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Evaluation of entomopathogenic fungi by larval and adult immersion method

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Agrosavia Biocontrol

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The search for commercially viable entomopathogenic fungi for use in integrated pest management programs involves several steps. Fungal species must first be obtained from diseased insects or the environment and identified. Then, they must be evaluated under laboratory conditions to identify the most promising candidates. Because of that, bioassays must be repeatable and reliable to determine accurate pathogenicity or virulence. Variability in results may be caused by the variation in the components of an assay. However, the availability of a standardized bioassay is limited. Few reports detail the methods used to develop bioassays for a specific purpose and, without these details, it is difficult to develop bioassay methodologies suitable to evaluate the fungus-host relationship. We described a protocol based on the immersion method to evaluate entomopathogenic fungi (larval and adult stages), that can be reproduced to reduce variability. This protocol can be used in several stages of biopesticide development: selection of the biological control agent, characterization of the microorganism, formulation compatibility, and *in vitro* evaluation of efficacy.

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Materials	
Larvae Bioassay	**Adults Bioassay**
Larvae of the required age. Feeding substrate Biocontrol agent Sterile brush Sterile paper towels 0.5 oz plastic cups with cap Sterile Petri dishes Sterile fine tulle fabric Sterile centrifuge tubes Sterile Eppendorf tubes 64 oz. plastic container Hemacytometer Improved (Bright-Line) Depth: 1/100mm	Adult insects required Feeding substrate Biocontrol agent Entomological forceps Sterile Eppendorf tubes Sterile Petri dishes 64 oz. plastic container Sterile centrifuge tubes Sterile Eppendorf tubes Sterile water Hemacytometer Improved (Bright-Line). Depth: 1/100mm

Equipment (larvae and adults bioassay)
Biological safety cabinet Analytical balance (Capacity: 0.5 g -1 g. Scale divisions: 0.01 g) Vortex mixer Microscope Stereoscope Micropipete 10 µL – 100 µL Micropipete 100 µL – 1000 µL Micropipete 1 mL µL – 10 mL Autoclave Freezer Thermohygrometer

Reagent preparation

1 ▪ **0.1%Tween® 80** (preparation to 1000 mL):

1 mL Tween® 80 (comercial concentration)
Complete volume with water up to 1000 mL
Autoclave at 121 °C ± 3 °C for 15 minutes at 15 psi

▪ **0.05% Sodium hypoclorite (NaClO)** (preparation to 1000 mL):

3.33 mL Sodium hypoclorite(comercial concentration)
Complete volume with water up to 1000 mL

Insect procurement

2 *Rearing:* Larvae for the experiment came from the insect rearing unit, maintained on artificial diet and under controlled environmental conditions at 25°C ± 2°C and a relative

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humidity of 60% ± 10% (these conditions vary according to the insect).

Field: The other possibility is to have the insects directly from the field. For this purpose, the insects should be sampled and placed in quarantine conditions (with the recommended temperature and humidity for the insect) for at least one week to discard individuals that have any sign of infection by pathogens or weakness due to handling and confinement conditions. Those individuals that remain mobile and active will be used for testing.

Fungal spore suspension

3 Cultivate fungal strains (biocontrol agent) on potato dextrose agar (PDA) or another media for fungi. Harvest the conidia by scraping them off the medium surface by a loop needle and transferring them to a centrifuge tube containing 9 mL of 0.1% Tween 80. Shake the tube on a vortex mixer for 3 min to homogenize the hydrophobic conidia. Filtrate the suspension through three layers of sterile fine tulle fabric to get hyphal-free spore suspension.

Determine spore concentration using a hemocytometer improved (Bright-Line) and adjust to a final concentration to determine biological activity (e.a. 1 x 10^7 conidia/mL).

Aspects to consider

4 Prior to setting up the bioassays, the most appropriate larval instar for the test must be established, since due to its intrinsic characteristics, the same instar is not always used in all species. For the same reason, the time the larvae will remain in immersion must be established.

The reconstitution of the formulated biological agents, the concentration to be applied and the diluent to be used, must be done as indicated in the instructions of the technical data sheet or on the label.

Bioassay set-up larvae

5

Larvae of Lepidoptera must be delivered on an artificial diet and be healthy, not overcrowded or stressed, and isolated if carnivorous or cannibalistic.

Fungal application.

Place batches of 15 larvae in a Petri dish. For each batch, 6 mL of the fungal suspension in 0.1% Tween 80 pour over the larvae. Shake gently the larvae for 20 seconds, then drained the suspension and put the insect in a piece of sterile fine tulle fabric, to remove excess moisture.

