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Pathogenicity and Genetic Variability of Five Entomopathogenic Fungi Against *Spodoptera littoralis*

[1]Amer, M.M., [1]T.I. El-Sayed, [2]H.K. Bakheit, [3]S.A. Moustafa and [2]Yasmin A. El-Sayed

[1]Department of Botany, Faculty of Sci., Banha Univ., A.R.C., Egypt.
[2]Plant Protection Institute, A.R.C., Egypt.
[3]Agric. Res. Engineering Institute, A.R.C., Egypt.

Abstract: Five entomophathogenic fungi *Beauveria bassiona*, *B. brongniartti, Paecilomyces farmosus, Metarhizium anisopliae* and *M. flavoviridae* were used as a biological control agents for *Spodoptera littoralis*. The fungi were grown on specific 3 media. Five concentrations were applied for each fungus. 2nd and 4th instar larvae of *S. littoralis* were exposed for 48 h. to treated castor bean leaves by using dipping technique with conidial suspensions. The significance of study is to illustrate the ability of fungi conidiospores to exhibit positive influences on larval mortality as well as malformation in different stages of *S. littoralis* treated. These effects showed that, the mortality percentage increased with increasing concentrations and time elapsed after treatment. The conidiospores effectiveness appeared that, *M. anisopliae* and *M. flavoviridae* have the most effective isolates. *M. anisopliae* gave the highest mortality % (60 and 55%) to the 2nd and 4th instar larvae with lethal time (LT_{50}) 7 and 10 days respectively. While *B. bassiona* and *B. brongniartti* appeared the lowest mortality % and with the lowest LT_{50} followed by *P. farinasus*. SDS-PAGE of protein analysis showed a distinct protein contents and fractions of 5 fungi whereas 11, 10, 5, 11 and 6 protein species with the positive markers, 5, 4, 1, 7 and 2 bonds respectively distinguished it from them. On the other hand they associated into 2 bands. The frequency of genetic variability (protein and DNA fingerprint) was detected in 5 fungi dependant, variations in the effectiveness as a biological control agents against *S. littoralis*. Five arbitrary primers were successfully used to amplify DNA extracted of 5 fungi isolates and have shown the existence of high polymorphism among 5 isolates with about 83% genetic variation. On the other hand primer, OPA-13 revealed more characteristic. The number of polymorphic amplicans with primer (OPA-13) 2,2,2,5 and 6 with % polymorphic 25, 29, 33, 55 and 66% respectively. The amplification reaction with the primer have shown the existence of the polymorphism about 44% among 5 fungi.

Key words: *S. littoralis*, Entomopathogenic fungi; Biological control, RAPD-PCR, SDS-PAGE

INTRODUCTION

The Egyptian cotton leaf worm *Spodoptera littoralis* is considered the major pest that causes great damage to cotton plants as well as other vegetable crops in Egypt. Great efforts have been made to control this pest chemically. Due to the continuous use of chemical pesticides against this pest, resistance to the action of pesticides had dramatically evolved. Also, the extensive use of these chemicals has given rise to problems such as residual toxicity (pollution) and harmful effects on beneficial insects, which are natural enemies of target or non-target pest species. Such problems have become a cause of search for safely pesticides including microbial agent as fungi, bacteria and viruses[21].

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 insect. Most are found ithin the deuteromycetes and entomophthoralis. Fungi infect individuals in all orders of nsects; most common are Hemiptera, Diptera, Coleoptera, Lipidoptera, Orthoptera and ymenoptera[8]. Some insect pathogenic fungi have restricted host ranges while other fungal species have a wide host range for example, *Metarhizium anisopliae, M. flavovrdiae, Paecilomyces farinosus, 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)[20]. The properties of the insect's integument with the nutritional requirements of the fungus[8,14] and the cellular defense of the host[11]. In contrast to bacteria and viruses that pass through the gut wall from contaminated food, fungi have a unique mode of infection. They reach the hamocoel through the cuticle. The infection process can be separated into three phases. (1) Adhesion and germination of the spores on the insect's cuticle. (2) Penetration of the spores into the hamocoel by the excretion of different enzymes such as lipase, chitinase and protease. (3) Development of fungus which results in the death of the insect.

Direct analysis of DNA polymorphisms is now a 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 the studies in

Corresponding Author: Amer, M.M, Botany Dept., Fac. Sci., Banha Univ., Egypt.

entomopathology, epizootics and insect biocontrol[13]. The analysis of random amplified polymorphic DNA (RAPD)[26] has been proposed to resolve genetic variations between fungal strains.

The Objectives of this Study Were To: Production of conidiospores of the five fungal isolates at laboratory level. Evaluate the effectiveness of the fungal isolates as a biological control agents against Egyptian cotton leaf worm *S. littoralis* under laboratory condition. Study the genetic diversity among these fungal isolates using SDS-PAGE and RAPD-PCR technique.

MATERIALS AND METHODS

Source of Entomopathogenic Fungi and Test Insect: Entomopathogenic fungi used in the present work were *Metarhizium anisopliae, Metarhizium flavovridae, Paecilomyces farinosis, Beauveria bassiana* and *Beauveria brongnirtu*. Laboratory strain of the cotton leaf-worm, *Spodoptera littoralis* has been reared for ten generations away from any insecticide contamination at 25±1ºC and 50-60% RH 1st and 2nd. All entomopathogenic fungi and test insect were provided by Insect Pathogen Unit (IPU) of Plant Protection Research Institute, Agriculture Research Center.

Mass Production of Fungi: *M. anisopliae, M. flavovridae* were produced on agar plates on Dox medium as described by[25]. As for *B. bassiana*, conidia were produced on agar plates of complete agar medium (CAM) was described by Riba *et al*[22]. Regarding *B. brongnirtii*, conidia were produced on agar plates of potato dextrase agar medium as described by Christensen[6].

