

Evaluating Toxicity of Extracted Destruxin from *Metarhizium Anisopliae* Against Citrus Leafminer, *Phyllocnistis citrella*

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Abstract: Problem statement: The fungus, *Metarhizium anisopliae* (Metschnikoff) Sorokin produces some cyclic peptide toxins, destruxins, which exhibit a variety of insecticidal actions. Citrus leafminer, *Phyllocnistis citrella* Stainton, is a major pest of young citrus trees; therefore its effective and safe control is a important issue. **Approach:** Five isolates of *M. anisopliae* were inoculated in Potato Dextrose Broth (PDB) mediums. Destruxin was extracted by adding chloroform. Citrus leafminer was bioassayed by using the leaves containing early stages larvae and the data were recorded after 1, 2, 3 and 4 days after treatment. **Results:** The range of mortality was between 30-65% based on the endpoint data. The minimum of three days to achieve 50% mortality was proved by probit analysis of time-mortality responses. The 10, 15 and 20 fold dilutions of extracted destruxins from isolates cause 17.2-48.5, 9.4-37.5 and 0-26.5%, respectively. **Conclusion:** Bioassay of destruxin by using citrus leafminer larvae shows its acceptable effect of destruxin. By considering biology of this species and calculated LT_{50S} , destruxin seems to be an effectiveness component of IPM programs. The 10, 15 and 20 fold dilutions of extracted destruxin have a reduced amount of mortality, but in some cases it can balance the expensive production process of microbial agents and their efficacy.

Key words: Destruxin, *Metarhizium anisopliae*, citrus leafminer, *Phyllocnistis citrella*, microbial agent

INTRODUCTION

The Citrus Leafminer (CLM), *Phyllocnistis citrella* Stainton is a major destructive insect pest of citrus orchards, especially in nurseries and new planting trees (Diez *et al.*, 2006). Larval damage may provide suitable condition for increasing the bacterial citrus canker infections in the area where the pathogen cause economic damage (Hoy *et al.*, 2007).

This pest was observed at 1994 in Mazandaran Province of Iran and cause remarkable damage in young trees, nowadays (Jafari, 1995). CLM control mostly relies on chemical pesticides (Diez *et al.*, 2006). Resistance to insecticides is a likely response of CLM individuals (Mafi and Ohbayashi, 2006). Apart from of undesirable effects of resistant populations, widely application of pesticides in citrus orchards lead to some difficulties in pest control measures and in the other hand to more significance of the IPM strategies which containing natural enemies and microbial agents.

The entomopathogenic fungus *Metarhizium anisopliae* is a well studied and applied species for microbial control of insect pests (Liu *et al.*, 2007;

Hoe *et al.*, 2009). This fungus produces some cyclic peptides, destruxins (Hsiao and Ko, 2001) which may play a role in its pathogenicity (Kershaw *et al.*, 1999). More than 35 different structurally related destruxins have been isolated from cultures of *M. anisopliae* (Pedras *et al.*, 2002). Destruxin capacity in control practices is a less studied matter, but some investigations have described its insecticidal properties (Brousseau *et al.*, 1996; Thomsen and Eilenberg, 2000).

In this study different dilutions of destruxins extracted from several fungal isolates were examined on citrus leafminer larvae.

MATERIALS AND METHODS

Mycotoxin preparation: Five *M. anisopliae* isolates (A-115, A-304, A-336, Bs-met and Met) were obtained in a series of soil screening experiments by using 200 samples (Ghanbary *et al.*, 2009). The isolates were inoculated in 50 mL Potato Dextrose Broth (PDB) mediums for destruxin production. The medium was filtered using filter paper seven days after culturing. Then 10 mL chloroform was added and shake

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vigorously for 10 min. The supernatant evaporate and the residue was containing destruxin. The residues were dissolved in 10 mL distilled water and stored at -20°C for further examinations.

Bioassays: The fresh citrus leaves containing CLM larvae were collected daily and the ones including at least 10 early stages larvae used in experiments. The rate of 10 larvae per leaf was provided by killing the additional larvae before treatment. The extracted destruxin and 10, 15 and 20-fold dilutions were used in bioassays. The prepared leaves were dipped in concentrations for 10 sec and allowed to dry for about one hour. The treated leaves were placed in Petri dishes and held in incubator conditions (27±1°C). The bioassays were replicated four times for any isolate and dilution and the control was containing only distilled water. Mortality was recorded at 1, 2, 3 and 4 days after treatment. The larvae with no movement were recorded as died ones. The probit and T-test options of SPSS software were used for analyzing time-mortality and comprising means of mortality, respectively.

