CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Pathogenicity of the biocontrol agent *Verticillium lecanii* against *Icerya seychellarum* on citrus trees in the greenhouse during October 2012

3.1.1. Selection of strain

The entomopathogenic fungus *Verticillium lecanii* (Zimmerman) Viegas which was reclassified by Zare and Gams (2001) as *Lecanicillium lecanii* with a high virulent pathogenicity against scale insect and mealy bugs originally isolated from Alaska, USA and EMCC Number: 919™ (Egypt microbial culture collection) was used in this study. The fungus was cultured and maintained on either potato dextrose agar (PDA) (250g potatoes, 25 g dextrose, 20 g agar and 1000 ml distilled water) or sabouraud dextrose agar (SDA) (10g mycopeptone, 40 g dextrose, 15 g agar and 1000 ml distilled water). The media were autoclaved at 120° C for 20 minutes and poured in Petri plates (9 cm diameter ×1.5 cm). The detected fungi were inoculated and incubated in the dark for 10-14 days. The fungal isolates were routinely re-cultured every 14-30 days and the isolates were kept at 4°C.

Taxonomic Position:

Lecanicillium lecanii (Verticillium lecanii)

Super Kingdom	Eukaryota
Kingdom	Fungi
Phylum	Ascomycota
Order	Hypocreales
Family	Hypocreaceae
Genus	*Verticillium*
Species	*lecanii*

3.1.2. Fungal maintenance and conidial preparations

To obtain conidial preparations, cultures were maintained on sabouraud dextrose agar (SDA). To encourage the fungal growth, malt agar with 0.1% yeast extract were added to SDA media and kept for 10 days at 25°C. Spores were harvested using an aqueous solution of 0.05%Triton -100®. The spore suspension was filtered through several layers of cheese cloth to remove

mycelial mats. The concentration of spores in the final suspension was determined by haemocytometer. The spore preparation used for bioassays was adjusted by diluting concentrated spores with 0.05% Triton -100® to a final stock concentration of $1.3X10^8$ spores/ml.

3.1.3. Rearing the tested insect

The immature individuals of the mealy bug *Icerya seychellarum* were reared on young citrus (orange) trees (1- year old). The trees were individually infested with 60 immature mealy bugs. Four weeks after initial infestation, the settled adults of *I. seychellarum* were observed and used for the bioassay experiments, as described by Rezk (2009).

Taxonomic Position:

Icerya seychellarum seychellarum

Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Hexapoda
Class	Insecta
Order	Hemiptera
Family	Monophlebidae
Genus	*Icerya*
Species	*seychellarum*
Subspecies	*seychellarum*

3.1.4. Bioassay procedure

Adult indiviuals of the tested insect (*I. seychellarum*) were assayed on citrus trees grown in a green house. The trees were sprayed with spore suspension (30 insects/ young tree). Five treatments (concentrations) were set up ($1.3X10^8$, $1.3X10^7$, $1.3X10^6$ and $1.3X10^5$ spores/ml, beside the untreated check using 3 replicates/ treatment. The data were daily recorded for a period of 14 days.

3.1.5. Correction of mortality

It quite often happens that a proportion of insects die during the experiment from natural cases or from causes unconnected with the tested concentration of the tested biocontrol fungus. The magnitude of this mortality may be estimated from "control" (untreated check) batches treated in exactly the same way as the test insects except for the exposure to the spores of the

tested fungus. This control mortality, if it is appreciable, will affect the precision of the results and a correction is applied using the formula of **Abbott (1925)** as follows:

$$P_r = \frac{P_o - P_c}{100 - P_c} \times 100 \quad , \quad \text{where}$$

P_r = corrected mortality, P_o = observed mortality and P_c = control mortality (all as %s).

