Aschersonia aleyrodis as a microbial control agent of greenhouse whitefly

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Proefschrift
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Omslag: Witte vlieg geïnfecteerd door de schimmel *Aschersonia aleyrodis* (oranje), geparasiteerd door de sluipwesp *Encarsia formosa* (zwart) en lege pophuidjes (doorzichtig)

Stellingen

1. Planteveredelaars zouden meer gebruik moeten maken van het gegeven dat de kwaliteit van de waardplant de vatbaarheid van insekten voor pathogenen beïnvloedt.
   Hare, J. D. & T. G. Andreadis (1983). Environ. Entomol. 12, 1892-1897.
   Herzog, D. C. & J. E. Funderburk (1985). In: „Biological Control in Agricultural IPM Systems" (eds.: M. A. Hoy & D. C. Herzog). Academic Press, 67-88.
   Kushner, D. J. & G. T. Harvey (1962). J. Insect Pathol. 4, 65.
2. Het feit dat een entomopathogeen micro-organisme evenals elk organisme aan genotypische en fenotypische veranderingen onderhevig is, heeft zijn positieve en negatieve kanten.
   Briese, D. T. (1986). In: „Biological Plant and Health Protection" (ed.: J. M. Franz). Gustav Fischer Verlag, Stuttgart, 233-256.
   Fargues, J. & P. H. Robert (1983). Mycopathologia 81, 145-154.
3. Aangezien biotoetsen voor evaluatie van entomopathogenen over het algemeen worden uitgevoerd onder optimale omstandigheden, geven deze toetsen uiteindelijk geen goed beeld van de effectiviteit van het organisme in de omgeving waar het wordt toegepast.
   Dit proefschrift.
4. De analyse van de samenstelling van de mucus afgescheiden door bepaalde schimmels bij de produktie van de sporen kan een bijdrage leveren tot de succesvolle formulering van deze organismen bij toepassing als microbieel pesticide.
   Louis, J. & R. C. Cooke (1985). Trans. Br. mycol. Soc. 84, 661-667.
   Dit proefschrift.
5. Voedingsstoffen die aan de formulering van infectieuze eenheden van entomopathogene schimmels worden toegevoegd ter stimulering van de kieming leiden niet vanzelfsprekend tot een toename van de infectie van de gastheer.
   Emmett, R. W. & D. G. Parbery (1975). Ann. Rev. Phytopathol. 13, 147-167.
   Grover, R. K. (1971). In: „Ecology of Leaf Surface Micro-organisms" (eds.: T. F. Preece & C. H. Dickinson). Academic Press, New York, 509-518.
6. Bij het bepalen van de invloed van natuurlijke vijanden op fluctuaties in populatieaantallen van plaaginsekten worden interacties tussen parasieten, predatoren én pathogenen vaak onderschat.
   Andreadis, T. G. (1987). In: „Epizootiology of Insect Diseases" (eds.: J. R. Fuxa & Y. Tanada). John Wiley and Sons, New York, 159-176.
7. De toepassing van remote-sensing technieken ter onderkenning van stress bij planten kan de automatisering van de teelt van bloemisterijgewassen stimuleren.
   Jackson, R. D. (1986). Ann. Rev. Phytopathol. 24, 265-287.
8. De huidige gang van zaken bij het toekennen van patenten is niet aantrekkelijk voor de ontwikkeling van een commerciële produktie van micro-organismen in het kader van de biologische bestrijding.
   Templeton, G. E., R. J. Smith & W. Klomparens (1980). Biocontrol News and Information 1, 291-294.
   Ricard, J. L. (1981). Biocontrol News and Information 2, 95-98.
9. Een landbouwbeleid dat streeft naar het terugdringen van overschotten en een grotere differentiatie aan landbouwprodukten zal invloed hebben op de gewasbeschermingsproblematiek en zal aanpassing vergen van de betrokken industrieën in deze sektor.
10. Overeenkomstig de relatiestructuur bij mensapen zou men kunnen aannemen dat de verhoudingen tussen vrouwen minder hiërarchisch geregeld en veel stabieler zijn dan die tussen mannen. Behoefte aan stabiliteit blijkt ook bij de mens uit de houding van de vrouwen ten opzichte van de mannelijke statuscompetitie.
    F. de Waal (1982). Chimpansee Politiek. Macht en Seks bij Mensapen. Becht, Amsterdam.
11. De massamedia veroorzaken momenteel meer storing in de communicatie dan dat zij deze bevorderen.
12. De mens leeft veeleer dankzij de natuur dan ondanks de natuur.

Stellingen behorende bij het proefschrift van mw. ir. J. J. Fransen, getiteld *„Aschersoniais aleyrodis* as a microbial control agent of greenhouse whitefly".

Wageningen, 7 december 1987

Aan mijn ouders

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CONTENTS

More or less revised versions of several of the chapters will be published as follows.

Chapter 3:

Fransen, J.J., C. Winkelman, & J.C. van Lenteren, 1987. The differential mortality at various life stages of the greenhouse whitefly, Trialeurodes vaporariorum (Homoptera: Aleyrodidae), by infection with the fungus Aschersonia aleyrodis (Deuteromycotina: Coelomycetes). J. Invertebr. Pathol. 50, 158-165.

Chapter 4:

Fransen, J.J. Age-specific dose-mortality effects of Aschersonia aleyrodis on greenhouse whitefly, Trialeurodes vaporariorum. Submitted.

Chapter 5:

Fransen, J.J. Effects of density on the germination of Aschersonia aleyrodis spores and their infectivity to greenhouse whitefly. Submitted.

Chapter 6:

Fransen, J.J. Survival of Aschersonia aleyrodis spores on leaf surfaces. Submitted.

Chapter 7:

Fransen, J.J. The influence of relative humidity and temperature on infection of greenhouse whitefly with the fungus Aschersonia aleyrodis. Submitted.

Chapter 8:

Fransen, J.J. & J.C. van Lenteren. Interaction between the parasitoid Encarsia formosa and the pathogen Aschersonia aleyrodis in the control of greenhouse whitefly, Trialeurodes vaporariorum: host selection and survival of the parasitoid in the presence of hosts infected with the fungus. Submitted.

Chapter 9:

Fransen, J.J. & J.C. van Lenteren. Interaction between the parasitoid Encarsia formosa and the pathogen Aschersonia aleyrodis in the control of greenhouse whitefly, Trialeurodes vaporariorum: survival of the parasitoid after treatment of parasitized hosts with fungal spores. Submitted.

CHAPTER 1

GENERAL INTRODUCTION

1.1 BIOLOGICAL CONTROL IN GLASSHOUSES

The first record of biological control in glasshouses was supposed to be on the use of the parasitoid Encarsia formosa for control of the greenhouse whitefly, Trialeurodes vaporariorum, in the United Kingdom (Speyer, 1927). The use of this natural enemy was abandoned after the Second World War when chemical control became widespread. The interest in the use of natural enemies in glasshouses revived after the appearance of resistance to insecticides in the two-spotted spider mite, Tetranychus urticae. An imported predatory mite Phytoseiulus persimilis, successfully kept the spider-mite populations below the economic injury level (Hussey & Bravenboer, 1971). This implied that broad spectrum insecticides could not be applied for control of other pests as these would negatively affect the natural enemy. Thus, attention was focused on the natural enemies of the other pests in the system. In the 1970's heavy infestations of whitefly caused problems because of increasing resistance against insecticides (Wardlow et al., 1972). The knowledge already available from previous applications of E. formosa earlier this century enhanced the development of introduction strategies for control of greenhouse whitefly with this parasitoid (Woets, 1973, 1978, Vet et al., 1980). During the last two decades the application of biological control against pests in glasshouses has increased steadily. Several predators and parasitoids became commercially available and others were tested (Table 1.1) (Ravensberg et al., 1983, Lenteren, 1983, 1987a). Nowadays, the application of natural enemies of pests in glasshouses still offers prospects for further expansion as the area of glasshouses worldwide amounts to 100,000 - 150,000 ha. Biological control is applied on 2250 ha. In some situations, however, application of biological control is not needed or not yet possible (Lenteren, 1983).

Table 1.1 Natural enemies presently being used against pests in glasshouses (Lenteren, 1987a).

Pest insect	Natural enemy	Application in glasshouses (ha) (1985)
Whitefly	Encarsia formosa	1600
Two-spotted spidermite	Phytoseiulus persimilis	1300
Leafminers	Dacnusa sibirica	460
	Diglyphus isaea	
Thrips	Amblyseius mackenziei	140*
Lepidoptera	Bacillus thuringiensis	875
Aphids	Aphidoletes aphidimyza	13

* in 1986

The biological-control programmes are influenced by changes in cultural techniques and economic circumstances. The introduction of rockwool, nutrient-film technique, the lowering of the temperature in glasshouses and the availability of different cultivars require studies for adaptation of biological-control programmes (Lenteren, 1987b). Moreover, new pest problems may appear. Presently, for example, Frankliniella occidentalis, Western Flower thrips, is a potential threat to ornamentals and vegetables, and natural enemies against this pest are being evaluated.

Besides parasitoids and predators, pathogens may play an important role in the future. The application of Bacillus thuringiensis strains against lepidopterous pests has increased spectacularly over the past three years (Lenteren, 1987a). The nematode Heterorhabditis sp. offers possibilities for control of the vine weevil, Othiorynchus sulcatus (Galle, pers.comm.). The utilization of a nuclear polyhedrosis virus and the nematode Steinernema feltiae against beet armyworm, Spodoptera exigua are under investigation (Smits, 1987, Begley, pers. comm.). The fungus Verticillium lecanii has been successfully applied against aphids on chrysanthemums. The effect was mainly due to the development of

epizootics over time brought about by prolonged periods of high relative humidity induced by plastic screens used to shorten daylength for the cultivation of chrysanthemums (Hall & Burges, 1979). Another strain of V. lecanii was applied against greenhouse whitefly (Hall, 1982, Kanagaratnam et al., 1982), but results of practical applications have been inconsistent. The development of an epizootic demands periods of over 85% relative humidity for at least 10 hr per day and this requirement is not always fulfilled. Another strain of V. lecanii is presently under investigation for control of Thrips tabaci (Gillespie, 1986). In the Netherlands and in Eastern European countries another fungus, Aschersonia aleyrodis, is considered as a control agent of greenhouse whitefly. The approach to its practical use is different from that of V. lecanii because it is not expected to cause an epizootic, but instantly to lower the whitefly population. Consequently, the dependence on environmental conditions will be less.

1.2 BIOLOGICAL CONTROL OF GREENHOUSE WHITEFLY

The greenhouse whitefly was first described by Westwood (1856). He assigned it to the genus Aleyrodes which has been altered in spelling to Aleurodes. Aleurodes vaporariorum was later transferred to Asterochiton and placed under the subgenus Trialeurodes. Quaintenance and Baker (1915) later transferred Asterochiton vaporariorum into Trialeurodes vaporariorum.

The greenhouse whitefly is a polyphagous species found on a wide range of host plants (Mound & Halsey, 1978). The region of origin is not known with certainty. It is thought to be native to Brazil or Mexico (Milliron, 1940). Nowadays, it is widely distributed, also occurring in temperate regions where it has become a persistent pest on vegetable and flower crops grown under protected cultivation. Damage to crops is mainly due to the excretion of honeydew by larval instars and adults. Accumulation of honeydew promotes the development of sooty moulds (Cladosporium spp.), thus reducing photosynthesis and transpiration of the plants and causing contamination of the fruits (O'Reilly, 1974). Additionally, greenhouse whitefly is reported to transmit viruses of vegetables and ornamentals (Duffus, 1965).

The adults are covered by white powdery wax and the females lay

their eggs on the undersurface of the leaves. These eggs turn black after one to three days. The first instar larvae are mobile at first but later on they settle down on the leaf surface, and the second, third and fourth instar larvae are all sedentary. After moulting into the fourth instar three substages can be distinguished: (1) early fourth instar (transparent, flattened), (2) transitional substage (larvae thickened and wax-ensheathed, dorsal and lateral spines), and (3) similar to substage 2 but with red eyes. The whitefly in these substages will be referred to as fourth larval instar, prepupa and pupa, respectively, according to Lenteren et al. (1976a).

There have been many studies of the development and behaviour of T. vaporariorum, the earliest being those of Hargreaves (1915), Lloyd (1922) and Weber (1931). As T. vaporariorum became a serious problem in the second half of this century, the investigations were continued. Different aspects of the whitefly-host-plant relationship have been studied, for example, the suitability of different host plants for development of whitefly (Boxtel et al., 1978, Merendonk & Lenteren, 1978, Sas et al., 1978) and host-plant selection (Verschoor-van der Poel & Lenteren, 1978). The literature on development of greenhouse whitefly on different host plants and under different environmental conditions was summarized by Lenteren & Hulspas-Jordaan (1983). In order to develop reliable sampling techniques for evaluation of the pest and the parasitoid populations the spatial distribution and movement were studied by Eggenkamp-Rotteveel Mansveld et al. (1982) and Noldus et al. (1985, 1986). Different aspects of biological control of greenhouse whitefly are under investigation. Extensive research on the parasitoid Encarsia formosa regarding host selection, searching behaviour and population development sofar was reviewed by Vet et al. (1980). Successful introduction schemes for the parasitoid E. formosa have been developed for several vegetable crops. In cucumbers however the whitefly population is not always kept below the economic injury level in contrast to the situation in tomato crops. This is due to the fact that the searching efficiency of the parasitoid is lower in cucumber than in tomato because the parasitoids are hindered by the hairiness of the cucumber leaves (Li Zhao Hua et al., in press). Besides, on cucumber the natural mortality of whitefly is lower, development is faster and the number of eggs laid is higher

than on tomato plants (Boxtel et al., 1978). Improvement of whitefly control by E. formosa in cucumber may be accomplished in several ways. Breeding new varieties of cucumber may offer possibilities, but short-term solutions are not expected. Therefore, a selective control method in addition to the parasitoid is desirable. To this end, Aschersonia aleyrodis offers perspectives because of its selective character. It may also be effective as a control agent of greenhouse whitefly in situations where the parasitoid cannot be introduced.

1.3 EVALUATION OF PATHOGENIC MICROORGANISMS FOR PEST CONTROL

Groups of microorganisms which are currently studied for suppression of insects are viruses, rickettsiae, bacteria, fungi, protozoa and nematodes. These microorganisms occur widely in nature and epizootics of natural origin are frequently observed. A combination of intrinsic and extrinsic factors provides the setting for conversion from an enzootic (i.e. low rate of incidence) condition to an epizootic (i.e. outbreak of disease) condition. Several approaches have evolved for the use of pathogens in integrated pest management: (1) permanent introduction and establishment which results in more or less permanent suppression of the pest; (2) inundative releases resulting in an immediate effect; (3) inoculative releases which are required on some recurring bases, although secondary infection occurs; (4) environmental manipulation involving the enhancement of naturally ocurring pest control by means other than direct addition of pathogen units to those already present (Fuxa, 1987). These methods have much in common with the release methods used for other natural enemies of pest insects, like parasitoids (Lenteren, 1986).

Many entomopathogenic fungi attack insects and mites of agricultural and medical importance. These fungi do not belong to a specific group, but are found in most of the taxonomic classes, viz. Chytridiomycetes, Oomycetes, Trichomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes (Hall & Papierok, 1982). Of these, only a few species have been investigated for their role as biological control agents. The fungi under development for practical application are listed in Table 1.2 (Burges & Hall, 1982, Jaques, 1983, Wilding, 1983, Roberts & Wraight, 1986, Zimmermann, 1986). For detailed

Table 1.2 Fungal pathosystems under investigation for practical application or already applied as such.

Fungal pathogen	Host insect	Crop	
Aschersonia spp.	Dialeurodes citri	citrus	**
Aschersonia aleyrodis	Trialeurodes vaporariorum	vegetables	**
Beauveria bassiana	Pachneus sp.	citrus	*
	Leptinotarsa decemlineata	potato	**
	Ostrinia nubilalis	corn	**
	Dendrolinus punctatus	pine	***
	Nephelotettix sp.	rice/tea	***
Beauveria brongnartii	Melolontha melolontha	pastures	**
Erynia spp.	Aphis fabae	bean	*
Erynia radicans	Empoasca sp.	alfalfa	*
Entomophthora aulicae	Lymantria dispar	pine	*
Entomophaga grylli	Camnula sp.	pasture	*
Hirsutella thompsonii	Phyllocoptruta oleivora	citrus	***
Hirsutella citriformis	Nilaparvata lugens	rice	*
Metarhizium anisopliae	Scotinophara coarctata	rice	**
	Aphodius tasmaniae	pasture	***
	Mahanarva posticata	sugar cane	***
	Oryctes rhinoceros	oil palm	***
	Curculio caryae	pecan nut	*
	Otiorhynchus sulcates	strawberry, pot plants	*
Nomuraea rileyi	Heliothis sp.	soybean, cotton	**
Verticillium lecanii	Myzus persicae	chrysanthemums	***
	Thrips tabaci	vegetables	*
	Trialeurodes vaporariorum	vegetables	***

* under investigation
** experimentally applied
*** (semi) commercially developed

information I refer to a few standard works on the subject (Steinhaus, 1949, Müller-Kögler, 1965, Burges & Hussey, 1971, Burges, 1981). Most of the fungi studied for use in inundative releases belong to the Deuteromycetes. In general, they are easily mass produced on (semi) artificial media. Entomophthorales, an important and worldwide distributed group of pathogens, are difficult to mass produce. The approach for use of these pathogens is mainly concentrated on the manipulation of the environment. However, recently production techniques have been devised based on stabilization of mycelia (marcescent process) (Rombach et al., in press). After spraying a suspension of fragmented mycelia these fragments may produce conidia in the field, thus initiating an epizootic.

Table 1.3 shows the factors which may be considered in the evaluation of fungi for control of insects. The importance of the factors mentioned are dependent upon the way in which the pathogen will be used. Inducing epizootics of naturally occurring pathogens in the field, for instance, may require the manipulation of environmental influences (Ignoffo, 1985), but not the artificial culturing of the pathogen. Using fungal pathogens in an inundative way as a microbial control agent involves mass production and formulation.

1.4 ASCHERSONIA SPECIES, FUNGAL PATHOGENS OF WHITEFLY

Webber (1897) was the first to demonstrate that species of the genus Aschersonia were parasitic on insects, as a result of his investigations of fungi and insects on citrus in Florida. Later on Petch (1921) described over 26 Aschersonia species from all over the world and Mains (1959) reported seven Aschersonia species occurring in North America. Aschersonia spp. form pycnidia and belong to the Coelomycetes of the Deuteromycotina. They are parasites of scale insects (Coccidae) and whiteflies (Aleyrodidae). The teleomorph stage of this fungus belongs to the genus Hypocrella (Clavicipitales; Ascomycotina) forming perithecia. The specific host range of the Aschersonia species within the Aleyrodidae and Coccidae is not exactly known. Petch (1921) remarked that it cannot be concluded whether a fungus is parasitic on a particular insect when that insect is found on a leaf with an entomogenous fungus, for several scale insects may

be found together on one leaf. According to Petch the only certain method of identification, is to detect the early stages of infection before the insect is completely covered by the fungus.

Table 1.3 Topics for evaluation of entomopathogenic fungi for control of insect pests.

Fungal characteristics	1.	specificity (host range)
	2.	virulence
	3.	sporulation
	4.	spread
	5.	persistence
	6.	possibilities for mass production - on (alternative) hosts - on (semi) artificial media in solid and/or liquid fermentation
	7.	suitability for storage and formulation
	8.	toxicological and safety aspects
	9.	effects on natural enemies
	10.	compatibility with insecticides and fungicides
Host characteristics	1.	susceptibility
	2.	economic injury level
	3.	age distribution, density and spatial distribution in relation to application or introduction strategy
Environmental influences	1.	abiotic factors: temperature, humidity, sunlight, rain, dew, irrigation, wind
	2.	biotic factors: host-plant quality, crop structure, intercropping, microbial interactions (antagonists, synergists), vectors.

Early this century Berger (1920, 1921) and Fawcett (1908, 1944) developed a method for the application of Aschersonia aleyrodis Webber ('red whitefly fungus') and A. goldiana Sacc. and Ellis ('yellow whitefly fungus') against the common whitefly Dialeurodes citri (Ashmead) and the cloudy-winged whitefly D. citrifolii Morgan on citrus in Florida. The authors recommended applying the entomopathogens during the periods of summer rains when insecticides were less successful. The already endemic fungi were used by pinning fungus-bearing leaves to fungus-free leaves. Also, spores from pure cultures on artificial medium (sweet potato agar) were sprayed directly onto the whitefly-infested citrus trees. Nowadays, whiteflies are abundant in Florida citrus groves, but chemical control is seldom applied specifically for these pests. Several factors may be responsible (Muma, 1969). Firstly, some of the whiteflies seem to be killed by the regularly applied insecticides and acaricides. Secondly, since copper and sulphur compounds are not used as extensively as they were before, it is possible that in combination with several hymenopterous parasitoids parasitic fungi are adequately controlling the whiteflies (McCoy, 1985).

In the 1970's interest in introduction of Aschersonia species in citrus arose also in other parts of the world. Successful application and establishment of the Aschersonia spp. is reported from citrus-growing areas around the Black Sea (Stolyarova, 1972, Ponomarenko et al., 1975). Experiments are also reported from Japan (Uchida, 1970). As a consequence of the success in citrus the fungus was tested against greenhouse whitefly in cucumber and tomato crops under protected cultivation in Eastern European countries. Different strains and species were tested, like Aschersonia placenta originating from China, Vietnam and India, Aschersonia tamurai and Aschersonia species referred to as 'Cuban orange' and 'Cuban red' (Zibulskaya et al., 1975, Osokina & Izevsky, 1976, Solovei & Koltsov, 1976, Spassova et al., 1980, Solovej, 1981). In different parts of the USSR the use of different strains is preferred, but the choice seems rather arbitrary and dependent on the success of cultivation of the fungus on artificial media (Solovej, 1981).

Landa (1984) successfully treated whitefly in a cucumber crop by application of 2.5 - 5.5 x 10^7 spores/ml of the Aschersonia 'Cuban

red' strain three times at seven-day intervals. The best results were achieved using E. formosa in addition to the fungus. The parasitoid was introduced first and the crop was treated with Aschersonia one month after the last introduction of the parasitoid. In the Netherlands studies of Aschersonia were started in 1979 as it appears to be a selective pathogen of whitefly, showing possibilities for use in a glasshouse situation (Ramakers & Samson, 1984).

1.5 INTRODUCTION TO THE CHAPTERS

In this thesis studies are described on different features of Aschersonia aleyrodis which are relevant to development of this fungus as a microbial control agent of greenhouse whitefly. A description of the infection process is given (Chapter 2). The impact of the fungus on greenhouse whitefly was studied in detail (Chapter 3) and dose-mortality responses were assessed by bioassay (Chapter 4). Various characteristics of the fungus in relation to survival of spores were investigated (Chapters 5 and 6), also the question of whether the temperature and relative humidity regimes in glasshouses are suitable for establishment of infection (Chapter 7). The interaction between the parasitoid E. formosa and the fungus was studied (Chapters 8 and 9). In the last chapter the implications of A. aleyrodis as a control agent of greenhouse whitefly are given and the results are summarized with regard to the different aspects mentioned in Table 1.3 (Chapter 10).

CHAPTER 2

THE INFECTION PROCESS OF GREENHOUSE WHITEFLY BY ASCHERSONIA ALEYRODIS

2.1 INTRODUCTION

The mechanisms of infection of insects by entomopathogenic fungi have been described by Müller-Kögler (1965), Roberts and Humber (1981) and Fargues (1984). In recent years techniques have become more advanced to study specific details of the host-pathogen interaction. Spore germination on the insect integument, penetration of the cuticle, colonization of the interior of the host, the role of enzymes and defense mechanisms are studied by means of histological techniques, scanning and transmission electron microscopy, biochemical and immunological analysis. The study of these aspects, however, is restricted to a small group of entomopathogenic fungi like Metarhizium anisopliae (Zacharuk 1970a, 1970b, 1971), Beauveria bassiana (Pekrul & Grula, 1979, Hunt et al., 1984, Woods & Grula, 1984), Nomuraea rileyi (Pendland & Boucias, 1985, Thorvilson et al., 1985) and several species of Entomophthoraceae (Brobyn & Wilding, 1977, Tomiyama & Aoki, 1982, Brey et al., 1986). The Aschersonia aleyrodis - greenhouse whitefly relationship has not been studied until now. Samson and McCoy (1983) observed advanced stages of infection of citrus whitefly by A. aleyrodis by means of scanning electron microscopy (SEM). Germination of spores on the host integument was not examined. This chapter presents details on various stages in the infection process of A. aleyrodis on greenhouse whitefly. The fungal structures on the insect integument were examined using SEM as well as light microscopy. The fungal structures inside the insect body were observed by means of light microscopy using a special staining technique. Additionally, an experiment was carried out in which a fungicide was applied at different time intervals after inoculation of greenhouse-whitefly larvae. Thus, the time period necessary for germination and penetration of the host was indirectly assessed.

2.2 MATERIALS AND METHODS

Scanning Electron Microscopy

Young fourth instar larvae on cucumber plants were inoculated with a spore suspension in sterile distilled water. The spores originated from a three-week-old culture on autoclaved millet grains. The treated larvae were incubated for four days at 20°C, 70% relative humidity (RH) and a 16 hr photoperiod.
The air-dried leaves were covered by transparent plastic bags for 24 hr to create a period of 100% RH. Pieces of leaves bearing the larvae were prepared for SEM by fixing for 24 hr in 3% glutaraldehyde in cacodylate buffer (0.1 M at pH 7.2), dehydration in ethanol, critical point drying and coating with gold-palladium.

Histological preparations

Third and fourth instar larvae on cucumber leaves were inoculated with spores of A. aleyrodis suspended in sterile distilled water. They were also exposed to a 24 hr period of 100% RH after air-drying by covering the plants with transparent plastic bags. Thereafter, the RH was kept at 70%. The temperature was 20 $\pm$ 1°C and the plants were exposed to a 16 hr photoperiod. After one to five days incubation the larvae were stained following a modification of the method of Phillips and Hayman (1970).
The larvae were heated at 90°C for 30 minutes in 10% KOH. This removed all insect tissues. Then the larvae were rinsed in water three times and acidified with 0.1 M HCl. Again they were rinsed in water three times and stained for 15 minutes in 0.03% trypan blue in lactophenol. After rinsing another three times in water they were kept in 50% lactic acid for 24 hr, after which they were stored in glycerine. The specimens were examined under the light microsope at 100 and 400 x magnification.

Assessment of penetration rate of A. aleyrodis on greenhouse whitefly

First, second and third instar larvae of greenhouse whitefly were inoculated with a suspension of A. aleyrodis spores in sterile distilled water. The spores originated from a three-week-old culture on autoclaved grains of millet. An amount of 1.0×10^7 spores in 2 ml was sprayed onto the leaves using a Potter spray tower set at 34.45 kPa. Two hours after spore application in some cases and 2-6 days after application in others, the air-dried leaves were sprayed to run-off with 0.63 gr /liter mancozeb (Dithane M45 ^{R} 80% wettable powder) using a DeVilbiss atomizer. The plants were kept under similar conditions as the ones used for histological observations. Control treatments included the application of A. aleyrodis spores alone to assess the level of infection, treatments with mancozeb alone or with water alone. Viability of A. aleyrodis spores was checked by examination of spores on water agar after 24 hr incubation at 25°C. Over 90% of the spores germinated on water agar. Three to seven leaves bearing whitefly larvae were used in each combination of spore application and fungicide treatment. In total, this amounted to about 130 first instar larvae, about 300 second instar larvae and about 700 third instar larvae per treatment.

To assess the fungicidal effect of mancozeb on the spores of A. aleyrodis, an in vitro bioassay was carried out as well. Mancozeb was added to the spore suspension. This "mixture" containing mancozeb in concentrations of 0, 0.0063, 0.063, 0.63 or 6.3 mgr/ml was applied to malt agar plates and incubated at 25°C and 16 hr photoperiod for 24, 48 and 72 hr. Three agar plates per concentration were used and 300 spores per plate were examined for germination. Spores were scored as germinated when the germ-tube length was similar to or exceeded the breadth of the spore. Spore germination was also examined on malt agar plates containing mancozeb in similar concentrations to those added to the spore suspension in the previous experiment.

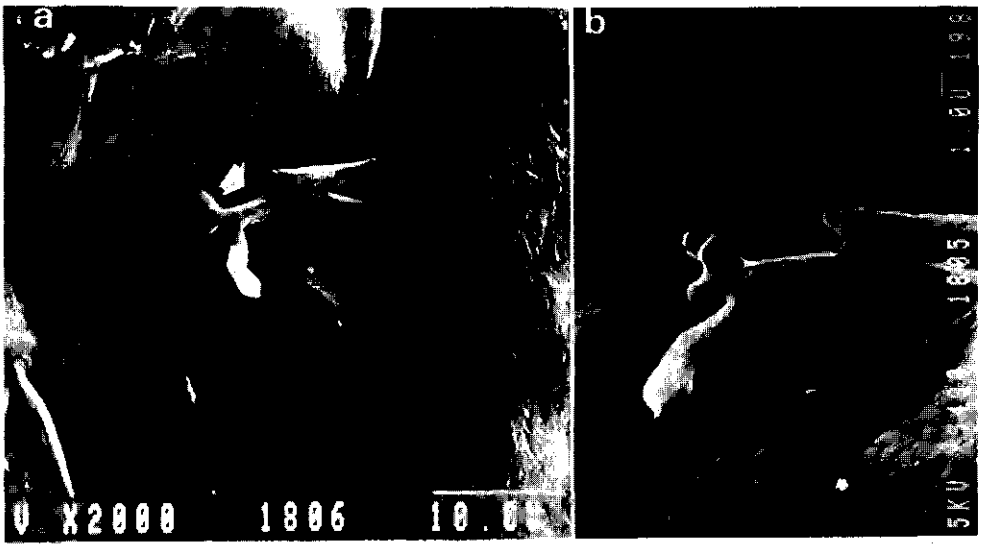


Fig. 2.1 (a) Germinating conidium of Aschersonia aleyrodis on the cuticle of a fourth instar larva of greenhouse whitefly (arrow indicates remnants of mucus); (b) close-up of germ tube and appressorium.

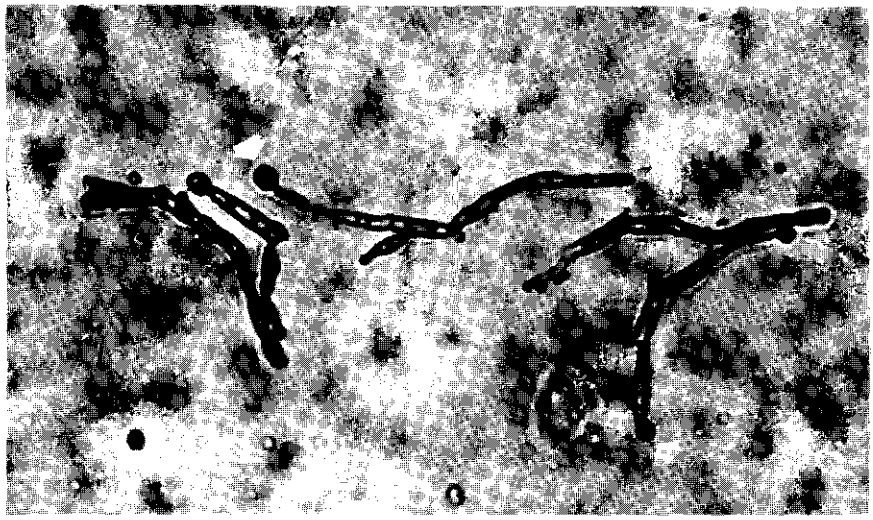


Fig. 2.2 Conidia of Aschersonia aleyrodis with germ tubes and appressorium-like structures (arrow) on cellophane (light microscope, 1300 x magn.).

2.3 RESULTS AND DISCUSSION

The infection process and the development of the disease have been divided into 10 stages according to Roberts & Humber (1981) and Charnley (1984). Following this classification the infection of greenhouse whitefly with A. aleyrodis will be discussed.

(1) The first stage after inoculation involves the attachment of the conidium to the insect cuticle. Substances present on the cuticle surface can change the attachment from passive to active by inducing enzymatic processes of secretion of mucilaginous substances from germinating conidia (Fargues, 1984). It has been shown that dormant conidia of M. anisopliae are coated with a mucilaginous substance which could play a role in the initial adhesion (Zacharuk, 1970a). A. aleyrodis spores are covered in slime when produced and this mucus may remain on the spores after they are suspended in water. Remnants of the mucilaginous layer can be observed on spores sprayed onto whitefly larvae (Fig. 2.1). Whether there is an interaction between spore and host cuticle resulting in adhesion is yet unclear.

(2) The second stage is related to the germination of spores on the host integument. A. aleyrodis produces short germ tubes on susceptible larvae of whitefly (Fig. 2.1), but aberrant growth of hyphae over the surface was observed on the more resistant prepupae. This aberrant growth has also been observed for other fungi on resistant hosts. For instance, Pekrul and Grula (1979) reported that low pathogenic mutants of B. bassiana show (i) inability of conidia to germinate, (ii) aberrant growth of hyphae over the surface of Heliothis zea without penetration, (iii) extensive elongation of hyphae prior to penetration.

(3) The third stage in the infection process refers to the penetration of the host cuticle. The germ tube penetrates directly into the cuticle or an appressorium is formed. Appressoria adhere to the host surface and a narrow infection peg is sent into the cuticle. Emmett and Parbery (1975) distinguished two main types of appressoria: simple appressoria formed from a single, modified cell and compound appressoria made up of many cells. A. aleyrodis forms an appressorium before penetration of the host integument (Fig. 2.1). From light-microscopic observations it is concluded that the appressorium consists

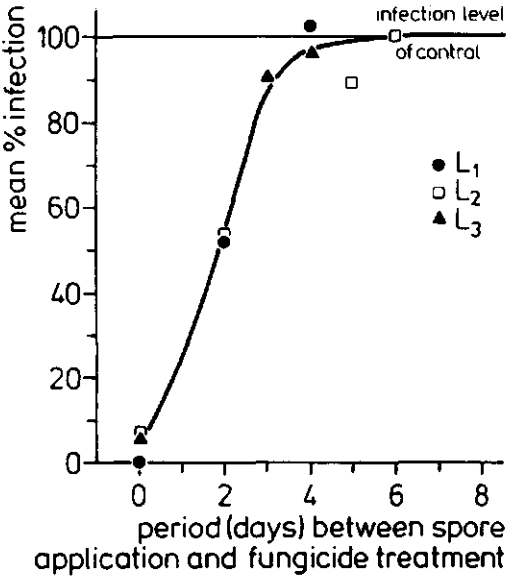


Fig. 2.3 Mean Aschersonia aleyrodis infection rates in first, second and third instar whitefly larvae, on leaves treated first with A. aleyrodis spores, then at various times afterwards with fungicide. The infection rates are expressed as percentages of the control (no fungicide) infection rate for larvae in the same instar (90.6% for first, 91.5% for second, and 86.1% for third instar larvae).

