Minireview Article

Bioassay Techniques in Entomological Research

ABSTRACT

BIOASSAY (Bio - life, Assay - determination) is the measurement of the potency of any stimulus (physical, chemical, biological, and psychological) and the reactions that it produces in living matter. In Entomology, to know about the potency, Specificity of any compounds, standardization of doses, and identify modes of action bioassay is needed. In addition to the basic bioassay methods such as poison food, dry film techniques, etc., depending upon the researcher's necessity, they are modified or newly developed. Diet covering and diet incorporation for lepidopterans and homopterans have been developed as advancements over the poison food assay method, by using insecticide-incorporated artificial diet. WHO cone, Wire ball test, Susceptibility test, CDC bottle assay and MCD assay developed for mosquito resistance monitoring and Larval Packet Technique (LPT) for cattle ticks, Sandwich method, TIBS, and Adult vial test are advancements over dry film technique. In Thrips Insecticide Bioassay System (TIBS), treated vials can be stored for up to 3 weeks at 4˚C for future efficacy assays at the field level. Adult Vial Tests have been developed for pentatomid and mirid adult bug bioassays. Larval Immersion Technique (LIT), Adult Immersion Technique (AIT) for cattle ticks, and MEBS have been developed as advancements over topical bioassay. In Mite Egg Bioassay System (MEBS), entomopathogenic fungi have been tested against *Tetranychus cinnabarinus*. MTT colorimetric assay and Trypan Blue Dye Exclusion assay helps to identify cell viability in insect cell line cultures. *Spodoptera frugiperda* cell line, *Sf* – 21 was tested against a scorpion, Leiurus *quinquestriatus* toxin β– BUTX – LQQ1a and the toxicity was determined through the viability of cell lines. Various basic and advanced bioassay techniques are discussed elaborately in this review.

Keywords: Bioassay – agricultural and household pests

Introduction

The term "BIOASSAY" was defined by Finney in the year of 1952, he stated that it is a measurement of potency of any stimulus (physical, chemical, and biological) and the reaction

which it produced in the living organism. Another definition by Hoskins and Craig in the year of 1962, it is the method to determine the concentration or potency of substances by its effect on living cells or tissues.

Principles of bioassay

- To compare the response of insects from treated samples under the same test condition
- A response may be anything it may be knockdown, mortality or any others
- To compare the test substance with international standard preparation of the same
- Intensity of the stimulus is varied by doses based on that a response will be followed by a subject

Characteristics of an ideal bioassay

- **Specificity:** The technique should produce the specific reaction to the test compound
- **Sensitivity:** It should be sensitive enough to detect even a small trace of a compound
- **Repeatability:** It should be feasible enough, so that it can be done by many times in order to reduce the errors
- **Reproducibility:** It should give the same results when we test under the same concentration irrespective of trails
- **Precision:** The results should be precise enough
- **Stability:** The method should be stable

Importance of bioassay in entomological research

Identification of the mode of action or fixing the doses for the unknown compound can be done through bioassay. Next, to that to determine the resistance of insect pests to a particular compound or to know about the compounds' selectivity towards natural enemies and to find the mode of action of a new compound.

Basic bioassay techniques in entomology

1. **Topical application**

In this method the known amount of insecticide is mixed with the suitable solvent and it is applied to the test insects by using a micro applicator. Advantage of this method is here; high degree of precise result and it requires a smaller number of test insects per replication.

2. **Potter's spray tower**

It was discovered by C. Potter at Rothamsted Experimental Station, Herpenden, Herts, England. It is mainly devised to give a uniform application of spraying or dusting to the target insects. The main advantage of this method is it will give the field exposure condition, enhance the safe handling, and purity of test compounds can be maintained during the operation. Disadvantage is it is more expensive.

3. Dipping method

This can be done in two ways: first, direct immersion of test insects with the help of forceps into the known concentration of insecticidal solution for a fixed period or else treat the material (leaf or any plant parts) with the insecticidal solution and allowed to dry then the test insects are allowed to feed on it.

4. Contact or dry film method

Here, a known quantity of a compound is mixed with the volatile solvents then it is coated inside the vial/petri plate. The solvent is allowed to evaporate completely by rotating the treated material horizontally followed by the test insects being allowed to expose it.

