvent and give definite dose response relationships.

Key words: *Meligethes aeneus*, susceptibility, organophosphates, bioassay

Zusammenfassung

Es wird ein Biotest vorgestellt, der die Bestimmung der Empfindlichkeit von in Deutschland gesammelten Rapsglanzkäfern gegen Organophosphate ermöglicht. In Serien mit unterschiedlichen Dosierungen wurde Chlorpyrifos-ethyl als technischer Wirkstoff oder formuliert getestet. Hierfür wurden die zu testenden Substanzen in Glassröhrchen pipettiert und bis zur vollständigen Verdunstung des Lösungsmittels auf einem Rollmischer gedreht. In einem weiteren Experiment wurden größere 'Rotilabo'-Polypropylengefäße mit Glasröhrchen verglichen. Für zukünftige Untersuchungen wird die Verwendung von technischen Wirkstoffen in Glassröhrchen empfohlen, deren Deckel einen Luftaustausch gestatten. Diese Gefäße sind kostengünstiger, benötigen weniger Platz, stehen wegen der reduzierten Trocknungszeit des Lösungsmittels schneller zur Verfügung und ermöglichen die Bestimmung einer Dosis-Wirkungs-Beziehung.

Stichwörter: *Meligethes aeneus*, Empfindlichkeit, Organophosphate, Biotest

Introduction

The oilseed rape pollen beetle, *Meligethes aeneus* (Coleoptera, Nitidulidae), is one of the most destructive pollen feeding pests of rape. The application of insecticides is the primary means of controlling this pest in Europe and North America. However, widespread and repeated use of chemicals can result in the selection for insecticide resistance. In Europe, nearly all the pyrethroid insecticides previously applied are no longer effective at reducing beetle populations in some oilseed rape producing areas. In France, Denmark, Switzerland and Germany the use of pyrethroids, like lambda-cyhalothrin, deltamethrin and beta-cyfluthrine, for controlling pollen beetles is widespread and resistance to these insecticides has developed in many areas (Ballanger et al., 2003; Derron et al., 2004; Hansen, 2003; Heimbach et al.,

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Accepted
June 2010

2006). Due to the development of resistance these pyrethroid-based insecticides have lost most of their efficacy during the last 5 years and new insecticides are also likely to quickly loose their effectiveness.

It is important to develop pesticides with new modes of action and without cross resistance, which are effective against oilseed rape pollen beetles. It was concluded that in order to reduce the level of resistance or the speed of development of resistance, insecticides with at least two different modes of action and no cross resistance to pyrethroids are required (ZLOF, 2008). Interesting candidates, among others, are organophosphates, which act as inhibitors of carboxyl ester hydrolases, particularly acetylcholinesterase (CHAMBERS and CARR, 1995).

The development and implementation of resistance management strategies to prevent or delay the evolution of resistance to organophosphates or other new insecticides is essential. An important step prior to the implementation of such programs is the establishment of bioassay techniques for the production of baseline susceptibility data for a pest species across its geographic range. These baseline studies should be carried out prior to the use of the active substance in the field so that any change in susceptibility is easily identified. Such baseline studies are today part of the pesticide regulation procedure. This paper reports a way of determining the dose-response relationships and the level of susceptibility of populations of the pollen beetle, *M. aeneus*, to organophosphate and recommends they are used in future studies on the susceptibility of this species to pesticides.

Methods

Adult pollen beetles were collected at random by sampling winter-oilseed rape flowers and shaking them above a plastic tray (2008: Keindorf, Saxony-Anhalt; Moosburg, Bavaria; Wendhausen, Lower Saxony; Köchelstorf, Thurow, Mecklenburg-Western Pomerania and 2009: Keindorf, Saxony-Anhalt; Dippersricht, Bavaria; Lübstorf, Mecklenburg-Western Pomerania; Wolsfeld, Rhineland-Palatinate) or by extracting soil samples from a hibernation site (2009: Sagerheide, Mecklenburg-Western Pomerania). All tests were performed within 12 days after collection on adult beetles showing coordinated movement.

