ISOLATION AND IDENTIFICATION OF THREE ENTOMOPATHOGENIC FUNGI

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Abstract

Entomopathogenic fungi used in the present study were *Metarhizium anisopliae, Beauveria bassiana* and *Paecilomyces fumosoroseus*. The first fungus was isolated from the *Rhynhophorus ferruginens* in Ismailiya governorate, the second from the white fly in the Sharkia and the third from *Aphis gossypii* in Al-Fayoom governorate. *Beauveria* colonies are usually slow growing, downy, at first white but later often becoming yellow. The spores as balls represent dense clusters of large numbers of conidiogenous cells and conidia. Paecilomyces colonies are usually slow growing, at first white but later often becoming rosy-tan to smoky-pink (or Grey) in mass. Ovoid to elongate. Metarhizium colonies are usually strong growing, olivaceous green with yellowish color in reverse. yellowished green in mass. RAPD amplified DNA products using 5 primers differentiated 3 isolates of entomopathogenic fungi belonging to different genera collected from different locations and host insects. Total of 91 amplified fragments generated 81 polymorphic bands can be used as RAPD markers. There are 81 specific markers out of 137 total amplified fragments. RAPD markers were found to be useful as isolate specific marker. The results showed the specific markers for the 3 entomopathogenic fungi isolates resulting from RAPD-PCR analysis. B. bassiana isolate exhibit 18 positive amplified fragments and 9 negative, while P. fumosoroseus isolate generated 22 positive amplified fragments as a highest number of specific positive markers and 12 negative amplified fragments. As well M. anisopliae isolate showed 15 positive amplified fragments and 8 negative. The proteins of three entomopathogenic isolates were extracted from their mycelium and fractionated by SDS-PAGE. The proteins of B. bassiana, P. fumosoroseus and M. anisopliae were separated into 11, 4 and 5 bands respectively.

INTRODUCTION

Entomopathogenic fungi were among the first organisms to be used for the biological control of pest. More than 700 species of fungi are pathogenic to insects. Most are found within the deuteromycetes and entomophthoralis. Fungi infect individuals in all orders of insects, most common are Hemiptera, Diptera, Coleoptera, Lepidoptera, Orthoptera and Hymenoptera (David, 1967). Some insect pathogenic

fungi have restricted host ranges while other fungal species have a wide host range for example, *Metarhizium anisopliae, M. flovordiae, Paecilomyces fumosoroseus, Beauveria bassiana* and *B. brongniartii*. This host specificity may be associated with the physiological state of the host system (i.e., insect maturation and host plant) (McCoy *et. al.*, 1988).

In many cases, these traits are variable and they generally yield insufficient information for comparative taxonomic studies. DNA techniques now permit the analysis of genetic markers to establish the identity of individuals. Direct analysis of DNA polymorphisms is now general approach to identify and compare fungi at intraspecific, species, genus or higher level. To date, these molecular genetic techniques which have proven most useful are RAPD-PCR technique. RAPD-PCR technique would be useful for studies in entomopathology, epizotics and insect biocontrol (Hegedus and Khachatourians 1995). The analysis of random amplified polymorphic DNA (RAPD) has been proposed to resolve genetic variations between fungal strains (Williams *et. al.*, 1990).

Many species of fungi are difficult to identify particularly, if they lack reproductive structure. Even when structures of sexual or a sexual reproduction are present, isolates may exhibit a typical, intermediate, variable or no diagnostic morphological characteristics which makes definite identification difficult. The principal reason for selecting protein patterns for additional diagnostic characters of fungi was that their diversity is directly to the diversity of the coding genes and may express specific differences or similarities among organisms (Gottlieb *et. al.*, 1971).

MATERIALS AND METHODS

Entomopathogenic fungi used in the present study are *Metarhizium anisopliae* isolated from the *Rhynhophorus ferruginens* at Ismailiya governorate, *Beauveria bassiana* from the white fly in the Sharkia and *Paecilomyces fumosoroseus* was kindly provided by Insect Pathogen Unit (IPU) of Plant protection Research Institute, Agriculture Research Center. The fungal isolates were grown on Czapek Dox Agar medium (CDA) and Sabouraud Dextrose Agar medium (SDA).

Isolation of the tested organisms from insects:

Insects showing signs of fungal infection were collected. After sporulation, fungi retrieved directly from surface of cadavers were amounted with lactophenol cotton blue, and examined microscopically.

Collected cadavers were kept in containers on filter papers that saturated daily with water at 25ºC to encourage fungal sporulation. Hyphal tips from the external growth

mycosed cadavers were taken on tip of a sterile wire loop and inoculated on appropriate Sabouraud Dextrose agar or Czapek Dox agar media, incubated at 25ºC. Inoculated dishes were inspected daily to observe the purified fungal growth and used to confirm the disease cycle. Then stored on slants of SDA or CDA media at 4ºC until they were used in subsequent experiments.

Identification of fungal isolates

Insect cadavers or mycelium were mounted on slides, stained with lactophenol cotton blue, examined microscopically and inoculated on SDA or CDA media. The fungi were isolated and incubated at 25ºC for 14 days. Infection of the host with fungi was manifested by mycosis symptoms and was examined to prove that they are the isolates of the first step (Abdel-Gwad 2000).