5. Injection method

It is done by injecting the known amount of insecticidal compound directly into the insect body by using the hypodermic needle.

6. Poison food technique

The test compound is directly mixed with the food material and the known number of insects is allowed to feed on it.

7. Fumigation method

This is pertaining to the stored product insect pests. It is carried out in the well airtight container at the temperature of 30˚C and 60% RH.

8. Aqueous solution method

Insecticide is mixed with small amount of water miscible solvent and the water is added to it. Aquatic immature insect or small fish is allowed on it for the desired period of time. Advantage of this method is the whole organism is constantly in contact with the test solution.

9. Photo migration method

It is another aqueous solution method. Here the mosquito larvae are exposed behind a porous barrier which is placed in the test tube containing test compounds. After some time, the

light is turned on and then the barrier is removed. Viable larvae will rapidly migrate away from the light source this is the principle of this method.

Improved or modified bioassays

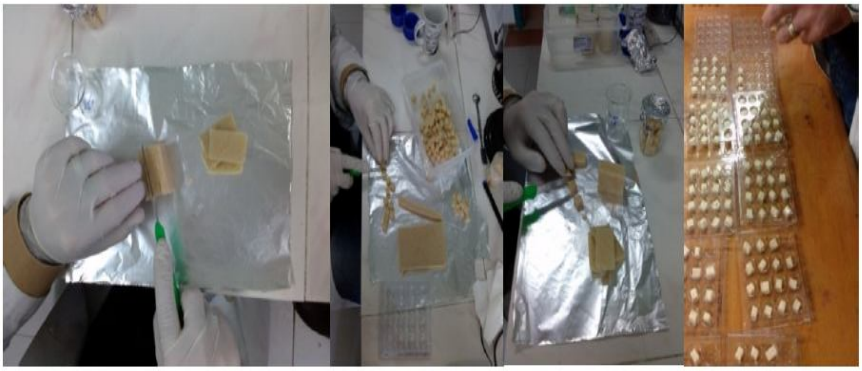

Fig. 1. Diet Covering and Diet Incorporation for European grapevine moth (*Lobesia botrana)* (Denis & Schiffermuller) (Lepidoptera: Torticidae) - (Durmusoglu *et al*. 2015)

Diet covering

In diet covering, 2 ml of insecticide is sprayed on the prepared artificial diet by using the potter's spray tower and then maintained at room temperature for about 30 mins. Then this treated diet was transferred into 16 well polystyrene trays followed by the 3rd instar larvae is released into each cell and maintained at 25 ± 2 ˚C and 60 – 65 % RH for the period of 72 hours and then mortality was noted.

Diet Incorporation

Here, the insecticide is mixed with the diet at a 1:9 ratio and then the temperature is maintained at 40˚C, because it is the lowest temperature at which the insecticide has not lost its integrity and then allowed to be maintained at room temperature for 24 hours. Next, cut the diet into 1cm^3 pieces and place it in the 16 well polystyrene tray followed by the 3rd instar larvae is released into each cell and maintained at 25 ± 2 ˚C and 60 – 65 % RH for the period of 72 hours and then mortality was recorded.

Inference

They have tested the Indoxacarb, Deltamethrin, Spinosad, and Chlorpyrifos-ethyl against the pest by using these two methods and they concluded that diet incorporation is the most appropriate method.

Diet Incorporation for pea aphid, *Acyrthosiphon pisum* - (Sadeghi *et al*. 2009)

This study tests flonicamid, a novel systemic insecticide on pea aphid, *Acyrthosiphon pisum*. A standard artificial diet for this pest is discovered by (Febvay *et al*. 1988). Prepare the stock concentration of 0.0001 µg/ml – 100 µg/ml and filter through the 0.2 µm filter.

