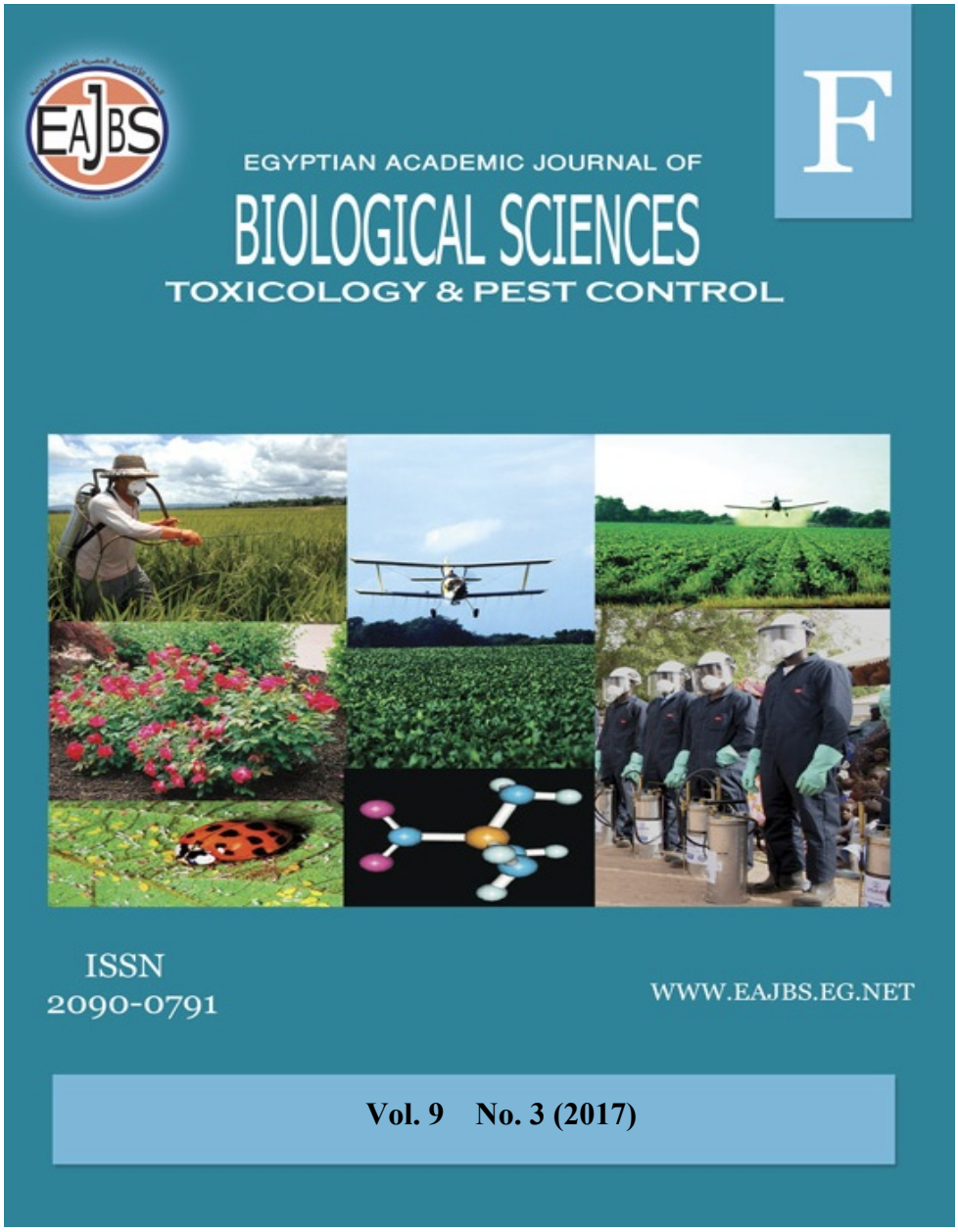


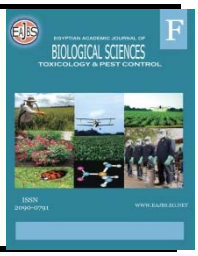
The journal of Toxicology and pest control is one of the series issued twice by the Egyptian Academic Journal of Biological Sciences, and is devoted to publication of original papers related to the interaction between insects and their environment.

The goal of the journal is to advance the scientific understanding of mechanisms of toxicity. Emphasis will be placed on toxic effects observed at relevant exposures, which have direct impact on safety evaluation and risk assessment. The journal therefore welcomes papers on biology ranging from molecular and cell biology, biochemistry and physiology to ecology and environment, also systematics, microbiology, toxicology, hydrobiology, radiobiology and biotechnology.

www.eajbs.eg.net

Egypt. Acad. J. Biolog. Sci., 9(3): 31-46 (2017)

Egyptian Academic Journal of Biological Sciences
F. Toxicology & Pest control
ISSN: 2090 - 0791
www.eajbs.eg.net



Microbial Control on *Sesamia cretica* Insects by *Beauveria bassiana* at Sharkia Governorate

Sherief, E. A. H. [1]; Ghaly, M.F.[2]; El-Mougith A. A. A[2]. and Abd El- Kader, M. A[1].
1-Department of Botany, Faculty of Science, Zagazig University, Egypt
2- Plant Protection Research Institute, Sharkia, Egypt.

ARTICLE INFO

Article History
Received: 25/8/2017
Accepted: 10/11/2017

Key words:
Microbial control – Sesamia cretica –Beauveria bassiama – Sharkia-Governorate

ABSTRACT

This research was performed in Sharkia Governorate during seasons of 2014and 2015 under laboratory conditions. *Beauveria bassiana* (Blas.) is an imperfect entomopathogenic fungus that attacks a wide range of agriculture pests causing disease named as white muscardine and currently used as biocontrol agents and substitute the harmful chemical insecticides. *Beauveria bassiana* isolate (Cairo MIRCEN) was evaluated as biocontrol agent against *Sesamia cretica* Led. under laboratory conditions. *B. bassiana* caused 100% mortality to *S. cretica* after 5 days. *B. bassiana* was screened for lytic enzyme production as it had the ability to produce chitinase, protease and no lipase production. Environmental and nutritional conditions were studied to detect the optimum conditions for growth, protease production. Fungal isolate was identified by both microscopic conditions. Culture filtrate of *B. bassiana* 1572 become concentrated (partial purified active compound by organic solvents (chloroform)) and tested on insects *Sesamia cretica* (larva at different ages) as bioinsecticide in comparison with diluted (original filtrate). Results were indicated after microscopic examinations as a complete change in insect body colour to be deep darker, Appearance of white growth of the fungal *B. bassiana* 1572 on the treated dead insect body in both treatments and the insect cadaver was solidified and there were some black lesions and some malformation. All symptoms were more in concentrated treatment than the other treatment.Also beauvericin toxin production was investigated by molecular weight using SDS-Protein Electrophoresis that indicated the presence of beauvericin toxin in both concentrated (partial purified active compound by organic solvents (chloroform)) and diluted (original filtrate). When conidia of *B. bassiana* 1572 became in contact with the insect cuticle surface under suitable ecological condition, it germinate, by the aid of both chemical (lytic enzyme secretion (chitinase, protease)) and mechanical effect (hyphal pressure on the penetration site. Then, hyphae penetrate the insect body cavity. That led to hyphae growth, division, beauvericin toxin production and production of spores spreaded in the hemolymph (in all parts of body cavity), after that physical and chemical changes occurred to the insect hemolymph by the aid of beauvericin toxin led to function disorder and death. The fungus grew outside the cadaver until the availability of suitable conditions to resume life cycle.

INTRODUCTION

Beauveria bassiana is a soil-borne entomopathogenic fungus found worldwide in diverse habitats (Zimmerman, 2007) from soil, to air and even as endophytes in some plants (Wagner & Lewis, 2000; Posada & Vega, 2005; Quesada-Moraga *et al*., 2006). Besides silkworm, the extensive list of hosts includes such important pests as whiteflies, aphids, grasshoppers, termites (Ownley *et al*., 2004). *B. bassiana* has a dimorphic mode of growth.

