USE OF THE FUNGAL PATHOGEN *HIRSUTELLA CRYPTOSCLEROTIUM* SP. NOV. FOR THE BIOCONTROL OF *RASTROCOCCUS INVADENS* (PSEUDOCOCCIDAE)

by

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A Phil,
por enseñarme a creer en mi misma

Abstract

The feasibility of using a fungal pathogen for the biocontrol of the mealybug pest *Rastrococcus invadens* was investigated during the course of the present study. A *Hirsutella* sp. isolated from mummified mealybugs collected in Togo, was identified as a new species and described as *Hirsutella cryptosclerotium*. All larval stages and adult females were found to be attacked by *H. cryptosclerotium*, however, alate males were not observed with confirmed fungal infection.

The pathogen causes death of the mealybug host before extensive colonization of the haemocoel and host tissues has taken place. Under optimal environmental conditions, *H. cryptosclerotium* protrudes through the cuticle forming phialides and conidia on the insect host. Sclerotia are only produced under adverse environmental field conditions. The pathogen could be dispersed in the field by rain and possibly by wind.

Mycelium of *H. cryptosclerotium* may be successfully mass-produced using defined liquid media and may be suitable for storage and formulation.

Under favourable environmental conditions, one or two applications of a mycelial suspension of *H. cryptosclerotium* could exert control over the mealybug populations on single plants for at least two months.

Mycelium of *H. cryptosclerotium* may be resistant to direct sunlight in the field. Infection of *R. invadens* by this pathogen may take place within a wide range of temperatures and high relative humidities may not be essential for infection to establish.

Results in the present study suggest that *Hirsutella cryptosclerotium* may be a promising biocontrol agent against *Rastrococcus invadens*.

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Chapter I

General Introduction

1.1 Biological control

Biological control consists of the use of natural enemies to reduce a plant or animal population that is inimical to man (Samways, 1981). Some authors include in this term various other non-chemical forms of control that are biology-based: (a) development of strains of crops that are resistant, or tolerant, to pests or diseases; (b) modification of cultural practices in a way that avoids or reduces infestation; (c) release of sterile males.

An undesirable organism may be eliminated locally or, more usually, it is suppressed to a level where it is no longer a nuisance and does not cause economic damage. Complete eradication is seldom achieved and besides, a natural enemy which completely eliminates its prey is then without food or host and cannot survive. In biocontrol it is more convenient to reduce the pest population to a level that is no longer of economic or health concern, yet leaves sufficient pests to allow survival of the control organism. This organism maintains its own population and prevents the pest from returning to damaging levels.

Most biological control programmes in the past have involved insect pests and weeds. However, the types of control agents (natural enemies) have been widened to include microorganisms (e.g., bacteria, viruses, protozoans, fungi), invertebrate (e.g., flat-worms, nematodes, mites, insects) and even some vertebrates (e.g., insectivorous birds) (Samways, 1981).

Biological control is a promising alternative to chemicals. Extensive use of chemicals has revealed a number of principles that should be taken into account: (a) the chemical must be active against the target species; (b) it should not be phytotoxic; (c) a lethal quantity must reach the pest in a suitable formulation; (d) application

should be made at a vulnerable stage of the pest, and so timed as to prevent economic damage to the crop; (e) the development of resistance by pests should assessed; (f) harm to natural enemies must be minimized; (g) dangerous residues on the plants and in the soil must be avoided; and (h) the chemical should be stable in storage before use. Disregard of these principles has already led to some failures which are largely responsible for the current interest in biological control (Burges & Hussey, 1971).

1.2 Microbial control

The term "microbial control" refers to that type of biological control involving the use of microorganisms for the control of pests (Steinhaus, 1949). Such agents can be used either as microbial insecticides, or as introductions leading to establishment and persistence of the pathogens in new environments (Fuxa, 1987). This distinction depends on the capabilities of the organisms. Those for introduction must spread from relatively small inocula and persist, so allowing nature to take its course. Those unable to spread and persist must be used as microbial insecticides and applied both regularly and in larger quantities.

Microorganisms can also be used as components of integrated pest management systems (Klassen, 1981; Hall, 1982a): (a) joint use of biological control agent and cultural methods of control; (b) biological control agents and pesticides; (c) biological control agents and resistant cultivars. Control techniques are selected which have minimal adverse effects on natural mortality factors of the pest such as parasites, predators and pathogens. In these techniques the microorganism can play a role as a desirable applied control agent or as a naturally occurring mortality factor which warrants protection (Roberts & Yendol, 1971).

1.3 Microbial control of arthropod pests by entomopathogenic fungi

Microorganisms have long been used against arthropods and mammals and recently against weeds. Most emphasis has been placed on arthropods and the study of their infectious diseases has expanded in recent years, revealing their potential for control (Simmonds *et al.*, 1976).

Infectious diseases of arthropods are usually grouped according to the type of agent involved. Thus, there are viral, rickettsial, bacterial, fungal, protozoan, and nematode diseases of insects (Steinhaus, 1963). The major research effort has been directed towards the study of bacteria and viruses whose potentialities as biological control agents for the microbial control of insects pests of crops were considered to be greater. However, lately, research with the entomopathogenic fungi has benefited from a renewed interest, which arises from a better understanding of the role of natural phenomena in the regulation of insect populations, among which epizootics caused by fungi have played a major part (Ferron, 1978).

The entomopathogenic fungi are much more common and are more effective in naturally reducing or controlling their hosts than most people realize. They constitute the largest single group of insect pathogens, represented by an estimated 750 species (Anonymous, 1979).

Entomopathogenic fungi have a particular advantage over many other microbial pathogens since they generally invade their hosts through the integument whereas viruses, bacteria and rickettsiae have to be fed to their hosts to cause disease (Carruthers & Soper, 1987). They can reasonably be expected to establish themselves in a host population and cause residual mortality through the period that the host population is available and, in many instances, into subsequent years, by means of residual stages (e.g., thick-walled spores, chlamydospores, sclerotia) which can survive inhospitable environmental conditions or periods when suitable hosts are

unavailable. The entomopathogenic fungi, like all fungi, usually have relatively efficient spore dispersal mechanisms which increase the likelihood of infecting more susceptible hosts.

Mycoses are common with certain taxonomic groups, e.g., Lepidoptera (moths and butterflies, particularly larvae), Homoptera (particularly aphids, cicadas, and scale insects), Hymenoptera (wasps, ants), Coleoptera (beetles), and Diptera (flies) (Roberts & Yendol, 1971).

All four major taxonomic groups of true fungi contain entomopathogenic species, with Zygomycotina and Deuteromycotina as the two most important ones. The fungal genera most frequently associated with diseases of arthropods are listed below:

Mastigomycotina: Zygomycotina:

Coelomomyces, Entomophthora, Massospora, Erynia, Entomophaga

Deuteromycotina:

Aschersonia, Aspergillus, Beauveria, Hirsutella, Metarhizium, Paecilomyces, Verticillium

Ascomycotina:

Cordyceps, Torrubiella, Hypocrella

Basidiomycotina:

Septobasidium

The host ranges of some species of *Coelomomyces*, *Entomophthora*, *Massospora*, *Hirsutella*, and *Septobasidium* are limited to a single family, genus, or a few species of insects or mites. Wide host ranges, however, are the rule with most commonly isolated fungi, e.g., the host range of *Metarhizium anisopliae* exceeds 200 species of insects (Veen, 1968, cited in Roberts & Yendol, 1971).

Some of the fungal species which have been investigated for their role as biological control agents are listed in Table 1.1. Most fungi studied for use as microbial insecticides belong to the Deuteromycotina. In general they are easily mass produced on (semi) artificial media, although the Entomophthorales, an important and world-wide distributed group of pathogens, are difficult to culture (Wilding, 1981).

Table 1.2 shows the factors which may be considered in the evaluation of fungi for control of insects (Fransen, 1987). The importance of the factors mentioned depends on the way in which the pathogen will be used: e.g., using fungal pathogens as microbial insecticides involves mass production and formulation, but this is not required when the pathogens are able to spread and persist from relatively small inocula (introductions).

1.4 Entomopathogenic fungi of mealybugs (Pseudococcidae)

Moore (1988) has recently reviewed the literature on pathogens of mealybugs. As yet only entomopathogenic fungi within the sub-divisions Zygomycotina and Deuteromycotina have been reported causing natural infestations among the Pseudococcidae. According to Moore (1988) some of the records from the literature may result from saprophytic growth on dead insects, since often the pathogens were recorded by entomologists rather than pathologists and pathogenicity was assumed rather than proved. Only three species are indicated with any confidence as pathogenic against mealybugs, *Neozygites fumosa* (Speare, 1922), a *Cephalosporium* sp. (Rotjer *et al.*, 1966), and a *Cladosporium* sp., possibly near *C. oxysporum* (Samways, 1983). The evidence for their pathogenicity varies. Speare (1922) considered *N. fumosa* to be largely responsible for the natural control of the citrus mealybug, *Pseudococcus citri,* in Florida, especially during periods of summer rain. The *Cephalosporium* sp. was isolated from *Planococcus citri* and was shown by

Table 1.1. Fungal species under investigation for their role as biological control agents of arthropods.

Fungal pathogen	Host insect	Crop	Reference
Aschersonia aleyrodis	*Trialeurodes vaporariorum*	vegetables	Fransen (1987)
Beauveria bassiana	*Nilaparvarta lugens*	rice	Rombach *et al.* (1986a)
Beauveria brongniartii	*Melolontha melolontha*	pastures	Ferron (1981)
Cephalosporium lecanii	*Coccus viridis*	coffee	Easwaramoorthy & Jayaraj (1978)
Entomophthora aphidis E. thaxteriana	*Acyrthosiphon pisum*	peas	Wilding (1970, 1973)
Entomophthora exitialis	*Galleria mellonella*	--	Krejzova (1973) (cited in Wilding, 1981)
Entomophthora sphaerosperma E. thaxteriana	aphids & spider mites	glasshouse plants	Tsinovskiy & Egina (1972) (cited in Wilding, 1981)
Hirsutella citriformis	*Nilaparvarta lugens*	rice	Rombach *et al.* (1986a)
Hirsutella thompsonii	*Abaracus hystrix*	vector ryegras mosaic virus	Hall & Lewis (1982)
Hirsutella thompsonii	*Phyllocoptruta oleivora*	citrus	McCoy *et al.* (1971, 1972)
Metarhizium anisopliae	*Mahanarva posticata*	sugar cane	Guagliumi *et al.* (1974) (cited in Ferron, 1981)
Metarhizium anisopliae	*Oryctes rhinoceros*	coconut palm	Young (1974)
Nomuraea rileyi	*Trichoplusia ni*	cabbage, soybean	Behnke & Paschke (1966)
Nomuraea rileyi	*Pseudoplusia includens*	cotton, soybean	Gudauskas & Canerday (1966)
Nomuraea rileyi	*Heliothis virescens Heliothis armigera*	cotton, soybean	Ignoffo (1981)
Paecilomyces lilacinus	*Scotinophara coarctata*	rice	Rombach *et al.* (1986b)
Paecilomyces farinosus	*Trichoplusia ni*	soybean	Agudelo & Falcon (1983)
Verticillium lecanii	*Coccus hesperidum*	citrus	Samsinakova & Kalalova (1975)
Verticillium lecanii	*Macrosiphoniella sanborni*	vegetables	Hall (1976, 1980)
Verticillium lecanii	*Trialeurodes vaporariorum*	vegetables	Hall (1982a)
Verticillium lecanii	*Thrips tabaci*	vegetables	Gillespie (1986)

Table 1.2. Topics for evaluation of entomopathogenic fungi for control of arthropod pests.

Fungal characteristics
-specificity -virulence -sporulation -spread -persistence -possibilities for mass production -suitability for storage and formulation -toxicological and safety aspects -effects on natural enemies -compatibility with insecticides and fungicides
Host characteristics
-susceptibility -economic injury level -age distribution, density and spatial distribution in relation to application or introduction strategy
Environmental aspects
-abiotic factors: temperature, humidity, sunlight, rain, dew, irrigation, wind -biotic factors: host-plant quality

Rotjer and his co-workers to be pathogenic to *Planococcoides njalensis* in laboratory experiments. Then in a field trial in Kenya, spraying cocoa plants with a mycelial suspension of this fungus induced an epizootic in the mealybug population with effects lasting at least three months (Rotjer *et al.*, 1966). The *Cladosporium* sp. was also isolated from infected *Planococcus citri*. Samways (1983) found that on guava trees in South Africa there was a mutualism between the ant *Anoplolepis custodiens* and *Planococcus citri*. This association was influenced to some extent by the *Cladosporium* sp., but the effects on the mealybug were apparently delayed by the presence of the ants. In the absence of ants, it caused a total crash in the mealybug population.

1.5 Introduction to the chapters

Studies were undertaken on different aspects of *Hirsutella* sp. nov. relevant to the development of this pathogen as a microbial control agent against the mealybug *Rastrococcus invadens*. The importance of *R. invadens* as a pest was reviewed and some aspects of the biology of this mealybug were studied for the first time on the host *Citrus microphylla* (Chapter II). *Hirsutella* sp. nov. was described as a new species (Chapter III) and the infection process on *R. invadens* was studied (Chapter IV). Studies were carried out on the importance of rain on the dispersion of the pathogen (Chapter V). The nutritional requirements of the fungus were investigated (Chapter VI) in order to develop a medium that could be used in routine mass production of this pathogen. The impact of *Hirsutella* sp. nov. on the mealybug was assessed by bioassay (Chapter VII). The effect of different environmental factors on the establishment of infection were investigated (Chapter VIII). Finally the feasibility of using *Hirsutella* sp. nov. as a control agent of *R. invadens* is considered and the results summarized (Chapter IX).

Chapter II

Aspects of the biology of *Rastrococcus invadens* on *Citrus microphylla*

2.1 Introduction

2.1.1 *Rastrococcus invadens*, an important pest of fruit crops in West Africa

Rastrococcus invadens is a newly discovered species described by Williams (1986) (Fig. 2.1). It was accidentally introduced into Ghana and Togo in 1981-1982, presumably on plant material, and has become a serious pest of citrus, mango and ornamental plants in those countries. The area of origin of this mealybug is the Oriental Region where it is widespread, but it has always been mistaken for *R. spinosus*, a closely related species (Williams, 1986).

R. invadens has been also recorded in Benin since 1986, and recently in Côte d'Ivoire, Nigeria, Congo, Gabon and Zaire (D. Moore, personal communication). In Togo the mealybug dispersed from the coastal area 500 km northwards in about 2 years. According to Agounké *et al.* (1988) people are likely to be responsible for most of the long distance movement by the transport of contaminated goods and plant material.

The mealybug has been recorded attacking 45 plant species in 22 families in Togo and Benin (Löhr, 1984, cited in Agounké *et al.*, 1988). Mango, citrus, banana, breadfruit and guava are the fruit crops particularly infested by the mealybug in these two countries. Yield losses have not been accurately quantified but have been estimated as up to 80% in mango in Ghana. Besides fruit trees, many horticultural and ornamental plants were recorded as being also badly infested (Agounké *et al.*, 1988).

Trees infested by *R. invadens* are characterized by black leaves due to sooty mould which develops on the honeydew secreted by the pest (Fig. 2.2). The colonies of *R. invadens* are generally located on the lower sides of the leaves, along the primary and second veins. The infestation begins at the base of the leaf and, progressively, the rest of the leaf is covered. This pest also infests petioles, flowers and fruits. *R. invadens* does not introduce toxins nor cause deformation of the attacked organs and it seems that it does not transmit diseases to the infested plants (Agounké *et al.*, 1988).

Control measures which have been used include pruning, burning, and application of pesticides such as cypermethrin, dimethoate and carbofuran. However, these attempts to control the mealybug do not provide long-lasting results (Agounké *et al.*, 1988).

2.1.2 Morphological characteristics of *R. invadens*

R. invadens is pale green covered with white wax with a bare area on the midline (Figs 2.3-2.4). The body length of female adults is 3.5-4.0 mm and the width is 2-2.5 mm. The mealybug bears long conspicuous wax filaments at the anterior and posterior ends and shorter lateral ones (Williams, 1986). The wax filament arrangement on the body is the best morphological character to distinguish between the different stages. Crawlers have one anterior and two posterior wax filaments; second nymphs are distinguished from crawlers by having four anterior and eight posterior wax filaments and shorter lateral filaments. It is impossible to separate the sexes in the first two instars, but it is possible after the second moult. The female looks the same in the third instar as it does in the second. The male, however, forms a cocoon with wax secretions and goes through two instars (third instar= prepupal stage; fourth instar= pupal stage) emerging as an alate adult (Willink & Moore, 1988).

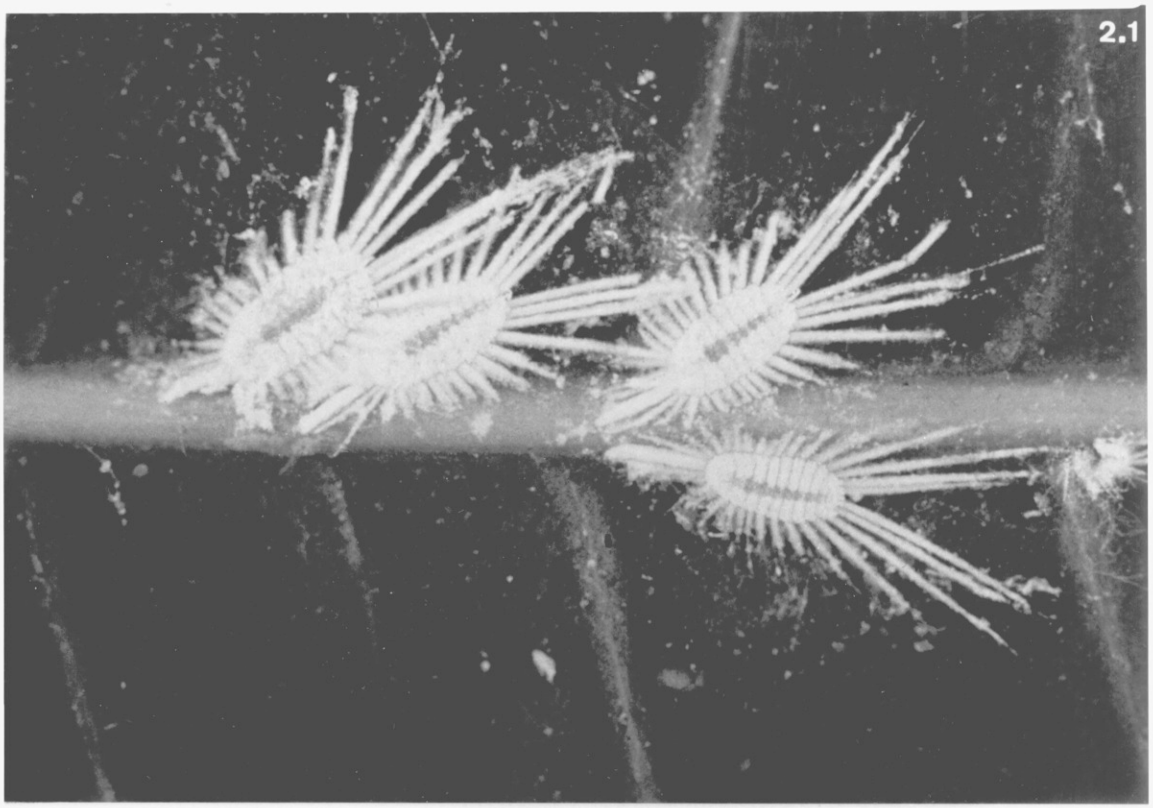
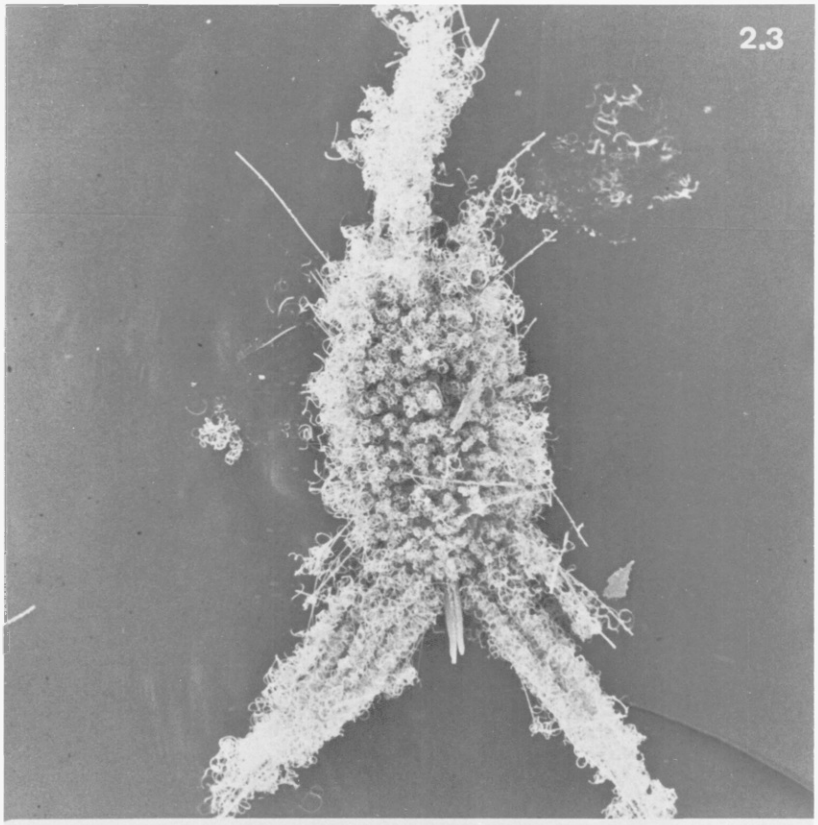


Fig. 2.1. *Rastrococcus invadens*, pest of fruit trees in West Africa.
Fig. 2.2. Mango plant showing heavy deposits of sooty mould developing on honeydew excreted by *R. invadens*.



Figs 2.3-2.4. Scanning electron micrographs revealing the waxy body covering of *R. invadens*. Fig. 2.4. Close-up to show the curly filaments which form the waxy cover.

2.1.3 Pseudococcidae: main characteristics and importance as pests

R. invadens belongs to the family Pseudococcidae in the group of families called Coccoidea. This group of plant sucking Homoptera includes the soft scales (Coccidae), the armoured scales (Diaspididae), the mealybugs (Pseudococcidae) and others not distinguished by common names (Margarodidae, Ortheziidae, Asterolecaniidae, Strictococcidae) but which also include common insects. Characteristics of coccoid families are wingless adult females in which the separation of head, thorax and abdomen are indistinct and the dorsum is covered with secretional material, the form of which varies from loose wax (Pseudococcidae) to a hard scale extending beyond the margin of the insect (Diaspididae).

In the Pseudococcidae, adult females are small oval insects with a soft body, the dorsal part of which is completely covered with filaments of white wax and the ventral one is only partially covered. On the lateral and terminal margins they carry filaments of white wax. Beneath the wax covering, which is secreted by dorsal and lateral glands, the body colour is often characteristic for a species and may be yellow, orange, pink, dark blue or black. Adult females have no wings and the points of junction of the head, thorax and abdomen are not externally distinct. The adult male is minute and very different from the female. It has one pair of functional flight wings and the posterior pair is reduced to small hook-like structures. It is unable to feed, having no mouth parts, and apart from a very long wax-covered hairs at the apex of the abdomen is naked. Production of young may be ovoviviparous (the eggs usually hatch within a few minutes of deposition) or oviparous. External differences between males and females do not usually appear until the second nymphal instar. At the end of the second instar males construct a small waxy cocoon in which they moult first to a prepupal and then to a pupal stage. The female, however, passes through only three pre-adult instars during all of which it feeds, before maturing without a pupal

stage. Reproductive females are therefore neotenic. After becoming adult females grow for a while without changing appearance and this make it difficult to distinguish last instar nymphs from young adults (Entwistle, 1972).

Excretions of plant sap (honey dew) rich in sugars and amino acids attract ants which tend (or attend) the coccids to obtain this fluid. Sooty mould grows saprophytically on this honey dew preventing photosynthesis when present in sufficient quantity (Entwistle, 1972).

Dispersal of the mealybug colony is mainly, but not exclusively, by the active first or second instar nymphs or "crawlers". The wind is also responsible for the dispersal of the nymphs, especially when they are moving. Ants are also accountable for local movements particularly when they attend the mealybug colony picking the nymphs up and carrying them to fresh feeding sites (Moore, 1988).

Mealybugs are one of the most important plant pests. There are between two and three thousand described species, with an estimate of a comparable number to be described (Beardsley, 1977, cited in Moore, 1988). They seem to reach their highest population levels in hot regions of moderate rainfall, where large numbers cause serious damage, especially on citrus and coffee (Entwistle, 1972). Mealybugs are difficult to control with pesticides because their waxy body repels contact chemicals (Greathead, 1986) and also because they move to high parts of the plant, where, especially in trees, pesticide application may be difficult (Panis, 1986, cited in Moore, 1988). Traditional agricultural practices such as good field hygiene, removal or destruction of crop residues, sterilization of planting material and removal of alternative hosts, have been recommended against mealybug pests (Dick, 1969). However, biological control has been the most promising approach to control mealybug pests in many cases (see Moore, 1988 for extensive review).

The aim of the work reported in this chapter was to study some aspects of the biology of *R. invadens* on *Citrus microphylla* in order to find out whether this plant was a suitable host for rearing the mealybug under laboratory conditions.

2.2 Materials and methods

2.2.1 Environmental conditions

Studies were carried out in a quarantine room with a 16 h light/8 h dark photoperiod, where the temperature was held constant at 25°C and the relative humidity at 75-80%.

2.2.2 Mealybug cultures

R. invadens was reared on *Citrus microphylla* seedlings, about 35 cm high (brought from Valencia, Spain), in ventilated Perspex cages (45x45x45 cm). Plants were infested by transferring adults onto leaves with a fine brush. Infestations of two-spotted mite, *Tetranychus urticae*, were controlled by regular applications of predatory phytoseiid mites (*Phytoseiulus* sp.).

2.2.3 Life cycle-studies

Life-cyle studies were carried out by placing one female on each of a number of leaves (16 leaves in total from 6 different plants) and waiting until they had settled and produced first instars. In preliminary experiments females were allowed to produce nymphs for 2-3 days which resulted in a range of densities of crawlers of different ages on the leaves. To avoid this, crawlers were taken away while daily production was less than ten. If a female produced ten or more crawlers on a particular day, she was taken away and the development of the nymphs was studied. Using this procedure a more synchronous development of individuals was obtained. Plants

were checked every two days and the number, stage and sex of the mealybugs present were recorded. Mortality was also assessed (mortality data included not only dead insects but also to those which fell off the plants or migrated to other leaves).

2.2.4 Fecundity

Female third instars were placed individually on leaves (14 leaves in total from 3 different plants) and were checked every day to note when they became adults and when crawlers were produced. It is more convenient to start the experiment with female third instars because disturbance of female adults by placing them on leaves leads, sometimes, to migration and scattered crawler production over the plant (E. Willink, personal communication). Later, leaves were checked every 4 days and the number of nymphs produced was recorded. The nymphs were then removed from each leaf. More frequent checks causes too much disturbance, resulting in lower crawler production or migration of females (A. Steenkiste, personal communication). Females were allowed to reproduce as long as they lived.

2.3 Results

2.3.1 Development time of *R. invadens* on *C. microphylla*.

Development took around 17 days for both males and females (Table 2.1). The first stage lasted about 5 days; development to the third instar took about another 6 days for both females and males; third instar females became then adults after about 6 days and it took a similar time for a male to undergo the two pupal stages and emerge as an alate individual.

The range of the development times is shown in Fig. 2.5, e.g., the shortest period to produce both male and female adults was 15 days and the longest 23 days. Development time was slightly (but not significantly) shorter for males than for females during the second instar: around 8% of the male population completed the

second instar after only 5 days whereas females always moulted to third instar after 7 days or more. As Fig. 2.5 shows, insects moulted gradually from one instar to another leading to overlapping of the different stages of *R. invadens* (Fig. 2.6).

Mortality (Table 2.2) was about 34% during the development to adult stage in both sexes. Of this mortality, about 28% occurred during first and second instars. First and second instars tended to stay on the leaf on which they were born (if the adults had not been removed, it is likely that the higher density of crawlers would have led to more migration (Willink & Moore, 1988)). However female third instars or young female adults migrated to other leaves (or fell off the plants while moving) whenever there were more than four or five individuals on the same leaf, accounting for the higher "mortalities" recorded during this two stages.

The ratio of males to females was about 75:25. The exact figures are shown in Table 2.3.

2.3.2 Female fecundity

Fig. 2.7 shows the overall pattern of first instar production up to 11 weeks (Table 2.4). About 70% of the progeny was produced within the first three weeks and 30% during the last 9 weeks. Females produce crawlers as long as they live and this contributes to overlapping generations. Observations while rearing *R. invadens* showed that when the progeny from the first cohort had reached adult stage, the female was still producing crawlers but at a lesser rate.

Data shown in Table 2.4 indicate that female adults had a pre-reproductive period of about 18 days, and produce an average of 121 first instars during a period of about 65 days.

Table 2.1. Mean development times of *R. invadens*.

stage of development:	mean number of days to complete stage (± s.e.)	
first instar	5.30	0.182
second instar, female	11.59	0.252
second instar, male	11.26	0.252
third instar, female	17.93	0.682
third-fourth instar, male	17.21	0.682

Table 2.2. Mortality of different stages of *R. invadens*.

	% mortality	
	mean	s.e.
first instar -> second instar	11.345	0.165
second instar -> male prepupal and pupal stages	17.221	0.165
male prepupal and pupal stages -> adult male	5.280	0.165
female third instar -> adult female	9.455	0.165

Table 2.3. Sex ratio of *R. invadens*.

	mean	s.e.
% males	72.300	69.81-74.72
% females	27.677	25.30-30.18

Table 2.4. Reproduction period and lifetime fecundity.

	mean	s.e.
Days to start of first instar production	35.91	1.985
reproduction period in days	65.55	2.895
number of progeny	121.16	7.567

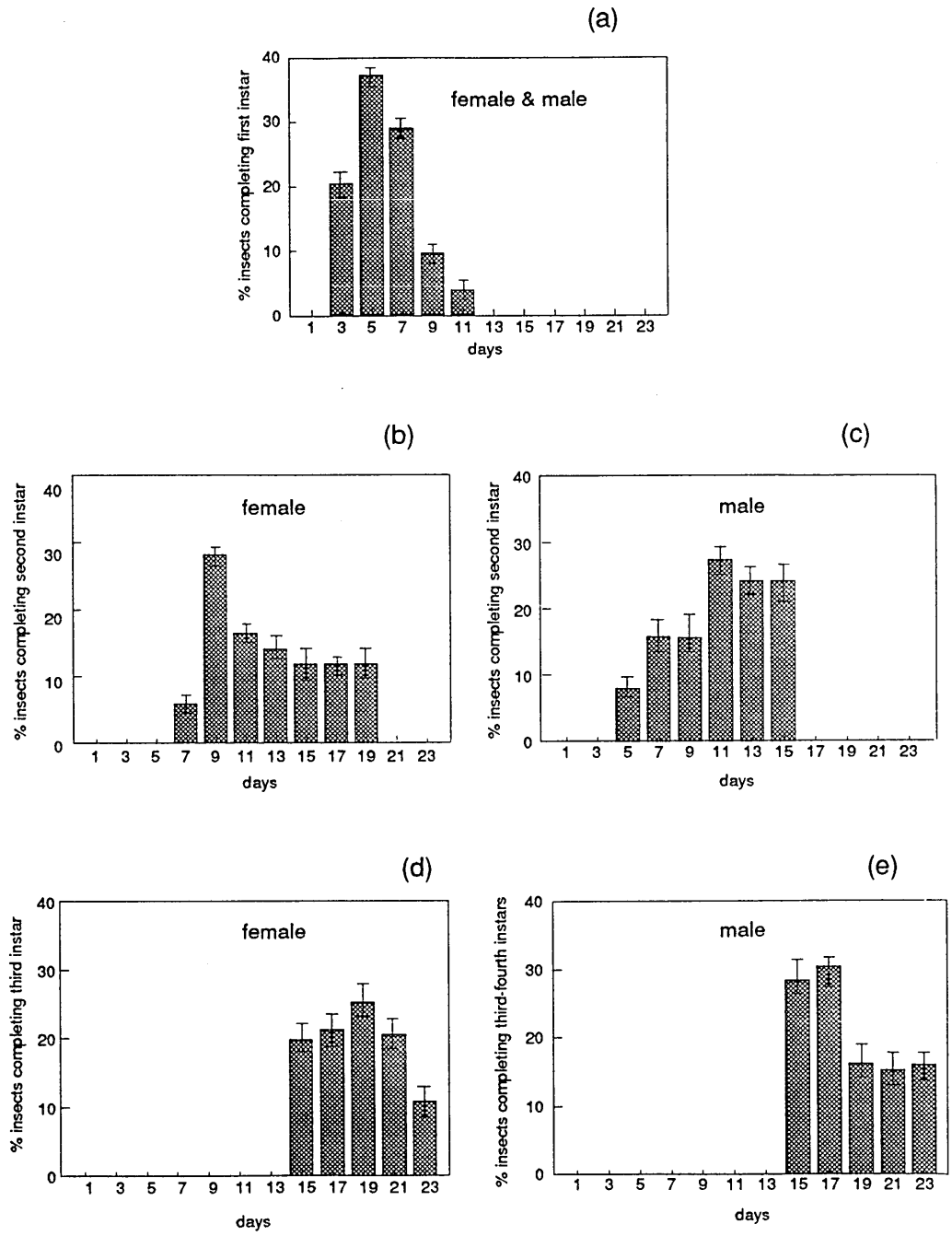


Fig. 2.5. Percentage of *R. invadens* nymphs completing first (a), second (b, female; c, male) and third instar (d, female; e, male). Note the gradual change from one instar to another for both females and males. Data are mean ± s.e. Data in App. 2.1.

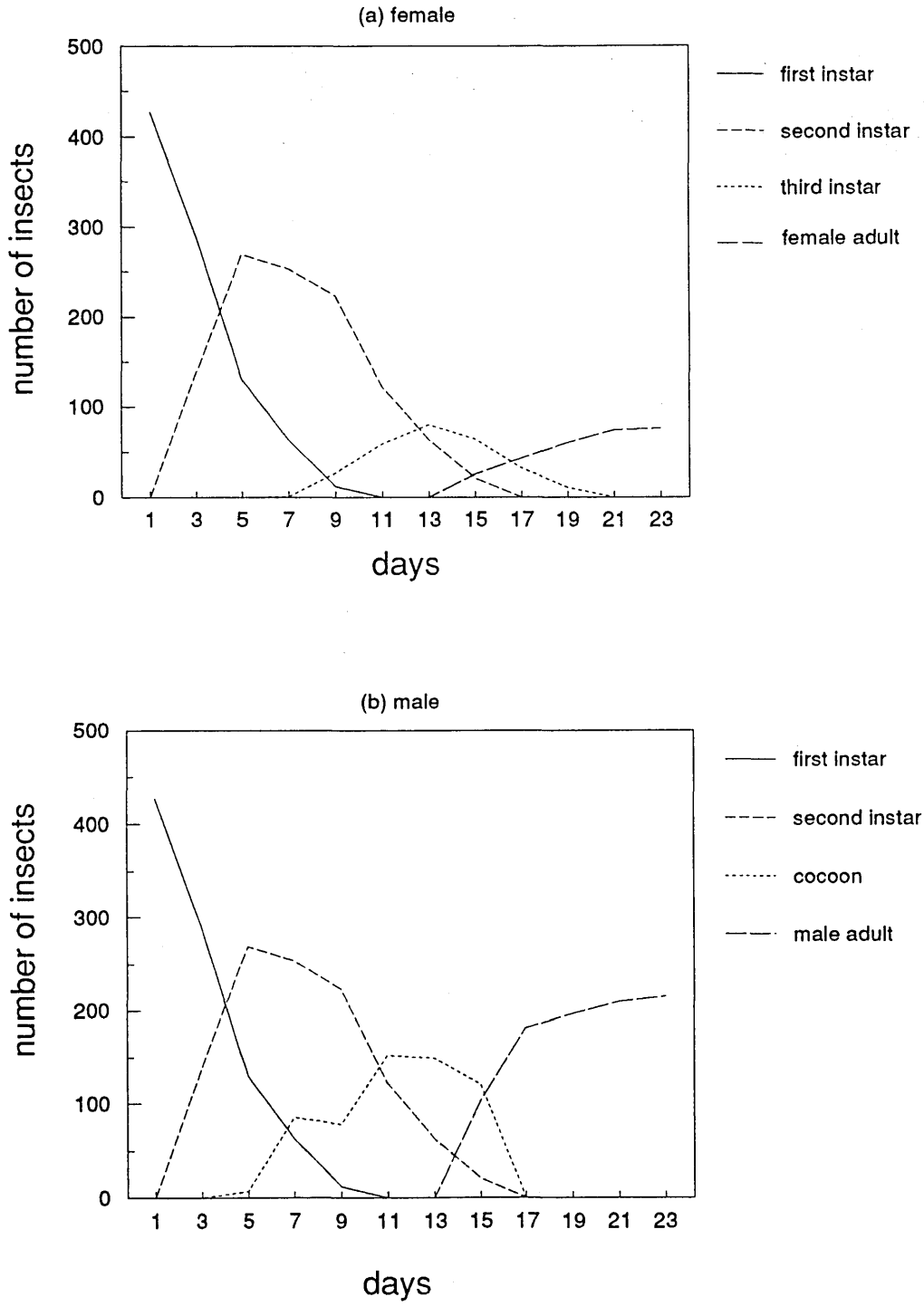


Fig. 2.6. Graph showing overlapping of the different stages of *R. invadens* (a, female; b, male) during development. Data in App. 2.2.

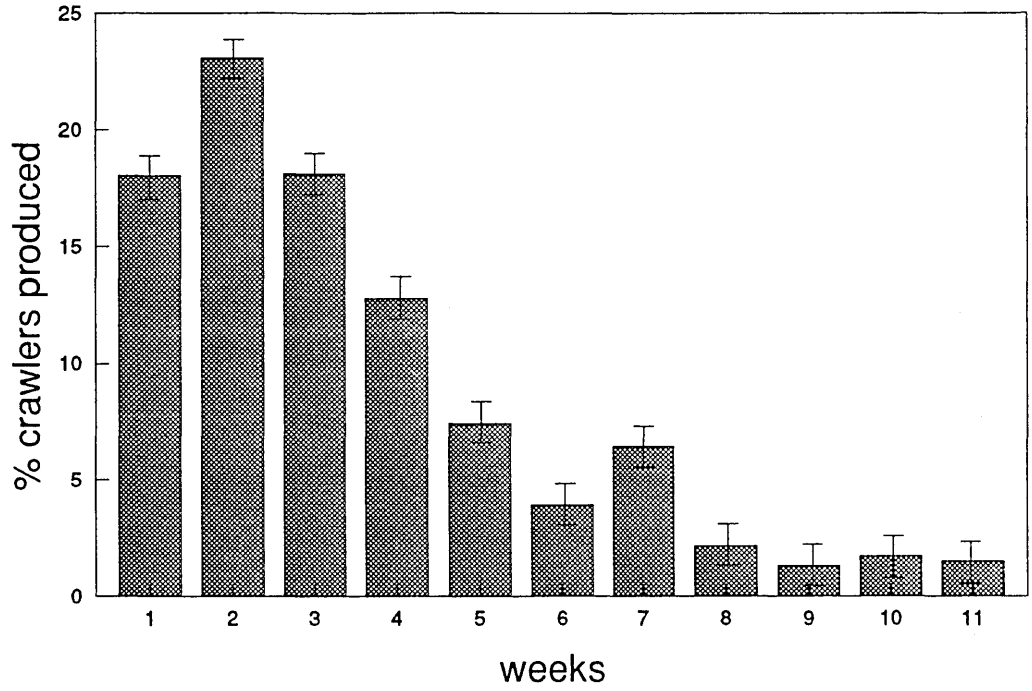


Fig. 2.7. First instar production per adult female of *R. invadens*. Females were allowed to reproduce for as long as they lived. Data are mean ± s.e. Data in App. 2.3.

2.4 Discussion

Studies on the biology of *R. invadens* have been previously carried out under laboratory conditions on the following host plants: *Nerium oleander*, *Mangifera indica*, *Manihot esculenta*, *Carica papaya*, *Citrus aurantifolia* and *Citrus x limon* (rough lemon, hybrid between *C. limon* and *C. medica*) (Laup, 1987; Willink & Moore, 1988). These studies showed that *Manihot esculenta* and *Carica papaya* are not suitable host plants for *R. invadens*. On most of the appropriate host plants the mealybug had a life cycle from first instar to reproductive adult of around 45-50 days, the exception being *Nerium oleander* on which the life cycle was completed after 68 days. Results from the present study indicated that *Citrus microphylla* is a suitable host plant for *R. invadens*, since development from first instar to reproductive adult took only around 40 days, and each female could produce nearly 121 first instars over an extended period. Data on mortality in the present study are lower than those previously reported. This may be a consequence of the shorter life cycle on *C. microphylla*. Lifetime fecundity has been only studied on rough lemon (Willink & Moore, 1988) where each female produced around 180 first instars.

