A STUDY OF SEVERAL PARAMETERS RELATING TO ENTOMOPATHOGENICITY OF *BEAUVERIA BASSIANA*

By

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CHAPTER I

INTRODUCTION

Biological control of insects has become increasingly important because of the toxic and carcinogenic effects of chemical insecticides on man and his environment. Because the chemicals used for insecticidal purposes have such deleterious effects, more emphasis is being placed on utilizing biological agents that possess insecticidal activity. These agents would be bacteria, such as Bacillus thuringiensis (Heimpel, 1959) and Pseudomonas aeruginosa (Lysenko, 1963); the polyhedrosal viruses that infect various insects, especially lepidoptera (Smith, 1959); and fungi, such as Beauveria bassiana (Bassi, 1835), and Aspergillus niger (Burnside, 1930).

Of particular interest here is Beauveria bassiana, which is capable of invading at least 30 species of insects (Pramer, 1965). B. bassiana has been a recognized entomopathogen since 1835, when Bassi showed the infection of the muscardine of the silkworm using the fungus (Steinhaus, 1949). Pospelov and others have done field studies using B. bassiana in the USSR for many years (cited in Ignoffo et al., 1979). Mass scale production of B. bassiana has been done in the USSR (20 tons of "Boverin" were produced in the USSR in 1978; Grula, personal communication), but only recently have the mycoinsecticidal capabilities of this fungus stirred interest in the United States.

Despite the variety of insects that are infected by B. bassiana, important parameters relating to the infective process have not been precisely defined. Investigators are still uncertain as to events occurring at the molecular level during invasion of certain insects by B. bassiana. The major question to be answered is, what makes B. bassiana an entomopathogen? The fungus obviously possesses some kind of invasive mechanism to which the insect is susceptible, but it has yet to be precisely defined.

It has been widely assumed that entomopathogenic fungi penetrate directly through the skin of insect larvae; however, the data to support such a mechanism are not conclusive (Lefebvre, 1934). Supporting this assumption are the studies by McCauley et al. (1968) of the fungus Metarrhizium anisopliae, which is pathogenic to wireworm larvae. They have shown that penetration of this larval integument occurs by an appressoria-like structure produced by the fungal hypha, and have concluded that penetration is facilitated by enzyme production. Cermakova and Samsinakova (1960) and Samsinakova (1971) also present evidence for direct penetration by B. bassiana through the soft skin of the Colorado beetle, and, also, the greater wax moth larvae.

Both of these latter studies indicated enzymatic degradation of the integument occurs during penetration. Lefebvre (1934) reported that B. bassiana could pass directly through the body wall of the corn borer, but he also indicated that the fungus could germinate and infect the alimentary canal upon injection of conidia.

It has also been reported by Burnside (1930) that infection of honey bees by Aspergillus niger occurs by germination of the fungus in the alimentary canal of the insect. These reports are either in conflict

or indicate that there are multiple modes of penetration; regardless, they point to the need for more precise information on the actual mechanism of penetration.

Work in our laboratory has centered on the biochemical events involved in entomopathogenicity of B. bassiana. The target insect used in these studies is the corn earworm (Heliothis zea), one of the most important agronomic pests in the United States. B. bassiana is pathogenic for this organism (Grula et al., 1979). There are three possible routes of infection of the corn earworm by this fungus: the digestive tract (gut area), direct penetration through the integument, or invasion via the spiracles.

The digestive tract of the corn earworm has a pH of 8.6 to 9.27 (Burton, unpublished data). This makes growth and survival of the conidia in this area highly unlikely. Furthermore, it is now known that B. bassiana can infect the corn earworm by either direct penetration into the integument or through the spiracles (Grula et al., 1979). Such penetration appears to be enzymatic, leading to the postulation that the fungus must produce exocellular enzymes that permit penetration through these complex surface layers.

Enzymatic Penetration of the Integument

The integument consists of three divisions, although there are layers within these divisions (Richards and Davies, 1977). The three divisions are: the cuticle, the epidermis, and the basement membrane. The cuticle is of primary concern, for it is this major structure which B. bassiana must enzymatically penetrate before invasion of the hemocoele is possible.

A cuticle both protects and determines the form of the insect. It is non-cellular, and flexible when first formed, but undergoes sclerotization and hardens upon aging. Its major components are proteins and chitin, a carbohydrate. Other components include phenolic precursors of quinones that link amino acid chains to form sclerotin, and various waxes and lipids, depending on the insect. These waxes and lipids are usually 80% hydrocarbons, with smaller amounts of fatty acids, alkyl esters, and other fatty alcohols (Richards and Davies, 1977).

A cuticle is comprised of two layers: the epicuticle (outer) and the inner procuticle. The epicuticle is itself composed of at least two layers. The first, which is devoid of chitin, is composed of cuticulin and is resistant to acids and most organic solvents. It is thought to be a highly polymerized lipid or a wax. The next layer, which is the principal one, contains proteins and is bound lipid. In some species of insects, there is also a superficial layer of lipid covered by a cement-like substance of unknown composition.

The procuticle, which makes up the bulk of the integument, also has two layers, the exocuticle and the endocuticle. They are composed of both chitin and protein, with chitin fibers joined to proteins by covalent linkages involving aspartic acid and histidine (Hackman, 1962). This means that the procuticle is actually a glycoprotein in which the chitin fibers are embedded in a protein matrix.

The integument also has enzyme systems for synthesis and degradation of major cuticular molecules. Processes at the surface of the cuticle are: secretion and repair of the wax layers, tanning of the endocuticle to form exocuticle, and repair of the surface cuticle after

abrasions (Locke, 1973). Figure 1 shows the structure of the integument, illustrating where the various chemical components are located.

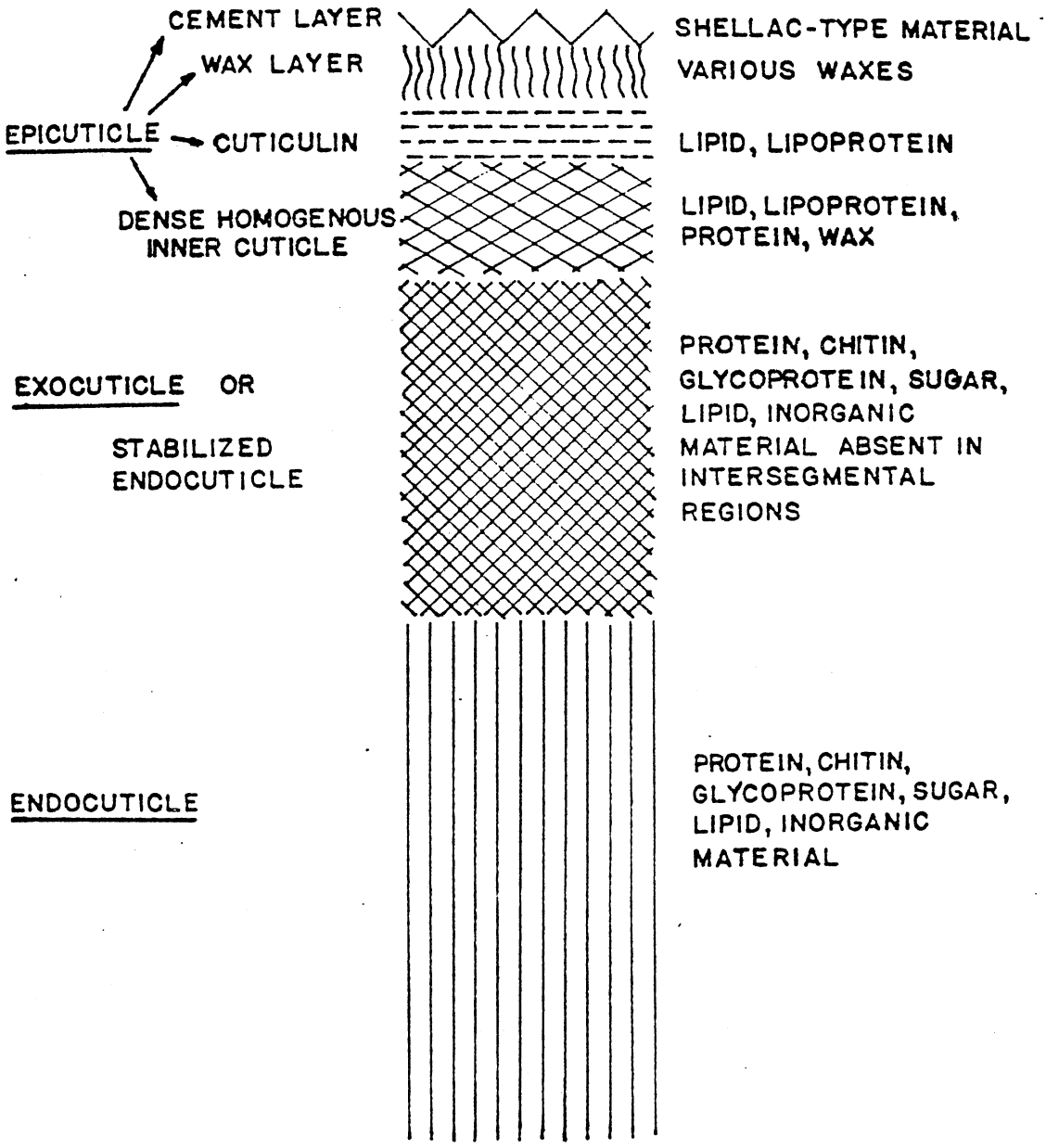
Considering that the integument is composed of these complex chemical constituents, it appears that a penetrating organism would have to produce exocellular enzymes to degrade some or all of these components. Such exocellular enzymes would include chitinases, proteases, lipases, and wax-degrading enzymes.

It is well documented that various fungi can produce chitinolytic and proteolytic enzymes. Huber (1958) showed that B. bassiana, as well as Metarrhizium anisopliae and Cordyceps militaris, can produce chitinolytic and proteolytic enzymes in vitro. Gabriel (1967) showed lipolytic, chitinolytic and proteolytic activity in four species of Entomophthorous fungi. More recently Blain et al. (1978) have shown the presence of lipolytic enzymes in species of Mucor, Rhizopus, and Aspergillus. Kucera and Samsinakova (1968), Kucera (1971) and Leopold (1970) presented evidence relating to the production of chitinases and proteases, as well as lipases by B. bassiana. Leopold (1970) has shown that a cellulase is also produced. In addition, B. bassiana synthesizes a wax-degrading enzyme (Grula et al., 1978).

In an interesting study reported by Samsinakova et al. (1971), it was shown that commercial preparations of chitinase, protease, and lipase will completely degrade water-washed integuments of the greater wax moth larvae. In addition, they obtained enzymatic extracts of B. bassiana in filtered lipid medium and found that the resulting degradation of the integument was similar to that caused by commercial enzyme preparations. It thus appears that B. bassiana is able to

Figure 1. A Diagramatic Representation of the Insect Integument

INSECT INTEGUMENT



produce the enzymes needed for penetration of the integument of insect larvae.

In addition to enzymes, B. bassiana also produces toxins which may be relevant to the infection process. These toxins alter cyst formation in the host, allowing mycelia to resume normal growth and invade the hemocoel (Ferron, 1978). Beauvericin is a cyclic depsipeptide produced by the fungus (Hamill et al., 1969; Sommerville, 1973; Prince et al., 1974). It is active against shrimp (Hamill et al., 1969) and bacteria (Champlin and Grula, 1979). Another toxin (bassianolide) is cidal to Bombyx mori larvae (Suzuki et al., 1977). Champlin and Grula (1979) have injected beauvericin and bassianolide into the hemocoele of the corn earworm and observed that neither toxin is lethal up to a concentration of six μg/larva.

