



## Biocontrol of *Planococcus citri* (Hemiptera: Pseudococcidae) by *Lecanicillium longisporum* and *Lecanicillium lecanii* under laboratory and greenhouse conditions



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### ABSTRACT

Much effort has been focused on the development of microbial biopesticides composed of entomopathogenic fungi as alternatives to synthetic insecticides for pest management programs. The citrus mealybug, *Planococcus citri* Risso (Hemiptera: Pseudococcidae), is an important pest in greenhouses and on citrus plants globally. The current study isolated and characterized two species of entomopathogenic fungi, *Lecanicillium longisporum* (Petch) Zare & W. Gams and *Lecanicillium lecanii* (Zimmere). Their virulence at the second nymph and adult stages of *P. citri* were documented using mycosis with scanning electron microscopy. *L. lecanii* was found to be more effective at different citrus mealybug growth stages. Nymphal *P. citri* were more susceptible than adults to fungal infection. Susceptibility at all stages was dose dependent. The results of laboratory trials used for greenhouse tests. In a comparison of wheat, beet pulp paddy rice and rice bran as substrates for solid substrate fermentation, wheat produced the most conidia for the *L. lecanii*. For *L. longisporum*, however, was the most appropriate media. The results of this study indicate that the entomopathogenic fungi *L. longisporum* and *L. lecanii* are potentially useful biological control agents for the citrus mealybug.

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### Introduction

The citrus mealybug, *Planococcus citri* Risso (Hemiptera: Pseudococcidae) is native to China and was first introduced to Europe and the United States as a pest on citrus and ornamental plants in 1813 and 1879, respectively (Bodenheimer, 1951). It is a pest on subtropical and tropical crops such as citrus (*Citrus* spp.), persimmon (*Diospyros kaki*) and banana (*Musa paradisiaca*) (Franco et al., 2009). It damages horticultural and ornamental crops that are primarily grown in greenhouses such as the oleander (*Nerium oleander*), garden croton (*Codiaeum variegatum*), lady slipper orchid (*Cymbidium orchid*) and chrysanthemum (*Chrysanthemum morifolium*) (Mafi Pashakolaei, 2010).

This mealybug feeds on plant nutrients in the stems, branches and leaves, excretes copious amounts of honeydew that give rise to sooty mold and injects toxins into the plant (Franco et al., 2009; Demirci et al., 2011). *P. citri* is also responsible for the transmission of a diverse group of viruses to a range of economically important crops (Cabaleiro and Segura, 1997). The citrus mealybug transmits grapevine leaf roll-associated virus-3 (GLRaV-3) and grapevine virus A (GVA) (Martelli,

2014). *P. citri* poses a serious threat, even at low densities (Franco et al., 2000; Gullan and Martin, 2003).

Treatment of mealybug infestations using chemical insecticides remains the main control tactic, but the pests protect themselves using different defense mechanisms (Franco et al., 2009). They hide in cracks and crevices in bark, leaf axils and root crowns, in the nodes of grass stems, under fruit sepals and within fruit navels. Their waxy body covering provides a natural barrier against insecticides (Mafi Pashakolaei, 2010; Lemawork et al., 2010). The broad use of insecticides, especially non-selective pesticides, adversely influences the mealybug's natural enemies and has led to its resurgence and to secondary pest outbreaks (Cloyd and Dickinson, 2006). Chemical insecticides are increasingly unacceptable because they present safety concerns and have low selectivity (Franco et al., 2009). Alternative methods of control, especially those that employ the natural enemies of mealybugs, are essential.

Entomopathogenic fungi (EPF) are the first microorganisms that were observed to be insect pathogens (Lacey et al., 2001). Hypocreales fungi are common entomopathogens that are effective against a diverse group of pests, especially species of Homoptera (Humber, 2008; Hajek and Delalibera, 2010). As piercing-sucking insects, mealybugs are rarely attacked by other viral or bacterial pathogens; only a few species of EPF have been reported effective against mealybugs (Lacey et al., 2011). These include *Beauveria bassiana* (Lemawork et al., 2010; Amnuaykanjanasin et al., 2012), *Metarhizium anisopliae* (Lemawork et

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al., 2010; Pereira et al., 2011), *Lecanicillium* cf. *lecanii*, *Lecanicillium muscarium* (Petch) (Naeimamini et al., 2010), *Lecanicillium lecanii* (Zimmere) (Naeimamini et al., 2010), *Isaria farinosa* (Demirci et al., 2011), *Aspergillus parasiticus* Speare, *Cladosporium oxysporum* Berk, *Hirsutella sphaerospora* Evans and Samson and *Neozygites fumosa* (Speare) Remaudière and Keller (Delalibera et al., 1997). Of these, *Lecanicillium* plays a major role as a biocontrol agent against Homoptera (Goettel et al., 2008).

*Verticillium lecanii* is a well-known and unique EPF with a wide range of hosts, including arthropods (scale insects and aphids), plant parasitic nematodes (root-knot nematode, *Meloidogyne incognita*; cysts and eggs of *Heterodera glycines*) and plant pathogens (biotrophic powdery mildew and rust fungi). It has been instrumental in the development of biopesticides (Zare and Gams, 2001; Goettel et al., 2008). Petch first exploited *V. lecanii* as an entomopathogenic fungus in 1925 to control green scale (*Coccus viridis*) (Alavo, 2015). Recent taxonomic studies on the genus *Lecanicillium* (*Verticillium*) using morphological characteristics and molecular analysis of internal transcribed spacer (ITS) sequences has shown that there are five clades, or clusters, within the *V. lecanii* complex (Zare and Gams, 2001). These are *L. attenuatum*, *L. lecanii*, *L. longisporum*, *L. muscarium* and *L. nodulosum* (Goettel et al., 2008). *L. lecanii* is exclusive to soft scale Lecaniidae and Coccidae. *L. muscarium* (Petch) Zare & W. Gams was developed as Mycotal® for use against whiteflies and thrips and as Verticillin® for use against whiteflies, aphids and mites. *L. longisporum* (Petch) Zare & W. Gams has been commercialized for use against aphids as Vertalec® and Vertirril® for whiteflies and thrips, respectively (de Faria and Wraight, 2007). *L. lecanii* and *L. longisporum* offer traits like a long shelf life, an effective mode of action, lack of toxicity in the environment, high host specificity. They are inexpensive to mass produce and non-persistent (Panahi and Loni, 2013).

