UNIVERSITY OF SOUTHAMPTON

THE POTENTIAL OF THE FUNGUS, *VERTICILLIUM LECANII*, AS A MICROBIAL CONTROL AGENT OF GLASSHOUSE APHID PESTS

by

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ABSTRACT

FACULTY OF SCIENCE

BIOLOGY

Doctor of Philosophy

THE POTENTIAL OF THE FUNGUS, VERTICILLIUM LECANII, AS A MICROBIAL CONTROL AGENT OF GLASSHOUSE APHID PESTS

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The ecology of the fungus, Verticillium lecanii, and its potential as a microbiological insecticide against the aphids, Myzus persicae, Macrosiphoniella sanborni and Brachycaudus helichrysi, on glasshouse chrysanthemums have been examined.

To achieve reproducibility, a single-spore isolate of V. lecanii, C-3, was used and designated as standard. It produced conidiospores on solid media and, more abundantly and readily, blastospores in liquid media. In nutritional studies, submerged production of conidiospores was not achieved. In aqueous suspension, conidiospores survived longer than blastospores. Conidiospores exposed below 80% relative humidity soon died, the speed of death depending on the spore microenvironment.

A bioassay technique was developed and is believed to be the first statistically reliable method of quantifying fungal spore pathogenicity towards insects. With M. sanborni, the semi-weighted mean lethal concentration 50% of 28 assays was 1.83×10^5 spores/ml suspension (fiducial limits, 1.45 and 2.31). All but one of 15 V. lecanii strains, some pathogens of other insects and some probable hyperparasites of rust fungi, were similar in pathogenicity to C-3. Five other strains, all from rust or mildew fungi, were apathogenic. Blastospores of C-3 were approximately twice as pathogenic as conidiospores. With single- and multi-spore isolates, repeated passage through aphids or subculture on agars scarcely altered pathogenicity, but subculture greatly changed colonial morphology.

In glasshouses, blastospores and conidiospores produced similar levels of aphid control. Spore sprays established excellent control of My. persicae within 2-3 weeks, maintaining control for up to 8 weeks. Also, B. helichrysi was controlled in vegetative plant tips but not on tightly closed flower buds. Control of M. sanborni was usually unsatisfactory. In laboratory bioassays, all 3 aphid species were equally susceptible to V. lecanii suggesting that behavioural and ecological factors explain differences in glasshouses. V. lecanii could be used advantageously in integrated control systems on chrysanthemums.

INTRODUCTION

In recent years, the intensive use of chemical insecticides has resulted in phytotoxicity and the selection of many insecticide-resistant strains of insect pests. This, together with the growing trend of avoiding the use of insecticides as a result of mounting concern about environmental pollution, has stimulated the investigation of alternative means of control. Predators, parasites and diseases of glasshouse insect pests have been considered for inclusion in integrated control programmes. Among insect diseases, entomogenous fungi have often been tested for insect control outside the glasshouse environment. Many such attempts failed probably because fungal epizootics in insect populations rely heavily on favourable weather conditions. However, the glasshouse offers a unique opportunity to manipulate critical physical parameters, particularly humidity and temperature. Natural infections by the hyphomycetous fungus, Verticillium lecanii occurred in dense populations of various pests on several glasshouse crops. These included the chrysanthemum aphid pests, Myzus persicae, Macrosiphoniella sanborni, and Brachycaudus helichrysi. The effectiveness of control produced by these natural infestations that had increased far beyond the level of commercial importance, prompted a study of the potential of V.lecanii in controlling aphid infestations that were still sparse and at a stage where commercial control would be applied. Chrysanthemums were chosen since it was possible to elevate night-time humidity under the black polythene sheets used to alter the daylength for the regulation of flowering dates.

CHAPTER I

MICROBIAL CONTROL OF INSECTS BY FUNGAL PATHOGENS - LITERATURE REVIEW

1. Historical Perspectives

The history and development of insect pathology and biological control has been the subject of excellent and detailed works by Steinhaus (1956, 1976). Fungus disease in insects has been recognised since pre-Christian times but its cause and nature were unknown until in 1836, Agosto di Bassi discovered that the white muscardine disease of silkworms was caused by a fungus (subsequently named Beauveria bassiana). With this discovery, Bassi became the first person to elucidate the aetiology of a contagious microbial disease and to effect the experimental infection of one living organism by another. He went so far as to suggest that micro-organisms could be artificially distributed to control insect pest populations. Some 30 years later, this idea was echoed by others, e.g. Bail, Pasteur and Thaxter, but Metschnikoff in 1879 was probably the first person to attempt insect control by artificial distribution of a fungus pathogen (Metarrhizium anisopliae) and certainly the first to propagate a fungus on artificial media on a large scale. Since then there have been numerous attempts outdoors to exploit entomogenous fungi for insect control. These attempts have been extensively reviewed by Steinhaus (1949, 1956), Baird (1958), Müller-Kögler (1965) , Ferron (1970), Roberts et al. (1971), and McCoy (1974). South was probably the first person to use Verticillium lecanii (cited as Cephalosporium lecanii) in 1910 (Baird, 1958). Despite many encouraging results with entomogenous fungi - Baird (1958) lists 41 successful attempts to control 28 species or groups of insects with fungi

- nobody, to the present day, has developed a fungal pathogen to a stage where it can be used for commercial crop protection. This is probably because concomitant failures and inconsistent field results have overshadowed the successes and cast doubts upon the reliability of fungal preparations. However, many of the causes of these failures can be attributed to a lack of understanding or consideration of important parameters such as temperature, humidity and viability of fungal inoculum. Only by appreciating the effects of these parameters can the potential of fungal pathogens be truly assessed.

2. Epizootiology of Fungal Disease in Insects

Insect-pathogenic fungi differ from all other groups of entomogenous micro-organisms in that their mode of invasion is usually through the cuticle (Lefebvre, 1934; Hurpin et al., 1958; Schaerffenberg, 1959; McCauley et al., 1968; Latgé et al., 1974; Zacharuk, 1970, 1973; Berisford et al., 1975). More rarely, invasion occurs through the spiracles or digestive tract (Sussmann, 1952; Gabriel, 1959; Veen, 1966; McCauley et al., 1968; Yendol et al., 1965; Broome et al., 1976). Other pathogens - bacteria, viruses, protozoa usually invade *via* the gut and must therefore first be ingested by the host. The predominantly external mode of infection by fungi explains why they comprise virtually all the known pathogens of aphid and scale insects, which imbibe plant sap, a nutrient source unlikely to contain any insect pathogens.