RESULTS

The results of the mortality caused by crude extracted from five isolates of *M. anisopliae* are presented (Table 1). Based on these results the isolate A-115 had the best effectiveness after four days. There was significance difference in mortalities only between A-115 and Bs-met (p<0.05) isolates and effect of these two and other isolates were similar (p>0.05 for all of the comparisons). Reducing the concentration of extracted destruxin were decreased mortalities (Table 2).

Table 1: The percent mortality cause by destruxin extracted from isolates of *Metarhizium anisopliae* on citrus leafminer larvae

Isolates	The days after treatment			
	1	2	3	4
A-115	5.0	30.0	47.5	65.0
A-304	10.0	22.5	45.0	57.5
A-336	5.0	22.5	45.0	52.5
Bs-met	0.0	2.5	7.5	30.0
Met	7.5	25.0	40.0	52.5

Table 2: The percent mortality cause by serial dilutions of destruxins extracted from isolates of *Metarhizium anisopliae* on citrus leafminer larvae

Dilutions	Isolates				
	A-115	A-304	A-336	Bs-met	Met
10-folds	48.5	37.2	28.6	22.8	17.2
15-folds	12.5	34.4	37.5	18.7	9.4
20-folds	26.5	6.0	20.5	3.0	0.0

Also, LT₅₀s of *M. anisopliae* isolates were evaluated (Table 3). These data show its long 3.04 days to achieve to 50 percent mortality by using A-115 isolate. This is the best value and Bs-met isolate had least effectiveness based on the LT₅₀s. Bs-met didn't cause any mortality at one day after treatment and we used the data of other three points (Table 1) for LT₅₀ calculating. At least four points is necessary for a valid statistical analysis (Robertson *et al.*, 2007), hence the value of Bs-met have not reliability. There were no significant differences between LT₅₀s of A-115, A-304, A-336 and Met isolates by 90 % confidence limit (Table 3).

DISCUSSION

Although the mode of action of destruxins in insects is an unclear issue but altering the calcium cannels function has proposed in some investigations (Dumas *et al.*, 1996; Samuels *et al.*, 1988). Primarily, tetanic paralysis is the common symptom in insects causing by application of destruxin (Samuels *et al.*, 1988). Opening the Ca²⁺ channels as a result of membrane depolarization by destruxin has been implicated as a cause of paralysis and death (James *et al.*, 1993). Humoral immune response seems to be specifically affected by destruxin (Pal *et al.*, 2007). Taken together these findings describe the probable reason for slow-acting this mycotoxin. Recording mortality data after the minimum of 72 h is a common procedure. Based on our data, three to four days after treatment appear to be appropriate final point for recording mortality.

Expensive production of microbial biopesticides is one of the limiting factors for wide application of these agents (Lacey, 2008; Dezianian *et al.*, 2010). Thus for investigating a cost effective procedure CLM larvae were examined by serial dilutions (10, 15 and 20 folds) of destruxins (Table 2). In many cases, entomopathogens are potential control agents against pests and use along with other component of integrated pest management (Kaya and Lacey, 2007). Therefore the dilutions of extracted destruxin are suitable candidates in these programs; they decrease application's cost of destruxin in an integrated procedure. 10-fold dilution of A-115 (Table 2) seems to be a good example of an accompanying agent in these programs.

Like other microbial agents, long period of lethal infection (Lacey, 2008) is a disadvantage of destruxins. This make the LT₅₀ value consider as a significance parameter. Citrus leaf miner produces four larval instars in shallow tunnels (mines) inside the leaves of young citrus trees (Godfrey and Grafton-Cardwell, 2002).