For use as biopesticides, large numbers of spores are needed. The growth and development of EPF relies on their food availability. If intensive and rapid production of conidia is possible using the same amount of substrate, this will reduce manufacturing costs (Vu et al., 2008). The present study was undertaken to evaluate the use of wheat grain, rice bran, rice husk and beet pulp for mass culturing of fungi. These have been selected for their worldwide availability, low cost and physical characteristics such as grain size and shape. The efficacy of *L. lecanii* and *L. longisporum* on the citrus mealybug at different life stages and different inoculum concentrations in laboratories and greenhouses has been investigated. SEM was used to determine the mode of infection by *L. longisporum* of the citrus mealybug.

## Materials and methods

### Mealybug

The mealybug was collected from oleander (*Nerium oleander*), in a greenhouse from Rasht University campus, north of Iran (37.2682° N, 49.5891° E). The final identity of mealybug specimens was confirmed by Masumeh Moghaddam, Iranian Research Institute of Plant Protection, Tehran, Iran. To obtain cohorts of different stages of the citrus mealybugs for laboratory and greenhouse experiments (second instars and newly molted adults), the ovisacs of mealybug were put on filter paper near a zucchini (*Cucurbita pepo*), and the emerged offsprings was considered as a cohort of first nymphal stage and collected daily.

### Fungi source

The first species, *Lecanicillium longisporum* strain LRC 190 was provided by Dr. Reza Talei-Hassanlouei, Tehran University, Iran. This strain had been isolated from *Macrosiphoniella sanborni* in England. The second fungus isolate strain Iran 822, originated from *Pulvinaria floccifera* in Iran.

### Characterization of fungal isolate

Fungal identification was carried out using classic manner as well as molecular analysis. For preliminary identification, macro- and micro-morphological features of fungal colonies like the colour of conidia and growth of the fungal colony in pure fungal cultures were determined. In microscopic observations, traits like size and shape of conidia were investigated and the species were characterized according to Humber's key (Humber, 2012). Twenty specimens were prepared, stained and were examined for the size, arrangement and shape of hyphal and conidial cells.

For molecular characterization, the fungal isolate was cultured on PDA plates, then the mycelial mass was removed and transferred to Erlenmeyer flasks, containing 50 ml sterile PDB liquid medium (200 g potato and 10 g dextrose in 1 L of sterilized water plus 0.035 g streptomycin). The containers were placed on a shaker at room temperature. Then the mycelia were harvested, eluted, transferred into a sterile 2-ml micro-tube, and finally stored at 4 °C until use. Genomic DNA was extracted using the QIAGEN DNeasy® kit (Venlo, the Netherlands). The ITS region was amplified with the ITS4 and ITS5 primers (White et al., 1990). Amplification was performed with a Biometra thermal cycler. The PCR products were sent to the MacroGen sequencing service facilities (MacroGen Inc., Seoul, Korea). The consensus sequence of the fungus was compared with the genbank database using the nBLAST search tool. The most similar and valid sequences were retrieved from the Genbank and were used to reconstruct the phylogenetic tree. Multiple alignments were performed using the Clustal W program and then phylogenetic trees were reconstructed using the neighbor-joining method in MEGA6 software (Tamura et al., 2013) with 1000 replicates of bootstrap.

### Determination of conidial concentration

For preparing serial dilution, the fungal isolates were cultured on Potato Dextrose Agar (PDA) medium, at 25 ± 1 °C for 15 days in darkness. The produced spores were suspended in 0.2% (v/v) Tween 20 in sterile distilled water, by gently scraping the culture surface using a sterile spatula and the suspension was then filtered through sterile cheese-cloth to remove mycelium. The conidia suspension was adjusted to 1 × 10<sup>7</sup> conidia/ml using an improved Neubauer haemocytometer (Neubauer improved, Superior Marienfeld, Germany).

### Determination of fungal viability

Both fungal isolates were checked for the viability percentage of conidia before initiation of each experiment. For viability test, concentration of 1 × 10<sup>6</sup> conidia/ml was prepared and 0.1 ml of the suspension was spread on water agar plates. After 24 h at 24 °C, cover slips were applied and the number of germinated conidia counted. 100 conidia per plate (a total of 400 conidia on four plates) were examined under a light microscope using 400× objective magnification power. A conidium was considered viable if a germ-tube emerged from the spore and a germ-tube length was two times the diameter of the conidium.

### Scanning electron microscopy (SEM)

In SEM studies, the adults of mealybug were inoculated by *L. longisporum* with 10 ml of 1 × 10<sup>7</sup> conidia/ml suspension. After 24 and 72 h of inoculation, the infected adults were assayed with SEM following the technique of Amnuaykanjanasin et al. (2012).