The plates were inoculated with entomogenous fungi and incubated for two weeks at 25ºC and 50-60% RH. At the end of the incubation period, the conidia were harvested from the surface of the cultures by scraping with a sterile solution of 0.01% Tween-80. The resulting suspension was counted according to hemocytometer counts technique.

The mycelium was separated from supernatant. The mycelium was stored at -20ºC till use for extraction of DNA and protein.

Efficiency of the Fungal Conidiospores Against *S. littoralis*: Fungal spore suspensions were used to prepare the different concentrations of entomopathogenic fungi as follows:

Five concentration 10^5, 10^6, 10^7 10^8 and 10^9 were prepared of conidospores mentioned above. Second and fourth instar larvae were exposed for 48 h to treated castor bean leaves, *Ricinus communis* and filter paper by using dipping technique with conidial suspensions. Effective host-pathogen contact takes place by cover the surface of castor bean leaves and filter paper with five concentrations of conidia of the entomogenous fungi. The larvae were placed on sprayed filter paper by conidial suspension. Four replicates were used, each replicate contain ten larvae. The control replicates consisted of forty larvae placed on filter paper sprayed with a sterile solution of -.01% Tween-80. After an exposure time of 48 h to the conidial suspension the infected larvae were placed in a sterile cups with cleaned castor bean leaves and filter paper. Mortality counts were recorded every two days for ten days.

Isolation of DNA from Entomopathogenic Fungi

Extraction of Total DNA: The DNA extracted from mycelium of five fungi using hexadecyl tri-methyl-ammonium bromide (CTAB) method of Doyle and Doyle. The dried DNA pellet was dissolved in 50 µl TE buffer. This suspension was taken as the DNA templet in the PCR mixture.

Polymerase Chain Reaction: Random amplification of DNA of *M. anisapliae, M. flavovridae, P. farinosus, B. bassiana* and *B. brongniartii* was performed according to the method of Williams *et al*[26] with some modification:

Reaction Mixture:	
DNA sample	1 µl
Primer	2 µl
dNTPs	1 µl
Taq DNA polymerase	0.5 µl
Taq DNA polymerase buffer (10x)	5 µl

The primers:
OPA 13 (CAGCACCCAC), OPA 20 (GTTGCGATCC), OPD 07 (TTGGCACGGG), OPD 08 (GTGTGCCCCA) OPE 20, AACGGTGACC

The mixture was completed to 50 µl total reaction volume using sterile distilled water.

Thermocycling Profile and Detection of the PCR Products: PCR amplification was performed in a Perkin-Elmer DNA Thermal Cycler 2400 (Norwalk, CT) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 37ºC for 2 min, and an elongation step at 72ºC for 2 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1 x TAE buffer at 95 volts for two hrs. PCR products were viualized on UV light (Biometra T13l = 302 nm) and photographed using a Polaroid camers (Gelcam – U.K). Amplified products were visually examined and the presence or absence of each size class was scored as 1 or 0 respectively.

Extraction of Protein from Entomopathogenic Fungi: Protein was extracted by homogenizing the mycelium in the extraction buffer[19] containing 1 mM (PMSI) as protease inhibitor. After homogenization the extract was incubated for about fifteen minutes on ice then centrifuged at 13000x g for five minutes. The pellet was discarded and the supernatant was kept on ince.

Preparation and Casting of SDS-PAGE: The gels were prepared from monomer solution of 30% Acrylamide and 2.7 Bis-Acrylamide according to Laemmli[15]. An equal concentrations of the samples were mixed with a 0.25 volume sample buffer, incubated at 95ºC water path for about 2 min to denature the protein and equal amount of all samples were loaded using Hamilton syringe on 12%. SDS-PAGE with a stacking 4% SDS-PAGE.

RESULTS AND DISCUSSION

Virulence of 5 Fungi Against Instar Larvae of *S. littoralis*: Data in Tables (1 & 2) and Fig. (1) show that the mortality percentage in the 2nd or 4th instar larvae of *S. littoralis* increased with increasing in time elapsed post-treatment, as well as concentration of conidiospore suspension. After 2 days, mortality percentages were almost low then gradual increase in mortality percentage appeared at different time intervals. Generally, positive correlation was obtained between mortality percentage and tested concentration.

The significance of the present study is to illustrates the ability of the suspension spores entomopathogenic fungi to exhibit positive influences on larval mortality as well as induced malformation in different stages 2nd and 4th of *S. littoralis* larval instars (Fig. 2). The effect of spore suspension mentioned above on *S. littoralis* showed that mortality percentage

Table 1: Mortality % of 2nd instar larvae *S. littoralis* feeded on caster bean leaves immersed in different concentration of conidiospores fungi.

		Feeding period (days)					
Conc. (spores/ml)	Fungi	2	4	6	8	10	12
10^5	*M. anisopliae*	2.60	7.40	12.52	17.32	21.76	25.83
	M. flavovridae	10.46	20.20	27.83	33.99	39.49	43.40
	P. farinosus	14.09	21.46	26.66	30.70	34.00	36.80
	B. bassiana	3.510	8.25	12.68	16.68	20.3	23.57
	B. brongniritii	5.980	13.42	19.95	25.56	30.43	34.69
10^6	*M. anisopliae*	1.05	6.60	14.98	24.06	32.77	40.70
	M. flavovridae	9.91	20.63	29.26	36.26	42.03	46.89
	P. farinosus	13.6	21.31	26.81	31.10	34.62	37.60
	B. bassiana	6.23	12.01	16.78	20.81	24.29	27.36
	B. brongniritii	10.7	16.88	21.40	24.49	27.98	30.55
10^7	*M. anisopliae*	4.16	12.83	21.64	29.59	36.56	42.61
	M. flavovridae	4.31	14.22	24.42	33.54	41.41	48.11
	P. farinosus	15.21	23.85	29.94	34.65	38.47	41.69
	B. bassiana	5.75	13.32	19.85	25.59	30.57	34.94
	B. brongniritii	9.36	20.44	29.53	36.94	43.06	48.20
10^8	*M. anisopliae*	12.64	26.35	36.92	45.15	51.7	57.02
	M. flavovridae	15.73	26.58	34.33	40.29	45.08	49.06
	P. farinosus	5.75	15.76	25.16	33.29	40.21	46.13
	B. bassiana	8.15	18.7	27.65	35.07	41.27	46.51
	B. brongniritii	9.35	19.42	27.59	34.26	39.82	44.52
10^9	*M. anisopliae*	19.38	33.43	43.14	50.32	55.9	60.38
	M. flavovridae	12.58	26.10	36.54	44.67	31.16	36.44
	P. farinosus	3.070	13.12	24.75	35.53	44.84	52.69
	B. bassiana	10.54	22.54	32.17	39.88	46.16	51.37
	B. brongniritii	11.69	23.14	32.02	39.06	44.79	49.57

