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Genetic analysis of the entomopathogenic fungus *Beauvaria bassiana* to the corn borers tested by UV as physical mutagen

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Abstract: The present investigation aims to study the impact of some genetic treatment (using mutation by UV). The influence of different doses of ultraviolet (UV) light on the pathogenicity of entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin to the European corn borer. The Mutant strains of *B. bassiana* (B.b) fungus showed different effect on *Ostrinia nubilalis, Chilo agamemnon* and *Sesamia cretica* of corn insect pests under laboratory conditions. The percentages of infestation were significantly decreased in all cases as compared with the wield type. Intracellular total soluble proteins of *Beauveria bassiana* are believed to play an important role in virulence against insect hosts. Twenty *B. bassiana* isolates, wild type (w.t) and their mutants were characterized by total soluble proteins present in cells, using the SDS_PAGE technique to differentiate the isolates based on virulence and host insect origin. Protein banding patterns SDS PAGE from w.t and their mutants show specific band of the different UV (doses of time) treatment may be referring to the occurred change of different character of *Beauveria bassiana.*
Keywords: *mutation, UV, Beauveria bassiana; corn borers, protein fingerprinting.*

Introduction:

The solar radiation, which includes visible light, UV radiation, infrared rays and radio waves have been the dominant source in which all organisms evolved and adapted. The effect of UV radiation on *B. bassiana* has been studied in natural and laboratory environments in a lot of work[1,2]. Radiation with UV at 280-320 nm wavelengths damages DNA and affects survival or percent germination[3,4]. The UV light exposure to *B. bassiana* cultures can interfere with their physiological properties from prototrophy to auxotrophy[5]. Also, (UV) radiation-induced DNA damage leading to entomopathogenic fungal inactivation is commonly measured by viable counts[6]. Early work found that *B. bassiana* conidiophores exposed to sunlight caused less mortality in insects[7].

Maize (*Zea mays*) is an important crop all over the world and also in Egypt. Corn is subjected to attack by many insect pests that affect the yield quality and quantity. Mediterranean countries which has 98% of the world's cultivated corn[8,9]. The corn borers moth develops three generations per year[8]. The fungus *Beauveria bassiana* exhibits host preferential infections in lepidopterous larvae, the fungi spores come in contact with the cuticle (skin) of susceptible insects, they germinate and grow into their bodies then penetrate the integuments; spread

rapidly blocked the respiratory pores lead to pest death[10]. The infected insect dead due to tissue destruction and by secretion of toxins inside the body of the insect[11].

Entomopathogenic fungi are found worldwide associated to insects and phytophagous mite populations, contributing to biological control of these arthropods on several economically important crops[12]. Fungal concentrations of 10^6 and 10^7 conidia/ml of *B. bassiana* and *N. rileyi* affected the larval development, movement and mobility of corn borers larvae during the seedlings and vegetative stages of corn plant under laboratory; greenhouse and field conditions. Conidia survival may be affected either by environmental factors or chemical products used to protect plants[13,14] . This is different than a lot of other broad-spectrum insecticides that are toxic if the insect merely comes in contact with dry insecticide residues[11]. Some studies had focused on identifying nutrient substrates that *B. bassiana* can utilize with application to industrial production, while others focused on the pathogenic processes of *B. bassiana* and interactions with insect cuticle[15]. Studies on total soluble proteins of fungal mycelium can also help in understanding relationships among different isolates of entomopathogens. *Helicoverpa armigera* (Hubner) is a highly destructive polyphagous pest causing severe loss to many economically important crops in the world[16]. The outbreak of this pest has been attributed to the development of insecticide resistance and the use of broad spectrum insecticides, which are known to have detrimental effects on populations of its natural enemies and nutritional and bioclimatic factors in host plants[17]. Total soluble protein profiles of all *B. bassiana* isolates under study were generated using step gradient SDS_PAGE for understanding the relation between protein profiles to their virulence pattern and host origin.

