RESEARCH

VIRULENCE AND PRE-LETHAL REPRODUCTIVE EFFECTS OF *Metarhizium anisopliae* var. *anisopliae* ON *Pseudococcus viburni* (HEMIPTERA: PSEUDOCOCCIDAE)

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Obscure mealybug, *Pseudococcus viburni* (Signoret), is a cosmopolitan pest that causes damage by suction of vascular juices and the production of honeydew, as well as for being a quarantine insect. Within control options, entomopathogenic fungi are a good alternative, nevertheless, more research is needed. In this research, the *Metarhizium anisopliae* var. *anisopliae* (Metschnikoff) isolate Qu-M984 was evaluated on *P. viburni* under laboratory conditions. Virulence was evaluated by lethal dose 50 (LD_{50}) and lethal time 50 (LT_{50}), for each of the four life stages of the female. The doses tested were 10^5, 10^6, 10^7 and 10^8 conidia mL^{-1}. Also fecundity, egg size, fertility, and longevity of adult females were evaluated at doses of 10^5 and 10^6 conidia mL^{-1}, the evaluations were made every 2 d throughout the insect life time. The LD_{50} and LT_{50} obtained were variable for each life stage, although without statistical differences among life stages (P > 0.05), ranging from $LD_{50} = 7.3 \times 10^5$ to 4.9×10^9 conidia mL^{-1} and $LT_{50} = 7.74$ to 9.97 d at 10^8 conidia mL^{-1}. Significant differences (P ≤ 0.05) were observed on longevity at 10^6 conidia mL^{-1}. Longevity was 29% less compared to the control. This result on longevity at relatively low dose is relevant due to the fact that decrease possibilities to find live quarantine insects at the moment of harvest. Fertility, fecundity and egg size showed no differences (P > 0.05).

Key words: Lethal dose, lethal time, biological control, entomopathogenic fungus, fecundity, fertility, longevity.

*P*seudococcus viburni Signoret (Hemiptera: Pseudo-coccidae), commonly named obscure mealybug, is a cosmopolitan insect present in temperate climates areas of the Northern Hemisphere, South Africa, Australia, New Zealand, and Chile (González *et al.*, 2001). It is polyphagous, attacking ornamentals, fruit trees, forest trees, and cactus. It has an oval and wax covered body. The direct damage is produced by suction of vascular juices and the production of sweet honeydew (González, 1989). The affected parts of the plant present a dark oily aspect, due to the sweet honeydew that is infected by fungus and dust, commonly named sooty mould (Artigas, 1994), which greatly depreciates the quality of fruit such as table grapes (*Vitis vinifera* L.) (Bournier, 1976). Obscure mealybug is a quarantine insect of mandatory control that hides in places of difficult access (González, 2003b). Most *Pseudococcus* species cause low direct damage and are considered minor plagues, and major plagues for being quarantine insects on grapes, pomaceous, and stone fruit (González, 2003a). The markets with quarantine restriction to *P. viburni* are Brazil, Mexico, Japan, and Korea (González and Volosky, 2006). Within insect pathogens, entomopathogenic fungi are the main contenders for commercial production

and use against homopterous pest insects (Lacey *et al.*, 2001). Entomopathogenic fungi do not cause immediate mortality. The activity of infected hosts can be altered just before death due to microbial infections (Hajek *et al.*, 2008).

A minimal amount of pathogen exists to start a disease (Alves and Lecuona, 1998), as well as a positive correlation between infectious conidia and mortality by mycosis (Ferron, 1978). There are sublethal effects that can affect the reproductive output of infected adult females with entomopathogenic fungi before death (Roy *et al.*, 2006). On the other hand, this infection does not have always the same effect on reproduction, being able to present a decrease or increase on fecundity, as well as a decrease on longevity (Hajek *et al.*, 2008). Whereas the majority of studies assessing biocontrol agents deal with their ability to produce mortality in the target pest, a considerable number have also being assessed for the impact that infection may produce on host behaviors. These include effects on developmental time, fecundity and feeding (Blanford and Thomas, 2001). Different studies with entomopathogenic fungi have shown effects over the reproductive potential of infected females. Castillo *et al.* (2000) show that *Metarhizium anisopliae* affect females fecundity of *Ceratitis capitata* Wiedemann, as well as *Blatella germanica* (Linneo) (Quesada-Moraga *et al.*, 2004) and *Anopheles gambiae* (Giles) (Scholte *et al.*, 2006), among other species.

