

Studies on the infection of honey bee larvae with
Ascosphaera apis

by

Sally Bamford

This thesis is submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy awarded by the CNA

Submitted March 1987

Department of Biological Sciences
Plymouth Polytechnic

PLYMOUTH POLYTECHNIC LIBRARY	
Accn No	55004358
Class No	T 595.799042 BAM
Contl No	X700560156

ACKNOWLEDGEMENT

The author wishes to thank Professor L.A.F. Heath for his unfailing enthusiasm and encouragement throughout the supervision of this project.

Grateful thanks are also extended to various members of the technical staff at Plymouth Polytechnic; and to the Devon L.E.A. which provided the funds.

CONTENTS

	page no.
ABSTRACT	1
CHAPTER 1. INTRODUCTION	2
1.1. Identification of the causal agents of chalk brood disease	2
1.2. Life cycle of <u>Ascosphaera apis</u>	
Figure 1	4
Development of an ascogenous system	5
Crozier development and ascospore formation	5
Figure 2	6
1.3. Disease description	8
1.4. Effects of the disease	10
1.5. Geographical distribution	11
1.6. Disease etiology	12
1.7. Disease transmission	15
1.8. Disease control	17
CHAPTER 2. FUNGAL SPORE PHYSIOLOGY	19
2.1. General introduction	19
2.2. General materials and methods	25
Inoculum preparation	25
Problems encountered using mummified larvae as inoculum source	25
Basic procedure for spore germination	26
Estimation of spore germination	27
2.3. Effect of temperature and pH on spore germination	28
Effect of temperature on spore germination	28

Introduction	28
Method	28
Results and Discussion	29
Figure 3	30
Figure 4	32
Effect of pH on spore germination	34
Introduction	34
Method	35
Results and Discussion	35
Figure 5	36
2.4. Nutritional requirements for spore germination	40
Introduction	40
Methods	42
Results and Discussion	43
The requirement by <u>A. apis</u> spores for exogenous nutrients to support germination	43
Figure 6	44
Carbon source requirements for spore germination	46
Figure 7	47
Nitrogen source requirements for spore germination	51
Figure 8	52
Figure 9	55
Figure 10	58
2.5. The spore ball phenomenon	61
Introduction	61
Production of mutual stimulating substances by germinating spores	63
Method	63

Results and Discussion	63
Figure 11	64
Presence of nutrient materials in spore washings	67
Method	67
Results and Discussion	68
Presence of amino acids in spore washings	68
Method	68
Figure 12	69
Results : Figure 13	71
Conclusion	72
Determination of nature of amino acids in spore washings	72
Method	72
Figure 14	73
Figure 15	74
Results and Discussion	75
Effect of washing spores on germination performance	75
Method	75
Results and Discussion	76
Figure 16	77
CHAPTER 3. INFECTION STUDIES	80
3.1. General Introduction	80
3.2. General methods	86
Collection and maintenance of bee larvae	86
3.3. Cuticular infections of honey bee larvae	87
Introduction	87
Dorsal inoculation of living larvae	89
Method	89

Results	89
Inoculation of dissected body wall	90
Method	90
Results	91
Inoculation of dead larvae	91
Method	91
Results	91
Discussion	92
3.4. Inhibitory properties of the cuticle	94
Introduction	94
Testing for chloroform- or water-soluble inhibitory factors on larval cuticle	95
Methods	95
Results and Discussion	96
Figure 17	97
Figure 18	99
Effects of some fatty acids on spore germination	100
Method	100
Results and Discussion	100
Figure 19	101
Effect of four larval food constituents on spore germination	104
Method	104
Results and Discussion	104
Figure 20	105
3.5. Mid-gut infection of honey bee larvae	108
Introduction	108
Methods	109
<u>In vitro</u> production of mid-gut infections of larvae	109

<u>In vivo</u> production of mid-gut infections of larvae	110
Preparation of larvae for histological study	110
Figure 21	111
Results and Discussion	112
Temperature and infection	112
Symptoms of disease	113
Disease development	114
Figure 22	115
Figure 23	117
Figure 24	120
Figure 25	122
Figure 26	126
Figure 27	128
Figure 28	130
Figure 29	132
Figure 30	134
Figure 31	136
Figure 32	138
Figure 33	140
3.6. Enzyme production by <u>A. apis</u>	142
Introduction	142
Determination of protease, chitinase and lipase activity by vegetative hyphae, germ-tubes and germinating spores	144
Method	144
Figure 34	145
Results and Discussion	146
Qualitative study of chitinase production by germinating spores	148
Method	148

	Results and Discussion	149
	Figure 35	150
CHAPTER 4.	REVIEW AND DISCUSSION	154
	4.1. Infection route utilised by <u>Ascospaera apis</u>	154
	4.2. Adaptations of spore physiology to larval environment	159
	4.3. Susceptibility of larvae to infection	163
	4.4. Disease prevention	168
	BIBLIOGRAPHY	170
APPENDIX I.	Germination of <u>in vitro</u> produced spores	(i)
	Introduction	(i)
	(A) Method	(i)
	Results and Discussion	(i)
	Figure A	(ii)
	(B) Method	(iii)
	Results and Discussion	(iii)
	Figure B	(iv)
APPENDIX II.	Isolation of bacteria commonly associated with spore cysts	(vi)
	Introduction	(vi)
	Method	(vi)
	Results and Discussion	(vii)
APPENDIX III.	CCP medium	(viii)
APPENDIX IV.	Histological preparation of larvae	(ix)
	Preparation of sections	(ix)
	Materials	(x)

APPENDIX V. Statistical analyses	(xi)
Mean	(xi)
Standard error of the mean	(xi)
T-test	(xi)
ANOVA	(xii)

Studies on the infection of honey bee larvae with *Ascosphaera apis*

by

Sally Bamford

The fungus *Ascosphaera apis* causes the disease chalk brood in larvae of the honey bee, *Apis mellifera*. Ascospores were recognised as the agents of disease, but the site of their germination to initiate infection had not been established. In this study larval surface cuticle was initially investigated as a possible site, but spores did not even activate here. Therefore, potential inhibitors of spore germination were studied, including water and chloroform washings of larval cuticle; 4 larval food constituents - pollen, honey, brood food, royal jelly; and a variety of medium-chain fatty acids at concentrations of 1, 0.1 and 0.01%. Royal jelly exhibited a severe inhibitory effect on all germination stages.

Larvae were successfully infected by feeding them food containing *A. apis* spores, both in vitro and in vivo. A histological study of infected larvae demonstrated germination of spores in the mid-gut lumen, followed by penetration of the peritrophic membrane and gut epithelium by developing hyphae; and subsequent invasion of larval tissues by mycelia.

Various aspects of spore physiology were investigated. Spore activation and enlargement were shown to be independent of temperature within the ranges of 10 to 40°C and 25 to 40°C respectively, while germ-tube production was closely temperature related, only occurring between 25 and 37°C - with an optimum between 31 and 35°C. However, all 3 germination stages were found to be independent of environmental pH within the range of pH 5 to 7.8. Studies on the nutritional requirements for germination revealed the need for exogenous supplies of both a carbon and nitrogen source to support germ-tube production. The 'spore-ball phenomenon' was investigated and a supplementary amino acid source was identified.

The etiology of chalk brood is discussed in the light of these findings.

CHAPTER 1

INTRODUCTION

1.1 IDENTIFICATION OF THE CAUSAL AGENTS OF CHALK BROOD DISEASE

The earliest recorded observation of chalk brood disease appears to come from Czechoslovakia in 1878 (Samšínáková et al., 1977). In 1913, Maassen, in Germany, described the disease and its causal agent, naming the fungus Pericystis apis, from its apparent similarity to a hive fungus described by Betts (1912) in England. This hive fungus, Pericystis alvei, grew on stored pollen and differed from P. apis by its production of chlamydospores, aleuriospores and by the spherical shape of its ascospores. Meanwhile, a bee-keeper in Hanover, Germany, had sent some of his affected comb to Dr Priess for examination. That the bee-keeper knew the disease as 'kalkbrut' suggests it was not then a new disease in his district. Dr Priess isolated the fungus and sent a sub-culture, along with the affected comb, to Claussen (1921), who made a detailed study, noting its morphological features and its heterothallism.

In 1935, Maurizio noted two different forms of P. apis, a small-fruited and a large-fruited form. The former was most frequently identified on larvae infected within the hive, whereas the latter most commonly developed on larvae and comb, once they were removed from the hive. Consequently, Maurizio considered the large-fruited form as a secondary invader. Statistical analyses determined the two forms were morphologically distinct and they were shown to be physiologically distinct too. Maurizio found the large-fruited form to have a temperature range of 11 to 25°C for fruit-body formation, whereas the small-fruited form sporulated from 15 to 37°C. Prökschl (1953) demonstrated the two forms could not be hybridised and named them as two varieties of the same species, Pericystis apis var. major (Maurizio)

Pröbkschl and Zobl and Pericystis apis var. minor (Maurizio) Pröbkschl and Zobl. He also discussed their systematic position as a possible link between the Phycomycetes and Ascomycetes. Speculation regarding their taxonomic position ended in 1955, when Spiltoir demonstrated the ascogenous system of croziers and asci; proof that the fungi were Ascomycetes. The asci do not remain separate, but become aggregated with loss of the ascial walls, to form the spore balls characteristic of these fungi. Also, in 1955, Spiltoir and Olive noted the generic name was already in use for a red alga, so they renamed the genus Ascospaera; creating a new family, Ascospaeraceae, to accommodate it. It is the only family in Ascospaerales, in the series Plectomycetes, subclass Euascomycetidae (Skou, 1972).

Skou (1972) compared and emended the descriptions of the characteristics of the Ascospaerales. The fungus attacking stored pollen, named by Betts (1912) as P. alvei, he renamed Bettsia alvei (Betts). He elevated P. apis var. minor to the species Ascospaera apis and P. apis var. major to Ascospaera major. He also created a new species name, Ascospaera proliperda, for the fungus causing chalk brood-like symptoms in the leaf-cutter bee Megachile centuncularis.

Later, Skou (1975) described two more species, Ascospaera aggregata and Ascospaera fimicola, the former a probable pathogen of Megachile pacifica-rotundata and M. centuncularis and of the mason bee Osmia rufa; and the latter a saprophyte on the faecal pellets of O. rufa. He also found a homothallic species which he named Ascospaera atra (Skou and Hackett, 1979) and also Ascospaera asterophora, which appeared as a saprophyte, although occurring with chalk brood in leaf-cutter bees (Skou, 1982).

Of all these species, the only one unequivocally demonstrated to be pathogenic to honey bee larvae is A. apis and this present study is entirely concerned with that species.

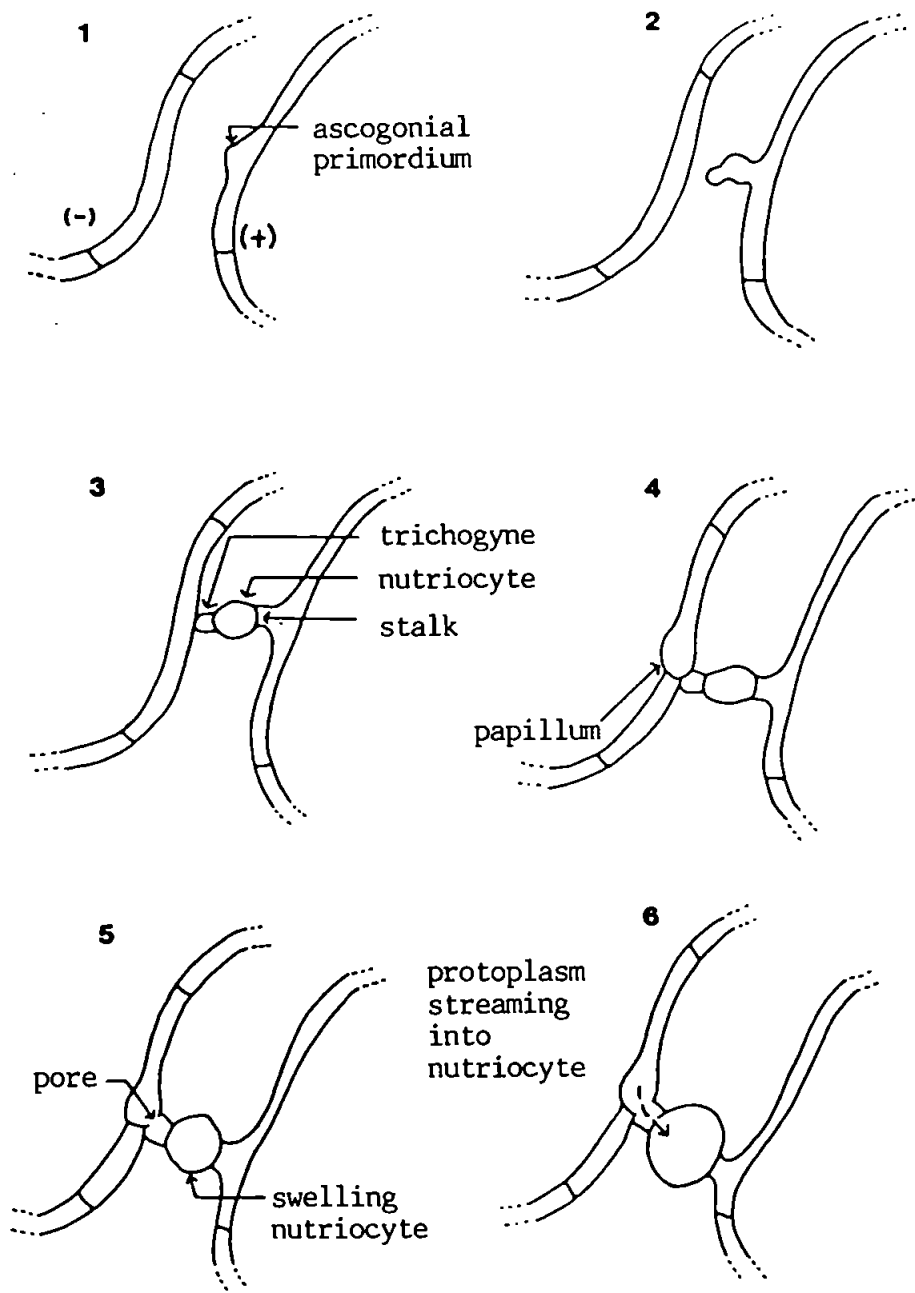


Figure 1: Development of an ascogenous system

1.2 LIFE CYCLE OF ASCOSPHAERA APIS

A. apis produces a white/grey mycelium, with hyphae 2.5 to 6 μm wide (Skou, 1972). The septa contain pores and the hyphae exhibit widely spaced dichotomous branching. No imperfect stage has ever been recorded, although Prökschl (1953) reported chlamydospores - an observation that has not been repeated despite extensive work on this fungus.

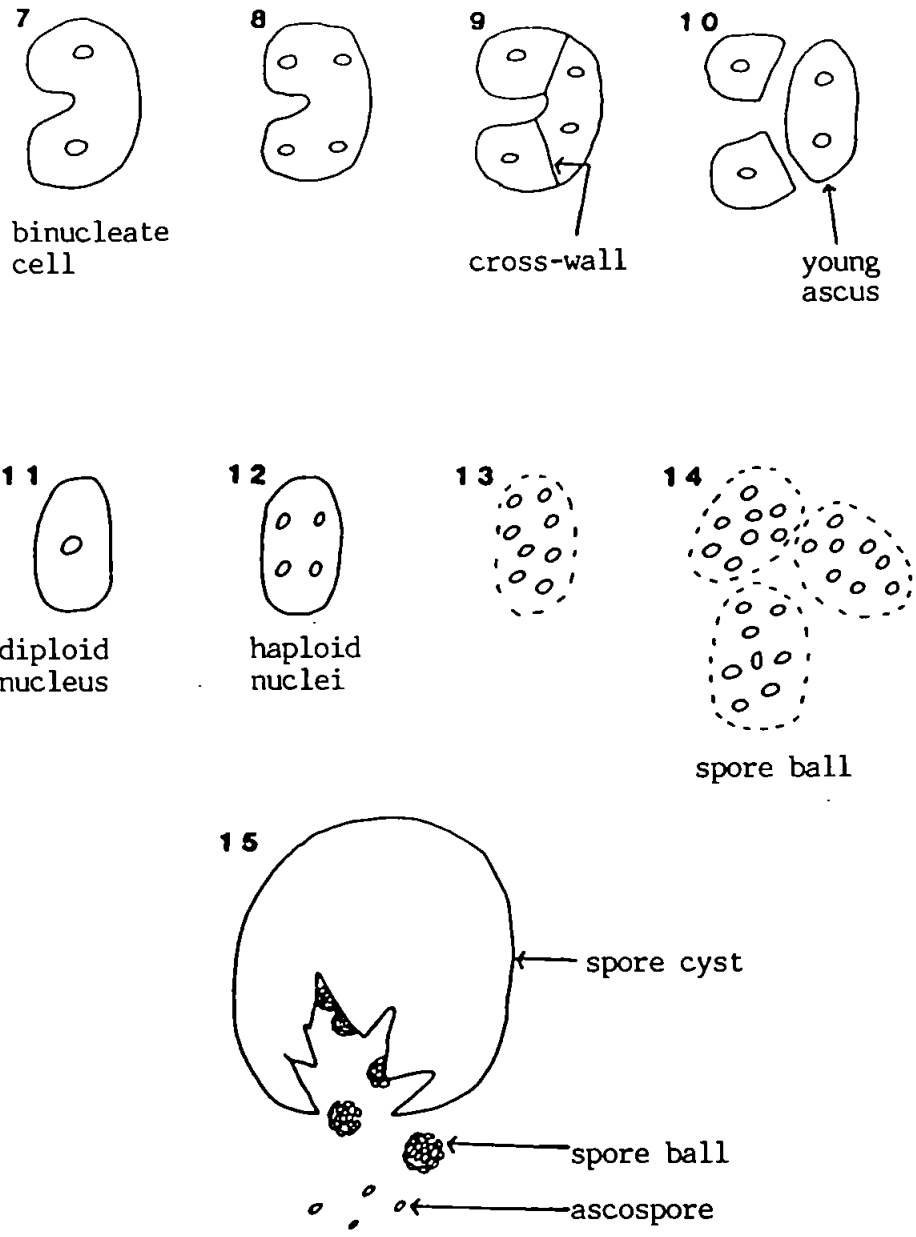
Development of an ascogenous system (Figure 1)

If (-) and (+) hyphae are present, the (+) produces an ascogonial primordium (1), which grows toward a nearby (-) hypha (2). On touching, cross-walls appear, dividing the primordium into a stalk-like portion, a central nutriocyte and a terminal trichogyne (3). All the structures are multinucleate. The (-) hypha sometimes responds by producing a small papillum, continuous with the hyphal cell (4). A pore appears where the trichogyne touches the papillum and the nutriocyte begins to swell (5). The trichogyne then receives protoplasm streaming from the (-) hypha, which continues into the nutriocyte through a pore in the septum (6). The nutriocyte then contains a number of nuclei, assumed to be (+) and (-). The protoplasm in the nutriocyte is delimited by a thin wall and constitutes an ascogenous hypha. The trichogyne empties completely, or the remaining protoplasm disintegrates. It must have supplied (+) nuclei, so acts as a gametangium as well as a trichogyne.

Initially, the primary ascogenous hypha is multinucleate, but it divides into cells containing variable numbers of nuclei. The cells proliferate to form an ascogenous system and some of the binucleate cells produce croziers.

Crozier development and ascospore formation (Figure 2)

One end of a binucleate cell increases in length and bends (7).



Adapted from Dade (1949) and Spiltoir (1955)

Figure 2: Crozier development and ascospore formation

The nuclei undergo a conjugate division (8) and walls are formed at right angles to each axis of division (9). These cut off a tip and a basal cell, leaving a binucleate cell which is the young ascus (10). Within this cell, the nuclei fuse, forming the diploid phase of the life cycle (11). The nucleus then undergoes a meiotic division, producing 4 haploid nuclei (12) and a mitotic division, resulting in 8 haploid nuclei. The ascus wall begins to disintegrate (13) and several of the asci group together, producing a spore ball (14). Spore walls then form around the individual nuclei. Bailey (1963) showed a membrane around each spore ball, but Gochnauer and Hughes (1976) did not observe any such membrane when spore balls were examined by scanning electron microscopy. The spores may remain in spore balls due to the viscous substance on the spore coat (Skou, 1972).

While the ascogenous system develops, the nuclei of the nutriocyte disintegrate and according to Spiltoir (1955), its protoplasm is probably absorbed, so once the spore balls are mature the nutriocyte is devoid of excess protoplasm. However, recent electron microscopy studies (Pulsford, unpublished data) show that this is not the case, excess protoplasm can be seen in the nutriocyte. The ripened nutriocyte is bounded by a thickened, dark-coloured wall and contains many spore balls (15). It is now called a spore cyst (Betts, 1912). The mature ascospores are ellipsoidal, unicellular, uninucleate, hyaline and on average measure $1.9 \times 3.2 \mu\text{m}$ (Spiltoir, 1955).

1.3 DISEASE DESCRIPTION

Chalk brood is a disease of bee larvae. Adult bees are not susceptible, but there are conflicting reports regarding disease development in eggs and pupae. Claussen (1921) and Betts (1932) considered eggs and pupae to be vulnerable, although the latter less so. Gilliam et al. (1978) failed to infect eggs and pupae in vitro, but in a later study several mummified pupae were found in hives (Gilliam et al., 1983). Since Nelson and Gochnauer (1982) found no evidence for the presence of A. apis in the pupae they examined, it seems likely, as suggested by Gilliam et al. (1983), that infection normally occurs in the larval stages, but in some cases metamorphosis is possible before the completion of disease development. As for the infection of younger stages, Maurizio (1934) had only observed the infection in stretched larvae, never coiled larvae. Wille (1975), however, notes that infections of earlier stages may not be seen since they would be easily detected and quickly removed by the worker bees.

Larvae of all castes are susceptible, although there are reports of drone larvae being preferentially attacked (Claussen, 1921; Betts, 1932; Kenward, 1932). This is most probably due to their position on the combs (Bailey, 1966; De Jong, 1977), rather than any intrinsic difference in resistance. Drone larvae are the first to be neglected when a colony is deficient in food or nurse bees. Infected queen larvae are rarely seen, as noted by Claussen (1921) and Betts (1932). Heath (1982) referred to a questionnaire returned by British bee-keepers, of which 7 out of 300 reported having seen queen larvae with the disease. He also cited Woyke and Bobrzecki (1978), who observed 20% of queen larvae with 'long queen cell disease' showing signs of chalk brood disease, which could introduce some confusion.

Within the hive, infected larvae die shortly after being sealed in their cells. Worker bees uncap these cells, exposing the white mycelia which surrounds the larvae. At first larvae are swollen, completely filling their cells, but they dry and shrink into mummified larvae, looking like small pieces of chalk. These 'mummies' appear white if mycelium only is present; if spores are produced they appear a grey/black colour. Since A. apis is heterothallic, it was assumed the former were infected with only one mating type, but both types can be isolated from white mummified larvae (Christensen and Gilliam, 1983; Heath, 1984).

Diseased larvae may be found in the hive from March to October, in the northern hemisphere, with maximum numbers occurring in May to June. Maurizio (1934) reported a peak in June in Switzerland, as did Toumanoff (1951) in France. A survey by Menapace and Wilson (1976) in the U.S.A. concluded most symptoms were observed from April to June, while De Jong (1977) observed maximum numbers in July, in New York State, U.S.A. In a full colony, therefore, this disease is most evident in the spring, when the colony is expanding.

1.4 EFFECTS OF THE DISEASE

Mummified larvae are detected and removed from their cells by house-cleaning bees, with varying degrees of efficiency, depending upon the strain of bee (De Jong, 1977). Some do so rapidly and to the bee-keeper the broodnest appears healthy (Mehr et al., 1978), unless a dead-bee trap is installed to collect mummified larvae thrown to the floor, prior to their ejection from the hive by cleaning bees (Menapace, 1978). In severe infections many cells containing mummies may remain capped (De Jong, 1977). This disease will rarely result in the death of a colony, although Anderson (1938) reported a case where every worker larva had been killed by it. Most commonly, the result is a decline in foraging force, due to loss of larvae. Rosenthal (1974) described colonies suffering a 49% reduction in gathering capacity as a result of chalk brood disease and Mehr et al. (1976) found only 23% of their infected colonies storing surplus honey, although less than 5% of the brood appeared to be infected. De Jong and Morse (1976) noted that in the first year the disease was detected in New York State over 1000 colonies were so badly weakened they failed to produce surplus honey. Consequently, the disease may be considered economically important. Also, Mehr et al. (1978) suggested colonies suffering from the disease during the summer are less likely to survive over-wintering, because the weakened population may contain too few young workers.

1.5 GEOGRAPHICAL DISTRIBUTION

Despite the early descriptions of chalk brood disease from England (Betts, 1915-24) and continental Europe, including Germany (Maassen, 1913) and Switzerland (Morgenthaler, 1918), it was not discovered in the U.S.A., in honey bee larvae, until 1971 (Hitchcock, 1972), although a chalk brood fungus was isolated from Megachile inermis in 1965 (Baker and Torchio, 1968). The importation of New Zealand queens to Canada (Pankiw, 1972) in 1968 may have provided a source of inoculum (De Jong, 1977) as may the importation of European pollen (Gochnauer, 1982), since the disease may be transmitted by contaminated pollen (Mehr et al., 1976; Menapace, 1978). Mraz (1973), however, stated that chalk brood was not a new disease in the U.S.A., but had been present there as long as European bees. In his view, it had been scarce for 50 years while the old, leather-coloured Italian bees were in use, since these were very resistant to the disease; but previously it had been common, when German black bees were kept. He suggested that it may have recently reappeared with the re-introduction of more susceptible strains of bees. Menapace and Wilson (1976) also reported early incidents of the disease in the U.S.A. in the 1920s and 1930s.

Chalk brood has now been reported from many countries and appears to be mainly a disease of bees in the north temperate regions, with recent out-posts within tropical (Belize, Mexico, Phillipines) and south temperate (New Zealand, Argentina) regions (Nixon, 1982; Heath, 1985a).

1.6 DISEASE ETIOLOGY

Maurizio (1934) described cases where apparently healthy larvae would develop disease symptoms, once they had been removed from their colony and were incubated in the laboratory. Also, the fungus was isolated from the vast majority of samples of brood comb examined by Heath (1985b); even when the supplier of the sample insisted that the colony was free of chalk brood disease. These observations suggest the fungus is often present in colonies, without manifesting itself; that certain pre-disposing conditions are required for disease development and that the disease may be much more widespread than first thought. Indeed, it may even be that the recent appearance of the disease in New Zealand, for example, resulted from a change of conditions in the colonies there, rather than from importation of the pathogen.

Rainy summers and consequent moist, cool apiaries have been blamed for precipitating the disease (Roussy, 1962; Dallman, 1974). Lunder (1971) reported the disease to be most prevalent in the damp regions of Norway and thought weakened colonies might be more susceptible, since they would be unable to maintain optimal brood temperature. Bailey (1966) considered chilling of the brood prior to cell sealing as a pre-requisite to chalk brood development and Cooper (1980) suggested that fluctuating brood-nest temperatures, as exhibited by some types of bee, might induce disease development through a chilling effect.

Albisetti and Brizard (1979) blamed dampness in the hive, resulting from evaporation of water from nectar, for disease development, but colonies maintain a constant relative humidity around brood (Moeller and Williams, 1976). Wille (1975) was also sceptical about the effects of high humidity.

Alternatively, inadequate pollen supplies have been blamed (De Jong, 1976). Mehr et al. (1976) and Herbert et al. (1977) induced chalk brood disease in colonies by feeding them 2 year old pollen, which according to Haydak (1961) would be of negligible nutritive value with respect to the development of the hypopharyngeal glands, so larvae may have been fed brood food of poor quality or inadequate quantity.

Deans (1940) considered colonies already weakened by acarine or paralysis to be vulnerable, as might colonies affected by sacbrood (Mehr et al., 1976; Moeller and Williams, 1976). Consequently, Wille (1975) regarded the fungus as a secondary invader, attacking those larvae already weakened by other circumstances.

De Jong (1977) commented on the varying susceptibility of bee strains to the disease and Menapace and Wilson (1976) thought inbreeding and uncontrolled genetic changes were leading to more susceptible strains of bees in the U.S.A. Seal (1957) found chalk brood only amongst darker races of bees, which may have been due to their 'excessive swarming', thereby leaving too large a brood nest for the remaining bees to tend. Wille (1975) suggested susceptibility was hereditary and recommended selection of resistant stocks. Nelson (1975), when crossing New Zealand and Californian bee stocks, found those crosses to have fewer colonies affected by chalk brood than local stocks. Gilliam et al. (1983) and Milne (1983) demonstrated resistance to infection related to nest cleaning behaviour in bees. This is controlled by genes for uncapping of cells and larval removal, as demonstrated by Rothenbuhler (1964) for American Foulbrood resistance.

Widespread use of oxytetracycline as treatment for other bee diseases was thought to have enhanced chalk brood (Samsinakova et al., 1977), but it has been demonstrated as ineffective at either inhibiting

or enhancing chalk brood symptoms (Menapace and Wilson, 1979, 1980).

1.7 DISEASE TRANSMISSION

Nelson and Gochnauer (1982) found comb-stored pollen contaminated with A. apis in infected colonies, as were the mid-guts of the adult bees they examined. Although nectar, ripened honey and royal jelly were found by them to be free of contamination, Yamazaki et al. (1975) isolated A. apis from domestic Japanese and imported honeys and Maurizio (1934) isolated the fungus from 2 year old, stored honey.

De Jong and Morse (1976) demonstrated transmission of the disease by the food-sharing behaviour of adult bees and Maurizio (1934) showed that the fungus may be present in the adult mid-gut long after disease symptoms have disappeared from the colony. Maurizio therefore suggested that the fungus over-wintered in adult mid-guts and in honey stores. Indeed, the spores of A. apis are long-lived (Borchert, 1939, cited by Toumanoff, 1951) and adhesive, so once within the colony they may well survive for years on hive surfaces.

It is unlikely that spores would be wind dispersed, as suggested by Moeller and Williams (1976) and Thorstensen (1976), due to their sticky surface. However, Moeller and Williams (1976) also thought spores could be picked up by foraging bees at sources of nectar, pollen and water and this is at least possible, since Nelson and Gochnauer (1982) isolated A. apis from pollen collected at the hive entrance. Although this contamination could have arisen from the body surface of the foraging bee itself, or from its honey stomach, considering the way in which pollen is packed into the pollen baskets.

Vandenberg et al. (1980) observed, by scanning electron microscopy, *Ascosphaera* spores adhering to the surface of M. rotundata. This may also be a means of transport for the sticky spores of A. apis on Apis mellifera. Consequently, robber and drifting bees may transport

spores between colonies (Barthel, 1971), either attached to themselves or within their mid-guts. Bee-keepers may transmit spores between colonies, either on their tools (Thorstensen, 1976), or by transference of queens, workers, sealed or unsealed brood from infected to uninfected colonies (Herbert et al., 1977). De Jong (1977) and Moeller and Williams (1976) also demonstrated that requeening with queens from contaminated colonies can transmit the disease. Using contaminated pollen supplies can also induce disease development (Mehr et al., 1976; Menapace, 1978). Also, the use of old, contaminated comb may contribute to the spread of the disease, according to Gochnauer (1982) and Koenig et al. (1986). The former, however, failed to isolate the fungus from swabs of brood cells from which infected larvae had been removed. This is also surprising given the frequency with which Heath (1985b) isolated the fungus from comb samples.

1.8 DISEASE CONTROL

Many antibiotics, antimycotics and disinfectants have been tested in vivo (Taber et al., 1975; Menapace and Wilson, 1979; Menapace and Hale, 1981; Stoner and Wilson, 1985), or in vitro (Giauffret and Taliercio, 1967; Dallman, 1974; Samsinakova et al., 1977; Gochnauer et al., 1979; Gochnauer and Margetts, 1980), or by both methods (Glinski, 1980; Herbert et al., 1985); but with little success in preventing fungal growth and therefore disease development. There remains no effective chemotherapy against this disease. Such a treatment would have to be easy to use; be no more expensive than the natural loss caused by chalk brood (Menapace and Hale, 1981) and should not induce the development of resistant strains of the fungus (Heath, 1982).

Others have recommended simple physical methods by which to maintain healthy colonies, for example, heating the hives in spring to prevent dampness (Pedersen, 1976), or ensuring adequate ventilation in the hives (Seal, 1957). Nelson and Gochnauer (1982) noted more infections in old than in new combs, but the destruction of infected combs is of no use, since Anderson (1938) reported extensive chalk brood in a comb so new it had not been fully formed. One simple treatment reported as successful is the coating of the insides of brood boxes with lime wash (Gochnauer, 1982).

The best means of control is good hive management, according to Morris (1977) and De Jong (1977) who recommended maintaining the strength of colonies, so there is an adequate adult to brood ratio. Many suggest requeening those colonies which appear most susceptible, in order to produce a more resistant stock (Roussy, 1962; Mraz, 1973; Wille, 1975). At present these are the only satisfactory recommendations as means of control.

Before any progress can be made in control methods, it is first necessary to fully understand the disease process, that is, exactly how the larvae become infected. Since the earliest stages of infection are likely to be the most vulnerable to any control régimes, an investigation of these stages is the purpose of this study.

CHAPTER 2

FUNGAL SPORE PHYSIOLOGY

2.1 GENERAL INTRODUCTION

Spores may be considered as the beginning or end of the life cycle for fungi. Sporulation usually occurs in response to unfavourable environmental conditions, in which the vegetative stage of the life cycle could not survive. Therefore, spores remove the fungus from adverse conditions, either in space or time, as agents of dispersal or survival respectively. The ascospores of A. apis are included in the latter category; increasing the chance of survival by germinating only when more favourable conditions prevail.

Such spores survive in a state of low metabolic activity, which may be constitutive or exogenous. In the former case germination is delayed by an innate property of the spore, until a 'trigger', an environmental stimulus normally associated with the biological function of the spore, initiates germination. The endogenous dormancy may be maintained in various ways; for example, a barrier to the transport of nutrients, that is, the impermeability of the cell membrane is thought to be the case in Lycogala epidendrum. Dimethylsulphoxide, which denatures proteins, stimulates its germination (Butterfield, 1968) and the activation of spores of Dictyostelium discoideum (Cotter and O'Connell, 1974, cited by Cotter et al., 1976). The spores of this latter fungus can also be stimulated by heat treatment (45°C for 30 minutes) and an analysis of freeze-fracture faces shows the drastic effect of heat on the distribution of particles in the plasma membrane (Hohl et al., 1978). These changes may trigger the sequence of events associated with germination. Indeed, a permeability barrier is the

classical explanation of dormancy and the changes in permeability associated with germination occur so rapidly that the membrane lipoproteins may well be the mediators (Griffin, 1981).

Endogenous dormancy may also be maintained by self-inhibitors produced within the spores. The most extensively studied are those of rust uredospores. These have been identified as derivatives of cinnamic acid and are thought to prevent the hydrolysis of the material plugging the pore in the spore wall, through which the germ-tube would emerge (Macko et al., 1976). Alternatively, a metabolic block may prevent germination. Key enzymes associated with respiration or other pathways of metabolism may be absent or not fully functional; for example, in Ustilago maydis the germination of teliospores is characterised by the appearance of key enzymes of both the hexose monophosphate shunt and the tricarboxylic acid cycle (Gottlieb and Caltrider, 1963); and in Phycomyces blakesleeanus trehalase activity increases ten to fifteen-fold when spores are activated (Van Assche et al., 1972). However, enzyme activation is generally associated with spore activation, therefore it is often difficult to determine whether a lack of activity in spores is a cause or consequence of dormancy.

On some occasions endogenously dormant spores may not respond to activation treatments until a certain time period has elapsed. This has been defined as 'after-ripening' and ensures the spores are mature before germination can begin. Other spores may be very short-lived and the percentage of germination may decrease with the increasing time spent in a given set of conditions. The rate of senescence of spores varies depending upon the nutrient source on which they were produced (Darby and Mandels, 1955) and humidity and temperature are other important factors.

The triggers which activate spores are varied and may work in combination. Periods of hot and cold, or of dry and wet, for example, simulate the 'over-wintering' conditions received by some spores in nature. Many spores respond to chemical treatments such as organic acids, ether and alcohols, as in the cases of Neurospora tetrasperma (Sussman et al., 1959) and Puccinia graminis (French et al., 1957). Such solvents are presumed to disrupt the lipoprotein bilayers and so alter membrane permeability. Some spores require heat treatment, temperatures of 40 to 75°C for a few minutes or sometimes hours. P. blakesleeanus requires 50°C for 3 minutes (Halbsguth and Rudolph, 1959) and Coprinus radiatus needs 44 to 46°C for 4 hours (Sussman, 1976). Other spores require cold temperatures, of -5 to 10°C, which is particularly common for Basidiomycetes (Sussman and Halvorson, 1966).

In the case of exogenous dormancy development is delayed by unfavourable chemical or physical conditions within the environment. Therefore, activation results when favourable conditions occur and again, these would be associated with the biological function of the spore. The conidia of Aspergillus niger require high intensity white or blue light to stimulate germination (Khan, 1977) and increased levels of carbon dioxide enhance the germination of Aspergillus nidulans (Trinci and Whittaker, 1968). Others may have absolute nutritional requirements, such as Penicillium griseofulvum for glucose (Fletcher and Morton, 1970). Alternatively, germination may be prevented by inhibitors within the environment, for example, those naturally occurring in plants, like hydroxylated cinnamic acid derivatives, universally present in plants as monomers or as part of a more complex product such as lignin. Other micro-organisms may produce compounds inhibitory to fungal spores or may compete with them for nutrients.

Some soils will exhibit fungistasis, where bacteria act as a 'nutrient sink', thriving on compounds released by fungal spores during enlargement, which would ordinarily be used by the fungus itself.

It should be noted that the distinction between the two types of dormancy is sometimes narrow and consequently, it is often difficult to designate a type of dormancy to a given spore.

Following activation many fungal spores, providing they are not excessively thick-walled, show an increase in size; a swelling process of mainly metabolically controlled growth, rather than the simple imbibition of water. Before reaching its maximum size, one or more germ-tubes emerge from the spore. The point of emergence may be random, or pre-determined, as in the ascospores of Chaetomium thermophile var. coprophyta. Here there is a germ-pore defined in the spore wall at which a thin-walled vesicle forms, which gives rise to a germ-tube (Cerelin and Fergus, 1971). Usually the cell wall ruptures during germ-tube formation (Hawker and Hendy, 1963), but whether this is caused by physical pressure from the increase in protoplasm or initiated by enzyme degradation is uncertain.

As well as the morphological changes associated with germ-tube production there are many physiological and biochemical changes in the spore, marking their transition to a metabolically active state. An increased respiratory rate is usually associated with germination. In Fusarium solani the endogenous respiratory rate in the macroconidia increased from 3.5 μl in the dormant state to 20 μl oxygen mg dry wt^{-1} hr^{-1} at germ-tube emergence (Griffin, 1981). There is also an increase in size and number of mitochondria to support this increase and an increased number of ribosomes to cope with the changes in macromolecular synthesis. By following the incorporation of radioactive precursors into proteins and nucleic acids, most spores studied have shown an

increase in RNA and protein synthesis 15 minutes after inoculation into the germination medium. In the case of Botryodiplodia theobromae protein synthesis preceded RNA synthesis during germination, suggesting ungerminated spores contained the necessary RNA for protein synthesis (Brambl and Van Etten, 1970). Indeed, pre-formed RNA has been reported for several fungi and the nuclear cap of zoospores of some aquatic fungi has been identified as a 'package' of ribosomes, which breaks down once germination begins (Lovett, 1963). All such changes result in the apical growth of the germ-tube and the resumption of the normal metabolic and physiological processes associated with the vegetative state.