The aim of this work to determine the influence of different doses of UV light to the pathogenicity of *B. bassiana* to corn borers and to determine the genetic differences among obtained strains and to compare them with wild type.

2. Materials and Methods:

2-1 Isolation of mutants;

2.1.1 *Beauvaria bassiana* culture: *B. bassiana* isolate (BR 3) kindly obtained from Prof. Dr. Alian Vey (mycology unite control, National De La Research Sientifique, Univ., Montpellier) was used in the experiment. The fungus was grown on potato dextrose agar medium at 27±2˚C for 15 days. The suspension of conidia filtered through cotton and sterile gauze in ceramic filter was used in the experiment. Portions of 20ml of an aqueous suspension containing 10^6 per ml conidia were placed in Petri dishes and irradiated. The source of ultra-violet light rays was a bactericidal lamp Philips with a dose rate at the level of irradiation 83µW.S/cm^2 and wavelength of 253.7nm. The distance between exposed suspensions and UV source was 30cm and the exposure duration was 30 and 60 minutes. The irradiated and control (non-irradiated wild type) suspensions were inoculated on potato dextrose agar medium in Petri dishes and incubated at 27±2˚C for 15 days and used for pathogenicity tests and for molecular identification.

2.2 Test insects:

The strains of irradiated and control (non-irradiated wild type) suspensions were tested against *Sesamia cretica*; *Ostrinia nubilalis* and *Chilo agamemnon* reared on corn leaves under laboratory conditions 25 ± 2ºC and 60± 5 RH. Leaves changed every two days. The fungus, was reproduced on potato dextrose agar (PDA) plus 0.4% yeast extracts (PDAY) and poured onto sterilized Petri-dishes[18]. Plating was performed according to the full dish method. The conidia were transferred from the vial to dish containing medium by platinum loop and then streaked. Plates were incubated at 25ºC with 12 hours photo phase for fungus growth and sporulation. After 15 days, conidia were scraped and transferred to conical flasks (250 ml) containing 200 ml sterilized distilled water with 0.02% the speeder sticker (tween, 80).Conidial concentrations in the suspensions were quantified directly under the optical microscope with a Haemacytometer. Then the suspensions were standardized until the direct concentration 1x10^7 conidia/ ml was obtained.

2.3 Laboratory tests:

Each irradiated and non-irradiated strains of *B. bassiana* suspensions ranged from 1x10^2 to 1x10^8 spores/ml were prepared by 1-10 fold dilution from the main stock culture and tested under controlled conditions (26 ± 2ºC and 65±5 %RH.) against third instar larvae of the tested insects. Fresh corn leaves were sprayed with the desired fungus concentration (3 shots as spurts/leaf)[19], left to dry and placed in 1 L plastic containers (one/each). Then, twenty larvae of different species were placed on each leaf. Five containers

(replicates) were used /concentration / microbial pathogen/strain. Each container was covered with muslin and incubated at 25 ºC, thereafter, untreated leaves were introduced in the plastic containers to allow gentle transfer of survivors to them and the previous treated leaves were discarded. Untreated leaves were placed in plastic containers sprayed with water only and used as control treatment. For each concentration (4 replicates/ each), ten L3 larvae of each of the tested insects were transferred into each Petri-dish. Control larvae were fed on un treated corn leaves. Percentages of mortality were calculated according to[20], while, LC50 was calculated throughout probit analysis. The experiment was carried out under laboratory conditions at 26°C± 2 and 60-70 % RH.

2.4. SDS PAGE for total soluble proteins:

2.4.1. Preparation of protein sample:

The mycelium of *B. bassiana* wild type and its mutants was ground to a fine powder using liquid nitrogen with a mortar and pestle, transferred to centrifuge tubes after adding protein extraction buffer (0.1M Phosphate buffer pH7.2). The homogenate was centrifuged at 10,000 rpm for 20 min at 4ºC in super spin (K12 Sigma) cooling centrifuge. The supernatant containing soluble proteins was transferred to sterilized eppendorf tubes and stored at -30ºC for further usage[21].