The aim of this research was to evaluate the virulence of different doses of *M. anisopliae* var. *anisopliae* isolate Qu-M984 on developmental stages of *P. viburni* and the pre-lethal effects fertility, egg size, fecundity, and longevity on the adult female.

MATERIALS AND METHODS

Insects
Pseudococcus viburni Signoret (Hemiptera: Pseudococcidae) were obtained from an artificial mass rearing unit maintained since 2005 by Instituto de Investigaciones Agropecuarias INIA Quilamapu (Chillán, Chile). Insects were reared on potato (*Solanum tuberosum* L.) buds and held in total darkness at 23 ± 1 ºC and 80% ± R.H. The four developmental stages were used for experimentation and the selection criteria was the movement capacity and acceptable healthy. The three nymph stages and adult females were identified under stereomicroscope of 40X, according to insect size and presence of marginal filaments, using the classification of the four developmental stages of *P. viburni* females described by Oyarzún (2004).

Conidial production
Metarhizium anisopliae var. *anisopliae* isolate Qu-M984, was obtained from the entomopathogenic fungi collection in INIA Quilamapu (Chillán, Chile). The fungus was cultivated on potato dextrose agar (PDA) medium in Petri dishes, inoculated in biological safety cabinet and placed in an incubator at 25 ºC for 2 wk in complete darkness until sporulation. Conidia were harvested directly by scraping from the surface culture and suspended in sterile distilled water containing 0.01% Tween 80 (Sigma®, Steinheim, Germany) to produce a homogenous suspension. The concentration of the suspension was determined by using a haemocytometer (BOECO, Neubauer, Germany) under microscope 1000X, and diluted with sterile distilled water plus 0.01% Tween 80 to obtain the conidial concentrations for each trial.

Virulence
Insects of the four developmental stages were placed on cylindrical flasks of 39 mL and sprayed with the doses 10^5, 10^6, 10^7 and 10^8 conidia mL^{-1}, the control was treated with sterile distilled water. All treatments contained 0.01% Tween 80. A Potter spray tower at 0.1 MPa was used to apply the conidia over the individuals that were immediately transferred to a piece of potato buds serving as food and maintained at 23 ± 1 ºC in the dark. Mortality was recorded for each individual every 2 d until 19 d post application. All dead insects were placed individually in a humid chamber at 25º C to facilitate the appropriate sporulation of *M. anisopliae* on the insect surface to confirm mycosis (Butt and Goettel, 2000). The criteria to determine mortality was the response to mechanical stimuli, being considered dead insects those with no evident reaction.

The experimental design was completely randomized, with five treatments and four replicates, each experimental unit consisting of 10 insects, for each of the four developmental stages of the female. Mortality rates were separated by Tukey test (P < 0.05) and compared by one-way ANOVA using the statistical program SAS (SAS Institute, 2003). The recorded means of mortality were corrected using Abbott formula (Abbott, 1925). Lethal dose (LD_{50}) and lethal time (LT_{50}) were estimated using Probit analysis (Statgraphics, 2000). Fit of the regression lines were verifying using χ^2 test (SAS Institute, 2003).

Pre-lethal effects
Fertility and egg size. The doses used on the trials of fertility, egg size, longevity, and fecundity were 10^5 and 10^6 conidia mL^{-1}. Control individuals were treated with sterile distilled water. All treatments contained Tween 80 at 0.01%. The low doses 10^5 and 10^6 conidia mL^{-1} were selected to increase possibilities of a slow infection by the fungus in the insect and analize sublethal effects before death, avoiding a high and quick mortality. Insects selected were adult females. Egg fertility was estimated registering the eclosion of eggs by number of nymphs emerged, checking the total eclosed and non eclosed eggs as well as the empty chorion left by eclosed eggs. The recorded means were analyzed using Chi-square test with an experimental unit of 25 eggs per female (SAS Institute, 2003). Egg size was recorded by taking samples of 25 eggs for each female, separating the wax and placing them in horizontal position, in order to record the length by digital pictures, using UTHSCSA ImageTool program (University of Texas Health Science Center, 1996). The recorded means of egg size were separated by Tukey test (P < 0.05) and compared by one-way ANOVA using the statistical program SAS (SAS Institute, 2003).