In the absence of the specific insect host *Beauveria* passes through an asexual vegetative life cycle that includes germination, filamentous growth. Although temperature and water availability have major impacts upon the conidial germination in *B. bassiana*, other environmental factors also influence germination and mycelial growth. Radial mycelial growth of *B. bassiana* is increased in the light, but density of the mycelium is reduced (Teng 1962). Germination capacity of spores of *B. bassiana* was not lost after accumulative exposure to direct sunlight for about 150h (Teng 1962). Spore germination was positively correlated with oxygen concentration. Mycelial growth under adequate oxygen supply was increased, but spore formation was decreased (Teng 1962). *Sesamia cretica*, the Corn stem borer, Greater sugarcane borer, Durra stem borer or Purple stem borer, is a moth of the Noctuidae family. It was described by. The range extends through the Middle East and Arabia to Pakistan, northern India and northern Africa. In the south, the range extends to northern Kenya and northern Cameroon (Tams and Bowden, 1953). .*Beauveria bassiana* had the ability to produce lytic enzymes (protease, chitinase and lipase) (Dias *et al*., 2008; St. Leger *et al*., 1996; Hegedus and Khachatourians, 1988). *Beauveria bassiana* produces several toxic compounds *in vitro* and *in vivo* (Strasser *et al*., 2000; Vey *et al*., 2001). A majority of these insecticidal molecules are low molecular weight secondary metabolites (Zimmermann, 2007). Beauvericin, bassianin, bassianolide, beauverolides, beauveriolides, tenellin, oosporein (Strasser *et al.,* 2000; Vey *et al.,* 2001), oxalic acid (Roberts, 1981) bassiacridin (Quesada-Moraga and Vey, 2004) are some of the important metabolites of *B. bassiana*. Among them, Beauvericin is the most important compound which was reported first from *B. bassiana*. Beauvericin was confirmed to have insecticidal, antimicrobial and anti-tumor activities (Logrieco *et al*., 2002). *Beauveria Bassiana* infected host insects by conidia that germinated and penetrated the host exoskeleton under favourable environmental conditions. The insect cuticle was made of chitin, lipid and other protein components that provide protection and structure to the insect (Richard *et al*. 2011). On the surface of a suitable host, fungal conidia became attached to its cuticle and germinated by means of a germ tube that penetrated it (Bateman *et al.* 1996). Penetration of the cuticle was achieved by mechanical and enzymatic degradation which allows the germ tube to grow into the haemocoel. Once in the haemocoel, growth continued by formation of mycelium and hyphae, that colonize the host organs and haemolymph. During the course of colonization, the fungi produced endotoxins. The toxins and physical rupture of internal organs by vegetative growth of the fungi killed the host. Hyphae exit first at intersegmental regions followed by appearance of fungi all over the body (Pekrul and Grula, 1979). *B. bassiana* may also infiltrate the host insect though the walls of the alimentary tract; this mode of entry has additional effects on the infected insect (Broome *et al*., 1976). After the host was dead, the hydrophobic conidia of the fungus *B. bassiana* emerges from the infected cadaver, the infection cycle began again (Hemmati *et al.,* 2001). Therefore, the present work to study the microbial effects of the fungi *B. bassiana* (Blas.) on the corn stem borer *S. cretica* Led., which is one of the most harmful insects in Egypt, as an initial step to be used in integrated pest management programs.

MATERIALS AND METHODS

Insect sampling: The samples of ***Sesamia cretica*** (larvae, pupa) were collected by hand from infested plants from different localities in Sharkia Governorate.

Insect Samples				
Insect name	**From**	**Host**	**Stage from field**	**Sampling Method**
***Sesamia cretica* Led.**	Elmesalamia – Zagazig - Sharkia	Maize	Larvae **(harmful different ages from 1st to 5th)** Pupa	Capture from infested plants

Biocontrol of *Beauveria bassiana* isolate on *Sesamia cretica* (under laboratory condition): The isolate *Beauveria bassiana* was tested on groups of *Sesamia cretica*, each group contained 20 insects by spraying the filtrate of *B. bassiana* (was grown on Potato dextrose broth for 7 days at 25ºC & pH 5.6) on these groups and their food (Maize) compared with control group and the mortality ratio was recorded that detect the effectiveness of isolate *Beauveria bassiana* to control *Sesamia cretica.*

Studying the effect of conditions to maximize the mortality on growth of *Beauveria bassiana*: In this experiment, the effect of some environmental factors(different incubation temperature , different pH values, and different incubation periods)on the growth of *B. bassiana* and production of protease enzyme. The effect of Nutritional factors on the growth of *B. bassiana* (Sabbour *et al*., 2011) and production of protease enzyme. Such as different carbon, nitrogen and sources against Sabouraud dextrose broth were studied. The protease production was measured as protease activity (Anson, 1938).

Identification of fungus *Beauveria bassiana*: Inoculums of *B. bassiana* was cultivated on potato-dextrose agar (Booth 1971) at 25°C for 7 days and then identified both under microscope after staining.

Simple extraction of active compound produced by *Beauveria bassiana*:

After the growth of *B. bassiana* in liquid Sabouraud dextrose (Sabouraud 1892) (for 7 days at 25ºC & pH 5.6) broth media it become grinded and filtered, then the filtrate containing the beauvericin extracted as follow:

Extraction of active compound by using organic solvents: 50ml of *B. bassiana* 1572 culture filtrate were mixed with a double volume of chloroform (Audhya and Russell 1973). The solvent layer was evaporated to dryness. The residue was dissolved in buffer. Then tested on insects *S. cretica* (larvae) as bioinsecticide in comparison with diluted (original culture filtrate of *B.bassiana* 1572).

Investigation of beauvericin toxin production by molecular weight using SDS- Protein Electrophoresis: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to make separation of proteins according to their molecular weight (Laemmli, 1970; Rais *et al.,* 2004). Both culture filtrate of *B. bassiana* 1572 and partially purified beauvericin extracted by chloroform were analyzed by SDS-Protein Electrophoresis against pure beauvericin toxin to ensure the beauvericin toxin production.

Prepration of Resolving Gel (10%): The gel was poured between the glass plates immediately and carefully overlaid with a layer of distilled water, then the gel was kept at room temperature to polymerize.

Preparation of stacking Gel (4%)

The surface of the polymerized resolving gel was rinsed with 5-7mL of stacking gel solution. The comb was aligned in the proper position, then, the stacking gel solution was added up to 2 mm from the top edge of the resolving gel, then left for polymerization at room temperature.

Sample Preparation:

30 µl of each active fraction were mixed with 10µl of loading sample dye. The samples were then boiled in a water bath for 2 minutes. The samples were then applied to the gel wells.

Running Conditions:

Electrophoresis was carried out with constant volt of 60V, the run was terminated when the bromophenol blue marker reached to the bottom of the gel.The separated proteins on polyacrylamide gels were then stained with Coomassie blue R-250 according to the method described by Andrews (1986); Schägger, (2003).

The electrophoresed gel was soaked in excess of staining solution for two hours, then the gel was rinsed with distilled water and destained with excess amount of destaining solution for several times until the excess stain was removed.

Effect of culture filtrate of *B. bassiana* on insect's morphology:

The control insect and that treated of *S. cretica* by the *B. bassiana* 1572 culture filtrate were examined under photomicroscope, to indicate the effect of the *B. bassiana* 1572 filtrate on the cuticle of the insect to indicate any growth of the fungus mycelium on surface of treated insect and observe any other symptom on treated instars of *S.cretica* (larvae and pupa). All symptoms were recorded before and after death.

RESULT AND DISCUSSION

Biocontrol of *Beauveria bassiana* isolate on *Sesamia cretica* (under laboratory condition):

Beauveria bassiana 1572 was used in the control of insects *Sesamia cretica* that attacked maize in Sharkia Governorate. Maize had economic importance especially in human food (Constable 1985; Wiersema and León, 1999).