2.4.2. SDS-PAGE

The total soluble protein profiles of *B. bassiana* isolates and its mutants subjected to the above mentioned were analyzed by SDS-PAGE. Protein samples were treated with treatment buffer (0.125M Tris pH 6.8, 20% glycerol, 2% SDS and 14.4mM β- Mercaptoethanol) for 10 minutes in a boiling water bath at 100°C. The samples were cooled to ambient temperature and 50µl of protein samples were loaded on Tris-glycine gels (5% stacking and 15% resolving). Electrophoresis was performed on Biotech vertical gel electrophoresis unit. Current of 50/100 volts for stacking and separating was applied. The gels were stained with 0.25% coomassie blue (0.25gms G250, 10ml acetic acid, 45ml methanol and 45 ml DD water) and visualized in the same solution excluding G250. The gels were photographed and scored for protein bands using Bio-rad Image Lab analysis Gel documentation system.

2.4.3. Data analysis:

Data from SDS-PAGE were pooled and transferred into 1 and 0. The data were then fed into the statistical data analysis software packing SPSS system (SPSS for windows, Ver. 11.5.1.2002,Chicago, SPSS Inc.). The statistical analysis of the data was carried out using the SPSS software package and utilizing the method described before with the least significant values calculated at the 5% probability level[21].

3. Results and Discussions*:*

Table (1) shows the effect of exposing the *B. bassiana* radiology for 30 and 60 minutes on the larvae of the *Chilo agamemnon.* The 30 and 60 minutes exposure by UV light positively increased the activity of *B. bassiana*. Under controlled laboratory conditions, the LC50 values of mutant isolates of *B. bassiana* of 30 minutes exposure ranged between 112 and 179 spores/ml as compared to 221 spores/ml in the non-irradiated wield type. While, the corresponding figures of LC50 values after 60 minutes recoded**,** 144, 181, and 240 spores/ml respectively.

Table 1. Activity of UV-light mutants of 30 and 60 minutes exposure time and wild type of *B. bassiana* toward *Chilo agamemnon* under laboratory conditions (26±2°C and65±5% relative humidity)

B.bassiana **(B.b.) wild type and mutants No.**	**LC 50**	**95% confidence limits**	***B.bassiana*** **(B.b.) wild type and mutants No.**	**LC 50**	**95% confidence limits**
1- B.b. 30/1	**112**	**101-132**	**1- B.b. 60/1**	**144**	**123-165**
2- B.b. 30/2	**156**	**144-170**	**2- B.b. 60/2**	**149**	**132-166**
3- B.b. 30/3	**167**	**153-188**	**3- B.b. 60/3**	**150**	**143-176**
4- B.b. 30/4	**169**	**146-189**	**4- B.b. 60/4**	**177**	**161-186**
5- B.b. 30/5	**179**	**158-199**	**5- B.b. 60/5**	**181**	**171-198**
Wt. (*B. bassiana)*	**221**	**189-256**	**Wt. (*B. bassiana)***	**240**	**201-253**

Fungal variants of 30 min. UV light exposure, the highest reduction in LC50 was found with *B. bassiana* mutant of 30/1, as the LC50 was decreased from 221 to 112 spores/ml in non-irradiated wild type and mutant (30/1) respectively. On the other hand mutagen of *B. bassiana* (60/1) of 60 minutes exposure was one of the highest impact on the larval isolates, as the LC50 was decreased from 240 to 144 spores/ml in non-irradiated wild type and mutant (60/1) respectively. Generally, fungal mutants are often generated by UV-mutagenesis[22,23].

Almost, the same results were obtained and also found that under laboratory conditions the LC50 obtained was 142 x104 , 146 x104, 166 x104 conidia/ml for *O. nubilalis, S. cretica* and *C. agamemnon* pests treated with different concentrations of *Nomuraea rileyi* fungi respectively[24]. Opposite results were observed[25] who discovered that with rising dose of UV-rays, the yield of variants of the fungus increased with reduced virulence, but the relation between fungus infectivity and UV irradiation was non-symmetrical.