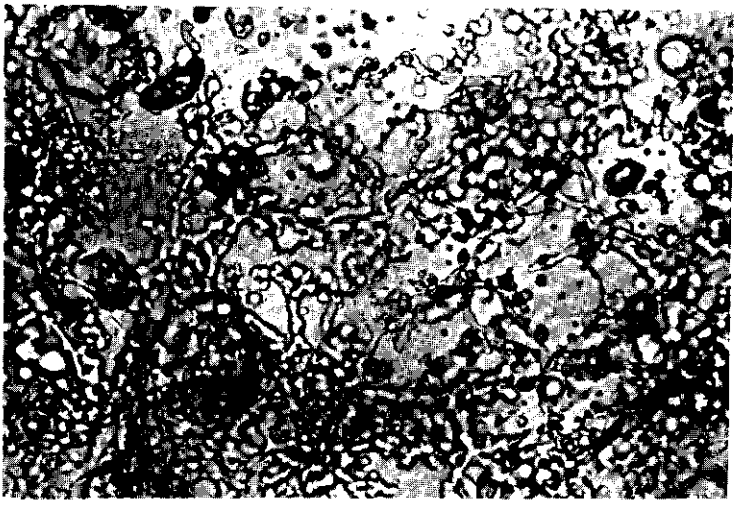


Fig. 2.4 Hyphal bodies and mycelial network of Aschersonia aleyrodis in the haemolymph of a third instar larva of greenhouse whitefly (light microscope, 900 x magn.).

of a single modified cell. Occasionally, I observed a narrow penetration peg through the cuticle. Adherence and penetration both involve enzymatic and physical activity. Several fungi form appressorial - like structures in vitro against solid surfaces such as glass and artificial wax membranes (Madelin et al., 1967). A. aleyrodis forms these structures on a cellophane sheet after four days incubation at 100% relative humidity and 20°C (Fig. 2.2).

A fungicide treatment was carried out to establish indirectly how long it took the fungus to penetrate the host. The fungicide mancozeb was chosen as a fungicide inhibiting spore germination as well as mycelium development (Ignoffo et al., 1975, Hall, 1981). The recommended rate for application in crops is 1.4-1.9 kg active ingredient (a.i.) per ha (Worthing & Walker, 1983). In this experiment the dosage of 0.63 gr WP/l (0.504 gr a.i./l) was used based on a rate of 1.5 kg a.i./ha for an application by means of a conventional spraying device.

Spores did not germinate on malt agar with different levels of fungicide. Germination of spores applied on malt agar in suspensions containing the fungicide was only observed at the lowest concentration of 0.0063 mgr(WP)/ml. The percentage germinated spores was 95.7% after 72 hr incubation compared with 97.1% in the control. However, at the concentration of 0.63 mgr/ml, which was used in the in vivo bioassay, the spores did not germinate.

A fungicide treatment immediately after inoculation of the larvae with A. aleyrodis spores reduced successful infection to a very low level, only about 5% of the infection rate of larvae not treated with mancozeb (Fig. 2.3). In the controls using mancozeb or water alone the mortality of whitefly larvae was about 10%, but fungicide treatment alone of young first instar larvae caused high mortality. Infection with Aschersonia aleyrodis after fungicide treatment of these first instar larvae at day 0 was absent. Application of the fungicide two days after inoculation of the larvae caused a reduction of the infection rate by about 50% in comparison with the infection rates after inoculation of the larvae with A. aleyrodis only. The mean infection rates of the larvae treated with A. aleyrodis only were 90.6%, 91.5% and 86.1% for first, second and third instar larvae, respectively. In other words, 50% of the larvae relative to the

Fig.2.5 Infected whitefly prepupae showing the orange colouration that indicates infection with Aschersonia aleyrodis. Two empty pupal cases from which adult whiteflies emerged and a black pupal case from which the parasitoid Encarsia formosa emerged may also be seen.

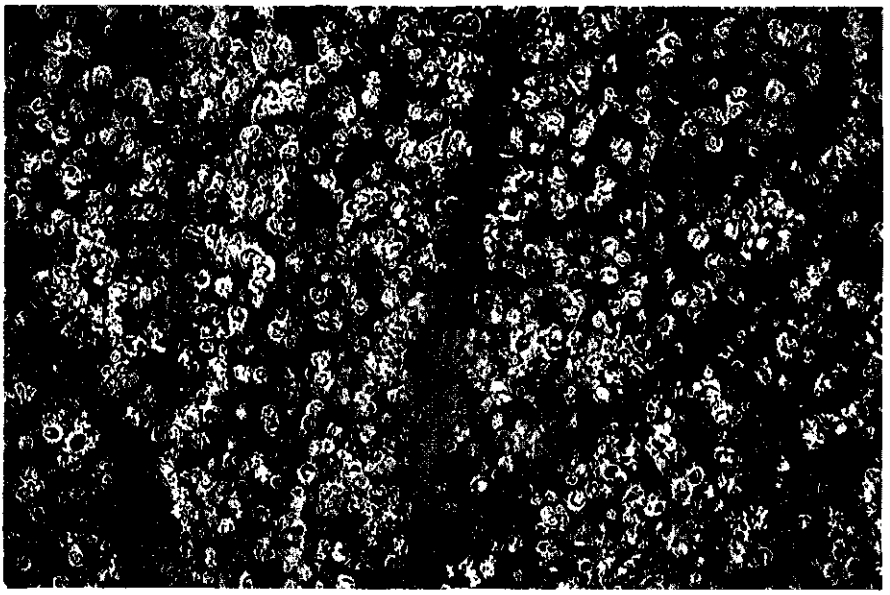


Fig. 2.6 Cucumber leaf showing whitefly larvae infected with Aschersonia aleyrodis in the sporulation phase.

Fig. 2.7 Larva of greenhouse whitefly colonized by Aschersonia aleyrodis producing orange-coloured slimy spore-masses.

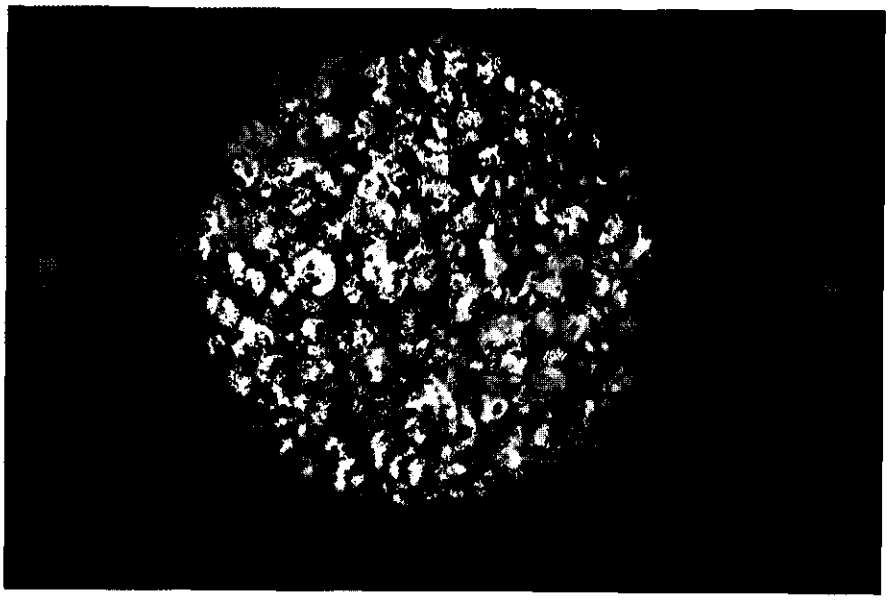


Fig. 2.8 Sporulating culture of Aschersonia aleyrodis on autoclaved millet grains.

respective infection rate of larvae not treated with mancozeb had successfully been penetrated by the fungus within two days at 20°C, thus escaping from the effect of the fungicide. A fungicide application three days after inoculation caused a mean reduction of 10% in the infection rate of third instar larvae (Fig. 2.3). In conclusion about 3 days after spore application most of the whitefly larvae had been penetrated by the fungus. Larvae, escaping from infection by moulting to the next instar stage, however, are still exposed to infective spores on the leaf surface with which they may come into contact after moulting and extending in size. A similar safety period for application of a fungicide after treatment with an entomopathogenic fungus was observed by Wilding & Brobyn (1980) for infection of pea aphids with Entomophthora aphidis. They found no reduction in infection rates when there was an interval of 70 hr between inoculation with spores and fungicide applications.

(4) Inside the host most entomopathogenic fungi sooner or later produce various structures, collectively referred to as hyphal bodies, which circulate in the haemolymph before germinating into mycelium. These are generally ovoid blastospores or truncated hyphal fragments produced by budding or separation from the initial penetrant hyphae, which often proliferate further by division (Charnley, 1984). After staining the hyphal bodies were observed to be present in the haemolymph of third instar larvae four days after inoculation with A. aleyrodis spores. These hyphal bodies formed proliferating mycelium throughout the haemolymph (Fig. 2.4). At the same time the haemolymph became floccose as was observed after dissection of whitefly larvae at various intervals after spore application (Chapter 8).

(5) Many entomopathogenic fungi overcome their hosts before extensive invasion of organs takes place and therefore toxins are presumed to be responsible for host mortality (Roberts, 1981). Whether toxins play a role in the greenhouse whitefly - A. aleyrodis relationship is unknown.

(6) The assessment of death of the insect is difficult before signs of discolouration become apparent. Whitefly larvae are sessile on the leaf and, therefore, symptoms of infection like slugginess or paralysis can not be observed. A decrease in honeydew production may indicate the time of cessation of host metabolism.

(7) The next phase involves the growth of mycelium with invasion of virtually all organs of the host. At this point larvae infected with A. aleyrodis are recognized by their orange colour, which may be either transparent or opaque (Fig. 2.5). The first insects showing signs of infection are present about 7 days after inoculation at 20°C. The orange colouration may appear in the stage in which the larva was treated or in later instars (see also Chapter 3).
(8) This stage refers to the protrusion of hyphae from the interior through the cuticle to the exterior of the insect. If the "mummy" (Fig. 2.5) is held under conditions of low RH the fungus will remain within the cadaver, but in a humid environment the mycelium will protrude. Usually, intersegmental membranes offer less resistance to penetration and this is the area of the first emergence of the fungi. White mycelium protrudes from the margin of the greenhouse whitefly larvae and pupae likewise as described by Samson and McCoy (1983).
(9) The production of conidia takes place in pycnidia formed on the stroma which increases in size. The fungus hardly spreads over the leaf surface and can easily be removed from the leaf surface. Pycnidia with orange-red slimy spore masses may be formed in a circle round the edge of the larvae (Fig. 2.6) or covering the stroma (Fig. 2.7) (Petch, 1921). The formation of pycnidia and the production of conidia on semi-artificial media is similar to that on the host insect (Fig. 2.8).
(10) The final step in the disease development cycle is the dispersal of infective units to locations where they are likely to encounter susceptible insects for the initiation of new cases of disease. The spores of A. aleyrodis are produced in slimy masses and are dispersed by means of water in a natural environment.

Finally, it can be concluded that A. aleyrodis causes the death of larvae of Trialeurodes vaporariorum by penetration and proliferation inside the host.

CHAPTER 3

THE DIFFERENTIAL MORTALITY AT VARIOUS LIFE STAGES OF GREENHOUSE WHITEFLY, TRIALEURODES VAPORARIORUM, BY INFECTION WITH THE FUNGUS ASCHERSONIA ALEYRODIS

3.1 INTRODUCTION

Biological control of pests occurring in glasshouses and orchards is an important research topic in the Netherlands and several successful methods have been developed (Minks & Gruys, 1980). For glasshouse crops, commercially applied integrated pest management (IPM) programs have been developed in which the predatory mite Phytoseiulus persimilis is used against the two-spotted spider mite Tetranychus urticae, and parasitoids against greenhouse whitefly and leafminers are introduced. Additionally, Bacillus thuringiensis is used against caterpillars (Ravensberg et al., 1983).

The parasitoid Encarsia formosa is effective against the greenhouse whitefly Trialeurodes vaporariorum, on tomatoes (Ravensberg et al., 1983), but on cucumber the excessive hairiness of the leaves impairs its efficacy (Hulspas-Jordaan & Lenteren, 1978). The need for frequent introductions and for careful supervision of the parasitoid's population development makes the use of E. formosa in cucumber economically unattractive. There is, therefore, a need for an additional biological control method, which would fit in the IPM schedule for cucumbers.

In preliminary glasshouse experiments Ramakers and Samson (1984), showed that Aschersonia aleyrodis is a promising natural enemy of greenhouse whitefly. This fungus has been reported from several subtropical and tropical areas. It infects Dialeurodes citrifolii and Dialeurodes citri in Florida (Berger, 1921, Fawcett, 1944) and Aleurodes woglumi in San Salvador (Quezada, 1974). The Deuteromycete Aschersonia aleyrodis forms orange-coloured spore masses in pycnidia. The spores are dispersed by water, in general by splash dispersion.

The results of applying different Aschersonia species to control

several whitefly species, either in glasshouses or in citrus orchards, have been reported from Japan, China and Eastern Europe (Uchida, 1970, Fang Qi-Xia et al., 1983, Primak & Chizhik, 1975). Spore suspensions are often sprayed in plots infested with mixtures of instars in order to evaluate infection by the fungus; however, exact data on the infection of different developmental stages cannot be obtained in this manner. Here, I present results aiming to elucidate the differential susceptibility of greenhouse whitefly at various developmental stages to infection by A. aleyrodis. This information will be useful for the development of an appropriate method for application of this entomopathogen in glasshouses.

3.2 MATERIALS AND METHODS

Plants and host insects

Greenhouse whitefly was reared on the mildew-resistant cucumber cv "Profito". Plants with four to five fully-grown leaves were used for the experiments. About 20 to 30 adult whiteflies put in clip cages were allowed to lay eggs on the undersides of the youngest fully-grown leaves for 24 hr at 20°C and a 16 hr photoperiod. Whitefly was treated with A. aleyrodis in the following stages: young eggs, old eggs, first, third and fourth instar larvae and socalled prepupae. Larvae are only mobile at the start of the first instar stage. After a period of time they settle on the leaf surface. The fourth larval stage is flattened, translucent to opaque-whitish, and gradually changes without moulting and becomes expanded, wax-ensheathed, opaque-white with dorsal and lateral waxy, spine-like protuberances (Nechols & Tauber, 1977). This transitional substage will be referred to as the prepupa. Further development takes place and the pharate adult with red eyes becomes visible beneath the integument. I will refer to this stage as the pupa (Lenteren et al., 1976a).

Because the egg laying was scheduled, whiteflies belonging to different developmental stages could be treated with a spore suspension of A. aleyrodis simultaneously.

Entomopathogenic fungus

The fungus A. aleyrodis (Rs.no.1088, the Centraalbureau voor Schimmelcultures at Baarn, the Netherlands) was cultured on autoclaved coarse cornflour and was subcultured five times. By rinsing with sterilized distilled water, spores were harvested from a three-week-old culture grown at 25°C. Spores suspended in sterilized distilled water were counted using a Schreck/Hofheim counting chamber. Two milliliters containing 4 x 10^6 spores/ml were applied to the underside of the leaf bearing the whiteflies with a Potter spray tower set at 34.45 kPa. The spore suspension was shaken at regular intervals using a table top shaker before it was applied. Ten leaves per age class were treated with a spore suspension of A. aleyrodis and two leaves were treated with distilled water as a control.

The viability of the spores was tested by spraying the suspension onto a water agar plate. The germination of spores was checked 24 hr after spraying: it appeared to be about 99% at 25°C throughout the experiments.

After the water had evaporated from the plants, they were covered in plastic bags to keep the relative humidity (RH) at 100% for 24 hr at 20 $\pm$ 2°C and a 16 hr photoperiod. Thereafter, the bags were removed and the plants were kept at 70 $\pm$ 10% RH.

The progress of infection was recorded at 3- to 4-day intervals until 90% of the uninfected instars had developed to adult whiteflies. Three categories of whitefly were distinguished: (1) instars showing signs of fungal infection, (2) healthy instars and empty pupal cases, (3) instars killed by other causes. The percentage infection was defined as the number of infected instars (1) divided by the sum of numbers belonging to (1), (2) and (3). In this experiment the natural mortality (3) in the control was negligible ($\leqslant$5%) and therefore no correction for natural mortality was made in the calculations.

Treatment of adult whiteflies under the Potter spray tower was difficult because they had to be anaesthetized and many drowned when sprayed. Therefore, adults were exposed directly to the surface of a sporulating culture of A. aleyrodis for 24 hr. After the exposure the adults were put in clip cages on cucumber plants at 20 $\pm$ 2°C and 70 $\pm$ 10% RH and examined for infection from 14 to 21 days post-treatment.

Because treated whitefly eggs hatched but the larvae were infected, studies were also made as to whether spores on the leaf surface might have caused this infection. Mobile first instar larvae were put on leaves that had been treated the same day or three or seven days earlier. In this experiment a spore suspension (2 ml, 1.6 x 10^7 spores/ml) from a culture on brown rice was used (82% germination on water agar). Two treatments were given: (1) plants were incubated for 72 hr at 100% RH after spraying; (2) crawlers (mobile first instar larvae) were put on the leaves before incubation for 72 hr at 100% RH.

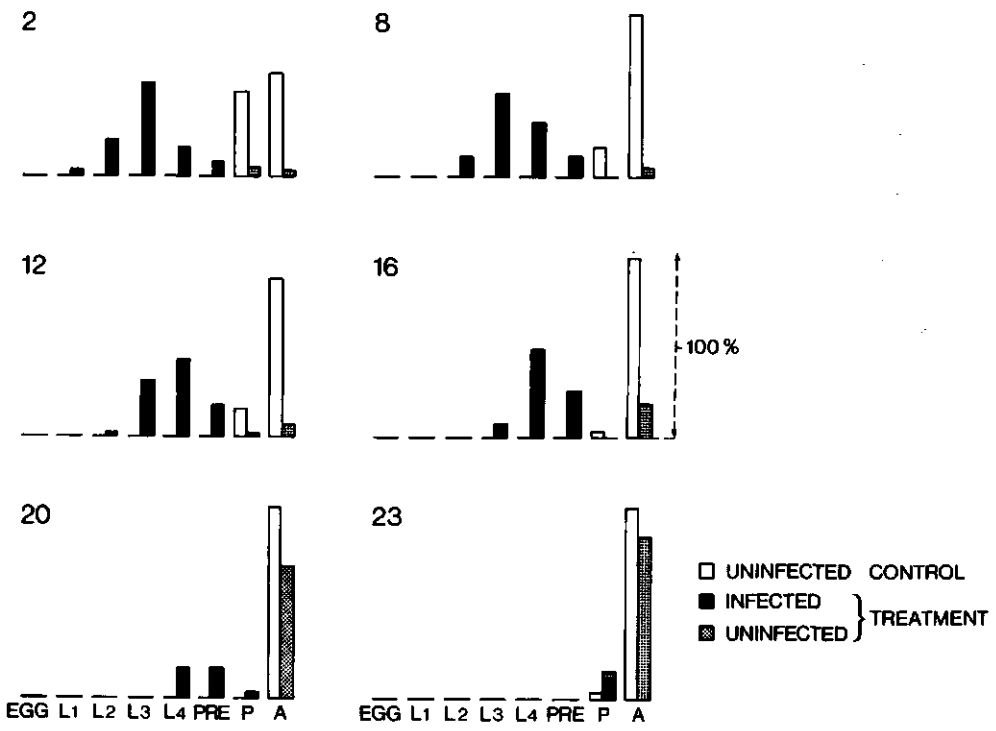


Fig. 3.1 Frequency distribution of proportions of infected instar stages at the end of the experiment after greenhouse whitefly was treated 2, 8, 12, 16, 20 and 23 days after egg laying corresponding to young or old eggs and first, third, fourth larval instars and prepupae, respectively.

3.3 RESULTS

Whitefly, which feeds on phloem, is infected when germinating spores of A. aleyrodis penetrate the insect's cuticle. The first sign of infection is a discolouration of the larvae from opaque white to bright orange. Under favourable circumstances the mycelium protrudes from the insect and forms a fringe around the insect's body. The following stage is an increase in mycelium, on which masses of spores are produced in pycnidia (Samson & McCoy, 1983).

Infection by A. aleyrodis declined with increasing age of whiteflies (Table 3.1a). Treatment of adults did not result in infection. No fixed time interval was found between the application of A. aleyrodis spores and the appearance of signs. The first sign of discolouration appeared four days after the larval stages had been treated, but this incubation period could extend to 10 to 14 days. Signs of infection could, therefore, still appear when whiteflies treated as young larvae had developed into prepupae (Fig. 3.1). When older instars were treated the overall incubation period was shorter.

When treated as 2-day-old eggs, about 7 days before hatching, signs of infection peaked at the third larval instar stage. There was a shift to a higher proportion of fourth instar larvae showing infection, when 8-day-old eggs and first instar larvae were treated. After young (2 days) and old (8 days) eggs had been treated, no mycelial growth, discolouration and/or sporulation were observed at the egg stage. In some cases, spores and germ tubes were found on empty egg skins, but penetration through the chorion was not found. The mortality of treated eggs did not differ from mortality of control eggs. However, larvae emerging from treated eggs later showed signs of infection (Table 3.1a). An additional experiment was carried out in which 1-day and 8-day-old eggs were treated showing similar results (Table 3.1b). No significant difference was found between the percentages of larvae hatched from eggs in the control treatment compared with those in the pathogen treatment. There was a difference between the numbers of larvae that hatched from 1-day-old eggs compared with those that hatched from 8-day-old eggs; this might have been because some eggs died during early development. The larvae that hatched from treated eggs became infected (Fig. 3.2). The signs of

Table 3.1a Mean final percentage infection of whitefly instars by Aschersonia aleyrodis when treated at different periods after egg laying.

Day of treatment after egg laying	Stage treated	Mean no. insects (SD) Control (n=2)	Mean no. insects (SD) Treated (n=10)[1]	Mean % infection (SD)	
2	Egg	79.5	46.4 (18.3)	94.4 (3.5)[3]	a[2]
8	Egg	117.0	104.7 (28.8)	92.5 (3.8)[3]	a
12	L_1	115.5	115.5 (39.1)	90.3 (4.0)	a
16	L_3	133.0	109.7 (30.7)	75.9 (13.0)	b
20	L_4	187.8	82.0 (31.3)	27.5 (12.1)	c
23	Prepupae	145.5	187.8 (67.0)	11.5 (9.8)	d

Table 3.1b Mean final percentage infection of whitefly instars by Aschersonia aleyrodis when treated as 1- or 8-day-old eggs.

Day of treatment after egg laying	Stage treated	% Larvae from eggs (n)[1] Control	% Larvae from eggs (n)[1] Treated	Mean no. insects (SD) Control	Mean no. insects (SD) Treated	Mean % infection (SD)
1	Egg	79 (3) a[2]	77 (3) a	439.7 (96.8)	632.7 (225.0)	95.4 (2.6)a
8	Egg	96 (3) b	95 (6) b	134.7 (51.6)	193.2 (45.0)	97.3 (2.8)a

1: n = number of leaves per treatment

2: values followed by the same letter are not significantly different (Wilcoxon, $P<0.05$).

3: infected larvae

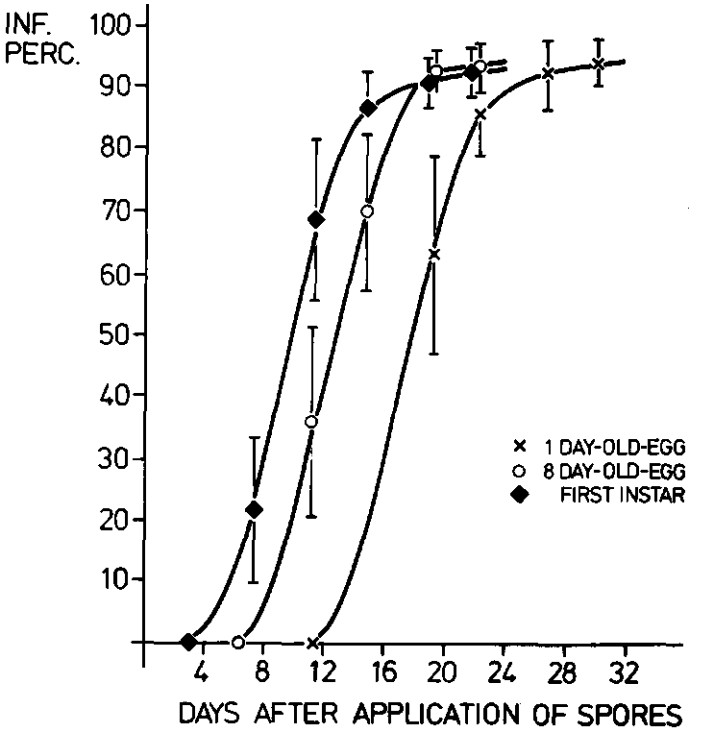


Fig. 3.2 Development of infection of whitefly larvae by Aschersonia aleyrodis over time when treated as 1-day-old eggs, 8-day-old eggs, and first instar larvae.

larval infection after treatment of eggs developed at the same rate and to the same extent as those from treated first instar larvae. The development of infection with time showed the same pattern. The appearance of the signs correlated with the developmental stage in which the whitefly was treated. The time lag before signs appeared comprised the time before the eggs hatched, about 2 days for old eggs and 8 days for young eggs. The final percentages of infected larvae did not differ significantly (Fig. 3.2).

The larvae hatching from treated eggs may have become infected during the egg stage, but the results presented above strongly suggest

Table 3.2 Mean final percentage infection of whitefly instars by residual activity of Aschersonia aleyrodis after crawlers had been put on treated leaves 0, 3 or 7 days after application.

Interval between treatment and placing crawlers on leaves (days)	% Infection	
	72 hr 100% RH after application	72 hr 100% RH after putting on crawlers
0	98% a (L = 4, P = 209)[1]	
3	97% a[2] (L = 2, P = 115)	75% b (L = 2, P = 92)
7	99% a (L = 2, P = 134)	76% b (L = 2, P = 143)

1: L = number of leaves per treatment
P = total number of pupae at the end of the experiment

2: Values followed by the same letter are not significantly different (Wilcoxon, $P<0.05$).

that they were infected by the A. aleyrodis spores present on the leaf surface with which these larvae came into contact when they were settling down.

In order to investigate the residual effect of the spores on the leaf, an experiment was carried out in which individual leaves of cucumber plants were treated with a spore suspension of A. aleyrodis. Immediately after the water component had evaporated from the leaf surface, or three or seven days later, crawlers were put on the leaves. The final percentage of infected larvae was the same in all three treatments (Table 3.2). The A. aleyrodis spores had a residual activity of at least seven days on the leaf surface. A humidity of 100% directly after application resulted in a better infection than when humidity was increased at the moment of crawler introduction.

3.4 DISCUSSION

Aschersonia aleyrodis successfully infects the first, second and third instar larvae of greenhouse whitefly. The fourth instar larvae, prepupae and pupae are successively less susceptible. The susceptible instars of whitefly do not always show signs of infection in the treated stage, but these may appear in subsequent developmental stages. That this general phenomenon of mortality is delayed until later stages can be ascribed to interactions between the insect integument being penetrated by the fungus and the moulting process of the host (Fargues & Rodriguez-Rueda, 1980, Fargues & Vey, 1974). This is also well illustrated by the results of Riba et al. (1983) and Feng et al. (1985) who showed that Ostrinia nubilalis is most susceptible to infection by Beauveria bassiana when exposed in the fifth larval stage which accounts for over 40% of the period of the entire larval stadium, compared to infection of younger larvae except for first instar larvae. A comparison between the infection of the treated whiteflies belonging to different age classes shows that there is a rapid decrease in infection from the fourth larval stage onwards. The last larval instar develops into the pupa without moulting. Inside the pupa the adult whitefly develops, and at the pupal stage the germinating spore has to penetrate not only the pupal case but also the cuticle of the adult. The development from the fourth larval stage to adult takes about 10 days at 20°C. During this time interval A. aleyrodis would have ample time to germinate and penetrate the cuticle of the host. Yet, mycosis does not appear, which may be due to resistance or immunity reactions of the host. Penetration may be difficult because the composition of the cuticle is changing and the adult whitefly is developing inside. The inability of the fungus to penetrate the cuticle of whitefly belonging to older instar stages may be due to failure of the fungus to produce enzymes or because increasing amounts of antifungal substances present in the cuticle inhibit the germination and penetration processes.

In my experiments Aschersonia aleyrodis was unable to infect and kill eggs and adults of greenhouse whitefly. In this latter case, this may be because the cuticle of the adult differs from that of the larvae. Also, the powdery wax that covers the adult may be an obstacle

to infection by the fungus. Furthermore, by grooming the adult insect may remove spores from its body, thus protecting itself in some way.

The effect of pathogenic agents is rarely tested on the insect egg, even though diseases of insect eggs may have an important impact on the mortality of this stage. For this reason, Steinhaus (1965) stressed the importance of investigating pathogenic agents of insect eggs. Signs caused by fungi are mainly discolouration and the appearance of the pathogen itself. In general, insect eggs are resistant to fungi, although sometimes they may become infected, particularly under damp conditions in the laboratory. There are, nevertheless, a number of Hyphomycetes that are specific to eggs. These include Oospora ovorum and species of Aspergillus, Fusarium, Penicillium and Macrosporidium (Madelin, 1963). The differences between a chorion and an insect cuticle presumably influence their susceptibility to infection by certain fungi. Chemically, layers of eggshells largely consist of protein. The hardening process of the eggshell is caused by crosslinking of these compounds (Margaritis, 1985). Zimmermann (1982) found that unmelanized young eggs of Otiorhynchus sulcatus were infected by Metarhizium anisopliae but older melanized eggs were less susceptible. Also larvae hatched from treated eggs were infected. He suggested that this could be due to infection of larvae within the eggshell or by picking up spores from the chorion during hatching.

Whitefly eggs are yellow white in colour when young but gradually turn dark purple indicating the melanization of the chorion. In my experiments neither young nor old whitefly eggs showed signs of infection by A. aleyrodis. The signs in newly hatched larvae were similar to those in treated larvae, whether the eggs had been treated when old or when young (Fig. 3.2). Thus, a difference in susceptibility between young unmelanized eggs and old melanized eggs was not found.

Infection of eggs and delayed infection of larvae was reported after egg masses of Spodoptera littoralis had been treated with Paecilomyces fumosoroseus (Rodriguez-Rueda & Fargues, 1980). Egg masses of Mamestra brassicae have also been found to be susceptible to infection by this fungus. Eggs of O. nubilalis were infected by M. anisopliae and P. fumosoroseus. Beauveria bassiana, however, only

infected up to 9% of O. nubilalis eggs, while Nomuraea rileyi failed to infect these eggs (Riba et al., 1983). Egg masses of M. brassicae and S. littoralis were not susceptible to N. rileyi either, but there was a delayed mortality of larvae which developed from treated eggs (Rodriguez-Rueda & Fargues, 1980).

The results of the experiment in which crawlers were placed on previously treated leaves, indicate that spores present on the leaf surface are capable of infecting larvae. Therefore, I believe that in T. vaporariorum the cause of infection should mainly be attributed to the viable spores still present on the leaf surface when larvae hatch, rather then to infection of the embryonic larvae in the egg before hatching. Very few data are available on the infectivity of spores of entomopathogenic fungi on plant surfaces. Brobyn et al. (1985) found that the infectivity of Erynia neoaphidis on abaxial leaf surfaces was greater than on adaxial leaf surfaces of Vicia fabae plants in the field. Also, the inoculum of conidia could remain infective for at least 14 days on a leaf surface in the field. A. aleyrodis was applied to the undersides of the leaves. This will positively affect the persistence of the spores, reducing the detrimental effects of sunlight. The results show that the spores remain viable for at least 7 days on the abaxial leaf surface at 20°C.

To evaluate the results for practical application of A. aleyrodis, the data have to be considered in a broader context in which spore dose, environmental conditions and virulence of the applied A. aleyrodis strain are also taken into account.

A. aleyrodis is most effective against larvae and control of whitefly can only be established in a glasshouse by repeated applications of spore suspensions. Guidelines for an optimal timing of the application of A. aleyrodis as a microbial insecticide against greenhouse whitefly should be developed.

CHAPTER 4

AGE-SPECIFIC DOSE-MORTALITY EFFECTS OF ASCHERSONIA ALEYRODIS ON GREENHOUSE WHITEFLY, TRIALEURODES VAPORARIORUM

4.1 INTRODUCTION

Entomopathogenic viruses, bacteria and fungi are being developed to control insect pests (Burges, 1981). The development of standardized bioassay techniques is essential for two main reasons. Firstly, the impact of a microorganism on an insect species needs to be measured. The assessment of dose-mortality relationships between pathogen and host enables one to estimate the amount of pathogen needed to obtain control of the insect pest in a practical situation. Additionally, the pathogenicity of strains and species of entomopathogens to the insect can be compared. Thus, bioassays can be used to select the most virulent strains. Secondly, bioassay methods should be developed to maintain consistency and accuracy in testing the quality of the microorganism. Especially, when these pathogens are produced in large quantities, the quality of the product must be guaranteed. The pathogenicity of a microorganism depends upon many interacting factors and is related to a specific set of conditions which exist between the microbial agent and a particular host (Burges & Thompson, 1971, Dulmage, 1973, Reichelderfer, 1985).

Reichelderfer (1985) describes five basic methods for execution of bioassays of fungal pathogens: (1) injection of suspensions into the insects; (2) topical application of spore preparations; (3) direct exposure of insects to conidia discharged from cultures produced in vitro; (4) direct exposure of insects to conidia discharged from infected insects; and (5) exposure of aquatic insects to contaminated water. Whitefly larvae, being phloem-feeding insects, can only be infected by germinating spores penetrating the insect cuticle. These larvae are sedentary except for the early first instars and they can not be removed from the leaves. There are few bioassays for testing pathogenic fungi against whitefly larvae or other sedentary phloem-feeding insects such as coccids. Hall (1982) applied Verticillium lecanii spores to second instar larvae of whitefly on leaf discs using

an immersion technique. More literature is available on testing chemical compounds against greenhouse whitefly (Yasui et al., 1985).

Aschersonia aleyrodis, a fungal pathogen of greenhouse whitefly, is being studied in the Netherlands for control of whitefly in glasshouses (Ramakers & Samson, 1984). Recent investigations have shown that the larvae are susceptible to infection by the fungus (Chapter 3). The aims of the experiments presented here were, firstly, to obtain information on the dose-mortality relationship of A. aleyrodis to greenhouse whitefly larvae of different instars. Secondly, to obtain information on the consistency of the bioassay results the tests were repeated after a certain period of time. Thirdly, an experiment was carried out to investigate the influence of in vitro subculturing on the pathogenicity of A. aleyrodis. Several reports have been published on the effects of in vitro subculturing of fungi on their pathogenicity, but no information is available on the stability of the virulence of A. aleyrodis after repeated subculturing on semi-artificial media.