Fungal Germination and Larval Surface Components

It has been shown that B. bassiana germinates on the surface of the corn earworm larva before penetration by the growing germ tube (Grula et al., 1978). Clearly a knowledge of the nutritional requirements for this germination event must be obtained. In addition, the chemical components on the larval surface that could be used nutritionally by the germinating spore must be further defined in order to comprehend the overall strategy and mechanism of growth and penetration by the fungus.

Lefebvre (1931) did the earliest study of germination and growth of B. bassiana conidia in various media. He reported that he could obtain 10-30% germination in the center of distilled water droplets and as high as 90% germination at the edge of water droplets. After germination,

extensive hyphal growth was observed in the droplets of distilled water within 24-48 hours. If his observations are correct, it could be concluded that germination of conidia of B. bassiana can occur in the complete absence of external nutrients. Because he further reported that 2% peptone greatly enhanced the percentage of germinating spores, one has to question the extensive germination and growth obtained in distilled water. Apparently, Lefebvre grew the fungus on potato-dextrose agar and directly transferred conidia to the distilled water without washing. Obviously, such procedures do not rule out the carry-over of nutrients that could be utilized for germination and growth. Since most of the nutritional work relating to germination of B. bassiana conidia has been carried out by Lefebvre, this area has since been further explored, utilizing more exacting procedures.

No reports have been found in the literature relating to release of chemicals by conidia that would signal the germination event in B. bassiana. Knowledge of such chemical signals would greatly enhance our ability to detect the exact moment of germination, and thus study the enzymes produced during penetration. Leighton (1970) reports that macroconidia of Microsporum gypseum release free amino acids and an alkaline phosphatase into the medium during germination.

It has been observed that carbon dioxide promotes the germination of Aspergillus niger conidia (Yanagita, 1957). Also, A. niger conidial germination can be stimulated by L-proline and L- or D-alanine (Miller, 1962).

It has been known for quite some time that fatty acids inhibit fungal germination and growth (Wyss et al., 1945). They found that undecylenic acid was a major inhibitor of fungal germination of

Aspergillus, Penicillium, and other fungi. Rolinson (1954) showed that mycelial growth of Penicillium chrysogenum was inhibited by fatty acids of intermediate chain length C_8-C_{10}. No mechanism for inhibition of germination and growth by fatty acids has received firm experimental support, although three major hypotheses have been proposed. These include: (a) direct action on the cell membrane, i.e., alteration of cell permeability (Teh, 1974); (b) nonspecific blocking of enzymatic sites (Teh, 1974); and (c) inhibition of specific reactions of cell metabolism (Lewis, 1970).

Koidsumi (1956) reported that the lipids of the cuticle of the silk worm and the rice stem-borer can inhibit fungal germination and growth of Aspergillus flavus. The most active anti-fungal constituents of cuticular lipids were free intermediate-chain saturated fatty acids, presumably caprylic or capric (C_8 and C_{10}), which he found to occur naturally in the cuticle. An ether extract of the cuticle of the silk worm inhibited germination of the fungus. Evalakova (1962), also using ether extracts of Eurygaster integriceps, showed inhibition of growth of B. bassiana at varying concentrations. These interesting findings permit us to postulate that the cuticle of the corn earworm may possess such compounds which would inhibit conidial germination and, therefore, eventual penetration of the structure by certain strains of B. bassiana susceptible to these components.

Therefore, to aid in establishing a molecular basis for the entomopathogenicity of B. bassiana against a target pest such as the corn earworm, the following information was sought: (a) exocellular enzymes produced by germinating and growing fungal hyphae, and (b)

nutritional requirements for germination and growth of the fungus on the larval surface, as well as the possible presence of inhibitory components.

CHAPTER II

MATERIALS AND METHODS

Test Organism

Mutants of Beauveria bassiana used in this study were obtained by others in this laboratory by irradiating (UV) suspensions of conidia. Such irradiated conidia were then assayed for differential proteolytic activity by plating on Sabouraud's Dextrose Agar (SDA, Difco) containing litmus milk (casein protein). Those organisms exhibiting varying levels of proteolytic enzyme activity were chosen as mutant forms. Larval passages have also been utilized to obtain mutants. These passages were accomplished by infecting corn earworm larvae with various highly pathogenic mutants and culturing the conidial growth after death of the larvae. Stock cultures were kept on SDA slants and usually transferred every three weeks for maximum viability. Cultures were always incubated at room temperature (25°C).

Target Insect

The larval form used in these studies was Heliothis zea (corn earworm). This species is a member of the Lepidoptera family. First instar larvae were obtained from the USDA Entomology Research Laboratory, Stillwater, Oklahoma, which is under the supervision of Dr. R. Burton. Larvae were allowed to grow in 1 oz clear plastic containers on a CSM

(Corn Soy Milk) diet developed by Burton (1970), with one larva in each container, because later instar forms of these larvae are cannibalistic. The containers were kept under constant light at 25°C, and allowed to grow until the desired instar (stage of growth) was reached.

Liquid Medium for Production of Different Enzyme Activities

A basal salt solution consisting of the following amounts per liter of distilled water was used in all defined medium formulations: NaCl, 0.30 g; $MgSO_4 \cdot 7\ H_2O$, 0.30 g; and K_2HPO_4, 0.30 g. Powdered chitin, gelatin, N-acetylglucosamine, D-glucosamine (all at the level of 1.0%) were added per 100 ml of the salts solution, depending on the type of enzyme protein response desired. The medium was sterilized in an autoclave for 15 minutes at 15 pounds pressure. N-Acetylglucosamine and D-glucosamine were filter sterilized and added separately. Two hundred ml of the medium was usually made up in a 250 ml Erlenmeyer flask, and inoculated with a loopful of Beauveria bassiana conidia from a SDA slant. The flask was incubated at 25°C on a rotary shaker for six days, except when proteolytic enzyme development was desired (four days).

Liquid Medium for Development of Integument-Degrading Enzyme Activity

Powered corn earworm integuments were added as the sole source of carbon, nitrogen, and energy to the basal salts medium. Powdered corn earworm integuments were obtained by skinning fifth instar larvae and boiling these skins in 1% sodium dodecyl sulfate for two hours. The

skins were then oven-dried (105°C) overnight, and ground into a fine powder with a mortar and pestle. Approximately 0.50 g of this product was used per 100 ml of medium. The medium (200 ml) was sterilized and inoculated as given previously and incubated for six days.

Liquid Medium for the Production of Inducible Chitinases

To induce enzyme activity against chitin, hyphae were grown for 24 hours in gelatin salts, Sabouraud's dextrose broth, powdered integument medium, or N-acetylglucosamine medium, after which 1.0 g of chitin (powdered) was added per 100 ml of the medium. The flasks were then reincubated under the same conditions for 24 hours.

Concentration of Supernatants

Each 250 ml Erlenmeyer flask containing 200 ml of liquid growth medium was centrifuged in a clinical centrifuge for 15 minutes to remove fungal growth. The supernatant was collected, placed in a dialysis bag (12,000 dalton exclusion limit), and concentrated overnight (with constant stirring) to approximately 1 ml against 20% Carbowax 6000 made up in distilled water. These concentrated supernatants were kept frozen until electrophoresis.

Protein Determination

A commercial BIO-RAD protein assay was used to determine total protein concentration of dialyzed and undialyzed supernatants from the liquid growth media.

Polyacrylamide Gel Electrophoresis

The simplified system of polyacrylamide gel electrophoresis by Clarke (1964) was used. Concentrated supernatants (approximately 50 µl) from the various growth media were layered directly onto gels with 20 µl glycerol (2:1 with distilled water). Tubes were run at two milliamps per tube for approximately one hour. Gels were stained with Coomassie Brilliant Blue and destained in acetic acid and methanol.

Larval Surface Extractions

Lipid extractions were obtained from third and fourth instar corn earworm larvae using hexane, ethanol, or a chloroform-methanol (2:1) solution. The larvae were grown on diet medium as previously described until the desired instar was reached, then separated into groups of 50. Each group was placed in a beaker of distilled water and washed for 30 minutes with constant stirring to remove any adhering diet. During this time the larvae defecate and regurgitate some food materials. These larvae were then placed in a beaker with 20 ml of the extraction solvent system and extracted with intermittent shaking for 30 minutes. The solvent extraction was filtered through Whatman No. 1 paper, and concentrated to dryness using a rotary evaporatory or under nitrogen gas. The residue was then methylated for gas-liquid chromatography by placing it in a screw-cap tube having a Teflon-coated cap. Sodium-dried benzene (4 ml) was added, along with 0.40 ml of 2,2 dimethoxy-propane, and 0.50 ml of methanolic hydrochloric acid (2.5% w/v methanol:HCl). The solution was kept at room temperature for 18 hours, after which time the resulting methyl esters were stored under nitrogen at -20°C until the time of analysis.

Gas-Liquid Chromatography

The gas-liquid chromatography unit used to separate the methyl esters of the fatty acids was a Perkin Elmer 990 unit equipped with a hydrogen flame detector. Column and instrument conditions are as follows:

Air Pressure ... 40 PSI

Hydrogen Pressure ... 15 PSI

Detector Temperature ... 250°C

Injection Port Temperature ... 250°C

Carrier Gas ... Nitrogen

Carrier Gas Flow Rate ... 50 ml/min.

Column Specifications ... 6' x 1/4" glass

Column Packing ... 20% Diethylene Glycol Succinate on a Chromosorb Support

Column Temperature ... Program, 80°C to 190°C, at a rate of 6°C/minute

Sample Specifications ... 4 µl to 8 µl in chloroform

The methyl esters of sample fatty acids were identified by comparison of retention times with known fatty acid methyl ester standards obtained from Sigma Chemical Company, St. Louis. The gas-liquid chromatograph is located in the Department of Biochemistry, Oklahoma State University, and is under the supervision of Dr. George Odell.

Preparation of Spore Suspensions

Either petri dishes or slants containing SDA were inoculated with the desired amount of B. bassiana. After sporulation had occurred (usually about nine days), 15 ml of 0.03% Triton X-100 was added to the

petri dish (or 5.0 ml added to the slants). This detergent aids in wetting the conidia for easier handling. The resulting spore suspension was centrifuged (clinical centrifuge) and washed two times with sterile distilled water to remove any possible residues of nutrients as well as excess Triton X-100. Aliquots of this suspension were used to inoculate various media for observation of germination and growth of the fungus.

Determination of Germination and Its Inhibition by Various Compounds

The growth media used for these studies consisted of basal salts solution, to which either N-acetylglucosamine or D-glucosamine was added to a final concentration of 0.10%. N-Acetylglycosamine was used in place of chitin because of the insolubility of chitin, which would make microscopic observation difficult. Germination was scored as the visible protuberance of a germ tube from the spherical conidium. Crystal violet (30 sec) was used to stain the preparations. Light photomicrography was performed on a Nikon research microscope equipped with phase contrast optics. Photographs were taken with a Nikon 35 mm camera. Kodak Panatomic X and Ectochrome 160 films were utilized for all photography.

