Short note [Nota corta]

BIOLOGICAL ACTIVITY OF *Bacillus thuringiensis* CULTURE SUPERNATANT ON *Bemisia tabaci* AND ITS PARASITOID *Eretmocerus eremicus*†

[ACTIVIDAD BIOLÓGICA DEL SOBRENADANTE DE CULTIVO DE *Bacillus thuringiensis* EN *Bemisia tabaci* Y SU PARASITOIDE *Eretmocerus eremicus*]

Esaú Ruiz-Sánchez[1], Alejandro García-Ramírez[1,*], Arturo Reyes-Ramírez[1], Marcela Gamboa-Angulo[2], José María Tun-Suárez[1] and Jairo Cristóbal-Alejo[1]

[1] Tecnológico Nacional de México, Instituto Tecnológico de Conkal, Avenida Tecnológico s/n, C.P. 97345, Conkal, Yucatán, México. Tel. 01 (999) 9-12-41-30 Ext. 121. Email: alejandro.garcia@itconkal.edu.mx
[2] Centro de Investigación Científica de Yucatán. Calle 43, No. 130 X 32 y 34, Col. Chuburná de Hidalgo, Mérida, C.P. 97205, Yucatán, México. Tel. 01 (999) 9-42-83-30 Ext. 192.
**Corresponding author*

SUMMARY

The effects of *Bacillus thuringiensis* culture supernatants and their derived products were evaluated on whitefly *Bemisia tabaci* and its parasitoid *Eretmocerus eremicus*. Evaluated variables on *B. tabaci* included adult repellence, oviposition deterrence and nymphal mortality. The most active products were evaluated on mortality of *E. eremicus*. Residual solid of strain ITCBT62 supernatant showed significant effects on adult repellence (RI, 0.42) and oviposition deterrence (ODI, -70.1). Residual solid of strain ITCBT62 supernatant and ethanol extract of strain ITCBT61 supernatant caused higher mortality of *B. tabaci* nymphs (53.8 % and 51.2 %, respectively). None of these supernatant derived products had toxic effects on the parasitoid *E. eremicus*. The analysis of ethanol extract of strain ITCBT61 supernatant by liquid chromatography/high resolution mass spectrometry (LC-HRMS) showed the presence of various compounds, with one particularly abundant metabolite of molecular formula $C_{15}H_{20}N_2O$, which has not been previously reported as product of the liquid culture of *B. thuringiensis*.
Keywords: *Bacillus thuringiensis*; whitefly; oviposition deterrence; ethanol extract; culture supernatant.

RESUMEN

Se evaluó el sobrenadante del cultivo de *Bacillus thuringiensis* en la mosca blanca *Bemisia tabaci* y *Eretmocerus eremicus*. Las variables evaluadas en *B. tabaci* incluyeron repelencia de adultos, disuasión de oviposición y mortalidad de ninfas. Los productos activos también se evaluaron en *E. eremicus*. En *B. tabaci*, el residuo sólido del sobrenadante de la cepa ITCBT62 causó repelencia de adultos (RI, 0.42) y disuasión de oviposición (ODI, -70.1). El residuo sólido del sobrenadante de la cepa ITCBT62 y el extracto etanólico del sobrenadante de la cepa ITCBT61 causaron mayor mortalidad de ninfas de *B. tabaci* (53.8 % y 51.2 %, respectivamente). Ninguno de estos dos productos tuvo efectos en *E. eremicus*. El análisis del extracto etanólico del sobrenadante de la cepa ITCBT61 por cromatografía líquida/espectrometría de masas de alta resolución (LC-HRMS) mostró la presencia de varios compuestos. El más abundate tiene fórmula molecular $C_{15}H_{20}N_2O$, que no ha sido previamente reportado como producto contenido en el cultivo líquido de *B. thuringiensis*.
Palabras clave: *Bacillus thuringiensis*; mosca blanca; disuasión de oviposición; extracto etanólico; sobrenadante de cultivo.

