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Original Article

BIOASSAY FOR DETECTION OF DICHLORVOS INSECTICIDE IN AIR IN ALFALFA LEAFCUTTING BEE (*MEGACHILE ROTUNDATA* F.) INCUBATORS

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Abstract

Dichlorvos is an insecticide used in slow-release plastic strips for controlling chalcid wasp parasites, such as *Pteromalus venustus* Walker, in incubators used to raise alfalfa leafcutting bees (*Megachile rotundata* F.). Beekeepers need a practical method to detect dichlorvos in air and verify that it has dissipated to levels acceptable for worker re-entry and for the bees to emerge. We evaluated three methods for analysis of the dichlorvos concentration in air. Vapor sampling tubes using a manually operated pump or diffusion collection had insufficient sensitivity in the concentration range of interest. Air samples collected using battery powered pumps were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS), which was accurate and sensitive, but too costly and slow for practical use. Finally, a convenient bioassay for detecting dichlorvos in air was developed using leafcutting bees and verified by comparison with the results obtained by LC/MS/MS for a series of dose levels. The bioassay is simple enough to be done by the beekeeper on-site, is inexpensive, and gives results within 1 h. The LC_{50} for dichlorvos vapor in air after 1 h of exposure was 273.2 µg/m³ by the probit regression method or 277.3 µg/m³ by the logit regression method.

Keywords: bioassay, dichlorvos, LC_{50}, *Megachile rotundata, Pteromalus venustus*.

INTRODUCTION

Alfalfa leafcutting bees, *Megachile rotundata* F., are the most important pollinators of alfalfa grown for seed production in Canada and are increasingly used for pollination of other crops, such as blueberries (Argall et al., 1996) and canola. In commercial operations that raise these bees for sale, large numbers of cocoons are removed from circular holes in nesting blocks and brought to maturity in incubators. The organophosphate insecticide, dichlorvos, is used to control parasitic wasps, such as *Pteromalus venustus* Walker (Grissell and Schauff, 1997; Wu and Smart, 2012), that emerge from alfalfa leafcutting bee cocoons in incubators about 3 - 8 days prior to the bees (Hill et al., 1984; Whitfield and Richards, 1987). Alternative control methods, such as black light/water traps do not work well enough alone because the wasps can re-parasitize healthy cocoons before being attracted to the light and caught in the trap. These wasps can cause enough damage to make the alfalfa leafcutting bee production operation uneconomical (Goerzen, 2010). Dichlorvos is registered as an insecticide for controlling flying insects in confined (indoor) spaces. It is dispensed in the form of a slow-release vapor from a plastic polymer strip and it rapidly kills flying insects (EPA, 2000). A commercially available dichlorvos slow-release insecticide strip may be used in incubators containing *M. rotundata* cocoons from day 7 - 13 of incubation (Hill et al., 1984; Goerzen, 2010). The dichlorvos concentration in the air at the levels used for wasp control has a minimal effect on the survival or emergence of *M. rotundata,* provided that the dichlorvos residues are thoroughly removed prior to the emergence of the adult bees (Hill et al., 1984). It is important to know if dichlorvos is present uniformly in the incubator at effective levels during treatment. It is also necessary to verify that the concentration has dissipated enough after treatment so that beekeepers can re-enter to handle the cocoons, the residual dichlorvos will not be harmful to emerging bees, and dichlorvos will not increase again in air after ventilation due to desorption of residues from surfaces (Goerzen, 2010). The cost and time delay for traditional sampling and analysis

has been a barrier to obtaining this information. In this work, we evaluated three methods and developed a reliable, practical, on-site bioassay to determine the dichlorvos concentration within 1 h. The method was calibrated by placing duplicate sets of 10 newly emerged adult alfalfa leafcutting bees in test chambers at a series of concentrations and using instruments to collect air samples to measure the actual concentration of dichlorvos averaged over 1 h. We also determined the LC_{50} of dichlorvos vapor in air for leafcutting bees.

MATERIAL AND METHODS

The mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply an affiliation, recommendation, or endorsement by the authors or their associations.

