Cytotoxic effects of entomopathogenic fungal crude filtrate on *Phthorimaea operculella* cell line

(Accepted: 10.06.2001)

[1*] O.Khamiss, [2] G.H.Sewify and [1] S.M. Abol-Ela

[1] Entomovirology Laboratory, Faculty of Agriculture Cairo University (IRD), Giza, Egypt.

* Genetic Engineering and Biotechnology Research Institut (GEBRI), Sadat City, Minufiya University, Egypt.

[2] Center of Biological Control, Department of Economic Entomology and Pesticides, Faculty of Agriculture, Cairo University, Giza, Egypt.

ABSTRACT

A Phthorimaea operculella cell line was used to study the cytotoxic effects of the entomopathogenic fungi Metahrizium anisopliae, Beauveria bassiana and Verticillium lecanii. P. operculella. Cell treatment with fungi crude filtrate (in shake culture and solid medium) showed a clear cellular cytopathic effect. Cells treated with M.anisopliae crude filtrate revealed granular contents, irregular forms and alterations on the cellular membrane. Treated cells of P.operculella with B. bassiana and V. lecanii crude filtrates showed elongation in cell shape. P.opercullela treated cells with sonicated conidospores filtrate of each of the three fungi isolates showed irregular forms and intensive vacuoles. However, treated cells with filtrated and then sonicated blastosopres of the fungi showed associated crystals in the culture medium and fibroplast elongated cells filled with intensive vacuoles. This study suggests that the sonicated blastospores induce more obvious cellular changes and higher toxic effect than crude broth filtrate secreted in fungi media.

Key words: *Phthorimaea operculella, cell line, Beauveria bassiana, Metahrizium anisopliae, Verticillium lecanii, cytotoxic effect, toxin.*

INTRODUCTION

The entomopathogenic fungi are known to cause diseases in species of insects, and most of them produce toxins such as destruxins (*Metahrizium anisopliae*) (Loutelier *et al.*, 1996, bassianolide (*Beauveria bassiana* and *Verticillium lecanii*) Kanaoka *et al.*, 1978), aspochracin (*Aspergillus ochraceus*), aflatoxins (*A. flavus*) (Ohtomo, 1975) and beauvericin (*B. bassiana*) (Hamill *et al.*, 1969).

The virulence of these fungi may depend upon the secretion of toxins (Vey, 1998). Vey *et al.* (1993) mentioned that crude broth filtrate produced by *Hirsutella thompsonii* var. *thompsonii* caused insecticidal effect to *Galleria melonella* larvae and *Drosophila melanogaster* adults *via* infection and *per os* application. Currently, there is a great deal of interest in the development of new insecticides from microbes, particularly with the emergence of the avermectins as broad spectrum natural pesticides derived from the actinomycete, *Streptomyces avermitilis*, (Putter *et al.*, 1981; McCoy *et al.*, (1982).

Thus, knowledge of fungal toxins is very much needed for a better understanding

of the mode of action of entomopathogenic fungi (Quiot *et al.*, 1985 and Vey *et al.*, (1987).

The insect cell lines have been used to describe the morphological changes induced by insect viruses and fungi infection (Khamiss *et al.*, 1998 and Vey, 1993). The use of cell culture for studies on entomopathogenic fungi toxins provides several advantages, including understanding the effects of entomotoxin at cellular and subcelluler levels, reduced time and cost for expermentation.

This work describes the cytotoxic effect of crude filtrate released in fungal media and filtrate sonicated spores of the entomopathogenic fungi *B. bassiana, M. anisopliae and V. lecanii* on a cell line derived from *P. operculella.*

MATERIALS AND METHODS

Fungal preparation

The three entomopathogenic fungi *Metahrizium anisopliae, Beauveria bassiana* and *Verticillium lecanii* were isolated from soil, potato tuber moth *Phthorimea operculella* and green peach aphid *Myzus persicae* by Dr.G. Sewify in Egypt. Fungi were cultured on an autoclaved Potato Dextrose medium to obtain the conidospores. Spores were harvested from two week old cultures grown at 25°C, by rinsing with sterilized distilled water. Collected spores were filtrated through cheese cloth. However, fungi were cultured in agitated biomalt liquid medium to obtain the blastospores. The cultures were allowed to grow for 6 days at 25°C, then blastospores were harvested by filtration through sheese cloth and homognization using magnetic strirres.