Adult pollen beetles were exposed in different ways to organophosphates (technical-grade or formulated chlorpyrifos-ethyl was applied at 8 concentrations, from 2.8 to 1875 ng ai/cm^2, which is equivalent to 100% of the intended field rate in Germany) and a control (acetone only). For this glass tubes of two different sizes (type 1: height: 5.3 cm, diameter: 2.2 cm; inside surface treated: 30.14 cm^2; type 2: height: 6.5 cm, diameter: 2.4 cm; inside surface treated: 53.53 cm^2) or polypropylene 'Rotilabo' cups (height: 5.9 cm, diameter: 4.4 cm; inside surface treated: 61.77 cm^2) were used. Into each of these containers was placed 1 ml of the insecticide dissolved in acetone and the solvent evaporated off by rotating the containers on a Roller Mixer. Whereas each glass tube slightly narrows towards the opening the 'Rotilabo' cups do not. Therefore, the latter were placed at an angle on the Roller Mixer to avoid leakage. This resulted in a greater area of the 'Rotilabo' cups remaining untreated. Pollen beetles were transferred into each container with the help of a pooter and then the lids were replaced. In order to ensure air exchange a large hole (diameter: 1.1 cm) was drilled in each lid, which were then covered with gauze (1 mm mesh size). During the bioassays the tubes were kept at 21 ± 1 °C and under continuous illumination. At least four replicates, each of 8–11 beetles, were used for assessing the effectiveness of each insecticide concentration.

The response of the beetles was determined by examining them under a binocular microscope. The number of living (responding normally, e.g. with coordinated movement, when gently touched) and dead or moribund pollen beetles (responding abnormally, e.g. unable to move in a coordinated way, when touched) were scored five and 24 hours after the start of the experiment. Thus, percentage mortality includes both dead and moribund beetles.

All the basic statistical analyses were done using the computer programme SYSTAT, Version 10.0. To identify the dose-response relationship the data were analysed by PoloPlus 1.0 (LeOra Software Company). The goodness-of-fit of the data (response of pollen beetles to different doses of insecticide) to a log-normal distribution (probit analysis) was determined using χ^2 tests.

To compare the concentrations resulting in 50 and 90% mortality of an insecticide applied in different ways, or the relative susceptibility of different populations of *M. aeneus*, a simple comparison of the ratio of one lethal dose to another does not include an estimate of the error involved in the calculation. To determine whether the lethal doses differ significantly the 95% confidence limits were calculated using PoloPlus 1.0 (LeOra Software Company).

Results and discussion

There are many laboratory bioassays for detecting resistance and although they provide little or no information about resistance mechanisms, they are a pre-condition for the identification of any change in susceptibility to a given insecticide. For this it is necessary to determine dose-response relationships. To study the susceptibility of pollen beetles to organophosphates many trials using 'IRAC Method No: 11' (IRAC, 2006) were carried out over the last few years. This method was developed for analyzing the susceptibility to pyrethroids and rarely gave definite dose-response relationships when used to analyze the susceptibility to organophosphates (unpublished results). Whereas both, pyrethroid and organophosphate insecticides act by contact and on ingestion, the last acts also via the respiratory system (ANONYMOUS, 1997). Because organophosphates have a high vapour pressure

glass tubes with air exchange or a larger container ('Rotilabo' cups with air exchange) were used in the following bioassays in order to reduce the effect of a build up of insecticide vapour.

First the susceptibility to Karate Zeon® of pollen beetles collected from oilseed rape fields, where insecticides were previously applied extensively (Keindorf) or intensively (Moosburg, Wendhausen, Köchelstorf, Thurow, Dippersricht, Wolsfeld, Lübstorf), was measured (Tab. 1). As the mortality recorded after 5 h incubation was below 100%, at 100% of the recommended field rate, all strains of the pollen beetles tested in 2008 and 2009 were resistant to lambda-cyhalothrin (according to HEIMBACH et al., 2006).

The results of the probit analysis of the mortality of pollen beetles from different sites in Germany collected in 2008 and 2009 exposed for five and 24 hours to different dosages of technical-grade chlorpyrifos-ethyl applied to the inner walls of glass tubes, with air exchange, are presented in Tab. 2. After five hours exposure the LC_{90}-values for all populations were always recorded at below 10% of the recommended field rate for chlorpyrifos-ethyl (187.5 ng ai/cm²). This indicates these beetles are susceptible to this insecticide.

A dose response was also found when beetles of a single population (Freising 2008) were exposed to both technical-grade and formulated chlorpyrifos-ethyl applied to the inner walls of glass tubes with air exchange

Tab. 1. Mean percentage mortality (standard deviation) of pollen beetles from different sites in Germany, exposed for 5 h to lambda-cyhalothrin, applied to the inner walls of glass tubes without air exchange.