Assimilation and growth efficiencies of phytophagous insects often improve with host plants containing a high nitrogen content (McNeill & Southwood, 1978; Mattson, 1980; Fernández Haeger *et al.*, 1987; Fernández García, 1988). The importance of chemical and physical defences in reducing assimilation has also been reported (Janzen, 1978; Strong *et al.*, 1984). The shorter life cycle of *R. invadens* on *C. microphylla* could be due either to a higher level of protein nitrogen in this plant or to a lower level of chemical defences. Obvious physical defences such as hooks, spines or trichomes are not present on leaves of those plants which were less suitable for *R. invadens*. However, the assimilation of nutrients from these plants might have been reduced if their leaves bore thicker cuticles (Strong *et al.*, 1984). Another factor, that may have contributed

to the efficacy of *C. microphylla* as a more suitable host plant, is that the original colony of *R. invadens* used in the present study had been reared in artificial laboratory conditions for a longer period than the colonies used in previous studies. It may be that those insects which developed quicker were selected to establish new colonies.

Chapter III

Hirsutella cryptosclerotium sp. nov., an entomopathogen of the mealybug pest, *Rastrococcus invadens*, in West Africa.

3.1 Introduction

The mealybug *Rastrococcus invadens* (Homoptera: Pseudococcidae) has recently become a serious pest of many fruit crops in West Africa (Agounké *et al.*, 1988) (see Chapter II). Because of the extremely rapid spread of this exotic insect and the types of smallholder farming systems affected, biological control was considered to offer the only viable method of containment and long-term control of the pest. Searches for the insect natural enemies were undertaken in India and Malaysia, the purported centres of origin of the mealybug (Narasimham & Chacko, 1988), and a host-specific hymenopteran parasitoid has since been released into West Africa (Willink & Moore, 1988; Agricola *et al.*, 1989). No pathogens were reported during these field surveys in Asia, but dead, mummified mealybugs, sent from Togo for analysis, were found to contain sclerotia internally. A *Hirsutella* sp. was subsequently isolated in culture from sclerotial tissue. This chapter reports on the *in vivo* and *in vitro* morphology of this fungus, which is described as a new species, *Hirsutella cryptosclerotium*.

3.2 Materials and methods

3.2.1 Fungal material

Two strains were isolated by H. C. Evans from moribund mealybugs received from collaborating scientists in Togo, West Africa. Isolates were grown on oatmeal agar (OA) in plastic Petri dishes in 16 h light/8 h dark at 25°C and also in a defined liquid medium (McCoy *et al.*, 1975) in both still and shake culture. Techniques for preparing the fungal material for SEM and TEM were the same as those of Beckett

et al. (1982) and O'Donnell *et al.* (1976) respectively. The material used for SEM examination consisted of small blocks of agar with sporulating fungus and for TEM examination a spore suspension.

3.2.2 Scanning electron microscopy

The material was frozen-hydrated using a EMscope Sputter Cryo system interface with a Hitachi S570 scanning electron microscope. This technique involved freezing the specimen on a copper stub in subcooled nitrogen under argon gas and then transferring it to an evacuated chamber in which it was sputter coated with gold after being defrosted. The frozen-hydrated material was coated with gold for 5 min. This was operated at 13.3 N m^{-2} argon pressure with a voltage of 1 kV on a current of 30 mA. Scanning electron micrographs were recorded on Kodak Plus X 125 ASA 120 roll film at a recording time of 100 s.

3.2.3 Transmission electron microscopy

The material was fixed in either (a) 1% $KMnO_4$ in distilled water for 1 h followed by a distilledwash for 30 min or (b) glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.2) at 0-4°C for 4 h, washed twice (15 min each time) in the same buffer, and postfixed for 2 h in 1 % aqueous OsO_4. The material fixed by either method was dehydrated in a graded alcohol series at 0-4°C as follows: 25, 50, 75, 90% ethanol, 25 min at each step; 100% ethanol twice, first for 30 min and overnight in the second change. The specimens were then embedded with ERL epoxy resin (Spurr's resin, Spurr, 1969) using a graded resin-ethanol series with continuous agitation. A thin layer of resin was polymerized in a 70°C oven for 8-10 h in aluminium dishes. Sections were cut with glass and diamond knives on a LKB ultramicrotome (Ultratome III 8802A). Glutaraldehyde-fixed material was stained with 0.5% uranyl acetate in

methanol-ethanol (70:30) for 2 h. All sections were examined at 80 Kv with a Philips 300 transmission electron microscope. Images were recorded on a 35 mm film (Kodak fine grain positive).

3.3 Description of *Hirsutella cryptosclerotium*

On host: mycelium sparse to abundant, cotton-like, difficult to observe amongst wax filaments (see Chapter IV, Fig. 4.9); vegetative hyphae hyaline, smooth, septate, 3.5-6 µm wide , mononematous, radiating out from the insect and attaching it to substrate, producing sessile conidiogenous cells. Conidiogenous cells, arising more or less at right angles, singly or in pairs, mono- to polyphialidic, hyaline, smooth, cylindrical, with an inflated base, 10-24x3.5-5 µm, tapering to a thin neck (3-6x0.9-1.2 µm), with an inflated apex (Figs 3.1-3.4). Conidia hyaline, aseptate, formed singly or rarely in groups at the apex, globose, 5-6 µm diam, initially smooth later appearing verrucose due to differential thickening of gelatinous sheath (Figs 3.5-3.12). Sclerotia formed within the host; black, pseudoparenchymatous (cells globose, 6-10 µm diam), undifferentiated, subglobose to irregular, 100-180x90-140 µm.

Colonies on agar (OA) slow-growing, attaining diam of 3-3.5 cm within 21 d at 25°C; white to pale gray, cotton-like; reverse olive to dark green; exudate and odour absent (Fig. 3.13). Black, irregular, pseudoparenchymatous sclerotia originally formed within agar, often larger than on the host.

Holotype *ex* Pseudococcidae (*Rastrococcus invadens*) on lower leaf surface of *Musa sapientum* L., Djágble, Togo, 7 December 1984, B. Löhr, IMI 334843.

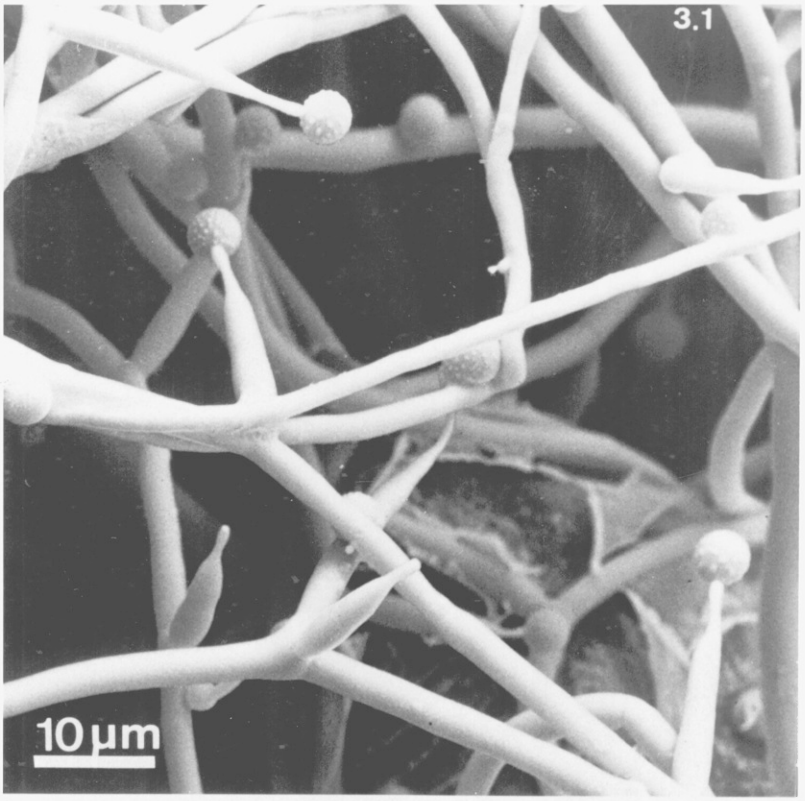
Paratype *ex R. invadens* on lower leaf surface of *Mangifera indica* L., Lomé, Togo, 15 July 1986, U. Agricola, IMI 334844.

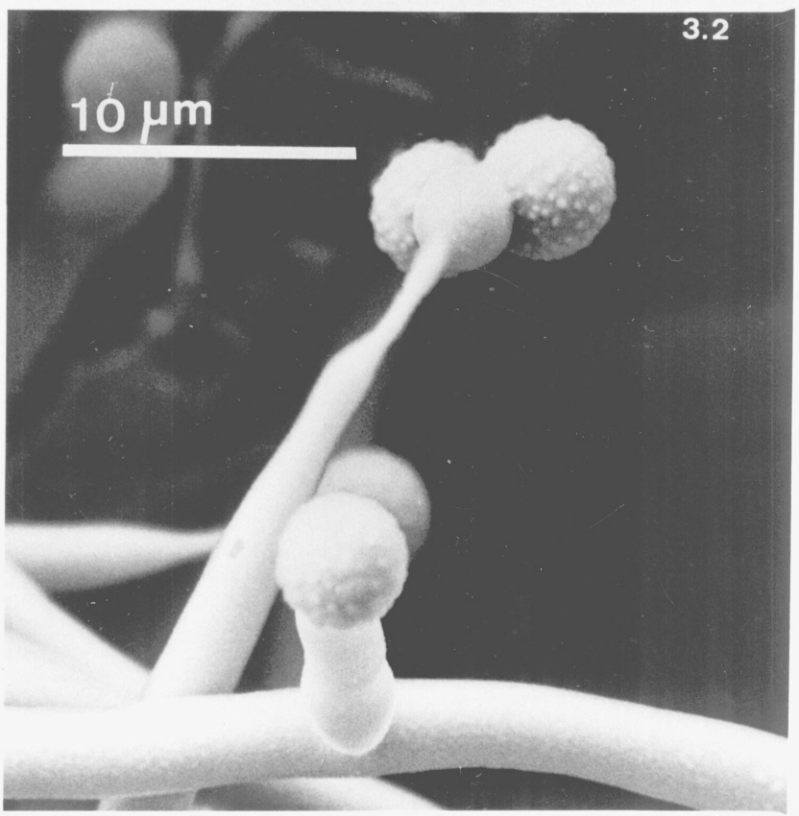
In unshaken liquid culture, sclerotia formed readily after 1 month (Figs 3.15-3.16), however, this ability was lost after subculturing. Abundant mycelial pellets regularly developed after two weeks in shake culture. On solid media containing yeast extract,

colonies were creamish-yellow, appressed and convoluted. On tap water agar (TWA), there was a high level of microcyclic conidiation (Fig. 3.14) (Latgé *et al.*, 1988). All larval stages and adult females have been found to be attacked by *H. cryptosclerotium*, although alate males have not been found with confirmed fungal infection.

3.4 Discussion

The genus *Hirsutella* Pat. is characterized by mono- or polyphialidic conidiogenous cells usually consisting of two parts: a swollen basal region and a gradually or abruptly tapering narrow apex, often with a long thin undulate neck. Conidia are formed singly or in groups, typically covered by a mucous or gelatinous sheath. The original description of the genus, as given by Speare (1920) and Mains (1951), included only species possessing synnemata. Minter & Brady (1980) recognized the artificial limitations of this generic concept and proposed a separate section (*Mononematosa*) to accommodate those taxa which lack synnemata. However, the presence or absence of synnemata is an unreliable character and cannot be used to separate sections. The formation of such structures is often physiologically rather than genetically determined (Samson & Evans, 1977; Samson *et al.*, 1980). For example, *Hirsutella* species forming conspicuous and complex synnemata on the host often fail to produce them *in vitro* whilst apparently mononematous species on small arthropods, particularly mites and thrips, readily produce highly-organized synnemata in culture (Samson *et al.*, 1980; H. C. Evans & R. A. Samson, personal communication). In the case of *H. cryptosclerotium*, synnemata have never been observed *in vitro* or *in vivo*, on a wide range of media and under varied environmental conditions. It may be that the nutrients are diverted into sclerotia rather than production of synnemata. The latter has been considered to be ecologically advantageous in those arthropods which hide away when infected (Samson & Evans,





Figs 3.1-3.6. Scanning electron microscopy of conidiogenous cells and conidia of *H. cryptosclerotium* from agar culture.

Fig. 3.1. General view of aerial mycelium and predominantly monophialidic conidiogenous cells.
Fig. 3.2. Conidiogenous cell bearing globose, apparently verrucose conidia, adhering in a group.

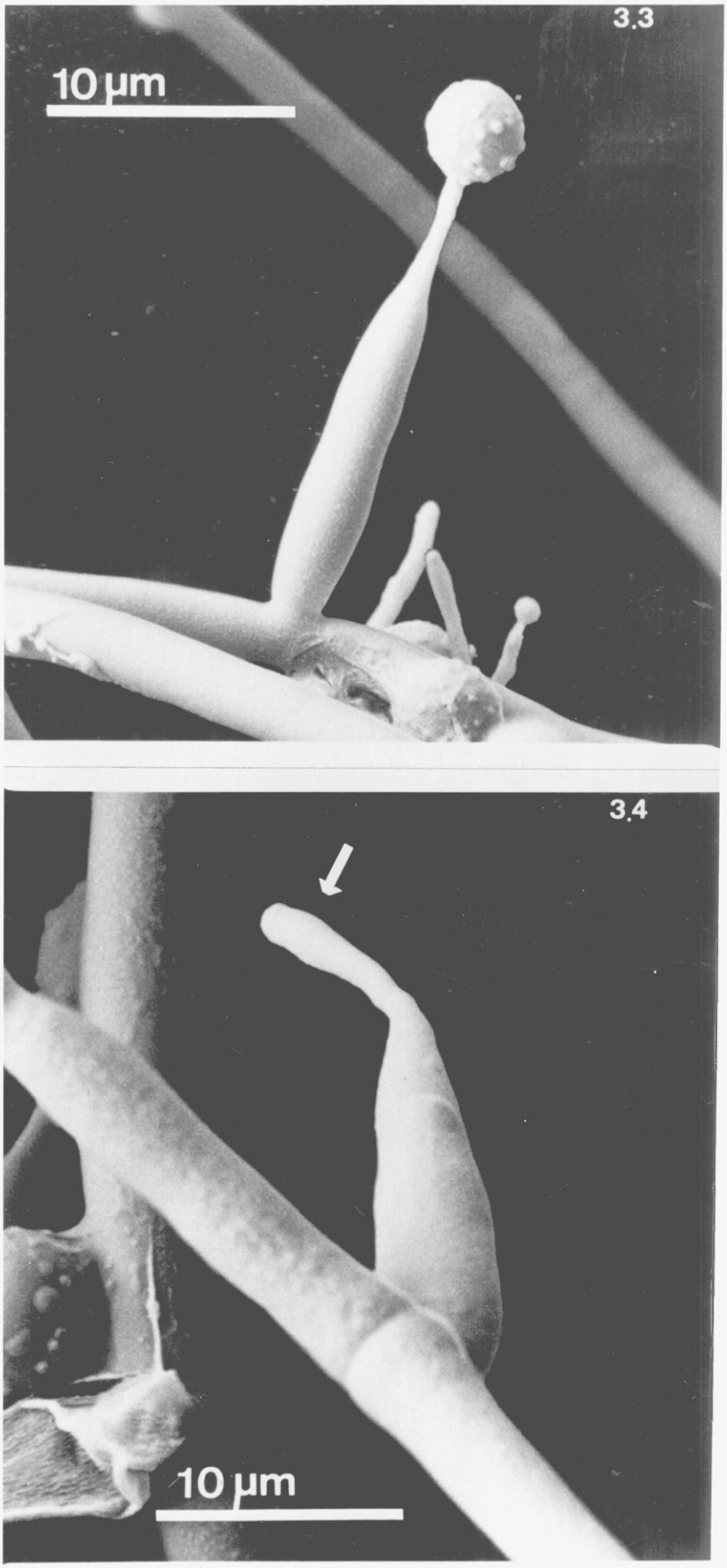
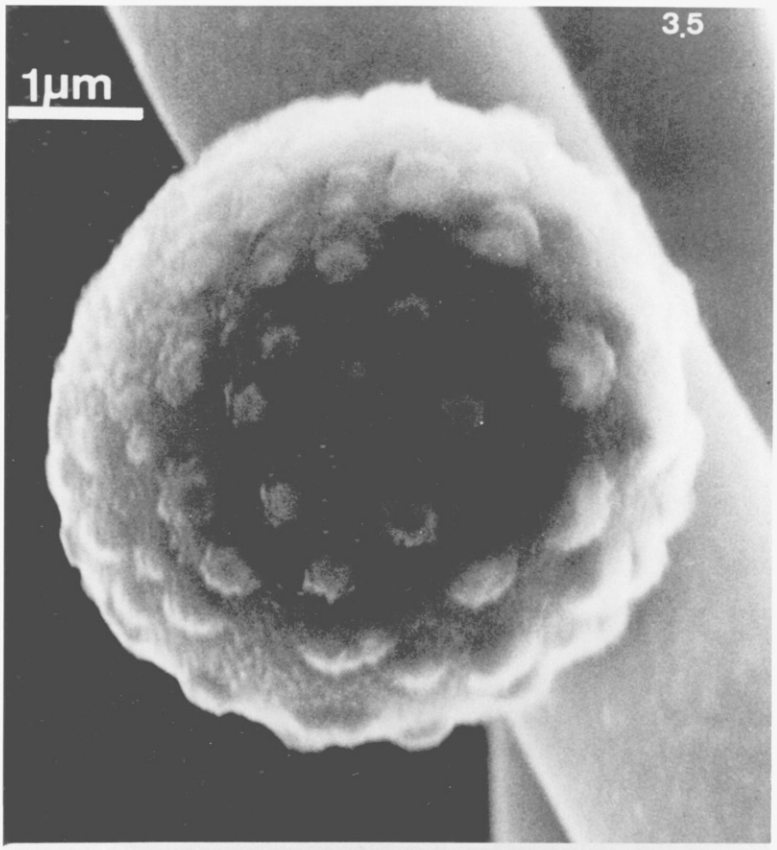


Fig. 3.3. Conidiogenous cell arising at a right angle revealing the cylindrical shape.
Fig. 3.4. Close-up of conidiogenous cell to show swollen tip (arrow).



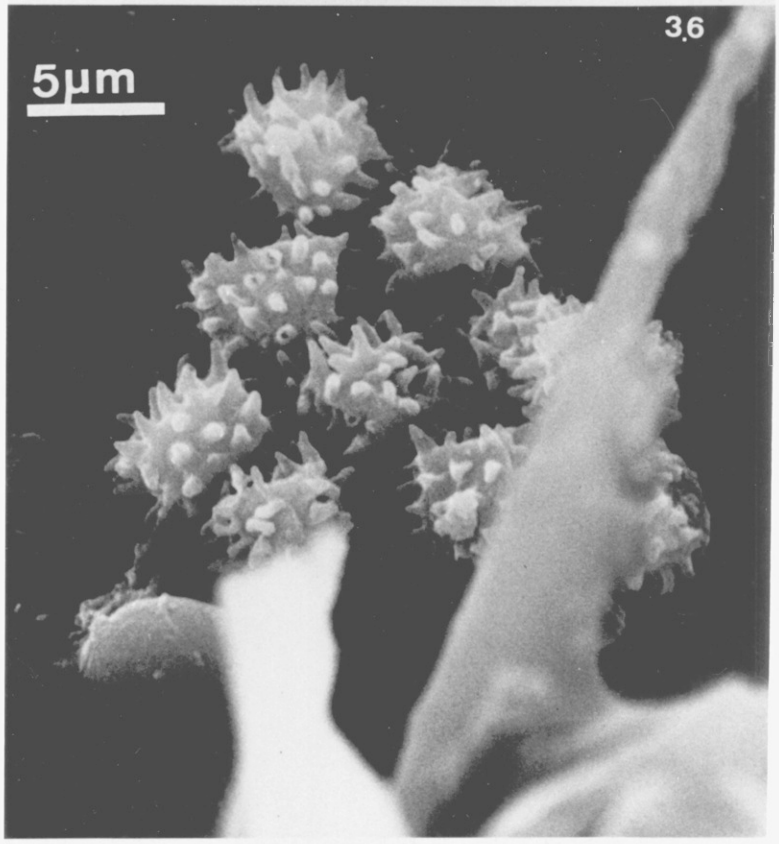
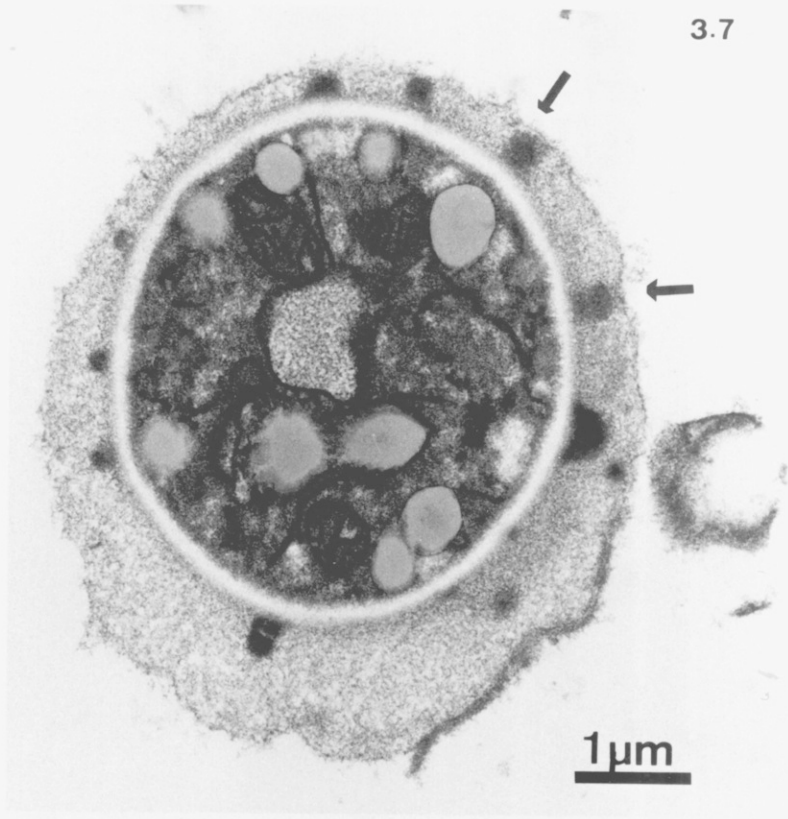
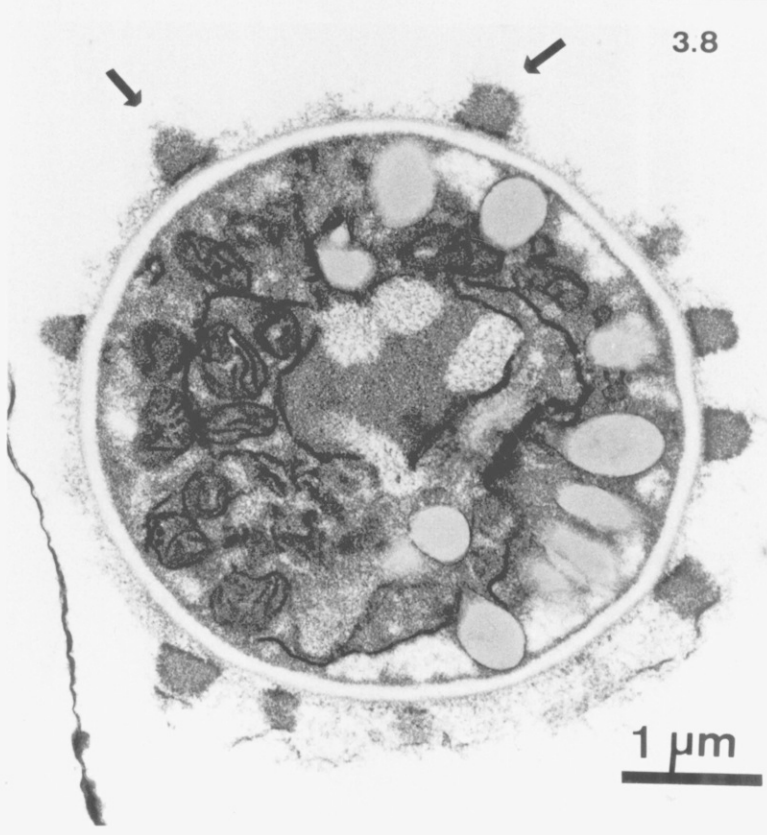


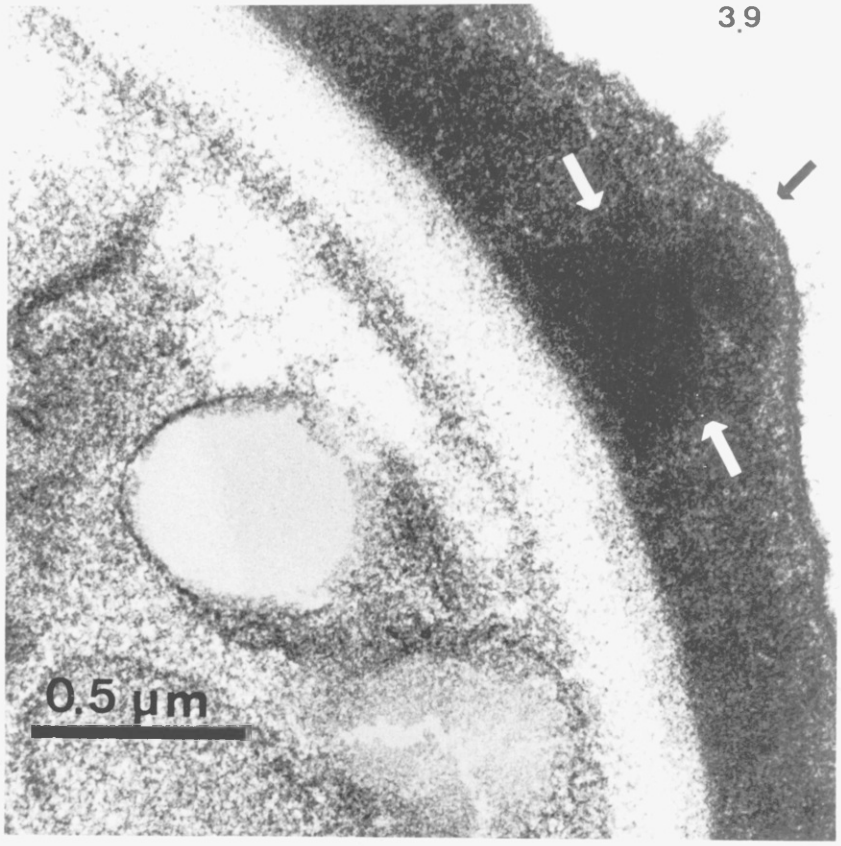
Fig. 3.5. Close-up of conidium showing ''warty'' surface.
Fig. 3.6. Conidia pre-treated with clove oil to remove free mucilage and reveal ''spines'' composed of dense or hardened mucilage.

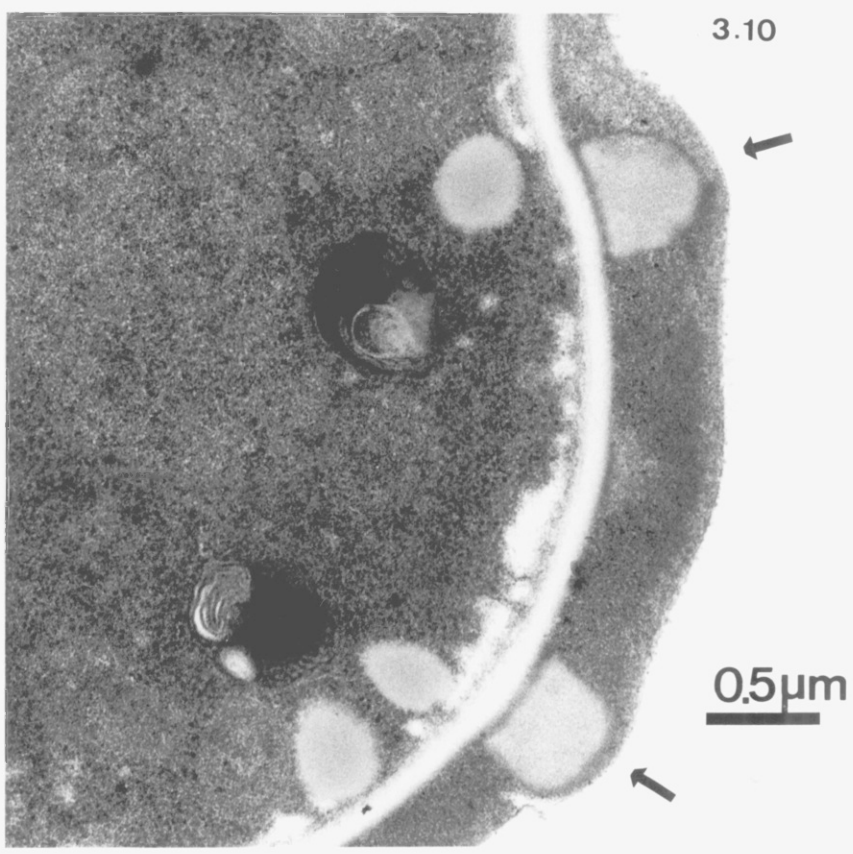




Figs 3.7-3.10. Transmission electron microscopy of conidia of *H. cryptosclerotium*.

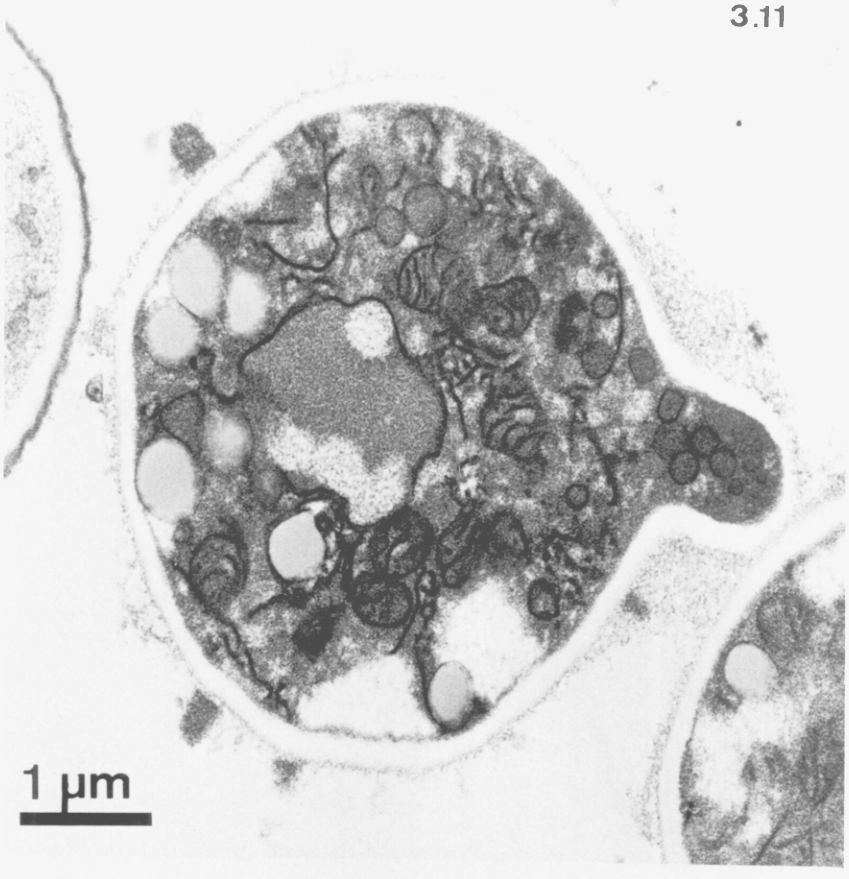
Figs 3.7-3.8. Spores treated with $KMnO_4$, showing outer layer of free, granular mucilage and darker mucilage dense areas (arrows).



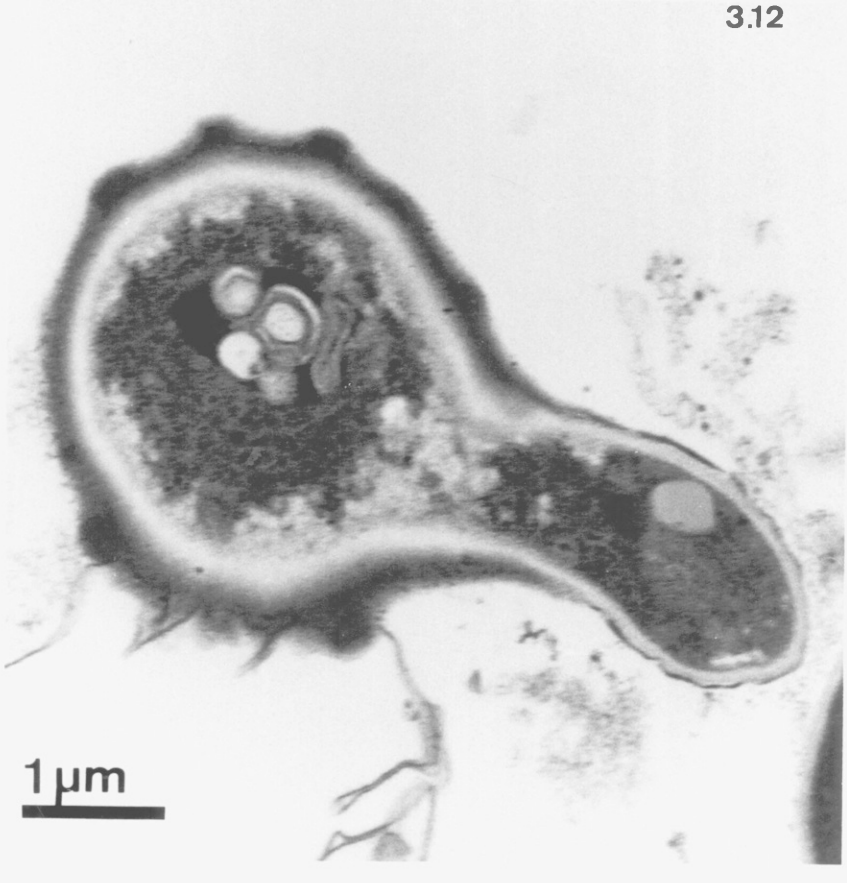


Figs 3.9-3.10. Close-ups of conidial wall to show origin of the "warts" (arrows) which can be distinguished from the free mucilage by darkly stained or (Fig. 3.9, $KMnO_4$) lightly-stained areas (Fig. 3.10, glutaraldehide).

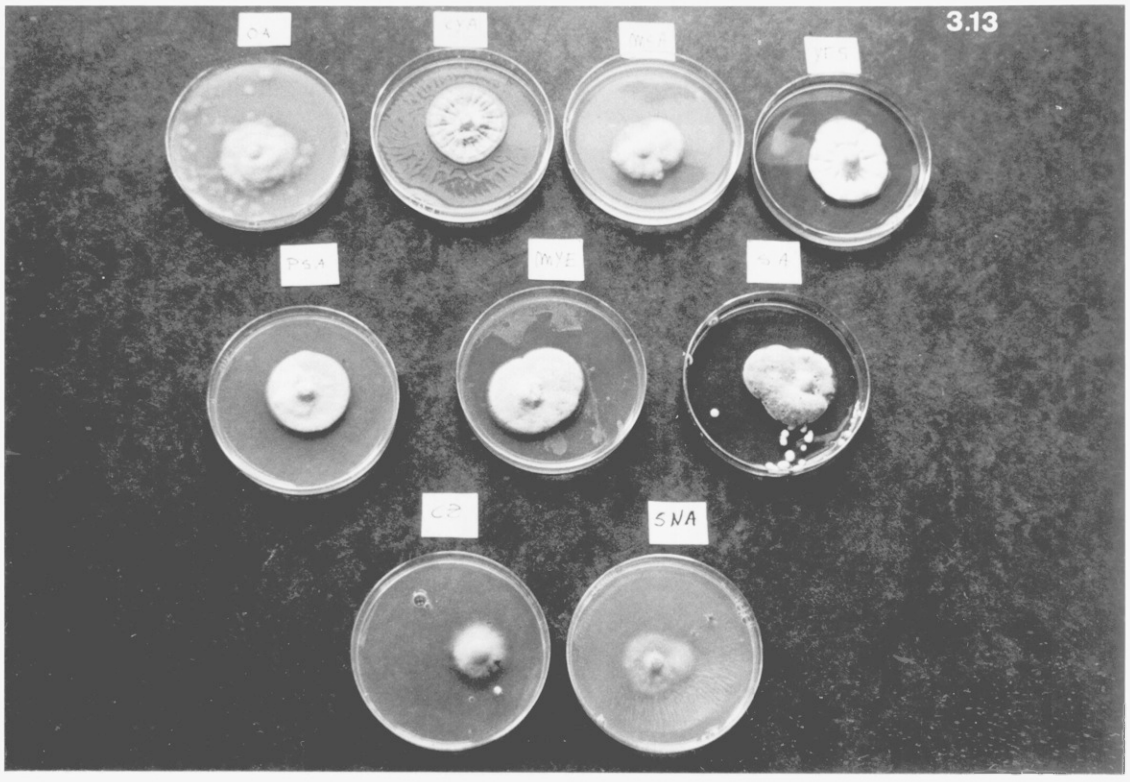
3.11



3.12



Figs 3.11-3.12. Germinating spores with the mucilage layer streching around the germ tube.