The insect cuticle is composed of several layers containing chitin, protein and lipids (Koidsumi et al., 1955; Rudall, 1963; Samšiňáková et al., 1971). Fungi probably penetrate this solid barrier by a combination of physical force and externally acting enzymes (Notini et

al., 1944; McCauley et al., 1968; Zacharuk, 1970; Berisford et al., 1975). Many workers, e.g. Notini et al. (1944), have attributed the biochemical activity in cuticle penetration to chitinase, although this has not yet been proved. Doubtlessly, other enzymes - proteases, lipases - are involved also (Leopold et al., 1970; Samšiňáková et al., 1971).

On penetrating the cuticle, fungal hyphae reach the hypodermis. Here they proliferate and invade the haemolymph, where most fungi form free hyphal bodies (Madelin, 1963). Often, hyphal bodies are restricted to the haemolymph until very shortly before the insect dies (Madelin, 1963); death may result from the selective action of toxins (Roberts et al., 1971; Lysenko et al., 1971) or perhaps from a combination of anoxia, nutritional disturbances and accumulation of toxic metabolites. In other instances, fungi invade subepidermal tissues and organs while the insect still lives (Madelin, 1963). After death, all tissues and organs are attacked and, given favourable external conditions of temperature and high humidity, the fungus emerges from the insect body and sporulates on its surface. With very few exceptions, sporulation of fungi on insects is a post-mortem event (Madelin, 1963; 1968). The Entomophthorales, probably in response to environmental changes (Wilding, 1972a), and one hyphomycetous fungus, Sorosporella uvella (Speare, 1920), also produce resting spores within the body cavity.

Diseases appearing in insect populations can be either epizootic or enzootic. An epizootic is characterised by very high mortalities in an insect population. Eventually, the epizootic dies out and gradually the population recovers, at which stage it might be difficult to detect the disease

in the population. In contrast, an enzootic disease is one that is always present in an insect population at a more or less constant level. How do physical and biotic factors affect the occurrence and development of epizootics?.

Entomogenous fungi, in common with fungi in general (Hawker, 1950; Cochrane, 1958), require humid or moist conditions for germination, growth and sporulation. Thus, it is not surprising that many natural fungal epizootics are preceded by damp or wet weather(Ullyett et al., 1940; Steinhaus, 1954; Baird, 1958; Müller-Kögler, 1965; Macleod et al., 1966;Missonier et al., 1970; Shands et al., 1972; Robert et al., 1973; Wilding, 1975). There are several reports of insects becoming infected at low ambient humidity (Macleod et al., 1966; Madelin, 1963; Müller - Kögler, (1965); Ferron personal communication).It is possible that, in these cases, the relative humidity (RH) was high in the microclimate around the host. The importance of microclimates must be emphasized. Localised humidity may encourage fungal epizootics even when dry weather predominates (Hall et al., 1957; York, 1958; Tanada, 1963; Shands et al., 1963) Irrigation programmes may encourage already enzootic diseases to become epizootic.

There are comparatively few field data on the influence of temperature on the development of epizootics. The field work of Missonier et al. (1970), and Robert et al. (1973), suggested that a mean maximum temperature of 20°C induced Entomophthora epizootics amongst aphids, whereas lower maximum temperatures did not (in spite of concomitant high humidity). In contrast, Wilding, (1975) found that epizootics of a different species, E.thaxteriana occured when the mean maximum temperature was only 12°C. Other authors (reviewed by MacLeod et al., 1966) reported that

Entomophthora epizootics almost eliminated insect populations during the cooler seasons of the year. Seasonal temperature variations may be responsible for the replacement of Beauveria bassiana epizootics in winter by Metarrhizium anisopliae epizootics in summer in the beetle, Rhopaea verreauxi (Milner, personal communication). Laboratory investigations on fungi and their hosts in carefully controlled conditions of temperature and humidity would greatly facilitate the understanding of the roles these physical parameters play in the field.

Given suitable weather conditions, epizootics generally occur in dense, large insect populations and an overwhelming body of observations exists to support this assertion (Steinhaus, 1954; Tanada, 1963; Müller-Kögler, 1965; Macleod et al., 1966). This generality has led many authors to refer to disease as being density-dependent. However, Steinhaus (1954), quoting from others, defined density-dependent mortality factors as those which "kill a greater percentage of insects" - presumably in a given time - "the greater the population density". Clearly this definition would allow diseases infecting a large proportion of insects in small, sparse populations also to be density-dependent. To observe the phenomenon of density-dependence in the field, disease must presumably be present in insect populations before they have exceeded the threshold at which disease will assume epizootic proportions. Recently, several workers have attempted to estimate the importance of aphid population density in relation to the spread of Entomophthora disease in the field prior to epizoosis. Wilding (1975) showed only a slight quantitative correlation between the percentage of aphids infected and the corresponding host density and Robert et al. (1973), observed that the percentage of diseased aphids increased

little prior to the outbreak of epizootics, although the size of insect populations increased. Probably in controlled conditions in the laboratory, disease may spread in a measurably density-dependent manner. Also, Robert et al., (1973) concluded that epizootics did not occur unless there was a threshold level of conidiospore inoculum present on diseased aphids and suggested that this was more important than the density of the aphid population in inducing epizootics. Similarly, Missonier et al. (1970) considered that epizootics depended upon threshold inoculum density rather than host population density. However, I consider that, in controlled laboratory conditions there would probably be a simple and exactly maintained relationship between threshold inoculum and host density. Natural threshold inoculum densities may often occur when the population density has exceeded the economic threshold of damage. Thus, in many habitats and situations, it may be necessary to artificially introduce, e.g. by spraying spores, a fungal inoculum to create an epizootic at low insect density.

3. Microbial Control by V.lecanii

Outdoors, in almost entirely tropical and subtropical regions, some degree of success was reported in each of 14 attempts (where results are given) to control scales and aphids with V.lecanii, listed under various synonyms (listed in Baird, 1958; Rojter et al., 1966). In glasshouses, 100% mortality of several target host species was reported in limited trials with V.lecanii (Neužilová, 1957; Rojter et al., 1966; Samšiňáková et al.,1976).In view of this potential of V.lecanii, it is surprising that this fungus has attracted few and limited studies compared to those of other entomogenous fungi e.g. Beauveria spp. and Metarrhizium anisopliae. This is perhaps because V.lecanii, although widespread (Petch, 1948; Leatherdale,

1970), has rarely caused epizootics outdoors except in the tropics (Baird, 1958), where probably resources and facilities for study in depth have been limited. However, V. lecanii epizootics amongst aphids and scales in temperate zones do occur in glasshouses where conditions approach those found in the tropics. Since, under glass, man can largely regulate otherwise variable physical parameters such as temperature and humidity, the glasshouse seems an ideal environment in which to develop the use of pathogens for reliable, consistent control of insects.