In the present work the attempted was carried out to test the biocontrol effect of *Beauveria bassiana* 1572 (under laboratory condition) tested on *S. cretica* as biological insecticides, clearly illustrated that the effect of *B. bassiana* 1572 on mortality ratio, as *B.bassiana* 1572 cause 100% mortality to *S. cretica* after 5 days (Table 1). So *B. bassiana* 1572 had biocontrol effect on *Sesamia cretica* (under laboratory condition). Also, *B. bassiana* 1572 caused different symptoms (loss of appetite- change in insect body colour to be darker- partial Paralysis- treated insects turned to pupa stage but never completed to adult stage- insect cadaver had some black lesions and malformation- appearance of white growth of the fungal *B. bassiana* 1572 on the treated dead insect body). (Pekrul and Grula, 1979; Bateman *et al*. 1996; Long *et al*. 2000) were in harmony with these results.

Table 1: Biocontrol of *B. bassiana* 1572 on *S. cretica* larvae (under laboratory condition).

Days / Treatment	0 day	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day
	Mortality ratio%	Mortality ratio%	Mortality ratio%	Mortality ratio%	Mortality ratio%	Mortality ratio%	Mortality ratio%	Mortality ratio%	Mortality ratio%
Sesamia cretica (larva) Treated by culture filtrate *Beauveria bassiana*	0	35	55	75	90	100			
Sesamia cretica (larva) Control	0	0	0	0	20	40	65	80	100

Maximize the entomopathogenic effect of Beauveria bassiana isolate 1572: Environmental condition:

Temperature:

Incubation temperatures varied in their effect on growth, protease activity and beauvericin toxin production, as shown in (Table 2), growth of *B. bassiana* 1572 at temperature 25 °C gave the highest growth , highest value for protease activity (0.563units/ml culture filtrate) and beauvericin production was maximum. While growth at temperatures 20 and 30°C which gave values less than that of 25°C but it was the most relative. At low temperature values (5, 10, 15°C),

the growth of *B. bassiana* 1572 was slow and as the protease activity ranged ascendingly from 0.03 to 0.288 units/ml culture filtrate. The same action appeared at higher temperature values (35, 40, 45 and 50°C) where the protease activity was ranged descendingly from 0.222 to 0.018units/ml culture filtrate. Mishra *et al*., 2013 reported that effect of temperature on enzyme activity of *B. bassiana* protease was represented as maximum activity of protease, pr1 was observed at 40 ºC (4.31 U/ml), followed by at 50 ºC (3.98 U/ml). For pr2 protease, maximum activity was observed at 50 ºC (4.52 U/ml). Minimum activity for both, pr1 (2.77 U/ml) and pr2 (2.21 U/ml) protease was observed at 10 ºC. For protease activity, preincubation at temperatures between 30-50 °C caused little loss in activity for Prl, while no loss in activity was reported for Pr2 (St. Leger *et al.,* 1987). Activity of enzyme was shown to be heat denaturated at the incubation temperatures over 50°C, while at 60°C <20% of initial activities of Prl or Pr2 were observed. Because most enzymes rapidly become denatured at temperatures above 40°C, widely the enzyme determinations were carried out somewhat below that temperature by (Natarajan and Murty, 2010). (Zibaee and Bandani, 2009) on *B. bassiana*, showed optimal temperatures of 30–45°C. Temperature could significantly affect the growth and survival of *B. bassiana*. Determining the temperature value in which the proteolytic activity of *B. bassiana* reaches its highest value is important when expecting pathogenicity on hosts.

Table 2: Effect of different temperature on *B. bassiana* 1572 proteases production

Incubation Temperatures (°C)	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**
Protease activity (units/ ml)	**0.03**	**0.079**	**0.288**	**0.439**	**0.563**	**0.455**	**0.222**	**0.139**	**0.066**	**0.018**

pH values: Different pH values varied in their effect on growth, protease activity and beauvericin toxin production. As summarized in (Table 3 2), the optimum pH value for growth, protease activity and beauvericin toxin production of *B. bassiana* 1572 was pH 5.5 which gave the highest growth, protease activity (0.607 units/ml culture filtrate) and beauvericin production was maximum. Also growth at pH 5 and 6 gave values less than pH 5.5but it was the most relative. It was clear that the growth, proteolytic effect of *B. bassiana* 1572 decreased at the pH values below or above the optimum pH values. (Mishra *et al*., 2013) illustrated that protease enzyme showed optimum activity between pH 5-7, while beyond this range decrease in enzyme activity was observed with increase in medium acidity or alkalinity. *B. bassiana* GK2016 Pr1 protease activity reached its maximum at pH 8.5 (Bidochka and Khachatourians, 1987). (Zibaee and Bandani, 2009) showed that *B. bassiana* optimal pH of 8–9.5. The optimum pH for beauvericin formation was pH 7.2 (Peeters *et al*., 1983, 1988).

Table 3: Effect of different pH on *B. bassiana* 1572 proteases production

pH values	**3**	**4**	**5**	**5.5**	**6**	**6.5**	**7**	**8**	**9**	**10**
Protease activity units/ml culture filtrate	**0.162**	**0.284**	**0.537**	**0.607**	**0.582**	**0.518**	**0.439**	**0.339**	**0.224**	**0.167**

Incubation periods: different incubation periods varied in their effect on the growth, protease activity and beauvericin toxin production. Data shown in (Table 4) clearly showed that from the first day until eight days of incubation the growth

and protease production by *B. bassiana* 1572 increased ascendingly protease activity ranged from 0.099 to 0.622 units/ml culture filtrate. The maximum value of beauvericin toxin production was obtained after 7 days of incubation. Then each item decreased descendingly. The optimum incubation period for growth, beauvericin production, protease production by *B. bassiana* 1572 was 7 days which gave value for protease activity (0.553 units/ml culture filtrate), and beauvericin production was maximum. In this respect for pr1 and pr2 proteases of a native *B. bassiana* (HQ917687) isolate, activity showed steady increase with incubation time (Mishra *et al*., 2013). Pr1 protease showed maximum activity of 4.66 U/ml, on 5th day of incubation while maximum activity for pr2 protease was recorded to be 5.48 U/ml on 4th day of incubation. The lipase activity varied between 0.24-2.26 U/ml for different incubation period, with maximum activity observed on 5th day of incubation. The maximal productions of beauvericin by *F. oxysporum* KFCC 11363P in culture media are summarized as beauvericin was maximal at the 8th and 25th days of cultivation in Yeast and malt extract broth and Potato dextrose broth (Lee *et al*., 2008).

Table 4: Effect of incubation period on *B. bassiana* 1572 proteases production

Incubation period(Days)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Protease activity units/ml culture filtrate	0.099	0.250	0.345	0.416	0.458	0.513	0.553 (max. beauvericin production)	0.622
Incubation period(Days)	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Protease activity units/ml culture filtrate	0.565	0.541	0.505	0.491	0.477	0.432	0.368	0.331
Incubation period(Days)	**17**	**18**	**19**	**20**				
Protease activity units/ml culture filtrate	0.285	0.233	0.219	0.186				

From the previous results, the optimum conditions for chitinolytic effect of *B. bassiana* 1572 were at (temperature 25°C, pH 5.5 and incubation period 7 days).

Nutritional conditions: Different nutritional requirements as different carbon sources, different nitrogen sources and different phosphorous sources were conducted to optimize the production of extracellular proteases, beauvericin toxin and growth of ***B. bassiana* 1572.**

Carbon sources: Different carbon sources (glucose-sucrose-fructose-galactose-starch-manitol- cellulose-xylose- sorbitol- carboxy methylcellulose (C.M.C) maltose) varied in their effect on growth, protease activity and beauvericin toxin production by *B. bassiana* 1572. As represented in (Table 5) growth on sucrose gave the highest growth, protease activity (0.553 units/ml culture filtrate) and beauvericin toxin production was maximum. Also growth on glucose gave lower values than sucrose but it was the most relative. Other carbon sources used differ in their effect on growth, proteolytic effect that measured by protease activity and beauvericin toxin production by *B. bassiana* 1572. Galactose, maltose, fructose, manitol, sorbitol and xylose gave moderate protease activity and beauvericin toxin production comparable to the optimum as the protease activity values were 0.412, 0.462, 0.481, 0.250, 0.458 and 0.458units/ml culture filtrate. Respectively, starch, cellulose and carboxy methyl cellulose (C.M.C) gave low protease activity comparable to the optimum and the values were 0.079, 0.065 and 0.099 units/ml culture filtrate .Also growth on starch gave low beauvericin toxin production. *Beauveria*

bassiana required a carbon-based energy source (sugars, etc) for growth (Smith and Grula, 1981). Carbon source screening for beauvericin production. A hexose or a pentose could be the carbon source instead of glucose. Of seven potential carbon sources, glucose was the most effective for beauvericin biosynthesis (Lee *et al*., 2008; Xu *et al*., 2010).