Paper Chromatography

Paper chromatography was performed using Whatman No. 1 chromatography paper cut into 8" x 8" sheets. The paper was spotted with 50 µl aliquots of germination medium that had been subjected to varying conditions, then placed in the chromatography jar in a solvent system consisting of isopropanol, formic acid, and deionized water (80-4-20;

v/v/v). The solvent was allowed to run about one inch from the top of the paper, and the chromatogram was then removed and air dried. Chromatograms were observed in several ways for specific compounds. These included observation under ultraviolet light, spraying with ninhydrin reagent or oxidizing reagent (Shaw, 1968), or a phosphate-detecting spray (Wade and Morgan, 1953).

Hestrin Test

The Hestrin test is used to detect carboxylic acids and their derivatives (Hestrin, 1949). The following reagents were prepared: hydroxylamine hydrochloride, 2.0 M (stored at 4°C); NaOH, 3.5 N; conc. HCl, sp. gr. 1.18, diluted with two parts by volume of distilled H_2O; $FeCl_3$, 0.37 M, in HCl, 0.1 N; standard solution of acetyl choline at 0.004 M in sodium acetate solution of 0.001 M with a pH of 4.5. Alkaline hydroxylamine was always prepared fresh by adding equal volumes of hydroxylamine hydrochloride and the NaOH. This solution (2 ml) was added to 1.0 ml of the solution to be analyzed. After at least one minute, the pH is adjusted to 1.2 ± 0.2 with 1 ml of HCl. Ferric chloride (1 ml) is then added, and the optical density of the solutions determined promptly at 540 nm. Quantitation is effected by comparison with a standard solution of acetyl choline.

Carbonyl Test

This reaction was used to test for keto acids or other compounds containing C=O groups. Reagents: 2,4 dinitrophenylhydrazine (100 mg in 100 ml of 2.0 N HCl, allowed to stand overnight at room temperature, and then stored in the cold indefinitely); ethanol (acetone-free, 95%);

and 6.0 N NaOH. The sample was added (0.10 ml is sufficient) to 0.5 ml of 2,4 dinitrophenylhydrazine reagent. Water (4 ml) was added and the solution was mixed by agitation in a vortex mixer. After approximately 20 minutes, 3 ml of 95% ethanol was added and the mixture again agitated then allowed to stand for another 20 minutes. Sodium hydroxide (1 ml) was then added, the mixture again agitated, and allowed to stand at least 10 minutes prior to reading at 540 nm against a reagent blank. A standard curve was prepared using sodium pyruvate.

Release of Radioactive Carbon Dioxide

Conidia were collected as previously described and inoculated into 15 ml of germination medium containing 0.10 μC/ml of the ^{14}C labeled D-glucosamine (specific activity 50 μC/1.24 mg). A carbon dioxide trapping system was devised wherein air was bubbled through the germination medium, and released CO_2 from the conidia was trapped in 15 ml of CO_2 Trapping Agent (Amersham, Arlington Heights, Illinois). At appropriate time intervals, aliquots of the CO_2 Trapping Agent (1.0 ml) were withdrawn and placed in scintillation vials containing 9 ml of PCS (Phase Combining System, Amersham/Seale, Arlington Heights, Illinois) counting cocktail. Samples were incubated to allow dissolution of material (one hour). The vials were counted for radioactive carbon using a liquid scintillation counter located in Life Sciences West under the supervision of Dr. Jack Bantle.

CHAPTER III

RESULTS

Exocellular Proteins of Beauveria Bassiana

To obtain information on exocellular proteins produced by Beauveria bassiana, concentrated spent growth media containing varying nutritional sources of carbon, nitrogen, and energy were analyzed by polyacrylamide gel electrophoresis (PAGE). These experiments were done to determine the number and amounts of exocellular proteins produced by the different fungal mutants. If any unique protein bands were observed in media from highly pathogenic mutants, it was theorized that these might represent enzymes that could be utilized for penetration of the corn earworm integument.

The method of approach was to utilize various growth media that would presumably induce production of enzymes needed for penetration. Because the integument is composed primarily of chitin, protein, and lipid, it was postulated that the exocellular enzymes needed for penetration of this structure would be chitinases, proteases, and/or lipases.

Once the production of exocellular enzymes by B. bassiana could be established, the various entomopathogenic mutants, differing in their abilities to infect, could then be compared. A rating of pathogenicity of the mutants has been determined in this laboratory using probit analysis of corn earworm larvae (Cheung, unpublished data).

Since chitin is a major component of the insect cuticle, the mutants were first analyzed for chitinolytic activity. A representation of the band patterns found when B. bassiana mutants were grown in the chitin salts medium is given in Figure 2. The gels are arranged in a decreasing order of pathogenicity for the corn earworm, with the most effective entomopathogen being E_1. It can readily be observed that no unique band pattern differences exist in the more effective entomopathogens; however, these band patterns become instructive from other aspects.

An exocellular band pattern representative of fungal growth in Sabouraud's dextrose broth, compared to growth in chitin salts is shown in Figure 3. It is evident that the patterns differ. More evidence for differences in secreted proteins can be shown when the fungus is grown in gelatin salts (Figure 4). These data are used to demonstrate that the production of exocellular proteins is dependent on composition of the growth medium.

The best simulation of the natural growth conditions that B. bassiana will encounter during infection of larvae is the powdered integument medium. The band representation of fungal proteins produced in this medium is given in Figure 5.

The above gel representations illustrate the inducibility of exocellular proteins produced by B. bassiana. Clearly, the fungus synthesizes different exocellular proteins depending on the nutrients provided.

To further establish the inducible nature of chitinase, powdered chitin was added to cultures growing in different media. Presumably the mold would exhaust the original nutrients present, and then utilize the chitin, while producing different proteins. The band pattern of

Figure 2. A Comparison of Proteins Obtained from Mutants of *Beauveria bassiana* Grown in Chitin Salts Medium. Mutants are arranged in a relative order of pathogenicity with E_1 being the most effective entomopathogen.

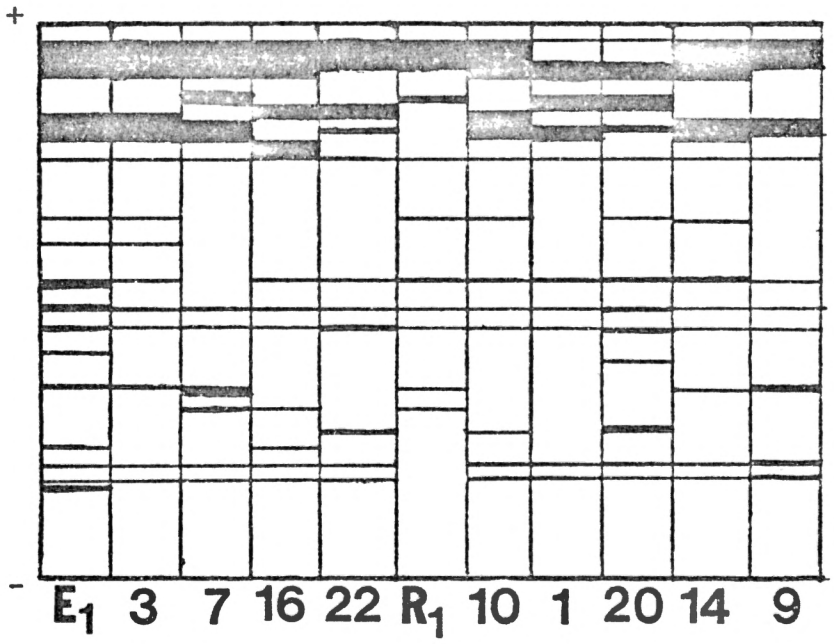

+
-
E1 3 7 16 22 R1 10 1 20 14 9

Figure 3. A Diagramatic Representation of Proteins Obtained from B. bassiana E_1 and 14 Grown in Chitin Salts Medium (Ch) and Sabouraud's Dextrose Broth (SDB).

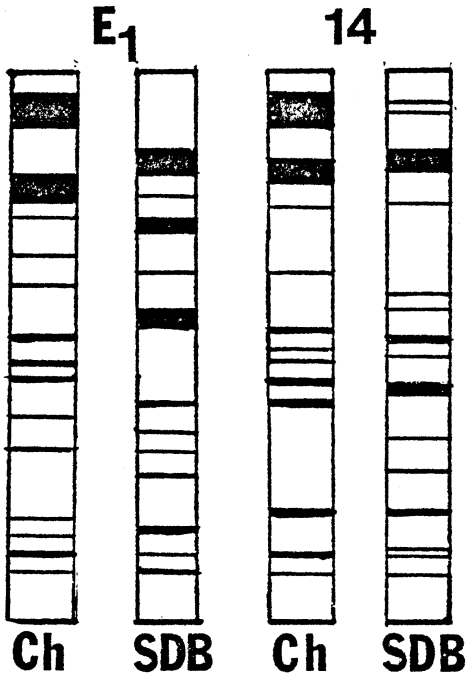

E1
14
Ch
SDB
Ch
SDB

Figure 4. A Representation of Proteins Obtained when B. bassiana E_1 is Grown in Chitin Salts Medium (Ch) and in Gelatin Salts Medium (Gel).

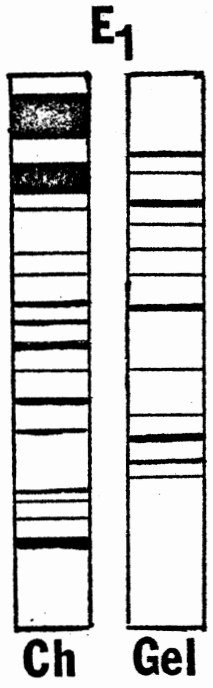
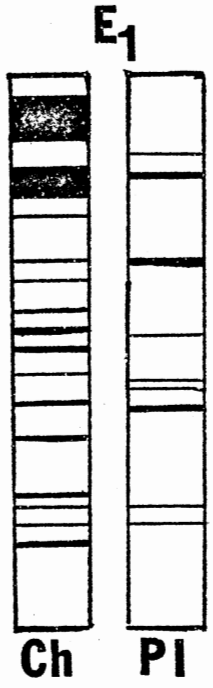

E1
Ch
Gel

Figure 5. A Protein Band Representation of *B. bassiana* E_1 Grown in Chitin Salts Medium (Ch) and in Powdered Integument (PI) Medium.


E1
Ch
Pl

B. bassiana grown in N-acetylglucosamine (NAG) and NAG with chitin added after 24 hours growth is shown in Figure 6. The chitin addition greatly enhances protein production and causes production of new proteins. Addition of chitin to Sabouraud's dextrose broth or powdered integument medium also results in altered exocellular protein production (Figures 7 and 8).

A summation of the various band patterns obtained when different nutritional conditions were utilized are presented in Figure 9. These data suggest that the protein present at position 1 may be a chitinase enzyme. This band is absent when the fungus is grown in the absence of chitin and always appears when chitin is added to growth medium.

Claus (1961) reported that chitinase is located intracellularly in the conidia and hyphae of B. bassiana. He demonstrated that it was not an exocellular enzyme by use of cell-free preparations. To show that the proteins analyzed by PAGE in this study were indeed exocellular, hyphae were sonicated using six 15-second bursts, with a one-minute rest period after each burst to permit cooling. Microscopic observation of the resulting suspension showed that the majority of hyphae were broken, although a few were still intact. The suspension was spun down (clinical centrifuge), and the supernatant concentrated and analyzed using PAGE. The gels did not reveal a great number of proteins, but seven major bands were present (Figure 10). None of these bands match up with the exocellular protein bands, allowing the conclusion that the proteins present in the supernatants of liquid growth media are indeed exocellular. In addition, we have been able to show that clearing occurs around colonies of B. bassiana growing on chitin salts agar (B. Abegaz, personal communication). Also, chitinase enzyme activity

Figure 6. A Comparison of Proteins Obtained from *B. bassiana* E_1 Grown in N-acetyl-glucosamine (NAG) With and Without the Addition of Chitin (Ch).

