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STUDIES ON THE BIOLOGICAL CONTROL OF THE COTTON LEAF WORM *Spodoptera littoralis* (Boisd.)

A thesis

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By

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To

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To my mother

Father
Sisters and brothers

And my fiancee

Ahmed Adly Mohamed Ibrahim

This **THESIS** has not been submitted for any degree at this or at any other university. The literature cited here after show how I have availed myself to the work of the others

Ahmed Adly Mohamed Ibrahim

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CONTENTS

ABSTRACT

In the present study, three entomopathogenic fungal isolates, *Metarhizium anisopliae*, *Metarhizium flavoviridae* and *Beauveria brongniartii* kindly provided by Insect Pathogen Unit at Plant Protection Research Institute were used for the biological control of Egyptian cotton leaf worm *Spodoptera littoralis*. The plan of work deals with four aspects, the first is the mass production of the conidiospores which takes place by growing the three fungal isolates on three different media. The second is concerned with the study of the efficiency of the fungal isolates against the Egyptian cotton leaf worm using two techniques (the first one is leaf and filter paper dipping technique and the second one is larval dipping technique), the third aspect is concerned with the detection of the enzymatic activities of the entomopathogenic fungi (including chitinases, lipases and proteases enzymes which could be used for the penetration of the spores into the larvae and causes mortality and / or malformation), and the fourth aspect is concerned with the study of the effects of the fungal pathogens *Metarhizium anisopliae*, *Metarhizium flavoviridae* and *Beauveria brongniartii* on the protein biosynthesis by the Egyptian cotton leaf worm using sodium dodecyle sulphate-polyacrylamide gel electrophoresis. Data indicated that protein contents of *Metarhizium anisopliae* was separated into ten bands whereas the control hemolymph of *Spodoptera littoralis* was separated into nineteen bands while the treated hemolymph was separated into nineteen bands after 24 and 48 hours, twenty bands after 60 hours and eighteen bands after 72 hours. On the other hand, protein contents of *Metarhizium flavoviridae* was separated into thirteen specific protein bands, whereas the control hemolymph of *Spodoptera littoralis* was separated into nineteen protein bands while the treated hemolymph was separated into sixteen bands after 24 h. , fifteen bands after 48 h. ,sixteen bands after 60 h. and seventeen bands after 72 hours. Finally, protein contents of *Beauveria brongniartii* was separated into eleven specific protein bands, whereas the control hemolymph of *Spodoptera littoralis* was separated into nineteen protein bands while the treated hemolymph was separated into twenty-one bands after 24 h. , sixteen bands after 48 h. , seventeen bands after both 60 and 72 hours.

INTRODUCTION

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Since the overuse or misuse of chemical pesticides and their negative impacts on soil, water quality, human health, wildlife and the ecological balance within agro-ecosystems have become increasingly a cause of concern underling the need for development of alternative and safe pest control methods, the arsenal of biological control drew the attention of man and is now of crucial interest in developed as well as in developing countries **(Rashed 1993)**.

The first microorganisms found to cause diseases in insects were fungi because of their conspicuous macroscopic growth on the surfaces of their hosts. Most entomogenous fungi are obligate or facultative pathogens and some are symbiotic. Their growth and development are limited mainly by the external environmental conditions, in particular, high humidity or moisture, adequate temperatures and to a lesser extent light conditions and the nutritional environment for sporulation and spore germination. The diseases caused by fungi are termed "mycosis". Fungi infect individuals in all orders of insects, most common are Hemiptera, Diptera, Coleoptera, Llepidoptera, Orthoptera, and Hymenoptera **(David 1967; Ferron 1975)**. In some insect orders, the immature (nymphal or larval) stages are more often infected than the mature or adult stage, in others, the reverse may be the case. The pupal stage is infrequently attacked and the egg stage is rarely infected by fungi.

Host specificity varies considerably, some fungi infect a broad range of hosts and others are restricted to a few or a single insect species, those with a broad host range may consist of a variety of pathotypes (**McCoy *et al.* 1988**). *Beauveria bassiana, B. brongniartii, Metarhizium anisopliae and M. flavovridae* infect over 100 different insect species in severa isolates of these

two fungi have a high degree of specificity (Feron *et al* 1972;Fargues *et al* 1976). Host specificity may be associated with the physiological state of the host system [i.e., insect maturation and host plant] (McCoy *et al.*, 1988), the properties of the insect's integument with the nutritional requirements of the fungus (David 1967; Kerwin and Washino 1986 a,c), and the cellular defense of the host (Fargues *et al.*, 1976, Ferron 1978). Three types of defense reaction have been observed in the insect's hemocoel phagocytosis, cellular encapsulation (capsule, model, 'giant' cell), and humored encapsulation (Charnley 1984).

