GROWTH AND SPORULATION OF *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE* ON CHEMICALLY DEFINED MEDIA

By

RUSSELL KENNETH CAMPBELL

Bachelor of Science in Agriculture

Oklahoma State University

Stilllwater, Oklahoma

1977

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 1987

GROWTH AND SPORULATION OF BEAUVERIA BASSIANA AND METARHIZIUM ANISOPLIAE ON CHEMICALLY DEFINED MEDIA

Thesis Approved:

F. L. Eckenbary
Thesis Adviser

George L. Barnes

John R Sauer

R. D. Morrison

Norman N. Durham
Dean of the Graduate College

PREFACE

This study was conducted in the hope that its findings would help further the development of pest control methodologies that are effective yet environmentally harmonious. Chapters II and III have already been published in the scientific literature as the following two citations, respectively:

Campbell, R.K., T.M. Perring, G.L. Barnes, R.D. Eikenbary, and C.R. Gentry. 1978. Growth and sporulation of Beauveria bassiana and Metarhizium anisopliae on media containing various amino acids. J. Invertebr. Pathol. 31: 289-295.

Campbell, R.K., G.L. Barnes, B.O. Cartwright, and R.D. Eikenbary. 1983. Growth and sporulation of Beauveria bassiana and Metarhizium anisopliae on media containing various carbohydrate sources. J. Invertebr. Pathol. 41: 117-121.

The author wishes to express his gratitude to his major adviser, Dr. Raymond D. Eikenbary, for his guidance, assistance, and encouragement over the course of many years. Appreciation is also expressed to my other committee members, Dr. George L. Barnes, Dr. Robert D. Morrison, and Dr. John R. Sauer for their assistance and continued advice.

A note of thanks is also given to the following for their help in data acquisition and analysis, or manuscript preparation: Dr. Tom Perring, Dr. Bob Cartwright, Dr. R.W. McNew, Dr. Jaime Yanes, Dr. C.R. Gentry, Kila Roggow, Becky Penrose, Mary Morris and Donnell Allen.

I also wish to thank the Oklahoma Agricultural Experiment Station for providing facilities and support during the course of this study.

Finally, special gratitude is expressed to my wife, Dr. Joyce Campbell, and my mother, Lucy T. Campbell, for their understanding, enthusiasm, and unflagging support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW AND CONCEPTS

Although it may at first appear that the area of insect pathogenic fungi is a very restricted topic, the literature in the area is vast. Muller-Kogler, (1965) in producing the most thorough review in the field cites approximately 1200 publications. In doing so he does not include non-pathogenic fungus-insect relationships or studies reported before 1937 or after 1963. The more recent literature is reviewed by Ferron (1978). In his review he indicates that the literature since 1963 comprises approximately 1000 citations. It is apparent that interest in this field is increasing.

B. bassiana holds a cardinal position of importance in insect pathology and indeed, in the science of pathology in general. Around 1800 a disease known as "mal del segno" or "calcino" or in France "muscardine" began to cause considerable difficulty in the silk industry by causing high mortalities among silkworm. Although the cause of this malady was unknown it was thought by various people to arise de novo, because of bad mulberry leaves used as food, or as a result of improper environmental conditions of temperature or humidity.

One of the people in Italy who became interested in this malady was Agostino Maria Bassi (1773-1856). Although much of the importance of his work has been overshadowed by the work of Pasteur a few years later, it was actually Bassi who was initially responsible for : (1.) helping to overthrow the theory of spontaneous generation, (2.) establishment of the germ theory of disease, and (3.) assisting in the foundation of the modern principles of disinfection.

These remarkable feats were derived from Bassi's experimental investigations of muscardine, which he showed to be caused by the fungus that we know today as Beauveria bassiana.

The findings of Bassi may be summarized as follows:

(1.) muscardine is a disease caused by B. bassiana.

(2.) B. bassiana grows and develops in living silkworms.

(3.) the diseased insect is rendered infectious to other individual insects and the infection may be transmitted.

(4.) growth and development of the fungus are facilitated by warm, humid conditions.

(5.) the pathogen can be destroyed by certain chemical and physical treatments such as lye, wine and brandy, boiling water, burning, and exposure to sunlight.

Thus, it was Bassi who first showed experimentally that a

microorganism can cause an infectious disease in an animal (Bassi, 1835). A few papers review the importance of Bassi's work (Major, 1944; Ainsworth, 1956; Steinhaus, 1956, 1975).

Bassi himself did not name the organism with which he worked. For this purpose he enlisted the assistance of Giuseppe Balsamo Crivelli. Balsamo (1835) placed the organism in the genus Botrytis and named it Botrytis paradoxa. He later changed the name to Botrytis bassiana in honor of Bassi. Later, Vuillemin (1912), as described in Steinhaus (1975), revised the taxonomy and made the fungus the type species of the genus Beauveria. This genus was recently reviewed and described as containing two species, B. bassiana and B. brongniartii (= B. tenella) (DeHoog, 1972). Even more recently, two more species (B. velata and B. amorpha) have been described (Samson and Evans, 1982).

As soon as the concept of infectious disease was developed it was not too great a leap to the idea of manipulating microorganisms to effect the control of pest insects. Indeed, Bassi (1835), during his experimental efforts to prove the infectious nature of the disease, was able to transmit the disease to insects other than the silkworm. He also was apparently the first to enunciate the idea of using the activities of microorganisms to control harmful insects (Bassi, 1836; as described in Steinhaus, 1975). This idea of "microbial control" was not followed up on immediately but similar suggestions were made later by

Leconte (1874), Pasteur (1874), and Hagen (1879).

About the same time that these suggestions were being made, the great scientist Elie Metschnikoff, who is most well known for his pioneering studies on phagocytosis and inflammation, was concerned with the destruction of cereal crops in Russia by the wheat cockchafer, Anisoplia austriaca. One of the naturally occurring population suppressants of A. austriaca was a fungus which Metschnikoff called the green muscardine. He named the fungus Entomophthora anisopliae and later changed the name to Isaria destructor. This is now known as Metarhizium anisopliae (reviewed by Tulloch, 1976). Metschnikoff suggested the use of M. anisopliae as a microbial control agent, but perhaps more importantly, he discovered that spores of the fungus could be produced on sterilized beer mash. Thus, he appears to have been the first to realize the importance of mass production on artificial media as an important characteristic of a microbial control agent (Metschnikoff, 1880).

Metschnikoff's observation the M. anisopliae is capable of growth on a medium such as beer mash points to the important fact that this fungus, as well as B. bassiana, are facultative pathogens. That is, they are capable of saprophytic growth on a wide variety of media as opposed to obligate pathogens, which require living hosts in order to grow. There is, of course, a complete spectrum of nutritional requirements among the fungi. Although there

are exceptions, a relationship seems to exist between a pathogen's nutritional requirements and the extent of this host range (MacLeod, 1954). There are two extremes: (1.) species with wide host ranges are usually capable of growing on a wide variety of simple media without absolute requirements for specific nutrients; they are thus metabolically diverse; and (2.) species with very narrow host ranges usually have nutritional requirements so complex that they have never been grown in culture.

Both B. bassiana and M. anisopliae fit into category number (1.) above. The host range for B. bassiana is in excess of 500 insect species and that of M. anisopliae is more than 200 species (Fargues and Remaudiere, 1977). Furthermore, both species are capable of growth and sporulation on a wide variety of natural and chemically defined media (Dresner, 1949; Samsinakova, 1966; Barnes et al., 1975; Campbell et al., 1978, 1983).

Although both B. bassiana and M. anisopliae have been known to cause disease in a wide variety of hosts, there exist numerous subspecific pathotypes within each species that are more closely linked to their hosts and exhibit a limited host range (Ferron et al., 1972; Fargues, 1976). It has been suggested that differences in the ability of different pathotypes to utilize various nutrients might be related to their differences in host specificity (Pelletier and Keitt, 1954; MacLeod, 1959).