Data in Table (2) showed that the LC50 values of *B. bassiana* mutants against *Sesamia cretica* for the five strains of the 30 minutes ranged between 133 and 168, as compared to 201 spores/ml in the non-irradiated wild type. The corresponding LC50 values for the five strains of the 60 minutes against *Sesamia cretica* were145 and 172 spores/ml compared to 201 spores/ml in the non-irradiated wild type.

Table 2. Activity of UV-light mutants of 30 and 60 minutes exposure time and wild type of *B. bassiana* toward *Sesamia cretica* under laboratory conditions (26±2°C and65±5% relative humidity)

B.bassiana **(B.b.) wild type and mutants No.**	**LC 50**	**95% confidence limits**	***B.bassiana*** **(B.b.) wild type and mutants No.**	**LC 50**	**95% confidence limits**
1- B.b. 30/1	**133**	**111-154**	**1- B.b. 60/1**	**145**	**133-165**
2- B.b. 30/2	**153**	**143-167**	**2- B.b. 60/2**	**149**	**122-167**
3- B.b. 30/3	**155**	**140-171**	**3- B.b. 60/3**	**151**	**133-177**
4- B.b. 30/4	**168**	**149-188**	**4- B.b. 60/4**	**160**	**149-182**
5- B.b. 30/5	**166**	**142-178**	**5- B.b. 60/5**	**172**	**163-196**
Wt. (*B. bassiana)*	**201**	**184-228**	**Wt. (*B. bassiana)***	**229**	**197-241**

The fungi; *B. bassiana* and *M. anisopliae* reduced the LC50 of *S. littoralis* under laboratory conditions has been found reported that UV light exposition significantly influenced the mortality effect of *B. bassiana* isolates to European corn borer larvae[26,5].

When *Ostrinia nubilalis* were treated with the different concentrations of the five mutant strains of the entomopathogenic fungi *B. bassiana,* the LC50 after 30 minutes exposure were ranged between 99 and152 spores/ml as compared to 223 spores/ml in the wild type (Table, 3). The corresponding LC50 values for the five strains of the 60 minutes exposure against the same pest were between 122 and 156 spores/ml compared to 210 spores/ml in the wild type.

Table 3. Activity of UV-light mutants of 30 and 60 minutes exposure time and wild type of *B. bassiana* toward *Ostrinia nubilalis* under laboratory conditions (26±2°C and65±5% relative humidity)

B.bassiana **(B.b.) wild type and mutants No.**	**LC 50**	**95% confidence limits**	***B.bassiana*** **(B.b.) wild type and mutants No.**	**LC 50**	**95% confidence limits**
1- B.b. 30/1	**99**	**87-115**	**1- B.b. 60/1**	**122**	**111-137**
2- B.b. 30/2	**134**	**111-151**	**2- B.b. 60/2**	**136**	**128-145**
3- B.b. 30/3	**143**	**133-165**	**3- B.b. 60/3**	**139**	**127-149**
4- B.b. 30/4	**144**	**131-170**	**4- B.b. 60/4**	**149**	**129-140**
5- B.b. 30/5	**152**	**145-187**	**5- B.b. 60/5**	**156**	**133-150**
Wt. (*B. bassiana)*	**223**	**195-265**	**Wt. (*B. bassiana)***	**210**	**188-226**

Studies on reduction in viability and germination or decrease in infectivity of *B. bassiana* without direct proof of DNA damage, have purported that DNA damage and repair are responsible for these effects[3,4 and 6] . Moreover, they found that entomopathogenic fungi reduced insect infestations of cabbage and tomato pests under laboratory and field conditions[12,27].