The fungal isolates were sub cultured every 14-30 days, kept at 4ºC and were passed through their natural host or through the wax moth larvae *Galleria mellonella* to maintain their virulence (Butt & Goettel 2000).

Identification of fungal species was carried out using a color Atlas of pathogenic fungi (Frey *et. al.*, 1979), key of (Humber 1997), Electron Microscope Unit, Ain Shams Univ., Egypt and by The Regional Center for Mycology and Biotechnology, Al-Azhar Univ., Egypt.

Comparison among fungal isolates by analysis of RAPD markers

The fungal isolates were grown on CDA medium for two weeks at 25-28˚C. A plug of the mycelium (1cm) from each isolate was transferred into a conical flask containing 50 ml CDA medium and allowed to grow on a rotary shaker (120 rpm) at room temperature. Mycelia were harvested after 4 days and stored at -20˚C until needed. (Tigano-Millani *et. al.*, 1995)

RAPD Reaction

Random Amplification of DNA of the fungi under study was performed according to the method of Williams *et. al.*, (1990) with some modification

Taq DNA polymerase, Taq DNA polymerase buffer (10x), $MgCl_2$,Primer

Primer name	Sequances (5′–3′)
OPA 13	CAGCACCCAC
OPA 20	GTTGCGATCC
OPD 07	TTGGCACGGG
OPD 08	GTGTGCCCCA
OPE 20	AACGGTGACC

-dNTPs: A mixture of dATP, dTTP, dCTP, dGTP

Reaction mixture

DNA , 1 ul , Primer , 2 ul , DNTPs , 1 ul , Taq DNA polymerase,0.5 ul and Taq DNA polymerase buffer (10X) , 5 ul .

Polymerase Chain Reaction Buffer

DNA extraction buffer (CTAB buffer, pH 8.0) has the following ingredients:

Hexadecyl trimethyl-ammonium bromide (CTA) , 2% (w/V) , NaCl , 1.4 M ,2-mercaptoethanol , 0.2% (V/V), EDTA, 20 mM and Tris-buffer , 100mM.

DNA washing buffer:consist of ethanol,76%(V/V) and ammonium acetate,10 mM.

Tris–EDTAbuffer consist of Tris –HCl(pH 8.0, 10 , and EDTA9pH8.0), 1.0 .

Tris–acetate-EDTA buffer(50x)(pH8.0)(volume/Later) consist of Tris base, 242g, glacial acetic acid , 57.1 ml and EDTA (0.5M), 100 ml.

Gel loading buffer (6x) :consist of bromophenol blue , 0.25%, xylene cyanol FF, 0.25% and glycerol in water, 30.

Polyacrylamide Gel Electrophoresis buffer

Protein extraction buffer :

Sodium Dodecyl Sluphate (1%(W/V), β-mercaptoethanol, 1%(V/V), Sodium phosphate (pH 7.0), 10 mM, Phenyl methyl sulphonyl fluoride (PMSF), 1 mM

Stock solution

Acrylamide/bis(30%):Acrylamider,27.5,N,N-bis-methylene-acrylamide

The solution was completed to 1000 ml with distilled water then filtered and stored at 4ºC in dark (30 days maximum)

1.5 M Tris-HCl (pH 8.8): Tris base, 18-5 g, Distilled water, 60 ml.

This solution was completed to 1000 ml with distilled water then filtered and stored at 4ºC.

0.5 M Tris–HCl (pH 6.8), 10% SDS, 10 % ammonium persulphate (APS, Sample buffer (62.5mMTris, 20%Glycerol, 2%SDS, 5%B-ME), (5X) Electrode buffer(1X25mMTris,192mMglycine, 0.1% SDS were prepared

Staining solution :Coomassie Brilliant blue R-250, 0.1 %, Methanol,40 %, Glacial acetic acid,10 %.

De-staining solution :Methanol, 40 %, Glacial acetic acid, 10 % Casting of SDS–Polyacrylamide Gel: Separating Gel (12%) and Stacking gel (4.0 %) were prepared:

Genomic DNA extraction

Extraction of genomic DNA was conducted using hexadecyltri-methyl-ammonium-bromide (CTAB) according to the method of Doyle and Doyle (1987). The mycelia were grounded to fine powder under liquid nitrogen then transferred to 30 ml microfuge tube. Five ml of preheated DNA extraction buffer (CTAB buffer) was added and mixed with the powdered mycelium using vigorous vortexing for 30-60 sec. This suspension was incubated at 60˚C for 30 min. with optional occasional gentle swirling. The DNA was then extracted with chloroform : isoamyl alcohol (24:1). The aqueous

phase containing DNA was collected after centrifugation at 1600 xg for 10 min. then the DNA was precipitated with two volumes of ice-cold Ethanol and left overnight at -20˚C. The DNA was precipitated by centrifugation at 500 xg for 2 min at 4˚C followed by washing buffer. Then the suspension was centrifuged at 1600 xg for 10 min, the pellet dried at room temperature for 1 hr. The dried DNA pellet was dissolved in 50 цl TE buffer. This suspension was taken as the template in the PCR mixture.