Fecundity and longevity. The treatments used on theses trials were the same than in fertility and egg size. Insects selected were adult females; the experimental unit consisted of 10 insects, replicated four times. The insects had contact with active males in the artificial rearing, prior to selection and, before fungi spraying, were added three males for each 10 females for 24 h to increase fecundity possibilities. According to Oyarzún (2004) one male is capable to fecundate six females daily. All individuals were sprayed with *M. anisopliae* Qu-M984 at the doses mentioned above using a Potter spray tower. Before spraying the insects, they were placed in cylindrical flasks of 39 mL in groups of 10, later each insect was located separately in flasks of the same characteristics and fed with potato buds held at 23 ± 1 ºC in dark and 80% ± R.H. Oviposition and mortality were individually recorded under stereomicroscope of 40X, every 48 h, until death of all insects. Fecundity of each female was determined

counting number of eggs every 48 h. To assess longevity mortality was recorded, considering dead insects those with no response to mechanical stimuli. The recorded means of fecundity and longevity were separated by Tukey test (P < 0.05) and compared by one-way ANOVA using the statistical program SAS (SAS Institute, 2003).

RESULTS AND DISCUSSION

Virulence

The isolate studied demonstrated pathogenicity for all developmental stages when clumped together (P ≤ 0.05), being mortality dose-dependent, mean values varying from 48% (10^5 conidia mL^{-1}) to 78% (10^8 conidia mL^{-1}), compared to 25.6% in the control (Figure 1). There were no differences among developmental stages (P > 0.05) with the only exception when using the dose 10^7 conidia mL^{-1} (P < 0.05) Likewise, there were differences (P < 0.05) within doses for nymph II, nymph III, and adult developmental stages, although no difference was found for nymph I (P > 0.05) (Table 1).

Lethal doses (LD$_{50}$) values ranged from 7.3×10^5 to 4.9×10^9 conidia mL^{-1} (adult and nymph I, respectively). Lethal time values at the higher dose (10^8 conidia mL^{-1}) ranged from 7.74 to 10 d among the different developmental stages (Table 2). All regression lines were verified and showed homogeneity for goodness of fit by χ^2. LT$_{50}$ obtained demonstrated that the dose 10^8 conidia mL^{-1} decreased life time period in all developmental stages, affecting the reproductive potential of females prior to death, decreasing fertile period and oviposition time. There is no evidence of TL$_{50}$ with entomopathogenic
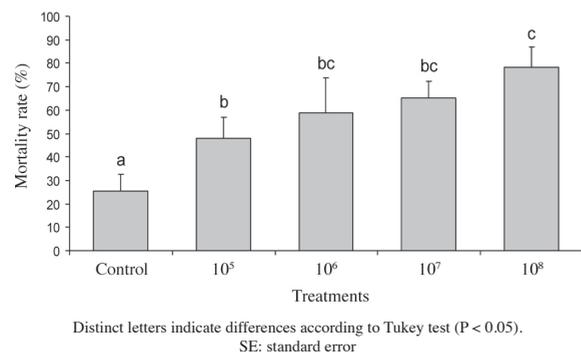
Table 2. LD$_{50}$ and LT$_{50}$ of *Metarhizium anisopliae* isolate QU-M984 on the different developmental stages of *Pseudococcus viburni*.

Development stage	LD$_{50}$	LT$_{50}$
	conidia mL^{-1}	d
Nymph I	$4.9 \times 10^9 \pm 3.93$NS	10.0 ± 1.02NS
Nymph II	$7.3 \times 10^6 \pm 3.75$	7.74 ± 1.10
Nymph III	$4.7 \times 10^6 \pm 4.19$	9.72 ± 1.18
Adult	$7.3 \times 10^5 \pm 4.02$	8.61 ± 1.36

LD$_{50}$ at 19 d post-inoculation; LT$_{50}$ at 10^8 conidia mL-1; NS: non significant (P = 0.05); ± Standard error.

fungi and *P. viburni*, but authors have documented that *Trialeurodes vaporariorum* and *Bemisia tabaci*, treated with *B. bassiana* obtained TL$_{50}$ ranging from 5.9 to 7.4 d with 10^7 conidia mL^{-1} on fourth stage (Quesada-Moraga *et al*., 2006). Therefore, the highest dose used (10^8 conidia mL^{-1}) needed a short time to reach 50% mortality on all developmental stages of *P. viburni*, with similar values obtained by entomopathogenic fungi in insects of the same kind.