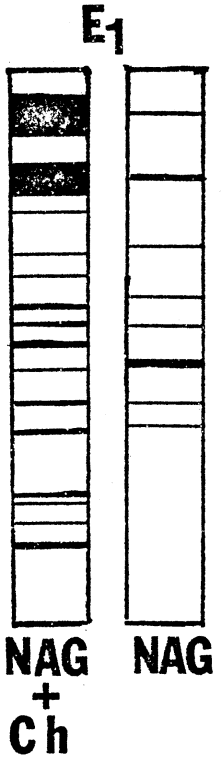

E1
NAG
+
Ch
NAG

Figure 7. A Similar Comparison of Proteins Obtained from B. bassiana E_1 Grown in Sabouraud's Dextrose Broth (SDB) and in SDB with the Addition of Chitin at 24 Hours.

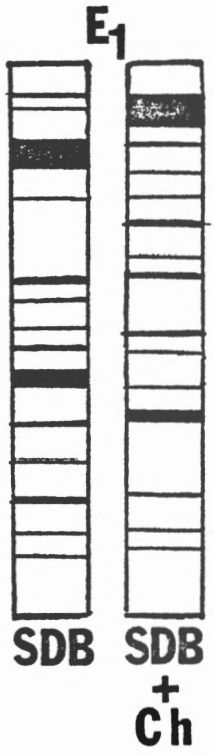

E1
SDB
SDB
+
Ch

Figure 8. A Protein Band Representation of Proteins from *B. bassiana* E_1 Grown in Powdered Integument Medium (PI), and in PI with the Addition of Chitin After 24 Hours.

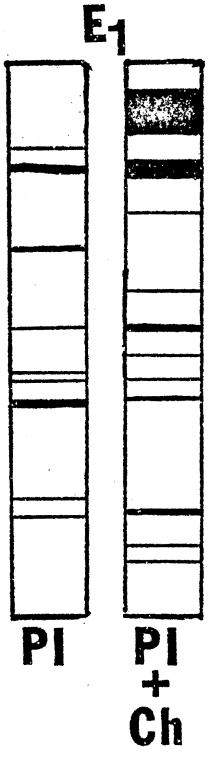

E1
PI
PI
+
Ch

Figure 9. A Comparison of Proteins Obtained from B. bassiana E_1 Grown in Chitin Salts (Ch), Powdered Integument Medium (PI), Gelatin Salts (Gel), Sabouraud's Dextrose Broth (SDB), N-Acetylglucosamine (NAG), and PI, SDB, NAG with the Addition of Chitin at 24 Hours. Pos 1 (arrows) indicates the presence of a possible chitinase band. (A protein band which migrates to a position approximately 1.5 cm from the bottom of the gel in control gels is not represented in the gelatin gel.)

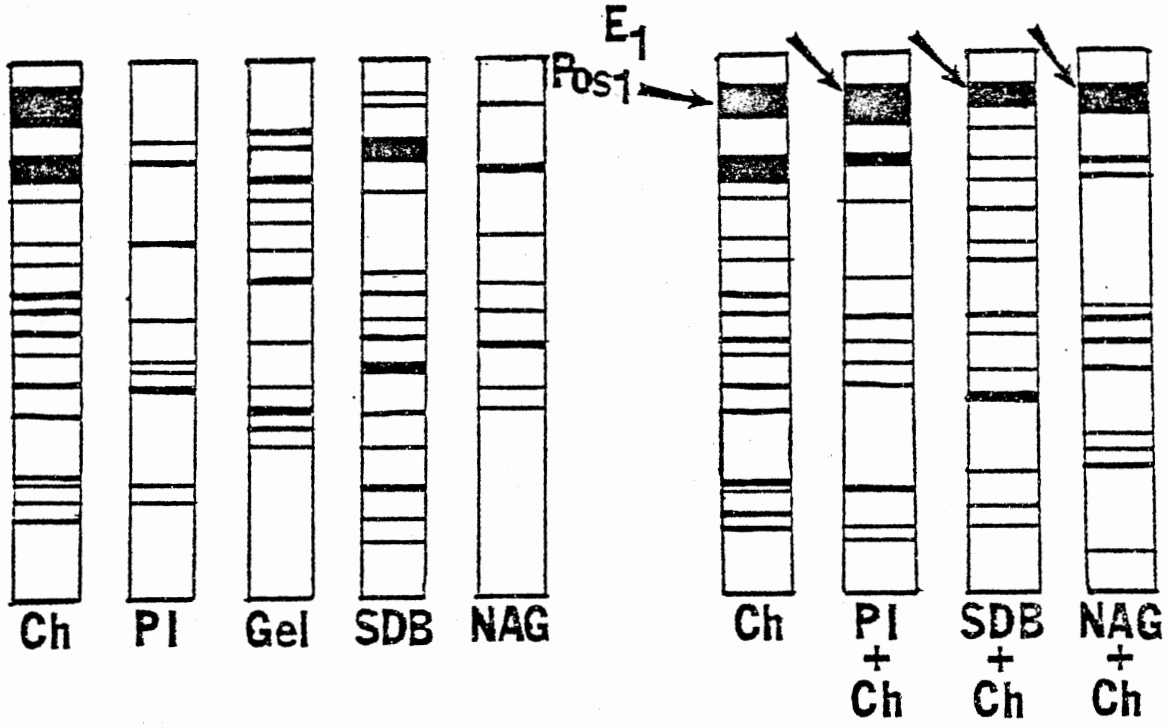
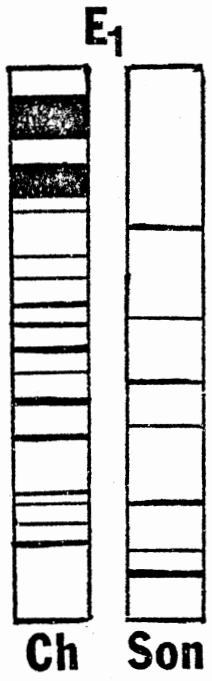

E1
Pos1
Ch
PI
Gel
SDB
NAG
Ch
PI + Ch
SDB + Ch
NAG + Ch

Figure 10. A Comparison of Exocellular and Cytoplasmic (Son) Proteins Obtained from B. bassiana E_1 Grown in Chitin Salts Medium (Ch).


E1
Ch
Son

can readily be demonstrated in concentrated supernatants by measurement of release of N-acetylglucosamine from powdered chitin (M. Grula, personal communication).

Another important point can be made concerning the production of exocellular enzymes and their role in entomopathogenicity. Growth of the fungus on isolated integuments was utilized to attempt to correlate the ability to grow with the relative entomopathogenicity of each mutant. During these studies, sterile conditions could not be maintained, and it was observed that these integuments had Aspergillus and Penicillium overgrowing B. bassiana. This was an interesting finding and led to a series of experiments utilizing these contaminants. The two fungi were extensively examined in regards to their pathogenic abilities for corn earworm larvae. Larvae were infected with Aspergillus and Penicillium (presumably sp. niger and chrysogenum, respectively) in the same manner as B. bassiana infections (Grula et al., 1978). In addition, larvae were injected with conidia from these fungi. In no instance were the Aspergillus and Penicillium sp. found to be pathogenic for the corn earworm. Enzyme analyses showed that the two fungi produced the same kinds of enzymes and were able to grow in the same types of media as B. bassiana (Table I). When inoculated into chitin salts medium, Penicillium chrysogenum and Aspergillus niger grew extensively and the band patterns are shown in Figure 11.

From these data, it is evident that the ability of an organism to utilize isolated integuments for growth does not mean that the organism is a pathogen. Aspergillus and Penicillium both synthesize the enzymes capable of degrading the corn earworm integument; i.e., chitinases, lipases, and proteases, and indeed they grow extensively on isolated

TABLE I

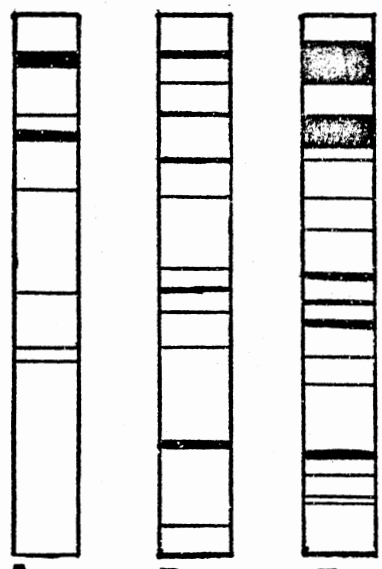
ENZYME PRODUCTION BY *ASPERGILLUS NIGER*, *PENICILLIUM CHRYSOGENUM*, AND *BEAUVERIA BASSIANA* ON DIFFERENT MEDIA

Fungus	Medium				
	Starch	Gelatin	Tributyrin	Chitin	Integument
Aspergillus	-	+	+	+	+
Penicillium	-	+	+	+	+
B. bassiana E_1	-	+	+	+	+
B. bassiana 16	-	+	+	+	+

+ denotes positive growth or enzyme production.

- denotes negative response.

Figure 11. A Representation of Protein Bands Obtained from *Aspergillus niger* (Asp), *Penicillium chrysogenum* (Pen), and *B. bassiana* E_1 Grown in Chitin Salts Medium.


Asp
Pen
E1
CHITIN
SALTS

integuments or in chitin salts medium. However, they are not pathogenic for the corn earworm. Pekrul (1979) has recently shown that conidia from Aspergillus and Penicillium are unable to germinate on the surface of the corn earworm. This finding aids in reinforcing the belief that prior to production of integument-degrading enzymes, conidia must germinate and produce hyphae that will secrete the enzymes needed to penetrate the integument.

Isolated integuments were also subjected to various commercial enzymatic preparations. Visible observation of integument degradation was the goal of these experiments. Commercial preparations of chitinase, protease, and lipase were added singly and in combinations to solutions containing one isolated corn earworm integument per tube. The solutions were buffered for the optimum pH of each combination of enzymes (Table II).

Samsinakova (1971) reported that with such enzymatic solutions, the integument of the greater wax moth larva was completely degraded. Our results using corn earworm larval integuments are of an opposing nature. No visible degradation of the integument was ever seen, particularly when the preparations were overlayed with toluene to prevent bacterial contamination during the long incubation periods (48 hours at 37°C).

Nutritional Requirements for Fungal Germination and Growth

The previous data suggest that the ability to produce exocellular enzymes capable of degrading the larval integument may not be the actual differentiating factor for the varying pathogenicities of mutants of B. bassiana. Pekrul and Grula (1979) have shown that B. bassiana

TABLE II

EFFECT OF COMMERCIAL ENZYMES ON ISOLATED LARVAL INTEGUMENTS

	Integument Preparation*		
	Water-Washed	Untreated	Triton X-100-Washed
LPC	-	-	-
PC	-	-	-
LP	-	-	-
LC	-	-	-
L	-	-	-
P	-	-	-
C	-	-	-
pH 6.8			
LPC	-	-	-
PC	-	-	-
LP	-	-	-
LC	-	-	-
L	-	-	-
P	-	-	-
C	-	-	-
pH 8.1			

L = Lipase (Sigma, E.C. No. 3.1.1.3).
P = Protease (Sigma, Type IV, from Streptomyces caespitosus).
C = Chitinase (Sigma, E.C. No. 3.2.1.14).
+ = Visible degradation of integument.
- = No visible degradation.