The results of the experiments will contribute to the development of bioassay methods for screening of entomopathogenic fungi for whitefly control. Although bioassay results can not entirely predict the effect of the pathogen in a practical situation, they may provide some information on the amount of the pathogen needed to control the pest successfully, so long as the experimental conditions are similar to those in the greenhouse.

4.2 MATERIALS AND METHODS

Cucumber plants of about 60 cm high were used for the experiments. These host plants were reared under controlled climate conditions and artificial light, so host-plant quality was not expected to be influenced by seasonal variations. The procedure for obtaining larvae in different instars was described in Chapter 3. Whitefly adults were allowed to lay eggs 10, 14 or 17 days before the experiment, which resulted in first, second and third instar larvae, respectively, at the time of spore application. The age of the fourth larval instars varied from 19 to 22 days after egg laying in the different experiments. Four leaves per plant were used, each bearing

about 100 first, second, third or fourth instar larvae. The older instars were on the lower leaves. Six to nine leaves per instar and dosage were treated.

Aschersonia aleyrodis was reared on autoclaved brown rice or millet grains. Spores were harvested from two to four week-old cultures grown at 25°C. The spore suspension was obtained by the procedure described in Chapter 3, and was sieved through sterile cotton wool to remove clumps of the rearing medium. The viability of the spores was checked by recording the germination rate of about 300 of them on water agar after 24 hr at 25°C (Table 4.1). A spray of 2 ml spore suspension was applied to the leaf surface in a Potter spray tower set at 34.45 kPa (Potter, 1952). The dosage range used per experiment is shown in Table 4.1. The amount of suspension reaching the plant surface was assessed, by spraying different volumes of water onto 9 cm diameter Petri dishes in the Potter spray tower. These Petri dishes were weighed before and after application. Thus, it was found

Table 4.1 Overview of the different experiments.

Experiment no.	A.aleyrodis number of subcultures	Date of experiment	% germination on water agar 24 hr	48 hr	Dosage (spores/2ml)
I	4	84-7-26	99.0		0, 10, 20, 100, 200, 1000 x 10^5
II	4	84-8-28	99.0		0, 1, 5, 10, 50, 100, 500 x 10^5
III	4	86-4-01	75.2	82.8	0, 5, 10, 50, 100, 500 x 10^5
IV	12	86-4-02	81.2	91.3	as in III
V*	2	85-1-17	95.6		as in II

*: only fourth instar larvae were treated

that only 10% of the original amount of 2 ml reached a surface of 63.61 cm^2. Several experiments were carried out as shown in Table 4.1. The whiteflies were reared on cucumber plants, of cultivar (cv) "Gele Tros" in experiment I and cv "Profito" in the other experiments. Two hours after spraying the water had evaporated from the leaf surface. The plants were then covered by transparent polythene bags for 40 hr, thus creating an atmosphere of 100% relative humidity (RH). Thereafter the bags were removed and the RH was kept at 70%. The plants were kept in a controlled climate room at 20 $\pm$ 1°C and 16 hr photoperiod. Some of the plants used in experiments III and IV were also kept in a glasshouse compartment. The temperature in this compartment fluctuated (20 $\pm$ 2°C) and the natural daylength was extended to 16 hr by means of artificial light. The RH was kept at 70%.

In experiments I, II, and V the leaves were inspected at intervals, starting 5 days after treatment for the three highest dosages, and on days 7, 10, 14 and 21 for all treatments until about 90% of the adult whiteflies had emerged from the healthy pupae. This took place around day 21 for first and second instars, around day 18 for third instars and day 14 for fourth instars. In experiments III and IV, infection rates were recorded at the end of the experiment, at a moment comparable to the final recordings in experiments I, II and V. The fraction of dead larvae was the number of larvae and pupae killed by A. aleyrodis infection and by any other causes, divided by the sum of numbers of infected, dead and healthy larvae, pupae and empty pupal cases. These fractions were transformed by the angular transformation. For each stadium in each experiment a regression analysis was performed of the transformed fractions on ln concentration. These analyses provided estimates of the median lethal concentrations (LC50) and their standard errors (SE) for each estimator. The fraction of dead larvae, killed by other causes in the control treatment was less than 5%. No signs of A. aleyrodis infection were found on whitefly larvae in the control treatment. The angular transformation was used for analysis of the data, since not only the dosage causing an overall mortality of 50% is of importance, but also the dosages causing lower and higher overall mortalities. In this case the angular transformation is a more suitable method for normalization of the data

(Snedecor & Cochran, 1980). Often, LC50's are estimated by use of a logit transformation which results in LC50's comparable to those estimated by angular transformation. The slope of the line obtained by angular transformation, however, can not be compared directly with the slope obtained by logit transformation of the data. The regression coefficient obtained by angular transformation has to be multiplied by approximately 2.5 for comparison with the regression coefficient obtained after logit transformation. Pairwise comparisons of LC50's for the different instars within experiments or for similar instars between experiments were made using (approximate) t-tests, each test at the 5% level of significance. Although four larval instar stages were present on separate leaves of one plant, all having been treated with the same dosage, plant effects were ignored since the variation between leaves of different plants was not substantially greater than the variation between leaves on the same plant. The tests concerning the differences in dose-response curves between instars within an experiment may therefore be considered conservative.

From the mortalities over time in experiments I, II and V the median lethal time (LT50) was calculated. As every leaf was evaluated over time, these mortalities were not independent. A separate regression analysis against time was therefore carried out for each leaf in relation to each of the higher dosages. This was done by logit transformation of the fractions of dead larvae against ln time. In each experiment the LT50 values were determined using dosage and instar as qualitative factors. The number of replicates ranged from seven to nine. Pooled-variance standard errors for means were estimated. Pairwise comparisons of LT50 values for the same dosage between different stages within or similar stages between experiments were carried out by (approximate) t-tests at the 5% level of significance.

4.3 RESULTS

In Table 4.2 the LC50 values for different instars and experiments are shown. Fig. 4.1 shows the relationship between the angular transformed mortality fractions of the different instars and the ln concentration for experiments I to V, respectively. In general, the first and second instar larvae were the most susceptible to infection

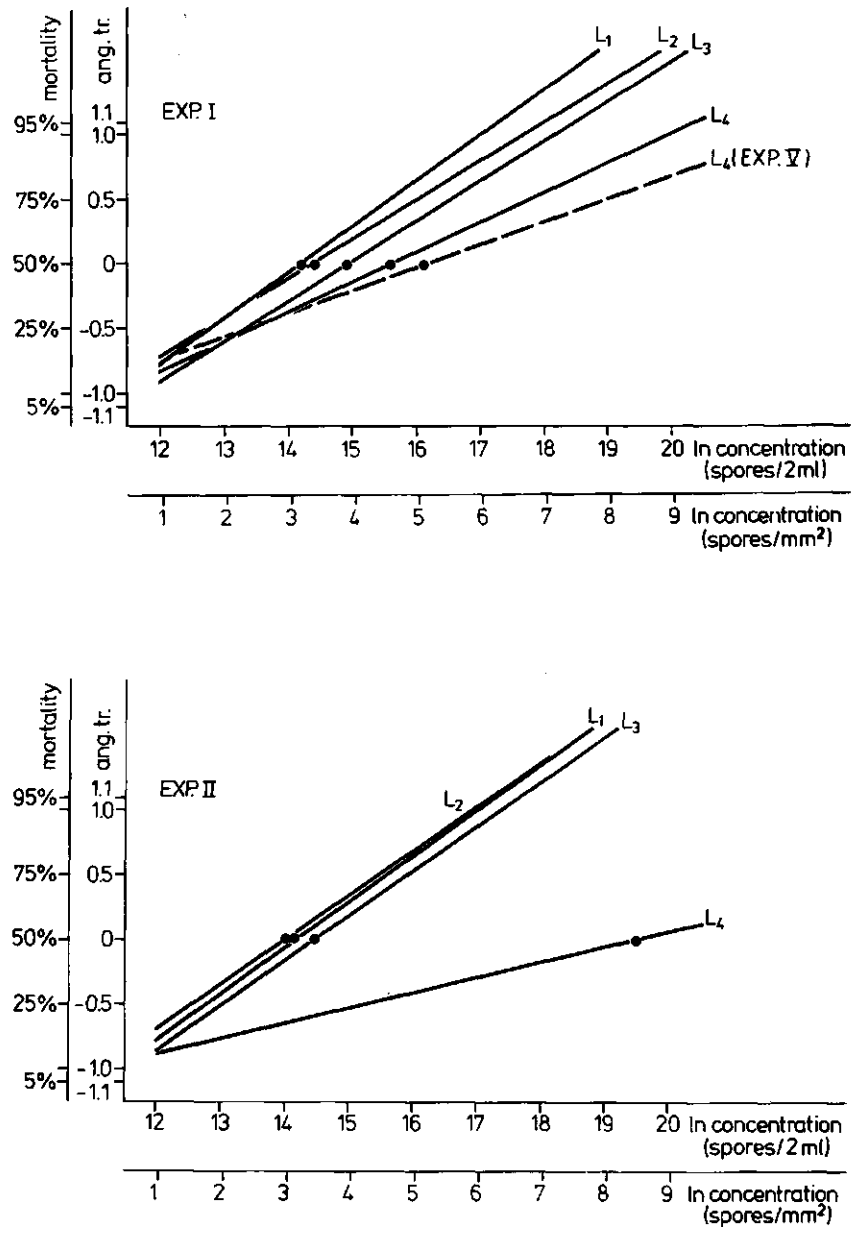
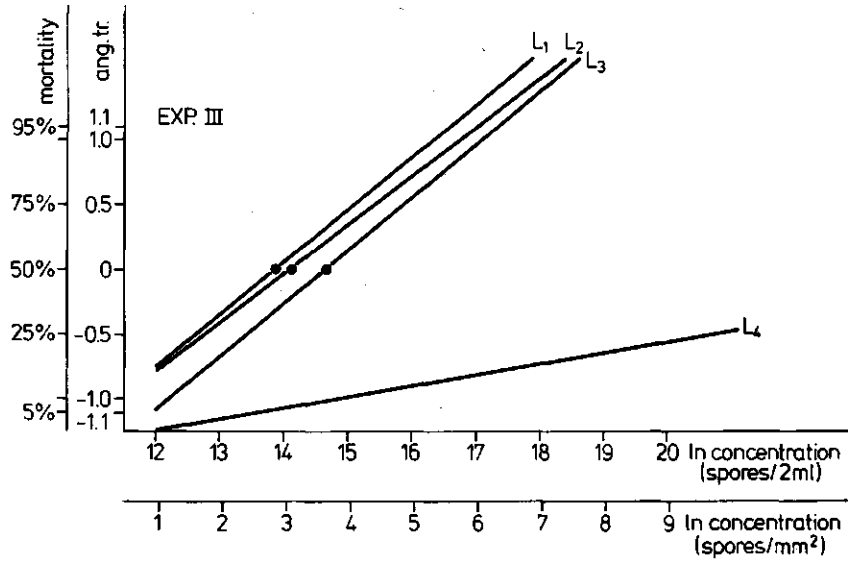


Fig. 4.1 The dose-mortality relations between Aschersonia aleyrodis and the four larval instars (L1, L2, L3, L4) of greenhouse whitefly in experiments I and V, II, III and IV, respectively. The data were normalized by angular transformation ($y=2$ arcsine $\sqrt{x} - \pi/2$).



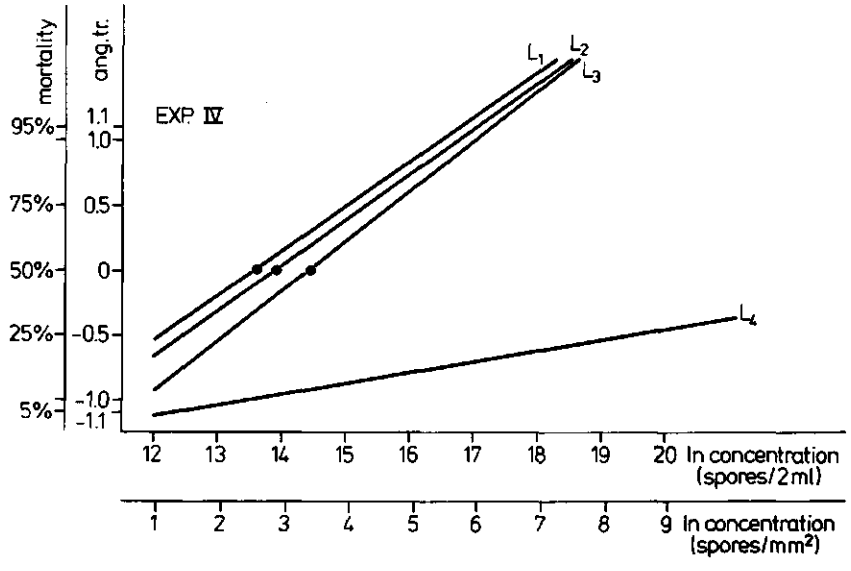


Fig. 4.1 Continued.

Table 4.2 LC50 (spores/2 ml), slope of the regression line of angular transformed mortality fractions against ln concentration and statistics for the four larval instars of greenhouse whitefly treated with Aschersonia aleyrodis.

Exp. I	L_1	L_2	L_3	L_4
LC50 ($x10^5$ spores/2 ml)	14.6 a[1]A[2]	18.8 abA	30.9 bcA	59.6 cA
95% confidence interval	10.1-21.0	12.0-29.3	21.5-44.6	32.4-109.9
Slope ± SE	0.36±0.04 a	0.31±0.04 ab	0.32±0.03 ab	0.24±0.04 b
Exp. II				
LC50 ($x10^5$ spores/2 ml)	14.1 aA	12.8 aA	19.1 bB	2646.5 cB
95% confidence interval	11.4-17.5	10.3-15.8	15.4-23.7	364.1-19235.6
Slope ± SE	0.37±0.02 a	0.37±0.02 a	0.37±0.02 a	0.13±0.02 b
Exp. III				
LC50 ($x10^5$ spores/2 ml)	10.7 aAB	14.0 aA	23.9 bAB	—[3]
95% confidence interval	8.3-14.0	11.0-17.9	20.4-28.0	--
Slope ± SE	0.42±0.03 a	0.39±0.02 a	0.43±0.02 a	0.09±0.02 b
Exp. IV				
LC50 ($x10^5$ spores/2 ml)	7.9 aB	11.2 aA	19.0 bB	—[3]
95% confidence interval	5.5-11.7	7.8-15.9	14.9-24.1	--
Slope ± SE	0.36±0.03 a	0.38±0.03 a	0.41±0.03 a	0.08±0.03 b
Exp. V				
LC50 ($x10^5$ spores/2 ml)	--	--	--	98.8 A
95% confidence interval	--	--	--	46.3-210.9
Slope ± SE	--	--	--	0.18±0.03

[1] values followed by the same lower case letter are not significantly different within one experiment ($P<0.05$).

[2] values followed by the same upper case letter are not significantly different between experiments ($P<0.05$).

[3] LC50 not within dose-range tested.

by A. aleyrodis. This phenomenon was consistent for all experiments. The LC50 for the third instar larvae was higher than for the younger instars. The slopes of the regression lines were, however, not significantly different for the first, second and third instar larvae within the different experiments (Table 4.2). Fourth larval instars showed a high variability in mortality. This may be explained by the difference in age when they were treated. Larvae were treated 19 and 21 days after egg laying in experiments I and II, respectively. Larvae were treated 20 to 21 days after egg laying in experiments III and IV and 19 to 20 days after egg laying in experiment V. During the fourth instar stage a fast transition from a susceptible to a resistant form takes place and therefore, treatments have to be timed carefully and one should use a population of homogenous age. In experiments I and V the larvae had moulted to the fourth instar just before treatment and there was no significant difference at the 5% level between the dosages needed to infect 25% and 50% (Fig. 4.1). In the other experiments, the fourth instar larvae were further developed and less susceptible. In experiments III and IV the germination of A. aleyrodis on water agar showed some retardation (Table 4.1). This may also have affected the infection of the fourth instar larvae. Fast germination and penetration may be of importance when the susceptibility of the insect is changing.

Overall the results of the experiments were consistent over time (Fig. 4.1). Higher spore dosages were needed for infection in experiment I than in experiments II, III and VI. This difference may be explained by the fact that a mildew susceptible cv of cucumber as host plant was used in experiment I (Table 4.1). Mildew present on the leaves at a later stage may have influenced either development of infection or host plant quality.

Experiments III and IV were carried out in an experimental glasshouse compartment as well as in a controlled-climate room. The final results for infection of the different stages were not significantly different. The pooled results from the glasshouse and the controlled-climate room were analyzed for differences between the two isolates. There were no significant differences in infection of the different larval instars by A. aleyrodis subcultured 4 times and subcultured 12 times (Table 4.2, Fig. 4.1). The mean estimated LC95 over experiments

Table 4.3 Logit mortality- ln time regression parameters and statistics for the four larval instars of greenhouse whitefly treated with Aschersonia aleyrodis.

Dosage x10^5 sp/2 ml	Exp. I LT50 (95% confidence limits)	Exp. I Slope±SE	Exp. II LT50 (95% confidence limits)	Exp. II Slope±SE
L_1				
100	10.9 (9.7-12.2) a[1]A[2]	3.5±0.4	13.7 (12.7-14.8) aB	6.5±0.7
200	9.9 (8.7-11.2) a	4.8±0.4	--	--
500	--	--	11.8 (10.9-12.8) b	7.6±0.7
1000	7.9 (7.0- 9.0) b	4.2±0.4	--	--
L_2				
100	9.2 (7.9-10.8) abC	1.7±0.5	9.8 (8.9-10.7) cC	4.0±0.3
200	8.9 (7.6-10.4) ab	3.5±0.5	--	--
500	--	--	9.5 (8.6-10.4) c	6.0±0.3
1000	8.9 (7.7-10.3) ab	4.9±0.5	--	--
L_3				
100	9.3 (7.6-11.4) abD	1.8±0.5	8.8 (7.7-9.9) cD	3.0±0.7
200	7.3 (5.9- 9.1) b	3.0±0.6	--	--
500	--	--	7.0 (6.2-8.0) d	5.2±0.7
1000	7.2 (5.8- 9.0) b	4.9±0.6	--	--

Dosage x10^5 sp/2 ml	Exp. I LT25 (95% confidence limits)	Exp. I Slope±SE	Exp. V LT25 (95% confidence limits)	Exp. V Slope±SE
L_4				
100	2.2 (1.2-3.9) aA	1.2±0.3	6.1 (4.1-9.1) aB	2.3±0.3
500	--	--	5.6 (3.7-8.5) a	2.0±0.3
1000	4.3 (2.2-8.4) a	2.2±0.4	--	--

[1] values followed by the same lower case letter are not significantly different within one experiment ($P<0.05$).

[2] values followed by the same upper case letter are not significantly different between experiments ($P<0.05$).

II, III and IV for first instar larvae is 1.98 x 10^7 spores sprayed in 2 ml onto the leaf surface, which is equivalent to 311.3 spores/mm^2. The LC95 for second and third instar larvae is 2.34 x 10^7 spores/2ml (367.9 spores/mm^2) and 3.27 x 10^7 spores/2ml (514 spores/mm^2), respectively.

In experiments I and II recordings were made of the infection of whitefly larvae by A. aleyrodis over time. In Table 4.3 the LT50 is shown for first to third instar stage in each experiment, and the LT25 for fourth instar larvae is shown for experiments I and V. The time at which 50% of the larvae showed signs of infection (orange colouring of the larvae) was shortest after treatment of third instar larvae followed by second and first instar larvae, in that order. However, the differences were not always significant. The LT25 for the fourth instar larvae varied between the two experiments. This may have been due to the changes in susceptibility taking place in this stage. Remarkably, applying a higher dose to fourth instar larvae in experiment I increased rather than decreased the LT25.

4.4 DISCUSSION

Bioassay technique

The procedure followed in these experiments for obtaining the whitefly larvae was similar to the one described by Busvine (1980), except that in these experiments the whiteflies laid eggs over 24 hr instead of 48 hr. It is preferable for standardization to restrict the egg-laying period even to 12 or 8 hr to obtain a uniform population of an instar stage. The fraction of the larvae still in the previous stage or already in the following stage should be minimized. Older instar larvae of A. aleyrodis become less susceptible to infection. This phenomenon was also shown when testing chemicals against whitefly of different stages (Yasui et al., 1985). It is important not to terminate the experiment before all the healthy larvae have developed to adult whiteflies, because signs of infection may also appear in the pupal stage just before adult emergence.

The LT50, measured as the time until the appearance of the orange colouring of the larvae and pupae, does not represent the exact time

of insect death. A more exact procedure for determination of the time of insect death would be the measurement of the honeydew production by the whitefly larvae (Yasui et al., 1985). It is recommended to use third instar larvae for screening fungal pathogens on greenhouse whitefly, because they give the most consistent results over time (Fig. 4.1).

Hall (1982) measured the susceptibility of second instar larvae to Verticillium lecanii isolated from whitefly, using an immersion technique and leaf discs. The leaf discs were kept at high humidity. The LC50 from several experiments ranged from 3.99 to 7.00 x 10^5 spores/ml. A comparison with the results in Table 4.2 cannot be made because different techniques were used. It is preferable to spray a standardized amount of a spore suspension onto the leaf surface because this technique provides information on the numbers of spores deposited on the leaf surface, in contrast to the immersion technique. When using leaf discs, honeydew produced by the larvae will contaminate the leaf surface, which is kept upside down. The honeydew may influence the bioassay by stimulating germination and growth of the entomopathogen or of other microorganisms (Fokkema et al., 1983). Thus, it is preferable to use intact plants, or leaves kept in a natural position to avoid contamination with honeydew. Primak and Chizhik (1975) conducted a bioassay and obtained an LC50 of 4.9 x 10^6 spores/ml, equivalent to the application of 0.015 ml on 100 mm^2. Consequently, supposing no liquid was lost, about 735 spores/mm^2 were present, which was considerably more than the LC50 found in the present study.

Analysis of the dose-response relation was carried out on data which include the mortality from other causes than fungal infection. One can distinguish larvae infected by A. aleyrodis, which are orange coloured, from larvae which do not show this sign of infection and may be presumed killed by other causes. The percentage of hosts killed by other causes in the treated groups may be similar to the percentage of dead insects in the control groups, but it is sometimes smaller. In the heavily-dosed treated groups, for example, most of the insects will be killed by the fungus, and so cease to be susceptible to those factors which killed some insects in the control group. Therefore, the overall mortality has been used for analysis of the dose-response

relation, rather than applying the Abbott's formula, which corrects the percent mortality in the treated group for the percent mortality in the control group.

In conclusion, the dose-mortality response after treatment of greenhouse whitefly reveals a linear relationship after transformation of the data. Repeated bioassays show consistency over time. The method is suitable for evaluation of the impact of A. aleyrodis on greenhouse whitefly.

Factors influencing dose-response relations

Fig. 4.1 shows the relation between the numbers of spores per mm^2 on the leaf surface and the mortality of whitefly. Using the estimated mean LC50 from experiment II and III for the first, second and third instar larvae, respectively, the number of spores on the leaf surface amounts to 19.53 spores/mm^2, 21.03 spores/mm^2 and 33.81 spores/mm^2, respectively. Taking into account the areas (length x breadth) of these larvae (Lenteren et al., 1976a), theoretically about 0.77 spores per first instar larva, 1.44 spores per second instar larva and 4.39 spores per third instar larva, would cause 50% of them to become infected. This would be the case if spores were evenly distributed over the leaf surface. Most of the water droplets, however, contain more than one spore and after the water has evaporated the spores are distributed in a clustered form. Applying different quantities of water or using additives may result in another distribution pattern of spores on the leaf surface (Easwaramoorthy & Jayaraj, 1978). Also, the use of different plant species with, for example, differences in hairiness or wax composition, may result in another distribution pattern of the spores and different dose-response relations. Host-plant quality may interfere with the susceptibility of the host to infection by an entomopathogen. Boucias et al. (1984) reported that Anticarsia gemmatalis larvae that fed on mature soy bean foliage (pot set stage) were more resistant to infection by Nomuraea rileyi than were larvae that fed on the prebloom and bloom foliage. Hare & Andreadis (1983) determined the susceptibility of Leptinotarsa decemlineata to Beauveria bassiana when the host insect was reared on Solanum tuberosum, S. dulcamara and S. carolinense. The host plant

species most suitable for L. decemlineata survival in the field also produced the L. decemlineata larvae least susceptible to B. bassiana. Cucumber is a good host plant for greenhouse whitefly (Boxtel et al., 1978), but tomato or sweet pepper, which are less suitable host plants, may reveal other dose-response relations. A higher natural mortality and a lower development rate of the larvae on these host plants may influence the susceptibility of greenhouse whitefly to infection with A. aleyrodis. Cucumber was used in the bioassay because A. aleyrodis is primarily intended for application against whitefly on cucumbers.

Virulence

The insects may show a variable susceptibility to fungal infection between seasons and years, because of a varying selection pressure from pathogens present under natural conditions or from previous introductions of the entomopathogen itself (Hall, 1976). On the other hand, the possible appearance and predominance of more resistant forms of the host insect provides a selection reservoir for the establishment of new isolates of the pathogen itself (Briese, 1986). Therefore, when testing a batch of fungal spores comparison between a laboratory-reared insect stock and second generation insects (pathogen free) originating from a field population may be useful. At the same time, fungal isolates can be collected from the field after application and compared to the original strain chosen for mass production. In case of Aschersonia aleyrodis this fungus is not endemic in European countries. Also, after introduction in a glass-house situation A. aleyrodis is not expected to maintain itself in an epizootic or enzootic form. Thus, the selection pressure is likely to be negligible.

A. aleyrodis subcultured on millet 12 times over about one year did not show changes in virulence towards first, second or third instar larvae. Culturing fungal entomopathogens on different media may cause differences in virulence (Fargues & Robert, 1983a). The influence of subculturing on virulence of fungal pathogens seems to be rather inconsistent, strains of the same fungal pathogen showing reduction of virulence in one situation and stability in others (Hall,

1980a, Daoust & Roberts, 1982). Loss of activity by in vitro serial passages may be due to an unstable pathotype subcultured on minimal media and/or grown under suboptimal conditions (Ignoffo et al., 1982). Rearing fungi on (semi) artificial media, however, will never provide the same conditions for growth and development as a host insect. Therefore, one should be aware of the fact that sooner or later subculturing may cause either phenotypic or genotypic alterations in the fungus.

Recommended doses

Bioassay results on dose-mortality relations of Aschersonia aleyrodis against greenhouse whitefly may not be extrapolated to a glasshouse situation. Nevertheless, the number of spores necessary to cause a high infection rate may be indicated. A dosage of about 3 x 10^7 spores/2ml is needed to infect more than 95% of first, second and third instar larvae. In a glasshouse, an insecticide or fungicide is applied in a high volume spray using about 2500 liter for 1 ha of cucumber plants. This is comparable to the application of 3.75 x 10^{13} spores in total. The use of improved spraying techniques and additives may influence the distribution of spores on the leaf surface positively, thus decreasing the dose of spores needed to obtain successful control of whitefly. Formulation and spraying technique are, therefore, important in relation to the numbers of spores applied in a glasshouse situation.

In general, from the bioassay results it can be concluded that A. aleyrodis has potential to be used against greenhouse whitefly.

CHAPTER 5

EFFECTS OF DENSITY ON THE GERMINATION OF ASCHERSONIA ALEYRODIS SPORES AND THEIR INFECTIVITY TO GREENHOUSE WHITEFLY

5.1 INTRODUCTION

Aschersonia aleyrodis, an entomopathogenic fungus of several whitefly species, is currently being considered for its potential as a microbial control agent of greenhouse whitefly, Trialeurodes vaporariorum. The spores are the infective units. They germinate on the cuticle of the larva and penetrate the host by means of an appressorial structure (Chapter 2). First to third larval instars of greenhouse whitefly are successfully infected by the fungus (Chapter 3). Spores are produced in pycnidia on the mycelium after growth in infected insects (Samson & McCoy, 1984) or (semi) artificial media (Fawcett, 1908).

Ideally, germination should only take place in circumstances which provide favourable conditions for growth and development. In general, spores have mechanisms to detect such a favourable environment and respond to it (Allen, 1976). Reduced germination in dense populations of spores, which can be considered an unfavourable environment, has long been known. Allen (1976) presents a list of fungi belonging to a wide range of taxonomic groups in which this reduction has been observed. A rest period of reversible interruption of the phenotypic development of an organism is defined as dormancy. When this is brought about by unfavourable chemical or physical conditions of the environment, it is called exogenous dormancy. Another type is constitutive dormancy, where development is delayed due to factors related to the cell's own structure and function, such as a barrier to penetration of nutrients, a metabolic block or the production of a self-inhibitor (Sussman & Halvorson, 1966). In many fungi self-inhibition has been traced to inhibitory substances released by the spores themselves, but in others competition for key substances such as oxygen or nutrients may be the factor (Macko et al., 1976).

Although self-inhibition of spore germination is a common pheno-

menon, it is rarely mentioned in relation to entomopathogenic fungi. A form of self-inhibition has been suggested for Beauveria bassiana spores, in which high doses of spores in soil resulted in a lower percentage infected pecan weevil larvae (Gottwald & Tedders, 1983). In the present study the relation of spore density to the germination of A. aleyrodis spores in vitro and the impact of this phenomenon on the infection of greenhouse whitefly has been investigated, and a bioassay method for testing infection is presented in which leaf discs are used. Results on dose-mortality relations determined on intact plants were presented in Chapter 4.

Since spore concentration is important in relation to mass production, formulation and practical application of spores, information on the presence of a density effect would be useful for the development of A. aleyrodis as a microbial control agent.

Additionally, the influence of the age of the cultures from which A. aleyrodis spores are obtained is examined in relation to spore germination. Park & Robinson (1970), for instance, found that arthrospores of Geotrichum candidum from cultures of different ages showed different germination behaviour.

5.2 MATERIALS AND METHODS

Spore germination in vitro

Aschersonia aleyrodis was isolated from greenhouse whitefly that had been infected by spores of the isolate RS. no. 1088, obtained from the Centraalbureau voor Schimmelcultures, Baarn, the Netherlands. After isolation from the insect the fungus was subcultured twice on millet. A 26-day old culture grown at 25°C and a 16 hr photoperiod was used. Spores produced on the medium were removed by suspending them in sterile distilled water. A part of this concentrated suspension of unwashed spores was used for the preparation of a range of five different spore dosages. Either 2, 10, 20, 100 or 200 x 10^6 spores were applied in 2 ml onto water agar by means of a Potter spray tower, set at 34.45 kPa. About 10% of the spore suspension reached the surface of water agar in a 9 cm diameter Petri dish. The other part of the original suspension was used to obtain washed spores. The

suspension was centrifuged for 15 minutes at 2000 rpm to remove any substances present in the suspension or on the surface of the spores. The supernatant was collected and the pellet (spores) was resuspended in sterile distilled water. This procedure was repeated twice. The spores were applied to water agar at the same dosages as for the unwashed spores. Some of the spores were resuspended after the final washing in the supernatant obtained after the first wash. These were applied at dosages of 82 and 1600 x 10^5 spores/2ml. Three agar plates per spore dosage were used. The percentage germination was determined after 40 hr at 25°C and a 16 hr photoperiod by counting 300 spores per Petri dish. Spores were considered to have germinated when the length of the germ tube was similar to or exceeded the breadth of the spore.

In vivo dose-response bioassay

Larvae of a uniform age were obtained by scheduling the egg laying of whitefly adults at 20°C. Either first instar larvae (9 - 10 days after egg laying), second instar larvae (13 - 14 days after egg laying), third instar larvae (17 - 18 days after egg laying) or fourth instar larvae (20 - 21 days after egg laying) were present on leaves of cucumber plants cv "Profito". From these leaves leaf discs (6.5 cm diameter) were obtained, bearing whitefly larvae in a concentrated area of about 4 cm in diameter. These leaf discs were treated with various dosages of A. aleyrodis spore suspension prepared according to the method described in Chapter 3. The isolate was subcultured three times after receipt at the laboratory. The spores originated from a 15-day-old culture. Up to 98.5% of the spores germinated after 24 hr at 25°C on water agar. The dosages of spores used in this experiment were 10, 20, 100, 200, and 1000 x 10^5 spores/2ml. Six leaf discs were used for each dosage and instar stage. The control treatment was carried out by spraying sterile distilled water instead of spore suspension.

Each leaf disc was supported by a perspex ring (5 cm diameter) with the whitefly larvae in their natural position on the lower leaf surface (Fig. 5.1). A Petri dish (5.5 cm diameter), filled one third with distilled water, was placed on top of the leaf disc. The Petri dish was covered with cellophane, held in place by a rubber band. The

water was taken up by the cellophane and part of it was transported to the area between the leaf and the Petri dish, thus wetting the leaf. Photosynthesis was not impeded and the leaf discs remained green for over 14 days at 20 ± 1°C and 16 hr photoperiod. The leaf discs were checked for infected larvae at 7, 10 and 14 days after treatment. The percentage of infected larvae and pupae was determined by dividing the number of infected larvae and pupae by the total of infected, healthy, hatched and dead larvae and pupae and of empty pupal cases per leaf disc.

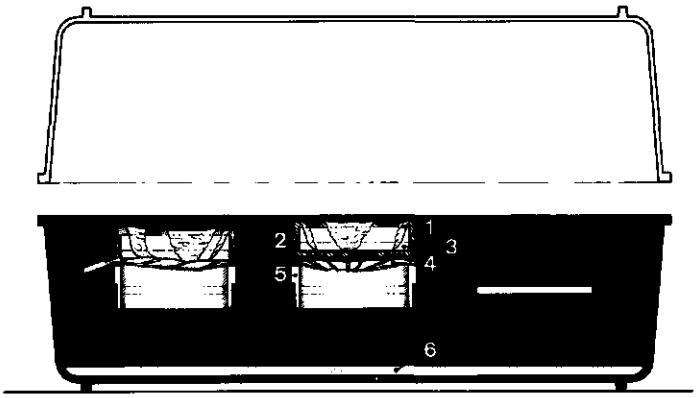


Fig. 5.1 Experimental set-up for the infection of greenhouse whitefly larvae by spores of Aschersonia aleyrodis on cucumber-leaf discs (1. cellophane; 2. rubber band; 3. Petri dish cover filled with water; 4. cucumber-leaf disc with whitefly larvae; 5. perspex ring; 6. water).