### Laboratory bioassays

Initial bioassays were conducted to quantify the susceptibility of adult and nymph stages of *P. citri* to both fungi. For each treatment, 10 individuals of adult or nymph stages were put into a sterile beaker

using a camel hairbrush. The individuals in each treatment were swirled for 30 s in 5 ml of conidial suspension and subsequently placed on a coleus (*Plectranthus scutellarioides*) leaf as diet. Each leaf was placed at the bottom of a Petri dish (diameter: 9 cm) lined with moistened filter paper. The leaves were incubated for 2 weeks at  $25 \pm 1$  °C, 14 L: 10 D photoperiod and 80% RH for each life stage of *P. citri*. Applied concentrations were  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$  and  $1 \times 10^7$  conidia/ml. Control coccids were treated only with sterile distilled water and 0.2% Tween 20. Each treatment had four replications and the whole experiment was conducted twice. Dead individuals were checked to confirm the Koch's postulates by transferring cadavers to moisturized filter paper to monitor the emergence of fungal hypha. The infected individuals counted under stereoscopic microscope and the percentage of mortality was calculated.

#### Greenhouse tests

The efficiency of *L. longisporum* and *L. lecanii* against the citrus mealybugs, *P. citri* was determined on coleus in the research greenhouse of the Ferdowsi University of Mashhad, Iran ( $27 \pm 1$  °C, 14 L: 10 D and 90% RH). The insects were placed on a coleus pot and allowed to settle on leaves for 3 h. Fifteen milliliter of conidia suspension were sprayed by a small handheld sprayer onto the coleus leaf surfaces and stems. The plants were then covered with a glass cylinder. Applied concentrations were  $2 \times 10^6$  and  $2 \times 10^7$  conidia/ml. Control pots were treated with sterile distilled water and 0.2% Tween 20 only. Each treatment had four replicates with thirty insects per replicate. The whole experiment was repeated twice.

#### Conidial production

For mass production of the fungal isolates, four different solid media were evaluated: rice bran, rice husk and wheat and beet pulp (Arian Trade Service Company, Tehran). One hundred grams of each solid media were taken in autoclavable bag. The bags were plugged with a ring and a cheese cloth covered cotton stopper. The media were augmented with tap water to the desire moisture (60%) and autoclaved at 121 °C for 20 min. After cooling, the media was inoculated with 14 ml spore suspension ( $10^7$  conidia/ml). Fermentation was carried out at  $25 \pm 1$  °C for 10 days under relatively humidity of 85%. Three replications were used for each substrate. To determine yields, two grams of conidiated media was suspended in 18 ml of 0.02% Tween 80. After 60 min of agitation in a 100 ml flask at 200 rpm, the mixture was filtered through sterile cheesecloth. The number of conidia was determined using an improved Neubauer haemocytometer.

#### Statistical analysis

Statistical analyses for all bioassays (nymphs and adult insects) were conducted with the SAS software version 9.1 (SAS Institute, 2002). Cumulative mortalities were corrected for control mortality (Abbott, 1925) and square-root transformed when required for assumptions of normality and homogeneity of variances. One-way analysis of variance (ANOVA) was used to determine the effect on mortality across fungal species and the effect on mortality across spore concentration. For calculation the mortality of *P. citri* treated with entomopathogenic fungi under laboratory and greenhouse, mortality of both stages of *P. citri* were analyzed using two-way analysis of variance (ANOVA) (fungi species \* spore concentration) followed by LSD test to compare larva mortality caused by interactive effects. In the laboratory bioassays the Kaplan–Meier estimate was used to derive the median survival time (MST) and the standard error (SE).

In solid state fermentation for aerial conidia production assay, the spore yield values resulted from the four mediums were subjected to One-Way ANOVA followed by Tukey multiple-comparison test with significance level at  $P < 0.05$ .

## Results

### Fungal identification

The fungal isolate was first determined to be the *Lecanicillium* species based on classic morphological criteria. Molecular analysis of the ITS sequence confirmed the identity of the fungus as *L. lecanii*. In phylogenetic analysis, the fungus isolate clustered with other isolates of *L. lecanii* in a well-supported clade (data not shown). Before conducting the assays, the conidial viabilities of *L. lecanii* and *L. longisporum* were 98% and 80%, respectively. The initial infectivity assay showed pathogenicity of both EPF species against the mealybug life stages. Therefore, it was confirmed that both *L. lecanii* and *L. longisporum* have basic pathogenicity against citrus mealybugs.

### SEM of mycosis

Scanning electron microscopy (SEM) was used to observe the mode of action of *L. longisporum* and investigate how it is able to colonize and infect the citrus mealybug. The modes of action of *L. longisporum* after infection of *P. citri* were: (1) conidia adhesion to the insect cuticle; (2) fungal development and conidia germination after 48 h (Fig. 1a); (3) appressorium formation (Fig. 1b) and; (4) production of a dense network of hyphae on the insect cuticle after 48 and 72 h (Fig. 1c, d, e).

### Fungal pathogenicity

#### Mortality of *P. citri* treated with EPF under laboratory conditions

##### Mortality of adult *P. citri*

*L. lecanii* and *L. longisporum* were able to infect and kill the citrus mealybug. Mortality began at 48 and 72 h after treatment for *L. lecanii* and *L. longisporum*, respectively. The cumulative mortality rates increased over time for both species (Table 1); however, differences were observed in the efficacy of the fungal species. *L. lecanii* caused the highest cumulative mortality (96.6%) and *L. longisporum* caused 66.8% mortality at the highest concentration ( $1 \times 10^7$  conidia/ml) at 10 days after infection (Table 1). The MST was 7.10 days and 10.36 days for *L. lecanii* and *L. longisporum* at the highest concentration, respectively. The control mealybugs lived significantly longer with a MST of 9.69 days for *L. lecanii* and 12.55 days for *L. longisporum* (Table 2).