Table 2: Mortality % of 4th instar larvae *S. littoralis* feeded on caster bean leaves immersed in different concentration of conidiospores fungi.

Conc. (spores/ml)	Fungi	Feeding period (days)			
		4	6	8	10
10^5	*M. anisopliae*	4.06	8.06	12.04	19.71
	M. flavovridae	4.53	10.91	18.3	32.89
	P. farinosus	20.45	25.97	30.31	36.91
	B. bassiana	2.11	5.05	8.66	16.59
	B. brongniritii	7.4	12.95	18.33	27.93
10^6	*M. anisopliae*	6.85	11.43	15.79	22.58
	M. flavovridae	1.87	7.12	15.12	33.82
	P. farinosus	24.7	29.86	38.8	39.65
	B. bassiana	4.35	9.36	14.91	25.85
	B. brongniritii	7.48	14.16	20.84	32.84
10^7	*M. anisopliae*	7.33	11.97	16.33	24.04
	M. flavovridae	6.31	14.48	23.17	39.45
	P. farinosus	25.96	30.98	34.79	40.41
	B. bassiana	3.58	9.04	15.62	29.22
	B. brongniritii	18.29	25.26	20.84	32.76
10^8	*M. anisopliae*	7.74	13.89	19.88	30.57
	M. flavovridae	4.27	13.79	26.04	49.54
	P. farinosus	22.54	28.95	33.79	41.5
	B. bassiana	5.17	13.35	22.9	41.15
	B. brongniritii	10.14	20.82	31.34	48.49
10^9	*M. anisopliae*	7.13	18.23	30.52	55.0
	M. flavovridae	10.71	23.04	32.22	54.98
	P. farinosus	27.36	36.77	44.01	54.51
	B. bassiana	8.58	18.55	29.67	48.15
	B. brongniritii	9.17	20.14	31.33	50.29

Table 3: Virulence of entomopathogenic fungi against the 2nd instar larvae of *S. littoralis* expressed as LT_{50} values.

Conc. (Scopers/ml)	Entomopathogenic Fungi	LT_{50}	95% (fiducial limit)		Intercept	Slope
			Lower	Upper		
10^5	*M. anisopliae*	29.4440	19.0676	74.20480	2.5560±0.3111	1.6637±0.3475
	M. flavovridae	15.7700	11.8163	27.35810	3.3225±0.2227	1.4005±0.2547
	P. farinosus	27.1628	15.7402	132.6001	3.6375±0.2116	0.9501±0.2449
	B. bassiana	39.1842	22.1402	174.8575	2.7678±0.2931	1.4012±0.3310
	B. brongniritii	22.0220	15.3566	46.57620	2.9942±0.2530	1.4437±0.2897
10^6	*M. anisopliae*	14.7035	12.3502	14.5465	1.8889±0.3566	2.6649±0.3889
	M. flavovridae	13.4714	10.6444	20.2115	3.2455±0.2243	1.5535±0.2611
	P. farinosus	24.7545	15.0539	94.6192	3.5991±0.2128	1.0052±0.2509

Table 3: Continued

	B. bassiana	38.0946	21.0357	140.4748	3.1034±0.2547	1.1447±0.2931
	B. brongniritii	41.5471	20.6212	411.955	3.4732±0.2255	0.9437±±0.2643
10^7	*M. anisopliae*	14.8895	12.0226	21.2926	2.6691±0.2604	1.9873±3.046
	M. flavovridae	12.6250	10.6192	16.4557	2.6383±0.2651	2.1446±±0.3004
	P. farinosus	19.0080	12.6708	50.6428	3.6562±0.2075	1.0507±0.2454
	B. bassiana	21.5037	15.1660	43.9351	2.9643±0.2550	1.5277±0.2416
	B. brongniritii	12.7867	10.2948	18.2983	3.1886±0.2266	1.6367±0.2642
10^8	*M. anisopliae*	9.43890	7.96280	11.9671	3.3457±0.2128	1.6469±0.2540
	M. flavovridae	12.5292	9.63130	20.3381	3.6146±0.2052	1.2618±0.2428
	P. farinosus	13.5004	11.0328	18.7181	2.8512±0.2508	1.9010±0.2864
	B. bassiana	13.5283	10.8311	19.6460	3.0994±0.2333	1.6801±0.2697
	B. brongniritii	14.7900	11.4226	23.5566	3.2232±0.2274	1.5187±0.2642
10^9	*M. anisopliae*	7.89790	6.5692	10.0032	3.6998±0.1974	7.4487±0.2361
	M. flavovridae	9.16030	8.0827	12.7880	3.3473±0.2130	1.6817±0.2506
	P. farinosus	11.2749	9.8198	13.70223	2.3796±0.2819	2.4906±0.3169
	B. bassiana	11.4376	9.3901	15.57780	3.2510±0.2208	1.6526±.25780
	B. brongniritii	12.1999	9.7679	17.67460	3.3530±0.2168	1.5161±0.2539

LT_{50} lethal time (time required to kill 50% of larvae.