Table 3: The LT₅₀s of *Metarhizium anisopliae* isolates on citrus leafminer

	Isolates				
	A-115	A-304	A-336	Bs-met	Met
LT ₅₀ (days)	3.04	3.47	3.59	5.16	3.77
(95% CL)	(2.61-3.73)	(2.84-4.85)	(2.99-4.87)	(4.25-10.02)	(3.04-5.56)

The first three instars are active and feed within the mines, while the fourth instar, no longer feed and produces silk from its mouthparts to form a pupal chamber. Growth of three stages takes about five to six days in summer (Beattie and Hardy, 2004), but in different environmental conditions it may be long one to three weeks (Ba-Angood, 1978; Godfrey and Grafton-Cardwell, 2002; Grafton-Cardwell, 2009). By considering the biology of CLM and the LT₅₀s of isolates (Table 3), destruxin have acceptable efficacy on CLM. Also, these measures suggest that three to four days after treatment is a suitable endpoint for destruxin bioassay.

CONCLUSION

Microbial agents are a suitable and naturally friend part of integrated pest management programs. Extracted destruxin from the fungi, *M. anisopliae* can be used as a constituent of citrus leafminer control measures. Like many of microbial agents, the slow acting property of destruxin should be considered in citrus leafminer measures. However, before it is used as a component of an IPM procedure, its compatibility with other biocontrol agents such as beneficial insects and the pesticides used in citrus orchards has to be examined.

REFERENCES

- Ba-Angood, S.A.S., 1978. On the biology and food preference of the citrus leaf miner, *Phyllocnistis citrella* Stainton (*Gracillariidae*, Lepidoptera) in PDR of Yemen. *J. Applied Entomol.*, 86: 53-57. DOI: 10.1111/j.1439-0418.1978.tb01910.x
- Beattie, A. and S. Hardy, 2004. Citrus leafminer. NSW Department of Primary Industries. http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0006/137634/citrus-leafminer.pdf
- Brousseau, C., G. Charpentier and S. Belloncik, 1996. Susceptibility of spruce budworm, *Choristoneura funiferana* Clemens, to destruxines, cyclopeptidic mycotoxins of *Metarhizium anisopliae*. *J. Invertebr. Pathol.*, 68: 180-183. DOI: 10.1006/jipa.1996.0079
- Dezianian, A. A.S. Sajap, W.H. Lau, D. Omar and H.A. Kadir *et al.*, 2010. Morphological characteristics of *P. xylostella* granulovirus and effects on its larval host diamondback moth *Plutella xylostella* L. (Lepidoptera, Plutellidae). *Am. J. Agric. Biol. Sci.*, 5: 43-49. <http://www.scipub.org/fulltext/AJAB/AJAB5143-49.pdf>
- Diez, P.A., J.E. Pena and P. Fidalgo, 2006. Population dynamics of *Phyllocnistis citrella* (Lepidoptera: *Gracillariidae*) and its parasitoids in Tafíviejo. Tucuman, Argentina. *Fla. Entomol.*, 89: 328-335. DOI: 10.1653/0015-4040(2006)89[328:PDOPCL]2.0.CO;2
- Dumas, C., M. Ravallec, V. Matha and A. Vey, 1996. Comparative study of the cytological aspects of the mode of action of Destruxins and other peptidic fungal metabolites on target epithelial cells. *J. Invertebr. Pathol.*, 67: 137-146. DOI: 10.1006/jipa.1996.0021
- Ghanbary, M.A.T., A. Asgharzadeh, A.R. Hadizadeh and M.M. Sharif, 2009. A quick method for *Metarhizium anisopliae* isolation from cultural soils. *Am. J. Agric. Biol. Sci.*, 4: 152-155. <http://www.scipub.org/fulltext/AJAB/AJAB42152-155.pdf>
- Godfrey, K. and B. Grafton-Cardwell, 2002. Citrus leafminer in California citrus. Biological Control Program. http://www.cdfa.ca.gov/phpps/ipc/biocontrol/pdf/insects/cleafminer_citrus-may6-02.pdf
- Grafton-Cardwell, E., 2009. Citrus leafminer. University of California IPM Online. <http://www.ipm.ucdavis.edu/PMG/r107303211.html>
- Hoe, P.K., C.F.J. Bong, K. Jugah and A. Rajan, 2009. Evaluation of *Metarhizium anisopliae* var. *anisopliae* (Deuteromycotina: Hyphomycete) Isolates and their Effects on Subterranean Termite *Coptotermes curvignathus* (Isoptera: Rhinotermitidae). *Am. J. Agric. Biol. Sci.*, 4: 289-297. <http://www.scipub.org/fulltext/AJAB/AJAB44289-297.pdf>
- Hoy, M.A., R. Singh and M.E. Rogers, 2007. Citrus leafminer, *Phyllocnistis citrella* (Lepidoptera: *Gracillariidae*) and natural enemy dynamics in central Florida during 2005. *Fla. Entomol.*, 90: 358-369. DOI: 10.1653/0015-4040(2007)90[358:CLPCLG]2.0.CO;2

