Evaluation of Bioassay Methods to Determine Mortality of Cabbage Loopers, *Trichoplusia ni* (Noctuidae), from Two Botanical Insecticides

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ABSTRACT Three types of bioassay for testing botanical insecticides against *Trichoplusia ni* (Hubner) (Noctuidae) larvae were compared for efficacy and sensitivity. A neem-based insecticide, Celaflor® (1% azadirachtin), and a pyrethrum-based insecticide, Exciter® (6% pyrethrins) were applied by dipping or painting cabbage leaf disks, or by spraying potted cabbage plants from which leaves were later detached and leaf disks taken. Significant differences in larval mortality were found between bioassays. Three days after treatment LC_{50} values for Exciter® via dipping and painting were 282 and 415 ppm, respectively, but only 1250 ppm following spraying. Six days after treatment LC_{50} values for Celaflor® via dipping and painting were 441 and 490 ppm, respectively; and with spraying, 863 ppm. Mortality was concentration-dependent for both insecticides. Exciter® caused more rapid mortality than Celaflor®, consistent with the modes-of-action of the respective active ingredients. Assays differed widely in variability, dipping being the least variable and spraying the most, presumably due to uneven foliar coverage from spraying. Our results indicate that dipping leaf discs remains an easy, rapid and effective means for screening botanical insecticides against chewing pest insects.

KEY WORDS : Bioassay, neem, azadirachtin, pyrethrum, toxicity, *Trichoplusia ni*

INTRODUCTION

The repeated use of synthetic chemical insecticides as crop protectants has posed serious hazards for humans and the environment, caused deleterious effects to natural enemies and led to resistance in pests to insecticides (Perry *et al.*, 1998). As synthetic insecticides cannot be used in organic crop production systems, organic growers need alternative tools for controlling devastating pests in crops (Yussefi and Willer, 2003). Therefore various attempts have been made to find new insecticides derived from plants, with preferably reduced human toxicity and less persistence in the environment (Ahmed *et al.*, 1984; Isman *et al.*, 2002). Botanical insecticides are one group of alternative pest-control agents that are generally more biodegradable, and less toxic to humans and natural enemies (Arnason *et al.*, 1989; Isman, 1994).

At present five botanical insecticides (neem, pyrethrum, rotenone, ryania and sabadilla) are approved for use in the United States (Isman, 1994; Jefferies *et al.*, 1992; Zang *et al.*, 1997). Widespread interest in the botanical insecticides has produced a demand for a standard bioassay that can be used to screen natural products found in plants (Alkofahi *et al.*, 1989). The appropriate bioassay technique is a key element in predicting the potential impact of active plant substances on herbivorous insects (Berlinger *et al.*, 1996).

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Many bioassay methods have been developed to screen useful natural chemicals from plants (Kubo, 1991; Escoubas, *et al.*, 1992; Miguel *et al.*, 1994; Dyer *et al.*, 2003). Artificial diets or treated leaf discs incorporating dry leaf powder, leaf extracts or secondary metabolites are widely used for screening plant extracts against chewing insects such as lepidopteran larvae and sucking insects such as aphids (Lewis and van Emden, 1986; Wolfson and Murdock, 1987; Xie *et al.*, 1996). These bioassays can provide information on feeding deterrent effects, growth-inhibition, oviposition perturbation, and toxicity. However, while such bioassays have been used to expedite screening of plant extracts, they vary widely in terms of predicting efficacy under field conditions (Stark *et al.*, 1995; Berlinger *et al.*, 1996; Banken and Stark, 1998). It has long been known that LC_{50} values obtained in feeding bioassays depend upon the characteristics of the bioassay used (Kubo, 1991). Because individual responses in bioassay vary widely, these bioassays require large numbers of insects to be sufficiently replicated, which can be laborious to conduct and evaluate (Robertson and Preisler, 1992). The objective of the present study was to compare and evaluate three different methods of applying insecticides to leaf discs for testing botanical insecticides in plants.

MATERIALS AND METHODS

Chemicals

Insecticides used were commercial formulations of the biopesticides neem and pyrethrum. The neem-based insecticide, Celaflor®, was obtained from Trifolio-M Gmbh, Germany. According to the label, it contains a seed extract from neem (*Azadirachta indica)* with 1 per cent azadirachtin as the active ingredient and its major modes-of-action include feeding deterrence and growth regulatory activity. The pyrethrum-based insecticide, Exciter® (Prentiss, USA), is a commercial formulation of an extract from pyrethrum flowers (*Tanacetum cinerariaefolium*) and contains 6 per cent pyrethrins. Pyrethrins are axonic neurotoxins known for their rapid knockdown properties, especially in flying insects.