PCR analysis

PCR amplification was performed in a Perkin–Elmer\DNA Thermal Cycler 2400 (Norwalk, CT) programmed to fulfill 40 cycles an initial denaturation cycle for 5 min at 94˚C. Each cycle consisted of a denaturation step at 94˚C for 1 min, an annealing step at 37˚C for 2 min, and an elongation step at 72˚C for 2 min. The primer extension segment was extended to 7 min at 72˚C in the final cycle. The amplification products were resolved by electrophoresis in agarose gel containing ethidium bromide (0.5 цg/ml) in 1x TAE buffer at 95 volts for two hrs. PCR products were visualized on UV light and photographed Amplified products were visually examined and the presence or absence of each size class was scored as 1 or 0 respectively.

Extraction of proteins from entomopathogenic fungi

The fungi under study were allowed to grow on Czapek Dox liquid medium, the mat of each fungus was collected, ground to a fine powder under liquid nitrogen. Protein was extracted by homogenizing the powder in protein extraction buffer (Marshall *et. al.*, 1984) containing 1 mM (PMSF) as protease inhibitor After homogenization the extract was incubated for fifteen minutes on ice then centrifuged at 15000 rpm for fifteen minutes. The pellet was discarded and the supernatant was kept in ice.

Preparation and casting of Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gels were prepared from monomer solution of 30% acrylamide and 2.7 Bis-acrylamide. An equal concentrations of the samples were mixed with 0.25 volume sample buffer, incubated at 95ºC water bath for about two minutes to denature the protein, and all samples were loaded using 12% SDS-PAGE. The separating gel solution was placed in 125 ml side arm vacuum flask, Stoppard flask and apply vacuum for several minutes to insure suction of oxygen from the solution. The gel was left to polymerize at room temperature for two hours. Stacking solution was added to the top of the separating gel combs, inserted and the gel was allowed to polymerize for at least half an hour. After polymerization the combs were removed slowly from the gel and each well was rinsed with tank buffer (Electrode buffer) using a Hamilton syringe.

Electrophoresis of Protein

The gel apparatus was assembled and the lower and upper chambers were filled with 1x tank buffer. A Hamliton syringe was used to load equal amount of sample protein in each well. High molecular weight standard Protein was applied to one well in the gel. Electrophoresis was carried out at about 100 volts till the dye of the samples was eluted from the gel. After electrophoresis, the gel was stained using 50 ml of staining solution for two hours and photographed.

RESULTS AND DISCUSSION

Identification and characterization of entomopathogenic fungi

(1) *B. bassiana* (Balsamo) Vuillemin

Beauveria colonies are usually slow growing, downy, at first white but later often becoming yellow. Microscopic examination showed that *B. bassiana* conidiogenous cells with globose bases and extended, denticulate rachis and the conidia is globose in shape, aseptate<3.5цm. The spores balls representing dense clusters of large numbers of conidiogenous cells and conidia (Fig.1).

(2) *P. fumosoroseus* (Bainier) Wize

Paecilomyces colonies are usually slow growing, at first white but later often becoming rosy-tan to smoky-pink (or Grey) in mass. Microscopic examination showed that *P. fumosoroseus* pigmented with a distinct neck and base flask-like (swollen base) to narrowly awl-shaped or nearly globose, borne single or in groups in whorls on conidiophores, on short side branches or in apical whorls, conidia aseptate, ≤ 4 цm, in long chains, one celled, ovoid to elongate (Fig. 1)

(3) *M. anisopliae* (Metschnikoff) Sorokin var. *anisopliae*

Metarhizium colonies are usually strong growing, olivaceous green with yellowish color in reverse. Microscopic examination showed that M. anisopliae conidiophores aggregated in dense tufts, more or less verticillate branching, 2.5 цm. Phaialides clavate 9-14 X 2-3.5 цm long, with rounded apex. Conidia produced in long chain, aseptate, cylindrical, 3.5-8 X 2.5-3.5 цm, thick walled, yellowished green in mass (Fig. 1).