CHAPTER II

THE FUNGUS, *VERTICILLIUM LECANII* (ZIMM.) VIÉGAS

1. Taxonomy

Corda in 1839 (Gams, 1971) placed all hyphomycetes bearing heads of single-celled spores at the tips of simple undifferentiated conidiophores in the genus *Cephalosporium*. However, this broad categorisation resulted in great confusion in the interpretation of this genus by several authors (Gams, 1971). In a recent and detailed examination, Gams (1971) reclassified the *Cephalosporia* and transferred some to a new section of *Verticillium*, sect. *Prostrata* sect. nov.. The salient characteristics of this new section are the occasional appearance of mesotonous to acrotonous verticillate whorls of phialides and the velvety-cottony, white or yellowish mycelium. In it,Gams (1971) placed all insect-parasitic, *Cephalosporium*-like fungi. Within the new section, *Verticillium lecanii* (Zimm.) Viégas contains many fungi[1]

[1]Barson (1976a) reported that Gams (1971) synonymised *Paecilomyces eriophytis* (Massee) Leatherdale with *V.lecanii*, because their determining characteristics were insufficient to justify their separation as distinct species. This is not so. Leatherdale (1965) isolated a fungus very different from *V.lecanii*, which he identified as *P. eriophytis* (Massee) Leatherdale, thus making it an obligate synonym with *Botrytis eriophytis* Massee *in* Taylor (1909). The latter fungus was identified by Petch (1931) as a *Cephalosporium* and subsequently by Gams (1971) as *V.lecanii*. Gams (1971) however, did include *P.eriophytis* in his synonymy list compiled from the literature but not in his list from examined material.

hitherto described as separate species (many of them insect pathogens - Petch, 1925, 1931, 1948) such as Cephalosporium aphidicola (Petch), C.coccorum (Petch), V.hemileiae (Bour.), C.muscarium (Petch), the complete list being given in Gams (1971).

The classifications of Petch were based in part, on the density of verticillate whorls and on spore sizes. Gams (1971), after experience with the section Prostrata, concluded that Petch's taxonomic criteria did not justify separations into species. In contrast, Ba*l*azy (1973) believed that Gams' work contained too far-reaching generalisations, even regarding his inclusion of certain entomogenous Cephalosporia in Verticillium sect. Prostrata as not fully justified. The latter criticism is based upon the almost total absence of verticillate whorls in some strains of V.lecanii (Zimm.) Viégas. In addition, Ba*l*azy (1973) separated some species from Gams' V.lecanii complex, e.g. Cephalosporium yvone (Dop) comb. nov., C.diversiphialidum sp. nov., and C.eriophytis (Massee) Petch. However, Ba*l*azy's breakdown of the complex into species was based upon morphological characteristics, e.g. colonial appearance on agar or phialide length. Within Gams' (1971) V.lecanii complex, phialide length varies continously from short to long and, in the present study, colonial morphologies (even of cultures derived from a single-spore isolate) were extremely variable (Fig. 5.16a,b). In view of my uncertainty over the validity of Ba*l*azy's taxonomic criteria, Gams' (1971) somewhat broader concept of V.lecanii will be used in the present study.

Gams (1971) described V.lecanii (Zimm.) Viégas colony morphology on Oatmeal and Malt extract agar media as follows:- Colonies, after 10 days at 20°C, white or pale yellow, cottony-

velvety. Underside, colourless but on Malt extract agar light yellow to chrome yellow to ochre. Phialides, awl-shaped, very variable in size, single or in small groups of verticillate whorls on hyphae of the aerial mycelium, occasionally with repeatedly branching conidiophores which do not differ in structure from vegetative hyphae.

In the present study, the morphology of the V.lecanii standard (C-3) used for all experiments closely resembled Gams' description above (Figs. 2.01 - 2.04). In liquid media, V.lecanii (C-3) becomes semi-yeast-like (blastospores; Fig. 2.05). Taxonomists have ignored the morphology of V.lecanii in liquid culture. Since not all strains in the V.lecanii complex produce blastospores like C-3 (IV, 14, b), this character may be of some use in future systematic reviews of the V.lecanii complex.

2. Occurrence and Host Range of V.lecanii

V.lecanii (sens lat. - Gams, 1971) has a broad geographical range (Evlakhova, 1939; Nagaich, 1973; McClelland et al., 1929; Viégas, 1939; Petch, 1948). Homoptera are by far the most common hosts (Baird, 1958; Ganhão, 1956; Viégas, 1939; Nagaich, 1973). Much less frequently V.lecanii has been reported parasitizing other orders of insects and arachnids - Coleoptera (Leatherdale, 1970; Barson, 1976a; Lipa, 1975), Collembola, Hymenoptera (Ichneumonidae), Diptera, spiders (Leatherdale, 1970; Gams, 1971; Petch, 1948), and mites (Massee, in Taylor, 1909; Balazy, 1973). V.lecanii has also been reported hyperparasitizing several genera of rust fungi (Gams, 1971), and powdery mildews (Ebben et al., unpublished). Finally, V.lecanii has been isolated from various other sources including earth, floorboards, baker's leaven, turnip seed, sweetcorn, Tuber maculatum, and Helvella lacunosa (Gams, 1971), a chrysanthemum leaf (VII, 2) and

Fig. 2.01 Colony of *Verticillium lecanii* on Sabouraud dextrose agar (C-3, third subculture from original diseased aphid, *Macrosiphoniella sanborni*).

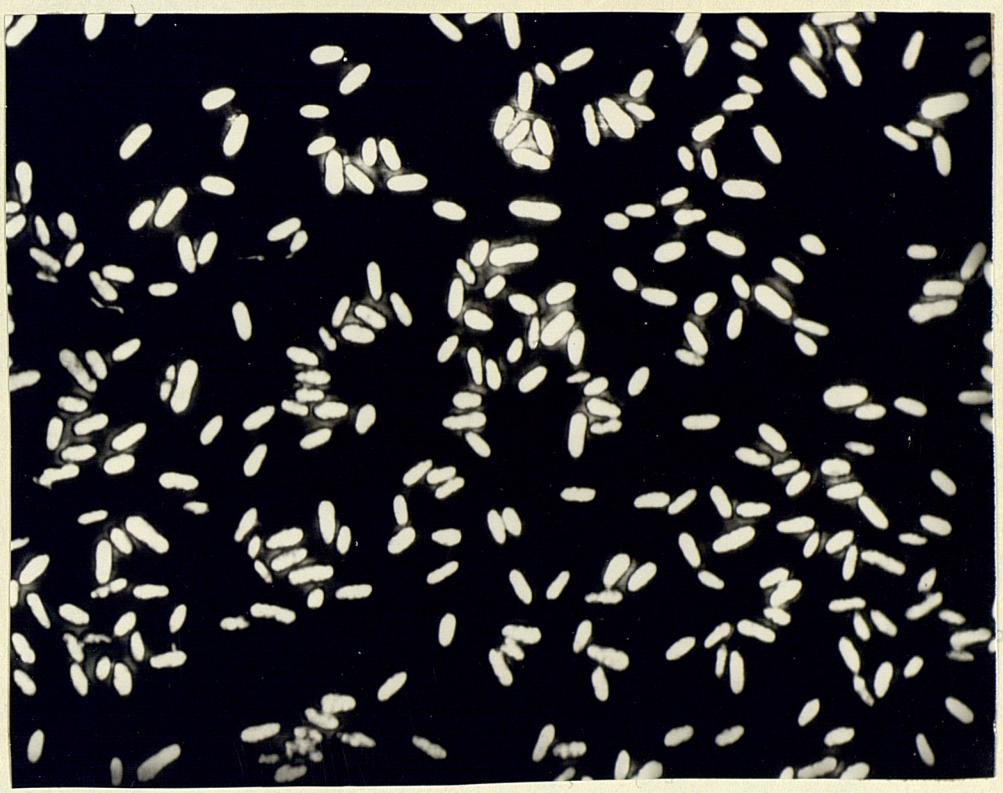


Fig. 2.02 Conidiospores of Verticillium lecanii
(X 500)

