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Development of a standard acute dietary toxicity test for the silkworm (*Bombyx mori* L.)

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ABSTRACT

Larvae of the silkworm (*Bombyx mori* L.) may be exposed to pesticide residues on the leaves of their food plant, the mulberry tree (*Morus* spp.), which can lead to adverse effects on silk production. A new acute dietary toxicity test method was evaluated as the basis for pesticide risk assessment. A series of 57 tests were carried out with six insecticides and six different silkworm strains. LC_{50} ranges were, in increasing order: cypermethrin 0.016–0.069 mg kg^{-1} fresh mulberry leaf; imidacloprid 0.13–0.29 mg kg^{-1}; monosultap 0.19–0.37 mg kg^{-1}; phoxim 0.42–0.48 mg kg^{-1}; dichlorvos 5.6–6.9 mg kg^{-1} and dimethoate 10.2–24.7 mg kg^{-1}. The Qiu Feng × Bai Yu and the Chun Lei × Zhen Zhu strains were overall most sensitive to the tested insecticides. Control mortality in the test system always remained below 5%, and precision of the LC_{50} estimates was high. The coefficient of variation of LC_{50} values among tests was consistently less than 20%. The acute dietary toxicity test was found to be highly reproducible and robust.

1. Introduction

Sericulture in China was initiated about 5000 years ago (Feng, 1996). Presently, China is the largest producer of silk in the world and it is estimated that China produced 300 000 metric tons of silkworm cocoons in 2007, with a total value of more than 1 billion US $ (FAO, 2009; Liu and Zhang, 2006).

Exposure of silkworm larvae to pesticides may cause acute effects on survival of the insects (Zhao et al., 2004; Dang and Li, 2005; Zhang et al., 2008), or sub-lethal effects on silk production and quality (Leonardi et al., 1998; Wang et al., 1999; Vassarmidaki et al., 2000), both of which could seriously affect the productivity of the silk industry. Exposure of the larvae to pesticide residues occurs via their food, the leaves of the mulberry tree (*Morus* spp.). Mulberry trees are grown in plantations, their leaves harvested and provided to silkworm larvae which are reared separately, generally in closed barns. Contamination of the mulberry leaves is primarily due to pesticide drift from crop fields that are close to mulberry plantations (Zhang et al., 2008), but may also be the result of direct control of mulberry pests or diseases.

Consequently, it is important that the risk of a pesticide to the silkworm is assessed whenever there is a possibility that mulberry leaves are exposed. Reliable toxicity tests resulting in relevant endpoints are an essential part of such pesticide risk assessment. Standard toxicity testing guidelines, which are part of the pesticide registration requirements, have, for instance, been issued in China and Japan (SEPA, 2004; Ma et al., 2005; ACIS, undated). However, these guidelines have a number of limitations with respect to their utility for pesticide risk assessment (Candolfi et al., 2001). For instance, a leaf-dipping bioassay is prescribed which results in a dose expression as quantity of active ingredient per unit of pesticide solution, rather than per weight unit of mulberry leaves. The former does not allow risk assessment of the pesticide, because silkworm larvae are exposed to a quantity of pesticides on the mulberry leaves and not directly to the spray solution. Furthermore, data on the precision, robustness and reproducibility of the test methods are lacking, which complicates the interpretation of the results of these tests when carried out by different laboratories.

The research presented here is a first step in the development of a standard acute dietary toxicity test which can be used to generate data for pesticide risk assessment. A series of toxicity tests was carried out with the following objectives: (1) assess the precision of

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results of the tests, using six insecticides comprising four different modes of action; (2) assess the variability in results between tests with one selected insecticide when carried out at different times over a period of two years; (3) assess the relative sensitivity of six different silkworm strains which are important in commercial use in China; and (4) compare two types of exposure cages.

2. Materials and methods

All experiments were carried out during 2008 and 2009 at the Division of Environmental Fate and Effects, Institute for the Control of Agrochemicals, Ministry of Agriculture (ICAMA), in Beijing. All toxicity tests followed the same principle: second instar larvae of the silkworm were continuously fed on mulberry leaves sprayed with a range of concentrations of the test insecticides. A spray tower was used to apply the pesticide, which allows exact quantification of the amount of pesticide applied per unit of leaves. Mortality was recorded daily for 96 h and compared to control values. The results were then analysed in order to calculate a 96 h-LC_{50}.

2.1. Sources and rearing of silkworm

Six hybrid silkworm strains which are used on a large scale in China for commercial silkworm farming were obtained for the tests: Chun Lei × Zhen Zhu, Jing Song × Hao Yue, Liang Guang 2, Qiu Feng × Bai Yu and 871 × 872 (all acquired from Shandong Guangtong Silkworm Eggs Group Co.), and Dong Ting × Bi Bo (acquired from the Sericulture Research Institute of Hunan). The silkworm strains were obtained as eggs and stored in a refrigerator until use. The Chun Lei × Zhen Zhu strain had already been used for several years at ICAMA, and considerable experience with its rearing and handling had been gained. Therefore, this strain was preferred as the main testing organism to assess robustness of the new testing method and for the identification of the toxic standard.