Table 4: Virulence of entomopathogenic fungi against the 4th instar larvae of *S. littoralis* expressed as LT_{50} values.

Conc. (Scopers/ml)	Entomopathogenic Fungi	LT_{50}	95% (fiducial limit) Lower	Upper	Intercept	Slope
10^5	*M. anisopliae*	35.1764	17.6372	466.8689	2.1794±0.7220	1.8242±0.7745
	M. flavovridae	15.770	11.8163	27.3581	3.3225±0.2227	1.4005±0.2597
	P. farinosus	27.1628	15.7402	132.6001	3.6375±0.2116	2.4906±0.3169
	B. bassiana	31.1842	22.1402	174.8579	2.7678±0.2931	1.4012±0.5310
	B. brongniritii	22.0220	15.3566	46.5763	2.9942±0.2530	1.4937±0.2897
10^6	*M. anisopliae*	33.8808	16.7658	2.80580E±03	2.6060±0.6337	1.5648±0.6884
	M. flavovridae	13.4714	10.6444	20.2115	3.2455±0.2243	1.5535±0.2611
	P. farinosus	24.7545	15.0539	44.6192	3.5991±0.2128	1.9010±0.2864
	B. bassiana	38.0946	21.0357	190.9748	3.1034±0.2547	1.1997±0.2931
	B. brongniritii	41.5471	20.6212	411.955	3.4732±0.2255	0.9433±0.2643
10^7	*M. anisopliae*	33.6309	16.823	1.13197E±03	2.5443±0.6434	1.6085±0.6978
	M. flavovridae	12.6250	10.6192	16.4557	2.6383±0.2651	2.1446±0.3004
	P. farinosus	19.0080	12.6708	50.6428	3.6562±0.2075	1.0507±0.2454
	B. bassiana	21.5037	15.166	43.9351	2.9643±0.2550	1.5277±0.2916
	B. brongniritii	12.7867	10.2448	18.2983	3.1886±0.2266	1.6367±0.2632

Table 4: Continued

10^8	*M. anisopliae*	22.0846	14.0949	198.5495	1.4223±0.6164	1.9178±0.6688
	M. flavovridae	12.5292	9.8313	20.3381	3.6146±0.2052	1.2618±0.2428
	P. farinosus	13.5004	11.0328	18.7181	3.5991±0.2128	1.0052±±0.2509
	B. bassiana	13.5283	10.8311	19.646	3.0994±0.2333	1.6801±0.2697
	B. brongniritii	14.740	11.4226	23.5566	3.2232±0.2274	1.5187±0.2642
10^9	*M. anisopliae*	10.5713	9.8782	15.5046	1.6204±0.6086	3.1782±0.6567
	M. flavovridae	9.6105	8.0827	12.2788	3.3473±0.2130	1.6817±0.2506
	P. farinosus	11.2745	9.8198	13.7023	3.6375±0.2116	0.9501±0.2499
	B. bassiana	11.4376	9.3901	15.5778	3.2510±0.2208	1.6526±0.2578
	B. brongniritii	12.1999	9.7679	17.6746	3.3530±0.2168	1.5161±0.2539

LT_{50}: Lethal time (time required to kill 50% of larvae.

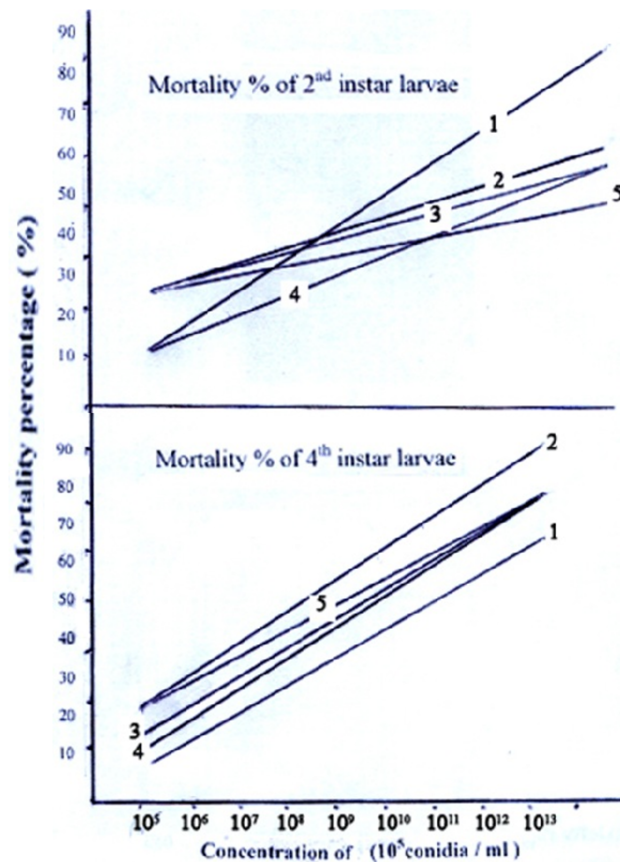


Fig. 1: Toxicity regression line of entomopathogenic fungi against 2nd and 4th instar larvae of S. littoralis.
1. M. anisopliae *3. P. farinosus* *5. B. brongniartii*
2, M. flavovidiae *4. B. bassiona*

of 2nd and 4th instar larvae *S. littoralis* increased with increasing concentrations and time elapsed other treatment. Results of the effectiveness of spore suspension of the five isolates mentioned above on the mortality 2nd and 4th instar larvare showed that *M. anisopliae* and *M. flavoviridae* were the most effective isolates. The spore suspension of *M. anisopliae* gave the highest mortality percentage

Fig. 2: Malformations induced by the entomopathogenic fungi in several stages of cotton leaf worm S. littoralis. 1(a): Normal larvae and b-infected intermediate stage between 2nd and 6th larval instars showing old and dark exuvium (b). 2(a): Normal pupae and b-pupae-moth intermediate stage (lpupae failed to be moth) and malformed pupae with small fragment of moth. 3(a) Normal adult and b-malformation of moth included crumpled wings and frizzled abdomen.