Fig. 2.03a Slime heads of Verticillium lecanii conidiospores (X200)

Fig. 2.03b Slime-heads of Verticillium lecanii conidiospores in a verticillate whorl (X1000).

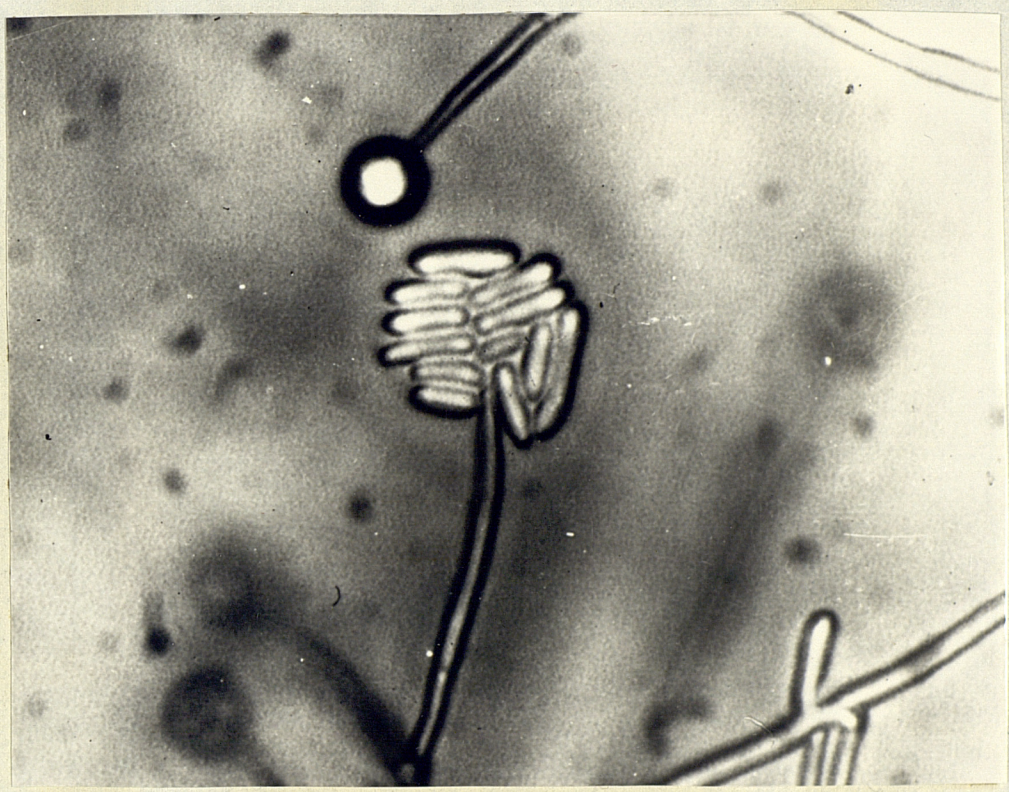


Fig. 2.04 Slime-heads of Verticillium lecanii conidiospores. One slime-head has become apposed to the cover-glass of the culture chamber showing conidiospores (X700)

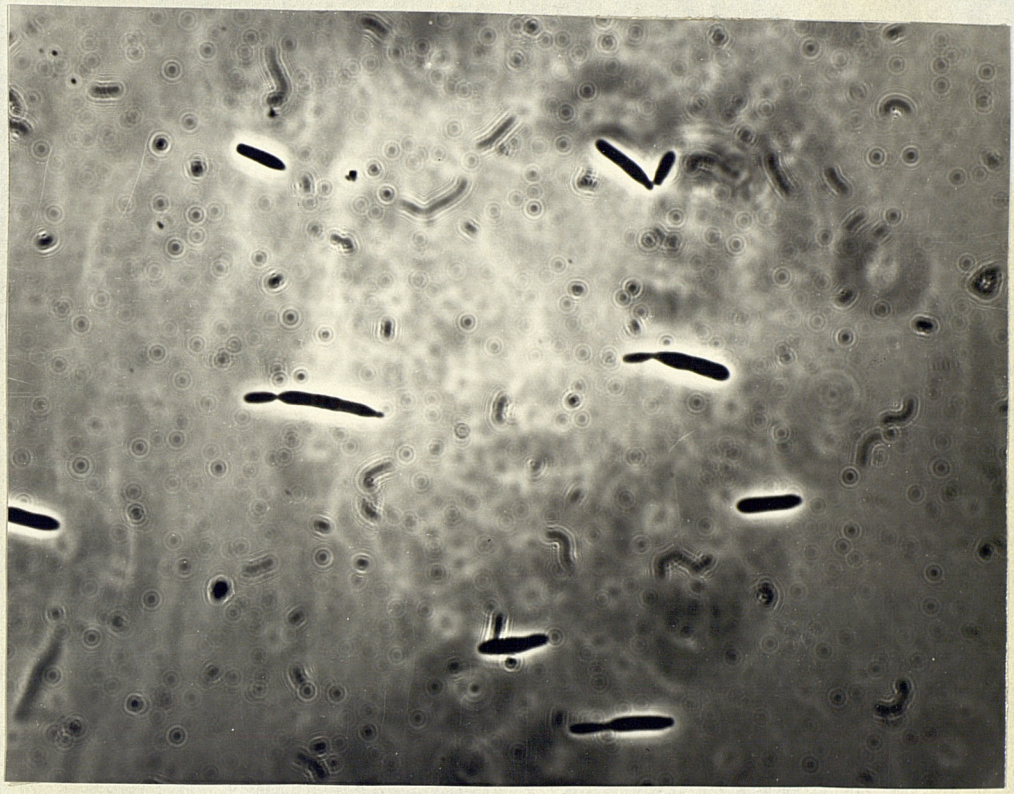


Fig. 2.05 Blastospores of *Verticillium lecanii* cultured in Sabouraud liquid medium (X500)

contact lenses (English, personal communication). There can be no doubt that the strains of the V.lecanii complex are extremely widespread in their occurence.