Insects

Cabbage loopers, *Trichoplusia ni* : (Hübner) were obtained from a laboratory culture maintained for > 50 generations at 22 ±1°C and a photoperiod of 16L:8D, and reared on artificial diet (velvetbean caterpillar diet, BioServ Inc., Frenchtown, NJ, USA).

Bioassay Experiments

Bioassays were conducted between December 2003 and July 2004, at the University of British Columbia, Vancouver, Canada. All bioassays were conducted in the early afternoon (1300-1600h), to reduce variation in larval feeding on leaf disks, which could obscure methodological differences (Hardee *et al.*, 1966). Preliminary studies were undertaken to determine the appropriate dosage range for test concentrations. Distilled water was used to dilute the stock solutions and acted as a control in all experiments. Test concentrations ranged from 0 to 3000 ppm (300, 500, 1000, 2000, 3000) with 0.125 per cent Triton X-100 as an emulsifier/surfactant. This concentration range was adjusted based on its LC_{50} value in preliminary tests.

Dipping. Cabbage plants *(Brassica oleracea* cultivar *'Stonehead'*) were started in plug trays and transplanted into 15cm plastic pots at 25 days. Cabbage leaves, 4 weeks old, were washed and leaf disks (1.5 cm dia) were cut from fully expanded and healthy leaves. Leaf disks were dipped into each solution for 5 seconds and allowed to air dry for 10 minutes. One leaf disk was placed in the center of a plastic Petri dish (0.7 x 5 cm dia) containing a filter paper disk (Whatman No.1) previously moistened with 0.2ml distilled water to maintain the turgidity of the leaf disks. Ten 1st instar larvae (4 days old) were carefully introduced with a fine brush onto the leaf disk and the dish was covered with a tight-fitting lid. The Petri dishes were placed in a covered 26 x 37 x 5.5 cm clear plastic box lined with moistened paper towels and placed in a growth chamber maintained at 25± 1°C with constant fluorescent light. Treatments (insecticides and concentrations) were replicated 6 times (each dish was a replicate).

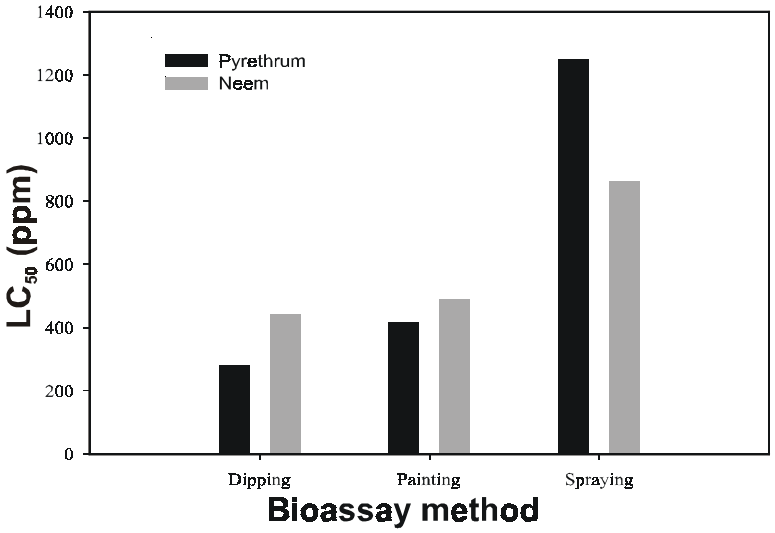

Fig. 1. LC_{50} for *Trichoplusia ni* larvae fed on leaf discs treated with various concentrations of the pyrethrum-based insecticide, Exciter®, and the neem-based insecticide, Celaflor® in different bioassays.

One day later, the treated leaf disks were replaced with aliquots of artificial diet (10-15 mg) and the tests continued for 6 days.

Painting. Cabbage leaf disks (1.5 cm dia) were painted with 17.5 µl of each treatment using a 5-20 µl pipettor (Gilson). The treated leaf disks were then dried for 10 minutes and placed in Petri dishes (0.7 x 5 cm diameter) containing a filter paper disk moistened with 0.5ml distlled water. Ten 1st instar larvae (4 days old) were placed in each Petri dish as described above. Treatments (insecticides and concentrations) were replicated 6 times (each dish was a replicate). One day later, the treated leaf disks were replaced with artificial diet as described above.