In order to understand how the pattern of nutrient utilization by the fungal pathogen might be related to host specificity and adaptation within the host-pathogen relationship, it will be instructive to review the disease cycle. There are ten steps in this cycle (after Roberts and Humber, 1981) as follows:

(1.) Attachment of the conidium (spore) to the insect cuticle. Since the major route of invasion in both fungi is through the cuticle this is the obvious first step. Conidia are likely adapted for attachment but the chemical and physical parameters involved are unknown.

(2.) Germination of the conidium on the insect cuticle. The cuticle normally provides a very effective barrier to microorganisms. There is now evidence that, in addition to serving as a physical barrier, the cuticle may contain substances which inhibit conidial germination. This interaction could therefore be the first having an influence upon host specificity. Koidsumi (1957) found medium chain saturated fatty acids, especially caprylic acid, to be present. When extracted these could inhibit the germination of Aspergillus flavus and B. bassiana conidia. Caprylic and other fatty acids were extracted from Heliothis zea and Spodoptera frugiperda larvae and found to inhibit germination of B. bassiana conidia (Smith

and Grula, 1982). Interestingly, these same workers were unable to extract such fatty acids from pecan weevil larval epicuticle and they found that the type of carbon and nitrogen nutrients present in media amended with caprylic acid influenced the amount of germination inhibition observed with B. bassiana. It is not known whether these antifungal materials exist in sufficient amounts at the intact cuticular surface to inhibit germination. Robinson (1966) used normal and dewaxed excised sclerites of Tenebrio molitor and found no difference in germination when spores of several entomopathogenic fungi were applied to the epicuticle. Furthermore, Smith and Grula (1981) could find no correlation between the abilities of B. bassiana mutants to germinate and grow on media containing caprylic acid and the pathogenicity of the mutants. It may, therefore, be that antifungal compounds in the epicuticle are not of great importance in some host-pathogen relationships.

(3.) Penetration of the cuticle. The germ tube may penetrate directly or an appressorium may be formed which attaches firmly to the cuticle and a narrow infection peg then sent into the cuticle (Zacharuk, 1973). This process involves both enzymatic and physical activities.

The enzymes which are produced by germinating conidia have not been identified but it is known that colonies may produce proteases, lipases, and chitinases when grown on various media. In B. bassiana these enzyme activities did not dissolve excised insect cuticle when applied alone but the cuticle was dissolved by a mixture (Samsinakova et al., 1971). It was also found that the amount of lipase activity was correlated with virulence (Samsinakova and Misikova, 1973).

As further penetration occurs, various defense reactions of the host may be brought into play. The most common of these reactions is melanization. This usually occurs upon contact with the hemolymph and disruption of basement membrane cells or hemocytes. Because free tyrosine and various prophenyl-oxidases are abundant in hemolymph, material released from cellular disruption may activate one or more of the enzymes' precursors thus resulting in melanin formation (Hackman, 1971; Jeuniaux, 1971). Once the hemolymph is reached cellular immune responses may also occur (Harshbarger and Faust, 1973; Salt, 1970). These may result in the formation of a cyst or granuloma. Both melanization reactions and granulomatous reactions may be overcome by successful pathogens. The ability to overcome

them is undoubtedly of great importance in determining host specificity.

(4.) Growth of the fungus in the hemocoel. Multiplication in the hemocoel usually occurs as yeast-like hyphal bodies which reproduce by budding.

In almost all insects studied to date a very high concentration of free amino acids (aminoacidemia) is characteristically found in the hemolymph (Florkin and Jeuniaux, 1974). However, variations in the pattern of amino acids present in different species and at different life stages are great. Generally, the peptide content is low although the protein concentration may be high. Hence, there is a readily available quantity of nitrogen present in the form of amino acids. An unusual aspect of insect hemolymph is the usual presence of large amounts of trehalose. This serves as the major form of carbohydrate transport.

Aoki and Chigusa (1968) studied the mycelial growth by B. bassiana on various amino acids. Furthermore, Aoki and Yanase (1970) found that those amino acids producing the best mycelial growth were also the best for the formation and multiplication of hyphal bodies.

With these facts in mind it is possible to hypothesize the following sequence of events (where -> means "leads to" or "implies"):

high nutrient utilization ability
↓
rapid hyphal body multiplication
↓
effective circumvention of host defenses and greater toxin production
↓
more rapid death of host (hence greater virulence)

This hypothesis therefore implies that a high degree of virulence is related to the pathogen's ability to utilize specific nutrients or patterns of nutrients present in the host's hemolymph. Of course, many other factors are probably involved, especially in the circumvention of host defenses.

(5.) The production of toxins. Both B. bassiana and M. anisopliae cause death in their hosts before extensive invasion of organs takes place. Toxins are thought to be responsible. To date, all known toxins were isolated and identified from mycelia or culture filtrates. Most of them are small molecules, although enzymes are also found in culture filtrates. It is usually assumed that toxins are produced by the fungus alone but it is also possible that toxic compounds are produced by the diseased host (Kucera and Samsinakova, 1968; Roberts, 1966, 1969).

(6.) Death of the host. This may be preceded by various abnormal behaviors such as sluggishness, tremors, or loss of coordination. Often small melanotic spots are observable on the cuticle which are sites of germ tube invasion. Also, in the case of B. bassiana infections, generalized color changes in the host may occur due to the production of bactericidal pigments (Basyouni et al., 1968).

(7.) Growth in the mycelial phase with invasion of virtually all organs of the host. Since both of these fungi may produce antibacterial compounds and since all of the host organs are replaced with mycelia the cadaver can sometimes appear quite normal.

(8.) Penetration of hyphae from the interior through the cuticle to the exterior of the insect. If the cadaver is held under conditions of low humidity the fungus will remain within the host but the fungus will grow through the cuticle if the humidity of the environment is high (Roberts and Yendol, 1971).

(9.) Sporulation of the mycelium on the exterior of the cadaver.

(10.) Dispersal of the infective conidia to other locations where they may encounter other hosts and so renew the disease cycle. This is accomplished

passively by the effects of wind, water, or other insects and animals.

As pointed out above, information concerning the utilization of nutrients by B. bassiana and M. anisopliae may be important in providing insight into the basic nature of the host-pathogen relationship. This would be especially true if there is a correlation between the ability to use nutrients and the presence and amounts of the same nutrients in the host's (pecan weevil) hemolymph.

Of course, nutritional information would also be of value for the development of more efficient and effective mass production media. The ability to mass produce conidia is an important component of any future microbial control technology using these fungi. The ability of these fungi to utilize unusual nutrients would also be of value in the development of selective media for their isolation from soil or plant surfaces. Such media would be of great assistance in the kinds of field studies that will be required for the further development of these fungi as control agents. Although media have been used for this in the past (Martin, 1950) and new media have been recently developed (Doberski and Tribe, 1980; Lingg and Donaldson, 1981) none of these are based on the kinds of nutritional data required to obtain the true selectivity and efficiency that are important in soil ecological studies.

LITERATURE CITED

Ainsworth, G.C. 1956. Agostino Bassi, 1773-1856. Nature (London) 177: 255-257.

Aoki, J. and K. Chigusa. 1968. Studies on nutrition and metabolism of pathogenic fungi of muscardine. I. Nitrogen utilization in synthetic media by Beauveria bassiana, Isaria farinosa, and I. fumoso-rosea. J. Sericult. Sci. (Japan). 37: 288-294.

Aoki, J. and K. Yanase. 1970. Studies on the nutrition and metabolism of pathogenic fungi of muscardine. II. The influence of amino acid for the formation and multiplication of hyphal bodies of Spicari fumosa-rosea and Beauveria bassiana. J. Sericult. Sci. (Japan). 39: 285-292.

Balsamo, C.G. 1835. Osservazione sopra una nuova specie di mucedinae del genere Botrytis, et cetera. Bibl. Ital. 79: 79-125. (In Italian).