Table 5: Effect different carbon sources on *B. bassiana* 1572 proteases production

Carbon sources	Sucrose	Glucose	Galactose	Maltose	Fructose	Starch	Cellulose	Manitol	Sorbitol	Xylose	C.M.C
Protease activity units/ml culture filtrate	0.553 (max. beauvericin production)	0.517	0.412	0.462	0.481	0.079	0.065	0.250	0.458	0.458	0.099

Nitrogen sources: different nitrogen sources (sodium nitrate $NaNO_3$-urea-peptone-yeast extract- beef extract-meat extract-ammonium sulphate- ammonium phosphate - (sodium nitrate-urea-ammonium sulphate) /dipotassium hydrogen phosphate)) varied in their effect on growth, protease activity and beauvericin toxin production by *B. bassiana* 1572. Aspresented in (Table 6), growth of *B. bassiana* 1572 on peptone gave the highest growth, protease activity (0.555 units/ml culture filtrate) and beauvericin toxin production was maximum. Also growth on meat extract gave values less than growth on peptone, but it was the most relative one. Other nitrogen sources used differ in their effect on growth, proteolytic effect and beauvericin toxin production by *B. bassiana* 1572 was remarkable. Beef extract, yeast extract, Nano3 and (Nano3 in the presence of K_2HPo_4) gave moderate protease activity and beauvericin toxin production by *B. bassiana* 1572 comparable to the optimum. Urea, Urea $(CO(NH_2)_2)$/ K_2HPo_4 , ammonium sulphate , ammonium sulphate / K_2HPo_4 and ammonium phosphate gave low protease activity and no beauvericin toxin production by *B. bassiana* 1572 comparable to the optimum. A series of 24 nitrogen sources including inorganic, organic non protein, proteins and complex natural media were used for the production of proteases of *B. bassiana* in submerged cultures demonstrated that the maximum amount of protease into the maize meal, yeast extract, ground maize and wheat bran broth was released 3 days after inoculation, It was found that the best sources are maize meal, yeast extract, and beef extract. The production optimum on these sources occurs on the third day of fermentation. The composition of the protease complex may be influenced by the type of nitrogen source (Kucera, 1971). Conidia had sufficient internal nitrogen-based reserves (proteins, etc) to germinate but additional external nitrogen sources were required for further growth and extension of fungal filaments, also referred to as hyphae or germ tubes (Smith and Grula, 1981). A wide variety of compounds in the host insect body supported luxuriant growth (Smith and Grula, 1981).Nitrogen source screening for beauvericin production was indicated that the optimal organic nitrogen source was peptone and the optimal inorganic nitrogen source was $NaNO_3$ (Lee *et al*., 2008 ;Xu *et al*., 2010).

Table 6: Effect different nitrogen source on *B. bassiana* 1572 proteases production

Nitrogen source	Peptone	Meat extract	Beef extract	Yeast extract	$NaNO_3$	$NaNO_3$/ K_2HPo_4	Urea	Urea/ K_2HPo_4	Amm. sulphate	Amm.sulphate / K_2HPo_4	Amm. phosphate
Protease activity units/ml culture filtrate	0.555 (max. beauvericin production)	0.498	0.452	0.415	0.081	0.215	0.025	0.041	0.049	0.5	0.03

Phosphorous sources: Different phosphorous sources varied in their effect on growth, protease activity and beauvericin toxin production by *B. bassiana* 1572. As in (Table7), growth of *B. bassiana* 1572 on peptone only (as nitrogen and phosphorus source) gave the highest growth, protease activity (0.555 units/ml culture filtrate) and beauvericin toxin production was maximum. Also growth on peptone /K_2HPO_4 and peptone /KH_2PO_4 gave values less than growth on peptone but it was the most relative one. Other phosphorous sources used differ in their effect on growth. While the effect of other phosphorous sources on protease activity and beauvericin toxin production by *B. bassiana* 1572 was remarkable. Peptone /Na_2HPO_4, peptone /NaH_2PO_4 and peptone /K_3PO_4 gave moderate protease activity and beauvericin toxin production comparable to the optimum. Ammonium phosphate gave low protease activity and no beauvericin toxin production by *B. bassiana* 1572. The influence of several other nutritional factors like phosphorous and trace element concentrations were only moderate (Lee *et al*., 2008).

Table 7: Effect of different phosphorous source on *B. bassiana* 1572 proteases production

phosphorous source	Peptone	K_2HPo_4/ peptone	KH_2Po_4/ peptone	NaH_2Po_4/ peptone	Na_2HPo_4/ peptone	K_3Po_4/ peptone	Amm. phosphate
Protease activity units/ml culture filtrate	0.555 **(max. beauvericin production)**	**0.528**	**0.488**	**0.429**	**0.415**	**0.393**	**0.03**

Identification of fungus *Beauveria bassiana*:

Under microscopic conditions, in culture, *B. bassiana* 1572 grows as a white mould. On most common cultural media, it produces many dry, powdery conidia in distinctive white spore balls. Each spore ball is composed of a cluster of conidiogenous cells. The conidiogenous cells of *B. bassiana* 1572 are short and ovoid; the conidia are single-celled, haploid.

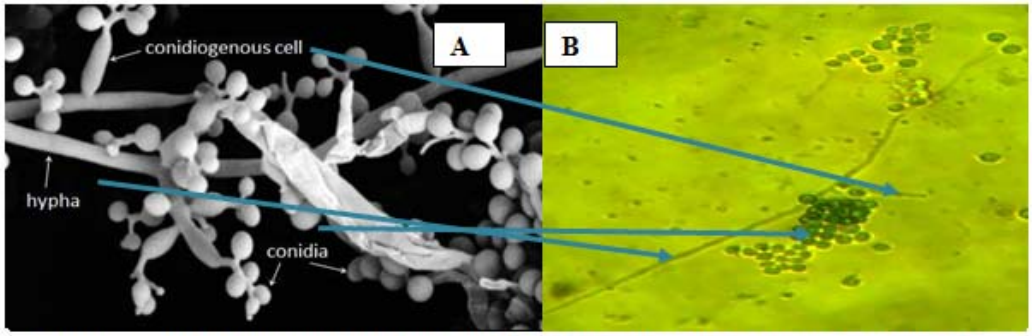


Photo (1): A- Scanning electron micrograph of *Beauveria bassiana* showing the structures (Glare, unpublished). B- Microscopic examination of *B. bassiana*1572

In this respect, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Ascomycota: Hypocreales: Cordycipitaceae) is an anamorphic specie of entomopathogenic fungi. *B. bassiana* colonies on full-strength Sabouraud's dextrose and potato by cottony or woolly appearance of white colour becoming yellowish or rarely pinkish, and the reverse of the culture is colourless or yellow or more rarely pinkish. *B. bassiana* produces vegetative hyphae 1-2 µm wide, on which conidiogenous cells are produced in dense clusters (Rehner *et al*., 2011). The conidiogenous cells are flask-shaped with long rachis forming a

zig-zag bearing the asexual spores called conidia (Brady, 1979; Inglis *et al*., 2001). Conidia are globose to broadly ellipsoidal and 2-3 µm × 2-3 µm (Rehner *et al*., 2011). *B. bassiana* reproduces asexually, however, the teleomorph *Cordyceps bassiana* has been discovered in Asia (Li *et al*., 2001 cited by Meyling *et al*., 2009) and has been proved to be linked developmentally and phylogenetically to *B. bassiana* (Rehner *et al*., 2011).

Concentration and partial purification of active compound produced by *Beauveria bassiana* 1572 The culture filtrate of *B. bassiana* 1572 become precipitated by chloroform, concentrated by evaporation to dryness and the rest was redissolved in buffer then become tested on insects *S. cretica* (larva) as bioinsecticide that indicated after microscopic examinations in photo (2) in comparison with diluted (original) as Complete change in insect body colour to be deep darker , Appearance of white growth of the fungal *B. bassiana* 1572 on the treated dead insect body and the insect cadaver was solidified and also there was some malformation .all symptoms were more in concentrated treatment than the other treatment.