Alfalfa leafcutting bees

For this study, alfalfa leafcutting bees (approximately 0.5 kg) were donated by a commercial bee supplier, Andrew Lindsay-Hawkins of the Saskatchewan Alfalfa Seed Producers Development Commission (SASPDC) in Saskatoon, Saskatchewan. They were shipped by courier in the form of cocoons. After receipt, the cocoons were kept in a 2 L glass bottle with a wire screen in place of the cap. When the adult bees started to emerge, the cocoons were spread in a clear plastic bin with the cover loosely set in place. The cover was lifted briefly to insert a vacuum collection device, which is described below, to collect the bees.

Bee cages and handling of bees

The containers used to hold the test bees during handling, exposure to the test conditions, and the bioassay procedure were 650 mL plastic drinking water bottles cut in half. The open end of the top half of the bottle was covered with a disc of metal window screen that was held in place with a narrow strip of adhesive "duct" tape around the perimeter. The screw cap of the bottle was used to keep the bees in after they were collected in the container. No attempt was made to separate male and female bees.

A battery powered, hand-held vacuum cleaner was used to collect the bees directly into the bee containers. To allow easy attachment of the test container to the vacuum cleaner, an adapter was made from a wide-mouthed plastic bottle with a diameter slightly larger than the diameter of the bee containers. The bottom of this bottle was cut out, and the bottle was attached to the hand-held vacuum cleaner securely using latex caulking compound, so that the air drawn into the apparatus came through the bottle. The screened end of the bee container was inserted into the end of the wide-mouthed bottle on the vacuum cleaner. The screw cap was removed from the bee container and bees were collected by gently drawing air and bees in through the neck of the test chamber. When 10 bees had been collected, the cap was replaced and the bee container was labeled, set aside, and replaced with a new container. Using this method, 20 sets of 10 bees could be collected and labeled within 30 min. These bee containers were used only once to avoid cross contamination. In some cases, when the bees were docile enough, they were collected by allowing them to crawl onto a wooden pencil and flicking them into the bee container without using the vacuum. Preliminary runs, prior to the study, showed that the use of low temperatures (-10° to -20°C) to sedate the leafcutting bees and make it easier to handle them, resulted in higher than acceptable mortality in untreated bees. Therefore, we did not use the low temperature sedation method.

The bees are best used immediately, but can be held overnight before use. Although it is easier to count out cocoons for use in an experiment than to handle emerged bees, the use of newly emerged adults removes any uncertainty about how many of the bees in a set will emerge from their cocoons in time for the test and how old the bees are if they were previously emerged.

Dichlorvos

Ortho Home Defense Max No-Pest Insecticide strips (Scott's Canada, Mississauga, ON, Canada) were obtained from a local hardware store. The initial weight of the strip without packaging was 59.5 g. The strip contained 19.2% dichlorvos by weight and 0.8% related active ingredients.

Test chambers

Six test chambers, each with a volume of approximately 1 m^3, were used to measure the susceptibility of the bees to a series of airborne concentrations of dichlorvos. This convenient, but minimal size allowed the dose to be applied as cut pieces of dichlorvos strip. These strips delivered the dose in a manner comparable to what happens when the product is used in leafcutting bee incubators. The wooden frames for the chambers had an outside dimension of 1 x 1 x 1 m, and were constructed

from standard spruce "2 in x 2 in" dimensional lumber (5.08 x 5.08 cm) held together by metal screws. The frames were wrapped neatly with 6 mil polyethylene film held in place with staples. An access flap ~ 30 x 30 cm was cut in one corner and closed with adhesive tape. The test chambers were set up indoors in a warm, well-ventilated area at an ambient temperature of ~ 30°C, which is the temperature used for incubation of leaf cutting bees (Goerzen, 2010).

Air sampling

Air samples were collected from each test chamber when the bees were being exposed. The air sampler pumps were battery powered Gilian Gil-air pumps (Sensidyne Gilian Corp, Clearwater, FL, USA) with electronic flow control to maintain a constant air flow over time. The pumps were fitted with industrial hygiene air sampler tubes containing OVS Tenax adsorbent (Cat. No. 220-56, SKC Inc., Eighty Four, PA, USA). The protective plastic caps that the tubes are supplied with were carefully removed and set aside for re-use. Each tube was connected to an air pump inlet using a short length of flexible plastic tubing. The air flow was set to 2.0 L/min measured at the inlet of the sampler tubes using a Gilian Gilibrator electronic bubble tube calibrator (Sensidyne Gilian Corp., Clearwater, FL, USA). Air flow calibrations were done in triplicate before each run and rechecked in triplicate at the end of the test run. The flows at the end of the runs were within 5% of the initial values and the overall average flow rate of 2.0 L/min was used for calculations.

