Video Article

Bioassays for Monitoring Insecticide Resistance

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Abstract

Pest resistance to pesticides is an increasing problem because pesticides are an integral part of high-yielding production agriculture. When few products are labeled for an individual pest within a particular crop system, chemical control options are limited. Therefore, the same product(s) are used repeatedly and continual selection pressure is placed on the target pest. There are both financial and environmental costs associated with the development of resistant populations. The cost of pesticide resistance has been estimated at approximately $ 1.5 billion annually in the United States. This paper will describe protocols, currently used to monitor arthropod (specifically insects) populations for the development of resistance. The adult vial test is used to measure the toxicity to contact insecticides and a modification of this test is used for plant-systemic insecticides. In these bioassays, insects are exposed to technical grade insecticide and responses (mortality) recorded at a specific post-exposure interval. The mortality data are subjected to Log Dose probit analysis to generate estimates of a lethal concentration that provides mortality to 50% (LC_{50}) of the target populations and a series of confidence limits (CL's) as estimates of data variability. When these data are collected for a range of insecticide-susceptible populations, the LC_{50} can be used as baseline data for future monitoring purposes. After populations have been exposed to products, the results can be compared to a previously determined LC_{50} using the same methodology.

Video Link

The video component of this article can be found at https://www.jove.com/video/2129/

Protocol

1. Introduction

Food production has become of the utmost importance with the realization that by 2025 the world will reach a population of 8.04 billion people[1]. There will be the need to supply a larger amount of food crops than is currently produced. Adequate food availability will not be capable without the use of plant protection products such as pesticides to increase the quantity of crop yields and maintain the quality. Based on the historic and current reliance on pesticides, instances of pesticide resistance will continue to occur and be reported in the scientific literature.

Pest resistance to pesticides is problematic because the products are an integral component of high-yielding and high quality production agriculture. However, overuse and/or misuse of a pesticide can lead to the development of resistance which can be detrimental to crop production. Pests (i.e., insects, weeds, pathogens, etc.) develop resistance by a variety of mechanisms but a major driving factor for the development of resistance is the lack of registered pesticides[1]with independent modes of action available for use. When few products are labeled for an individual pest within a particular crop system, chemical control options are limited. Therefore, the same chemical(s) is used repeatedly and continual selection pressure is placed on the pest. This problem is exacerbated when the pest has multiple generations in a single year and each generation is exposed to the pesticide.

There are both financial and enrivornmental costs associated with resistance[2]. Pest resistance leads to higher rates and more frequent applications of pesticide required to achieve satisfactory control. Yield losses are likely to occur even after increasing pesticide use because of failure to control the target pest. Pimentel[2] estimates these costs of pesticide resistance in the Unites States to be approximately $1.5 billion annually.

Surveys of pesticide susceptibility among pest populations is a proactive approach to detect any shift in insecticide performance and provide an early warning to modify chemical control strategies. By modifying overall IPM strategies, the viability of a given pesticide may be extended, which in turn, is important for agriculture to continue to provide enough food and fiber for the world. This paper will describe a protocol that can be used to monitor insecticide susceptibility and detect the development of insecticide-resistant populations using the adult vial test[3] for contact insecticides and a modification of this test for plant systemic insecticides [4].

2. Methods

2.1 Safety Statement

Using proper safety precautions is important when dealing with insecticides. Consult the Material Safety Data Sheet (MSDS) for appropriate Personal Protective Equipment (PPE) before handling pesticides. Specific laboratory training on handling pesticides in laboratories should be required by personnel before attempting any bioassays.

2.2 Making Stock Solution

Develop a stock solution of a known concentration from a source of insecticide active ingredient (AI). All desired concentrations included as treatments in the bioassay will be made from the original stock solution. For the interest of this paper, 100mls of a 100 µg/ml solution will be developed as a stock solution; however, any concentration can be diluted from this initial concentration. Adjust the amount of technical grade AI to be weighed based on the percent purity.

Amount to be weighed = (volume to make) X ([# µg / ml solution] / [% purity/100])

For example, it would require 10152.284 µg of technical AI to make 100 mls of a 100 µg/ml solution of a technical grade insecticide of 98.5% purity:

(100 ml) X ([100µg/ml] / 0.985) = 10152.284 µg

2.2.1 Adult Vial Test for Contact Insecticides

Partially fill a volumetric flask with acetone and weigh the adjusted amount of insecticide AI. Rinse the weight boat with acetone into the flask to remove all insecticide from the weigh boat. Fill the volumetric flask to the graduation line with acetone. Most solutions held in refrigerated conditions will maintain activity with no appreciable loss of efficacy for about one month.