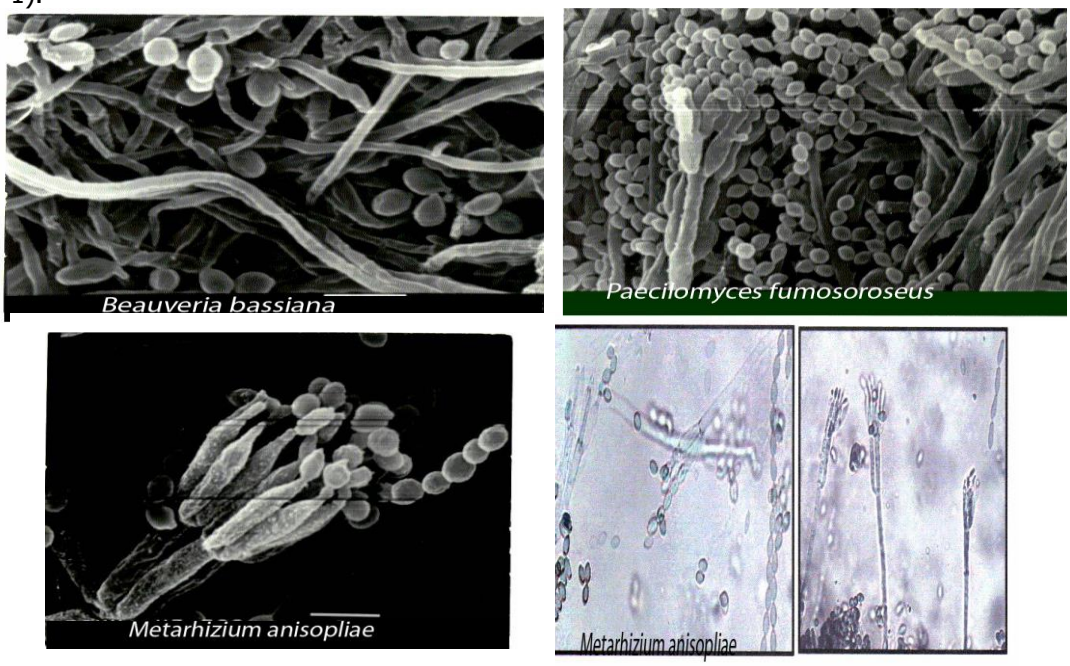

Fig. 1. morphological characteristics of Entomopathogenic fungi isolates.

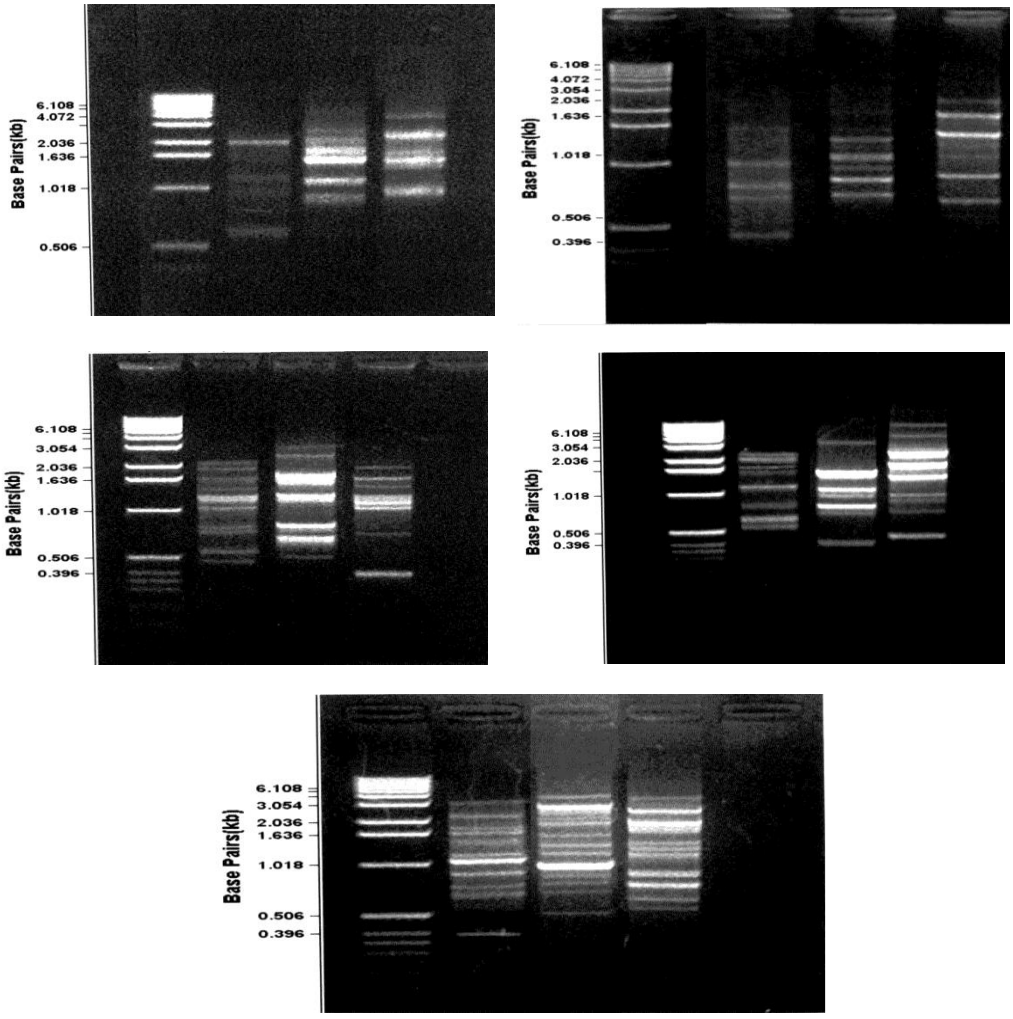

Fig. 2. RAPD fingerprint profile of 3- entomopathogenic fungi isolates (Lanes 1-3, *Beauveria bassiana, Paecilomyces fumosoroseus* and *Metarhizium anisopliae)* using OPA- 13, OPA- 20, OPD- 07, OPD, 0 8 and OPE- 20 primers. M, DNA marker ladder as a molecular size standar