After calibration, the air pump was turned off, but the sample tubes were left attached to the pump and a protective cap was put on the inlet end of the tube. The tubes were used within 1 h after calibration. They were uncapped and the pumps were turned on immediately before they were placed in the test chamber for 1 h. When the pump was in the test chamber and running, the exhaust from the pump was released back into the chamber. The total sample of approximately 120 L represents only 12% of the volume of the test chamber, so it was expected that the release rate of the dichlorvos would easily compensate for this over the time of the run, and maintain the level of dichlorvos in the air. Nonetheless, any reduction in the concentration would be reflected in the measured average residue value, which was used in the calculation of the LC_{50}. After the sampling time, the actual run time was recorded from the air pump timer. The OVS tubes were detached from the pumps and recapped at both ends. They were packed in an insulated cooler with ice for shipment to the analytical laboratory. Sealed, unused tubes were also sent to the lab for calibration and quality control.

Dose administration

A series of five target dose levels was chosen based on the product label application rate of one strip per 40 m^3 or 1.49 g of plastic strip/m^3. Based on an initial range-finding test with caged bees in the test chambers, the target dose levels were set at 1.49, 0.74, 0.37, 0.19, and 0.10 g of plastic strip/m^3 or per test chamber. These levels correspond to 100, 50, 25, 12.5, and 6.75 % of the recommended application rate, rounded to 2 decimals. Three pieces of dichlorvos strip, of similar size and shape, were cut for each target dose level and weighed on an electronic balance. One piece was used for each dose level in each of the three runs. The above-mentioned target values helped to keep the actual dose levels within the desired range. The measured concentrations for each run and the average measured concentration over the three runs are presented in Table 1. A control test chamber, with no dichlorvos in it, was also set up for each run.

The test pieces were placed on a 5 x 5 cm metal screen with the corners bent down to form legs that would hold the piece of dichlorvos strip ~ 1 cm above the polyethylene bottom surface of the chamber. For all handling, the control sets, which contained no dichlorvos, were sampled first, followed by increasingly higher concentrations of dichlorvos. After inserting the test piece, the access flap was taped closed and the dichlorvos vapor in the chamber was allowed to equilibrate for 1 h. Then, two arbitrarily chosen sets of 10 bees were placed in the chamber along with a calibrated air pump fitted with an uncapped OVS air sample tube, and the pump was turned on. The access flap was promptly taped shut. After 1 h, the bee containers were collected, and the air pumps were removed and turned off. The total number of bees and the number of live and dead bees in each test container were recorded (Tab. 1). The air temperature and time interval were also recorded.

Chemical analysis

The OVS tube samples were sent to Australian Laboratory Services Inc. in Edmonton, Alberta, an independent commercial laboratory, to determine the quantity of dichlorvos collected. The samples were extracted with 10 mL of 50% acetone/methanol following the manufacturer's recommendations. The

analytical method was adapted from ASTM D4861 by using liquid chromatography/tandem mass spectrometry (LC/MS/MS), with a Sciex multistage linear ion trap quadrupole mass spectrometric detector, model 1022643D (AB Sciex, Concord, ON, Canada,) LC column: Atlantis T3 C - 18, 5 µm, 3.0 x 100 mm (Waters Corp., Milford, MA, USA); and mobile phases: A = 0.2% acetic acid in water, B = methanol. The limit of quantitation (LOQ) of the method was 0.03 µg/sample. The average recovery was 95.8 ± 11% from quality control samples run at 1, 5, and 10 times the LOQ. The analytical results of the samples collected from the experimental test chambers are listed in Table 1.

Evaluation of alternative detection methods

In addition to the bioassay method, several additional dichlorvos detection methods were evaluated to see if they would meet the needs of the bee producers. The most accurate method involved the use of calibrated air pumps to collect a known volume of air through a sample collection tube and quantification of dichlorvos in the sample using LC/MS/MS as described above. However, there are also two commercially available indicator tubes that are used to verify that dichlorvos concentrations are low enough to be acceptable for workers to enter an area.