Preparation of cell culture dishes

The cell line used in this work was obtained from an embryon primary culture of *Phthorimea operculella.* The establishment, cloning, passages and experimental techniques were carried out in the cell culture unit in the center of virology -IRD- Faculty of Agriculture, Cairo University.

The cell line obtained from primary culture of *P. operculella* was maintained as an attached cell line at 27°C in Grace s modified media (Lery and Fediere, 1990) with 15% FBS (Fetal bovine serum). The Pop cells were harvested from late-log-phase growth and seeded in Petri dishes (35 mm in diameter) 2 x 10^5 cells / dish.

The dishes were incubated at 27°C in a constant humidity chamber. The impact on the cell growth or any cytopathological effect was determined by observations and photography under normal or phase contrast inverted microscope, at a magnification of 150 to 450 x.

Studying the effect of crude filtrate of entomopathogenic fungi on *P.operculella* cell line

Suspensions containing the spores described above (conidosopres and / or blastospores) were filtered using Whatman filter paper and then prepared for cell culture treatment by deviding the volume of each suspension separately in 2 plastic test tubes. The first treatment was conducted by filtering the suspension through 0.45 seringe strile filter under sterilized conditions. The second treatment was carried out by sonicating the suspension for 1.5-2 min, then filtering the sonicated suspension through 0.45 seringe strile filter under the lamnar-air flow. The pre-prepared monoattached layer dishes of Pop cells (containing $2x10^5$ cells / dish) were treated by adding 250l of one of the prepared toxin suspension (filtrated or sonicated). Every treatment was represented in 3 replicates. Untreated control dishes were identically tested including appropriate

additions of fungi-free media or distilled water as a positive control and untreated dishes as a negative control. The concentrations of tested spore suspensions were $4x10^7$, $1x10^5$ and $2x10^7$ spores / ml for *B. bassiana, M. anisopliae* and *V. lecanii*, respectively. The cell dishes were checked daily and any observed effects on the cells were recorded and photographed.

RESULTS

The effect of crude filtrate on the cells of a *P. operculella* cell line

The treatment of the cells of *P. operculella* with crude filtrate produced by the entomopathogenic fungi *M. anisopliae, B. bassiana* and *V. lecanii* in both shake culture and solid medium (PDA), showed cellular changes. The cells which were treated with *M. anisopliae* crude filtrate revealed granular content, irregular form and particular alterations of the cellular membrane (Fig.1). Different cytotoxic effects by *B. bassiana* crude filtrate were observed on *P. operculella* cell line. The examined cells revealed elongated shape and agglomeration of dead cells (Fig. 2). The cytotoxic effects of *V. lecanii* crude filtrate were rather similar to the case of *B. bassiana* filtrate. Great reduction in cell titers treated with different concentrations of the three entomopathogenic fungi crude filtrates were observed compared with untreated cells.