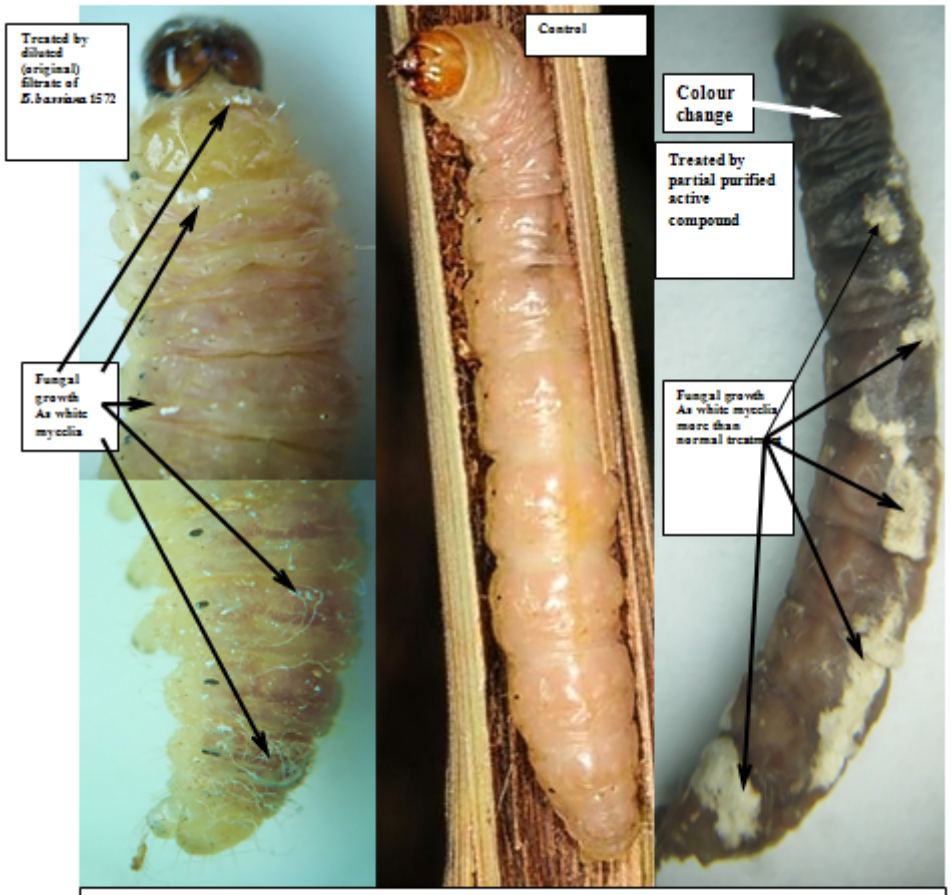


Photo (2): Dorsal view of control non treated insect (*Sesamia cretica*) larvae), treated insect (*Sesamia cretica*) larvae) (Treated by diluted (original) filtrate of *B.bassiana* 1572) and treated insect (*Sesamia cretica*) larvae) (Treated by partial purified active compound)

Investigation of beauvericin toxin production by molecular weight using SDS-Protein Electrophoresis:

Both culture filtrate of *B. bassiana* 1572 and partially purified beauvericin extracted by chloroform were analyzed by SDS-Protein Electrophoresis against pure beauvericin toxin to ensure the beauvericin toxin production. The obtained results in photo (3) clearly indicated that beauvericin toxin was produced by *B. bassiana* 1572 as in both

culture filtrate of *B. bassiana* 1572 and partially purified beauvericin extracted by chloroform, beauvericin appears clearly in the band of molecular weight =783.95 as same as the pure beauvericin toxin. SDS protein analysis for culture filtrate of *B. bassiana* 1572 led to separation into 19 bands including beauvericin, while in case of partially purified beauvericin extracted by chloroform, SDS protein analysis led to separation into only 9 bands including beauvericin that mean extraction and partial purification by chloroform exclude 10 protein compounds.

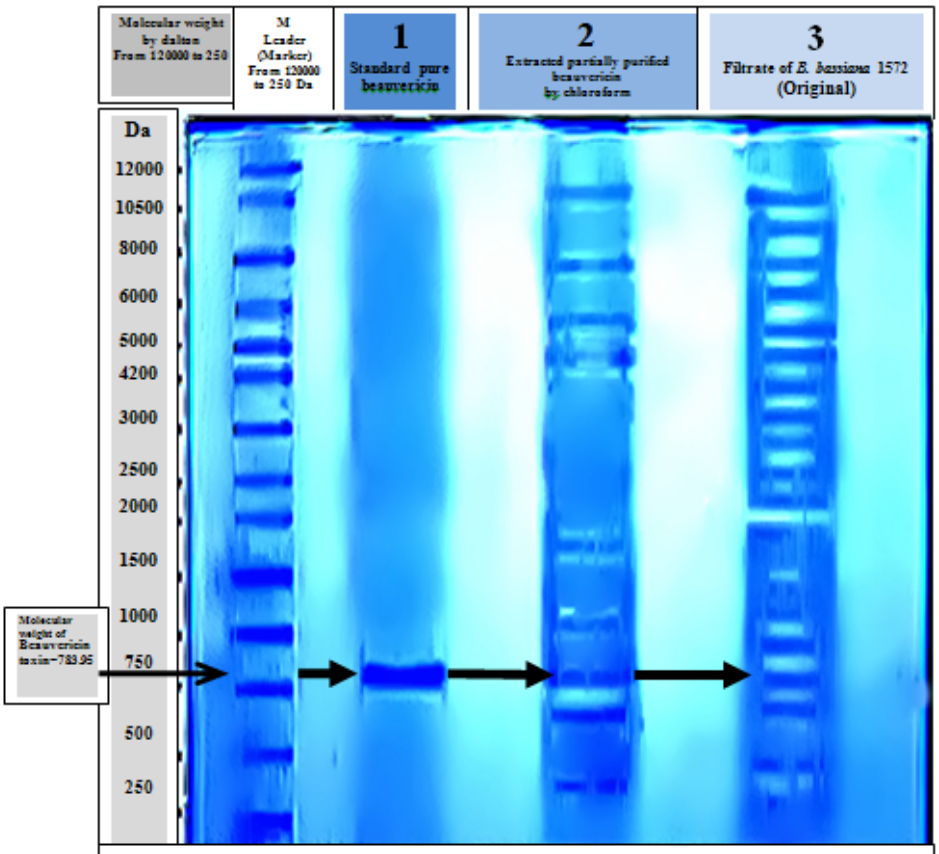
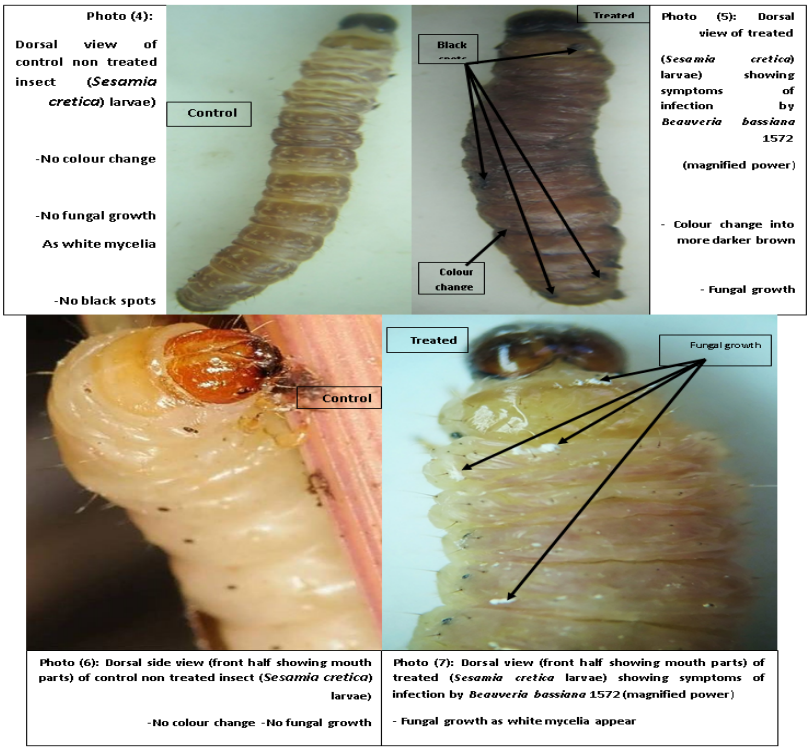


Photo (3): SDS Electrophoresis of the molecular weight of original filtrate of *B. bassiana* 1572(3), extracted partially purified beauvericin by chloroform (2) and standard pure beauvericin (1) (Ensure the presence of beauvericin in both filtrate original filtrate of *B. bassiana* 1572 and extracted partially purified beauvericin by chloroform) .