Influence of the culture age on spore germination

Aschersonia aleyrodis was subcultured three times on autoclaved millet after isolation from greenhouse whitefly and then used to test the spore viability at various periods after inoculation of the medium. Three cultures were used. Sectors of the colonized and sporulating medium were removed and the spores were suspended in sterile distilled water. The germination of spores from each culture

was assayed at 7, 11, 19, 25, 32 and 39 days after inoculation of the medium and subsequent incubation at 25°C and 16 hr photoperiod. A dosage of 3 ml of 1 x 10^6 spores/ml was sprayed onto water agar. Three agar plates were used for each culture and sampling date. After application of the spore suspension the agar plates were kept at 25°C and 16 hr photoperiod for 24 hr. For each plate 400 spores were examined for germination and recorded in the following categories: ungerminated; germ tube shorter than the breadth of the spore; germ tube longer than or equal to the breadth but shorter than the length of the spore; germ tube equal to and longer than the length of the spore.

5.3 RESULTS

The spores of A. aleyrodis germinate on water agar without an additional nutrient source. Fig. 5.2 shows the germination at different densities on water agar. The germination rate decreased with increasing density of spores. The percentage germination of washed spores was significantly lower than that of unwashed spores at low densities ($P<0.05$). At high spore densities the reverse was observed

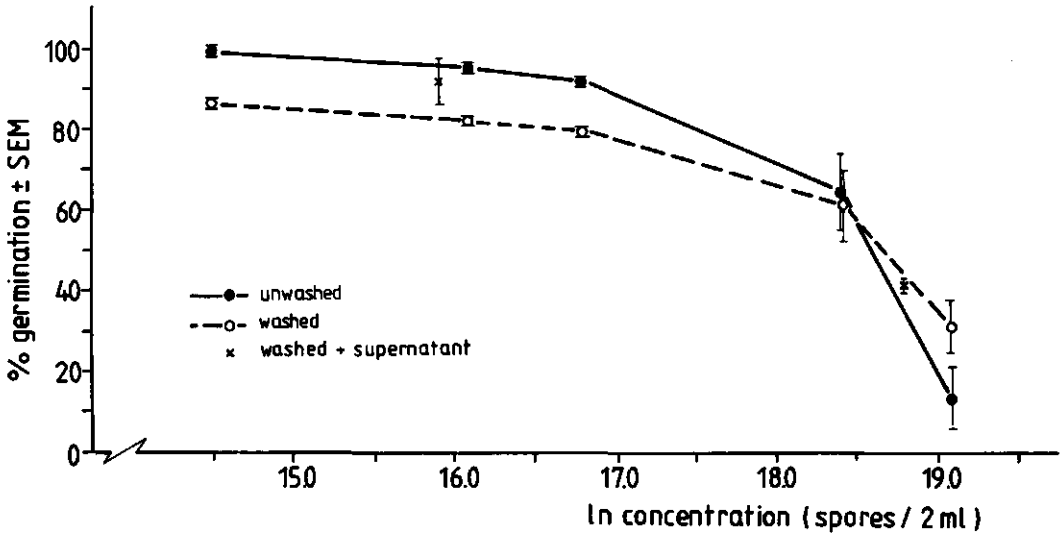


Fig. 5.2 Mean germination rates (%) and standard errors of the mean (SEM) for washed and unwashed spores of Aschersonia aleyrodis, 40 hr after application at different dosages to water agar.

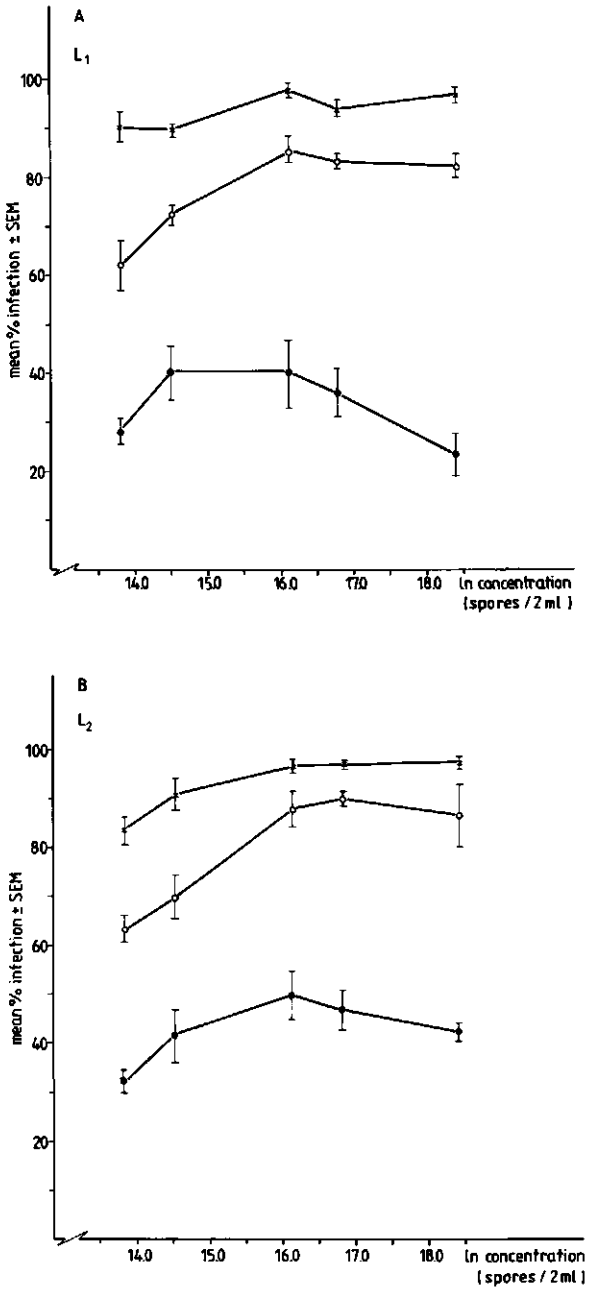


Fig. 5.3 Mean infection rates (%) and standard errors of the mean (SEM) for whiteflies treated as first (L_1), second (L_2), third (L_3), or fourth (L_4) instar larvae, with different dosages of Aschersonia aleyrodis spores, 7 (●), 10 (o) and 14 (*) days after treatment.

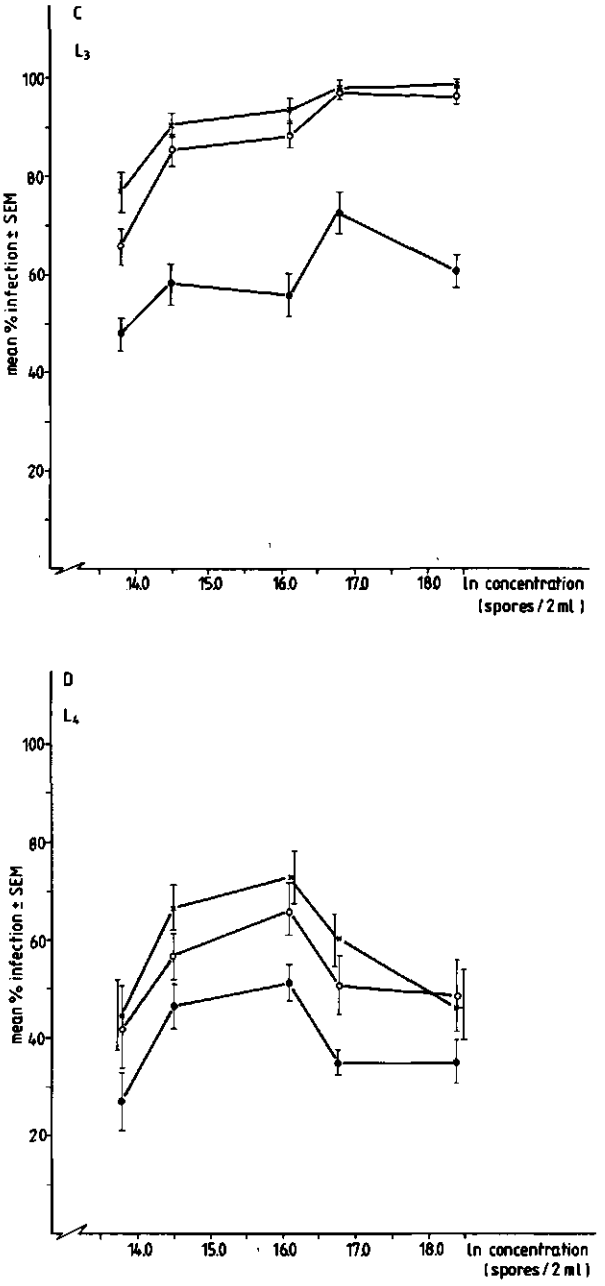


Fig. 5.3 Continued.

although the difference in percentage germination was not significant (Fig. 5.2). The corresponding densities on the agar surface were 314 spores/mm^2 for a suspension containing 2 x 10^7 spores/2ml and 3100 spores/mm^2 for a suspension with 2 x 10^8 spores/2ml. Addition of supernatant to the washed spores stimulated germination at low spore density.

A density effect of the spores on infection of whitefly larvae was found in the dose-response bioassay (Fig. 5.3). One would expect an increase in percentage infected with increasing spore concentration. This phenomenon was observed in the range of 10^6 to 10^7 spores/2ml, but at higher concentrations there was a levelling-off or even a decrease in the percentage infected after a seven-day incubation period. Seven days after application of 10^8 spores/2ml, the percentage of infected first instar larvae was significantly ($P<0.05$) lower than the percentage infected after application of 1 x 10^7 spores/2ml and 2 x 10^6 spores/2ml. Infection rates in second instar larvae were also lower at high doses of spores, but the percentages were not significantly different. The percentage infected third instar larvae after application of 10^8 spores/2ml was also significantly lower than after application of 2 x 10^7 spores/2ml. The density effect did not result in an overall lower infection rate at the end of the experiment, except in fourth instar larvae, where the effect was most evident. Infection percentages obtained after application of 2 x 10^7 and 1 x 10^8 spores/2ml were significantly lower than the percentages obtained at 1 x 10^7 spores/2ml. These older larvae become less susceptible over time (Chapter 3) and retardation of germination and penetration apparently had an important impact on the infection of fourth instar larvae. The experiment was terminated after 14 days when sporulation was observed on the infected larvae, favoured by the high relative humidity (100%). Thus, recording of mortality due to secondary infection was avoided. Treated young larvae may show signs of primary infection later than 14 days after treatment (Chapter 3), but in this experiment the infection rate of young larvae was very high after 14 days, even with low doses. The increase in infection after day 14 was, therefore, considered negligible. In general, the percentage infection was very high even at low spore densities. Application of 10^6 spores/2ml even resulted in 90% infection in larvae

and pupae treated as first instars, 83% infection in larvae and pupae treated as second instars and 77% infection in larvae and pupae treated as third instars (Fig. 5.3).

When the experiment was terminated after 14 days, 93% of the control whitefly larvae treated as fourth instars, 78% of those treated as third instars and 1.3% of those treated as second instars had become adults, and most of the second instars had developed into pupae. About 37% of first instars in the controls had developed into pupae and the rest were fourth instar larvae or prepupae. At day 14 the mortality in the control was less than 2% for all stages. The leaf discs with the water treated whiteflies were maintained until 21 days after treatment, when also the younger larvae had developed into adults. The mortality in the controls by that time was on average 5% (mean of 5-6 leaf discs) for first, third and fourth instars. The mean mortality for second instars was 8.8%, which may have been due to drowning of the larvae in free water on the leaf surface. In general, the larvae on the leaf discs were able to develop normally.

Fig. 5.4 Mean percentages of ungerminated spores and standard errors of the mean (SEM) for Aschersonia aleyrodis originating from cultures of different age.

Figure 5.4 shows the mean percentage of ungerminated spores of A. aleyrodis from three different cultures over time. A culture of A. aleyrodis can be used from 7 to about 32 days after inoculation of the medium. After that spores do not germinate. The proportions of germinated spores in the different categories stayed constant over time; more than 90% of the spores had a germ tube longer than the length of the spores (>10 μ) after incubation for 24 hr at 25°C. The mean proportion of spores with germ tubes shorter than the breadth of the spore at day 32 and day 39 was 2.0% and 4.0%, respectively, while the proportion of ungerminated spores showed a nine fold increase. Cultures grown on millet older than 32 days should not be used, because autolysis of the spores occurs when fungal growth stops on depletion of the medium and, presumably, harmful waste products are produced. Spores are continuously produced in pycnidia over time and new pycnidia are also formed. Therefore, spores from a culture are a mixture of young and older spores. Maturation of spores does not seem necessary for germination.

5.4 DISCUSSION

A density effect on germination of A. aleyrodis spores is evident. Conidial masses on sporulating colonies do not germinate, and spores on water agar show a reduction in the germination rate at high densities. Competition for oxygen, carbon dioxide or nutrients may play a role at high spore densities, but self-inhibitors produced by the spores may also be present. Since A. aleyrodis spores germinated well on water agar, the absence of an exogenous nutrient source does not prevent germination. Oxygen deficiency is considered unlikely to influence spore germination either; the spores are present in a thin layer on top of the water agar. A. aleyrodis spores at high concentrations germinated readily on malt agar (Fransen, unpubl. results). Within a range of 314 spores/mm^2 to about 3100 spores/mm^2 the decrease in spore germination was apparent, which agrees with the findings of Lingappa and Lingappa (1965) for Glomerella cingulata.

At low spore dosages, the germination rate for unwashed spores of A. aleyrodis was higher than for washed spores. Washing may remove compounds needed for germination, such as nutrients or a specific

stimulant leaching out of the spores. This was observed for Cochliobolus victoriae conidia as removal of exudates from around the spores resulted in restriction of spore germination (Sneh & Lockwood, 1976). However, washing of spores may also remove inhibitory substances. The final effect of these two counteracting phenomena is influenced by spore density. For instance, the uredospores of Puccinia graminis and other rusts have an optimum density for spore germination. Up to the optimum, increasing concentrations of a stimulant enhance germination and above the optimum increasing concentrations of inhibitors reduce it (Allen, 1976, Manners, 1981). At high densities of spores, inhibitory substances may affect the germination of A. aleyrodis spores and subsequent removal of nutrients by washing has less effect on germination. Thus, in the absence of a self-inhibitor, washed spores of A. aleyrodis would be expected to show a germination rate lower than or similar to that of unwashed spores. In contrast, the percentage germination of washed spores was higher than that of unwashed spores at the high density of 3100 spores/mm^2. This suggests a partial removal of an inhibitory substance by washing. Repeated washings of spores with water eliminated the inhibitory effect on spore germination in G. cingulata (Lingappa & Lingappa, 1966) and in Colletotrichum muscae (Parbery, 1981). However, a reduction in the germination rate of A. aleyrodis spores was still found after washing, possibly due to failure to remove all the inhibitor or to its further production by the spores. Macko et al. (1976) mentioned that the inhibitors of rust fungi were extracted directly from the spores with water but not with non-polar solvents. The self-inhibitors were analysed as cinnamatic esters and should have been readily soluble in non-polar solvents. It was concluded that water was apparently necessary for the release of the inhibitor.

When high doses of spores were applied to whitefly larvae a slowing down of the infection process was the result. This phenomenon was not observed in the dose-response experiment using intact plants with only a short period of high relative humidity (Chapter 4), although the LT25 of infection of fourth instars increased slightly when a higher spore dose was used. On the leaf discs a high humidity was maintained, and even free water was present on the leaf surface. This water film may have allowed the diffusion of an inhibitor pro-

duced by the spores which affected germination. Chung & Wilcoxson (1969) showed that prolonged storage of spores of Phoma medicaginis in water reduced the germination of newly added spores to the suspension after removal of the original spores.

The final overall infection rate after treatment of the whitefly larvae was much higher at low doses than it was for larvae on intact plants (Chapter 4). This is possibly due to the constant relative humidity of 100% and the presence of free water. Germination and penetration of the insect may be faster in the presence of free water. Moreover, after spore application the water from the spore droplets normally evaporates and the spores on leaves and larvae have a more or less clumped distribution. Condensation of water on the leaf discs creates a water film which may lead to redistribution of the spores and increase the proportion of spores that contact larvae. The density effect of A. aleyrodis spores takes place only at very high dose levels and under specific conditions. Investigations of the causes of the reduction in spore germination rate at high densities may be useful with regard to the formulation of a product. In a glasshouse situation, however, this density effect will not be seen because the recommended dosage (about 3×10^7 spores/2ml) will give lower spore densities (Chapter 4) and condensation on leaf surfaces does not usually occur.

Ageing of the cultures of A. aleyrodis on millet did not influence the germination of the spores up to 32 days after inoculation of the medium. Older cultures should not be used because autolysis of the spores occurs.

CHAPTER 6

SURVIVAL OF ASCHERSONIA ALEYRODIS SPORES ON LEAF SURFACES

6.1 INTRODUCTION

The survival of fungal structures of Aschersonia aleyrodis is important for colonization of new hosts. Before the contact with new hosts is established, the infective units are dispersed mainly by water or vectors, after survival through host-free periods. The host-free period may be spent in or on colonized insects, on plants, in soil or other habitats. The survival of inoculum directly influences the success of epizootic development of the entomopathogen in the field. Also, when entomopathogenic fungi are used as microbial insecticides, residual activity of the infective units can be useful. Natural epizootics of A. aleyrodis occur during the summer months in citrus groves in Florida causing mortality of Dialeurodes citri and D. citrifolii (Fawcett, 1944). High ambient relative humidity promotes infection and sporulation on the host; frequent rains cause effective dispersal of the infective units. However, the onset of the epizootic is related to the survival of infective units during the winter months. No reports on survival of A. aleyrodis in the original habitat are available. Studies on the survival of entomopathogens in a soil environment have been carried out by Ignoffo et al. (1978) and Fargues et al. (1983). Fargues and Robert (1985) reported that spores of Metarhizium anisopliae may even survive in soil up to 21 months, but spores on plant parts survived for only a few days. The detrimental effect of ultraviolet light (UV) is considered the main factor causing short longevity of fungal spores on plant parts (Ignoffo et al., 1977, 1979, Gardner et al., 1977). Nevertheless, fungi seem to be more resistant to UV than other entomopathogens are. Krieg et al. (1981) showed that Beauveria bassiana spores could withstand near UV (285-380 nm) better than nuclear polyhedrosis virus and spores of Bacillus thuringiensis. Additionally, spores on abaxial leaf surfaces and on lower leaves can survive longer, as was shown for Erynia neoaphidis on bean plants (Brobyn et al., 1985) and for Entomophthora muscae on carrot plants (Carruthers & Haynes, 1986). Apart from climatological

influences, the plant structure and the characteristics of the canopy may have effect on the survival of spores or other infective units. Moreover, the interaction between the entomopathogen and other micro-organisms, either antagonistic or synergistic, should be taken into account. This aspect has not yet received much attention in insect-pathogen studies.

Interest in Aschersonia aleyrodis, a fungal pathogen of greenhouse whitefly, Trialeurodes vaporariorum, is focused on its use as a microbial control agent by applying the spores, which cause direct infection of whitefly larvae. In Chapter 3 it was shown that spores of A. aleyrodis remained viable on leaf surfaces for at least seven days after application. Thus, the residual effect of surviving spores on leaf surfaces may be of importance, and, therefore, it was studied more closely.

6.2 MATERIALS AND METHODS

Bioassay

Whitefly adults were allowed to lay eggs on cucumber plants cv "Profito" for 24 hr. This period was designated day 0. Separate sets of leaves from the plants were treated with a spore suspension of A. aleyrodis, starting 18 days before egg laying was allowed. The other treatments took place at three-day intervals up to 9 days after egg laying. The last treatment took place just before the first instar larvae would have hatched from the eggs. Six leaves (two per plant) were sprayed, each with 3 ml of 1.0×10^7 spores/ml, and four leaves were sprayed with 3 ml of sterile distilled water as a control.

The A. aleyrodis isolate was subcultured twice before use, either on autoclaved millet grains or cornflour as a medium. The viability of the spores originating from these cultures was assessed after 24 hr incubation at 25°C (see also Chapter 3 for methods). The germination rate on water agar exceeded 90%.

After application of the spore suspension to the abaxial leaf surfaces, the cucumber plants were left to dry for two hours and then covered by transparent plastic bags for 24 hr to keep the relative humidity (RH) at 100%. After removal of the bags, the RH was kept at

70%. The temperature was 20° ± 1°C and artificial light (11000 lux) was supplied with a 16 hr photoperiod. On days 7 and 8 the numbers of whitefly eggs on the leaves were counted. On day 27, the numbers of infected larvae and pupae, empty pupal cases and larvae killed by other causes were recorded. The fraction infected hosts was defined as the ratio of the total number of infected hosts to the total number of whiteflies in the above-mentioned categories.

Leaf impression technique

Cucumber plants cv "Profito" with second instar whitefly larvae or plants without larvae were treated with an A. aleyrodis spore suspension. Again 3 ml of 1.0×10^7 spores/ml were sprayed onto the abaxial leaf surfaces. The environmental conditions were similar to those in the bioassay experiment. At 0, 4, 6, 12, 15, 20, 26, 35 and 43 days after application of the spore suspension, leaf impressions on water agar plates were made from six separate leaves (two leaves per plant). Immediately after the impressions were made, the numbers of germinated and ungerminated spores in a total of 300 per agar plate were recorded. The plates were incubated at 25°C for 24 hr (16 hr light/8 hr dark), which gave the viable ungerminated spores time to germinate, then 300 spores per plate were re-examined for germination. To test for a possible temperature effect, treated plants bearing larvae were also kept at 25° ± 1°C and spores were examined 1, 4, 6, 12, 15 and 20 days after application of A. aleyrodis. A spore was arbitrarily defined as germinated if the length of the germ tube was equal to or exceeded the breadth of the spore.

6.3 RESULTS

Bioassay

Table 6.1 shows the percentage of infected whitefly larvae and pupae on the leaves which were treated with A. aleyrodis before or after the eggs were laid. It is apparent that the spores could survive host-free periods of up to 28 days on the leaf surfaces, i.e. a period of 18 days before eggs were laid plus a period of 10 days before the

eggs hatched and the whitefly larvae could be infected. The percentage infected larvae did not show a significant decrease over time, indicating the survival of a high percentage of the spores on the leaf surface. Infected larvae were not found on control leaves. The proportions of eggs resulting in whitefly larvae, pupae and adults at the end of the experiment were highly variable, in the treated as well as in the control groups.

Table 6.1 Mean percentage infected whitefly larvae and pupae after application of a spore suspension of Aschersonia aleyrodis at different times before (-) or after (+) egg laying (0) at 20°C.

Time of treatment (days)	Residual activity (days)	Total no. of larvae	No. of leaves	%Infected larvae (SEM)
+9	1	662	7	98.0 (1.4)
+6	4	727	6	99.7 (0.2)
+3	7	563	6	98.0 (1.6)
0	10	562	8	98.0 (1.1)
-3	13	1022	8	96.0 (1.8)
-6	16	825	6	94.0 (3.8)
-9	19	405	6	97.0 (1.1)
-12	22	613	6	96.5 (1.1)
-15	25	246	2	78.0 -
-18	28	69	1	90.0 -

Leaf impressions

Figs. 6.1, 6.2 and 6.3 show the mean percentage of germinated spores on water agar after impression from leaf surfaces of cucumber plants incubated at 20°C and at 25°C. Apparently, only a low number of the spores germinated on the leaf surfaces. Comparing Fig. 6.1 and 6.2 the presence of whitefly larvae did not influence the germination of the spores significantly ($P<0.05$). There was no increase in the

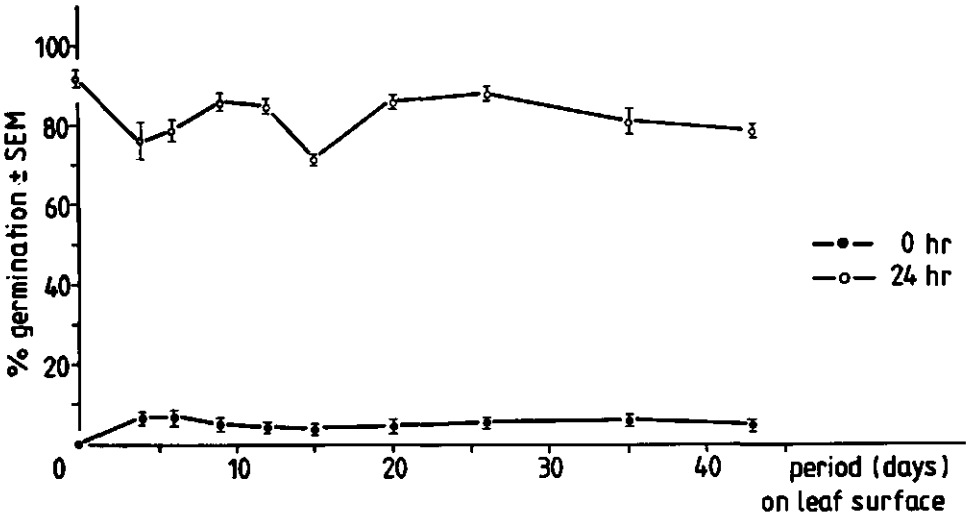


Fig. 6.1 Mean germination rates (%) and standard errors of the mean (SEM) for spores of Aschersonia aleyrodis from cucumber leaves incubated at 20°C without greenhouse whitefly larvae recorded immediately after (●) and 24 hr after (o) impression of the leaves on water agar at 25°C.

numbers of germinated spores over time. At 25°C, an overall percentage of 7.6% spores had germinated on the leaf surface which appeared to be just significantly higher (P=0.05) than the overall percentage of 4.5% at 20°C.

In this test, it was assumed that the microbial propagules stuck better to the agar medium than to the leaf surfaces when the leaves were removed from the agar, thus resulting in a representative sample of microorganisms from the leaf surface on the water agar (Parbery et al., 1981). To test this assumption cellulose acetate (collodion) impressions were also taken from the leaf surfaces kept at 25°C and examined for germinated spores after staining the strips with lactophenol blue. The overall percentage of germinated spores was 4.0% when samples were taken on ten subsequent days after application. Moreover, direct observations of the spores on the leaf surfaces were made using the fluorescent technique described by Drummond & Heale (1985). By these direct observations it was also found that few of the spores, either clustered or singly distributed on the leaf surface, had germinated. It may be concluded that the leaf impression technique

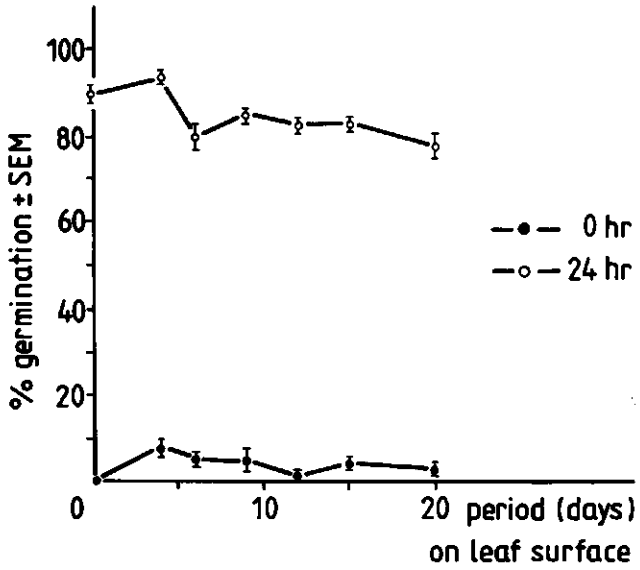


Fig. 6.2 Mean germination rates (%) and standard errors of the mean (SEM) for spores of Aschersonia aleyrodis from cucumber leaves incubated at 20°C with greenhouse whitefly larvae, recorded immediately after (●) and 24 hr after (o) impression of the leaves on water agar at 25°C.

can be used to evaluate the presence of both germinated and ungerminated spores on the cucumber leaf surface. Fig. 6.4 shows the formation of a germ tube by one spore out of a cluster on a cucumber leaf. Definitely more spores germinated on the cuticle of young fourth instar larvae kept at 100% RH and 25°C for 18 hr. The overall percentage germination of 277 spores on 6 insects was 52.0%, with germination rates on individual insects of 11.5 to 94.6%.

Spores from the leaf impressions incubated on water agar at 25°C for 24 hr showed a high percentage of germinated spores which indicates the viability of the spores on the leaf surface over time (Fig. 6.1, 6.2, 6.3). Spores from leaves showed a decline in viability after about 12 days at 25°C. This was due to an increasing amount of microorganisms present on the agar which probably negatively influenced the germination of the spores.

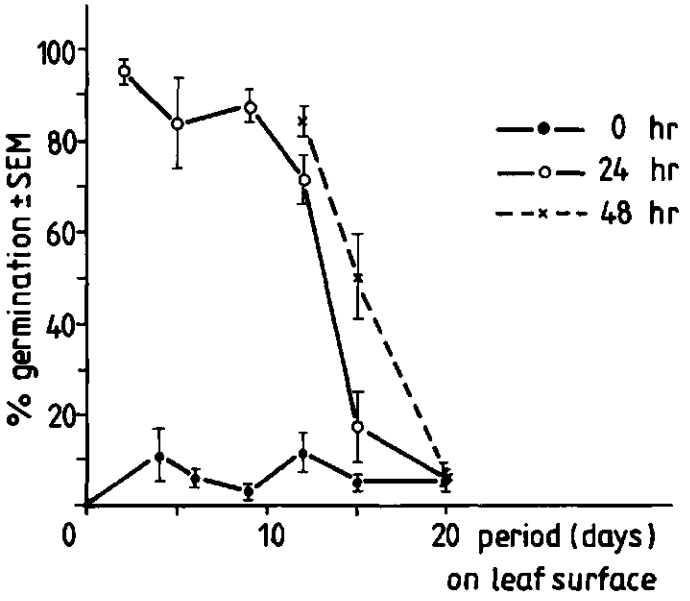


Fig. 6.3 Mean germination rates (%) and standard errors of the mean (SEM) for spores of Aschersonia aleyrodis from cucumber leaves incubated at 25°C with greenhouse whitefly larvae, recorded immediately after (●), 24 hr after (o) and 48 hr after (*) impression of the leaves on water agar at 25°C.

6.2 DISCUSSION

The ability of an entomopathogen to remain viable in a potential habitat of the host insect is an important factor affecting the effectiveness of naturally occurring and introduced pathogens (Jaques, 1985). The main factors influencing spore survival on plant surfaces are temperature, relative humidity, light, plant surface and canopy characteristics, and the presence of other microorganisms on the leaf. Leaves are open to infection or saprophytic colonization by air-borne or splash-dispersed microorganisms (Campbell, 1985). Positional effects such as height in the canopy, under or upper leaf surface or orientation to north, south, east or west influence the presence of populations of microorganisms (Andrews et al., 1980, Brobyn et al., 1985, Carruthers & Haynes, 1986). The numbers of all microorganisms are low when leaves open, but the population soon increases and peak numbers are reached as the leaves senesce (Campbell, 1985). Spores of A. aleyrodis remain viable for up to 28 days on the abaxial leaf

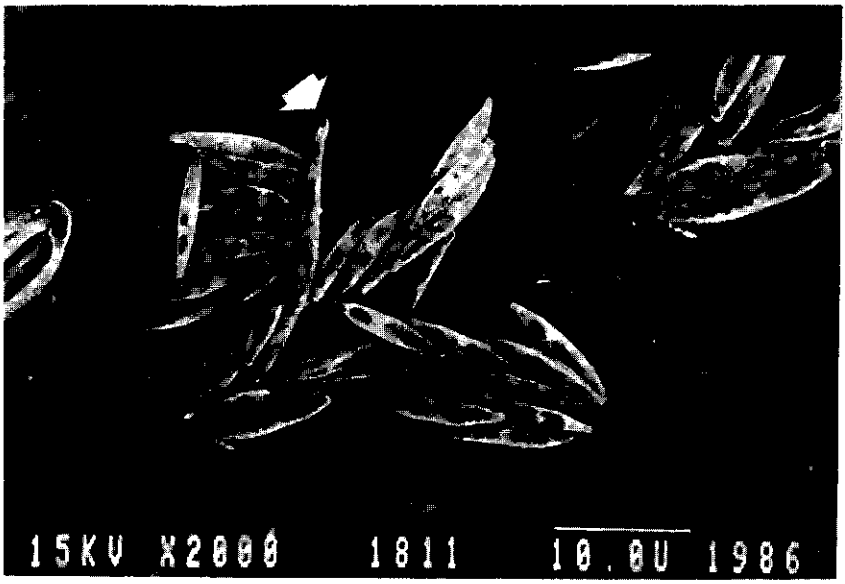


Fig. 6.4 Scanning electron micrograph of Aschersonia aleyrodis spores on a cucumber leaf (arrow: germ tube); inset: remnants of mucilaginous substance sticking to the spores.

surface of cucumber plants at 70% RH and 20°C. At 25°C the percentage of viable germinated spores on water agar from leaf impressions declined after about 12 days but this was assumed to be mainly caused by interference from other microorganisms on the agar. The cucumber plants kept at 25°C showed signs of senescence earlier than plants kept at 20°C, which explains the increase of microorganisms on the leaf surface and consequently on the agar plates. Additional information on the infectivity of spores on leaf surfaces at higher temperatures can be obtained by use of bioassays. Spores inhibited from germinating on water agar by interference from other microorganisms may germinate on the host cuticle. Remarkably, spores on cucumber leaves did not germinate in great numbers although they were exposed to 100% RH for 24 hr. Most of the spores on whitefly larvae, however, did germinate. Also, spores on water agar or cellophane (Chapter 7) are able to germinate, indicating the endogenous character of the germination process. It is yet unknown whether the low germination rate on leaves is due to lack of stimulation or to inhibition

by factors such as leaf exudates, pH or surface structure. The use of leaf impressions was preferred to the use of leaf washings, which involve the counts of colony forming units from agar plates. A. aleyrodis is a slow growing fungus and cannot easily be distinguished from other fungi unless spores are formed. Therefore, other microorganisms may have colonized the agar before the presence of A. aleyrodis can be recorded. The observations on leaf impressions provide direct information regarding spore viability.

A. aleyrodis is evaluated for use against greenhouse whitefly in glasshouses where environmental conditions are less extreme than in the open field. Jaques (1972) found that deposits of Trichoplusia ni nuclear polyhedrosis virus and Pieris rapae granulosis virus on leaves of collard plants in a greenhouse were inactivated more slowly than those exposed to sunlight outside, but more quickly than those exposed only to artificial light in a growth chamber. Poor survival of Verticillium lecanii on chrysanthemums in glasshouses was indicated by a reduction in the infection rate of Myzus persicae at about 5 days after application (Gardner et al., 1984). Nevertheless, conidia of this fungus in slime heads attached to aphid cadavers showed good survival, even after 39 days on the under surface of chrysanthemum leaves under humid conditions (Hall, 1980b). Also Daoust & Pereira (1986) observed that Beauveria bassiana condidia exposed to rain and sunlight survived for at least two weeks attached to cowpea curculio cadavers (Chalcodermus sp.), but conidia sprayed onto foliage became inviable in one day or less. A. aleyrodis infects the whitefly species Dialeurodes citrifolii and D. citri in citrus groves in Florida (Fawcett, 1944). During the summer season in which A. aleyrodis is predominant, bright sunshine alternates with cloudy rainy weather, causing periods of high temperature and humidity. Although whitefly is present on the under surface of the citrus leaves, these surfaces are also exposed to direct and indirect sunlight. The fungus is not known to produce resting spores or other special structures to survive unsuitable environmental conditions. Spores are produced in pycnidia on the colonized insect, and slimy masses of conidia are readily suspended in water. During the sunny periods, protection of the spores may be related to three factors. Firstly, being in a mass the spores on the outside may protect the inner spores from sunlight and drought.