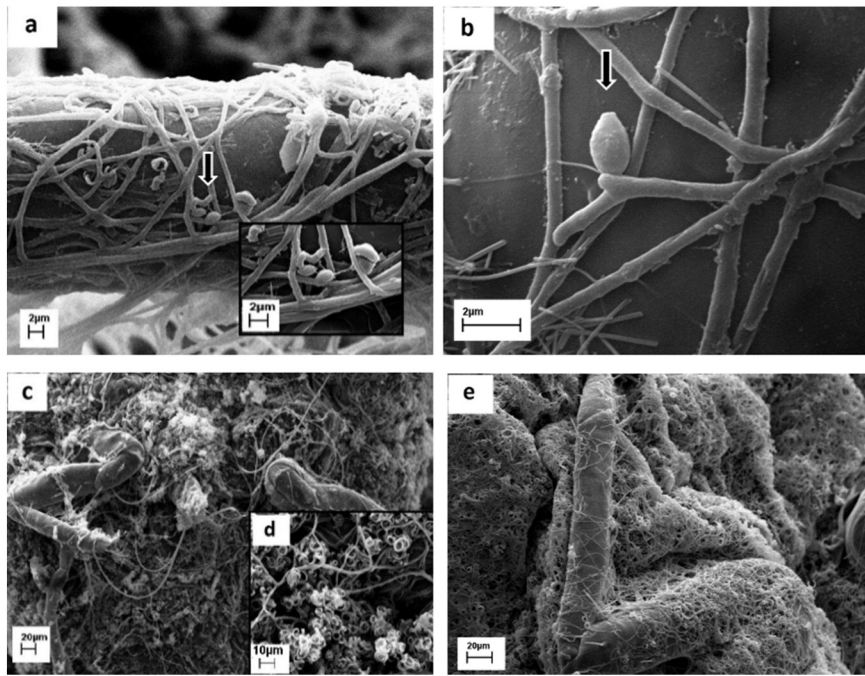
The results showed the significant effects of fungal species ( $F = 87.97$ ;  $df = 24, 1$ ;  $P < 0.0001$ ) and spore concentration ( $F = 12.87$ ;  $df = 24, 3$ ;  $P < 0.0001$ ) on *P. citri* mortality but no significant interactive effect ( $F = 0.53$ ;  $df = 24, 3$ ;  $P = 0.6677$ ). Of the two fungal species, significant differences in mealybug mortality occurred when the mealybugs were treated with spore concentrations (conidia/ml) of  $1 \times 10^4$  ( $F = 61.44$ ;  $df = 6, 1$ ;  $P = 0.0002$ ),  $1 \times 10^5$  ( $F = 57.71$ ;  $df = 6, 1$ ;  $P = 0.0003$ ),  $1 \times 10^6$  ( $F = 18.45$ ;  $df = 6, 1$ ;  $P = 0.0051$ ) and  $1 \times 10^7$  ( $F = 8.02$ ;  $df = 6, 1$ ;  $P = 0.0299$ ) (Table 1). There were significant differences for mortality caused by each fungal species at all concentrations of  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$  and  $1 \times 10^7$  conidia/ml for *L. lecanii* ( $F = 8.89$ ;  $df = 12, 3$ ;  $P = 0.0022$ ) and *L. longisporum* ( $F = 5.83$ ;  $df = 12, 3$ ;  $P = 0.0107$ ) (Table 1).

The mycosis level was dose-dependent. The highest mortality was recorded at the highest concentration. Cumulative mortality in groups treated with low to high doses on the last day of observation were 66.9% to 96.6% for *L. lecanii* and 29.7% to 66.8% for *L. longisporum* (Table 1).

##### Mortality of nymphal stage of *P. citri*

Mycosis in the citrus mealybug nymphs was similar to that in adults. Nymphal mortality started to appear at 48 and 72 h after treatment for *L. lecanii* and *L. longisporum*, respectively. The cumulative mortality also increased over time for both species. *L. lecanii* caused the highest cumulative mortality rate (96.5%) and *L. longisporum* caused a mean 59.14%





**Fig. 1.** SEM of surface body of adult *Planococcus citri* inoculated with conidia suspension of *Lecanicillium longisporum* showing: (a) hyphal network with conidia adhesion and germination on tibia; (b) close-up of appressorium; (c) hyphal network of *Lecanicillium longisporum* over *Planococcus citri* cuticle at 24 h; (d) detail of hyphal development on *Planococcus citri* cuticle at 24 h; (e) dense hyphal network growing on cuticle of *Planococcus citri* at 72 h.

mortality at the highest concentration ( $1 \times 10^7$  conidia/ml) (Table 1). The MST was 4.61 days and 8.87 days for *L. lecanii* and *L. longisporum* at the highest concentration, respectively. The control mealybugs lived significantly longer with a MST of 5.80 days for *L. lecanii* and 11.53 days for *L. longisporum* (Table 2).

The results demonstrated significant main effects for both fungal species ( $F = 458.27$ ;  $df = 24, 1$ ;  $P < 0.0001$ ) and spore concentration ( $F = 19.40$ ;  $df = 24, 3$ ;  $P < 0.0001$ ) on *P. citri* mortality with a significant interactive effect ( $F = 4.32$ ;  $df = 24, 3$ ;  $P = 0.0143$ ). Significant differences in mealybug mortality occurred when the coccid were treated with the following spore concentrations (conidia/ml):  $1 \times 10^4$  ( $F = 215.93$ ;  $df = 6, 1$ ;  $P < 0.0001$ ),  $1 \times 10^5$  ( $F = 144.55$ ;  $df = 6, 1$ ;  $P < 0.0001$ ),  $1 \times 10^6$  ( $F = 88.30$ ;  $df = 6, 1$ ;  $P < 0.0001$ ) and  $1 \times 10^7$  ( $F = 60.63$ ;  $df = 6, 1$ ;  $P = 0.0002$ ) (Table 1). Both fungal species at concentrations of  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$  and  $1 \times 10^7$  conidia/ml showed significant differences for efficacy between *L. lecanii* ( $F = 4.84$ ;  $df = 12, 3$ ;  $P = 0.0196$ ) and *L. longisporum* ( $F = 15.04$ ;  $df = 12, 3$ ;  $P = 0.0002$ )

(Table 1). The mortality caused by these fungi increased as the spore concentration increased. The mean mortality caused by each fungal species was 84.2% to 96.5% for *L. lecanii* and 24.8% to 59.47% for *L. longisporum* (Table 1).