Materials needed for the experiment

- ✓ Plexiglass
- ✓ Piece of parafilm
- ✓ Rubber ring
- ✓ Small petri dish

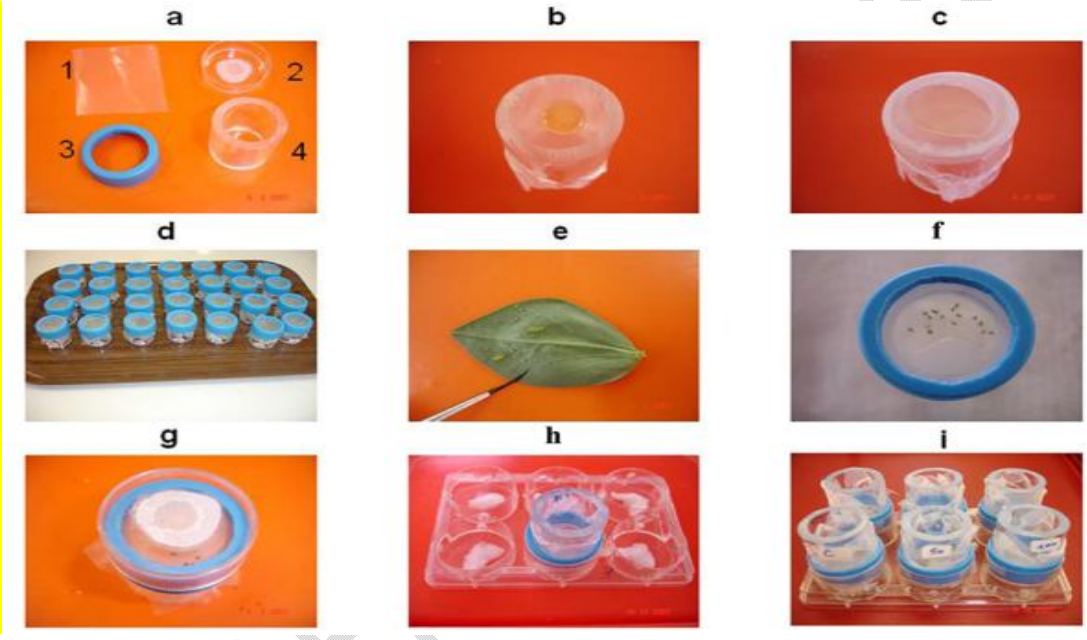

Fig. 2. Materials used for the study

Procedure

A piece of parafilm is placed on the top of plexiglass then stretched slightly. 200 µl of solution that contains artificial diet along with respective dilution of insecticide is poured on the parafilm, followed by another piece of parafilm being placed over it**.** Then we fixed the rubber ring around the top of the plexiglass**.** Release the known number of aphids into it**.** To prevent the escape of aphids from the structure place a small petri dish over it. For ventilation make a hole in the center of the petri dish and covered it with the net cloth. Mortality was assessed from 24, 48, and 72 hours after feeding**.** Replace the food provision every 2 days till the completion of the experiment.

Inference

Flonicamid is effective against the first instar nymphs with a record of LC_{50} of 20.4 µg/ml after 24 hours and 0.24 µg/ml after 72 hours.

Fig. 3. Sandwich method for western flower thrips, Frankliniella occidentalis against entomopathogenic fungus Beauveria bassiana - (Ugine et al. 2005)

Materials needed

- ✓ 30 ml polystyrene cup
- ✓ Two filter paper disks and two leaf disks

Procedure

Keep the following materials inside the polystyrene cup in the following order. First place one filter disk at the bottom of the cup then adds two leaf disks which are previously coated with the fungal application at the rate of 1.09×10^8 by using the Burgerjon spray tower and allowed to dry for some time, the disks should face abaxial surface each other and the group of 12-second instar thrips is exposed on it, finally place another filter paper over this. Both the filter papers are poured with Deionized water of 35 µl for humidity and for prevention of leaf desiccation**.** Then the cup was sealed with parafilm and held at 25˚C for about 5 days after this the mortality was assessed.

Determination of the number of conidia acquired by the individual thrips

For this a drop of lactic acid (85 %) which contains acid fuchsin 1mg/ml was placed at the center of the cover slip then five thrips were collected from the assay cup and immersed on the stain and place another cover slip over it. It should be gently placed and pressed in a way it does not rupture the insects**.** Observe under a phase contrast microscope at 400 x magnification.

Inference

It was revealed that the entomopathogenic fungus *Beauveria bassiana* was found to be effective at low doses which has LC_{50} values of 33 – 66 conidia/ insect.