Effect of *Beauveria bassiana* 1572 on *S. cretica* (larvae, pupa):

Sesamia cretica was treated by the filtrate of *Beauveria bassiana* 1572 and was examined under photomicroscope against the control insect to study the action of the filtrate of *B. bassiana* 1572 on the insect cuticle and insect morphology that indicated white colonies of the fungus grown on both sides of the insect and cause complete colour change to the insect cuticle be deep darker with some malformation, some black lesions on the skin. Due to insect response to infection by the precipitation of black colour melanin material in and around the fungal hyphae penetration points and partial paralysis appears in some treated insects. Due to the fungus attacked body tissues (fatty and muscular) and grew as dense hyphae and filamentous mycelia. The insect movement reduced that led to Partial paralysis, so the insect lost the response ability to external effects, reduced or stop feeding, then completely stop movement and death. When conidia of *B. bassiana* 1572 became in contact with the insect cuticle surface under

suitable ecological condition, it germinate and by the formation of germ tube, by the aid of both chemical (lytic enzyme secretion (chitinase, protease)) and mechanical effect (hyphal pressure on the penetration site. Then, hyphae penetrate the insect body cavity. That led to hyphae growth, division, beauvericin toxin production and production of spores spreaded in the hemolymph (in all parts of body cavity), after that physical and chemical changes occurred to the insect hemolymph led to function disorder and death. After insect death, the fungus grew outside the cadaver and might rest for a while until the availability of suitable growth conditions to resume life cycle. *B. bassiana* 1572caused also Loss of appetite, partial paralysis and prevent molting proprieties of treated larvae (photos 4-14).



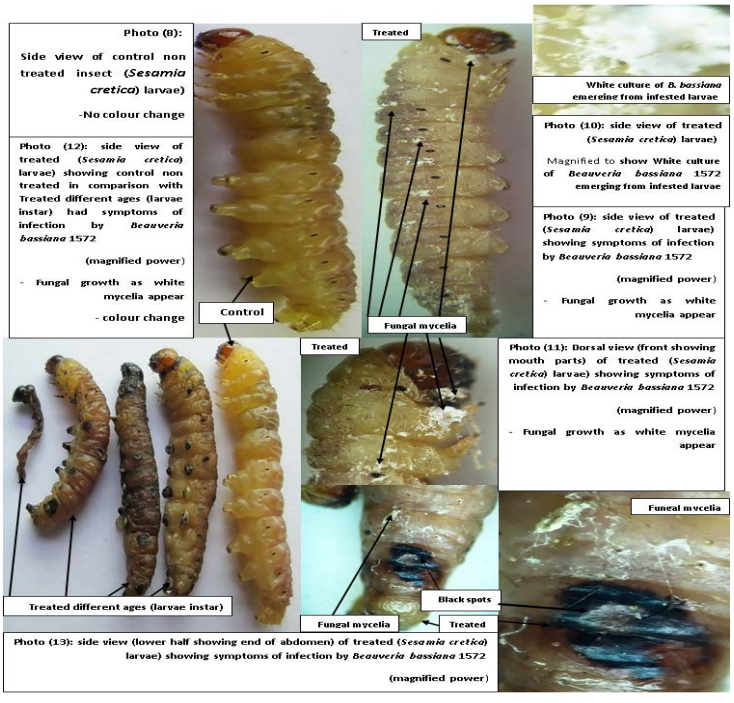
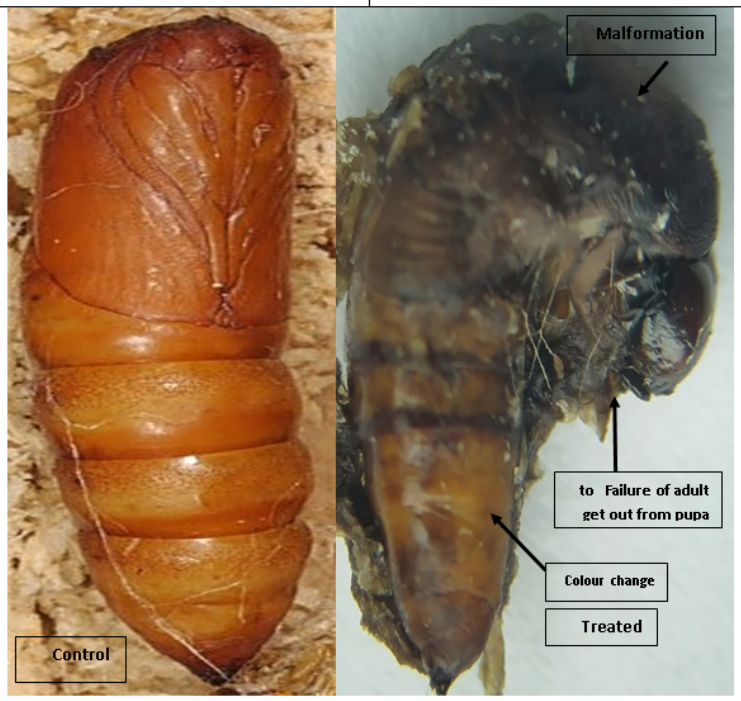


Photo (14): Control non treated insect (*Sesamia cretica*) pupa) -No colour change-No fungal growth as white mycelia -No black spots	Photo (15): Treated insect (*Sesamia cretica*) pupa) showing symptoms of infection by *Beauveria bassiana* 1572 growing (magnified power) - colour change - Fungal growth as white mycelia -Malformation -No black spots



Beauveria bassiana kills the pest by infection as a result of the insect coming into contact with fungal spores. An insect can come into contact with the fungal spores in several ways: by having the spray droplets land on its body, by moving on a treated surface, or by consuming plant tissue treated with the fungus (the latter is not a major method of uptake). For *B. bassiana* to successfully infect an insect host, a sufficient number of conidia must adhere to its cuticle (Pekrul and Grula, 1979). Conidia germinated on all regions of the cuticle, and the growing germ tubes showed a positive chemotaxis toward cuticle; thus it was possible for several infections to occur simultaneously. Once the fungal spores attach to the insect's skin (cuticle), they germinate sending out structures (hyphae) that penetrate the insect's body and proliferate. During cuticle penetration, fungal enzymes are secreted in the immediate vicinity of the hyphal tip. In addition they mentioned that penetration may occur as early as 16 - 18 hr post inoculation (p.i.) for highly pathogenic strains (Pekrul and Grula, 1979). The germ tube creates a clean hole at the point of penetration. The fungus may also enter the respiratory system via conidial contamination and germination, or via germ tube penetration through the opening or side of the spiracle. They added also, by 48 hr p.i., extensive fungal growth might be present, with minimal tissue damage. At 72 hr p.i., the first tissue to show degradation was the fat body followed by the gut tissue and Malpighian tubules. At 4 to 6 days p.i., death and mummification had occurred, with the hemocoel completely filled with fungal growth (Pekrul and Grula, 1979). Long *et al.*

2000 stated that it might take 3-5 days for insects to die, but infected cadavers may serve as a source of spores for secondary spread of the fungus. Insects can also spread the fungus through mating. High humidity and free water enhance activity of the conidia and the subsequent infection of the insect as Hyphal re-emergence occurs when a relative humidity of at least 70% is reached (MacLeod *et al*., 1966).