Table (4) the different between the molecular weight and their mutants of *Beauvaria bassianaI*

RF	MW	w.t	B.b1/30	B.b1/60	B.b2/30	B.b2/60	B.b3/30	B.b3/60	B.b4/30	B.b4/60	Frequency	Polymorphism
0.099	161.120	-	-	-	+	+	-	-	-	-	0.222	Polymorphic
0.102	159.504	+	+	-	-	-	+	+	+	-	0.556	Polymorphic
0.105	157.904	-	-	+	-	-	-	-	-	-	0.111	Unique
0.129	145.669	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
0.154	133.930	-	-	-	-	-	-	-	-	+	0.111	Unique
0.171	126.493	-	-	+	-	-	-	-	-	-	0.111	Unique
0.176	124.386	-	+	-	-	-	-	-	-	-	0.111	Unique
0.179	123.138	-	-	-	-	-	+	-	-	-	0.111	Unique
0.185	120.680	-	-	-	-	+	-	-	-	-	0.111	Unique
0.187	119.871	-	-	-	+	-	-	-	-	-	0.111	Unique
0.207	112.079	-	+	-	-	-	-	-	-	-	0.111	Unique
0.229	104.092	-	-	-	-	-	-	-	+	-	0.111	Unique
0.248	97.653	-	-	-	+	-	-	-	-	-	0.111	Unique
0.292	84.231	-	-	-	-	-	+	-	-	-	0.111	Unique
0.295	83.386	-	-	-	-	+	-	-	-	-	0.111	Unique
0.300	81.996	-	-	-	-	-	-	-	-	+	0.111	Unique
0.309	79.553	-	-	+	+	-	-	-	-	-	0.222	Polymorphic
0.342	71.202	-	-	-	-	-	+	-	-	-	0.111	Unique
0.353	68.618	-	-	-	-	+	-	-	-	-	0.111	Unique
0.383	62.038	-	+	-	+	-	-	-	-	-	0.222	Polymorphic
0.421	54.600	-	-	-	-	-	+	-	-	-	0.111	Unique
0.424	54.053	-	-	-	-	-	-	+	-	-	0.111	Unique
0.446	50.201	-	-	-	-	+	-	-	-	+	0.222	Polymorphic
0.457	48.379	-	-	-	-	-	+	-	-	-	0.111	Unique
0.493	42.866	-	+	-	-	-	-	-	-	-	0.111	Unique
0.501	41.729	-	-	-	-	-	+	-	-	-	0.111	Unique
0.526	38.366	-	-	-	-	-	-	-	-	+	0.111	Unique
0.554	34.921	-	-	+	-	-	-	-	-	-	0.111	Unique
0.559	34.339	-	-	-	-	-	-	-	+	-	0.111	Unique
0.576	32.432	-	-	-	+	-	-	-	-	-	0.111	Unique
0.614	28.544	-	-	-	-	+	-	-	-	-	0.111	Unique
0.623	27.694	-	-	-	-	-	+	+	-	-	0.222	Polymorphic
0.625	27.508	-	-	-	-	-	-	-	+	-	0.111	Unique
0.661	24.374	-	-	-	-	-	-	-	-	+	0.111	Unique
0.711	20.604	-	-	-	-	-	-	+	-	-	0.111	Unique
0.738	18.816	-	-	-	-	-	-	-	-	+	0.111	Unique
0.749	18.134	-	-	-	+	-	-	-	-	-	0.111	Unique
0.752	17.952	-	-	-	-	-	+	-	-	-	0.111	Unique
0.766	17.127	-	-	-	-	-	-	-	+	-	0.111	Unique
0.774	16.672	-	-	-	-	-	-	+	-	-	0.111	Unique
0.777	16.505	+	-	-	-	-	+	-	-	-	0.222	Polymorphic
0.780	16.340	-	+	+	-	-	-	-	-	-	0.222	Polymorphic
0.782	16.230	-	-	-	+	+	-	-	-	-	0.222	Polymorphic
0.793	15.641	-	-	-	-	-	-	-	-	+	0.111	Unique

SDS-PAGE protein finger printing:

Total soluble proteins in all isolates were resolved in 44 bands (Table, 4) thought some were very faint to be seen in the photograph (Fig. 1). All isolates showed one monomorphic bands at molecular weight 145.66 kDa. Wild type of *B.b* isolate showed only three polymorphic bands at different molecular weight. Isolates of *B.b* lan 3 , 7 and 8 showed 6 bands of which 3 were specific for lane 3 and 7 respectively, and four specific bands for *B.b* lane 8.

