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

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**ABSTRACT**

This research was performed in Sharkia Governorate during seasons of 2014 and 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* isolate became 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 (Owney 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.

<table>
<thead>
<tr>
<th>Insect Samples</th>
<th>Insect name</th>
<th>From</th>
<th>Host</th>
<th>Stage from field</th>
<th>Sampling Method</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sesamia cretica</em> Led.</td>
<td></td>
<td>Elmesalamia – Zagazig - Sharkia</td>
<td>Maize</td>
<td>Larvae (harmful different ages from 1st to 5th) Pupa</td>
<td>Capture from infested plants</td>
</tr>
</tbody>
</table>

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 25°C & 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 25°C & 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.

Preparation 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).

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

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 (0.563 units/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 0.018 units/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 °C (4.31 U/ml), followed by at 50 °C (3.98 U/ml). For pr2 protease, maximum activity was observed at 50 °C (4.52 U/ml). Minimum activity for both, pr1 (2.77 U/ml) and pr2 (2.21 U/ml) protease was observed at 10 °C. For protease activity, preincubation at temperatures between 30-50 °C caused little loss in activity for Pr1, 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 Pr1 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

<table>
<thead>
<tr>
<th>Incubation Temperatures (°C)</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
<th>40</th>
<th>45</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease activity (units/ml)</td>
<td>0.03</td>
<td>0.079</td>
<td>0.288</td>
<td>0.439</td>
<td>0.563</td>
<td>0.455</td>
<td>0.222</td>
<td>0.139</td>
<td>0.066</td>
<td>0.018</td>
</tr>
</tbody>
</table>

### 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

<table>
<thead>
<tr>
<th>pH values</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>5.5</th>
<th>6</th>
<th>6.5</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro tease activity units/ml culture filtrate</td>
<td>0.162</td>
<td>0.284</td>
<td>0.537</td>
<td>0.607</td>
<td>0.582</td>
<td>0.518</td>
<td>0.439</td>
<td>0.339</td>
<td>0.224</td>
<td>0.167</td>
</tr>
</tbody>
</table>

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

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 0.458 units/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

<table>
<thead>
<tr>
<th>Carbon sources</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Galactose</th>
<th>Maltose</th>
<th>Fructose</th>
<th>Starch</th>
<th>Cellulose</th>
<th>Mannitol</th>
<th>Sorbitol</th>
<th>Xylose</th>
<th>C.M.C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease activity units/ml culture filtrate</td>
<td>0.553 (max. beauvericin production)</td>
<td>0.517</td>
<td>0.412</td>
<td>0.462</td>
<td>0.481</td>
<td>0.079</td>
<td>0.065</td>
<td>0.250</td>
<td>0.458</td>
<td>0.099</td>
<td></td>
</tr>
</tbody>
</table>

Nitrogen sources: different nitrogen sources (sodium nitrate NaNO₃-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. As presented 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 K₂HPO₄) gave moderate protease activity and beauvericin toxin production by B. bassiana 1572 comparable to the optimum. Urea, Urea (CO(NH₂)₂)/K₂HPO₄ , ammonium sulphate, ammonium sulphate / K₂HPO₄ 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 NaNO₃ (Lee et al., 2008 ;Xu et al., 2010).

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

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Peptone</th>
<th>Meat extract</th>
<th>Beef extract</th>
<th>Yeast extract</th>
<th>Na₅O₃</th>
<th>NaNO₃/K₂HPO₄</th>
<th>Urea</th>
<th>Urea/K₂HPO₄</th>
<th>Ammonium sulphate</th>
<th>Ammonium sulphate/K₂HPO₄</th>
<th>Ammonium phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease activity units/ml culture filtrate</td>
<td>0.555 (max. beauvericin production)</td>
<td>0.498</td>
<td>0.452</td>
<td>0.415</td>
<td>0.081</td>
<td>0.215</td>
<td>0.025</td>
<td>0.041</td>
<td>0.049</td>
<td>0.5</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Phosphorous sources: Different phosphorous sources varied in their effect on growth, protease activity and beauvericin toxin production by *B. bassiana* 1572. As in (Table 7), 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$_2$HPO$_4$ and peptone /KH$_2$PO$_4$ 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$_2$HPO$_4$, peptone /NaH$_2$PO$_4$ and peptone /K$_3$PO$_4$ 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>
<thead>
<tr>
<th>phosphorous source</th>
<th>Peptone</th>
<th>K$_2$HPO$_4$/peptone</th>
<th>KH$_2$PO$_4$/peptone</th>
<th>Na$_2$HPO$_4$/peptone</th>
<th>NaH$_2$PO$_4$/peptone</th>
<th>K$_3$PO$_4$/peptone</th>
<th>Amm. phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease activity units/ml culture filtrate</td>
<td>0.555 (max. beauvericin production)</td>
<td>0.528</td>
<td>0.488</td>
<td>0.429</td>
<td>0.415</td>
<td>0.393</td>
<td>0.03</td>
</tr>
</tbody>
</table>

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. In this respect, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Ascomycota: Hypocreales: Cordycipitaceae) is an anamorphic species 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.

**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.

**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).
*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


Microbial control on Sesamia cretica insects by Beauveria bassiana at Sharkia Governorate


Reference. CRC Press LLC, pp 75-76.

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