3.1.6. Bioassay analysis:

The percentages of corrected cumulative mortalities **(Abbott, 1925)** of each treatment were analyzed with the acomputer program to calculate LT_{50} values **(Finney, 1971).**

3.2. Virulence of *Verticillium lecanii* (Zimm.) Viegas subcultures grown on an artificial medium or its natural host *Icerya seychellarum* (Hemiptera: Monophlebidae)

3.2.1. The tested fungus and insect:

The entomopathogenic fungus *Verticillium lecanii* (Zimmerman) viegas which was reclassified by Zare and Gams (2001) as *Lecanicillium lecanii* with a high virulent pathogenicity against scale insects and mealy bugs was originally isolated from Alaska (USA and EMCC Number: 919™). The culture was obtained from Egypt microbial culture collection (Ain Shams Univ., Egypt). The adult individuals of the mealy bug *Icerya seychellarum* were reared on citrus leaves which have been replaced in Petri dish surrounded with a wet tissue paper to keep it fresh as insect host medium.

3.2.2. Fungal maintenance and conidial preparations:

Cultures of the fungus were maintained on sabouraud dextrose agar (SDA) media (10g mycopeptone, 40 g dextrose, 15 g agar and 1000 ml distilled water). For the fungal growth, malt agar with 0.1% yeast extract were added to SDA medium and kept for 10 days at 25°C. Spores were harvested with an aqueous solution of 0.05% Triton -100®. The spore suspension was filtered through several layers of cheese cloth to remove mycelial mats. The concentration of spores in the final suspension was determined using a haemocytometer and adjusted for bioassay was by dilution with 0.05% Triton

-100 to a final stock concentration of $1.7X10^8$spores/ml. This procedure was done according to **Mehta *et al.* (2012).**

3.2.2.1. Conidial subculturing on artificial media:

For subculturing and repeated *in vitro* spore transfers, the spores were harvested from the surface of 10 days old culture by scraping with a loop and subcultured to fresh molasses yeast broth (MYB) (30g molasses, 5g yeast and 1000 ml distilled water). Cultured fungi were incubated at 28°C. This multi-spore *in vitro* transfer was repeated up to 4 times. Before subculturing, a suspension of harvested spores was prepared to be used for bioassay and inoculate other insects. This procedure was done according to **Asghar, (2013).**

3.2.2.2. Conidial passaging through insect:

For the *in vivo* passage of the fungus maintained in the insect-host, spores of the 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} *in vitro* subcultures were recovered from Petri dishes and suspended in sterile distilled water. Spore concentration of the suspension was adjusted to 1.7×10^8 spores/ml. Adult individuals of the mealy bug *Icerya seychellarum* were dipped in the spore's suspension (1.7×10^8spores/ml) containing 0.02% Tween 80® solution and placed individually in a small plastic container. Inoculated insects were maintained on a lettuce until response (mortality) occurred. Dead insects were cleaned with cotton wool soaked with 70% ethanol and incubated at 28°C with 80-90% R.H to stimulate sporulation of the fungi. The spores from cadavers were harvested by scraping with a pointed needle and suspended in sterile distilled water to inoculate other insects. This insect passage was repeated and virulence of the fungi was evaluated by mortality through a bioassay. This procedure was done according to **Asghar, (2013).**

3.2.3. Bioassay procedure:

The virulence of the evaluated fungi was assayed (i) with the original isolates before subculturing on artificial media (ii) after subculturing on artificial media, and (iii) after *in vivo* passage in the insect.

Adult individuals of the mealy bug, *I. seychellarum* were assayed on citrus trees (30 insects/ young tree) grown in a green house and the trees were sprayed with spore suspension ($1.7X10^8$ spores /ml). Six treatments (sub cultures) were used: mother culture, 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} passage culture through artificial medium and an insect host (*Icerya seychellarum*) (Hemiptera: Monophlebidae) beside the untreated check. Three replicates were considered for each treatment. The fungi virulence was measured through daily calculation of mortality percentage within 14 days post fungal application. This procedure was done according to **Asghar, (2013).**