† *Submitted October 22, 2018 – Accepted March 12, 2019.*
ISSN: 1870-0462

INTRODUCTION

The whitefly *Bemisia tabaci* is a highly harmful pest on tropical and subtropical agroecosystems. Whitefly infestations in horticultural crops result in crop yield losses up to 100 % when severe transmission of Begomovirus occurs (Brown *et al*., 1995; de Barro *et al*., 2011). The frequent use of neonicotinoids and other systemic insecticides has selected *B. tabaci* resistant populations (Horowitz and Ishaaya, 2014). To face the increasing concern on the use of synthetic insecticides, other strategies to manage *B. tabaci* have been implemented, such as the use of plant derived compounds and microbial insecticides (Lacey *et al*., 2008; Baldin *et al*., 2013). Microbial derived products might offer a feasible alternative to suppress population of *B. tabaci* in the field (Wang *et al*., 2014). The biological activity of *Bacillus* spp. culture filtrates on *B. tabaci* has been reported in previous studies. Ateyyat *et al*. (2009) found that culture supernatants of *Bacillus pumilis*, *B. subtilis* and *B. licheniformis* caused 15 – 20% mortality of *B. tabaci* nymphs. Salazar-Magallon *et al*. (2015) found that the complex spore-crystal of *B. thuringiensis* caused more than 90% nymphal mortality. These studies open new avenues to explore the use of *B. thuringiensis* derived products to manage whitefly infestations. Thus far, the use of *B. thuringiensis* as biorrational agent to manage pest insects has been restricted to Lepidoptera and Coleoptera larvae (Sanahuja *et al*., 2011). In the present work, the effects of *Bacillus thuringiensis* culture supernatants and their derived products were evaluated on whitefly *Bemisia tabaci* and its parasitoid *Eretmocerus eremicus.*

MATERIALS AND METHODS

The present work was carried out to evaluate the effect of the ethanolic extracts and residual solids of *B. thuringiensis* culture supernatants on adult repellence, oviposition deterrence and mortality of nymphs of *B. tabaci*. The chromatographic analyses and the toxic effect of the active extracts on the *B. tabaci* parasitoid, *Eretmocerus eremicus*, were also evaluated.

Adult whiteflies were obtained from a stock rearing colony maintained on eggplant *Solanum melongena* in a greenhouse. The colony has been maintained since 2013 (Ballina-Gómez *et al*., 2013). All *B. thuringiensis* strains were obtained from a collection maintained in the Laboratory of Microbiology. Identity of the strains has been confirmed by molecular characterization and reported elsewhere (García-Ramírez *et al*., 2013). Bacteria were grown in nutrient broth (BD Bioxon, Mexico) with yeast extract (Dibico, México). Cultures were incubated at 29 °C for 72 h in an orbital shaker MaxQ 4450 (Thermo Scientific, USA), and then centrifuged at 3000 rpm for15 min in a C 600 Centrifuge (Solbat, Mexico). Supernatants of eight bacterial strains were recovered and use directly for preliminary screening. Subsequently, four strains (NT5-39, NT12-51, ITCBT61 and ITCBT62) were selected based on their activity. Lastly, extracts of culture supernatants of only two strains (ITCBT61 and ITCBT62) were evaluated on *B. tabaci* and *E. eremicus*.

Crude culture supernatants were lyophilized in a FreeZone$^{2.5}$ (Labconco, Mexico), and then macerated with ethanol at room temperature, and filtered (3×) through Whatman filter paper #1. Two types of products were obtained from each lyophilized supernatant: Ethanol Extract (EtE) and Residual Solid (ReS). Solvent of EtE was eliminated in a rotatory evaporator Buchi RV 10 (IKA, USA) at 40 °C. Products were kept in glass vials at 4 °C until use. Blank was obtained from non-inoculated culture media processed exactly as those containig the *B. thuringiensis* strains. For positive controls, two commercial products were used: the neonicotinoid imidacloprid (Confidor, 2 mL L^{-1}. Bayer Crop Science) and a botanical insecticide (Biodie, 3.6 mL L^{-1}. Ultraquimia Mexico). For negative control, distilled water with 1 % tween 20 (v/v) was used. The bioassays included: adult repellence, oviposition deterrence and nymphal mortality for *B. tabaci*, and pupal mortality for *E. eremicus*.