Isolates of *B.b* 2 lane 5 and *B.b*. 4 lane 9 showed eight bands with 4 and 6 specific bands, respectively isolate of *B.b* 3 lane 6 showed 11 bands of which 7 were specific. Isolates of *B.b* 1 lane 2 and *B.b* 2 lane 4 scored seven and nine bands of which three and four band were specific respectively, for instance band with molecular weight 145.66 kDa was present in w.t, B.b1/30, B.b3/30, B.b3/60 and B.b4/30. Likewise protein band with molecular weight 79.553kDa was shared by isolates B.b1/60 and B.b2/60 and protein band with molecular weight 62.038kDa was shared by isolates B.b1/30 and B.b2/30. Polymorphic bands were showed in isolates B.b2/60 and B.b4/60 at molecular weight 50.201kDa. Isolates B.b3/30 and B.b3/60 recorded the same band at molecular weight 27.694 kDa. Also, the isolate B.b1/30 and B.b1/60 have the polymorphic band at molecular weight 16.34kDa. The same found in the isolates B.b2/30 and B.b2/60 with molecular weight 16.23kDa (Table, 4).

Data on Rf values of the protein profiles for the nine isolates were subjected to Cluster analysis. A total of 45 reproducible bands were recorded with molecular weights ranging from 15.64 KDa to 161.20 KDa (Table, 4). The nine isolates clustered into two phenetic groups (Figure, 1).

Electrophoretic analysis of total soluble protein demonstrated extensive intraspecific variability among the 30 *B. bassiana* isolates. The 30 isolates shared only 22% similarity in their protein profiles. Thus, the isolates are genetically very distant. Extracellular protease from *B. bassiana* in the presence of *Eurygaster intergriceps* Puton (Hemiptera: Scutelleridae) cuticle were characterised using SDS-PAGE[28]. Such clustering of isolates from the related hosts and from the same geographic area has been observed in *B. bassiana* based on an analysis of esterase isozyme profiles[29] .Similarity and clustering of isolates to the type of insect species from which the isolates originate and the geographic origin. They also observed that *B. bassiana* isolates from the weevil species *Sitonia humeralis* Stephens (Coleoptera: Curculionidae) and *S. lineatus* collected in different years were monomorphic, showing close similarity in isoesterase profiles, while the isolates from *S. discoideus* isolated from the same field on the same day, showed a significant heterogeneity. The clustering observed in the analysis of total protein profiles seemed to depend on the geographic origin. Characterized 40 isolates of indigenous strains of *B. bassiana* from various insect hosts collected from Central India by employing protease zymography and RAPD analysis. This molecular study showed that the strains of *B. bassiana* in general are distantly related. The intraspecific variation corroborated findings suggesting that due to the overall degree of genetic diversity, *B. bassiana* had maintained a large effective population size over a long period[30].

Despite the great variability in the total protein profiles in the *B. bassiana* isolates, the largest phenetic group consisted of isolates geographically widespread (across continents in both the hemispheres). The magnitude of genetic distances between isolates, their diversity in protein profiles and the pattern of distribution of the genotypic classes has an overall similarity to the observations made by St. Leger through a study of isozyme profiles of a larger sample of *B. bassiana* isolates. Great genetic distances indicate reproductive isolation and a clonal mode of reproduction[31].

Cluster analysis for SDS-PAGE data

Similarity analysis for the protein profiles of w.t and the eight isolates grouped into two major phenetic groups , two separate were evident, one consisting of w.t, B.b4/30, B.b3/60, B.b1/30 and B.b1/60 and the other consisting of B.b4/60, B.b2/30, B.b2/60 and B.b3/30 (Figure, 1). Isolate B.b4/30 more related to w.t. It's evident from the tree shown in fig. (2), two separate clusters were consisting of closely related isolates.