For A. apis very little is known of the physiology of the ascospores and their germination, despite them being long assumed to be the infective agents of chalk brood disease. In order to fully understand the infection of honey bee larvae by this fungus these processes must be elucidated. Perhaps there is a dearth of information in the literature due to the difficulties encountered in their in vitro germination. Claussen (1921) was first to note 'the low germinating capacity of the spores', followed by Betts (1932), who germinated the spores 'with difficulty'.

Bailey (1966) found the spores of A. apis to germinate best in vitro beneath the surface of semi-solid agar and concluded their requirement was an anaerobic atmosphere for germination, although the mycelium required an aerobic atmosphere for development. These observations were confirmed by Thomas and Luce (1972), who produced growth from spores incubated initially in anaerobic conditions and subsequently placed in aerobic or carbon dioxide enhanced conditions. Kish (1980) attempted to germinate spores of other Ascosphaera species under reduced oxygen, either by adding spores to an agar medium at 40 to 45°C and mixing, so the spores became submerged in medium, or

by placing them on agar onto which a second layer was added. Alternatively, spores were placed on Riddell mounts (Riddell, 1950), which were incubated in a desiccation chamber that was either evacuated only, or evacuated and subsequently purged with carbon dioxide. Best germination was achieved using this latter method, where germination levels ranged from 4 to 80% under high concentrations of carbon dioxide. Stephen et al. (1982) used a similar method to germinate spores of A. aggregata, a 2 litre chamber was purged with carbon dioxide to a final concentration of $60 \pm 5\%$. However, the gaseous requirements of germinating spores of A. apis were not elucidated until Heath and Gaze (1987, in press) determined that it was the capneic, rather than anaerobic conditions, which would activate the spores to germinate. Maximum spore activation could be achieved in an atmosphere of 12.5% carbon dioxide in air.

Carbon dioxide certainly breaks the dormancy of A. apis spores, but whether it activates the spores by breaking an exogenous or constitutive dormancy is not clear at this stage. The carbon dioxide may trigger activation by preventing the production of self-inhibitors, or by releasing a metabolic block, or it may be a constituent of the favourable environment required by an exogenously dormant spore.

However, now a method is available by which these spores can be successfully and reliably activated in vitro, the further requirements for germination can be studied.

2.2 GENERAL MATERIALS AND METHODS

Inoculum preparation

Chalk brood mummified larvae were collected by bee-keepers, from colonies of Apis mellifera throughout the U.K. and were sent to Plymouth Polytechnic. Here they were stored in petri-dishes at room-temperature, until required.

When inoculum was required the spore cysts were scraped from the surface of mummified larvae onto a sterile microscope slide, using a sterile mounted needle. The spore cysts were crushed, using a razor blade, to release spore balls and individual spores.

Problems encountered using mummified larvae as inoculum source

The main problem was inherent variability amongst the spores. Even those spores produced on a single larva would exhibit genetic variability arising from the various combinations of a multitude of strains, depending on the number of spores that had germinated and grown throughout the larva. This problem would be overcome if germinable spores could be produced in vitro. However, De Jong (1976) observed that few infections of larvae could be induced using cultured spores and in this present study such spores could not be germinated in those conditions which supported germination of in vivo produced spores (Appendix I). The constituents of the medium on which 2 strains sporulate appears to be important in the production of spores that will germinate similarly to in vivo produced spores (Appendix I). This requires further investigation in order to determine the environmental conditions needed to culture germinable spores. This would then allow better controlled experiments and the differences between strains could be examined, since biochemical differences may be indicative of variation in virulence of strains. Chmielewski and Glinski (1980/1981) studied the biochemical properties of 109 strains

of A. apis and their results revealed 10 biochemical types based on urease production, liquefaction of gelatine, lipolytic activity and haemolysis of sheep erythrocytes. Sporulating strains were later tested for their virulence and significant differences in the LD₅₀ values were obtained (Glinski and Chmielewski, 1982).

A second problem also arose with the use of the natural source of spores. This was the bacteria which were commonly associated with spore cysts on the mummified larvae. Some of these bacteria have been isolated (Appendix II), but require further study to determine their relationship with A. apis. If germinable spores could be cultured in vitro the bacteria would no longer present a problem. This assumes these bacteria have no association with the germination of A. apis spores or with the infection process, but are secondary invaders on the infected larvae. However, other hypotheses are tenable at this stage in our knowledge.

Basic procedure for spore germination

A 1% (w/v) glucose and 1% (w/v) yeast extract (Oxoid) solution in distilled water was prepared and autoclaved. Once cool, oxytetracycline, sterilised by membrane filtration, was added to give a final concentration of 0.005%. This would inactivate most bacteria which might be contaminating the spore cysts, but would not affect fungal spore germination (Oakes, 1984). This medium is referred to as GYO throughout this thesis.

Germination in liquid culture:-

5 cm³ aliquots of GYO medium were aseptically dispensed into sterile 25 cm³ conical flasks and spore inoculum added, from 1 mummified larva to each flask. The flasks were stoppered with sterile suba-seal rubber bungs and injected with carbon dioxide gas to 10% of the total flask volume. Flasks were incubated for 24 hours at 35°C.

Germination on a solid medium:-

15 cm³ aliquots of GYO medium, to which 1.5% agar had been added prior to sterilisation, were aseptically dispensed into sterile 9 cm diameter petri-dishes. Riddell mounts (Riddell, 1950) as adapted by Heath and Gaze (1987, in press) were prepared. These were further adapted by using a 1 cm diameter cork borer to cut agar discs, of which 4 could be evenly spaced on a sterile microscope slide. Spore inoculum was added to each disc. This consisted of the contents of crushed spore cysts carried on the tip of a sterile inoculating loop. The microscope slide was then inserted into a humidity chamber. This was a sterile petri-dish containing 4 x 9 cm Whatman filter papers soaked in 10 cm³ sterile distilled water. Incubation was for 24 hours at 35°C, in an ASSAB carbon dioxide incubator set at 10% carbon dioxide.

Estimation of spore germination

Spores were stained with lactophenol cotton blue stain (Appendix IV) and the stages of germination were estimated by counting the numbers of spores which remained unactivated (i.e. remained impermeable to stain), had activated (permeable to stain), enlarged, and produced germ-tubes, from a sample of 200 spores on an agar disc, or in a drop of suspension. 2 samples of spore suspension were counted from each flask and 1 sample from each of 4 agar discs incubated in 1 humidity chamber. Activation, enlargement and germ-tube production were calculated as percentages of the total number of spores counted.

2.3 EFFECT OF TEMPERATURE AND pH ON SPORE GERMINATION

Effect of temperature on spore germination

Introduction

The effect of temperature on vegetative growth and sporulation of A. apis has been noted by a few researchers. The earliest, Betts (1915-24), observed the fungus to grow better at 32°C than at room temperature; producing cysts within 7 days when incubated on bee pupae bouillon agar. In 1935, Maurizio grew A. apis on beer wort agar at temperatures ranging from 0 to 42°C and found an optimum of 30°C. She also noted (-) hyphae grew faster than (+), which had also been observed by Claussen (1921). As for sporulation, a range of 15° to 37°C was determined, with an optimum of 30°C. The importance of the type of nutrient substrate used for growth, plus pH, aeration and temperatures was noted by Prökschl (1953), who determined an optimum of 30°C for sporulation, with an upper limit of 37°C, and found best vegetative growth at 25° to 35°C. More recently Furuya et al. (1981) found a range of 25° to 35°C for vegetative growth and 30° to 35°C for sporulation. Consequently, an overview of such data suggests an optimum temperature between 25° and 35°C for vegetative growth and an optimum for sporulation at 30° to 35°C.

These stages of the life cycle of A. apis appear well-adapted to a life within the beehive, where the temperature of the brood area is maintained around 35°C by the bees. However, the adaptation of the germination stages to this habitat has not been investigated, despite its importance in the reactivation of the fungal life cycle each spring. Roussy (1962) stated an optimum of 20° to 25°C for spore germination and Bailey (1966) quoted 35°C as the optimum, but gave no indication of the range he tested.

Method

Spores were germinated in GYO liquid medium in flasks incubated

at a range of temperatures between 4 and $65 \pm 1^\circ\text{C}$. Once the overall temperature range for each stage of germination had been determined, the optimum temperature for germ-tube production was investigated, using a closer range of temperatures.

Results and Discussion

From Figure 3 it can be seen that the temperature range differs for each stage of the germination process. Activation occurred within the full range investigated, i.e. 4 to 65°C , although it was reduced at 4°C and erratic beyond 40°C . At temperatures between 10 and 40°C there were no significant differences between the percentages of activation ($F = 0.88$; $P > 0.05$, $df = 8,20$), which accounted for approximately 90% of the spores in each case. Enlargement, however, occurred within a narrower temperature range, from 25° to 40°C , between which there were no significant differences in the percentage of spores enlarged ($F = 0.7$; $P > 0.05$, $df = 5,14$) and below or above which, the percentages of enlarged spores were negligible. Both activation and enlargement appear independent of temperature within their specific temperature ranges. This is not surprising for activation, since it probably involves mostly physical processes, but it is unexpected to find the metabolically active enlargement stage exhibiting a similar trend. Germ-tube production was shown to be closely temperature related, only occurring within the relatively narrow range of 25° to 37°C . A second experiment studied the temperature range of germ-tube production in more detail and it can be seen from Figure 4 that the optimum temperature lies between 31° and 35°C . There were no significant differences between the percentages of germ-tubes produced at these temperatures ($F = 1.3$; $P > 0.05$, $df = 4,37$).

The results of these experiments disagree with those of Roussy (1962), in that no germ-tube production was seen at 20°C and although some germ-tubes were produced at 25°C , the optimum was certainly not

Figure 3: Graphs showing the mean percentages, \pm the standard error of the mean (SE), of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in liquid GYO medium in 10% CO₂ at temperatures between 4 and 65 \pm 1°C.

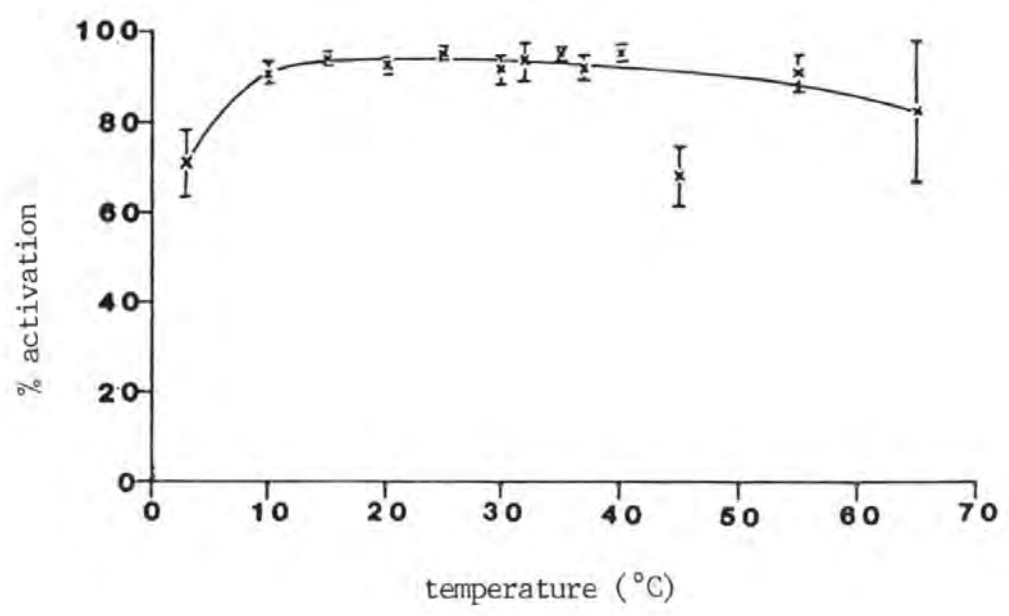
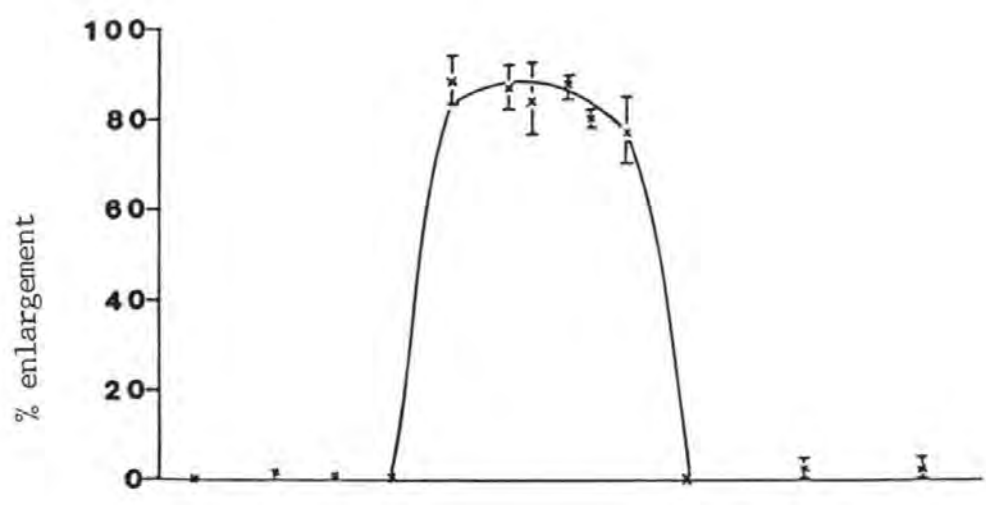
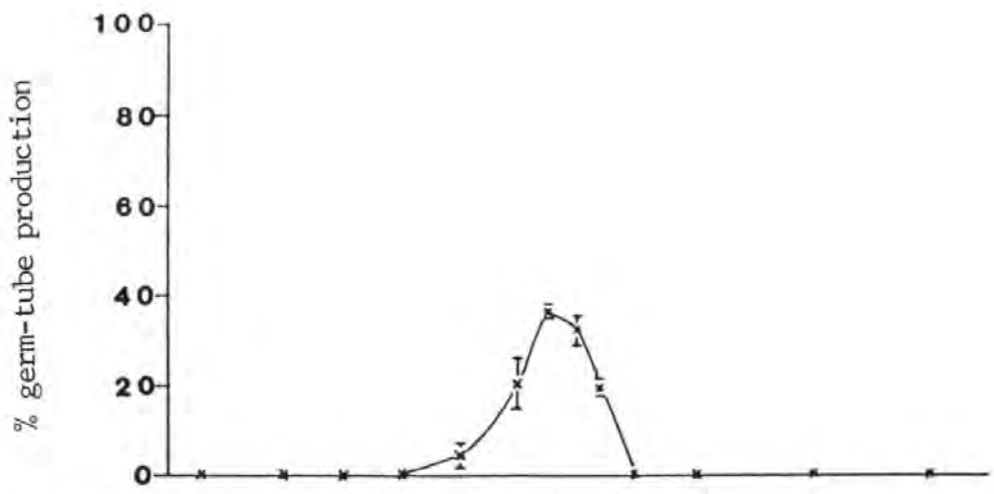
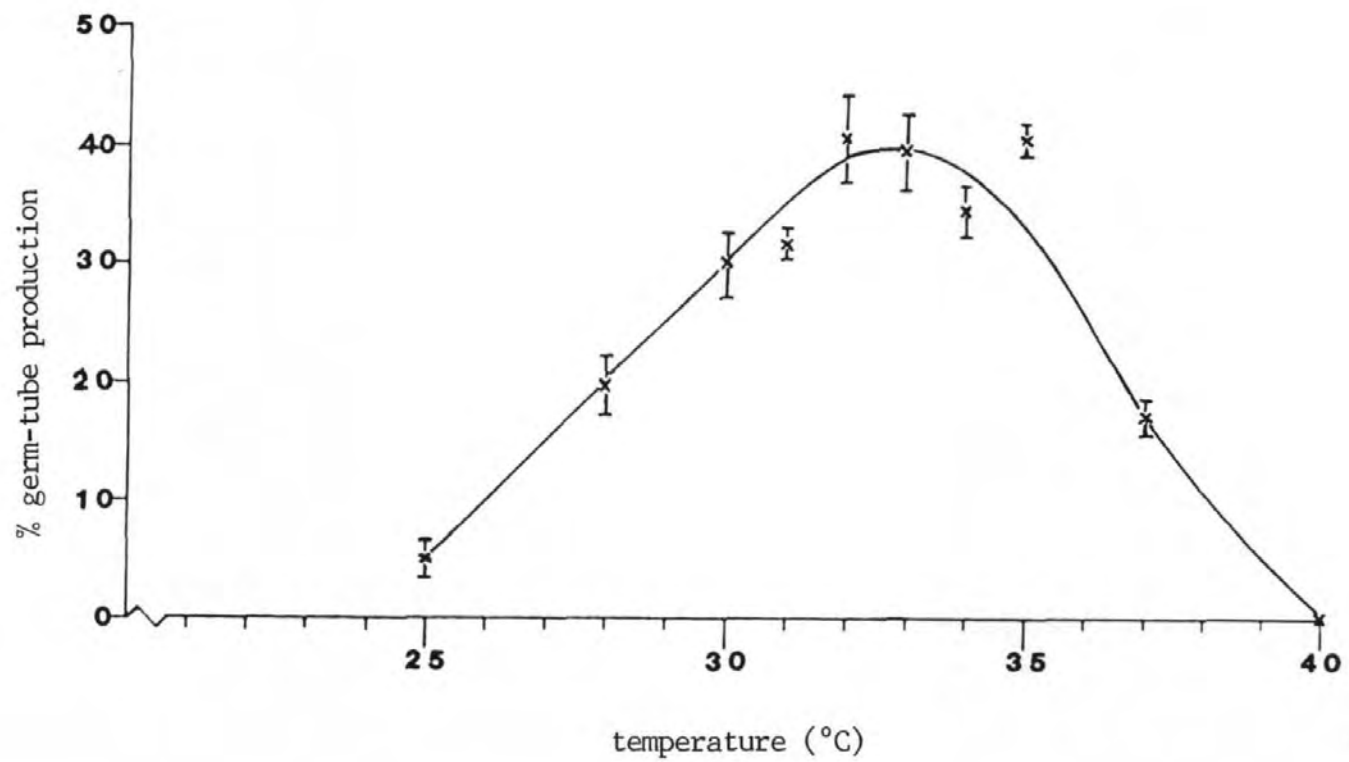


Figure 4: Graph showing the mean percentages, \pm SE , of spores producing germ-tubes after 24 hours incubation in liquid GYO medium in 10% CO₂ at temperatures between 25 and 40 \pm 1°C.



that low. The results here agree with Bailey (1966), that the optimum for spore germination is close to that temperature maintained within the brood area of the bee colony, i.e. 35°C. The slight difference between his result of 35°C and that reported here, 31° to 35°C, may be accounted for by the differences in medium used for spore germination and possibly strain differences inherent in the spores, depending on their source. In addition, Bailey does not give sufficient details of his experiment to determine whether he studied such a close range of temperatures as those reported here.

The spores of A. apis appear well-adapted to their ecological niche, as is required of a successful pathogen. The optimum temperature for germination of this fungus is similar to that required for its sporulation and is within the range for vegetative growth. This is not always the case for fungi, but depends upon their life cycles. For A. apis all stages occur within the brood area of the bee colony and so it is fitting that all stages of the life cycle reach optimum at 30° to 35°C.

Effect of pH on spore germination

Introduction

There is even less known of the effect of environmental pH on germination or on vegetative growth and sporulation. Huber (1958) described the hyphal growth of A. apis as sensitive to alkali, since it developed poorly at pH 7.2 under his experimental conditions and Pedersen (1974) stated an optimum of pH 5 for hyphal growth at 30°C. Vegetative growth, therefore, appears to do best in acidic conditions, although the optimum pH value may depend upon other prevailing environmental conditions, for example, nutrient availability and temperature. Classey (1980) and Smith (1984) demonstrated that optimum temperatures for hyphal growth of A. apis varied according to environmental pH. There is no information to indicate whether this

is also the case for sporulation, and more importantly, in the current context, for germination.

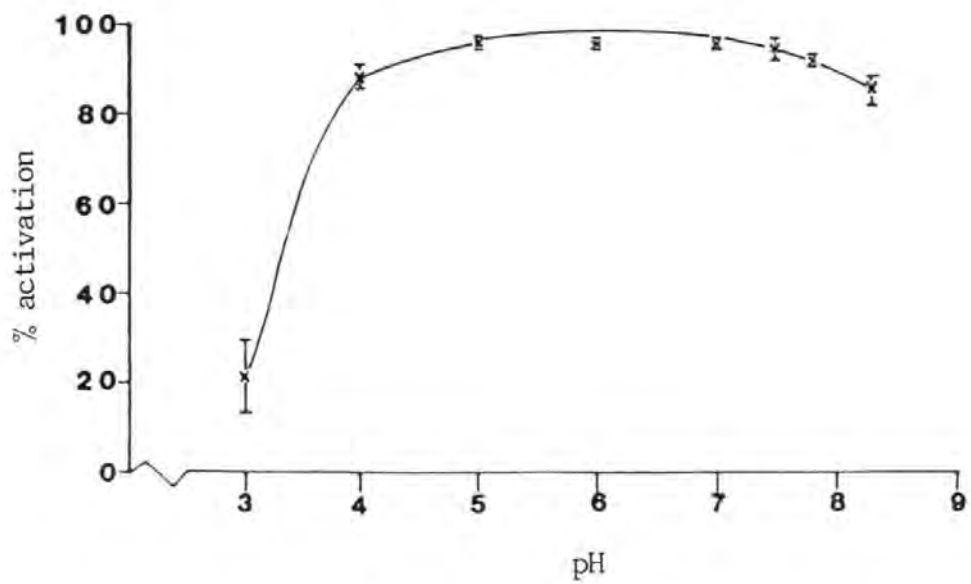
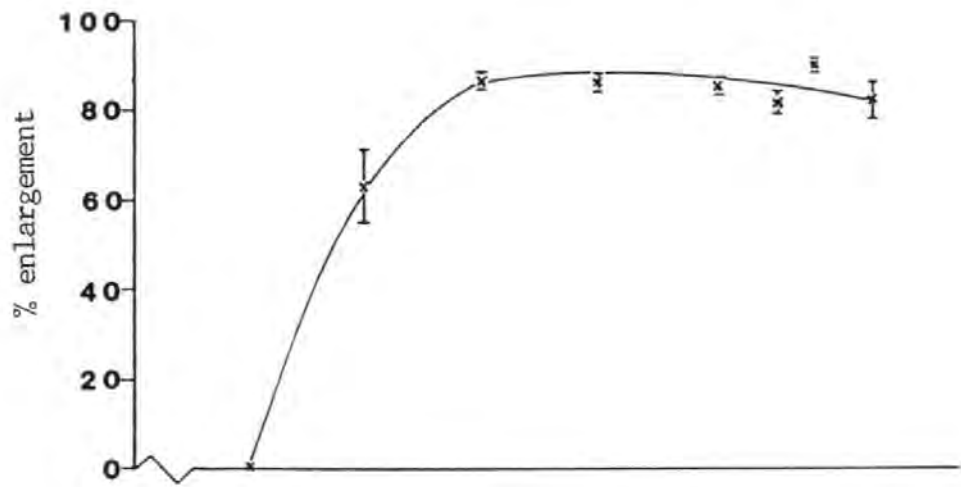
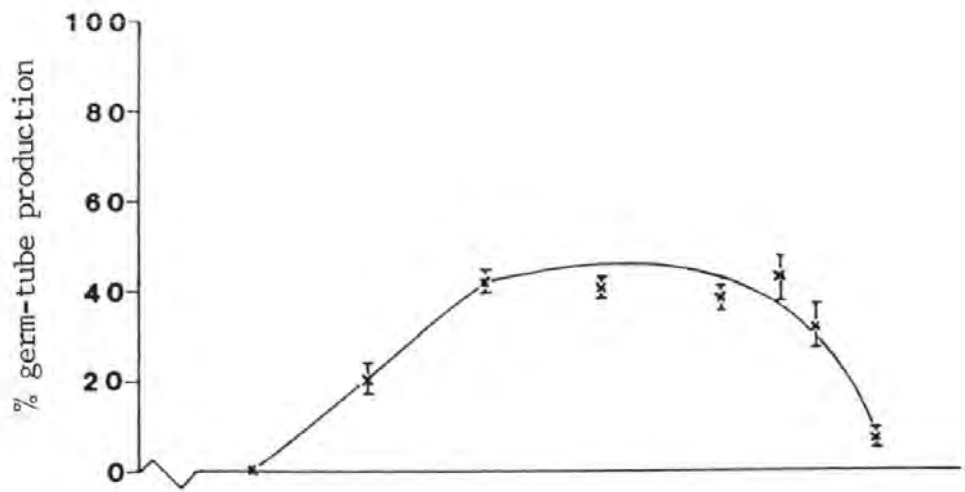
Method

Spores were germinated in GYO liquid media, the pH of which were altered, prior to autoclaving, using 0.1 M HCl and 0.1 M NaOH. The pH of the media were again tested after sterilisation. Buffering was regarded as unnecessary while studying these brief, early stages of growth.

Results and Discussion

The effect of environmental pH on spore germination is indicated in Figure 5. This shows that activation was supported at all the pH values tested, with no significant differences in the percentages achieved at pH 5 to 7.8 ($F = 0.73$; $P > 0.05$, $df = 4,44$), but a slight reduction at pH 4 and pH 8.3 and a dramatic decrease at pH 3. There was a similar trend for enlargement, where there appeared to be no significant differences in the percentages achieved at pH 5 to 8.3 ($F = 1.65$; $P > 0.05$, $df = 5,50$), but a reduction at pH 4 and a negligible percentage at pH 3. Again, for germ-tube production, there were no significant differences between the percentages produced at pH 5 to 7.8 ($F = 1.31$; $P > 0.05$, $df = 4,40$); reduced percentages at pH 4 and 8.3; and no germ-tube production at pH 3. Therefore, the 3 stages of germination appear independent of pH within the range of pH 5 to 7.8. Activation is probably less affected by lower values of pH, since it largely involves only physical processes, rather than the complexes of biochemical reactions involved in enlargement and germ-tube production. The upper limits of pH which would support activation and enlargement were not determined, since maintaining high pH values during sterilisation of media required buffered solutions and the results would not then be comparable to those

Figure 5: Graphs showing the mean percentages, \pm SE, of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in liquid GYO media of various pH in 10% CO₂ at 35°C.



obtained from the non-buffered systems. Also, media of high pH would react with the carbon dioxide required by the spores for activation.

There can be no comparison of these results with previous work for this particular fungus, but a broad pH optimum for germination is characteristic of many fungi, although the optima for most occur on the acidic side (Sussman and Halvorson, 1966). Deacon (1980) noted that in vitro many fungi will grow over a wide pH range and show broad optima, but this is in pure culture with readily available nutrients. The nature of the optima, however, may alter should environmental conditions be different. In this case A. apis spores germinated well at pH 5 to 7.8, when yeast extract and glucose were readily available to support germination, but in vivo these are unlikely to be the conditions and the spores may consequently respond differently to changes in environmental pH.

In the hive environment there are several substances with which the spores are likely to come into contact, which have acidic properties. Honeys exhibit a pH range of pH 3.2 to 4.5 (White, 1962); pollens, a range of pH 3.64 to 4.58 and brood food, pH 4 to 4.3 (Herbert and Shimanuki, 1983). According to the results obtained here, all are too acidic to support maximum germ-tube production. Indeed, the acidic property of honey is one of its defences against the growth of contaminating micro-organisms and this may be so for pollen and brood food too. Also, A. apis spores may not germinate in these environmental conditions, since they are not necessarily associated with a living larva, which the fungus requires in order to complete its life cycle. The pH of larval mid-gut contents, however, at pH 7.5 (Bignell and Heath, 1985) and of the haemolymph of a mature larva, prior to metamorphosis, at pH 6.8 (Bishop, 1923), appear much more favourable to spore germination.

Unfortunately, the effects of the interaction of pH and temperature on spore germination have not been investigated. The effect of temperature on germination was studied at the pH of GYO medium (pH 6.8) and the effect of pH was studied at the optimum temperature determined for germination in a medium of pH 6.8. It is not realistic to study environmental conditions in isolation, since in vivo all conditions will interact, for example, optimum temperature may vary according to environmental pH, as demonstrated for vegetative growth (Classey, 1980). However, such studies do give initial information regarding the types of environmental conditions which are favourable or unfavourable to spore germination.

2.4 NUTRITIONAL REQUIREMENTS FOR SPORE GERMINATION

Introduction

A. apis has been grown vegetatively on numerous types of medium, for example, potato bouillon agar (Betts, 1915-24); Coon medium plus yeast extract (Maurizio, 1935); malt agar (Spiltoir, 1955; Bailey, 1963); Czapek-Dox agar plus yeast extract, milk agar (Huber, 1958) and Sabouraud agar plus yeast extract (Thomas and Luce, 1972; Marin et al., 1977). All of these have contained complex materials. There is no published defined medium, but undergraduate students at Plymouth Polytechnic (Craven, 1980; Castle, 1981; Patel, 1983) have examined the nutritional requirements for growth of A. apis and designed a fully synthetic medium containing glucose, asparagine, salts (KCl, K_2HPO_4 , $MgSO_4$) and vitamins (biotin, nicotinic acid, pyridoxine, riboflavin, thiamine). This is called CCP medium, after the originators, and is described in Appendix III.

There is no such defined medium to support the germination of A. apis spores. Bailey (1966) germinated the spores under the surface of semi-solid agar containing glucose, potassium hydrogen phosphate, starch and yeast extract, a medium designed for Streptococcus pluton (now Melissococcus pluton) (Bailey, 1963). Kish (1980) attempted to germinate the spores of A. proliferperda using media containing various materials, such as amino acids, biotin, casein and found most germination was achieved using V-8 juice (an extract of 8 vegetables), glucose and yeast extract, at pH 7. This medium was also used by Stephen et al. (1982) to germinate spores of A. aggregata.

Most recently, Heath and Gaze (1987, in press) adapted Bailey's medium, for the activation of A. apis spores, by omitting the starch component, since this did not appear to enhance the amount of activation achieved with incubation in a 12.5% carbon dioxide

atmosphere at 30°C. Subsequently, potassium hydrogen phosphate has also been excluded because as much activation is achieved using glucose and yeast extract only in 1.5% agar.

In the light of the requirement of A. apis spores for carbon dioxide for activation it has not been determined whether exogenous nutrient supplies are vital to the spores for the later stages of germination, or whether after initial activation the germination process can be supported by endogenous reserves stored in the spores during their formation. If exogenous supplies are required, their exact nature requires investigation to elucidate interactions at the infection site.

The nutritional requirements for the germination of fungal spores vary considerably. Occasionally such spores have sufficient endogenous reserves to support germination; for example, the uredospores of the rust fungus Puccinia graminis tritici require only oxygen and water (Shu et al., 1954) and the conidia of Cochliobolus miyabeanus germinate in water, utilising primarily carbohydrate reserves (Oku, 1960); but most commonly exogenous supplies are required. Indeed, some fungi have been shown to contain endogenous reserves, yet still require some nutrients to be supplied by the environment to support germination. Fusarium conidia, for example, contain substantial quantities of lipid, but require carbon and nitrogen sources for germination (Cochrane et al., 1963a, b). Similarly, 20% of the dry weight of spores of Myrothecium verrucaria are nutrient reserves, but exogenous supplies are necessary for germination (Mandels et al., 1965). Such requirements may serve to ensure spores only germinate where there are sufficient nutrients to support further growth.

Most frequently an exogenous carbon source is needed, for

example, Ustilago maydis requires sucrose (Caltrider and Gottlieb, 1966). Others require a nitrogen source as well as a carbon source, for example, the conidia of Penicillium notatum and Trichoderma lignorum (Martin and Nicolás, 1970). Some have specific requirements for amino acids, for example, Aspergillus niger requires alanine or proline (Yanagita, 1957); and some for vitamins, thus, biotin and thiamine stimulate the germination of Botrytis cinerea (Hawker, 1950, cited by Gottlieb, 1978) and thiamine stimulates Gigaspora margarita (Siquiera and Hubbell, 1982). The nutritional requirements may be for the complete germination process or for one of the stages. Penicillium atrovirens, for instance, requires exogenous glucose and phosphate to support the enlargement phase, but requires an additional nutrient - a nitrogen source, for germ-tube production (Gottlieb and Tripathi, 1968).

In the case of entomogenous fungi, it was noted by MacLeod (1954) that genera such as Beauveria or Metarrhizium, which infected a wide host range, had relatively simple nutritional requirements for growth - a salts and glucose medium with an inorganic nitrogen source - whereas those fungal genera with a narrow host range, such as Hirsutella and Spicaria had complex nutritional requirements, including an organic nitrogen source. Since A. apis has such a narrow host range, it might, on this basis, be expected to have fairly complex nutritional requirements.

Methods

Media solidified with 1.5% agar, and containing 0.005% oxytetracycline plus various nutrients, were prepared and the pH adjusted to that of GYO (pH 6.8), using 0.1 M HCl and 0.1 M NaOH. In the preliminary experiment GYO was compared with media lacking either or both of its major constituents. Subsequently, a range of

individual carbon and nitrogen sources were incorporated. Each medium was then tested for its relative support of the stages of germination, by inoculating it with spores from mummified larvae and making counts, after incubation in 10% carbon dioxide at 35°C, as described in section 2.2.

Results and Discussion

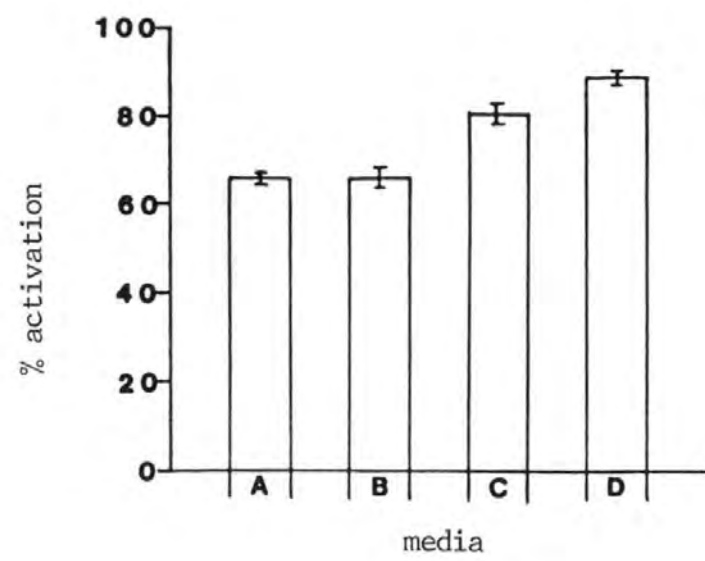
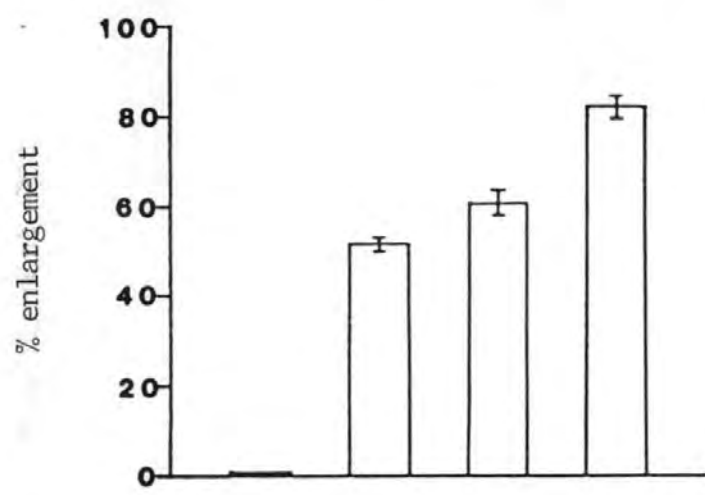
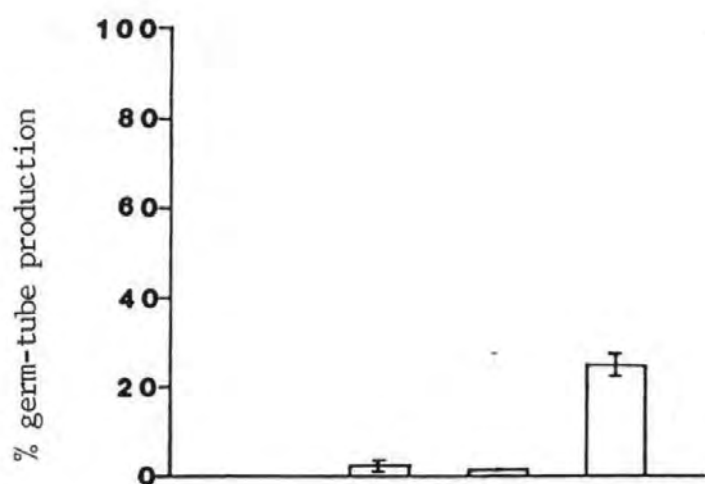
The requirement by *A. apis* spores for exogenous nutrients to support germination

The results of the preliminary experiment, as shown in Figure 6, establish that exogenous nutrients are required by *A. apis* spores for the two later stages of germination, enlargement and germ-tube production. Activation, however, occurred on a medium containing no nutrients, supporting the idea that this is only a physical process, with no energy requirements. A simple trigger, in this case carbon dioxide, is sufficient to activate the germination process, but in order for the process to continue nutrients must be supplied by the environment. This reinforces the idea that the spore enlargement stage is not merely a physical swelling phase caused by the imbibition of water, but is a metabolically active growth phase with energy requirements. Mandels and Darby (1953) demonstrated this by using metabolic inhibitors such as sodium azide to prevent the swelling of spores of *Myrothecium verrucaria*.

For *A. apis* spores, a carbon source alone, glucose in this experiment, supported spore enlargement, but a negligible amount of germ-tube production. Similarly, with only a potential nitrogen source, yeast extract, there was no substantial germ-tube production, although there were significantly higher percentages of activated and enlarged spores than supported by either water (activation: $t = 4.05$; $P < 0.005$, $df = 30$; enlargement: $t = 17.47$; $P < 0.005$, $df = 30$) or glucose only

Figure 6: Histograms representing the mean percentages, \pm SE , of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in 10% CO₂ at 35°C on solid media containing:-

- A: no added nutrients
- B: 1% glucose
- C: 1% yeast extract
- D: 1% glucose and 1% yeast extract



(activation: $t = 4.4$; $P < 0.005$, $df = 36$; enlargement: $t = 2.88$; $P < 0.005$, $df = 36$). A medium containing both glucose and yeast extract supported most activation and enlargement, plus 24% germ-tube production. Therefore, the results indicate that a carbon or nitrogen source alone is insufficient to support maximum activation or enlargement, or any germ-tube production - a combination of nutrients is required.

A dependency on exogenous nutrients to support germination may be considered ecologically advantageous, since it ensures germination will not occur in an environment unable to support further growth and development. Such nutrients may be required quantitatively, to supplement insufficient endogenous reserves, or qualitatively, to provide specific chemicals or key respiratory intermediates not available within the spore. The two types, however, are not necessarily exclusive (Cochrane et al., 1963a).