The development of mycosis can be separated into three phases :

1- adhesion and germination of the spores on the insect's cuticle.

2- penetration of the spores into the hemocoel via the excretion of different enzymes, such as lipase, chitinase and protease.

3- development of the fungus, which generally results in the death of the insect and finally reverse penetration of hyphae from the interior to the exterior through the cuticle in order to produce the infective units on the dorsal side of the insect. (McCoy *et al.*, 1988; Samson *et al.*, 1988).

The process of penetration through the insect's integument by a hyphae germinating from a spore involves chemical (enzymatic) and physical forces. The mechanical force is noticeable at the tip of an invading hyphae where the cuticular layers are distorted from pressure. The enzymes detected on germ tubes are protease, aminopeptidase, lipase, esterase and N-acetyl-glucosamidase (chitinase) (Ratault and Vey 1977). The in vitro studies indicate that the digestion of the integument follows a sequential lipase, protease, chitinase process of digestion (Samsinakova *et al.*, 1971; Smith *et al.*, 1981; St. Leger *et al.*, 1986 a). The proteases are the major cuticle degrading enzymes and their activities appear to precede those of chitinases

(**Samsinakova *et al.*, 1977; Smith *et al.* 1981**; **St. Leger *et al.*, 1987a,b**). The chitinase activity occurs mainly at the time of fungal growth, conidia formation and sporulation of conidiophores (**Coudron *et al*, 1984**). Aggressive or virulent strains may produce large amounts of extra-cellular enzymes (**Paris and Segretain 1978; Paris and Ferron, 1979**) (e.g., lipase, elastase, protease and alpha - glucanase), but in many cases no clear relationship has been established between such enzymes and aggressiveness of a fungal strain (**Samson *et al.*, 1988**).

Accordingly, The main aims of the present work could be summarized in the following :

1- Mass production of conidiospores of the three fungal isolates undrr study in different growth media.

2- Efficiency of the fungal isolates as a biological control agents against the Egyptian cotton leaf worm *Spodoptera littoralis* (Boisd.) under laboratory conditions.

3- Detection of enzymatic activities of three an extracellular enzymes including Chitinases, Lipases and Proteases qualitatively.

4- Effects of the fungal pathogens *Metarhizium anisopliae* , *M. flavovridae* & *Beauveria brongniartii* on the protein biosynthesis of the infected *Spodoptera littoralis* larvae obtained by sodium dodecyle sulphate polyacrelamide gel electrophoresis (SDS – PAGE).

LITERATURE REVIEW

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I-Entomopathogenic fungi metabolites:

Bidochka and Khachatourians (1993) found that *Beauveria bassiana* grown in a liquid medium containing N-acetyl-D-glucosamine and colloidal chitin produced two distinct N-acetyl-D-glucosaminidases, NAGase 1 and NAGase 2. NAGase 1 had a molecular weight of 97 000 and NAGase 2 was found to be composed of two subunits, of molecular weights 64 000 and 66 000. **Bidochka *et al.*, (1997)** reported that the entomopathogenic fungus *Beauveria bassiana* produces two extracellular N-acetylglucosaminidases (NAGase) in liquid medium containing colloidal chitin as the sole source of carbon and nitrogen. When they measured the regulation of NAGase synthesis, N-acetyl-D-glucosamine (GlcNAc), glucose NH_4 NO_3, or amino acids were added to the colloidal chitin medium and NAGase activity. They appeared that synthesis of NAGase is sensitive to cell energy and the carbon and nitrogen requirements. **Emmerich *et al.*, (1993)** found that there were four frequently occuring lichen compounds, (-) and (+)-usnic acid, vulpinic acid, and stictic acid (isolated from *Cladonia convoluta, C. stellaris, Usnea lapponica, U. filipendula,* and *Letharia vulpina*), were studied for toxicity and antifeedant activity against larvae of *S. littoralis*. Stictic acid caused no larval mortality, but reduced larval growth (ED_{50} of 56.2 mol/g DW). The other 3 lichen constituents exhibited significant toxic and anti-feedant activities against *S littoralis*. (-)-Usnic acid was the most active compound, with an LD_{50} value of 8.6 mol/g DW and an ED_{50} value of 1.2 mol/g DW. The LD_{50} and ED_{50} values for (+)-usnic acid were 90.8 and 4.5 ,mol/g DW respectively. The LD_{50} and ED_{50} values for vulpinic acid were 111.0 and 11.4 ,mol/g DW,