(60 and 55%) to the 2nd and 4th instar larvae with lethal time (LT_{50}) 7 and 10 days for the same instar respectively. On the other hand, *B. bassiona* and *B. brongniartii* gave the lowest mortality percentage. The mortality percentage of spore suspension of *B. bassiona* and *B. brongniartii* against 2nd and 4th instar larvae was (51 and 55%) and (49 and 57%) with lethal time (11 and 9) and (12 and 9) days respectively (Table, 3 and 4).

The entomopathogenic fungi were reduced malformation in different stages 2nd and 4th of *S. littoralis*, showing old, dark exuviam, small crumpled; Frizzled and dwarfism larvae; small fragment of moth; abnormal pupae attached with moth; body shrankage and moth falled in emerging from pupal case of pupal-moth intermediate stage, and crumpled and twested wings, frizzled abdomen; small size moth without wings attracted with pupae in adult slage (Fig. 2).

Detection genetic variability in virulence among five entomopathogenic fungi provides an important tool for initiating further study of the protein and DNA fingerprint using SDS-PAGE and RAPD/PCR respectively.

SDS-PAGE Profiles: Total cellular proteins of entomopathogenic fungi isolates, 11, 10, 5, 11, 6 bands of *M. anisopliae, M. flavoridae*, P. farinosus, B. bassiong and *B. brongniartii* respectively were fractionated in denaturing gel electrophoresis (SDS-PAGE) (Table 5). Comparison of protein patterns from five fungi isolates there were 2 common bands 95.25 and 20.10 KDa with Rf 0.258 and 0.955, and amount percentage 6.0 and 9.7. In addition to 6 bands 95.25, 74.10, 34.71; 33.0, 29.91 and 20.10 KDa were detected between *B. bassiona* and *B. brongniartii*, 4 bands, 95.25, 74.10; 31.18 and 20.19 KDa between *M. anisopliae, M. flavovridiae* and *P. farinosus* (Table 5 and Fig. 3). Similarity relationship of protein content and fractions among 5 fungi is shown in Fig. (4). Results indicated a weak similarity of protein content, ($R \leq 0.13$), this result confirm the ability of using SDS-PAGE to appear genetic variability of 5 fungi as a biological control.

RAPD-PCR Profiles: DNA sample preparation for RAPD-PCR amplification was found crucial for finger print of five entomopathogenic fungi. The total DNA genome was extracted by using CTAB method. The yield of DNA was spectrophotometrically as 10 µg/0.05 of cells. The purity of DNA genome samples as indicated by A_{260}/A_{280} ratio was 1.8. After optimization of the reaction PCR-condition polymorphism among the different species of fungi were detected using five random primers. RAPD analysis gave the best results of amplification expressed as average number of band per primers. Five random primers screened (operon random primers) were

surveyed. For the reproducibility of RAPD patterns, two independent experiments were performed for each primer. Repetition of the experiments conformed the stability and reproducibility of the results, of the 5 random primers that were screened in RAPD-PCR analysis for their ability to produce sufficient amplification products, one primer, OPA 13 was more stable and producible and gave sufficient polymorphism among five fungi, the distribution of the polymorph bands were generated using selected random

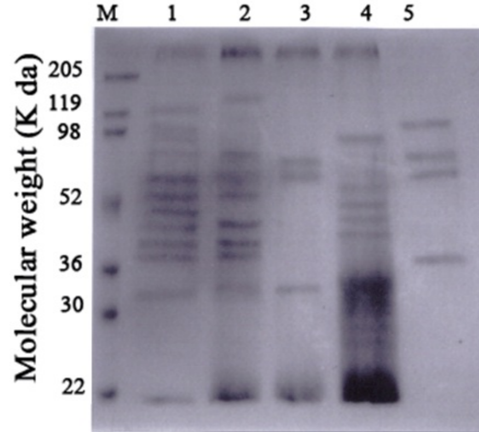
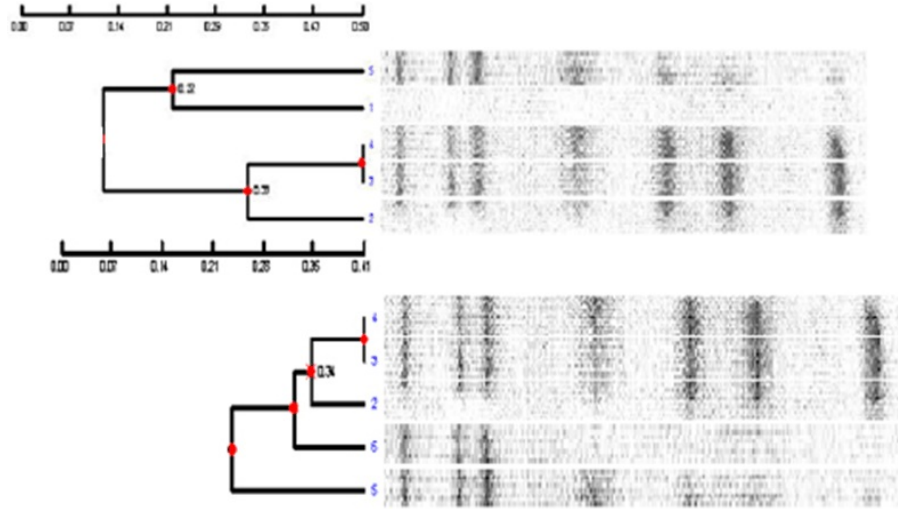