#### Mortality of *P. citri* treated with EPF under greenhouse conditions

##### Mortality of adult *P. citri*

The results of the greenhouse assay shows the potential of both EPF species. Both were capable of infecting and killing mealybugs under greenhouse conditions. The mortality of the treated coccids started at 24 and 48 h after treatment for *L. lecanii* and *L. longisporum*, respectively (Fig. 2). *L. lecanii* caused the highest cumulative mortality (96.5%), followed by *L. longisporum* (66.8%) at the highest concentration (Fig. 2 and Table 3).

The results demonstrated significant main effects for *P. citri* mortality by both species ( $F = 112.09$ ;  $df = 12, 1$ ;  $P < 0.0001$ ) and at two

**Table 1**  
Mean percentage of corrected mortality by *Lecanicillium lecanii* and *Lecanicillium longisporum* on adult and nymphal *Planococcus citri* following exposure to  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$  and  $1 \times 10^7$  conidia/ml under laboratory conditions.

Developmental stages	Adult					Nymph		
	2	4	6	8	10	2	4	6
% mortality ( $\pm$ SE)								
Fungal species: <i>L. lecanii</i>								
$1 \times 10^4$ conidia/ml	9.9 $\pm$ 4.0	22.2 $\pm$ 4.7	39.6 $\pm$ 4.0	54.5 $\pm$ 6.3	66.9 $\pm$ 4.7 Ac	5 $\pm$ 2.8	56.9 $\pm$ 6.2	84.2 $\pm$ 2.8 Ab
$1 \times 10^5$	17.3 $\pm$ 4.7	37.1 $\pm$ 2.4	54.5 $\pm$ 2.8	69.3 $\pm$ 4.0	76.8 $\pm$ 4.7 Abc	5 $\pm$ 2.8	56.9 $\pm$ 4.7	86.6 $\pm$ 2.4 Aab
$1 \times 10^6$	24.7 $\pm$ 6.3	49.5 $\pm$ 5.7	66.8 $\pm$ 2.4	79.2 $\pm$ 4.0	86.7 $\pm$ 4.7 Aab	7.4 $\pm$ 4.7	61.9 $\pm$ 4.7	94.1 $\pm$ 2.8 Aab
$1 \times 10^7$	27.2 $\pm$ 4.7	59.4 $\pm$ 6.9	74.3 $\pm$ 6.3	86.7 $\pm$ 2.4	96.6 $\pm$ 2.4 Aa	9.9 $\pm$ 4	71.8 $\pm$ 8.4	96.5 $\pm$ 2.4 Aa
Fungal species: <i>L. longisporum</i>								
$1 \times 10^4$ conidia/ml	0	4.9 $\pm$ 2.8	9.9 $\pm$ 0.02	19.8 $\pm$ 0.02	29.7 $\pm$ 0.02 Bb	0	17.3 $\pm$ 6.2	24.8 $\pm$ 2.8 Bc
$1 \times 10^5$	0	7.4 $\pm$ 4.7	14.9 $\pm$ 2.8	24.8 $\pm$ 4.9	34.7 $\pm$ 2.8 Bb	0	19.8 $\pm$ 4.0	29.7 $\pm$ 4.0 Bbc
$1 \times 10^6$	0	17.3 $\pm$ 10.1	22.3 $\pm$ 10.9	29.7 $\pm$ 12.7	44.6 $\pm$ 8.5 Bab	0	29.7 $\pm$ 5.7	42.14 $\pm$ 4.7 Bb
$1 \times 10^7$	0	17.3 $\pm$ 6.2	29.7 $\pm$ 8.08	49.5 $\pm$ 8.08	66.8 $\pm$ 10.2 Ba	0	37.1 $\pm$ 2.4	59.47 $\pm$ 4.0 Ba

Mean values within last collected day (8th. for adult and 6th. For nymph) bearing the different upper case letter showing significant difference among fungal species for each concentration. Mean values within last collected day (8th. for adult and 6th. for nymph) bearing the different lower case indicated significant difference between concentrations for each fungus species (Tukey test,  $P > 0.05$ ). The total numbers of replicates were 8 per treatment.

**Table 2**Effect of various conidia concentrations of *Lecanicillium lecanii* and *Lecanicillium longisporum* on adult and nymph of *Planococcus citri*.