Gastec 132D Diffusion Vapor Sampler Tubes (SKC Inc., PA, USA)

These tubes are designed to measure vapor concentrations of dichlorvos in air down to the Threshold Limit Value (TLV). The term, TLV, is reserved by the American Conference of Governmental Industrial Hygienists (ACGIH) to indicate an 8 h time-weighted average concentration that a typical worker may be exposed to for a working lifetime without adverse effects. The TLV for dichlorvos is 1 mg/m^3 or 1000 µg/m^3 (EPA, 2000). During use, the dichlorvos is allowed to diffuse into the tubes and react to form a yellow indicator color. The length of the band of yellow color that develops in the tube is proportional to the concentration of dichlorvos in the air being sampled. In this study, air samples were collected in the test chambers by breaking off the seal at the end of the glass tube and placing a tube in the chamber along with the calibrated air pump samplers. At the measured concentrations listed in Table 1, no yellow color developed within 1 h. Even when the tubes were left in the test chambers with the piece of insecticide strip for 8 h, no yellow color developed. The color did develop within 1 h when a tube was placed in a covered 1 L glass jar with a piece of insecticide strip.

Gastec 132LL Sample Tubes (SKC Inc., PA, USA)

These are similar to the 132D tubes, but are designed for use with a manually operated vacuum pump to draw a measured volume of air into the sample tube. These 132LL sample tubes were also used to sample the air in the test chambers during the study. No color developed in these tubes at any of the concentrations in Table 1.

Statistical analysis

For the statistical analysis, the bee mortality per concentration results were converted to survival per concentration. Data analyses to determine LC_{50} values were conducted according to the Statistical Guidance recommended by Environment Canada (EC, 2007). According to research by Hubert, for data sets with fewer than 30 organisms per treatment, χ^2 is not "statistically justified" (Hubert, 1984). Therefore, models for quantal endpoints were chosen based on the approximate χ^2 and closeness to LC_{50} estimation via hand graphed regression. The R program (R Development Core Team, 2010) results using probit and logit regression methods are presented in Table 2.

RESULTS

The vacuum collection method or the use of a probe, such as wooden pencil, worked well to transfer bees into the test containers. With these methods, mortality among untreated bees was <10% (Tab. 1). Using the highest dose as 100 % of the label application rate, the average measured concentrations (Tab. 1) in the next two lower dose levels were 50.2% and 28.5% of the label rate. These values are close to the target values of 50% and 25% of the label rate, respectively. The remaining two levels were 19.8% and 21.5% of the label rate, instead of the expected 12.5% and 6.25%, respectively. This implies that the dichlorvos was released faster than expected from the smaller pieces of plastic used for the lower dose levels, possibly due to a higher surface area to volume ratio. In any case, only the measured concentrations were used to calculate the LC_{50}.

The average temperatures inside the test chambers were 26°C, 30°C, and 30°C for the three runs, respectively. These temperatures were close to the temperature of 30°C used for commercial leafcutter bee incubation (Goerzen, 2010). The results presented in Table 1 do not show a significant trend with temperature. The calculated LC_{50} (1 h) for the leafcutting bees was 273.2 µg/m^3 by

Table 1.

Mortality of Leafcutting Bees per Concentration of Dichlorvos

Dose	Run #1		Run #2		Run #3		Average[c]	
Level	C[a]	M[b]	C	M	C	M	C (µg/m³)	M (%)
1 Control	0.082	0,0	0.078	0,0	0.35	1,0	1.4	1.7
2	43[d]	0,0	31	0,1	23	5,4	224	16.7
3	19	1,1	26	7,9	30	5,5	206	46.7
4	36	3,0	36	3,8	33	10,7	293	51.7
5	69	9,4	40	9,9	79	9,10	523	83.3
6	260	10,9	72	8,10	44	10,10	1045	95.0

a) C = Concentration - µg per sample. Level 6 is equivalent to the label use rate.
b) M = Mortality number of dead bees out of 10 in the two replicates separated by a comma.
c) The average concentrations for the 120 L air samples were converted to µg/m³ by multiplying by 8.33.
d) The 43 µg per sample run with no mortality of bees in either replicate is likely an outlier.

Table 2.