Fig. 3: SDS-PAGE patterns of total protein of different isolates of entomopathogenic fungi. Lane M: Standard marker protein, Lane 1: B. brongniartti, Lane 2: B. bassiona Lane 3: M. anisophae, Lane 4: M. flavovrdiae, Lane 5: P. formosus



	Dice coefficient				
	B. bassiona	B. bronguiartii	P. farmosus	M. anisopliae	M. flavoviridae
B. bassiona	-	66.67	66.67	50.00	00.00
B. bronguiartii	85.71	-	00.00	00.00	00.00
P. farmosus	85.71	100.00	-	00.00	00.00
M. anisopliae	71.43	57.14	57.14	-	00.00
M. flavoviridae	71.43	85.71	85.71	71.43	-
	Frequency similarity				

Fig. 4: Similarity matrix calculated by frequency similarity and Dice coefficient. Matric Adj-RF.Marker protein. Tolerance 100%.

primer (OPA-13) among 5 fungi are summarized in Tables (6, 7) and Fig. (5). The result, revealed that by using the primer (OPA-13) 9, 7, 7, 9 and 9 TAF bands). The result, revealed 3,3,2,5 and 6, PAF band as well as % polymorphic 33, 29, 33, 55 and 66% were detected in *B. bassiana, B. brongiartii; P. farinosus, M. flavovirdiea* and *M. anisopliae* respectively (Tables 6, 7 and Fig. 5). Similarity correlationship and the Dice coefficient presented in Fig. 6 is low between *Beauveria* sp. *Pacilomyces* sp. And *Metarhizium* sp., but it is high between *B. bassiana, B. brongniartti* and *M. flavovrdiae* and *M. anisapliae* (Fig, 6).

Table 5: Scored protein fractions of the five entomopathogenic fungi.

B. bronaniartti			*B. bassiana*			*M. anisopliae*			*M. flavovrdiae*			*P, farinosus*		
RF	%	MW	RF	%	MW	RF	%	MW	RF	%	MW	RF	%	MW
-	-	-	0.099	11.6	155.32	-	-	-	-	-	-	-	-	-
0.135	9.2	140.57	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	0.166	31.0	127.72
0.202	6.4	114.57	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	0.211	12.8	111.03	-	-	-
-	-	-	0.253	7.3	97.08	-	-	-	-	-	-	-	-	-
0.258	6.0	95.25	02.58	8.7	95.25	0.258	2.5	95.25	0.258	2.5	95.25	0.258	14.9	95.25
-	-	-	-	-	-	0.276	11.3	89.43	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	0.303	3.0	81.73	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	0.312	29.4	78.40
0.329	8.4	73.71	0.328	8.7	74.10	0.326	7.1	74.10	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	0.342	2.4	70.27	-	-	-
-	-	-	0.377	10.5	61.28	-	-	-	-	-	-	-	-	-
0.384	11.1	59.58	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	0.398	3.6	58.28	0.398	2.5	56.28
0.424	10.0	50.68	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	0.439	6.8	47.48	-	-	-
-	-	-	0.461	17.3	43.10	-	-	-	-	-	-	-	-	-
0.473	16.6	41.04	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	0.4	12.9	39.06	-	-	-
0.512	11.7	34.50	0.512	13.8	34.71	-	-	-	-	-	-	-	-	-
0.549	13.8	33.18	0.544	10.1	33.6	0.544	35.6	31.18	-	-	-	-	-	-

Table 5: Continued.

0.662	8.5	29.18	0.662	6.9	29.18	-	-	-	0.650	14.7	29.18	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	0.554	24.7	28.65
0.955	9.7	29.10	0.955	13.8	20.10	0.941	46.0	20.14	0.752	283	20.10	0.752	2.75	20.10
-	-	-	-	-	-	-	-	-	0.802	7.1	18.58	-	-	-
-	-	-	-	-	-	-	-	-	0.931	5.9	17.25	-	-	-

Table 6: Genotype variability among five entomopathogenic isolatesL DNA fingerprint; number and percentage molecular size (bp) of amplicans bands releaved by RAPD primer.

RAPD primer	M.W. (k.pb)	Percentage of band *B. bassina*	*B. bronaniartti*	*P. furinosus*	*M. flavovrdiae*	*M amsoplia*
OPA-13 ACGCACCCAC	5.84	-	-	-	-	13.8
	4.58	-	-	-	-	7.7
	3.91	-	-	10.2		-
	3.45	-	10.9	-	-	-
	3.30	-	-	-	22.4	-
	3.02	11.5	-	-	-	-
	2.88	-	-	-	10.7	-
	2.73	-	-	-	-	32.4
	2.54	-	35.2	-	-	-
	2.47	12.2	-	-	21.9	-
	1.95	8.0	-	-	-	-
	1.88	-	-	-	6.8	-
	1.77	-	-	-	-	4.4
	1.60	7.0	2.0	-	-	-
	1.51	-	-	-	2.5	-
	1.33	-	-	30.1	-	-
	1.05	-	-	-	0.7	-
	1.00	-	-	-	-	12.3
	0.74	23.0	26.7	5.4	-	-
	0.55	-	-	27.0	18.8	12.7
	0.37	-	-	-	-	5.8
	0.21	-	3.1	-	-	3.9
	0.16	7.1	4.9	-	-	-
	0.14	7.1	-	18.6	5.6	-
	0.10	-	17.2	-	10.6	-
	0.08	14.1	-	8.7	-	7.0

Table 7: Genotype polymorphic (% of polymorphic) among five entomopathogenic fungi.