Barnes, G.L., D.J. Boethel, R.D. Eikenbary, J.T. Criswell, and C.R. Gentry. 1975. Growth and sporulation of Metarrhizium anisopliae and Beauveria bassiana on media containing various peptone sources. J. Invertebr. Pathol. 25: 301-305.

Bassi, A. 1835. Del mal del segno calcinaccio o moscardino. Parte prima. Lodi. (In Italian). Translated in: G.C. Ainsworth and P.J. Yarrow Phytopathological Classics no. 10 Monumental Printing Co. Baltimore. 49 pp.

Bassi, A. 1836. Maladies des vers a soi: Recherches sur la muscardine. C. R. Seances Hebd. Acad. Sci. 2: 434-436. (In French).

Basyouni, S.H. El, D. Brewer, and L.C. Vining. 1968. Pigments of the genus Beauveria. Can. J. Bot. 46: 441-448.

Campbell, R.K., G.L. Barnes, B.O. Cartwright, and R.D. Eikenbary. 1983. Growth and sporulation of Beauveria bassiana and Metarhizium anisopliae in a basal medium containing various carbohydrate sources. J. Invertebr. Pathol. 41: 117-121.

Campbell, R.K., T.M. Perring, G.L. Barnes, R.D. Eikenbary, and C.R. Gentry. 1978. Growth and sporulation of Beauveria bassiana and Metarrhizium anisopliae on media containing various amino acids. J. Invertebr. Pathol. 31: 289-295.

DeHoog, G.S. 1972. The genera Beauveria, Isaria, Tritirachium, and Acrodontium. Stud. Mycol. 1: 1-41.

Doberski, J.W. and H.T. Tribe. 1980. Isolation of entomogenous fungi from elm bark and soil with reference to ecology of Beauveria bassiana and Metarhizium anisopliae. Trans. Brit. Mycol. Soc. 74(1): 95-100.

Dresner, E. 1949. Culture and use of entomogenous fungi for the control of insect pests. Contrib. Boyce Thompson Inst. 15: 319-335.

Fargues, J. 1976. Specificite des champignons pathogenes imparfaits (hyphomycetes) puor les larves de Coleopteres (Scarabaeidae et Chrysomelidae). Entomophaga. 21: 313-323. (In French with English summary).

Fargues, J. and G. Remaudiere. 1977. Considerations on the specificity of entomopathogenic fungi. Mycopathologia. 62(1): 31-37.

Ferron, P. 1978. Biological control of insect pests by entomogenous fungi. Ann. Rev. Entomol. 23: 409-442.

Ferron, P., B. Hurpin, and P.H. Robert. 1972. Sur la specificite de Metarhizium anisopliae (Metsch.) Sor. Entomophaga. 17: 165-178. (In French with English summary).

Florkin, M. and C. Jeuniaux. 1974. Hemolymph: composition. In: The Physiology of Insecta Vol. V. 2nd Ed. M. Rockstein, ed. Academic Press, New York.

Hackman, R.H. 1971. The integument of Arthropoda. In: Chemical Zoology Vol. 6 (Arthropoda) Part B. p. 1-62. M. Florkin and B. T. Scheer, eds. Academic Press, New York.

Hagen, H.A. 1879. Obnoxious pests-suggestions relative to their destruction. Can. Ent. 11: 110-114.

Harshbarger, J.C. and R.M. Faust. 1973. Environmental factors internal to the host that affect the success of microbial insecticides. Ann. N.Y. Acad. Sci. 217: 131-140.

Jeuniaux, C. 1971. Hemolymph-Arthropoda. In: Chemical Zoology. Vol. 6. (Arthropoda) Part B. p. 63-118. M. Florkin and B. T. Scheer, eds. Academic Press, New York.

Koidsumi, K. 1957. Antifungal action of cuticular lipids in insects. J. Insect Physiol. 1: 40-51.

Kucera, M. and A. Samsinakova. 1968. Toxins of the entomophagous fungus Beauveria bassiana. J. Invertebr. Pathol. 12: 316-320.

LeConte, J.L. 1874. Hints for the promotion of economic entomology. Proc. Am. Assoc. Adv. Sci. 22: 10-22.

Lingg, A.J. and M.D. Donaldson. 1981. Biotic and abiotic factors affecting stability of Beauveria bassiana conidia in soil. J. Invertebr. Pathol. 38: 191-200.

MacLeod, D.M. 1954. Natural and cultural variation in entomogenous Fungi Imperfecti. Ann. N.Y. Acad. Sci. 60: 58-70.

MacLeod, D.M. 1959. Nutritional studies on the genus Hirsutella. (I.) Growth response in an enriched liquid medium. Can. J. Bot. 37: 695-714.

Major, R.H. 1944. Agostino Bassi and the parasitic theory of disease. Bull. Hist. Med. 16: 97-107.

Martin, J.P. 1950. Use of acid, rose bengal, and streptomycin in the plate method for estimating soil fungi. Soil Sci. 69: 215-232.

Metschnikoff, E. 1880. Zur Lehre uber Insecktenkrankheiten. Zool Anz. 3: 44-47. (In German).

Muller-Kogler, E. 1965. Pilzekrankheiten bei Insekten. P. Parey, Berlin. 444 pp. (In German).

Neel, W.W. and P.P. Sikorowski. 1971. Pecan weevil susceptibility to fungus. The Pecan Quart. 6: 14-15.

Osburn, M.R., W.C. Pierce, A.M. Phillips, J.R. Cole, and G.L. Barnes. 1963. Controlling insects and diseases of the pecan. USDA Handbook 240. 52 pp.

Pasteur, L. 1874. (On the use of fungi against Phylloxera). C.R. Seances Hebd. Acad. Sci. 79: 1233-1234. (In French).

Pelletier, R.L. and G.W. Keitt. 1954. Venturia inaequalis (Cke.) Wint. (VI): Amino acids as sources of nitrogen. Am. J. Bot. 41: 362-371.

Roberts, D.W. 1966. Toxins from the entomogenous fungus Metarrhizium anisopliae II. Symptoms and detection in moribund hosts. J. Invertebr. Pathol. 8: 222-227.

Roberts, D.W. 1969. Toxins from the entomogenous fungus Metarrhizium anisopliae: Isolation of destruxins from submerged cultures. J. Invertebr. Pathol. 14: 82-88.

Roberts, D.W. and W.G. Yendol. 1971. Use of fungi for microbial control of insects. In: Microbial Control of Insects and Mites. H.D. Burges and N.W. Hussey, eds. Academic Press, New York.

Roberts, D.W. and R.A. Humber. 1981. Entomogenous fungi. In: Biology of Conidial Fungi. G.T. Cole and B. Kendrick, eds. 2 vols. Academic Press, New York.

Robinson, R.K. 1966. Studies on penetration of insect integument by fungi. Pest Articles News Sum. Sect. B. 12: 131-142.

Salt, G. 1970. The Cellular Defense Reaction of Insects. (Cambridge Monographs of Experimental Biology). Vol. 16. Cambridge Univ. Press. Cambridge, England.

Samsinakova, A. 1966. Growth and sporulation of submersed cultures of the fungus Beauveria bassiana in various media. J. Invertebr. Pathol. 8: 395-400.

Samsinakova, A., S. Misikova, and J. Leopold. 1971. Action of enzymatic systems of Beauveria bassiana on cuticle of the greater wax moth larvae (Galleria melonella). J. Invertebr. Pathol. 18: 322-330.

Samsinakova, A. and S. Misikova. 1973. Enzyme activities in certain entomophagous representatives of Deuteromycetes in relationship to their virulence. Ceska Mykol. 27: 55-60.

Samson, R.A. and H.C. Evans. 1982. Two new Beauveria spp. from South America. J. Invertebr. Pathol. 39: 93-97.

Smith, R.J. and E.A. Grula. 1981. Nutritional requirements for conidial germination and hyphal growth of Beauveria bassiana. J. Invertebr. Pathol. 37: 222-230.