Spraying. Cabbage plants (4 weeks old) were sprayed with the treatments using a 500 ml hand-held sprayer to the point of run off. Control plants were sprayed with an aqueous solution of 0.125 per cent Triton X-100. The treated leaves were then dried for 10 minutes. Leaf disks (1.5 cm dia) were cut from the treated leaves and placed in Petri dishes (1 disk per dish). Ten first instar larvae (4 days old) were placed in each dish and the dishes placed in a plastic box as described above . Treatments (insecticides and concentrations) were replicated 6 times (each dish was a replicate).

Statistical Analysis

Mortality was recorded 1, 2, and 3 days after treatments of Exciter® and scored as a percentage. Unlike Exciter®, observations of treatments of Celaflor® were made at 2, 4, and 6 days after, because of the slow-acting toxicity of neem. LC_{50} values were calculated using probit analysis (Finney, 1971). Mortality was compared to that of the distilled water + emulsifier control. LC_{50} values for each type of bioassay were considered significantly different where there is no overlap of their respective 95 per cent confidence limits.

RESULTS

Based on non-overlap of 95 per cent confidence limits, there are significant differences among the bioassays with Exciter^R for method of application (Fig. 1). Overall LC_{50} values were significantly lower when leaf disks were dipped or painted than when sprayed. LC_{50} values at 3 days for Exciter^R were 282 (95% CL: 127-420) and 415 (95% CL: 159-679) ppm for dipped and painted discs, respectively, whereas the LC_{50} for sprayed discs was 1250 (95% CL: 890-1524) ppm. For Celaflor®, LC_{50} values for dipped and painted discs were 441 (137-771) and 490 (140-881) ppm, respectively, and with sprayed discs, 863 (310-1572) ppm. Differences between