2.2.2 Modification of Adult Vial Test for Systemic Insecticides

Intoxication of insects with systemic insecticides is distinctly different from that of contact products. Systemic products usually must be ingested by the insect to become active instead of the insect having direct exposure to the product as with contact insecticides. Therefore, the adult vial test was modified in order for systemic insecticides to be tested. The weight of the AI needed for the stock solution is calculated similar to that mentioned in section 2.2. The methodology is similar, except the AI is dissolved in a 10% by weight honey:water solution. This solution should be made within 24 hours of initiation of the bioassay. If the technical grade material is not water soluble, a high concentration of insecticide solution can be made with acetone such that only a small volume of the insecticide:acetone mixture is added to the honey:water solution[4].

2.3 Determining a Range of Concentrations and Developing Concentrations

Determining the appropriate concentrations needed to establish the range of responses for the bioassay can be difficult. Several replications may be needed to define the range of concentrations and this is done by trial and error. Additionally, the range may change over time if the population changes levels of susceptibility. Numerous other factors should be considered in establishing final concentrations and include: family of insect, class of insecticide, size of insect, etc. Previously published results of adult vial tests can assist with the initial selection of insecticide concentrations. Bioassays have been published for: Hemiptera: Aleyrodiidae[5-6], Aphididae[7], Pentatomidae[8-10] and Miridae[11-14]; Thysanaptera: Thripidae[15]; Coleoptera: Brentidae[16], Curculionidae[17], Coccinellidae[18-19] and Cybocephalidae[19]; Diptera: Culicidae[20]; Lepidoptera: Tortricidae[21] and Noctuidae[3], [22-26]; Hymenoptera: Braconidae[7,18], Ichneumonidae[26-27], Aphidiidae[18], Encyrtidae[7], and Aphelinidae[18].

Concentrations are made based on "X" µg/ml solution. However, when vials are prepared for the adult vial test, only 0.5ml will be added to each vial; therefore, the concentration of the vial is half the concentration made. Once concentrations are chosen, the following equation can assist in determining the amount of solution added to make the desired concentration.
(C1)(V1) = (C2)(V2)
Where C1 is the concentration of stock solution; V1 is the volume of stock needed to make the new concentration; C2 is the concentration being prepared; and V2 is the volume of the new concentration. For example, 5mls of a 100µg/ml stock solution is needed to make a 100mls of a 5µg/ml. This 5µg/ml solution would result in vials coated at a concentration of 2.5µg/vial.
5mls = [(5µg/ml) (100mls)] / (100µg/ml)

2.3.1 Adult Vial Test for Contact Insecticides

Partially fill volumetric flaks, and aliquot the calculated amount of stock solution necessary to make the desired concentration. Fill the volumetric flask with acetone to the graduation line and proceed to section 2.4 or seal with Parafilm M (Alcan Inc, Neenah, WI) for storage in the refrigerator for later use. Most solutions are good for 1 month.

2.3.2 Modification of Adult Vial Test for Systemic Insecticides

This process is the same as previously mentioned in 2.3.1 except a 10% by weight honey:water solution is substituted for acetone. Mix enough honey:water solution to make all desired concentrations. These solutions should be used within 24 hours of preparation.

2.4 Preparation of Vials

2.4.1 Adult Vial Test for Contact Insecticides

If concentrations were stored in the refrigerator, allow to warm to ambient temperature. The volume of acetone changes based on temperature which can affect the insecticide concentration. Color code 20 ml glass vials (fitted for screw caps) for concentrations using paint or markers.

Adjust a repeating pipettor to deliver 0.5ml/concentration. Initiate work with acetone control, following with the lowest concentration and continuing with increasing concentrations until all concentrations have been used. Pour approximately the half volume of solution as the actual number of vials to be treated (i.e., 20mls for 40 vials) in a small beaker. When the solution is not in use, cover it to minimize evaporation which can cause changes in concentration. Draw the insecticide solution into the repeating pipettor and dispense 0.5ml solution into individual glass vials.