Molecular characteristics using RAPD analysis

The RAPD markers are DNA fragments generated by the amplification of genomic DNA through PCR reaction using a single primer of arbitrary nucleotide sequences in each reaction. RAPD amplified DNA products using primers 1-5 with 3 isolates of entomopathogenic fungi belonging to different genera collected from different locations, several host insects were used in RAPD analysis (Fig.2). The segregating RAPD bands amplified in the 3 isolates using 5 primers, the bands were scored as present (1) and absent (0). Primer (1) generated 17 amplified fragments ranged between 3.63 and 0.36 bp (figure 1) the highest number of detectable fragments (11

bands) appears in the isolate *P. fumosoroseus*, while the lowest number of bands was record by isolate *M. anisopliae* (7bands) followed by *B. bassiana* (9 bands)

Primer (2) scored 10 RAPD amplified bands sized between 3.70 and 0.69 bp. Isolates *M. anisopliae* and *P. fumosoroseus* showed 7and 6 bands respectively as a highest number of bands. Isolate *B. bassiana* recorded 4 bands as the lowest number of bands (figure. 2).

The results in (figure 1), showed that primer (3) generated 19 amplified bands for all isolates with 19 markers sized from 7.34 to 0.58. *M. anisopliae* showed 10 bands, as the largest number of bands, followed by *B. bassiana* 8 bands, while isolate P*. fumosoroseus* showed 7 bands as the lowest number of bands.

In figure 2, 21 RAPD amplified markers were generated by primer (4), all ranged between 3.29 to 0.33 bp. The isolate *P. fumosoroseus* recorded the highest number of bands (12 bands) followed by *B. bassiana* isolate (11 bands), while the lowest number of bands (8 bands) was recorded for isolate *M. anisopliae*.

Primer (5) scored 24 RAPD amplified bands sized between 4.03 and 0.60 bp. Isolates *B. bassiana* and *P. fumosoroseus* showed 12 bands, while the isolate *M. anisopliae* was recorded 13 bands as shown in figure 1.

Numbers of amplified fragments, polymorphic bands and specific markers of the 3 isolates were generated from RAPD analysis. Total 91 amplified fragments generated 81 polymorphic bands.

The size of amplified fragments ranged from 7.34 bp in primer 3 to 0.33 bp in primer 4. Primer 2 detected the lowest number of total polymorphic bands (PB), 6 markers out of 10 total amplified fragments, while the largest number was detected for primer 5 (21 markers out of 24 total amplified fragments).

All isolates showed number of common bands (CB). These bands were 3 for both primers (1 & 5), 2 for both primers (2 & 4). No common bands appeared with primer 3. *B. bassiana* isolate generated 9 amplified fragments from (primer 1), 4 from (primer 2), 8 from (primer 3), 11 from (primer 4) and 12 from (primer 5). While *P. fumosoroseus* isolate generated 11 amplified fragments from (primer1), 6 from (primer 2), 7 from (primer 3), 12 from both primers (5 & 6). As well as *M. anisopliae* isolate generated 7 amplified fragments from both primers (1 & 2), 10 from (primer 3), 8 from (primer 4) and 13 from (primer 5). RAPD analysis generated specific marker either in the presence or absence of a band for the 3-entomopathogenic fungi. There are 81 specific markers out of 137 total amplified fragments. RAPD markers were found to be useful as isolate specific marker. The results showed the specific markers for the 3-entomopathogenic fungi isolates resulting from RAPD-PCR analysis.

B. bassiana isolate exhibit 18 positive amplified fragments and 9 negative, while *P. fumosoroseus* isolate generated 22 positive amplified fragments as a highest number of specific positive markers and 12 negative amplified fragments. As well as *M. anisopliae* isolate appeared 15 positive amplified fragments and 8 negative.

Many major species of fungal entomopathogens have basic diagnostic characters making them quickly identifiable, it must be remembered that species such as *B. bassiana* (Bals.) Vuill., *M. anisopliae* (Sorok.) Metsch., and *P. fumusoroseus* (Bainier) Wize are widely agreed to be species complexes whose resolutions will. Depend on correlating molecular, morphological, pathological and other characters (Humber 1996).

In the present study, the morphological identification with microscopic examination showed that *B. bassiana* conidiogenous cells with globosely bases and extended denticulate rachis. The conidia is globose in shape, aseptate < 3.5 µm. While microscopic examination of *P. fumosoroseus* showed that the conidiogenous cells with a distinct neck and base flask-like (swollen base) to narrowly awl-shaped or nearly globose. Conidiophores, on short side branches or in apical whorls. Conidia aseptate, ≤ 4 µm in long chains, one celled, ovoid to elongate. As for *M. anisopliae* microscopic examination showed that conidiophores aggregated in dense tufts, more or less verticillate branching, 2.5 µm. Phaialides clavate 9-14 X 2-3.5 µm long, with rounded apex. Conidia produced in long chain, aseptate, cylindrical, 3.5-8 X 2.5-3.5 µm, and thick walled yellowish green in mass. About absent of relationship appeared between spore size of entomopathogenic fungi isolates and their virulence. No relationship appeared between virulence of *Verticillium lecanii* isolates and their spore size, which agrees with (Yokomi & Gottwald 1988).