Smith, R.J. and E.A. Grula. 1982. Toxic components on the larval surface of the corn earworm (Heliothis zea) and their effects on germination and growth of Beauveria bassiana. J. Invertebr. Pathol. 39: 15-22.

Sri-Arunotai, S., P.P. Sikorowski, and W.W. Neel. 1975. Study of the pathogens of the pecan weevil larvae. Environ. Entomol. 4(5): 790-792.

Steinhaus, E.A. 1956. Microbial control - The emergence of an idea: A brief history of insect pathology through the nineteenth century. Hilgardia. 26: 107-157.

Steinhaus, E.A. 1975. Disease in a Minor Chord. Ohio State Univ. Press, Columbus. 488 pp.

Tedders, W.L., D.J. Weaver, and E.J. Wehunt. 1973. Pecan weevil: Suppression of larvae with the fungi Metarrhizium anisopliae and Beauveria bassiana and the nematode Neoaplectana dutkyi. J. Econ. Entomol. 66(3): 723-725.

Tulloch, M. 1976. The genus Metarhizium. Trans. Brit. Mycol. Soc. 66: 407-411.

Vuillemin, P. 1912. Beauveria, nouveau genre de Verticilliacees. Soc. Bot. de France (Paris) Bull. 59; 34-40. (In French).

Zacharuk, R.Y. 1973. Electron microscope studies of the histopathology of fungal infections by Metarrhizium anisopliae. Misc. Pub. Entomol. Soc. Am. 9: 112-119.

CHAPTER II

GROWTH AND SPORULATION OF *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE* ON MEDIA CONTAINING VARIOUS AMINO ACIDS

Introduction

Initial observations of the entomopathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* were made well over a century ago. However, since that time control efforts utilizing these fungi have met with slow development due to two major factors: (1.) the necessity of selecting and maintaining highly virulent isolates for a particular insect pest, and (2.) the overriding importance of the microenvironment on the development of epizootics. Although field trials with these fungi to control foliar pests have met with only sporadic success, their use in the naturally more favorable soil environment appears highly promising (Hurpin and Robert, 1972). The authors have been interested in the control of the pecan weevil, *Curculio caryae*, a major pest of pecans which diapauses for 1-2 years in the soil and for which a high degree of control in laboratory tests with these fungi has been observed (Neel and Sikorowski, 1972; Swingle and Seal, 1931; Tedders et al., 1973). Knowledge of nutrient utilization for growth and sporulation by these

fungi has two possible applications to the development of control methodology. The first of these is the development of efficient mass production media. Roberts (1966) investigated the effect of various inorganic and organic (peptone) nitrogen sources on the growth and toxin production of M. anisopliae and found that Neopeptone (Difco) produced greater mycelial yields than sodium nitrate. Barnes et al. (1975) studied the growth and sporulation of B. bassiana and M. anisopliae on a number of peptone sources and found several that induced both good mycelial growth and sporulation. The preservation of, and selection for, highly virulent isolates is a second possible application of nutritional data. Pelletier and Keitt (1954) made an extensive study of the amino acid nutrition of Venturia inaequalis. They pointed out numerous variables that may influence the growth of fungi and suggested that interisolate variability of amino acid utilization may be of potential importance in relation to pathogenicity and host specificity of the fungus. The differential effects of amino acid nitrogen sources on the formation of hyphal bodies of B. bassiana and Spicaria fumoso-rosea were studied by Aoki and Yanase (1970), and Aoki (1968) found that in general there is a correlation between the amino acid content in silkworm larval body fluid and the suitability of amino acids for the growth of a B. bassiana isolate. Such a correlation could possibly be the basis of an isolate selection technique that would be simpler and more cost-

effective than the currently used bioassay technique. The current inability to mass-rear the pecan weevil presents problems in procuring uniform larvae for bioassay and an alternative technique is highly desirable. Also desirable is the development of specialized mass-production media. Growth curve determinations for B. bassiana and M. anisopliae on an asparagine medium and their mycelial growth and sporulation responses on different amino acids and KNO_3 are reported in this chapter.

Materials and Methods

Mycelial Growth and pH Curves

Preparation of media. Because a literature search revealed no quantitative comparison of mycelial growth and concomitant medium pH changes between B. bassiana and M. anisopliae isolates, we elected to obtain such data. For this purpose L-asparagine was provided as the sole nitrogen source in a basal synthetic liquid medium containing the following per liter: 2.000 g of asparagine, 10.000 g of D-glucose, 1.000 g of KH_2PO_4, 0.500 g of $MgSO_4 \cdot 7H_2O$, 1.447 mg of $Fe(NO_3)_3 \cdot 9H_2O$, 0.880 mg of $ZnSO_4 \cdot 7H_2O$, 0.406 mg of $MnSO_4 \cdot 4H_2O$, 0.005 µg of biotin, 0.100 µg of thiamine, and distilled water to 1 liter. This amount of asparagine is equivalent to 0.425 g of elemental nitrogen/liter. After thorough mixing, the pH was standardized at 7.0 using 1 N NaOH. Fifty-milliliter aliquots were dispensed into 250-ml Erlenmeyer flasks that had been precleaned using 1 N HCl and

then plugged with cotton stoppers. The flasks of media were sterilized for 20 min at 121°C, resulting in a slight lowering of the medium pH to an average of 6.8.

Inoculum and inoculation of media. The isolates of B. bassiana (Bb Re No. 4SS) and M. anisopliae (Ma Re No. 4SS) used in this investigation were obtained from infected pecan weevil larvae removed from pecan orchard soil near Stillwater, Oklahoma and stored under mineral oil on slants of Proteose Peptone No. 2 (Difco)-dextrose agar (PP2DA). Both isolates were single-spore-derived, and conidiospores for inoculum preparation were obtained from 2-week-old PP2DA cultures grown in Petri dishes. Each Erlenmeyer growth flask received 1 ml of inoculum containing 70,000 spores/ml as standardized with a hemacytometer. The inoculum-suspending fluid was modified from that recommended by Burges and Thomson (1971) and prepared as follows: 0.0425 g of KH_2PO_4, pH adjusted to 7.2 with 1 N NaOH, 0.2 ml of Triton X-100 , 0.05 ml of Dow Anti-Foam A , and distilled water to 1 liter. In the case of M. anisopliae, the spore suspension required a subsequent blending for 1 min to break apart clumps of spores.

Culture and harvesting techniques. The construction of growth curves for each fungus was divided into two phases: Days 0-20 and Days 21-40. For each phase a sufficient number of flasks was inoculated to allow the harvest of five flasks per day plus an additional number for "check"

harvests on a few days not included in that phase. Harvests in the first phase did not begin until Day 3. In nearly all cases the check harvests correlated quite closely with the harvests for those days, and hence only one mean was calculated for ten replicates on those days. Most of the values graphed are the means of five replicates. In all cases the five flasks harvested each day were randomly selected from the remainder.

The fungi were still cultured at 24°C. Mycelial yields were separated from the media with predried and weighed filter papers in polyethylene funnels, and filtrates were saved for pH determinations. All yields were washed with 250 ml of distilled water after which the papers and yields were dried for 24 hr at 100°C and weighed.

Growth and Sporulation on Various Amino Acids

Twenty-four amino acids and KNO_3 were utilized to determine their effects on growth and sporulation of *B. bassiana* and *M. anisopliae*. Inoculation and harvesting procedures used were the same as those outlined above except for necessary differences pointed out below.

The same basal medium was utilized in every case with only the different amino acids being changed. Each medium tested received a sufficient amount of amino acid to be equivalent to 0.425 g of elemental nitrogen/liter. All flasks were inoculated as above and still-cultured for 2 weeks at 24°C. Each amino acid test consisted of nine

flasks, five of which were harvested to determine mycelial mass and filtrate pH. To quantitate spore production, 20 ml of a 0.1% Triton X-100 solution was added to each of the four remaining flasks. Each was then agitated for 1 min on a reciprocal shaker set a 100 strokes/min. A 10-ml aliquot of fluid was removed from each of the four flasks, and these aliquots were pooled to give one spore sample per amino acid. The spore concentration of each agitated sample was determined with a hemacytometer thus providing a relative determination of sporulation. All data for mycelial growth, sporulation, and filtrate pH were subjected to analysis of variance.