Adult repellence was evaluated in multi-choice assay using 30 d old *Capsicum chinense* plants. The products were evaluated at concentrations of 10 mg. mL^{-1}. In all cases 1 % of Tween 20 (v/v) was added to the solutions. For treatment, two young fully expanded leaves per plant were immersed for 10 s in the products. Plants were placed in 1.2 × 1.2 × 1.0 m cages made by anti-aphid mesh with aluminium frame, and then 300 non-sexed *B. tabaci* adults were released into the cage. The number of adults on the abaxial surface of leaves was counted at 48 h of adult release. Immediately after evaluation, leaves were excised, measured and the number of eggs counted with the aid of a stereoscopic microscope. The number of adults or eggs were reported as individuals per cm^2. Twenty replicates (leaves) were used per treatment.

The adult repellent index (RI) was calculated by using the equation RI = 2T/(T + C), where T is the number of insects attracted to the treated leaf, and C is the number of insects attracted to the control leaf (distilled water). Values of RI lower than 1 indicate repellency. The oviposition deterrence index (ODI) was calculated by the equation ODI = [(T − C)/(T + C)]/100, where T is the number of eggs counted on the treated leaf, and C is the number of eggs counted on the control leaf. Values of ODI from 0 to −100

indicate deterrence (Baldin *et al*., 2013). For bioassay of nymphal mortality, *C. chinense* plants were infested with *B. tabaci*. Leaves with third instar nymphs were cut from plants and taken to the laboratory. To treat nymphs, leaves were immersed in the products for 10 s and placed in Petri dishes that had a layer of wet cotton on the bottom. Petri dishes were kept in the laboratory at 26 °C ± 2 and 14 h :10 h ligh:dark photoperiod for 48 h and then nymphal mortality was evaluated. Sixteen replicates (Petri dishes) were set per treatment. Corrected mortality was calculated as described by Abbott (1925). Nymphs were considered dead if body color changed from yellowish to dark brown or if the body appeared dry (Ateyyat *et al*., 2009). To evaluate the toxicity on *E. eremicus*, pupae of the parasitoid hosted in *B. tabaci* mommies (Koppert B. S. Mexico), were immersed for 10 s in the products, dried for 1 h and placed in Petri dishes (60 × 15 mm). Two weeks later, mortality of parasitoids was recorded base on non-emerged adults (Sugiyama *et al*., 2011).

Data were analyzed using one-way analysis of variance (ANOVA) in the software package InfoStat version 2016a (www.infostat.com). All data were checked for normality and homogeneity of variance before ANOVA. Means were grouped using Scott-Knott test. Samples of EtE and ReS obtained from strains ITCBT61 and ITCBT62 were analyzed (2 µL, 0.4%, w/v) by LC-UV-MS using data-dependent acquisition protocol (Martin *et al*., 2014). Chromatograms and mass spectra were obtained in LC/MS Agilent (Santa Clara, CA) coupled to a mass detector Bruker maxis HR-QTOF (Bruker Daltonics GmbH, Germany), using a column Zorbax SB-C8 (2.1 × 30 mm) at 40 ºC. Briefly, mobile phase was a mixture of solvent A (water: acetronitrile 90:10 with 0.01% trifluoroacetic acid and 1.3 mM ammonium formate) and solvent B (water:acetonitrile 10:90 with 0.01% trifluoroacetic acid and 1.3 mM ammonium formate) with a flow of 300 µL/min. The gradient was at constant flow rate of 10% B to 100% B in 6 min, 100% B for 2 min, and then returned to 10% B for 2 min. Mass spectra (150 *m/z* to 2000 *m/z*) were acquired in positive mode. The components detected were compared with MEDINA database of microbial metabolites and the Chapman & Hall Dictionary of Natural Products (v25.1).

RESULTS AND DISCUSSION

Initial evaluation showed that culture supernatants of four bacterial strains of *B. thuringiensis* (NT5-39, NT12-51, ITCBT61 and ITCBT62) caused significant adult repellence and oviposition deterrence. Culture supernatants of strains ITCBT61 and ITCBT62 were the most active. The calculated RI values for ITCBT61 was 0.55 and for that of ITCBT62 was 0.47. The ODI values for ITCBT61 was -42.3 and for that of ITCB62 was -71.4.

The supernatant derived products, EtE and ReS, from ITCBT62 and ReS from ITCBT61 caused significant adult repellence and oviposition deterrence compared to that of the control, but not compared to their blanks. Ethanol extracts of ITCBT62, as well as ReS of both strains caused a significant decrease in the number of *B. tabaci* adults in *C. chinense* leaves compared to the control. Similarly, these products caused significant decrease in the number of eggs laid by *B. tabaci* (Table 1). Calculation of RI and ODI showed that ReS of ITCBT62 was the most active for both variables if compared to its blank (Table 1).