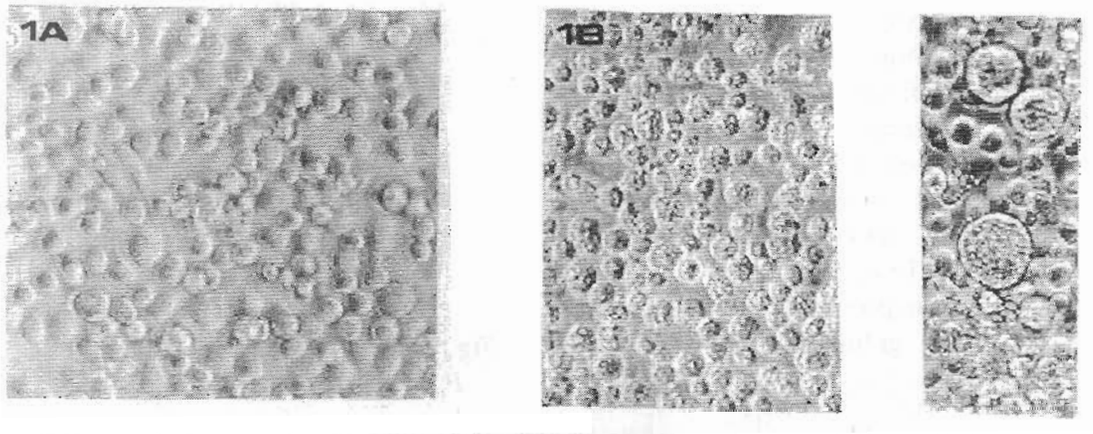

Fig. (1): Effect of the crude filtrate of M.anisopliae on P.operculella cell line (A) control unteated cells, (B) treated cells showing granular contents and alteration on the cellular membrane. (phase contrast x450).

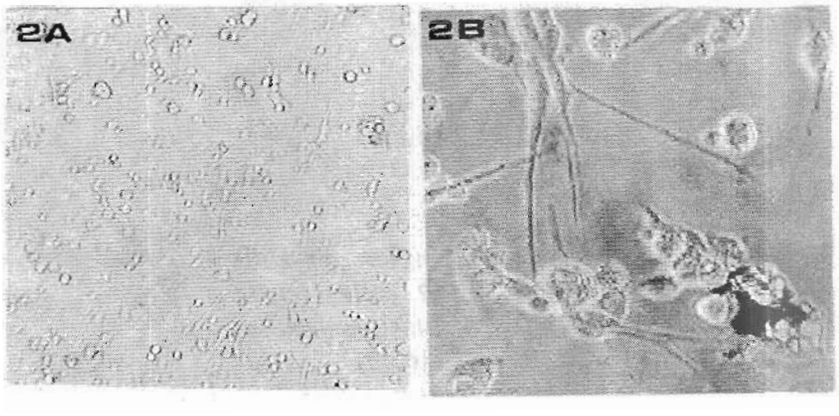

Fig.(2):Effect of the crude filtrate of B.bassiana on P.operculella cells, (A) Treated cells showing elongated cells (fibroplast) (x150), (B) Elongated cells and agglomeration of dead cells (x450).

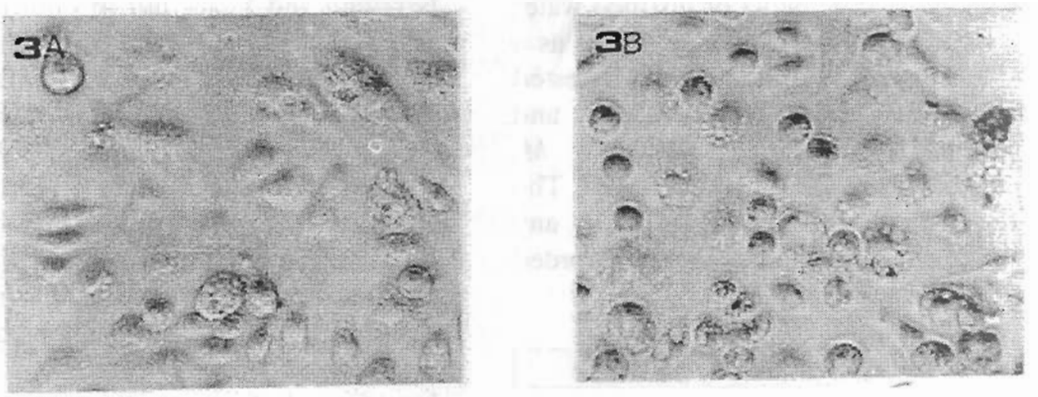

Fig. (3): Effects of sonicated conidospores of M.anisopliae on P.operculella cells, note the intensive vacuoles and irregular forms of cells (x450 (A) phase contrast, (B) normal objective).