*400 µg of each enzyme was added to 5.0 ml of either 0.02 M phosphate buffer (pH 6.8) or 0.5 M Tris buffer (pH 8.1) containing one integument. Integuments were either untreated, washed in distilled water (1X--five minutes), or washed in 1% Triton X-100 for 15 minutes and rinsed in distilled water for five minutes. Tubes were incubated under toluene at 37°C for 48 hours.

germinates on the surface of corn earworm larvae with subsequent penetration into the hemocoel by the growing germ tube. Therefore, the germination event of the fungus requires definition with regard to three parameters: (a) nutritional requirements, (b) presence of components on the larval surface that might retard the germination event, and (c) compounds produced by germinating conidia that could be used as chemical signals of the event. These areas were explored in detail.

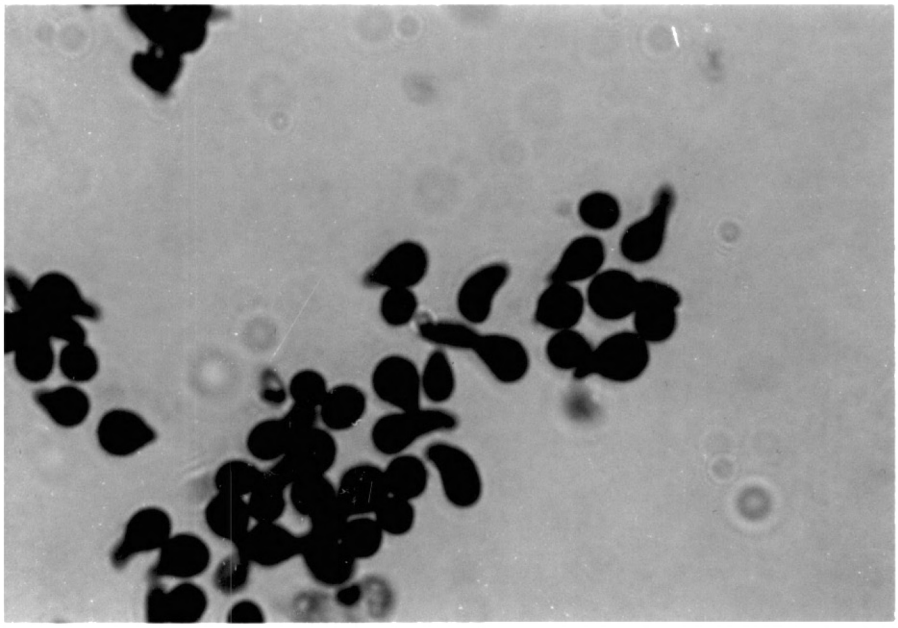
Of primary importance was the establishment of a defined medium that could be used for the observation of germination and outgrowth of fungal hyphae. Chitin is a major component of the larval integument, as well as being a very good substrate for the production of exocellular enzymes by B. bassiana. Chitin is, however, insoluble, thereby making optical microscopic observation difficult. N-Acetylglucosamine (NAG) was chosen as the sole carbon, nitrogen, and energy source for the germination medium, because it is a hydrolytic product of chitin; in addition, NAG is very soluble in water.

Lefebvre (1931) reported that B. bassiana could germinate and grow in distilled water. The observations reported in this study differ greatly from those of Lefebvre. Washed conidia (2X) cannot grow in distilled water; indeed conidial lysis occurs (Figure 12).

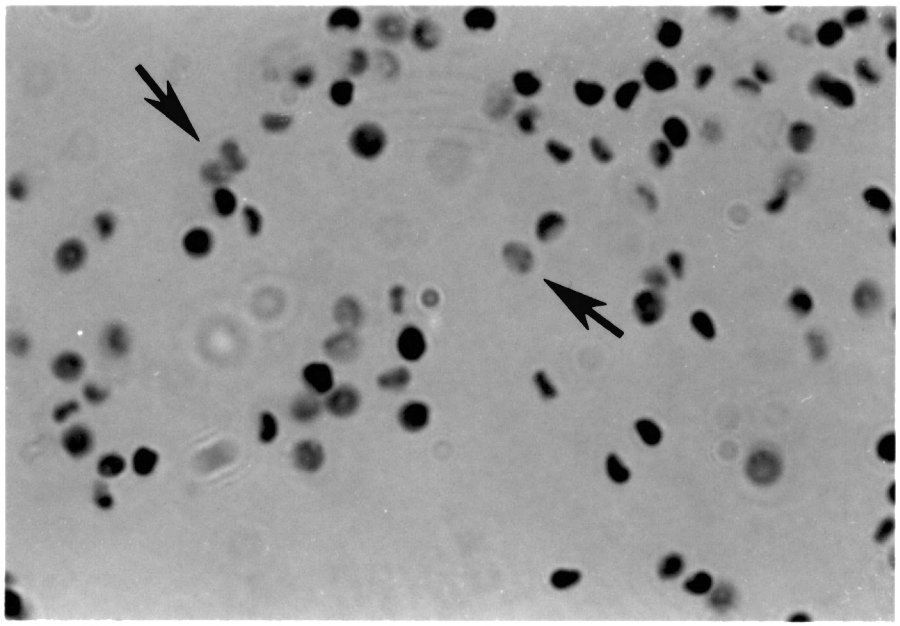
A variety of nutritional situations were examined using the basal salts medium. It was observed that the fungus cannot germinate or grow in the basal salts medium even after addition of ammonium chloride. Addition of glucose to the basal salts medium allows germination; however, lysis of the outgrowing germ tubes ensues. When the conidia are grown in the basal salts medium plus glucose and ammonium chloride, extensive germination and growth are obtained equal to that seen in the

Figure 12. Conidia of B. bassiana E_1 Germinating in NAG Medium (a), and Lysing (Arrows) in Distilled Water (b), at 16 Hours. (2000X).

a)



b)



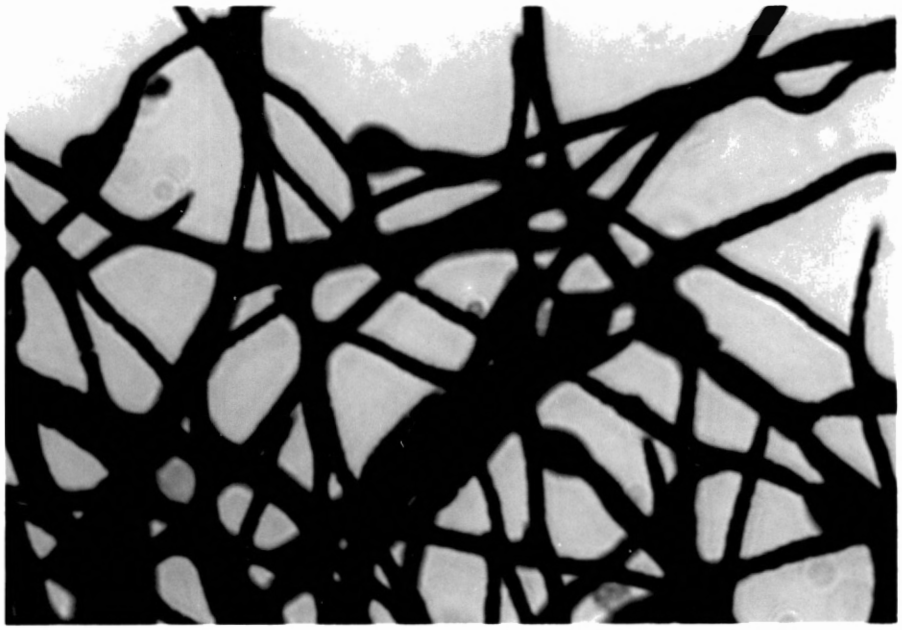
NAG medium. These findings (Figure 13) illustrate that a carbon and energy source such as glucose will allow germination, but a nitrogen source must be present to sustain growth and prevent autolysis of the outgrowing hyphae.

Once the general requirements for germination and growth were ascertained, in-depth studies of various carbon and nitrogen sources were made. Amino acid studies were initiated and conducted in the following manner: (a) amino acids were grouped according to their metabolic families for investigation (Table III), (b) individual amino acids were tested at the level of 0.01% in the basal salts solution, (c) an inoculum of a washed conidial suspension (2X) was used, and (d) microscopic observations were made for germination and growth of the conidia. Results are given in Table IV.

The amino acids in families 1 and 4 allowed the best germination and hyphal growth. The amino acids in these families were then tested individually and in all possible combinations (Table IV). A minimum of three amino acids allowed good germination and subsequent growth. Two amino acids would not support both germination and growth, and no individual amino acid was found to allow either germination or growth. The best combinations were alanine, phenylalanine, and valine, or alanine, phenylalanine, and leucine. Leucine and valine appeared to be interchangeable, as were phenylalanine and tyrosine. When the concentration of valine was increased (from 0.01% to 0.03% and 0.05%), germination and growth were enhanced. Using a combination of alanine, phenylalanine, and valine, various amino acids were then added to check for either a toxic or enhanced growth effect. No amino acid was found

Figure 13. Conidia of *B. bassiana* E_1 Growing in Glucose and Ammonium Chloride (a), and Hyphal and Germ Tube Lysis in Glucose Alone (b) at 22 Hours. (2000X).

a)



b)

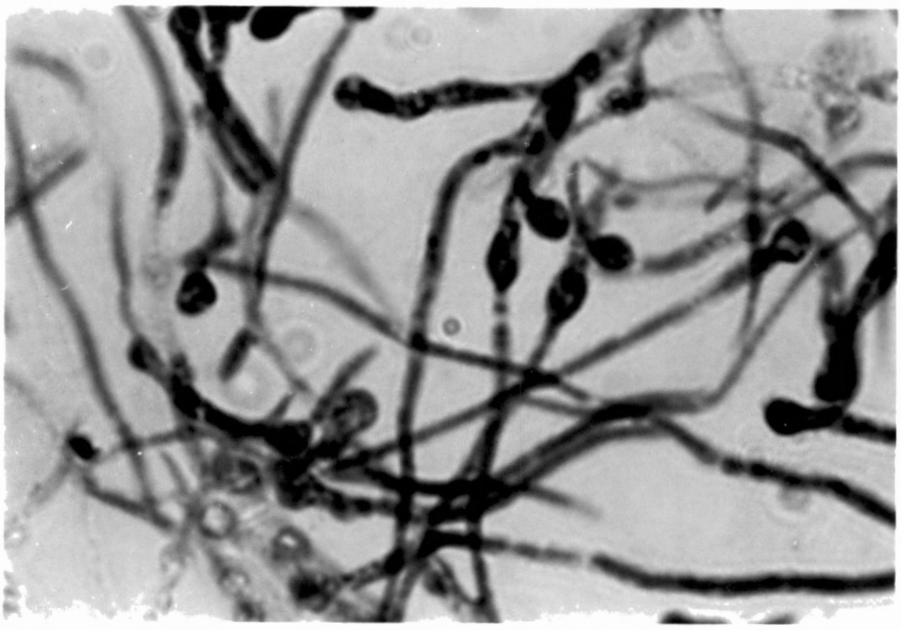


TABLE III

AMINO ACID GROUPINGS FOR B. BASSIANA NUTRITION STUDIES

Family	Amino Acids
1	Alanine, valine, leucine
2	Aspartic acid, lysine, methionine, isoleucine, and threonine
3	Serine, glycine, cysteine
4	Phenylalanine, tyrosine, tryptophan
5	Glutamic acid, proline, arginine
6	Histidine

Each family was added (0.01%*) into the basal salts medium, and the pH was adjusted to 6.0. All families were tested separately and in all possible combinations for their abilities to allow germination and growth of B. bassiana. Families 1 and 4 allowed the best germination and growth of conidia.