REFERENCES

Andrews, A. T. (1986): Electrophoresis: Theory, techniques, biochemical and clinical applications. 2nd edn. Oxford University Press, New York.

Anson, M.L. (1938): The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. J. Gen. physiol., 22(1):79-89.

Audhya, T. K. and Russell, D. W. (1973): Spectrophotometric Determination of Enniatin A and Valinomycin in Fungal Extracts by Ion Complexation. Analytical Letters, 6(3): 265-274.

Bateman, R. P.; Carey, M.; Batt, D.; Prior, C.; Abraham, Y.; Moore, D.; Jenkins, N. and Fenlon, J. (1996): Screening for virulent isolates of entomopathogenic fungi against the desert locust, *Schistocerca gregaria* (Forskål). Biocontrol Science and Technology, 6: 549-560.

Bidochka M. J. and Khachatourians, G. G. (1987): Purification and Properties of an Extracellular Protease Produced by the Entomopathogenic Fungus Beauveria bassiana. Applied and Environmental Microbiology, 53(7): 1679-1684.

Booth, C. (1971): The Genus *Fusarium*. Commonwealth Mycological Institute, Kew, Surrey, England.

Brady, B. L. K. (1979): *Beauveria bassiana*. CMI Descriptions of Fungi and Bacteria n°602. Set n° 61. CAB International 1998.

Broome, J. R.; Sikorowski, P. P. and Norment, B. R. (1976): A Mechanism of Pathogenicity of *Beauveria bassiana* on Larvae of the Imported Fire Ant, *Solenopsis richteri*. Journal of Invertebrate Pathology, 28: 87 – 91.

Constable, G. (1985): Grasslands and Tundra. Planet Earth. Time Life Books, pp. 19.

Dias, B.A.; Neves, P.M.O.J.; Furlaneto-Maia, L. and Furlaneto, M.C. (2008): Cuticle-degrading proteases produced by the entomopathogenic fungus Beauveria bassiana in the presence of coffee berry borer cuticle. Braz J Microbiol 39: 301-306.

Hegedus, D.D. and Khachatourians, G.G. (1988): Production of an extracellular lipase by *Beauveria bassiana*. Biotechnol Lett, 10:637 - 42.

Hemmati, F.; Pell, J. K.; McCartney, H. A. and Deadman M.L. (2001): Airborne concentrations of conidia of *Erynia neoaphidis* above cereal fields. Mycol. Res., 105:485–489.

Inglis, D. G.; Goettel, M. S.; Butt, T. M. and Strasser, H. (2001): Use of Hyphomycete fungi for managing insect pests. In: Butt TM, Jackson CW, Magan N (eds.). Fungi Biocontrol Agents-Progress Problems and Potential. Wallingford, UK: CAB International pp.23-69.

Kucera, M. (1971): Toxins of the entomophagous fungus *Beauveria bassiana*. II. Effect of nitrogen sources on formation of the toxic protease in submerged culture. Journal of Invertebrate Pathology, 17: 211-215.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lee, H.S.; Song, H.H.; Ahn, J.H.; Shin, C.G.; Lee, G.P.; Lee, C. (2008): Statistical optimization of growth medium for the production of the

entomopathogenic and phytotoxic cyclic depsipeptide beauvericin from *Fusarium oxysporum* KFCC 11363P. J. Microbiol. Biotechnol., 18 (1)138–144.

Li, Z. Z.; Li, C. R.; Huang, B. and Fan, M. Z. (2001): Discovery and demonstration of the teleomorph of *Beauveria bassiana* (Bals.) Vuill., an important entomogenous fungus. Chinese Science Bulletin, 46(9): 751-753.

Logrieco, A.; Moretti, A.; Ritieni, A.; Caiaffa, M.F.; Macchia, L. (2002): Beauvericin: chemistry, biology and significance. In Advances in Microbial Toxin Research and Its Biotechnological Exploitation; Upadhyay, R., Ed.; Kluwer Academic: New York, NY, USA, 2002; pp. 23–30.

Long, D.W.; Drummond, G.A. and Groden, E. (2000): Horizontal transmission of *Beauveria bassiana*. Agriculture and Forest Entomology 2: 11-17.

MacLeod, D. M.; MacBain Cameron, J. W. and Soper, R. S. (1966): The influence of environmental conditions on epizootics caused by entomogenous fungi. Rev. Roum. Biol. Botanique, 11:125-134.

Meyling, N. V.; Lubeck, M.; Buckley, E. P.; Eilenberg, J. and Rehner, S. A. (2009): Community composition, host range and genetic structure of the fungal entomopathogen *Beauveria* in adjoining agricultural and seminatural habitats. Molecular Ecology, 18(6):1282-1293.

Mishra, S.; Kumar, P. and Malik, A. (2013): Effect of process parameters on the enzyme activity of a novel *Beauveria bassiana* isolate. Int. J. Curr. Microbiol. App. Sci., 2(9): 49-56.

Natarajan, K. and Murty, V. R. (2010): Optimization of chitinase production from *Serratia marcescens* . A classical approach. Biological Segment, 1(1):1510.

Ownley, B. H.; Pereira, R. M.; Klingeman, W. E.; Quigley, N. B. Leckie, B. M. (2004): *Beauveria bassiana*, a dual purpose biocontrol organism with activity against insect pests and plant pathogens. In: R.T. Lartey and A.J. Caesar (eds.), Emerging Concepts in Plant Health Management, Research Signpost, T.C., Kerala, India: 255-269.

Peeters, H.; Zocher, R.; Kleinkauf, H. (1988): Synthesis beauvericin by a multifunctional enzyme.J. Antibiot., 12: 352–359.

Peeters, H.; Zocher, R.; Madry, N.; Kleinkauf, H. (1983): Incorporation of radioactive precursors into beauvericin produced by *Paecilomyces fumoso-roseus*. Phytochemistry, 22: 1719–1720.

Pekrul, S. and Grula, E. A. (1979): Mode of infection of the Corn Earworm (*Heliolithis zea*) by *Beauveria bassiana* as Revealed by Scanning Electron Microscopy. Journal of Invertebrate Pathology, 34: 238–247.

Posada, F., Vega, F.E., (2005): Establishment of the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) as an entophyte in cocoa seedlings (*Theobroma cacao*). Mycologia, 97:1195–1200.

Quesada-Moraga, E. and Vey, A. (2004): Bassiacridin, a protein toxic for locusts secreted by the entomopathogenic fungus *Beauveria bassiana* Mycology Research 108: 441–452.

Quesada-Moraga, E.; Landa, B. B.; Munoz-Ledesma, J.; Jimenez-Dıaz, R. M. and Santiago-Alvarez, C. (2006): Endophytic colonization of opium poppy, *Papaver somniferum*, by an entomopathogenic *Beauveria bassiana* strain. Mycopathologia 161: 323–329.

Rais, I.; Karas, M. and Schagger, H. (2004): Two- dimensional electrophoresis for the isolation of integral membrane proteins and mass spectrometric identification. Proteomics, 4:2567-2571.

Rehner, S. A.; Minnis, A. M.; Sung, Gi.; Luangsa-ard, J. J.; Devotto, L. and Humber, R. A. (2011): Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. Mycologia, 103(5): 1055-1073.

Richards, E. H.; Jones, B. and Bowman, A. (2011): Salivary secretions from the honey bee mite, *Varroa destructor*: effects on insect haemocytes and preliminary biochemical characterization. Parasitology, 138: 602–608.

Sabbour, M.M.; Ragei, M. and Abd-El Rahman, A. (2011): Effect of Some Ecological Factors on the growth of *Beauveria bassiana* and *Paecilomyces fumosoroseus* against Corn Borers. Australian J. Basic and Applied Sci., 5(11): 228-235.

Roberts, D. W. (1981): Toxins of entomopathogenic fungi. in "Microbial control of pests and plant diseases", (Ed.): Burges, H. D. Academic Press, London, PP. 441-464.

Sabouraud, R. (1892): Sabouraud Dextrose Broth .Ann. Dermatol. Syphilol, 3:1061.

Schägger, H. (2003): SDS electrophoresis techniques in membrane protein purification and crystallization. A practical guide 2nd edn. (eds. Hunte, C., von Jagow, G. & Schägger, H.) 4.85–4.103 (Academic, San Diego, California).