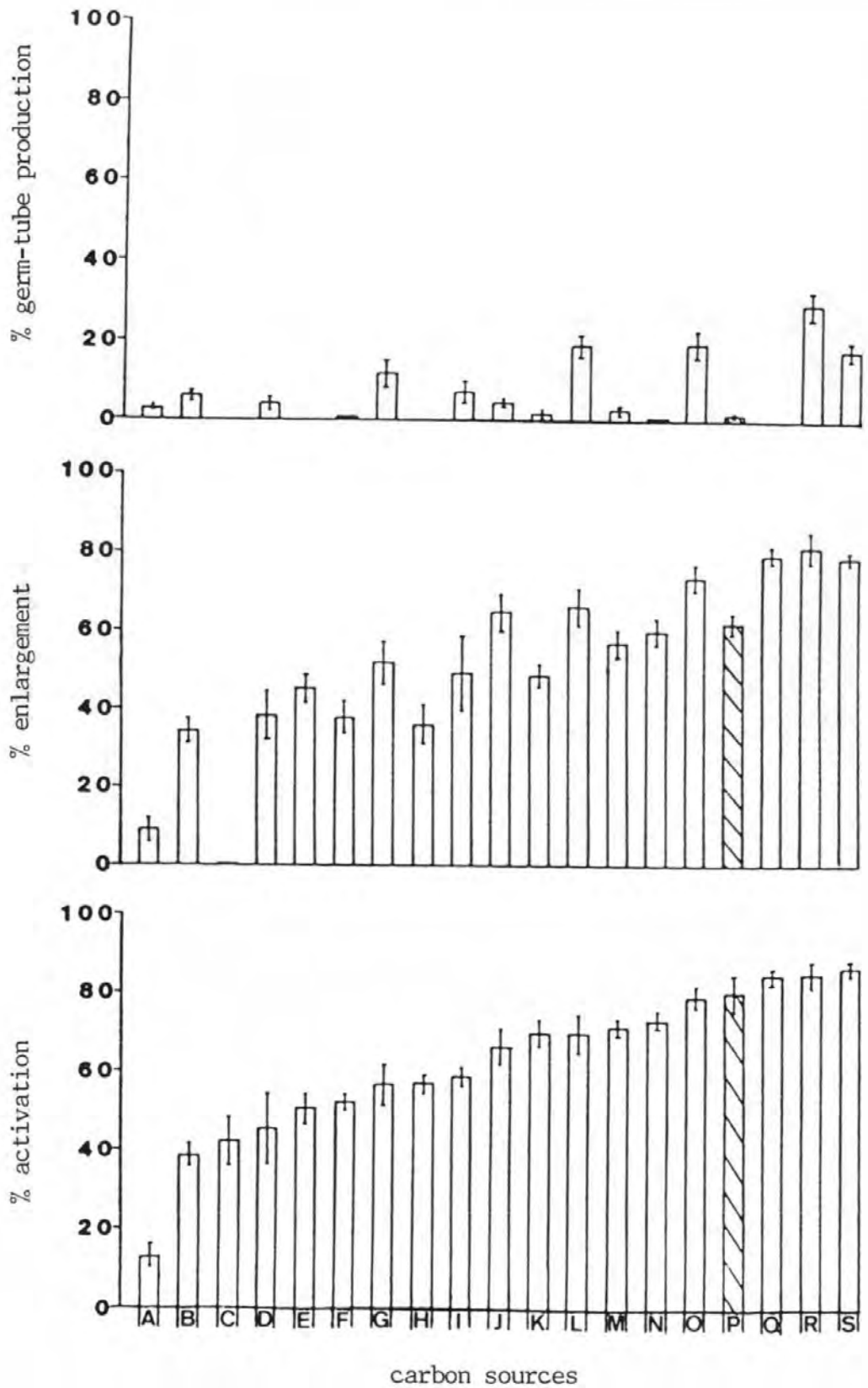
Carbon source requirements for spore germination

The most common nutrient deficiency for germination is for a carbon source, specifically a carbohydrate (Gottlieb, 1978) and the ability to overcome this, once it is supplied by the environment, is dependent on the existence and proper functioning of (a) specific carrier molecules to transport the nutrient across the plasma membrane into the cell and (b) biochemical pathways to metabolise the nutrient molecules (Garraway and Evans, 1984). The carrier molecules, or permeases, may be specific for specific molecules or for a group of similar molecules, or may be non-specific.

Of the various chemicals tested here as potential carbon sources to support germination, it can be seen from Figure 7 that only 5 supported percentages of germ-tube production above 10% - galactose (11.5%), trehalose (17.9%), mannose (18.6%), fructose (19%)

Figure 7: Histograms representing the mean percentages, \pm SE , of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in 10% CO₂ at 35°C on solid media containing 1% yeast extract and 1% of one of the following carbon sources:-

- A: starch
- B: n-acetyl glucosamine
- C: acetic acid
- D: mannitol
- E: xylose
- F: maltose
- G: galactose
- H: ribose
- I: chitin
- J: glycogen
- K: arabinose
- L: mannose
- M: sucrose
- N: glycerol
- O: fructose
- P: no carbon source
- Q: citric acid
- R: glucose
- S: trehalose



and glucose (29%). Glucose, fructose and trehalose all supported percentages of activation not significantly different from each other or from that produced by yeast extract only ($F = 1.08$; $P > 0.05$, $df = 3,40$), but each supported percentages of enlargement significantly higher than that produced by yeast extract only (glucose: $t = 3.94$; $P < 0.005$, $df = 26$; fructose: $t = 2.75$; $P < 0.01$, $df = 26$; trehalose: $t = 3.86$; $P < 0.005$, $df = 26$). Many of the alternative carbon sources significantly reduced the percentages of activation to below that which occurred with yeast extract only. This includes galactose ($t = 4.6$; $P < 0.005$, $df = 26$), which also supported some germ-tube production. Others severely inhibited enlargement too and this was most noticeable for acetic acid and starch. The former completely inhibited enlargement, although 42% activation was maintained, while the latter only supported 13% activation and 9% enlargement. Such substances must interfere with and prevent the normal utilisation of yeast extract, resulting in an apparent inhibition of germination. This interference may occur at the transportation stage, where non-specific permeases may transport or may be blocked by unsuitable carbon source molecules. Those substances which also cause a reduction in activation must interfere with what are probably simple physical changes, that lead to spore activation.

The carbon sources supporting most germination are those that are readily utilisable, that is, readily convertible to glucose or acetyl CoA. Since glucose, a six-carbon monosaccharide, supported the highest levels of germ-tube production, an efficient transport system must be available to the activated spore to transport the glucose molecules into the spore ready for glycolysis. The changes in spore wall permeability associated with activation may be involved,

as changes in the spore wall may reveal the necessary permease. This same system may also transport fructose, mannose and galactose, all six-carbon monosaccharides, although once inside the cell some may be less efficiently metabolised than others, leading to the observed differences in germ-tube production. Trehalose, a glucose disaccharide, was also utilised by the spores, but is unlikely to be transported into the cell as a disaccharide, since fungi tend to produce exoenzymes to cleave complex molecules prior to their transportation. Consequently, activation may have altered cell wall permeability such that exoenzymes, for example, trehalase, can be released into the environment. Once trehalose is degraded, the glucose moieties could be transported by the monosaccharide transport system.

Not only are the preferred carbohydrates readily metabolisable molecules, but they are also to be found in the hive environment. Glucose and fructose are the major components of honey. According to a survey by White (1962) glucose accounts, on average, for 31.3% of honey, while fructose accounts for 38.2%. Maltose was also present at an average level of 7.3%, sucrose at 1.3% and there were negligible amounts of various other sugars depending upon the nectar source for the honey. *A. apis* spores did not appear able to utilise the disaccharides sucrose and maltose, presumably since they lacked the necessary cleaving enzymes. The non-reducing disaccharide trehalose was utilised. This is a common blood sugar in many insects, and although not as abundant in the honey bee as in some other insects, it is present (Wyatt, 1961, cited by Wigglesworth, 1972). This source, however, would only be available once the spores had infected the larvae. Trehalose is common in fungi and especially large amounts are often found in spores, where it acts as a storage compound for

glucose. In the spores of M. verrucaria 18.6% of the dry weight is trehalose (Mandels et al., 1965). If the spores of A. apis utilise trehalose in this way, they would probably contain a constitutive trehalase. Mannose and galactose, the remaining carbohydrates apparently utilised by germinating spores, are unlikely to be found in any significant quantities in the hive environment. In vitro they may be transported and metabolised due to their similarity to glucose and fructose, but this situation would not arise in vivo.

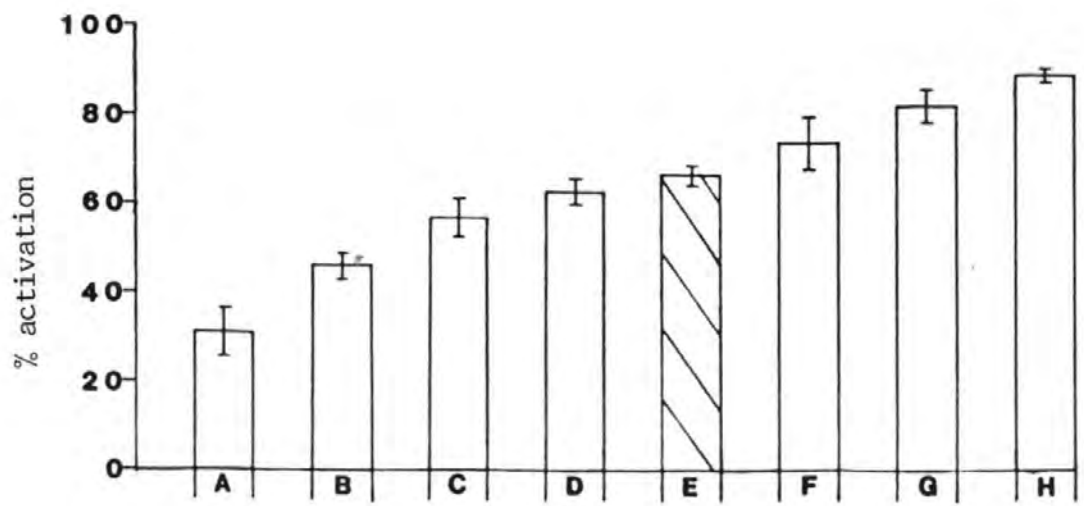
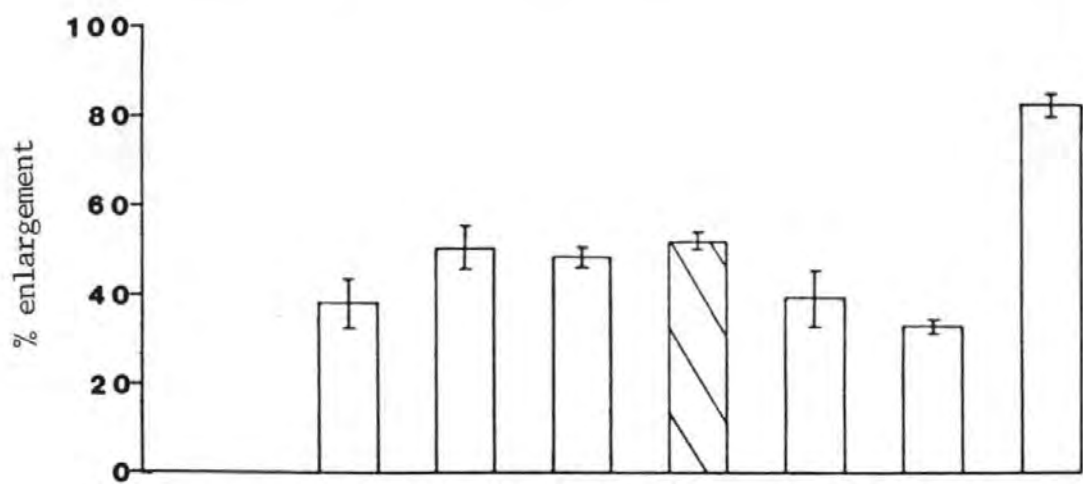
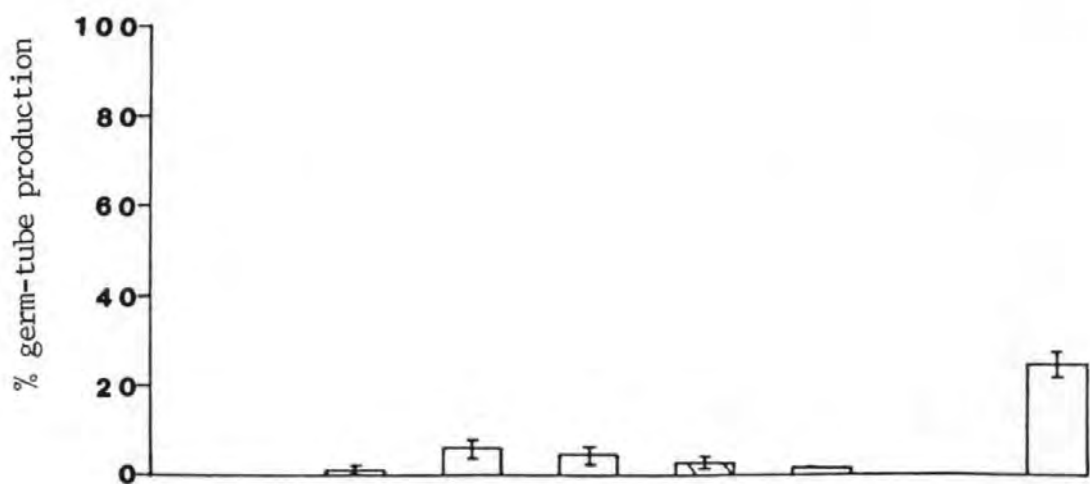
Indeed, the carbon sources recognised here as those best supporting germination are similar to those identified as best supporting vegetative growth. Gochnauer and Margetts (1979) found glucose, fructose and galactose to produce the most vegetative growth from the few carbohydrates they tested and found these three to support sporulation, but trehalose to support most sporulation. Meanwhile, Craven (1980) showed glucose, fructose and trehalose to be the best carbohydrate sources for the strains of A. apis she tested. Again, these would be expected to be the preferred sources, since they are the most abundant carbohydrates to be found in the larva, from which the vegetative stage must obtain sufficient nutrients for growth and sporulation.

Nitrogen source requirements for spore germination

The nitrogen requirements of the spores appear to be fairly complex. Of the inorganic sources tested, as shown in Figure 8, only ammonium sulphate supported significantly greater activation than glucose only ($t = 3.4$; $P < 0.005$, $df = 22$), a percentage not significantly different from that supported by GYO medium ($t = 2.0$; $P > 0.05$, $df = 20$). Sodium, potassium and ammonium nitrates supported percentages of activation not significantly different from each other or from that supported by glucose only ($F = 2.93$; $P > 0.05$, $df = 3,38$), while

Figure 8: Histograms representing the mean percentages, \pm SE , of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in 10% CO₂ at 35°C on solid media containing 1% glucose and 1% of one of the following inorganic nitrogen sources:-

- A: ammonium chloride
- B: calcium nitrate
- C: potassium nitrate
- D: sodium nitrate
- E: no nitrogen source
- F: ammonium nitrate
- G: ammonium sulphate
- H: yeast extract



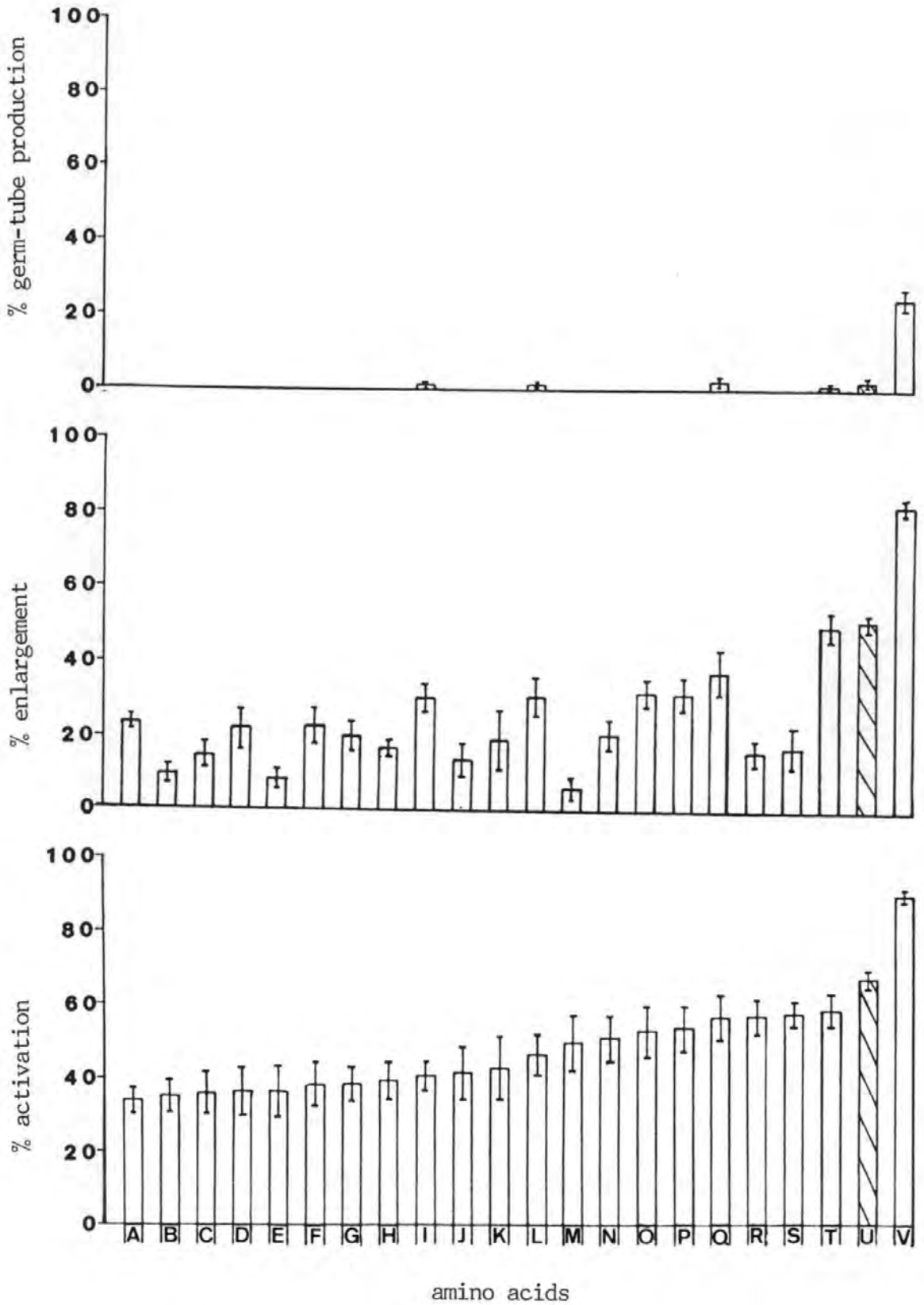
inorganic nitrogen sources

calcium nitrate and ammonium chloride both supported significantly less than glucose only ($\text{Ca}(\text{NO}_3)_2$: $t = 3.94$; $P < 0.005$, $df = 20$; NH_4Cl : $t = 6.49$; $P < 0.005$, $df = 20$). A slightly different pattern emerged for spore enlargement. Ammonium sulphate and calcium nitrate both supported significantly lower percentages than glucose only ($(\text{NH}_4)_2\text{SO}_4$: $t = 5.84$; $P < 0.005$, $df = 22$; $\text{Ca}(\text{NO}_3)_2$: $t = 3.06$; $P < 0.005$, $df = 20$) and ammonium chloride completely inhibited enlargement. Ammonium, sodium and potassium nitrates supported percentages not significantly different from each other or from that supported by glucose only ($F = 2.54$; $P > 0.05$, $df = 3,41$), but no inorganic source supported the 82% enlargement produced by yeast extract. This pattern was repeated for germ-tube production: ammonium sulphate, ammonium and calcium nitrates, and ammonium chloride supported negligible amounts, or completely inhibited outgrowth; sodium and potassium nitrates supported 4 to 5%, not significantly different from glucose only ($F = 0.93$; $P > 0.05$, $df = 2,33$); whereas yeast extract supported 25% germ-tube production. In conclusion, no inorganic nitrogen source tested here was capable of supporting the amount of germination produced by yeast extract. Indeed, some of the inorganic sources appeared to interfere with the utilisation of glucose, resulting in reduced percentages of activation and enlargement when their performance was compared to that obtained with glucose only.

Individual amino acids were also tested as potential nitrogen sources, but all proved unsuccessful at supporting germ-tube production, as shown in Figure 9. All twenty of the acids tested appeared to produce reduced activation percentages when these were compared to the results obtained with glucose only, although the amount of reduction was variable depending on the amino acid. There was a

Figure 9: Histograms representing the mean percentages, \pm SE , of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in 10% CO₂ at 35°C on solid media containing 1% glucose and 0.1% of one of the following amino acids:-

- A: histidine
- B: asparagine
- C: leucine
- D: isoleucine
- E: phenylalanine
- F: alanine
- G: valine
- H: glycine
- I: proline (imino acid)
- J: cysteine
- K: threonine
- L: arginine
- M: tryptophan
- N: serine
- O: glutamic acid
- P: methionine
- Q: lysine
- R: tyrosine
- S: aspartic acid
- T: glutamine
- U: no nitrogen source
- V: yeast extract



similar pattern for enlargement, although glutamine supported a percentage not significantly different from that supported by glucose only ($t = 0.44$; $P > 0.05$, $df = 24$). The remaining amino acids supported less enlargement. All supported a negligible amount or no germ-tube production. An important result was obtained when a mixture of amino acids was included in the medium and this can be seen in Figure 10. This mixture supported an equal amount of germ-tube production as yeast extract ($t = 0.59$; $P > 0.05$, $df = 25$), but the percentages of activation and enlargement were not significantly different from those produced by glucose only (activation: $t = 1.48$; $P > 0.05$, $df = 26$; enlargement: $t = 0.29$; $P > 0.05$, $df = 26$). This suggests an amino acid mixture is sufficient to support germ-tube production, but another constituent of yeast extract may be responsible for enhancing spore enlargement. Consequently, a mixture of B vitamins (biotin, nicotinic acid, pyridoxine, riboflavin, thiamine) was added to a medium containing glucose and an amino acid mixture and this was tested for spore germination. The results are shown in Figure 10. There proved to be no significant differences in the percentages achieved at any stage of germination, whether or not vitamins were included with the glucose and amino acids (activation: $t = 0.3$; $P > 0.05$, $df = 19$; enlargement: $t = 0.6$; $P > 0.05$, $df = 19$; germ-tube production: $t = 0.64$; $P > 0.05$, $df = 19$). Therefore, it may be the concentrations or ratios of amino acids in the yeast extract, which are not reflected in the amino acid mixture, that are responsible for the observed differences in enlargement; or the mineral salts included in yeast extract may be important.

If a mixture of amino acids is required for the production of germ-tubes, there are plenty in the hive environment. Pollen is a rich proteinaceous source, and is vital to nurse bees for the

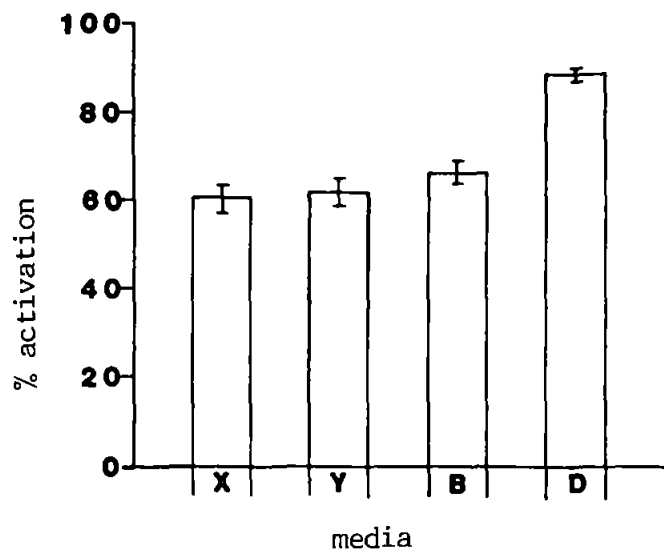
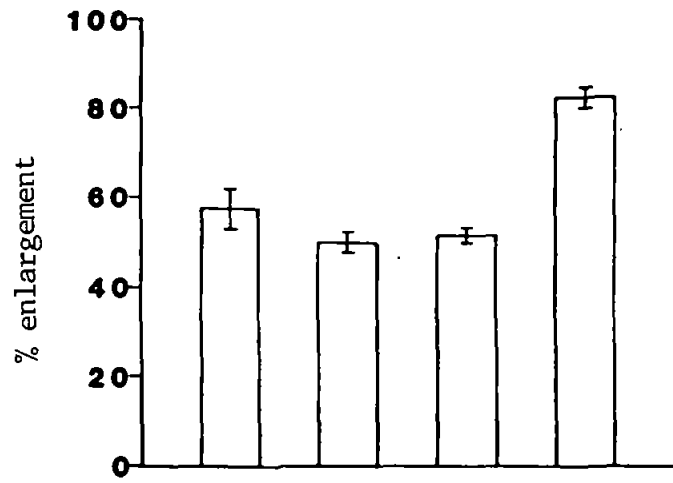
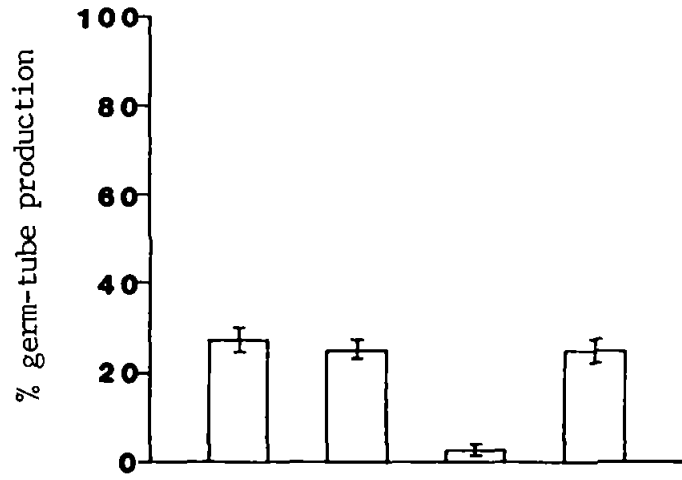
Figure 10: Histograms representing the mean percentages, \pm SE, of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in 10% CO₂ at 35°C on solid media containing:-

X: 1% glucose and a mixture of 19 amino acids (alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, valine) and proline, each at a concentration of 0.01%.

Y: Medium X plus a mixture of 5 vitamins (biotin, nicotinic acid, pyridoxine, riboflavin, thiamine) each at a concentration of 10 $\mu\text{g}/\text{cm}^3$.

B: 1% glucose

D: 1% glucose and 1% yeast extract.



production of 'bee milk', which is fed to worker bee larvae as brood food or to queen larvae as 'royal jelly'. The latter has been more commonly chemically analysed and Howe et al. (1984) quoted a protein content of 11.9% for royal jelly. Since the larval stage is the growth phase of insect development the larvae can be considered a rich source of protein too, although this is only available once the larvae are infected. However, young larvae are constantly fed brood food and for the first three days of larval development may be seen floating on a pool of food within their cells - a potentially rich nutritional environment for fungal development.

Consequently, the results here confirm the hypothesis of MacLeod (1954), in that A. apis spores appear to have fairly complex nutritional requirements for germination. Although those carbohydrates which support enlargement were determined, the remaining nutrients necessary to support germ-tube emergence were not elucidated. Indeed, from the practical aspect no better medium than GYO was found. However, this only supports approximately 25% germ-tube production. In vivo, germ-tube production must be much higher than this in order that the pathogen is efficient in infecting larvae. It follows that the honey bee environment must supply additional nutrients not yet identified.

2.5 THE SPORE BALL PHENOMENON

Introduction

During experiments involving the germination of A. apis spores on a solid medium, it was observed that spore balls often showed enhanced germination when compared with individual spores. Some spores become displaced from their original spore ball when they enlarge, but a group of spores in the advanced stages of germination may still be recognised as part of a spore ball, showing more advanced germination than individual spores scattered on the agar. There appears to be an additional factor affecting germination within the spore ball, resulting in faster germination. Since a method of germinating these spores has only recently been established there is no published information describing the germination of spore balls and individual spores. Also, Ascospaera species are unusual in their spore ball arrangement, where several asci have aggregated to form a spore ball, so Ascospaera cannot easily be compared to other Ascomycetes.

When germination counts were made, as reported in the earlier parts of this chapter, only individual spores were counted, in order to minimise the influence of this spore ball effect. However, this is an interesting observation, requiring investigation.

Three possibilities to explain this effect have been considered:-

(a) Within the spore ball the carbon dioxide concentration increases as a result of the accumulation of respiratory carbon dioxide from germinating spores, and this subsequently stimulates the germination of adjacent spores. If this were the case, incubation of spores in high carbon dioxide concentrations would equalise the availability of carbon dioxide to each spore and so eliminate the spore ball effect. However, even in high carbon dioxide concentrations the spore ball

effect remains (Gaze, unpublished data).

(b) The spores are stimulated by a product of the metabolism of adjacent spores. Once biochemical pathways in one spore have been induced, a metabolite may be released which stimulates the germination of those spores in close proximity. The action of this stimulatory substance may be direct, possibly as an essential nutrient, or indirect by counter-acting a self-inhibitory substance within dormant spores.

The stimulatory and inhibitory effects of germinating spores, on spores of the same species or of another species, has long been recognised. Several observations suggest the presence of self-inhibitors, for example, the failure of spores to germinate on the parent mycelium or within the spore fructification. Such inhibition is considered as a possible mechanism for the maintenance of constitutive dormancy, increasing the chances of survival by ensuring spores will only germinate after dispersal (Gottlieb, 1978). Exogenous dormancy may also be maintained by inhibitory substances indicating an unfavourable environment.

(c) The existence of supplementary nutrients within the spore balls may stimulate germination. These may be components of some excess protoplasm which was not completely absorbed during the formation of individual spores within the spore cyst; or may even be the products of commensal bacteria. At germination these may supplement nutrients supplied by the environment. Chung and Wilcoxson (1969) found the conidial matrix of Phoma medicaginis to promote the germination of conidia and speculated this material either contained a nutrient to support growth; made the conidia resistant to the action of a self-inhibitor; or aided survival in adverse conditions. This latter theory was the case for Empusa muscae, where a protoplasmic coating on primary conidia served as a means of protection against desiccation.

If the conidia were placed in a droplet of water, the coating was washed away and the conidia would not germinate completely (Baird, 1957).

Experiments were, therefore, performed to investigate the cause of the spore ball phenomenon.

Production of mutual stimulating substances by germinating spores

Method

Spores in a concentrated suspension (the spore scrapings from 2 mummified larvae in 5 cm³ GYO medium) were germinated and the suspension then centrifuged (5 minutes at 5000 r.p.m.) in an MSE bench centrifuge. The supernatant was decanted and filtered through a sterile Millipore filter (Whatman cellulose nitrate membrane filters, pore size 0.45 μm). A sample was removed aseptically, using a sterile Pasteur pipette, and observed microscopically, to ensure no spores were present in the filtrate. The filtrate was returned to a sterile 25 cm³ conical flask, re-inoculated and re-incubated. A control medium, no constituent of which had been in contact with germinating spores, was also inoculated and re-incubated.

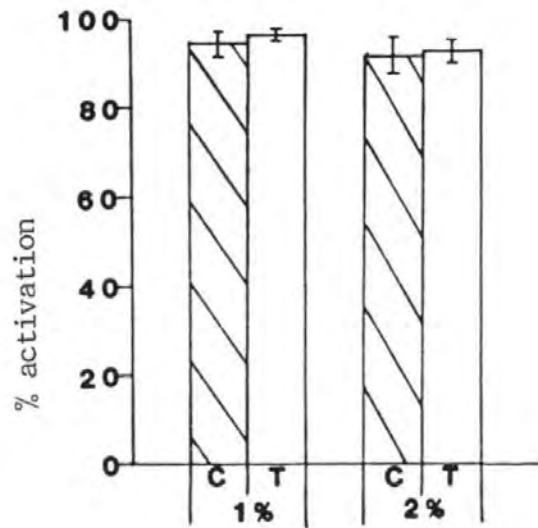
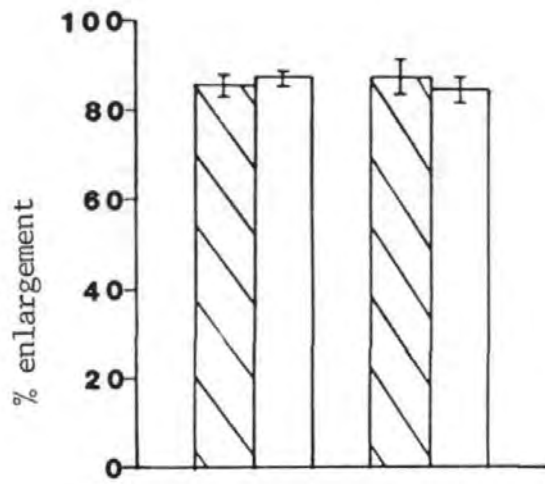
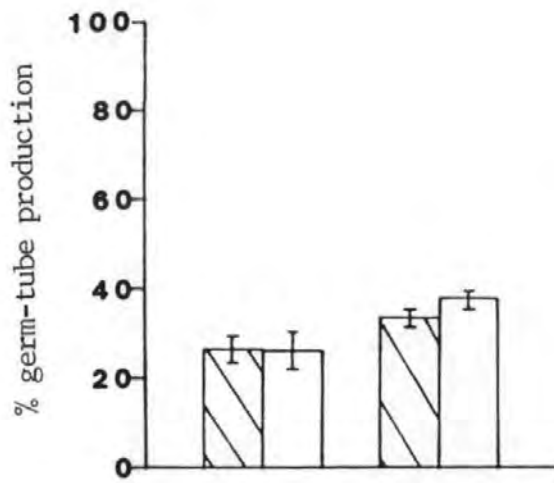
After this second incubation period spore samples were removed from the flasks and estimations of percentages of activation, enlargement and germ-tube production were made.

This experiment was repeated using a 2% glucose and yeast extract medium.

Results and Discussion

The results from this experiment, as shown in Figure 11, indicate there is no stimulatory effect produced by germinating spores. If a stimulatory metabolite, or a positive balance between stimulator and inhibitor, had resulted from germination, an increase in germination when spores were incubated in a previously utilised

Figure 11: Histograms representing the mean percentages, \pm SE , of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in 10% CO₂ at 35°C in fresh (C) or previously utilised (T) 1% or 2% GYO liquid medium.



media

medium would have been expected. However, this was not the case, there were no significant differences in the percentages of spores activated ($t = 0.77$; $P > 0.05$, $df = 14$), enlarged ($t = 0.51$; $P > 0.05$, $df = 14$) or producing germ-tubes ($t = 2.12$; $P > 0.05$, $df = 14$) after incubation in fresh or previously utilised medium. To ensure there were sufficient nutrients in the medium to support the germination of a second batch of spores, a 2% glucose and yeast extract medium was also tested. If a 1% medium had been insufficient, the levels of germination may have resulted from a stimulatory effect, despite some nutrient deficiency. But this did not appear to be so, even with a 2% medium there were no significant differences in the percentages of spores activated ($t = 0.17$; $P > 0.05$, $df = 13$), enlarged ($t = 0.6$; $P > 0.05$, $df = 13$), or producing germ-tubes ($t = 1.35$; $P > 0.05$, $df = 13$) after incubation in fresh or previously utilised medium.

This experiment also suggested that no inhibitory effects were produced by the germinating spores, since germination in a previously utilised medium did not result in a reduction in any stage of germination.

The spore ball phenomenon observed with A. apis might have been the result of self-stimulation. Although a high spore concentration usually results in the inhibition of germination, ascospores occasionally appear as exceptions, where germination is sometimes enhanced or unchanged by spore concentration (Cerelin and Fergus, 1971; Wong and Bau, 1978). Also, in the Basidiomycete Agaricus campestris, spores were found to germinate best in groups or in close proximity to a growing mycelium and this was thought to be due to a self-stimulator (Ferguson, 1902, cited by De Zeeuw, 1943). There is also evidence for low spore concentrations limiting germination, which might be attributed to a deficiency in each spore of some

metabolite vital for activation, therefore the spore concentration needs to be increased until the critical level for this diffusate is reached (Gottlieb, 1978). In some instances, self-inhibitors and self-stimulators may be released simultaneously from spores with the final result depending on spore concentration. This was observed for Uromyces phaseoli (Yarwood, 1956) and for Uromyces ciceris-arietini where self-stimulation resulted in an increased elongation of germ-tubes (Bahadar and Sinha, 1966). Self-stimulation may also play a role in the formation of infection apparatus, such as appressoria or infection pegs, after germ-tube emergence (Allen, 1957; French et al., 1957). Several chemical stimulators have been isolated from uredospores, including phenolic compounds and nonanal, which stimulate germination by counter-acting the effects of inhibitory substances. Therefore, rather than the presence or absence of a stimulatory or inhibitory substance, the balance between them may be of more importance. In the experimental conditions tested here, however, no evidence was obtained for either effect.

Presence of nutrient materials in spore washings

Method

Using a sterile mounted needle spore cysts were scraped from 10 to 15 mummified larvae into a sterile Potter homogeniser and were homogenised in 5 cm³ of sterile distilled water, until the majority of spore balls had been disrupted. This was checked by aseptically removing a sample of spore suspension, using a sterile Pasteur pipette, and examining it microscopically. No evidence was found for the disruption of individual spores by the homogenisation process. The spore suspension was then centrifuged (5 minutes at 5000 r.p.m.) and the supernatant decanted and filtered through a Millipore filter (pore size 0.45 µm). A sample of filtrate was removed aseptically

and observed microscopically to ensure it was spore-free.

A 30 μ l drop of filtrate was pipetted onto agar discs of 1% glucose or 1% yeast extract, both containing 0.005% oxytetracycline, prior to their inoculation with spores and incubation for germination. Control discs were included, where sterile distilled water replaced filtrate. Agar discs containing GYO were also included.

After incubation, percentages of activation, enlargement, and germ-tube production were calculated for test and control agar discs.

Results and Discussion

This experiment tested spore washings as replacements for glucose or yeast extract in a germination medium. The results, as shown in Figure 12, show the washings were able to replace yeast extract, but not glucose. The addition of washings to yeast extract agar resulted in no significant differences in activation ($t = 1.41$; $P > 0.05$, $df = 14$), enlargement ($t = 0.51$; $P > 0.05$, $df = 14$) or germ-tube production ($t = 1.9$; $P > 0.05$, $df = 14$) when compared to the control agar, but the addition to glucose agar resulted in significant increases in all 3 stages of germination (activation: $t = 4.84$; $P < 0.005$, $df = 14$; enlargement: $t = 5.53$; $P < 0.005$, $df = 14$; germ-tube production: $t = 17.1$; $P < 0.005$, $df = 14$). This suggested the presence of amino acids in the washings, which were able to replace yeast extract in the germination medium.