- Hsiao, Y.M. and J.L. Ko, 2001. Determination of destruxins, cyclic peptide toxins produced by different strains of *Metarhizium anisopliae* and their mutants induced by ethyl methane sulfonate and ultraviolet using HPLC method. *Toxicon*, 39: 837-841. PMID: 11137544
- Jafari, M.E., 1995. Citrus leafminer a new pest of citrus in Mazandaran. Proceeding of the 12th Plant Protection Congress, Sept. 2-7, University Tehran, Karaj, Iran, pp: 211-211.
- James, P.J., M.J. Kershaw, S.E. Reynolds and A.K. Charnley, 1993. Inhibition of desert locust (*Schistocerca gregaria*) malpighian tubule fluid secretion by destruxins, cyclic peptide toxins from the insect pathogenic fungus *Metarhizium anisopliae*. *J. Insect. Physiol.*, 39: 797-804. DOI: 10.1016/0022-1910(93)90056-W
- Kaya, H.K. and L. Lacey, 2007. Introduction to Microbial Control. In: Field Manual of Techniques in Invertebrate Pathology, Lacey, L.A. and H.K. Kaya (Eds.). Springer, Dordrecht, The Netherlands, ISBN: 978-1402059322, pp: 3-7.
- Kershaw, M.J., E.R. Moorhouse, R. Bateman, S.E. Reynolds and A.K. Charnley, 1999. The Role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. *J. Invertebr. Pathol.*, 74: 213-223. DOI: 10.1006/jipa.1999.4884
- Lacey, L.A., 2008. Microbial Control of Insects. In: Encyclopedia of Entomology. Capinera, J.L. (Eds.). Springer, new York, ISBN: 978-1402062421, pp: 2367-2373.
- Liu, B.L., T.M. Rou, Y.K. Rao and Y.M. Tzeng, 2007. Effect of pH and aeration rate on the production of Destruxins A and B from *Metarhizium anisopliae*. *Int. J. Applied Sci. Eng.*, 5: 17-26. <http://www.cyut.edu.tw/~ijase/2007/012-003-3.pdf>
- Mafi, S.A. and N. Ohbayashi, 2006. Toxicity of insecticides to the citrus leafminer, *Phyllocnistis citrella* and its parasitoids, *Chrysocharis pentheus* and *Sympiesis striatipes* (Hymenoptera: Eulophidae). *Applied Entomol. Zool.*, 41: 33-39. DOI: 10.1303/aez.2006.33
- Pal, S., R.J.S. Leger and L.P. Wu, 2007. Fungal peptide Destruxin A plays a specific role in suppressing the innate immune response in *Drosophila melanogaster*. *J. Biol. Chem.*, 282: 8969-8977. DOI: 10.1074/jbc.M605927200
- Pedras, M.S.C., L.I. Zaharia and D.E. Ward, 2002. The destruxins: Synthesis, biosynthesis, biotransformation and biological activity. *Phytochemistry*, 59: 579-596. DOI: 10.1016/S0031-9422(02)00016-X
- Robertson, J.L., N.E. Savin, H.K. Preisler and R.M. Russell, 2007. Bioassays with Arthropods. 2nd Edn., CRC Press, New York, ISBN: 978-0849323317, pp: 224.
- Samuels, R.I., S.E. Reynolds and A.K. Charnley, 1988. Calcium channel activation of insect muscle by destruxins, insecticidal compounds produced by the entomopathogenic fungus, *Metarhizium anisopliae*. *Comp. Biochem. Physiol. Part C: Comp. Pharm.*, 90: 403-412. DOI: 10.1016/0742-8413(88)90018-7
- Thomsen, L. and J. Eilenberg, 2000. Time-concentration mortality of *Pieris brassicae* (Lepidoptera: Pieridae) and *Agrotis segetum* (Lepidoptera: Noctuidae) larvae from different destruxines. *Environ. Entomol.*, 29: 1041-1047. DOI: 10.1603/0046-225X-29.5.1041