Effect of filtrated sonicated spores on the *P. operculella* cell line

The cytotoxic effect of filtrated sonicated conidospores and blastospores of entomopathogenic fungi *M. anisopliae, B. bassiana* and *V. lecanii* on the cells of *P. operculella* were observed. The *M. anisopliae* filtrated sonicated conidospores induced cellular alterations, and irregular intensive vacuoles (Fig.3). However, the cells treated with sonicated blastospores showed associated crystals (Fig.4) and granular content in the

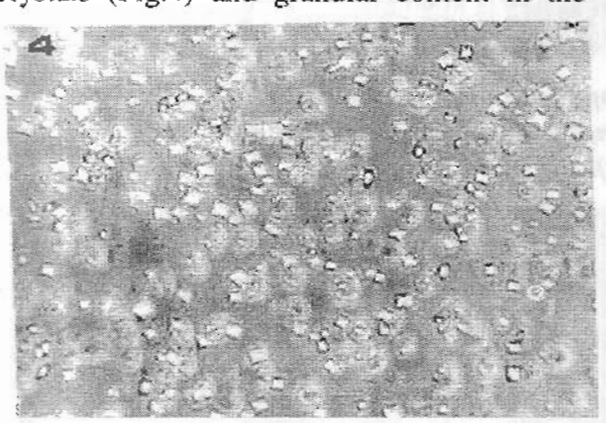

Fig. (4): Associated rectangler crystals observed in P. O cells treated with sonicated blastospores of M.anisopliae (x450).

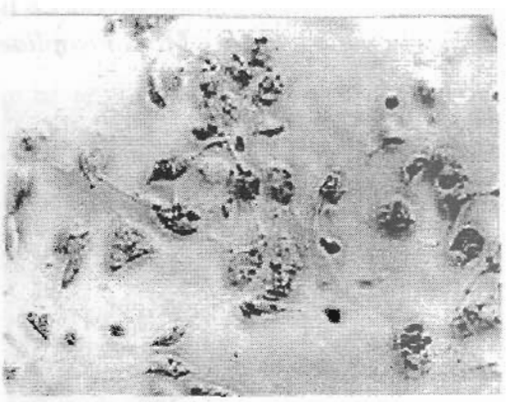

Fig. (5): Fibroplast elongated cells of P.operculella treated with sonicated lastospores of B. bassiana. (x450)

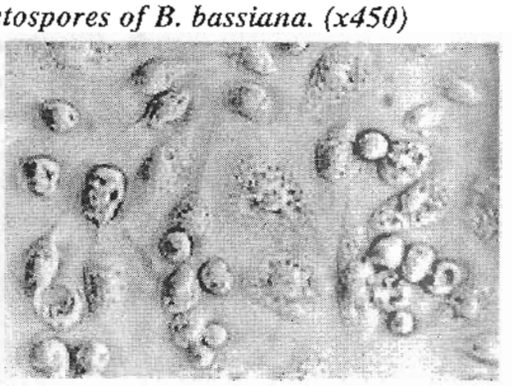

Fig. (6): Vacuoles formed in P.o cells treated with sonicated blastospores of B.bassiana (450).

cells. No cytotoxic effects of *B. bassiana* sonicated conidospores appeared on the cell culture of *P. operculella*., while the treatment of *P. operculella* cell culture with *B. bassiana* sonicated blastospores showed spectacular symptoms; mainly very deformed cells. These cells were transformed to fibroplast elongated cells (Fig. 5) filled with intensive vacuoles (Fig. 6). The same effects were noticed when the *P. operculella* cell line was treated with *V. lecanii* filtrated sonicated spores.

In addition to these cytopathic effects, the number of cells of *P. operculella* cell line were greatly reduced compared with the untreated control cells (Fig. 7).

treated cell line with sonicated blastospores of V.lecanii.