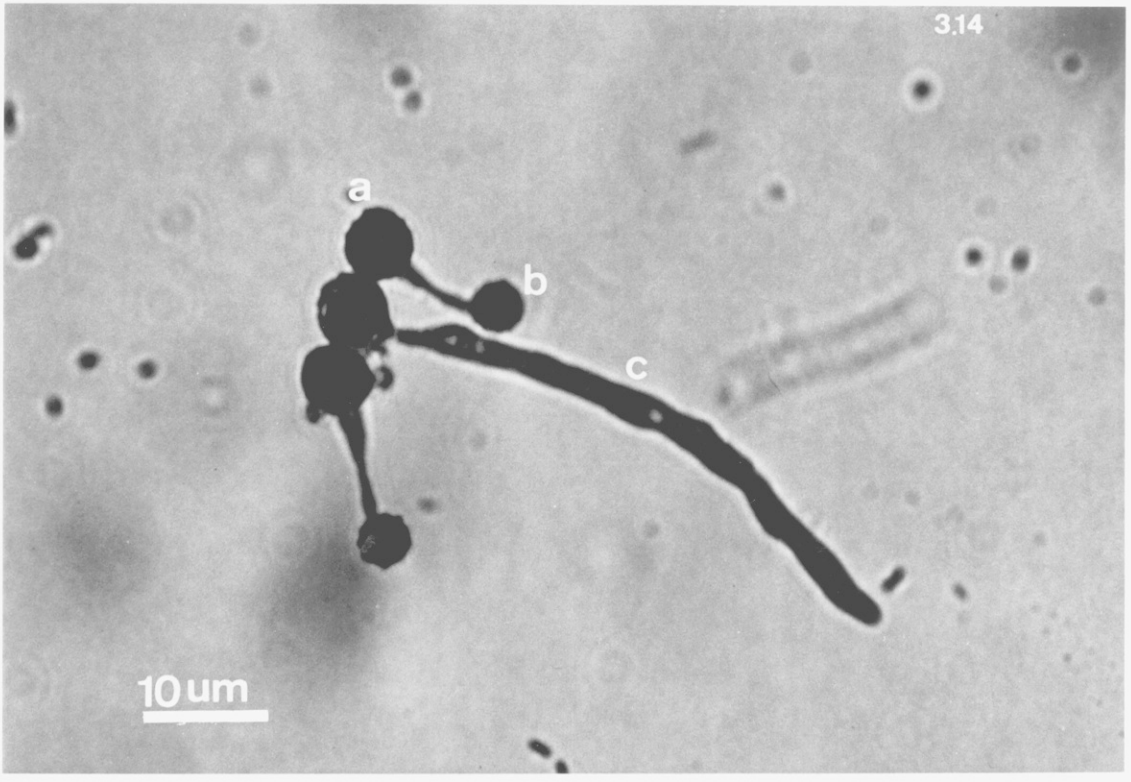
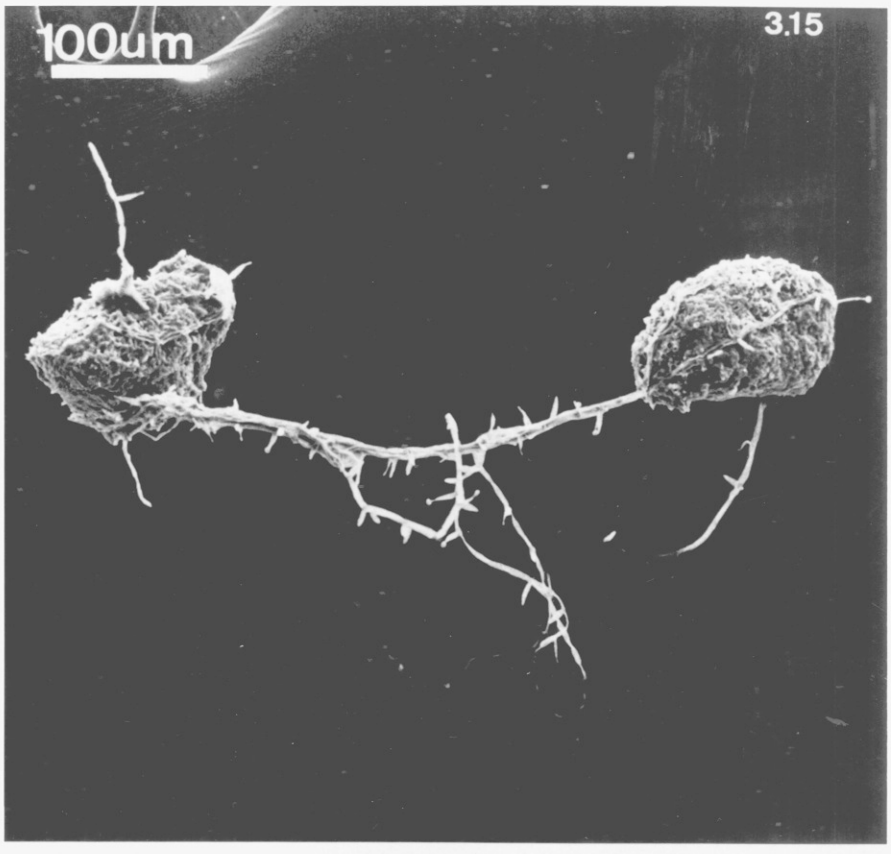
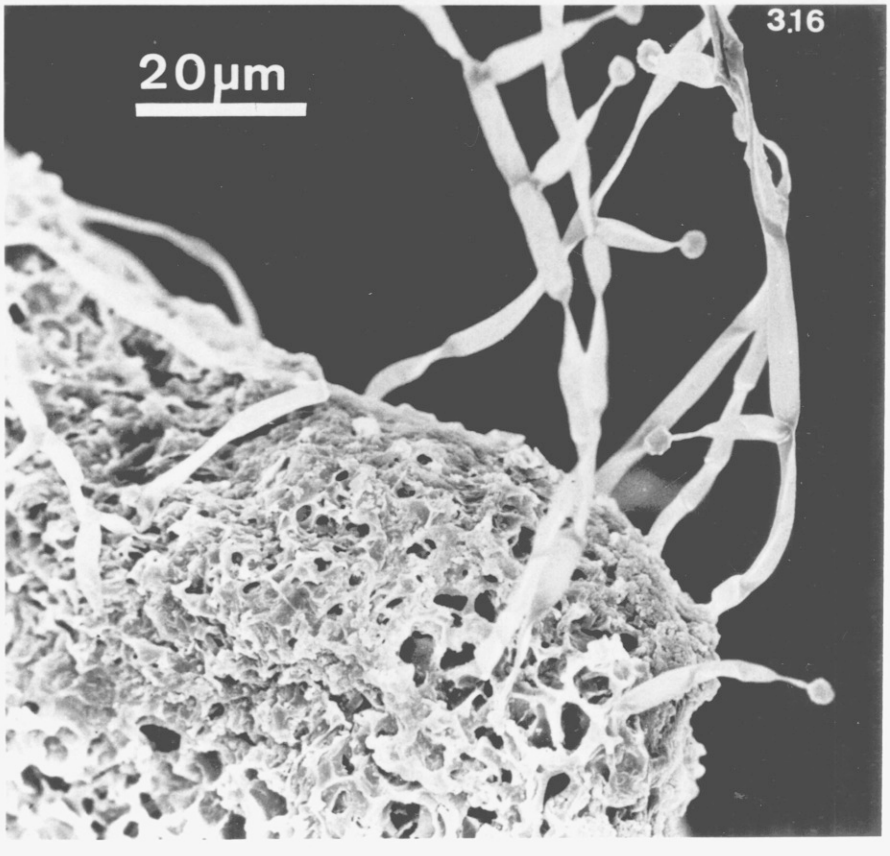


Fig. 3.13. Growth of *H. cryptosclerotium* on different agar media after 21 d at 25°C. On solid media containing high levels of yeast extract (5 g/l or more), colonies are creamish-yellow appressed and convoluted (CYA, YES). Colonies on media with low levels of yeast extract pale gray with a cotton-like appearance.

Fig. 3.14. Microcyclic conidiation on tap water agar: (a) primary conidium, (b) secondary conidium, (c) germinating tube from primary conidium.





Figs 3.15-3.16. Sclerotia of *H. cryptosclerotium* produced in liquid culture. SEM of germinating sclerotia: note irregularly-shaped poorly differentiated sclerotia forming vegetative mycelium with conidiogenous cells.

1977; Evans, 1989). But, in the case of *H. cryptosclerotium*, where the insect host is freely exposed at death in an environment subjected to extended dry periods, the formation of resistant resting structures seems to have received priority.

Field-collected, infected mealybugs are usually grossly swollen, hard to the touch, and initially brownish orange in colour becoming black with age. As the infected insect becomes rigid, the body arches away from the substrate; this attitude being a characteristic symptom. Internally the entire contents are replaced by sclerotia, which only become exposed when the host wall is ruptured (see Chapter IV, Fig. 4.14-4.15). It is thought that these propagules are dispersed by wind or gravity. Under conditions of high humidity, the sclerotia germinate forming vegetative hyphae, conidiogenous cells and conidia (Figs 3.15-3.16). The latter probably reach new hosts following contact or rain splash, and once within the mealybug colony it is likely that further spread occurs as the crawlers migrate within and between host plants. This has been simulated in the laboratory where mealybugs were allowed to wander over sporulating cultures and subsequently incubated at high humidity (>95%) for several days. Death occurred within three days and sporulation was observed on the host after a further two days at 25°C. Organized sclerotia have not been found within laboratory-inoculated insects, possibly due to the stable environmental conditions which favour the formation of conidia rather than resting spores.

H. cryptosclerotium resembles *H. thompsonii*, a pathogen of phytophagous mites (Samson *et al.*, 1980; McCoy, 1981; Latgé *et al.*, 1988) and *H. sphaerospora*, recorded only once on larvae of Eriococcidae (Evans & Samson, 1982a), now identified as *Eriococcus papillosus*, a species restricted to the Galapagos Islands (D. Williams, personal communication). SEM and TEM comparisons clearly show that the mucous or gelatinous sheath covering the conidia of both *H. cryptosclerotium* and *H. thompsonii* is morphologically identical (Figs 3.7-3.8), and there are indications, from the limited herbarium material available, that the older conidia of *H. sphaerospora* sometimes

develop a similar outer gelatinous layer (H. C. Evans, unpublished). The micrographs revealed that the "warts" are in fact no more than condensed areas of mucilage and are not true extensions of the cell wall (Figs 3.9-3.10). Whether or not this condition can be accurately described as verrucose remains in doubt. A further similarity between *H. cryptosclerotium* and the two species mentioned above is suggested by the occurrence of compact hyphal bodies or chlamydospores within the host, although distinct sclerotia have not been found. The following key is proposed to separate these closely related *Hirsutella* spp.:

(1)	Host body containing hard, irregular to subglobose sclerotia. Apex of conidiogenous cells typically inflated (Fig. 3.4), on Pseudoccocidae	*H. cryptosclerotium*
(1)'	Host body occasionally containing irregular to globose chlamydospores, sclerotia absent	(2)
(2)	Base of conidiogenous cells 12-22 μm long, conidia predominantly smooth becoming verrucose, on Eriococcidae	*H. sphaerospora*
(2)'	Base of conidiogenous cells 5-17 μm long, conidia verrucose, on phytophagous mites (mainly Eriophyidae)	*H. thompsonii*

There are many similarities between *H. sphaerospora* and the mealybug pathogen which prompted a re-examination of the type of *H. sphaerospora*. However, the presence of internal sclerotia, so characteristic of the *Rastrococcus* pathogen, was not detected. It could be argued that *H. cryptosclerotium* represents a mature form of *H. sphaerospora* in which the chlamydospores have become organized into pseudoparenchymatous structures. Nevertheless, they are considered here to be distinct taxa, based on host and

geographic differences, in addition to morphological criteria, particularly the inflated apices of the conidiogenous cells of *H. cryptosclerotium* and the production of integral sclerotia. Several other *Hirsutella* spp., e.g. *H. jonesii* (Speare, 1920; Evans & Samson, 1982b); *H. subramanianii* (Samson & Evans, 1985), are known to produce sclerotia but sclerotia of these species are always produced externally on the host and are more highly organized than those of *H. cryptosclerotium.*

If, as it appears, *R. invadens* reached West Africa from Asia within the last 10 years, it must be concluded that either the pathogen was originally introduced with its specific host or that it has rapidly switched from an indigenous mealybug host. This has been the case in West Africa with the cassava mealybug, *Phenacoccus manihoti* (a New World species), and the entomophthoralean fungus, *Neozygites fumosa* (Le Rü, 1986). The same pathogen has since been found infecting *Rastrococcus* in Togo (H. C. Evans, unpublished), so it would seem that this is a plurivorous entomopathogen, at least within the Pseudococcidae. It remains to be established whether *H. cryptosclerotium* is restricted to *Rastrococcus* or has a wider host range than the results of the preliminary pathogenicity tests suggest (App. 3.1).

Field trials had been planned in Togo to assess the effectiveness of *H. cryptosclerotium* against *R. invadens*, using a mycelial preparation. However, the hymenopteran parasitoid, introduced into Togo in 1987, has proved to be exerting such a high degree of control of the mealybug pest (Moore, 1988; D. Moore, personal communication), that these plans have been suspended.

Chapter IV

The infection of *Rastrococcus invadens* by *Hirsutella cryptosclerotium*

4.1 Introduction

Many fungal species have been recorded from a variety of dead terrestrial insects (Madelin, 1963, 1966; Ferron, 1978), but for most of them there is no experimental evidence to determine whether they are parasitic or saprophytic. Of the relatively few fungi that have been shown to be primary pathogens, detailed information on their pathogenesis is available only for the most important species in the genera *Metarhizium, Beauveria, Nomuraea,* and *Entomophthora* (Zacharuk, 1971; Brobyn & Wilding, 1977; Pekrul & Grula, 1979). The morphology of the infection process of insects by fungi has been described by Ferron (1978), Roberts & Humber (1981) and Zacharuk (1981). Recently, Charnley (1984) and Samson *et al.* (1988) have reviewed the physiological and biochemical mechanisms determining the susceptibility or resistance of an arthropod to a potentially active fungus.

In general, disease caused by entomogenous fungi is induced as follows. The infective unit in most fungi is a spore and disease is usually established when it germinates and the germ tube penetrates the insect cuticle. Invasion through the respiratory and alimentary tracts has been reported (Yendol & Pascke, 1965), but these are relatively rare invasion sites.

The attachment of the conidium to the insect cuticle is the prerequisite for the establishment of a mycosis. Once host contact is made, the nature of the spore cell wall and/or its extracellular materials are of great importance in ensuring successful attachment and subsequent infection. Many of the Entomophthorales produce spores in an amorphous mucus, and slimy conidia are commonly formed by anamorphs of the Clavicipitales, providing an adaptation for adhering to waxy epicuticle. The nature of

this mucilaginous matrix is unknown in the entomopathogenic fungi. Similar matrices found in phytopathogenic fungi are of primary importance in the adhesion process. These matrices protect the spores from desiccation and from potentially toxic substances present on the host (Nicholson & Moraes, 1980). Dry spores, such as most hyphomycete conidia, appear to be unsuited for adhesion to waxy epicuticle. However, the dry spores of some hyphomycetes are apparently covered by interwoven fascicles of rodlets which may play a key role in binding the spore to the cuticle (Evans, 1989). To some extent these types of attachment might be considered "passive" in the sense that they result from the configuration of the spore wall. However, other more "active" processes may follow as there are interactions at the interface between the pregerminating fungal propagule and insect cuticle. These can involve surface antibodies, carbohydrate-binding proteins, glycoproteins, sugars, mucilaginous substances from germinating conidia and other less well defined mechanisms (Fargues, 1984; Boucias & Latgé, 1986; Samson *et al.*, 1988).

Following spore attachment, the germination and growth of the fungus on the cuticular surface leads to the production of penetration pegs. Cuticular invasion involves both enzymatic and physical activities (Roberts & Humber, 1981). The germ tube or penetrating hypha forms appressorial-like structures - swellings at hyphal apices formed on the cessation of germ tube elongation (Hawksworth *et al.*, 1983) - or penetrate directly through the cuticle. Germinating spores of several entomopathogenic species, such as *Metarhizium anisopliae*, *Neozygites fresenii* and *Beauveria bassiana*, produce an appressorial cell at the germ tube-epicuticle interface. Some fungi appear to require appressoria only under certain conditions. *B. bassiana* produces appressorial-like structures during infection of *Leptinotarsa decemlineata* (Vey & Fargues, 1977); however infection of *Heliothis zea* by this fungus occurs as a result of direct penetration of the integument of the growing hyphae (Pekrul & Grula, 1979). In the light of such variation, Emmett & Parbery (1975) considered that it may be useful to adopt a broader

definition of the term appressorium: a germ tube or hyphal tip, without regard to morphology, which has the ability to adhere to a host surface and ability to grow and penetrate the host. Samson *et al.* (1988) reported that mucilaginous material can be observed around germ tubes of conidia of some species which do not produce appressoria. They also suggested that this material may be responsible for the attachment of the penetrating hypha to the cuticle.

Initial growth of the fungus may be localized around the epidermis at the point of entry but finally the fungus proceeds to invade the rest of the insect. On entering the haemocoel penetrant hyphae of certain entomopathogenic fungi continue filamentous development, e.g., *Entomophthora coronata* in the termite, *Reticulitermes flavipes*. However, most entomopathogenic fungi grow in the haemocoel as yeast-like cells. These are generally ovoid or truncated hyphal segments produced from the initial penetrant hyphae which proliferate further by division (Zacharuk, 1971; Charnley, 1984). A further specialized case is the production of wall-less protoplasts, e.g., by *Entomophthora egressa* in the haemocoel of *Choristoneura fumiferana* (Charnley, 1984).

The growth of the fungus eventually leads to the death of the insect though it is often difficult to define the exact point of death of an insect. It may be preceded by behavioural changes such as tremors, loss of coordination, or climbing to an elevated point (Charnley, 1984; Evans, 1989).

Because of the replacement of internal organs with mycelia, the insect immediately after death is very nearly normal in appearance. These fungus-filled insects, called "mummies", can serve as reservoirs of the fungus through periods of adverse environmental conditions (Roberts & Humber, 1981). A better understanding of pathogen reservoirs may lead to a more effective use of pathogens as biological control agents. Hochberg (1989) has recently shown that host populations may be regulated to low and relatively constant densities if sufficient numbers of pathogens are translocated

from pathogen reservoirs to habitats where transmission can occur. When the insect is totally colonized by fungal hyphae, fructifications are then produced which erupt through the cuticle and produce spores on the outside of the insect. With most Deuteromycotina, conidiophores and conidia are not produced unless the insect is in a moist environment (Roberts & Yendol, 1971).

The development of fungal infection in arthropods is closely connected with the dose of fungal spores, the physiological stage of the host, and abiotic conditions such as temperature, humidity and light (Ferron, 1978).

This chapter presents information on the various stages in the infection of *R. invadens* by *H. cryptosclerotium*.

4.2 Materials and methods

4.2.1 Inoculation procedure

Third instar nymphs and female adults were allowed to crawl on 4-week old sporulating cultures of the fungus in dishes of oatmeal agar. Adequate inoculum uptake was ensured by leaving the insects for at least 10 min.

4.2.2 Histological preparations

Insects were suspended in oil of cloves for 1 min in order to remove the wax layer from the surface. Then they were rinsed in water and stained for 1 min in 0.03% Trypan blue in lactophenol. The specimens were examined under the light microscope.

4.2.3 Scanning electron microscopy

Techniques for preparing the fungal material were the same as used by O'Donnell & Hooper (1974). The insects were immersed in 0.2 *M* phosphate-buffered 3% glutaraldehyde at pH 7.2 for 5 h. This was followed by a wash in the phosphate

buffer for 30 min. The buffer-washed material was then dehydrated with ethanol at 0-4°C using the following series: 10, 20, 30, 40, 50, 60, 70, 80, 90% ethanol, 5 min at each step; 100% twice , 10 min each time. The specimens were then dried in a Polaron E3000 critical-point dryer using CO_2 as the carrier gas. The critical-point-dried specimens were coated with gold in a Polaron E5000 sputter coater and stored in a desiccator. They were observed in a Philips 500 SEM operated at 25 kV. Images were recorded on 35 mm Ilford Pan F film Asa 50.

4.3 Results

The infection process and the development of the disease have been divided into different stages according to Roberts & Humber (1981). The infection of *R. invadens* by *H. cryptosclerotium* is presented below following this classification.

4.3.1 Attachment of the conidium to the cuticle

Conidia adhered anywhere on the host cuticle. Transmission electron micrographs of *H. cryptosclerotium* spores showed the presence of a mucoid sheath, which presumably aided attachment to insect cuticle (see Figs 3.7-3.8). Usually the mucoid sheath remained on the spores when they were suspended in water but was easily removed with oil of cloves (see Fig. 3.6). Whether there is a chemical and\or physical interaction between spore and host cuticle resulting in adhesion is still unclear.

4.3.2 Germination of spores on the host integument

Conidia of *H. cryptosclerotium* were able to germinate on all regions of the cuticle. Penetrating hyphae of conidia germinating on legs and antennae varied noticeably in length. Germ tubes of conidia germinating on both dorsal and ventral

surfaces presented a shorter and more constant length with an average of 45 µm (Figs 4.1-4.2). Secondary conidial production from primary conidia was never observed on the cuticle.

4.3.3 Penetration of the cuticle

H. cryptosclerotium appeared to enter *R. invadens* directly without the aid of any special visible structure (Figs 4.3-4.4). A circular hole could be seen at the point of penetration of the cuticle (Figs 4.1-4.2). Amorphous mucilaginous material was often present around germ tubes (see Fig 3.11-3.12). A dark patch was observed on the cuticle surrounding the penetrant hypha (Figs 4.1-4.2).

4.3.4 Growth of the fungus in the haemocoel

Yeast-like cells were observed in the haemocoel of female adults 72 h after inoculation with *H. cryptosclerotium* at 25°C (Fig. 4.5). Later, these cells formed proliferating mycelium throughout the haemocoel (Fig. 4.6).

4.3.5 Death of the host

Behavioural changes of the host have not been observed during the infection process of *R. invadens* by *H. cryptosclerotium*. However, symptoms of infection such as sluggishness and paralysis as well as a decrease in honeydew production may indicate the time of cessation of host metabolism. Death of *R. invadens* (insects were considered to be dead when they completely paralysed) was observed 60 h after inoculation at 25°C.

4.3.6 Mycelial growth

Growth of *H. cryptosclerotium* occurs in the mycelial phase with invasion of virtually all organs of the host (Fig. 4.7). When *R. invadens* is totally colonized by

H. cryptosclerotium nymphs and female adults are recognized by their darker colour (Fig. 4.8). In adverse field conditions black-coloured sclerotia are produced at this stage inside the host (Figs 4.14-15).

4.3.7 Protrusion of hyphae from the interior through the cuticle to the exterior of the insect

If the mummy is held under conditions of low relative humidity *H. cryptosclerotium* will remain within the cadaver, but in a humid environment the mycelium will protrude (Fig. 4.9). A study of mummified mealybugs revealed that hyphae emerged indiscriminately through the host surface without major damage of the cuticle around the point of exit (Fig. 4.10-4.11).

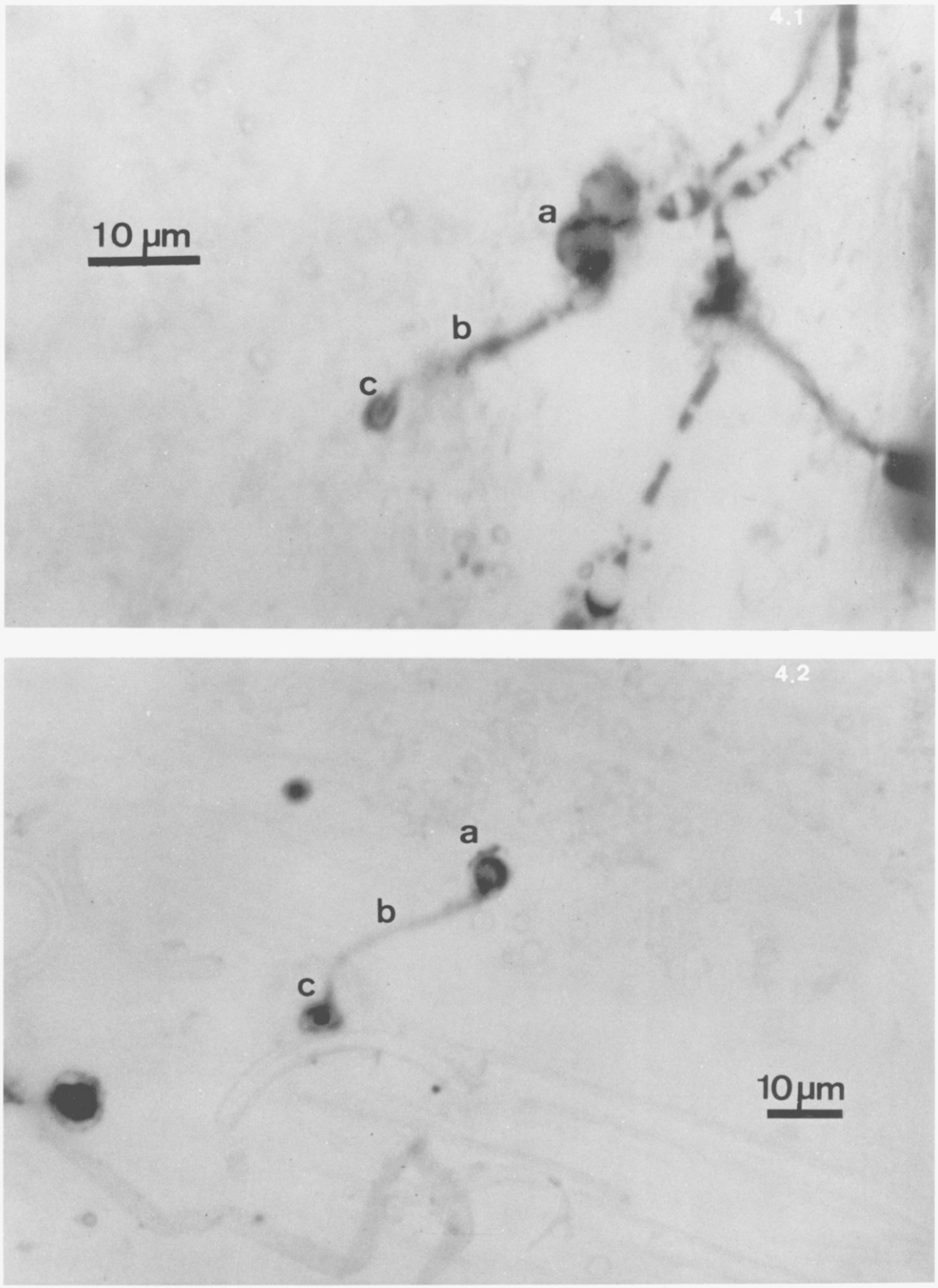
4.3.8 Production of infective units on the exterior of the host

Conidia were produced from phialides formed laterally on the mycelium covering the exterior of the host (Figs 4.12-4.13). Phialides were produced soon after emergence.

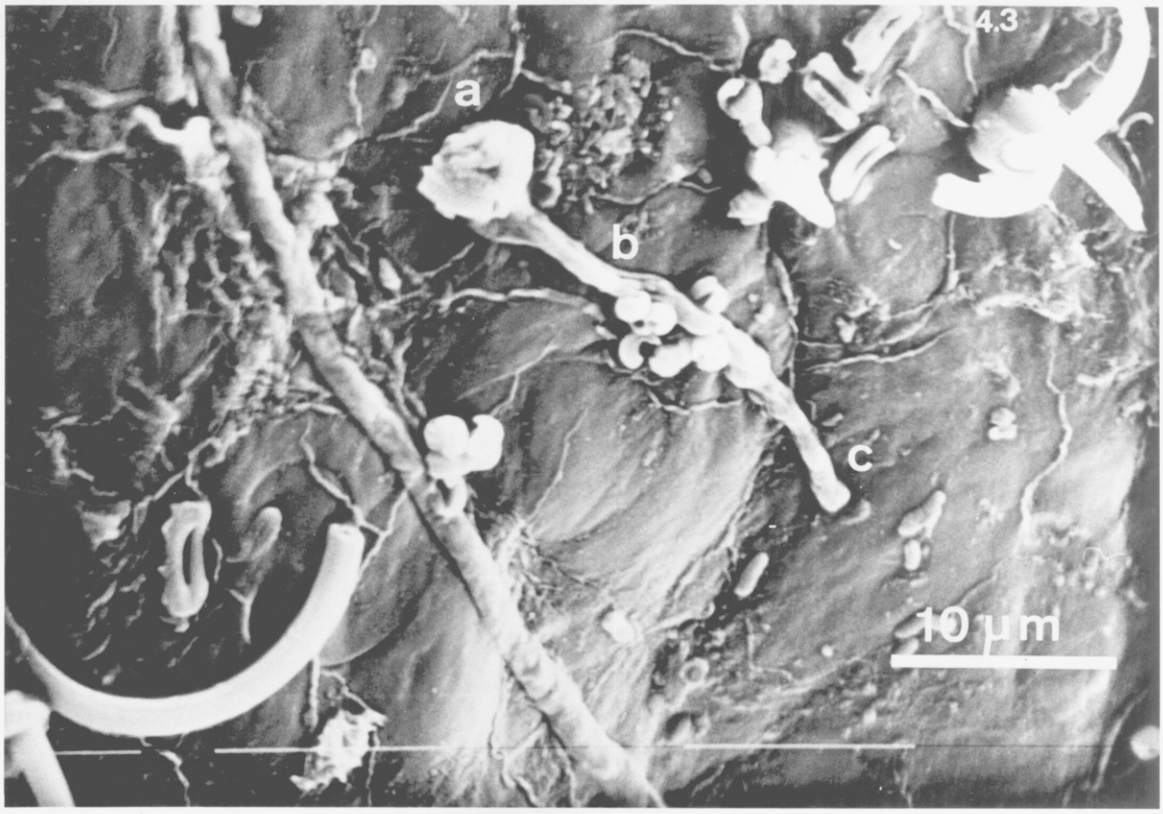
4.3.9 Dispersal

The final stage in the disease development cycle is the dispersal of the infective units to locations where they are likely to encounter susceptible hosts for the initiation of new cases of disease. This aspect of the infection process is studied in detail in Chapter V.

Fig. 4.16 summarizes the sequence of the main stages in the development of *H. cryptosclerotium* on *R. invadens* at 25°C, >95% relative humidity in 16 h light/8 h dark.



Figs 4.1-4.2. Germinating conidia of *H. cryptosclerotium* on the cuticle of female adults; (a) conidium, (b) germ tube, (c) site of penetration on the integument. Note a dark patch on the cuticle surrounding the penetrant hypha.



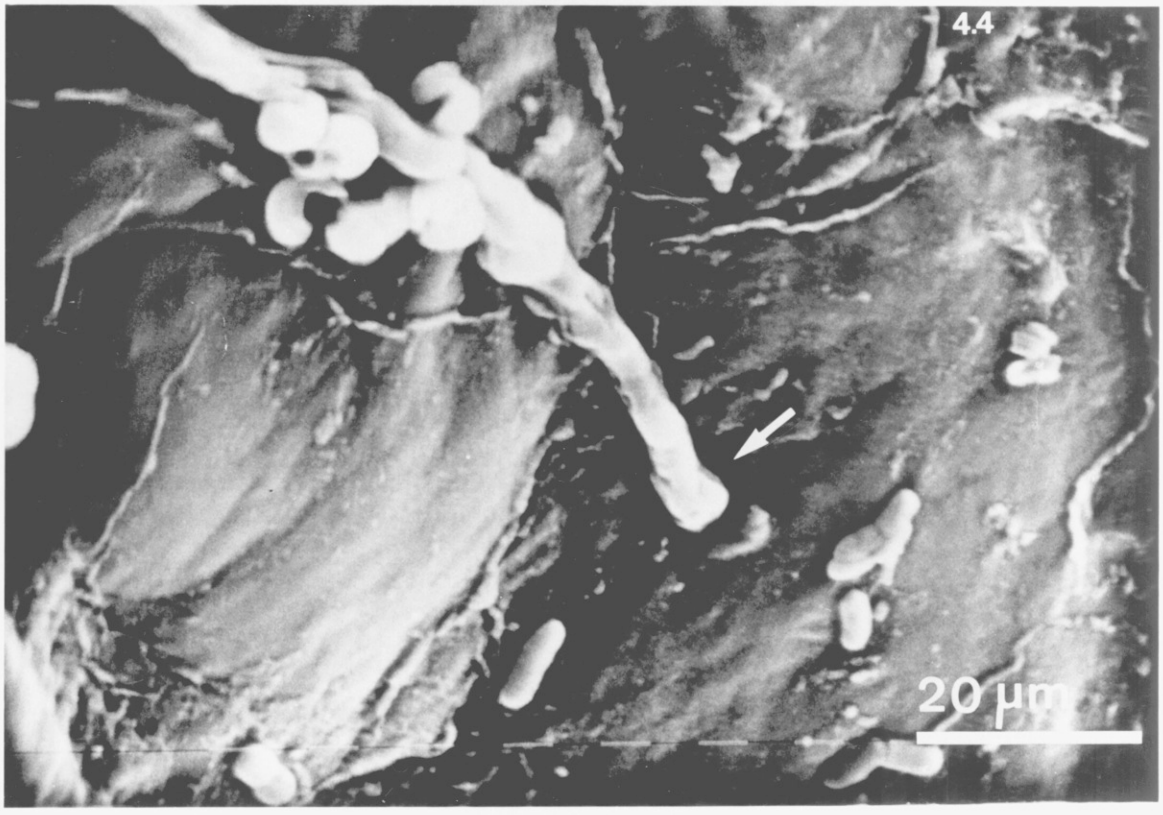
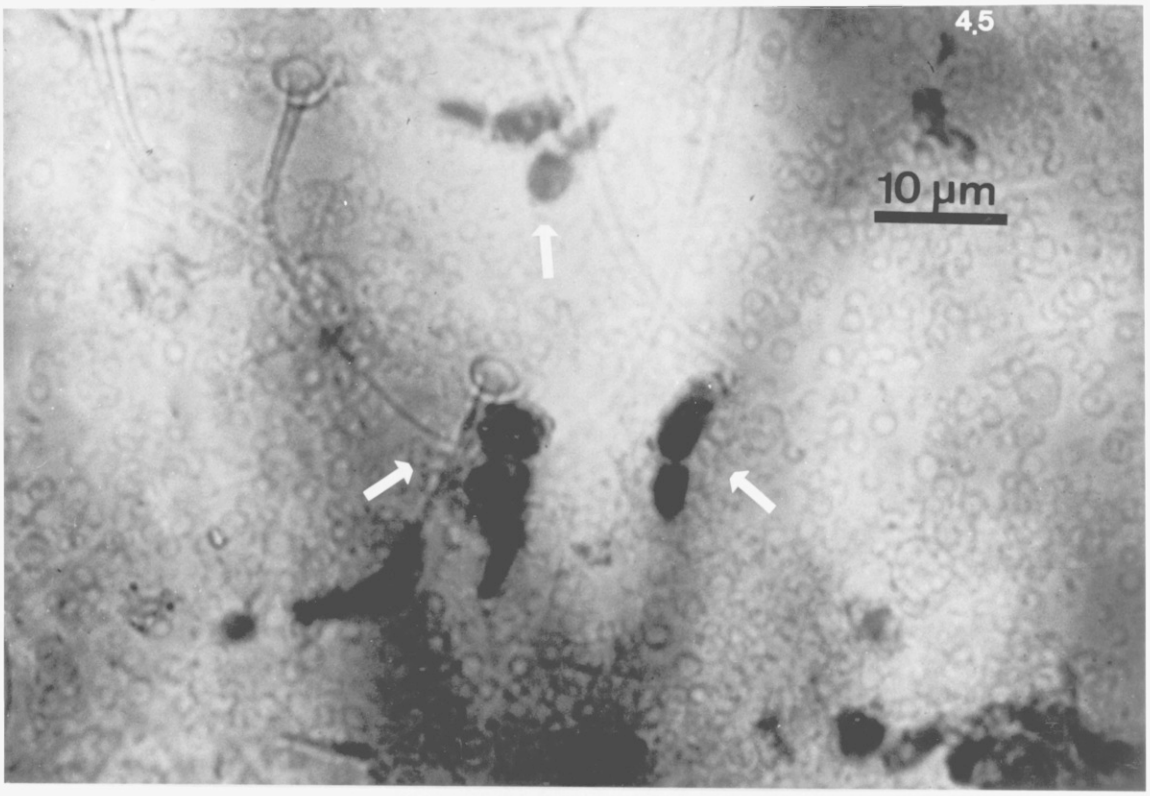


Fig. 4.3. Scanning electron micrograph of a germinating conidium; (a) conidium, (b) germ tube, (c) penetration site. Fig. 4.4. Close-up of germ tube showing no evidence of appressorium at the point of penetration (arrow).



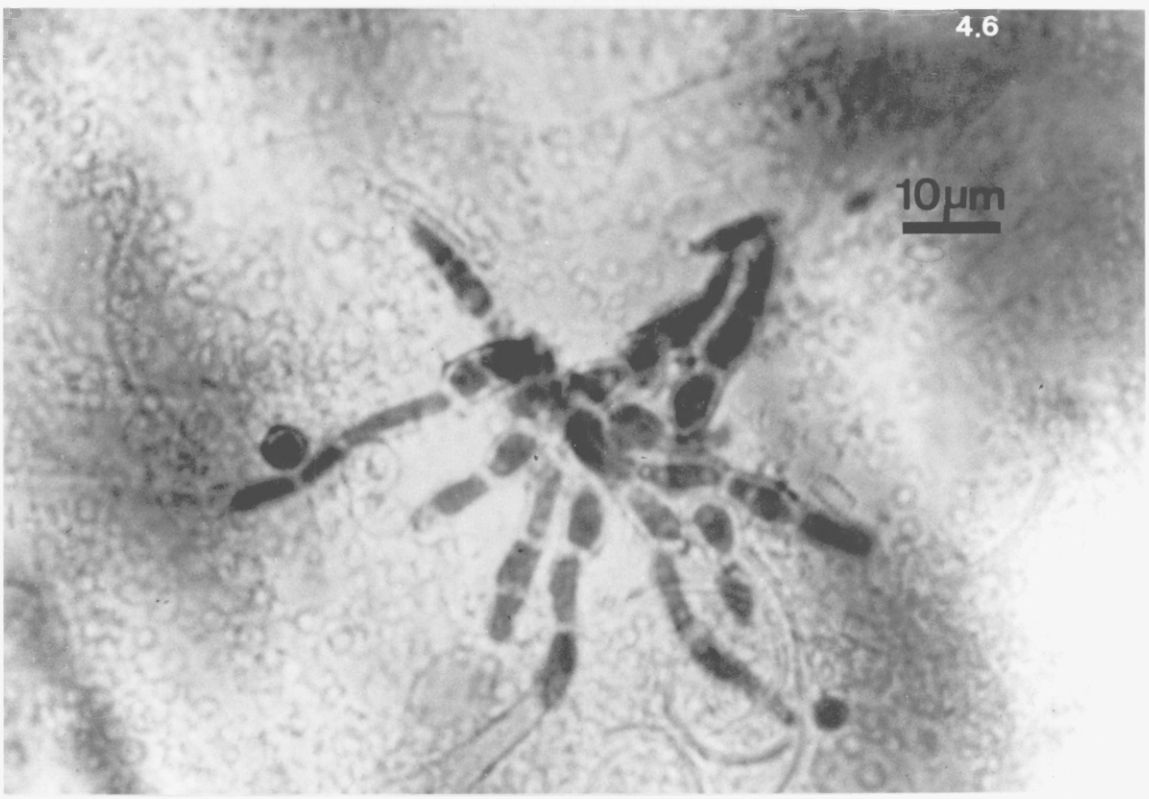


Fig. 4.5. Yeast-like cells of *H. cryptosclerotium* (arrows) in the haemocoel of a third instar nymph, 60 h after inoculation at 25°C.
Fig. 4.6. Proliferating mycelium in the haemocoel from yeast-like cells, 96 h after inoculation at 25°C.

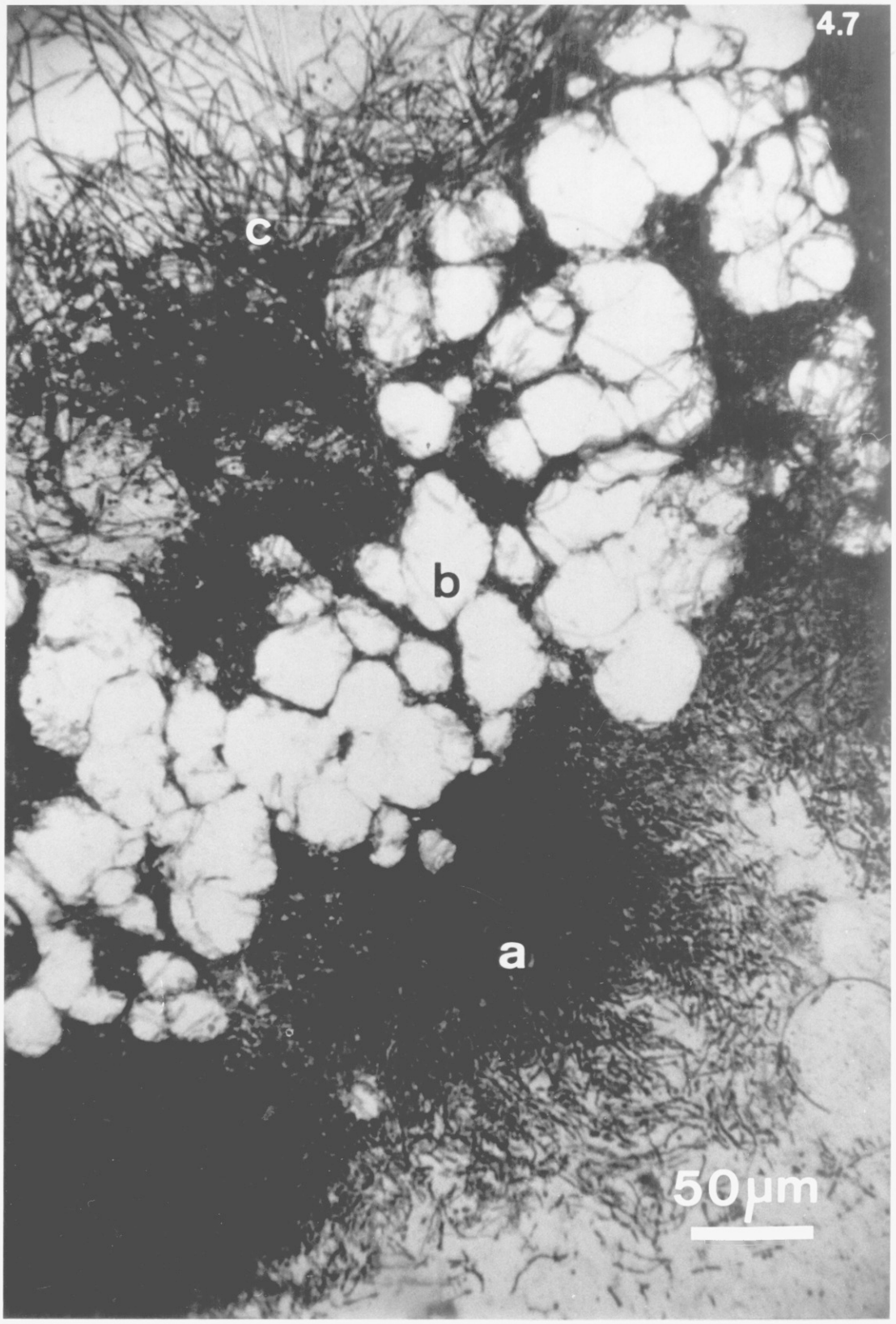
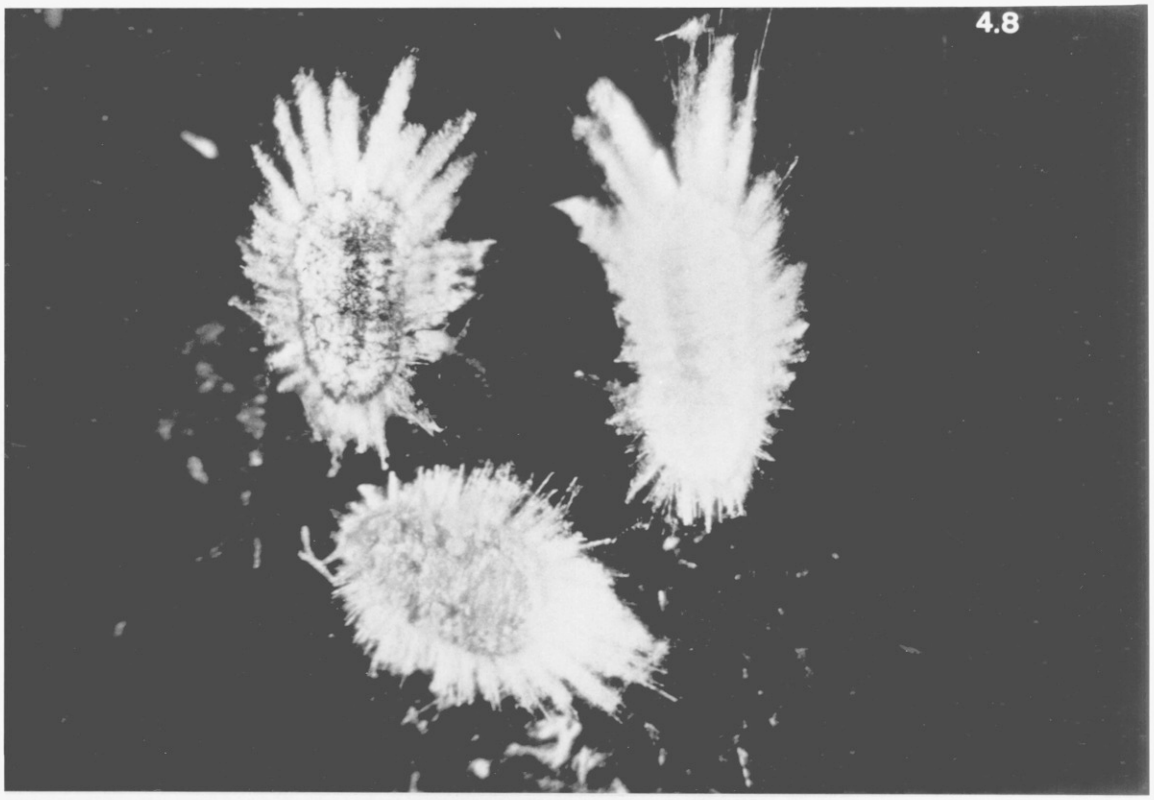


Fig. 4.7. Cross-section of a infected mealybug, 96 h after inoculation, showing: (a) proliferation of yeast-like cells in the haemolymph; (b) invasion of the fat body; (c) and protrusion of hyphae through the cuticle to form sporulating mycelium on the surface of the insect.