Presence of amino acids in spore washings

Method

To 0.5 cm^3 of filtrate, obtained as previously described, were added 0.5 cm^3 of 0.1 M NaOH plus 4 cm^3 of Coomassie blue stain (Fazekas de St.Groth et al., 1963). Two controls were run simultaneously with the test, one replacing filtrate with distilled

Figure 12: Histograms representing the mean percentages, \pm SE , of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in 10% CO₂ at 35°C on one of the following solid media:-

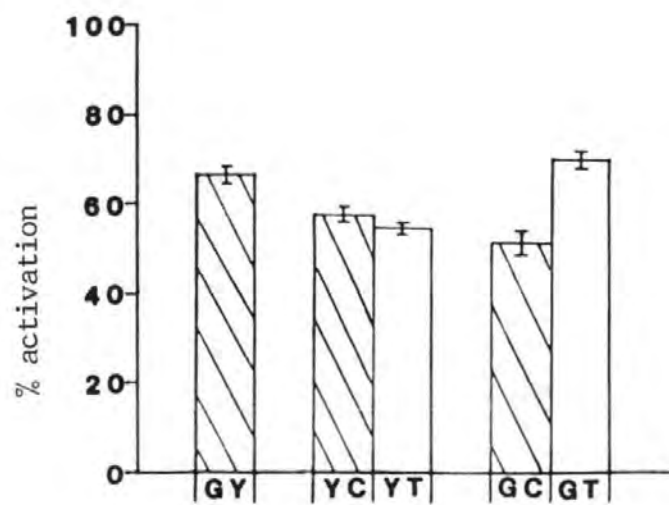
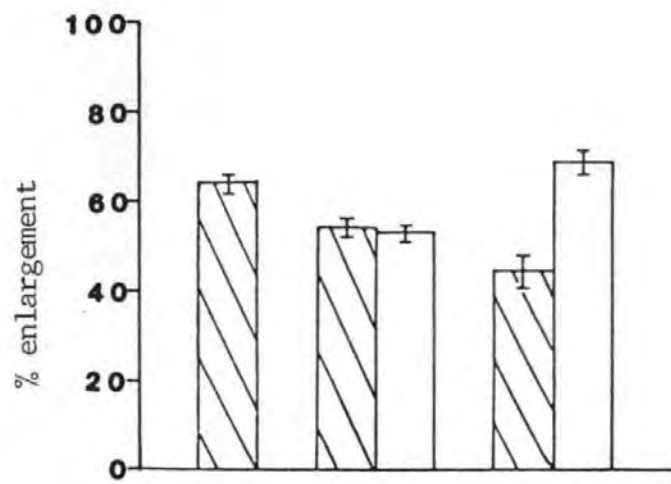
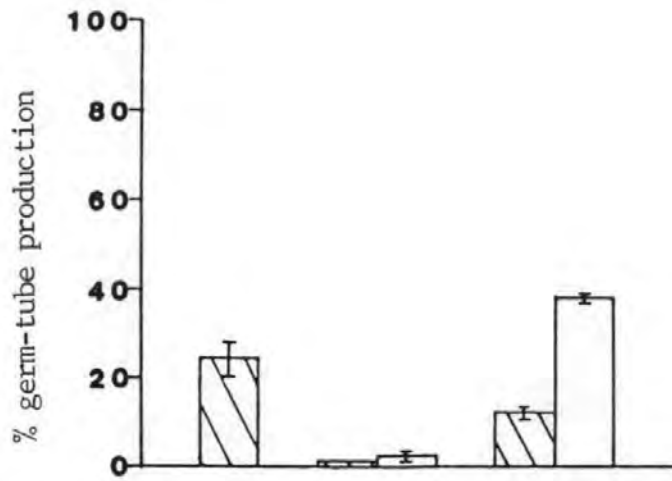
GY: GYO

YC: 1% yeast extract agar plus water

YT: 1% yeast extract agar plus spore washings

GC: 1% glucose agar plus water

GT: 1% glucose agar plus spore washings



media

	COLOUR CHANGE		
BIOCHEMICAL TEST	POSITIVE CONTROL	NEGATIVE CONTROL	TEST SOLUTION
Coomassie blue	blue	red/brown	blue

Figure 13: Table showing the result of the addition of Coomassie blue stain, for amino acid detection, to an amino acid solution (positive control), distilled water (negative control) and spore washings (test solution).

water and the second with a solution of amino acid (10 mg/cm^3) glycine .

Conclusion

The results, as shown in Figure 13, confirm the presence of amino acids in the spore washings.

Determination of nature of amino acids in spore washings

Method

Thin layer chromatography (t.l.c.) plates were prepared - coated with a 0.25 mm layer of silica gel. Standard solutions (1 mg/cm^3) of 19 amino acids (alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, valine) and the imino acid proline were prepared. These were 'spotted' onto t.l.c. plates using capillary tubing, with samples positioned 2 cm apart. 7 individual amino acids were 'spotted' per plate; also 1 spot including all 7 acids and 1 spot of the test filtrate. The spots were air-dried before the plates were positioned in tanks containing the solvent, n-butanol-acetic acid-water (4:1:4), which had been equilibrating for at least 1 hour.

Once developed, the plates were removed from the tanks and dried in a blower fume cupboard. They were then sprayed with a 0.2% solution of ninhydrin in acetone. After air-drying, the plates were developed in an oven at 120°C for 15 minutes.

The R_f values ($R_f = \text{distance travelled by sample}/\text{distance travelled by solvent front}$) for each amino acid were calculated and compared to those values obtained for the ninhydrin-positive 'spots' that developed from the filtrate.

Those amino acids identified in the test filtrate were included in a 1% glucose agar medium, each at a concentration of 0.1%

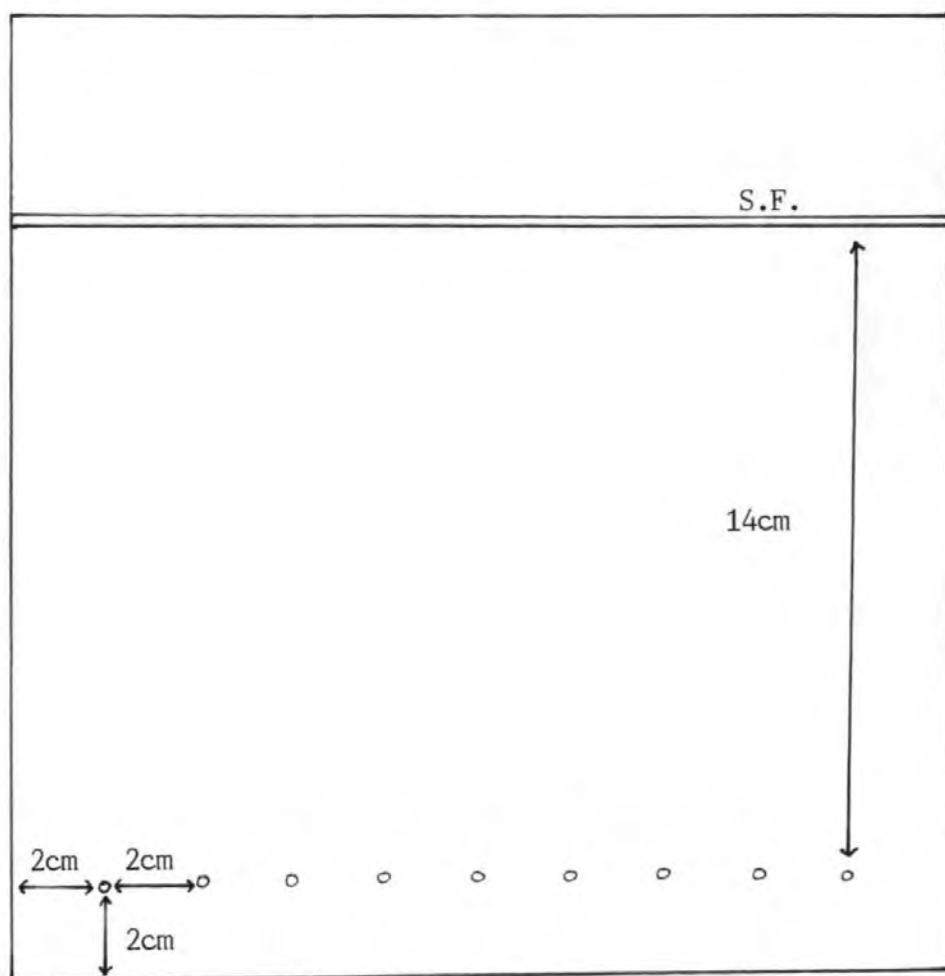


Figure 14: Diagram of thin layer chromatography plate showing position of samples (o) and solvent front (S.F.).

	MEAN PERCENTAGES (\pm SEM) OF SPORES		
MEDIUM	ACTIVATED	ENLARGED	PRODUCING GERM-TUBES
A	88.4 \pm 1.6	82.3 \pm 2.4	24.7 \pm 2.8
B	60.5 \pm 3.2	57.4 \pm 4.6	27.2 \pm 2.8
C	65.1 \pm 4.5	63.4 \pm 4.5	28.3 \pm 3.9

Figure 15: Table showing the mean (\pm standard error of the mean) percentages of spores activated, enlarged and producing germ-tubes after 24 hours incubation on GYO agar (A); 1% glucose agar plus 19 amino acids and proline (B); and 1% glucose agar plus 12 amino acids (C) in 10% carbon dioxide at 35°C. (All acids at a concentration of 0.01%).

and the medium was then tested for support of the germination stages.

Results and Discussion

The chromatographic analysis showed the presence of several amino acids in the spore washings. This was narrowed, by deduction, to a mixture of 12 amino acids (alanine, arginine, cysteine, glutamate, glycine, histidine, leucine, lysine, methionine, threonine, tyrosine, valine). When this mixture was included in a germination medium with 1% glucose, it supported equivalent germ-tube production ($t = 0.73$; $P > 0.05$, $df = 23$), but significantly reduced enlargement ($t = 4.06$; $P < 0.005$, $df = 23$) and activation ($t = 6.1$; $P < 0.005$, $df = 23$), compared with yeast extract; as shown in Figure 15. These 12 amino acids supported percentages of activation, enlargement and germ-tube production not significantly different from those supported by 19 amino acids, plus proline (activation: $t = 0.86$; $P > 0.05$, $df = 16$; enlargement: $t = 1.79$; $P > 0.05$, $df = 16$; germ-tube production: $t = 0.22$; $P > 0.05$, $df = 16$). The number of amino acids essential for germ-tube production may be fewer than 12, since the analysis of washings by chromatography repeatedly produced 6 ninhydrin-positive 'spots', but separation was never sufficient to determine whether these were 6 individuals or whether they included some merging of spots. A more accurate separation technique or the testing of various combinations of the amino acids already identified would show the exact amino acid requirements.

Effect of washing spores on germination performance

Method

The germination performances, on solid GYO medium, of spores after 3 different treatments were compared:-

- (i) dry spores scraped from mummified larvae;
- (ii) spores washed in a Potter homogeniser, as described on page 67;

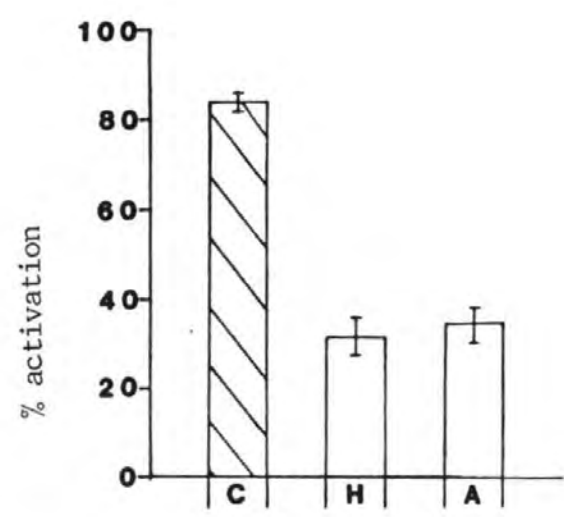
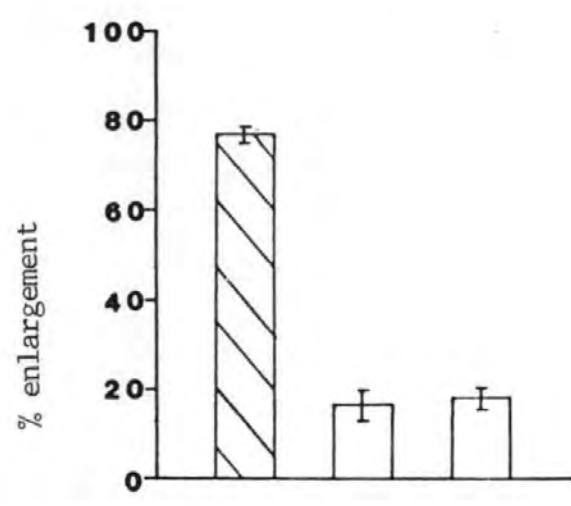
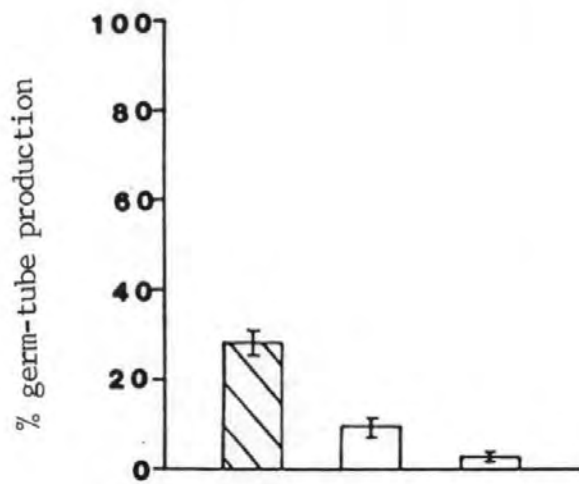
- (iii) spores washed by repeated agitation in 2 cm³ of distilled water, followed by centrifugation (5 minutes at 5000 r.p.m.)

Results and Discussion

Sheridan et al. (1978) studied the effects of washing on the spores of 5 species of fungi and found the result on 4 to be a reduction in germination, caused by the leakage of endogenous nutrients, and on the fifth to be enhanced germination, attributed to the removal of a self-inhibitor by the washing process. The former result was achieved when spores of A. apis were washed, as shown in Figure 16, both washing processes resulted in a severe reduction at all 3 stages of germination, when compared to the results obtained with dry, unwashed spores. However, it seems unlikely that the washings would contain endogenous reserves leaked from the spores during the washing processes. In order for reserves to diffuse from the dormant spore, the very thick, impermeable spore wall would have to be damaged. If this occurred, the washed spores would appear permeable to stain even prior to activation, but the results here (Figure 16) show that even after activation by carbon dioxide, far fewer washed spores are permeable to stain than the unwashed spores. Therefore, any substances in the spore washings must arise from outside the spore, either closely associated with the outer spore coat, or perhaps part of the sticky coating surrounding each spore, described by Skou (1972).

In conclusion, the spore ball phenomenon does not appear to be the result of an interaction between self-stimulators and self-inhibitors produced by germinating spores, but appears to result from an accumulation of vital amino acids within the spore ball. Their importance may be more significant when considered in vivo, rather than in vitro. Once germination has been activated the process will only continue if

Figure 16: Histograms representing the mean percentages, \pm SE , of spores activated, enlarged, and producing germ-tubes after their washing by homogenisation (H), or by agitation (A), or having received no treatment (C) prior to their 24 hour incubation in GYO liquid medium in 10% CO₂ at 35°C.



spore treatments

nutrients are supplied by the environment. In vitro a carbohydrate source and an amino acid mixture are required, but in vivo these may not be so readily available, perhaps the essential amino acids less so than the carbohydrate source. Those accumulated within the spore ball may be sufficient to supplement those available at the site of germination, ensuring germ-tube growth until the developing hypha reaches a plentiful nutrient supply within the host's tissues. Single spores, dissociated from their spore balls, may not always have sufficient of the additional amino acids.

If this hypothesis is correct, a successful infection of a larva may involve the germination of a spore ball or group of spores, rather than individuals. The germination of several spores, from a spore ball, on one host may be more successful than depending on several single spores to simultaneously infect and overcome one host, by chance. In which case, the dissemination of spore balls, rather than individuals may be more important for this fungus.

CHAPTER 3

INFECTION STUDIES

3.1 GENERAL INTRODUCTION

Although entomopathogenic fungi are to be found amongst all four fungal taxonomic groups, the genera Entomophthora, Beauveria, Metarrhizium and Aspergillus are those most commonly encountered in nature and are consequently the best studied. The characteristics of each group give variety to their modes of action and hence their ability to develop under different ecological conditions. The host ranges of such fungi may be limited to a single family, genus, or to a few species of insect; although those most commonly isolated have wide host ranges.

Roberts and Yendol (1971) considered entomogenous fungi to be unique amongst entomopathogenic micro-organisms, mainly due to their route of infection, which they believed to be invariably directly through the host cuticle. This suggests wider tolerances of environmental conditions permitting disease development compared to an infection arising from the relatively stable conditions within a host. Spores are usually the infective unit, germinating on the cuticle and producing an appressorium - a swelling on the end of a short germ-tube, which attaches to the cuticle and from which grows an infection peg. This extends through the cuticle, either penetrating between lamellae of the cuticle or directly into the host's haemocoel. Penetration may be enzymatic or by mechanical means or both. Once within the host many fungi can multiply by the formation of 'hyphal bodies'. These are yeast-like cells which multiply by budding or fragmentation and so rapidly spread throughout the host's haemolymph. In some cases toxins are produced and no major organs are attacked before host death. When the organs are invaded the insect's fat body is a

preferred site, since it contains large amounts of stored nutrients. The mycelia will ramify through all organs, either before or after host death, filling the insect with hyphae before rupturing the integument. Sporulation may occur underneath the integument or after it has been ruptured and depends upon suitable environmental conditions, particularly relative humidity. The resultant mummified insect appears resistant to bacterial degradation, due in some cases, at least, to antibiotics produced by the fungus. For example, species of *Beauveria* produce oosporein, a red pigment which has bactericidal properties and which is also responsible for the colour changes of cadavers (Ferron, 1978).

Host death may be the result of any of a number of developments, for example, histolytic action; mechanical action, whereby hyphal bodies prevent blood circulation or mycelia block the gut; by progressive destruction of blood cells; or by toxin production. Once a pathogen has gained entry, it is met by the host's main line of defence and becomes surrounded by phagocytes. The phagocytosis of fungal cells is a general phenomenon in mycoses, whereby small fungal particles, such as hyphal bodies, are drawn into the cells by endocytosis. When microbes are injected into an insect body cavity they can soon be found in the cytoplasm of the phagocytes, although few researchers have claimed to have observed the actual process in insect cells and it is only assumed to be the same as in other animals. Haemolymph containing the microbes may be engulfed within pinocytotic vesicles or the microbes themselves may be encircled by pseudopodia. Plasmatocytes have been identified as the principal phagocytotic cells in the haemolymph of most insects, but it is not known whether their initial contact with a foreign body is by chance or whether a stimulus is involved. The upper limit for the size of particle which may be phagocytosed has not been fixed and there are reports of spores of

size $4.2 \times 1.8 \mu\text{m}$ and of schizonts of $5.4 \times 2.2 \mu\text{m}$ being engulfed (Salt, 1970). As for fungi, Ermin (1939, cited by Salt, 1970) observed the phagocytosis of yeast cells, while Sussman (1952) observed the phagocytosis of spores of Aspergillus niger in Platysamia cecropia and Kawakami (1965) reported the spores and hyphal bodies of Isaria fumosoroseus and Harziella entomophila being phagocytosed during the early stages of their infection of larvae of Bombyx mori. However, as noted by Donaubaueer (1959, cited by Salt, 1970), who studied B. bassiana in sawfly larvae, despite the phagocytosis of pathogen cells early during the infection process, the phagocytes themselves may be attacked by the fungus and the disease become lethal. Consequently, when Sussman (1952) infected P. cecropia with a more virulent species of Aspergillus, A. flavus, there was little phagocytosis and the insect's blood cells were attacked by the fungus. It would be expected, therefore, that phagocytosis as a means of defence would prove ineffective against a relatively large pathogen such as a fungal hypha, but the insects have a second defence mechanism. Encapsulation involves numerous blood cells surrounding a foreign body and adhering to its surface, so constructing a 'capsule'. The blood cells within the capsule flatten, producing lamellae of cells which effectively restrict and smother the invader. The inner-most cells may undergo melanisation, resulting from the action of tyrosinase on tyrosine within the cells, so depositing melanin on the foreign body. Whatever causes the blood cells to adhere and aggregate around the invader is not fully understood. Reik (1968, cited by Salt, 1970) thought an acid mucopolysaccharide on the surface of the blood cells, the production of which was stimulated by contact with the capsule surface, was involved. However, these processes and the way in which the insect defence system recognises foreign bodies are still not clear. Since

all insects are susceptible to fungal diseases, these methods and others, such as toxin detoxification and segregation, must be ineffective against the most virulent fungal strains.

Although a cuticular route of infection is usual for entomopathogenic fungi, there are conflicting reports concerning the route utilised by Ascosphaera apis. Maurizio (1934) made a histological study of externally healthy brood taken from cells neighbouring mummified larvae and found an isolated case where the tissues suffered only a little destruction. The fungus was restricted mainly to the gut lumen and the immediate vicinity of the gut. Maurizio believed this to be evidence for a gut infection, but the remaining larvae that were examined either proved healthy or too far infected, so the results were inconclusive. Conversely, Roussy (1962) thought the cuticle was the site of infection. He observed the fungus at successive stages of development on fragments of larval cuticle. A maximum number of spores germinated on larvae of 3 to 4 days old, but larval development was not affected until the sixth or seventh day when they became flabby and progressively covered in mycelia. Tumanoff (1951) noted that larvae could survive for some time with an infected cuticle, but died once internal organs were penetrated, so implying the cuticle was the site of infection. When testing the effects of chilling on larvae fed with food containing spores of A. apis, Bailey (1966) concluded chalk brood disease was only lethal when larvae had been chilled for a brief time after cell-sealing. He thought chilling enabled oxygen to diffuse into the otherwise anaerobic gut, so reactivating the spores for growth once the temperature was restored to 35°C. The youngest larvae would not be susceptible, since there would be too long a period of anaerobiosis, and neither would older larvae, since there would not be enough time for germination before the

gut contents were voided, when the mid-gut/hind-gut junction opened, prior to pupation. The ideal larval age for infection was therefore 3 to 4 days.

Feeding larvae with food containing spores has proved to be an effective method of inoculation. De Jong (1976) fed 3 day old larvae a 35% sucrose solution containing spores and successfully infected them. He found the best inoculum source to be from mummified larvae from infected colonies, rather than from incubator-reared mummies or spores cultured in vitro. Moffet et al. (1978) fed commercially produced pollen, contaminated with spores, to colonies and this appeared to induce infections, although they only used a small number of colonies. However, Gochnauer (1982) also found pollen to be an effective means of introducing chalk brood into a colony, rather than using syrup.

In a recent study by Glinski (1981) on the infection of honey bee larvae with A. apis, larvae were dorsally inoculated or fed with a 35% solution of sucrose containing spores. Both methods resulted in infections, although larvae of all ages were found to be more susceptible to oral inoculations. However, Glinski decided chilling was not a pre-requisite to disease development, despite routinely incubating larvae at 25°C! Also, he assumed the sites of infection without following the process from spore germination to disease development. There is no histological evidence to support his conclusions and there is the possibility that spores placed on the larval cuticle may have been ingested as larvae moved about during the incubation time. Similarly, spores placed on the mouthparts could finally be deposited on the cuticle and have germinated there. Indeed, such evidence is also lacking from the work of Gilliam et al. (1978), who inoculated the cuticle and mouthparts of larvae which subsequently developed the disease, and who concluded infection could occur from

either route, without having followed the course of infection.

The modes of infection utilised by other species of Ascosphaera have been more critically investigated. Vandenberg and Stephen (1983) studied the infection of the alfalfa leaf-cutting bee Megachile rotundata with A. aggregata by feeding third and fourth instar larvae on a pollen-based diet plus spores and then chemically fixing larvae, ready for histological study, at intervals after inoculation. The infection was found to start in the mid-gut. Within 1 to 3 days germ-tubes emerged within the mid-gut and the peritrophic membrane was attacked. After 2 to 4 days hyphae were present in the haemocoel, invading tracheae and the epidermis. Within 1 to 2 weeks sporulation occurred beneath the cuticle. External signs of infection were not apparent until the host was nearly dead and filled with mycelia. No spores were observed to germinate on the outer surface of the host.

A similar investigation was also undertaken by Youssef et al. (1984) on the infection of first instar larvae of M. rotundata by A. proliperda. Again, a gut infection was discovered and no evidence of germination on the cuticle or in a respiratory tract was found.

Therefore, the route of infection utilised by A. apis remains open to question; with only Roussy (1962) having reported actually observing spore germination on the larval surface cuticle, plus the inconclusive histological evidence for a mid-gut infection provided by Maurizio (1934). In order for chalk brood disease and the life cycle of A. apis to be fully understood this question must be answered. Similarly, this information may prove vital in the search for a means of disease control.

3.2 GENERAL METHODS

Collection and maintenance of bee larvae

Larvae required for experiments were collected from colonies of Apis mellifera at Rumleigh Experimental Station, 15 miles from Plymouth. These colonies had previously appeared susceptible to chalk brood disease. Pieces of comb containing larvae of the appropriate age were cut from the combs, placed in a plastic box lined with damp paper and taken to the laboratory as quickly as possible.

Larvae could not be maintained in their cells without constant feeding, therefore they were removed from their cells by carefully cutting away one side of the cell and gently easing the larva out, using fine, blunt-ended forceps. They were placed in a self-feeding chamber, which consisted of a petri-dish containing a wad of sterile, absorbent cotton wool soaked in 30 cm³ of a sterile 35% (w/v) sucrose solution and overlain with a fine nylon mesh, which allowed the larvae to feed but prevented them from drowning (Michael and Abramovitz, 1954). 20 to 30 larvae could be maintained in 1 dish. They could survive for several days when incubated at 35°C, although this was dependent upon the age and health of each larva.

3.3 CUTICULAR INFECTIONS OF HONEY BEE LARVAE

Introduction

At the beginning of this investigation the information concerning the routes of infection used by other species of *Ascosphaera* was not published in the literature. Consequently, when capneic rather than anaerobic conditions were determined as one of the requirements for spore germination in *A. apis* (Heath and Gaze, 1987), it seemed most likely that spore germination did occur on the surface of the cuticle, activated by respiratory carbon dioxide from the larva. The emerging germ-tubes might be attracted by an increasing carbon dioxide gradient to a spiracle, which would provide an ideal entrance into the larva. McCauley et al. (1968), for example, noted that while *Metarrhizium anisopliae* most commonly penetrated the cuticle of its wire-worm hosts directly, occasionally the fungus entered through the spiracles and pores of sense organs. In these instances there was a lack of appressorial development, which was undoubtedly related to the easy access these routes provided.

The theory of Bailey (1966), wherein an anaerobic gut was the site of spore activation by anaerobiosis, could no longer be considered the case once Bignell and Heath (1985) showed the fifth instar larval gut to have a positive redox potential. Indeed, few mycopathogens of insects utilise this route, which would involve penetration of the numerous thin lamellae of the peritrophic membrane. Although this membrane, in insects generally, is described as consisting of chitin mixed or combined with protein (Wigglesworth, 1984), its chemical nature, in the honey bee at least, is uncertain. Snodgrass (1956) commented on its questionable nature, whether or not it was chitinous. He quoted Campbell (1929) and von Dehn (1933) who reported it to contain chitin, but also cited numerous others who thought otherwise.

He also mentioned Waterhouse (1953) who found chitin in the peritrophic membrane of many other insects, thereby suggesting it could also be present in the honey bee. However, Mello et al. (1971) found negative results for the histochemical tests for chitin they performed on the peritrophic membrane of a stingless bee, Melipona quadrifasciata. To date, the membrane in larvae of A. mellifera can only be assumed to be a chitin-protein complex, until its composition is fully investigated. In these larvae 6 membranes are shed daily (Rengel, 1903, cited by Roeder, 1953), so a germ-tube would need to remain in contact with the membrane at one site while secreting exoenzymes in order to successfully penetrate, despite the continuous shedding of membranes and movements of the larva while it continues to feed.

However, there are also problems encountered in an infection via the cuticle, such as the ecdyses associated with insect growth. A spore may adhere to a host's cuticle and have begun germination, only to be discarded with an old cuticle after ecdysis. Consequently, some infection processes vary to coincide with these moulting stages. For example, Beauveria bassiana penetrates the cuticle of third instar larvae of Leptinotarsa decemlineata just prior to moulting; hyphae and blastospores invade the exuvial fluid and so infect the newly formed cuticle (Ferron, 1978). Similarly, penetrant hyphae of M. anisopliae, on reaching the exuvial fluid of moulting wire-worms, produce a second appressorium on the developing cuticle. If the fungal infection is more advanced, the new cuticle may be damaged during ecdysis as old and new cuticle are held together by mycelia. The resultant wounds provide easy access for further infection (Zacharuk, 1973). Such problems need not arise in the cuticular infection of honey bee larvae, since the fifth instar stage provides an ideal opportunity for infection. Until this time cuticle is shed

daily, but after the fourth moult the cuticle remains intact for 3 to 4 days, so providing a suitable time lapse for a successful infection.

Dorsal inoculation of living larvae

Method

Once placed in self-feeding chambers, fifth instar larvae were inoculated with spores of A. apis. Using a sterile mounted needle, spore cysts were scraped from mummified larvae onto a sterile microscope slide, where they were crushed using a razor blade. The released spores and spore balls were then carefully brushed onto the cuticles of larvae, using a fine paint brush. Chambers containing inoculated larvae were incubated at 35°C, as were those containing control larvae, which had received no spore inoculum. Some test and control chambers were also incubated in atmospheric conditions at 30°C and in 5 and 10% carbon dioxide at 35°C.

After incubation for 24 and 48 hours larvae were observed under the stereoscopic microscope at x20 magnification and pieces of body wall dissected from some larvae were stained with lactophenol cotton blue and observed, using an Olympus CH microscope at x100 and x400 magnification, for any signs of spore germination.

Results

Of 40 fifth instar larvae that were dorsally inoculated and incubated, in 2 humidity chambers, 30 survived at 35°C for 24 hours. Larvae were considered dead when they failed to respond to a stimulus such as a gentle prod and were then removed from the chambers. Of the remaining larvae 5 from each chamber were observed for signs of germination on their surface cuticle, but no evidence was found. This was also the case after 48 hours incubation, when the 12 larvae still living were checked for signs of spore germination. 20 control larvae

had also been incubated, of which 16 survived for 24 hours. 6 were checked for signs of germination, which would have resulted from inherent infections, but no germinating spores were found. This was also the case for the 8 living larvae checked after 48 hours incubation.

40 larvae were also dorsally inoculated and incubated at 30°C. Although these appeared to survive better, with 35 living after 24 hours and 15 after 48 hours, there were no signs of germination on any of the larvae checked. Again, 20 control larvae were also incubated of which 17 survived for 24 hours, but no signs of germination were found on the 6 larvae checked. There was a similar result after 48 hours.

The spores did not even activate on the surface cuticle of larvae in these initial incubation conditions, so larvae were inoculated and then incubated in an atmosphere of enhanced carbon dioxide. 40 larvae were incubated at 35°C in 5% carbon dioxide. Of the 28 surviving after 24 hours, 8 were observed for activated spores, but none were found. After 48 hours incubation 14 larvae had survived and there was no evidence of spore activation on the 8 observed. Some larvae were, therefore, incubated at 10% carbon dioxide, but again no evidence of spore activation could be seen. Control larvae were included in each case, but always exhibited the same result, no activation on the surface cuticle.

Inoculation of dissected body wall

Method

Pieces of body wall, approximately 4 x 2 mm were dissected from fifth instar larvae and placed, external surface uppermost, on a sterile microscope slide. They were inoculated with spores of A. apis, obtained by crushing the scrapings from mummified larvae, and placed in

a humidity chamber, prior to their incubation at 35°C in an atmosphere of 10% carbon dioxide. After incubation the pieces of body wall were stained and observed as previously described.

Results

A total of 20 pieces of body wall were inoculated and incubated. After 24 hours, 10 pieces were stained and observed for spore germination, but none was evident. However, after 48 hours when the remaining 10 pieces were checked, 1 showed extensive hyphal growth. After microscopic examination this growth appeared to be the result of a contaminating fungus, a species of Penicillium.

10 control pieces of body wall were also incubated and spores were found on 1 piece, but these had not even activated after 24 hours incubation.

Inoculation of dead larvae

Method

Fifth instar larvae were killed by freezing at -4 or -70°C for 24 hours. They were thawed at room temperature and placed on sterile microscope slides where they were inoculated with the 'crushed' scrapings from mummified larvae and then incubated at 35°C in an atmosphere of 10% carbon dioxide.

After 24 and 48 hours incubation larvae were observed under the stereoscopic microscope at x20 magnification and pieces of body wall were dissected, stained and observed as previously described.

Results

20 larvae killed by freezing at -4°C and 20 killed at -70°C were tested. After 24 hours incubation 10 of each were observed for signs of spore germination. Of those larvae frozen at -4°C germinating spores were observed on 8, while there were no such signs on those

frozen at -70°C . After 48 hours incubation all 10 of those frozen at -4°C showed extensive hyphal growth, which on closer examination proved to be A. apis, but no growth was observed on the larvae that had been frozen at -70°C .

Control larvae were also incubated and 2 of 10 that were frozen at -4°C showed signs of spore germination after 24 hours, while 1 was covered in hyphal growth, confirmed to be A. apis, after 48 hours incubation. On those frozen at -70°C there were no signs of spore germination or hyphal growth.

Discussion

Under the experimental conditions tested here spore germination was only achieved on larvae killed by freezing at -4°C . However, it could not be determined whether these spores had germinated on the surface cuticle, because these larvae collapsed, losing all structure, during incubation. They appeared as watery sacs, some of which had burst, so spores may have been in contact with larval contents throughout the incubation period. Those larvae frozen at -70°C , meanwhile, retained their structure during incubation and failed to support any spore germination.

On the surface cuticle of living larvae or on pieces of dissected body wall spore activation was not even achieved, despite incubation in atmospheres of enhanced carbon dioxide. Yet spores incubated in vitro on only water agar will activate when placed in 10% carbon dioxide. This result may be explained in either of two ways. Firstly, the cuticle may fail to provide additional environmental factors required by the spores for their germination, although this would not explain the inhibition of spore activation; therefore, secondly, the cuticle may contain a substance preventing spore activation.

Although several researchers have thought the route of infection to be via the surface cuticle, only Roussy (1962) claimed to have seen spores germinating on the cuticle. He examined and autopsied infected larvae, presumably those visibly infected, so the infection was well in progress, but from which he concluded the infection occurred on the cuticle, rather than per os. No details are given for the methods by which the larvae were examined. He also noted spores which were not germinating were often seen on cuticles, but did not know the mechanism which induced their germination. Although the fungus was apparently observed in its successive stages of development on fragments of cuticle, Roussy does not describe the penetration stages he observed, the most important development after spore germination. Also, he quotes an optimum temperature of 20° to 25°C for spore germination in vitro, compared to 31° to 35°C determined in this study (page 29). The latter is more likely since the temperature in the brood area is maintained around 35°C. Consequently, it is possible that Roussy was observing the wrong fungus, perhaps it was one capable of growing on the surface cuticle of larvae, but not of infecting them.

3.4 INHIBITORY PROPERTIES OF THE CUTICLE

Introduction

There is evidence to support the theory that the cuticle of an insect provides a chemical as well as a physical barrier for protection against potential pathogens. Sussman (1951) discovered that washing pupae of the giant silk-worm Platysamia cecropia in ether prior to their inoculation with A. flavus significantly increased the numbers that became infected. Similarly, the susceptibility of Eurygaster integriceps to B. bassiana was increased sharply after the epicuticle was removed, while the ether extracts of the epicuticular lipids also depressed spore germination (Evlakhova and Shekhurina, 1963, cited by Madelin, 1966). Koidsumi (1957), too, found larvae of the silk-worm B. mori and of the rice stem-borer Chilo simplex to become highly susceptible to muscardine when their cuticular lipids were either mechanically or chemically removed. The most active anti-fungal constituents of the cuticular lipids were free medium-chain saturated fatty acids, presumed to be caprylic and capric. Therefore, there is the possibility that spores of A. apis are prevented from germinating on the cuticle of honey bee larvae by the lipids present in the epicuticle.

An alternative source of inhibition to spore germination may be the constituents of larval food. During the early stages of larval development the larvae float upon a pool of food, which is constantly replenished by nurse bees. The larval cuticle therefore becomes coated in larval food and this may offer some protection against infection. By the fifth instar larvae are being fed by the nurse bees on a mixture of brood food, pollen, and regurgitated nectar or diluted honey. Honey has some anti-bacterial properties, such as its low pH, hyperosmoticity and hydrogen peroxide content, produced by the action of

glucose oxidase on glucose during honey dilution. Brood food and the equivalent substance fed to queens - royal jelly, also possess anti-bacterial properties. They, too, have a low pH and have been shown to prevent the in vitro growth of Melissococcus pluton, the causative agent of European Foulbrood and Bacillus alvei, a frequent secondary invader in larvae attacked by M. pluton, although the food of older worker larvae did not retain this ability (Krasikova, 1955). Therefore, in this instance, these properties may also prove effective against fungal spore germination.

Testing for chloroform- or water-soluble inhibitory factors in larval cuticle

Methods

5 fifth instar larvae were washed in 2 cm³ of chloroform, a lipid solvent, for 2 minutes, before pieces of body wall, approximately 4 x 2 mm, were dissected from them and placed, external surface uppermost, on sterile microscope slides. Spores and spore balls, released from spore cysts by crushing them with a razor blade, were brushed onto the pieces of body wall, using a fine paint-brush. The slides were placed in humidity chambers and incubated at 35°C in an atmosphere of 10% carbon dioxide for 24 hours. Pieces of body wall from larvae, similarly washed in distilled water were also inoculated and incubated. After which the pieces of body wall were stained with lactophenol cotton blue and observed, using an Olympus CH microscope at x100 and x400 magnification, for any signs of spore germination.

The chloroform and water used to wash larvae were tested for their effects on spore germination. A drop (approximately 30 µl) from a Pasteur pipette was placed on a disc of GYO agar, prior to its inoculation and incubation for spore germination. Fresh chloroform and

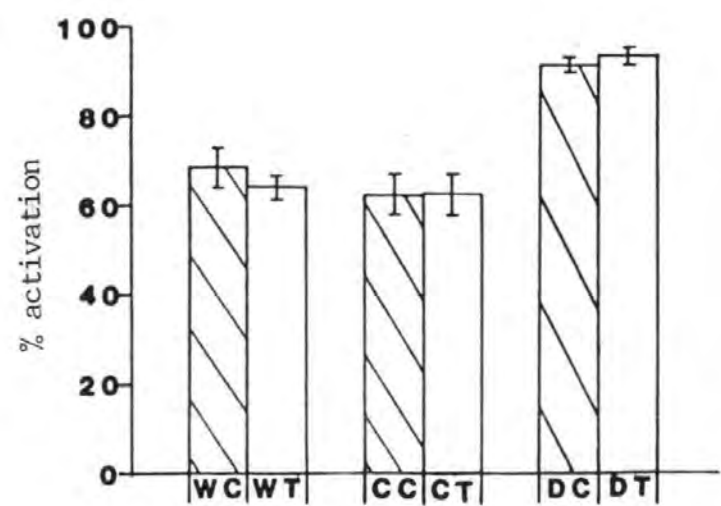
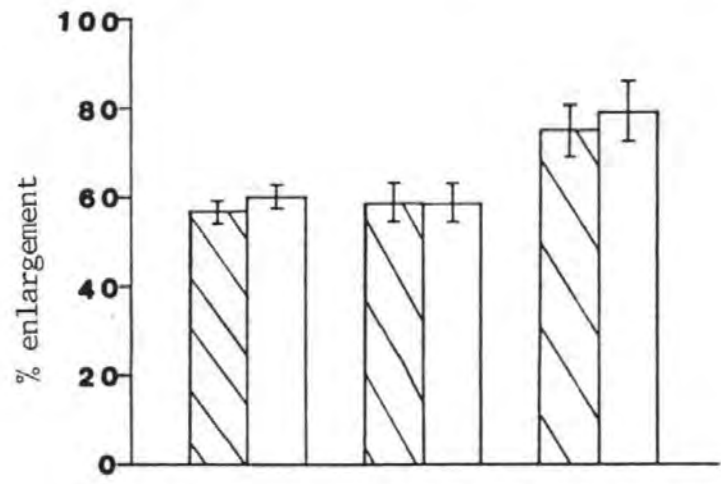
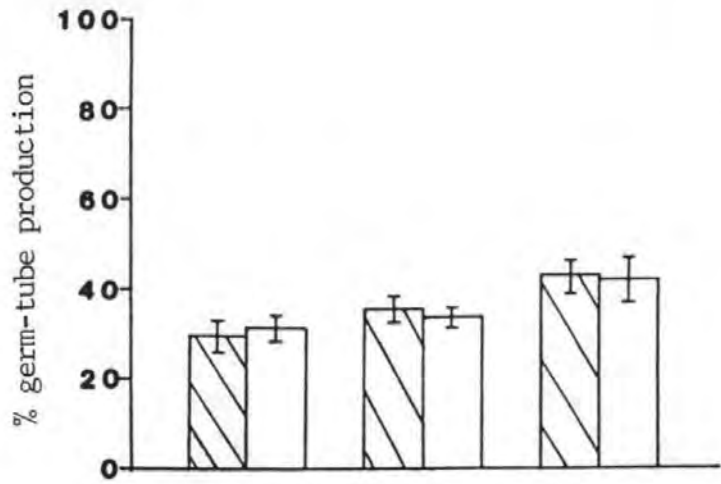
distilled water were also tested as controls.