DISCUSSION

The obtained results demonstrate the *in vitro* impact of the entomopathogenic fungi crude filtrates of *M. anisopliae*, *B. bassiana* and *V. lecanii* on *P. operculella* cell line which reflect the effect of toxins related to the mycelium growth of the three entomofungi. These exotoxins induced cellular changes in the treated cells. The cellular responses differed according to fungal species. The *M. anisopliae* crude filtrate caused cell granular contents, irregular form and alteration in the cellular membrane. However, the *B. bassiana* and *V. lecanii* crude filtrate caused elongated cell shape (fibroplast). These effects agree with the results observed by Vey *et al.* (1993) who reported that the exotoxins were secreted by the fungal mycelia *Hirsutella thompsonii* var *thompsonii* in a defined culture broth in shake. These toxins caused granular contents, less refringence and irregular form to *Bombyx mori* cell culture. Similar cytotoxic effects were noticed in the epithelial cells of malpighian tubules and hemocytes after *G. mellonella* interahemocelic injection with destruxin toxins (Dumas *et al.*, 1996). These cells showed an intense vacuolization consisting of the accumulation of small rounded vacuoles. Different cytotoxic effects were noticed when *P. operculella* cell cultures were treated with filtrated sonicated conidospores and blastospores of the three entomopathogenic fungi. The *P. operculella* treated cells with sonicated conidospores of each one of the three fungi showed irregular forms and intensive vacuoles. However, these cells, treated with sonicated blastospores of the three fungi, showed associated crystals in culture medium and (fibroplast) elongated cells filled with intensive vacuoles. Therefore, it seemed that the sonicated blastospores induced more obvious cellular and stronger cytotoxic effects than crude broth filtrate secreted in fungi media. The recent study suggests a specificity in the cytotoxic action of both crude filtrate produced in fungi media and filtrated sonicated spores. Several studies on the purification, characterization and mode of action of the toxins secreted in the filtrated crude culture of *M. anisopliae, B bassiana* and *V. lecanii* have been reported by Dumas *et al.* (1996), Vey *et al.* (1987), Kiessling (1986) and Murakoshi *et al.* (1987) . However, the mycotoxins released from the sonicated spores of these fungi appear to be new and need more deep toxicological investigations, identification and characterization (Sewify *et al.*, 2000).

REFERRENCES

Dumas, C, Ravalleg M., Matha V., and Vey A. (1996). Comparative study on 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.

Hamill, R. L., Higgins C. E., Boat H. E.,and Gormann M. (1969). The structure of beauvericin, a new depsipeptide antibiotics toxic to *Artema salina* Tetrahedron letters 49 : 4255 4258 .

Kanaoka, M. Akira A., Murakoshi S., Ichinoe M., Suzuki A., and Tamura S. (1978). Bassianolide, a new insecticidal cyclodepsipeptode from *Beauveria bassiana* and *Verticillium lecanii* . Agric. Biol. Chem., 42 (3): 629 635.

Khamiss, O., Lery X., Belal M.H., Badawy H. A., Gianotti J.,and Abol-Ela S.M., (1998). Effects of some insecticides on the division of a *Spodoptera littoralis* cell line and on the replication of *Sl* Baculovirus (NPV). Appl. Entomol. Zool . 33(3): 349 355.

Kiessling, K. H. (1986). Biochemical mechanism of action of mycotoxins. Pure. Appl. Chem. 58: 327- 333.

Lery, X. and Fediere G. (1990). Effect of different amino acids and vitamins on Lepidopteran cell cultures . J. Invertebr . Pathol . 55: 47 51 .

Loutelier, C., Cherton J. C., Lange C., Taris M., and Vey A. (1996). Studies on the dynamics of the production of destruxins by *Metahrizium anisopliae* Direct high performance liquid chromatographic and fast atom bombardment mass spectrometric analysis correlated with biological activity tests. Journal of chromatography A, 738: 181 189 .

McCoy, C. W., Bullock R. C., and Dybas R. A. (1982). Avermectin B_1 : A noval miticide active against citrus mites in Florida . Proc. Fla. State Hortic. Soc. 95, 51 56.

Murakoshi, S., Ichinoe M., Suzuki A., Kanoaka M., Isogai A., and Tamura S. (1987). Presence of toxic substance in fungus bodies of the entomopathogenic fungi, *Beauveria bassiana* and *Verticillium lecanii*. Appl. Ent. Zool. 13 (2): 97 102.