Immediately after treatment, the glass vials are placed on a commercial hot dog roller. As the rollers turn, glass vials rotate and the acetone evaporates, leaving the inside of the vial coated with the technical grade insecticide. Heat can degrade insecticide; therefore, it is important for the heat to be off. This can be done either by disconnecting the heating element on a model that heats and rolls simultaneously and is controlled by the same switch or by using a model of hot dog roller that has independently functioning switches for the heating element and rollers. Avoid treating more vials than the roller holds; the acetone may evaporate before the glass vials are rotated and they may not receive an even coating on the vial walls.

Allow the vials to rotate until all acetone has evaporated. There maybe a fine layer of acetone-condensation on vial walls; therefore, vials need to be examined individually. Time required to roll vials varies based upon laboratory conditions. Once the vials are dry, cap and store them either in chilled or dark conditions. Insects have been observed resting on lids with conical liners or torn foil liners that hang below the lip of the vial (i.e., avoiding treated surfaces); therefore, it is important to use un-lined lids.

Any remaining solution not used to prepare vials should be disposed properly. The solution remaining in the volumetric flask can be sealed with Parafilm and stored in the refrigerator. Date the vials when they are prepared, because different insecticides have different shelf lives. For example, pyrethroid-coated vials are good for approximately one month; whereas vials coated with a more unstable insecticide, like an organophosphate, have shelf lives of two weeks or less.

2.4.2 Modified Adult Vial Test for Systemic Insecticides

Floral foam is needed to serve as a substrate for delivering the insecticide solution to insects [4]. Cut floral foam (12mm X 12mm) pieces using a cork borer. Place one piece of floral foam into the glass vials previously described. Fill the repeating pipettor adjusted to deliver 0.5ml solution and dispense onto the floral foam. This volume of liquid should saturate the piece of floral foam, but should not exceed the level of the foam in the vial. Again work in sequence from the control (10% by weight honey:water solution only) and lowest to highest concentrations.

2.5. Storing Vials

2.5.1 Adult Vial Test for Contact Insecticides

When storing insecticide coated vials, know the properties of the insecticide. Different insecticides have different storage requirements such as: pyrethroids are light sensitive and can be stored at room temperature but in the dark; however, organophosphate insecticides are temperature sensitive and need to be stored in a freezer. If insecticides are required to be stored in the freezer, they must be warmed to room temperature before exposing the insects.

2.6 Bioassay

Collect the insects to be tested. This process can be done using pheromone-baited traps, sweep nets, or any other means of mass capture. Insects should be held for 8-24h to allow for natural mortality to occur for those injured during the collection process; provide them with food and a moisture source during this period. Test 10-25 insects per concentration (10 minimum); the larger the number of insects tested, the more robust the data set will be. When selecting insects for the bioassay, choose healthy, active individuals and discard lethargic or abnormal individuals. Place insects in the vials such that they are exposed to the full range of the concentrations and not just one concentration at a time. This procedure will prevent placing the most healthy and active individuals in one or a few concentrations. Additionally, if it is not possible to conduct the experiment with a minimum of 10 insects per concentration, expose as many as possible to each concentration. Then collect more insects from the same sample area within a short time (two-three weeks) and repeat the experiment. Some insects can lose susceptibility (build resistance) as the crop season progresses; therefore, the response of individuals collected in the early spring may be different from those collected in the fall[28].

Develop criteria to assess mortality. The most commonly used criteria used to classify insects as moribund or dead are a lack of coordinated movement. These observations may include the inability to right itself if placed on its dorsal surface, unable to sustain coordinated flight of 1m or lack of coordinated movement when gently prodded with a blunt instrument. If the individual can right itself but falls over, there is no coordinated movement; the insect should be considered dead. The insect may experience difficulties righting itself on a slick surface; therefore, it maybe necessary to supply the insect a surface so that it is able to gain the traction necessary to right itself. Record the number of survivors and dead individuals to calculate the survivorship for each concentration.