CHAPTER III

SALIENT FEATURES OF THE CHRYSANTHEMUM CROP AND ITS APHID PESTS

1. All-Year-Round Chrysanthemums

The chrysanthemum is primarily a short day plant and does not normally form its flower buds when the day-length exceeds $14\frac{1}{2}$h. In the British Isles, chrysanthemums will not therefore initiate their flower buds until mid-August and will not naturally commence to develop these buds until early September. However, in the glasshouse, plants are illuminated artificially or the night period is lengthened. This keeps plants vegetative on the one hand and initiates buds on the other. Chrysanthemum plants are given artificial short days by covering the beds with black polythene sheeting (Fig. 3.01) to extend the normal night period during March to September. Condensation water collects on the underside during the night and the RH is substantially raised. Since fungi in general require a high humidity for spore germination and vegetative growth (Chapter I), this overnight period of high humidity offers an opportunity to utilise entomogenous fungi against chrysanthemum insect pests. The principal pests of chrysanthemums are leaf miner (*Phytomyza syngenisiae*), red spider mite (*Tetranychus urticae*), thrips (*Thrips tabaci*), and aphids. The latter group, being the subject of the present study, will be described in some detail below.

2. Aphid Pests of Chrysanthemums

(a) Generalised Life Cycle and Biology of Aphids

The usual life cycle of aphids in temperate regions is as follows: winter is passed on a plant species known as a primary host. The eggs hatch in spring to produce

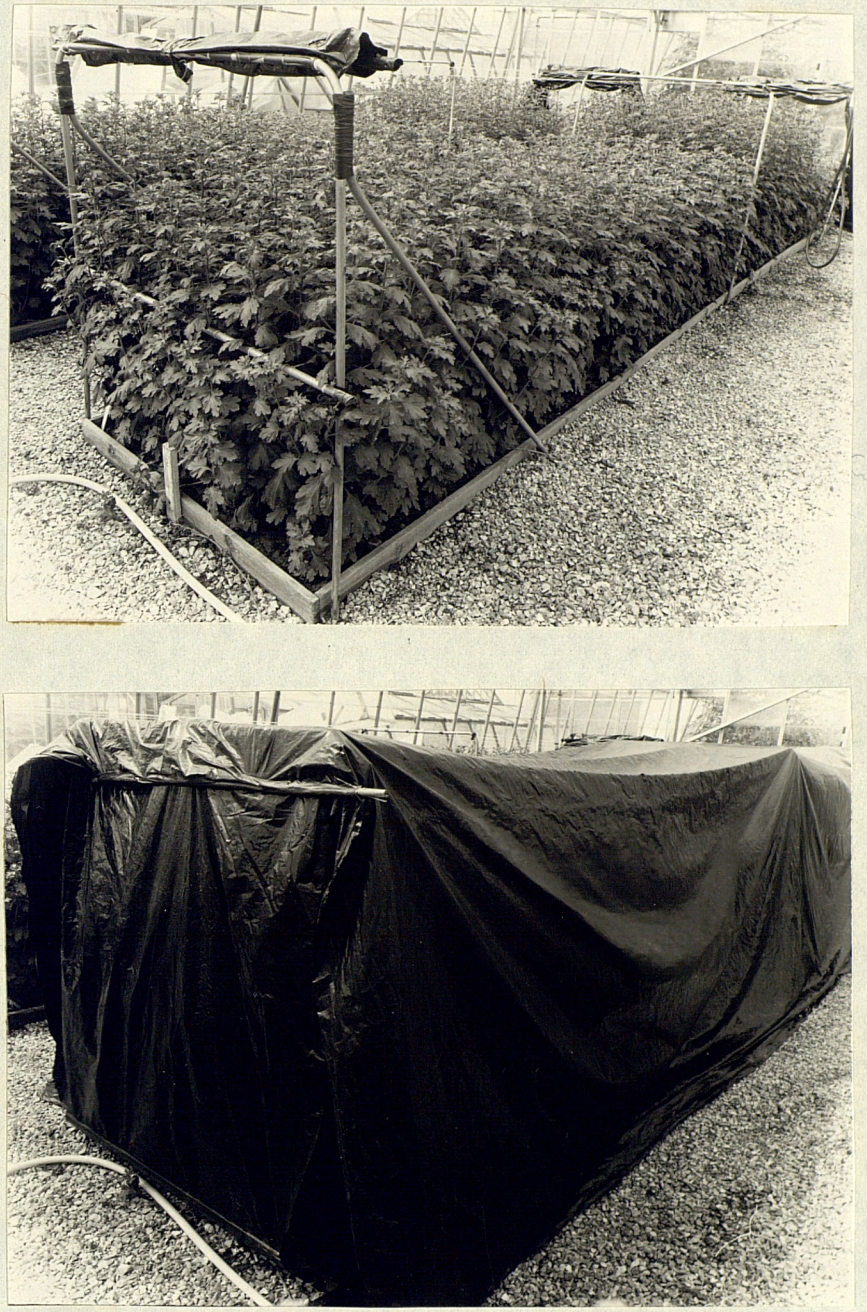


Fig. 3.01 Polythene blackout covers on chrysanthemum bed

apterous, viviparous, parthenogenetic females (fundatrices). The fundatrices produce similar forms but, as population density increases, winged forms (alatae) appear and increase in numbers. Alatae migrate to other plant species, (secondary hosts). On these plants, generations of apterae and alatae are produced, the winged forms migrating again to colonize more plants. Up to this time, all adults have been viviparous and parthenogenetic. In autumn, winged parthenogenetic females, in response to certain physical stimuli, e.g. temperature, light, produce males and oviparous females (sexuales). These mate and females lay the winter eggs on the primary hosts. Under glass, reproduction with many aphid species is normally continuously parthenogenetic throughout the year on herbaceous secondary host plant species, producing both apterae and alatae.

Aphids damage plants in several ways and have sucking mouthparts for feeding from plant phloëm. As with many Homoptera, a sweet substance (honeydew) is discharged continuously from the anus. Honeydew usually falls onto leaves and, with dense aphid populations, covers the leaves impairing their respiration and encouraging the growth of phytophagous fungi, ultimately disfiguring and damaging the plants. In addition, the feeding of many aphids distorts leaves, buds and flowers, and may stunt the plants. However, aphid-transmitted viruses may be more injurious to many crops than direct feeding damage.

On commercial flower crops, even single aphids of some species in the open blooms tend to lower the market value. Many flower crops in the British Isles are grown under glass where, in the absence of natural enemies and unfavourable weather, aphids multiply extremely rapidly. My.persicae produces at least 50 progeny/female and under average

conditions each aphid matures in about a week (Hussey et al., 1969).