Ohtomo, T., Murakoshi S., Sugiyama J., and Kurate H. (1975). Detection of aflatoxin B_1 in silkworm larvae attached by an *Aspergillus flaves* isolates from a sericultural farm. Applied Microbiology (30): 1034 1035.

Putter, I. J., Macconnell G., Preiser F. A., Haidri A. A., Ristich S.S. and Dybas R. A. (1981). A vermectins: Novel insecticides, acaricides and nematicidies from a soil microorganism. Experienta 37: 963 964.

Quiot, J. M., Vey A., and vago C. (1985). Effects of mycotoxins on invertebrate cells *in vitro*. Adv. Cell. Cult. 4: 199 212.

Sewify, G.H, Abol-Ela S. and Eldin M.S. (2000). Effects of the entomopathogenic fungus *Metarizium anisopliae* (METSCH.) and granulosis virus (GV) combinations on the potato tuber moth *Phthorimaea operculella* (ZELLER) (Lepidoptera: Gelechidae). Bull.Fac.Agric., Cairo Univ., (51) : 95-106.

Vey, A. (1998). Mycotoxins as pathogenicity determinats secreted by entomogenous fungi, and as tools for improvement of pest control. Abstracts of British Mycological Society, International Symposium The future of fungi in the control of pests, weeds and diseases 5-9 April 1998, Southampton, England.

Vey, A., Quiot J. M., and Vago C. (1987). Mode d action insecticide d une mycotoxins, la destruxine, sur lesdepteres vecteurs et disseminateurs de germes. C. R. Acad. Sci. Ser. 3, 354: 229 234.

Vey, A., Quiot J. M., Mazet I., and McCoy C. W. (1993). Toxicity and pathology of crude broth filtrate produced by *Hirsutella thompsonii* var. *thompsonii* in shake culture. J. Invertber . Pathol. 61: 131 137.

الملخص العربي

التأثيرات الخلوية السامة لراشح بعض الفطريات الممرضة للحشرات علي مزارع الخلايا من فراشات درنات البطاطس

اميمة خميس [1*] ، جمال السويفي [2] ، سعيد أبو العلا [1]
[1] مركز بحوث الفيروسات ، كلية الزراعة جامعة القاهرة.
[2] قسم الحشرات الاقتصادية والمبيدات ، كلية الزراعة ، جامعة القاهرة.
* معهد الهندسة الوراثية ، جامعة المنوفية.

استخدم في هذه الدراسة خط انتاج لخلايا حشرة فراشة درنات البطاطس لدراسة التأثيرات السامة لفطريات الميتاريزيوم ، البوفاريا ، الفيرتسيليوم الممرضة للحشرات علي الخلايا المختبرة.

تم معاملة الخلايا بالراشح الناتج من كل من هذه الفطريات علي حدة سواء الناتجة من البيئة السائلة بالرج أو من غسيل الجراثيم علي البيئة الصلبة.

أوضحت معاملة الخلايا بالراشح من فطر الميتاريزيوم اعراض واضحة حيث أظهرت الخلايا بالفحص الميكروسكوبي محتويات حبيبية متكتلة مع اختلاف وتشوه وتعرج الجدار الخلوي الخارجي ، أما في حالة المعاملة براشح كل من البوفاريا والفيرتسيليوم فقد نتج عن ذلك ظهور الشكل الاستطالي المغزلي للخلايا كرد فعل حلوي.

اما في حالة تعريض الراشح من الفطريات السابقة الي الموجات فوق الصوتية قبيل معاملة الخلايا بها فقد اظهرت النتائج أعراض مختلفة حيث ظهرت الخلايا مليئة بالفجوات بالاضافة الي ظهور عرض مميز في حالة الميتاريزيوم حيث ظهر بكثافة انتشار بللورات كريستالية في اطباق الخلايا بعد المعاملة بالاضافة الي ظهور الفجوات والشكل الغير منتظم للخلايا.

هذه النتائج تقترح ان الجراثيم البلاستوسبورية المعرضة للموجات فوق الصوتية ذات تأثير قوي وتسبب تغيرات جوهرية سامة علي الخلايا اكثر من الراشح الناتج في البيئة السائلة.