Fig. 4. Thrips Insecticide Bioassay System (TIBS) - (Rueda and Shelton, 2003)

In this method, a simple 0.5 ml microcentrifuge tube is used, which has a flexible cap. A cap was provided with a small well-like structure and it is used to fill the food for the thrips. 3 drops of 10 % sugar solution are filled in this well along with red food colorants (which indicate contamination that happen during the trials) and the well was closed with thin parafilm. Desired chemical compounds with solvent are coated inside the vial and allowed for 4 hours to remain inside and after that dry for about 8 hours at room temperature. For the introduction of thrips into this tube make the hole at narrow end of the tube by using the heated pin. Then aspirate the thrips into the tube and mortality can be assessed after 24 hours by using the dissecting microscope. The main advantage of this method is that treated tubes can be stored for up to 3 weeks at 4 ˚C.

Adult vial test

It is one of the simplest methods here, 0.5 ml of the desired compound with suitable solvent is coated inside the 20 ml glass vial. For evaporation of the solvent and uniform application of compound throughout the material roll the vial in a horizontal position manually or by using a roller. After this process, known numbers of test insects are exposed to the vial. During the trials, the vial should be in the upright position, and after 24 hours mortality and morbidity (Inability of the insect to right itself when inverted or incapable to cling on the surface of the vial) is measured.

Case studies

1. **Toxicity of insecticide to *Halyomorpha halys* (Hemiptera: Pentatomidae) – (Nielsen *et al*. 2008)**

Among the tested insecticides, Pyrethroid especially bifenthrin, caused mortality at low doses with LC_{50} values of 0.03 - 0.49 (g (AI)/cm^2).

2. **Insecticidal toxicity against cotton fleahopper, *Pseudatomoscelis seriatus* (Hemiptera: Miridae) – (Lopez *et al*. 2008)**

Here, out of 17 insecticides tested bifenthrin, dicrotophos, thiamethoxam, and methomyl were recorded as the most toxic insecticides based on the LC_{50} values.

Mite Egg Bioassay System (MEBS) – lethal effect of *Beauveria bassiana, Metarhizium anisopliae,* and *Paecilomyces fumosoroseus* on eggs of *Tetranychus cinnabarinus* – (Shi *et al.* 2004)

Procedure for production of conidia

Aerial conidia of the above entomopathogenic fungus are produced in steamed rice in liquid culture containing 3 % glucose, 1 % peptone, and 1 % yeast extract. Then this was transferred into a large petri dish and maintained at 25˚C in the period of 12:12 L:D hours for 6 to 9 days. Followed by the conidia were harvested via a vibrating sieve of 74 µm/mesh.

Assay method

Dissolve the dry conidia in 0.02 % tween 80 and then standardize the suspension of 1×10^8, 2×10^7 and 4×10^6 conidia/ml. Eggs on 3 - 4 leaves were taken and transferred into the uncovered petri dish then place the dish at the bottom of the large bucket. Spraying of conidia can be done by using a micro hand-held droplet application sprayer. The sprayer should hold at the position of 1 m above the bucket and hold for 15 – 20 seconds for spraying followed by it is allowed for deposition for 5 mins. To determine the conidia, count on eggs, three glass slips were placed behind the leaf in the petri dish. Counting of conidia can be carried out at compound microscope at 400 x magnification. After the exposure period the Petri plates were covered with a lid and incubated at 25˚C and 90 % RH. Egg hatchability was noted daily for the three consecutive days after this transfer of those unhatched eggs into the moist petri plate for the period of 2 to 3 days. Verify the presence of fungal pathogens by examining the spore's outgrowth on the surface of eggs by using the dissecting microscope at 50 x magnification and confirm the results.

Inference

It was noted that Bb 2860 strain was highly infectious with LC_{50} values of 548 conidia / mm^2 followed by Pfr.116 – 1848 conidia / mm^2 and Pfr.153 – 9183 conidia / mm^2.

Insect Cell Line Bioassays

It is the best alternative method to normal bioassays with live insects. Here, it has many advantages like less time consumption, more precise results, and assays can be done for organs or parts by using specific cells thereby getting the appropriate results.

Commonly used assays techniques in insect cell lines

MTT colorimetric assay – cell proliferation and survival test – (Mosman, 1983)

MTT is nothing but the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt, which is exclusively used in toxicological and cytotoxic studies due to its low cost and quick response mechanism.