	Origin	Dosage (ng ai/cm²)		
		0	15	75
2008	Keindorf*	0.0 ± 0.0 (45)	11.4 ± 13.6 (39)	39.7 ± 19.4 (41)
	Moosburg**	0.0 ± 0.0 (50)	44.0 ± 18.2 (50)	88.0 ± 13.0 (50)
	Wendhausen**	0.0 ± 0.0 (51)	51.1 ± 8.9 (51)	96.7 ± 7.5 (49)
	Köchelstorf**	0.0 ± 0.0 (50)	28.5 ± 9.0 (47)	96.0 ± 8.9 (52)
	Thurow**	0.0 ± 0.0 (44)	15.5 ± 17.7 (52)	88.7 ± 7.7 (52)
2009	Keindorf*	0.0 ± 0.0 (39)	0.0 ± 0.0 (39)	18.3 ± 11.1 (39)
	Dippersricht*	0.0 ± 0.0 (39)	10.6 ± 8.3 (38)	19.9 ± 7.3 (40)
	Wolsfeld*	0.0 ± 0.0 (40)	28.2 ± 16.7 (40)	35.0 ± 12.9 (40)
	Lübstorf**	0.0 ± 0.0 (46)	17.6 ± 8.3 (41)	66.0 ± 23.0 (50)

* exposed in glass tube type 1, height: 5.3 cm, diameter: 2.2 cm; ** exposed in glass tube type 2, height: 6.5 cm, diameter: 2.4 cm

Tab. 2. LC_{50} and LC_{90} (95% confidence limits) of pollen beetles collected in 2008 and 2009 from different sites in Germany, exposed for 5 and 24 h to technical-grade chlorpyrifos-ethyl, applied to inner walls of glass tubes with air exchange. Percentage mortality in control was zero, except for beetles from Keindorf (2009) exposed 24 h (2.5 ± 5.00).

	Origin		LC_{50} (ng ai/cm²)	LC_{90} (ng ai/cm²)
2008	Keindorf	05h*	8.42 (5.978 – 9.814)	14.10 (12.04 – 20.42)
		24h	ndr	
	Moosburg	05h**	10.62 (5.55 – 15.69)	55.71 (40.83 – 84.91)
	Wendhausen	05h**	18.88 (16.03 – 22.11)	38.74 (31.67 – 52.63)
	Köchelstorf	05h**	18.33 (16.03 – 20.98)	38.43 (32.14 – 49.29)
	Thurow	05h**	19.22 (17.08 – 21.58)	33.52 (28.93 – 41.45)
2009	Keindorf	05h*	13.00 (7.40 – 26.41)	41.85 (22.19 – 401.01)
		24h	4.35 (3.55 – 5.30)	7.93 (6.37 – 11.14)
	Dippersricht	05h*	8.68 (7.03 – 10.32	17.65 (14.42 – 24.23)
		24h	4.99 (4.18 – 6.01)	8.29 (6.78 – 11.19)
	Wolsfeld	05h*	12.04 (7.96 – 19.76)	49.83 (27.58 – 187.60)
		24h	5.61 (4.17 – 7.16)	18.50 (13.46 – 31.61)
	Lübstorf	05h**	16.93 (13.06 – 22.24)	50.45 (34.97 – 99.71)

LC, lethal concentration; * glass tube type 1, height: 5.3 cm, diameter: 2.2 cm, assessed after 5 and 24 h; ** glass tube type 2, height: 6.5 cm, diameter: 2.4 cm, assessed after 5 h only; ndr, no dose response

(Tab. 3). Whereas the technical-grade substance can be dissolved in pure acetone, the formulated product needs to be dissolved in a mixture of 5% water and 95% acetone, which resulted in longer period for the evaporation of the solvent. The smaller glass tubes (type 1) treated with formulated chlorpyrifos-ethyl resulted in slightly reduced LC-values. After 24 h exposure to chlorpyrifos-ethyl the hypothesis of equality ($\chi^2 = 49.64$; d.f. = 2; $P < 0.005$) was rejected, but not parallelism ($\chi^2 = 1.36$; d.f. = 1; $P > 0.05$). These differences may be caused by a stronger concentration of organophosphate volatiles in the smaller glass tubes, indicating that in long term studies (e.g. baseline studies) the size of the glass tubes should not be changed.