Also, individual fifth instar larvae, held in fine forceps, were 'dabbed' onto discs of GYO agar, prior to their inoculation and incubation for spore germination. Agar discs receiving no treatment were also inoculated and incubated.

Results and Discussion

As shown in Figure 17, by using this method neither water-soluble nor chloroform-soluble substances having any significant effect on any stage of spore germination were washed from larval cuticles. (Water, activation: $t = 0.81$; $P > 0.05$, $df = 14$; enlargement: $t = 0.88$; $P > 0.05$, $df = 14$; germ-tube production: $t = 0.4$; $P > 0.05$, $df = 14$. Chloroform, activation: $t = 0.85$; $P > 0.05$, $df = 13$; enlargement: $t = 0.8$; $P > 0.05$, $df = 13$; germ-tube production: $t = 0.5$; $P > 0.05$, $df = 13$). Neither did the washing of larvae render their cuticles more acceptable for spore germination, as shown in Figure 18. 'Dabbing' larvae onto agar also resulted in no significant differences at any stage of germination, when compared with the control agar (activation: $t = 0.82$; $P > 0.05$, $df = 12$; enlargement: $t = 0.31$; $P > 0.05$, $df = 12$; germ-tube production: $t = 0.12$; $P > 0.05$, $df = 12$). These experiments do not, however, give any evidence for the presence or absence of lipids on the epicuticle of honey bee larvae, but merely indicate that any that might have been deposited on the agar discs, by either method, were not inhibitory to the germination of A. apis spores. Perhaps washing or 'dabbing' larvae without abrasion would not remove the lipids if they are closely associated with other components of the epicuticle.

Figure 17: Histograms representing the mean percentages, \pm SE , of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in 10% CO₂ at 35°C on solid GYO medium treated with water (WC) or water-soluble washings from larval cuticle (WT), or with chloroform (CC) or chloroform-soluble washings (CT); or 'dabbed' with larval cuticle (DT) or untreated (DC).



media

TREATMENT OF LARVAL CUTICLE	EFFECT ON SPORE GERMINATION
Washed in water	No activation
Washed in chloroform	No activation

Figure 18: Table showing the effect of washing larval cuticle in water or chloroform on A. apis spore germination on larval cuticle.

Effects of some fatty acids on spore germination

Method

Solutions of fatty acids of 1, 0.1 and 0.01% were prepared in 50% ethanol. A 30 μ l drop of each was pipetted onto discs of GYO agar, prior to their inoculation and incubation for spore germination. The effect of the addition of 50% ethanol to the agar discs was also tested.

Results and Discussion

Figure 19 shows the effects of the various concentrations of fatty acids tested on spore germination. At the highest concentration all the acids tested totally inhibited spore enlargement and germ-tube production. Activation was significantly reduced by 5 of the acids tested (C6: $t = 6.25$; $P < 0.005$, $df = 14$; C7: $t = 9.11$; $P < 0.005$, $df = 14$; C8: $t = 15.9$; $P < 0.005$, $df = 14$; C9: $t = 8.0$; $P < 0.005$, $df = 14$; C10: $t = 3.3$; $P < 0.005$, $df = 11$), but undecanoic (C11) and lauric (C12) acids supported percentages of activation not significantly different from each other or from the control ($F = 1.65$; $P > 0.05$, $df = 2,13$). At 0.1% germ-tube production was either totally inhibited or reduced to a negligible percentage by all acids, except lauric. Spore enlargement was reduced to a negligible amount by undecanoic acid and was greatly reduced by the acids nonanoic (C9) and capric (C10). Capnoic (C6) and lauric acids also significantly reduced enlargement (C6: $t = 3.82$, $P < 0.005$, $df = 14$; C12: $t = 2.34$; $P < 0.05$, $df = 15$), while heptanoic (C7) and caprylic (C8) acids maintained percentages not significantly different from the control ($F = 0.95$; $P > 0.05$, $df = 2,19$). Percentages of activation not significantly different from those supported by each other or the control were produced by heptanoic, caprylic, nonanoic and lauric acids ($F = 2.76$; $P > 0.05$, $df = 4,32$), while the percentage was significantly reduced by capnoic

Figure 19: Histograms representing the mean percentages, \pm SE , of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in 10% CO₂ at 35°C on solid GYO medium treated with 50% ethanol (C) or with a 1, 0.1 or 0.01% solution of one of the following fatty acids in 50% ethanol:-

C6: capnoic

C7: heptanoic

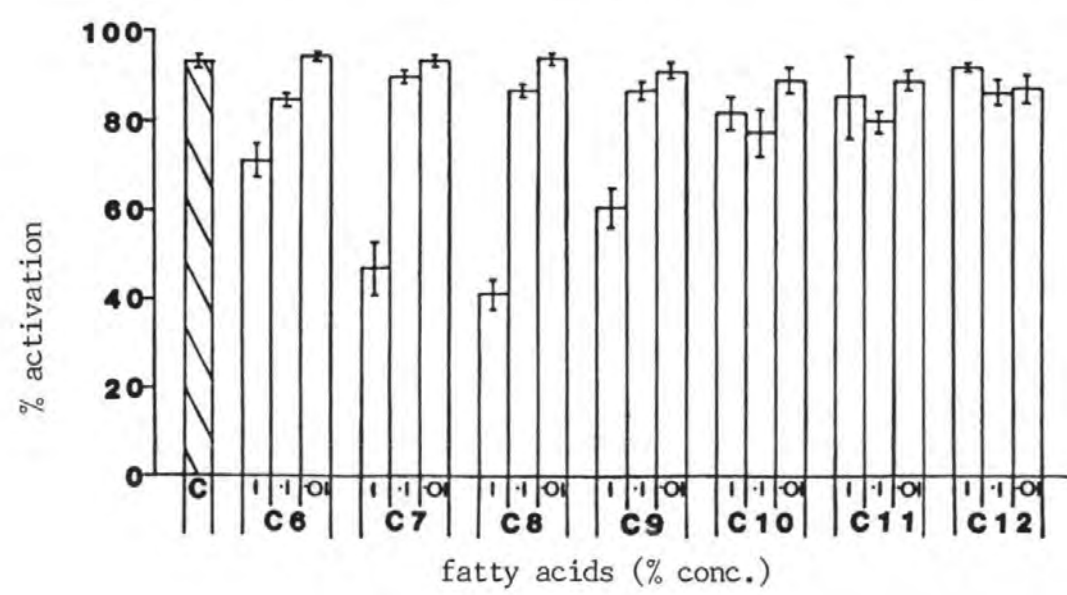
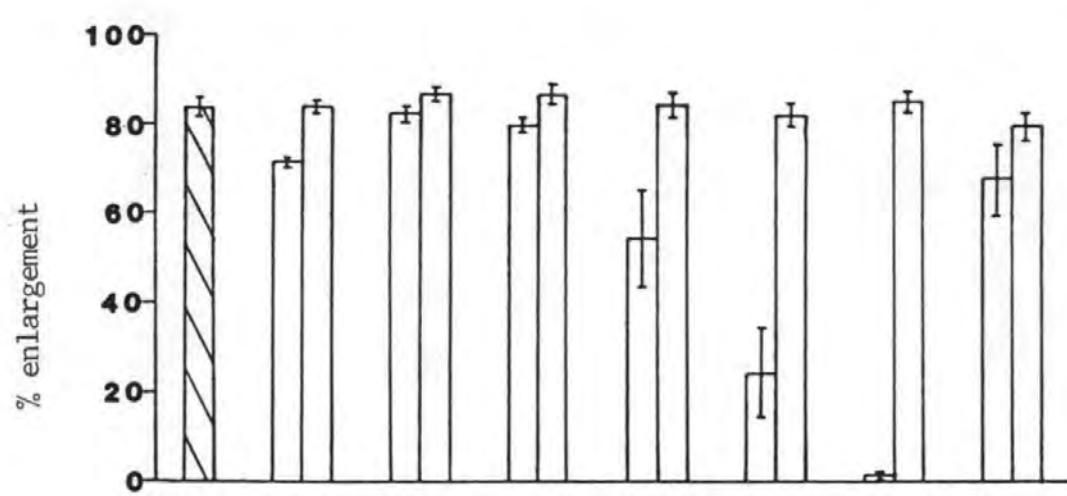
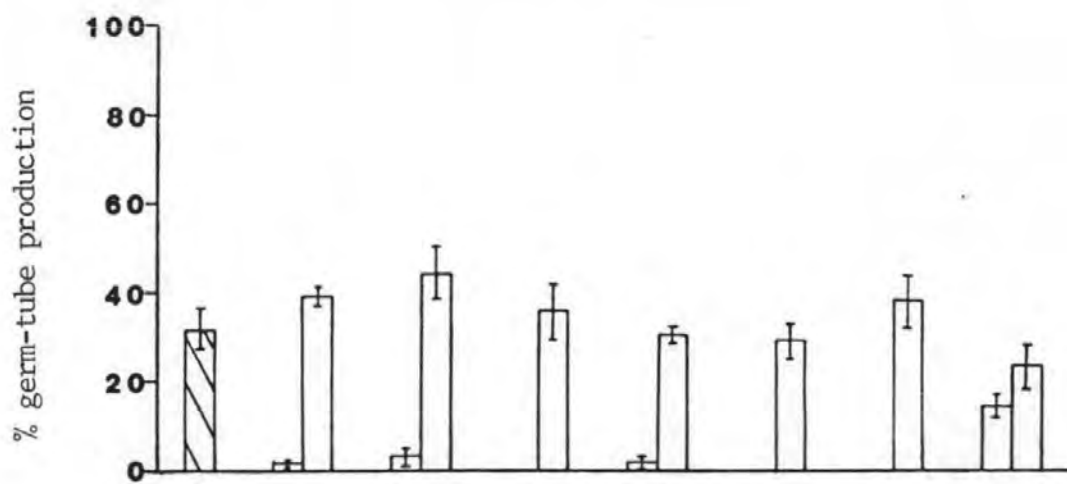
C8: caprylic

C9: nonanoic

C10: capric

C11: undecanoic

C12: lauric



($t = 3.47$; $P < 0.005$, $df = 14$), capric ($t = 3.4$; $P < 0.005$, $df = 15$) and undecanoic ($t = 4.35$; $P < 0.005$, $df = 15$) acids. At the lowest concentration of acids, 0.01%, there were no significant differences in the percentages of activation, enlargement or germ-tube production supported by any of the acids when compared to each other and the control (activation: $F = 1.53$; $P > 0.05$, $df = 7,52$; enlargement: $F = 0.88$; $P > 0.05$, $df = 7,52$; germ-tube production: $F = 1.85$; $P > 0.05$, $df = 7,52$).

Although the highest concentration tested here proved effective against spore germination, it is unlikely to be encountered by the spores in vivo. The lowest concentration proved ineffective and therefore there would need to be a site of fairly concentrated acids on the larval cuticle to prove inhibitory. The fatty acids appearing most effective at 0.1% were nonanoic, capric and undecanoic, reducing spore enlargement and inhibiting germ-tube production. Perhaps a mixture of these acids should be tested, since they may prove as effective at lower concentrations when working in combination.

These results are similar to those achieved by Stephen et al. (1982), who tested the effects of some fatty acids on the spore germination of A. aggregata. They also found the acids to significantly reduce germ-tube production and enlargement at 1%; to significantly reduce germ-tube production, but to maintain some enlargement at 0.1%; and to be relatively ineffective at 0.01%. They also found undecanoic acid to be one of the most effective and lauric to be the least effective. They also tested tridecanoic (C13) and myristic (C14) acids and found them to be ineffective even at 1%. Consequently, spore germination in both of these species of Ascospaera appears to be inhibited by some fatty acids, which may still prove important in the inhibition of germination on larval cuticle.

Effect of four larval food constituents on spore germination

Method

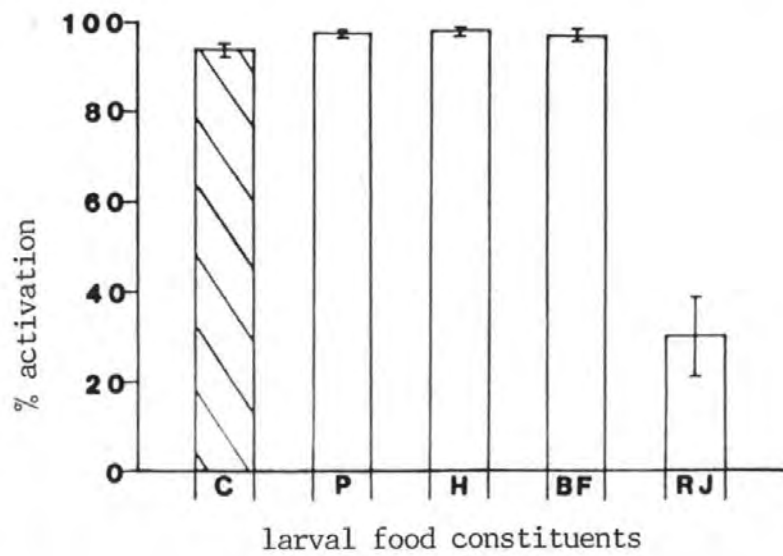
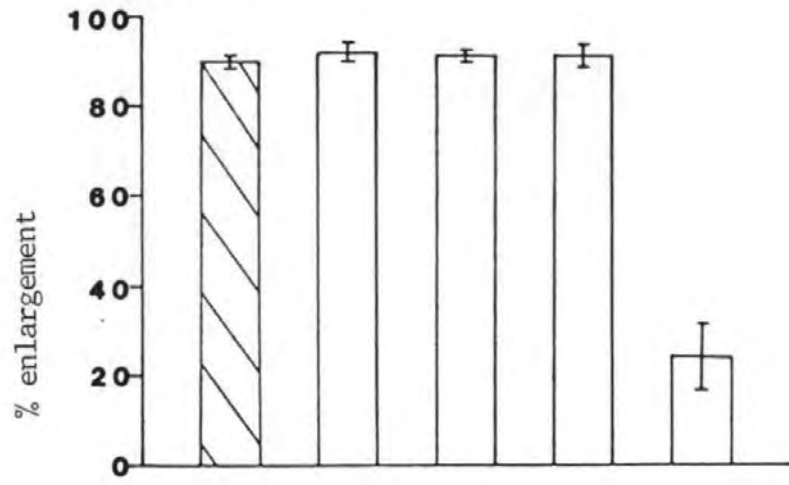
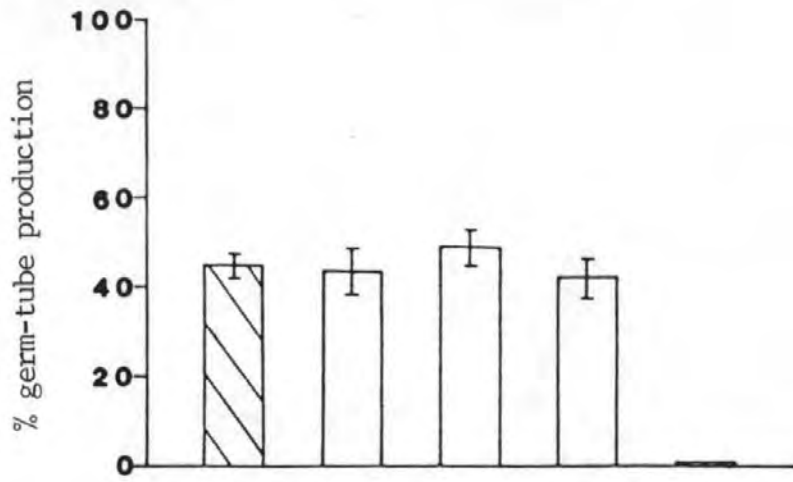
The upper surfaces of individual discs of GYO agar were spread with visibly similar amounts of pollen, honey, brood food or royal jelly, using an inoculating loop. The pollen was freshly collected from colony stores and crushed using a razor blade; the honey was also collected from colony stores and the brood food was collected from the cells of 1 to 2 day old larvae and the royal jelly from queen cells. The agar discs were then inoculated and incubated for spore germination. Discs receiving no larval nutrients were also included.

Results and Discussion

The effects of the various larval food constituents on spore germination can be seen in Figure 20. Neither pollen, honey nor brood food appeared to have any significant effect on any stage of germination when compared to the controls (activation: $F = 3.06$; $P > 0.05$, $df = 3,24$; enlargement: $F = 0.27$; $P > 0.05$, $df = 3,24$; germ-tube production: $F = 0.56$; $P > 0.05$, $df = 3,24$). However, royal jelly produced a most dramatic effect. Activation, enlargement and germ-tube production were greatly reduced by it. The anti-bacterial properties of honey, which may have been expected to prevent spore germination did not do so. The low pH of honey, pollen and brood food may have been buffered by the relatively neutral pH of the GYO medium. The rich amino acid source provided by pollen and the abundant carbohydrates in honey might have been expected to increase germ-tube production, but this was not the case.

The most surprising result is that brood food failed to have any effect on enlargement and germ-tube production, while royal jelly severely inhibited both. The low pH of royal jelly cannot be the cause, since honey, pollen and brood food exhibit a similarly low pH.

Figure 20: Histograms representing the mean percentages, \pm SE, of spores activated, enlarged, and producing germ-tubes after 24 hours incubation in 10% CO₂ at 35°C on solid GYO medium treated with pollen (P), honey (H), brood food (BF), royal jelly (RJ) or untreated (C).



The effect must be due to a constituent of royal jelly, which makes it qualitatively or quantitatively different from brood food. The differences between the two types of food have long been of interest with reference to caste differentiation. Several differences have been identified such as vitamin content, royal jelly containing more pantothenic acid, bioppterin and neoppterin compared with brood food (Rembold, 1965); or the presence of meso-inositol in royal jelly (Helleu, 1956). Also, the isolation of 10-hydroxy-2-decenoic acid from royal jelly and its lower concentration in brood food (Barker et al., 1959). Indeed, the lipid component of royal jelly seems favourite as an inhibitory factor, since some fatty acids have been found to inhibit spore germination in A. apis (Figure 19). Lipid accounts for 4.3% of total royal jelly content, with 10-hydroxy-2-decenoic acid accounting for 50.3% of this (Howe et al., 1985). Yatsunami and Echigo (1984), however, showed effective antibacterial action of both the ether-soluble and -insoluble fractions of royal jelly. Further experiments are therefore required to determine the anti-fungal component of royal jelly.

3.5 MID-GUT INFECTION OF HONEY BEE LARVAE

Introduction

Since the attempts to infect larvae via the cuticle or indeed to germinate spores on larval cuticle proved unsuccessful (section 3.3), it was decided to investigate the possibility of an infection arising in the mid-gut. Until Vandenberg and Stephen (1983) and Youssef et al. (1984) reported this as the route utilised by other species of Ascospaera, there were no substantiated records of insect mycopathogens infecting in such a way (Steinhaus, 1963). They demonstrated the route of infection by histological studies, providing the evidence which was lacking from the work of other researchers trying to demonstrate an infection via the mid-gut in A. apis (Gilliam et al., 1978; Glinski, 1981). Such evidence is required to exclude the possibility of an integumental infection.

There are reports of insects being infected in parts of the gut other than the mid-gut. Veen (1966), for example, studied the infection of second instar nymphs of Schistocerca gregaria by M. anisopliae and found the nymphs to be readily infected when spores were included in their food. After a histological examination hyphae were discovered in the proximal part of the maxillary palps of some nymphs, while others showed hyphae elsewhere in their heads. Consequently, Veen speculated this was actually an oral infection. Mycopathogens which infect in such a way are of interest as agents of biological control, since this would alleviate the problems of correct environmental conditions for infection, as the pathogen would be adapted to the internal environment of the host. Schabel (1976) also demonstrated a strictly oral infection of the pales weevil Hylobius pales by M. anisopliae. No direct evidence could be found for a gut invasion, despite conidia germinating in mid-gut contents in vitro.

Fixed sections demonstrated an infection in the buccal cavity. Sweeney (1975) found the infection of larvae of the mosquito Culex fatigans by Culicinomyces to begin in the fore- and hind-guts. The conidia of these fungi are suitably shaped so that they adhere to the site of infection. They pierce the cuticle by a slender penetration hypha which extends through the gut epithelium to the haemocoel, where the fungus then spreads by means of hyphal bodies. Such an infection, within the fore- and hind-guts, avoids the problem of penetrating the peritrophic membrane, that is, growing against the direction of synthesis and maintaining a position despite gut movements while the larva continues to feed. The peritrophic membrane is confined to the mid-gut. Therefore, Ascospaera species must be unique amongst insect mycopathogens if a route of infection through the mid-gut is demonstrated.

Methods

In vitro production of mid-gut infections of larvae

Fifth instar larvae were collected, removed from their cells and placed in petri-dishes. Here they were each fed a drop (approximately 5 μ l) of 35% sucrose solution containing crushed spore cysts of A. apis. The drop was placed on the mouthparts of each larva, using a sterile 2 cm³ microsyringe and needle (16mm) and the larvae could be observed consuming the food if placed under a stereoscopic microscope at x20 magnification. The larvae were left to feed for 30 minutes at room temperature, before being placed in self-feeding chambers and incubated at 25°, 30° or 35°C. Each day for 4 days the numbers of living larvae were counted and any showing signs of hyphal growth were removed and placed on malt agar (Oxoid) plates and incubated at 30°C. The fungus could then be observed and identified as A. apis. In such a way the best incubation temperature for survival

of larvae and development of chalk brood symptoms was determined.

Third, fourth and fifth instar larvae were fed inoculated food and incubated at the appropriate temperature. At 18 and 24 hours post-inoculation and daily thereafter, living larvae were removed from the chambers and prepared for histological study.

In vivo production of mid-gut infections of larvae

In a honey bee colony, known to be susceptible to chalk brood disease, a frame was chosen which included an area of cells containing 2 to 3 day old larvae. The position of this area was marked by scratching the boundary cells with a knife and was mapped onto a piece of paper, so that it could be relocated. Into each cell within this area was syringed a drop (approximately 5 μ l) of 35% sucrose solution inoculated with crushed spore cysts of A. apis. The frame was then replaced and the colony left for 2 days. The area of cells, now containing larvae of age 4 to 5 days, was relocated, cut out and returned to the laboratory in a humid box. Here the larvae were removed from their cells and prepared for histological study.

Preparation of larvae for histological studies

The larval cuticle was slit slightly on the dorsal side of the larva to allow penetration of the fixative. Larvae were fixed in Gendre's fluid, dehydrated in alcohol and cleared in xylene. They were then infiltrated with paraffin wax, under vacuum, and embedded in wax. Longitudinal and transverse sections (thickness 8 μ m) were cut using a R. Jung (Heidelberg) microtome. The sections were mounted on microscope slides coated with egg albumen and left to dry overnight.

The sections were cleared in xylene, rehydrated and stained with either lactophenol cotton blue, counter-staining with eosin; or with periodic acid-Schiff reagent, counter-staining with Light green. They were then dehydrated and mounted in DPX. (See Appendix IV for

INCUBATION TEMPERATURE (°C)	INITIAL No. OF LARVAE IN CHAMBER	% OF LARVAE ALIVE AFTER 4 DAYS	MEAN $\bar{x} \pm SE$ OF LARVAE ALIVE AFTER 4 DAYS	% OF LARVAE VISIBLY INFECTED AFTER 4 DAYS	MEAN $\bar{x} \pm SE$ OF LARVAE VISIBLY INFECTED AFTER 4 DAYS
25	15	40	31.5 ± 3.8	26.7	22.5 ± 5.5
	20	35		0	
	30	26.7		13.3	
	25	24		32	
	25	20		36	
	30	43		27	
30	25	40	23.9 ± 7.1	20	23.2 ± 6.7
	25	16		32	
	25	20		24	
	30	3.3		40	
	15	40		0	
35	20	0	6.8 ± 5.0	0	8.3 ± 3.9
	30	26.7		13.3	
	25	4		8	
	30	3		20	
	15	0		0	

N.B. SE. represents 'standard error of the mean'.

Figure 21: Table showing the mean percentage of larvae living and visibly infected with A. apis after 4 days incubation in self-feeding chambers at 25, 30 and 35°C.

procedures and materials).

Results and Discussion

Temperature and infection

From the results shown in Figure 21 it can be shown that there was no significant difference between the mean percentages of larvae living after incubation for 4 days at 25° or 30°C ($t = 0.98$; $P > 0.05$, $df = 9$), whereas the percentage was significantly reduced at 35°C when compared to that at 30°C ($t = 1.96$; $P < 0.05$, $df = 8$). It was observed that of those larvae not showing any visible signs of infection, the most healthy, retaining their pearly-white appearance, were those incubated at 25° or 30°C. Of those larvae which became visibly infected after 4 days, most were incubated at 25° or 30°C. There was no significant difference between the percentages of infection achieved at these temperatures ($t = 0.08$; $P > 0.05$, $df = 8$), but a significantly reduced percentage was achieved at 35°C when compared to that obtained at 30°C ($t = 1.92$; $P < 0.05$, $df = 8$). This was to be expected since it was the percentages of infections arising from live larvae that were counted, therefore if more larvae remained alive at the lower temperatures then more could become visibly infected. At this stage the site of infection could not be determined. The lower temperature of 25°C was used by Glinski (1981) who claimed to induce oral and cuticular infections, but this may have been a result of the low incubation temperature, which is unlikely to be frequently encountered in vivo. Therefore, in future experiments here, where larvae were to be infected, they were incubated at 30°C - close to that temperature maintained in their natural environment, but at which they seemed to survive better in vitro and which supported more infections.

Symptoms of disease

Once larvae became infected in vitro they lost their pearly-white appearance, becoming cream-coloured. They also became hardened, which was noticed when larvae were gently 'prodded' to determine whether they were still responsive. This hardening was presumably due to the numerous hyphae in the larval tissues by this stage. The first visible signs of the hyphae themselves were usually at the posterior-dorsal side, where fine, white hyphae penetrated the cuticle and grew aeri ally. This could be as early as 3 days post-inoculation and the larva would not necessarily be dead. During the next few days more hyphae would penetrate the dorsal side, until the larva was covered in a white mycelial mass and was then invariably dead. The hyphae continued to grow aeri ally and would sporulate within the following week.

The symptoms observed here appear to be similar to those seen in M. rotundata infected with A. aggregata or A. proli perda. In the former case, according to Vandenberg and Stephen (1982), chalk brood signs invariably became apparent after cessation of movement and within 24 hours the larva developed a tan or pink or grey cast internally, in one body area, which spread throughout the whole host in another 24 to 48 hours. The diseased larva remained tan or changed to a chalk-white colour and finally assumed a mottled appearance as dark fungal cysts formed beneath the cuticle. Usually the cuticle remained intact, but occasionally the fungus erupted through it and grew to a limited extent on the residual pollen diet of the infected larva, an observation disputed by Youssef et al. (1984). It was also noted that those larvae inoculated at one day old, which had developed chalk brood by the fourth instar, exhibited different symptoms. Internal colour changes were not observed, but the opaque cream colour

was retained during hardening of the cadaver. Such young larvae were not infected in the experiments here, so no comparison can be made to this particular observation. The symptoms caused by A. proliperda in M. rotundata were described by Youssef et al. (1984). They noted that dying larvae frequently defecated a small number of soft pellets, but this was not observed in the infections by A. apis or A. aggregata. Similarly dead larvae became flaccid, whereas honey bee larvae infected with A. apis hardened. The larvae infected with A. proliperda did, however, undergo a colour change to cream-brown. During the week following death larvae became desiccated and shrivelled slightly, prior to the development of a light dimpling of the integument which was apparently due to the pressure of the growing hyphae that eventually penetrated the integument. The larvae then appeared 'fuzzy' as they were overgrown with mycelia. Approximately 5 to 8 days later nutriocytes were visible, which darkened and developed as spore cysts in another 5 to 7 days.

Disease development

Germinating spores in larval mid-guts as well as more advanced infections were observed in stained sections of larvae which had been infected in vitro and in vivo. In both cases, however, it was not known whether these were induced infections, as a result of feeding larvae sucrose solution containing spores, or whether they were natural infections. In vitro infections were observed in third, fourth and fifth instar larvae.

In sections of mid-gut individual activated and enlarged spores and germ-tubes were observed (Figure 22), as well as those spores germinating in spore balls (Figure 23), amongst the contents of the mid-gut lumen. Activated and enlarged spores were observed 18 hours post-inoculation in larvae fed spores in vitro, while germ-tubes were

Figure 22: Photomicrograph of a longitudinal section through a larval mid-gut, stained with eosin and lactophenol cotton blue. This shows the epithelial cells (EC) and the peritrophic membrane (PM) lining the mid-gut; also enlarged spores (E) of A. apis and germ-tubes (G) amongst the contents of the mid-gut lumen. (x 336).

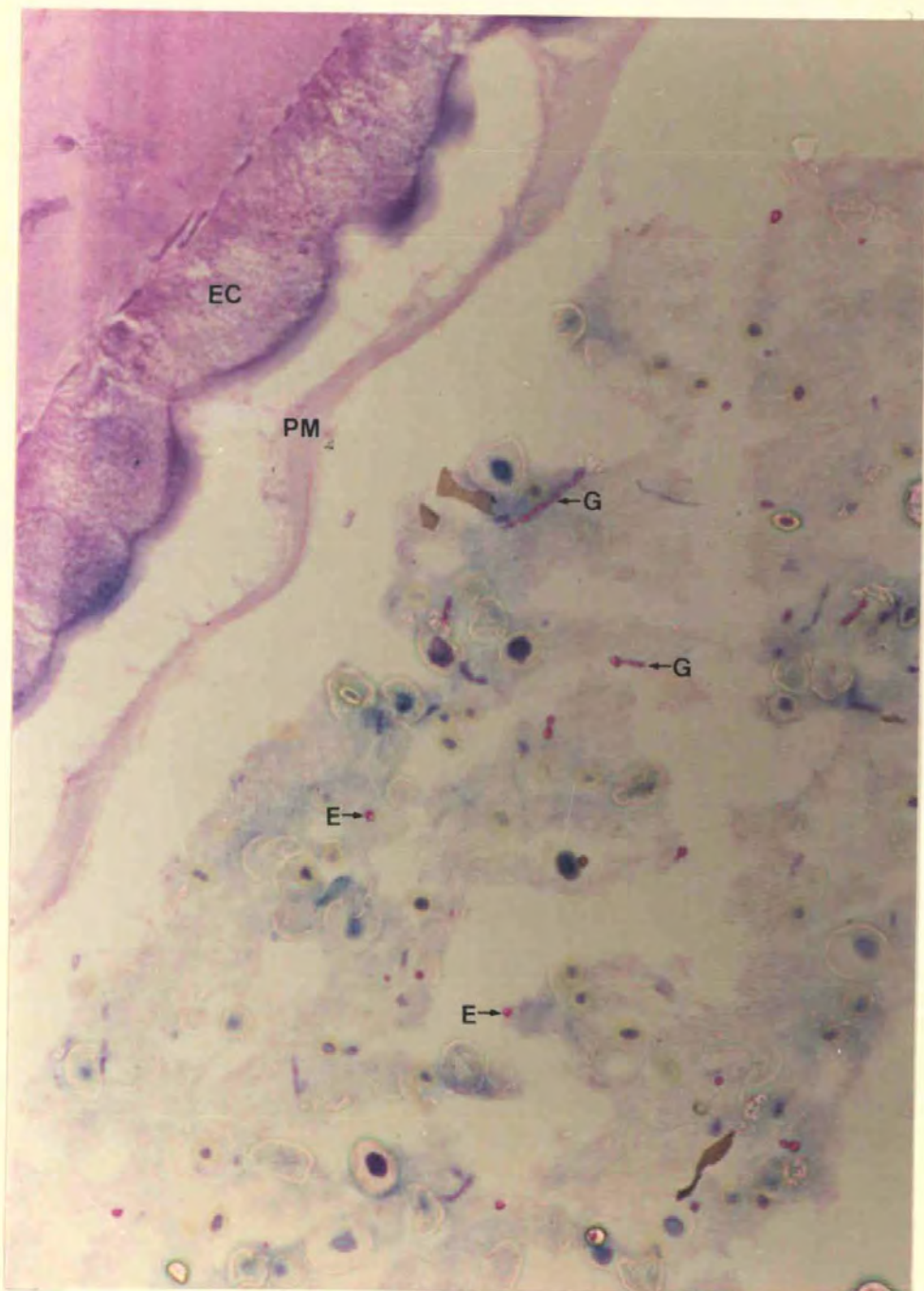
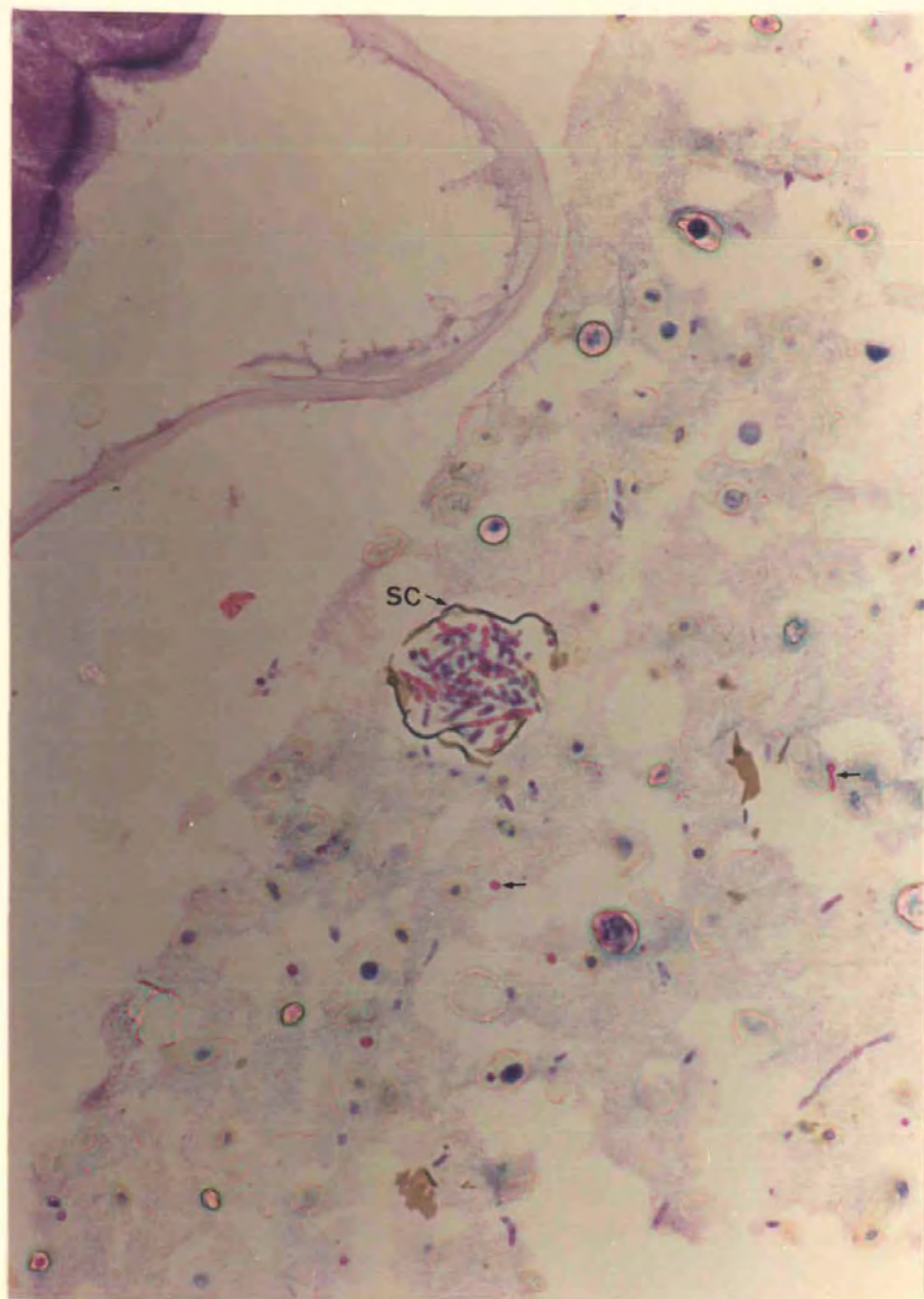


Figure 23: Photomicrograph of a longitudinal section through a larval mid-gut, stained with eosin and lactophenol cotton blue. The spores of A. apis have germinated within the spore cyst (SC). There are also individual enlarged spores and germ-tubes (arrows). (x 336).



seen after 24 hours. By 48 hours these had penetrated the peritrophic membrane and gut epithelial cells. The young hyphae appeared to grow directly through the peritrophic membrane and to digest the epithelial cells, rather than using only mechanical means to force their way through the gut lining. Figure 24 shows a hypha growing through a gut epithelial cell, while in Figure 25 the developing hypha has reached the basal membrane and has apparently caused the complete disruption of the epithelial cell through which it has grown. It was also interesting to note that germ-tubes within the mid-gut lumen grew directly out of the gut, rather than first filling the lumen with hyphae. This was also observed by Youssef et al. (1984) in the mid-guts of M. rotundata infected with A. proliferda. They noted that developing hyphae immediately penetrated the gut wall, regardless of their location. However, they did not speculate on any reason or cause for this observation, yet it is not the expected reaction of fungal hyphae, which usually exploit the most readily available nutrient source. The gut contents of a growing larva would presumably be rich with suitable nutrients and much more accessible than the gut lining. Perhaps the directional growth exhibited by the developing hyphae is due to a chemotropic response to a gradient existing between the gut lumen and the haemocoel. This may take various forms, including an ionic gradient. In some insects the mid-gut plays an active role in ionic regulation, with goblet cells believed to be involved in the active transport of potassium ions from the haemocoel to the gut lumen. Consequently, such gradients may serve to attract developing hyphae to the haemocoel or perhaps to repel them from the gut lumen. Unfortunately little is known of chemotropism in fungi, let alone specific evidence for its role in entomopathogenic infections. This may be due to the lack of a reliable quantitative assay (Griffin, 1981).

Figure 24: Photomicrograph of a transverse section through a larval mid-gut, stained with eosin and lactophenol cotton blue. Young hyphae are growing through the peritrophic membrane (PM) towards the epithelial cells (EC). The arrows indicate hyphae that are beginning to penetrate epithelial cells. (x 336).