(b) Control of Aphids on Chrysanthemums under Glass

Common insecticides used to control aphids on chrysanthemums include nicotine, gamma - BHC, the organophosphates - particularly demeton-S-Methyl- and pirimicarb. Application of these chemicals is recommended weekly in summer but less frequently in winter (Framptons Cultural Information, 1971).

However, in recent decades, commercial control of aphids under glass has been embarrassed by resistance of aphids to pesticides. Resistant aphids evolved in the glasshouses of commercial suppliers of chrysanthemum cuttings on which they were spread around the country. Thus, recently, interest has been focussed on biological control of aphids by parasitic and predatory insects and pathogenic fungi (virtually all recorded pathogens of aphids have been fungi Chapter I). So far, only one biological agent, a hymenopterous parasite (Aphidius matricariae) of the most serious aphid pest, Myzus persicae, has been successfully used on chrysanthemums. Biological methods are sometimes more efficient than chemical control (Hussey et al., 1969) and certainly do not involve phytotoxic side effects that accompany many chemicals.

(c) Annotated List of Aphid Pests of Chrysanthemums

Species marked with * are known to be susceptible to V.lecanii disease. Species marked with ** are susceptible and have also been observed by the author to have succumbed to natural epizootics of V.lecanii in the glasshouse.

(i) Myzus persicae (the peach-potato aphid)**

This ubiquitous aphid (Fig. 3.02) is a major pest of many crops throughout the world. It is variable in colour - light red to green - and, being very restless, populations are comparatively mobile (Hussey et al., 1969). This aphid is a vector of many plant virus diseases, including some in horticultural crops (Hussey et al., 1969).

(ii) Macrosiphoniella sanborni** (the chrysanthemum aphid)

This common aphid (Fig. 3.03) infests chrysanthemums outdoors and commonly infests glasshouse chrysanthemums in late summer. It is a shiny, red-brown aphid and, at low densities feeds high on the chrysanthemum stem (Fig. 3.03).

(iii) Brachycaudus helichrysi** (the leaf-curling plum aphid)

This small pale green aphid (picture - Fig. 6.15) feeds, at low densities, exclusively at the tips of plants, causing severe distortion and mottling of the young leaves. It deposits crystalline honeydew which may be extensive enough to render plants unmarketable (Fig. 3.04).

(iv) Aulocorthum solani (glasshouse potato aphid)

This large green aphid is a minor pest of chrysanthemums and, in the author's experience, has never been a problem.

(v) Aphis fabae* (the black bean aphid)

Groups of this mat black aphid occasionally infest chrysanthemums and cluster on stems like M.sanborni (Fig. 3.03).

Fig. 3.02 Myzus persicae on chrysanthemum leaf

Fig. 3.03 Macrosiphoniella sanborni on chrysanthemum stem

Fig. 3.04 Chrysanthemum badly infested with Brachycaudus helichrysi. Note waxy appearance of honeydew on leaves.

CHAPTER IV

BIOLOGY OF VERTICILLIUM LECANII

MATERIALS AND METHODS

1. General

(a) Media

Complex media were obtained from Oxoid Ltd., London, unless otherwise stated.

Sabouraud dextrose agar

mycological Peptone	10 g
glucose	40 g
agar	15 g

pH approximately 5.2

in one litre of distilled water

Sabouraud liquid medium

pancreatic digest of casein	5 g
peptic digest of fresh meat	5 g
glucose	20 g

pH approximately 5.7

in one litre of distilled water

Potato dextrose agar

potato extract	4 g
glucose	20 g
agar	15 g

pH approximately 5.6

in one litre of distilled water

Czapeck-Dox agar and liquid medium

KNO_3	2.0 g
KCl	0.5 g
Magnesium Glycerophosphate	0.5 g
$FeSO_4$	0.01 g
K_2SO_4	0.35 g
sucrose	30.0 g
Oxoid agar No. 3	12.0 g

with or without

pH approximately 6.8

in one litre of distilled water

Malt extract agar and liquid medium

malt extract (Boots Pure Drug Co.Ltd.,Nottingham)	25 g
KH_2PO_4	2 g
$MgSO_4.7H_2O$	0.123 g
$FeSO_4.7H_2O$	1 mg
$ZnSO_4.7H_2O$	1 mg
$CaCl_2.2H_2O$	0.147 g
with or without Oxoid agar No. 3	15 g

pH 7.0

in one litre of distilled water

Corn steep liquor (Blachère et al.,1973)

Corn steep liquor (Corn Products Ltd. Manchester)	20 g
sucrose	30 g
KH_2PO_4	2 g
$Na_2HPO_4.12H_2O$	2 g
$MgSO_4.7H_2O$	123 g
$FeSO_4.7H_2O$	0.023 g
$ZnSO_4.7H_2O$	0.02 g
K_2SO_4	0.174 g
$CaCl_2.2H_2O$	0.147 g

pH 7.2

in one litre of distilled water

Basal liquid medium

glucose	30 g
$MgSO_4.7H_2O$	2 g
KH_2PO_4	5.4 g
$FeSO_4.7H_2O$	1 mg
$ZnSO_4.7H_2O$	1 mg
H_3BO_4	0.06 mg
$CuSO_4.5H_2O$	0.5 mg
$MnSO_4.4H_2O$	0.04 mg
$Na_2MoO_4.2H_2O$	0.05 mg
$CaCl_2.2H_2O$	0.5 g

pH 7.2

in one litre of distilled water

(b) Wetting and Dispersal Agents

Triton X-100 (BDH Chemicals Ltd., U.K.) was used as a wetting and dispersal agent of spores. Concentrations in excess of those used in experiments did not reduce the short-term viability of V.lecanii spores (IV, 9, b).

(c) Buffer

Phosphate buffer (0.0003 M KH_2PO_4) was prepared from a concentrated stock solution adjusted to pH 7.2 with NaOH.

(d) Antifoams

Foam produced by agitation tends to trap spores (Ingold, 1974). Foaming was prevented by 2 types of antifoam which were non-toxic at concentrations in excess of those normally used; polyethylene glycol (PEG), a water-soluble reagent (BDH Chemicals Ltd., U.K.) and "Antifoam A" (Sigma Chemicals Ltd.) a silicone based reagent. Soluble antifoams increase the solubility of the foaming agent and, being biologically inert, are suitable for use in biological systems. PEG was used to suppress foaming in vigorously agitated liquid cultures at the rate of one drop/100 ml of culture medium.

Antifoam A was used to suppress the foam of Triton X-100 since PEG was ineffective against this detergent. Silicone-based antifoams prevent interactions at the interface between air and detergent by forming a very thin layer on the surface of the liquid. Since this layer must be complete, the quantities used thus varied, but approximated to a rate of one drop/100 ml of liquid in a 250 ml conical flask i.e. approximately 40 cm^2 of surface area. Antifoam A was used in germination experiments and in spraying spores in glasshouses, but not for sensitive laboratory assays, because the encapsulating properties of this antifoam

might adversely affect the respiration of insects. In assays, foaming was minimised by use of only 0.02% Triton X-100.