Secondly, the spores are covered by a mucilaginous substance which may offer some protection from sunlight and drought (see inset Fig. 6.4) (Louis & Cooke, 1985, Dickinson, 1986). Thirdly, the spores are bright orange coloured due to pigments, mainly carotenoids (Eijk et al., 1979). The sensitivity of spores to (ultraviolet) light is frequently related to their colour, and pigmented spores often survive longer periods of exposure than colourless ones (Leach, 1971). Krinsky (1976) stated that one of the major functions of carotenoids in nature is to protect cells against potentially harmful or lethal photodynamic effects. Thus, A. aleyrodis seems well equiped to withstand negative environmental conditons, some of which may still occur in a glasshouse.

In conclusion, the prospects of survival of A. aleyrodis spores on the abaxial surfaces of cucumber leaves are good. Spores may infect larvae hatching from eggs laid after the spores are applied. However, one should consider the population structure of greenhouse whitefly in a cucumber crop. Generally, whitefly adults move to the younger (top) leaves to deposit eggs shortly after emergence (Noldus et al., 1985), although some eggs may be laid at the site of emergence. The top leaves usually will be small when eggs are deposited and thus, the residual effect of surviving A. aleyrodis spores on these leaves will be diminished by the increase in leaf surface over time. Nevertheless, when A. aleyrodis was applied to a crop of cucumber plants with whitefly eggs on the young leaves, up to 30% of the larvae which hatched became infected (Fransen, unpubl. results). On host plants which show a less distinct whitefly distribution, the impact of the residual effect after application of A. aleyrodis should be taken into account.

CHAPTER 7.

THE INFLUENCE OF RELATIVE HUMIDITY AND TEMPERATURE ON INFECTION OF GREENHOUSE WHITEFLY WITH THE FUNGUS ASCHERSONIA ALEYRODIS

7.1 INTRODUCTION

Various abiotic environmental conditions influence the different phases of the fungal-pathogen-host relationship. Temperature and humidity are important, also air movement, light and the presence of free water. Those phases in the disease-development cycle which involve the presence of the fungus outside the host are influenced by these factors, especially the initial infection phases when the spore germinates on the host integument and penetrates its cuticle by means of the formation of an appressorium (Chapter 2). Later on, protrusion of the fungus from the interior of the host and subsequent sporulation on the host depend on suitable environmental conditions. The environment also plays a role in the dispersal and survival of the infective units. When mass production of the fungus is considered for application of the pathogen, the infective units have to survive periods of storage after being formulated, in a dry form or otherwise. The process of mass production and formulation involves research on the dependence of temperature and relative humidity in relation to the retention of the viability and virulence of the spores (Roberts & Campbell, 1977, Daoust & Roberts, 1983b).

In this chapter the attention is focused on the influence of temperature and relative humidity (RH) on the infection by the fungus Aschersonia aleyrodis and subsequent death of the host insect, greenhouse whitefly. Information of these aspects is needed to determine whether A. aleyrodis can succeed in reducing the whitefly population in a glasshouse environment. The control of a whitefly population will depend on the immediate effect of spore introduction, because the dispersal of the pathogen and secondary infection is limited under glasshouse conditions.

The influence of RH on germination has been studied in vitro using agar media with a different water activity (a_w). Gillespie & Crawford (1986) tested several entomopathogenic fungi for their

ability to germinate on agar media with a specified a_w. The isolates examined proved to be remarkably uniform in their response to a_w, with an optimum of 1.00 at 23°C. However, the interpretation of these results should be made with caution, because the influence of a_w on germination is dependent on several factors such as the medium used, temperature and pH (Magan & Lacey, 1984).

Reports on the requirement of high humidity for infection of the host insects are contradictory. In some cases, the discrepancies can be explained by the fact that the microclimate around the fungal spore was humid when the macroclimate was dry (Fargues, 1972, Ferron, 1977, Ramoska, 1984, Riba & Entcheva, 1984). These experiments provide information on the infection of hosts by pathogens under constant relative humidity regimes. However, in a natural environment periods of high ambient RH alternate with periods of low RH. Interruption of periods with high RH may accelerate the death of germinating spores but when these periods are short, they may survive (Cohen, 1977, Jeger et al., 1985, Jones, 1986). Solovei and Koltsov (1976) mention successful applications of Aschersonia sp. on glasshouse tomatoes and cucumbers at a RH regime of 70 to 100%. However, further investigation of the influence of relative humidity is needed for application of Aschersonia aleyrodis in Dutch glasshouses.

Information on the ability of A. aleyrodis to infect greenhouse whitefly at different temperature regimes is also lacking. Solovei and Koltsov (1976) and Spassova et al. (1980) mention successful infection of greenhouse whitefly in glasshouses over a temperature range of 18° to 32°C. Most entomopathogenic fungi show maximal germination within the range of 20° to 30°C (Roberts & Campbell, 1977). In general, both the rate of disease development and the final infection rate are higher at the optimum temperature of the fungus than at suboptimal temperatures. There are, however, exceptions, Fargues (1972) found that the number of third and fourth instar larvae of Leptinotarsa decemlineata successfully infected with Beauveria 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. 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 (Fargues, 1972). Another phenomenon

was described by Mohammed et al. (1977) and Boucias et al. (1984) who found a reduction in the rate of development of Nomuraea rileyi disease in treated Heliothis zea and Anticarsia gemmatalis larvae, respectively, at suboptimum temperatures, but the total mortality rates were not different from those at the optimal temperature. Information on the influence of temperature and humidity on the infection of greenhouse whitefly with A. aleyrodis is needed to predict the impact of fungal applications under glasshouse conditions. In this chapter results are presented of a laboratory study designed to provide this information.

Also in this chapter, the effects of repeated subculturing of A. aleyrodis and the influence of a single host passage in the temperature and humidity experiments are reported. The dose-mortality relations for A. aleyrodis subcultured four times and twelve times were similar (Chapter 4). Bioassays have been reported for different fungal biotypes against a range of various insects (Boucias et al., 1982, Boucias et al., 1984, Ignoffo & Garcia, 1985) and the modifying effect of successive in vitro and in vivo passages on the virulence of fungal pathogens has been established (Daoust & Roberts, 1982, Ignoffo et al., 1982, Fargues & Robert, 1983b). However, the influence of temperature and humidity on the virulence of different strains, host-passaged or subcultured isolates of the same fungus has not often been investigated. Soares et al. (1983) compared different strains of Metarhizium anisopliae with respect to their ability to infect Othiorynchus sulcatus at two temperatures. The strains induced equivalent mortalities at 20°C, but at 15°C one strain was significantly better than any of the others. It is worthwhile to consider the effect of host passaging and subculturing in this context as it may influence the choice of the pathogen to be applied in a practical situation.

7.2 MATERIALS AND METHODS

Influence of relative humidity on germination

The effects of air humidity on spore germination of A. aleyrodis was studied in an experiment in which a spore suspension was sprayed

onto sheets of cellophane. The sheets of cellophane were kept in air tight 100 ml jars in which a constant relative humidity was obtained by a mixture of glycerol and water (Anonymous, 1983). The RH in the individual jars was kept at 100.0, 98.2, 96.0, 93.9, 91.9, 89.3, 87.3 and 84.8%. The temperature was kept at 20-22°C and the jars were kept in darkness. After exposure periods of 24, 96 and 168 hr about 300 spores per exposure period were examined under the microscope for germination. For this purpose, a piece of cellophane was cut from the original sheet and the spores were stained with lactophenol aniline blue. A spore was considered to have germinated when the length of the germ tube was equal to or exceeded the breadth of the spore.

Influence of relative humidity on infection of whitefly larvae on plants

The experiment was carried out to obtain information on the minimum period of high RH needed for A. aleyrodis to infect whitefly larvae when the ambient RH was kept at 50%. For these experiments A. aleyrodis (Rs.no. 1088) was subcultured three times on autoclaved millet grains. Thereafter, whitefly larvae were inoculated by spores from this subculture and subsequently spores were obtained from pycnidia on fungal outgrowth of the insects on malt agar. This host-passaged culture is referred to as C_0. The same isolate (Rs.no. 1088) was subcultured four times and ten times on autoclaved millet grains and these subcultures will be referred to as C_4 and C_{10}, respectively. Cultures were incubated at 25°C and a 16 hr photoperiod and used after three to four weeks. Two ml of the suspension was applied on the abaxial leaf surfaces of cucumber plants bearing third instar whitefly larvae. The spore dosage was 2.5×10^7 spores/ml for C_4 and C_{10} and 1.3×10^7 spores/ml for C_0 (see Chapter 3 for procedure). The germination rate of the spores was checked on water agar. The plates were incubated at 25°C for 24 hr. The germination rates were 99.7%, 81.1% and 97.7% for C_0, C_4 and C_{10}, respectively. After evaporation of the water from the treated leaves the plants were covered by transparent plastic bags for 0, 3, 6, 12 or 24 hr. After removal of the bags, the plants were exposed to a RH of 50%. The temperature was 20 $\pm$ 1°C and the photoperiod 16 hr. The soil surface of the pots and

the trays were covered by a plastic sheet during the experiment, thus preventing influences on ambient RH by evaporation of water from the soil or from the trays. The plants were widely spaced without overlapping leaves. The RH between the plants was 50%, and air movement was 0.1 - 0.2 m/s. In total 7 to 12 leaves were used (two or three leaves per plant) per culture and RH regime. The control consisted of three to six leaves bearing whitefly larvae treated with sterile distilled water. The total number of hosts per treatment ranged from 100 to 150 and per control from 50 to 100. The mortality fraction was defined by the number of infected larvae and pupae and those killed by other causes divided by the total number of healthy larvae and pupae, empty pupal cases, infected and otherwise killed larvae and pupae. Recordings were made 7, 10, 14 and 21 days after application of the spore suspension.

The results were analyzed by angular transformation of the mean mortality fraction per plant. Testing on effects of RH and cultures was done by analysis of variance. The LT50 was estimated by logit transformation of the data (see Chapter 4) and tested in an analysis of variance on differences in RH treatments and cultures.

Influence of temperature on germination

A suspension of A. aleyrodis spores originating from a culture, subcultured five times after isolation from infected whitefly larvae, was sprayed onto water agar plates (2 ml, 2.0-2.5 x 10^6 spores/ml). The agar plates (three per temperature) were kept at 11, 16, 20, 21, 25, 28, and 33°C and a 16 hr photoperiod. Observations were made after 12, 24, 48 and 72 hr incubation. The percentage germination was determined by recordings over 300 spores per agar plate. Spores were considered germinated when the length of the germ tube was equal to or exceeded the breadth of the spore.

Influence of temperature on infection of whitefly larvae on leaf discs and plants

The influence of temperature on infectivity of A. aleyrodis on greenhouse whitefly was studied in experiments, one on leaf discs and another one on intact plants. The same host-passaged culture (C_0) was used as in the RH experiment described above. The original isolate (Rs. no 1088) was subcultured three times and 19 times on autoclaved millet grains and these subcultures will be referred to as C_3 and C_{19}, respectively. An amount of 2 ml was sprayed on to the abaxial leaf surface of cucumber plants bearing second and third instar larvae. The dosage applied was 2.5×10^6 spores/ml. The viability of the spores was checked after incubation on water agar at 25°C and 16 hr. The percentage germination over 300 spores exceeded 90% in all cases.
Experiment 1. Leaf discs were used in the same manner as described in Chapter 5, but instead of water a mixture of glycerol and water was present at the bottom of the tray. Thus, the environment was kept at 96.0% RH which prevented condensation of water on the leaf discs. The subcultures C_0 and C_{19} were used in this experiment. Treated leaf discs were kept at 15, 20, 25 and 30°C. Six leaf discs were treated with spore suspension and four discs were treated with sterilized distilled water as a control. The recordings were made 4, 7 and 11 days after treatment.
Experiment 2. Two leaves per cucumber plant were treated with spores of C_3. In total five plants per treatment were used and three plants per control. The plants were kept at 100% RH for 24 hr by covering them with transparent plastic bags. After removal of the plastic bags, the RH was kept at 70% at all temperatures. The plants were kept at temperatures of 15, 20, 25 and 30°C. Recordings were made 4, 7, 11 and 14 days after treatment, and also at 21 and 28 days after treatment for plants kept at 15°C. Analysis of the results was carried out by angular transformation of the mean mortality fraction (see above) per plant and the separate mortality fractions per leaf disc.
Differences associated with temperature and culture type were analyzed by an analysis of variance within the experiments. LT50 values were estimated by logit transformation of the data (see Chapter 4) and tested by an analysis of variance for temperature and culture effects.

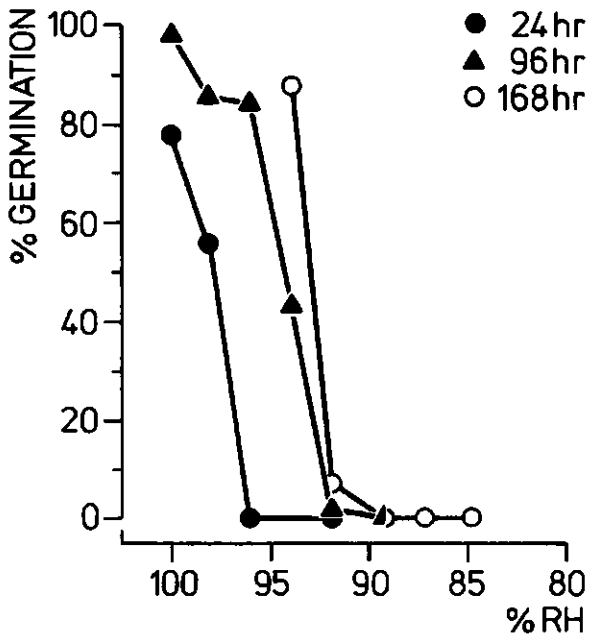


Fig. 7.1 Germination rates (%) of Aschersonia aleyrodis spores on cellophane under different RH conditions.

7.3 RESULTS

Influence of relative humidity on spore germination and infection of hosts

The germination rate of A. aleyrodis spores on cellophane in the environment of 100% RH was 78% after 24 hr (Fig. 7.1). At lower RH values the germination of the spores was retarded. At 91.9% RH the percentage germination was still below 10% after 168 hr incubation. Successful infection of the larvae with A. aleyrodis was obtained at all RH regimes, even when the treated plants were exposed to 50% RH for the whole experimental period (Fig. 7.2). The final infection rates for the different cultures C_0, C_4 and C_{10} were all high, exceeding 75% in all cases, but there was a significant difference in infection rates between the cultures ($P \leqslant 0.001$). The influence of high RH during different periods was apparent at day 7 ($P \leqslant 0.001$).

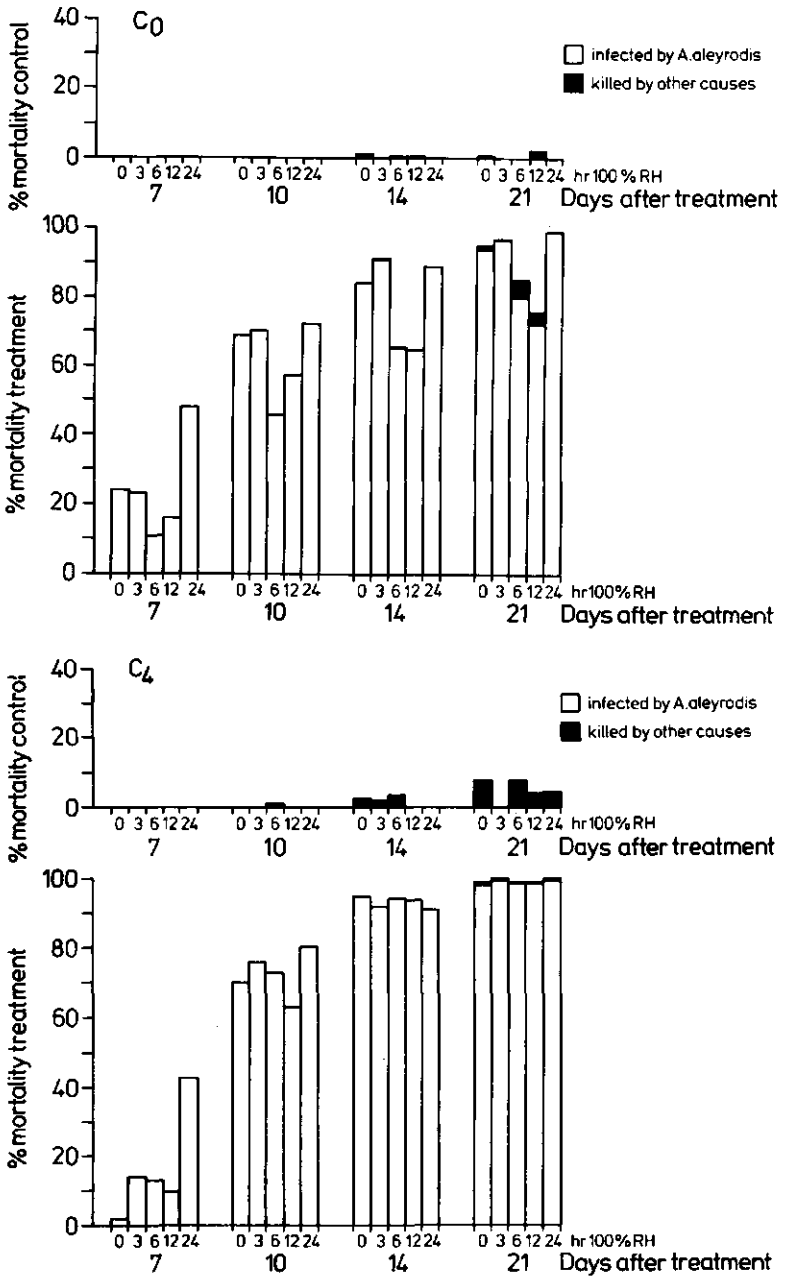


Fig. 7.2 Mortality rates (%) of greenhouse whitefly treated with Aschersonia aleyrodis from three different cultures C_0, C_4 and C_{10} and kept at 100% RH for different periods after treatment. The ambient RH was 50% at 20°C. (for details of the cultures, see text).

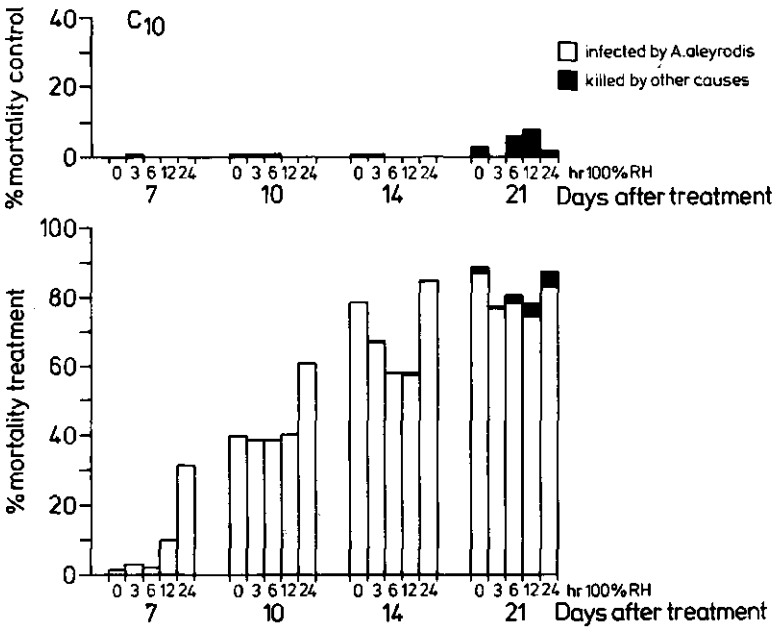


Fig. 7.2 Continued.

Signs of infection appeared earliest when insects had been exposed to 100% RH for a period of 24 hr. The relationship between LT50 and the length of the 100% RH period is, however, not linear (Fig. 7.3). Exposure to a period of 100% RH for 6 or 12 hr resulted in a delay in the appearance of signs of infection, reflected in the higher LT50 values for these exposure periods than those for the exposure periods 0, 3 and 24 hr. The differences in LT50 in relation to the different periods of high RH were significant at the 10% level, and the differences in LT50 in relation to the different cultures were significant at the 5% level.

Influence of temperature on spore germination and infection of hosts

The influence of temperature on germination of A. aleyrodis spores on water agar is shown in Fig. 7.4. Maximal germination within 24 hr took place at 25°C. At 15°C, 53% of the spores germinated within 24 hr, but their germ tubes were shorter than those of spores exposed to higher temperatures. After 48 hr over 90% of the spores had ger-

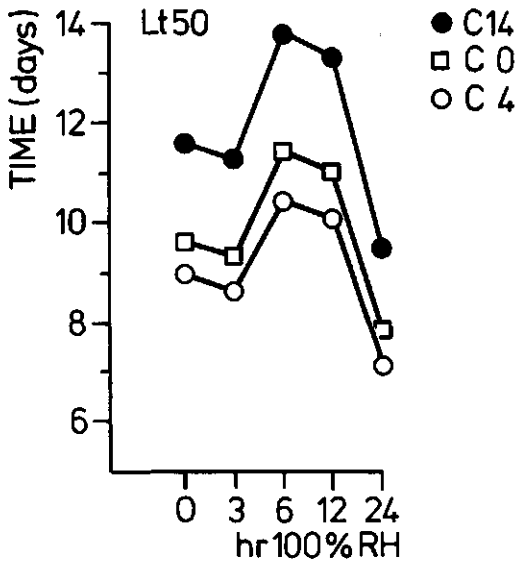


Fig. 7.3 LT50 values for whitefly larvae treated with Aschersonia aleyrodis from different cultures (C_0, C_4 and C_{10}) after exposure to different RH regimes (for details of the cultures, see text).

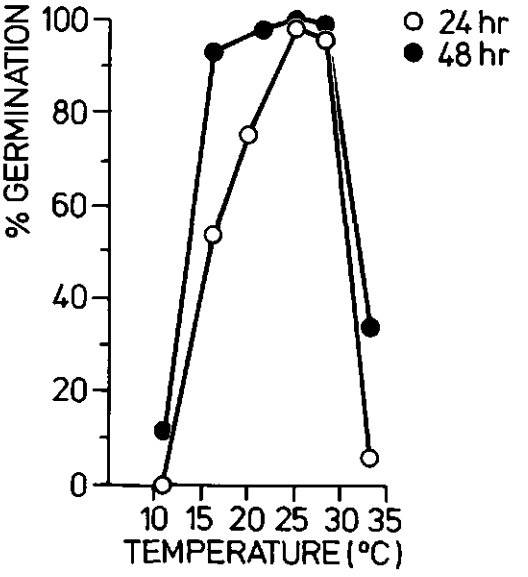


Fig. 7.4 Germination rates (%) of Aschersonia aleyrodis spores on water agar after incubation at different temperatures.

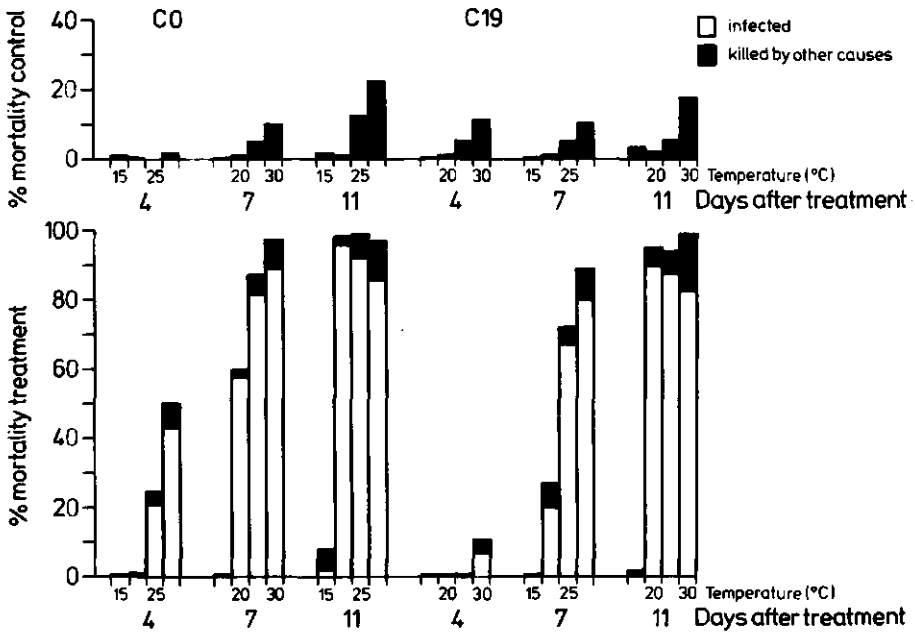


Fig. 7.5 Mortality rates (%) of greenhouse whitefly on leaf discs after infection with Aschersonia aleyrodis, from different cultures (C_0 and C_{19}), at different temperatures (for details of the cultures, see text).

minated at all temperatures from 16 to 28°C. Fig. 7.5 and 7.6 show the results of the experiments in which treated whitefly larvae on leaf discs and plants, respectively, were exposed to different temperatures. The development of infection was fastest at 30°C followed by that at 25°C. At 15°C the infection developed slowly and the leaf disc experiment was terminated before high levels of infection were reached. However, the whitefly larvae had not yet developed into adults at this temperature. Results of the experiment with plants show that at 15°C infection of whitefly larvae does take place, and may reach about the same level as the infection at higher temperatures (Fig. 7.6). In the leaf disc experiment a difference in infection rates for the two cultures C_0 and C_{19} can be observed. Treatment with spores of C_0 resulted in a higher percentage mortality. In the control of both the plant and leaf disc experiment the mortality rate was generally below 15%, except for the whiteflies

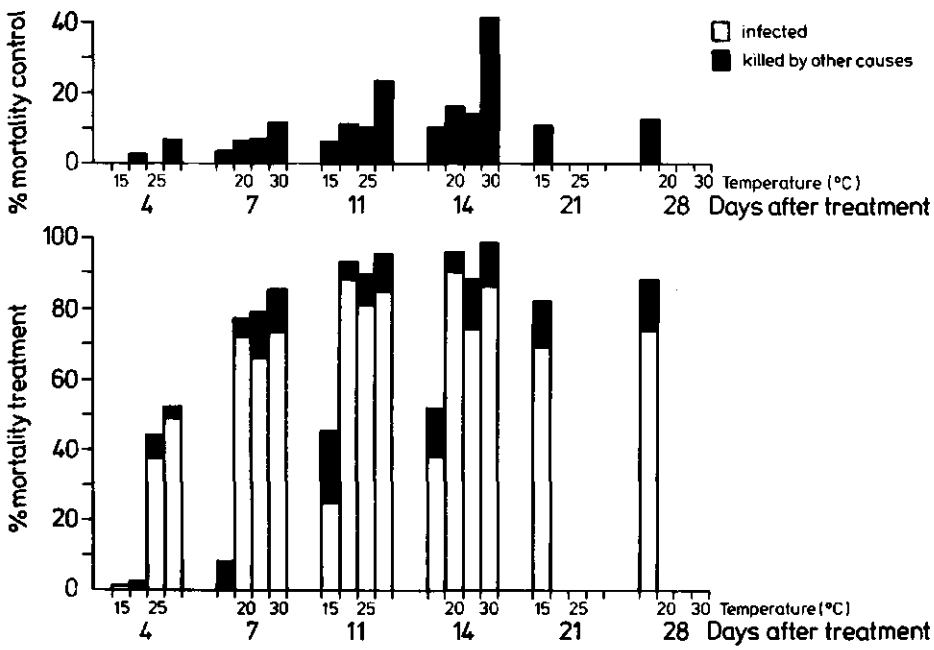


Fig. 7.6 Mortality rates (%) of greenhouse whitefly on plants after infection with Aschersonia aleyrodis, subcultured three times (C_3), at different temperatures.

exposed to 30°C. Thus, a significant temperature effect on control mortality was recorded ($P \leqslant 0.001$).

Information of the influence of temperature on the time needed for the expression of signs of infection (orange colouration of the hosts) in 50% of the whitefly population is presented in Fig. 7.7. Application of A. aleyrodis spores from C_{19} resulted in higher LT50 values, implying a lower infection rate over time.

7.4 DISCUSSION

Influence of relative humidity

Aschersonia aleyrodis shows optimal germination at 100% RH. Free water is not necessary for spore germination. Other entomopathogenic fungi, Verticillium lecanii, Metarhizium anisopliae and Beauveria

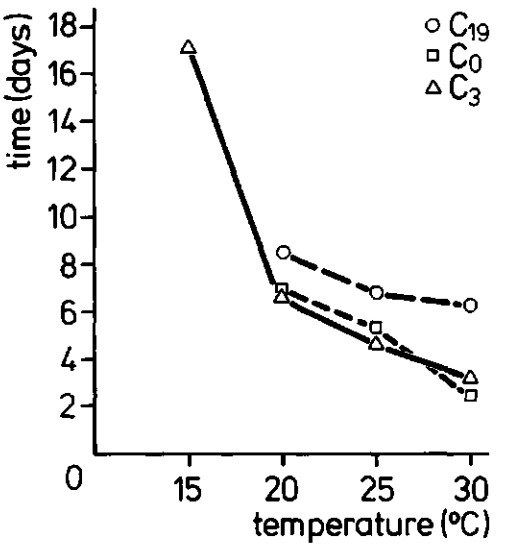


Fig. 7.7 LT50 values for whitefly larvae treated with Aschersonia aleyrodis, from different cultures (C_0, C_3 and C_{19}), after exposure to different temperature conditions (for details of the cultures, see text).

bassiana, show a similar optimum for spore germination (Schneider, 1954, Walstad et al., 1970, Gillespie & Crawford, 1986). Although germination is retarded at lower humidities, A. aleyrodis spores can germinate at 93.9% RH, resulting in 88% germination after incubation for 168 hr (Fig. 7.1). Although relative humidity definitively influences spore germination, reports have been published on successful infection of host insects at low ambient RH (Fargues, 1972, Ferron, 1977, Ramoska, 1984). It is suggested that spores may germinate at low ambient RH on the insect cuticle as a result of stimulating substances, nutrients and possible transpiration of water from the insect body.

Fang Qi-Xia et al. (1983) found that Aschersonia papillata on greenhouse whitefly showed a decrease in infection at decreasing RH. RH studies carried out by Uchida (1970) using an Aschersonia sp. against citrus whitefly gave inconsistent results. Riba and Entcheva (1984) reported an effect of RH on the infection of greenhouse whitefly on bean plants treated with V. lecanii. The mortality of the

larvae decreased from 80% at 100% RH to 45% at 92% RH. The results presented in this chapter show that the final percentage mortality of whitefly larvae on cucumber caused by A. aleyrodis spores at conditions of 50% RH after 24 hr 100% RH is similar to the percentage mortality at conditions of constant 50% RH (Fig. 7.2). This suggests the influence of a boundary layer on the leaf and insect surfaces, creating RH conditions sufficient for germination of the spores. The humidity at the leaf surface depends upon the rate at which water vapour is transferred through the boundary layer. The humidity at the surface is controlled by the thickness of the boundary layer, stomatal aperture and the ambient RH (Burrage, 1971). It took longer before signs of infection appeared at constant low RH of 50% than when a period of 100% RH for 24 hr was given (Fig. 7.3). This may suggest differences in rate of germination in relation to the different RH regimes.

Alternation of periods of high humidity with periods of low humidity may accelerate the death of germinating spores. Diem (1971) studied the effect of low humidity on the survival of germinated spores. Survival of Cladosporium spp. was attributed to the resistance of the germ tube. Few germinated spores of Aspergillus sp. and Penicillium sp. survived at a relative humidity of 85%. The development of infection with A. aleyrodis did show differences in relation to the high humidity periods. Exposure to 100% RH for 6 and 12 hr resulted in lower infection rates than exposure to 100% for 0 and 3 hr. This suggests that the germinating spores are in a phase more vulnerable to RH changes after a period of 6 to 12 hr at 100% RH and 20°C. It is likely that after 3 hr at 100% RH most of the spores are still not showing a germ tube. The change from high to low RH by removal of the plastic bags, being rather sudden, may also have induced a reaction of the stomata as their aperture may be controlled by their individual transpiration conditions (Lange et al., 1971). In a glasshouse environment, however, the changes of RH will be more gradual.

The effect of humidity on infection of whitefly with A. aleyrodis is influenced by several factors. Firstly, plant characteristics like hairiness and leaf size may influence the microclimatic boundary layer, indirectly affecting spore germination. Secondly, retardation

of spore germination at suboptimal RH conditions may allow the insect to escape by moulting. However, after moulting the whitefly larva may still come into contact with spores present on the leaf surface. Thirdly, temperature influences spore germination. At higher temperatures the rate of germination increases and shorter periods of high RH will be required. The phase in germination vulnerable to RH changes will become apparent after a shorter incubation period (Cohen, 1977, Jones, 1986). Fourthly, culture characteristics may play a role. A uniform level of whitefly mortality was achieved with spores originating from C_0, C_4 or C_{10}. The percentage germinated spores on water agar at 25°C was 99.7, 81.1 and 97.7% for C_0, C_4 and C_{10}, respectively, after 24 hr, but the spores of C_0 showed the longest germ tubes. Nevertheless this did not result in a shorter LT50 compared with the LT50 for C_4 in this experiment. The dosage of spores of C_0, however, was lower than the dosage of spores of C_4 and C_{10}.

Presoaking of spores in water reduced the time to germination of M. anisopliae and V. lecanii (Charnley, 1984, Ekbom, 1981). Thus, spores may become less dependent on long periods of high RH for germination to take place. However, activated spores should not be used because they are probably more vulnerable to external conditions than nonsoaked spores.

Influence of temperature

More time is needed for infection to become apparent at 15°C than at 20°C or higher temperatures. Fig. 7.7 shows a nonlinear relationship between LT50 values and temperature. This correlates with the development of the host itself, because whitefly takes unproportionally longer to develop at 15°C than at 20°C or higher temperatures (Lenteren & Hulspas-Jordaan, 1983). At 28 days after spore application also at 15°C a high infection percentage was achieved.

High temperatures may influence the susceptibility of the host insects. Fargues (1972) found that Leptinotarsa decemlineata larvae became more resistant to B. bassiana infection at 25° and 30°C than at 20°C, although the optimum temperature for germination as found by Walstadt et al. (1970) ranged from 25° to 30°C. A similar phenomenon

was reported by Boucias et al. (1984): Anticarsia gemmatalis, which has a developmental optimum of 31°C, was resistant to Nomuraea rileyi when reared under high temperature regimes. Also Mohamed et al. (1977) found a decrease in the infection rate of Heliothis zea larvae with N. rileyi at 30°C in comparison with lower temperatures.
T. vaporariorum does not become resistant to A. aleyrodis at higher temperatures. A. aleyrodis successfully infected the larvae at 30°C. The development of infection is even fastest at this temperature (Fig. 7.7) although 30°C is higher than the optimum for spore germination (Fig. 7.4). This temperature, however, is suboptimal for whitefly development as shown by the relatively high mortality rates in the control (Fig. 7.5 and 7.6).