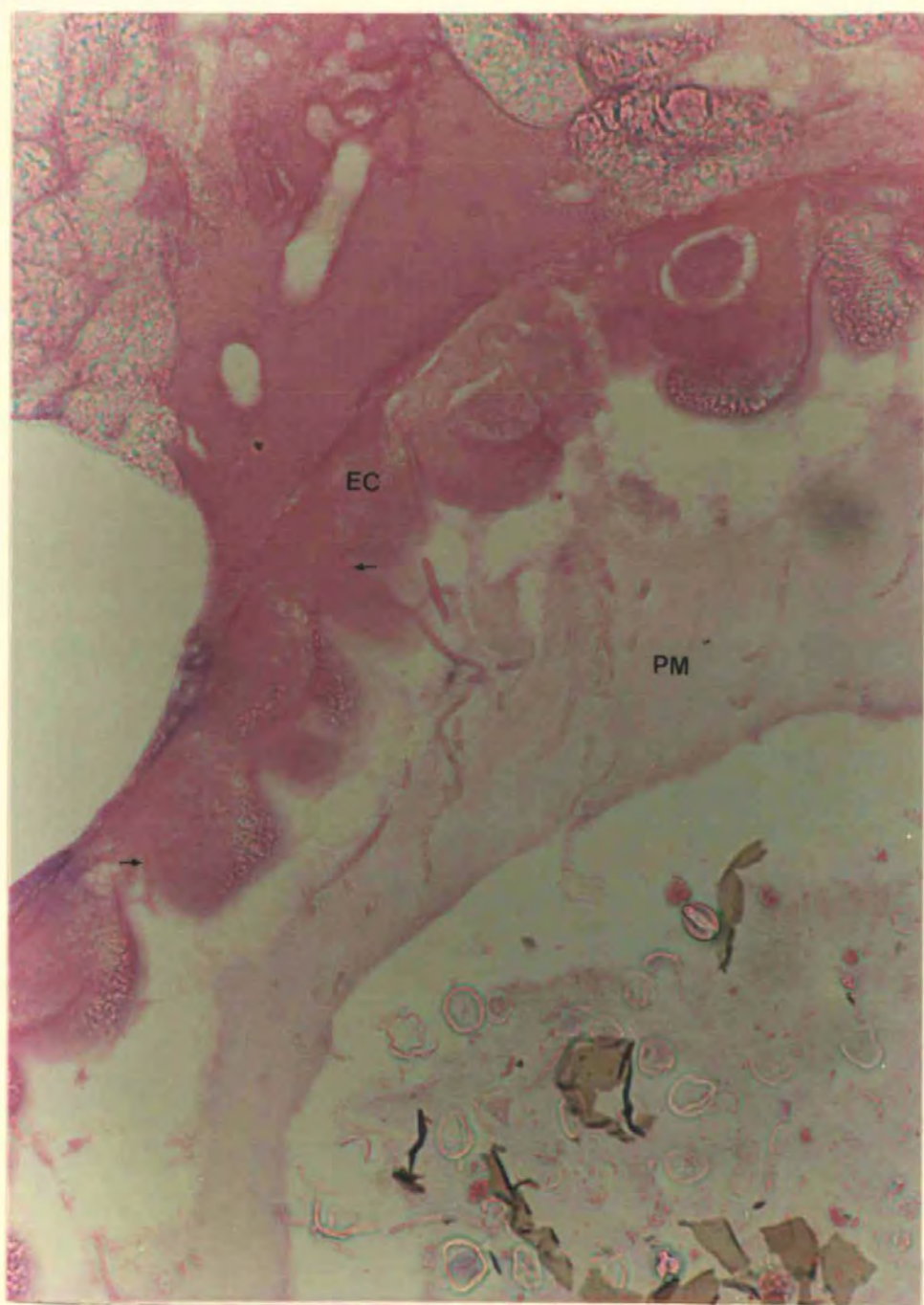
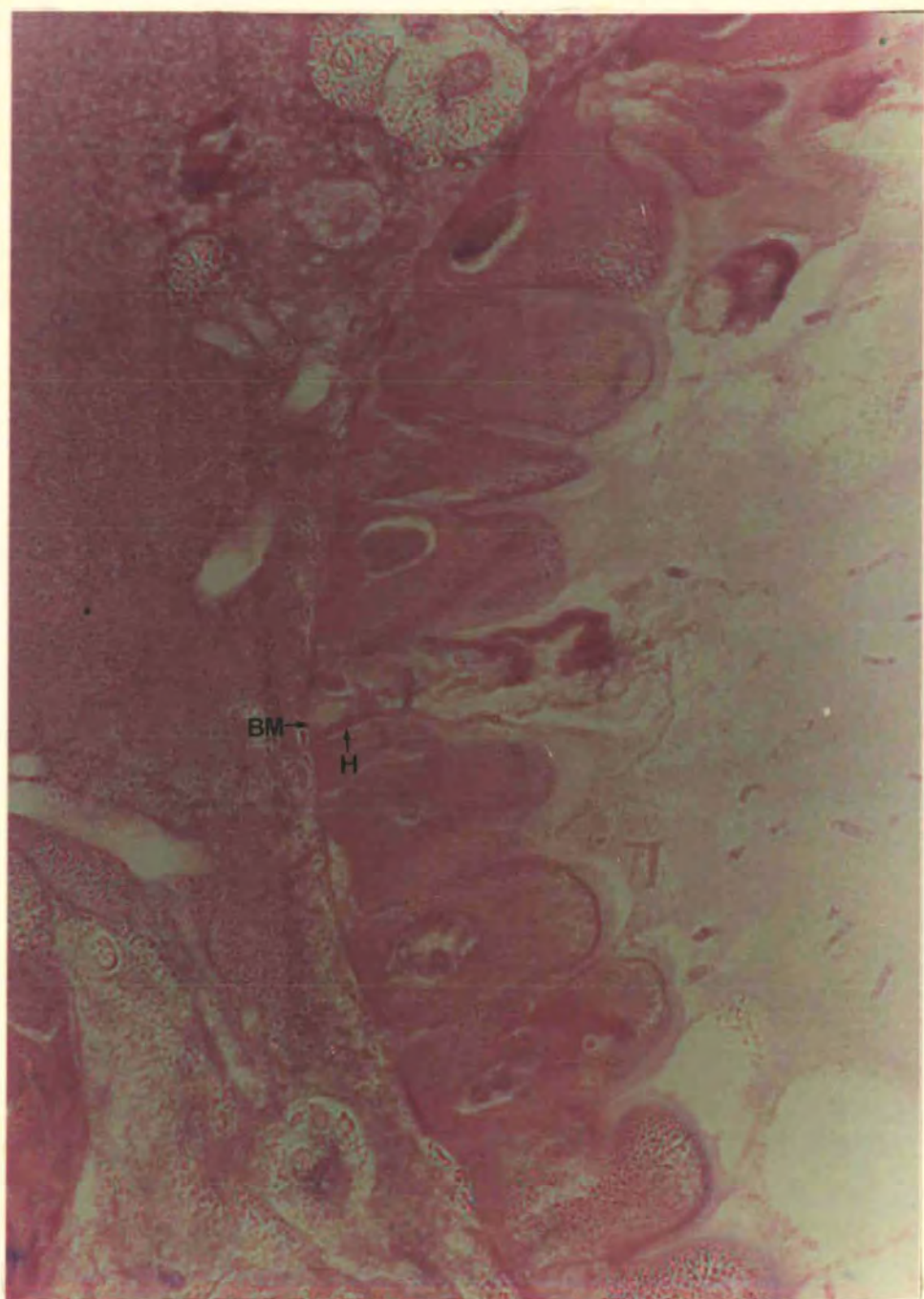


Figure 25: Photomicrograph of a transverse section through a larval mid-gut, stained with eosin and lactophenol cotton blue. A developing hypha (H) has penetrated an epithelial cell and reached the basal membrane (BM). The attacked cell is completely disrupted. (x 336).



Chemotaxis, however, has received more attention and has a recognised role in the attraction of pathogenic zoospores to the roots of their host plants. Therefore, it seems fair to speculate that chemotropism could play an important part in these insect infections.

Once the epithelial cells have been penetrated, hyphae grow throughout the haemocoel. Figure 26 shows a longitudinal section through a partly infected mid-gut, where hyphae have just penetrated some of the epithelial cells, but have not yet extended through the haemocoel. A weft of hyphae is often observed at the basal membrane of the epithelium, where hyphae probably fill the haemocoelomic spaces prior to further development. Figures 29 and 30 illustrate this. They show transverse sections through fully infected larvae and this weft is clear in each instance. This can also be seen in Figure 31, which also demonstrates the directional growth of the developing hyphae away from the gut lumen. Although Figure 28 apparently shows a hypha penetrating a fat cell such an observation was rare and most commonly the hyphae appeared to push their way between cells, as illustrated in Figure 27. No evidence was found of any kind of resistance by the host cells towards the invader and no signs of encapsulation were identified. The hyphae grew throughout the haemolymph and did not appear to attack the fat body cells to any extent until later in the infection process. Tracheae (Figure 32), malpighian tubules and muscles were also attacked. Youssef et al. (1984) commented on the invasion of all tissues by A. proliperda and noted all cuticular elements, regardless of system, were infected. In A. mellifera, once A. apis reached the outer cuticle this did not present a barrier, but the hyphae were able to grow through this (Figure 33) and produce a mass of hyphae on the integument. The points of penetration of the cuticle appeared random, in that intersegmental regions or spiracles

did not appear to be used preferentially, which was also the case for A. proliperda in M. rotundata. . . However, 'bundles' of hyphae were observed to penetrate the integument for this fungus (Youssef et al., 1984), but this was not so for A. apis, where individual hyphae appeared to be capable of piercing the outer cuticle. Youssef et al. (1984) also claimed ascocysts were produced by A. apis beneath the cuticle as well as outside the host, but sporulation was only observed outside the host in this investigation.

The apparent ease and speed by which A. apis grows throughout its host initially suggests toxins are being produced to incapacitate the larva and that no evidence was found for any defence mechanism in action also suggests that the haemocytes may have been inactivated by a substance secreted by the invader. However, this would be expected to be associated with extensive tissue damage throughout the host early in infection and this is not the case; there is a striking lack of tissue damage until late in infection. Indeed, this fungus may not need to rely upon toxin production but may instead possess a well-adapted enzyme complement which enables it to quickly and efficiently digest and penetrate its host. Consequently, the enzyme complement of A. apis requires investigation.

Figure 26: Photomicrograph of a longitudinal section through a larval mid-gut, stained with PAS and Light green. Hyphae have penetrated the epithelium (arrows), but the infection has not spread far into the fat body. The fibrous nature of the peritrophic membrane can be seen(L).(x 170).

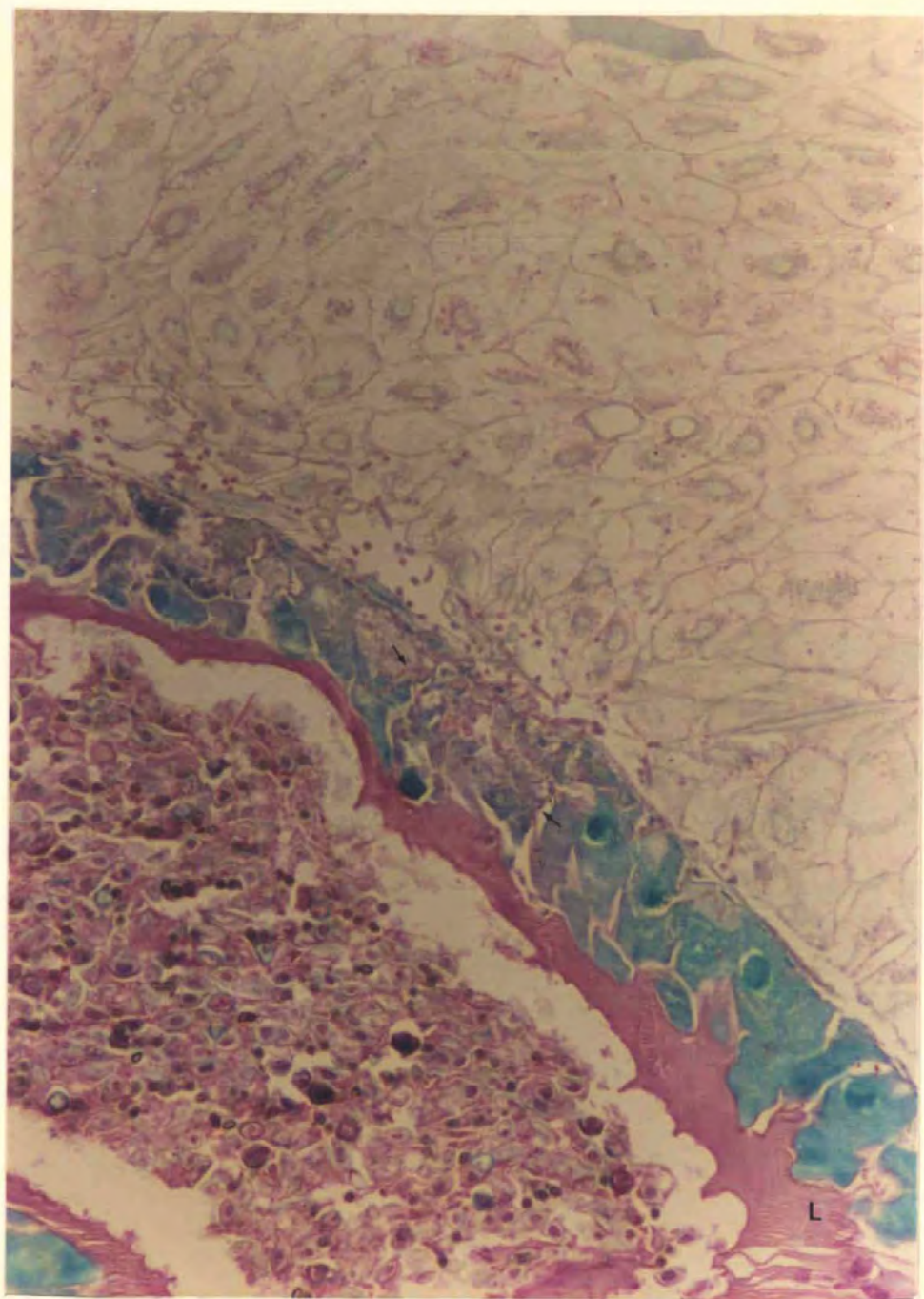


Figure 27: Photomicrograph of a longitudinal section through a larval mid-gut, stained with PAS and Light green. The young hyphae have disrupted the epithelial cells and are beginning to grow in the fat body - apparently pushing their way between the cells (arrow). (x 336).

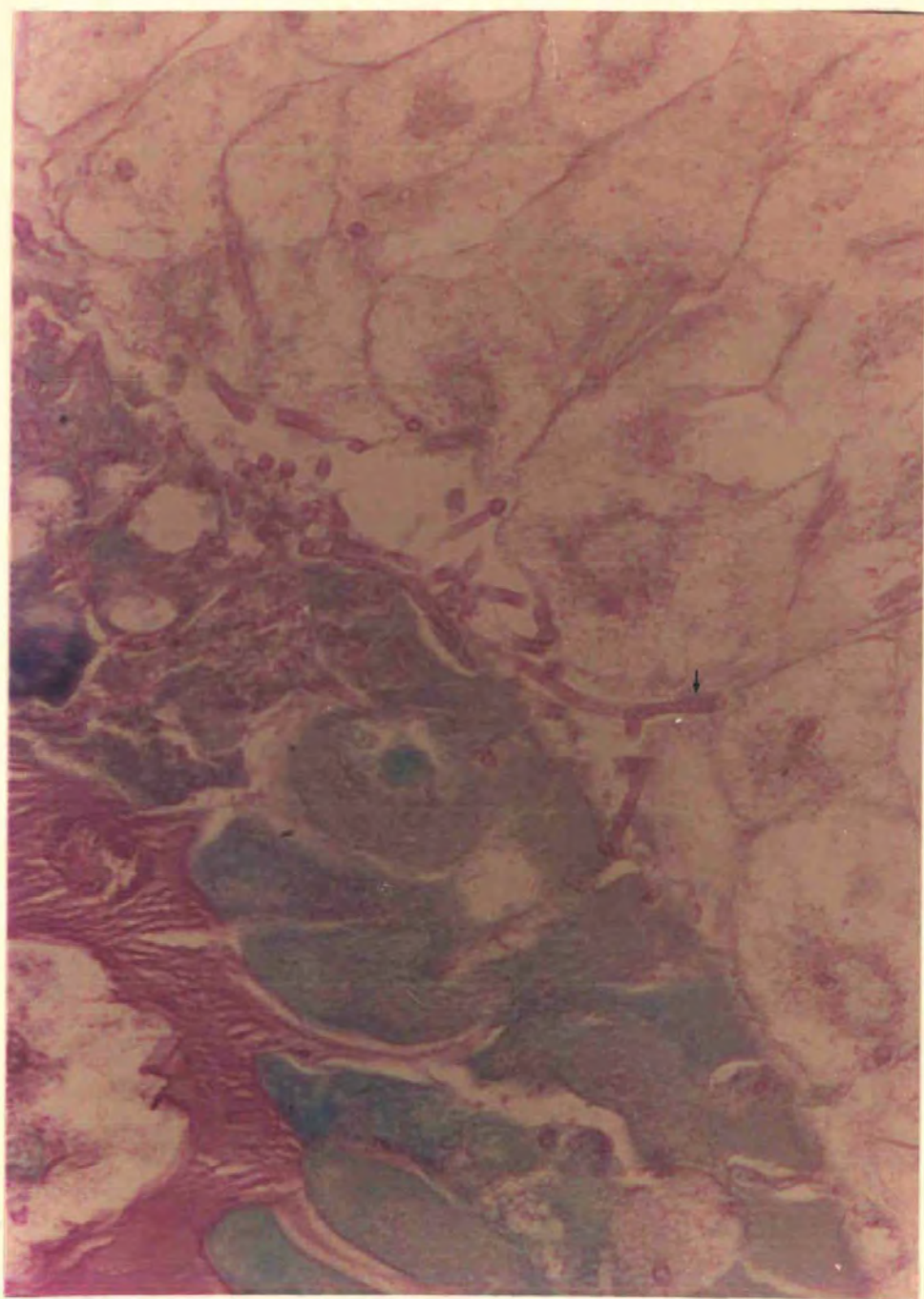


Figure 28: Photomicrograph of a longitudinal section through a larval mid-gut, stained with PAS and Light green. The young hyphae have penetrated the gut epithelium. The tip of one hypha (H) can be seen penetrating a fat cell. (x 430).

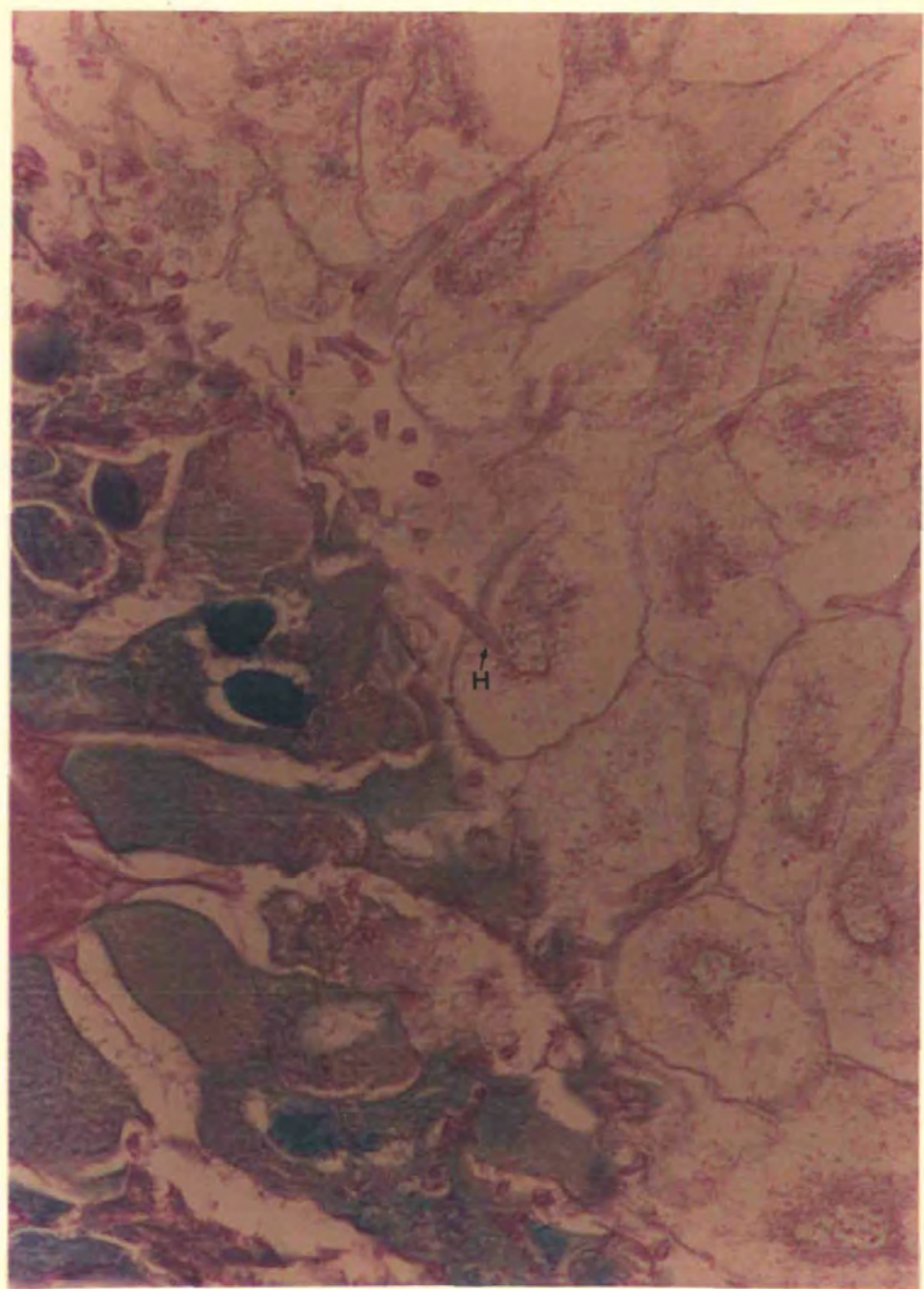


Figure 29: Photomicrograph of a transverse section through an infected larval mid-gut, stained with PAS and Light green. The hyphae have completely disrupted the gut epithelium (EC) and are growing throughout the haemocoel. A weft of hyphae (W) is apparent at the basal membrane of the epithelium, where hyphae grow profusely in the blood spaces prior to their advancement throughout the larval tissues. It can be seen that the mid-gut lumen does not become full of hyphae before the infection spreads beyond the gut. Young hyphae can be seen radiating outward from the peritrophic membrane towards the epithelium (arrows). (x 132).

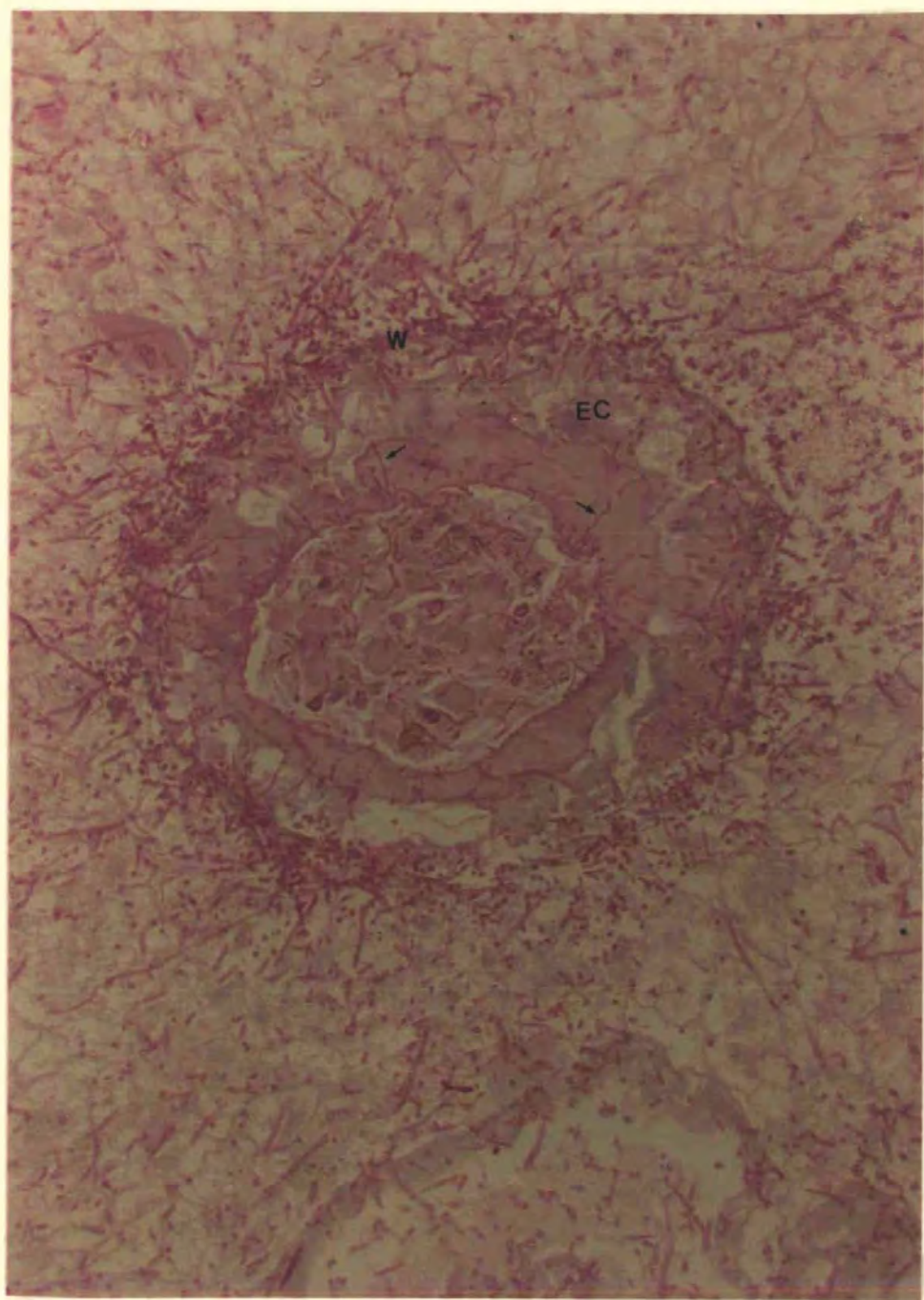


Figure 30: Photomicrograph of a transverse section through an infected larval mid-gut, stained with PAS and Light green. The hyphae have grown throughout the haemocoel and a weft of hyphae (W) is present at the basal membrane of the gut epithelium. The peritrophic membrane is not severely disrupted. (x 132).

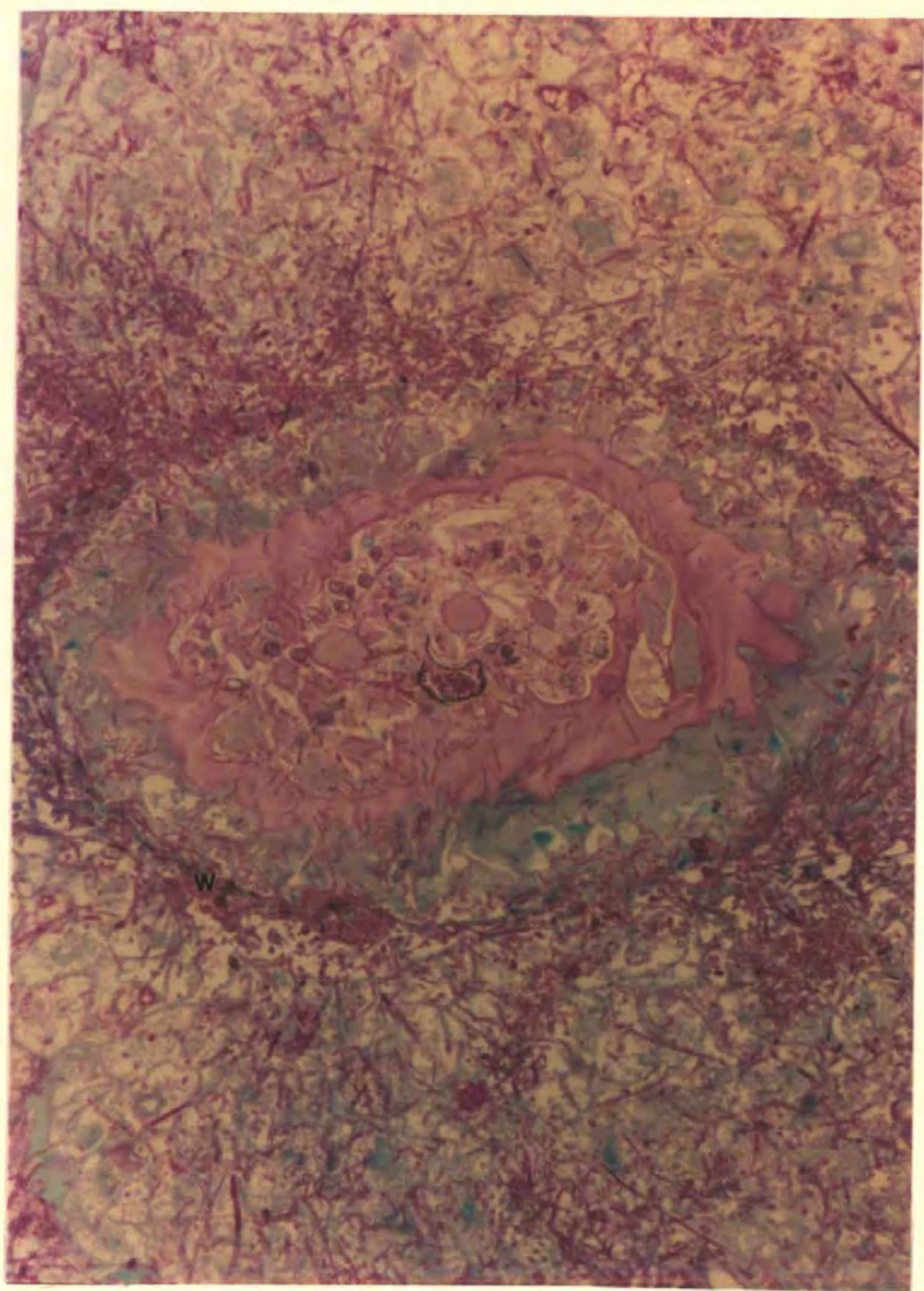


Figure 31: Photomicrograph of a transverse section through an infected larval mid-gut, stained with PAS and Light green. This also shows the weft of hyphae growing in the blood spaces surrounding the mid-gut and the lack of hyphal development within the mid-gut lumen. A spore cyst (SC) containing germinating spores is present amongst the mid-gut contents. (x 336).

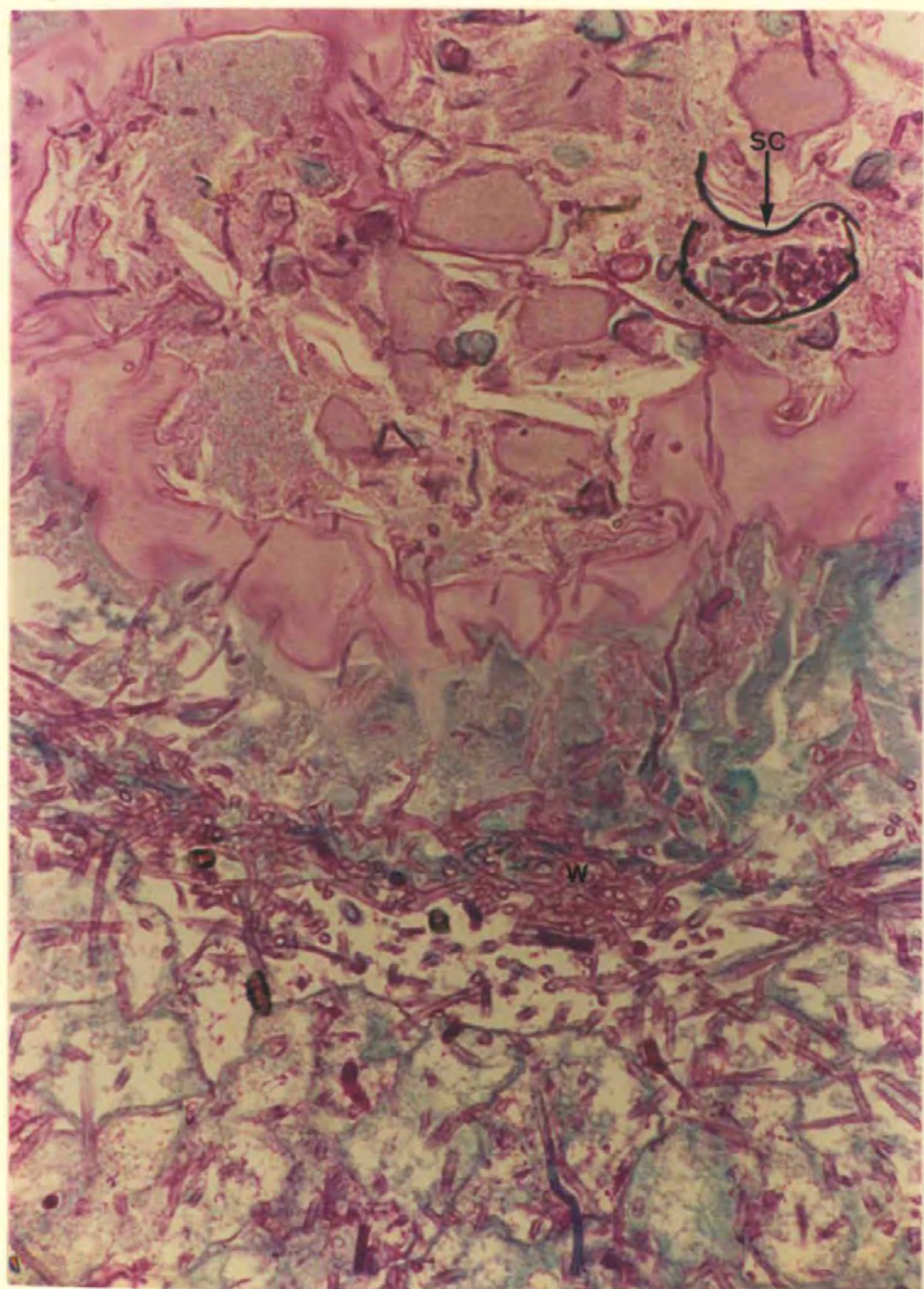
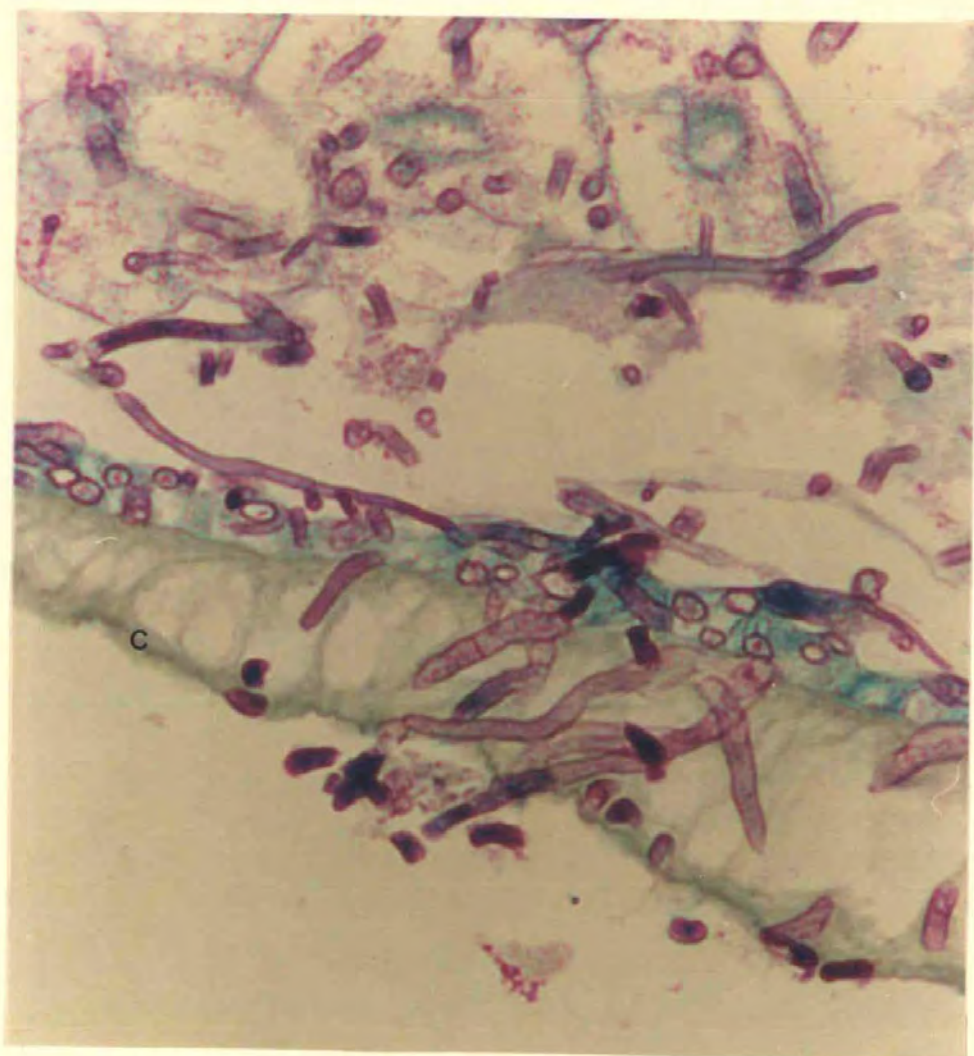


Figure 32: Photomicrograph of a longitudinal section through an infected larva, stained with PAS and Light green. A trachea is shown and hyphae appear to be encircling its cuticular lining (arrows). (x 336).



Figure 33: Photomicrograph of a longitudinal section through an infected larva, stained with PAS and Light green. The hyphae have reached the larval integument where the cuticular layers (C) do not present a problem to the penetrating hyphae. (x 538).



3.6 ENZYME PRODUCTION BY ASCOSPHAERA APIS

Introduction

Since there is now convincing evidence that A. apis spores infect honey bee larvae via the mid-gut, it seems unlikely that this fungus possesses the poor enzymic ability suggested by the work of Huber (1958) and Gochnauer and Margetts (1979). The former studied the nutrient requirements of several insect mycopathogens - M. anisopliae, Cordyceps militaris, B. bassiana, Aspergillus flavus, A. apis - and found the latter to be the most difficult to culture. Although the other fungi were able to utilise such nutrients as fat, glycogen, urea, milk and chitin, A. apis could only be grown on milk agar. Recent studies, however, on the nutritional requirements of A. apis for vegetative growth have shown that vitamins are required by this fungus (Castle, 1981) and these were probably vital constituents missing from the culture media tested by Huber. He also used ammonium nitrate as a nitrogen source, but this fungus requires an organic nitrogen source (Patel, 1983). Gochnauer and Margetts also attempted to grow A. apis on various media containing substances such as starch, chitin, Tween 20 and gelatine for the detection of enzymic activity by vegetative hyphae, but found all expected lytic enzymes, except phosphatase, to be absent from the growing cultures. Both of these investigations studied the enzymic activities of vegetative hyphae rather than germ-tubes or young developing hyphae, which may prove to be more enzymically active in order to initiate the infection process. There are several theories concerning the most enzymically active phase of a fungal life cycle. Enzymes may be produced at any stage, providing the necessary conditions for enzyme synthesis are fulfilled; alternatively, the youngest stage, such as the germinating spore, may be most active. Consequently, the germinating spores of

A. apis may have a different enzyme complement to that of the vegetative hyphae.

The enzyme activities of other entomogenous fungi have been studied and most researchers have concentrated on those enzymes required to penetrate the host cuticle, namely chitinases, proteases and perhaps lipases to attack the protective epicuticle. Robinson (1966) suggested the epicuticle was penetrated mechanically, followed by enzymic attack of the subsequent layers of cuticle. Conversely, by studying the histochemistry of the invasion of the cuticle of Galleria mellonella and B. mori by Entomophthora coronata Gabriel (1968a) thought lipases were produced at the point of penetration. He believed the chitin was not extensively dissolved, but that protein hydrolysis, restricted to the area surrounding the invading hypha, digested certain proteins thereby loosening the exo- and endo-cuticular lamellae, which could then be easily penetrated by mechanical means. Gabriel (1968b) had detected chitinolytic, proteolytic, and lipolytic activity in vitro by Entomophthora apiculata, Entomophthora thaxteria, Entomophthora virulenta and 4 strains of E. coronata, but considered histochemical studies necessary to confirm his initial observations. Other studies of the enzyme activities of entomopathogenic fungi include an investigation of B. bassiana (Leopold and Samsinakova, 1970); 13 members of the Deuteromycetes (Samšičáková and Mišiková, 1973) and of B. bassiana, M. anisopliae and Paecilomyces viridis (Pabst and Sikorowski, 1980).