3.3. Mass-production of *Verticillium lecanii* on the different media types

3.3.1. Mass production of *V. lecanii* on liquid media

In liquid state production, five liquid media (Potato dextrose broth (PDB), Potato sucrose broth (PSB), Molasses yeast broth (MYB), Sucrose yeast broth (SYB) and Potato carrot broth (PCB)) were tested. Conical flasks containing 100 ml sterilized liquid media were inoculated with 1ml of spore suspension (1×10^7spores/ ml with three replications and incubated in growth chamber at 25±1 °C and 90±3% RH for 14 days. To determine the spores production, culture were agitated vigorously and filtered through whatman NO. 2 filter paper and the fungal mat was oven-dried at 40-45°C until constant weight was achieved and liquid nitrogen – dried technique was also used with other three replications. To illustrate the difference between the ways of drying on the amount of spores produced and viability of the total of spores/ml, Naeubauer improved haemocytometer was used to determine spore germination. Spore suspension (0.1ml of 1×10^6 spores/ ml) was spread on PDA petri plates and incubated at 25±1°C, 90±3% RH. After 24 h, germination percentage was estimated by counting 300 spores for each plate using microscope at 400x magnification. A spore was considered germinated when the germ tube was at least equal to its width. Three plates were used per treatment and each plate served as a replicate.

3.3.2. Mass production of *V. lecanii* on solid media

In solid state production, four cereal grains (rice, wheat, corn and barley) and two solid agricultural wastes (rice husk and rice straw) were evaluated. Raw grains were taken in autoclavable polypropylene bags (20×28cm) at 100 g/bag and were soaked with tap water for 12h and the excess water was drained completely to each of this bags, 2g of calcium carbonate and 2g of calcium sulphate were added and mixed thoroughly to get uniform coating of salts over grains . The bags were then sterilized by autoclaving twice at 121°C for 20 min. The grain media was inoculated with 1ml of spore suspension (1×10^7 spores/ ml) under aseptic conditions. The bags were once again sealed manually and incubated for 14 days at 25±1°C, 90±3% RH for biomass production of conidia. Three replications were used for each substrate. After 14 day of incubation, grains with fungal growth were dried under aseptic conditions at 30°C. After drying, the spore production of each grain was estimated by Naeubauer improved haemocytometer. Then, the grains with fungal growth were sieved through a sterile filter and the coarse dust thus collected was further sieved through a sterile 105 μ sieve to get fine spores

dust. The quantity of spores obtained per 100g of each substrate media was estimated.

3.3.4. Mass production on diphasic system

In diphasic system of production, five liquid media (Potato dextrose broth (PDB), Potato sucrose broth (PSB), Molasses yeast broth (MYB), Sucrose yeast broth (SYB) and Potato carrot broth (PCB)) and crtain broken grains (broken rice, broken corn, broken wheat and barley) as solid substrates were tested. One hundred milliliter of each above mentioned broths were prepared in 250 ml conical flasks and inoculated with 1 ml of fungal suspension (1×10^7 spores/ ml) in a shaker maintained at 120 rpm for 3 days. Fifteen milliliters of 3 day-old shake cultures were inoculated to each of the bags containing 100g grain and mixed well with the grains. The inoculated bags incubated as mentioned earlier for production of spore. Spore dust biomass production and viability of the total of spores/ ml was assessed.

3.4. Formulation, storage conditions and shelf life

3.4.1. Wettable powder formulations of *V. lecanii*

A weight of 2.5 g. of fresh conidial powder of *Verticillium lecanii* represented concentration of 2×10^9 conidia/ ml. Conidia were mixed with silica gel powder in 2.5:10 weight ratios. Before mixing the silica gel powder, carrier materials was sieved through 355 mesh size to maintain uniformity of particle size of conidial powder and the carrier material. This carrier material was sterilized in an autoclave at 120°C and 15 Psi for 30 min and mixed with conidial powder after two days; Casein UV-protectant was added at the rate of 2 % by weight and the wetting agent Tween 80® was also added to the mixture of conidia at the rate of 0.5 % by weight. Carboxy methyl cellulose (CMC) was added at the rate of 2 % by weight as a sticker. Both talc powder and starch were added up to gain a final weight of 100 g. as filler, Ten grams of each of these formulations were packed in small polyethylene bags. Approximately 12 g. of dry powder was obtained for each case and poured onto 50 ml falcon plastic tubes. These tubes were covered with aluminum foil and stored under different conditions: in laboratory at a temperature ranging from 30–35°C and in a refrigerator at a temperature of 4±2°C for 180 days. For examining the effect of storage time, filler media types of mass production on formulation efficacy and spores viability (germination percentage), storability, conidial germination and viability under laboratory conditions in each of the prepared formulations were assessed before storing and at monthly

interval for the first six months of storage. One gram of the sample was suspended in 10 ml of sterilized distilled water containing 0.02 % Tween-80® were estimated.