Pre-lethal effects

Fertility and egg size. No significant differences were found (P > 0.05) (Table 3) in egg fertility and egg size. In fertility was recorded 94%, 95% and 93% of eclosed eggs (control, 10^5 and 10^6 conidia mL^{-1}, respectively). The eggs of infected insects by entomopathogens can result in total or partial infertility, or contain infected embryos that will be inoculum for the dispersion of the pathogen (Alves and Pereira, 1998). In this study effects of decreasing egg fertility on survival females at doses of 10^5 and 10^6 conidia mL^{-1} were not detected. Similar results on egg fertility were observed on *B. tabaci* (Hemiptera: Aleyrodidae) and *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) using *B. bassiana* (Fargues *et al*., 1991). Likewise, inoculating directly on eggs of *Bemisia argentifolii* with *Verticillium lecanii* Zimmermann, this showed no pathogenic effects, however all other developmental stages of the species were affected by the pathogen (Gindin *et al*., 2000). On the other hand, other studies have shown effects on egg fertility of survival females infected by *M. anisopliae*, as

Distinct letters indicate differences according to Tukey test (P < 0.05).
SE: standard error

Figure 1. Mortality rate (± SE) 19 d after aplication of *Metarhizium anisopliae* (conidia mL^{-1}) for all developmental stages of *Pseudococcus viburni*.

Table 3. Mean (± SE) total eggs, eggs d^{-1}, and egg size for *Pseudococcus viburni* treated with *Metarhizium anisopliae* isolate QU-M984.

Dose (conidia mL^{-1})	Total eggs	Eggs d^{-1}	Egg size (mm)
10^6	133.6 ± 18.29NS	13.4 ± 2.16NS	0.382 ± 0.0023NS
10^5	120.4 ± 18.46	9.1 ± 1.17	0.376 ± 0.0029
Control	176.9 ± 17.79	12.3 ± 1.18	0.380 ± 0.0021

NS: non significant (P = 0.05); SE: standard error.

Table 1. Virulence of *Metarhizium anisopliae* isolate QU-M984 on the different developmental stages of *Pseudococcus viburni*.

Development stage	Dose (conidia mL^{-1})				
	Control	10^5	10^6	10^7	10^8
Nymph I	37.5 ± 4.8aA	50.0 ± 10.8aA	62.5 ± 21.7aA	52.5 ± 11.1abA	65.0 ± 8.7aA
Nymph II	25.0 ± 5.0aA	52.5 ± 2.5aABC	70.0 ± 12.2aBC	42.5 ± 2.5aAB	80.0 ± 10.8aC
Nymph III	27.5 ± 11.1aA	40.0 ± 5.8aAB	52.5 ± 12.5aABC	85.0 ± 6.5cC	80.0 ± 8.2aBC
Adult	12.5 ± 4.8aA	50.0 ± 12.9aAB	50.0 ± 10.8aAB	80.0 ± 7.1bcB	87.5 ± 6.3aB

Means (± SE) within a column followed by the same lower case letter and within row followed by the same upper case letter are not significantly different according to Tukey test (P = 0.05).

in the case of *Megalurothrips sjostedti* at concentrations of 10^6 to 10^8 conidia mL^{-1} (Ekesi and Maniania, 2000) and *Cylas puncticollis* at concentrations from 1×10^6 to 3.0×10^7 conidia mL^{-1} (Ondiaka *et al*., 2008).

Fecundity. Oviposition throughout the time reached a peak on the fourth day, and then decreased gradually, giving the same response in all treatments. No significant difference was found (P > 0.05) (CV = 77%) (Figure 2). The total laid eggs counted throughout the lifetime of each adult female was 177 eggs to control, 134 eggs with 10^6 and 120 with 10^5 conidia mL^{-1}. Likewise, in the number of eggs d^{-1} by female ranged among 9.1 and 13.4 eggs and no significant differences were found (P > 0.05, CV = 83%) (Table 3).