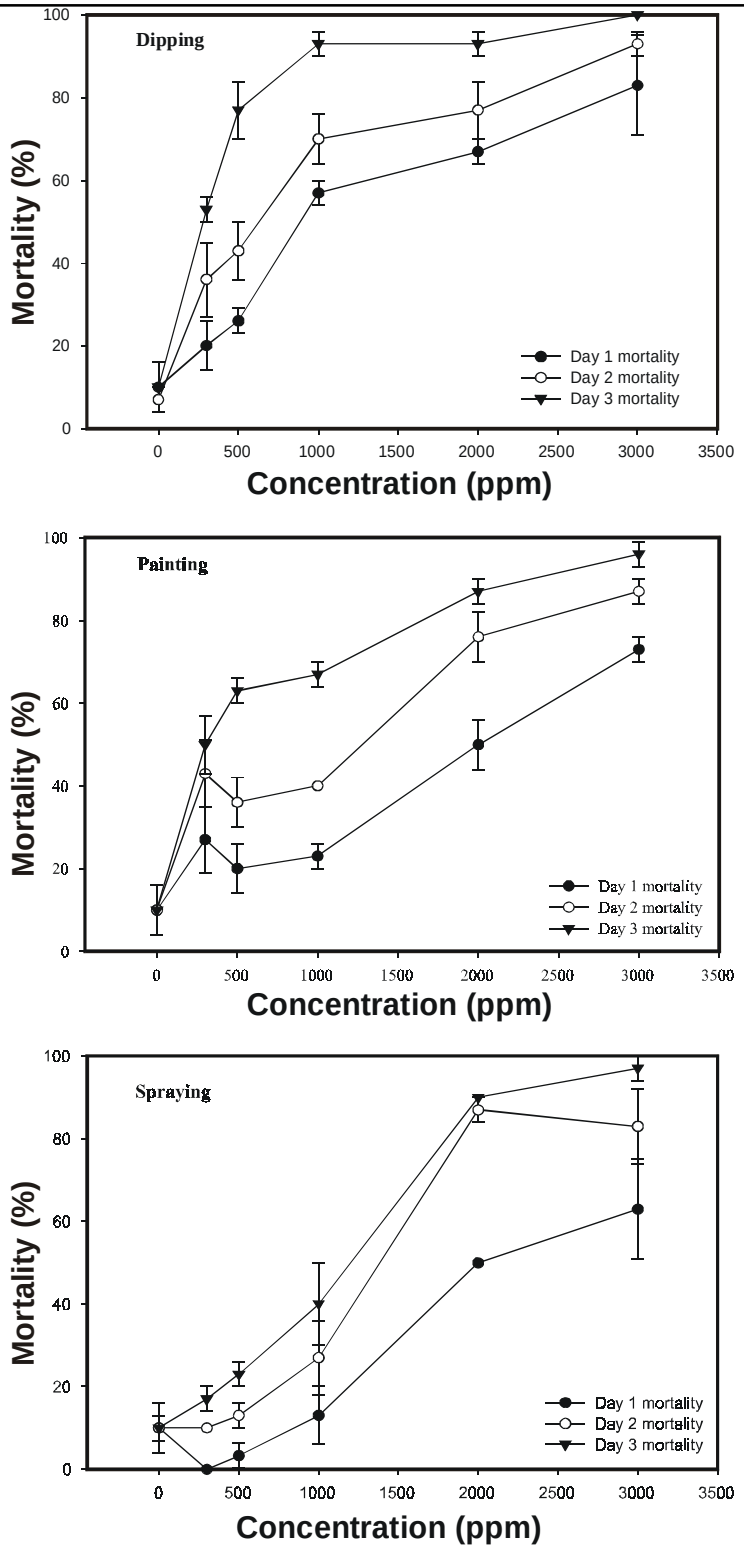

Fig. 2. Mortality of *Trichoplusia ni* larvae fed on leaf discs treated with various concentrations of the pyrethrum-based insecticide, Exciter® in different bioassays. Values are means ± SE; n=6. Some error bars are small and covered by symbols.

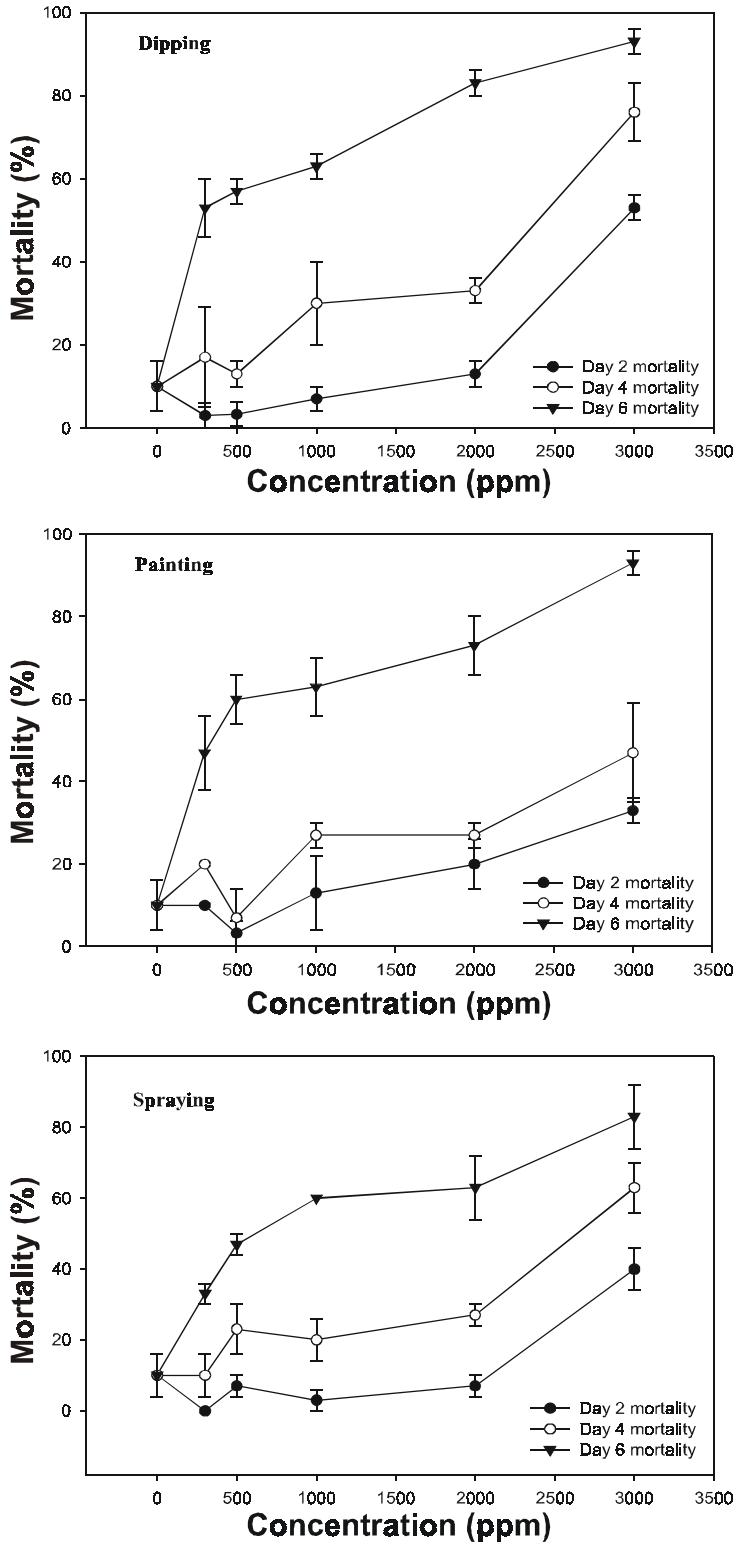

Fig. 3. Mortality of *Trichoplusia ni* larvae fed on leaf disks treated with various concentrations of the neem-based insecticide, Celaflor® in different bioassays. Values are means ± SE; n=6. Some error bars are small and covered by symbols.

methods with Celaflor® were obscured by the greater variation in bioassays (wider confidence intervals) using this product.