After inoculation, place the treated insects individually in 0.5oz plastic cups previously prepared with a paper towel in the bottom and feeding substrate. Put them in a 64 oz. plastic container and incubated each batch at 25 °C, and 40% relative humidity (RH).

Record insect mortality every two days, starting with the control and then the fungus

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treatment. In each day, it is necessary to change the feeding substrate. Transferer the dead larvae into a microscope slide in a Petri dish containing a piece of moistened paper towel to promote sporulation of the fungi (Figures 1 and 2).

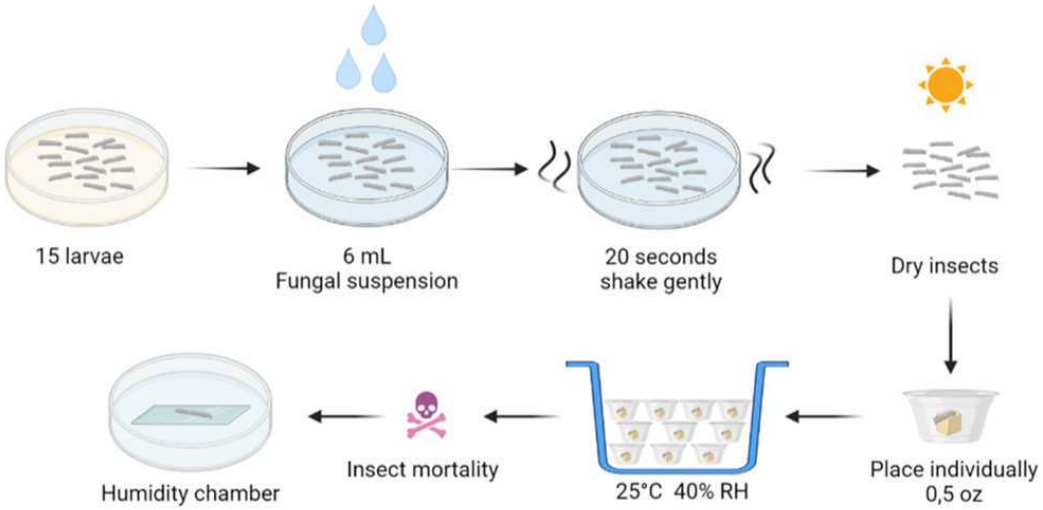

Figure 1. Schematic representation of larval infection process. Diagram: Carlos Espinel-Correal and Lissette Torres-Torres.

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Figure 2. Larvae of *Plutella xylostella* (Lepidoptera: Plutellidae) infected by *Beauveria bassiana* (Hypocreales: Cordycipitaceae) on broccoli leave. Photo: Carlos Espinel-Correal.

Experimental design: Completely randomized design including a control and application of biocontrol agent treatments each with three replicates minimum. Each replicate with 10 to 15 insects.

Experimental units: The experimental unit consists of a 0.5 oz plastic cups with cap, with a larva and feeding substrate (artificial or natural diet, ensure that it is free of contamination and in the case of the natural diet, it must be kept fresh).

Bioassay set-up adults

6 Before setting up a bioassay with adult insects, it is recommended to disinfect them, especially those belonging to the Curculionidae family

Superficial disinfection.

For this type of insects (Coleoptera:Curculionidae) it is possible to perform an initial superficial disinfection process, due to their resistant structure. It is not recommended for

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other types of adult insects.

Prepare two Petri dishes. Place up to 10 adults of the insect (per replica) in the first Petri dish and add 20 mL of a 0.05% NaClO solution, shaking gently for 20 seconds. Then, transfer the adults to the second Petri dish and rinse with 20 mL of sterile distilled water, shaking gently for 20 seconds. Then, transfer the insects to a paper towel to dry at room temperature.

Fungal application.

Place the 10 disinfected adults in a Petri dish. Place the 10 disinfected adults in a Petri dish. Add 20 mL of the fungal suspension in 0.1% Tween 80, at the concentration estimated above (the concentration depends on the objective of the assay. It can be a standard concentration used to determine biological activity e.g. 1 x 10^7conidia/mL).

Shake gently the insects for 20 seconds, then place them on a paper towel to dry at room temperature. Place them in a 64 oz. plastic container previously prepared with a paper towel in the bottom and feeding substrate.

Incubate at 25°C, and 60% relative humidity (RH) and a light/darkness photoperiod of 12 h. Record insect mortality diary, starting with the control and then the fungus treatment (Figures 3 and 4).