*Final concentration.

TABLE IV

AMINO ACID REQUIREMENTS FOR GERMINATION AND GROWTH OF *B. BASSIANA* E_1

Amino Acid*					Germination**	Growth**
Alanine	Valine	Leucine	Phenylalanine	Tyrosine		
+	+	+	+	+	4	4
+	+	+	+	-	2	3
+	+	+	-	+	2	2
+	+	-	+	+	3	3
+	-	+	+	+	3	3
-	+	+	+	+	1	1
+	+	+	-	-	2	2
+	-	-	+	+	2	3
+	+	-	+	-	4	4
+	+	-	-	+	3	4
+	-	+	+	-	4	4
+	-	+	-	+	1	0
-	+	+	+	-	1	2
-	+	+	-	+	1	0
-	-	+	+	+	2	1

*Level of each amino acid was 0.01%.

**4 = maximum germination or growth response; 0 = no growth. Cultures were observed at 16 hours for germination and 72 hours for growth.

to be toxic to B. bassiana and addition of other amino acids did not significantly enhance growth or germination.

Germination and growth of B. bassiana were also examined utilizing various fatty acids as carbon and energy sources. Apparently a few fatty acids can support germination; however, none support further growth of the fungus (Table V).

A summation of the compounds that will allow germination and growth of B. bassiana E_1 is given in Table VI. Also listed are those compounds that will allow germination but no growth, as well as those that will not even support or allow germination.

It is apparent from these data that B. bassiana can germinate and sustain hyphal growth using a wide variety of carbon, nitrogen, and energy sources. Carbohydrates such as dextrose, glucosamine, N-acetyl-glucosamine, and chitin, as well as various amino acid combinations can all be utilized. Therefore, it would appear that this fungus has an excellent probability of survival in nature due to its relatively non-specific requirements for germination and growth. Thus, the chances for infecting various insects are maximized.

Inhibition of the Germination Event and Larval Surface Components

After investigating nutritional requirements for germination, we next examined compounds that inhibit germination of the fungus. As previously mentioned in Chapter I, the integument is covered with a waxy layer. It was postulated that there would be free fatty acids present on the larval surface, and these could be inhibitory to fungal germination and growth; thus preventing secretion of exocellular enzymes and

TABLE V

NUTRITIONAL STUDIES OF B. BASSIANA
USING FATTY ACIDS

Fatty Acid**	Germination	Growth***
Butyric (C:4)	-	-
Valeric (C:5)	-	-
Caproic (C:6)	-	-
Heptanoic (C:7)	-	-
Caprylic (C:8)	-	-
Nonanoic (C:9)	-	-
Capric (C:10)	+	-
Undecanoic (C:11)	+	-
Stearic (C:18)	+	-
Oleic (C:18-1)	+	-

+ = Germination has occurred (at least 50% of conidia were germinating).

- = No visible observation of germination.

*NH_4Cl (0.01%) was present in all tubes in addition to the basal salts. Mutant E_1 was the test organism.

**Final concentration of fatty acid was 1.0%.

***Growth was observed at 72 hours.

TABLE VI

NUTRITIONAL REQUIREMENTS FOR GERMINATION AND GROWTH OF B. BASSIANA E_1*

Compound and Concentration	Germination	Growth
Basal salts solution**	-	-
Chitin (1.0%)	+	+
N-Acetylglucosamine (1.0%)	+	+
D-Glucosamine (1.0%)	+	+
Alanine, phenylalanine, valine (0.01% of each)	+	+
Glucose (1.0%)	+	-
Glucose (1.0%) + NH_4Cl***	+	+
Undecanoic acid (1.0%) + NH_4Cl	+	-
Oleic acid (1.0%) + NH_4Cl	+	-
Stearic acid (1.0%) + NH_4Cl	+	-
Distilled water	-	-
Butyric acid (1.0%) + NH_4Cl	-	-
Valeric acid (1.0%) + NH_4Cl	-	-
Caproic acid (1.0%) + NH_4Cl	-	-
Caprylic acid (1.0%) + NH_4Cl	-	-
Nonanoic acid (1.0%) + NH_4Cl	-	-
Capric acid (1.0%) + NH_4Cl	+	-
Uracil (1.0%)	-	-
Guanosine (1.0%)	-	-
Cytosine (1.0%)	-	-
Adenosine (1.0%)	-	-
Urea (1.0%)	-	-

*+ = A positive response; - = a negative response.

**Composition of basal salts solution: 0.30 g NaCl, 0.30 g $MgSO_4 \cdot 7 H_2O$, 0.30 g K_2HPO_4/liter distilled water.

***NH_4Cl was always present at a final concentration of 0.01%.

penetration of the structure by hyphae. This working hypothesis had even greater significance when it was observed that most fatty acids plus ammonium chloride cannot support germination and growth of B. bassiana (Table VI).

Data presented in Table VII summarize the effect of some saturated fatty acids when added to conidia of B. bassiana in the NAG-basal salts medium. Saturated short chain fatty acids are fungistatic, inhibiting conidial germination up to 36 hours, depending on concentration of the fatty acid. Germination and normal outgrowth as well as inhibition of these processes in the presence of 0.02% caprylic acid (16 hours) are shown in Figure 14.

After establishing the fungistatic effect of short length fatty acids on germination of B. bassiana conidia, extractions of fatty acids from the surface of corn earworm larvae were done. Hexane, ethanol, and chloroform-methanol extractions from both fourth and fifth instar larvae all inhibit conidial germination, whereas water washings of the larval surface do not (Figure 15). Apparently an inhibitory compound (or compounds) is present on the larval surface that is readily extracted into solvents having a lower polarity than water.

The minimal concentration of commercial fatty acids, such as caprylic acid, needed for inhibition of the germination event is 0.02%. At lower concentrations, there appears to be little or no effect on either germination or growth in NAG-salts medium. Also, both commercial fatty acids and larval surface extracts are fungistatic. Additional testing revealed that these compounds (at levels up to 0.1%) have no effect on a growing culture of B. bassiana E_1.

TABLE VII

FATTY ACID INHIBITION OF GERMINATION OF *B. BASSIANA* E_1

Fatty Acid	Inhibitory**
Butyric acid (C:4)	Yes
Valeric acid (C:5)	Yes
Caproic acid (C:6)	Yes
Caprylic acid (C:8)	Yes
Nonanoic acid (C:9)***	Yes
Capric acid (C:10)	No
Undecanoic acid (C:11)	No
Stearic acid (C:18)	No
Oleic acid (C:18-1)	No

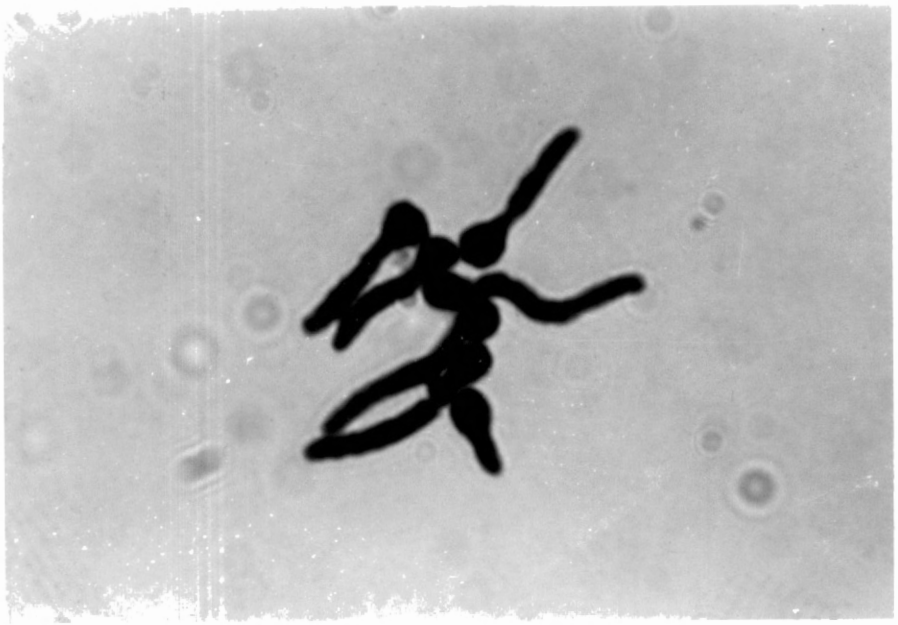
*Fatty acids were present at a final concentration of 0.02% in the NAG-salts medium (1.0%).

**Effects were ascertained at 16 hours.

***Causes extensive lysis of conidia.

Figure 14. *B. bassiana* E_1 Germinating in NAG Medium (a), and Dormant Conidia in NAG Medium After the Addition of 0.2% Caprylic Acid (C:8) (b). Observations were made at 16 hours. (2000X).

a)



b)