Final considerations

The LT50 value of 7.1 days recorded in the RH experiment using subculture C_4 (24 hr 100% RH, incubation at 20°C) agrees with the LT50 value reported in Chapter 4 for treatment of third instar larvae with a similar dosage. The LT50 value of 6.7 days recorded in the temperature experiment using C_3 (incubation at 20°C) is somewhat lower than reported in Chapter 4. Previously, I had found that A. aleyrodis did not show any decrease in virulence after 12 serial passages on semi-artificial medium (see Chapter 4). Ignoffo et al. (1982) obtained similar results for N. rileyi after 12 serial passages on artificial medium. However, in both experiments the LT50 values were not assessed. The repeated in vitro passaging of the isolate (C_{10} and C_{19}) used in the RH and temperature experiments, respectively, accounted for some decrease in overall mortality and an increase in median lethal time. A single host passage of A. aleyrodis on greenhouse whitefly did not seem to increase the virulence of the isolate. Nevertheless, further research of the influence of in vivo serial passages on the virulence of A. aleyrodis by assessment of LC50 and LT50 data may be worthwhile.

In the Netherlands, cucumbers are grown at temperatures which do not go below ca. 18°C during the night, and may reach about 32°C during a few hours of bright sunshine at midday. Ambient relative humidity may fluctuate from about 50% under bright sunshine to about

95% for a period of several hours at night. Based on the data presented in this chapter, which were obtained over temperature and humidity ranges similar to those in glasshouses in the Netherlands, it can be concluded that Aschersonia aleyrodis would be able to infect most Trialeurodes vaporariorum under normal glasshouse conditions.

CHAPTER 8

INTERACTION BETWEEN THE PARASITOID ENCARSIA FORMOSA AND THE PATHOGEN ASCHERSONIA ALEYRODIS, IN THE CONTROL OF GREENHOUSE WHITEFLY, TRIALEURODES VAPORARIORUM: HOST SELECTION AND SURVIVAL OF THE PARASITOID IN THE PRESENCE OF HOSTS INFECTED WITH THE FUNGUS

8.1 INTRODUCTION

Greenhouse whitefly, Trialeurodes vaporariorum (Westwood), is an important pest of several glasshouse crops including tomatoes and cucumbers. Damage is mainly caused by contamination of leaves and fruits by honeydew produced by larval instars and adults which stimulates prolific growth of sooty moulds (Cladosporium spp.). This fungal growth reduces photosynthesis and respiration of the plant (O'Reilly, 1974) and the presence of moulds necessitates expensive washing procedures of the fruit before sale. Encarsia formosa Gahan is successfully applied against whitefly in tomato crops, in cucumber crops control is not always achieved because the parasitization efficiency of E. formosa in cucumber is lower. The parasitoid's movement is hampered by the hairiness of the cucumber leaves (Hulspas-Jordaan & Lenteren, 1978, Li Zhao Hua et al., 1987). Moreover, cucumber is a more suitable host plant for whitefly than tomato (Boxtel et al., 1978).

An additional selective control method for whitefly is needed which would not disturb other biological-pest-control components in cucumber crops like the use of the predatory mite Phytoseiulus persimilis against the two-spotted spider mite Tetranychus urticae. A candidate for this additional control is the entomopathogenic fungus Aschersonia aleyrodis Webber, which infects insects of several whitefly species (Berger, 1921). Infection of greenhouse whitefly with this fungus has been reported from Eastern European countries (Primak & Chizhik, 1975). Preliminary experiments with A. aleyrodis in glasshouses in the Netherlands have yielded encouraging results (Ramakers & Samson, 1984).

In Chapter 3 it was shown that the first, second and third larval

instars are successfully infected by the fungus, but the fourth larval instar and the socalled prepupal and pupal stages are less susceptible. Unfortunately, epizootic development of A. aleyrodis is unlikely to occur in a "rain-free" glasshouse without overhead irrigation, because the fungus is dispersed by means of splash dispersion. Therefore, effective use of A. aleyrodis alone probably requires frequent spray applications.

Combined use of E. formosa and A. aleyrodis may be more efficient than using them separately and may result in complementary mortality of whitefly. Extensive research conducted on the host finding, host selection and host discrimination by E. formosa (Nell et al., 1976, Lenteren et al., 1976a, 1980, Fransen & Montfort, 1987) indicates that the parasitoid prefers third and fourth instar hosts to younger instars. As mentioned above, A. aleyrodis is more pathogenic on early instars of whitefly. The mortality of whitefly caused by complementary activity of parasitoid and fungus will depend on the ability to parasitize or infect hosts which have not been attacked previously. Therefore, the timing and frequency of the application of A. aleyrodis in relation to the introduction of E. formosa then become important. For this reason detailed knowledge is needed on the interaction of these two natural enemies.

In this chapter I present results from experiments in which E. formosa encounters whiteflies which have been treated beforehand with spore suspension of A. aleyrodis and in which E. formosa is permitted to select between infected and noninfected hosts.

8.2 MATERIALS AND METHODS

Hosts

Greenhouse whitefly was reared on cucumber plants cv "Profito" (experiment 1, 3 and 4) and cv "Gele Tros" (experiment 2) at 20°C and 16 hr photoperiod. About 20 to 30 adult whiteflies were put in clipcages (2.8 cm diameter) on the plants and were given the opportunity to lay eggs during a 24 hr period. Instars of different developmental stages were treated either with a spore suspension of

A. aleyrodis or water and offered to the E. formosa females when they had developed to the third and fourth instar.

Encarsia formosa

Black pupae which developed from parasitized whitefly larvae were put in Petri dishes at 25°C and a 16 hr photoperiod. After the adult E. formosa females emerged they were offered whitefly hosts for parasitization and feeding for one to two days. The parasitoids were then kept individually in gelatine capsules with honey as a food source for about 12 hr before use in the experiments.

Aschersonia aleyrodis

A. aleyrodis was obtained from the Centraalbureau voor Schimmelcultures at Baarn, the Netherlands. The isolate (Rs.no. 1088) originated from Columbia. Spores were harvested from two to four week old cultures on brown rice grown at 25°C and 16 hr photoperiod by rinsing with sterilized distilled water. A dosage of $2.2 - 2.3 \times 10^7$ spores/ml was used in experiment 1 and 3 and a dosage of $1.0 - 4.3 \times 10^7$ spores/ml was used in experiment 2. A Potter spray tower set at 34.45 kPa was used to spray 4 ml of the spore suspension onto the underside of each leaf with hosts. In each treatment individual leaves still attached to plants were treated with spore suspension and similar leaves were treated with sterilized distilled water as a control.

The spore suspensions were checked for spore viability by spraying 2 ml onto water agar plates. After 24 hr at 25°C the germination was checked; this amounted to 85-98% in experiment 1 and 3 and to 91-99% in experiment 2.

After treatment the plants bearing whitefly were kept at 100% relative humidity (RH) and 20 $\pm$ 1°C for 36 to 48 hr by covering them with transparent polythene bags. After removal of the polythene bags RH was kept at 50% to 70%.

Experiment 1: Behavioural Observations

Whiteflies in the third and fourth larval instar stage which had been treated with a spore suspension 0, 4, 7, 10 or 14 days beforehand in the stage as shown in Fig. 8.1 were offered to the parasitoid females. This was done in order to obtain different stages of infection when the hosts were offered to E. formosa.

The parasitoids were observed individually for one hr at 25 ± 1°C. Two persons carried out the observations simultaneously, each observing the parasitoid's behaviour on either water-sprayed or suspension-treated hosts (5-6 observations per treatment and per control). Recordings were made of encounters with hosts, oviposition behaviour and host feeding, according to the classification of behaviour by Lenteren et al. (1976a, 1976b, 1980). Afterwards the larvae which were encountered, and on which oviposition behaviour was observed, were dissected and checked for parasitoid eggs.

Infection was classified in different categories: some larvae showed signs of infection (orange colour) visible from the outside, others had to be dissected under the microscope. By means of dissection a distinction could be made between larvae showing no (detectable) signs of infection, and those containing cloudy floccose haemolymph in contrast to the clear haemolymph in healthy larvae.

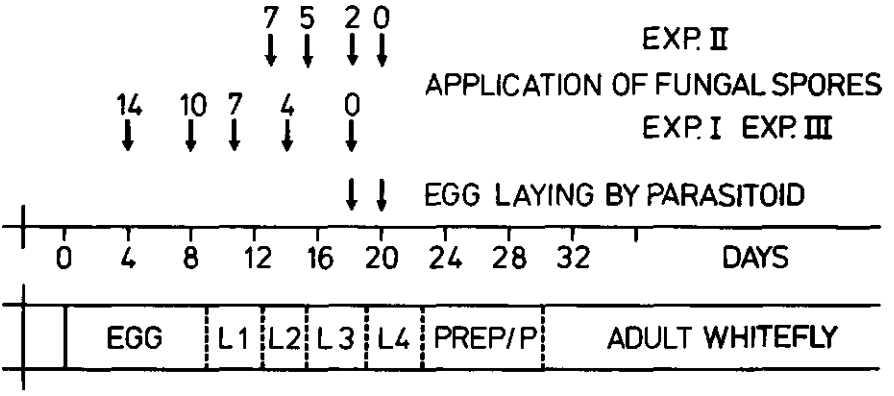


Fig. 8.1 Schedule of treatment of whitefly by Aschersonia aleyrodis and parasitization by Encarsia formosa related to the different whitefly stages.

Experiment 2: Dissection

Fourth instar larvae were offered to E. formosa either 0, 2, 5 or 7 days after treatment with A. aleyrodis (Fig. 8.1). The parasitoids were kept individually in clip cages on the site where larvae were present for 24 hr at 25 ± 1°C. After removal of the parasitoids all larvae in the clip cage area were dissected for parasitoid eggs and the condition of the hosts was recorded as in experiment 1. The number of hosts offered to the parasitoids ranged from about 40 to 120 per parasitoid. Dissection was time consuming, so some of the larvae were dissected later after storage at 4°C which halts the process of fungal and insect development.

The proportion of detectably infected larvae divided by the total number of larvae offered was used to determine the percentage infection just after removal of the parasitoids. Four to six E. formosa females were used per treatment and the experiments were carried out three to four times.

Experiment 3: Survival of Encarsia formosa

The treatment of greenhouse whitefly with a spore suspension of A. aleyrodis was carried out as in experiment 1 (Fig. 8.1). The parasitoid females were kept individually in clip cages on the leaves bearing hosts either treated with A. aleyrodis spore suspension or with distilled sterilized water, at 25 ± 1°C and 55% RH. After 24 hr the parasitoids were removed and the plants were kept at 20 ± 2°C until adult whitefly and parasitoids emerged. This experiment was carried out twice. The number of parasitoid females used and the total number of whitefly per treatment are shown in Table 8.3.

At the end of the experimental period (about 20 days after parasitization) the numbers of insects in the following categories were recorded: (1) whitefly larvae and pupae showing signs of infection with A. aleyrodis, (2) pupal cases of whitefly, (3) pupal cases of parasitized whitefly, (4) whitefly larvae killed by other causes. Parasitized pupae showing signs of infection were only observed twice and therefore it was not considered worthwhile to place these in a separate category. The percentage infected larvae and

larvae killed by other causes was determined by dividing the numbers of whiteflies belonging to category (1) and (4) by the sum of the numbers of insects belonging to all categories (1) to (4).

Experiment 4: Transmission of Aschersonia aleyrodis by Encarsia formosa.

When E. formosa shows oviposition behaviour on infected hosts, the ovipositor will make contact with the fungus inside the larvae. To study the ability of E. formosa to perform as a vector of the fungus, parasitoids were offered either orange-coloured hosts or healthy untreated hosts for 24 hr, after which they were offered healthy hosts for another 24 hr at 25°C. The infected hosts were still intact and no mycelium had protruded from the colonized insect body. A. aleyrodis spores are formed in pycnidia on mycelium on the exterior of the host. In this experiment, the parasitoid could only come into contact with fungal structures inside the host where spores are absent. Parasitoids were removed and whitefly larvae were reared to adult emergence. The numbers of emerging whiteflies, black pupae and infected larvae were recorded.

8.3 RESULTS

Experiment 1: Behavioural Observations

The different stages of A. aleyrodis infection of whitefly on the day of exposure to the parasitoids can be divided as follows. Treatment with fungal spores just before parasitization (day 0): spores of A. aleyrodis are present on the surface of larvae and the leaf. Treatment four days before parasitization (day 4): spores have germinated and penetration of the larval cuticle may have taken place. The haemolymph of some larvae showed a floccose composition. Treatment seven days before parasitization (day 7): colonization of the interior of larvae has taken place to some extent. Other experiments have shown that the LT50 is about seven to eight days at 20°C and at the dose used (Chapter 4). Treatment 10 or 14 days before parasitization (day 10 or 14): eggs were sprayed during hatching and about four days

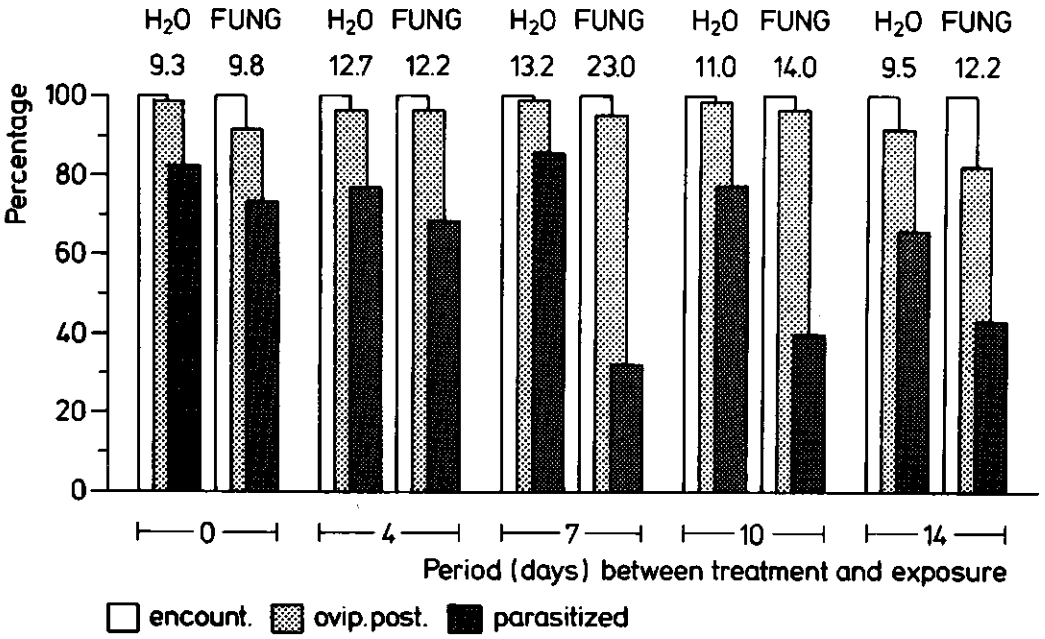


Fig. 8.2 Percentage oviposition postures and parasitized larvae related to the mean number of encounters (considered as 100%) of either water treated hosts or hosts treated by A. aleyrodis at different periods before exposure to E. formosa females (n=6 per spore- and control treatment).

before hatching. Infection does not occur in the egg stage, but after hatching the larvae will become infected by spores present on the leaf surface (Chapter 3 and 6). As a consequence, the stage of infection at exposure to the parasitoid is similar to the stage after treatment seven days before exposure to the parasitoid. A high number of whitefly treated as first instar larvae or eggs reach the third and fourth instar stage before they are killed by the fungus (Chapter 3). Therefore, treated as well as untreated larvae were offered in the preferred stages to E. formosa.

No differences in behaviour were found between wasps searching for hosts on treated leaves and those on untreated leaves. The numbers of hosts encountered were in most cases the same in the control and the fungal treatment. When hosts were offered seven to ten days after

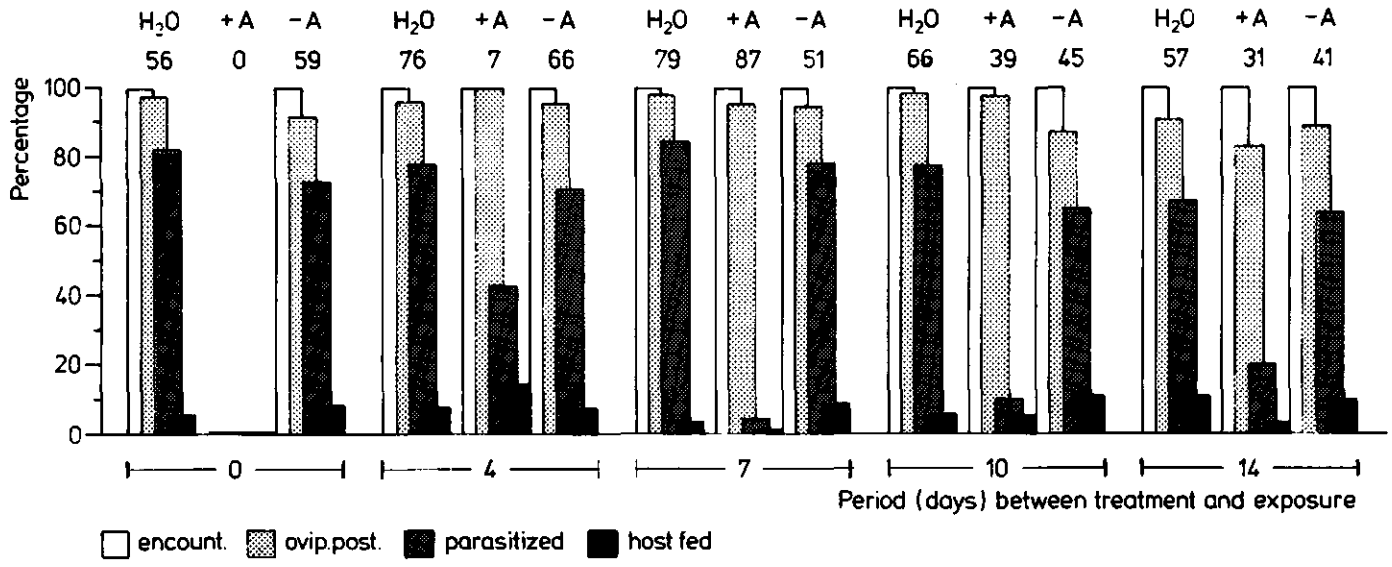


Fig. 8.3 Percentage oviposition postures, parasitized larvae and host fed larvae related to the overall number of encounters (considered as 100%) of either water treated hosts or hosts treated by A. aleyrodis at different periods before exposure to E. formosa. Spore-treated hosts were distinguished in infected (+A) and noninfected (-A) after one hour exposure to the parasitoids.

fungal treatment, however, there were significantly more encounters in the treatment than in the control (analysis of variance after a square root transformation of the numbers of encounters, P=0.003) (Fig. 8.2).

The parasitoids drummed the hosts with their antennae and the oviposition posture was adopted by wasps on treated hosts as well as on untreated hosts. Furthermore, parasitoids encountering hosts adopted the oviposition posture irrespective of the different stages of infection in the hosts. This suggests that they do not react to the presence of the fungus after external examination of the host (Fig. 8.2). Nevertheless, significantly more hosts were parasitized by E. formosa in the control treatment than in the fungal treatment ($P \leqslant 0.01$). The mean number of parasitized larvae was 7.4 when they were offered to E. formosa 7 days after treatment. This amounts to 32.2% of the mean number of encountered hosts. In the control the mean number of parasitized larvae was 11.2, being 84.8% of the mean number of encountered hosts (Fig. 8.2).

When offered hosts seven days after A. aleyrodis treatment, parasitoids encountered a total of 87 hosts showing detectable signs of infection, and 51 hosts that showed no detectable signs (Fig. 8.3). The latter category, referred to as noninfected hosts, consists of noninfected hosts and hosts possibly being infected but not yet showing detectable signs of infection. The parasitoids showed an oviposition posture on a total of 83 (95.4%) out of the 87 infected larvae, but laid only 4 eggs (4.6%). In contrast, on 48 (94.1%) out of 51 noninfected hosts an oviposition posture was adopted, and 40 eggs (78.4%) were found after dissection (Fig. 8.3). The data on numbers of oviposition postures on infected and noninfected hosts and the number of eggs laid in the hosts referring to the different treatments were analyzed by analysis of variance of the preference index u, being the normal deviate of kl for the normal approximation of the Fischer exact probability test in a 2 x 2 table. The rejection of infected hosts (u being negative in all cases) was significant for all parasitoids offered hosts 4, 7, 10 or 14 days after treatment (simultaneous $P \leqslant 0.001$). The rejection of hosts is highly correlated with the presence of -for the observer- detectable infection in the host.

Table 8.1 shows the mean number of rejections, defined as the number of times the oviposition posture is adopted which did not

Table 8.1. Mean number of rejections, defined as adoption of the oviposition posture not resulting in egg laying or host feeding by Encarsia formosa (n=6 per treatment), taking more or less than 1 minute 40 seconds.

Time of treatment (days) before parasitization		H_2O treatment		A. aleyrodis treatment			
				< 1'40"		≥1'40"	
		<1'40"	≥1'40"	+ Inf.	- Inf.	+ Inf.	- Inf.
0	x̄	0	1.0	0	0	0	1.2
	range	-	0-3	-	-	-	0-2
4	x̄	0	1.2	0.3	0	0.7	0.5
	range	-	0-3	0-1	-	0-2	0-1
7	x̄	0	1.3	12.5	0	1.2	0
	range	-	0-2	3-21	-	0-2	-
10	x̄	0	1.3	5.2	0.5	0.3	0.2
	range	-	0-3	2-10	0-1	0-1	0-1
14	x̄	0	1.3	1.5	0	1.7	1.0
	range	-	1-2	1-3	-	0-5	0-2

result in egg laying nor in host feeding. Lenteren et al. (1976b) found that egg laying by E. formosa took at least 1 minute and 40 seconds. Thus, a distinction was made between oviposition postures lasting less than 1'40" and oviposition postures that took 1'40" or longer. In the control treatment rejections belonging to the former category did not occur (Table 8.1). When infected hosts were encountered, most of the rejections occurred within 1'40". Apparently less time is spent when probing infected whitefly larvae than probing noninfected larvae. This may be a positive factor considering the parasitoid's searching efficiency. Less time is needed to reject infected hosts than to reject noninfected hosts. In this context the higher number of encounters, compared with the control when hosts were offered 7, 10 or 14 days after treatment to the parasitoids, could be explained by a difference in time allocation. In the treatment more time was spent by the parasitoid searching and encountering unsuitable hosts as most of the hosts were rejected within 1'40", whereas in the

control more time was spent actually laying eggs. Rejection of infected larvae which took more than 1'40" was correlated with a floccose appearance of the host's haemolymph, while orange coloured larvae always were rejected within 1'40".

E. formosa generally fed on hosts during the observation period (Fig. 8.3). On some occasions host feeding was observed on infected whitefly larvae. In total host feeding was observed on 5 infected hosts out of 154 on which the oviposition posture was adopted and on 24 noninfected hosts out of 240 on which the oviposition posture was adopted. Significantly less feeding took place on infected hosts compared with feeding on noninfected hosts (critical level P=0.01). This may suggest that parasitoids are less inclined to feed on infected larvae than on noninfected larvae.

Experiment 2: Dissection

Parasitoids were given the opportunity to parasitize hosts for 24 hours. Only the final oviposition result was determined. Table 8.2 shows the results of experiments in which E. formosa females were offered hosts sprayed with a spore suspension of A. aleyrodis 0, 2, 5 or 7 days beforehand. Five females were used per treatment and the treatments were carried out three or four times, resulting in a total of 15 to 20 females per interval between spore treatment and parasitization. Hosts were classified as in experiment 1.

Infection cannot be detected shortly after application of the spore suspension. In this experiment high percentages of infection appeared within five days of treatment (Table 8.2) which is faster than in experiment 1, where hardly any infected hosts were encountered 4 days after treatment. This may have been caused by the difference in temperature. E. formosa females were given the opportunity to parasitize hosts at 25°C for 24 hr, instead of one hour in experiment 1. The infection may, therefore, have developed faster at this temperature, which may also have influenced the high variation of infection percentages five days after treatment (Table 8.2).

The total numbers of eggs laid by E. formosa females in the treated hosts did not differ significantly from the numbers of eggs laid in water-treated hosts (nonorthogonal analysis of variance of the

Table 8.2. Mean number of eggs laid by E. formosa (n=5) in two categories of treated hosts determined after dissection: infected hosts (+ Inf) and noninfected hosts (- Inf). The percentage infection was determined by dissection of all hosts immediately after parasitization.

		H_2O treatment					A. aleyrodis treatment														
Time of treatment (days) before parasitization	Test no.	1	2	3	4	5	1			2			3			4			5		
		No. eggs					No. eggs		%Inf.	No. eggs		%Inf.	No. eggs		%Inf.	No. eggs		%Inf.	No. eggs		%Inf.
							+Inf	-Inf		+Inf	-Inf		+Inf	-Inf		+Inf	-Inf		+Inf	-Inf	
0	x̄	4.8*	9.0	6.2	-	-	0.0	9.0	0.0	0.0	11.6	0.0	0.0	7.8	0.0	-	-	-	-	-	-
	range	3	7	4			0	6	0	0	6	0	0	5	0						
		6	11	8			0	11	0	0	4	0	0	11	0						
2	x̄	10.2	9.2	4.6	-	-	0.0	11.0	0.0	0.0	12.6	2.0	0.0	6.4	0.0	-	-	-	-	-	-
	range	7	5	1			0	8	0	0	8	0	0	0	0						
		12	14	7			0	15	0	0	16	10	0	12	0						
5	x̄	7.4	11.8	-	10.0	12.2	0.0	11.8	5.6	6.4[a]	3.0	89.4	-	-	-	0.0	9.8	7.4	0.6	6.4	84.2
	range	5	7		4	8	0	5	0	0	0	47				0	3	0	0	1	56
		11	18		14	17	0	25	13	11	15	100				0	16	26	3	11	99
7	x̄	6.2	9.2	-	8.2	11.0	0.0	11.2	35.4	0.0	6.8[b]	65.6	-	-	-	0.3**	8.5**	39.0**	0.6	8.2[b]	83.2
	range	4	5		0	7	0	7	8	0	3	44				0	3	0	0	5	49
		10	14		15	14	0	15	56	0	9	73				2	14	82	2	16	96

* : mean of four recordings
** : mean of six recordings
[a] : in four out of five observations 100% infection was found, therefore, no choice
[b] : hosts were superparasitized

difference between the mean number of parasitized larvae in the treatment and the mean number of parasitized larvae in the control). This suggest that the presence of fungal spores does not inhibit oviposition by E. formosa. However, infected hosts and noninfected hosts were distinguished when treatment of larvae occurs 5 or 7 days before parasitization, and significantly more eggs were laid in the latter category (statistical analysis using the preference index u). On the leaf level E. formosa searches at random (Lenteren et al., 1976a) and therefore is expected to encounter hosts from the two categories at a frequency proportional to their abundance. But apparently, the distribution of parasitoid eggs in the two categories of hosts does not follow this pattern. These results are consistent with the results of experiment 1.

When the proportion of infected hosts is high, the parasitoids will accept such hosts and deposit eggs, as shown in the results for hosts treated five days before exposure to E. formosa (Test 2, Table 8.2). In test 2, in four out of five recordings 100% infection was found, so the parasitoids had no choice between infected and noninfected hosts.

Another parasitization strategy was evident when noninfected hosts were available in limited numbers. In tests 2 and 5 when spore treatment of hosts was carried out seven days before parasitoids were released, very few eggs were laid in infected hosts, but superparasitization of noninfected whitefly larvae took place (Table 8.2). In a situation where healthy hosts are abundant superparasitization will not occur, as E. formosa is able to discriminate between parasitized and unparasitized hosts (Lenteren et al., 1976b).

Experiment 3: Survival of Encarsia formosa.

The results in Table 8.3 show the mean number of surviving parasitized whitefly larvae when E. formosa was given the opportunity to parasitize hosts 0, 4, 7, 10 or 14 days after A. aleyrodis treatment, respectively. Parasitoid females can discriminate between infected and noninfected hosts (experiment 1 and 2). In this experiment, evaluation took place after healthy whiteflies and

Table 8.3. Mean number of surviving parasitized whitefly pupae (black), offered to Encarsia formosa at different time intervals after treatment of the hosts with a spore suspensions of Aschersonia aleyrodis.

Time of treatment (days) before parasitization	Control					A. aleyrodis treatment				
	n[1]	No. larvae[2]	Mort.%[3]	Mean no. black pupae (range)	% Emergence E. formosa	n[1]	No. larvae[2]	Mort.%[4]	Mean no. black pupae (range)	% Emergence E. formosa
0	5	529	40.4	13.4 (2-29)	94.0	6	795	96.0	0	0
	10	1311	4.4	14.3 (9-33)	82.5	11	1490	40.7	8.3 (1-17)	59.3
4	5	407	12.6	21.2 (13-29)	94.3	6	448	95.4	0.2 (0-1)	100.0
	7	679	15.2	15.0 (7-20)	75.7	12	971	95.8	0.1 (0-1)	100.0
7	7	374	12.2	6.4 (0-18)	95.6	7	383	97.0	0.1 (0-1)	100.0
	10	1713	14.8	13.8 (7-19)	86.2	12	1790	92.9	0.3 (0-2)	50.0
10	7	942	2.6	17.7 (8-23)	98.4	7	1257	98.0	0.4 (0-2)	100.0
	10	551	9.2	19.0 (11-26)	94.2	10	382	91.8	0.3 (0-2)	100.0
14	5	652	7.0	14.2 (1-20)	95.8	7	1072	96.9	0.6 (0-2)	100.0
	12	1186	5.4	16.3 (2-22)	93.9	11	773	92.7	1.0 (0-3)	72.7

[1]: Number of parasitoid females per treatment.
[2]: Total number of whitefly larvae treated.
[3]: Mortality of whitefly larvae by other causes than A. aleyrodis infection.
[4]: Mortality of whitefly larvae by other causes and mortality caused by infection of A. aleyrodis.

parasitoids had hatched. The mean number of surviving whitefly and parasitized pupae is very low in all treatments except one when parasitization took place just after application of the spore suspension of A. aleyrodis. The lower percentage infection of 41% (Table 8.3), was caused by omitting the period of high humidity. In this experiment the relative humidity was low (55%) and the temperature was 25°C.

During the early stage of infection, parasitoids do not detect infection. Parasitized infected hosts usually do not result in healthy parasitoids. If discrimination between healthy and infected larvae occurs, then a higher number of parasitized healthy hosts should be found than expected from a random distribution of parasitoid eggs over healthy and infected hosts. However, the mean number of parasitized healthy pupae in the treatments (Table 8.3) was too low to allow statistical analysis.

Table 8.4 shows the mean number of healthy hosts available per parasitoid as recorded at the end of the experiment. The mean percentage parasitization of noninfected hosts may be compared across treatments because these are derived from about the same numbers of healthy hosts available to the parasitoid. This percentage increases based on comparison of A. aleyrodis treatment 4 days before parasitization with the treatments 7, 10 or 14 days before parasitization. This may suggest selection by parasitoids for noninfected hosts, as was found in experiment 1 and 2.

Experiment 4: Transmission of Aschersonia aleyrodis by Encarsia formosa.

As has been shown in experiment 1 E. formosa can detect infection of T. vaporariorum by A. aleyrodis only by means of ovipositor probing inside the body of the host. In this situation in which contact with fungal structures is probable, the parasitoid's ovipositor may become contaminated with fungal remnants which may infect healthy hosts probed thereafter. Spread of A. aleyrodis by the parasitoid is possible to some extent because some whitefly larvae offered to the 'contaminated' female parasitoids were infected by A. aleyrodis. Parasitoids were either offered orange-coloured hosts or healthy hosts

Table 8.4. Mean number of healthy hosts, available per parasitoid and the mean percentage parasitization of the available hosts per parasitoid as recorded at the end of experiment 3.

	H_2O treatment		*A. aleyrodis* treatment		
Time of treatment (days) before parasitization	n	Mean no. hosts/parasitoid	n	Mean no. hosts/parasitoid	Mean % parasitization healthy hosts
0	5	65.8	6	7.5	0.0
	10	126.5	11	78.0	10.6
4	5	87.3	6	3.5	4.8
	7	82.4	12	3.1	2.7
7	7	46.3	7	1.1	12.5
	10	145.6	12	3.2	10.5
10	7	131.4	7	3.7	11.5
	10	50.6	10	2.7	11.1
14	5	141.8	7	4.1	10.3
	12	77.0	11	7.4	13.6

for 24 hr, after which they were offered healthy hosts for another 24 hr. Two parasitoids out of seven caused infection of two hosts each, two parasitoids caused infection of one whitefly host each and three parasitoids did not transmit the fungus. The total number of parsitized hosts per female was the same when parasitoids were offered healthy hosts or infected ones beforehand. The infected larvae may have been the first hosts to be parasitized by *E. formosa* after contact with infected hosts. It is conceivable that the parasitoid may contact spores present on the leaf surface remaining from the previous treatment; however, the chance that viable spores would be transported by the parasitoid's legs and be deposited on a healthy larva seems rather small.

8.4 DISCUSSION

The interaction between parasitoids and fungal pathogens on the same host has not yet been studied extensively (Verenini, 1982). More is known about the interaction between Bacillus thuringiensis or several insect viruses and parasitoids. Sublethal doses of B. thuringiensis caused a delay in the development of Gypsy moth larve, Lymantria dispar. As a consequence more parasitization was observed by Apanteles melanoscelus because this parasitoid prefers small larvae for parasitization (Weseloh & Andreadis, 1982, Weseloh et al., 1983). The same phenomenon was observed by Wallner et al. (1983) using B. thuringiensis in combination with Rogas lymantria, another parasitoid of Gypsy moth larvae. Applying sublethal doses of B. thuringiensis consistently increased total parasitism and mummy production and the adult parasitoid emergence was not significantly less compared with the emergence of parasitoids from control hosts.