Smith, R. J. and Grula, E. A. (1981): Nutritional Requirements for Conidial Germination and Hyphal Growth of *Beauveria bassiana*. Journal of Invertebrate Pathology, 37: 222 – 230.

St Leger, R.J.; Cooper, R.M. and Charnley, A.K. (1987): Production of cuticle-degrading enzymes the by entomopathogen *Metarhizium anisopliae* during infection of cuticles from *Calliphora vomitoria* and *Manduca sexta*. J Gen Microbiol., 133: 1371-1382.

St. Leger, R.J.; Joshi, L.; Bidochka, M.J.; Rizzo, N.W. and Roberts, D.W. (1996): Characterization and ultra structural localization of chitinases from *Metarhizium anisopliae, Metarhizium flavoviridae* and *Beauveria bassiana* during fungal invasion of host (*Manduca Sexta*) cuticle. Appl Environ Microbiol, 62: 907-912.

Strasser, H.; Vey, A. and Butt, T.M. (2000): Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium, Tolypocladium* and *Beauveria Species*?. Biocontr. Sci. Technol., 10: 717-735.

Tams, W.H.T. and Bowden, J. (1953): A revision of the African species of *Sesamia Guenée* and related genera (Agrotidae-Lepidoptera). Bulletin of Entomological Research, 43:645-678.

Teng, C. (1962): Studies on the biology of *Beauveria bassiana* (Bals.) Vuill. with the reference to microbial control of insects. Acta Botanica Sinica 10: 210–232.

Vey, A.; Hoagland, R. and Butt T. M. (2001): Toxic metabolites of fungal biocontrol agents. in "Fungi as biocontrol agents: progress, problems and potential", (EdS.): Butt, T. M., Jackson, C. W. and Magan, N.. CAB International, Wallingford, pp., 311-346.

Wagner, B.L., Lewis, L.C., (2000): Colonization of corn, *Zea mays*, by the entomopathogenic fungus *Beauveria bassiana*. Applied and Environmental Microbiology., 66: 3468–3473.

Wiersema, J. H. and León, B. (1999): World Economic Plants A Standard

Reference. CRC Press LLC, pp 75-76.

Xu, L.; Liu, Y.; Zhou, L.; Wu, J. (2010): Optimization of a liquid medium for beauvericin production in *Fusarium redolens* Dzf2 mycelial culture. Biotechnol. Bioproc. Eng., 15: 460–466.

Zibaee A., Bandani A.R. (2009): Purification and characterization of the cuticle-degrading protease produced by the ento-mopathogenic fungus, *Beauveria bassiana* in the presence of sunn pest, *Eurygaster integriceps* (Hemiptera: Scute-lleridae) cuticle. Biocontrol Science Technology, 19(7–8): 797–808.

Zimmermann, G. (2007): Review on Safety of the Entomopathogenic Fungi *Beauveria bassiana* and *Beauveria brongniartii*. Biocontr. Sci. Technol., 17(5/6): 553-596.

RABIC SUMMERY

المكافحة الميكروبية لدودة القصب الكبيرة باستخدام فطر بيوفاريا باسيانا بمحافظة الشرقية

السيد على حسن شريف[١]- محمد فاروق غالى[٢] -عبده أحمد عبدالرازق المغيث[٢] – محمد عبدالله عبدالقادر[١]

١- قسم النبات- كلية العلوم- جامعة الزقازيق

٢- معهد بحوث وقاية النباتات- الشرقية- مصر

تم إجراء هذا البحث بمحافظة الشرقية خلال موسمي ٢٠١٤، ٢٠١٥ في المعمل. حيث تم استخدام فطر بيوفاريا باسينا الممرض للحشرات (عزلة ١٥٧٢) فى المكافحة الحيوية لحشرة دودة القصب الكبيرة تحت الظروف المعملية و تمت تجربة راشح الفطر *بيبوفاريا باسيانا* ١٥٧٢ على دودة القصب الكبيرة وكانت نسبة الموت وصلت ١٠٠% في مجموعة الاعمار المختلفة ليرقات دودة القصب الكبيرة بعد ٥ أيام. تم اختبارقدرة فطر *بيبوفاريا باسيانا* ١٥٧٢ على انتاج الانزيمات المحللة (الكيتنيزو البروتييزواللييز) وذلك عن طريق نموها على بيئات غذائية مخصصة لذلك وأوضحت النتائج ان فطر *بيبوفاريا باسيانا* ١٥٧٢ لديها انتاج جيد جدا من البروتييز وانتاج جيد نسبيا من الكيتنيزولا تنتج اللييز. ثم تمت دراسة اثر الظروف البيئية وعناصر التغذية على انتاج انزيم البروتييز لاختيار الظروف المثلى للنمولانتاج اكبر كمية من انزيم البروتييز. تم تعريف الفطر وتم فحصه بواسطة الميكروسكوب ووجد ان الفطر *بيبوفاريا باسيانا* ١٥٧٢ ينمو كمستعمرات بيضاء. على الوسط الغذائى الأكثر شيوعا ، وتنتج العديد من الكونيديات الدقيقة الجافة الفردية احادية الخلية ، فى كرات جراثيم بيضاء. تم تركيز راشح الفطر *بيبوفاريا باسيانا* ١٥٧٢ باستخدام المذيبات (الكلوروفورم) ليصبح راشح مركز (شبه نقى) .وتم التحقق من وجود سم البيوفيرسين فى الراشح المركز والغير مركز بواسطة الفصل الكهربى على الجل والذى اثبت وجود سم البيوفيرسين على الراشحين ثم تمت المعاملة بكلا الراشحين على حشرة دودة القصب الكبرى (يرقات) وتم فحصه تحت الميكروسكوب بالمقارنة بالحشرات الكنترول ووجد ان الحشرات المعاملة بالفطر يوجد عليها نموات لمستعمرات لفطر *بيبوفاريا باسيانا* ١٥٧٢ على جسد الحشرات الميتة وتغير فى لون الحشرة الخارجى الى لون اغمق فى كل الحشرات. وجود بقع سوداء وتشوهات فى اليرقات(عموما جميع الاعراض كانت اشد فى الراشح المركز). تم فحص اثر راشح الفطر على حشـــرات دودة القصب الكبرى (يرقات وعذارى) تحت الميكروسكوب بالمقارنة بالحشرات الكنترول ووجد ان الحشرات المعاملة بالفطر يوجد عليها نموات لمستعمرات لفطر *بيبوفاريا باسيانا* ١٥٧٢ على جسد الحشرات الميتة وتغير فى لون الحشرة الخارجى الى لون اغمق فى كل الحشرات. وجود تشوهات خاصة فى يرقات وعذارى و دودة القصب الكبيرة.عندما تلتصق كونيديات الفطر بجسم الحشرةتبدأ فى النمو عند توافر الظروف المناسبة خصوصا الرطوبة تبدأ فى النمو واختراق جسم الحشرة من الاماكن اللينة عن طريق الافراز الكيميائى للانزيمات المحللة (الكيتنيزو البروتييز) والضغط الميكانيكى لنمو الهيفات على مكان الاختراق . بعد الاختراق تنمو هيفات الفطر وتنقسم وتنتج انزيم البروتييز وسم البيوفيرسين ثم الجراثيم وتنتشر فى الهيموليمف فى كل اجزاء تجويف الحشرة . بعد ذلك تحدث تغيرات فيزيائية وكيميائية للهيمولمف والتى تؤدى الى اختلال وظائف جسم الحشرة وتموت الحشرة. بعد حدوث موت الحشرة ينمو الفطر خاج جسم الحشرة الميتة (الجثة) ويمكن ان يكمن قليلا لحين توافر الظروف المناسبة للنمو.فطر (*بيبوفاريا باسيانا* ١٥٧٢) يسبب ايضا بعض الاعراض الاخرى مثل فقدان الشهيةوشلل مؤقت ويوقف عمليات الانسلاخ فى اليرقات المصابة. ويمكن الاستفادة من نتائج هذا البحث عند وضع استراتيجية المكافحة المتكاملة لهذه الآفة (دودة القصب الكبرى) لزيادة المحصول كما ونوعا.