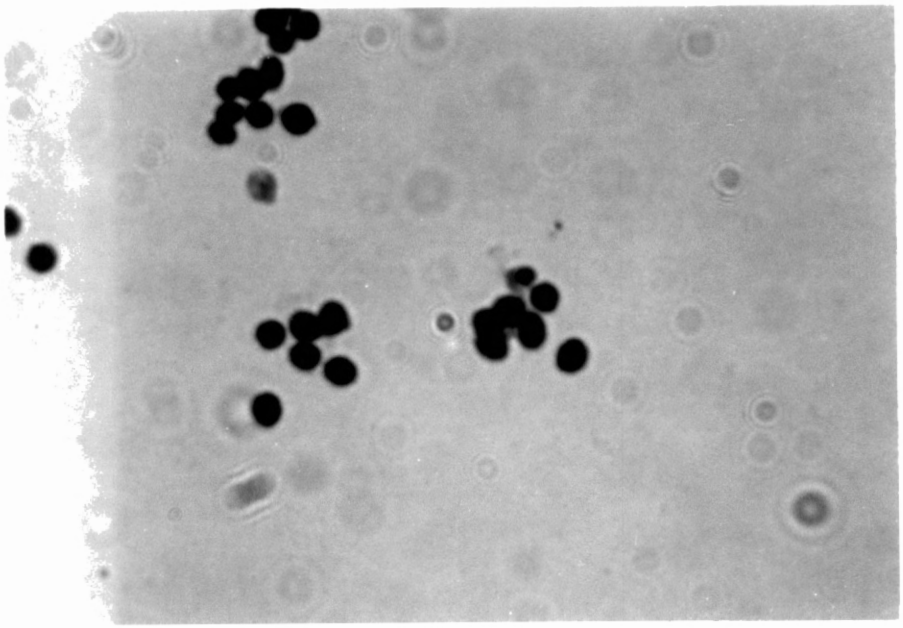
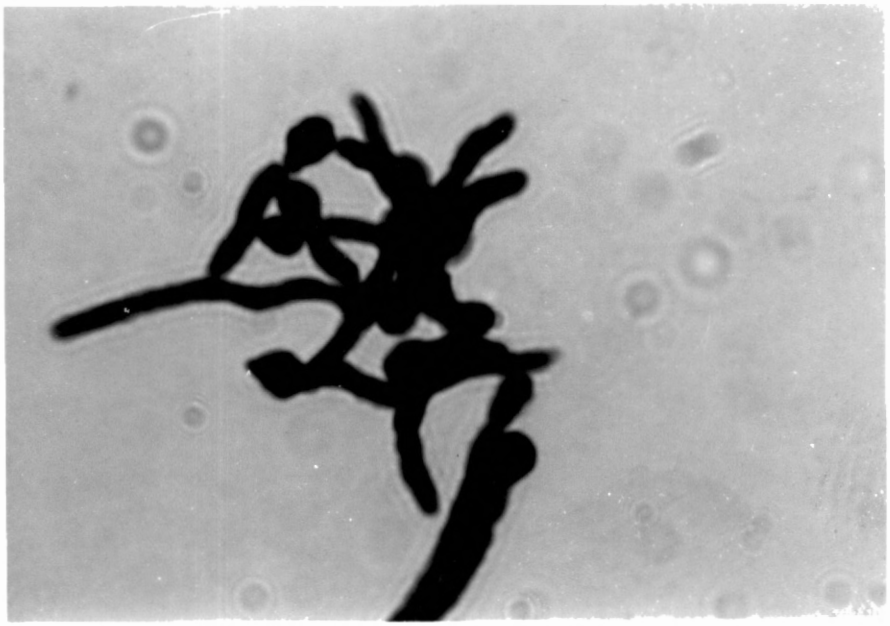
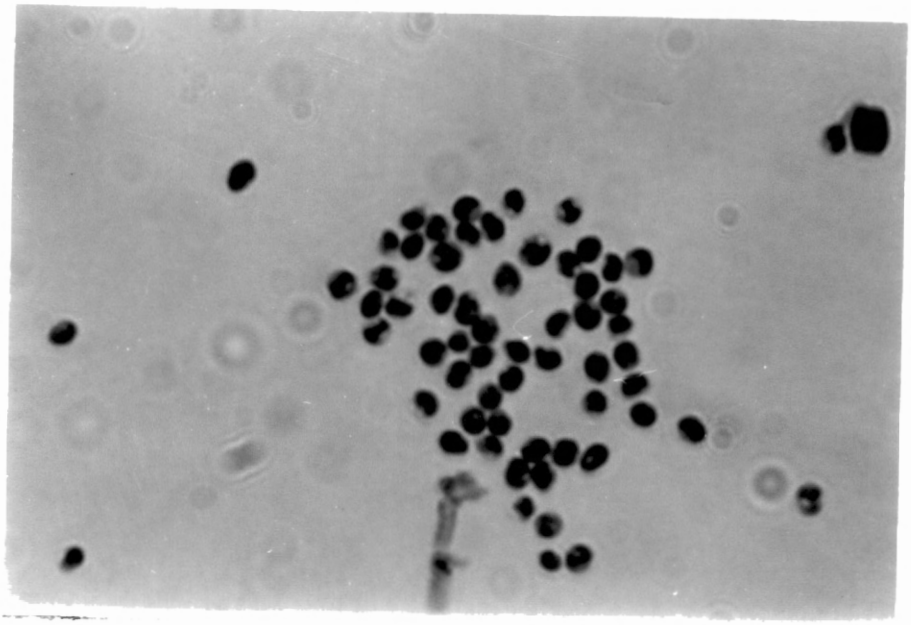


Figure 15. *B. bassiana* E_1 Germinating and Growing in NAG Medium (a) and Dormant Conidia in NAG Medium After the Addition of Larval Surface Components Extracted with Hexane (b). Observations were made at 24 hours. (2000X).

a)



b)



For identification of the compounds present on the larval surface, gas-liquid chromatography was utilized. By comparison to commercial fatty acid standards, information was obtained relating to possible identification of the inhibitory components. A representation of the peaks obtained when the larval surface extraction was subjected to gas-liquid chromatography is given in Figure 14. There are three well-defined peaks, with the middle peak having the greatest area. A comparison of the retention times of the larval extraction peaks with the fatty acid standards is show in Table VIII. Using these data, the fatty acids extracted from the surface of corn earworm larvae have tentatively been identified as valeric (C:5), caprylic (C:8), and nonanoic (C:9) acids, respectively. Caprylic acid is the major component.

Testing of caprylic acid (Sigma) at concentrations of 0.05% and 0.1% revealed that this compound can inhibit germination of conidia from all mutants of B. bassiana for up to 48 hours (Table IX). These tests were accomplished utilizing agar media.

In summation, relatively short chain fatty acids are present on the surface of corn earworm larvae and these compounds are fungistatic to B. bassiana in low concentrations. These fatty acids are not present in the diet medium. Although identifications are still tentative, testing of commercial preparations of the suspected compounds shows that they are fungistatic for germination.

Chemical Signals for Germination

Another aspect of germination that required investigation related to possible release of some chemical immediately before or during the

Figure 16. Gas-Liquid Chromatography Tracing of the Scan Obtained from a Fungistatic Hexane Larval Surface Extraction. S = solvent peak, C_5 = valeric acid peak, C_8 = caprylic acid peak, C_9 = nonanoic acid peak.

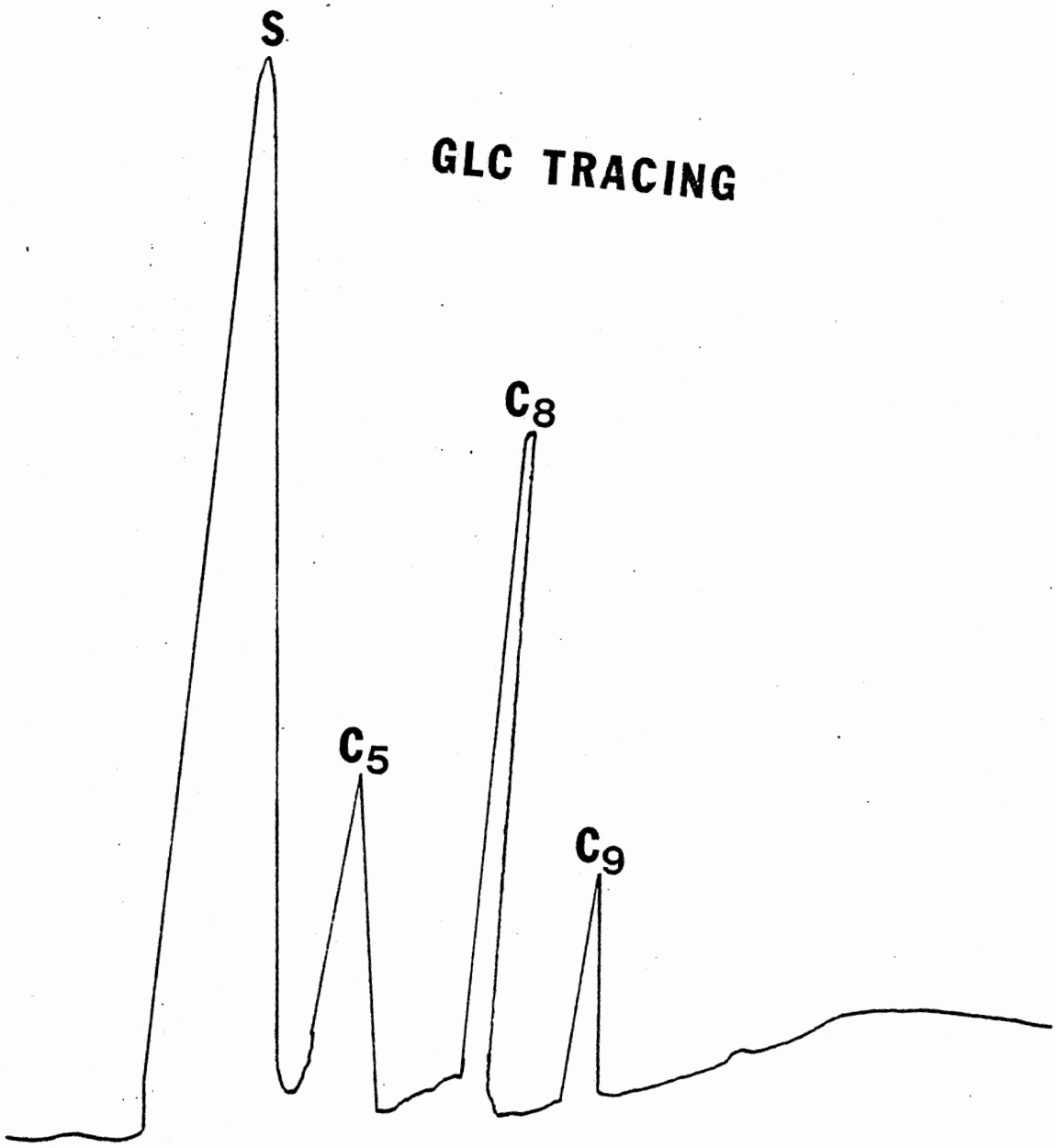

S
GLC TRACING
C_8
C_5
C_9

TABLE VIII

TENTATIVE IDENTIFICATION USING GLC OF COMPOUNDS EXTRACTED FROM THE SURFACE OF FIFTH INSTAR CORN EARWORM LARVAE*

Standards**	Retention Times (Minutes)
Valeric (C:5)	2.5 ± 0.4
Caproic (C:6)	2.9 ± 0.4
Heptanoic (C:7)	3.7 ± 0.4
Caprylic (C:8)	4.5 ± 0.4
Nonanoic (C:9)	6.1 ± 0.4
Capric (C:10)	7.2 ± 0.4
Undecanoic (C:11)	8.5 ± 0.4
Hexane Extraction	**Retention Times (Minutes**
Peak 1	2.5 ± 0.6
Peak 2	4.4 ± 0.6
Peak 3	6.2 ± 0.6

*Procedure used for preparation of methyl esters, columns employed, etc., are given in the Materials and Methods section.

**Commercial (Sigma) standard saturated fatty acids.

TABLE IX

GROWTH OF VARIOUS MUTANTS OF B. BASSIANA IN THE PRESENCE OF CAPRYLIC ACID (C:8)*

Mutant	Concentration	
	0.05%	0.1%
6	48 hr	316 hr
16	48 hr	316 hr
20	48 hr	316 hr
R_1	48 hr	316 hr
7	72 hr	-
8	72 hr	-
10	72 hr	-
13	72 hr	316 hr
E_1	72 hr	148 hr
23	72 hr	316 hr
24	72 hr	148 hr
1	96 hr	-
2	96 hr	-
3	96 hr	-
5	96 hr	-
14	96 hr	-
22	96 hr	-
9	120 hr	-
12	120 hr	-
21	120 hr	-

*The time given indicates the approximate number of hours the fatty acid exerted its fungistatic effect at each concentration. Conidia from Aspergillus niger and Penicillium chrysogenum germinated within 48 hours at both concentrations.

first stages of this event. Basically, the objective was to determine if release of a unique chemical signals initiation of germination. Bacterial spores produce dipicolinic acid when they germinate (Sussman and Halvorson, 1966). Leighton (1970) reported that amino acids and alkaline phosphatase are released by Microsporum gypseum at the onset of germination.