respectively. **Havukkala *et al.*, (1993)** investigated chitinolytic enzyme production and induction in entomopathogenic fungus *Beauveria bassiana* (an isolate from a *Praying mantis*) in liquid culture medium. They appeared that extracellular chitinolytic proteins could be induced in ca. 20 h after washing mycelia and then, transferred to a medium containing colloidal chitin as a sole carbon and nitrogen source. Chitinolytic enzymes were partially purified by ammonium sulfate precipitation followed by gel filtration with a Cellulose fine GCL 2000-sf column. Two main degradation activity peaks toward glycol-chitin were eluted out. Peak (1) degraded glycol-chitin and p-nitrophenyl-beta-D-N-acetylglucosaminide (pNP-NAG1), indicating mixed activities of endo- and exo-chitinase. Peak (2) degraded glycol-chitin but not pNP-NAG1, suggesting endochitinase activity. Peak (2) was further purified to homogeneity by ion-exchange chromatography on a Mono Q column and gel filtration through a joint-column of TSK gel G3000SW and G2000SW. The molecular weight of the endochitinase thus purified was estimated to be 45 kDa by SDS-polyacrylamide gel electrophoresis.

St.-Leger *et al.*, (1993) detected multiple extracellular chitinase isozymes in culture filtrate from *Metarhizium anisopliae*, *Beauveria bassiana*, and *Aspergillus flavus* following electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. They found that following renaturation, lytic zones of glycol chitin included in the gel revealed by UV illumination after staining with Calcofluor White M2R. The method was applied to a study on the mode of regulation of chitinolytic enzymes. High chitinase activities were present only in chitin-containing media. Addition of alanine, a readily utilized nutrient source, repressed chitinase production in *M. anisopliae* indicating that each isozyme was similarly regulated by products of chitin degradation by an

inducer-repressor mechanism. However, a chitinase-deficient mutant of *M. anisopliae* demonstrated reduced production of all but one of the chitinase isozyme *S. littoralis* The remaining activity (48 kDa) is evidently under separate control from the other isozymes which presumably are jointly controlled from a single regulatory gene.

Kagamizono *et al.*, (1995) isolated a new platelet aggregation inhibitor, bassiatin, from the cultured broth of *Beauveria bassiana* which had been isolated from a soil sample collected in Yunnan province, China. They found that the structure of bassiatin was (3S,6R)-4-methyl-6-(1-methylethyl) 3-phenylmethyl-1,4-perhydrooxazine 2,5-dio ne by NMR analysis, X-ray crystallographic analysis and chemical synthesis Bassiatin inhibited ADP-induced aggregation of rabbit platelets with the IC_{50} being 1.9×10^{4}.

St.-Leger *et al.*, **(1996)** suggested that extracellular chitinases have been to be virulence factors in fungal entomopathogenicity. By isoelectric focusing and a set of three fluorescent substrates, they found chitinolytic enzymes; N-acetyl- beta -D-glucosaminidases and endochitinases produced by the entomopathogenic fungi *Metarhizium anisopliae, Metarhizium flavoviride,* and *Beauveria bassiana* during growth in media containing insect cuticle. *M. flavoviride* also produced 1,4- beta -chitobiosidases. The endochitinases could be divided according to whether they had basic or acidic isoelectric points In contrast to those of the other two species, the predominant endochitinases of *M. anisopliae* were acidic, with isoelectric points of about 4.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis resolved the acidic chitinases of *M. anisopliae* into two major bands (43.5 and 45 kDa) with identical N-terminal sequences (AGGYVNAVYFY TNGLYLSNYQPA) similar to an endochitinase from the mycoparasite *Trichoderma harzianum.*