Williams *et. al.*, (1990) proposed that RAPD, consist of dominant molecular markers identify species and strains of entomopathogenic fungi. In the present study five decamer primers (OPA-13, OPA-20, OPD-07, OPD-08, OPE-20) of arbitrary sequence were used to differentiate among three entomopathogenic fungi *B. bassiana*, *P. fumosoroseus* and *M. anisopliae*. Regarding data that employs 5 primers with 3-entomopathogenic fungi isolates. The results showed that the specific markers for the 3-entomopathogenic fungi isolates resulting from RAPD-PCR analysis. Where *B. bassiana* isolate exhibit 18 positive amplified fragments and 9 negative, while *P. fumosoroseus* isolate generated 22 positive amplified fragments as a highest number of specific positive markers and 12 negative amplified fragments. As well as *M. anisopliae* isolate appeared 15 positive amplified fragments and 8 negative.

Total proteins analysis using Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE).

Total cellular proteins of entomopathogenic fungal isolates under study were fractionated on denaturing gels by electrophoresis (Laemmli 1970). Data in table (1) showed the scored bands detected in each fungus. The bands were

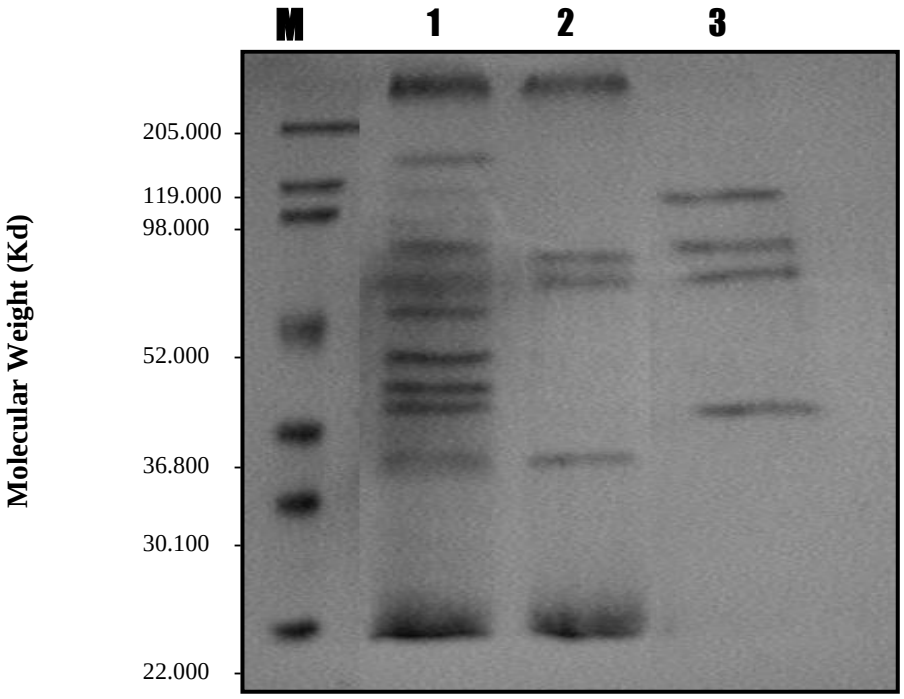

Fig 3. Electrophoretic patterns of total cellular protein of different isolates of entomopathogenic fungi. The numbers beside the gel indicate molecular masses of standard marker proteins. Lane 1, *Beauveria bassiana*, Lane 2, *Metarhizium anisopliae*, Lane 3, *Pacilomyces fumosoroseus*; Lane M, Standard molecular masses.

Scored as present (1) and absent (0), the scored bands were (11, 5, 4) in *B. bassiana, M. anisopliae* and *P. fumusoroseus* respectively.

Results in table (2) exhibit the molecular weight of protein bands detected in each fungus, where in *B. bassiana*, the molecular weight ranged between (184.437 and 17.3 M.W.), while in *M. anisopliae* the molecular weight of detected bands ranged between (184.092 and 17.245 M.W.). However in *P. fumusoroseus* the molecular weight were ranged as (110.088 to 43.642 M.W.).

In the study the protein of three isolates were extracted from their mycelium and fractioned by SDS-PAGE revealed a maximum number of 19 bands, which were not necessarily present in all samples. The protein of *B. bassina* was separated into 11 bands ranging in molecular mass from (17 KDa to 183 Kda). This results disagree with (Gregs, *et. al.*, 2005) who extracted the protein from *B. bassina* using (SDS-PAGE) and found that (12-15) distinct reactive protein bands, ranging in molecularmass from (12 KDa to > 95 KDa) (under denaturing conditions). The protein of *M. anisopliae* was

separated into 5 bands ranging in molecular mass from (17 KDa to 183 KDa). Also these results disagree with (Carvalho Freire, *et. al.*, 2001) who studied total protein in mutants of *M. anisopliae* and found that the concentration of total protein in the mycelium extracts of the wild strain presented 7 bands, and the mutant 5 bands. In the present study *P. fumosoroseus* fractionated in 4 bands rangingin molecular mass from (43 KDa to 110KDa). *M. anisopliae* was the most virulent isolates against *S. littoralis* (Boisd.) than the remaining isolates this is may be due to the presence or absence of some protein bands from those two isolates*. M. anisopliae* has only one positive marker (band No. 2 with M.W. 87 KDa) that not present in remaining isolates. (Berny and Hennebert 1990)

Table 1. Scored bands of *B. bassiana, M. anisopliae* and *P. fumosoroseus* isolates obtained by (SDS-PAGE).