RAPD primer	OPA-13 : CAGCACCCAC				
Genotype	*B. bassiana*	*B. bronaniartti*	*P. forinosus*	*M. flavovrdiae*	*M anisopliae*
TAF	8	7	6	9	9
MAF	6	5	4	4	3
PAF	2	2	2	5	6
Polymorphic %	25	29	33	55	66

TFA = Total amplification fragments

MAF = Monomorphic amplification fragments (common amplification fragments)

PAF = Polymorphic amplification fragments or specific amplification fragments

$$PAF\ \% = \frac{\text{No. of PAF}}{\text{No. of TAF}} \times 100$$

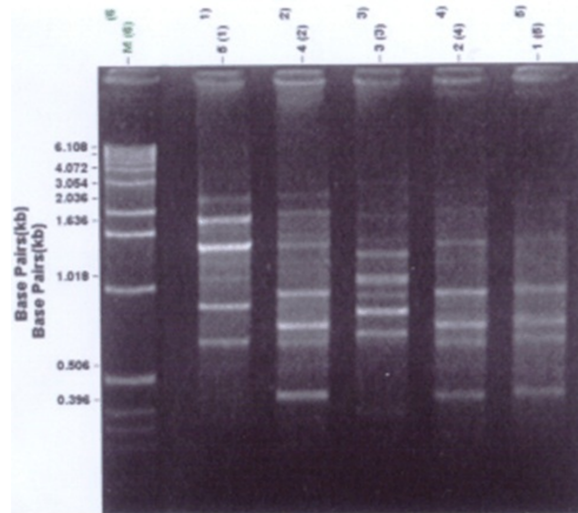


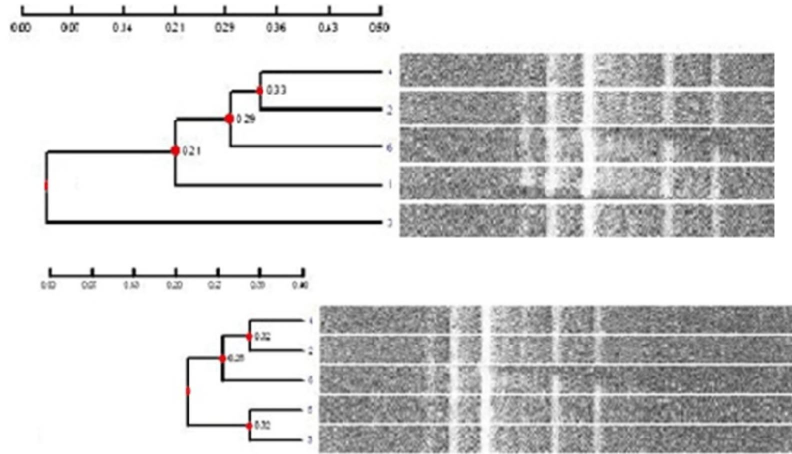
Fig. 5: Agarose gel electrophoresis (12) showing RAPD profiles obtained by PCR-amplification of different isolates of entomopathogenic fungi using the primer OPD 07.
Lane M: DNA marker, Lane 1: B. bassiana, Lane 2: B. brongniartti,
Lane 3: P. forinosus, Lane 4: M. flavoridae, Lane 5: M. anisopliae.

Discussion: Chemical insecticides have been used worldwide to control insect pests. The extensive use of chemical insecticides has created several and serious problems e.g. effect on man, his animal, non target insect pest and effect on beneficial insects and natural enemies[3]. Such problems of chemical insecticides have triggered a potential search for safely alternatives including the use of microbial agents as bacteria, viruses and fungi. The first microorganisms found to cause diseases in insects were fungi because their specificity for pest control and their high persistence in the environment.

The significance of the present study is to illustrate the ability of the entomopathogenic fungi spore suspension *M. anisopliae, M. flavovrdiae, B. bassiana, B. brongniartii* and *P. farinosus* to exhibit positive influences on larval mortality as well as induced malformation in different stages of *S. littoralis* treated as 2nd and 4th larval in stars. The effect of spore suspension mentioned above on *S. littoralis* showed that mortality percentage increased with increasing concentrations and time elapsed after treatment.

Results of the effectiveness of the spore suspension of the five isolates on the mortality of 2nd and 4th instar larvae showed that *M. anisopliae* and *M. flavoridae* were the most effective isolates. While *B. bassiana* and *B. brongniartii* gave the lowest mortality percentage followed by *P. farinosus*. The use of spore suspension depends on the host pathogen contact. According to the insect anatomy the insect cruicle contains chitin fibrils within a protein matrix together with lipids, waxes, small quantities of phenols, inorganic and pigments. The infection of the insect host starts with the unspecific adhesion of the fungal spores to the insect cuticle. Successful adhesion depends on the characteristics of mucilage, enzymes, lectins, hydrophobic bonding and electrostatic forces[1]. Spore germination is the second step of the infection process and many factors have been found to play an important role in conidial germination[2]. In most cases the germination is followed by the formation of an appressorium. Low levels of nutrients enhance the formation of appressoria whereas high levels do not[24]. The final penetration is a combination of mechanic and enzymatic processes and occurs mainly through intact cuticle, as opposed to through wounds or natural orifices[2]. After penetration most of the entomopathogens change their structure into yeast like propagules (Hyphae) which spread in the host haemolyph. The death of the insect host is a result of the penetration of the tissue, extensive growth in the haemolymph and the production of toxins[5]. It is clear from the results that the susceptibility of insect instar differs with the difference in entomopathogenic fungi. This is correlated to the biologically active metabolites which released in the haemolymph of the insect and disrupt the host immuno-response and metamorphosis[1].

The results also showed that 2nd instar larvae were more susceptible to mycoinsecticide than 4th instar larvae. This due to the 3th instar larvae formed



	Dice coefficient				
	B. bassiona	B. bronguiartii	P. farmosus	M. anisopliae	M. flavoviridae
B. bassiona	-	28.57	28.57	50.00	0.00
B. bronguiartii	16.67	-	0.00	66.67	0.00
P. farmosus	16.67	66.67	-	0.00	0.00
M. anisopliae	33.33	83.33	50.00	-	0.00
M. flavoviridae	00.00	83.33	83.33	66.67	-
	Frequency similarity				

Fig. 6: Similarity matrix calculated by: Dice coefficient and frequency similarity.
Matric: Adj Rf Reference marker DNA Tolerance: 1.00%.

immunity called maturation immunity which made them more resistance than 2nd instar larvae.