(e) Temperature Measurement

Temperature where possible, was recorded by a thermograph but otherwise spot thermometer readings were taken over a period of time. The observed means and ranges are given with experimental data.

(f) Humidity Control

A range of humidities, maintained in glass containers, was provided by 3 types of aqueous solutions; sulphuric acid (Solomon, 1951), glycerol (Johnson, 1940) and saturated salt solutions (Solomon, 1951; Hickman, 1970; Judd et al., 1972[1]):-

Saturated salt	Relative humidity(%) at 20°C
K_2SO_4	97
KH_2PO_4	96
KNO_3	93(Solomon,1951;
$(NH_4)_2SO_4$	81 Hickman,1979)
NaCl	76
NaBr	58
P_2O_5 (anhydrous powder)	0

[1]The humidity over KNO_3 quoted in this paper is incorrect

(g) Glassware

Glassware was normally washed in detergent solution and rinsed thoroughly in distilled water. Ultra-clean slides were obtained by boiling them for 20 min. in detergent ("Daz"), dropping them one by one into boiling distilled water, repeating this procedure once, then rinsing with ethanol (95%) and leaving them to dry on clean glass rods over filter paper. The ultra-clean slides were sterilized in clean glass Petri-dishes and sterilized by dry-heat at 120°C for at least 4h.

(h) Total Spore Counts

Total counts of conidiospores and blastospores were made in an improved Neubauer haemocytometer. The moistened coverslip was worked to and fro on the slide until interference colour patterns became visible, a guide to the correct chamber depth. A concentration of approximately 10^6 spores/ml was used to flood the chambers. The number of spores in each of four 1 mm^3 squares were counted in each of 2 chambers and this usually encompassed about 400 spores altogether. Above this figure, accuracy does not improve much because the standard error due to random sampling decreases at a progressively lower rate in proportion to the number of spores counted (Meynell et al.,1965). Owing to imperfect adhesion of the coverslip to the slide, variation in chamber depth can give high variation between counts (Norris et al.,1961). Therefore, each count was repeated at least once on the same slide and again if the 2 counts differed by more than 10%.

(i) Assessment of percentage Viability of Conidiospores ("Agar-slide technique").

Preliminary tests indicated that the most satisfactory and speedy method for assessing the viability of

conidiospores consisted of spreading a layer of sterile, Sabouraud dextrose agar (SDA) on a slide and spreading on it 3 drops of the test suspension, diluted approximately to 10^6 spores/ml. Such slides were incubated overnight in a Petri-dish lined with water-saturated filter paper to boost humidity. Viable spores produced a germ tube visible under phase contrast microscopy. Approximately 100 spores were counted in each of the counts on agar slides.

(j) Assessment of Percentage Viability of Blastospores

Since distinguishing between germinated and ungerminated blastospores was virtually impossible by the agar-slide technique, colonies produced on agar by viable spores were counted.

The test suspension was serially diluted and a total spore count performed. Also, aliquots (0.25ml) of each dilution were spread on to each of 4 replicate Petri - plates of SDA, care being taken not to touch the edges of the dish with the glass spreader, because spores tended to aggregate at any such points of contact. After incubation for 4 days at 23.5 $\pm$ 0.5°C, colonies were enumerated and the percentage viability of the original suspension was calculated with reference to the total spore count.

(k) Single-Spore Isolate

Conidiospores for experiments were produced from cultures derived from a single-spore isolate of V.lecanii (G.C.R.I.code=C-3; IMI 179172[1]) which itself was derived from a population of diseased M.sanborni infesting chrysanthemums at the Glasshouse Crops Research Institute. The single-spore isolate was obtained by a method similar to that of Veen (1967). Giemsa's stain revealed that V.lecanii

1 Deposited at the Commonwealth Mycological Institute, Kew, Surrey, U.K.

conidiospores were uninucleate (Fig. 4.01). The chances of variation upon successive subculturing were thus smaller than if the spores had been multinucleate and possibly heterokaryotic as with other Fungi Imperfecti (Hansen, 1938).

The method of producing the single-spore isolate was as follows. A dead specimen of M.sanborni sporulating with V.lecanii, was vigorously agitated in phosphate buffer with 0.02% Triton X-100. Serial dilutions were made and 0.25 ml aliquots were spread on to SDA plates, and incubated overnight at 23 ± 0.5°C. Next day, they were examined at X100 magnification; isolated germinated spores were removed on their surrounding agar with an inoculating loop and transferred to a clean agar plate. These single spores were allowed to develop into colonies and from one, many more SDA Petri-dishes were inoculated, incubated at 23 ± 0.5°C for 7 days and then stored at -17°C.

(L) Culture and Harvesting of Conidiospores

Conidiospores were cultured by spreading a spore suspension made from one of the deep-frozen cultures (see above) on more agar plates or for larger experiments, on covered trays of SDA and incubating at 23 ± 0.5°C. Spores were harvested by flooding the culture with distilled water buffered at pH 7.2, containing 0.02% Triton X-100 and agitating with a bent glass rod. Hyphal debris was filtered off through 4 layers of coarse-meshed cheesecloth and the spores centrifuged and washed 4 times in buffer.