No.	Marker (Kda)	Lane 1	Lane 2	Lane 3
1	183.574	1	1	0
2	136.21	1	0	0
3	123.908	0	0	0
4	110.872	0	0	1
5	101.752	1	0	0
6	90.673	1	0	1
7	87.261	0	1	0
8	78.199	1	1	1
9	70.42	1	0	0
10	63.759	0	0	0
11	57.228	1	0	0
12	50.656	1	0	0
13	46.075	1	0	0
14	43.642	0	0	1
15	36.584	1	1	0
16	32.065	0	0	0
17	27.395	0	0	0
18	24.667	0	0	0
19	17.268	1	1	0

Table 2. Molecular weight of SDS Protein bands detected in *B. bassiana, M. anisolpiae* and *P. fumosoroseus* .

Marker	Lane 1	Lane 2	Lane 3
(M. W.)	(M. W.)	(M. W.)	(M. W.)
205	184.437	184.092	110.088
119	136.21	87.261	88.124
98	101.773	79.024	77.927
52	91.103	36.808	43.642
36.8	77.837	17.245	
30.1	69.676		
22	57.15		
	49.979		
	46.332		
	36.911		
	17.3		

Table 3. Relative fragmentation (R_f) values of SDS protein bands detected in *B. bassiana, M. anisolpiae* and *P. fumosoroseus*.

Lane 1	Lane 2	Lane 3
R_f	R_f	R_f
0.036	0.039	0.24
0.16	0.336	0.329
0.283	0.369	0.381
0.327	0.65	0.595
0.375	0.915	
0.408		
0.49		
0.535		
0.561		
0.648		
0.917		

REFERENCES

1. Abdel-Gwad, H.A. 2000. Studies on entomopathogenic fungi for control certain lepidopterous insects on corn. Ph.D. Thesis, Cairo Univ., 229p.
2. Berny, J.F. and G.L. Hennebert. 1990. Variants of *Penicillium expansum* analysis of cultural and microscopic character as taxonomic criteria. In: Samson RA, Pitt JI, eds. Modern Concepts in *Penicillium* and *Aspergillus* classification. NATO AIS series A, Life Sciences 185, London: Plenum Press, 49-65.
3. Butt, T. M. and T. M. Goettel. 2000. Bioassay of entomogenous fungi. In: Bioassay of entomopathogenic microbes and nematodes. A. Navon and K. R. S. Ascher (eds.). CABI publishing, UK.
4. Carvalho Freire, L.L., A.B.L. Costa, L.B. Goes and N.T. Oliveira. 2001. DNA polymorphism and total protein in mutants of *Metarhizium* var *anisopliae* (Metsch.) Sorokin strain E_9. Braz. J. Microbiol. 32(2).
5. David, E.M. 1967. The physiology of the insect integument in relation to invasion of pathogens. In "insects and physiology" (J.W.L. Beament and J.E. Trehern, eds.) Oliver and Boyd. Edinburgh., 17-35.
6. Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11-15.
7. Frey, D., R.J. Oldfield and R.C. Bridger. 1979. A color atlas of pathogenic fungi. Wolfe Medical Publication, Holland, 168 p.
8. Gottlieb, L.D. 1971. Gel electrophoresis: new approach to the study of evolution. Bio-Science 21: 939-944.
9. Gregs, S.W., S.W. Huang and N.O. Keyhani. 2005. Allergens of the entomopathogenic fungus *Beauveria bassiana*. Clinical and Molecular Allergy 3:1.
10. Humber, R.A. 1996. Fungal pathogens of the Chrysomelidae and prospects for their use in biological control. In Biology of Chrysomelidae (eds. P. H. Jolivet, M. L. Cox & T. H. Hsiao). Vol. IV, in press. SPB Academic Publishing, The Hague
11. Humber, R.A. 1997. Fungi: Identification, pp. 153-185. In: Manual of techniques in insect pathology, L. Lacey (ed.) Academic Presses, USA.
12. Hegedus, D.D. and G.G. Khachatourians. 1995. The impact of bio-technology on hyphmycetous fungal insect biocontrol agents. Bio-technol. Adv. 13, 455-490.
13. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head Bacteriophage T4. Nature. 277: 680-685.
14. Marshal, H.F., M.A. Shirer and J.P. Cherry. 1984. Characterization of glandless storage proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Cereal Chem. 61: 166-169.