Several analytical procedures were employed in an attempt to detect the production of some compound by germinating spores. Paper chromatography was utilized to check for the production of amino acids, sugars, fluorescent compounds, or phosphate-containing substances. All results were negative. The Hestrin and carbonyl tests were used to check for the production of any type of keto or carboxylic acid, and again, no such compound was detected. The Bio-Rad method for protein determination also showed lack of production of any protein by germinating spores for up to 30 hours. A summation of these studies is given in Table X.

Because none of the analytical methods used gave positive information relating to the release of a unqiue chemical, more sensitive methodologies involving ^{14}C were utilized. Incorporation of ^{14}C into conidia of B. bassiana was accomplished by growing the fungus for 14 days in the presence of uniformly labeled ^{14}C D-glucosamine. The labeled conidia were allowed to germinate in NAG salts medium and aliquots were removed every two hours for counting and paper chromatography in different solvent systems. The chromatograms were analyzed in two ways: (a) they were exposed to X-ray film and examined for any radioactive spots that might indicate a labeled compound had been released, and (b) the chromatograms were cut into various sized pieces and these pieces then counted using a liquid scintillation counter. Data are shown in Tables

TABLE X

ANALYTICAL PROCEDURES EMPLOYED TO DETECT PRODUCTION AND RELEASE OF COMPOUNDS FROM GERMINATING CONIDIA

Method Used	Types of Compounds Detected	Results
Hestrin	Carboxylic acids	Negative
Carbonyl	Keto acids	Negative
Bio-Rad	Protein	Negative
Paper Chromatography	Amino acids	Negative
	Sugars	Negative
	Fluorescent compounds	Negative
	Phosphate compounds	Negative

XI and XII. In no instance could significant radioactivity be detected in the germination medium or in specific compounds released during the process.

Because no unique compound could be detected using various analytical procedures or chromatography involving ^{14}C, it was theorized that the spores might be producing CO_2 and this could be used as an indicator of germination. To study CO_2 release, conidia of B. bassiana were obtained by growth on SDA agar. These conidia were then placed in basal salts medium containing ^{14}C D-glucosamine. Hopefully, the spores would assimilate the ^{14}C-glucosamine and release some of it in the form of $^{14}CO_2$. For these experiments, a CO_2 trapping solvent from Amersham was utilized, to effectively trap any released carbon dioxide. The CO_2 trapping solvent was then counted using a scintillation counter at timed intervals, before, during, and after the germination event. Results of these experiments are given in Table XIII. It is apparent that the spores assimilated some radioactive carbon (9.5% to 13.7%), but the amount released (approximately 1.5% to 2.0% of that assimilated) is not considered great enough to be used as a signal of the germination event. Because some CO_2 is released even at three hours, it indicates that the conidia are probably metabolically active long before germination is actually seen using optical microscopy.

In summation, no chemical that would signal initiation of germination was found. All subsequent studies of germination had to utilize microscopic examination of conidia to determine whether or not germination had occurred. As a working parameter, we conclude that germination has occurred when the germ tube is visibly protruding from the spherical conidium.

TABLE XI

EXPERIMENTS INVOLVING ^{14}C LABELED CONIDIA OF B. BASSIANA

Time (Hours)	Germination Medium (cpm/ml)
0	18*
10	29
12	80
14	31
20	35
30	48
0	30**
2	141
4	96
6	161
8	72
10	119
11	87
12	113
13	235
24	239

*Aliquots of germination medium containing labeled conidia were counted (1.0 ml). One ml of the labeled spore suspension had a total count of 6,849 cpm. All counts were corrected for background.

**One ml of the labeled spore suspension had a total count of 11,550 cpm. All counts were corrected for background.

TABLE XII

ANALYSIS OF CHROMATOGRAMS FOR AREAS OF RADIOACTIVITY

	Time of Sampling			
	0 Hours	10 Hours	14 Hours	24 Hours
Origin	14*	24	26	35
1st	11	12	19	12
2nd	7	14	20	18
3rd	21	23	18	14
4th	16	15	15	18

Total spore cpm/ml = 3302.

*Denotes cpm/inch chromatogram. 70 µl of medium was chromatographed at each sampling time. Optical microscopy revealed the conidia had germinated by 14 hours.

TABLE XIII

RELEASE OF $^{14}CO_2$ FROM GERMINATING CONIDIA OF B. BASSIANA*

Time (Hours)	CO_2 Trapping Agent (Amersham) cpm/ml
0	8**
11	76
12	61
13	74
14	101
16	98
0	5***
3	44
6	53
12	142
18	161
24	493

*All counts were corrected for background.

**The germination medium had 37,400 cpm/ml at T = 0 hours; at T = 16 hours, the cpm was 32,300; therefore, the conidia took up 13.7% (5,100) of the total count available and released 2% (98) of those counts.

***The germination medium had 334,900 cpm/ml at T = 0 hours; at T = 24 hours, the cpm was 302,600; therefore, the conidia took up 9.6% (32,700) of the total count available and released 1.5% (493) of those counts.

CHAPTER IV

DISCUSSION

The data presented suggest that the biochemical basis for entomopathogenicity of Beauveria bassiana probably cannot be attributed to one factor. B. bassiana must germinate and grow before it can penetrate the integument of corn earworm (Heliothis zea) larvae, indicating the importance of all of these events for pathogenicity. Once penetration of the integument is accomplished by the growing germ tube, growth of fungal conidia continues in the hemocoel. There is no variation of infective ability of the mutants once they are in the hemocoel (Cheung, unpublished data), again suggesting that germination and penetration are key events for the infective process. Using scanning electron microscopy, Grula et al. (1978) have shown the presence of holes produced by the penetrating germ tubes, and these holes appear to be enzymatic because of the appearance of the surrounding integument.

Cuticular components of the integument include chitin, protein, and lipid; thus, the enzymes needed for penetration of this structure would be chitinases, proteases, and/or lipases. Data presented in this study show that the enzymes needed for penetration of the insect cuticle can indeed be produced by B. bassiana. A chitin salts medium allows the greatest amount of exocellular protein secretion. When mutants of B. bassiana are grown in the chitin salts medium, there are no unique protein bands or band patterns present after PAGE analysis that might

indicate a difference in exocellular enzyme production by the more effective entomopathogens. Thus, it appears that all mutants can synthesize and produce exocellular chitinases and none of the better entomopathogens produce any different proteins that might be used as a "penetrating" enzyme.

Exocellular enzyme production by the fungus is largely dependent on the nutrients present in growth media. When the fungus is grown in Sabouraud's dextrose broth, N-acetylglucosamine-salts, gelatin-salts, or powdered-integument-salts medium, band patterns vary greatly (Figure 9). These variations in protein patterns illustrate the inducibility of the proteins in response to differing nutrients. Thus, the enzymes needed to penetrate the corn earworm integument (chitinases, proteases, and lipases) can be induced and synthesized by the fungus soon after germination on the larval surface.

Aspergillus and Penicillium sp. (most likely niger and chrysogenum) can produce these same types of exocellular enzymes, and both fungi grow luxuriantly on isolated integuments of corn earworm larvae. However, conidia of Aspergillus and Penicillium cannot germinate on the surface of live larvae nor can growing hyphae penetrate the integument of live larvae.

The above information suggests two hypotheses: (1) there may be a compound present on the integument of live larvae that cannot be utilized by Aspergillus or Penicillium, but can be used by B. bassiana for both germination and growth; or (2) there may be some kind of inhibitory compound on the surface of a live larva that prevents the germination of Aspergillus and Penicillium. It is apparent that isolated

integuments can lead to misconceptions of what is actually occurring on a live larval surface.

Based on the data presented, it is evident that although B. bassiana produces exocellular enzymes capable of degrading and penetrating the larval integument, Aspergillus and Penicillium can also produce such enzymes. Since only B. bassiana is pathogenic to the corn earworm, it appears logical to suggest that entomopathogenicity is more complex than the mere ability to produce exocellular enzymes capable of degrading the insect integument. Unquestionably, penetrating enzymes are an important part of the invasive mechanism, but the requirements for B. bassiana to first germinate and then grow on the larval surface are parameters of critical importance and must be elucidated before a molecular basis for entomopathogenicity can be established.

Three parameters were examined relating to the germination process of B. bassiana: (1) nutritional requirements for the process, (2) inhibition of germination by certain fungistatic compounds present on the surface of corn earworm larvae, and (3) the release of a chemical that could signal initiation of germination in order to study the process more closely at the molecular level.

Previous nutritional work relating to germination and growth of B. bassiana has been minimal. Lefebvre (1934) reported that conidia could germinate and the resulting hyphae would grow in distilled water droplets. No contradiction of his early report appears in the literature. Data presented here show that washed (2X) conidia of B. bassiana cannot germinate in distilled water. Presumably, in his studies, nutrients were carried over with the conidia since he did not report any washing of them.

It has now been shown that B. bassiana will germinate and grow using a wide variety of compounds. These include: N-acetylglucosamine, D-glucosamine, combinations of amino acids, chitin, and glucose. Apparently only a utilizable carbon-energy source is needed for germination. Non-utilizable carbon-energy sources include most of the short and intermediate chain fatty acids. Thus, further addition of nitrogen (ammonium salt) to glucose must occur before hyphal growth can continue, otherwise the germ tubes and nascent hyphae lyse. It is obvious that hyphal extension requires synthesis of protein. No requirements for B vitamins, nucleic acid bases, or unusual compounds exist. The ability to use a relatively wide variety of compounds for germination and growth enhances survival and infectivity of B. bassiana in nature.

There appears to be a fungistatic component(s) present on the surface of corn earworm larvae capable of inhibiting conidial germination for up to 36 hours. Gas chromatography revealed that compounds having retention times similar to valeric (C:5), caprylic (C:8), and nonanoic (C:9) acids are present in larval surface lipid extractions (Figure 16). The caprylic acid-like compound is the most abundant component of these extractions. Testing of commercial caprylic acid (Sigma) has shown that it is toxic to conidial germination of B. bassiana at a minimum inhibitory concentration of 0.02% in the NAG medium, and inhibitory to growth at a concentration of 0.01% in NAG-salts agar. Since these larval surface extractions contain a compound(s) that is toxic and a major component appears to be caprylic acid, it is postulated that the surface components can decrease the invasive ability of B. bassiana by inhibiting its germination and subsequent growth and penetration.

These studies indicate that any invading fungus must encounter and overcome these toxic components before conidia can germinate and pentrate the integument.

Conidial germination was monitored and analyzed in an attempt to identify some kind of chemical signal produced by the germinating spores. Chromatographic analyses of defined media, as well as chemical detection tests, were done both before and soon after the onset of germination, and no type of compound could be detected. In addition, no significant amount of carbon dioxide was found to be released by germinating conidia. Only 1.5% to 2.0% of assimilated ^{14}C was released as carbon dioxide. This amount was not adequate for use as a chemical detection signal.

Although several parameters relating to germination, growth and invasiveness of fungal hyphae have been defined, more extensive investigation of larval surface components are needed to establish a solid molecular basis for the entomopathogenicity of B. bassiana. The larval surface must now be closely examined to determine what soluble compounds are present that can be used for germination and growth of B. bassiana. The inhibitory components on the larval surface must also be completely identified and their effects on germination and growth should be determined in the presence of those nutrients present on the larval surface. Additionally, identification of those enzymes secreted immediately (or soon) after germination must be accomplished.

Obviously, the molecular events involved in entomopathogenicity of B. bassiana are more complex than previously imagined. The need for biological (as opposed to chemical) control of insects is all too obvious, however, and progress must continue.

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