It is considered that enhanced enzymic activity in a fungal strain is related to its virulence. Samšičáková and Mišiková (1973) examined the production of degradative enzymes by fungal strains of diverse origins in relation to their virulence against G. mellonella, finding the production of chitinase, protease and lipase to vary

together and that those strains producing most enzyme activity were likely to be the most virulent. Al-Aidroos and Seifert (1980) tested the relationship between enzyme activity and virulence by using mutants of M. anisopliae altered in a single enzyme activity. They found the ability to degrade starch was correlated with virulence towards the mosquito Culex pipiens pipiens, whereas the ability to degrade 2 protein sources, gelatine and milk, was not. They thought the starch-degrading activity could, in vivo, be related to the use of glycogen or other insect substrates and to the invasion of insect tissues. Consequently, strains of A. apis may vary in their virulence according to their enzyme activities, which may also help to explain the spread of chalk brood disease, as strains mutate and the most virulent, that is, those with the most appropriate enzyme complement for infecting larvae, are those that survive. These enzymes would be expected to be related to the route of infection, therefore, those required to penetrate the peritrophic membrane and gut epithelium initially. However, before the relationship of virulence and enzyme activity can be considered for this fungus, its typical enzyme complement requires investigation.

Determination of protease, chitinase and lipase activity by vegetative hyphae, germ-tubes and germinating spores

Method

Plates containing 1.5% agar and 0.005% oxytetracycline plus one of the following were prepared:

- (i) 5% skimmed milk
- (ii) 0.1% colloidal chitin (prepared by the method of Lingappa and Lockwood, 1962)
- (iii) 2% tributyrin

STAGE OF FUNGAL DEVELOPMENT	PROTEOLYTIC ACTIVITY	CHITINOLYTIC ACTIVITY	LIPOLYTIC ACTIVITY
Vegetative hyphae	++++	-----	-----
Germ-tubes	++++	-----+	-----+
Germinating spores	++++-	-----	-----

N.B. Each (+) or (-) represents the result observed on one test agar plate.

Figure 34: Table showing the presence (+) or absence (-) of proteolytic, chitinolytic and lipolytic activity by vegetative hyphae, germ tubes and germinating spores of A. apis.

Each type of media was then inoculated with each of the following:

- (i) A 1 cm diameter plug of vegetative hyphae taken from a growing culture on CCP medium (Appendix III)
- (ii) A 30 μ l drop of spores previously germinated in GYO liquid medium for 30 hours at 35°C in 10% carbon dioxide
- (iii) The 'crushed' scrapings from a mummified larva

Those plates inoculated with vegetative hyphae or germinated spores were incubated at 30°C for 3 days. The plates inoculated with dormant spores were incubated at 35°C for 24 hours in 10% carbon dioxide and then observed for signs of medium utilisation. Each day for 3 days the remaining plates were observed for signs of medium utilisation, that is, a clear halo in the medium surrounding the inoculum.

Results and Discussion

As seen in Figure 34 this simple method failed to conclusively detect any lipolytic or chitinolytic activities at any of the 3 developmental stages investigated. The tests for proteolytic activity, however, proved positive in the majority of trials. Firstly, considering vegetative hyphae, these would be expected to produce a wide range of enzymes in order to utilise all the chemical constituents of the infected larva to the greatest advantage, but this was not reflected in the results. Thus suggesting this experimental method is too simple to be used to determine enzyme activity, considering the complexities of substrate-enzyme specificities and interactions, although similarly basic techniques were successfully used by Gabriel (1968, b) and Pabst and Sikorowski (1980) to determine enzyme production by other entomopathogenic fungi. It should be noted, however, that the results shown here were obtained using only one strain of A. apis and that the enzyme complement will vary between strains, so the enzyme production by several strains from a variety of sources

ought to be tested. Since A. apis is unlikely to produce a caseinase as casein would not be encountered in vivo, a non-specific protease must have been responsible for the digestion of casein in the milk agar. Perhaps the results obtained here reflect the production of non-specific proteases by A. apis and its secretion of specific lipases and chitinases. The latter would be expected to be highly specific, since the larval chitin only must be digested and not that present in hyphal walls. This could be overcome by membrane-bound chitinases on the hyphal walls, which would also account for the apparent lack of degradation observed in infected peritrophic membranes and integuments.

In the case of germinating spores these would not be expected to produce an extensive enzyme complement, but rather to utilise the simplest, most readily metabolisable nutrients available to support and include in their extensive biochemical reactions. Earlier investigations on the nutritional requirements of germinating spores indicated an amino acid mixture is required as well as a carbon source (section 2.4). During the enlargement stage, however, proteases may be produced or activated in order to supply additional amino acids to support germination, possibly by providing the building blocks for further enzyme synthesis. Once the spores have germinated, the germ-tubes would require a range of enzymes in order to initiate infection. Although amounts of lipid may be present in the mid-gut, depending upon the pollen source on which the larva was fed, the developing hyphae may not utilise these. However, they would be expected to produce a chitinase in order to penetrate the peritrophic membrane, which is assumed to be a chitin-protein complex. The fast rate of penetration suggests the membrane is enzymically weakened, although there is little degradative damage surrounding the young hyphae, suggesting the enzymes may be bound to the hyphal walls. Indeed, these need not be chitinases,

but as noted by Gabriel (1968a) for a cuticular infection, the chitin component may not be extensively digested, rather certain associated proteins are attacked, so weakening the membrane allowing easy mechanical penetration. It was, therefore, decided to investigate the possible chitinase production by germinating spores using a more sensitive method, in order to help determine whether this was necessary for penetration of the peritrophic membrane.

Qualitative study of chitinase production by germinating spores

Method

2 x 25 cm³ conical flasks containing 5 cm³ of one of the following liquid media were prepared:

- (i) 1% glucose and 1% yeast extract (GY)
- (ii) 1% glucose (G)
- (iii) 1% yeast extract (Y)
- (iv) distilled water (W)

To each flask was added 0.05 g of colloidal chitin, prior to sterilisation. Once cool, oxytetracycline, sterilised by membrane filtration, was added to give a final concentration of 0.005%. Each flask was then inoculated with the 'crushed' scrapings from 1 mummified larva. They were then sealed with suba-seal bungs and injected with carbon dioxide to give a final concentration of 10% and were incubated at 35°C for 30 hours. Control flasks from which either chitin or spores were omitted were also incubated.

After incubation, the media were separated from their spores by centrifugation (5000 r.p.m. for 5 minutes) and filtration through sterile Millipore filters (pore size 4.5 µm). A drop from each pellet containing spores was observed microscopically for signs of spore germination and contamination. 0.5 cm³ of each filtrate was

tested for the presence of N-acetylglucosamine using the method of Reissig et al. (1955) and reading samples at wavelength 585 nm in a CE 303 Grating spectrophotometer, against the appropriate germination medium blanks.

The filtrates were also tested for their enzymic activity. To 0.5 cm³ of filtrate in a sterile test tube was added 1 cm³ of sterile colloidal chitin suspension (approximately 10 mg/cm³) in acetate buffer (pH 5, 0.08 M). This was incubated in a water bath at 33°C for 24 hours. Filtrates containing inactivated enzyme were also tested. The enzyme was inactivated by placing the filtrate in a boiling water-bath for 15 minutes prior to its incubation with the chitin suspension. After incubation all filtrates were observed for signs of contamination by examining a drop microscopically and then tested for an increase in amount of N-acetylglucosamine, using the Reissig method and reading the test filtrates against the 'boiled' controls at 585 nm.

Results and Discussion

Although quantitative results were obtained there proved to be great variability between the absorbence readings acquired during each experimental run. Therefore, the results could only be compared within each run, not between runs. This variation was probably due to the spores, either in the amounts added to each flask, since this was only quantified as the scrapings from 1 mummified larva, or was due to inherent differences amongst the strains of spores used. Consequently, the data has been represented in a qualitative form in order that the trend of the results from the experimental runs can be seen.

In the first part of the experiment the presence of n-acetylglucosamine in the various germination media was investigated

INCUBATION CONDITIONS	EXTENT OF GERMINATION	DETECTION OF NAG	DETECTION OF CHITINASE ACTIVITY
GY + C + S	germ-tubes	++	+
GY + S	germ-tubes	++	++
GY + C		-	-
G + C + S	enlargement	++	-
G + S	enlargement	+	+
G + C		-	-
Y + C + S	enlargement	+	+
Y + S	enlargement	-/+	++
Y + C		-	-
W + C + S	activation	++++	-/+
W + S	activation	+++	-
W + C		-	-

N.B. (GY), (G), (Y) and (W) represent different media
(see method)

(C) represents chitin

(S) represents spores

(-) indicates undetectable quantities

(+) indicates detectable quantities

↓

(++++) increasing quantities detected

Each result represents the trend observed from 3 experimental runs.

Figure 35: Table showing the extent of germination of A. apis spores and the detection of n-acetylglucosamine (NAG) and chitinase activity in their various germination media.

and in the second part the media were used as enzyme preparations. Samšišňáková et al. (1971) used the medium in which B. bassiana was grown as an enzymic solution and found the activity identical to that obtained using a homogenate of hyphal mat. In that instance the majority of enzymes were released into the medium and this was assumed to be the case for A. apis. Different incubation media were used here in order to determine whether spores could utilise chitin as a carbon source, nitrogen source or both.

As shown in Figure 35, in the first part of the experiment, the greatest amount of n-acetylglucosamine (NAG) was detected where spores were incubated in water and chitin. The control, containing no inoculum, produced a negative result and that where chitin was omitted produced a positive result. Thus suggesting the NAG was not a result of enzymic action on chitin, but arose from the spores themselves. Perhaps NAG is washed from the surface of the spore walls during incubation and more is lost after activation when there is a change in spore wall permeability. If this does occur at activation, similar amounts of NAG ought to be detected in each instance where spores have activated, but this is not the case. Far less is detectable once the incubation medium contains nutrients, which would at least support the enlargement stage of germination. It is possible that these spores 'mop-up' the NAG lost during activation for use during the growth of the spores, hence its disappearance from the medium. The extent to which this occurs seems variable and may be less efficient when glucose is included in the medium and could be utilised instead.

Although the spores incubated in water only produced a comparatively high reading for NAG, an even greater reading was achieved when chitin was included, suggesting there was also some

degradation of chitin producing more NAG. Since the spores in this medium only activated, the chitin being unable to be used as a carbon or nitrogen source, they remained in a metabolically inactive state. As such, any enzymes produced would be constitutive, possibly released from the spores once they had activated; or perhaps membrane-bound and brought into contact with the medium as a result of the changes in spore wall permeability at activation. Again, such high amounts of NAG would not be detectable once spores enlarge and can transport those molecules into the spore for utilisation.

Some of the theories suggested above to explain the first set of results are confirmed in the second part of the experiment. As shown in Figure 35, where spores were incubated in water, with or without chitin, there does not appear to be any significant enzyme activity. Perhaps the only enzymes available immediately after activation are membrane-bound, therefore their activity would only be detectable if the spores themselves were incubated with the chitin. The remaining results, where spores had been incubated with nutrients, indicate the presence of enzyme in the media, although there is an exception - where spores were incubated with glucose and chitin. Here, the original supply of chitinase may have been utilised during the initial incubation and without a nitrogen source the spores may be unable to replenish this supply. However, when chitin was lacking from the original medium the initial supply of enzyme was still available during the second incubation period.

The results reported here suggest germinating spores do have the ability to secrete chitinase, which appears to be constitutively produced. However, many of the above explanations are speculative and require much further experimental evidence in order to be validated. Also, these observations were made in vitro and under such experimental

conditions the spores may act differently to those in vivo.

Consequently, a histochemical study of infected larval mid-guts might best provide evidence for the production of enzymes by germinating spores in vivo and this would also indicate the composition of the honey bee larva peritrophic membrane.

CHAPTER 4

REVIEW AND DISCUSSION

4.1 INFECTION ROUTE UTILISED BY ASCOSPHAERA APIS

Just prior to this present investigation of the infection of larvae of A. mellifera by A. apis, chalk brood disease was thought to develop once larvae had ingested A. apis spores with their food and subsequently become chilled. It was considered that the spores required anaerobic conditions for germination and that such conditions were found in the larval mid-gut. However, hyphal growth was known to require aerobic conditions, so it was assumed that the chilling of larvae increased their permeability to oxygen and so made hyphal growth possible. The hyphae were then thought to grow throughout the larval tissues, eventually producing a mummified larva. Such accounts of chalk brood disease are to be found in bee pathology books by Morse (1978) and Bailey (1981). This explanation of disease development, however, lacked substantial evidence. Apart from Maurizio (1934) who began, but did not complete, a search for histological evidence for a mid-gut infection, there were no recorded observations or photographs of germinating spores in the mid-guts of larvae. To further complicate the issue, no other insect mycopathogen was known, at that stage, to attack its host through the mid-gut. This was thought to be due to the protective peritrophic membrane lining the ventriculus. It therefore seemed odd that A. apis was assumed to be unique amongst entomopathogenic fungi and to pursue this route of infection, when it was known that other fungi could not.

Doubts concerning this generally accepted picture of infection arose when Bignell and Heath (1985) demonstrated that fifth instar larval guts were electropositive; and Heath and Gaze (1987, in press) showed that high local carbon dioxide levels rather than anaerobic

conditions were the 'trigger' for activation of A. apis spores. These findings resurrected the observations of Roussy (1962), who claimed to have seen spores germinating on the larval surface cuticle. If high carbon dioxide levels rather than anaerobic conditions were important, then an infection arising from the surface, rather than the mid-gut, seemed more likely and in accordance with the known infection routes of other entomogenous fungi.

However, in this present study any attempts to germinate spores of A. apis on the surface of living larvae or on pieces of dissected body wall proved unsuccessful. Even incubation in atmospheres enhanced with carbon dioxide up to 10% failed to even activate spores on surface cuticle. This indicated the cuticle was inhibiting activation in some way. Although attempts here to isolate an inhibitory substance from larval epicuticle were unsuccessful, some fatty acids, particularly nonanoic, capric and undecanoic at 0.1%, were found to inhibit spore germination. Also, of the 4 constituents of larval food that were tested for their effects on spore germination, royal jelly proved to have a dramatic effect - greatly reducing all 3 stages of germination. It was very surprising to find brood food did not produce the same result. It is suggested that a component of the royal jelly, which makes it different from brood food, may be responsible for the inhibitory effects observed. One such component is 10-hydroxy-2-decenoic acid, a derivative of a 10-carbon fatty acid (capric or decanoic acid). As indicated above, another substance in larval epicuticle appears to have a similar inhibitory effect. Previous researchers, such as Koidsumi (1957), considered short to medium chain fatty acids to be responsible for the anti-fungal effect exhibited by other insect cuticles. Therefore, since royal jelly and epicuticle contain similar fatty acids, it is possible that

the mixtures of substances involved in inhibition in each case are similar. A comparison of the effects of combinations of the substances present in royal jelly and epicuticle may enable duplication of this effect in vitro.

This inability of spores to even activate on larval cuticle contradicts those researchers who claimed germination could occur on the larval surface. Roussy's observations have already been discussed (page 93). He was the only one to have claimed to have observed the germination process on larval cuticle. Others, such as Gilliam et al. (1978) and Glinski (1981) thought infection could occur via either route - the surface cuticle or mid-gut. However, this was surmised from unsatisfactory experiments. In both instances germination was assumed to have occurred in the mid-gut after observations of hyphal growth on larvae whose mouthparts were inoculated with food and spores, without attempting to observe the actual germination process and discounting the possibility of the spores germinating on the cuticle, rather than having been ingested. Similarly, germination on the cuticle was assumed once hyphal growth was observed on larvae whose dorsal side had been inoculated, without considering the possibility of larval movement by which the spores might have been ingested. Nobody has claimed to have observed the infection apparatus by which the surface cuticle is penetrated and searches for fungal chitinases had proved negative (Huber, 1958; Gochnauer and Margetts, 1979).

The discovery that A. apis spores would not germinate on the surface of honey bee larvae, under the conditions in this study, implied that the theory of a mid-gut infection was possible and this coincided with the publication of results concerning the infection of leaf-cutting bees with other species of Ascosphaera, where germinating

spores were found in the mid-guts of larvae. Therefore, histological evidence was sought for the germination of spores of A. apis in larval mid-guts, in order that the infection site would be finally determined. As a result of this work, spores at various stages of germination and developing hyphae have been observed in the mid-gut. This evidence would probably have been provided much earlier, had Maurizio (1934) published the results of her completed histological study.

The infection of A. mellifera larvae by A. apis follows a similar pattern to that shown by A. aggregata and A. proliferda in M. rotundata. (Vandenberg and Stephen, 1983; Youssef et al., 1984). Symptoms of disease are similar, as are the descriptions of disease development, from germination of spores in the mid-gut lumen to the immediate penetration of the peritrophic membrane and gut epithelium, followed by the invasion of all larval tissues. In no instance has penetration of the surface cuticle been observed, so these species of Ascospaera are confirmed as unique amongst entomopathogenic fungi. However, it is possible that there are other insect mycopathogens which can also utilise the mid-gut as a route for infection, but these have not yet been identified; perhaps because this route is not always considered as a viable alternative to the surface cuticle, so is not fully investigated.

It is interesting to note that Stephen et al. (1981), when considering the germination of A. aggregata spores in M. rotundata recognised the requirement for carbon dioxide for spore activation and thought carbon dioxide resulting from digestive processes might accumulate in the mid-gut while it was blind-ended, during the first to third instars. This theory was subsequently dismissed, once older larvae of M. rotundata, third to fifth instars, with mid- and hind-guts joined were shown to be highly susceptible to the disease (Vandenberg

and Stephen, 1982). However, carbon dioxide is now recognised as the trigger for activation of A. apis spores in vitro and since the mid-guts of larvae of A. mellifera remain blind until the end of the fifth instar, the spores have a longer time period in which to germinate and initiate an infection prior to pupation. Respiratory carbon dioxide may, therefore, be of sufficient levels to activate spores, whether the mid-gut is blind or not, but the spores must have sufficient time within the mid-gut after activation for developing hyphae to become attached to the gut epithelium or they would be evacuated when the mid-gut / hind-gut junction opened. Maurizio (1934) and Bailey (1966) observed A. apis to initially penetrate the body wall of larvae of A. mellifera at the hind end, which suggests this region within the mid-gut might provide most favourable conditions for germination. Waste products of digestion and spores probably accumulate here as the larva continues to feed and prior to faecal evacuation.

The effect of carbon dioxide observed in vitro is almost certainly not direct, but as a result of the reaction of carbon dioxide with water, producing carbonate and bicarbonate ions, which in turn may be responsible for activation. In vivo these ions or others may interfere with spore cell membranes, perhaps having a de-ionising effect on constituent molecules, resulting in a change in membrane permeability. Alternatively carbon dioxide may not have a role in vivo, but in vitro it simulates the action of the in vivo 'trigger'. Further conclusions are not possible until the activation process itself is fully understood. Investigations to determine the action of carbon dioxide in vitro, that is, whether it is metabolised and so induces respiration, or counter-acts an inhibitor, are needed before any role in vivo can be analysed.

4.2 ADAPTATIONS OF SPORE PHYSIOLOGY TO LARVAL ENVIRONMENT

On reflection, the results from some of the spore physiology experiments indicate the adaptation of the fungus to a life-cycle within the bee colony and particularly to its route of infection. The temperatures supporting most germination, for example, are close to that maintained within the brood area; and pH requirements for germination appear to be indicative of a mid-gut infection, rather than cuticular. If the infection arose on the cuticle the spores would have been in contact with the acidic constituents of the larval food. The pH of these substances is too low to support germination (page 38), but the pH of larval mid-gut contents has been shown to be near neutral, which from these results can be seen to be a much more favourable environment for germination. Since larval food is acidic and mid-gut contents near neutral, there must be a buffering capacity within the mid-gut, perhaps alkaline secretions from the epithelial cells. This is another area requiring further investigation, in order to provide information concerning larval physiology and its relationship with spore physiology.

The requirement of spores for exogenous nutrients for their germination ensures this process will only proceed in a nutritionally favourable environment. Those carbohydrates utilised by the germinating spores - glucose, fructose, trehalose, are those in most plentiful supply in the hive environment. Similarly, there is a rich supply of amino acids, from pollen and brood food, which seem most important in the production of germ-tubes. Within the mid-gut the constituents of larval food may be in more favourable concentrations or forms than found on the surface cuticle. Some of the larval nutrients will be digested in the gut lumen and in such forms may be more readily utilised by the spores. An analysis of mid-gut contents may confirm

and provide additional information concerning the nutritional requirements of germinating spores. At present only 25% of germ-tube production is achieved in vitro, using 1% glucose and yeast extract agar. It would be expected that the percentage germ-tube emergence in vivo would be much greater than this, in order to increase the chances of a successful infection. Therefore, the mid-gut may be providing additional nutrients, not yet identified in vitro. The importance of minerals and trace elements to A. apis spore germination have not yet been investigated.

This present investigation also studied the spore ball phenomenon and some speculation has been made regarding its importance in vivo. When histologically prepared sections of in vitro infected larvae were studied, germinating spore balls as well as individuals were observed. This was the case when larvae were fed a large number of spores, but in vivo the larvae must receive a much smaller dosage. Unfortunately, naturally infected larvae were not studied in order to determine their spore content.

Vandenberg et al. (1980) observed spores of other *Ascosphaera* species on newly emergent adults of M. rotundata by scanning electron microscopy, to show how the spores would adhere to the bees and so be transmitted. Nurse bees of A. mellifera could be similarly studied to determine whether they carry individual spores or spore balls. Although the presence of A. apis in the ventriculus of adult bees has been demonstrated by culturing the fungus from excised adult guts (Nelson and Gochnauer, 1982), the form in which the fungus was present has not been determined. Finally, the role of the sticky coating on the spores may be three-fold; to keep the spores together in spore balls, for their individual dispersal, and perhaps to provide important nutrients, as discussed earlier (page 79).

The arrangement of the spores into balls presented practical problems. For example, homogeneous spore suspensions could not be prepared in order to study the effects of spore concentration on the germination of individual spores, nor to calculate the spore dosage for infection of larvae, since the artificial disruption of spore balls appeared to have a detrimental effect on spore germination (page 76). It is interesting to observe that this has not prevented researchers in the past from preparing spore suspensions of various concentrations. For example, Bailey (1966) used a suspension of 10^4 spores in 1 μ l to inoculate larvae. This concentration was measured using a haemocytometer and presumably spore balls were disrupted in order to achieve a homogeneous suspension. Later Gilliam et al. (1978) prepared suspensions of A. apis spores for inoculation by grinding black or white mummified larvae in distilled water, using a tissue grinder. Again, the spore balls were artificially disrupted. Lastly, Glinski (1981) used in vitro produced spores, which were prepared to a concentration of 2000 spores in 1 μ l, for infection experiments. Unfortunately, in this present study, the in vivo germination of spores from spore balls disrupted by homogenisation was not studied. It is possible that these spores retain their germinability when they are deposited in suitable conditions, within the larval mid-gut and the loss of this ability is confined to the less favourable conditions encountered in vitro. Further study to establish the importance of the spore ball phenomenon in vivo is required.

Lastly, a preliminary study of the enzyme complement of germinating spores indicated proteases were produced, but lipases and chitinases were not. A second experiment suggested that chitinases could be produced by the spores, but these results require confirmation

by a more reliable and sensitive technique. Further research in this particular area is paramount, in order to elucidate the means by which developing hyphae penetrate the larval peritrophic lining and epithelium and so induce infection. Also, enzyme production might indicate the composition of the peritrophic membrane and could be important in determining fungal strain virulence.

4.3 SUSCEPTIBILITY OF LARVAE TO INFECTION

Maurizio (1934) was the first to note that apparently healthy larvae, moved from hives to the laboratory, would develop chalk brood disease. This was later observed by Bailey (1966) and from this he postulated his chilling theory - that those larvae chilled briefly after being sealed in their cells would develop the disease, if previously infected with spores. Maurizio had also commented that drone larvae appeared to be more affected by the disease than worker larvae and Bailey thought this was due to the position of their cells on the periphery of the combs, where they were more likely to be chilled. Similarly, the disease is often more evident in the spring, when the colony is expanding and the adult to brood ratio is least favourable. While testing his theory, Bailey found more larvae became infected if they were incubated at 30° rather than 35°C. This was also found to be the case in this present study and is not a surprising result, since larvae would be physiologically stressed at the lower temperature and consequently more vulnerable to infections. Bailey also found very brief periods of chilling at 22°C for inoculated larvae to induce disease development. However, as he himself noted, few larvae are likely to encounter such unfavourable conditions in vivo. Bailey thought chilling enabled oxygen to diffuse into the otherwise anaerobic mid-gut, so re-activating the growth of the mycelium. Now that the mid-guts of fifth instar larvae, at least, have been shown to be mildly oxidising, chilling can no longer be considered as a pre-requisite to infection. As noted by Heath (1982), it is difficult to understand how chilling could affect those bees in America, where the mean monthly temperature was 29°C (Gilliam, 1978). Similarly, Mehr et al. (1976) observed chalk brood disease in hot, dry weather.

Indeed, the remaining pre-disposing conditions considered for infection development may now be explored in the light of this investigation. It is unlikely that environmental conditions such as hot weather (Betts, 1932; Roussy, 1962), or damp (Lunder, 1971; Dallman, 1974) directly affect the development of the disease, since the spores germinate within the relatively stable environs of the larval mid-gut. If germination had occurred on the surface cuticle, environmental fluctuations may have had greater influence. However, conditions such as temperature, humidity and carbon dioxide levels within the brood area are maintained at relatively constant levels by the worker bees, provided there are sufficient numbers of appropriate bees. If there are too few, then increased levels of carbon dioxide within the brood area may be an important factor, now that spores of A. apis are known to require carbon dioxide to trigger their activation. Not only might they initiate germination, but would also stress larvae and so possibly increase their susceptibility to infections.

The possibility that inadequate pollen supplies or pollen of poor quality increases larval susceptibility (De Jong, 1976; Herbert et al., 1977) now appears to be a viable alternative. Such deficiencies would lead to inadequate quantity or quality of brood food produced by the nurse bees, which would manifest as protein or amino acid deficiencies in the larvae. Consequently, larval development would be stunted, including the secretion of peritrophic membrane, which is at least partly proteinaceous. This may render it and the gut epithelial cells more vulnerable to attack from the fungus. Such a lack of amino acids would also explain the supplementary nutrients associated with spore balls (page 68), to ensure there are sufficient to support germination and growth until alternative nutrient sources are reached. A pollen deficiency could, therefore, explain the

variability in susceptibility of larvae, but the trigger for germination must, in some way, be associated with this state in order that infections, once initiated, are successful.

A pollen deficiency might be most likely in the spring, once winter stores have been depleted and prior to the build-up of the new season's supplies. It might also explain why infections would develop more readily within the laboratory, where the larvae, in this investigation, were fed only sucrose and no protein source. This could lead to deterioration of the mid-gut lining and initiate appropriate germination conditions. Perhaps the chilling treatment of larvae and high carbon dioxide levels result in similar deterioration and so increase susceptibility of larvae.

Associated with this theory is that relating to the genetic variability in the resistance of bee strains to chalk brood disease. This was thought to be related to house-cleaning behaviour, that is, to the detection and removal of infected larvae (Gilliam et al., 1983; Milne, 1983). Efficient house-cleaning may certainly help to reduce the numbers of spores within a colony, but fails to explain the variation in susceptibility of larvae (De Jong, 1977). The genetic variability may instead be associated with feeding, that is, the quality of food given to the larvae. Those strains appearing resistant to the disease may produce better quality brood food, perhaps with more of the qualities of royal jelly, which exhibited an inhibitory effect on A. apis spore germination in vitro. Alternatively, if a protein deficiency is significant in disease development, resistant strains may prove to be more efficient pollen gatherers or storers, or to have better control over brood numbers and food supply. A third possibility is based simply on genetic variations in peritrophic membrane quantity or composition. Very

little is known of the constituents in this membrane, but it is possible that in some bee strains it proves impenetrable to the fungus. Symptoms of disease would not develop, although the spores may be present and may even germinate within the mid-guts of the larvae. Meanwhile, the means of the resistance exhibited by some strains remains open to question.

An increased virulence amongst strains of the chalk brood fungus has been blamed for the apparent spread of the disease (Mraz, 1973; Moeller and Williams, 1976). Again, this could be related to the means of resistance inherent to some strains of bee. If, for instance, susceptibility of the peritrophic membrane to fungal attack is a factor determining vulnerability of a bee strain, a modification in the enzyme complement of developing hyphae may result in previously resistant bee larvae becoming vulnerable. This fungal strain would subsequently become dominant.

The image of A. apis as an opportunistic pathogen, as described by Bailey (1966), still stands. It does not, however, appear to kill the larvae by competition for primary nutrients as suggested by Gochnauer and Margetts (1979), but actively infects and destroys larval tissues, in favourable conditions. Neither does it appear to be a secondary invader, in that it was observed to infect apparently healthy larvae. No signs of other infections were observed in the hives from which larvae were taken or in the histologically prepared sections of larvae infected with A. apis. However, chalk brood disease may develop indirectly from other infections, if these weaken the larvae, increasing the susceptibility of the mid-gut to infection, or if the balance of the colony is disturbed such that larvae are 'stressed' and thus made more susceptible; but these are not the only circumstances in which the disease will develop. It is this

'stress-factor' which appears to be most important in determining larval susceptibility and which still requires definition.

4.4 DISEASE PREVENTION

The spores of A. apis are probably so ubiquitous in areas where colonies are infected, that to completely eradicate the spores, and so the disease, would be an impossible task. However, it must be possible to find a chemical treatment to inhibit spore germination. At this stage of its life-cycle the fungus could be considered to be at its most vulnerable, with the many physiological and biochemical changes in progress. One type of chemotherapy presently under consideration is that which interferes with sterol synthesis within fungi. Any such treatment must be economic and easy to use, as well as non-toxic to bees and have no detrimental effects on hive products. Any treatment to which the fungus could rapidly develop resistance would be of no use.

While an effective means of chemotherapy is a practical method by which to control the disease in the short-term, it must be better to fully understand the disease process and so be able to prevent disease development, rather than resorting to a programme of continuous chemotherapy. Indeed, this study has successfully determined the route of infection utilised by A. apis and has explored some aspects of fungal physiology, in order that the relationship of the fungus and its host might be better understood. Inevitably, during this initial investigation a complete explanation of chalk brood disease could not be compiled, but it has taken several steps in improving our knowledge of the disease. In future studies the extent of the adaptation of A. apis to its ecological niche requires investigation, with particular reference to fungal enzymology. Also, the 'stress-factor' which appears to be most important in determining susceptibility of larvae to the disease, and which is now thought to be related to the physiological condition of the larval gut, needs to be determined. This report

can be taken to confirm those suggestions made by previous researchers, who recommended maintaining adequate quantity and quality of food in order to maintain healthy and resistant larvae. Only by a quest for greater understanding of the fungal and host relationship can chalk brood disease be fully elucidated and further recommendations be made for disease prevention.

BIBLIOGRAPHY

- Al-aidroos, K. and A.M. Seifert 1980. Polysaccharide and protein degradation, germination, and virulence against mosquitoes in the entomopathogenic fungus Metarrhizium anisopliae. J. Invert. Path. 35 : 29-34.
- Albisetti, J. and A. Brizard 1979. Les mycoses de la colonie d'abeilles. Bull. tech. apic. 6 (1) : 43-48.
- Allen, D.J. 1957. Properties of a volatile fraction from uredospores of Puccinia graminis var. tritici affecting their germination and development. I. Biological activity. Plant Physiol. 32 : 385-389.
- Anderson, J. 1938. Chalk brood. Scott. Beekpr. 14 : 106.
- Bahadur, P. and S. Sinha 1966. Self-inhibition and self-stimulation of uredospore germination in Uromyces ciceris-arietini (Grogn.) Jacz. Proc. 53rd. Ind. Sc. Cong. Part III Abstracts 8.
- Bailey, L. 1963. Infectious diseases of the honeybee. London, Land Books.
- Bailey, L. 1966. The effect of temperature on the pathogenicity of the fungus Ascosphaera apis, for larvae of the honey bee; Apis mellifera. Proc. Int. Coll. Insect Path. 162-167.
- Bailey, L. 1981. Honey bee pathology. London, Academic Press.
- Baird, R.B. 1957. Notes on laboratory infection of diptera caused by the fungus Empusa muscae Cohn. Can. Entomologist 89 : 432-435.
- Baker, G.M. and P.F. Torchio 1968. New records for Ascosphaera apis from North America. Mycologia 60 : 189-190.
- Barker, S.A., A.B. Foster, C.D. Lamb and N. Hodgson 1959. Identification of 10-hydroxy- Δ^2 decenoic acid in royal jelly. Nature 183 : 996-997.

- Barthel, B. 1971. Der Kalkbrut auf der Spur. Garten u. Kleintierz.
C. Imker 10 (4) : 12-13.
- Betts, A. 1912. A beehive fungus, Pericystis alvei gen. et sp. nov.
Ann. Bot. 26 (103) : 795-799.
- Betts, A.D. 1915-1924. Unpublished notebook on fungi from beehives and
a few other habitats. Vol. 1 - 1915-1919; Vol. 2 - 1919;
Vol. 3 - 1924. Bee Research Association Library.
- Betts, A.D. 1932. Chalk brood. Bee World 13 (7) : 78-80.
- Bigliani, D.E. and L.A.F. Heath 1985. Electropositive redox state of
the fifth instar larval gut of Apis mellifera. J. Apic. Res.
24 (4) : 211-213.
- Bishop, G.H. 1923. Body fluid of the honey bee larva. I. Osmotic
pressure, specific gravity, pH, O₂ capacity, CO₂ capacity,
and buffer value, and their changes with larval activity and
metamorphosis. J. Biol. Chem. 58 : 543-563.
- Borchert, A. 1939. Krankheiten der Honigbiene. Richard Schoetz, Berlin.
- Braithwaite, G.R. and C.O.D. Titmus 1967. Lanchester short statistical
tables. The English Universities Press.
- Brambl, R.M. and J.L. Van Etten 1970. Protein synthesis during fungal
spore germination V. Evidence that the ungerminated conidiospores
of Botryodiplodia theobromae contain messenger ribonucleic acid.
Arch. Biochem. Biophys. 137 : 442-452.
- Butterfield, W. 1968. Effect of dimethylsulphoxide on the germination
of spores of Lycogala epidendrum. Nature 218 : 494-504.
- Caltrider, P.G. and D. Gottlieb 1966. Effect of sugars on germination
and metabolism of teliospores of Ustilago maydis. Phytopath.
56 : 479-484.
- Campbell, F.L. 1929. The detection and estimation of insect chitin;
and the irrelation of chitinization to hardness and pigmentation

- of the American cockroach, Periplaneta americana. Ann. Ent. Soc. Am. 22 : 401-426.
- Castle, I. 1981. Growth of Ascosphaera apis in the presence of B vitamins and two N sources. B.Sc. Thesis, Plymouth Polytechnic.
- Cerelin, E.M. and C.L. Fergus 1971. Effects of nutrients, temperature and relative humidity on germination and longevity of the ascospores of Chaetomium thermophile var. coprophile. Mycologia 63 : 1030-1045.
- Chmielewski, M. and Z. Glinski 1980/1981. Studies on the pathogenicity of Ascosphaera apis for honey bee larvae Part I. Biochemical properties of A. apis. Annales Universitatis Mariae Curie-Sklodowska, DD 35/36 (8) : 77-82. Apicultural abstract 1323/85.
- Christensen, M. and M. Gilliam 1983. Notes on the Ascosphaera species inciting chalkbrood in honey bees. Apidologie 14 (4) : 291-297.
- Chung, H.S. and R.D. Wilcoxson 1969. Effect of conidial number and matrix on germination of conidia in Phoma medicaginis. Phytopath. 59 : 440-442.
- Classey, D. 1980. The effects of variation in temperature and pH on growth of the chalk brood causing fungus Ascosphaera apis. B.Sc. Thesis, Plymouth Polytechnic.
- Claussen, P. 1921. Entwicklungsgeschichtliche Untersuchungen über den Erreger der als 'Kalkbrut' bezeichneten Krankheit der Bienen. Arbeiten Biol. Reichsanst. Land. u. Forstw. 10 (6) : 467-521.
- Cochrane, J.C., V.W. Cochrane, F.G. Simon and J. Spaeth 1963 (a). Spore germination and carbon metabolism in Fusarium solani I. Requirements for spore germination. Phytopathology 53 : 1155-1160.
- Cochrane, V.W., J.C. Cochrane, C.B. Collins and F.G. Serefin 1963 (b). Spore germination and carbon respiration in relation to germination. Am. J. Bot. 50 : 806-814.

- Collins, C.H. and P.M. Lyne 1985. Microbiological methods. London
Butterworth and Co. Fifth edition.
- Cooper, B. 1980. Fluctuating broodnest temperature rhythm. Br. Isles.
Bee Breeders News 18 : 12-16.
- Cotter, D.A. and R. O'Connell 1974. Activation of Dictyostelium
discoideum spores with dimethyl sulfoxide and urea. Abs.
Ann. Meeting Amer. Soc. Microbiol. : 29.
- Cotter, D.A., J.W. Morin and R.W. O'Connell 1976. Spore germination
in Dictyostelium discoideum II. Effects of dimethyl sulfoxide
on post-activation lag as evidence for the multistate model of
activation. Arch. Microbiol. 108 : 93-98.
- Craven, V. 1980. An investigation into the carbohydrate source of the
fungus Ascospaera apis. B.Sc. Thesis, Plymouth Polytechnic.
- Dade, H.A. 1949. The laboratory diagnosis of honeybee diseases.
Monogr. Queckett Micro Club 4 : 272-285.
- Dallman, H. 1974. Neue Wege bei der Bekämpfung der Kalkbrut in
Bienenstöcken. Merkblatt, Imker des Verbandes der Kleingärtner,
Siedler und Kleintierzüchter No. 8.
- Darby, R.T. and G.R. Mandels 1955. Effects of sporulation medium and
age on fungus spore physiology. Plant Physiol. 30 : 360-366.
- Deacon, J.W. 1980. Introduction to modern mycology. Oxford,
Blackwell Scientific Publications.
- Deans, A.S.C. 1940. Chalk brood. Bee World 21 (4) : 46.
- De Jong, D. 1976. Experimental enhancement of chalk brood infections
Bee World 57 (3) : 114-115.
- De Jong, D. 1977. A study of chalk brood disease of honey bees.
M.Sc. Thesis, Cornell University, Ithaca, U.S.A.
- De Jong, D. and R.A. Morse 1976. Chalk brood - a new disease of honey
bees in the U.S. N.Y. J. Life Sci. Q. 9 (2) : 12-14.