Identification of variation in virulence among different species of pathogen provides an important tool for initiating further study of the genetic and biochemical properties of a pathogen which determines its potential for causing disease. Williams *et al*.[26] proposed that $RAPD_S$ consist of entomopathogenic fungi. In the present study five decamer primers (OPA 13, OPA 20, OPD07, OPD08 and OPE 20) of arbitrary sequence were used to differentiate among five entomopathogenic fungi *B. bassiana, B. brongniartii, M. anisopliae, M. flavoviridae* and *P. farinosus*.

Many workers have studied the DNA of the five isolates by using RAPD-PCR. Louela *et al*.[17] made differentiation of *B. bassiana* isolates by RAPD analysis using ten primers and found that the number of bands generated by each primer was variable ranging from 7 to 18 bands. While in the present study we disagree with them in which the five random primers mentioned above the OPA-13 gave 8 and 7 bands for *B. bassiana* and *B. brongniartii* ranged. There were 15 shared bands between *B. bassiana* and *B. brongniartii* while 4 bands can distinguish them from each other, in which *B. bassiana* has 2 characteristic bands and 2 characteristic bands for *B. brongniartii* for the remaining bands.

Cobb and Clarkson[7] detected molecular variation in the insect pathogenic fungus *M. anisopliae* using RAPD-PCR. They investigated DNA polymorphism between *M. anisopliae* and *M. flavovridiae* by RAPD-PCR and found that DNA fragments ranged between 0.3 and 2.7 Kb by using eight primers of arbitrary nucleotide sequence and each isolate differed in the size and number of RAPD products, indicating considerable polymorphism. Laurineide lopesde carvalho freire *et al*.[16] showed DNA polymorphism in mutants of *M. anisopliae* used seven RAPD primers which provided a total of 113 bands with an average of 16 bands per primer. Also, we disagree with them in which a total of 18 bands was scored from the OPA-13 primer for *M. anisopliae* and *M. flavoridae* the bands pattern ranged between 9 and the size of amplified DNA varied.

There were 18 shared bands between *M. anisopliae* and *M. flavovridae*, while 11 bands can distinguish them from each other, *M. flavovridae* has 5

characteristic bands, while *M. anisopliae* has 6 characteristic bands among the five isolates mentioned above, the polymorphism percentage (%) was 83 for five primers. OPA-13 primer showed the highest polymorphism percentage 44 (%) among the five isolates. Also there was polymorphism between *B. bassiana* and *B. brongniartii* it was 25 and 29%, while it was 55 and 66% between *M. anisopliae* and *M. flavovridae*. In many cases, a single primer produced band profiles able to differentiate all species being examined[18].

The two highly virulent isolates against *S. littoralis, M. anisopliae* and *M. flavovridae* showed two unique RAPD markers that were not present in the other pathogens. The RAPD markers (OPA-13) may have diagnostic potential in identifying highly virulent isolates. *M. anisopliae* showed 5 unique RAPD markers that were not present in the other isolates. Also *M. flavovridae* showed 6 unique RAPD markers that were not present in the other isolates.

Many workers have studied the protein of fungi, Chang *et al*.[4] were the first that apply the SDS-PAGE technique to the fungi.

In the present study the protein of five isolates mentioned above was extracted from their mycelium and fractioned by SDS-PAGE. SDS-PAGE revealed a maximum number of 20 bands, which were not necessarily present in all samples. The protein of *B. bassiana* was separated into 10 bands this result disagree with Gregs West Wood *et al*.[12] who extracted the protein from *B. bassiana*, analyzed it by sodium-dodecyl sulphat-polyacrylamide gel electrophoresis (SDS-PAGE) and found 12-15 distinct reactive protein bands, ranging in molecular mass from 12KDa to >95 KDa (under denaturing conditions). The protein of *M. anisopliae* was separated into 5 bands also this result disagree with Laurineide lopes de carvalho frire *et al*.[16] 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 seven bands, and the mutant five bands.

In the present study *B/ brongniartii, M. flavovridae* and *P. farinosus* showed 11, 11 and 6 bands respectively. *B. brongniartii* was distinguished from *B. bassiana* by 5 positive markers (M.W 140.57; 114.57, 59.58, 50.68, 41.4 KDa). While *B. bassiana* has 4 positive markers 155.32, 97.08, 61.28 and 43.10 KDa) can distinguished it from *B. brongniartii, M. anisopliae* was distinguished from *M. flavovridae* by 3 positive markers (89.43; 74.10; 31.18 KDa). While *M. flavovridae* has eight positive marker (M.W. 111.03, 81.73, 70.77, 56.28, 47.48, 39.06, 24.18, 18.58 and 17.25 that distinguished it from *M. anisopliae*. *M. anisopliae* and *M. flavovridae* were the highly virulent isolates against *S. littoralis* than the remaining isolates this is may be due to the presence or the absence of some protein from these two isolates, *M. anisopliae* has only one positive marker (M.W 9.43 KDa) that not present in the remaining isolates, while *M. flavovridae* has 7 positive markers (M.W 111.03, 81.73, 70.27, 47.48, 39.06, 18.58 and 17.25 KDa) also not present in the remaining isolates. The reasons of the absence of some bands and decreasing in band intensity in some isolates like *M. anisopliae* correlated to Snider[23].

Culture, either maintained on agar slants or immersed for a longer term in mineral oil or frozen through in adequate procedures, are subject to mutation and such mutation may alter protein composition. Altering or absence of protein from *M. anisopliae* may made it the highly virulent isolates also the presence of protein in *M. flavovridae* that not found in the other isolate may made it the highly virulent isolate.

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