Principle

A live cell can cleave these MMT salts and reduce their yellow color to insoluble purple crystals which indicates the live cells and proliferation rate.

Trypan Blue Dye Exclusion – cell viability assay - (Fornelli *et al.* 2004)

It is used to quantify the reduction in percent viable cells and cytotoxic effect.

Principle

Here the live cells can exclude the acid dye like TBD by thus identifying the live cells easily.

Case studies

Cytotoxic and lethal effects of Scorpion, *Leiurus quinquestriatus* toxin - β – BUTX – LQQ1a against *Spodoptera frugiperda Sf* – 21 cell lines

A toxin, β – BUTX – LQQ1a was procured from the National Center for Biotechnology Information and *Sf* – 21 cell lines from National Center for Cell Science, Pune.

Procedure

Transfer the 5 µl of *Sf* – 21 into the 96 well plates and then the already prepared concentrations (2.47, 4.95, and 7.42 µg) of toxin, β – BUTX – LQQ1a were added to it. Add the 5 µl of MTT salt into the plate and allowed to incubate for 4 to 6 hours at 27˚C un till the formation of purple color crystals. Then centrifuge those purple crystals at 1000 g for 15 mins. After this, solubilize the crystals in 100 µl of dimethyl sulfoxide and read the absorbance by using the tecan multi-plate reader.

Intra haemocoel injection assay

Here the 4^{th} and 5^{th} instar larvae of both *Spodoptera litura* and *Helicoverpa armigera* were tested against the toxin, β – BUTX – LQQ1a**.** 5 µl of β – BUTX – LQQ1a at the concentrations of (2.0, 1.0, and 0.2 µg) for *Helicoverpa armigera* and (2.0, 1.0, 0.5, and 0.2 µg) for *Spodoptera litura* is injected into the respective larvae**.** Mortality was assessed after 12, 24, 36, 48, and 72 h of post-injection. The result showed the Inhibition concentration, IC_{50} values of 0.13 µg for *Helicoverpa armigera* and 0.147 µg for *Spodoptera litura* with symptoms of immediate paralysis and feed reduction was observed.

Cytotoxicity of fungal metabolites to lepidopteran *Spodoptera frugiperda* (*Sf* – 9) cell line

Inference

The toxicity of sixteen fungal metabolites produced by some entomopathogenic fungi or biological control fungi agents was evaluated on lepidopteran *Spodoptera frugiperda* (Sf-9) cell line by Trypan blue dye exclusion and MTT-colorimetric assay, after 48 h of incubation. No statistical difference was found between IC_{50} values (50 % Inhibiting Concentration) and CC_{50} values (50% Cytotoxicity Concentration) obtained by the MTT test and Trypan blue dye exclusion for each fungal metabolite. The comparison with other bioassays showed that the Sf-9 insect cell line could represent a further tool to screen for the toxic effects of fungal metabolites, especially for beauvericin, gliotoxin, and zearalenone.

Toxicity of pyrrolizidine alkaloids to *Spodoptera exigua* cell lines

Inference

Pyrrolizidine alkaloids (PAs) are feeding deterrents and toxic compounds to generalist herbivores. Among the PAs of *Jacobaea vulgaris* Gaertn, jacobine and erucifoline are the most effective against insect herbivores indicating that the cell lines are a valuable tool for a first toxicity screen.

Fig. 5 . ADVANCED INSECTICIDE RESISTANCE ASSAYS FOR MOSQUITOES

WHO cone test - (WHO, 2006)

This method is used to determine the insecticide resistance in mosquitoes. Here, the insecticide-impregnated net is taken and a cone-like structure is placed above this net. There is provision for the introduction of mosquitoes on the top of the cone after the process is done it is closed with the cotton ball.

Procedure

For this assay, two to five days old non – blood-fed susceptible female mosquitoes were taken and allowed inside the cone for the exposure period of 3 minutes. After the exposure, transfer these mosquitoes into the 150 ml plastic cups which were provided with sucrose solution which serves as food and maintained a climatic chamber for 24 hours at 27 ± 2°C and 80 ± 10 % RH. Percent knockdown was noted after 60 minutes and after 24 hours percent mortality can be recorded.