Results

Mycelial growth and growth medium pH changes are illustrated in Figure 1 for both B. bassiana and M. anisopliae. Both fungi attained essentially the same growth maximum of about 0.22 g on the 20th day of growth. However, B. bassiana maintained a more rapid growth rate than M. anisopliae during about the first 12 days, after which M. anisopliae grew faster until Day 20. In both fungi this rapid growth phase was accompanied by a decline in filtrate pH which was quite precipitous in the case of B. bassiana. The minimum pH in both cases occurred prior to the time maximum growth was achieved. A decline phase was evident for both fungi concurrent with an increase in pH to a level equal to or greater than the initial pH of the medium. The

onset of this pH rise seems to be correlated with the decline in the initial rapid growth rate but nevertheless occurs before Day 20.

Yield and sporulation rates for B. bassiana are shown in Table I. Tryptophan and alanine were definitely the best overall nitrogen sources for both growth and sporulation. Other sources eliciting high sporulation rates were glutamine, KNO_3, phenylalanine, and norleucine. However, in the case of the latter two sources, a large number of the spores produced were irregularly shaped. It seems probable that for B. bassiana those amino acids well utilized for growth are not also automatically well utilized for spore production.

Yield and sporulation data for M. anisopliae are shown in Table II. Again, it may be seen that tryptophan was the best for production of mycelial mass, with glutamic acid and histidine producing slightly less. All three of these sources elicited very good sporulation rates. Nitrogen sources containing sulfur were very poorly utilized for spore production by M. anisopliae.

By comparing overall means of Table I to those of Table II, it may be seen that in general B. bassiana produces much greater numbers of spores and significantly greater mycelial mass than M. anisopliae. This general trend is accompanied by an increased amount of variability as evidenced by the greater values for least significant differences for B. bassiana.

Figure 1. Comparison of mycelial growth and medium pH changes over time for Beauveria bassiana and Metarhizium anisopliae. A liquid medium with L-asparagine as the sole nitrogen source was utilized. Vertical bars represent one standard deviation. Curves were drawn by eye.

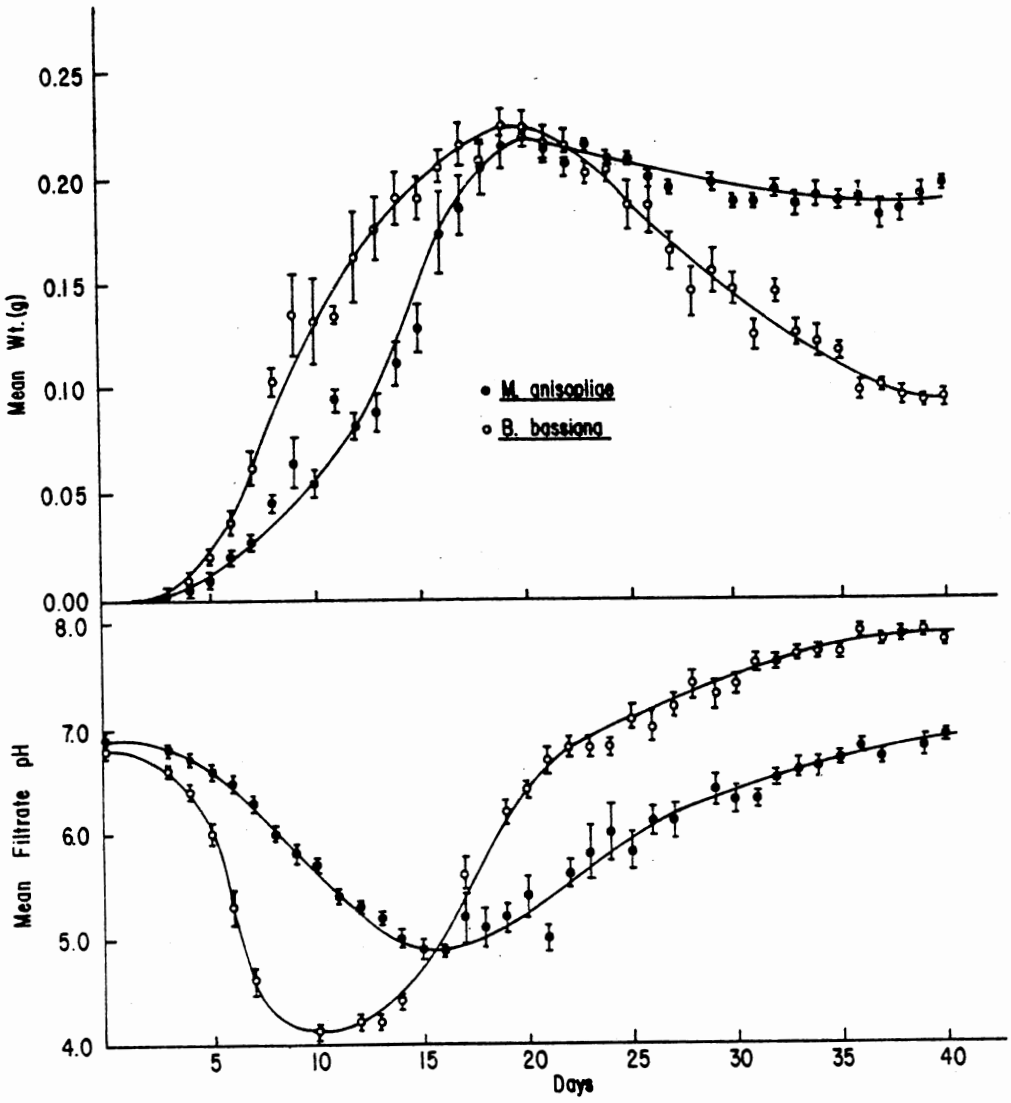
Mean Wt.(g)
0.25
0.20
0.15
0.10
0.05
0.00
M. anisopliae
B. bassiana
Mean Filtrate pH
8.0
7.0
6.0
5.0
4.0
5
10
15
20
25
30
35
40
Days

TABLE I

GROWTH AND SPORULATION OF *BEAUVERIA BASSIANA* ON MEDIA CONTAINING DIFFERENT SINGLE AMINO ACIDS, KNO_3, AND CONTROL

Amino Acid	Mean Weight[a]	Mean pH[b]	Sporulation[c]
L-tryptophan[d]	0.2222	6.29	625.2
L-alanine[d]	0.2218	4.41	483.0
L-asparagine[d]	0.1948	5.68	191.0
L-arginine[d]	0.1870	4.06	414.4
L-serine[d]	0.1854	4.30	96.8
L-glutamine[d]	0.1804	4.75	612.4
L-valine[e]	0.1726	5.14	269.1
glycine[d]	0.1686	4.29	389.0
L-cysteine[d]	0.1638	3.93	88.7
L-histidine[d]	0.1508	3.75	229.2
L-hydroxyproline[e]	0.1490	4.21	141.3
L-threonine[d]	0.1370	4.38	201.8
L-proline[d]	0.1338	4.10	45.3
L-lysine[d]	0.1282	3.24	367.9
L-ornithine[d]	0.1270	2.93	205.9
KNO_3[f]	0.1174	7.43	659.2
L-aspartic acid[d]	0.1140	6.91	410.5
L-norvaline[e]	0.1110	4.15	317.2
L-leucine[d]	0.1092	4.40	414.3
L-phenylalanine[d]	0.1084	4.75	750.0
L-glutamic acid[d]	0.0964	4.74	120.5
L-norleucine[d]	0.0890	4.12	829.6
L-isoleucine[d]	0.0470	6.67	443.0
L-methionine[d]	0.0452	3.78	450.9
L-cysteic Acid[d]	0.0084	6.07	244.0
Control (no nitrogen source)	0.0012	6.38	76.2
LSD (P = 0.05)	0.0173	0.18	27.4
Overall means	0.1296	4.80	349.1

[a]Mean weight = mean dry weight (grams) of spores and mycelia produced for five replicates.
[b]Mean pH = mean filtrate pH for five replicates.
[c]Sporulation = mean number of conidiospores in thousands for 10 fields counted (using a hemacytometer) of a pooled sample from four test flasks.
[d]Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.
[e]Obtained from Sigma Chemical Co., St. Louis, Missouri.
[f]Obtained from Fisher Scientific Co., Fairlawn, New Jersey.