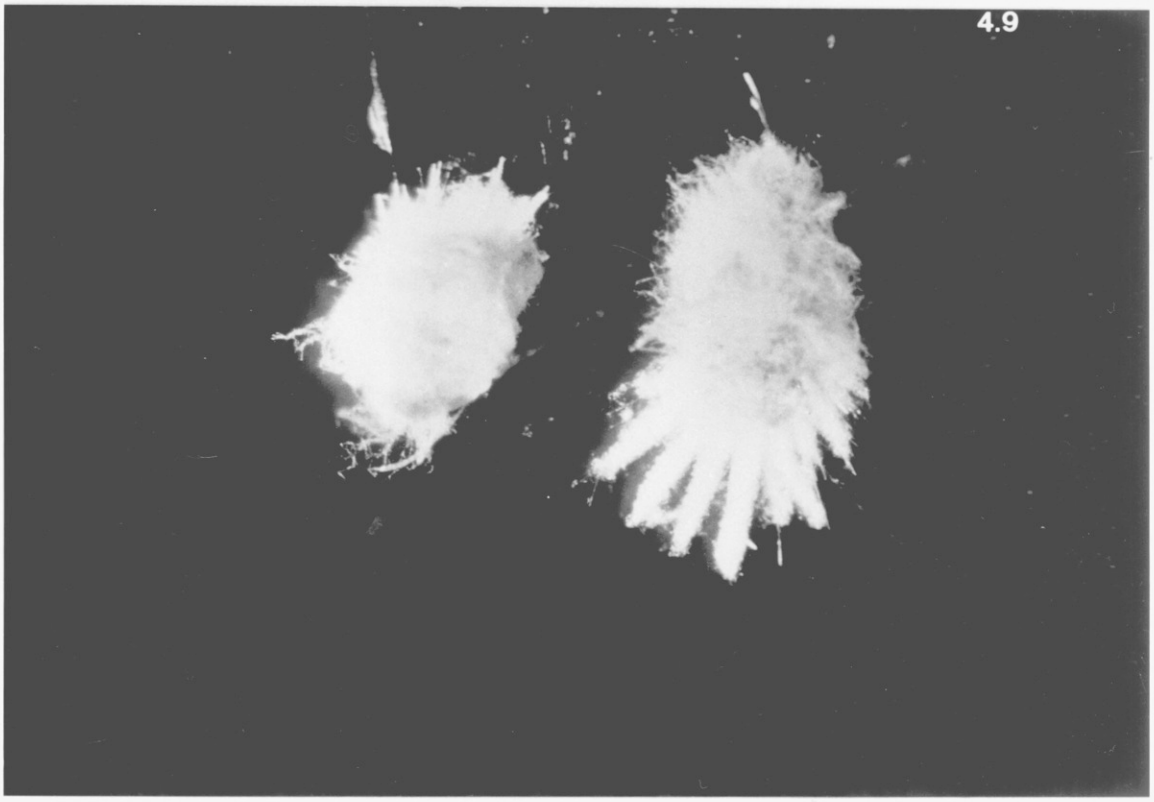
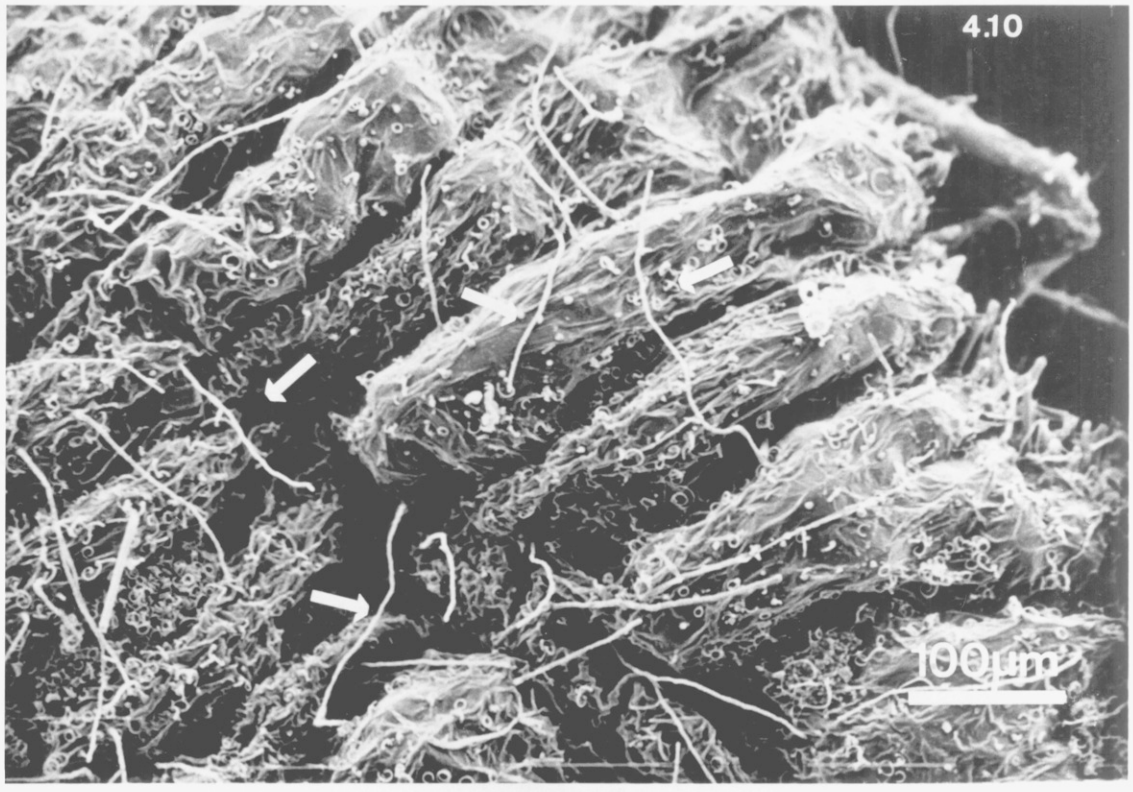


Fig. 4.8. Two infected, swollen and dark female adults 72 h after inoculation at 25°C. Note the white, healthy mealybug on the right (x *ca* 15).

Fig. 4.9. Mealybugs covered with mycelium of *H. cryptosclerotium* after being kept for 96 h in a humid environment after inoculation (x *ca* 15). Note the cotton-like appearance, difficult to observe amongst wax filaments of the hosts.



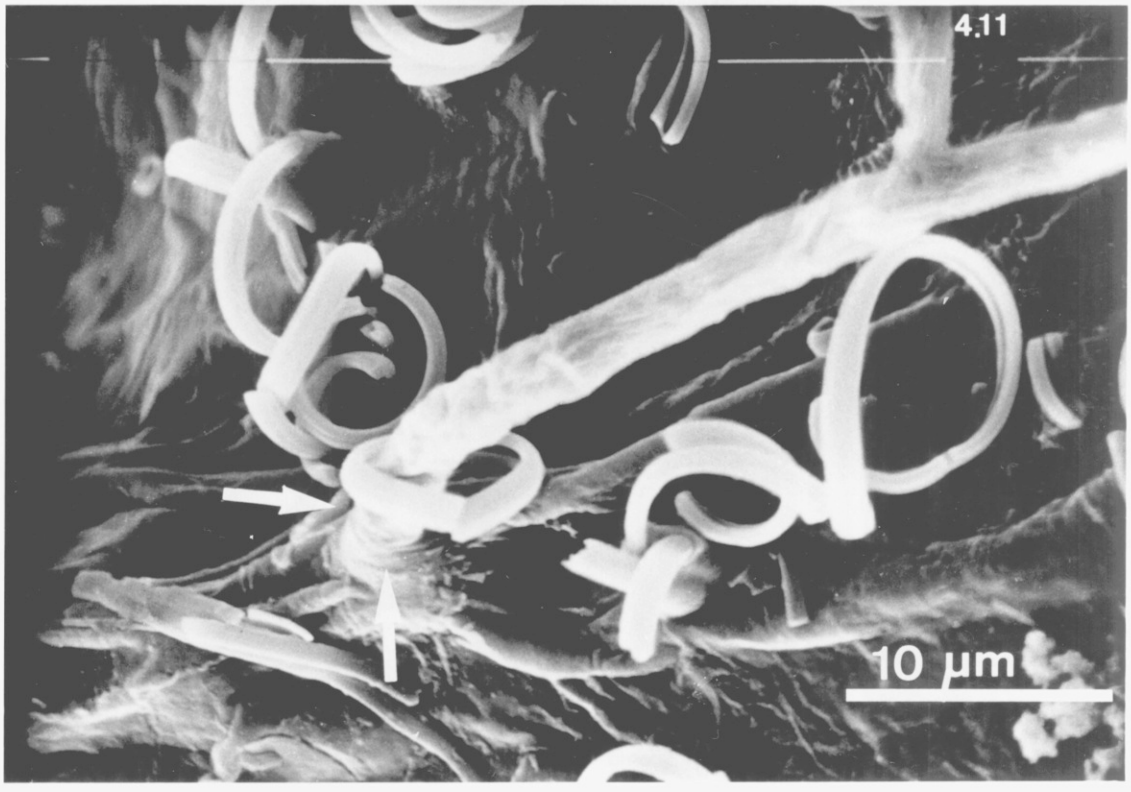
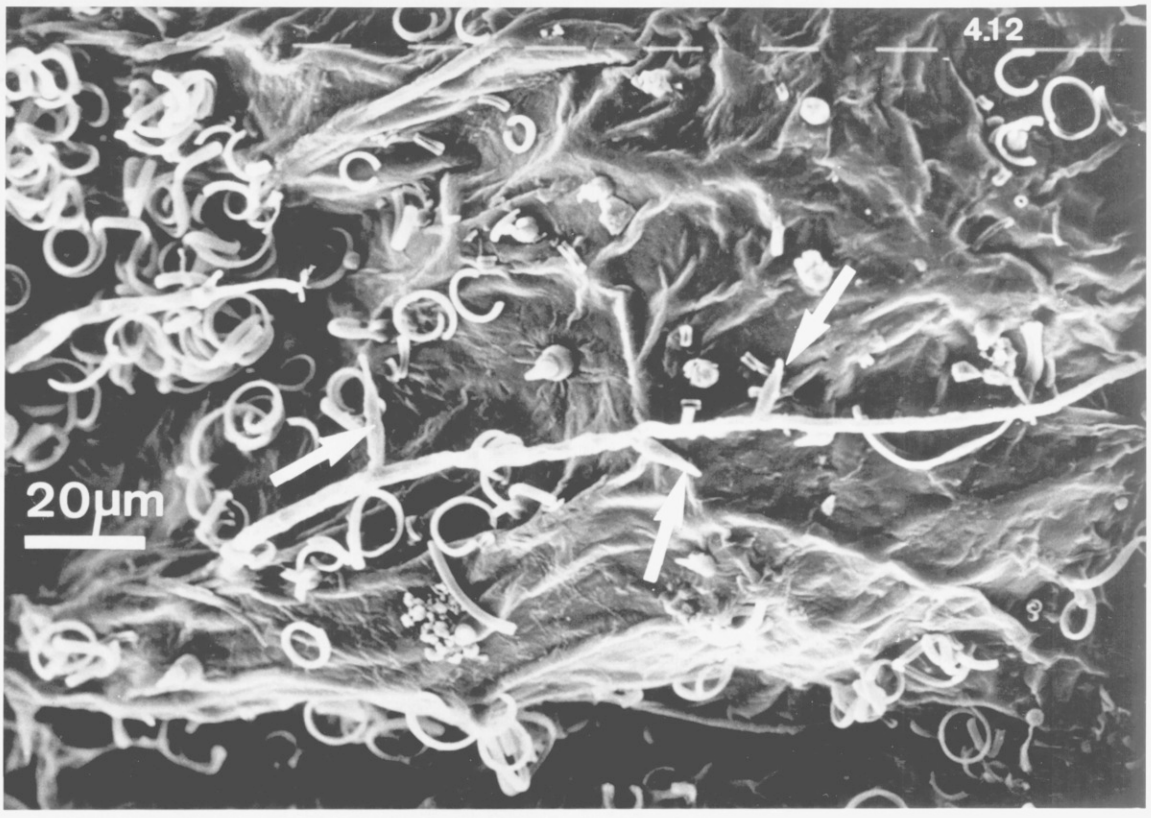
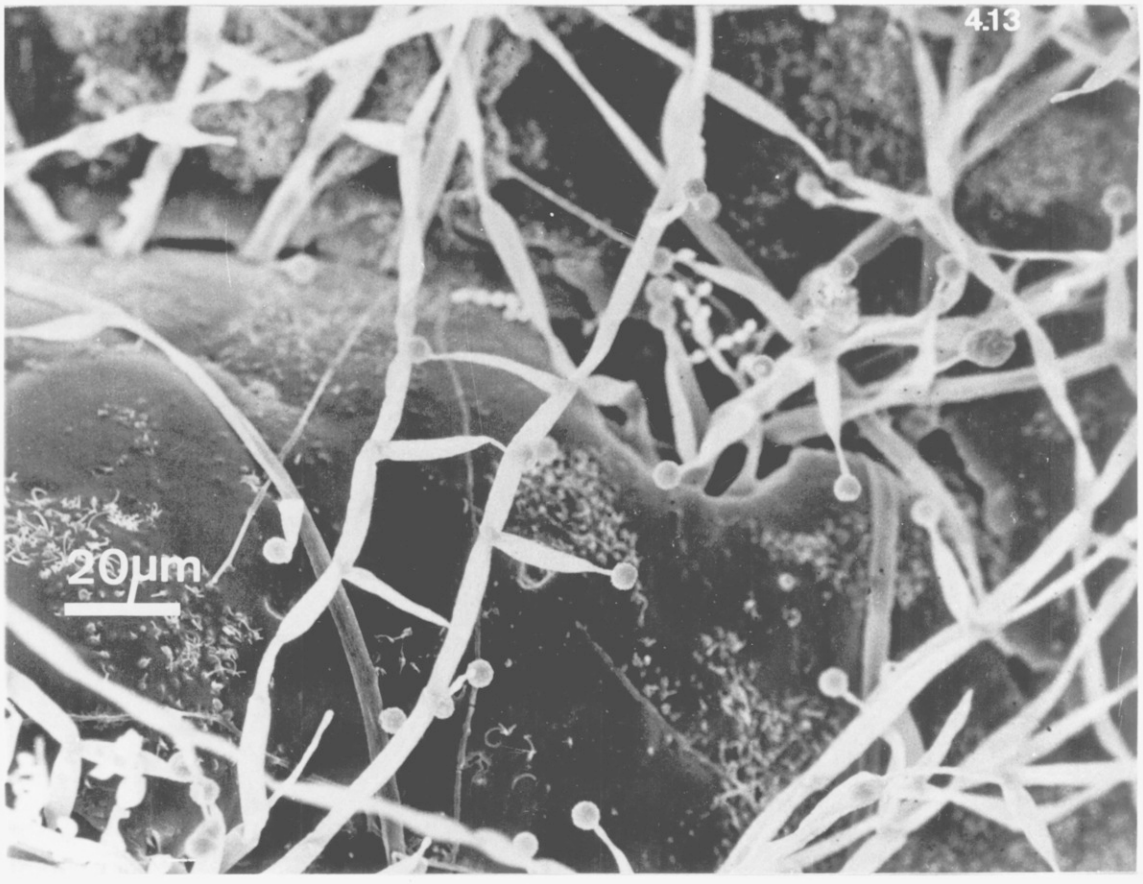
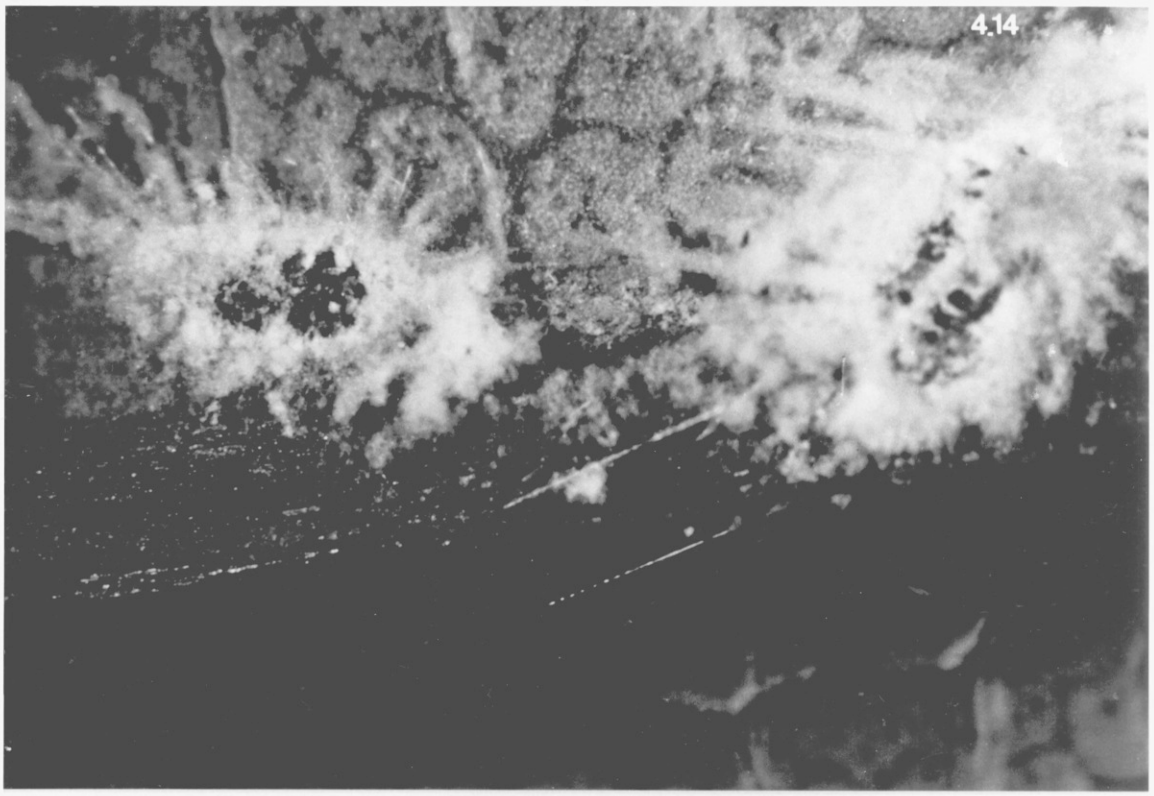


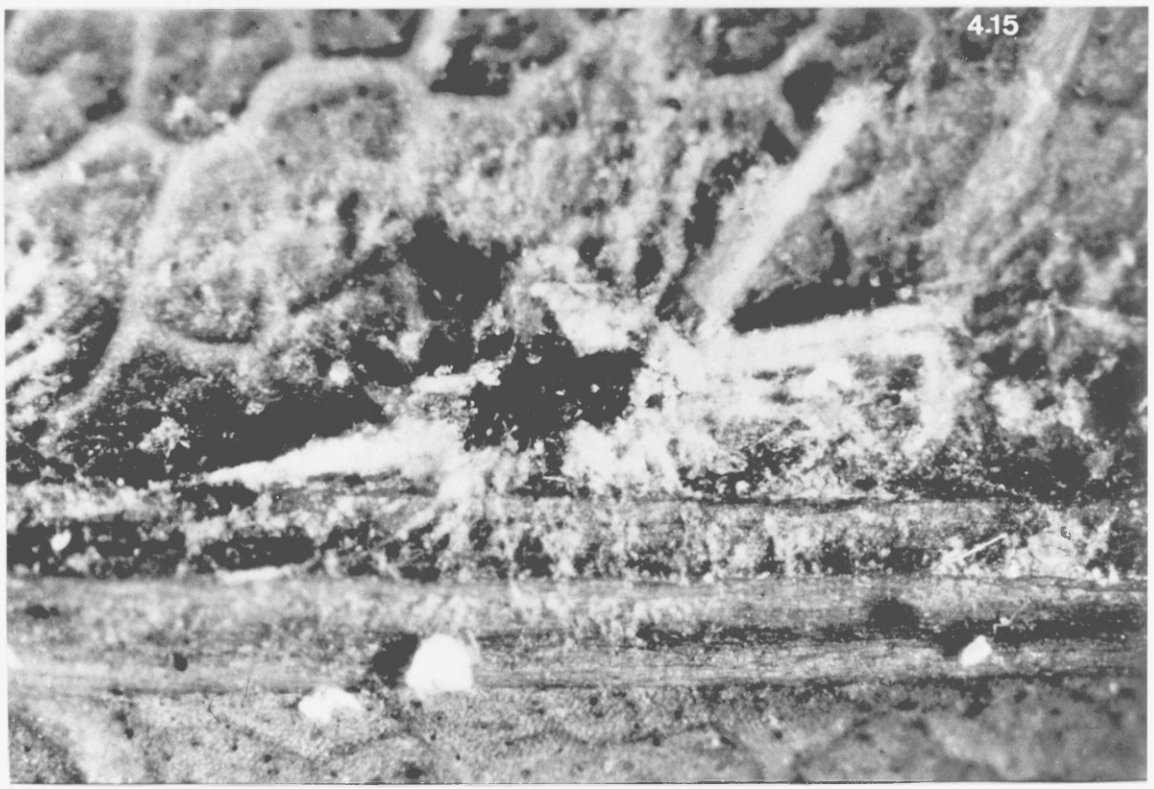
Fig. 4.10. Scanning electron micrograph showing the protrusion of hyphae from the interior through the cuticle to the exterior of the insect (84 h after inoculation) (arrows).
Fig. 4.11. Exit of fungal hypha on a mummified nymph (arrows). Note the lack of cuticle damage around the point of exit.





Figs 4.12-4.13. Production of conidia from phialides formed on the mycelium covering the exterior of the host after emergence through the cuticle (96 h after inoculation). Phialides (arrows in Fig. 4.12) are produced soon after emergence.





Figs 4.14-4.15. Mealybug adults attached to lower leaf surface of mango leaf, Togo. The mummified bodies have broken open to reveal black sclerotia within (Fig. 4.14, x *ca* 10; Fig. 4.15, x *ca* 15).

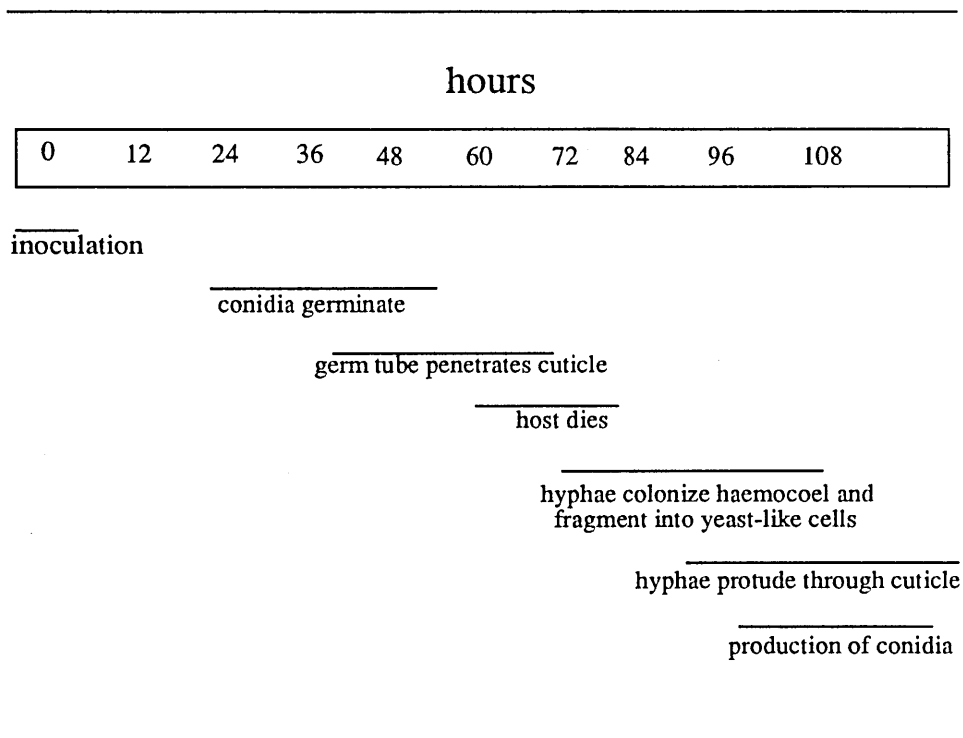


Fig. 4.16. Stages in the colonization of *R. invadens* by *H. cryptosclerotium* at 25°C, >95% relative humidity in 16 h light/8 h dark.

4.4 Discussion

The infection process of *R. invadens* by *H. cryptosclerotium* is rapid, since death of the host takes place within 60-72 h and it only takes 96 h for the development of the infection to be completed to sporulation on the host surface. McCoy (1978) reported similar results on the infection process of *Phyllocoptruta oleivora* by *H. thompsonii*. Spores of *H. thompsonii* penetrated the mite cuticle after 4 h and the infection process was completed after 72 h, the death of the host occurring within 48 h. Another similarity between the two pathogens is that they both overcame their host after limited growth in the haemocoel and without major invasion of the tissues. It seems likely that in both cases the hosts were killed mainly by fungal toxins, however this has not been proved yet for either pathogen. Brobyn & Wilding (1977) studied in detail the infection of the aphid *Acyrthosiphon pisum* by two *Entomophthora* species finding out that the infected aphids died when "most of their tissues were invaded and much of the body cavity was packed with hyphal bodies". The biochemical changes occurring in infected insects have been poorly studied (Domnas, 1981). The extensive fungal development prior to death of some fungus-insect combinations indicates that toxins may not be produced (Roberts & Humber, 1981). Several authors (Roberts, 1981; Domnas, 1981) have suggested that in these cases the main cause of death could be a physiological starvation caused by deprivation of insect metabolites by the pathogen. In those cases where fungi overcome their hosts after limited growth, toxins may play a significant part in host death (Charnley, 1984). Toxins have been claimed to be responsible for the death of numerous insects infected by Deuteromycotina (Ferron, 1981). For most Entomophthorales, however, death seems to occur only when all the tissues of the insect have been substituted by the fungus (Samson *et al.*, 1988).

Abnormal growth of penetrating hyphae of *H. cryptosclerotium* over the surface of *R. invadens* nymphs or female adults was never observed. This may indicate that the

cuticle of both nymphs and female adults has similar chemical and physical characteristics which act as a barrier against fungal attack by this fungus once host contact is achieved. Fransen (1987) noticed abnormal growth of hyphae of *Aschersonia aleyrodis* over the surface of prepupae of whitefly which had been shown to be more resistant than larvae. This errant growth has also been described for other fungi on resistant hosts (Pekrul & Grula, 1979).

Germinating conidia of *H. cryptosclerotium* were able to penetrate anywhere on the mealybug cuticle, however, surface growth was more extensive on legs and antennae. Charnley (1984) reported that surface growth is often most extensive on hard cuticle. Pekrul & Gula (1979) observed that hyphae from *Beauveria bassiana* penetrated the cuticle of non-cephalic regions of *Heliothis zea* soon after germination but not on the head where germination was followed by abnormal hyphal extension with no penetration.

The darkening of *R. invadens* cuticle where it was penetrated by a germinating hypha may imply the release of prophenoloxidases which might be involved in the mealybug defence reactions (Taylor, 1969; Richards, 1978; Götz & Boman, 1985). Melanization is the most common cuticular reaction to fungal penetration (Brobyn & Wilding, 1977; Travland, 1979); this melanization is enough to stop some fungi, but successful pathogens overcome any such reaction and penetrate to the haemocoel.

Further work needs to be carried out to study the biochemical and physiological factors which determine the susceptibility of *R. invadens* to *H. cryptosclerotium*: e.g., enzymatic activity at the germ tube-epicuticle interface and inside the host, sequence of the colonization of the different tissues of *R. invadens*.

Finally it can be concluded that *H. cryptosclerotium* is a primary pathogen causing the death of nymphs and female adults of *R. invadens* by penetration and proliferation inside the host.

Chapter V

Dispersal of *Hirsutella cryptosclerotium* by simulated rain

5.1 Introduction

There are many fungi in which spore liberation is apparently a passive process, being unaided by any special discharge mechanism. In such cases, rain and wind may be important factors influencing liberation (Grace & Collins, 1976). The dispersal of pathogen inoculum may be considered in three stages: removal from the host, transport through the air, and deposition on a new host. Rain and/or wind may be involved in all three stages to a greater or lesser extent for different pathogens. The two modes of dispersal are not mutually exclusive; rain may be involved in the dispersal of typically "dry-dispersed" pathogens and wind in the dispersal of typically "splash-dispersed" pathogens. Hirst & Stedman (1963) pointed out that when a raindrop strikes a surface bearing dry spores, the puff of air which precedes the spreading drop may dislodge spores. Additionally, if the surface is an infected leaf, the impact of the drop will shake it and this may also remove spores. Wind is also important in the dispersal of pathogens removed from the host in splash droplets. Field trials (Faulkner & Colhoun, 1976) and laboratory experiments with simulated rain and wind (Fitt & Nijman, 1983) showed that spores carried in droplets small enough to be influenced by wind may be dispersed higher and further than in still air.

In general, spores dispersed by rain are produced in mucilage or are individually surrounded by a mucoid sheath (Gregory, 1973). These spores are generally wettable due to the fact that the mucilage surrounding them is able to absorb water, making possible the dispersal of the spores within splash droplets. This mucilage protects spores from desiccation and loss of viability during dry weather and confines dispersal to periods of rainfall when conditions favour disease spread because free water is available for germination/reproduction on host surfaces (Fournet, 1969, cited in Fitt *et al.*, 1989).

The importance of rain in the dispersal of microorganisms was first demonstrated by Miquel in the 1880's during studies on the microflora of the Parc Montsouris in Paris. He found that collected rainwater contained large numbers of fungal spores and bacteria and also that numbers of airborne spores and bacteria decreased while rain was falling (Miquel, 1883). Since then, the role of rain in dispersal of many plant pathogens has been extensively studied. Splash-dispersed plant pathogens include *Phoma exigua, Phytophthora megakarya, Pseudocercosporella herpotrichoides* and *Pyrenopeziza brassicae*. However, little is known concerning the significance of rain in insect pathogen dispersal (Fitt *et al.*, 1989).

The aim of the present study was to find out whether *H. cryptosclerotium* could be dispersed by rainfall under field conditions. Experiments to investigate the horizontal dispersal of *H. cryptosclerotium* by simulated rain are described in this chapter.

5.2 Materials and methods

5.2.1 Rain tower

The rain tower (Fig. 5.1) used for these experiments at Rothamsted Experimental Station is 1 m^2 in internal cross-section and is constructed of wood lined with asbestos board. It is open at the top and traversed by two rolled steel joists for supporting rain-generating equipment. The height of the rain tower is 13 m.

5.2.2 Drop generator

Water drops (3.3 mm) were produced by allowing them to fall from a hypodermic needle (18 gauge) (Fig. 5.2). Water was fed to the needle at about 30 ml/h using a syringe infusion pump (Braun, Perfursor VI) (Fig. 5.3). At this flow rate drops were formed at about one per second.

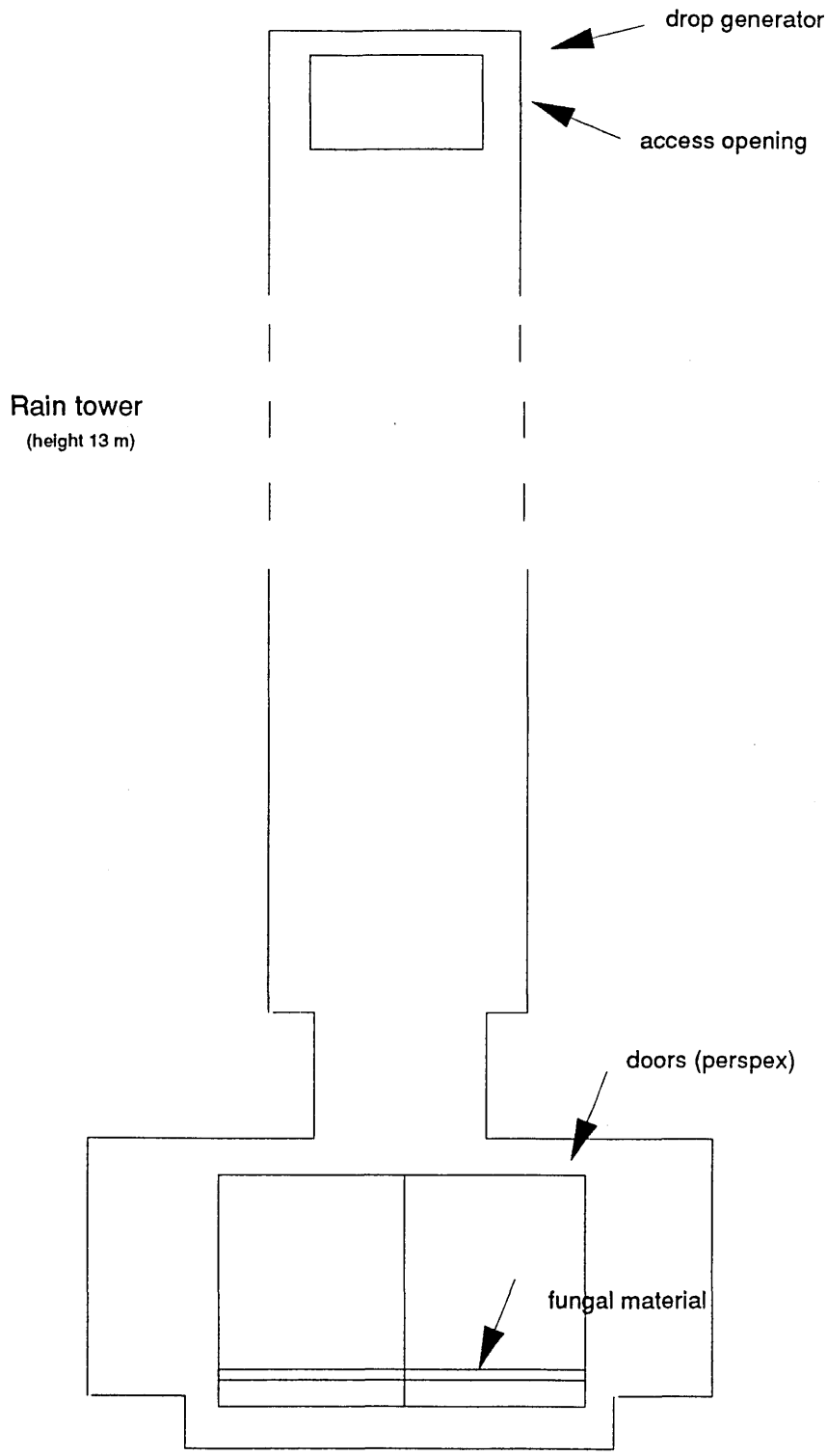


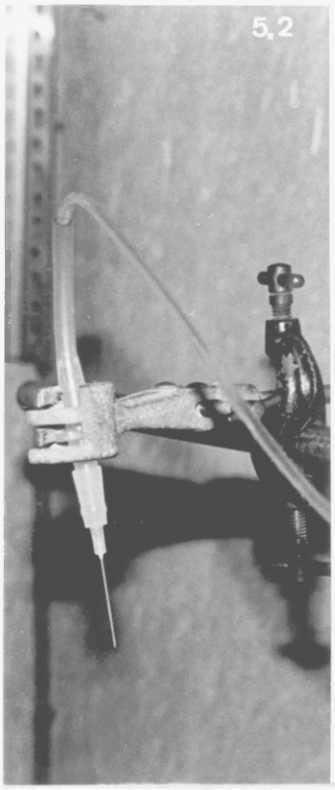
Fig. 5.1. Rain tower for studying the dispersal of pathogens by rain.

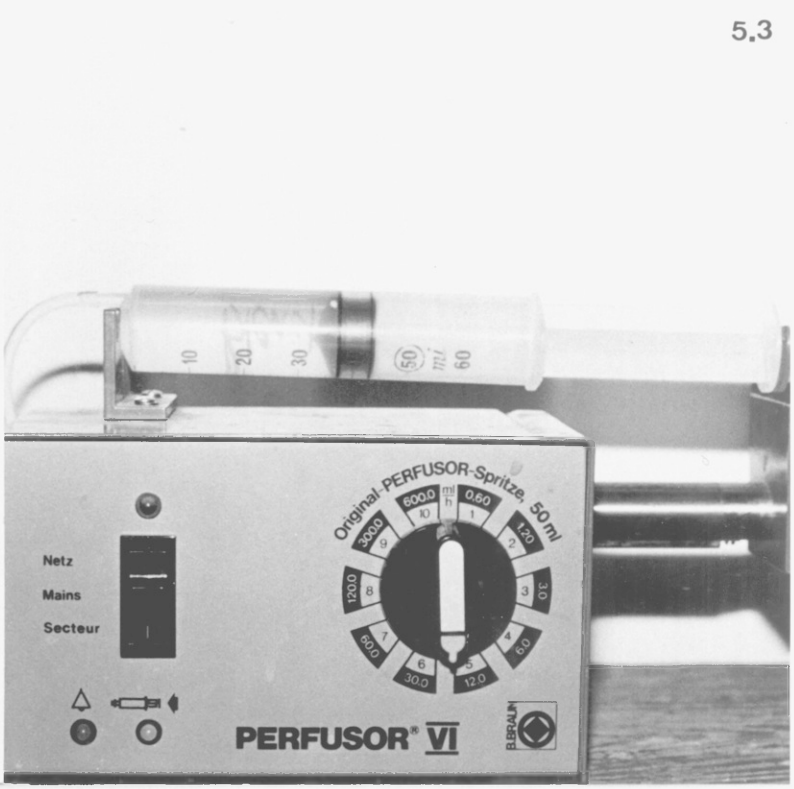
5.2.3 Splash experiments

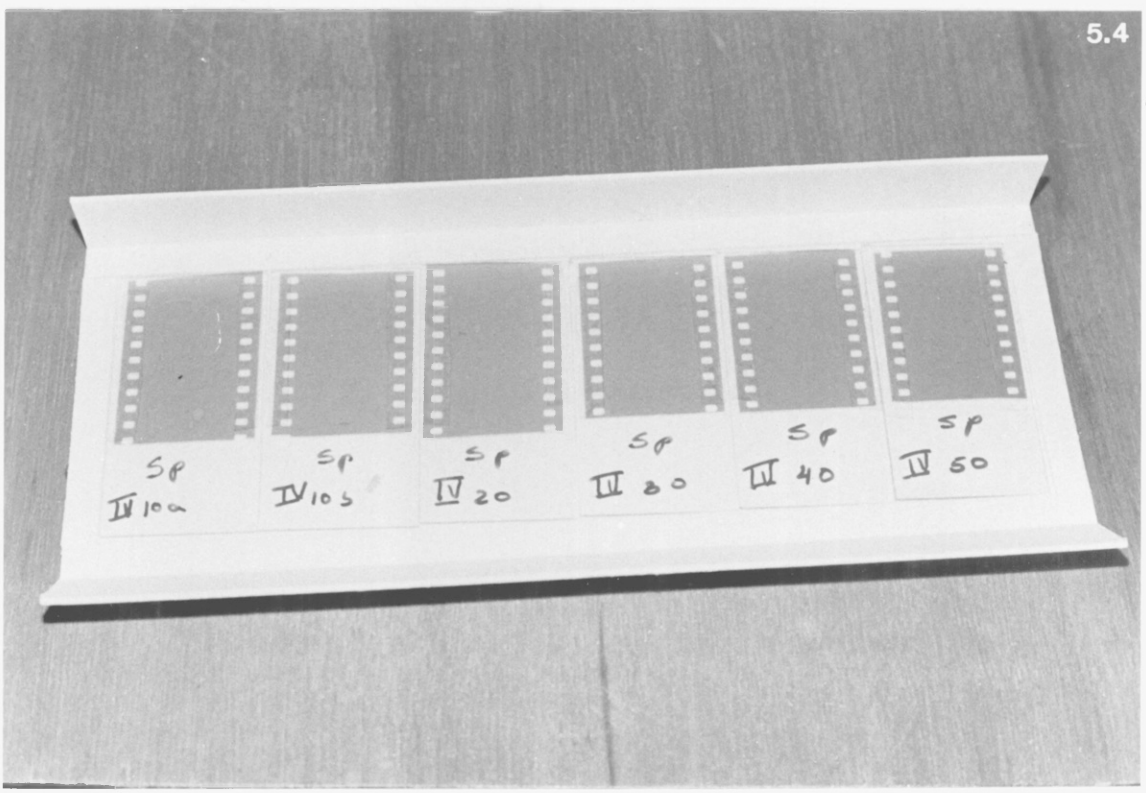
In still air, water drops fell 13 m down the rain tower onto two different targets. One of them consisted of dishes (9 cm diam) of malt agar plus antibiotics (Penicillin at 50 units/ml and Streptomycin at 100 units/ml) bearing one-month old sporulating mycelium of *H. cryptosclerotium*, with an estimated number of spores per dish of $6.3x10^7$. The second target was aqueous suspensions of *H. cryptosclerotium* spores on flat glass plates. A volume of 2.5 ml was placed in a 50 cm^2 circle marked with a wax pen on a glass plate (8 cm diam). The thickness of the suspension was about 0.5 mm and the spore concentration was $3.9x10^6$ spores/ml. Spore concentrations were determined with a haemocytometer slide.