Fig. 6. WHO wire ball test

Procedure

Insecticide-impregnated net is wrapped around a wire frame of 15 x 15 x 15 cm. While netting around the frame leave a small sleeve through which the mosquitoes were introduced into the wire ball. As in the cone method, allow the mosquitoes for an exposure period of 3 minutes and then transfer into them the holding cups. After 24 hours mortality was assessed.

WHO susceptibility test - (Owusu *et al.* 2015)

In this assay, the same procedure as in the cone and wire ball test i.e., introduce the 2 to 5 days old non – blood-fed susceptible female mosquitoes for the exposure period of 3 mins. But, here release into the WHO exposure tube which was lined with a diagnostic dose of insecticide-impregnated filter paper. Knockdown was recorded every 5 minutes for the period of one hour**.** After this period, transfer the mosquitoes into another tube with 10 percent sugar solution at 27 ± 2˚C and 80 ± 10 RH for 24 hours then the mortality was noted.

Fig. 7. CDC bottle assay – (Aizoun et al. 2013)

Brogdon and McAllister, in the year of 1998 discovered the alternative method to the WHO susceptibility test which is the CDC bottle assay. This method was developed under the Center for Disease Control hence referred to as CDC. 250 ml of glass bottle was coated with 500 µl of insecticide along with acetone. Then release the 3 to 5 days old female mosquitoes into it. Knockdown were measured after 120 minutes followed by the treated mosquitoes were transferred into the 30 ml plastic beaker with 10 per cent sugar solution. After 24 hours mortality was recorded.

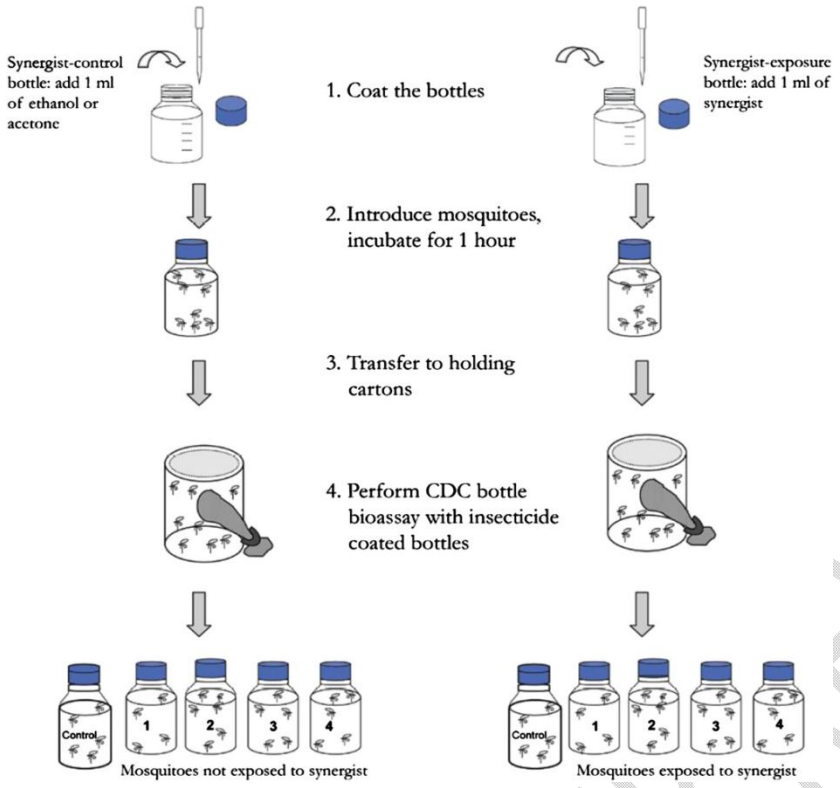

Fig. 8. Use of synergist

Two synergists were used one is S.S.S-tributylphosphorotrithioate (DEF) at the rate of (125 µg/bottle), which inhibits the esterase activity and another one is piperonyl butoxide (PBO) at the rate of (400 µg/bottle) which inhibit oxidase activity. For this assay 125 mosquitoes were used. Mortality rate was recorded during the different time intervals 0, 15, 30, 35, 40, 45, 60, 75, 90, 105, and 120 minutes. To test the synergistic effect, compare the obtained mortality (before and after the addition of synergist.