Newly moulted second instar larvae were used for the tests. Silkworm were reared in a controlled climate incubation room at 26 ± 1 °C, with relative humidity 80 ± 5% and a 16:8 h light:dark photoperiod (Wan, 2003; Ma and Li, 2004a, 2004b). Care was taken to ensure simultaneous moulting and minimize differences in development between larvae. All silkworm larvae were fed with fresh mulberry leaves. Larvae collected for the test were of similar size (approximately 0.65 ± 0.1 cm) and weight (approximately 5.5 ± 1 mg).

2.2. Source of mulberry leaves

Mulberry leaves were harvested from a small open plantation adjacent to the laboratory, which had not been treated with any pesticides. Mulberry leaves were freshly picked from the top branches of the trees shortly before each test, and were relatively young, light green and tender. Leaf size and weight were kept as similar as possible within each test, and ranged from 1.7 to 2.3 g fresh weight per leaf.

2.3. Insecticides

The following insecticides were selected for the test, belonging to different insecticide classes with four distinct modes of action: cypermethrin (98% technical grade (t.g.); obtained from Jiangsu Changlong Chemicals Co. Ltd.), dichlorvos (99% t.g.; National Centre for Quality Supervision and Testing of Pesticides), dimethoate (84.1% t.g.; National Centre for Quality Supervision and Testing of Pesticides), imidacloprid (98% t.g.; Jiangsu Tianrong Group Co.), monosultap (99.7% t.g.; Shandong Province United Pesticides Industry Co. Ltd.) and phoxim (99% t.g.; National Centre for Quality Supervision and Testing of Pesticides). Dimethoate is recommended by OECD as toxic standard for the bee toxicity tests (OECD, 1998) and was therefore selected as the main test chemical for the comparison of the sensitivity of the silkworm strains.

A fresh stock solution was prepared from the technical grade insecticide for each test, using a precision balance or a calibrated micro-pipette and volumetric flasks. Dichlorvos, dimethoate and monosultap were diluted with distilled water. The stock solution of phoxim was prepared with acetone and Tween80 (0.1% primary solution), imidacloprid with acetone (0.1% primary solution), and cypermethrin with acetone and ethanol (0.1% primary solution). Concentrations in the stock solutions were not confirmed through chemical analysis. Subsequent dilutions were made with distilled water, using calibrated micro-pipettes and volumetric flasks.

2.4. Experimental procedure

2.4.1. Application of test substance to the mulberry leaves

The insecticide solutions were applied to one side of the mulberry leaves using a laboratory spray tower (Potter Tower®, Burkhard Scientific, UK). The spray tower was calibrated to apply a constant volume of pesticide solution and even droplet deposition pattern on the leaf (Lin et al., 2004). Water sensitive papers were used to evaluate the droplet density. An initial spray solution of 2 mL, a spray pressure of 7 bar and a droplet deposition time of 20 s were adopted as standard application parameters for all tests. These settings resulted in an even and dense droplet deposition pattern without too much confluence of individual droplets, and not causing run-off of the spray solution from the leaf.

Mulberry leaves were individually weighed immediately before pesticide application, using a precision balance, and weighed again immediately after, so that the exact quantity of applied pesticide could be determined for each leaf. Leaves were subsequently air-dried for 5–10 min. The leaf stalk of each mulberry leaf was then inserted in a small centrifuge tube filled with agar (1% w/v in water), to reduce the speed of desiccation of the leaf, before being placed in the exposure cages.

2.4.2. Administration of doses

Two or three treated mulberry leaves were placed flat in each exposure cage, with the treated side up, covering the entire bottom surface. Silkworm larvae were then carefully placed on top of the leaves, using a soft brush. Treated mulberry leaves were only introduced in the cages at the start of the test. A total leaf weight of 3.5–5 g was provided per 30 larvae, as this had been shown to be sufficient food for 96 h. In general, two replicate test groups, each of 30 silkworm larvae, were exposed per dose. In a few cases, three test groups with 20 silkworm larvae each were used.

Five or six doses were prepared for each toxicity test, in an approximately geometric series with a factor of about 1.5, and never exceeding 2.25. In addition, a water-control and, where required, a solvent-control were included in the test. Range-finding tests were used to establish appropriate dose ranges, which are listed for each test in Tables 1–3.

2.4.3. Exposure conditions

Two types of exposure cages were used for the tests: open culture dishes and closed ventilated exposure cages. For the majority of the test, glass culture dishes with a diameter of 20 cm and a height of 3 cm were used as exposure cages. Culture dishes were left open during the test to avoid possible build-up of pesticide vapour inside the dish. Because all culture dishes were placed