Fungi	Concentration (conidia/ml)	Adult			Nymph		
		MST <sup>1</sup>	SE <sup>2</sup>	95% confidence interval	MST	SE	95% confidence interval
<i>L. lecanii</i>	0	9.69 d <sup>3</sup>	0.07	(9.55–9.83)	5.80 c	0.06	(5.68–5.91)
	1 × 10 <sup>4</sup>	8.24 c	0.13	(7.98–8.49)	4.93 b	0.09	(4.74–5.11)
	1 × 10 <sup>5</sup>	7.74 b	0.13	(7.47–7.10)	4.85 ab	0.10	(4.66–5.04)
	1 × 10 <sup>6</sup>	7.36 a	0.13	(7.10–7.63)	4.72 ab	0.10	(4.52–4.91)
	1 × 10 <sup>7</sup>	7.10 a	0.13	(6.83–7.36)	4.61 a	0.10	(4.41–4.80)
<i>L. longisporum</i>	0	12.55 d	0.09	(12.38–12.73)	11.53 d	0.09	(11.36–11.71)
	1 × 10 <sup>4</sup>	11.82 c	0.12	(11.58–12.06)	10.29 c	0.14	(10.03–10.56)
	1 × 10 <sup>5</sup>	11.49 c	0.13	(11.23–11.75)	10.00 c	0.14	(9.73–10.28)
	1 × 10 <sup>6</sup>	10.94 b	0.14	(10.66–11.22)	9.45 b	0.15	(9.16–9.74)
	1 × 10 <sup>7</sup>	10.36 a	0.14	(10.08–10.64)	8.87 a	0.15	(8.59–9.16)

<sup>1</sup> Median survival time in days.<sup>2</sup> Standard error of the MST in days after analyses with the Kaplan-Meier-test.<sup>3</sup> Numbers followed by the same letter are not significantly different ( $P \leq 0.05$ ).

spore concentration ( $F = 18.25$ ;  $df = 12, 1$ ;  $P = 0.0011$ ) without a significant interactive effect ( $F = 2.79$ ;  $df = 12, 1$ ;  $P = 0.1204$ ). There was a significant difference between the mean mortality of the citrus mealybug at spore concentrations (conidia/ml) of  $1 \times 10^6$  ( $F = 84.01$ ;  $df = 6, 1$ ;  $P < 0.0001$ ) and  $1 \times 10^7$  ( $F = 35.95$ ;  $df = 6, 1$ ;  $P = 0.0010$ ) (Table 3). There were also significant differences for mean mortality by *L. longisporum* ( $F = 19.13$ ;  $df = 6, 1$ ;  $P = 0.0047$ ) at  $1 \times 10^6$  and  $1 \times 10^7$  conidia/ml concentrations, but no significant differences within mean mortalities by *L. lecanii* ( $F = 3.14$ ;  $df = 6, 1$ ;  $P = 0.1268$ ) at the same concentrations (Table 3).

#### Mortality of nymphal stage of *P. citri*

The greenhouse assay of the fungal species indicated that mortality started at 24 and 48 h post-treatment with *L. lecanii* and *L. longisporum*, respectively (Fig. 3). The highest mortality was caused by *L. lecanii* (99.9%) followed by *L. longisporum* (85.4%) at the highest concentration at 8 days (Fig. 3 and Table 3).

The bioassay demonstrated significant main effects on *P. citri* mortality by species ( $F = 41.59$ ;  $df = 12, 1$ ;  $P < 0.0001$ ) and spore concentration ( $F = 16.23$ ;  $df = 12, 1$ ;  $P = 0.0017$ ) with a significant interactive effect ( $F = 4.06$ ;  $df = 12, 1$ ;  $P = 0.670$ ). There were significant differences in mealybug mortality for both species when the insects were treated at spore concentrations (conidia/ml) of  $1 \times 10^6$  ( $F = 21.03$ ;  $df = 6, 1$ ;  $P = 0.0037$ ) and  $1 \times 10^7$  ( $F = 33.05$ ;  $df = 6, 1$ ;  $P = 0.0012$ ) (Table 3).

There were no significant differences for mean mortality caused by *L. lecanii* ( $F = 2.78$ ;  $df = 6, 1$ ;  $P = 0.1463$ ) but there were significant differences for *L. longisporum* ( $F = 14.36$ ;  $df = 6, 1$ ;  $P = 0.0091$ ) when treated with  $1 \times 10^6$  and  $1 \times 10^7$  conidia/ml concentrations (Table 3).

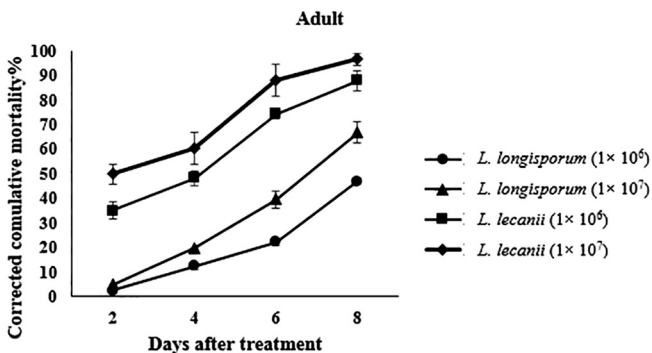
#### Mass production potential of solid substrates

There were significant differences between substrates ( $F = 36.25$ ;  $df = 24, 3$ ;  $P < 0.0001$ ) and fungal species ( $F = 167.91$ ;  $df = 24, 1$ ;  $P < 0.0001$ ) and the interaction between them ( $F = 49.49$ ;  $df = 24, 3$ ;  $P < 0.0001$ ). For *L. lecanii*, the wheat substrate with  $1.90 \times 10^8$  conidia/g showed maximum conidial production. For *L. longisporum*, maximum conidial production occurred using rice bran and beet pulp at  $2.41 \times 10^8$  and  $2.32 \times 10^8$  conidia/g, respectively (Fig. 4).

#### Discussion

The data indicates that *L. lecanii* and *L. longisporum* are pathogenic to the adult and nymphal stages of the citrus mealybug. To explore the precise interactions between host and pathogen, a disease triangle was assumed for determination of the complex interactions between the host, pathogen and the environment. The affecting factors of temperature, humidity, duration of infection, prevalence of pathogen, virulence of pathogen and age or maturity of the host were considered (Tanada and Kaya, 1993; Scholthof, 2007; Boucias and Pendland, 1998).