In both experiments, microscope slides and pieces of fixed photographic film were used to collect the splashed fungal material. The film pieces were used to sample splash droplets carrying fungal material (spores or sporulating mycelium) and the microslides to count spores since individual spores were difficult to see on the film pieces due to their small size. The film was prepared following the methods described by Fitt *et al.* (1982). Three metres of photographic film (FP4 35 mm film, Ilford Ltd) were fixed in the dark to remove the silver coating, rinsed and left to dry. Droplets which fell onto this fixed film left a permanent trace by dissolving the thin layer of gelatine which was left on the film after removal of the silver coating. The droplet spread factor was calculated to be 0.56 by Fitt *et al.* (1982). According to Fitt *et al.* (1982) the spread factor is independent of the droplet size and is constant throughout the film because the gelatine layer is uniform. Fixed film was cut into 5 cm lengths and mounted onto 7x4 cm microscope slides using double-sided sticky tape (Fig. 5.4).

Microscope slides and pieces of film were arranged along one radius from the target at 10, 20, 30, 40 and 50 cm. At each particular distance a microslide and a







Figs 5.2-5.3. Drop generator used to simulate rain. Water drops were produced by allowing them to fall from a hypodermic needle (Fig. 5.2). Water was fed to the needle using a syringe infusion pump (Fig. 5.3).
Fig. 5.4. Fixed film pieces used to sample number and size of splash droplets carrying fungal material.

film piece were placed side by side. Sixty drops were allowed to fall onto each Petri dish and thirty drops onto each glass plate. Each test was replicated four times. To avoid overloading with splash droplets, those microslides and pieces of film nearest to the source (10 cm) were changed once during each test. When the splash droplets had dried, spores and sporulating mycelia collected on the microslides were stained with 0.03% Trypan blue in lactophenol to count the total number of spores dispersed by each method. Film pieces were examined with a light microscope under a magnification of x100. Droplets collected on film pieces which were carrying fungal material (sporulating mycelium or spores) were recorded and their size calculated by multiplying the diameters of droplet traces by 0.56, the droplet spread factor of the film.

5.2.4 Data analysis

Horizontal deposition gradients for spores and droplets carrying fungal material were studied by fitting to the data sets a negative exponential model in its linear form:

$$\ln y = \ln(a) - bx$$

where y is the number of spores or droplets carrying fungal material, x is distance, a is related to the source strength and b is a measure of the gradient. Data which followed a Poisson distribution were tested for significance with the parameter χ^2 and those which were normally distributed with the F parameter.

5.3 Results

5.3.1 Gradient of droplet deposition

The numbers of splash droplets carrying either spores or sporulating mycelium decreased sharply with increasing distance from the source in all tests (Figs 5.5,5.7). A negative exponential model gave a good fit to both of the data sets (spore-carrying droplets, χ^2=129.34, d.f. 1, p<0.001; mycelium-carrying droplets, χ^2=61.23, d.f. 1,

$p<0.001$). About 45% of the spore-carrying droplets were deposited at 10 cm from the source and none were collected beyond 40 cm (Fig. 5.6). With regard to mycelium-carrying droplets, 65% were deposited within 10 cm, however, there was some deposition beyond 40 cm (Fig. 5.8).

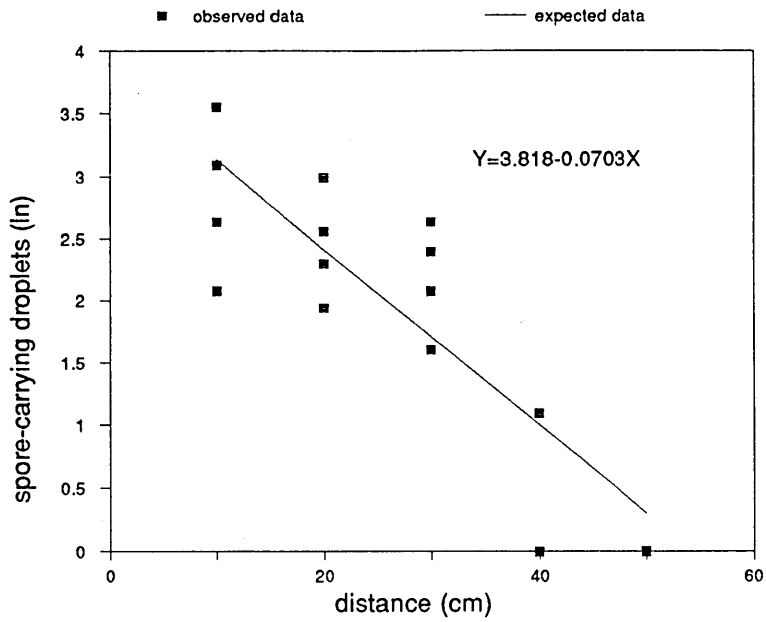
5.3.2 Gradient of spore deposition

It was not possible to count the number of spores borne by dispersed sporulating mycelium, since a high number of hyphae were usually transported within the same droplet making it impossible to record the high number of spores frequently found. The number of spores dispersed from spore suspensions was easily counted and it decreased significantly with increasing distance ($\chi^2=4499$, d.f. 1, $p<0.001$) (Fig. 5.9). Approximately 68% of the spores were collected at 10 cm and none at 50 cm (Fig. 5.10). Fig. 5.11 shows the number of conidia per droplet at different distances. The mean number of spores per droplet at 10 cm was 97.86, however, the range was very broad (4-500). At other distances the ranges were narrower and the maximum number of spores in a single droplet was never higher than 90.

5.3.3 Size of splash droplets carrying fungal material

Figs 5.12-5.13 show that droplets carrying spores were smaller than those transporting sporulating mycelium at all distances. When the difference was quantified it was found to be highly significant ($F_{(1,109)}=22.44$, $p<0.001$). However, within each type of splash droplet size did not change significantly with different distances (spore-carrying droplets, $F_{(4,51)}=0.57$, n.s.; mycelium-carrying droplets, $F_{(4,59)}=0.82$, n.s.).

Splash droplet diameters varied depending on the source used: the droplet size category containing the greatest proportion of spore-carrying droplets was 200-400 μm (Fig. 5.14). However, there was a larger proportion of mycelium-carrying droplets



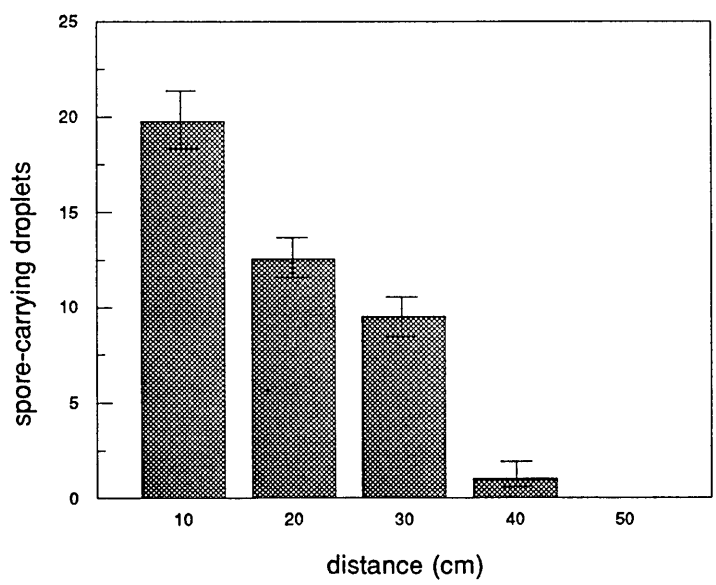
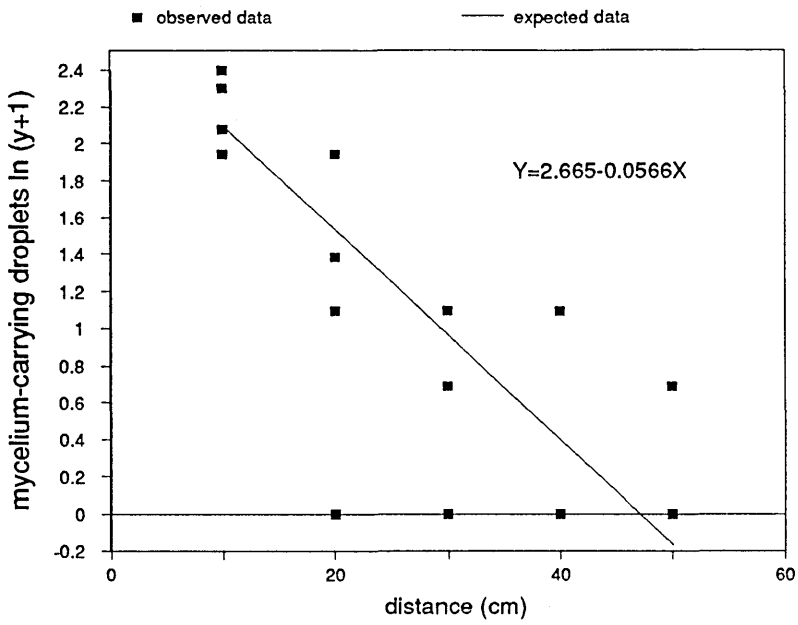


Fig. 5.5. Linear relationship between spore-carrying splash droplets (ln) and distance (cm) when simulated rain fell onto a spore suspension of *H. cryptosclerotium*. 67% variance accounted for.

Fig. 5.6. Number of spore-carrying splash droplets at different distances from the source (spore suspension of *H. cryptosclerotium*). Data are mean ± s.e. Data in App. 5.1.



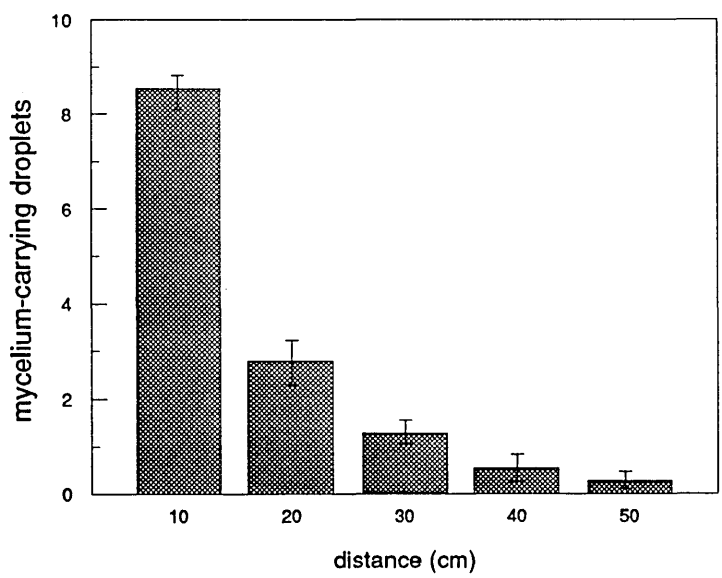
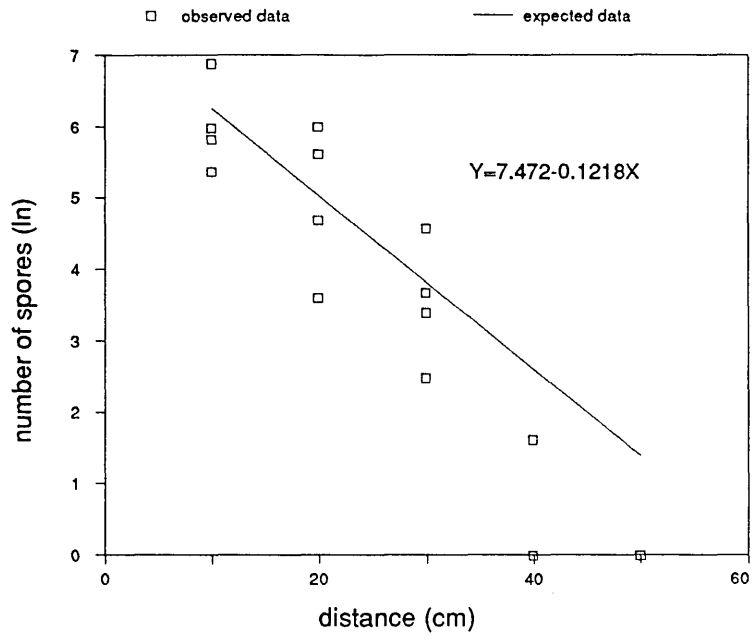


Fig. 5.7. Linear relationship between mycelium-carrying splash droplets (ln) and distance (cm) when simulated rain fell onto surface grown cultures of *H. cryptosclerotium*. 72% variance accounted for.

Fig. 5.8. Number of mycelium-carrying splash droplets at different distances from the source (surface grown cultures of *H. cryptosclerotium*). Data are mean ± s.e. Data in App. 5.2.



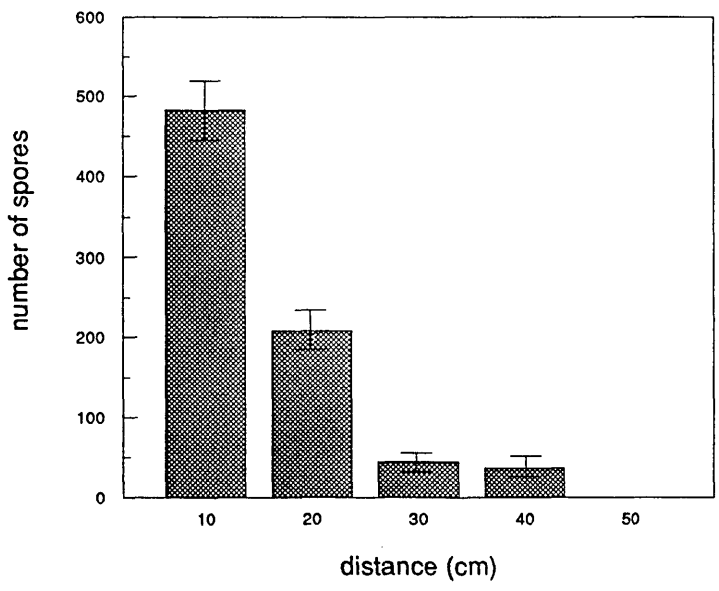


Fig. 5.9. Linear relationship between number of spores (ln) and distance (cm) when simulated rain fell onto a spore suspension of *H. cryptosclerotium*. 76% variance accounted for.

Fig. 5.10. Number of spores dispersed by simulated rain at different distances from the source (spore suspension of *H. cryptosclerotium*). Data are mean ± s.e. Data in App. 5.3.

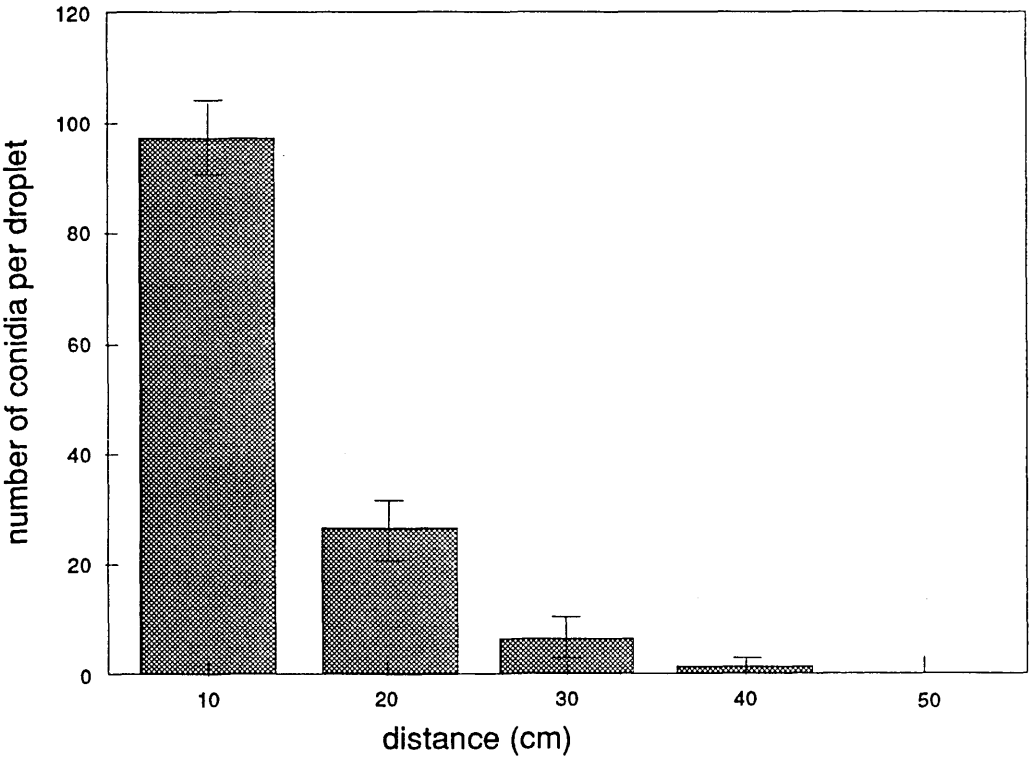
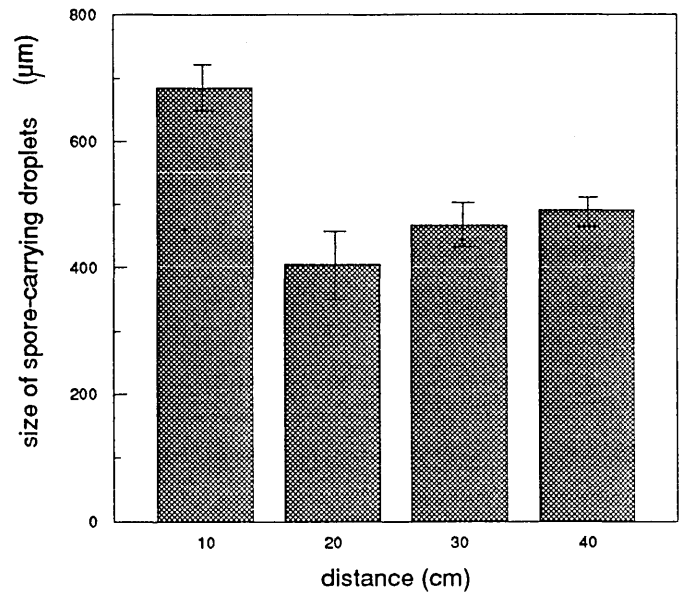


Fig. 5.11. Number of conidia per splash droplet at different distances from the source (spore suspension of *H. cryptosclerotium*). Data are mean ± s.e. Data in App. 5.4.



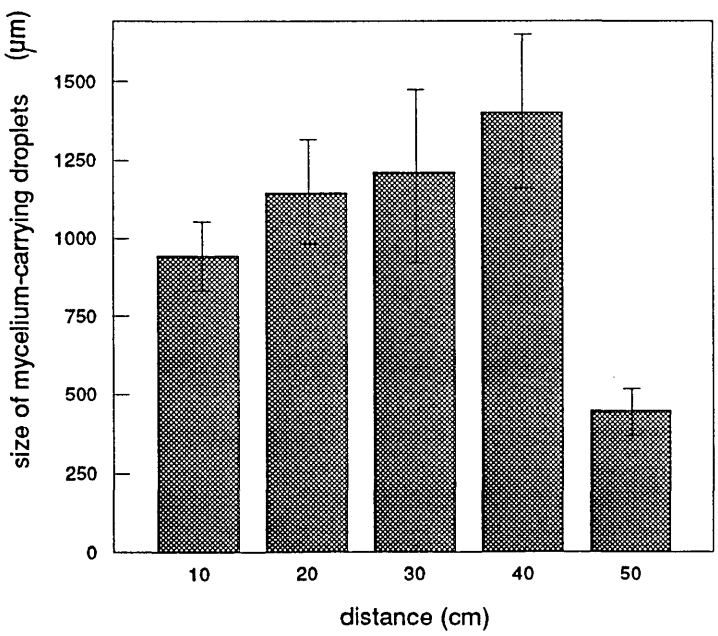
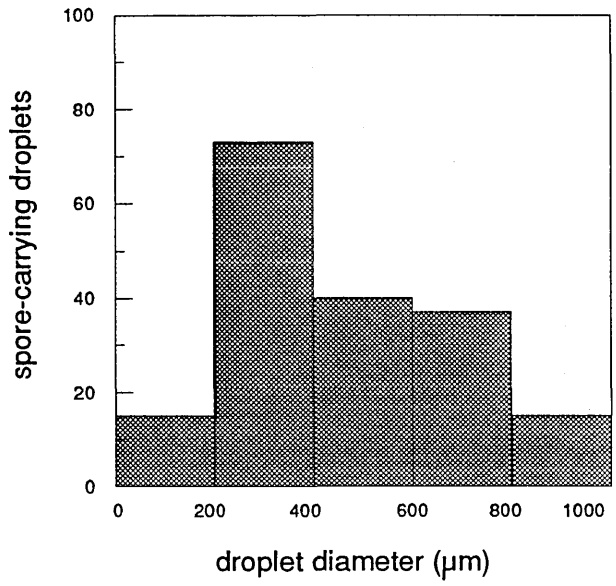


Fig. 5.12. Mean size of spore-carrying droplets at different distances from the source (spore suspension of *H. cryptosclerotium*). Data are mean ± s.e. Data in App. 5.5.

Fig. 5.13. Mean size of mycelium-carrying droplets at different distances from the source (surface grown cultures of *H. cryptosclerotium*). Data are mean ± s.e. Data in App. 5.6.



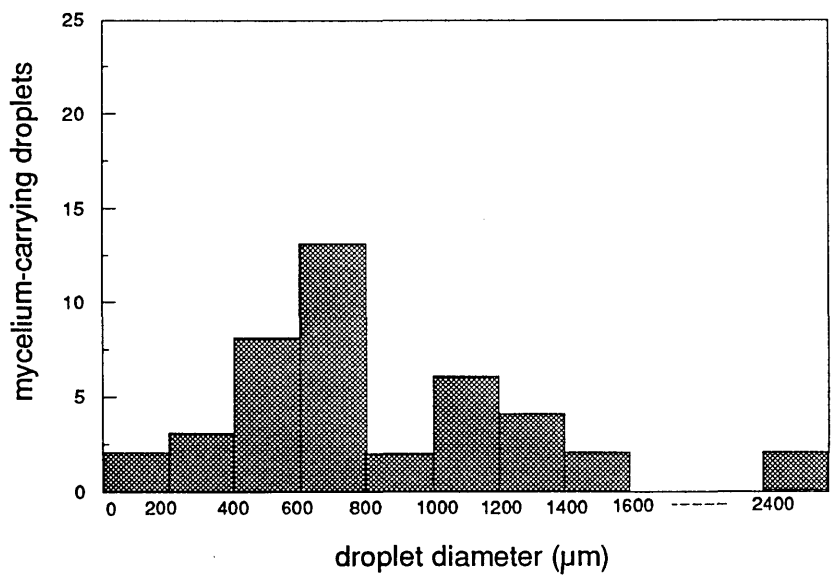


Fig. 5.14. Number of spore-carrying splash droplets in different droplet size categories when simulated rain fell onto a spore suspension of *H. cryptosclerotium*.

Fig. 5.15. Number of mycelium-carrying splash droplets in different droplet size categories when simulated rain fell onto surface grown cultures of *H. cryptosclerotium*.

with sizes between 600-800 μm (Fig. 5.15). The ranges also differed: diameters of splash droplets carrying spores were from 112 to 990 μm and those of splash droplets bearing mycelium ranged from 36 to 2550 μm.

5.4 Discussion

Results obtained in this study must be interpreted with care since it has been shown that splash dispersal from leaves is significantly different from splash from liquid films or rigid surfaces (Macdonald & McCartney, 1988). However, they demonstrate the potential of rain to spread inoculum of *H. cryptosclerotium*. In still air, few inoculum-carrying droplets travelled beyond 40 cm from either source and the number of droplets deposited decreased steeply with increasing distances from the sources. According to Fitt *et al.* (1989) the importance of wind in the dispersal of pathogens removed from the host in splash droplets becomes greater as the size of the droplets decreases. Droplets larger than 100 μm are not likely to be dispersed by wind (Fitt & McCartney, 1986a,b). In this study some inoculum-carrying droplets were <100 μm and might become airborne in moving air. Thus inoculum from *H. cryptosclerotium* could potentially be spread over long distances.

Chapter VI

Culture, mass production and storage of *Hirsutella cryptosclerotium*

6.1 Introduction

6.1.1 Nutrition as a basis for the study of fungi

Investigations on the nutrition of entomogenous fungi are important as a basis for understanding the nature of the host-fungus relationship. It might be supposed that in cases of obligate parasitism or symbiosis the heterotrophic requirements of the fungi concerned would be more complex than if they were saprophytes or facultative in their parasitism or symbiosis. All entomogenous fungi are heterotrophic for carbon sources and some are heterotrophic for materials such as nitrogen compounds, vitamins, growth factors etc. (Burnett, 1968; Garraway & Evans, 1984). Entomogenous genera with simple nutritional requirements (*Beauveria*, *Metarhizium*) are associated with a variety of host species, whereas those with more exacting requirements (*Hirsutella*, *Gibellula*) appear to be limited to a particular family, genus, or a few species of arthropods (MacLeod, 1954).

6.1.2 Mass production of entomogenous fungi

The increasing interest of insect pathologists in trying to exploit entomopathogens for biological control has resulted in more *in vitro* studies of these microorganisms. Many techniques have been described for cultivation and mass production of fungal cultures in quantities large enough for field use against pest insects (Ignoffo, 1967; Soper & Ward, 1981; Samson, 1982; Roberts & Wraight, 1986; Latgé & Moletta, 1988). Despite these developments, only a few entomogenous fungi have been commercially developed (Table 6.1) (Aquino *et al.*, 1977; Ferron, 1978; Ignoffo *et al.*, 1979; Soper & Ward, 1981; Hall, 1982b; Lysansky & Hall, 1983;

Table 6.1. Fungal pathogens commercially developed

Fungal pathogen	**Pest**	**Commercial product**	**Country**
Aschersonia aleyrodis	*Trialeurodes vaporariorum*	Aseronija	USSR
Beauveria bassiana	*Leptinotarsa decemlineata*	Boverin	USSR
Hirsutella thompsonii	*Phyllocoptruta oleivora*	Mycar	USA
Metarhizium anisopliae	*Mahanarva posticata*	Metaquino	BRAZIL
Verticilliun lecanii	*Myzus persicae* *Macrosiphoniella sanborni*	Vertalec	UK
Verticillium lecanii	*Trialeurodes vaporariorum*	Mycotal	UK

Rombach & Gillespie, 1988). The lack of progress can be attributed in part to the scarcity of precise data on the nutritional requirements of these pathogens and lack of information on production methodology.

6.1.3 What fungal propagule should be produced?

Since the beginning of biological control, spores have been considered to be the most suitable fungal propagule to produce for insect application because of their primary role in the infection process.

Conidia of the Entomophthorales are fragile and short lived when stored. Conversely, conidia of the Deuteromycotina in general can be readily mass-produced on solid media under aerated conditions (Roberts & Wraight, 1986). However, the sporulation cycle *in vitro* is relatively long (2-4 weeks) (Couch, 1982). Conidia can also be obtained in liquid media, being produced on typical conidiophores arising from hyphal filaments or directly from the spore through a sporulation microcycle, i.e. conidiophores are formed by the germ tube (Latgé & Moletta, 1988). In the USSR, an industrial technique has been developed for the mass production of conidia of *Beauveria bassiana* in submerged culture. Conidia are produced on conidiophores and yields vary from 5.10^8 to 2.10^9 conidia/ml. The unformulated preparation, however, is only viable for 2-3 months (Goral, 1979). The potential of microcyclic sporogenesis has been investigated by Latgé *et al.* (1988). This technique is of particular interest in the case of entomopathogenic fungi since it shortens the culture time and increases spore yields.

The resting spore of the Mastigomycotina and Zygomycotina is another type of spore which might be mass produced. These spores offer the advantage of being highly resistant and can survive for several months both *in vitro* and in nature.

However, like mycelium, these spores, are not directly infectious, their pathogenicity being dependent upon their ability to produce infective spores by germination (Latgé & Moletta, 1988).

The production of mycelium of entomopathogens on both solid and liquid media has also been investigated. On solid media, a continuous segmented mycelium is usually produced, whilst in shake liquid cultures, fungal development can also be characterized by the formation of yeast-like cells able to reproduce by fission (Soper & Ward, 1981).

6.1.4 Liquid or solid media?

Soper and Ward (1981) list four general methods for the *in vitro* production of fungi: solid media, semi-solid media, diphasic fermentation, and submerged fermentation. Table 6.2 records some of the fungi which have been mass produced using each technique.

6.1.4.1 Solid media

Although production on solid media is the most expensive method, it is also the simplest. The solid substrate used is usually agar containing different nutrients. Goettel (1984) used cellophane to separate mould colonies from the agar substrate on which they were grown. By adopting the use of cellophane as a barrier between the mycelium and nutrient source, large quantities of fungus free from substrate contamination were obtained. Conidia yields of 10^{10} per g of dry biomass were obtained for *Tolypocladium cylindrosporum*, *Verticillium lecanii* and *Beauveria bassiana* using this technique. Soper *et al.* (1975) produced large quantities of pure entomophthoralean resting spores on egg yolk media with approximately 2 to 3 g of spores for each egg yolk. Autoclavable plastic bags were used for laboratory production of *Beauveria bassiana* (Soper & Ward, 1981). Agar

Table 6.2. Mass produced entomogenous fungi.

Solid media		
Fungus	**Propagule produced**	**Reference**
Beauveria bassiana	conidia	Soper *et al.* (1975); Goettel (1984)
Metarhizium anisopliae	conidia	Marques *et al.* (1981)
Metarhizium anisopliae	conidia	Ferron (1981)
Nomuraea rileyi	conidia	Ignoffo (1981)
Tolypocladium cylindrosporum	conidia	Goettel (1984)
Verticillium lecanii	conidia	Goettel (1984)

Semi-solid media		
Aschersonia aleyrodis	conidia	Samson (1982)
Beauveria bassiana	conidia	Couch (1982)
Hirsutella thompsonii	conidia	Couch (1982)
Verticillium lecanii	conidia	Hall (1981)

Diphasic fermentation		
Beauveria bassiana	conidia	Soper & Ward (1981)

Table 6.2 (cont.) Mass produced entomogenous fungi.

Submerged fermentation		
Fungus	**Propagule produced**	**Reference**
Beauveria bassiana	mycelium	Rombach *et al.* (1988)
Beauveria bassiana	blastospores	Adamek (1965)
Beauveria bassiana	conidia	Goral (1979); Rombach (1989) Thomas *et al.* (1987)
Hirsutella thompsonii	mycelium and conidia	McCoy *et al.* (1971)
Hirsutella thompsonii	mycelium	McCoy *et al.* (1972,1975)
Hirsutella thompsonii	blastospores	Hall & Lewis (1982)
Hirsutella thompsonii var. *synnematosa*	conidia	Van Winkelhoff & McCoy (1984)
Hirsutella thompsonii	conidia (*)	Latge *et al.* (1988)
Metarhizium anisopliae	mycelium	Ferron (1981); Rombach *et al.* (1986)
Nomuraea rileyi	blastospores	Holdom & Van De Klashorst (1986)
Verticillium lecanii	blastospores	Latge & Moletta (1988)

(*) conidia produced directly from the spore through a sporulation microcycle.

medium was added to each bag and autoclaved. After the medium had solidified, the bags were inflated with sterile air. Inoculation was achieved by spraying the surface of the medium in the bag with mycelium grown in liquid medium. Sporulation was complete after 14 days, and conidia were harvested by vacuum. This technique has been adopted in Brazil for the mass production of conidia of *Metarhizium anisopliae*, using rice as substrate, is incubated in autoclavable plastics bags (Marques *et al.*, 1981).

6.1.4.2 Semi-solid media

Production of fungi on semi-solid media involves the use of cereal grains or other granular substrates impregnated or not with nutrients. The difference between solid media and semi-solid media is that oxygen may be forced through the latter by rotation or bubbling (Latgé & Moletta, 1988). Inorganic material can be added, such as vermiculite, to provide a large surface area for growth. Inoculum is usually obtained from mycelium grown in liquid medium. The fermentation process takes place in trays (tray reactors) or large vessels (homogeneous solid reactors). Tray reactors consist of several trays containing the substrate. Constant temperature and humidity are maintained by the addition of water or nutrient and the system can be illuminated if this is essential for sporulation. The major disadvantage of the tray reactors is the long fermentation cycle (3-4 weeks), the risk of contamination being therefore high. The homogeneous solid reactors are temperature controlled vessels in which the medium is mixed by rotation. The homogenization of the substrate is superior to that of the tray but the illumination is lower. Rotary fermenters have been used for the mass production of *Verticillium lecanii* using grains as solid substrate (Hall, 1981).

6.1.4.3 Diphasic fermentation

Diphasic fermentation is a system which attempts to take advantage of both solid and liquid media. The fungus is grown in fermentation tanks and then the mycelia are spread on trays placed in rooms with high relative humidity, and allowed to produced conidia. This technique is used in the USSR to produce conidia of *Beauveria bassiana* (Soper & Ward, 1981).

6.1.4.4 Submerged fermentation

Submerged culture offers a level of control superior to that obtained by other methods, as well as usually being much faster. Substrate, temperature, aeration, mixing, pH or light can be manipulated, thus leading to the possibility of harvesting cultures at the precise physiological age best suited to processing and storage. The major disadvantage of fermentation in liquid media is that the type of propagule formed may not be suitable for subsequent field application due to low pathogenicity and/or stability (Latgé & Moletta, 1988).

6.2 Materials and methods

6.2.1 Inoculum

The strains IMI 334843 and IMI 334844 of *H. cryptosclerotium* were used in this study (hereafter referred to as strains A and B respectively). The two isolates were maintained in the laboratory by periodic transfers of mycelia from one-month old cultures growing in Petri dishes containing oatmeal medium.

6.2.2 General procedure on solid media

The two strains were evaluated on nine different types of agar medium currently used at the Commonwealth Micological Institute (Johnston & Booth, 1983): Czapek-yeast extract agar (CYA), potato-sucrose agar (PSA), Czapek agar (CZ),

malt-salts agar (MSA), oatmeal agar (OA), yeast extract-sucrose agar (YES), Sabouraud agar (SA), malt-yeast extract agar (MYE) and Sabouraud-nitrate agar (SNA). Six Petri dishes (9 cm diam) per medium were prepared each with 25 ml of medium. Each Petri dish was then inoculated with the same amount of inoculum using a cork-borer (4 mm diam) and incubated at 25°C, 16 h light/8 h dark. Growth was determined by measuring the diameter of the colony after three weeks. A comparative study was also made of spore production on each medium. The composition of the different media used is shown in App. 6.1.

6.2.3 General procedure in liquid media

The basal medium adopted for further refinement was similar to the yeast-extract glucose medium used by McCoy *et al.* (1975) for large scale production of *Hirsutella thompsonii*. It consisted of 1.5 g potassium dihydrogen phosphate (KH_2PO_4) (anhydrous), 0.5 g magnesium sulphate ($MgSO_4$) (anhydrous), 0.01 g calcium chloride ($CaCL_2$), 10 g glucose, 5 g yeast extract, 0.5 g peptone and distilled water to 1 l. Two sources of carbon (glucose and sucrose) and two sources of nitrogen (yeast extract and peptone) were tested. Fifty ml of the various preparations tested were transferred to 100 ml Erlenmeyer flasks which were autoclaved for 20 min at 121°C. Each flask was inoculated with the same amount of inoculum using a cork-borer (4 mm diam). Mycelia used as inoculum were obtained from 1-month-old stock cultures. Flasks were incubated on a rotatory shaker (150 rev/min) in a small air-conditioned room maintained at 22-26°C. After 15 days, the mycelia were separated from the medium by vacuum filtration, dried at 60°C for 24 h and weighed. Absorption of water by the dried mycelium was avoided by wrapping each sample in foil and keeping them in a desiccator while they were weighed. Each test was carried out in triplicate, the results presented being the mean of the three determinations.

6.3 Results

6.3.1 Data analysis

Data followed a normal distribution. One way analysis of variance and two way analysis of variance were carried out to measure the effect of the different media used on growth of *H. cryptosclerotium*. Results were tested for significance with the *F* parameter.

6.3.2 Culture on solid media

Although both strains of *H. cryptosclerotium* were cultured successfully on all eight media, vegetative growth varied significantly among the different media (ANOVA strain A, $F_{(8,45)}$= 25,32, p<0.001; ANOVA strain B, $F_{(8,45)}$=22.97, p<0.001) (Fig. 6.1) and sporulation patterns (Table 6.3) differed depending on the solid media utilized.

No single factor appeared to account for the differential pattern of vegetative growth, but it appeared that media high in carbohydrates and also containing yeast extract led to maximum growth (CYA, YES, MYE). PSA, SA, and OA, all high in carbohydrates were also optimum media for growth of *H. cryptosclerotium*.

As shown in Table 6.3, for both strains maximum sporulation occurred on media containing high levels of carbon but lacking yeast extract such as OA, PSA, and SA. Those with least sporulation (MYE, CYA, YES) contained yeast extract and gave the best radial growth.

6.3.3 Culture in liquid media

6.3.3.1 Effect of varying glucose and sucrose concentrations on yield of mycelium

The quantity of glucose and sucrose necessary for optimum yield of

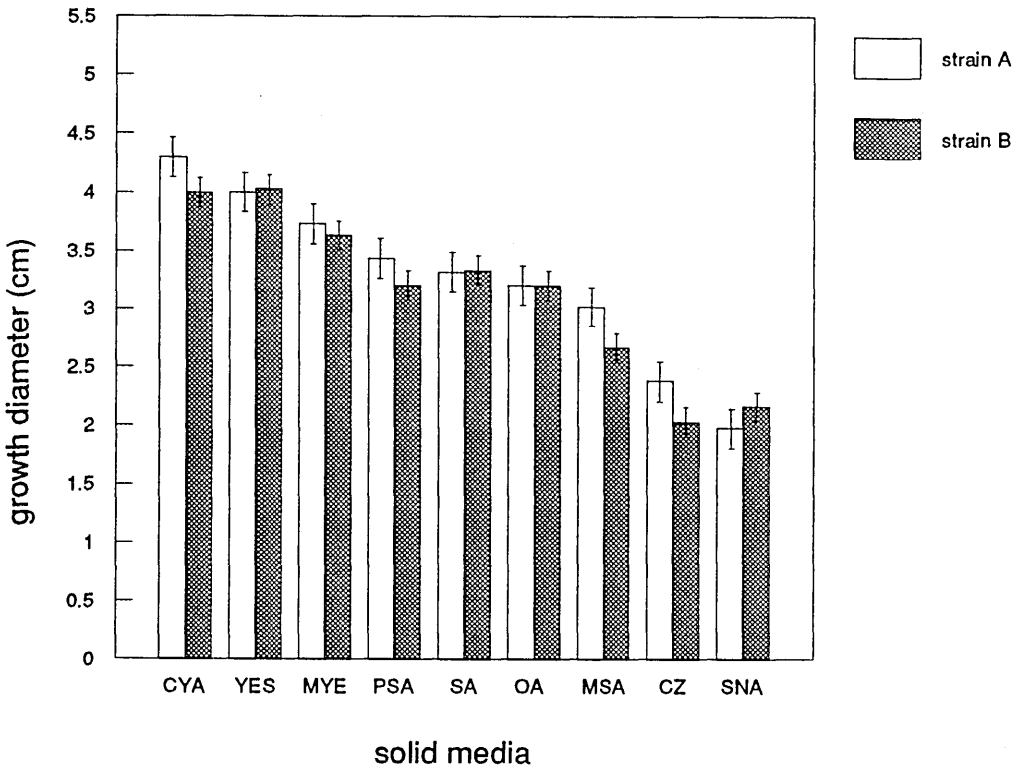


Fig. 6.1. Growth of two strains (A, B) of *H. cryptosclerotium* on different solid media (Czapek-yeast extract agar (CYA), yeast extract-sucrose agar (YES), malt-yeast extract agar (MYE), potato-sucrose agar (PSA), Sabouraud agar (SA), oatmeal agar (OA), malt-salts agar (MSA), Czapek agar (CZ), Sabouraud-nitrate agar (SNA)) after 21 d at 25°C. Data are mean ± s.e. Data in App. 6.2.