Mortality from Exciter® differed with concentrations, application method and observation time (Fig. 2). Mortality was greater on dipped and painted discs than on sprayed discs over the range of concentrations from 500 ppm to 1000 ppm, but at the higher concentrations tested, (2000 - 3000 ppm), there were no differences among methods. However, significant differences in mortality between methods were evident at lower concentrations (< 2000 ppm) at 3 days. Mortality increased with time of observation for all 3 methods. At 3000 ppm, mortality approached 100 per cent for all methods, and control mortality never exceeded 10 per cent.

The toxic action of Celaflor® was slow, compared to Exciter® and mortality also varied among bioassays. All three methods led to concentration-dependant mortality, although mortality was low in all treatments until the 6th day after treatment. Only at 3000 ppm did mortality clearly differ at 6 days, where dipping > painting > spraying. After 6 days at 2000 ppm treatment, mortality was 81 per cent for dipping, 76 per cent for painting and 62 per cent for spraying (Fig. 3).

DISCUSSION

Our results indicate that significant variation exists among bioassay methods. Differences in the rate and extent of larval mortality among bioassays may indicate differences in the bioavailability of neem and pyrethrum to the larvae. The increased activity of both compounds via dipping and painting of leaf disks suggests that larvae were ingesting more active ingredient by this method than when the compounds were applied by spraying. The amount of pesticide applied by painting leaf disks was that which covered the surface, but a greater quantity may have been obtained by dipping, because the solution could also infuse the leaf disks. Spraying provides the least amount to the leaf disks. This observation is important because it indicates that the measured mortality of natural insecticides to insects in the laboratory could be a function of the bioassay method (George *et al.*, 1996; Jonathon *et al.*, 1996; Pilar *et al.*, 2002; Raudonis *et al.*, 2004).

Generally both neem and pyrethrum cause mortality of many arthropod pests by toxic and some antifeedent (starvation) effects. The results of our study show that Exciter® caused more rapid mortality than Celaflor® in all bioassays. Several authors (Banerjee *et al.*, 1977; Hasis *et al.*, 2003; Simmonds *et al.*, 2002) have found that pyrethrum products are strong contact insecticides; i.e. they are toxic immediately upon application. Pyrethrum acts on the nervous system in insects, causing such symptoms as excitation leading to paralysis and knockdown, and eventually, death of the insects (Banerjee *et al.*, 1977). Our results show that toxicity existed for 3 days and then toxic effects disappeared. The bioassay for residual effects of Exciter® to insects needs further study.

The results of our study confirm the slow action of neem as efficacy was only apparent 4 days after treatment. Mulla and Su (1999) showed that neem has minimal contact toxicity and requires ingestion to be fully effective. In contrast, Stark and Rangus (1994) found that a neem insecticide (Margosan-O) was very toxic when applied topically to pea aphids. It is well known that the physiological action of neem (azadirachtin) primarily results from interference with the synthesis and release of ecdysteroids, causing disruption of moulting in hemi-and holometabolous insects (Bruce *et al.*, 2004).

Variations in bioassay mortality can arise from many causes, including insect age, moisture level, plant age and temperature. Our results complement previous studies (Schalk and Stoner, 1976; Berlinger *et al.*, 1996; George *et al.*, 1996). Treatments and observations require a high degree of accuracy, and proficiency is needed to distinguish between living and moribund insects. The most consistent results are obtained when one person performs all bioassays. The most important means of reducing experimental variability is to increase the number of experimental units (replicates and treatments). Painting and dipping leaf disks offer a level of speed, precision and reproducibility that spraying may not always be able to match. On the other

hand, spraying intact plants with subsequent removal of leaf disks better represents the real world application of insecticide to plants. We therefore recommend the use of dipped leaf disks for laboratory bioassays aimed at quantifying toxicity of experimental materials, with the best leads then subjected to greenhouse trials wherein those materials are sprayed on intact plants.

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