3.5. Field experiment

In a trial to investigate the effects of some commercial bio-pesticides, a natural botanical insecticide and some chemical insecticides comparative with *Verticillium lecanii* as a formulated WP 2.5% on the selected mealy bug (*I. seychellarum*), a field experiment was conducted in Alexandria governorate. Certain treatments were evaluated included a bioinsecticide (Bio- Catch® 1.15 % - *Verticillium lecanii*), a botanical insecticide (Nimbecidine® 0.03%), two chemical insecticides (thiamethoxam® 25 W.G and Malathion® 57%E.C) and the mineral oil Kz oil® 95% E.C .These tested materials were sprayed on orange C. *sinensis* trees (10-years old) to assess their effect on the mealy bug *(Icerya seychellarum*). The experiment was arranged in Randomized Complete Block Design with three replicates for each treatment and each replicate contain three trees of citrus in two different application timing (in the morning and night). Ten leaves were randomly collected from four direction of each treatment. The samples were collected weekly for four weeks and examined by a binocular at the laboratory to calculate the reduction percentage according to **Henderson and Tilton (1955)** equation as follows:

$$\textbf{Reduction \%} = \left(1 - \frac{a}{b} \times \frac{c}{d}\right) \times 100$$, where

a = Population in treatment after spraying
b = Population in treatment before spraying
c = Population in untreated check (control) before spraying
d = Population in untreated check after spraying

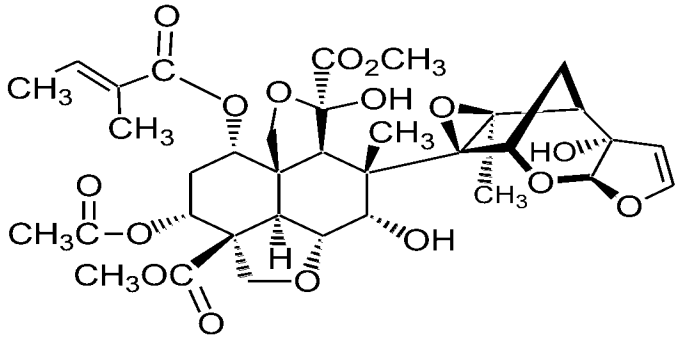
Reduction percentages were calculated after 1,2,3and 4 weeks post-treatment.

3.6. Botanical, Bio and Chemical insecticides used

3.6.1. Nimbecidine® 0.03% (Azadirachtin)

Azadirachtin is the principal insecticidal ingredient of neem seed extracts (extracted from the neem tree, *Azadirachta indica.*); these extracts also contain a variety of limonoids, such as nimbolide, nimbin and salannin.

Chemical group: Terpenoids
Chemical name: dimethyl (3*S*,3a*R*,4*S*,5*S*,5a*R*,5a^{1}*R*,7a*S*,8*R*,10*S*,10a*S*)-8-acetoxy-3,3a,4,5,5a,5a^{1},7a,8,9,10-decahydro-3,5-dihydroxy-4-{(1*S*,3*S*,7*S*,8*R*,9*S*,11*R*)-7-hydroxy-9-methyl-2,4,10 trioxatetracyclo[6.3.1.0^{3,7}.0^{9,11}]dodec-5-en-11-yl}-4-methyl-10[(*E*)-2-methylbut-2-enoyloxy]-1*H*,7*H*-naphtho[1,8a,8-*bc*:4,4a-*c*']difuran-3,7a-dicarboxylate
Chemical Structure:



Biochemistry: Ecdysone agonist / antagonist.