Table 6.3. Sporulation on various agar media by two strains of *H. cryptosclerotium* (A, B) at 25°C, 16 h light/8 h dark (oatmeal agar (OA), potato-sucrose agar (PSA), Sabouraud agar (SA), Sabouraud-nitrate agar (SNA), Czapek agar (CZ), malt-salts agar (MSA), malt-yeast extract agar (MYE), Czapek-yeast extract agar (CYA), yeast extract-sucrose agar (YES)).

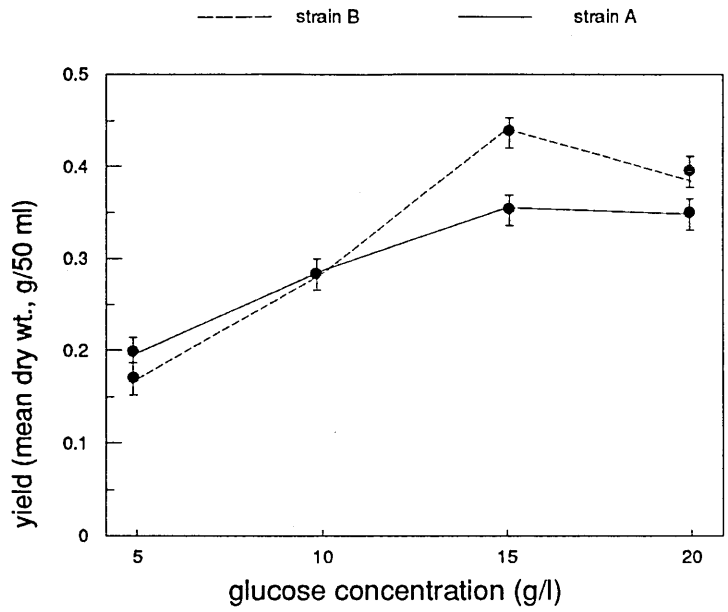
	days							
	2		4		14		21	
media	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
OA	0	0	+	+	+++	++	+++	++
PSA	0	0	+	+	+++	+++	+++	++
SA	0	0	+	+	+++	++	+++	++
SNA	0	0	+	+	++	++	++	++
CZ	0	0	+	+	++	++	++	++
MSA	0	0	+	+	++	++	++	++
MYE	0	0	0	0	+	+	+	+
CYA	0	0	0	0	+	+	+	+
YES	0	0	0	0	0	0	0	0

(0) no sporulation; (+) poor, only a few scattered spores;
(++) good, numerous spores; (+++) abundant, large number of spores.

mycelium was investigated using 5, 10, 15, and 20 g of each sugar per litre of basal medium. Glucose determined a significant effect on growth of both strains (ANOVA strain A, $F_{(3,8)}$=53.26, p<0.001; ANOVA strain B, $F_{(3,8)}$=62.49, p<0.001) (Fig. 6.2). Yield increased with increasing concentrations of glucose to 15 g/l for both strains. Thereafter, yield was similar to that obtained with 15 g/l for strain A and inferior for strain B. Increase in concentration of sucrose led to a very different pattern of growth. Yield did not increase when sucrose was augmented up to 15 g/l. However, dry weight of mycelium at a concentration of 20 g/l gave a similar yield to that obtained with 15 g/l of glucose. The effect of sucrose on yield of mycelium was found to be significant for both strains (ANOVA strain A, $F_{(3,8)}$=20.16, p<0.001; ANOVA strain B, $F_{(3,8)}$=16.56, p<0.001) (Fig. 6.3).

6.3.3.2 Influence of yeast extract and peptone on growth

To find out the amount of each organic nitrogen source necessary to achieve optimum yield, both compounds were tested alone, yeast extract at 6 concentrations (5, 10, 15, 20, 25, 30 g per litre of basal medium) and peptone at 4 concentrations (0, 0.5, 1, 1.5 g per litre of basal medium). As shown in Fig. 6.4, yields of both strains were increased by the addition of higher concentrations of yeast extract to 20 g/l, thereafter, the strains grew less well. The effect of yeast-extract on growth was highly significant for both strains (ANOVA strain A, $F_{(5,12)}$=65.53, p<0.001; ANOVA strain B, $F_{(5,12)}$=34.72, p<0.001). Yields also increased with an increase in the concentration of peptone to 1 g/l (ANOVA strain A, $F_{(3,8)}$=17,14 p<0.001; ANOVA strain B, $F_{(3,8)}$=12.64, p<0.001) (Fig. 6.5). A further increase in concentration of peptone (5 g/l), did not give significantly different yields (strain A= 100±8* mg, dry wt./50 ml; strain B= 88±6 mg, dry



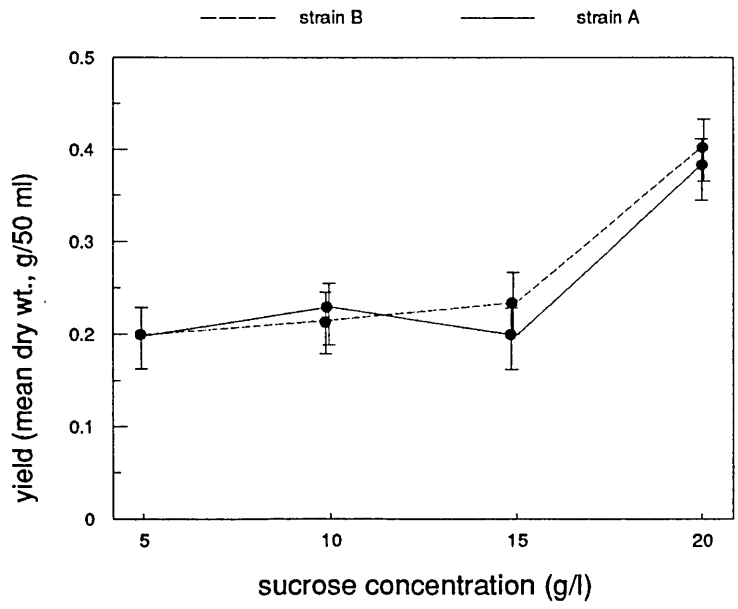


Fig. 6.2. Effect of different concentrations of glucose on yield of mycelium (dry wt., g/50 ml) of two strains (A, B) of *H. cryptosclerotium*. Data are mean ± s.e. Data in App. 6.3.

Fig. 6.3. Influence of sucrose on yield of mycelium (dry wt., g/50 ml) of two strains (A, B) of *H. cryptosclerotium*. Data are mean ± s.e. Data in App. 6.4.

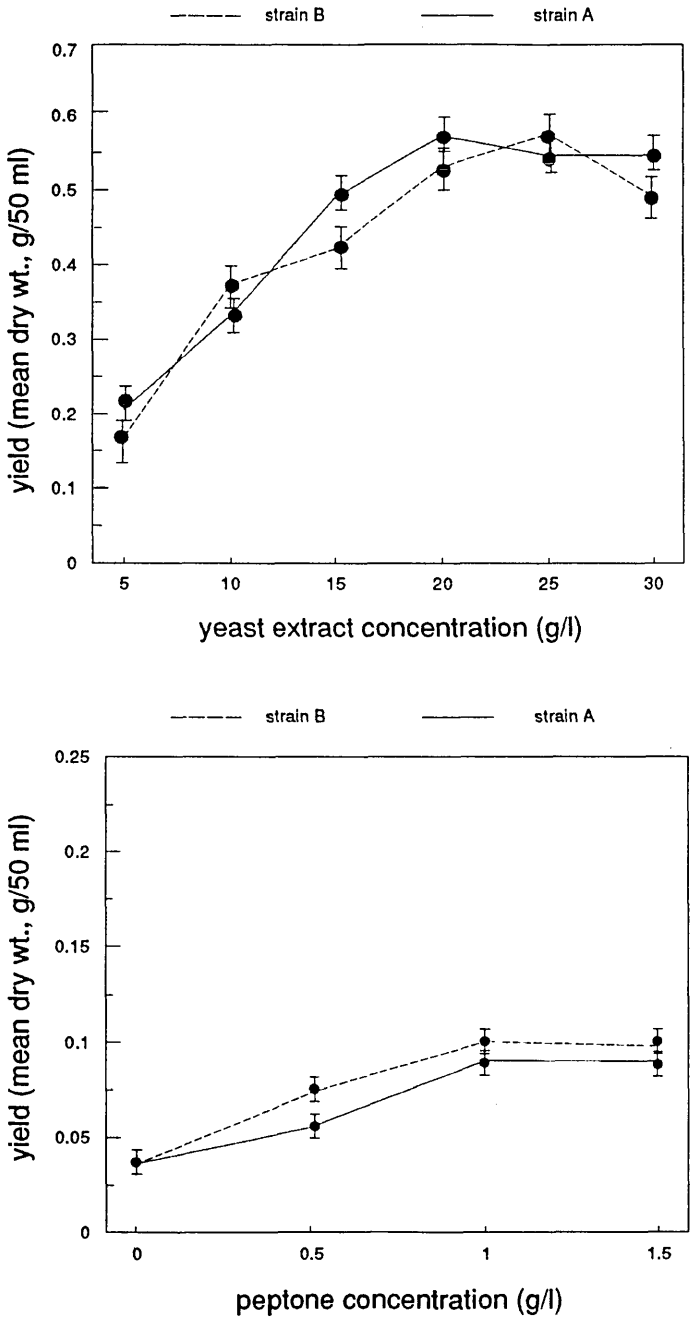


Fig. 6.4. Effect of varying yeast extract concentrations on yield of mycelium (dry wt., g/50 ml) of two strains (A, B) of *H. cryptosclerotium*. Data are mean ± s.e. Data in App. 6.5.

Fig. 6.5. Influence of peptone on yield of mycelium (dry wt., g/50 ml) of two strains (A, B) of *H. cryptosclerotium*. Data are mean ± s.e. Data in App. 6.6.

wt./50 ml) to those obtained with 1 g/l (strain A= 90±6 mg, dry wt./50 ml; strain B= 100±8 mg, dry wt./50 ml).

(*) values are mean ± s. e.

6.3.3.3 Growth response to varying yeast extract - peptone concentrations

The combined effect of yeast extract and peptone was studied as in some fungi the utilisation of nitrogen sources is more effective in mixtures (McCoy *et al.*, 1972). For this purpose a factorial experiment was carried out in which yeast extract was supplied at 0, 5 and 10 g and peptone at 0, 0.5, 1 and 1.5 g per litre of basal medium. Twelve possible combinations were set up for each strain. The analysis of variances for both strains is given in Table 6.4. Yields of both strains increased significantly with an increase in concentration of yeast extract, but not with an increase of peptone. There was no synergism between the two nitrogen sources.

6.3.4 Summary of the results

Maximum growth of *H. cryptosclerotium* on solid medium was achieved with those media containing yeast extract, however, sporulation was greater in the absence of this nutrient. Growth was rather slow even on the best media in comparison with other fungi imperfecti (P. Bridge, personal communication).

Nutritional studies in submerged culture showed that glucose is a more effective carbon source than sucrose for use in large-scale production of *H. cryptosclerotium*. With glucose, optimum yield (strain A= 355±28* mg, dry wt./50 ml; strain B= 440±15 mg, dry wt./50 ml) was obtained at a concentration of 15 g/l, whereas sucrose was needed at a concentration of 20 g/l to produce a similar yield (strain A= 384±13 mg, dry wt./50 ml; strain B= 402±17 mg, dry wt./50 ml). With regard to the organic

Table 6.4. Effect of varying yeast extract-peptone concentrations on the growth of *H. cryptosclerotium*. Data in App. 6.7.

analysis of variance (strain A)				
source of variation	d.f.	variance	observed *F*	*p*
between yeast extract concentrations	1	0.0445	10.3	<0.001
between peptone concentrations	2	$6.78\ 10^{-3}$	1.5	n.s.
interaction between yeast extract and peptone	2	$1.70\ 10^{-3}$	0.3	n.s.
total	17	$4.29\ 10^{-3}$		
residual	12	$9.53\ 10^{-4}$		

analysis of variance (strain B)				
source of variation	d.f.	variance	observed *F*	*p*
between yeast extract concentrations	1	0.0856	9.67	<0.001
between peptone concentrations	2	0.0238	2.69	n.s.
interaction between yeast extract and peptone	2	$5.10\ 10^{-3}$	0.57	n.s.
total	17	$8.84\ 10^{-3}$		
residual	12	$5.76\ 10^{-4}$		

nitrogen sources, yeast extract was much more effective for growth of *H. cryptosclerotium* than peptone. The addition of peptone to a simple glucose-salts medium gave only limited growth; however, yeast extract gave a significantly greater yield than peptone at an equivalent concentration (at 5 g/l: strain A= 205±18 mg, dry wt./50 ml; strain B= 169±24 mg, dry wt./50 ml). Yield was not improved when peptone was added to a liquid medium containing yeast extract.

(*) values are mean ± s. e.

In view of the promising results obtained in submerged culture it was decided to mass produce *H. cryptosclerotium* using this technique.

6.3.5 Mass production of *H. cryptosclerotium*

6.3.5.1 Preparation of the culture medium

The final composition of the medium used in the production of *H. cryptosclerotium* was:

-10 g glucose

-20 g yeast extract

-1.5 g (KH_2PO_4) (anhydrous)

-0.5 g ($MgSO_4$) (anhydrous)

-0.01 g ($CaCl_2$)

-distilled water to 1 l

Four litres of liquid medium were placed into 10 litre Pyrex fermentation vessels, which were sterilized for 30 min at 121°C. After sterilization the vessels were allowed to cool overnight.

6.3.5.2 Preparation of the inoculum

The original inoculum used in the mass production of *H. cryptosclerotium* was one-month old sporulating cultures growing in Petri dishes containing

oatmeal medium. Flasks containing 125 ml of the selected medium were inoculated with the same amount of inoculum using a cork-borer (4 mm diam) and were incubated for 3 days at 22-26°C on a shaker rotated at 150 rev/min. Fermentation vessels were inoculated by pouring the contents of one flask into each vessel.

6.3.5.3 Incubation

The incubation of both strains of *H. cryptosclerotiun* was conducted at 22-26°C on a shaker at 110 rev/min. Maximum yield per fermentation vessel was obtained after 18 days with no significant difference between strains (strain A= 19.09±0.19 g, dry wt.; strain B= 20.23±0.23 g, dry wt.).

To improve yield, another technique was used which consisted of introducing filtered air into the vessels while they were rotating. The filtered air was produced by an air-pump connected to a Swinnex 47 Millipore^{R} filter containing an 0.45 µm paper, by means of silicone rubber tubing. The vessels which had been originally acquired for this purpose had an aeration head with several outlets. One of them was used as an intake passage for the filtered air, another one as an exhaust (stoppered with non-absorbent cotton) and the rest were stoppered with non-absorbent cotton and foil. An aerator stone attached to a 20 cm Pyrex tube was connected to the intake tube to receive the filtered air and distribute it in the liquid medium. This technique was unsuccessful since the fungus started growing around the aerator stone, blocking the passage of air after 3 or 4 days of incubation.

6.3.5.4 Preparation of mycelia for insect application

H. cryptosclerotium grew forming mycelial spheres as well as short-branch strands with a light tan colour. Sporulation was never observed in submerged

culture. The techniques used to prepare mycelia for insect application were similar to those described by McCabe & Soper (1985). The mycelia produced were recovered from the culture broth using a strainer and kept in a refrigerator at 5°C overnight. They were then dried rapidly (6-8 h) in a laminar air flow cabinet at room temperature (20-26°C). The dried mats were ground using an electric blendor (Waring Mc 3) and the powder was sealed in a sterile container and stored at 5°C (mycelial mats stored wet or at higher temperatures became contaminated with bacteria and were non-viable). The wettable powder obtained contained $0.76x10^5 \pm 0.023$ colony forming units (cfu) of *H. cryptosclerotium* per gramme. This was determined by plating one ml of a 0.5% suspension of fragmented mycelia (g, dry wt./100 ml distilled water) on each of ten 4.5 cm Petri dishes containing malt agar plus antibiotics (Penicillin at 50 units/ml and Streptomycin at 100 units/ml) and counting the number of colonies per plate five days later. The dried mycelia taken from cold storage were prepared as a sprayable suspension for use on *Rastrococcus invadens* on the same day the application was to be made.

6.4 Discussion

Previous nutritional studies on the genus *Hirsutella* (MacLeod, 1954, 1959a, 1959b, 1960; McCoy & Kanavel, 1969; McCoy *et al.*, 1972, 1975; Kenneth *et al.*, 1979) indicated that it has more exacting nutritional requirements than many entomogenous hyphomycetous fungi with wider host ranges. The present study on *H. cryptosclerotium* was carried out to develop a standard medium that could be used in routine mass production of the fungus rather than to investigate its physiology and nutrition; however, the results obtained appear to support previous findings.

Media containing yeast extract appeared inferior for sporulation: e.g., sporulation never occurred on YES during the experimental period of 21 days but was observed after three months. These results appear to indicate that lack or depletion of nutrients enhanced

sporulation: YES contains not only a higher level of yeast extract (20 g/l) than CYA or MYE, which were suitable media for sporulation, but also a higher level of glucose (150 g/l) than any other media. McCoy & Kanavel (1969) also found that media high in nitrogen seemed inferior for sporulation of *H. thompsonii*.

Certain factors present in yeast extract seem to be essential for growth of *H. cryptosclerotium*, since growth in submerged culture was rapid and vigorous in the presence of this nutrient in comparison with the lower yields obtained with a simple glucose-salt solution. McLeod (1959b) and McCoy *et al.* (1972) observed similar results with *H. gigantea* and *H. thompsonii* respectively. They also found that yeast extract appear to have an inhibitory effect at high concentrations. Garraway & Evans (1984) pointed out that the most common causes for growth inhibition are: (1) the depletion of other nutrients, (2) the accumulation of toxic metabolites, and (3) adverse osmotic effects.

McCoy *et al.* (1972) reported that yeast extract and peptone in combination gave a significantly greater yield than either yeast extract or peptone alone. In the current study growth of *H. cryptosclerotium* was not improved when yeast extract was used in conjunction with peptone.

Both MacLeod (1959a) and McCoy *et al.* (1972) found that glucose was a better carbohydrate source for growth of *H. gigantea* and *H. thompsonii*, in comparison with other sources of carbon. McCoy *et al.* (1972) found that sucrose was also a satisfactory carbon source for *H. thompsonii*, but a concentration of 10 g/l was needed to obtain a similar yield to that obtained with glucose at 5 g/l. In the present study glucose also appeared to be utilized by *H. cryptosclerotium* more readily than sucrose. Garraway & Evans (1984) reported similar patterns of growth with glucose and sucrose for *Coprinus lagopus* to those found in the present study (Figs 6.2-6.3). According to Garraway &

Evans many fungal species can utilize monosaccharides better than larger molecules composed of these same monosaccharide units: e.g., *Rhizopus nigricans* and *Sordaria fimicola* grow poorly on sucrose but well on glucose and fructose.

Growth of *H. cryptosclerotium* was studied on both solid and liquid media to find out the best technique for large-scale production of this pathogen. Cultures of *H. cryptosclerotium* on solid media grew slowly, however, abundant sporulation was obtained on most media after two weeks. Although best results were obtained in liquid culture, conidial production did not occur. The most common mass production technique used today involves either a surface cultivation, or a two stage cultivation in which the fungus is first grown under submerged conditions and then allowed to sporulate as a surface culture in still-liquid or semi-solid medium (Roberts & Sweeney, 1982). These methods of cultivation yield conidia which are the desired propagule to be produced. Blastospores are less virulent and resistant to adverse environmental conditions, and mycelium is not infective by itself. The surface method of cultivation, however, is very time consuming and not economical. The preferred method for mass production is one-stage submerged cultivation of the fungus to produce spores in a relatively short time. Most fungi imperfecti do not produce conida in submerged culture, despite the fact that they produce mycelia and balstospores in abundance (Thomas *et al.*, 1987). However, recently there have been attempts to produce conidia under submerged cultivation with certain fungi (Thomas *et al.*, 1987; Van Winkelhoff & McCoy, 1984). Thomas *et al.* (1987) have shown that production of "submerged" conidia of *Beauveria bassiana* depends on the nature of the medium used: media containing complex nitrogen sources, such as peptone or yeast extract, do not support conidiation, however, it is abundant in media containing nitrate as a nitrogen source. Future research on mass production of *H. cryptosclerotium* should be directed towards the study of the feasibility of submerged conidial production.

McCoy *et al.* (1975), showed that yield of *H. thompsonii* in submerged culture was improved by a continuous supply of oxygen. Future work should be undertaken with the use of compressed air or with a more powerful air-pump.

Chapter VII

Mortality effects of *Hirsutella cryptosclerotium* on *Rastrococcus invadens*

7.1 Introduction

7.1.1 Bioassay: probit transformation

The development of standardized bioassay techniques is a prerequisite to measure the impact of a microorganism on an insect. The evaluation of dose-response relationships between pathogen and host enables us to estimate the amount of pathogen inoculum needed to obtain control of the insect pest in a practical situation. Moreover, the pathogenicity of different strains and species of entomopathogens to the insect can be compared in order to select those which are more virulent (Hall, 1982a). The desired effect in the field is death of the insect, so mortality is the most commonly selected factor in bioassays (Burges & Thomson, 1971; Pinnock, 1981).

A typical test used in the evaluation of a mycoinsecticide is one in which successive batches of insects are exposed to different concentrations of pathogen inoculum for a constant time and, after a suitable interval, scored for the number dead or alive to obtain for each dose the proportion (p) of insects responding. As an alternative to varying concentration, a standard concentration may be used throughout, but different total volumes given. Another factor which is sometimes studied at different levels is the period of exposure, the concentration and quantity of pathogen being kept constant (Finney, 1964).

The dose which will produce a response in half the insect population is the median lethal dose (LD50) or mean response (μ). The reliability of this estimate is shown by the limits within which the true values are likely to lie with a selected degree of certainty, usually 95% (confidence intervals). The LD50 alone does not fully describe the effectiveness of the pathogen. Two pathogens may be applied at the same rates in order

to be lethal to half the population, however, the responses at higher or lower rates may be very different. If the distribution of responses has a lesser "spread" for one than for the other, any increase or decrease from this dose will produce a greater change in mortality for the first than for the second. This "spread" is measured by the variance, σ^2: the smaller the value of σ^2, the greater is the effect on mortality of any change in dose (Finney, 1964).

To estimate μ and σ^2 of the distribution of responses the **probit transformation** of the results is required. The probit of the proportion p is defined as the abscissa which corresponds to a probability P in a normal distribution with mean 5 and variance 1. The LD50 is estimated as that value of x which gives $y = 5$.

Normalization can often be achieved by expressing the responses to the pathogen inoculum in terms of the logarithms of the concentrations instead of the absolute values. Representing mortality against dose gives an S-shaped curve, but it is approximated to a straight line when response is converted to a probit scale and dose to logs (Finney, 1964).

7.1.2 Age-specific mortality

To understand the impact of a pathogen on its insect host, the differential susceptibility at various developmental stages must be determined. This study will provide an initial experimental base upon which further studies can be developed (Feng *et al.*, 1985).

7.1.3 Shelf-life

Tests of viability of stored fungal products are essential since the use of material with low viability for microbial control is a waste of effort (Burges & Thomson, 1971). A microbial insecticide must be produced, formulated and stabilized so that storage conditions do not affect insecticidal properties. In general, at least 18 months stability

under ambient storage conditions is required to be commercially economical. If the pathogen is to be supplied by contract for application at a specific time, shelf-life is less of a problem and stability for 3 to 6 months may be acceptable (Couch & Ignoffo, 1981).

7.1.4 Formulation and application

According to Van Walkenburg (1973), "a pesticide formulation is defined as the resultant composition when the candidate pesticide is mixed with anything, including water". The object of a formulation is to provide the correct combination of ingredients so that the active component is suitable for agricultural application. The formulation must present several characteristics to be successful: (a) when applied, the pathogen should spread uniformly and remain for a period of time (spreader-sticker qualities) (Soper & Ward, 1981); (b) care should be taken to insure the formulation at least does not interfere with the infection process, and at best will favour it (Hall, 1982c).

Entomopathogens can be applied as dry or liquid formulations. The term "dry" refers to the form in which the active ingredient is formulated and stored until used, although such formulations may later be mixed with water and applied as a spray. Types of dry formulations are dusts, granules and wettable powders. Liquid formulations include emulsifiable and nonemulsifiable suspensions, pastes or gels and foams. The type of formulation to be used will depend upon the mechanisms of the infection process and the habitat of the host (soil, terrestrial or aquatic) (Couch & Ignoffo, 1981; Soper & Ward, 1981).

7.1.5 Persistence

The survival of inoculum will directly influence the chances of successful epizootic development of an entomopathogen in the field. Formulations can increase the field persistence of fungal pathogens by stabilizing their infective units (Gardner *et al.*, 1977).

The aim of the present study was to evaluate the feasibility of using *Hirsutella cryptosclerotium* to control *Rastrococcus invadens*. Different aspects were considered which may provide some insight into the use of this fungal pathogen for the control of *R. invadens*.

7.2 Materials and methods

7.2.1 Environmental conditions

Assays were undertaken in a quarantine controlled environment room, with 16 h light/8 h dark, where the temperature was held constant at 25°C and the relative humidity at 75-80%.

7.2.2 Mealybug cultures

Insects were reared on *Citrus microphylla* seedlings in ventilated Perspex cages (45x45x45 cm). Plants were infested by transferring adults onto leaves with a fine brush or by placing infested leaves on uninfested plants. Infestations of two-spotted spider mite, *Tetranychus urticae*, were controlled by regular applications of predatory mites (*Phytoseiulus* sp.)

7.2.3 Assay procedure

Experiments were carried out either with infested seedlings or with detached citrus leaves bearing mealybugs which were removed from the plants and placed in plastic containers (5x5x15 cm). Whole plants were used in those experiments where only numbers of live insects were recorded, whereas detached citrus leaves were used in those experiments where numbers of both live and dead insects were assessed. The reason for using detached leaves was to prevent dead insects from falling from the plant and

being lost. The relative humidity in the plastic containers was kept at >95% by placing wet filter paper underneath the detached leaves. In those experiments where whole plants were used, humidity was boosted by covering the plants with plastic bags at night.

7.2.4 Preparation and application of inoculum

Except where indicated, isolate IMI 334843 of *Hirsutella cryptosclerotium* was used in this study. The fungal material used as inoculum consisted of dried fragmented mycelium which was sprayed onto infested plants or leaves in water containing 0.01% of Tween 20 as wetting agent. Dried fragmented mycelium was obtained as indicated in Chapter VI. Application of the inoculum was carried out with a hand sprayer (Philip Harris C-42 180, capacity 250 ml) designed for chromatography studies. The amount of inoculum that reached each leaf was standardized by spraying it three consecutive times from a distance of 10 cm. In all experiments, an original volume of 25 ml was prepared per treatment and was sprayed in this way. The amount of suspension reaching the leaf surface was assessed by spraying Petri dishes (5.7 cm diameter) three times with a standard volume of water (25 ml). These dishes were weighed before and after the application, thus, it was found that only 0.8% of the original amount of 25 ml reached a surface of 25.51 cm^2.

7.2.5 Dose-mortality relationship

Citrus leaves infested with mealybugs were sprayed with different solutions of fragmented mycelium: 0.5, 1, 2, 3, and 4 g dry weight/100ml of distilled water plus 0.01% Tween 20. Ten leaves were used per treatment. Natural mortality was assessed by spraying ten leaves with water and wetting agent. Mortality was checked three days after inoculation. For statistical analysis, the percentage mortality was obtained by comparing the number of dead insects with the total for each group of leaves and correcting with Abbott's formula (Abbott, 1925) for natural mortality. The results were

subjected to probit analysis (Finney, 1964).

7.2.6 Effect of inoculum age on mortality

Six batches of ten infested leaves were sprayed with a 2% solution of fragmented mycelium (2 g dry wt./100 ml of distilled water plus 0.01% Tween 20) which had been stored at 4°C for different lengths of time (see Fig. 7.2). Mortality was recorded three days after inoculation.

7.2.7 Relative susceptibility of juveniles and adults

Leaves infested solely with first, second and third instar or female adults of *R. invadens* were treated with a 2% solution of fragmented mycelium (as in 7.2.6). Ten leaves were used per treatment. Three days after inoculation, leaves were examined and the number of dead insects per leaf was recorded and compared with the original number. Control mortality for each particular stage was evaluated by spraying leaves, bearing either first, second and third instar or female adults, with water and wetting agent.

7.2.8 Effect of different wetting agents on mortality

The pathogenic effect of the fungus was tested alone and in combination with three different wetting agents: Tween 20 (emulsifier), Triton X-405 (spreader-sticker) and Triton X-114 (emulsifier). Each one was tested at three different concentrations: 0.01%, 0.05% and 0.1%. A 2% mycelial suspension was used in all treatments. The wetting agents were also applied alone as controls. Ten leaves were used per assay.

7.2.9 Influence of the number of applications on mortality

Plants infested with mealybugs were sprayed with a 4% solution of fragmented mycelium (4 g dry wt./100 ml of distilled water plus 0.01% Tween 20) until run-off. Plants were sprayed either once, twice or three times. The interval between applications was fifteen days. To reduce variability, the initial number of insects per plant was kept as constant

as possible by infesting each plant with eight reproductive female adults and allowing them to produce crawlers for four weeks. Plants were examined weekly for two months and the total number of live insects per plant was recorded. Each treatment was replicated three times. Plants sprayed only with water and wetting agent were used as control. The total volume of inoculum needed to spray one plant until run-off was *ca* 5 ml.

7.2.10 Pathogen persistence on the plant surface

Two different experiments were undertaken. One of them was based on the infestation of plants the leaves of which had been treated previously with a 4% mycelial suspension of *H. cryptosclerotium* (as in 7.2.9) applied until run-off on three consecutive days. To assess the effect of the pathogen, untreated plants were also infested and used as controls. The second experiment was carried out by reinfesting plants which had supported a mealybug population for two months and had recently been eradicated with a 4% mycelial suspension of the pathogen applied until run-off on three consecutive days (mummified dead hosts remained attached to the plants at the moment of reinfestation). Controls consisted of plants which had been supporting a mealybug colony for two months and which had then been eliminated using soapy water. Plants were infested or reinfested by placing eight female adults of *R. invadens* on each plant seven days after treatment with the fungus. Both experiments were replicated three times. Development of the mealybug populations was assessed weekly for two months by scoring numbers of live insects.

7.2.11 Data analysis

Data followed either a binomial or a Poisson distribution. In general, results were tested for significance with the χ^2 parameter.

7.3 Results

7.3.1 Dose-mortality relationship

Fig. 7.1 shows the regression lines between probit mortality and log dose for two strains of *H. cryptosclerotium* (A, IMI 334843 and B, IMI 334844). For both strains this relationship was found to be highly significant (strain A, χ^2=236.32, d.f. 1, p<0.001; strain B, χ^2=216.52, d.f. 1, p<0.001). A high proportion of the variance was accounted for in both cases (strain A, r^2=97.38; strain B, r^2=95.90). The slope of the regression line for strain A was 1.79±0.13 and for strain B 3.01±0.24, these two values being significantly different (t-test, t=9.80, d.f. 8, p<0.001). Strain B was therefore significantly more virulent than strain A. The LD50 and LD95 values and 95% confidence intervals of both values are presented in Table 7.1 (LD95, dose required to reduce the insect population by 95%). For both strains the LD50 is around 1.9 (dry wt., g/100 ml of distilled water plus 0.01% Tween 20), however, the LD95 is 2.4 times higher in strain A than in strain B. This also indicates that strain B is more virulent than A since a much lower dose is required to reduce the mealybug population by 95% with strain B.

Table 7.1. LD50 and LD95 and doses associated with 95% confidence intervals around those values for both strains (A, B) of *H. cryptosclerotium*.

	A	B
LD50	1.92	1.87
95% c.i.	1.75-2.11	1.71-2.02
LD95	15.87	6.57
95% c.i.	11.95-23.19	5.61-8.12

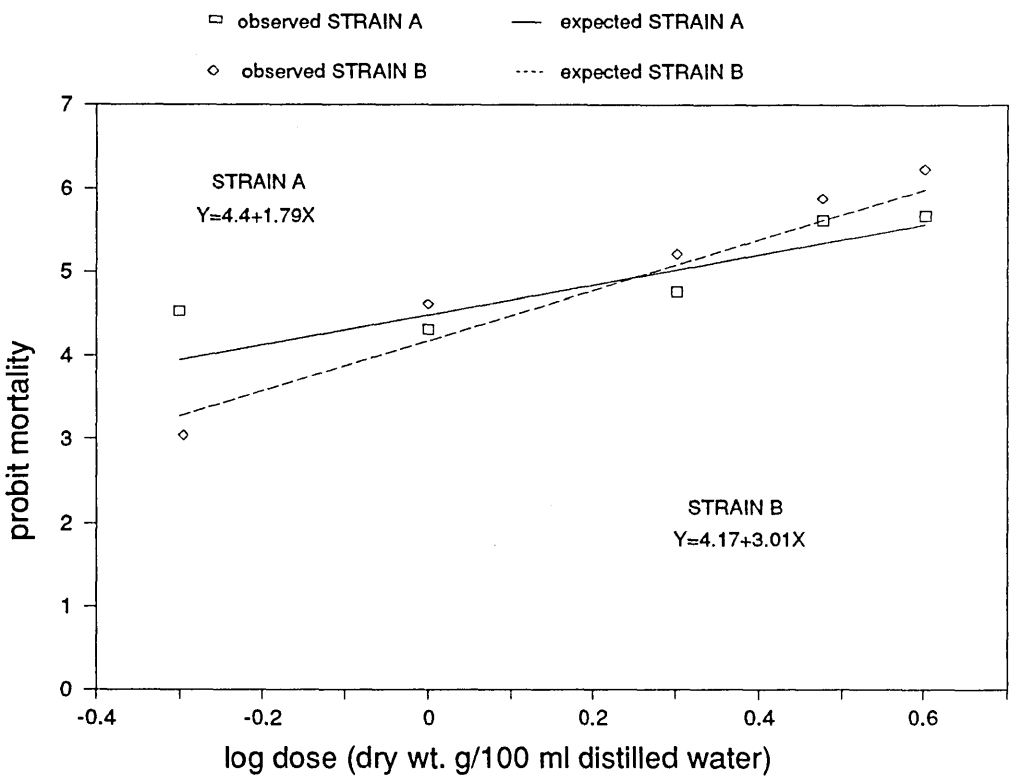


Fig. 7.1. Dose-mortality relationships between *H. cryptosclerotium* and *R. invadens*: regression lines between probit mortality and log dose for two strains (A, B) of *H. cryptosclerotium*. Strain A, 97% of the variance accounted for; strain B, 95% of the variance accounted for. Data in App. 7.1.

7.3.2 Effect of inoculum age on mortality

Fig. 7.2 shows that fragmented mycelium of *H. cryptosclerotium* stored as a dry powder at 4°C did not lose virulence against *R. invadens* within a period of 12 months (ANOVA χ^2=2.82, d.f. 5, n.s.).

7.3.3 Relative susceptibility of juveniles and adults

First and second nymphal stages were the most susceptible to infection by *H. cryptosclerotium* (Fig. 7.3). Third instar nymphs showed a slightly higher mortality when the fungus was applied to them, but female adults did not respond significantly to it at the concentration used in this experiment (2 g dry wt./100 ml of distilled water plus 0.01% Tween 20).

7.3.4 Effect of different wetting agents on mortality

In general, results in Fig. 7.4 indicate that the infectivity of *H. cryptosclerotium* against *R. invadens* was not significantly increased by the addition of three wetting agents at different concentrations. In most cases where mortality was significantly higher with the addition of wetting agents, mealybugs died from drowning within a few hours after application. This is shown by the high control mortalities recorded. However, with the addition of Triton X-405 at 0.05% (arrow) mortality was significantly increased (*t*-test, t=231.25, d.f. 18, p<0.001), the control mortality being significantly lower.

7.3.5 Influence of the number of applications on mortality

Figure 7.5 reveals the effect of the number of fungal applications on mortality. In all trials the control results are very similar, with the different mealybug populations fluctuating around the initial values. However, the number of live mealybugs dropped rapidly after treatment with *H. cryptosclerotium*. In all experiments most mortality was caused in the first week after treatment. In experiment 1, in which the initial mealybug population was treated only once, there was some recovery and the populations increased

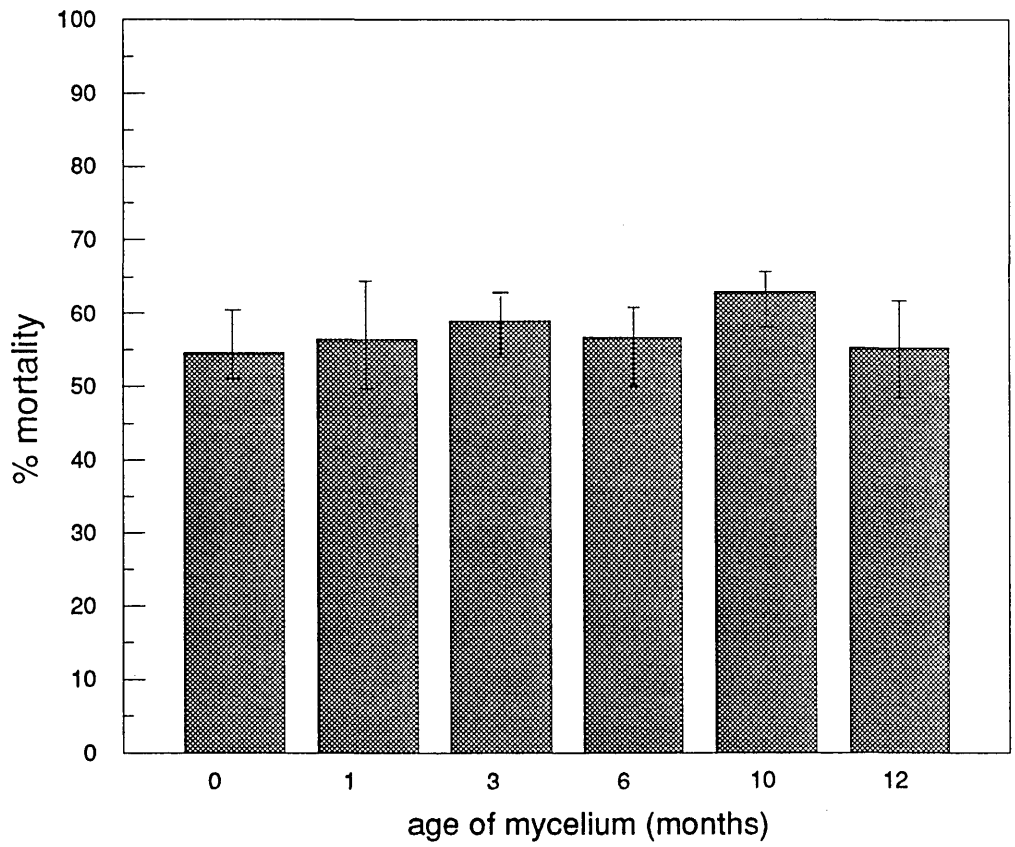


Fig. 7.2. Effect of inoculum age on *R. invadens* mortality. Data are mean ± s.e. Data in App. 7.2.