TABLE II

GROWTH AND SPORULATION OF *METARHIZIUM ANISOPLIAE* ON MEDIA CONTAINING DIFFERENT SINGLE AMINO ACIDS, KNO_3, AND CONTROL

Amino Acid	Mean Weight[a]	Mean pH[b]	Sporulation[c]
L-tryptophan[d]	0.2568	6.39	329.0
L-glutamic acid[d]	0.1760	4.92	400.4
L-histidine[d]	0.1722	5.89	230.7
L-glutamine[d]	0.1490	3.71	58.4
L-cysteine[d]	0.1444	4.85	28.5
L-asparagine[d]	0.1178	4.86	84.2
glycine[d]	0.1130	5.80	72.3
L-arginine[d]	0.1080	4.68	43.3
L-isoleucine[d]	0.1040	5.47	107.2
KNO_3[e]	0.0934	6.41	49.1
L-ornithine[d]	0.0902	3.97	83.8
L-serine[d]	0.0896	5.28	59.8
L-leucine[d]	0.0876	4.44	17.5
L-alanine[d]	0.0858	4.85	50.1
L-proline[d]	0.0804	5.20	69.0
L-phenylalanine[d]	0.0804	6.00	31.3
L-valine[f]	0.0698	4.22	20.9
L-aspartic acid[d]	0.0684	5.91	28.0
L-lysine[d]	0.0396	4.44	161.0
L-norleucine[d]	0.0390	5.59	90.6
L-norvaline[f]	0.0296	4.82	58.3
L-methionine[d]	0.0238	4.50	10.5
L-hydroxyproline[f]	0.0210	5.62	13.7
L-threonine[d]	0.0206	4.85	39.0
L-cysteic Acid[d]	0.0078	5.11	13.0
Control (no nitrogen source)	0.0004	6.51	1.6
LSD (P = 0.05)	0.0153	0.14	11.5
Overall means	0.0872	5.17	82.7

[a]Mean weight = mean dry weight (grams) of spores and mycelia produced for five replicates.
[b]Mean pH = mean filtrate pH for five replicates.
[c]Sporulation = mean number of conidiospores in thousands for 10 fields counted (using a hemacytometer) of a pooled sample from four test flasks.
[d]Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.
[e]Obtained from Sigma Chemical Co., St. Louis, Missouri.
[f]Obtained from Fisher Scientific Co., Fairlawn, New Jersey.

Discussion

As is evident in Figure 1, M. anisopliae appears less likely to suffer cellular lysis and loss of soluble cellular components than B. bassiana when both are grown on an asparagine medium. Lysis with increasing age of the culture is probably responsible both for the decline in mycelial weight and the rise in medium pH (Roberts, 1966). The sporulation data of Table I suggest the existence of a survival response in B. bassiana when this fungus is provided with a less than satisfactory nitrogen source for mycelial growth. Note that a significant number of spores were produced even when no nitrogen source was provided. No evidence for a similar mechanism in M. anisopliae was uncovered (Table II).

The excellent utilization of tryptophan in this study by both fungal isolates is of some interest. Pelletier and Keitt (1954) surveyed the results of amino acid utilization studies with 22 fungal organisms. They then grouped the amino acids into "good," "fair," or "poor" sources of nitrogen, finding that generally, tryptophan was a "poor" source of nitrogen. It has been known for some time that wide vs narrow host ranges in entomogenous fungi are correlated with simple vs complex nutritional requirements (MacLeod, 1954). The authors are interested in determining if interisolate variability of nutritional factor utilization, e.g., tryptophan, may be used as an indicator of relative virulence of isolates to a particular pest

species. Additional research with different fungal isolates will be necessary to answer this question.

Other research in this area should include assessments of virulence and environmental stability of spores produced on various nutritional factors. It is possible that a synthetic medium may be developed especially designed for high spore production with only minimal mycelial growth.

Summary

Natural isolates of two entomogenous fungi, Beauveria bassiana and Metarhizium anisopliae, were cultured in liquid culture media containing 24 amino acids and KNO_3 to determine their effect on growth and sporulation. In addition, the growth and medium pH changes for each isolate grown on an asparagine-containing medium were compared. Tryptophan and alanine were most effective for growth and sporulation of B. bassiana, although glutamine and KNO_3 also produced large numbers of regularly shaped spores. Tryptophan, glutamic acid, and histidine were all well utilized for both growth and sporulation of M. anisopliae. Nitrogen sources containing sulfur were poorly utilized for sporulation by M. anisopliae. In general, B. bassiana produces greater mycelial mass and much larger numbers of spores than M. anisopliae. Both fungi attained nearly the same growth maximum on asparagine medium though B. bassiana exhibited an initially more rapid growth rate. In both fungi this rapid growth phase was accompanied by a decline

in medium pH followed by a rise in pH during the decline phase of growth.

Literature Cited

Aoki, J. 1968. Some considerations on the infection mechanism of insect pathogenic fungi: Nitrogen utilization of Beauveria bassiana, Isaria farinosa, and Isaria fumoso-rosea. In: Proceedings Joint U.S. - Japan Seminar on Microbial Control of Insect Pests, pp. 107-113. U.S.-Japan Committee on Scientific Cooperation, Fukuoka.

Aoki, J. and K. Yanase. 1970. Studies on the nutrition and metabolism of pathogenic fungi of muscardine. II. The influence of amino acid for the formation and multiplication of hyphal bodies of Spicari fumosa-rosea and Beauveria bassiana. J. Sericult. Sci. (Japan). 39:285-292.

Barnes, G.L., D.J. Boethel, R.D. Eikenbary, J.T. Criswell, and C.R. Gentry. 1975. Growth and sporulation of Metarrhizium anisopliae and Beauveria bassiana on media containing various peptone sources. J. Invertebr. Pathol. 25: 301-305.

Burges, H.D., and E.M. Thomson. 1971. Standardization and assay of microbial insecticides. In: Microbial Control of Insects and Mites. H.D. Burges and N.W. Hussey, eds. Academic Press, New York.

Hurpin, B., and P.H. Robert. 1972. Comparison of the activity of certain pathogens of the cockchafer Melolontha melolontha in plots of natural meadowland. J. Invertebr. Pathol. 19: 291-298.

MacLeod, D.M. 1954. Natural and cultural variation in entomogenous Fungi Imperfecti. Ann. N.Y. Acad. Sci. 60: 58-70.

Neel, W.W., and P.P. Sikorowski. 1972. Pecan weevil susceptibility to fungus. Pecan Quart. 6: 14-15.

Pelletier, R.L. and G.W. Keitt. 1954. Venturia inaequalis (Cke.) Wint. (VI): Amino acids as sources of nitrogen. Am. J. Bot. 41: 362-371.

Roberts, D.W. 1966. Toxins from the entomogenous fungus Metarrhizium anisopliae II. Symptoms and detection in moribund hosts. J. Invertebr. Pathol. 8: 222-227.

Swingle, H.S., and J.L. Seal. 1931. Some fungus and bacterial diseases of pecan weevil larvae. J. Econ. Entomol. 24: 917.

Tedders, W.L., D.J. Weaver and E.J. Wehunt. 1973. Pecan weevil: Suppression of larvae with the fungi Metarrhizium anisopliae and Beauveria bassiana and the nematode Neoaplectana dutkyi. J. Econ. Entomol. 66: 723-725.