This was also supported by experiments in which the results obtained using glass tubes of type 1 (small) were compared with those obtained using the much larger 'Rotilabo' cups. For the beetles from a hibernation site, linear regressions were calculated for the probit mortality recorded after exposure to technical grade chlorpyrifos-ethyl applied to the inner walls of glass tubes and 'Rotilabo' cups, both with air exchange (Fig. 1 and Tab. 4). The results differed depending on the type of container. Pollen beetles were susceptible to this insecticide, but more so when assayed in glass tubes than 'Rotilabo' cups. After 5 h exposure to chlorpyrifos-ethyl the hypothesis of equality ($\chi^2 = 287.0$; d.f. = 2; $P < 0.005$) was rejected, but not parallelism ($\chi^2 = 2.72$; d.f. = 1; $P > 0.05$). This difference may be due to the greater area of untreated surface within the 'Rotilabo' cups, or, more likely, a higher concentration of organophosphate volatiles in the smaller glass tubes. By choosing technical-grade organophosphates it should be possible to generate data for several years in a standardised way and identify any change in susceptibility. Modifications in the formulation of an insecticide may cause problems within time when datasets are analyzed. Therefore, wherever possible it is recommended that technical-grade substances are used instead of formulated products for determining dose-response relationships.

These results demonstrate that all the pollen beetle populations tested were very susceptible to organophosphates. Dose-response relationships are best obtained by testing the response to these insecticides in glass tubes.

Recommendation of a bioassay suitable for determining the susceptibility of pollen beetles to organophosphates

Several different bioassays are used to determine the susceptibility of pollen beetles to organophosphates. Here the following method is recommend.

Tab. 3. LC_{50} and LC_{90} (95% confidence limits) of pollen beetles collected in 2008 on winter-oilseed rape near Freising in Germany, exposed for 5 and 24 h to chlorpyrifos-ethyl formulated* or as technical-grade substance applied to inner walls of glass tubes with air exchange. Percentage mortality in control was always zero, except for beetles exposed for 24 h to technical-grade chlorpyrifos-ethyl** (3.6 ± 4.97).**

		LC_{50} (ng ai/cm²)	LC_{90} (ng ai/cm²)
Glass tube type 1*	05h	23.79 (19.35 – 29.57)	57.28 (43.41 – 89.51)
	24h	12.19 (10.13 – 14.59)	27.23 (21.66 – 38.53)
Glass tube type 2**	05h	ndr	
	24h	26.18 (20.44 – 30.96)	49.86 (40.97 – 72.47)

LC, lethal concentration; glass tube type 1, height: 5.3 cm, diameter: 2.2 cm; glass tube type 2, height: 6.5 cm, diameter: 2.4 cm; ndr, no dose response

Tab. 4. LC_{50} and LC_{90} (95% confidence limits) of pollen beetles collected in 2009 at a hibernation site near Sagerheide, exposed for 5 and 24 h to technical-grade chlorpyrifos-ethyl, applied to inner walls of glass tubes and 'Rotilabo' cups, both with air exchange. Percentage mortality in control was zero, except for beetles exposed for 24 h in glass tubes (5.1 ± 7.20) or 'Rotilabo' cups (7.1 ± 9.82).

		LC_{50} (ng ai/cm²)	LC_{90} (ng ai/cm²)
Glass tube type 1	5h	9.43 (8.10 – 10.82)	21.41 (17.78 – 28.09)
	24h	3.60 (3.02 – 4.14)	7.08 (6.10 – 8.64)
'Rotilabo' cup	5h	63.51 (53.40 – 77.93)	178.46 (132.90 – 280.84)
	24h	ndr	