There was no effect of *M. anisopliae* isolate Qu-M984 on fecundity of *P. viburni* in the assessed doses. This could be due to sample heterogeneity, evidenced by the high coefficient of variation (CV= 77%) not being possible to detect statistical differences. The distribution of the number of eggs laid throughout the female life span does not change among treatments when exposed to *M. anisopliae* (Figure 2), which differs to other studies where insects exposed to entomopathogenic fungi have shown higher initial number of eggs than the control, however, without statistical differences (Arthurs and Thomas, 2000; Pires *et al*., 2008). The development of a disease produced by a pathogen could be affected by biotic factors on the host, such as reproductive properties characteristics of the insect, population characteristics (individual susceptibility), and insect habits, among others (Alves and Lecuona, 1998). On the other hand, it should be considered the number of the individuals of the population inoculated, since the higher densities of insects increase the contact possibilities among them and consequently horizontal transmission, something that was not observed in this study, because the insects under study were isolated individuals. Also, the fitness of a specimen is directly dependent on the number of viable offspring produced, and both the pathogen and the host adopt strategies to maximize reproductive output (Baverstock *et al*., 2006; Roy *et al*., 2006). Also temperature can
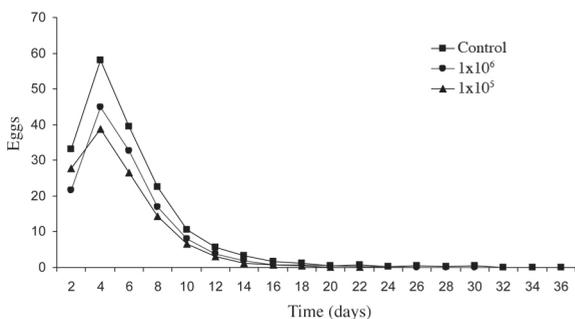
influence fecundity response. *Leptinotarsa decemlineata* treated with *B. bassiana* decreased its reproductive output at 23 ºC, but not at 25 ºC (Fargues *et al*., 1991). In our research this effect was not evaluated, since a constant 23 ± 1 °C was used.

Any measurable behavior presents response thresholds and the form or magnitude often increases with dose (Hoy *et al*., 1998), therefore, it is possible that when doses are increased the reproductive output decreases. There are no registers of the effects of entomopathogens on *P. viburni*, however, it is possible to make comparisons with other insects. Similar results to this study were observed in the reproductive output of *Tutta absoluta* (Pires *et al*., 2008), using doses of 10^7 conidia mL^{-1} of *M. anisopliae* and *Diuraphis noxia* (Wang and Knudsen, 1993) using *B. bassiana*. Other studies have shown positive results on this topic, for example *Cylas puncticollis* (Ondiaka *et al*., 2008), *Anoplophora glabripennis* (Hajek *et al*., 2008), *Megalurothrips sjostedti* (Ekesi and Maniania, 2000), among others.

Longevity. A significant difference was found in longevity of adult females among treatments (P ≤ 0.05) (Table 4, Figure 3). At 10^6 conidia mL^{-1} there were differences compared to the control, however, no differences were detected between 10^5 conidia mL^{-1} and control. With the dose 10^6 conidia mL^{-1} was recorded a decrease of 11.8 d compared to the control (29%) (CV = 62%; P ≤ 0.05). The oviposition time was not significantly (P > 0.05) affected by the fungus, varying among 12 to 14.7 d (Table 4). Longevity of adult females was affected by *M. anisopliae* isolate Qu-M984 at 10^6 conidia mL^{-1}. This could be the threshold concentration when the fungi start to affect longevity in females of the obscure mealybug. The decreased longevity at relatively low dose (10^6

Table 4. Average (± SE) longevity and oviposition time for *Pseudococcus viburni* treated with *Metarhizium anisopliae* isolate QU-M984.