Behavioural studies on host selection by parasitoids in relation to virus infections have been conducted. Beegle and Oatman (1975) observed that female Hyposoter exiguae parasitoids laid as many eggs in Trichoplusia ni larvae infected by nuclear polyhedrosis virus as in noninfected T. ni larvae. In infected hosts these parasitoids perished when their hosts died of virus infection. Apanteles glomeratus parasitoids did not discriminate between healthy larvae of Pieris rapae or those treated by granulosis virus (Levin et al., 1983). Nevertheless, it was observed that healthy larvae were attacked first with a greater frequency than GV-treated larvae. A. glomeratus does distinguish between parasitized and nonparasitized healthy hosts, but not between infected parasitized hosts and infected nonparasitized hosts. Versoi and Yendol (1982) reported results which showed that Apanteles melanoscelus preferred healthy Gypsy moth larvae to those infected by nuclear polyhedrosis virus. Although the number of host contacts in noninfected and virus-infected larval treatments were not significantly different, the percentages of ovipositional attempts following host contact were significantly greater in the noninfected larvae than in the infected larvae. In this situation the parasitoid, A. melanoscelus, rejected hosts before the oviposition posture was adopted, which suggests alterations in or on the host which are

externally detectable to the parasitoid. In contrast, E. formosa was not able to discriminate between whitefly larvae infected by the fungus and noninfected hosts until the ovipositor had contacted the interior of the host.

Host feeding by E. formosa was observed on infected whitefly larvae although to a lesser extent than on noninfected whitefly larvae. Whether host feeding on infected larvae influenced the survival of the female parasitoids was not investigated, although the ability of A. aleyrodis spores to infect E. formosa through the gut epithelium is not likely to occur. Mück et al. (1981) critizised the research on the toxicity of B. thuringiensis to parasitoids because the risk that parasitoids fed on high concentrations of B. thuringiensis in a field situation, is very low. Nevertheless, parasitoids feeding on infected hosts were not considered in their study. Whitefly larvae colonized by A. aleyrodis sometimes served as an alternative food source for Phytoseiulus persimilis, the predatory mite of Tetranychus urtica. No detrimental effect on the predatory mite was observed.

Aschersonia aleyrodis spores were applied in high doses, resulting in high numbers of infected whitefly larvae. When hosts were parasitized shortly after A. aleyrodis spore application, the fungus became the 'winner of the competition'. Even when fungal application took place on the same day as parasitization, the fungus was successful in colonizing the host. This was underscored by the fact that during all experiments only two whitefly pupae were found showing both blackening as a sign of advanced development of the parasitoid larva and signs of A. aleyrodis infection at the same time. Parasitization at an early phase of infection is, therefore, detrimental to the survival of the parasitoid's progeny. The ability to discriminate between infected and noninfected hosts at a later phase, gives the parasitoid the opportunity to successfully parasitize healthy whitefly larvae, and thereby to cause host mortality complementary to the fungal treatment.

Fungal remnants sticking to the ovipositor caused infection of healthy hosts, and consequently concurrent parasitization of these contaminated hosts resulted in the death of E. formosa's progency. However, infection of healthy hosts resulting from probing by

parasitoids with ovipositors contaminated with the fungus occurred infrequently. Therefore, the impact of this type of vectoring on the survival of the progeny of E. formosa is not expected to be large. Levin et al. (1979), Irabagon and Brooks (1974) and Beegle and Oatman (1975) also mentioned the transmission of virus by parasitoids from infected to healthy hosts. Transmission of virus was related to the degree of infection in the larvae to which each parasitoid was exposed prior to ovipositing in healthy larvae. In the case of A. aleyrodis, only infected larvae were used which were orange and thus totally colonized by the fungus. Using infected hosts in an earlier stage of infection may result in less vectoring because the chance of contamination of the ovipositor may increase with the development of the infection inside the host.

In conclusion, A. aleyrodis and E. formosa can be used together in a glasshouses situation. E. formosa will be most effective when introduced seven to ten days after application of A. aleyrodis. Application of the fungus also involves the exposure of already parasitized whitefly larvae to infection by the fungus. The results of this research will be discussed in the following chapter.

CHAPTER 9

INTERACTION BETWEEN THE PARASITOID ENCARSIA FORMOSA AND THE PATHOGEN ASCHERSONIA ALEYRODIS IN THE CONTROL OF GREENHOUSE WHITEFLY, TRIALEURODES VAPORARIORUM: SURVIVAL OF THE PARASITOID AFTER TREATMENT OF PARASITIZED HOSTS WITH FUNGAL SPORES

9.1 INTRODUCTION

Greenhouse whitefly, Trialeurodes vaporariorum (Westwood), is an important pest in greenhouse crops like vegetables and ornamentals. In the 1970's biological control of this pest using the parasitoid Encarsia formosa Gahan was developed. This natural enemy is presently available commercially in 18 countries and in 1985 it was applied on 1600 ha, primarily vegetables (Lenteren, 1987a). In tomato, effective control is achieved by only few introductions of E. formosa, whereas in cucumbers E. formosa has to be introduced frequently and it is more difficult to keep the whitefly population below the economic damage level (Lenteren et al., 1979).

Aschersonia aleyrodis Webber, an entomopathogenic fungus, is presently under investigation as an alternative or as a complement to E. formosa for control of greenhouse whitefly. This fungus is reported to be selective on whitefly species (Petch, 1921). In the greenhouse whitefly infection is limited to the larval instars (Chapter 3). In order to develop and optimize the combined use of the parasitoid and the fungus the interrelation between these two organisms on whitefly must be investigated.

Preliminary glasshouse trials have been carried out by Ramakers and Samson (1984). Although the numbers of parasitized hosts decreased after application of A. aleyrodis, the overall percentage of whitefly larvae killed either by the parasitoid or the fungus exceeded the percentage of whitefly larvae killed by the parasitoid population alone. Also Landa (1984) reported that a combined application of parasitoid and fungus gave better results than that of A. aleyrodis or of the parasitoid alone.

A. aleyrodis is a well-known natural enemy of citrus whitefly

Dialeurodes citri in Florida (Berger, 1921, Fawcett, 1944). Because A. aleyrodis generally is only effective when citrus whitefly reaches high densities, the parasitoid Encarsia (Prospaltella) lahorensis was introduced into the citrus-growing regions for whitefly control (Meyerdink et al., 1980). The parasitoid has become well established according to McCoy et al. (1980). Information on the interaction between the parasitoid and the fungus in citrus groves is not available. There is, however, information on other systems in which fungi and parasitoids occur on the same host insect (Fabres, 1977, Powell et al., 1986, Burleigh, 1975, Los & Allen, 1983). From these studies it was concluded that a negative correlation between fungal infection and parasitization by the parasitoids may be related to an interaction between these two types of natural control agents in the field.

In a glasshouse where A. aleyrodis is introduced at a time when parasitoids are present and the same host is attacked, an interaction between the two organisms may occur. In Chapter 8 attention is focused on the effect of treatment of larval instars of greenhouse whitefly by A. aleyrodis spores before releasing E. formosa. It is concluded that the parasitoid females are able to discriminate between infected and noninfected hosts. The consequences for the parasitoid's progeny after treatment of parasitized whitefly larvae by A. aleyrodis spores are discussed in this chapter.

9.2 MATERIALS AND METHODS

Hosts

Greenhouse whitefly was reared on cucumber plants cv "Gele Tros" at 25°C and a 16 hr photoperiod (experiment 1 and 3) and cv "Profito" at 20°C and a 16 hr photoperiod (experiment 2). About 20 to 30 adult whiteflies, a mixture of males and females, were put in clip cages (2.8 cm diameter) on the plants and were given the opportunity to lay eggs during a 24 hr period (experiment 2) or a 48 hr period (experiment 1 and 3). Hosts were offered to E. formosa as a mixture of second and third instar larvae or as a mixture of third and fourth instar larvae (experiment 1) or as third instar larvae (experiment 2 and 3).

Encarsia formosa

The parasitoids used in experiment 1 were obtained as black pupae which developed from parasitized whitefly larvae on bean leaves from a mass rearing at the Plant Protection Service at Wageningen, the Netherlands. The stock originated from Koppert BV, Berkel and Rodenrijs, the Netherlands. The parasitoids used in experiment 2 were obtained directly from this firm as black pupae on cardboard cards. The black pupae were put in Petri dishes at 25°C and a 16 hr photoperiod. The emerging adult E. formosa females were offered whitefly larvae for parasitization and feeding for one to two days. Thereafter they were kept individually in gelatine capsules with honey as food for about 12 hr before use in the experiments. Then they were put in clip cages on the patches with hosts and were given the opportunity to parasitize hosts for 24 or 48 hr.

The hosts were treated at a certain time period after parasitization by a spore suspension of A. aleyrodis or by sterilized distilled water as a control. The number of black pupae was recorded 14 days after parasitization and eight days later the number of empty black pupae from which healthy E. formosa adults had emerged, was recorded, giving information on the survival of parasitoids.

Aschersonia aleyrodis

Aschersonia aleyrodis, isolate from the Centraalbureau voor Schimmelcultures (CBS) at Baarn, the Netherlands, was subcultured three times before use in the experiments. By rinsing with sterilized distilled water, spores were harvested from two to four weeks old cultures on brown rice or cornmeal grown at 25°C and a 16 hr photoperiod. A dosage of 1.0 to 3.4 x 10^7 spores/ml was used in experiment 1 and a dosage of 2.2 x 10^7 spores/ml was used in experiment 2. Four milliliters of the spore suspension were sprayed onto the underside of each leaf bearing parasitized and unparasitized hosts, using a Potter spray tower set at 34.45 kPa. An amount of 4 ml of sterilized distilled water was sprayed as a control. The spore suspensions were checked for spore viability by spraying 2 ml onto water agar plates. After 24 hr at 25°C the percentage of germination was determined by

observing 300 spores. In most cases this percentage exceeded 90%, but in some cases the percentage was 67% to 77%.

After evaporation of the water from the suspension on the leaf surface, the plants were covered by transparent plastic bags to create a condition of 100% relative humidity (RH) for 72 hr (experiment 1) or for 36 hr (experiment 2). After removal of the bags RH was 50%-60%, the temperature 25 ± 1°C and the photoperiod 16 hr.

Experiment 1

This experiment was carried out to obtain information on the survival of parasitized hosts when treated by a spore suspension of A. aleyrodis. Parasitoids were put in clip cages on patches of hosts on day 0 and removed after 24 hr or 48 hr. The leaves were treated with a spore suspension of A. aleyrodis immediately after removal of the parasitoids (day 1 or day 2) or on day 4, 7 or 10. Two patches of hosts were present per leaf and 2 or 3 leaves per plant were used for a total of ten patches in the spore treatment and six in the control treatment. The patches were considered as independent experimental units. The experiment was carried out four times with slight modifications which are summarized as follows.

Exp. 1a. Two parasitoid females per patch were offered hosts of the third and fourth larval instar stage for 24 hr.

Exp. 1b. Two parasitoid females per patch were offered hosts of the third and fourth larval instar stage for 48 hr. The number of patches was six instead of ten.

Exp. 1c. Three parasitoid females per patch were offered hosts of the second and third larval instar stage for 24 hr.

Exp. 1d. Two parasitoid females per patch were offered hosts of the second and third larval instar stage for 48 hr.

During assessment the following categories of whitefly hosts were distinguished: (1) black pupae (parasitized hosts) surviving the treatment by A. aleyrodis, signs of infection being absent; (2) empty pupal skins from which whiteflies emerged; (3) whitefly larvae killed by other causes than fungal infection or parasitization; and (4) whitefly larvae infected by A. aleyrodis.

In addition to the control using distilled water instead of

A. aleyrodis spore suspension another control using a spore suspension of A. aleyrodis applied to unparasitized whitefly larvae was included.

The percentage infection was defined as the number of infected whitefly larvae (category 4) divided by the sum of the numbers of insects belonging to categories (1), (2) and (4). Category (1) was included in the calculation of the percentage infection. The numbers of insects belonging to category (3) were omitted from the calculations because the percentages of larvae killed by other causes in the spore treatment and the two different control treatments showed the same trends.

Experiment 2

Experiment 2 was carried out to obtain information on the survival of parasitized hosts when treated with a spore suspension of A. aleyrodis on day 1, 2, 3, or 4 after parasitization. One female parasitoid per patch was given the opportunity to parasitize hosts in the third larval instar for 24 hr. One patch of hosts per leaf, two to three leaves per plant and three to five plants per spore or control treatment were used. Eight to 16 E. formosa females were used per A. aleyrodis treatment and eight to ten females per control. All categories of whitefly larvae distinguished were identical to the ones defined in experiment 1.

Experiment 3

The parasitoids which developed from treated parasitized hosts were offered fresh hosts in order to determine the parasitization capacity of these parasitoids. Hosts containing parasitoids in the egg, larval or pupal stage were treated with A. aleyrodis (treatment at day 1, 4, 7 or 10 after parasitization). Three parasitoids originating from A. aleyrodis-treated hosts as well as from water-treated hosts were used per treatment day. Each parasitoid was offered third instar larvae for 48 hr at 25 $\pm$ 1°C and a 16 hr photoperiod. The number of black pupae and the percentages emergence were determined as in experiment 1.

Table 9.1 Mean number of parasitized hosts which developed into black pupae after treatment with Aschersonia aleyrodis at different times after parasitization.

Time interval (days) between parasitization and treatment	H_2O treatment			A. aleyrodis treatment		
	n[1]	Mean	Range	n[1]	Mean	Range
Exp. 1a (L4, 2 parasitoids/ patch, 24 hr)						
1	6	14.8	8–21	10	1.1	0–3
4	6	16.3	9–26	10	16.8	6–32
7	6	14.8	9–24	10	14.9	5–28
10	6	14.5	7–22	10	14.5	2–27
Exp. 1d (L3, 2 parasitoids/ patch, 48 hr)						
2	6	29.0	14–44	8	1.0	0–4
4	6	28.2	17–45	10	24.3	14–45
7	6	27.8	19–37	9	25.0	18–38
10	5	21.0	13–30	7	27.4	13–36
Exp. 2 (L3, 1 parasitoid/ patch, 24 hr)						
1	8	10.6	4–20	15	3.9	1–7
2	10	13.5	7–21	8	2.1	0–4
3	10	11.6	2–21	16	1.5	0–7
4	9	10.6	4–15	10	8.0	4–16

1: number of patches.

9.3 RESULTS

Table 9.1 shows the results of experiment 1a, 1d and 2. In experiment 1 the number of parasitized whitefly larvae developing into black pupae is significantly higher in the control treatment than in the spore treatment when A. aleyrodis was applied at day one or two after parasitization by E. formosa. This phenomenon was consistent for experiment 1a to d. The mean number of black pupae was 4.5 in experiment 1b and 0.7 in experiment 1c when treatment took place on day 2 and day 1, respectively. The mean number of black pupae in the additional controls was 29.3 and 17.5, respectively. The results of experiment 2 show that the number of parasitized larvae surviving an A. aleyrodis treatment was low when a treatment was applied 1, 2 or 3 days after after parasitization (Table 9.1). However, no significant difference between the number of parasitized larvae in control and treatment was observed when A. aleyrodis spores were applied on day 4 after parasitization in experiment 1 and 2. In experiment 1b the mean number of black pupae in control and treatment was 35.8 and 33.3, respectively, when A. aleyrodis spores were applied 4 days after parasitization. In experiment 1c the mean number of black pupae in control and treatment was 20.5 and 19.6, respectively. Also, treatment of hosts on day 7 and day 10 after parasitization did not result in different numbers of black pupae compared with the control (Table 9.1). Thus, the fungus is able to infect and colonize parasitized hosts only during the first three days after parasitization.

The emergence of parasitoids from black pupae was not significantly different comparing the weighed fractions from the different treatments and the control data in experiment 1a to d. Table 9.2 shows the total number of emerged E. formosa females for experiment 1a and experiment 1d. The survival of parasitized larvae (about 80%) was consistent with the survival recorded by Nechols & Tauber (1977) and Arakawa (1982). A. aleyrodis was never isolated from non-emerged black pupae put on malt agar. Only two black pupae in experiment 1 and one in experiment 2 previously exposed to A. aleyrodis showed signs of infection by A. aleyrodis. In these cases, the fungus succeeded in protruding from the melanized pupal cuticle and formed a fringe of mycelium around the black pupal case.

Table 9.2 Total number of emerged Encarsia formosa females (em.) and total number of black pupae (b.p.) after treatment of parasitized hosts by Aschersonia aleyrodis or water at different times after parasitization.

Time interval (days) betweem parasitization and treatment	Control		A. aleyrodis	
	em.	b.p.	em.	b.p.
Exp. 1a (L4, 2 parasitoids/patch, 24 hr)				
1	76	89	9	11
2	--	--	---	---
4	75	98	130	168
7	70	89	113	149
10	72	87	116	145
Exp. 1d (L3, 2 parasitoids/patch, 48 hr)				
1	---	---	---	---
2	142	174	6	8
4	153	169	197	243
7	140	167	158	225
10	75	105	149	192

The mean percentage infection of whitefly larvae is shown in Table 9.3. Parasitized as well as unparasitized larvae can be infected by the fungus and infected hosts belonging to these two groups can not be distinguished afterwards. The percentage infection decreased as the interval between parasitization and treatment with A. aleyrodis increased. This phenomenon is associated with a decrease in susceptibility of the last larval instar and the pupal stage of whitefly

to infection by the fungus (Chapter 3 and 4). Nevertheless a number of the older instar whitefly did become infected because of the high spore dose used. As the overall percentage of infection decreased the probability of survival of parasitized larvae increased. However, the survival of parasitized larvae could not merely be accounted for by this phenomenon. Another mechanism may be present because in the treatment 4 days after parasitization a rather high percentage of infection was observed even though the number of surviving parasitized pupae was not different from the number in the control (Table 9.3, Table 9.1). Comparing the percentage infection in the control without parasitized hosts and the treatment with parasitized hosts, the former

Table 9.3 Mean percentage infection of greenhouse whitefly with Aschersonia aleyrodis at different times after exposure to E. formosa parasitoids (+P) and mean percentage infection of hosts not exposed to parasitoids (-P) as a control.

Time interval (days) between parasitization and treatment:		1	2	4	7	10
Exp. 1a	+P	88.0a[1]	-	40.0a	10.9a	11.0a
	-P	85.5a	-	48.9a	11.4a	13.9a
Exp. 1b	+P	-	84.6a	53.5a	16.7a	6.7a
	-P	-	91.2a	74.8b	21.4a	10.9b
Exp. 1c	+P	91.5a	-	66.7a	30.9a	13.6a
	-P	89.6a	-	71.7b	32.4a	18.1a
Exp. 1d	+P	-	94.4a	67.4a	34.3a	6.7a
	-P	-	95.3a	90.4b	47.3b	10.1a

[1]: Values of +P and -P followed by the same letter are not significantly different (Wilcoxon two sample test, $P<0.05$).

Table 9.4 Development of Encarsia formosa in larvae of greenhouse whitefly at 25°C.

Days after parasitization of third instar larvae:	2	3	4	5	6
parasitoid egg	14	21	0	0	0
parasitoid larva	0	0	30	27	23
total	14	21	30	27	23

was significantly higher when treatment took place 4 days after parasitization (Table 9.3). The hypothesis that the probability of successful infection by the fungus of parasitized hosts was equal to that of unparasitized hosts was tested. Statistical analysis showed that the probability of parasitized hosts becoming infected and colonized by the fungus is less than the probability of unparasitized hosts becoming infected when the fungus is applied more than 3 days after parasitization.

Remarkably few whitefly larvae were found showing signs of both infection and parasitization in any of the treatments. The change from low to high survival of parasitized hosts when A. aleyrodis was applied 3 and 4 days after parasitization suggests a correlation with certain changes in the host related to the parasitoid's development. Therefore, parasitized larvae were dissected to record the developmental stage of the parasitoid inside the host (Table 9.4). When whitefly larvae belonging to the third and fourth larval instar were parasitized, all E. formosa eggs hatched between day 3 and day 4 after parasitization at 25°C. Survival of parasitized larvae treated at the fourth day after parasitization seems to coincide with the hatching of the parasitoid larva.

E. formosa females from hosts exposed to A. aleyrodis were offered third instar larvae to determine their reproductive capacity during 48 hr. The number of whitefly larvae parasitized by these parasitoids was the same as the number of hosts parasitized by parasitoids which emerged from hosts not exposed to the fungus (Table 9.5). Also, the moment of treatment did not influence the number of

Table 9.5 Mean number of black pupae (b.p.) and emerged parasitoids (em.) that are progeny of Encarsia formosa females treated with Aschersonia aleyrodis at different times during their development inside the whitefly host.

Time interval between parasitization and treatment of hosts containing parental parasitoids		Control	A. aleyrodis
1	b.p.	21.3	26.3
	em.	20.3	23.0
4	b.p.	26.7	25.0
	em.	25.3	23.0
7	b.p.	24.3	27.0
	em.	23.0	25.3
10	b.p.	28.0	24.3
	em.	25.7	23.0

hosts parasitized afterwards nor the emergence of the second generation. As the parasitoids are still able to develop normally and produce normal progeny after A. aleyrodis treatment, it can be concluded that no direct or indirect effects on the parasitoids and their progeny occur.

9.4 DISCUSSION

The probability of parasitized hosts to become infected by A. aleyrodis is lower than that of unparasitized hosts when the fungus is applied 4 days or longer after parasitization. This suggests the presence of factors making parasitized hosts less susceptible to infection by the fungus than unparasitized hosts. Vinson & Iwantsch

(1980a) stated that the ovipositing female parasitoid or her progeny may cause changes in the host, a phenomenon generally defined as host regulation. It may include changes in growth rate, food consumption, development, morphology, behaviour, respiration and other biochemical, physiological or physical activities within the host. Little is known about the changes caused by parasitization of greenhouse whitefly by E. formosa. Melanization of the host's cuticle takes place about 9 days after parasitization at 25°C. The decrease in susceptibility of parasitized hosts did not synchronize with the melanization of the host's pupal cuticle, but instead took place 4 days after parasitization. This change in susceptibility correlates with the emergence of the parasitoid larva from the egg inside the host.

Several factors can be suggested as influencing the effective colonization of a parasitized host.

(A.) The increase in survival of parasitized hosts when treated with an entomopathogen a long period after parasitization can be caused by a decrease in susceptibility of the host for infection, which may also be apparent in unparasitized hosts (see Chapter 3).

(B.) Penetration of parasitized larvae by the fungus may be more or less difficult than of unparasitized hosts because of changes in the host cuticle (indirectly) caused by the parasitoid inside the host (El-Sufty & Führer, 1981a). E.g., unparasitized Cydia pomonella larvae showed a melanization of the cuticle as a reaction against penetration of Beauveria bassiana, but this defensive reaction is suppressed in hosts parasitized by Ascogaster quadridentatus, therefore, making penetration easier (El-Sufty & Führer, 1985).

(C.) Competition for food between the parasitoid larva and the fungus may be present. If competition for hosts plays a role, one expects a gradual change from a high number of hosts killed by the fungus if applied shortly after parasitization and an increase in number of hosts killed by the parasitoid when this time period increases. Additionally, a category of hosts may be present showing both signs of infection and of parasitoid development. The former phenomenon was described by Levin et al. (1981) for the interaction between Apanteles glomeratus and granulosis virus in Pieris rapae. The latter phenomenon was described by Powell et al. (1986) for the interaction between Aphidius rhopalosiphum and Erynia neoaphidis on Metopolophium

dirhodum. In the relationship between E. formosa and A. aleyrodis, however, there appears to be an abrupt change from infected parasitized hosts to parasitized hosts successfully surviving fungal treatment. If the fungal treatment takes place when the parasitoid is still in the egg stage, the fungus may have the advantage colonizing the haemolymph of the host before the parasitoid is able to start feeding. This kind of competition for food may play a role in entomopathogen-parasitoid relations. For instance, significant numbers of the parasitoid Campoletis sonorensis developed in nuclear-polyhedrosis-virus-infected Heliothis virescens hosts only if exposure to the virus was delayed by at least 48 hr following parasitization (Irabagon & Brooks, 1974). Also Beegle & Oatman (1975) found that the proportion of the parasitoid Hyposoter exigua which survived increased as the time between parasitization and nuclear-polyhedrosis-virus exposure of Trichoplusia ni increased. Parasitoid larvae which developed in T. ni exposed to the virus soon after parasitization spent significantly less time in their hosts than did parasitoids which developed in noninfected hosts. Keller (1975) also observed this influence on Aphidius sp. parasitoids in the aphid Acyrthosiphon pisum infected by Entomophthora spp. These parasitoids were small and sometimes showed malformations. Most of the parasitoids died as older instar larvae or as pupa, but colonization by the fungus was restricted to the tissues of the host insect.

(D.) After successful penetration of the parasitized host, subsequent colonization by the fungus may be hampered by defence mechanisms (Vinson & Iwantsch, 1980a and b). Willers et al. (1982) found an inhibitory effect of the anal secretion of Pimpla turionella larvae on B. bassiana. Not only was the length of the hyphae affected, but also the shape and spirally swollen and excessively branched hyphae were observed. The development of B. bassiana in Carpocapsa pomonella and in Pieris brassicae parasitized by Ascogaster quadridentatus and Apanteles glomeratus, respectively, was inhibited (El-Sufty, 1978). Antimicrobial compounds may be produced by the developing parasitoid to protect the host as a food source from being a victim of septecaemia caused by the flora from the gut lumen of the host. Another mechanism by which the parasitoids may protect themselves is by releasing teratocytes in the haemolymph of the host at the same

time as the first instar larva hatches from the egg (El-Sufty & Führer, 1981b).

A decrease in susceptibility of parasitized whitefly larvae to infection by A. aleyrodis may be explained by the supposition that: (1) the composition of the host cuticle changes, thus inhibiting the penetration of A. aleyrodis or (2) fungistatic/fungitoxic substances are present in the haemolymph of the host produced or induced by the parasitoid larva. More detailed research is needed to find the exact explanation for this interaction in the E. formosa-A. aleyrodis-whitefly relationship.

Introduction of A. aleyrodis to a commercial market requires registration of the product which will depend on providing information on, for instance, side effects on other organisms. Guidelines have been developed to test the side effects of pesticides on beneficial insects. According to these, parasitized whitefly pupae should be treated with different amounts of the test agent three days before the adult parasitoids would emerge (Oomen, 1985). From the results in this chapter it can be concluded that A. aleyrodis spores do not cause any harm to the parasitoids' progeny when treatment would be carried out following these guidelines. The parasitoids emerging from treated hosts are well capable of laying as many eggs as parasitoids emerging from untreated hosts.

With regard to a practical glasshouse situation, an application of A. aleyrodis should not be combined with simultaneous introduction of E. formosa adults. When a population of all developmental stages of E. formosa is well established, an application of A. aleyrodis will only negatively affect a small part of the parasitoids' progeny. The question is whether the parasitoids' progeny is able to suppress the whitefly population which escapes infection by the fungus. Parasitization will be optimal when parasitoids are able to distinguish infected from noninfected hosts. Such a discrimination phenomenon is present about five to seven days after the spore application when the colonization of whitefly larvae by the fungus has advanced (Chapter 8). Therefore, eggs laid by parasitoids just after a spore application may succumb by fungal infection. The age structure of the whitefly population will play an important part in the establishment of a new equilibrium between the parasitoid and its

host. Otherwise an introduction of E. formosa at least ten days after the spore application can be considered. Another possibility is the application of A. aleyrodis early in the season, thus postponing the introduction of the parasitoid. Finally, the conclusion can be drawn that E. formosa and A. aleyrodis are compatible to a great extent and prospects for control of whitefly by introductions of E. formosa and applications of A. aleyrodis are promising.

CHAPTER 10

GENERAL DISCUSSION

In this chapter the previously described results are summarized and put in a general framework (Table 10.1) relating to the subjects for evaluation shown in the general introduction (Table 1.3 of Chapter 1). Furthermore the implications for A. aleyrodis as a control agent for greenhouse whitefly are given and some suggestions for future research are made.

10.1 FUNGAL CHARACTERISTICS

Specificity of Aschersonia aleyrodis

Aschersonia aleyrodis germinates on the cuticle of greenhouse whitefly and penetrates the integument by means of an appressorium (Chapter 2). It causes death of the insect host by colonization of the haemolymph and host tissues. When environmental conditions are favourable the fungus protrudes from the insect and pycnidia are formed in the stroma on the insect. Aschersonia spp. are known to be parasitic on whitefly species (Aleyrodidae) and scale insects (Coccidae). A. aleyrodis has been observed to infect Dialeurodes citri, D. citrifolii, Bemisia tabaci, Trialeurodes floridensis and T. abutiloneus (Berger, 1921, Morrill & Back, 1912) and Aleurocanthus woglumi (Quezada, 1974). Petch (1921) stated that a single species of Aleyrodid may be attacked by several species of Aschersonia. Further research of Aschersonia species and their host range would be useful to find more species pathogenic on economically important whitefly and coccid pests.

Virulence of the pathogen

To obtain information on the virulence of A. aleyrodis on greenhouse whitefly, bioassays were carried out (Chapters 4 and 5). The LC95 for first, second and third larval instars was 311.3, 367.9, and 514.0 spores/mm^2 respectively. The infection of fourth instar

larvae varied with age; 150 spores/mm^2 resulted in 50% mortality of young fourth instar larvae. Entomopathogens in general show dose-mortality relations with low slopes in comparison with dose-mortality relations for chemical compounds (Burges & Thompson, 1971). An exception is the activity of Bacillus thuringiensis of which the dose-response relation may also show steep slopes, due to the action of toxins. The dosages needed to achieve high mortality of whitefly are such that the development of a mass production of the spores seems feasible. Moreover, optimization of spraying techniques and formulation may allow high mortality levels at even lower dosages. The availability of more virulent strains and strains effective against several important whitefly pests may be economically attractive. For this purpose, selection of naturally occurring strains, mutagenesis or the use of recombinant genetic techniques may open perspectives for the future.

The LT50 was 11.8, 9.5 and 7.0 days for first, second and third instar larvae, respectively, at 20°C after application of 2 ml of 2.5 x 10^7 spores/ml. The LT25 for fourth instar larvae was 5.6 days at application of the same dosage. The LT50 and LT25 were determined by the appearance of signs of infection which involved the change in colour of the larvae and pupae from transparent yellow green or opaque white to clear orange or opaque orange. The actual death of the larvae or pupae will have taken place some time before these signs occur.

Bioassay methods depend on the consistency of the method when comparing different isolates or species of fungal pathogens. In the case of greenhouse whitefly the use of intact plants appeared suitable, since consistent results were found over time. Other relations may be found when other host plants are used which indirectly influence the susceptibility of the insects (Hare & Andreadis, 1983) or when incubation after treatment takes place at different environmental conditions. Another bioassay using leaf discs was carried out (Chapter 5) resulting in very high infection rates even after application at the low dosage of 1 x 10^6 spores/2ml. This was due to free water present on the leaf surface, which stimulates spore germination and also causes redispersal of the spores. In this situation a negative density-dependent effect was noticed on the germination of the spores and subsequent infection of the larvae.

However, this only resulted in a lower overall mortality of the fourth instar larvae at the highest dosages used of 2 x 10^7 spores/2ml and 1 x 10^8 spores/2ml. This phenomenon did not occur when intact plants were used with no free water on the leaf surface. The former bioassay method using plants should be preferred as this bioassay is carried out under environmental conditions which show similarity to some extent to those present in a practical situation.

Sporulation, spread and persistence of the pathogen

Greenhouse whitefly larvae infected with A. aleyrodis may show extensive sporulation (Fig. 2.6, Chapter 2) if environmental conditions are favorable. Secondary infection is limited, however, and is not expected to contribute to an effective control of whitefly after a single application on a cucumber crop. Spread of the disease is negligible and an epizootic does not develop in a glasshouse situation. The spores produced in slimy masses are dispersed by means of water. Because it does not rain in a glasshouse and overhead irrigation is not applied, only condensation of water on the leaf surface may enable spores to be transported from a sporulating insect to a susceptible insect on the same leaf. Insects may transport spores from one site to another. For instance, adult whiteflies or other insects walking over sporulating insects may pick up spores on their legs. Encarsia formosa can transmit the fungus by means of her ovipositor. However, this will not contribute to a high rate of infection within the short time period necessary to control a whitefly population.

A. aleyrodis is intended to be used in glasshouses, where detrimental influences of sunlight are limited (Krieg et al., 1981, Jaques, 1972). However, A. aleyrodis may also be applicable in the field, because the spores are directed against whitefly larvae which are present at the underside of leaves, and thus protected from sunlight. Moreover, the spores of A. aleyrodis contain pigments which may make them less vulnerable to UV radiation than hyaline spores (Leach, 1971). The mucilaginous layer in which the spores are embedded after formation in the pycnidia may also offer protection (Louis & Cooke, 1985).

Spores survive on the abaxial leaf surfaces of cucumber plants for over 28 days at 20°C and 70% RH after an initial period of 100% for 24 hr (Chapter 6). Few spores germinate on the leaf surface, but when spores contact a host several days after application germination can still occur. Thus, leaves sprayed before whitefly females laid their eggs, can bear infected larvae afterwards. However, the extent of this residual effect is dependent on host distribution over the plants. On a cucumber crop whiteflies tend to lay eggs on the young top leaves of the plants which will increase in size later. Thus, after treatment with spore suspension a dilution of spores will take place. It is, therefore, advisable to repeat the application aimed at these top leaves and newly developed leaves after a certain time interval.

Possibilities for mass production

Subculturing of A. aleyrodis on a semi-artificial medium did not significantly affect the virulence of the fungus. A. aleyrodis subcultured 12 times showed a dose-response relation similar to A. aleyrodis subcultured four times (Chapter 4). In the experiment in which the influence of relative humidity and temperature was studied (Chapter 7) A. aleyrodis was used after three, four, ten or 19 serial in vitro passages and after a single passage in greenhouse whitefly. Passaging through the insect did not seem to affect the final percentage infection nor the LT50. After the fungus had gone through ten and 19 serial in vitro passages, however, it showed somewhat lower infection rates and longer prepatent periods in whitefly larvae than fungus that had gone through only three to four in vitro passages. Changes in virulence were found in Metarhizium anisopliae after one host passage over a homologous host but this increase was lost after three to four subculturings on artificial medium (Fargues & Robert, 1983b). However, Ignoffo et al. (1982) did not find any significant decrease or increase in LC50 values after 12 in vivo passages of N. rileyi on Trichoplusia ni or 12 in vitro serial passages on semi-synthetic medium. Kogan and Serjapin (1978) advise reisolating A. aleyrodis once a year after three to four host passages. Whether subsequent host passages would influence the virulence of A. aleyrodis

was not stated in their article. Subsequent subculturings may gradually reduce the spore production of the fungus on millet or brown rice and host passaging was noticed to restore the production on semi-artificial medium in my experiments. Changes in the median lethal time (LT50) after serial in vitro passages may be due to gradual changes in the fungus adapting to the medium. However, this will not be a problem, because a mass production can be started from stock material which retains viability and virulence when stored in liquid nitrogen.