CHAPTER III

GROWTH AND SPORULATION OF *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE* ON MEDIA CONTAINING VARIOUS CARBOHYDRATES

Introduction

Knowledge of carbohydrate utilization for growth and sporulation by these fungi has two possible applications to the development of control methodology: (1.) Selection and preservation of highly virulent isolates, and (2.) development of efficient mass production media. Roberts (1966) investigated the effect of several inorganic and organic (peptone) nitrogen sources on mycelial growth and toxin production of *M. anisopliae* and found that Neopeptone (Difco) produced greater yields of mycelium than did sodium nitrate. Barnes et al. (1975) determined growth and sporulation of *B. bassiana* and *M. anisopliae* on many peptone sources and found several that induced both good mycelial growth and spore production: Tryptone (Difco), Casitone (Difco), and yeast extract (Difco) for *M. anisopliae*; Soytone (Difco), Casitone (Difco), and Neopeptone (Difco) for *B. bassiana*. Campbell et al. (1978) studied utilization and sporulation of both fungi on a wide variety of amino acids and amides. Both fungi responded well on several

compounds; L-tryptophan and L-alanine for B. bassiana; and L-tryptophan, L-glutamic acid, and L-histidine for M. anisopliae. Since no previous comprehensive study has been reported it was deemed necessary to investigate growth and sporulation of these fungi on a wide variety of carbohydrate compounds. Research on 22 mono- and oligosaccharides is reported in this chapter.

Materials and Methods

Preparation of test media

The basal medium was an autoclaved, chemically defined, nutrient solution to which was aseptically added a fritted glass filter sterilized, single, water soluble carbohydrate compound. This basal medium contained 0.5 g $MgSo_4 \cdot 7H_2O$, 3.1 g KNO_3, 1.0 g KH_2PO_4, 100 µg thiamine, 5 µg biotin, 0.2 mg Fe^{3+}, 0.2 mg Zn^{2+}, and 0.1 mg Mn^{2+} in 800 ml of distilled water (Lilly and Barnett, 1951). The pH of the medium was 4.3 $\pm$ 0.2. Forty-five-milliliter aliquots were dispensed into 250-ml Erlenmeyer flasks. Six replicate flasks were utilized for each carbohydrate. Flasks were plugged with DiSPo foam-plastic closures and autoclaved at 121°C for 15 min. After cooling, 5 ml of a fritted glass-filter sterilized carbohydrate solution was pipetted aseptically into each of six flasks of sterile basal medium to yield a final carbohydrate concentration equivalent to 20 g of glucose/liter. Sterilization of carbohydrates by filtration prevents hydrolysis and chemical reactions (Cochrane, 1958).

Glass filtration also prevents pH changes which can occur when Seitz filters are used (Browne, 1942). Sterilizing temperatures sustained for 15 min do not degrade amino acids and their amides (Windholz, 1976). Of the vitamins used, biotin is heat stable and thiamine is heat stable at acid pH during autoclaving (Lilly and Barnett, 1951).

Inoculum and inoculation of media

The isolates of B. bassiana (Bb Re No. 4SS) and M. anisopliae (Ma Re No. 4SS) used in this investigation were obtained from infected pecan weevil larvae removed from pecan orchard soil near Stillwater, Oklahoma and stored under mineral oil on slants of Proteose Peptone No. 2 (Difco)-dextrose agar (PP2DA). Both isolates were single-spore-derived, and conidiospores for inoculum preparation were obtained from 2-week-old PP2DA cultures grown in Petri dishes. Other procedures were the same as reported earlier (Chapter 2 and in Campbell et al., 1978) with one exception: Each flask received 0.5 ml of spore suspension (50,000 spores/ml).

Culture and harvesting techniques

The fungi were still cultured at 24°C for 2 weeks. Before harvesting, the flasks were hand shaken and a sample of the filtrate from each treatment was placed on a hemacytometer slide and the spore concentration was determined. Mycelia were vacuum filtered onto predried and

TABLE III

GROWTH AND SPORULATION OF *BEAUVERIA BASSIANA* ON MEDIA CONTAINING DIFFERENT SINGLE CARBOHYDRATE COMPOUNDS AND CONTROL

Carbohydrate	Mean weight[a]	Duncan's multiple range values	Sporulation response[b]	Duncan's multiple range values
D-melezitose	0.5152 (0.1677)	A	11.24 (2.86)	ABC
D-sucrose	0.4524 (0.1644)	AB	31.08 (16.69)	A
D-trehalose	0.3770 (0.0682)	ABC	31.02 (12.15)	A
glycerol	0.3689 (0.0224)	ABC	6.78 (2.61)	ABC
D-glucose	0.3667 (0.0080)	ABC	30.02 (8.91)	A
D-mannose	0.3660 (0.0117)	ABC	22.58 (6.58)	ABC
D-mannitol	0.3565 (0.1497)	ABC	16.55 (3.73)	ABC
D-melibiose	0.3117 (0.0217)	ABCD	24.18 (7.15)	ABC
D-sorbitol	0.2876 (0.0167)	BCD	24.28 (7.84)	ABC
D-raffinose	0.2850 (0.0198)	BCD	13.13 (5.25)	AB
D-maltose	0.2283 (0.0431)	CDE	27.48 (19.35)	AB
D-ribose	0.1728 (0.1334)	CDEF	8.56 (3.61)	ABC
i-erythritol	0.1667 (0.1208)	CDEF	5.45 (0.80)	BC
D-lactose	0.1600 (0.1264)	CDEF	6.13 (1.94)	BC
D-fructose	0.1575 (0.0665)	CDEF	20.78 (7.94)	ABC
D-arabinose	0.1560 (0.1461)	CDEF	5.62 (2.38)	BC
None (Control)	0.1540 (0.1392)	CDEF	1.44 (0.62)	C
D-galactose	0.1433 (0.0279)	CDEF	17.08 (7.07)	ABC
D-sorbose	0.1220 (0.1160)	DEF	4.87 (1.02)	BC
D-xylose	0.1203 (0.1163)	DEF	18.04 (9.05)	ABC
D-cellobiose	0.1203 (0.1163)	DEF	5.72 (1.94)	BC
i-inositol	0.0516 (0.0079)	EF	11.98 (5.22)	ABC
L-rhamnose	0.0080 (0.0058)	F	6.02 (3.85)	BC

[a]Mean weight = average dry weight (g) of mycelium produced from six replicates. Values in parentheses are standard errors.

[b]Sporulation response = mean number of conidiospores in thousands/10 fields counted (using a hemacytometer) of a pooled sample from six flasks. Values in parentheses are standard errors.

TABLE IV

GROWTH AND SPORULATION OF METARHIZIUM ANISOPLIAE ON MEDIA CONTAINING DIFFERENT SINGLE CARBOHYDRATE COMPOUNDS AND CONTROL

Carbohydrate	Mean weight[a]	Duncan's multiple range values	Sporulation response[b]	Duncan's multiple range values
D-mannose	0.1720 (0.1125)	A	38.52 (15.05)	AB
D-glucose	0.1317 (0.0606)	AB	21.57 (6.93)	ABC
D-sucrose	0.0968 (0.0108)	ABC	19.68 (4.35)	ABC
D-trehalose	0.0883 (0.0114)	ABC	22.20 (12.63)	ABC
D-maltose	0.0840 (0.0150)	ABC	19.96 (8.89)	ABC
D-mannitol	0.0650 (0.0138)	BC	12.73 (6.09)	BC
glycerol	0.0616 (0.0079)	BC	43.93 (19.63)	A
D-sorbitol	0.0567 (0.0049)	BC	14.32 (5.53)	BC
D-xylose	0.0533 (0.0156)	BC	20.58 (8.45)	ABC
D-raffinose	0.0360 (0.0121)	BC	12.76 (8.75	BC
D-sorbose	0.0283 (0.0145)	C	0.73 (0.66)	C
D-fructose	0.0271 (0.0047)	C	23.71 (10.60)	ABC
D-melezitose	0.0240 (0.0051)	C	8.78 (3.47)	BC
i-erythritol	0.0220 (0.0150)	C	1.60 (0.90)	C
i-inositol	0.0217 (0.0060)	C	45.55 (22.90)	A
D-galactose	0.0133 (0.0042)	C	8.28 (3.41)	C
D-melibiose	0.0102 (0.0026)	C	16.70 (7.80)	ABC
D-ribose	0.0100 (0.0045)	C	3.77 (1.51)	C
L-rhamnose	0.0050 (0.0029)	C	2.18 (1.06)	C
D-cellobiose	0.0050 (0.0022)	C	1.17 (0.40)	C
D-lactose	0.0040 (0.0024)	C	3.78 (1.20)	C
D-arabinol	0.0020 (0.0020)	C	12.42 (10.38)	BC
None (Control)	0.0000 (0.0000)	C	1.70 (1.13)	C

[a]Mean weight = average dry weight (g) of mycelium produced from six replicates. Values in parentheses are standard errors.