The role of the first element of the triangle, the pathogen, was addressed using SEM and infectivity assays. The SEM images and data confirmed successful infectivity of the fungus on the coccid. The infection observed by SEM was similar to that described previously (Kim and Roberts, 2012; Güerri-Agulló et al., 2010). Dense networks of *L. longisporum* hyphae covered the cuticle of the citrus mealybug. EPF such as *Metarhizium anisopliae*, *B. bassiana* and most fungi from the order Entomophthorales penetrate their host after germination or the onset of hyphae production (Charmley, 1989), but *L. longisporum* hyphae produced a compact network on its host cuticle before or at the time of



**Fig. 2.** Cumulative mortality of adult *Planococcus citri* at 1–8 days post-treatment by *Lecanicillium lecanii* and *Lecanicillium longisporum* at  $1 \times 10^6$  and  $1 \times 10^7$  conidia/ml under greenhouse conditions (Mean  $\pm$  Standard error of the mean).

**Table 3**Susceptibility of different developmental stages of *Planococcus citri* to *Lecanicillium lecanii* and *Lecanicillium longisporum*. Mean percentage of corrected mortality ( $\pm$  SE) at 8 days post-inoculation under greenhouse conditions.

Developmental stages	Adult		Nymph	
	Number of days after treatment			
	8		8	
Fungal species	<i>L. lecanii</i>	<i>L. longisporum</i>	<i>L. lecanii</i>	<i>L. longisporum</i>
1 × 10 <sup>6</sup> conidia/ml	87.8 $\pm$ 4.2	47.05 $\pm$ 1.4	92.8 $\pm$ 3.7	66.8 $\pm$ 4.2
	Aa	Bb	Aa	Bb
1 × 10 <sup>7</sup>	96.5 $\pm$ 2.4	66.8 $\pm$ 4.2	99.05 $\pm$	85.4 $\pm$ 2.3
	Aa	Ba	0.01 Aa	Ba

Mean values of each stage within row bearing different upper case letter are significantly different among fungal species and within column bearing the different lower case letters are significantly different among concentrations (Tukey test,  $P > 0.05$ ). The total numbers of replicates were 8 per treatment.

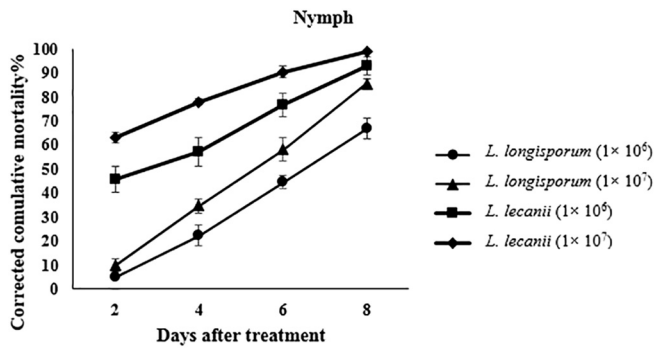


Fig. 3. Cumulative mortality of nymph stage of *Planococcus citri* at 1–8 days post-treatment by *Lecanicillium lecanii* and *Lecanicillium longisporum* at  $1 \times 10^6$  and  $1 \times 10^7$  conidia/ml under greenhouse conditions (Mean  $\pm$  Standard error of the mean).

penetration, an event that confirms earlier observations (Schreiter et al., 1994).

Hall (1976) stated that *V. lecanii* is unusual compared to other hyphomycetous fungi. He reported that, at high concentrations, microscopical examination showed abundant hyphal growth and sporulation over the entire surface of the host, but no invasion of internal tissue. The growth of dense hyphae on the surface may depend on factors such as relative humidity and food availability. Successful penetration by EPF of the cuticle requires energy, and this strategy allows *L. longisporum* to use components of the epicuticle as amino acids, fatty acids and hydrocarbons (Charnley, 1989). It was assumed that the dense networks of *L. longisporum* hyphae increased the possibility of propagules finding appropriate sites to enter the host as stated by Wraight et al. (1990).

Knowing the infection process in the host can be useful to understanding the mechanism of EPF influence. Güerri-Agulló et al. (2010) found that SEM data aided understanding possible methods for development of fungal-based insecticides.

One criterion associated with the pathogen properties is the difference between various species and strains. *Lecanicillium* spp. have been reported to produce secondary metabolites with insecticidal activity (Kumar et al., 2015), such as bassianolide, which plays a notable role in virulence and overcomes its hosts (Goettel et al., 2008). EPF host specificity is an innate ability and various classes of pathogenic genes enable the fungus to escape host defense mechanisms, penetrate the host cuticle or grow rapidly (Boucias and Pendland, 1998). Different species of EPF and strains (isolates) within the species show a high degree of specificity (Boucias and Pendland, 1998; Kim and Roberts, 2012). Different strains of *Lecanicillium* spp. have differently sized conidia (Sugimoto et al., 2003), different speeds of germination, germination rates and capacities for sporulation (Fazeli-Dinan et al., 2016; Feng et al., 2002). These physiological characteristics affect pathogenic

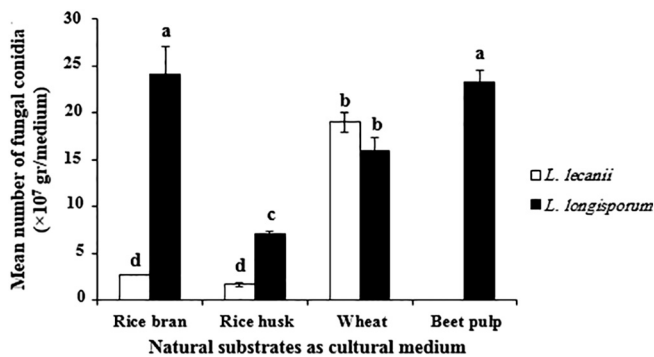


Fig. 4. Mean number of conidia ( $\pm$  SE) produced by four solid substances (rice bran, rice husk, wheat, and beet pulp) for *Lecanicillium lecanii* and *Lecanicillium longisporum*. Letters denote significant differences between interactive fungal species and substance; Tukey's test;  $p < 0.05$ .

abilities and allow their selection as an entomopathogen for biological control.