Evaluation of mortality on *B. tabaci* nymphs and its parasitoid *E. eremicus* showed that EtE of ITCBT61 and ITCBT62 caused higher mortality than that of the control. Residual solid of ITCBT62 caused significantly higher mortality than that of ITCBT61 (Table 2).

Table 1. Effects of derived products from *B. thuringiensis* culture supernatant on number (means ± standard error) of adults and eggs on *C. chinense* leaves, adult repellent index (RI) and oviposition deterrence index (ODI) of *B. tabaci* at 48 h after treatments.

Treatment	Adults·cm^2	Eggs·cm^2	RI	ODI
Blank EtE	2.1 ± 0.7 b	0.8 ± 0.2 b	0.37	-60.4
ITCBT61 EtE	7.7 ± 2.2 a	3.7 ± 1.1 a	0.92	06.4
ITCBT62 EtE	3.7 ± 1.7 b	1.4 ± 0.5 b	0.58	-39.5
Blank ReS	4.3 ± 1.6 b	2.4 ± 0.8 a	0.64	-16.1
ITCBT61 ReS	4.1 ± 2.4 b	1.6 ± 0.7 b	0.63	-33.3
ITCBT62 ReS	2.4 ± 1.1 b	0.6 ± 0.2 b	0.42	-70.1
Imidacloprid	2.4 ± 0.6 b	0.9 ± 0.2 b	0.42	-57.9
Botanical insecticide	1.4 ± 0.7 b	0.7 ± 0.5 b	0.26	-64.4
Control	9.0 ± 3.6 a	3.3 ± 0.9 a	1.00	00.0

Means followed by the same letter with a column are not significantly different (Skott-Knott, P, 0.05). EtE indicates ethanol extracts and ReS indicates residual solids. RI values range from 1 to 0. The lower the value the higher the repellence. ODI values range from 100 to -100. Oviposition deterrence is given by values from 0 to -100, lower values indicate higher effects.

When applied to *E. eremicus* pupae, neither EtE nor ReS caused any mortality. Blanks of both type of products caused 5-10 % mortality, whereas the botanical insecticide and imidacloprid caused 37.5 ± 5.1 and 61.8 ± 4.7 % mortality of *E. eremicus* pupae.

To identify possible active metabolites, samples of EtE and ReS of ITCBT61 and ITCBT62 were analyzed by LC-HRMS. Chromatograms showed similar pattern of EtE and ReS with their respective blanks, except for EtE of ITCBT61 that showed the presence of various signals, with one particularly abundant metabolite. The metabolite was observed at retention time of 3.6 min, which showed a molecular formula of $C_{15}H_{20}N_2O$ according with its molecular ion at *m/z* 245.1645 $[M+H]^+$ (calc 245.3259) (Figure 1).

Table 2. Percent mortality (means ± standard error) of *B. tabaci* nymphs 48 h after treated with ethanol extracts of *B. thuringiensis* culture supernatant.

Treatment	Mortality (%)
Blank EtE	29.44 ± 6.37 c
ITCBT61 EtE	51.21 ± 8.16 b
ITCBT62 EtE	42.02 ± 10.56 b
Blank ReS	23.78 ± 4.07 c
ITCBT61 ReS	34.84 ± 7.40 c
ITCBT62 ReS	53.75 ± 6.66 b
Imidacloprid	32.61 ± 4.99 c
Botanical insecticide	71.41 ± 6.83 a
Control	5.78 ± 1.46 d

Means followed by the same letter within a column are not significantly different (Scott & Knott; P, 0.05). EtE indicates ethanol extracts and ReS indicates residual solids.

The use of *B. thuringiensis* derived products has been previously proposed as a feasible alternative to manage *B. tabaci*. Salazar-Magallon *et al*. (2015) reported more than 92 % nymphal mortality after treated with culture broth containing spore-crystal complex. Al-Shayji and Shaheen, (2008) reported 50 to 60% mortality of *B. tabaci* nymphs treated with a strains of *B. thuringiensis*. In the present work, EtE of ITCBT61 and ITCBT62 caused 42 to 51 % mortality and ReS of the latter also caused 53.7 % mortality of *B. tabaci* nymphs, suggesting moderate toxicity. It is important to note that EtE had also effects on adult repellence, and ReS on oviposition deterrence, suggesting that the bacterial culture supernatants may have compounds with different bioactivity. Interestingly, the effects of both types of products caused no toxic effects on the *B. tabaci* parasitoid, *E. eremicus*, suggesting that the bioactive compounds against *B. tabaci* might be harmless to this parasitoid species.