[b]Sporulation response = mean number of conidiospores in thousands/10 fields counted (using a hemacytometer) of a pooled sample from six flasks. Values in parentheses are standard errors.

weighed filter papers. All mycelia were washed with 250 ml of distilled water after which papers and mycelia were dried 24 hr at 100°C and weighed. All data were subjected to analysis of variance.

Results

Differential mycelial growth and sporulation for B. bassiana and M. anisopliae when grown on each carbohydrate are presented in Tables III and IV. D-melezitose provided the greatest mycelial yield of any of the carbohydrates used for B. bassiana. Three other compounds, D-sucrose, D-trehalose, and D-glucose induced sporulation statistically greater than that induced by the other compounds. With M. anisopliae, mannose afforded the most mycelium; and inositol and glycerol induced the greatest spore production.

Interestingly, B. bassiana proved capable of producing some mycelial growth without the presence of a carbon source (control), while M. anisopliae was unable to do the same. However, B. bassiana could not sporulate under such conditions.

Discussion

Carbohydrate utilization patterns for B. bassiana and M. anisopliae strains investigated in this study appear to be different from those of most fungi. For most fungi, D-glucose, D-mannose, D-fructose, D-xylose, D-maltose, and D-raffinose are better utilized (Cochrane 1958). B. bassiana

exhibits a greater departure from the utilization pattern of most fungi (Cochrane, 1958) than does M. anisopliae in its ability to utilize D-trehalose. Most fungi utilize tryptophan poorly or not at all but these species utilize this amino acid quite well (Campbell et al. 1978). The emerging nutritional information implies that these entomopathogens have different nitrogen and carbon utilization patterns than most fungi. This may have significance in relation to their pathogenicity to insects. For instance, D-trehalose promoted high growth and sporulation rates by both fungi. D-trehalose is the principal carbohydrate component of hemolymph of many species of insects (Florkin and Jeuniaux, 1974).

The good results obtained when using D-melezitose for growth for B. bassiana implies the fungus may produce an enzyme capable of hydrolyzing D-turanose (Cochrane, 1958). The fungus also produces sucrase and trehalase as evidenced by the good utilization of sucrose and trehalose, respectively. M. anisopliae produces sucrase, trehalase, cellobiase, and maltase. Slight utilization of many carbohydrates implies production of various "induced" enzymes. This could better be determined if a much longer growth period had been provided.

This basic nutritional research on carbohydrate sources complements earlier studies in which peptone and amino acid sources were evaluated for inducing mycelial growth and sporulation of B. bassiana and M. anisopliae (Barnes et al.

1975; Campbell et al. 1978). These studies, coupled with nutrient analyses of the insects infected by these fungi, may provide insights concerning the host specificity of these fungi at the subspecific level. Future research should include studies of these pathogens in their natural soil environment, where certain factors such as soil humus can be increased, soil moisture can be regulated through irrigation, and the presence of other soil organisms can be manipulated. A recent study has indicated the importance of a number of these factors on conidial survival of B. bassiana in soil (Lingg and Donaldson, 1981).

Summary

Naturally occurring isolates of two entomopathogenic fungi, Beauveria bassiana and Metarhizium anisopliae, pathogenic to the pecan weevil, Curculio caryae, were cultured in flasks of a liquid synthetic culture medium containing one of 22 individual carbohydrate sources to determine growth and sporulation responses. B. bassiana grew best on D-melizitose but sporulated best on D-sucrose, D-trehalose, and D-glucose. M. anisopliae grew best on D-mannose but sporulated best on i-inositol and glycerol. B. bassiana grew least on D-sorbose. Both fungi utilized D-trehalose, a major component of insect hemolymph.

Literature Cited

Barnes, G.L., D.J. Boethel, R.D. Eikenbary, J.T. Criswell, and C.R. Gentry. 1975. Growth and sporulation of Metarrhizium anisopliae and Beauveria bassiana on media containing various peptone sources. J. Invertebr. Pathol. 25: 301-305.

Browne, H.H. 1942. Changes in reaction caused by filtration through Seitz filters. J. Bacteriol. 43: 189-192.

Campbell, R.K., T.M. Perring, G.L. Barnes, R.D. Eikenbary, and C.R. Gentry. 1978. Growth and sporulation of Beauveria bassiana and Metarrhizium anisopliae on media containing various amino acids. J. Invertebr. Pathol. 31: 289-295.

Cochrane, V.W. 1958. Physiology of Fungi. Wiley, New York.

Florkin, M. and C. Jeuniaux. 1974. Hemolymph: composition. In: The Physiology of Insecta Vol. V. 2nd Ed. M. Rockstein, ed. Academic Press, New York.

Lilly, V.G. and H.L. Barnett. 1951. Physiology of the Fungi. McGraw-Hill, New York.

Lingg, A.J. and M.D. Donaldson. 1981. Biotic and abiotic factors affecting stability of Beauveria bassiana conidia in soil. J. Invertebr. Pathol. 38: 191-200.

Roberts, D.W. 1966. Toxins from the entomogenous fungus Metarhizium anisopliae. I. Production in submerged and surface cultures, and in inorganic and organic nitrogen media. J. Invertebr. Pathol. 8: 212-221.

Windholz, M. (ed.). 1976. The Merck Index: An Encyclopedia of Chemicals and Drugs. 9th ed. Merck & Co., Rahway, N.J.

VITA

Russell Kenneth Campbell

Candidate for the Degree of

Master of Science

Thesis: GROWTH AND SPORULATION OF BEAUVERIA BASSIANA AND METARHIZIUM ANISOPLIAE ON CHEMICALLY DEFINED MEDIA.

Major Field: Entomology

Biographical:

Personal Data: Born in Enid, Oklahoma, August 30, 1954, the son of Lucy and Kenneth Campbell.

Education: Graduated from Kremlin High School, Kremlin, Oklahoma, in May, 1972; attended Phillips University, Enid, Oklahoma, 1972-1973; recieved a Bachelor of Science Degree from Okahoma State University, Stillwater, Oklahoma, May, 1977; enrolled in doctoral program at Cornell University, Ithaca, New York, 1980-1981; completed requirements for the Master of Science Degree at Oklahoma State University, Stillwater, Oklahoma, May 1987.

Professional Experience: Laboratory Technologist I, Department of Entomology, Oklahoma State University, Stillwater, Oklahoma, 1977-1979; Research Assistant, Boyce Thompson Institute for Plant Research, Ithaca, New York, 1979-1980; Graduate Research Assistant, Department of Entomology, Cornell University, Ithaca, New York, 1980-1981; Senior Agriculturist, Department of Entomology, Oklahoma State University, Stillwater, Oklahoma, 1986-1987.

Organizations: Entomological Society of America, International Organization for Biological Control of Noxious Animals and Plants, Sigma Xi, Phi Kappa Phi.