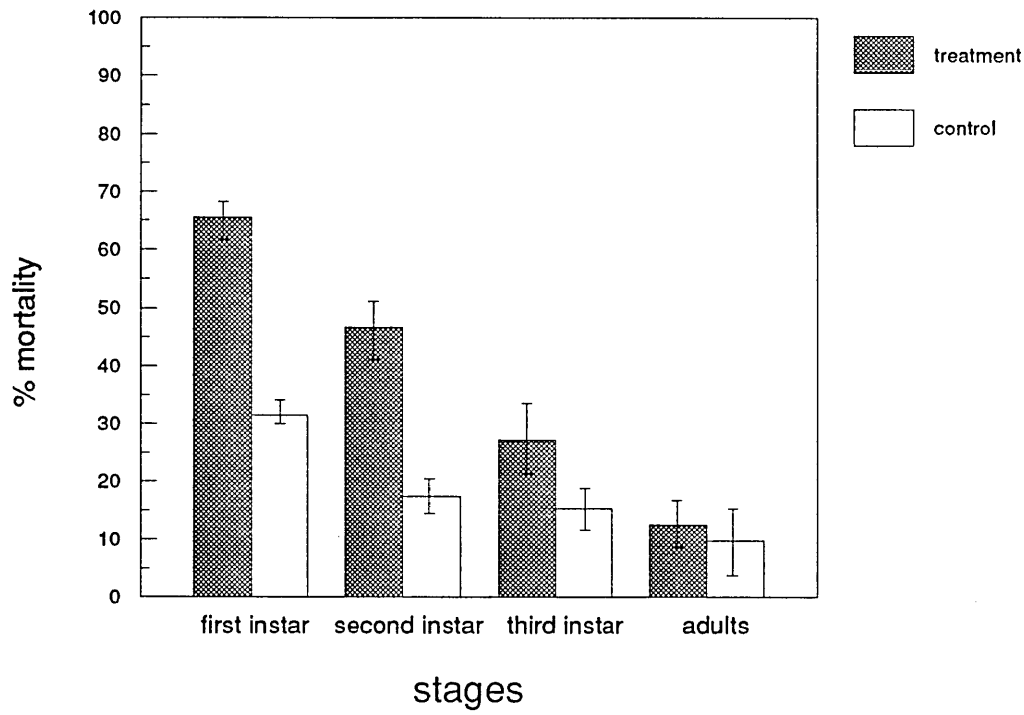


Fig. 7.3. Relative susceptibility of juveniles and adults of *R. invadens* after application of a 2% mycelial suspension of *H. cryptosclerotium*. Data are mean ± s.e. Data in App. 7.3.

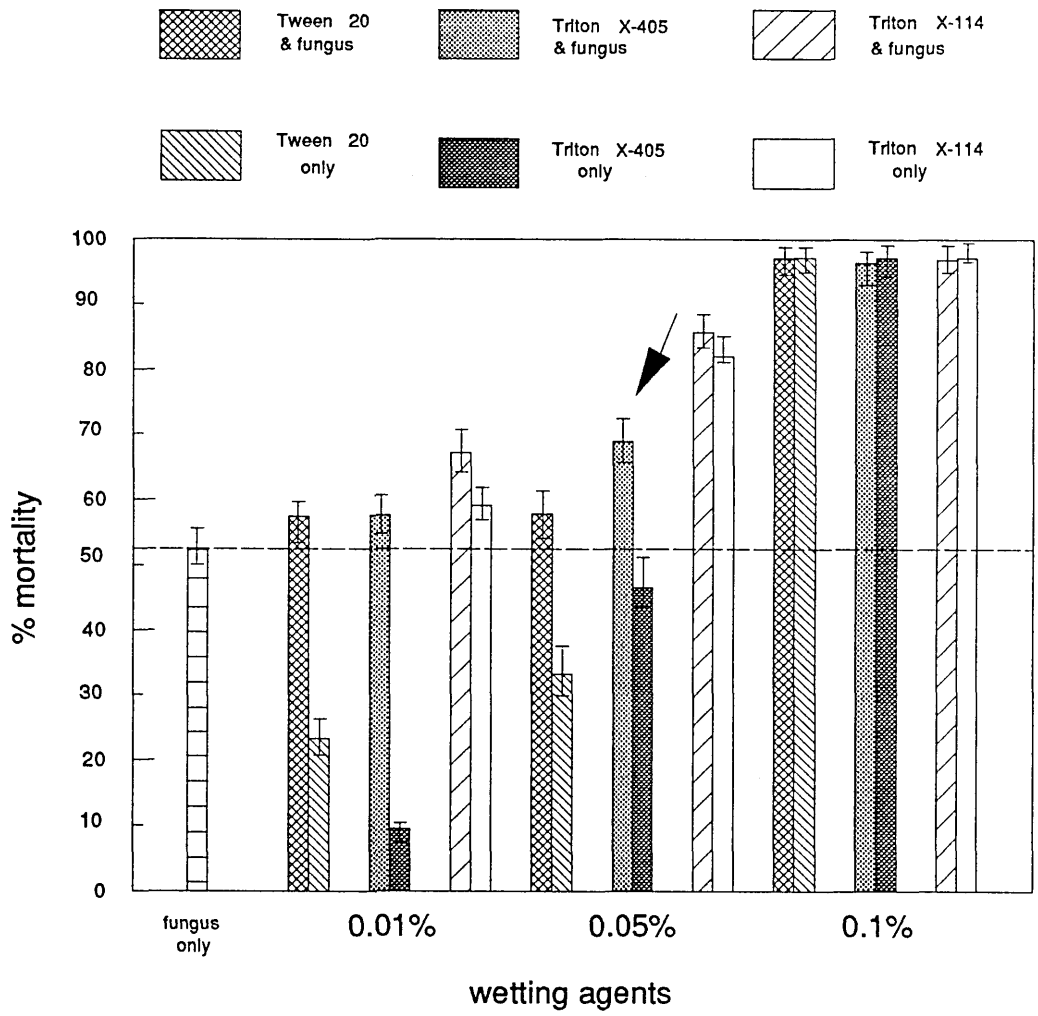


Fig. 7.4. Effect of using different wetting agents on mortality of *R. invadens* by *H. cryptosclerotium*. Data are mean ± s.e. Data in App. 7.4.

during the four weeks after application, then decreased due to secondary infections. There was no difference between the mealybug populations treated two or three times nor was there any definite recovery amongst the multiple-treated populations.

7.3.6 Pathogen persistence on the plant surface

Results in Fig. 7.6, show that after both infestation and reinfestation, mealybug numbers began to rise, but later populations were depressed compared with the control and held at a low level by fungal attack. This indicates that sufficient inoculum had persisted on the plants to establish an epizootic. It is important to note that mealybug populations on those plants which had been reinfested were kept at a lower level by the fungus than on those which had been infested for the first time. This may indicate that the amount of inoculum on the former plants was possibly greater due to *in vivo* sporulation on the dead hosts already present on them.

7.4 Discussion

One aim of this study was to demonstrate the pathogenic nature of *H. cryptosclerotium* against *R. invadens*. A number of fungi have been recorded as pathogenic to mealybugs (Dick, 1969), but many of these records are unsubstantiated and were made by entomologists rather than pathologists (Moore, 1988). *H. cryptosclerotium* is thus one of the very few fungi proven to be pathogenic to mealybugs.

The bioassay results on dose-mortality relations of *H. cryptosclerotium* against mealybug populations may not necessarily be applicable under field conditions. However, from these results it can be concluded that this pathogen has potential for use against *R. invadens* in a practical situation. Nevertheless, it is useful to indicate the dose necessary to cause a high infection rate: a dose of 6.57% is needed to infect 95% of the population using strain B under laboratory conditions.

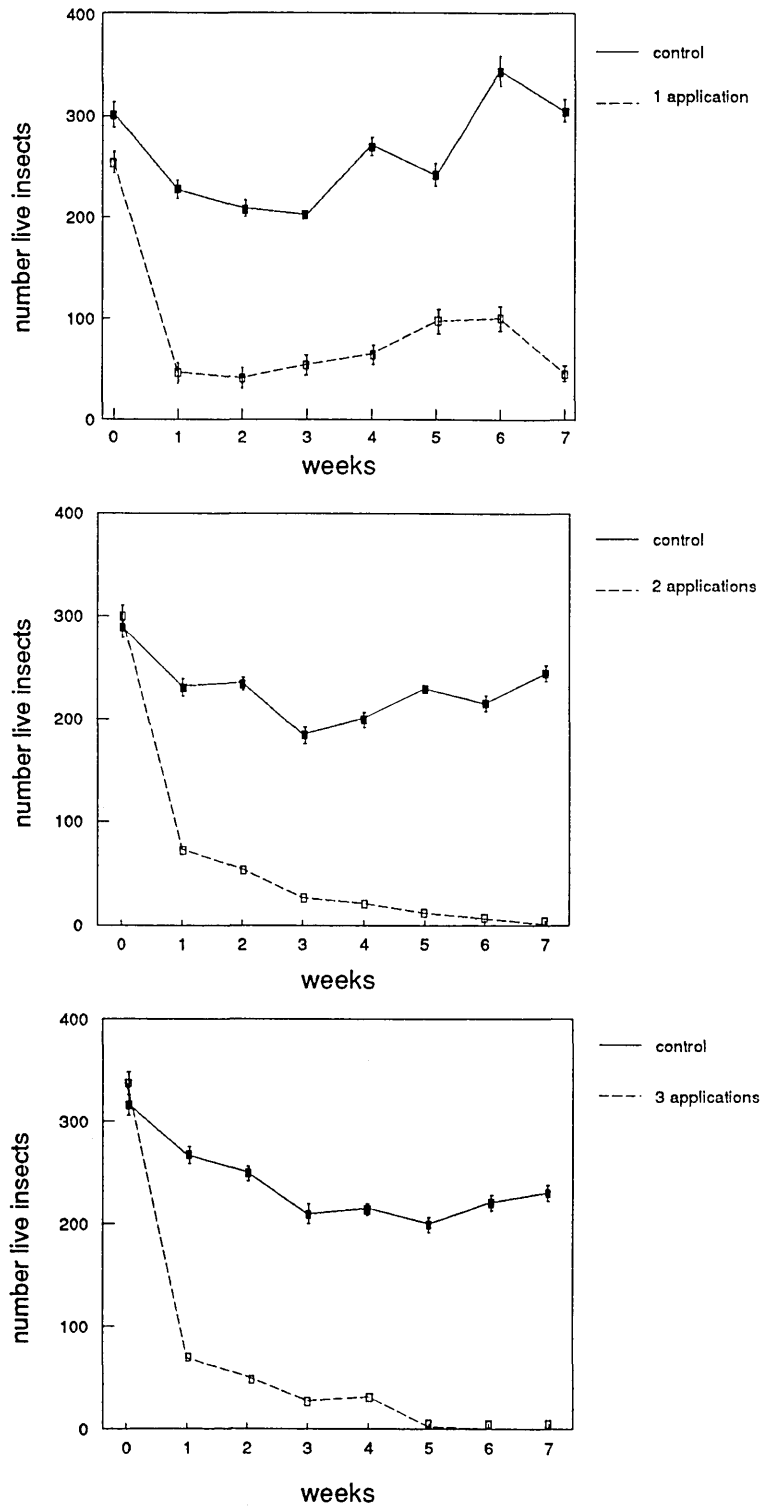


Fig. 7.5. Influence of the number of applications of inoculum of *H. cryptosclerotium* on *R. invadens* mortality. Data are mean ± s.e. Standard errors (not drawn) were very small; see App. 7.5.

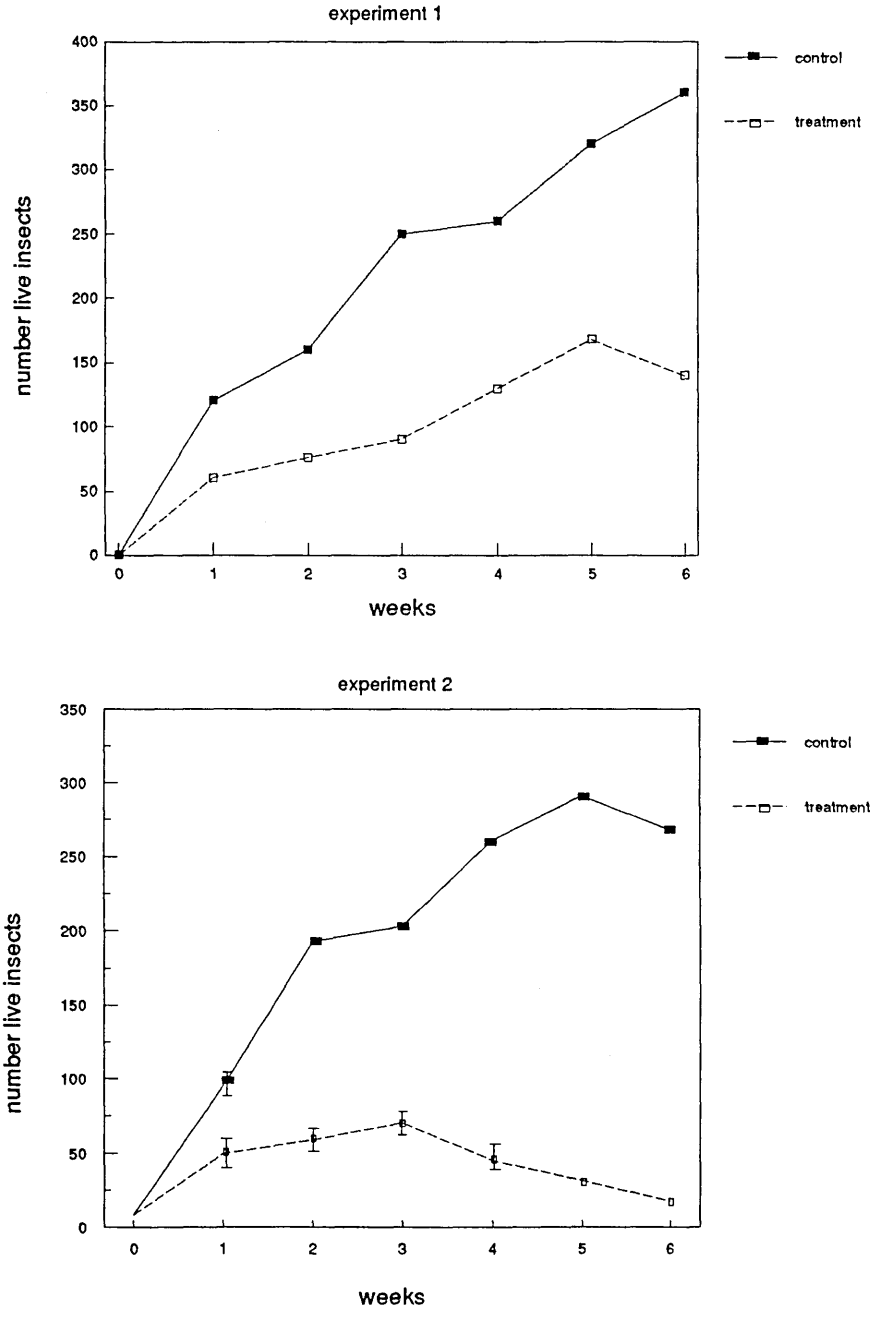


Fig. 7.6. Persistence of *H. cryptosclerotium* inoculum on the plant surface. Experiment 1: Infestation with *R. invadens* of plants which leaves had been previously treated with three applications of a 4% mycelial suspension of *H. cryptosclerotium*. Experiment 2: Reinfestation of plants which had supported a mealybug population for two months and was eradicated with three applications of a 4% mycelial suspension of *H. cryptosclerotium*. Data are mean ± s.e. Standard errors (not drawn) were very small; see App. 7.6.

A differential susceptibility of juveniles and adults of *R. invadens* has been clearly demonstrated in the present study. The young may be more susceptible to the fungus because their cuticle has a thinner wax layer than adults as revealed with the scanning electron microscope (personal observation).

Tests of viability of stored fragmented mycelium of *H. cryptosclerotium* revealed that virulence was not lost after 12 months. Further work needs to be carried out to find out whether the mycelium could remain stable for 18 months, the recommended shelf-life to be commercially economical (Couch & Ignoffo, 1981).

Formulation and spraying techniques may improve the performance of the fungus under field conditions and thus it may be possible to reduce the dose needed to achieve successful control. Wetting agents can affect the LD50 by facilitating better adhesion of the inoculum to the insect cuticle (Hall, 1982c). The results of this study indicate that the wetting agent Triton X-405 (spreader-sticker) at a concentration of 0.05% could be used to increase fungal mortality. The use of spreader-stickers, rather than emulsifiers, has also been recommended by McCoy & Couch (1982) to improve the effectiveness of *H. thompsonii* against the citrus rust mite *Phyllocoptruta oleivora*.

The effect of the number of applications has also been demonstrated. The study suggested that one or two applications could exert control over the mealybug populations for an appreciable time. This is probably due to subsequent populations becoming infected from fungal propagules sporulating on dead hosts.

The results of the investigations on inoculum persistence indicated that under optimal environmental conditions *H. cryptosclerotium* can survive on the plant surface for two months. The ability of an entomopathogen to remain viable in a potential habitat of the host insect for prolonged periods is an important factor affecting the effectiveness of naturally occurring and introduced pathogens. The main factors influencing survival on plant surfaces are temperature, relative humidity and light (Campbell, 1985). These aspects will be studied in detail in Chapter VIII.

Chapter VIII

Environmental factors affecting the stability of *Hirsutella cryptosclerotium*

8.1 Introduction

8.1.1 Ultraviolet radiation

Natural sunlight constitutes a natural environmental factor which affects the stability of microorganisms (Ignoffo & Hostetter, 1977; Roberts & Campbell, 1977). This is mainly due to the absorption of UV radiation by nucleic acids and, to a much lesser extent, by proteins and other biologically important molecules present in the cells (Harm, 1980). Visible light also causes mortality through photooxidation and/or simple overheating (Leach, 1971).

Since it is known that the insecticidal properties of microorganisms can be inactivated by natural or artificial sunlight, research has been undertaken on the influence of UV radiation on viruses (Cantwell, 1967; David, 1969; Witt & Stairs, 1975), bacteria (Cantwell, 1967; Ignoffo *et al.*, 1977), protozoa (Sikorowski & Lashomb, 1977; Kaya, 1977; Kelly & Anthony, 1979), nematodes (Gaugler & Boush, 1978) and fungi (Tuveson & McCoy, 1982; Zimmermann, 1982; Carruthers *et al.*, 1988) to maximize their effectiveness as microbial control agents. Recently, emphasis has been placed on the use of effective UV protectants (Ignoffo & Batzer, 1971; Shapiro, 1985; Killick, 1986)

Light affects many aspects of growth, development, reproduction and behaviour of fungi (for reviews see, Hollaender, 1955; Carlile, 1965; Page, 1965; Leach, 1971; Tan, 1978). These effects have been classified by Page (1965) as morphogenetic and non-morphogenetic. Morphogenetic effects imply the inhibition or formation of a structure (e.g., fruiting bodies, sclerotia, germinating structures) (Chang & Calpouzos, 1971; Leach, 1971; Bergman, 1972). In

non-morphogenetic effects, only the rate or the direction of movement or growth of a structure are affected. Non-morphogenetic responses may be orientated, the response having a spatial relationship to the source of illumination, e.g., phototropism (Carlile, 1965), phototaxis (Carlile, 1975), or non-orientated, either a stimulation or an inhibition of the rate of growth or the synthesis of a compound (Batra, 1971).

In nature, most fungi are exposed to a little far ultraviolet radiation (FUV) (mainly 290-300 nm wavelength), and to considerable near ultraviolet (NUV) (300-380 nm) and visible (UV) (380-750 nm) radiation. However, most work has concentrated on the actions of wavelengths below 300 nm which are not present in the solar UV reaching the surface of the earth (Peak & Peak, 1982). This presents a limitation to our knowledge of ultraviolet effects in nature, since responses induced by NUV differ greatly from those induced by FUV (Webb, 1977).

8.1.2 Temperature

There are strict temperature limits within which the growth, development and metabolic processes of fungi can take place (Anderson & Smith, 1976; Anderson, 1978). The optimum temperature of most entomogenous fungi for growth, sporulation and spore germination lies between 20°C and 30°C. The optimum can vary with the native locality of the fungal isolates, e.g., isolates of *Beauveria bassiana* collected in northern Russia had lower optimum temperatures than isolates from southern Russia (Roberts & Campbell, 1977).

The relationship between temperature and development of insect mycoses under laboratory conditions has been studied in detail for many organisms (Roberts & Campbell, 1977). In general, both the rate of disease development and the level of disease are higher at the optimum temperature of a fungus than at lower temperatures. In some cases, however, there are exceptions: reduced

temperatures increased the time from infection to mortality without reducing total mortality (e.g., *Entomophthora aphidis* infecting the pea aphid, *Acyrthosiphon pisum*, Wilding, 1970). Another phenomenon was described by Fargues (1972), who found that the number of third and fourth instar larvae of *Leptinotarsa decemlineata* successfully infected with *B. bassiana* at 25°C was less than the number infected at 20°C, although the optimum temperature for growth of this fungus on agar was 25°C. He argued that asynchronous development of the fungus and the host resulted in an escape of the insect from infection by moulting to the next stage, thus leaving the spores behind on the empty exuviae. Temperatures above the optimum may reduce or eliminate insect mortality due to the low infectivity of the fungus.

8.1.3 Relative humidity

High relative humidity is presumed to be essential for the success of fungi in insect control, since it is required for spore germination. However, reports on the requirements of different fungi for high relative humidity for infection of their host insects, indicated that very high humidity levels apparently were not essential (Moore, 1973). In some cases, the discrepancies can be explained by noting that the microclimate of the fungal spore was humid even though the macroclimate was rather dry, e.g., leaf surface (Burrage, 1976; Dickinson, 1986). However, most entomopathogens required the presence of high humidity to sporulate on the surface of their hosts. Sporulation of the fungus on the host after death is critical for the establishment of epizootics (Roberts & Campbell, 1977).

Different techniques have been described to maintain a constant relative humidity in biological systems (Johnson, 1940; Wiston & Bates, 1960; Solomon, 1971; Anonymous, 1983). There are two alternative ways of providing a series of humidities by the use of solutions. The first is to make up, from a suitable

compound, a series of solutions at concentrations adjusted to provide the appropriate water vapour pressures. The second is to use saturated solutions of different salts which can be selected according to the humidities required. According to Solomon (1971), only the first method will give a precise and evenly graded series.

In this chapter, attention was focused on the influence of ultraviolet radiation, temperature, and relative humidity on the infectivity of *Rastrococcus invadens* by *H. cryptosclerotium*.

8.2 Materials and methods

8.2.1 Ultraviolet radiation

8.2.1.1 Ultraviolet source

The ultraviolet radiation source used consisted of a single 40 w sunlamp (Philips Tl 40w/09, peak 365 nm; range 290-500 nm), 60 cm in length. The intensity of the sunlamp at the plane of the different materials (agar medium or leaves) was measured using a laboratory light meter designed by Young *et al.* (1987) (Table 8.1). The lamp was positioned 28 cm above the incident surfaces. All UV experiments were carried out in a dark quarantine growth chamber with constant air temperature (25±1°C) and relative humidity (75-80%), between 21.00 and 09.00 hours.

Table 8.1 Measurement of the radiation intensity emitted at 330-375 nm by a NUV source reaching different incident surfaces.

	intensity (w/cm^2)
agar medium	$41.03\ 10^{-3}$
leaves	$44.76\ 10^{-3}$

8.2.1.2 Fragmented mycelium and spore production

Fragmented mycelium was produced as described in Chapter VI, and 1 ml of a 0.5% suspension (dry wt., g/100 ml sterilized distilled water) was plated onto Petri dishes (9 cm diam) containing malt agar with antibiotics (Penicillin at 50 units/ml and Streptomycin at 100 units/ml). The Petri dishes were kept in a 25°C incubator with a light regime of 16 h light /8 h dark. After 10 days, the sporulating mycelium from two dishes was removed from the agar and suspended in sterilized distilled water plus 0.01% Tween 20 as wetting agent. The suspension was agitated by hand for 5 min and filtered with a 180 μm mesh. The resultant solution contained $1.5x10^5$ spores/ml, as determined by haemocytometer counts.

8.2.1.3 Treatments

In the first series of tests, the survival of spores and fragmented mycelium after exposure to UV radiation was determined. Petri dishes (5 cm diam) containing malt agar plus antibiotics were inoculated, with either 0.5 ml of a 0.5% solution of fragmented mycelium (dry wt., g/100 ml distilled water) or 0.5 ml of the spore solution described above. A sterilized bacteriological glass spreader was used to spread the different inocula evenly over the surface of the agar. The dishes were left to dry without the lids for 30 min in a laminar flow cabinet, and then with lids replaced were exposed to the UV source for different periods (see Figs 8.2-8.3). Petri dishes wrapped in aluminium foil were used as controls. Each treatment was replicated three times. After exposure to UV radiation, the treated Petri dishes were also wrapped in aluminium foil and held in a 25°C incubator together with the controls. Those inoculated with spores were kept for 24 h and those inoculated with fragmented mycelium for 96 h.

Following the incubation period, the survival of the inoculum was evaluated. Survival of the fragmented mycelium was assessed by counting the number of colony forming units per dish after 24, 48, 72 and 96 h incubation. To determine spore survival about 100 spores per dish were examined for germination under a light microscope (x400 magnification). For this purpose, three drops of 0.03% Trypan blue in lactophenol were added to each Petri dish and a circular coverslip (19 mm) was placed over each drop. A spore was considered to have germinated when the length of the germ tube was equal to or exceeded the breadth of the spore.

In the second series of experiments, groups of detached citrus leaves infested with *R. invadens* were sprayed with a 2% mycelial suspension (dry wt. g/100 ml distilled water plus 0.01% Tween 20) and placed in plastic containers (5x5x15 cm). The sprayed leaves were exposed to UV radiation for different lengths of time (see Fig. 8.3). After exposure to UV radiation, the leaves were kept in the dark. Ten sprayed leaves were used as controls. Mortality of *R. invadens* was assessed three days later.

8.2.2 Temperature

8.2.2.1 Viability of *H. cryptosclerotium* mycelium at different temperatures

The effect of temperature on *H. cryptosclerotium* was studied by plating 0.5 ml of a 0.5% solution of fragmented mycelium on each of 5 Petri dishes (9 cm diam) containing malt agar plus antibiotics, and counting the number of colony forming units per dish after 24, 48, 72 and 96 h.

8.2.2.2 Influence of temperature on the infectivity and incubation period of *H. cryptosclerotium* on *R. invadens*

Detached citrus leaves infested with *R. invadens* were sprayed with a 2% mycelial suspension of *H. cryptosclerotium* and placed in incubators at 15, 20, 25, 30, 35 and 37°C, each with a 16 h light/8 h dark photoperiod. Mortality was assessed three days later. Leaves sprayed only with water and wetting agent were used as controls. There were ten leaves per treatment. To evaluate the influence of different temperatures on the incubation period, numbers of dead mealybugs bearing sporulating mycelium were simultaneously recorded three and six days after inoculation.

8.2.3 Relative humidity

8.2.3.1 Effect of relative humidity on sporulation of *H. cryptosclerotium* mycelium

A 2% mycelial suspension was sprayed on to the bases of Petri dishes (5 cm diam) which were then placed in desiccators kept at 60, 70, 80, 85, 90, 95 and 100% r.h. (Anonymous, 1983). The desiccators were later placed in incubators at 25°C with a 16 h light/8 h dark photoperiod. Five Petri dishes were used per level of relative humidity and were checked after 2, 4, 6, 8, 10, and 12 h following inoculation to record the time needed for the mycelium to start sporulating at different relative humidities.

8.2.3.2 Influence of relative humidity on the infectivity of *H. cryptosclerotium* on *R. invadens*

Detached citrus leaves, infested with *R. invadens*, were sprayed with a 2% mycelial suspension of *H. cryptosclerotium* and placed in desiccators kept

at 60, 70, 80, 85, 90, 95 and 100% r.h. There were ten leaves per treatment. Leaves sprayed only with water and wetting agent were used as controls. The desiccators were then placed in incubators at 25°C with a 16 h light/8 h dark photoperiod. Mortality was assessed three days later. The relative humidity required for sporulation on the dead hosts was also recorded.

8.2.4 Data analysis

In general, results followed a binomial distribution. Except where indicated, significance was tested with the χ^2 parameter.

8.3 Results

8.3.1 Effect of ultraviolet radiation on *H. cryptosclerotium* viability

8.3.1.1 Mycelium

Fig. 8.1 clearly indicates that the viability of mycelium of *H. cryptosclerotium* was not affected by exposure to a source of near ultraviolet radiation for up to 12 h (ANOVA, χ^2=7.30, d.f. 5, n.s.). Percentages of colony forming units increased to 100% after incubation for 96 h in all treatments.

8.3.1.2 Spores

No spores germinated after direct exposure to NUV radiation for 2 or more hours (Fig. 8.2). Spore viability was reduced to 56% and 29% respectively after 30 min and one hour's exposure.

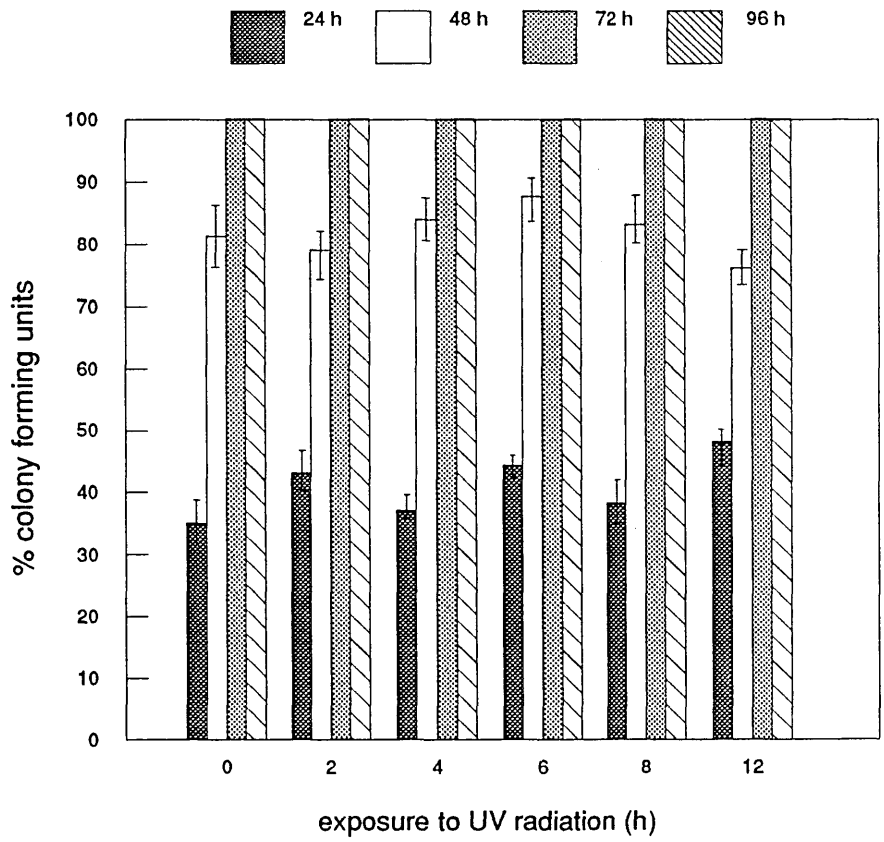


Fig. 8.1. Effect of ultraviolet radiation on the viability of mycelium of *H. cryptosclerotium*. Data are mean ± s.e. Data in App. 8.1.

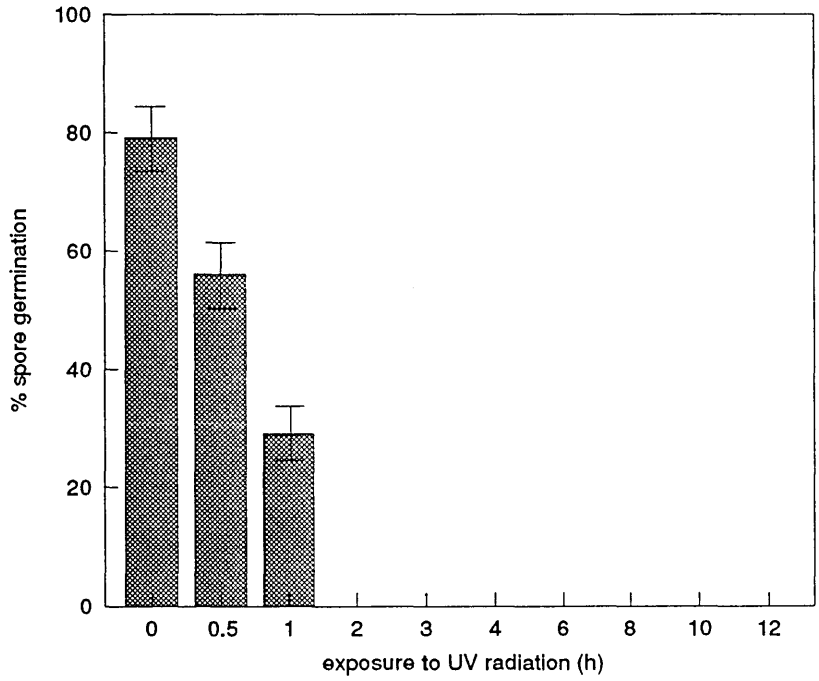


Fig. 8.2. Effect of ultraviolet radiation on the viability of spores of *H. cryptosclerotium*. Data are mean ± s.e. Data App. 8.2.

8.3.2 Influence of ultraviolet radiation on the infectivity of *H. cryptosclerotium* to *R. invadens*

Results in Fig. 8.3 show that the infectivity of *H. cryptosclerotium* to *R. invadens* was not influenced by exposures to a NUV source for periods up to 12 h (ANOVA, χ^2=6.98, d.f. 5, n.s.).

8.3.3 Viability of *H. cryptosclerotium* mycelium at different temperatures

Temperature significantly affected the viability of mycelium of *H. cryptosclerotium* (Fig. 8.4; ANOVA, χ^2=150.23, d.f. 5, p<0.001). At 15°C, colony-forming units were observed after 96 h and at 20°C after 48 h, but, at higher temperatures, they were observed after 24 h. At 37°C, no colonies developed, however, some colonies formed when these Petri dishes were transferred to 25°C after the experiment was concluded. It interesting to note that, although *H. cryptosclerotium* grows better at 25°C on malt agar (Fig. 8.5), higher percentages of colony-forming units were recorded at 30°C and 35°C after 24 h.

8.3.4 Influence of temperature on the infectivity and incubation period of *H. cryptosclerotium* on *R. invadens*

Infection of *R. invadens* took place at all temperatures except 37°C (Fig. 8.6; ANOVA, χ^2=100.31, d.f. 6, p<0.001). Mortalities at 20, 25, 30 and 35°C did not differ significantly.

Temperature significantly affected the incubation period of *H. cryptosclerotium* infecting *R. invadens* (Fig. 8.7; ANOVA, χ^2=131.16, d.f. 5, p<0.001). More time was needed to complete infection at both 15°C and 20°C

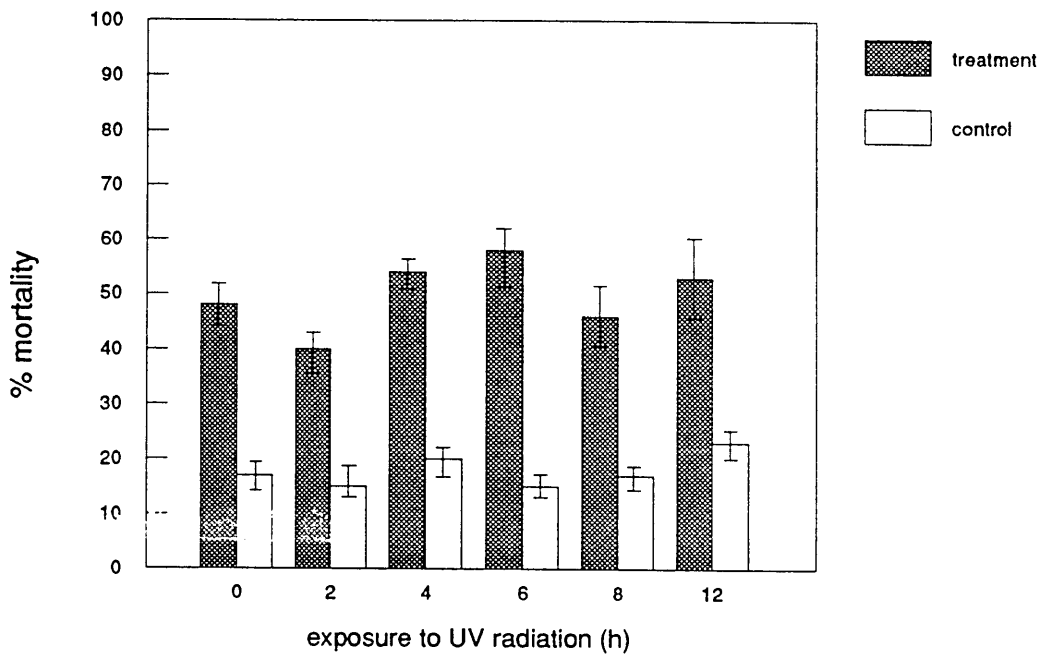


Fig. 8.3. Influence of ultraviolet radiation on the infectivity of *H. cryptosclerotium* to *R. invadens*. Data are mean ± s.e. Data in App. 8.3.

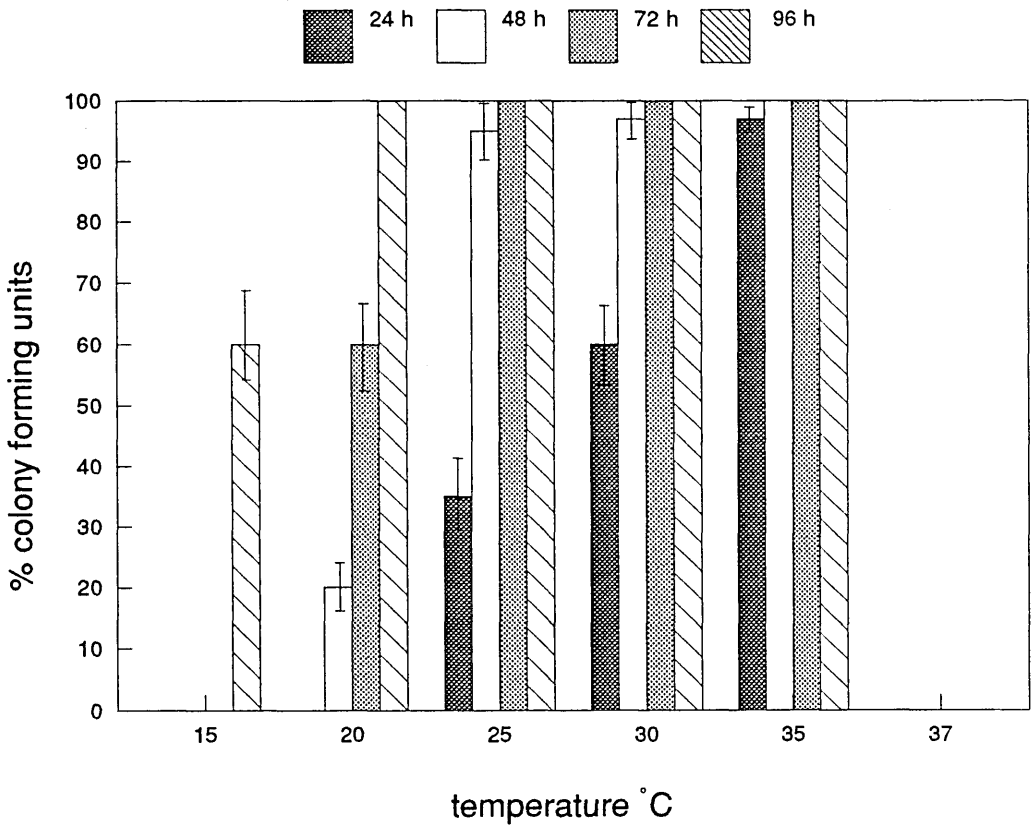


Fig. 8.4. Viability of *H. cryptosclerotium* mycelium at different temperatures. Data are mean ± s.e. Data in App. 8.4.