Mortality at all stages was dose-dependent, with the highest mortality occurring at the highest dose. If the conidia concentration released into the environment increases, the possibility of the host coming into contact with infective conidia will increase (Hajek and Delalibera, 2010). The virulence of fungal treatments observed on the *P. citri* were in line with results reported elsewhere. Demirci et al. (2011) stated that the percentage of mortality at all stages of *P. citri* inoculated with *Isaria farinosa* increased as the humidity and inoculum density increased under laboratory conditions. Shrestha et al. (2015) found that susceptibility at all stages of the lettuce aphid (*Nasonovia ribisnigri*) infected by *B. bassiana* was dose-dependent in the laboratory and under semi-field evaluations. Panahi and Loni (2013) maximized control of *Trialeurodes vaporariorum* at the highest concentration of *L. longisporum*. In another study, the number of surviving individuals of *T. vaporariorum* decreased as the concentration of *L. longisporum* increased (Fazeli-Dinan et al., 2016). Kim and Roberts (2012) also showed that the same result was true for *Lecanicillium attenuatum* treated with *Aphis gossypii*.

The potential of various media as inexpensive sources for semi-mass rearing was explored. The successful implementation of EPFs in IPM (Integrated Pest Management) programs of greenhouses required a high conidial concentration. The results of the current study proved that the amount of spore production differed by fungal species and strains or according to the media on which the fungi grow. This fact was supported by Bhadauria et al. (2012), who studied mass production of *B. bassiana* on 15 whole grains and found that the highest conidial count was on cowpea media ( $9.06 \times 10^7$  conidia/ml) and that the amount of conidia on wheat bran was  $2.66 \times 10^7$  conidia/ml. Similar to the current findings, Grajek (1994) calculated that conidial production of *V. lecanii* on wheat bran, sugar-beet and mixed media were  $3.0 \times 10^9$ ,  $1.6 \times 10^9$  and  $3.2 \times 10^9$  conidia/g. Vu et al. (2008) evaluated spore production by *L. lecanii* 41,185 on wheat bran powder, polished rice, rice bran, rice husks, peat moss and polished rice mixed with rice bran and found that the best substrate was polished rice with  $5.7 \times 10^9$  conidia/g.

Different grains or other solid media have different amounts of nutrients to supply for the growth of EPF (Bhadauria et al., 2012). Surface-to-volume ratio is also a factor in spore production (Bena-Molaei et al., 2000). Different solid substrates need different water contents for spore production (Feng et al., 2000). In the current study, wheat and rice bran recorded the highest spore production for *L. lecanii* and *L. longisporum*, respectively. These media have larger surface-to-volume ratios and their coarse particles maintain sufficient water for spore production. Coarse particles of wheat and rice bran provide enough space for the exchange of oxygen and CO<sub>2</sub>, which was also observed by Bena-Molaei et al. (2000).

Environmental conditions, especially very high humidity (>90% RH) and temperature, affect spore germination of the infective propagules (Demirci et al., 2011) as they multiply in the host and spread to other susceptible hosts (Boucias and Pendland, 1998). The recommended humidity for commercial *Lecanicillium* spp. is 80% to 95% at the leaf surface for 10–12 h per day for several days (Jaronski, 2009). The area between the mealybug body and leaf surface and the waxy structure on the cuticle of mealybug bodies provide sufficient humidity for germination of conidia. To a lesser extent, air movement also affects the infection and sporulation of EPF (Demirci et al., 2011) prepared in a greenhouse. This was tested under controlled conditions in the greenhouse.

The main element related to the host was the influence of immature versus adult stages of the coccid. Susceptibility of insects to pathogens differs according to the developmental stage of the host. These differences between stages of the citrus mealybug may also relate to differences in physiology or exoskeleton biochemical composition as protective barriers that change with the developmental stage of the insect, as for other arthropods (Boucias and Pendland, 1998; Willis et al., 2005). EPF, after entry into the host and reaching the hemolymph,



must evade immune responses that have developed gradually (Willis et al., 2005; Vega et al., 2012). The current study illustrated the importance of the life stages of the host in relation to EPF and showed that the second instar nymph of *P. citri* is generally more susceptible to infection by both fungi than the adult.

EPF infects by cuticular penetration, making the molting time a factor influencing fungal infection (Kim and Roberts, 2012; Vega et al., 2012). The speed of fungal germination is also an important criteria for reduction of exposure to unfavorable environmental factors and insect molting (Vega et al., 2012). Because citrus mealybugs molt their cuticle at long intervals (every 7 to 16 days) (Kerns et al., 2001), *L. lecanii* and *L. longisporum* have ample opportunity for infection. The presence of a waxy coating on the third nymphal instar and adult of citrus mealybugs reduces the chance of conidia coming into contact with and entering the cuticle (Hogendorp et al., 2009).

The current study provided both basic and applied data about the interaction of EPF and the citrus mealybug. This is an initial insight into the pathology and efficacy of *Lecanicillium* spp. against *P. citri*. The results answered major questions about the ability of *Lecanicillium* spp. to infect the citrus mealybug and the potency of the fungi against important life stages of the pest in the laboratory and greenhouse conditions. For semi-mass rearing of virulent species/strain of fungi, it is critical to understand, in relation to the cost/benefits of the microbial control program, which material can provide a higher conidia yield.

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