3.6.2. Bio- Catch® 1.15%

It was used as a liquid formulation contains 1×10^{9} cfu / ml of the fungus *Verticillium lecanii* .
Other trade names: Bioline® (Biotech International Ltd.)
Mycotal® Vertalec® *(*Koppert Biological Systems)
Ecocill® *(*Margo Biocontrols Pvt. Ltd.)

Both of the above-mentioned formulation (Bio- Catch® 1.15% and Nimbecidine® 0.03% (Azadirachtin)) were kindely donated by T. Stanes & Company Ltd., India.

3.6.3. *Verticillium lecanii* as a formulated WP 2.5%

This prepared formulation contains 1.3×10^{8} spores/g. The entomopathogenic fungus *Verticillium lecanii* (Zimmerman) Viegas originally isolated from Alaska, USA and EMCC Number: 919^{TM} (Egypt microbial culture collection) was used in this study. This fungus was prepared as a 2.5% WP formulation as described in section 3.4.1.

3.6.4. Mineral Oil

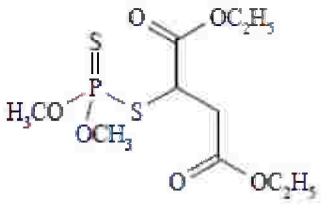
Kz Oil® 98% is a mineral oil produced by Kafr El-Zayat Co. for Pesticides and Chemicals. It was used at the application rate of 1.5 %.

3.6.5. Malathion® 57% EC

Commercial formulation of the insecticide Malathion® (57% EC) is distributed by Kafr El-Zayat Pesticides and Chemicals Co., Egypt) and it was used at the application rate of 300 ml /100 l.

Chemical Class: Organophosphate
Common name: Malathion
Chemical name: *O, O*-dimethyl dithiophosphate of diethyl mercaptosuccinate

Chemical Structure:

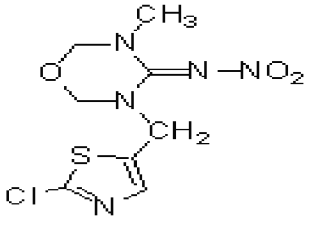


3.6.6. Actara® 25% WG

It is the commercial formulation of the insecticides thiamethoxam produced by Syngenta Crop Protection AG Postfach CH-4002 Basel Switzerland. It was used at the application rate of 25g. /100l.

Chemical class: Neonicotinoid
Common name: Thiamethoxam
Chemical Name: 3-(2-chloro-1, 3-thiazol-5-ylmethyl)-5-methyl-1, 3, 5-oxadiazinan-4-ylidene (nitro) amine

Chemical structure



3. 7. Treatments application

To evaluate the performance of the tested foliar bioinsecticides, botanical insecticide and chemicals insecticides on the pest population, trees were sprayed with those suggested compounds to show to what extent they might be included in an IPM program of citrus. Treatments included the seven tested compounds plus an untreated check (control). Treatments were applied with a hand sprayer (10 liters) at the recommended dose / Feddan. The untreated (control) trees were chosen to be little away from those treated trees to avoid any contamination or interference of spray drift. Also, two rows were used as a barrier between each treatment and another. Treatments were arranged in complete randomized block design with three replicates for each treatment. The mean number of alive individuals was considered to calculate the reduction percentages over different periods.

3.7.1. Sampling technique and pest inspection

Insect-pests populations attacking citrus trees during the period of the study were recorded as an indicator of the effectiveness of the tested compounds through the inspection of the mealy bugs on citrus trees. The samples were collected before and after treatment at periods of 1, 2, 3, and 4 weeks post-treatment to determine the numbers of pests on 3 trees (30 leaves from each plot from the lower, medium and upper parts of each tree). The leaves samples were transferred to the laboratory and examined with the aid of a binocular microscope to determine the degree of infestation by counting the number of living individuals.

3.8. Statistical analysis

Data were subjected to the analysis of variance test (ANOVA) as a Randomized Complete Block Design (F. test). The least significant differences (LSD) at the 5% level were determined according to a computer program "Costat" and Duncan's Multiple Range testes **(Duncan, 1955)** modified by **Steel and Torrie (1981)** and they were used to compare the differences between obtained the means.