Dose	Longevity rate	Oviposition time
conidia mL^{-1}		d
10^6	29.62 ± 2.25a	12.06 ± 1.29a
10^5	35.68 ± 2.30ab	12.18 ± 1.03a
Control	41.43 ± 1.36b	14.74 ± 1.11a

d: days after treatment, SE: standard error. Means within columns followed by the same letters are not significantly different according to Tukey test (P = 0.05).
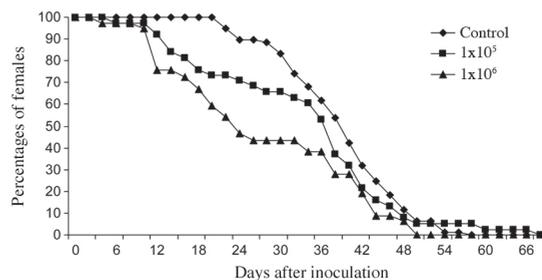

Figure 2. Mean eggs laid by adult females of *Pseudococcus viburni* exposed to *Metarhizium anisopliae* isolate Qu-M984.

Figure 3. Longevity of adult females of *Pseudococcus viburni* treated with *Metarhizium anisopliae* isolate QU-M984.

conidia mL^{-1}), reduces the direct damage caused by the insect, which does not have economic importance, but takes relevance when decreasing possibilities to find live quarantine insects at the moment of harvest. Similar results to this study were obtained with *Diuraphis noxia* Kurdiumov, when pre-lethal effects of entomopathogenic fungi were evaluated and found effects only on longevity (Wang and Knudsen, 1993).

CONCLUSIONS

Metarhizium anisopliae var. *anisopliae* isolate Qu-M984 demonstrated pathogenicity for *Pseudococcus viburni*, under controlled laboratory conditions. Virulence was dose-dependent and showed high mortality in short term. Low doses of the isolate do not affect pre-lethal reproductive effects, such as fecundity, fertility and egg size, however, longevity is affected to a low dose. These results showed that *M. anisopliae* isolate Qu-M984 is an option to control this pest; nevertheless research at field conditions is needed.

ACKNOWLEDGEMENTS

Virulencia y efectos pre-letales en la reproducción de *Metarhizium anisopliae* var. *anisopliae* en *Pseudococcus viburni* (Hemiptera: Pseudococcidae). Chanchito blanco de la vid, *Pseudococcus viburni* (Signoret), es una plaga cosmopolita que causa daños tanto por succión de jugos vasculares como por su producción de mielecilla, así como también por ser un insecto cuarentenario. Dentro de las opciones de control, hongos entomopatógenos son una buena alternativa, sin embargo, más investigación es necesaria. En esta investigación fue evaluado *Metarhizium anisopliae* var. *anisopliae* (Metschnikoff) aislamiento Qu-M984 en *P. viburni* bajo condiciones de laboratorio. Fue evaluada virulencia según dosis letal 50 (LD$_{50}$) y tiempo letal 50 (LT$_{50}$) para cada uno de los cuatro estados de desarrollo de la hembra. Las dosis evaluadas fueron 10^5, 10^6, 10^7 y 10^8 conidias mL^{-1}. Fecundidad, tamaño de huevos, fertilidad y longevidad de hembras adultas fueron evaluados con dosis 10^5 y 10^6 conidias mL^{-1}, las evaluaciones fueron realizadas cada 2 d durante todo el período de vida de los insectos. Las LD$_{50}$ y LT$_{50}$ obtenidas fueron variables para cada estado de desarrollo, sin mostrar diferencias significativas (P > 0.05) entre estados de desarrollo, fluctuando entre LD$_{50}$ = 7.3 × 10^5 - 4.9 × 10^9 conidias mL^{-1} y LT$_{50}$ =7.74 - 9.97 d con 10^8 conidias mL^{-1}. Diferencias significativas (P ≤ 0.05) fueron observadas en longevidad a 10^6 conidias mL^{-1}. Longevidad fue 29% menor comparado con el testigo. Esta disminución en longevidad a una dosis relativamente baja es relevante debido a que disminuyen las posibilidades de encontrar insectos cuarentenarios vivos al momento de cosecha. Fertilidad, fecundidad y tamaño de huevos no mostraron diferencias (P > 0.05).

Palabras clave: dosis letal, tiempo letal, control biológico, hongo entomopatógeno, fecundidad, fertilidad, longevidad.

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