2.6.1 Adult Vial Test for Contact Insecticides

Place the insect(s) into the vial and secure the cap loosely. The lid needs to prevent the insect from escaping, but be loose enough to allow air flow. For most insect bioassays, only one insect is placed in each vial; however, small insects, e.g. whiteflies or thrips, may be exposed at a rate of as many as 30 individuals per vial[6, 15]. Place the vials upright at room temperature until the insects are assessed for mortality at the endpoint of the bioassay. Exposure times can differ with species and possibly the insecticide[3,5-27]. In the process of establishing initial toxicity levels with

a new insect or chemical, monitor the subjects at multiple scheduled time points after exposure. Examinations can be terminated when mortality at the highest concentration is 100% while still maintaining high survivorship (<10% mortality) in the non-insecticide treated control. If 100% mortality is not achieved at the highest concentration with high survivorship in the control, the bioassay should be repeated using a range of higher concentrations. Ideally, mortality levels should increase as the insecticide concentration increases.

Some insects, when exposed for 24 hours, require a source of moisture (i.e., a small piece of plant material) [12]. When working with a delicate insect, it is beneficial to determine how long the insect can survive in the vial without a source of moisture prior to insecticide exposure in the adult vial test. This information can be determined by placing the insect in a vial with and without a moisture source and monitoring its survival over time before conducting the actual insecticide surveys.

2.6.2 Modified Adult Vial Test for Systemic Insecticides

Prior to the initiation of the bioassay, determine if the insect can feed and survive on floral foam saturated with the honey:water solution in the absence of insecticide. Place insects in vials with floral foam saturated with honey:water only (or honey: water with the greatest volume of acetone used in preparing concentrations with non-water soluble technical grade insecticide) and monitor survivorship for several days. This modification of the adult vial test has only been examined with a Miridae [4] and a Pentatomidae [29]; therefore, determining the duration of the test requires more experimentation than for the contact insecticide bioassay. For example, *Lygus lineolaris* (Palisot de Beauvois) was assessed for mortality at 24 hours after exposure to thiamethoxam, but 72 hours for imidacloprid[4]. Mortality of *Oebalus pugax* F. was assessed at 96 hours when exposed to dinotefuran[29]. Therefore, insect survivorship in control vials must be consistently high for several days before an accurate mortality rating from insecticide intoxication can be made.

Place the insect(s) into the scintillation vial with the saturated floral foam. Instead of sealing the vials with lids, seal vials with a cotton ball. Place the vials upright at room temperature until the insects are assessed for mortality. As previously mentioned, rate mortality at regular intervals. Examination can be terminated when there is 100% mortality at the highest concentration with high survivorship (<10% mortality) in the control. It may be necessary to test additional concentrations.

2.6.3 Data Analysis

Correct for mortality in the control treatment using a formula according to Abbott[30].
Corrected mortality (%) = ((% survival control - % survival treated)/% survival control) x 100
Analyze data using Log Dose probit to determine the lethal concentration required to kill 50% of a population (LC_{50}) and establish 95% confidence intervals (CL). Multiple software programs are available to determine the LC_{50} (SAS: PROC PROBIT [31], Polo-Plus [32]).

3. Notes

1. Work with acetone and insecticides under a negative-flow air hood.
2. When filling volumetric flasks, it is best to have two wash bottles of acetone. One wash bottle that has been adapted for fast delivery of liquids and one for slow delivery. Remove part of the delivery arm to create a larger opening and this bottle can be used for delivery of a large volume quickly. Once the acetone nears to the graduation line, change bottles and use the wash bottle with slower delivery so that you have more control over the amount that is dispensed. If a flask is filled above the graduation line, leave the top off and allow acetone evaporate to the graduation line.
3. Note the amount needed of the stock solution, needed to prepare a concentration, i.e., if you fill the flask ¾ the way but need to add 30mls, you may go over graduation line.

Discussion

The LC_{50} value can be used to establish as baseline susceptibility of a target population(s). The value of this data can be in future monitoring surveys or for the immediate purpose of comparing the current results to that of a previously determined LC_{50} to determine the susceptibility of the target population has shifted. Actual LC_{50} values can be compared among populations by examining the 95% confidence intervals; if the upper and lower limits do not overlap, then it is likely that the population has experienced a significant change in susceptibility and in some situations is an indication of resistance[33]. The LC_{50}s can also be used to examine seasonal changes in insecticide esusceptibilitye[28], or compare responses among species or insecticide AI s. Considerable work has also use these data to compare responses between males and females[10] or between adults and immature stages,[10,34]. Sometimes confidence intervals are wide or not able to be calculated. To gain tighter confidence intervals conduct the bioassay with more insects and/or more concentrations.

Disclosures

No conflicts of interest declared.

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