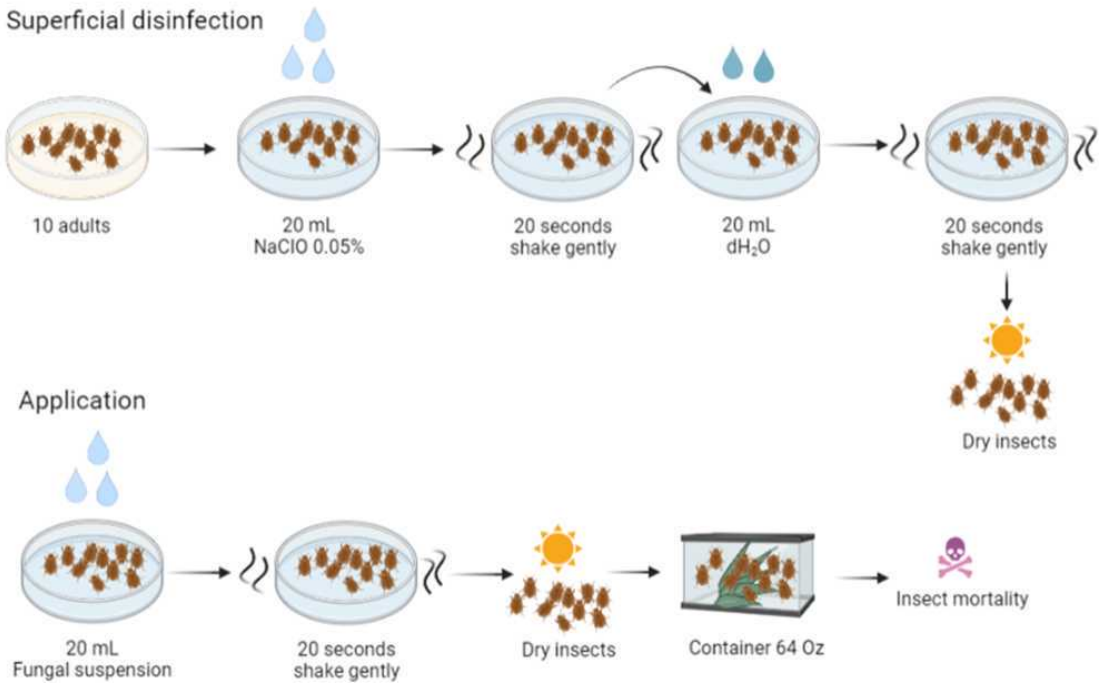

Figure 3. Schematic representation of adult insects infection process. Diagram: Carlos Espinel-Correal and Lissette Torres-Torres.

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Figure 4. Adult of *Gonipterus platensis* (Coleoptera: Curculionidae) infected by *Beauveria bassiana* (Hypocreales: Cordycipitaceae) on eucaliptus leave. Photo: Carlos Espinel-Correal.

Experimental design: Completely randomized design including a control and application of biocontrol agent treatments each with three replicates minimum. Each replicate with 10 to 15 insects.

Experimental units: Consisted of 10 adults per cage (Petri dish or plastic container) with feeding substrate that may be artificial or natural diet. For example, to *Gonipterus platensis* (Coleoptera: Curculionidae) an eucalyptus branch, whose front end should be introduced in an Eppendorf tube with water, to ensure wetting of the foliage.

Data analysis

7 Insect bioassays can focus on the evaluation of the biological activity of a control agent or the determination of lethal times and concentrations. The data analysis for the two cases is as follows:

Biological activity bioassay

Biological activity of a control agent is determined at a pre-defined concentration and for a pre-defined time on a target insect. At the end of the evaluation, the percentage mortality is determined by considering the number of live insects, dead insects, and total insects for

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each of the treatments (control and application of biocontrol agent).

Finally, the efficiency percentage is determined according to the Schneider-Orelli formula (Zar, 1999):

$$Efficacy(\%) = \left(\frac{(b-k)}{(100-k)}\right) * 100$$

b: % of dead insects in the treatment where the control agent was applied.
k: % of dead insects in the control treatment.

Concentration and lethal time bioassay

Bioassay to determine efficacy of biological control agents in terms of Lethal Concentration (LC) and Lethal Time (LT) against an insect pest requires Probit analysis.

8 A bioassay of biological activity on an insect pest should include acceptance parameters related to:

- Mortality in the control treatment less than or equal to 20 %.
- Coefficient of variation between replicates of the same treatment (except for the control treatment) is less than or equal to 30 %.
- In the quality control of biological control agents, it is recommended to evaluate as an additional treatment a positive control (reference strain) with a known efficacy rate. The efficacy may not be lower than defined.
- The bioassay must be carried out under the predefined temperature and humidity conditions.

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