Total protein extraction banding patterns SDS PAGE for four mutant resulting from using UV rays as physical mutagenic on B. *bassiana* and its mutant illustrated in Table (4) and Fig. (1). There are observable differences in protein banding pattern for all mutant and control.

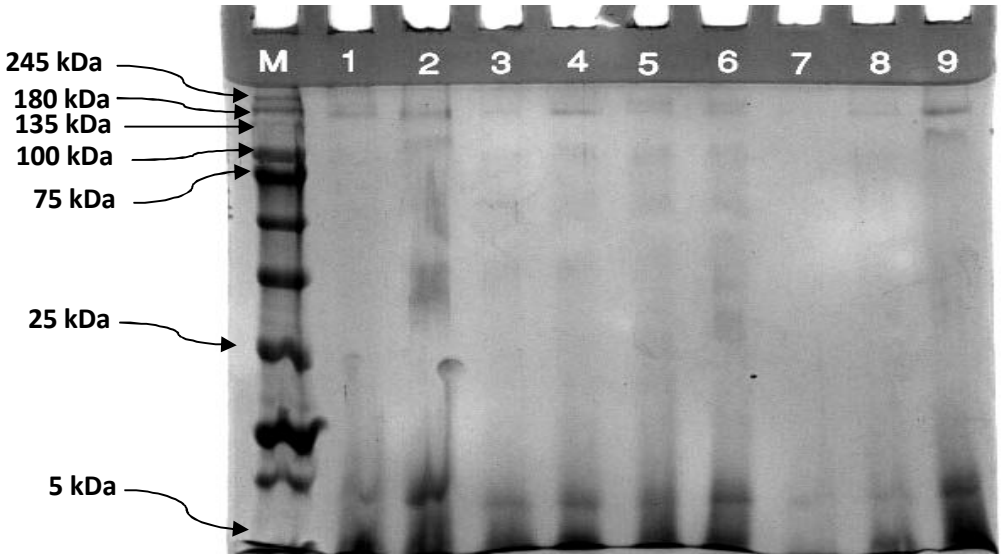

Fig 1: SDS-PAGE protein banding pattern of B. *bassiana* and their selected mutant.

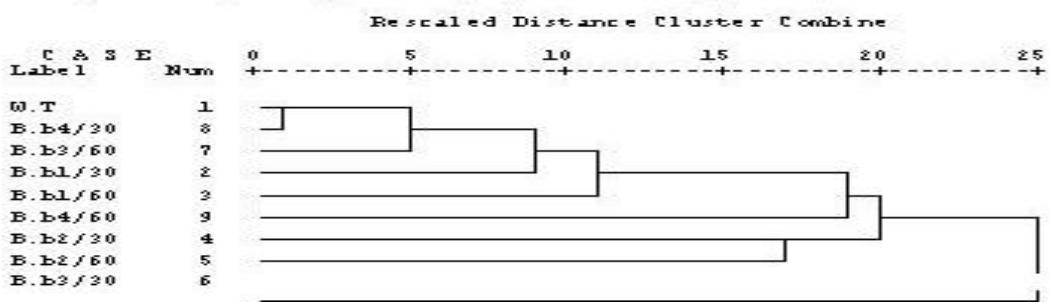

Fig 2: Dendrogram using average linkage (Between Groups)

4. Conclusion

The mutant strains of *B. bassiana* fungus showed different effect on corn borers *Ostrinia nubilalis, Chilo agamemnon and Sesamia cretica* of corn insect pests under laboratory conditions. Also, UV light positively influenced the activity of *B. bassiana* mutant isolates in all cases as compared with the wild type. It is real possibility to use UV irradiation for development of new *B. bassiana* strains. The overall picture of the genetic structure of *B. bassiana* obtained from the DNA fingerprints, biochemical marker like isozymes and the total soluble protein profiles seems to be similar. Total soluble protein profiles are as characteristic of an isolate as its DNA fingerprints.

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