The chromatography profile analysis showed that all compounds present in the EtE and ReS were also observed in their respective blanks. There were various compounds observed in the EtE of ITCBT61, with a particularly abundant metabolite of molecular formula $C_{15}H_{20}N_2O$. This compound match with 25 possible compounds in the Chapman & Hall Dictionary of Natural Products (v25.1). Four of them have been reported for fungi and only one of them for *Bacillus cereus* 0411381. The latter identified as *R*(+)-2-(heptan-3-yl) quinazolin-4(3*H*)-one. More studies are required to reveal the identity of this compound and to verify its effect on *B. tabaci*.

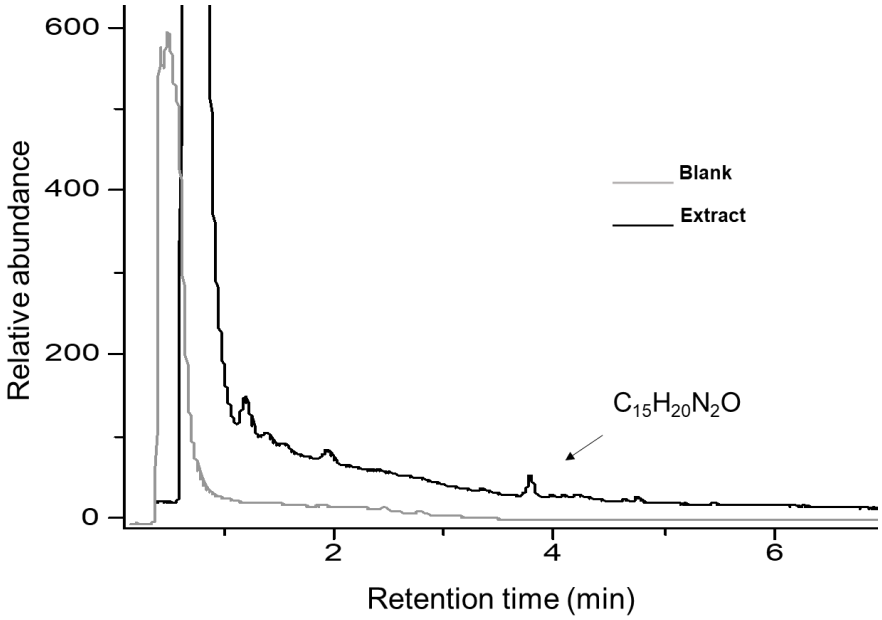

Figure 1. LC-UV chromatogram (210 nm) of the ethanol extract of ITCBT61 supernatant. The arrow shows the compound at retention time of 3.6 min.

CONCLUSIONS

In conclusion, supernatant derived products of *B. thuringiensis* culture might represent a good alternative for future research on screening of natural products for *B. tabaci* management. Ethanol extracts and ReS of two strains, ITCBT61 and ITCBT62, showed a modest, but consistent effect on adult repellence, oviposition deterrence and nymphal mortality of *B. tabaci*. The products had no effect on *E. eremicus*. Chromatography profile showed no conclusive evidence of the presence of compounds that could be pointed as possible active metabolites.

ACKNOWLEDGEMENT

Authors thank J. Martín and Fundación MEDINA (Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, Granada, Spain) for the assistance with the chromatograms and mass spectra. Authors also thank Irma Leticia Medina Baizabal for her assistance in the preparation of the crude culture supernatants.

REFERENCES

Abott, W.S. 1925. A method of computing the effectiveness of an insecticide. Journal Economy Entomology. 18:265-267. https://doi.org/10.1093/jee/18.2.265a.

Al-Shayji, Y., Shaheen, N. 2008. Isolation of *Bacillus thuringiensis* strain from Kuwait's soil effective against whitefly nymphs. Journal of Insect Science. 8(4):4.

Ateyyat, M.A., Shatnawi, M., Al-Mazra'awi, M.S. 2009. Culturable whitefly associated bacteria and their potential as biological control agents. Jordan Journal of Biological Sciences. 2(3):139-144.