Advantages of WHO susceptibility test

- Knockdown was easily measured
- Standard diagnostic doses were followed in the assay

Disadvantages

- There is no provision for the use of synergist
- High cost of WHO kit when compared to other methods

Advantages of CDC bottle bioassay

- Simple, rapid, and cheap method
- Use of synergist is feasible here

Disadvantages

- Shelf life and re-use of the bottle is not well documented till now
- We need to coat insecticide for each bioassay

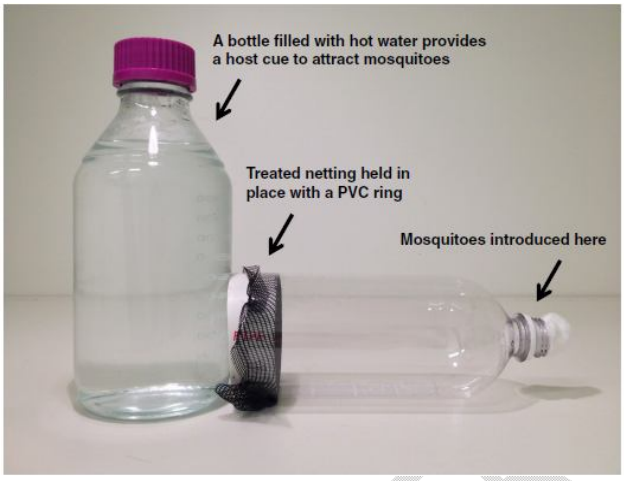

Fig. 9. MCD BOTTLE BIOASSAY – (Sternberg et al. 2014)

This method was developed under the Mosquito Contamination Project hence, it is named as MCD bottle assay.

In this method, a 1-liter plastic bottle is used in place of a glass bottle with the bottom cut off and then fix insecticide-impregnated net along with a rubber band for support. Then this structure was attached to a glass bottle which has a cue for mosquitoes. *Anopheles stephensi* was used and allowed for the exposure period of 3 minutes. Exposure time is calculated from the time when mosquitoes are introduced into the bottle not the exact time when mosquitoes contact the IIN. After the exposure period, transfer them into the paper cups with sugar solution and replace the food daily. Knockdowns were measured after one hour and mortality monitoring was extended up to the one-week post-exposure.

Bioassay techniques - cattle ticks, *Boophilus microplus* - (Castro - Janer *et al*. 2009)

Larval Packet Technique

1 % of fipronil along with the solvent, acetone was tested against cattle ticks, B*oophilus microplus*, and make this into 100 ml**.** 670 µl of the above dilution was poured on the filter paper and then maintain at room temperature for about 24 hours**.** Followed by making the treated paper into packets by folding and clipping on the sides then release the 15 days old, 100 larval ticks into this and incubate at 27 – 28˚C / 80 – 85 % RH for 24 hours**.** Finally, no dead and live ticks were recorded.

Larval Immersion Technique

Here, 1 ml of fipronil in 10 ml solution that contains 9 % of acetone and 0.04 % triton x – 100 is taken. From the above solution take the 1 ml and then transfer this into the 2 ml Eppendorf tubes. Transfer 500 tick larvae into the solution and immerse for 10 minutes by agitating vigorously. Finally drain the content and remove the larvae from the tube and place them in filter paper at 27 to 28˚C and 80 – 81 % RH for 24 hours followed by mortality was assessed.

Adult Immersion Technique

In this method, 0.01 % fipronil is taken in 10 % of acetone. Make the concentration of 3, 2.5, 2, 1.7, 1.5, 1.2, 1, 0.8, 0.7, 0.6, 0.4 and 0.2 ppm. In control 10 percent of acetone is used. Transfer the 10 engorged female ticks into the 20 ml of respective dilution in a 50 ml beaker followed by immersing for 1 minute by gentle agitation and drying rapidly in filter paper and incubate at 27 – 28˚C, 80 – 85 % RH. On the 7^{th} and 14^{th} days – the number of eggs and weight were recorded.

Conclusion

Bioassay is a mandatory tool for screening of insect management compounds and it offers scope for innovations and need-based modifications. Hence, bioassay techniques are ever-evolving and advancing. This makes the study of bioassay a dynamic one. Our understanding of bioassays needs to be updated often.

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