Statistical Analysis

Degrees of Freedom	Intercept	Slope	LC_{50} (µg/sample)	LC_{50} (µg/m³)	Standard Error	Residual Deviance
Logit method						
30	1.59393	-0.04858	32.8	273.2	2.64	150
Probit method						
30	0.64581	-0.01939	33.3	277.4	3.87	166.3

The outlier from Run # 1 was excluded in these results.
The calculations were run using the "R" software for statistics (R Development CoreTeam, 2010).

the probit regression method or 277.3 µg/m³ by the logit regression method (Tab. 2). There is no theoretical basis for selecting which method to use (EC, 2007); therefore, the results from both methods are presented to show that they agree quite closely. It is interesting to note that these values are less than 1/3 of the TLV (1000 µg/m³) for worker re-entry (EPA, 2000). For comparison, the range of concentration typically used for control of flying insects is 180 µg/m³ (0.02 ppm) (Hayes, 1982).

The appearance of low levels of dichlorvos residues in the control samples resulted from cross contamination during handling of the samples, and was attributed to the movement of dichlorvos vapors. The levels of cross contamination did not exceed 2% of the lowest measured dose level. The control mortality did not exceed 10%, so this level of cross contamination was considered insignificant. The levels of dichlorvos measured in the test chambers and the mortality observed at dose levels near the LC_{50} were highly variable. The variability of the dose levels was expected for the dosing procedure but it was considered essential to apply the dose in a manner that was representative of the actual use of the product. As noted previously, this variability was corrected for since the results depend on the measured concentrations and not the arbitrary target concentrations. The mortality at each dose level was also highly variable, as expected for a biological end-point.

DISCUSSION

In this study, we found that test containers used to collect and hold bees for the measurement of the LC_{50} can also be used in a convenient bioassay for detecting harmful levels of dichlorvos in air. The bee producer can place sets of 10 emerged bees in such a container in the area to be tested for 1 h and then count the surviving or dead bees. Alfalfa leafcutting bees for use in the bioassay are readily available because the producer can warm a small sample of bees to get them to emerge sooner than the remainder of the bees in the incubator. The bioassay does not measure residues on surfaces, but the airborne concentration of dichlorvos reaches equilibrium with the surface residues in an enclosed space rapidly, as determined by the vapor pressure.

At equilibrium, the concentration on the surface is constant and proportional to the concentration in the air. When the level present in the air in an enclosed space is below the lethal level, it is expected that the residues on surfaces inside the space are also very low and will further decrease over time (EPA, 2000). Thus, it is unlikely surface residues would emit enough dichlorvos to kill the emerging bees at a later time. Nonetheless, if the beekeeper is concerned about this, the bioassay can be repeated to verify that residual dichlorvos on surfaces is not being released into the air in harmful amounts.

Range of sensitivity

The bioassay was sensitive down to the level that does not kill leafcutting bees, which is approximately 0.7 µg/m^3 and up to the level that kills 100% of the bees within 1 h, which is approximately 366 µg/m^3. Above this level, the test will not show differences in the concentration of dichlorvos, but will show that the dichlorvos is present at levels effective against flying insects, because as noted above, the typical level used for insect control is 180 µg/m^3. If the number of dead bees in the bioassay sample from the test area exceeds that in the control, then the concentration of dichlorvos is still high enough to be harmful to the emerging bees and further ventilation is required. For beekeeper re-entry, if less than 100% of the bees died, the concentration is below 900 µg/m^3, the safe level recommended for re-entry (EPA, 2000).

Evaluation of Alternative Detection Methods

The LC/MS/MS chromatographic method for airborne concentrations is the most accurate and sensitive method and is free from possible interferences. This method is linear over a wide range of concentrations and is very sensitive compared with other methods, as the LOQ is 0.03 µg per sample. However, this method costs over $200 per sample and it may take more than 30 days to get the results, so it is impractical for the needs of the alfalfa leafcutting bee growers. In addition, there are two types of industrial hygiene sample tubes designed for detecting and measuring dichlorvos in air. We tried these tubes because they would be relatively inexpensive, easy to use, and would give immediate results. Unfortunately, they were not sufficiently sensitive in the range of concentrations that were present in the incubators when using the dichlorvos insecticide strips. None of the tubes gave a visible color change under the conditions of this study.

CONCLUSIONS

A simple bioassay using adult alfalfa leafcutting bees was developed to determine if dichlorvos residues had dissipated to safe levels for emerging bees and human re-entry. The LC_{50} for dichlorvos vapor in air after 1 h of exposure was 273.2 µg/m^3 by the probit regression method or 277.3 µg/m^3 by the logit regression method.

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