Baldin, E.L., Crotti, A.E., Wakabayashi, K.A., Silva, J.P., Aguiar, G.P., Souza, E.S., Veneziani, R.C., Groppo, M. 2013. Plant-derived essential oils affecting settlement and oviposition of *Bemisia tabaci* (Genn.) biotype B on tomato. Journal of Pest Science. 86:301-308. https://doi.org/10.1007/s10340-012-0462-x.

Ballina-Gómez, H.S., Ruiz-Sanchez, E., Chan-Cupul, W., Latournerie-Moreno, L., Hernández-Alvarado, L., Islas-Flores, I., Zuñiga-Aguilar, J.J. 2013. Response of *Bemisia tabaci* Genn. (Hemiptera: Aleyrodidae) Biotype B to genotypes of pepper *Capsicum annuum* (Solanales: Solanaceae). Neotropical Entomology. 42:205-210. https://doi.org/10.1007/s13744-012-0106-0.

Brown, J.K., Frohlich, D.R., Rosell, R.C. 1995. The sweetpotato or silverleaf whiteflies: Biotypes of *Bemisia tabaci* or a species complex? Annual Review of Entomology. 40:511-534. https://doi.org/10.1146/annurev.en.40.010195.002455.

De Barro, P.J., Liu, S.S., Boykin, L.M., Dinsdale, A.B. 2011. *Bemisia tabaci*: a statement of species status. Annual Review of Entomology. 56:1-19. https://doi.org/10.1146/annurev-ento-112408-085504.

García-Ramírez, A., Tun-Suárez, J.M., Reyes-Ramírez, A. 2013. Aislamiento y presencia de genes Cry en cepas nativas de *Bacillus thuringiensis*. XXXVI National Conference on Biological Control. Oaxaca, Mexico. p. 99-102.

Horowitz, A.R., Ishaaya, I. 2014. Dynamics of biotypes B and Q of the whitefly *Bemisia tabaci* and its impact on insecticide resistance. Pest management science. 70:1568-1572. https://doi.org/10.1002/ps.3752.

Lacey, L.A., Wraight, S.P., Kirk, A.A. 2008. Entomopathogenic Fungi for Control of *Bemisia tabaci* Biotype B: Foreign Exploration, Research and Implementation. In: Gould J., Hoelmer K., Goolsby J. (eds) Classical Biological Control of *Bemisia tabaci* in the United States - A Review of Interagency Research and Implementation. Progress in Biological Control, vol 4. Springer, Dordrecht: E- Publishing Inc. p. 33-69. https://doi.org/10.1007/978-1-4020-6740-2_3.

Martín, J., Crespo, G., González-Menéndez, V., Pérez-Moreno, G., Sánchez-Carrasco, P., Pérez-Victoria, I. 2014. MDN-0104, an antiplasmodial betaine lipid from *Heterospora chenopodii*. Journal of Natural Products. 77:2118–2123. https://dx.doi.org/10.1021/np500577v.

Salazar-Magallon, J.A., Hernandez-Velazquez, V.M., Alvear-Garcia, A., Arenas-Sosa, I., Pena-Chora, G. 2015. Evaluation of industrial by-products for the production of *Bacillus thuringiensis* strain GP139 and the pathogenicity when applied to *Bemisia tabaci* nymphs. Bulletin of Insectology. 68(1):103-109.

Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T., Christou, P. 2011. *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnology Journal. 9:283-300. https://doi.org/10.1111/j.1467-7652.2011.00595.x.

Sugiyama, K., Katayama, H., Saito, T. 2011. Effect of insecticides on the mortalities of three whitefly parasitoid species, *Eretmocerus mundus*, *Eretmocerus eremicus* and *Encarsia formosa* (Hymenoptera: Aphelinidae). Applied entomology and zoology. 46(3):311-317. https://doi.org/10.1007/s13355-011-0044-z.

Wang, L., Huang, J., You, M., Guan, X., Liu, B. 2007. Toxicity and feeding deterrence of crude toxin extracts of *Lecanicillium* (*Verticillium*) *lecanii* (Hyphomycetes) against sweet potato whitefly, *Bemisia tabaci* (Homoptera: Aleyrodidae). Pest management science. 63:381-387. https://doi.org/10.1002/ps.1359.