- De Zeeuw, D.J. 1943. A method for obtaining single-spore cultures of Agaricus campestris. *Phytopath.* 33 : 530-531.
- Donaubauer, E. 1959. Über eine Mykose der Latenzlarve von Cephaleia abietis L. *Sydowia* 13 : 183-222.
- Drury, R.A.B. and E.A. Wallington (ed.) 1980. Carletons histological technique. Oxford University Press. Fifth edition.
- Ermin, R. 1939. Über Bau und Funktion der Lymphocyten bei Insekten (Periplaneta americana L.). *Z. Zellforsch. mikrosk. Anat.* 29 : 613-669.
- Evlakhova, A.A. and T.A. Shekhurina 1963. Antifungal action of the cuticle of Eurygaster ingriceps Put. *Dokl. Akad., Nauk SSSR* 148 : 977-978.
- Fazekas de St. Groth, S., R.G. Webster and A. Datyner 1963. Two new staining procedures for quantitative estimation of proteins on electrophoresis strips. *Biochim. Biophys. Acta* 71 : 377-391.
- Ferguson, M. 1902. A preliminary study of the germination of the spores of Agaricus campestris and other basidiomycetes. U.S. Dept. Agric., Bur. Pl. Ind..Bull. 16.
- Ferron, P. 1978. Biological control of insect pests by entomogenous fungi. *Ann. Rev. Ent.* 23 : 409-442.
- Fletcher, J. and A.G. Morton 1970. Physiology of germination of Penicillium griseofulvum conidia. *Trans. Br. Myc. Soc.* 54 : 65-81.
- French, R.C., L.M. Massey Jr. and R.L. Weintraub 1957. Properties of a volatile fraction from uredospores of Puccinia graminis var. tritici affecting their germination and development. II. Some physical and chemical properties. *Plant Physiol.* 32 : 389-393.
- Furuya, K., K. Takatori, O. Sonobe and T. Mabuchi 1981. Occurrence of chalk brood disease in honey bee larvae in Japan. *Trans. Myc. Soc. Jap.* 22 : 127-133.

- Gabriel, B.P. 1968 (a). Histochemical study of insect cuticle infected by the fungus Entomophthora coronata. J. Invert. Path. 11 : 82-89.
- Gabriel, B.P. 1968 (b). Enzymatic activities of some entomophthorous fungi. J. Invert. Path. 11 : 70-81.
- Garraway, M.O. and R.C. Evans 1984. Fungal nutrition and physiology. U.S.A., John Wiley and Sons.
- Gaze, B. 1983. Unpublished data.
- Giauffret, A. and Y. Taliercio 1967. Les mycoses de l'abeille (Apis mellifera L.), étude de quelques antimycosiques. Bull. apic. Doc. Sci. tech. Inf. 10 (2) : 163-174.
- Gilliam, M. 1978. Chalkbrood-status today and hopes for control. Am. Bee J. 118 (7) : 468-471.
- Gilliam, M., S. Taber III and J.B. Bray Rose 1978. Chalkbrood disease of honeybees Apis mellifera L.; A progress report. Apidologie 9 (1) : 75-89; Apiacta 13 (2) : 67-75; Proc. XXVI int. Beekeep. Congr. : 451-458.
- Gilliam, M., S. Taber and G.V. Richardson 1983. Chalkbrood disease and hygienic behaviour of honeybees. Gleanings in bee culture III (3) : 258, 264, 265.
- Glinski, Z. 1980. Laboratory and field studies on the therapy of chalk brood with the use of polyene antibiotics. Proc. XXVII int. Beekeep. Congr. : 337-341.
- Glinski, Z. 1981. Studies on the effect of the fungus Ascosphaera apis on larvae of the honey bee Apis mellifera L. Polskie Archiwum Weterynaryjne 23 (1) : 9-15.
- Glinski, Z. and M. Chmielewski, 1982. Studies on the pathogenicity of Ascosphaera apis for honey bee larvae Part II. Relationships between biochemical type and virulence of A. apis. Annales Universitatis Mariae Curie-Sklodowska, DD 37 (8) : 69-77. Apicultural abstract 1324/85.

- Gochnauer, T. 1982. Communications and bee diseases. Can. Beekeep. 10 (4) : 76-77.
- Gochnauer, T.A., R. Boch and V.J. Margetts 1979. Inhibition of Ascosphaera apis by citral and geraniol. J. Invert. Path. 34 : 57-61.
- Gochnauer, T.A. and S.J. Hughes 1976. Detection of Ascosphaera apis in honeybee larvae (Hymenoptera : Apidae) from Eastern Canada. Can. Ent. 108 : 985-988.
- Gochnauer, T.A. and V.J. Margetts 1979. Properties of honeybee larvae killed by chalkbrood disease. J. Apic. Res. 18 (3) : 212-216.
- Gochnauer, T.A. and V.J. Margetts 1980. Decontaminating effect of ethylene oxide on honeybee larvae previously killed by chalkbrood disease. J. Apic. Res. 19 (4) : 261-264.
- Gottlieb, D. 1978. The germination of fungus spores. Meadowfield Press Ltd., Co. Durham, England.
- Gottlieb, D. and P.G. Caltrider 1963. Synthesis of enzymes during germination of fungus spores. Nature 197 : 916-917.
- Gottlieb, D. and R.K. Tripathi 1968. The physiology of swelling phase of spore germination in Penicillium atrovenetum. Mycologia 60 : 571-590.
- Griffin, D.H. 1981. Fungal physiology. U.S.A.: John Wiley and Sons.
- Halbsguth, W. and H. Rudolph 1959. Untersuchungen über die Warmeaktivierung der Sporangiosporen von Phycomyces blakesleeianus I. Arch. Mikrobiol. 32 : 296-308.
- Hawker, L.E. 1950. Physiology of fungi. London, University of London Press.
- Hawker, L.E. and R.J. Hendy 1963. An electron microscope study of germination of conidia of Botrytis cinerea. J. Gen. Micro 33: 43-46.

- Haydak, M.H. 1961. Influence of storage on the nutritive value of pollens for newly emerged honey bees. *Am. Bee J.* 101 : 354-355.
- Heath, L.A.F. 1982. Development of chalkbrood in a honeybee colony: a review. *Bee World* 63 (3) : 119-130; 130-135.
- Heath, L.A.F. 1984. The chalk brood enigma. Central Association of Bee-Keepers, Ilford, Essex.
- Heath, L.A.F. 1985 (a). Occurrence and distribution of chalk brood disease of honeybees. *Bee world* 66 (1) : 9-15.
- Heath, L.A.F. 1985 (b). Chalk brood research at Plymouth. *Bee Craft* 67 (5) : 139-140.
- Heath, L.A.F. and B. Gaze 1987. Carbon dioxide activation of spores of the chalk brood fungus, *Ascosphaera apis*. In press, *J. Apic. Res.*
- Helleu, C. 1956. The presence of meso-inositol in royal jelly. *Profession méd.* 718. Apicultural abstract 137/60.
- Herbert, E.W., D.J. Chitwood and H. Shimanuki 1985. New compounds with potential for the control of chalkbrood. *Am. Bee J.* 125 (6) : 430-431.
- Herbert, E.W. and H. Shimanuki 1983. Effect of diet pH on the consumption, brood rearing and pH of worker jelly produced by caged honey bees. *Apidologie* 14 (3) : 191-196.
- Herbert, E.W., H. Shimanuki and D.A. Knox 1977. Transmission of chalk brood disease of honeybees by infected queens, and worker brood and adults. *J. Apic. Res.* 16 (4) : 204-205.
- Hitchcock, J.D. 1972. Chalk brood disease of honeybees - a review. *Am. Bee J.* 112 (8) : 300-301.
- Hohl, H.R., M. Bülmann and E. Wehrli 1978. Plasma membrane alterations as a result of heat activation in *Dictyostelium* spores. *Arch. Microbiol.* 116 : 239-244.
- Howe, S.R., P.S. Dimick and A.W. Benton 1985. Composition of freshly

- harvested and commercial royal jelly. *J. Apic. Res.* 24 (1) : 52-61.
- Huber, J. 1958. Untersuchungen zur Physiologie Insektentötender Pilze. *Arch. Mikrobiol.* 29 (3) : 257-276.
- Humason, G.L. 1967. *Animal tissue techniques*. London, W.H. Freeman and Co.
- Jones, F.R. 1919. U.S. Dept. Agric. Bull. 759 : 1-38.
- Kawakami, K. 1965. Phagocytosis in muscardine-diseased larvae of the silkworm, Bombyx mori (Linnaeus.) *J. Invert. Path.* 7 : 203-208.
- Kenward, F. 1932. Foul brood. *Br. Bee J.* 60 (2609) : 290-291.
- Khan, M. 1977. Light stimulation of fungal spore germination. *Z. Pflanzenphysiol.* 81 : 374-376.
- Kish, L.P. 1980. Spore germination of *Ascosphaera* spp. associated with the alfalfa leafcutting bee, Megachile rotundata. *J. Invert. Path.* 36 : 125-128.
- Koenig, J.P., G. Mallory Boush and E.H. Erickson Jr. 1986. Old comb may contribute to chalkbrood disease. *Am. Bee J.* 126 (3) : 191-192.
- Koidsumi, K. 1957. Antifungal action of cuticular lipids in insects. *J. Ins. Physiol.* 1 : 40-51.
- Krasikova, V.I. 1955. Bactericidal properties of brood food. *Pchelovodstvo* 8 : 50-53. *Apicultural Abstract* 160/57.
- Leopold, J. and A. Samsňáková 1970. Quantitative estimation of chitinase and several other enzymes in the fungus Beauveria bassiana. *J. Invert. Path.* 15 : 34-42.
- Lillie, R.D. 1965. *Histopathologic technic and practical histochemistry*. New York, The Blakiston Co.. Third edition.
- Lingappa, Y. and J.L. Lockwood 1962. Chitin media for selective isolation and culture of actinomycetes. *Phytopath.* 52 : 317-323.
- Lovett, J.S. 1963. Chemical and physical characterisation of 'nuclear

- caps' isolated from *Blastocladiella* zoospores. *J. Bacteriol.*
85 : 1235-1246.
- Lunder, R. 1971. Undersøkelse av kalkyngel. *Birøkteren* 88 (4) : 55-60.
- Maassen, A. 1913. Weitere Mitteilungen über die seuchenhaften
Brutkrankheiten der Bienen. *Mitt. biol. Zent. Anst.* 14 : 48-58.
- Macko, V., R.C. Staples, Z. Yaniv and R.R. Granados 1976. Self-inhibitors
of fungal spore germination. In *The fungal spore: form and
function* (ed. by D.J. Weber and W.M. Hess) pp. 73-100. John
Wiley and Sons.
- MacLeod, D.M. 1954. Natural and cultural variation in entomogenous
Fungi Imperfecti. *Ann. N.Y. Acad. Sci.* 60 : 58-70.
- Madelin, M.F. 1966. Fungal parasites of insects. *Ann. Rev. Entomol.*
11 : 423-448.
- Mandels, G.R. and R.T. Darby 1953. A rapid cell volume assay for
fungitoxicity using fungus spores. *J. Bacteriol.* 65 : 16-26.
- Mandels, G.R. and A.B. Norton 1948. Res. Rept., Quartermaster Genl.
Lab., U.S.A., Microbiol. Ser. 11
- Mandels, G.R., R. Vitols and F.W. Parrish 1965. Trehalose acts as an
endogenous reserve in spores of the fungus *Myrothecium verrucaria*.
J. Bacteriol. 90 : 1589-1598.
- Marin, M., G. Velescu, P. Mitrow, C. Gilca, D. Anastasiu, D. Ionescu,
I. Rusu and I. Orgada 1977. Mycosis investigations in apiculture.
Proc. XXVI int. Beekeep. Congr. : 470-473.
- Martin, J.F. and G. Nicolás 1970. Physiology of spore germination in
Penicillium notatum and *Trichoderma lignorum*. *Trans. Br. Myc.*
55 (1) : 141-148.
- Maurizio, A. 1934. Über die Kalkbrut (Pericystis-Mykose) der Bienen.
Arch. Bienenk. 15 (5) : 165-193.
- Maurizio, A. 1935. Beiträge zur Kenntnis der Pilzflora im Bienestock I.

- Die Pericystis-infektion der Bienenlarven. Ber. Schweiz. bot. Ges. 44 : 133-156.
- McCauley, V.J.E., R.Y. Zacharuk and R.D. Tinline 1968. Histopathology of green muscardine in larvae of four species of Elateridae (Coleoptera). J. Invert. Path. 12 : 444-459.
- McManus, J.F.A. 1948. Histological and histochemical uses of periodic acid. Stain Technology 23 : 99-108.
- Mehr, Z., D.M. Menapace, W.T. Wilson and R.R. Sacket 1976. Studies on the initiation and spread of chalkbrood within an apiary. Am. Bee J. 116 (6) : 266-268.
- Mehr, Z., W.T. Wilson and R.R. Sacket 1978. Persistence of chalkbrood (Ascospaera apis) in some North American honey bee colonies one year after infection. Apiacta 13 (3) : 99-102.
- Mello, M.L.S., B.C. Vidal and L. Valdrighi 1971. The larval peritrophic membrane of Melipona quadrifasciata (Hymenoptera Apoidea). Protoplasma 73 : 349-365.
- Menapace, D.M. 1978. Chalkbrood infection and detection in colonies of honey bees, Apis mellifera. Am. Bee J. 118 (3) : 158-159.
- Menapace, D.M. and P.J. Hale 1981. Citral and a combination of sodium propionate and potassium sorbate did not control chalk brood. Am. Bee J. 121 (12) : 889-891.
- Menapace, D.M. and W.T. Wilson 1976. The spread of chalk brood in the North American honey bee, Apis mellifera. Am. Bee J. 116 (12) : 570.
- Menapace, D.M. and W.T. Wilson 1979. Feeding oxytetracyclines as Terramycin does not aggravate chalk brood infections. Apidologie 10 (2) : 167-174.
- Menapace, D.M. and W.T. Wilson 1980. Terramycin does not inhibit or enhance chalk brood infections. Am. Bee J. 120 (2) : 115-117.
- Michael, A.S. and M. Abramovitz 1954. A method of rearing honeybee

- larvae in vitro. J. Econom. Ent. 48 (1) : 43-44.
- Milne, C.P. 1983. Estimations of the heritabilities of and genetic correlation between two components of honey bee (Hymenoptera : Apidae) hygienic behaviour: uncapping and removing. Ann. Ent. Soc. Am. 78 (6) : 841-844.
- Moeller, F.E. and P.H. Williams 1976. Chalkbrood research at Madison, Wisconsin. Am. Bee J. 116 (10) : 484, 486, 495.
- Moffet, J.O., W.T. Wilson, A. Stoner and A. Wardecker 1978. Feeding commercially purchased pollen containing mummies caused chalkbrood. Am. Bee J. 118 (6) : 412-414.
- Morgenthaler, O. 1918. Bienen-und Wabenuntersuchungen in Jahre 1917. Schweiz. Bienenztg. 41 (4) : 103-104.
- Morris, H. 1977. Chalkbrood. Glean. Bee. Cult. 105 (5) : 215.
- Morse, R.A. (Editor) 1978. Honey bee pests, predators and diseases. London, Cornell University Press. Including 'Fungi' by M. Gilliam pp. 78-101.
- Mraz, C. 1973. Chalk brood. Glean. Bee Cult. 101 (4) : 115,129.
- Nelson, D.L. 1975. An evaluation: a cross between New Zealand and Californian honey bee stocks. Am. Bee J. 115 (6) : 228-229, 334.
- Nelson, D.L. and T.A. Gochnauer 1982. Field and laboratory studies on chalkbrood disease of honey bees. Am. Bee J. 122 (1) : 29-34.
- Nixon, M. 1982. Preliminary world maps of honey bee diseases and parasites. Bee World 63 (1) : 23-42.
- Oakes, S.C. 1984. The effects of various antibiotics and antimycotics on the stages of the germination process of Ascosphaera apis spores, in vitro. B.Sc. Thesis, Plymouth Polytechnic.
- Oku, H. 1960. Biochemical studies on Cochliobolus miyabeanus VII. Cellular carbohydrate as an endogenous substrate for conidia germination in Cochliobolus miyabeanus. Plant and Cell Physiol. 1 : 231-239.

- Pabst, G.S. and P.P. Sikorowski 1980. Susceptibility of southern pine beetle (Dendroctonus frontalis) on oligidic medium to Paecilomyces viridis and also Beauveria bassiana and Metarhizium anisopliae. J. Georgia Entomol. Soc. 15 (3) : 235-241.
- Pankiw, P. 1972. The place for New Zealand queen bees in Canada. Can. Agric. 17 (2) : 34-35.
- Patel, G. 1983. An investigation into the nitrogen source of the fungus Ascosphaera apis. B.Sc. Thesis, Plymouth Polytechnic.
- Pedersen, K. 1974. Chalkbrood: determination of fungus, fungicides and mycelium growth in vitro and possible curation of bee colonies. In Beekeeping in cold climatic zones. pp. 73-75. Bucharest, Romania: Apimondia Publishing House.
- Pedersen, K. 1976. Kalkyngel: mulige bekjempelsesmetoder og virkning av tilleggsvarme. Birøkteren 92 : 18-22.
- Pröckschl, H. 1953. Beiträge zur Kenntnis der Entwicklungsgeschichte von Pericystis apis Maassen. Arch. Mikrobiol. 18 : 198-209.
- Pulsford, A. 1985. Unpublished data.
- Reik, L. 1968. Contacts between insect blood cells, with special reference to the structure of capsules formed about parasites. M.Sc. Thesis, University of Cambridge.
- Reissig, J.L., J.L. Strominger and L.F. Leloir 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. 217 : 959-966.
- Rembold, H. 1965. Biologically active substances in royal jelly. Vitam. Horm. 23 : 359-382.
- Rengel, C. 1903. Über den Zusammenhang von Mitteldarm und Enddarm bei den Larven der aculeaten Hymenopteren. Zeitschr. wiss. Zool. 75 : 221-232.
- Riddell, R.W. 1950. Permanent stained mycological preparations obtained

- by slide culture. *Mycologia* 42 : 265-270.
- Roberts D.W. and W.E. Yendol 1971. Use of fungi for microbial control of insects. In *Microbial control of insects and mites.* (ed. by H.D. Burges and N.W. Hussey). pp. 125-149. London, Academic Press.
- Robinson, R.K. 1966. Studies on penetration of insect integument by fungi. *Pest articles and news summaries* 12 : 131-142.
- Roeder, K.D. 1953. *Insect physiology.* Wiley.
- Rosenthal, C. 1974. Potentialul biologic si productiv la familiile de albine atinse de Ascosphaera apis. *Lucr. Ses. ştiint. Inst. agron. Nicolae Bălcescu Ser. D17* : 86-88.
- Rothenbuhler, W.C. 1964. Behaviour genetics of nest cleaning in honey bees IV. Responses of F₁ and backcross generations to disease killed brood. *Am. Zool.* 4 : 111-123.
- Roussy, L. 1962. Nouvelle contribution a l'etude du Pericystis apis. *Gaz. apic.* 63 : 101-105.
- Salt, G. 1970. *The cellular defence reactions of insects.* London, Cambridge University Press.
- Samšičáková, A., S. Kalalova and D. Haragsin 1977. Effects of some antimycotics and disinfectants on the Ascosphaera apis Maassen fungus in vitro. *Z. angew. Ent.* 84 : 225-232.
- Samšičáková, A. and S. Mišiková 1973. Enzyme activities in certain entomophagous representatives of Deuteromycetes (Moniliales) in relationship to their virulence. *Česká Mykologie* 27 (1) : 55-60.
- Samšičáková, A., S. Mišiková and J. Leopold 1971. Action of enzymatic systems of Beauveria bassiana on the cuticle of the greater wax moth larvae (Galleria mellonella). *J. Invert. Path.* 18 : 322-330.
- Schabel, H.G. 1976. Oral infection of Hylobius pales by Metarrhizium anisopliae. *J. Invert. Path.* 27 : 377-383.

- Seal, D.W.A. 1967. Chalk brood disease of bees. *N.Z. J. Agric.* 95 (6) : 562.
- Sheridan, J.J., D. McGrath and J. Sherrington (1978). The effects of washing and concentration on fungal spore germination. *J. Life Sci. Royal Dublin Soc.* 2 (2) : 161-170.
- Shu, P., K.G. Tanner and G.A. Ledingham 1954. Studies on the respiration of resting and germinating uredospores of wheat stem rust. *Can. J. Bot.* 32 : 16-23.
- Siquiera, J.O., D.H. Hubbell and N.C. Schenck 1982. Spore germination and germ-tube growth of a vesicular-arbuscular mycorrhizal fungus in vitro: *Mycologia* 74 (6) : 952-959.
- Skou, J.P. 1972. *Ascospaerales*. *Friesia* 10 (1) : 1-24.
- Skou, J.P. 1975. Two new species of *Ascospaera* and notes on the conidial state of Bettsia alvei. *Friesia* 11 (1) : 62-74.
- Skou, J.P. 1982. Ascospaera asterophora species nova. *Mycotaxon* 14 (1) : 149-159.
- Skou, J.P. and K. Hackett 1979. A new homothallic species of *Ascospaera*. *Friesia* 11 (5) : 265-271.
- Smith, J. 1984. An investigation into the effects of temperature and pH on the growth of the fungus Ascospaera apis. B.Sc. Thesis, Plymouth Polytechnic.
- Snodgrass, R.E. 1956. *Anatomy of the honey bee*. New York; Comstock Publishing Associates, Cornell University Press.
- Spiltoir, C.F. 1955. Life cycle of Ascospaera apis (Pericystis apis). *Am. J. Bot.* 42 (6) : 501-508.
- Spiltoir, C.F. and L.S. Olive 1955. A reclassification of the genus *Pericystis* Betts. *Mycologia* 47 : 238-244.
- Steinhaus, E.A. (ed.) 1963. *Insect pathology - An advanced treatise*. London; Academic Press.

- Stephen, W.P., J.D. Vandenberg and B.L. Fichter 1981. Etiology and epizootiology of chalkbrood in the leafcutting bee, Megachile rotundata (Fabricus) with notes on Ascosphaera species. Agricultural Experiment Station, Oregon State University, Corvallis. Station Bulletin 653.
- Stephen, W.P., J.D. Vandenberg, B.L. Fichter and G. Lahm 1982. Inhibition of chalkbrood spore germination in vitro (Ascosphaera aggregata : Ascosphaerales). Agricultural Experiment Station, Oregon State University, Corvallis. Station Bulletin 656.
- Stoner, A. and W.T. Wilson 1985. Toxicity effects of chalkbrood incidence in honey bee colonies fed controlled doses of fungicides. J. Ent. Soc. 20 (2) : 172-178.
- Sussman, A.S. 1951. Studies of an insect mycosis I. Etiology of the disease. Mycologia 43: 338-350.
- Sussman, A.S. 1952. Studies of an insect mycosis III. Histopathology of an aspergillosis of Platysamia cecropia L. Ann. Ent. Soc. Am. 45 : 233-245.
- Sussman, A.S. 1976. Activators of fungal spore germination. In The fungal spore: form and function (ed. by D.J. Weber and W.M. Hess) pp. 101-139. John Wiley and Sons.
- Sussman, A.S. and H.O. Halvorson 1966. Spores: their dormancy and germination. New York; Harper and Row.
- Sussman, A.S., R.J. Lowry and E. Tyrrell 1959. Activation of Neurospora ascospores by organic solvents and furans. Mycologia 51 : 237-247.
- Sweeney, A.W. 1975. The mode of infection of the insect pathogenic fungus Culcinomyces in larvae of the mosquito Culex fatigans. Aust. J. Zool. 23 : 49-57.
- Taber, S., R. Sacket and J. Mills 1975. A possible control for chalk

- brood disease. Am. Bee J. 115 (1) : 20.
- Thomas, G.M. and A. Luce 1972. An epizootic of chalk brood, Ascosphaera apis (Maassen ex Claussen) Olive and Spiltoir in the honey bee, Apis mellifera L. in California. Am. Bee J. 112 (3) : 88-90.
- Thorstensen, K. 1976. Kalkyngel, en soppsykdom hos bier. Birøkteren 92 (1/2) : 14-17.
- Toumanoff, C. 1951. Les maladies des abeilles. Rev. fr. Apic. Numéro spécial no. 68.
- Trinci, A.P.J. and C. Whittaker, 1968. Self-inhibition of spore germination in Aspergillus nidulans. Trans. Br. Myc. Soc. 51 : 594-596.
- Van Assche, J.A., A.R. Carlier and H.I. Dekeersmaeker 1972. Trehalase activity in dormant and activated spores of Phycomyces blakesleeanus. Planta 103 : 327-333.
- Vandenberg, J.D., B.L. Fichter and W.P. Stephen 1980. Spore load of Ascosphaera species on emerging adults of the alfalfa leafcutting bee, Megachile rotundata. Appl. Envir. Microbiol. 39 (3) : 650-655.
- Vandenberg, J.D. and W.P. Stephen 1982. Etiology and symptomatology of chalkbrood in the alfalfa leafcutting bee, Megachile rotundata. J. Invert. Path. 39 : 133-137.
- Vandenberg, J.D. and W.P. Stephen 1983. Pathogenesis of chalkbrood in the alfalfa leafcutting bee, Megachile rotundata. Apidologie 14 (4) : 333-341.
- Veen, K.H. 1966. Oral infection of second-instar nymphs of Schistocerca gregaria by Metarrhizium anisopliae. J. Invert. Path. 8 : 254-256.
- Von Dehn, M. 1933. Untersuchungen über die Bildung der peritrophischen Membran bei den Insekten. Z. Zellforsch. mikr. Anat. 19 : 79-105.

- Waterhouse, D.F. 1953. The occurrence and significance of the peritrophic membrane, with special reference to adult Lepidoptera and Diptera. Aust. J. Zool. 1 : 299-318.
- White, J.W. Jr. 1962. Composition of American honeys. U.S. Dept. Agriculture Technical Bulletin No. 1261.
- Wigglesworth, V.B. 1972. The principles of insect physiology. London, Chapman and Hall. Seventh edition.
- Wigglesworth, V.B. 1984. Insect physiology. London, Chapman and Hall. Eighth edition.
- Wille, H. 1975. Couvain calcifié, maladie bénigne ou dangereuse? J. Suisse Apic. 72 (11) : 287-295.
- Wong, H.C. and Y.S. Bau 1978. A comparison of conidial and ascospore germination of Monascus purpureus. Trans. Br. Mycol. Soc. 70 : 277-282.
- Woyke, J. and J. Bobrzecki 1978. Long queen cell disease. Pszczel. Zesz. nauk. 22 : 59-69.
- Wyatt, G.R. 1961. The biochemistry of insect haemolymph. Ann. Rev. Ent. 65 : 75-102.
- Yamazaki, M., S. Horie, T. Udagawa, T. Echigo and M. Kimi 1975. Mycoflora of honey. J. Fd. hyg. Soc. Jap. 16 : 1-6.
- Yanagita, T. 1957. Biochemical aspects on the germination of conidiospores of Aspergillus niger. Arch. Mikrobiol. 26 : 329-344.
- Yarwood, C.E. 1956. Simultaneous self-stimulation and self-inhibition of uredospore germination. Mycologia 48 : 20-24.
- Yatsunami, K. and T. Echigo 1984. Antibacterial action of honey and royal jelly. Honeybee Science 5 (3) : 125-130.
- Youssef, N.N., C.F. Roush and W.R. McManus 1984. In vivo development and pathogenicity of Ascospaera proliperda (Ascospaeraceae) to the alfalfa leafcutting bee, Megachile rotundata. J. Invert. Path. 43 : 11-20.

Zacharuk, R.Y. 1973. Penetration of the cuticular layers of elaterid larvae (Coleoptera) by the fungus Metarrhizium anisopliae, and notes on a bacterial invasion. J. Invert. Path. 21 : 101-106.

APPENDIX I

GERMINATION OF IN VITRO PRODUCED SPORES

Introduction

De Jong (1976) noted that few chalk brood infections of honey bee larvae could be induced if cultured spores were used as an inoculum source, suggesting such spores differed to those produced in vivo. The aim of this study was to initially test the germinability of in vitro produced spores and to culture germinable spores on a suitable artificial medium.

(A) Method

1 cm diameter discs of inoculum, of a(-) and a(+) strain of A. apis, were placed approximately 5 cm apart on a malt agar (Oxoid) plate and were incubated at 30°C. After 7 days both strains had grown across the plate and a black line of spore cysts had developed, where the 2 strains had met. These cysts were scraped from the plate using a sterile fungal wire and were used to inoculate GYO agar discs. These were then incubated for germination at 35°C in 10% carbon dioxide for 24 hours, as described on page 27.

Tests were carried out on spores produced on malt agar; CCP medium (Appendix III) and modified CCP, where yeast extract or mycological peptone (lab m) replaced asparagine. In each case spores from mummified larvae were also tested.

(A) Results and Discussion

As shown in Figure A the spores formed after 7 days growth on artificial media failed to germinate. CCP medium containing peptone or yeast extract was included in case CCP alone or malt agar failed to provide some nutrients required by the sporulating fungus to include in the spores and which might prove vital for their

SPORULATION MEDIUM	GERMINATION
malt agar	-
CCP	-
CCP with yeast extract	-
CCP with peptone	-
mummified larva	+

Figure A: Table showing the presence (+) or absence (-) of germination of spores produced after incubation of mycelia for 7 days on various media.

subsequent germination. However, the results achieved here indicated that this was not the case.

An alternative theory is that the spores require a maturation period after their formation. The age of the spores on the mummified larvae was not known, therefore these may have undergone a maturation phase, whereas those spores produced in vitro after 7 days incubation would be immature. As early as 1919, Jones (cited by Gottlieb, 1978) noted the ascospores of Pseudopeziza trifoli germinate readily if naturally discharged, but not if obtained by crushing the ascus before that time. Mandels and Norton (1948, cited by Gottlieb, 1978) observed that the spores of young cultures of Myrothecium verrucaria germinate very little when first produced, but the percentage germination increased to a maximum at 30 days, after which a decline began. Consequently, this maturation hypothesis was tested for A. apis.

(B) Method

Spores grown on CCP medium were aseptically removed and germination attempted at weekly intervals for 6 weeks. Similarly, the spores scraped from a newly mummified larva, produced in vivo, were also tested for germination at weekly intervals.

(B) Results and Discussion

From the results shown in Figure B it can be seen that the spores produced on larvae did not require a maturation period, since they were capable of germ-tube production 1 week after collection from the hive. These spores may have been slightly older than 1 week, but probably by only a week or so, since the mummified larvae had not been removed from the comb by the nurse bees and were among sealed brood. The percentages of germ-tubes produced by such spores during the 6 weeks were not significantly

	MEAN % \pm SE OF GERM-TUBE PRODUCTION	
WEEK No.	ARTIFICIAL MEDIUM	LARVAE
1	0	18.7 \pm 3.2
2	0	23.6 \pm 2.1
3	0	33.0 \pm 8.4
4	12.4 \pm 5.1	31.1 \pm 3.8
5	22.0 \pm 7.6	30.9 \pm 3.1
6	21.6 \pm 4.3	31.2 \pm 4.0

N.B. Each result represents the mean calculated from the germination tests of at least 3 trials.

Figure B: Table showing the mean percentages (\pm standard error of the mean) of germ-tubes produced by spores cultured on artificial medium (CCP) or on bee larvae, at weekly intervals for 6 weeks.

different ($F = 1.66$; $P > 0.05$, $df = 5,37$). By contrast, those spores produced in vitro failed to produce any germ-tubes until the fourth week. This percentage was maintained in the fifth and sixth weeks ($F = 0.84$; $P > 0.05$, $df = 2,9$). Although the percentage of germ-tube production by the in vivo produced spores was significantly higher than that exhibited by the in vitro produced spores in week 4 ($t = 2.88$; $P < 0.01$, $df = 10$), at weeks 5 and 6 there were no significant differences between percentages (week 5: $t = 1.44$; $P > 0.05$, $df = 9$; week 6: $t = 1.47$; $P > 0.05$, $df = 10$).

It would, therefore, appear that in vitro produced spores are capable of germinating, but their delayed response may not be entirely due to a maturation period. Another explanation may be the production of an inhibitory substance by the vegetative hyphae. Such a substance ensures the spores will not germinate while conditions are still favourable for the present hyphal growth. Once environmental conditions change and the vegetative hyphae die, the spores are then capable of germinating once favourable conditions return. In vivo the spores will be germinable once all the nutrients within the infected larvae have been utilised by the fungus and the hyphae have died. The infected larvae then appear as completely dehydrated, mummified masses, covered with spore cysts. In vitro, however, the spores are formed on a rich nutrient medium which supports vegetative growth and sporulation. The hyphae may be producing an inhibitory substance until nutrients are depleted and the hyphae die, at approximately 4 weeks in this instance. Consequently, spores may become germinable sooner if a less rich growth medium were used, which would support sporulation, but maintain vegetative growth for a shorter time period.

APPENDIX II

ISOLATION OF BACTERIA COMMONLY ASSOCIATED WITH SPORE CYSTS

Introduction

When spores from mummified larvae were germinated on agar or in liquid culture containing no oxytetracycline, bacterial contamination was common and of a similar type in each instance. Consequently, these bacteria were thought to be associated with the spore cysts. The aim of this investigation was to isolate these bacteria and to initiate their identification by Gram staining.

Method

Spore cysts were scraped from a mummified larva, using a sterile mounted needle, onto a sterile microscope slide, where they were crushed using a razor blade. A plate of nutrient agar (Oxoid) was inoculated with the scrapings and was then incubated for 24 hours at 35°C. After incubation, a sample of the bacterial growth was streaked onto another nutrient agar plate and re-incubated for 24 hours. The separated colonies could then be isolated by producing further streak plates and the bacteria examined by Gram staining.

The bacteria isolated from several mummified larvae were examined.

Gram staining:-

Fixed smears were prepared from 24 hour cultures and were stained for 2 minutes with 0.5% crystal violet. They were then flooded with iodine (1% iodine and 2% KI in distilled water) for 2 minutes and subsequently washed and decolorised with 70% alcohol. After rinsing with water, the smears were counter-stained with 1% safranin for 2 minutes and washed with water; air-dried; and observed by oil immersion using a CH Olympus microscope, at x1000 magnification.

Results and Discussion

2 different colonies of bacteria were commonly isolated from streak plates. The first were cream coloured colonies of Gram-negative long rods and the second were white coloured colonies of Gram-positive short rods.

The identity of these bacteria could not be determined without further diagnostic tests and this, unfortunately, was beyond the scope of this particular project. However, a more extensive investigation should be undertaken to determine the frequency with which these and other bacteria occur with chalk brood infections and their relationship with the infecting fungus.

APPENDIX III

CCP MEDIUM

CCP is a defined synthetic medium which supports the vegetative growth and sporulation of Ascosphaera apis. The name CCP is derived from the names of 3 undergraduate project students at Plymouth Polytechnic, who contributed to the design of the medium. Craven (1980) investigated possible carbon sources utilised by A. apis; Castle (1981) identified the requirement for vitamins; and Patel (1983) tested various amino acids as nitrogen sources.

All constituents of the medium can be autoclaved, including the vitamins (Patel, 1983). The composition of CCP medium is as follows:-

<u>Constituent</u>	<u>Quantity (g/l)</u>
Glucose	100
l-asparagine	0.5
KCl	0.125
K ₂ HPO ₄	0.25
MgSO ₄	0.125
biotin	2 x 10 ⁻⁶
nicotinic acid	5.6 x 10 ⁻⁴
pyridoxine	4 x 10 ⁻⁵
riboflavin	4 x 10 ⁻⁵
thiamine	6 x 10 ⁻⁶
(agar for solidified medium 15g)	

APPENDIX IV

HISTOLOGICAL PREPARATION OF LARVAE

Fixation: Gendre's fluid, minimum 24 hours

Dehydration: 90% alcohol, 10 hours (12)
90% alcohol, 10 hours (12)
100% alcohol, 2.5 hours (2)
100% alcohol, 1.5 hours (2)

Clearing: xylene, 30 minutes (1 hr.)
xylene, 15 minutes (1 hr.)

Samples were normally agitated in a tissue processor for the times shown. Those not so agitated were treated for the longer periods shown in brackets.

Infiltration of wax: paraffin wax, 1 hour under vacuum, then embedded in wax blocks

Preparation of sections:-

Dewaxed: xylene, 2 minutes
xylene, 2 minutes

rehydrated: 100% alcohol, 2 minutes
100% alcohol, 2 minutes
90% alcohol, 2 minutes
70% alcohol, 1 minute
50% alcohol, 1 minute
30% alcohol, 1 minute
distilled water, 1 minute

Staining: lactophenol cotton blue, 1 minute
distilled water, 10 seconds
eosin, 30 seconds

OR 1% periodic acid, 5 minutes
running water, 15 minutes
Schiffs reagent, 10 minutes
differentiator, 2.5 minutes
differentiator, 2.5 minutes
running water, 10 minutes
Light green, 10 minutes

(PAS with Light green adapted from the method of McManus, 1948)

Dehydrate: distilled water, 1 minute
 70% alcohol, 1 minute
 90% alcohol, 1 minute
 100% alcohol, 2 minutes
 100% alcohol, 2 minutes
 xylene, 2 minutes
 xylene, 2 minutes
 and mounted in DPX

Materials

Gendre's fluid (Lillie, 1954, cited by Humason, 1967):-

95% ethanol saturated with picric acid	80 cm ³
formalin	15 cm ³
glacial acetic acid	5 cm ³

Lactophenol cotton blue (Collins and Lyne, 1985):-

phenol, melted	20 g
lactic acid	20 cm ³
glycerol	40 cm ³
water	20 cm ³

Warm the water and add the melted phenol, followed by the lactic acid, glycerol and 0.05% cotton blue (methyl blue).

Eosin (Drury and Wallington, 1980):-

eosin Y	1 g
distilled water	1000 cm ³

Periodic acid (McManus, 1948, cited by Humason, 1967):-

periodic acid	1 g
distilled water	100 cm ³

Schiffs reagent (BDH Chemicals Ltd.)

Differentiator (McManus, 1948):-

potassium metabisulphite, 10% aqueous	5 cm ³
1 M HCl	5 cm ³
distilled water	100 cm ³

Light green (McManus, 1948):-

light green SF	0.2 g
distilled water	100 cm ³
glacial acetic acid	0.2 cm ³

APPENDIX V

STATISTICAL ANALYSES

SAMPLE MEAN

$$\bar{x} = \frac{\sum x}{n}$$

where \bar{x} = sample mean

x = variable

n = sample size

STANDARD ERROR OF THE MEAN (SE)

The standard deviation of the distribution of sample means is called the standard error of the mean and can be calculated as follows:-

$$S_{\bar{x}} = \frac{S_x}{\sqrt{n}}$$

where $S_{\bar{x}}$ = standard error (or deviation) of the mean

S_x = standard deviation

SIGNIFICANCE TESTS

T-TEST

In order to compare 2 samples of sizes n_1 and n_2 with means of \bar{x} and \bar{y} , a t-test is performed. A null hypothesis ($H_0 = \mu_1 = \mu_2$) determines that there is no significant difference between the mean of one population (μ_1) and the mean of the other (μ_2), i.e. the 2 samples could be from the same population. A value for 't' is calculated as follows:-

$$t = \frac{\bar{x} - \bar{y}}{S} \sqrt{\frac{n_1 n_2}{n_1 + n_2}} \quad \text{where degrees of freedom (v)}$$
$$= n_1 + n_2 - 2$$

The null hypothesis is accepted if the value for 't' is less than the critical value at P 0.05.

ANOVA

A one factor analysis of variance is performed if the means of 3 or more samples are to be compared. Again, a null hypothesis determines there is no significant difference between the means.

A variance ratio, or F-value is calculated as follows:-

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	Ratio
between groups	$\sum nj (\bar{x}_j - \bar{x})^2$	n-1	$SS/n-1 = \hat{\sigma}_1^2$	$F_{N-n}^{n-1} = \hat{\sigma}_1^2 / \hat{\sigma}_2^2$
within groups	by subtraction	N-n	$SS/N-n = \hat{\sigma}_2^2$	
total variation	$\sum \sum (x_{ij} - \bar{x})^2$	N-1		

where N = total number of observations

n = number of observations in each group (j)

$\hat{\sigma}_1^2$ = population variance estimate

$$V_1 = n-1$$

$$V_2 = N-n$$

The null hypothesis is accepted if the 'F' value is less than the critical values at P 0.05.

All calculations were computed on a BBC Microcomputer using a biological statistical package. Probability values for t and F were read from statistical tables (Braithwaite and Titmus, 1967).