LC, lethal concentration; ndr, no dose response

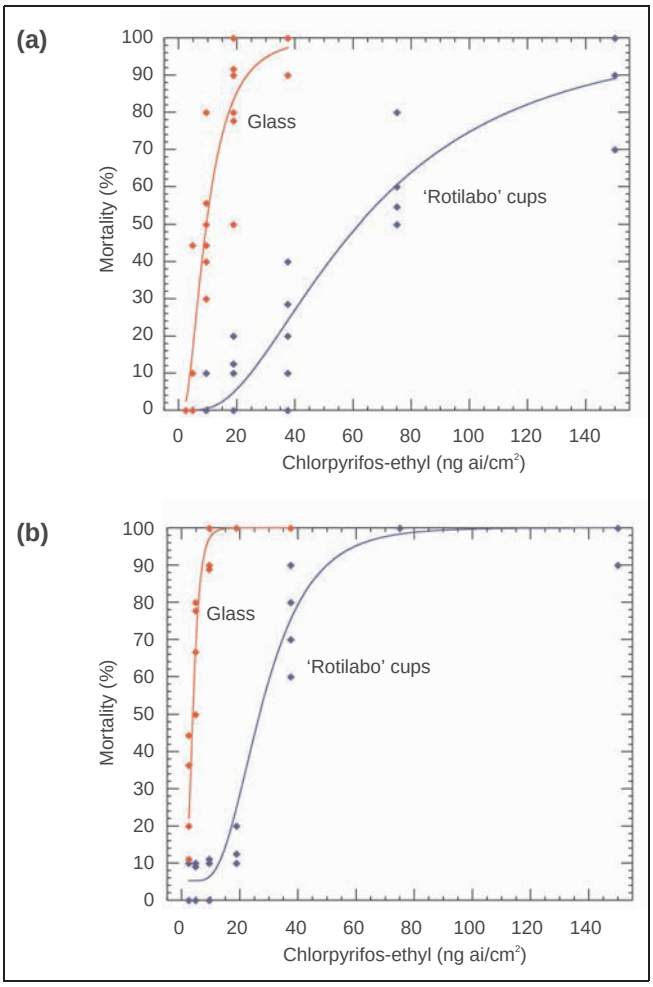

Fig. 1. Percentage mortality of pollen beetles after **(a)** 5 h and **(b)** 24 h exposure to technical-grade chlorpyrifos-ethyl applied to the inner walls of glass tubes (type 1) and 'Rotilabo' cups, both with air exchange. Beetles were collected in 2009 from a hibernation site near Sagerheide.

Materials needed

Insect-proof containers, a fine artist's paint brush, beakers for test liquids, syringes/pipettes for liquids, balance for weighing solids, acetone, syringes/pipettes for making dilutions, 20–30 ml glass vials, gauze to cover the holes in the lids of containers, vial roller, pooter or small funnel for transferring beetles to vials, binocular microscope or hand lens, paper towels, maximum/minimum thermometer.

Method

- For analysing dose-response relationships at least 300 adult beetles should be collected at different locations across an infested field. Store beetles in aerated plastic containers or in large (10 l) plastic bags. Some dry paper towel should be placed at the bottom of the container or bag, and as food for the adults two or three oil seed rape inflorescences should be added. Avoid keeping the insects at temperatures above 25 °C or at a high humidity, which will result in water droplets forming on the inner surface of the containers/bags, or stressing of the beetles.
- A form for recording the details of the sample and other information should be attached to each sample to facilitate the tracking of samples and interpretation of results.
- The collected beetles should be transferred as quickly as possible to the test laboratory. Containers with beetles can be stored in a refrigerator overnight or for some days but not for prolonged periods of time.
- The standard test organophosphate is technical-grade chlorpyrifos-ethyl. Other synthetic organophosphates may be used, but the concentrations applied may need to be adjusted to take account of differences in the inherent potency of different organophosphates.
- The test containers are glass vials with an internal surface area of 30–60 cm^2. Newly purchased vials should be cleaned of any residues resulting from their manufacture by soaking them overnight in soapy water, rinsing with acetone and air drying for at least 4 hours before use.
- Dilutions of organophosphates should be prepared with acetone. For chlorpyrifos-ethyl suitable test concentrations in ng ai per cm^2 glass surface are as follows: 48, 24, 12, 6 and 3, with acetone only as a control. Freshly prepared test solutions can be stored sealed and cooled for approximately 8 hours.
- Glass vials should be filled with 0.5–1.5 ml (depending on vial size) of solution and rotated on a vial roller at room temperature until the acetone has completely evaporated. At the start of the coating process the inner surface of the vials should be completely covered with the test solution.
- Four replicates of each concentration and a control are required (i.e. 24 vials per test).
- Using a pooter or a funnel ten beetles are placed into each vial and the lids replaced. There should be a hole of approximately 1 cm diameter covered with gauze in the lid of each vial and the vials stored at 20 ± 2 °C.
- Five and 24 hours after the start of the experiment briefly shake the vials and then record the number of dead or moribund and live beetles. Mortality includes both dead and moribund (those not capable of coordinated movement) beetles.
- Results should be expressed as percentage mortality corrected for the mortality in the "untreated" control using Abbot's formula. Mortality in the controls should also be recorded. The results of trials in which the mortality in the control is more than 20% should be rejected.

Acknowledgements

The authors wish to thank R. BULL, L. DUWE, I. ROGOTZKI and K. BURLAK for technical assistance. We are deeply grateful to Tony DIXON whose comments and suggestions helped to improve the manuscript. A. MÜLLER was financially supported by the German Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) through the Federal Institute for Agriculture and Nutrition (BLE), grant number 06HS038.

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