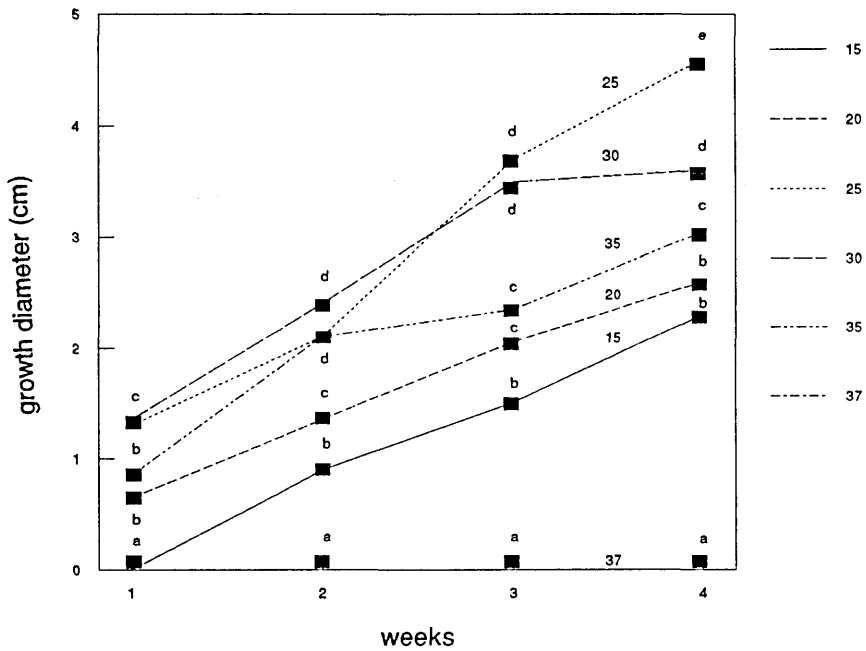
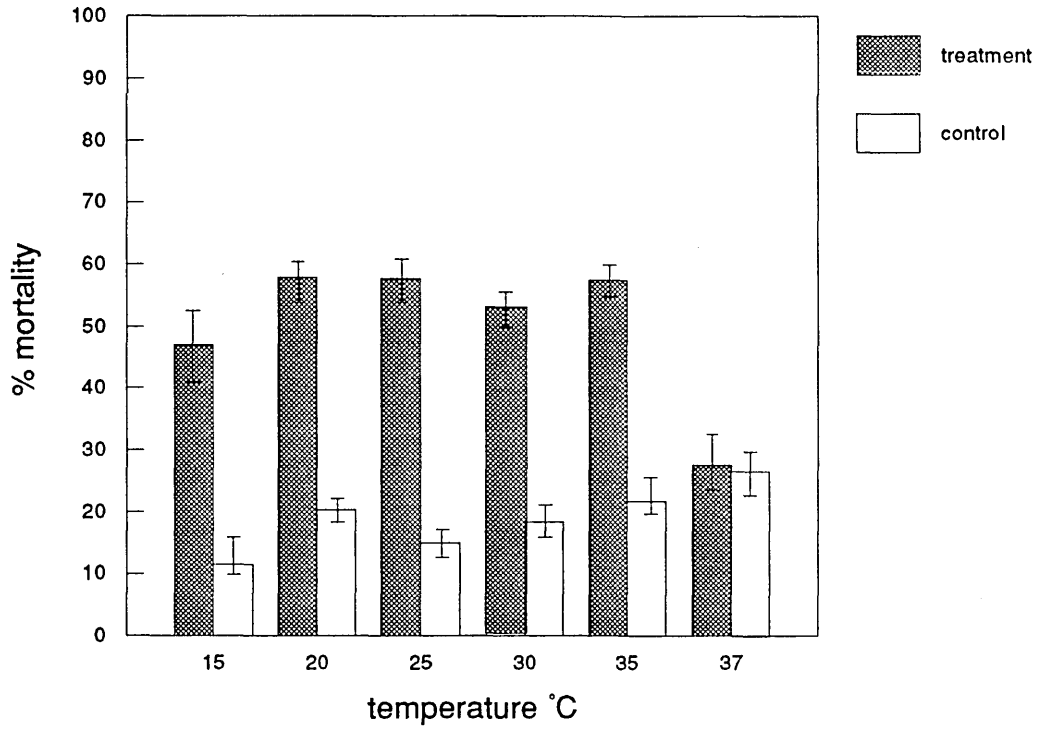
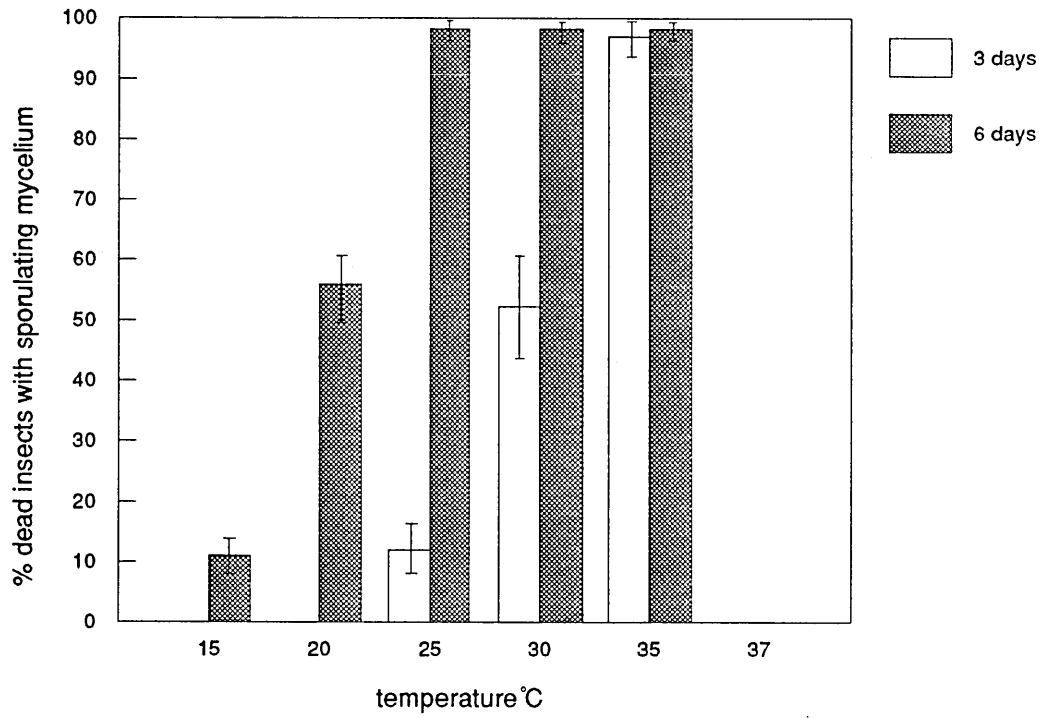


Fig. 8.5. Growth of *H. cryptosclerotium* on malt agar at different temperatures (°C) (ANOVA, $F_{(5,45)}$=5.275, p<0.001). Data marked by different letters are significantly different (t-test, p<0.001). Data are mean (from 5 replicates). Data in App. 8.5.



Figs 8.6. Influence of temperature on the infectivity of *H. cryptosclerotium* to *R. invadens*. Data are mean ± s.e. Data in App. 8.6.



Figs 8.7. Influence of temperature on the incubation period of *H. cryptosclerotium* on *R. invadens*. Data are mean ± s.e. Data in App. 8.7.

than at higher temperatures. Results in Fig. 8.7 also show that the incubation period is shorter at 35°C than at 30°C or 25°C, although this is a suboptimal temperature for prolonged growth of the fungus on agar (Fig. 8.5).

8.3.5 Effect of relative humidity on sporulation of *H. cryptosclerotium* mycelium

H. cryptosclerotium mycelium started sporulating after 12 h at all humidities except 60%. A nonquantified observation was that the number of phialides produced per individual fragmented unit of mycelium decreased with decreasing relative humidity

8.3.6 Influence of relative humidity on the infectivity of *H. cryptosclerotium* to *R. invadens*

Maximum mortality was obtained at 80% r.h. and above (Fig. 8.8) suggesting that in excess of 70% may be required to obtain successful infection of *R. invadens* by *H. cryptosclerotium* (ANOVA, χ^2=114.56, d.f. 6, p<0.001). Sporulation on the host surface only took place at 100% r.h.

8.4 Discussion

Experiments on the effect of NUV radiation on the viability of *H. cryptosclerotium* in this study revealed that: (a) spore viability was completely lost after 2 h of exposure; (b) fragmented mycelium was not affected after 12 h of exposure as indicated by the viability and pathogenicity tests. Kenneth *et al.* (1979) reported that *H. thompsonii* mycelia treated with potentially damaging doses of far-ultraviolet radiation were unaffected. However, Tuveson & McCoy (1982) found that *H. thompsonii* spores exposed to the same radiation were not viable after 1 h of exposure. According to Roberts & Campbell (1977), UV radiation is usually detrimental to spore survival, but effects on vegetative mycelium are, in general, not

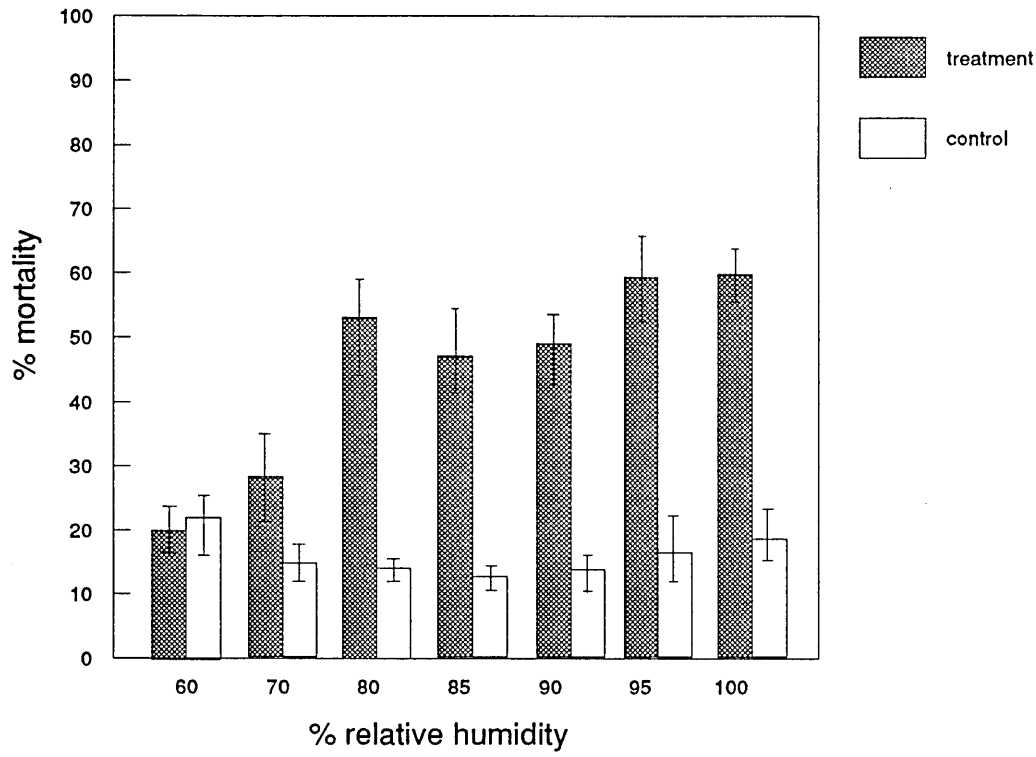


Fig. 8.8. Influence of relative humidity on the infectivity of *H. cryptosclerotium* to *R. invadens*. Data are mean ± s.e. Data in App. 8.8.

so damaging. Sensitivity of spores is frequently related to pigmentation; in addition, thickness of the spore wall may also be involved in the ability of fungi to tolerate radiation (Leach, 1971). The resistance shown by *H. cryptosclerotium* mycelium to NUV, could be due to the fact that the surface mycelium in the fragmented units was protecting the mycelium underneath.

Studies on the survival of *Entomophaga grylli* conidia, both under laboratory conditions (exposure to artificial light) and in the field (sunlight), have revealed that fungal spores may survive for a longer period in the field. Conidia exposed in lower canopy areas and in canopies with more dense foliage survived significantly longer (Carruthers *et al.*, 1988). Similarly, Brobyn *et al.* (1985) found that *Erynia neoaphidis* conidia may remain infective for at least 14 days in bean canopies. They also found that infectivity persisted longest when there was less sun. Therefore, it should be taken into consideration that *H. cryptosclerotium* conidia in the field, under highly cloudy conditions or conidia located deeper in the foliage, may remain viable significantly longer than the times indicated in the present study.

Results on the effect of temperature on mortality of *R. invadens* by *H. cryptosclerotium* clearly indicated that although 25°C is the optimum temperature for long term growth of this pathogen, infection is possible within a broad range of temperatures (15°C to 35°C). However, the infection process was slower with lower temperatures. Fransen (1987) reported that *Trialeurodes vaporariorum* was successfully infected by spores of *Aschersonia aleyrodis* at 30°C and that the development of the infection was faster than at 25°C, the optimum temperature for spore germination. She pointed out that high temperatures might have influenced the susceptibility of the host insects, since relatively higher mortalities were recorded in the controls at 30°C. In the present study, however, control mortalities did not significantly increase at temperatures higher than 25°C, the optimum temperature for *R. invadens* development (A. Steenkiste, unpublished). This may be beacuse the

experiment lasted only three days, since Steenkiste (unpublished) found that survival of all different stages of *R. invadens* was significantly reduced after long term exposure to 30°C.

In the present study it has been shown that very high humidities may not be required for *H. cryptosclerotium* mycelium to sporulate and cause mortality on *R. invadens*. Different factors may account for this. Preliminary experiments revealed that fragmented mycelium applied as an aqueous suspension was able to retain water (acting as a sponge) for a period of 24 h at 70% r.h. or longer at higher relatives humidities; however, it was found that only 12 h are required for the fragmented mycelium to sporulate. Alternatively, the relative humidity in the microclimate around the fragmented mycelium could have been higher than in the surrounding air, due possibly to transpiration of water from the insect body and the leaf surface (Burrage, 1976; Dickinson, 1986; Fransen, 1987).

Chapter IX

Development of *Hirsutella cryptosclerotium* as a microbial control agent of *Rastrococcus invadens*

In this chapter, the results already described are considered in relation to the different aspects for evaluation of entomopathogens as biological control agents, shown in Chapter I (Table 1.2). Additionally, suggestions for future research are presented.

9.1 Topics for consideration in the evaluation of *H. cryptosclerotium* for control of *R. invadens*

9.1.1 Fungal characteristics

(a) Specificity of *H. cryptosclerotium*

A fungal pathogen from mummified mealybugs collected in Togo, was identified during the course of the present study as a new *Hirsutella* sp. The pathogen was described as *Hirsutella cryptosclerotium* since it produces sclerotia within the body of the host, a characteristic not found in other *Hirsutella* spp. (Speare, 1920; Evans & Samson, 1982b). As yet, there is no evidence that this pathogen attacks other mealybugs or related Homoptera (Chapter III).

A study of the infection process showed that spores of *H. cryptosclerotium* germinate on the cuticle of the mealybug and appear to penetrate the integument without forming an appressorium. *H. cryptosclerotium* causes death of the insect host before extensive colonization of the haemocoel and host tissues has taken place. When environmental conditions are favourable, the fungus protrudes through the cuticle forming phialides and conidia on the insect host. Sclerotia are produced only under adverse environmental field conditions and have not been observed in laboratory-inoculated insects (H. C. Evans, personal communication)

(Chapter IV).

(b) Virulence of *H. cryptosclerotium*

Bioassays were carried out to obtain information on the virulence of *H. cryptosclerotium* to *Rastrococcus invadens*. The LD50 and LD95 for a mixed population were 1.87 (mycelial suspension, dry wt. g/100 ml water) and 6.57 respectively using strain IMI 334844 of *H. cryptosclerotium*.

One or two applications could exert control over the mealybug populations on single plants for an appreciable time (2 months), under optimal environmental conditions. It was also shown that *H. cryptosclerotium* mycelium applied to the mealybugs' host plants may protect them against mealybug attack (Chapter VII).

(c) Sporulation, spread and persistence

If environmental conditions are favourable, *H. cryptosclerotium* will sporulate extensively on dead infected mealybugs and remain viable for a considerable time, causing secondary infections (Chapter VII). The pathogen could be spread in the field by rain and possibly by wind (wind may disperse infected crawlers (Chapter II) and possibly also small rain-splash droplets carrying inoculum) (Chapter V). Sclerotia formed within the host may constitute an effective reservoir of pathogen inoculum under adverse field conditions (Hochberg, 1989).

(d) Possibilities for mass production

Subculturing of *H. cryptosclerotium* on solid media did not significantly affect the viability and virulence of the fungus. Mycelium of *H. cryptosclerotium* was successfully mass-produced using defined liquid media (abundant mycelial pellets regularly developed after two weeks in shake culture using optimum media) (Chapter VI).

(e) Suitability for storage and formulation

Unformulated mycelium of *H. cryptosclerotium* must be stored at low temperatures and under low relative humidities to remain viable for a considerable length of time. Inoculum stored as a dry powder at 4°C for different lengths of time (up to one year) remained highly pathogenic to *R. invadens*. Formulation might increase mortality of *R. invadens* by *H. cryptosclerotium*; different wetting agents were added to a mycelial suspension at various concentrations, however, only Triton X-405 at a concentration of 0.05% increased fungal mortality significantly (Chapter VII).

(f) Safety and compatibility with other control procedures

H. cryptosclerotium is not expected to cause any negative effects on the environment because of its high specificity. However, this pathogen should be evaluated for possible adverse effects on man, other animals and plants. Special attention must be paid to allergenicity tests (Mier *et al.*, 1989).

Investigation into the use of *H. cryptosclerotium* in conjunction with the parasitoid *Gyranusoidea tebygi* have revealed that the use of both natural enemies together is feasible (Akalach *et al.*, submitted). Compatibility of the pathogen with chemical pesticides needs to be tested (Jaques & Morris, 1981).

9.1.2 Host characteristics

Susceptibility

Results indicated that the different stages of the host are not equally susceptible. First and second nymphal stages were significantly more susceptible than third stage or female adults. Alate males have not been found with confirmed fungal infection (Chapter VII).

R. invadens is less likely to develop resistance to *H. cryptosclerotium* than to chemical insecticides. Pathogens are living organisms that are subject to selection, and a coevolutionary relationship exists between pathogens and hosts under field conditions (Evans, 1989).

9.1.3 Environmental aspects

H. cryptosclerotium spores may lose viability after exposure to direct sunlight in the field. However, mycelium is likely to be less affected by sunlight. Infection of *R. invadens* by *H. cryptosclerotium* occurs within a broad range of temperatures. A very high relative humidity is not required for infection to develop (Chapter VIII).

9.2 Future research

Further work is required on the following aspects, especially under field conditions:

aspects for evaluation	suggestions for future research
toxicological and safety aspects:	tests needed for commercialization
compatibility with insecticides and fungicides:	selection of resistant strains
economic injury level:	timing of application
density and spatial distribution:	application strategy
effects of biotic factors:	host plant quality ant-mealybug mutualism

Finally, it can be concluded that *H. cryptosclerotium* has many of the attributes desirable in a microbial control agent (Burges & Hussey, 1971) and may have potential as a mycopesticide; both spores and mycelium can be used to infect *R.*

invadens, it is relatively easy to culture and appears suitable for mass production, possibly on a commercial scale.

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APPENDICES

	days											
% insects completing:	1	3	5	7	9	11	13	15	17	19	21	23
first instar		20.03 (19.2-22.1)	38.25 (36.5-39.5)	28.59 (26.8-31.7)	9.25 (8.5-11.2)	5.87 (4.5-6.9)						
second instar, male			9.47 (7.5-11.2)	16.52 (14.2-18.1)	16.10 (13.5-18.7)	27.84 (25.7-29.0)	23.02 (21.5-25.4)	23.15 (20.9-26.7)				
second instar, female				6.56 (4.5-7.6)	29.83 (27.8-30.1)	18.01 (17.3-19.6)	16.23 (15.2-17.8)	12.66 (9.5-15.4)	12.83 (10.1-13.8)	12.60 (9.6-14.5)		
third-fourth instars, male								28.89 (27.5-32.9)	31.01 (29.5-33.0)	14.00 (11.9-17.5)	12.75 (9.4-15.4)	13.01 (10.8-15.1)
third instar, female								19.52 (17.6-23.8)	22.61 (18.7-24.5)	25.21 (23.5-27.4)	21.78 (17.5-23.8)	12.01 (9.8-14.5)

App. 2.1. Percentage of nymphs completing first, second and third instar during development of *R. invadens*. Data are mean ± s.e. Data for Fig. 2.5.

App. 2.2. Total number of insects recorded for different stages during development of *R. invadens*. Data for Fig. 2.6.

	number of insects											
	days											
instars	1	3	5	7	9	11	13	15	17	19	21	23
N1	427	289	130	63	12							
N2		138	269	253	223	122	63	21				
C			7	86	78	152	149	121				
N3				1	27	59	80	64	32	11		
male								104	181	197	210	215
female								26	43	60	74	76

App. 2.3. First instar production per adult female of *R. invadens*. Data are mean ± s.e. Data for Fig. 2.7.

	number of crawlers	
weeks	mean	s.e.
1	18.0001	2.6110
2	23.0715	2.7911
3	18.0812	2.7911
4	12.7961	2.7911
5	7.4231	2.7911
6	3.9261	2.8962
7	6.4260	2.8962
8	2.1540	2.8962
9	1.5420	2.8962
10	1.9256	2.8962
11	1.8524	2.8962

App. 3.1. Preliminary results on *H. cryptosclerotium* host range.

species	family	origin	response
Rastrococcus invadens	Pseudococcidae	Togo	positive
Pseudoccocus obscurus	"	UK	negative
Pseudococcus aphinis	"	UK	negative
Phenococcus manihoti	"	Kenya	negative
Phenococcus herreri	"	Kenya	negative
Planococcus citri	"	UK	negative
Coccus celatus	Coccidae	Kenya	negative

App. 5.1. Number of spore carrying droplets at different distances from the source (spore suspension of *H. cryptosclerotium*). Data for Fig. 5.6.

	spore-carrying droplets	
distance (cm)	mean	s.e.
10	19.22	18.41-22.33
20	12.54	11.72-13.83
30	9.10	7.37-11.31
40	1.25	2.20-0.82
50	0	-

App. 5.2. Number of mycelium-carrying droplets at different distances from the source (surface grown cultures of *H. cryptosclerotium*). Data for Fig. 5.8.

	mycelium-carrying droplets	
distance (cm)	mean	s.e.
10	8.54	8.62-8.91
20	2.76	2.47-3.20
30	1.46	0.92-1.65
40	0.56	0.31-0.60
50	0.25	0.13-0.31

App. 5.3. Number of spores collected at different distances from the source (spore suspension of *H. cryptosclerotium*). Data for Fig. 5.10.

	number of spores	
distance (cm)	mean	s.e.
10	470.76	448.13-526.26
20	210.34	175.23-232.52
30	47.87	39.22-52.32
40	45.66	34.26-50.22
50	0	-

App. 5.4. Number of condia per droplet at different distances from the source (spore suspension of *H. cryptosclerotium*). Data for Fig. 5.11.

	number of conidia per droplet	
distance (cm)	mean	s.e.
10	98.34	87.23-106.34
20	26.78	19.32-30.12
30	9.23	4.92-12.45
40	3.76	1.24-4.21
50	0	-

App. 5.5. Size of spore-carrying droplets at different distances from the source (spore suspension of *H. cryptosclerotium*). Data for Fig. 5.12.

	size (µm)	
distance (cm)	mean	s.e.
10	678.34	649.31-734.22
20	410.76	348.20-426.32
30	427.36	418.41-451.05
40	442.57	426.09-452.59
50	0	-

App. 5.6. Size of mycelium-carrying droplets at different distances from the source (surface grown cultures of *H. cryptosclerotium*). Data for Fig. 5.13.

	size (µm)	
distance (cm)	mean	s.e.
10	830.45	769.46-840.32
20	1127.58	838.76-1332.71
30	1234.73	824.52-1412.23
40	1278.10	1129.27-1228.21
50	380.97	302.81-519.01

App. 6.1. Mycological agar media used in Chapter VI.

CYA:

-1 g dipotassium hydrogen phosphate (K_2HP_4)
-10 ml Czapek concentrate (30 g $NaNO_3$, 5 g KCl, 5 g $MgSO_4.7H_2O$, 0.1 g $FeSO_4.7H_2O$ and distilled water to 100 ml)
-5 g yeast extract (Difco)
-30 g sucrose
-15 g agar
-distilled water to 1 l

PSA:

-500 ml potato water (boil 1,800 g peeled potatoes in 4.5 l of water)
-20 g sucrose
-20 g agar
-distilled water to 1 l

CZ:

-50 ml stock solution A (40 g $NaNO_3$, 10 g KCl, 10 g $MgSO_4.7H_2O$, 0.2 g $FeSO_7.7H_2O$ and distilled water to 100 ml)
-50 ml stock solution B (20 g K_2PO_4 and distilled water to 100 ml)
-30 g sucrose
-20 g agar
-900 ml distilled water
To each litre of stock solutions A and B add 1 ml of stock solution C (1 g $ZnSO_4.7H_2O$, 0.5 g $CuSO_4.5H_2O$ and 100 ml of distilled water)

MSA:

-5 g malt extract (Oxoid)
-1 g ammonium nitrate (NH_4NO_3)
-1 g potassium dihydrogen phosphate (KH_2PO_4)
-1 g magnesium chloride
-17 g agar
-distilled water to 1 l

OA:

-30 g oatmeal
-20 g agar
-distilled water to 1 l

YES:

-20 g yeast extract (Difco)
-150 g sucrose
-20 g agar
-distilled water to 1 l

SA:

-10 g sucrose
-10 g peptone
-15 g agar
-distilled water to 1 l

MYE:

-20 g malt extract (Oxoid)
-2 g yeast extract (Difco)
-20 g agar
-double distilled water to 1 l

SNA:

-1 g potassium dihydrogen phosphate (KH_2PO_4)
-1 g potassium nitrate (KNO_3)
-0.5 g magnesium sulphate ($MgSO_4.7H_2O$)
-0.5 g potassium chloride (KCl)
-0.2 g glucose
-0.2 sucrose
-20 g agar
-distilled water to 1 l

App. 6.2. Growth of two strains of *H. cryptosclerotium* (A, B) on different solid media after 21 d at 25°C. Data for Fig. 6.1.

	growth diameter (cm) strain A	
solid media	mean	s.e.
SNA	1.983	0.1721
CZ	2.383	0.1721
MSA	3.017	0.1721
OM	3.200	0.1721
SA	3.317	0.1721
PSA	3.433	0.1721
MYE	3.733	0.1721
YES	4.000	0.1721
CYA	4.300	0.1721

	growth diameter (cm) strain B	
solid media	mean	s.e.
SNA	2.167	O.1532
CZ	2.033	O.1532
MSA	2.667	O.1532
OM	3.200	O.1532
SA	3.333	O.1532
PSA	3.200	0.1532
MYE	3.633	O.1532
YES	4.033	O.1532
CYA	4.050	O.1532

App. 6.3. Effect of different concentrations of glucose on yield of mycelium of two strains (A, B) of *H. cryptosclerotium*. Data for Fig. 6.2.

	yield (g, dry wt.) strain A	
glucose (g/l)	mean	s.e.
5	0.1969	0.0284
10	0.2871	0.0284
15	0.3551	0.0284
20	0.3485	0.0284

	yield (g, dry wt.) strain B	
glucose (g/l)	mean	s.e.
5	0.1687	0.0151
10	0.2834	0.0151
15	0.4406	0.0151
20	0.3844	0.0151

App. 6.4. Influence of sucrose on yield of mycelium of two strains (A, B) of *H. cryptosclerotium*. Data for Fig. 6.3.

	yield (g, dry wt.) strain A	
sucrose (g/l)	mean	s.e.
5	0.1976	0.0127
10	0.2295	0.0127
15	0.1982	0.0127
20	0.3835	0.0127

	yield (g, dry wt.) strain B	
sucrose (g/l)	mean	s.e.
5	0.1995	0.0166
10	0.2152	0.0166
15	0.2342	0.0166
20	0.4024	0.0166

App. 6.5. Effect of varying yeast extract concentrations on yield of mycelium of two strains (A, B) of *H. cryptosclerotium*. Data for Fig. 6.4.

	yield (g, dry wt.) strain A	
yeast extract (g/l)	mean	s.e.
5	0.2055	0.0182
10	0.3320	0.0182
15	0.4897	0.0182
20	0.5724	0.0182
25	0.5459	0.0182
30	0.5462	0.0182

	yield (g, dry wt.) strain B	
yeast extract (g/l)	mean	s.e.
5	0.1687	0.0247
10	0.3739	0.0247
15	0.4213	0.0247
20	0.5307	0.0247
25	0.5758	0.0247
30	0.4909	0.0247

App. 6.6. Influence of peptone on yield of mycelium of two strains (A, B) of *H. cryptosclerotium*. Data for Fig. 6.5.

	yield (g, dry wt.) strain A	
peptone (g/l)	mean	s.e.
0	0.0362	0.0064
0.5	0.0551	0.0064
1	0.0902	0.0064
1.5	0.0899	0.0064

	yield (g, dry wt.) strain B	
peptone (g/l)	mean	s.e.
0	0.0357	0.0084
0.5	0.0735	0.0084
1	0.1003	0.0084
1.5	0.0981	0.0084

App. 6.7. Effect of varying yeast extract-peptone concentrations on yield of mycelium of two strains (A, B) of *H. cryptosclerotium*. Data are mean ± s.e. Data for Table 6.4.

	yield (g, dry wt.) strain A		
	yeast (g/l)		
peptone (g/l)	0	5	10
0	0.0362±0.0143	0.2069±0.0143	0.3320±0.0143
0.5	0.0551±0.0143	0.1969±0.0143	0.3319±0.0143
1	0.0902±0.0143	0.2887±0.0143	0.3565±0.0143
1.5	0.0899±0.0143	0.2748±0.0143	0.3706±0.0143

	yield (g, dry wt.) strain B		
	yeast (g/l)		
peptone (g/l)	0	5	10
0	0.0357±0.0172	0.1978±0.0172	0.3739±0.0172
0.5	0.0735±0.0172	0.1687±0.0172	0.3737±0.0172
1	0.1002±0.0172	0.3172±0.0172	0.4237±0.0172
1.5	0.0981±0.0172	0.3372±0.0172	0.4395±0.0172

App. 7.1. Effect of using different doses of inoculum of *H. cryptosclerotium* on *R. invadens* mortality. Data were pooled for statistical analysis. Data for Fig. 7.1.

	strain A					
	dose (dry wt. g/100 ml distilled water plus 0.01% Tween 20)					
	0	0.5	1	2	3	4
DEAD	14	63	43	17	54	17
TOTAL	75	93	77	38	74	19
DEAD	15	16	26	48	9	24
TOTAL	46	57	49	68	12	26
DEAD	4	11	10	36	12	47
TOTAL	12	51	30	80	15	67
DEAD	3	7	15	28	53	30
TOTAL	28	30	25	59	63	40
DEAD	0	5	20	20	32	14
TOTAL	13	34	59	33	42	19
DEAD	5	0	5	28	60	27
TOTAL	28	14	22	60	97	38
DEAD	4	17	8	0	48	8
TOTAL	30	84	18	13	62	14
DEAD	7	4	2	4	6	23
TOTAL	27	20	9	7	11	25
DEAD	0	3	24	37	17	17
TOTAL	14	11	63	60	26	25
DEAD	4	4	6	10	51	26
TOTAL	20	10	11	23	64	36
	strain B					
	0	0.5	1	2	3	4
DEAD	6	14	29	22	4	32
TOTAL	58	33	49	36	4	32
DEAD	7	18	6	25	59	73
TOTAL	20	40	9	54	64	77
DEAD	14	0	33	32	24	57
TOTAL	46	8	81	53	28	68
DEAD	12	35	18	40	3	7
TOTAL	59	65	35	60	6	8
DEAD	19	30	15	39	2	60
TOTAL	20	80	46	44	5	64
DEAD	10	8	8	11	32	3
TOTAL	40	22	18	19	33	5
DEAD	10	25	10	20	19	36
TOTAL	44	67	22	32	25	42
DEAD	23	20	17	10	23	64
TOTAL	39	50	55	20	30	75
DEAD	5	4	20	5	6	9
TOTAL	66	15	45	5	8	11
DEAD	10	7	12	16	7	5
TOTAL	46	20	23	30	9	6

App. 7.2. Effect of inoculum age on *R. invadens* mortality. Data for Fig. 7.2.

	% mortality	
age ofinoculum (months)	mean	s.e.
0	54.56	52.02-61.51
1	56.39	49.41-64.31
3	58.83	54.42-62.62
6	56.60	53.25-62.17
10	62.87	57.53-65.09
12	55.19	49.09-61.41

App. 7.3. Relative susceptibility of juveniles and female adults of *R. invadens*. Data are mean ± s.e. Data for Fig. 7.3.

	% mortality	
	treatment	control
first instar	65.54 (62.31-68.12)	31.44 (30.52-34.12)
second instar	46.61 (41.56-51.00)	17.39 15.27-21.11)
third instar	27.15 (21.31-34.22)	15.39 (11.55-19.23)
adults	12.49 (8.35-18.63)	9.72 (4.16-16.35)

Appx. 7.4. Effect of using different wetting agents on mortality of *R. invadens* by *H. cryptosclerotium*. Data are mean ± s.e. Data for Fig. 7.4.

	% mortality					
	wetting agents					
	Triton X-405		Tween 20		Triton X-114	
concentration % (v/w)	treatment	control	treatment	control	treatment	control
0.01	57.61 (55.40-60.01)	9.49 (7.85-11.42)	57.45 (53.74-59.73)	23.39 (20.69-25.81)	67.17 (64.60-69.54)	59.15 (57.20-61.12)
0.05	68.31 (65.92-71.31)	46.65 (44.00-49.13)	57.82 (55.35-60.87)	33.20 (30.62-35.82)	85.72 (83.32-87.84)	82.12 (80.00-84.12)
0.1	93.00 (90.81-94.72)	96.40 (94.11-97.70)	97.54 (95.81-97.90)	97.01 (96.10-97.75)	94.45 (92.71-97.92)	96.90 (95.93-97.61)

App. 7.5. Influence of the number of applications of inoculum of *H. cryptosclerotium* on *R. invadens* mortality. Data are mean ± s.e. Data for Fig. 7.5.

	number live insects					
	n° of fungal applications					
weeks	1 appl.	control 1	2 appl.	control 2	3 appl.	control 3
0	254.34 (250.2-258.6)	302.32 (227.3-307.1)	301.10 (226.5-305.6)	290.30 (285.4-294.7)	338.12 (336.7-341.5)	318.30±4.61
1	47.62 (45.23-49.34)	227.30 (223.8-232.7)	73.62 (70.11-75.82)	232.12 (321.6-235.4)	69.12 (67.89-71.23)	237.20±3.72
2	37.64 (35.23-39.32)	209.51 (206.7-301.4)	54.67 (53.23-55.43)	336.60 (333.1-339.7)	51.00 (48.90-53.21)	250.50±3.91
3	54.61 (51.29-56.97)	203.32 (201.5-205.8)	27.39 (25.34-29.46)	185.40 (182.2-188.5)	27.31 (25.80-29.42)	210.60±3.82
4	65.63 (63.28-67.10)	271.45 (268.6-274.3)	21.30 (19.32-23.76)	201.00 (198.2-203.5)	31.50 (29.97-32.78)	215.01±2.52
5	98.00 (96.54-100.3)	242.20 (240.1-242.9)	12.13 (11.54-13.42)	230.12 (229.9-231.2)	2.00 (1.57-2.78)	200.00±3.12
6	101.60 (98.38-103.7)	345.43 (342.2-347.6)	6.40 (5.01-7.36)	215.30 (213.2-217.4)	0	220.11±3.60
7	88.41 (86.24-89.97)	305.60 (303.3-306.8)	1.00 (0.76-1.34)	245.00 (242.3-247.3)	0	230.50 (227.4-233.6)

App. 7.6. Pathogen persistence on the plant surface. Data are mean ± s.e. Data for Fig. 7.6 (experiment 1).

	number live insects	
weeks	control	treatment
0	8.00	8.00
1	123.91 (121.53-124.78)	53.10 (51.12-55.02)
2	160.27 (157.24-163.20)	74.85 (71.23-77.97)
3	251.17 (249.20-253.09)	85.25 (83.89-87.10)
4	253.98 (250.67-256.70)	137.01 (133.20-141.02)
5	338.56 (334.08-342.70)	163.23 (159.20-166.53)
6	362.28 (357.01-365.16)	146.67 (143.10-147.01)

App. 7.6 (cont.). Pathogen persistence on the plant surface. Data are mean ± s.e. Data for Fig. 7.6 (experiment 2).

	number live insects	
weeks	control	treatment
0	8.00	8.00
1	95.12 (91.23-98.51)	49.31 (37.20-55.24)
2	193.13 (188.98-197.23)	58.66 (53.23-64.20)
3	203.51 (199.23-207.61)	70.01 (64.23-76.73)
4	261.61 (259.12-263.41)	45.34 (42.80-47.01)
5	290.95 (287.54-293.71)	31.14 (29.30-32.65)
6	268.12 (264.10-271.01)	17.64 (15.21-19.03)

App. 8.1. Effect of UV radiation on the viability of *H. cryptosclerotium* mycelium. Data are mean ± s.e. Data for Fig. 8.1.

	% colony forming units			
	hours			
UV radiation exposure (h)	24	48	72	96
0	34.01 (31.34-38.56)	81.23 (76.03-87.56)	100	100
2	40.58 (43.04-46.39)	77.56 (73.35-81.23)	100	100
4	36.58 (34.00-38.37)	83.26 (79.56-87.67)	100	100
6	43.27 (41.79-45.25)	86.45 (83.47-89.56)	100	100
8	38.27 (34.21-41.35)	81.47 (78.67-86.02)	100	100
12	47.35 (44.25-59.45)	74.46 (73.33-79.88)	100	100

App. 8.2. Effect of UV radiation on the viability of *H. cryptosclerotium* spores. Data are mean ± s.e. Data for Fig. 8.2.

	% spore germination	
UV radiation exposure (h)	mean	s.e.
0 (control)	79.01	74.10-83.21
0.5	56.23	51.43-60.80
1	29.89	25.31-32.65
2	0	-
3	0	-
4	0	-
6	0	-
8	0	-
10	0	-
12	0	-

App. 8.3. Influence of UV radiation on the infectivity of *H. cryptosclerotium* to *R. invadens*. Data are mean ± s.e. Data for Fig. 8.3

	% mortality	
UV radiation exposure (h)	treatment (fungus)	control (water)
0 (control)	47.31 (45.12-52.45)	16.23 (14.78-19.98)
2	40.45 (36.35-44.20)	14.00 (13.24-18.01)
4	53.25 (49.67-56.72)	19.26 (17.58-22.01)
6	57.10 (50.00-62.33)	15.30 (13.35-17.98)
8	45.47 (41.67-51.78)	17.45 (15.67-19.78)
12	52.78 (46.22-60.78)	22.36 (19.67-24.89)

App. 8.4. Viability of *H. cryptosclerotium* mycelium at different temperatures. Data are mean ± s.e. Data for Fig. 8.4.

	% colony forming units			
	hours			
°C	24	48	72	96
15	0	0	0	59.21 (53.42-68.00)
20	0	18.72 (16.15-24.37)	58.32 (53.22-66.73)	100
25	34.25 (28.90-41.85)	96.00 (93.22-98.97)	100	100
30	61.34 (54.27-66.52)	95.52 (92.14-98.67)	100	100
35	96.00 (93.41-97.78)	100	100	100
37	0	0	0	0

App. 8.5. Growth of *H. cryptosclerotium* on malt agar at different temperatures. Data are mean ± s.e. Data for Fig. 8.5.

	growth diameter (cm)			
	weeks			
°C	1	2	3	4
15	0	0.91±0.17	1.52±0.17	2.35±0.17
20	0.65±0.17	1.35±0.17	2.05±0.17	2.61±0.17
25	1.32±0.17	2.10±0.17	3.75±0.17	4.65±0.17
30	1.35±0.17	2.41±0.17	3.53±0.17	3.65±0.17
35	0.85±0.17	2.15±0.17	2.35±0.17	3.05±0.17
37	0	0	0	0

App. 8.6. Influence of temperature on the infectivity of *H. cryptosclerotium* to *R. invadens*. Data are ± s.e. Data for Fig. 8.6.

	% mortality	
°C	treatment	control
15	48.00 (41.23-53.00)	11.52 (9.91-15.23)
20	56.55 (54.32-61.33)	21.00 (18.31-21.92)
25	57.12 (54.00-62.31)	14.22 (12.52-16.51)
30	57.37 (55.23-62.52)	18.24 (16.72-22.19)
35	56.11 (53.98-61.73)	23.37 (21.56-25.94)
37	24.49 (23.93-33.20)	23.45 (20.30-26.09)

App. 8.7. Influence of temperature on the incubation period of *H. cryptosclerotium* on *R. invadens*. Data are mean ± s.e. Data for Fig. 8.7.

	% dead insects with sporulating mycelium	
	days	
°C	3	6
15	0	11.10 (9.12-14.89)
20	0	56.42 (50.00-61.32)
25	12.00 (9.72-17.78)	97.32 (94.17-99.02)
30	52.32 (43.00-58.12)	96.54 (92.01-98.31)
35	97.00 (93.12-99.27)	97.82 (93.11-98.72)
37	0	0

App. 8.8. Influence of relative humidity on the infectivity of *H. cryptosclerotium* to *R. invadens*. Data are mean ± s.e. Data for Fig. 8.8.

	% mortality	
% r. h.	treatment	control
60	19.95 (18.19-21.85)	21.57 (18.02-24.45)
70	28.61 (20.94-32.28)	14.54 (12.56-16.76)
80	53.38 (50.33-56.94)	14.43 (12.49-15.61)
85	46.76 (41.25-57.25)	13.57 (11.35-15.34)
90	48.65 (42.77-54.55)	14.35 (11.65-16.68)
95	59.00 (53.40-66.01)	15.73 (11.25-19.65)
100	59.34 (54.95-63.25)	16.79 (14.82-21.91)