15. McCoy, C.W., R.A. Samson and D.G. Boucias. 1988. Entomogenous fungi in "CRC Handbook of Natural Pesticides, Part A. Entomogenous Protozoa and Fungi." (C.M. Ignoffo, ed.).
16. Tigno-Milani, M.S., R.J. Honeycutt, L.A. Lacey, R. Assis, M. McClelland and B.W.S. Sobral. 1995. Genetic variability of Paecilomyces fumosoroseus isolates revealed by molecular markers. J. Invertebr. Pathol., 67: 147-152.
17. Williams, J.G., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid, 18: 6531-6535.
18. Yokomi, R.K. and T.R. Gottwald. 1988. Virulence of *Verticillium lecanii* isolates in aphids determined by detached-leaf bioassay. J. Invertebr. Pathol., 51: 250-258.

عزل و تعريف ثلاثة فطريات ممرضة للحشرات

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تعتبر الفطريات الممرضة للحشرات من الأعداء الحيوية الطبيعية القادرة علي إمراض الحشرات وقتلها دون أن تسبب ضررا للإنسان أو الحيوان أو النبات أو عناصر البيئة الأخرى.

لإجراء هذا البحث تم استخدام ثلاث عزلات من الفطريات الممرضة للحشرات وهي *Beauveria bassiana*, *Paecilomyces fumosoroseus* و*Metarhizium anisopliae* وقد استخدمت هذه الفطريات الثلاث كمبيدات حيوية لمكافحة آفة خطيرة هي دودة ورق القطن الكبري *Spodoptera littoralis* (Boisd.), والتي تعتبر من اخطر الآفات الزراعية التي تسبب ضررا كبيرا.

ولقد تضمنت هذه الدراسة النقاط التالية:

1- عزل سلالات من الفطريات الممرضة للحشرات

تم عزل فطر الـ*Metarhizium anisopliae* من حشرة سوسة النخيل الحمراء بمحافظة الاسماعيلية. أما فطر *Beauveria bassiana* فقد تم عزله من حشرة الذبابة البيضاء من محافظة الشرقية. وفطر *Paecilomyces fusmosoroseu* تم عزله من حشرة من القطن من محافظة الفيوم.

2- تعريف وتوصيف عزلات الفطريات الممرضة للحشرات

- صفات الشكل الظاهري:

أوضحت النتائج أن فطر الـ *Metarhizium anisopliae* ينمو بصورة كثيفة علي البيئات الصناعية، ذات لون اخضر زيتوني ذو كونيديات اسطوانية او بيضاوية عريضة النهايتين (truncate) وابعاد الكونيديات3.5-2.5 x 8-5. µm.

وفطر *Beauveria bassiana* ذو مزارع بيضاء قطنية المظهر وللخلايا المكونة للكونيديات جزء قاعدي منتفخ ينتهي طرفيا بمحور متعرج (زجزاجي) والكونيديات تاخذ شكلا بيضاويا غير مقسم بأبعاد أقل من 3.5 µm.

وفطر *Paecilomyces fumosoroseus* ذو مزارع بنفسجية، غامقة اللون وللخلايا المكونة للكونيديات جزء قاعدي منتفخ مخروطي الشكل بأبعاد أقل من أو تساوي 4 µm.

الصفات الوراثية:

-إستخدام تحليل التضاعف العشوائي لمناطق متباينة من الحمض النووي:

تم استخدام خمس بادئات عشوائية لإ جراء تحليل التضاعف العشوائي لمناطق متباينة من الحمض النووي (RAPD) لثلاث عزلات فطرية تابعة لأجناس مختلفة عزلت من عوائل حشرية متباينة من مناطق جغرافية مختلفة. وقد تم تحديد احدي وثمانين حزمة (81)

دنا متعددة المظاهر (Polymorphic DNA bands) من مجمل احدي وتسعين شظية متضاعفة (Amplifiid fragments) يمكن اعتبارها كواسمات (Markers). كما أظهرت النتائج وجود احدي وثمانين واسمة مميزة (Specific markars). أظهرت العزلات الأ ولي التي تنتمي لجنس *Metarhizium*

*anisoplia*e وجود خمسة عشر واسمة مميزة موجبة وثمان واسمات مميزة سالبة. بينما اظهرت العزلة الثانية التي تنتمي لجنس *Beauveria bassiana* وجود ثمانية عشر واسمة مميزة موجبة وتسع واسمات مميزة سالبة. أظهرت العزلة الثالثة التي تنتمي لجنس *Paecilomyces fumosoroseus* وجود 22 واسمة مميزة موجبة و12 واسمة سالبة.

-تحليل بروتين للسلالات المعزولة باستخدام طريقة الفصل الكهربي للبروتينات باستخدام جهاز الالكتروفوريسيس (SDS-PAGE)

تم تحليل البروتينات للعزلات الثلاثة أوضحت النتائج المحصل عليها من الفصل الكهربي انه تم فصل فطر *M. anisopliae* الي خمسة خمسة حزم بروتينية، اما فطر *B. bassiana* فقد تم فصله الي إحدى عشر حزمة بروتينية. كما تم فصل أربع حزم بروتينية فى فطر *P. fumosoroseus*.