A. aleyrodis can be successfully grown on several kinds of medium. In Florida Berger (1920) developed a method growing cultures of A. aleyrodis in one-pint bottles on sweet potato agar from which 8.0×10^6 spores/cm^2 were obtained. One bottle would be enough for 70-100 gallons (264-378 liter) of water, sufficient to treat 70 to 100 citrus trees. The cultures were sold to the growers for 75 dollar cents per culture. In 1918 a total of 1156 cultures were sold. Spassova (1980) describes the production of fungal material using beer wort, 10% sugar, 0.1% manganese, 0.01% iron or magnesium, at 24-26°C. However, scaling up of production is necessary to develop a marketable product. Cereal grains offer a uniform, readily available substrate. The fungus may be fermented on trays, in autoclavable polypropylene bags or in large fermentation vessels. Also inert support material soaked with nutrients can be used in conventional upright fermentors (Lysansky & Hall, 1983, Ignoffo & Anderson, 1979). A. aleyrodis produces 1 to 2 $\times 10^9$ spores per gram on solid substrate (millet) which results in about 2 to 4 kg of substrate necessary to produce enough spores to treat 1 ha of cucumbers. These figures indicate that the costs of production and the practicality of using these quantities are likely to be reasonable (Burges & Hall, 1982).

Suitability for storage and formulation

When a fungal pathogen is used as a microbial control agent the retention of viability and virulence of infective units during storage and application is of importance. The development of an adequate formulation is therefore needed. The type of formulation ultimately selected depends upon the biology and the physical properties of the pathogen, host insect and target crop (Soper & Ward, 1981, Couch &

Ignoffo, 1981). For instance, the addition of nutrients has been considered for stimulation of germination and infection. However, one should be aware of the processes that can be influenced by the introduction of these formulations in a crop. Firstly, the addition of nutrients may stimulate germination but may induce errant and non-penetrating growth of the germ tubes (Hunt et al., 1984). Secondly, the addition of nutrients may activate germination of A. aleyrodis spores on the leaf surface which makes them more vulnerable to environmental conditions and may reduce their survival over time. Thirdly, the microorganisms on the phylloplane will also be influenced, resulting in positive or negative effects on the plants (Blakeman & Fokkema, 1982). Also, nutrients are added to a formulated product containing spores or mycelium to stimulate growth and sporulation on the leaf surface. In addition these spores may contact host insects and subsequently cause infection (Rombach et al., 1986, Hall, 1982). The success of this method is dependent on favourable environmental conditions during a prolonged period of time.

Safety and compatibility with other control procedures

A. aleyrodis is not expected to cause any negative effects on the environment because of its high specificity. The fungus originates from (sub)tropical regions and it is unlikely that it will be able to spread and propagate in temperate regions in such a manner that it will eradicate its host. Entomopathogens under consideration as microbial insecticides must be evaluated for possible deleterious effects to man, other animals and plants. Since fungal agents are potentially allergenic, special attention has to be devoted to allergenicity tests. Hall et al. (1982) recommend a safety-testing to warm-blooded animals arranged in a 3-tier system. Positive results in Tier 1 may lead to rejection of the product or to further testing in Tiers 2 and/or 3. The nature of tests beyond Tier 1 should enable any effects to be quantified and where appropriate risk/benefit analyses to be performed leading to rejection of the product or acceptance with or without lable restrictions as necessary. Because of its specific nature A. aleyrodis is not expected to give cause for danger to producer, grower or consumer. The risk of development of allergic

responses is reduced as the spores are not air-borne but produced in slimy masses.

The use of both natural enemies of greenhouse whitefly, E. formosa and A. aleyrodis, is feasible (Chapters 8 and 9). E. formosa is able to distinguish infected hosts from noninfected hosts when the haemolymph of the whitefly larvae is colonized by the fungus. In addition, E. formosa parasitizes fourth instar larvae, while these are less susceptible to infection with A. aleyrodis. In this way the effect of both natural enemies will be complementary. The parasitoid population survives a treatment with A. aleyrodis. Only whitefly hosts parasitized less than four days before inoculation with the spores may be infected by the fungus. Hosts parasitized four or more days before application will produce healthy parasitoids. Also, no detrimental effect has been observed on adult E. formosa females. Thus, A. aleyrodis can be considered as a selective additional control agent of greenhouse whitefly. I did not observe infection with A. aleyrodis of other insects or arachnids present in a glasshouse environment.

The application of A. aleyrodis has to be integrated into the management system of applying other control measures against pests and diseases. The use of fungicides especially may impair the effectivity of A. aleyrodis. Application of the detrimental fungicide mancozeb did not influence the infection of third instar larvae when applied three days after inoculation of the host with the spores (Chapter 2). Thus, after a certain safety period fungicides can be applied. Besides, there are fungicides available which are compatible with the use of A. aleyrodis (Ramakers, pers.comm.) or other entomopathogenic fungi (Hall, 1981) for application in glasshouses though they should not actually be mixed together. However, in the future the manipulation of A. aleyrodis may result in fungicide-resistant strains which may be applied in combination with the fungicide concerned.

10.2 HOST CHARACTERISTICS

Susceptibility of greenhouse whitefly

First, second and third instar larvae of greenhouse whitefly are highly susceptible to infection with A. aleyrodis (Chapter 3). Eggs are not infected and whitefly adults do not succumb to infection either. Sporadically, a whitefly adult was found showing sporulation in an environment of 100% RH. The fourth instar larvae become less susceptible over time when they develop into the so-called prepupae and pupae. This coincides with changes in the host cuticle, and increased resistance is probably due to the inability of the fungus to penetrate this cuticle. Investigation of this phenomenon in relation to the pathogenicity of the fungus may contribute to the knowledge of the mechanisms involved and may be helpful in the selection of more virulent strains of the pathogen.

Resistance in whitefly to infection with A. aleyrodis is less likely to develop than resistance to chemical insecticides. Pathogens are living evolving organisms that are also subject to selection and a coevolutionairy relationship exists between pathogen and host under field conditions (Briese, 1986). Although application as a microbial insecticide causes high rates of mortality within a short time period and, therefore, may result in a high selection pressure, resistance is unlikely to develop because A. aleyrodis is not an endemic pathogen constantly present in the whitefly population. Combined use of the parasitoid E. formosa and the pathogen reduces this probability even more.

Age of whitefly and spatial distribution

Adult greenhouse whiteflies aggregate on the underside of the young (upper) leaves of cucumber plants where they feed and oviposit. The combination of restricted oviposition sites and plant growth leads to a typical vertical distribution of the immature sessile whiteflies within the plant. Emergence of adults takes place on the lower leaves of the plants, and these migrate to the upper leaves (Noldus et al., 1987). Whitefly in different stages of development show differences in

susceptibility to infection with A. aleyrodis. This phenomenon can be related to the spatial distribution of the age groups of the whitefly population within the plant. In small glasshouse trials using full-grown cucumber plants heavily infested with greenhouse whitefly A. aleyrodis spores were applied in 1.3 ml (4×10^7 spores/ml + 0.1 vol. % Tween 80) per leaf. The RH varied from 60 to 95% and the temperature remained at 20°C with occasional peaks of 25°C on sunny days. Two applications within three days were carried out and after two weeks the infection rate indicated successful control of the susceptible whitefly larvae (Fig. 10.1) (Fransen, unpubl. results). The lower leaves bearing mainly pupae, prepupae and fourth instar larvae show variable infection rates. The younger instar larvae predominating on the higher leaves are successfully infected. The small top leaves bearing eggs at the time of treatment were sampled four weeks after application. Although spore numbers present per area

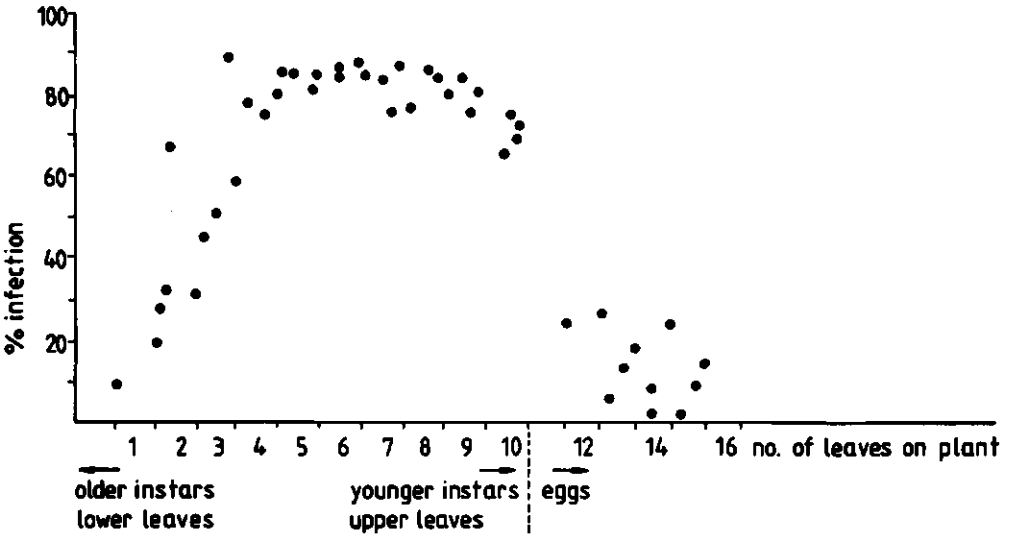


Fig. 10.1 Infection rates (%) of greenhouse whitefly on cucumber plants treated with Aschersonia aleyrodis spores in relation to the age of the larvae and their positions on the plant (on the abscissa, no. 1 represents the lowest and no. 16 the highest leaf on the plant; larvae on leaves 1-10 were examined 2 weeks after treatment and those on leaves 11-16, which were still in the egg stage at the time of treatment, were examined 4 weeks after treatment).

leaf surface decrease as the leaves increase in size, infection rates up to 30% of young whitefly larvae after eggs hatched were recorded. This was due to the residual activity of the spores on the leaves. Another spore application may be considered after a certain period of time aimed at the newly formed leaves.

The differential mortality of greenhouse whitefly at the various life stages makes several applications of the pathogen necessary to achieve good control of a whitefly population. This may be considered a disadvantage, but it may also be advantageous in that it opens possibilities for use of the parasitoid Encarsia formosa in combination with the pathogen, by optimal timing of the application of the pathogen and releases of the parasitoids.

10.3 INFLUENCE OF THE ENVIRONMENT

A. aleyrodis spores are sensitive to relative humidity for germination. They are able to germinate in absence of free water but a retardation of spore germination takes place at decreasing relative humidities (Chapter 7). On the plant surface the humidity may be higher than ambient humidity. This will depend on crop structure, leaf structure and size, hairiness of the leaves, wind speed and other factors (Burrage, 1971). In an environment of 50% RH and 20°C high infection rates of larvae on cucumber plants were established (Chapter 7). The final infection rates were not different from those on plants exposed to 50% RH after a period of 24 hr at 100% RH, but it took longer before the signs of infection became apparent. Periods of 6 or 12 hr at 100% RH resulted in even higher LT50's. Thus, there is no linear relationship between period of high humidity and LT50. It is assumed that the germinating spores are more vulnerable to a sudden decrease of RH after 6 and 12 hr periods of high humidity (Diem, 1971). Practical application of the spores at the middle of the day at 30-35°C and 50% RH resulted in high infection rates and it was concluded from other experiments that spores can be applied at any period of the day (Fransen, unpubl. results). In a glasshouse, the RH fluctuates from 50% during the middle of the day to 95% during some hours at night. In this situation the infection develops faster than when only a single short period of high humidity is given (Fransen,

unpubl. results).

A. aleyrodis is able to infect greenhouse whitefly at constant temperatures of 20, 25 and 30°C (Chapter 7). At 15°C the infection process takes longer but the whitefly larvae do not escape from infection at this low temperature, because the developmental period of whitefly also increases substantially (Lenteren & Hulspas-Jordaan, 1983). The temperature regime in a cucumber crop ranges from 18° to 20°C during the night to a minimum temperature of 20° to 22°C during the day. Periods of bright sunshine may cause the temperature to increase occasionally to 30-35°C for some hours during the middle of the day. It can be concluded that the temperature and relative humidity regime in a cucumber crop does not impair the infection of greenhouse whitefly by A. aleyrodis. It is intended to use A. aleyrodis as a microbial insecticide and for this application only the phases of germination and penetration depend on the external conditions. It seems that special measures are not necessary to create optimal conditions in a cucumber crop to establish infection of whitefly after application of the fungal spores. Continuation of research studying the interaction of host plant, microclimate and infection with A. aleyrodis is worthwhile to define the conditions necessary to successfully control whiteflies in other glasshouse crops, as well as in field crops. Manipulation of the environment may be possible in some situations, provided that stimulation of plant pathogens is avoided.

10.4 FINAL CONSIDERATIONS

Another fungus, Verticillium lecanii, is also pathogenic on greenhouse whitefly and a special strain was produced commercially from 1982 until recently as Mycotal ^{R} for control of whitefly. Successful applications have been reported under certain environmental conditions (Hall, 1982). Temperature should not be too low or too high (Kanagaratnam et al., 1982) and prolonged periods of high humidity (10 hr 85%) should prevail during the night for more than a week (Quinlan, pers.comm.). These conditions are necessary to obtain infection of the hosts and sporulation. Additionally, the fungus may sporulate on the leaf surface by means of the nutrient substrate added

to the formulation. Thus, the overall inoculum level is increased and spores may contaminate newly emerged whitefly adults. Infected sporulating adults may act as new sources of inoculum for young instar larvae on unsprayed foliage (Hall, 1982). Because of the expected spread of the fungus only one or two applications should be sufficient for control of whitefly in a cucumber crop. Nevertheless a considerable lag phase may be present before the effect can be noticed according to Hall (1985) who advices to reduce a high whitefly population with a chemical insecticide prior to introduction of V. lecanii. Results in commercial glasshouses have been inconsistent and Mycotal ^{R} has not been registered in the Netherlands (Koppert, pers.comm.). The results of application of Vertalec ^{R}, the aphid strain of V. lecanii, have been more positive. Control of Myzus persicae has been achieved in chrysanthemum crops where overhead irrigation is present and blackouts are used. These conditions enhance spread of the fungus and besides mature and immature aphids are mobile whereas whitefly larvae are sessile. Control of another aphid Macrosiphoniella sanborni was variable and this is thought to be due to a combination of feeding-site preference on the exposed parts of the plants, where humidity is low, and the relative immobility of this aphid (Hall & Burges, 1979). V. lecanii has also been tested for control of whitefly on tomato crops. When tomatoes are grown on nutrient-film, rock-wool or grow-bags, conditions tend to be too dry for use of V. lecanii (Hall, 1985). When night-time temperatures are reduced to 12°C it is not likely that V. lecanii will establish either.

Aschersonia aleyrodis shows better perspectives for control of whitefly compared with V. lecanii. The fungus is specific on whitefly and appears to be more virulent on first to fourth instar whitefly larvae than V. lecanii (Fransen, unpubl. results). Survival of unformulated A. aleyrodis spores was high; up to 96,5% of the larvae were infected by spores present on cucumber leaves for 22 days at 20°C. Only 50% of V. lecanii spores survived for four days on chrysanthemum foliage (Gardner et al., 1984). Successful infection of whitefly with A. aleyrodis was established at low temperature and low humidity regimes. In Table 10.1 an overview is given of the different aspects which have been investigated in relation to the development of

Table 10.1 Results of study of Aschersonia aleyrodis as a microbial agent against greenhouse whitefly.

ASPECTS FOR EVALUATION	RESULTS	SUGGESTIONS FOR FURTHER RESEARCH
FUNGAL CHARACTERISTICS		
1. specificity	specific to whitefly	infection of other Aleyrodids
2. virulence	highly virulent	influence of host passage
3. sporulation	abundant at high RH	epizootic effect in field situation
4. spread	little in glass-houses	epizootic effect in field situation
5. persistence	spores stay viable and infective	influence of host plant
6. possibilities for mass production	feasible on (semi) artificial media	optimization and up-scaling
7. suitability for storage and formulation	not tested	influence of additive on viability and virulence
8. toxicological aspects	not considered	tests needed for registration
9. effects on natural enemies	no effects, complementory to other natural enemy E. formosa	-
10. compatibility with insecticides and fungicides	safety period sometimes needed	fungicide resistant strains of the entomo-pathogen
HOST CHARACTERISTICS		
1. susceptibility	L1,L2,L3 very susceptible, L4 prepupa, pupa less, eggs and adults not	-
2. economic injury level	not considered	timing of application
3. age distribution, density and spatial distribution	infection correlated with age and spatial distribution	application strategy
INFLUENCE OF THE ENVIRONMENT		
1. abiotic factors	infection at 50% RH; at 15,20,25 30°C	relation of microclimate and host plant structure
2. biotic factors	not considered	-

A. aleyrodis as a microbial control agent of greenhouse whitefly. Finally, it can be concluded that A. aleyrodis shows perspective to be commercially developed as a microbial control agent. Trials in commercial tomato and cucumber crops have resulted in successful infection of greenhouse whitefly (Koppert, pers.comm.). Future research should be directed at mass production and formulation. Optimal timing and frequency of application should also be assessed. The use of both E. formosa and A. aleyrodis is feasible for control of greenhouse whitefly in cucumber crops, and may also be considered for other crops. It may well be possible that A. aleyrodis and other Aschersonia species show potential for application against other economically important whitefly species infesting open field crops, such as Bemisia tabaci on cotton.

Until recently, industry was not interested in the development of microorganisms for pest control, but the advances made in genetic engineering extend the potential for use of these organisms. Safety considerations, the induction of host resistance by chemicals and the increasing costs of development of chemical pesticides makes other ways of pest control more attractive even from a commercial point of view. The potential market for fungi in plant protection is not restricted to insects, but they may also be developed as antagonists of plant pathogens, as herbicides and as pathogens of plant parasitic nematodes.

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SUMMARY

Various aspects of the development of the entomopathogenic fungus Aschersonia aleyrodis as a control agent of greenhouse whitefly, Trialeurodes vaporariorum, were investigated. For control of greenhouse whitefly in tomato crops the parasitoid Encarsia formosa has been successful, but in cucumber crops a successful suppression of the whitefly population is often not achieved. Therefore, an additional selective control method is needed. The attention was focused on the fungal pathogen Aschersonia aleyrodis (Chapter 1).

The spores of A. aleyrodis germinated on the integument of whitefly larvae. Penetration of the cuticle took place after formation of an appressorium. The haemolymph and insect tissues were colonized by the fungus and the insect changed in colour from transparent yellow green to clear or opaque orange. Under favourable conditions the mycelium protruded from the insect and orange-coloured spore masses were produced in a mucilaginous layer (Chapter 2).

Information on the susceptibility of greenhouse whitefly at the various life stages is of importance for application of A. aleyrodis. Eggs of the host did not become infected. First instar, second instar and third instar larvae were highly susceptible to infection. Fourth instar larvae were susceptible to a lesser extent. When these larvae developed into the so-called prepupal and pupal stage the cuticle changed and the whitefly became more resistant. Generally, adults did not become infected (Chapter 3).

Dose-mortality responses were determined for the first, second, third and fourth instar larvae. Several experiments over time were carried out which gave consistent results. The dosage of spores on leaves needed to obtain 50% mortality (LC50) of first, second and third instar larvae was 19.53 spores/mm^2, 21.03 spores/mm^2 and 33.81 spores/mm^2, respectively. This represents a dose of about 0.77 spores per first instar larva, 1.44 per second instar larva and 4.39 spores per third instar larvae. The LC95 values, expressed as number of spores per amount sprayed, were 1.98×10^7 spores/2ml for first instar larvae, 2.34×10^7 spores/2ml for second instar larvae and 3.27×10^7 spores/2ml for third instar larvae. The LC95 for fourth

instar larvae was outside the dosage range tested. The LC50 varied with the age of the fourth instar larvae, from 6.0 x 10^6 spores/2ml to 2.6 x 10^8 spores/2ml in two different bioassays. The period before 50% of the larvae showed signs of infection (orange colouration) (LT50) at 20°C, was 11.8 days for first instar larvae, 9.5 days for second instar larvae and 7.0 days for third instar larvae, after application of 5.0 x 10^7 spores in 2 ml. The LT25 for fourth instar larvae was 5.6 days (Chapter 4).

Another bioassay method was tested using cucumber leaf discs (6.5 cm diameter). The presence of free water on the leaf surface enhanced the infection to such a degree that 77 to 90% of the larvae became infected after application of 1.0 x 10^6 spores in 2 ml per leaf disc. No LC50 values could be derived. After exposure of larvae to dosages of 5.0 x 10^7 and 1.0 x 10^8 spores in 2 ml a delay in the development of infection was noticed. After treatment of fourth instar larvae the final percentage infection was lower at the higher dosages than at the lower dosages. This was different from the linear relationship found in previous bioassays on plants. On water agar the spores of A. aleyrodis showed reduced germination at high densities (3100 spores/mm2). This density-dependent effect of the spores on the germination was apparently also present when leaf discs were used under conditions of high humidity and free water on the leaf surface. This may indicate the presence of a self-inhibitor (Chapter 5).

Impressions of cucumber leaves treated with A. aleyrodis spore suspension on water agar showed that only a low percentage of the spores (4.5%) germinated on the leaf surface. However, the ungerminated spores remained viable and infective for a long time. Spores from leaves treated 43 days before showed 78% germination after incubation on water agar for 24 hr. Whitefly treated in the egg stage became infected when young larvae contacted spores on the leaf surface after hatching. Nearly all young larvae (96%) contacting spores present on the leaf surface for about 22 days became infected (Chapter 6).

When aiming to apply A. aleyrodis in a glasshouse environment knowledge on the influence of temperature and relative humidity (RH) is wanted. Over 90% of the A. aleyrodis spores germinated within 48 hr

on water agar in the temperature range of 15 to 28°C. Larvae were infected at 15, 20, 25 and 30°C, with the most rapid development at 30°C (LT50: 3.3 days), though the final mortality rates of whitefly at the different temperatures were the same. Spores on cellophane sheets were exposed to various relative humidities. Germination was fastest at 100% RH and 20°C (78% within 24 hr), but after 168 hr 88% of the spores germinated at 93.9% RH. In experiments using cucumber plants it was found that successful infection of the larvae occurred at a RH of 50% and 20°C. A period of 100% RH for 24 hr enhanced the development of infection (LT50: 7.1 days). After exposure of the plants bearing treated larvae to 0, 3, 6, 12 or 24 hr 100% RH a linear relationship between these periods of high humidity and the LT50 values was not observed. The LT50 value amounted to 8.9, 8.6, 10.4 and 10.1 days for periods of 0, 3, 6 and 12 hr high RH, respectively. It is suggested that germinating spores are in a vulnerable phase after the periods of 6 and 12 hr at 100% RH, and are then highly susceptible to the decrease in RH from 100% to 50% (Chapter 7).

Serial in vitro passages of the fungus influenced the rate in which signs of infection became apparent. One in vivo passage of A. aleyrodis on greenhouse whitefly did not influence the infection rate but this needs further investigation (Chapter 7).

The interaction between A. aleyrodis and the parasitoid Encarsia formosa was studied in relation to the introduction of both natural enemies for control of whitefly (Chapters 8 and 9). From behavioural observations it could be concluded that the parasitoid was able to distinguish infected hosts from noninfected hosts if the fungus is present in the haemolymph of the host. Infected larvae were rejected for oviposition after the ovipositor penetrated the host. From four days onwards after inoculation of the spores the parasitoid could detect the fungus in the host. By distinguishing between infected and noninfected hosts the parasitoid is able to complement the fungal pathogen. E. formosa was able to transmit A. aleyrodis from infected hosts to noninfected hosts by the contaminated ovipositor. The transmission of the fungus was restricted to one or two healthy hosts (Chapter 8).

Whitefly larvae parasitized by E. formosa more than three days before were not susceptible to infection by the fungus. Nonparasitized larvae, however, were still infected by the fungus. The regulation of this phenomenon of reduced susceptibility in parasitized larvae is yet unknown. It may be related to the hatching of the parasitoid larva from the egg in the host (Chapter 9).

From the presented results of the experiments it can be concluded that A. aleyrodis shows promise as a microbial control agent of greenhouse whitefly in glasshouses (Chapter 10). Further research should be concentrated on the development of mass production, formulation of a product and application strategies.

SAMENVATTING

Verscheidene aspecten die van belang zijn voor de ontwikkeling van de entomopathogene schimmel Aschersonia aleyrodis ter bestrijding van kas-wittevlieg, Trialeurodes vaporariorum, zijn onderzocht. Kas-wittevlieg kan effectief worden bestreden door de introductie van de parasitaire sluipwesp Encarsia formosa in de tomatenteelt. Echter, in de teelt van komkommers blijft een succesvolle onderdrukking van de witte-vliegpopulatie vaak uit. Een aanvullende selectieve bestrijdingsmethode is daarom gewenst. De aandacht werd gevestigd op de selectieve pathogene schimmel Aschersonia aleyrodis waarvan bekend is dat zij verschillende witte-vliegsoorten infecteert (Hoofdstuk 1).

A. aleyrodis infecteerde de larven van kas-wittevlieg. De sporen van de schimmel, de infectieve eenheden, kiemden op het insekt en het insekt werd na vorming van een appressorium door de schimmel gepenetreerd. Het inwendige van het insekt werd gekoloniseerd en veranderde van kleur van transparant groen-geel naar helder of mat oranje. Onder gunstige omstandigheden groeide de schimmel naar buiten en produceerde oranje kleurige massa's sporen in slijm (Hoofdstuk 2).

Bij toepassing van A. aleyrodis tegen kas-wittevlieg is het van belang te weten in welke ontwikkelingsfasen de gastheer vatbaar is voor infectie. Eieren van de gastheer werden niet geinfecteerd. De larven van het eerste, tweede en derde larvale stadium waren zeer vatbaar voor infectie. De larven van het vierde stadium, daarentegen, waren in mindere mate vatbaar. Naarmate de cuticula van structuur veranderde en het insekt zich ontwikkelde tot prepupa en pupa, nam de vatbaarheid verder af. Volwassen witte vliegen werden over het algemeen niet geinfecteerd (Hoofdstuk 3).

De dosis-mortaliteitsrelaties werden vastgesteld, waarbij witte vlieg in de verschillende larvale stadia werd blootgesteld aan suspensies met A. aleyrodis sporen. Verschillende proeven werden uitgevoerd die een consistent beeld gaven van de virulentie van A. aleyrodis. Er waren 19,53 sporen per mm^2 bladoppervlak nodig om 50% van de eerste stadium larven te infecteren, 21,03 sporen per mm^2 voor infectie van 50% van de tweede stadium larven en 33,81 sporen per mm^2 voor infectie van 50% van de derde stadium larven. Dit komt omgerekend

neer op een dosis van ongeveer 0,77 sporen per eerste stadium larve, 1,44 per tweede stadium larve en 4,39 sporen per derde stadium larve. Om 95% doding (LC95) van de eerste stadium larven te krijgen werden $1,98 \times 10^7$ sporen verspoten in 2 ml per blad. Voor tweede en derde stadium larven waren hiervoor respectievelijk $2,34 \times 10^7$ en $3,27 \times 10^7$ sporen per 2 ml nodig. De LC95 voor vierde stadium larven viel buiten de geteste concentratie reeks. De LC50 was sterk afhankelijk van de ouderdom van de vierde stadium larven en varieerde in twee biotoetsen van $6,0 \times 10^6$ sporen/2ml tot $2,6 \times 10^8$ sporen/2ml. De tijdsduur voordat oranje kleuring van 50% van de larven (LT50) zichtbaar werd bij 20°C, nam 11,8 dagen in beslag voor eerste stadium larven, 9,5 dagen voor tweede stadium larven en 7,0 dagen voor derde stadium larven bij toepassing van $5,0 \times 10^7$ sporen in 2 ml. De LT25 voor vierde stadium larven bedroeg 5,6 dagen (Hoofdstuk 4).

Een andere biotoets methode werd uitgetest, waarbij gebruik werd gemaakt van bladponsen. De aanwezigheid van vrij water bevorderde de infectie dermate, dat 77 tot 90% infectie optrad bij toediening van $1,0 \times 10^6$ sporen in 2 ml per bladpons. Uit de reeks van concentraties die getest worden, kon geen LC50 worden afgeleid. Bij toediening van de hoogste concentraties werd een vertraging in de ontwikkeling van de infectie waargenomen. Bij behandeling van vierde stadium larven was het uiteindelijke mortaliteitspercentage na toepassing van hogere concentraties zelfs lager dan bij lagere concentraties. Dit was afwijkend van het rechtlijnig verband wat bij de voorgaande biotoetsen op intacte planten werd gevonden. Echter, op water agar werd bij aanwezigheid van hoge dichtheden van A. aleyrodis sporen (3100 sporen/mm^2) een reductie in het kiemingspercentage waargenomen. Dit dichtheidsafhankelijke effect van de sporen op de kieming was blijkbaar ook aanwezig bij gebruik van de bladponsen in aanwezigheid van vrij water. Het verschijnsel duidt op de aanwezigheid van een remstof (Hoofdstuk 5).

Afdrukken van met A. aleyrodis bespoten bladeren op wateragar gaven een beeld van het aantal sporen dat op het bladoppervlak kiemde. De sporen kiemden maar voor ongeveer 4,5% op het bladoppervlak. De ongekiemde sporen bleven echter voor lange tijd kiemkrachtig en infectieus. Na incubatie op wateragar vertoonden sporen afkomstig van

bladeren die 43 dagen tevoren bespoten waren, nog voor 78% kieming. Behandeling van bladeren 22 dagen voordat de sporen in aanraking kwamen met jonge larven resulteerde in 96% infectie van de witte-vlieglarven (Hoofdstuk 6).

Voor toepassing van A. aleyrodis ter bestrijding van witte vlieg in kassen is informatie over de invloed van temperatuur en relatieve luchtvochtigheid van belang. Minstens 90% van de A. aleyrodis sporen kiemde binnen 48 uur op wateragar in het temperatuur traject van 15 tot 28°C. Infectie vond plaats bij 15, 20, 25 en 30°C. Hoewel de infectie zich het snelst ontwikkelde bij 30°C (LT50: 3,3 dagen), was het uiteindelijke mortaliteitspercentage van witte vlieg bij 15°C niet verschillend van de percentages bij de andere temperaturen. Sporen aangebracht op cellofaan kiemden bij verschillende relatieve luchtvochtigheden (rlv). Het snelst kiemden sporen bij 100% rlv (78% na 24 uur bij 20°C). Desalniettemin was na 168 uur 88% van de sporen gekiemd bij 93,9% rlv. Er werden tevens proeven met planten uitgevoerd. Infectie van larven vond plaats bij een constante rlv van 50% en 20°C. De infectie verliep het snelst bij blootstelling aan een periode van 24 uur 100% rlv (LT50: 7,1 dagen). Toch bestond er geen lineair verband tussen de periode van hoge luchtvochtigheid en de LT50 waarden. De LT50 waarden bedroegen bij perioden van 0, 3, 6 en 12 uur 100% rlv 8,9 , 8,6 , 10,4 en 10,1 dagen, respectievelijk. Het wordt aangenomen dat de kiemende sporen in een kwetsbare fase zijn na 6 en 12 uur 100% rlv, waardoor zij sterk beinvloed worden door de overgang van 100 naar 50% rlv (Hoofdstuk 7).

Het overenten van de schimmel beinvloedde de snelheid waarmee de infectie zichtbaar werd in de temperatuur- en rlv experimenten. Het herisoleren van de schimmel van geinfecteerde witte-vlieglarven had geen invloed op de ontwikkeling van infectie, maar verder onderzoek hiernaar is gewenst.

De interactie tussen de schimmel A. aleyrodis en de parasitaire sluipwesp Encarsia formosa werd bestudeerd in verband met de integratie van beide natuurlijke vijanden ter bestrijding van witte vlieg (Hoofdstuk 8 en 9). Uit gedragsstudies van de sluipwesp kwam naar voren dat de parasiet geinfecteerde larven kon onderscheiden van ongeinfecteerde indien de schimmel aanwezig was in de hemolymf van de

gastheer. De aanwezigheid van de schimmel werd opgemerkt nadat de parasiet haar legboor in de gastheer had gebracht. De legboor werd weer teruggetrokken zonder dat een ei in de gastheer was afgezet. Vanaf vier dagen na inoculatie van de gastheren met A. aleyrodis sporen kon de parasiet het onderscheid tussen geinfecteerde en ongeinfecteerde larven maken. Door dit onderscheidingsgedrag kan de parasiet complementair werken na toepassing van de entomopathogene schimmel. E. formosa kon A. aleyrodis overbrengen van een geinfecteerde gastheer naar een gezonde door contaminatie van de legboor. De transmissie van de schimmel gebeurde op beperkte schaal aangezien na contact met een door de schimmel gekoloniseerde larve hooguit twee gezonde larven geinfecteerd raakten na ovipositie gedrag van de parasiet (Hoofdstuk 8).

Bij toepassing van de schimmelsporen op reeds geparasiteerde witte-vlieglarven bleek dat gastheren die meer dan drie dagen eerder geparasiteerd waren, niet meer vatbaar waren voor infectie met A. aleyrodis. Ongeparasiteerde larven gingen daarentegen nog wel aan de schimmel te gronde. Het mechanisme van verminderde vatbaarheid van de geparasiteerde larven is nog onbekend. Het uitkomen van de E. formosa larve in de gastheer heeft hier vermoedelijk iets mee te maken (Hoofdstuk 9).

Uit het onderzoek kan geconcludeerd worden dat A. aleyrodis goede vooruitzichten biedt voor toepassing ter bestrijding van witte vlieg onder kasomstandigheden (Hoofdstuk 10). Het duidt er op dat een integratie van sluipwesp en parasiet mogelijkheden biedt. Verder onderzoek naar de massaproductie, formulering van een houdbaar product en applicatie strategieën is gewenst voor ontwikkeling van A. aleyrodis als microbieel bestrijdingsmiddel.

CURRICULUM VITAE

Johanna Jacoba Fransen werd op 18 juli 1956 te Geldrop geboren. In 1974 werd het eindexamen Gymnasium B aan het Lorentz Lyceum te Eindhoven behaald. Datzelfde jaar werd begonnen met de studie in de richting Planteziektenkunde aan de Landbouwuniversiteit te Wageningen. Het kandidaatsexamen werd afgelegd in 1978 en begin 1982 werd de ingenieursstudie afgerond. Het verzwaarde doctoraal examen omvatte de drie hoofdvakken entomologie, fytopathologie en toxicologie. In het kader van de stageperiode werd entomologisch onderzoek uitgevoerd op het 'Department of Applied Biology' te Cambridge in Engeland.
In 1982 was zij werkzaam als practicum assistent op de vakgroep Toxicologie en in 1983 werd in tijdelijk verband onderzoek naar de bestrijding van aardrupsen met entomofage nematoden uitgevoerd op het Instituut voor Planteziektenkundig Onderzoek te Wageningen. Vanaf september 1983 tot september 1987 werd onderzoek verricht op de vakgroep Entomologie in de functie van wetenschappelijk assistent in tijdelijke dienst van de Stichting voor Technische Wetenschappen (STW) en de Stichting voor Biologisch Onderzoek (BION). De resultaten van dit onderzoek zijn verwerkt in deze dissertatie.
Vanaf september 1987 is de promovenda aangesteld als 'senior onderzoeker gewasbescherming' in dienst van het Proefstation voor de Bloemisterij in Nederland, te Aalsmeer.