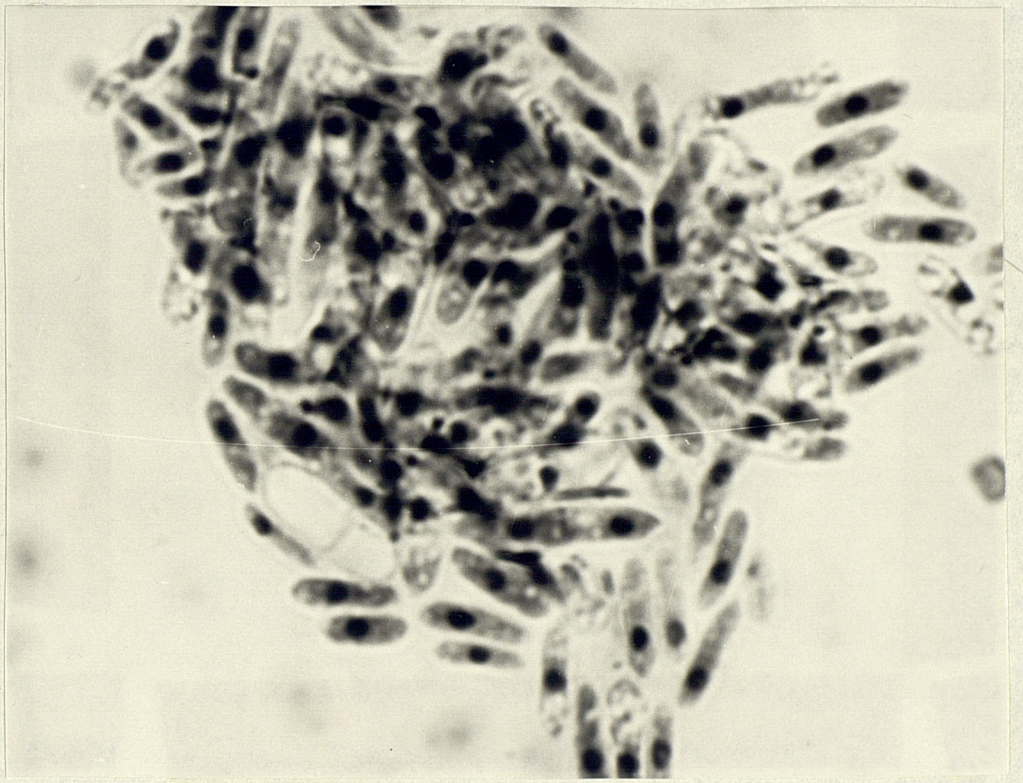


Fig. 4.01 Mononuclear conidiospores of Verticillium lecanii (Giemsa's stain; X1000)

(m) Harvesting of Blastospores

Blastospore cultures in liquid medium were filtered through 4 layers of coarse-meshed cheesecloth and the spores centrifuged and washed 4 times in buffer.

2. Growth and Sporulation of V.lecanii on Solid Media

Mycelial growth and sporulation were compared on various media in light and darkness, and at a range of temperatures. Growth was estimated by measuring periodically the diameter of colonies spreading from inoculum plugs, 12 m m in diameter, cut from a 7-day uniform confluent growth of V.lecanii and placed in a hole on a new agar plate. Sporulation was estimated by removing three 12 m m diameter plugs from plates of confluent growth, placing each plug in 10 ml distilled water containing 5% Triton X-100 and agitating it very vigorously for 2 min on a vortex mixer. Total spore counts of the resulting spore suspensions were made (IV, 1, h). Rough subjective visual assessments of the degree of sporulation were also made at X200 magnification.

No attempts were made to avoid small temperature rises in Petri-plates incubated under light, because the effects of such rises would be insignificant since these experiments were performed at 20 $\pm$ 1°C and growth rates do not differ greatly over the range 20 - 25°C (IV,7,b).

3. Germination of V.lecanii Conidiospores

(a) Temperature

Batches of inoculated agar-slides were incubated at temperatures between 11.5 and 32°C. At progressively longer intervals, 2 slides were removed from each batch at each temperature and spore viabilities determined. Also, at some temperatures, germ tube lengths were measured using an eyepiece micrometer.

(b) pH

Liquid SLM medium was preferred because of the ease of pH adjustment with sterile 1M HCl or NaOH, which was performed after sterilization of SLM because its pH, adjusted with NaOH, was altered by autoclaving. Each 100 ml aliquot of medium in a conical flask was inoculated and vigorously aerated for 20h at 20 ± 1°C. The percentage germination was then assessed using phase contrast microscopy.

(c) Relative Humidity and Free Water

Sterile slides, held by forceps, were dipped briefly into sterile SDA. When the agar had set, the slides were equilibrated for 48h at the desired humidities at 20 ± 1°C on plastic supports in sterile Kilner jars containing the appropriate saturated salt solutions. After equilibration, agar-slides were gently pressed on to sporulating cultures of V.lecanii, returned to their Kilner jars and incubated overnight at 20°C. Next morning, the slides were agitated(in distilled water containing 1% Triton X-100)vigorously on a vortex mixer. The resulting suspension was finely dispersed by sonication for 2 min (20 Kc/s, 62W) and the proportion of germinated spores determined.

Extremely accurate temperature control (± 0.1°C) is necessary to reliably determine whether a fungus requires free water for spore germination (Schein, 1964). Such accuracy was not possible in this study, but the results of the following experiment gave an indication as to whether free water was necessary.

Five drops of spore suspension (10^6 spores/ml) in SLM containing various concentrations of the thixotrophic agent, carboxymethyl cellulose [1](CMC) were placed on 2

1 "Cellosize", Union Carbide Ltd.

sets of slides. On one set, the suspension was thinly layered with a glass spreader along the length of the slide. Both sets of slides were placed in Petri-dishes lined with filter paper saturated with distilled water for 16h at 20°C and the percentage germination determined next day using phase microscopy.

4. Longevity of Conidiospores and Cultures

(a) Temperature

Conidiospores from 10-day old V.lecanii cultures were harvested (IV, 1, L), extra care being taken to prevent contamination. The suspensions were diluted with sterile distilled water to approximately 10^6 spores/ml and distributed into 5 ml screw cap "bijoux" bottles. Batches of bottles were then stored at -17, 2 and 20°C. Periodically, 3 "bijoux" bottles were removed at each temperature, the -17°C samples being thawed at room temperature and the proportion of viable spores in each determined using the agar-slide technique (IV, 1, i).

(b) Antibiotics and Wetting Agents

Other bottles of spores containing 50 - 100 µg/ml of either tetracycline or streptomycin (Sigma Chemicals Ltd.) or a wide range of concentrations of Triton X-100 or Tween 80, (Sigma Chemicals Ltd.), were stored at 2°C. Bottles were removed periodically to assess spore viability as in (a) above.

(c) Relative Humidity

Washed spores were exposed at 20 ± 1°C to different humidities by spreading a concentrated conidiospore suspension (ca. 10^8 spores/ml) on to 1 cm^2 pieces of cellophane[1] which were placed on filter papers on stands

1 - 500P cellophane. D.J.Parry & Co., Ltd., London.

in Kilner jars containing saturated salts (IV, 1, f). Periodically, cellophane squares were transferred to agar plates, spores uppermost, and incubated at 15 ± 1°C overnight. Spores were then washed off the cellophane in distilled water containing 1% Triton X-100 and agitated briefly on a vortex mixer. Clumps were dispersed by sonication for 2 min (20 Kc/s, 62 W). An incubation temperature of 15°C was used because after 14h at this temperature, the germ tubes were short enough to permit effective dispersal of clumps.

The cellophane technique was also used for unwashed spores; squares were pressed gently onto a confluent culture of V.lecanii, transferred to Kilner jars and treated as above. Spores, still enveloped in the slime matrix, adhered to these squares.

The survival of spores on aphid (M.sanborni) bodies, and on 10-day old mycelium grown on 1 cm^2 cellophane squares on SDA, was also studied. These were placed in the Kilner jars and treated as above.

In these 4 studies, unwashed cellophane squares were employed, since when washed, they lost their smooth consistency and would not appose evenly to the agar surface. A check experiment to guard against the possibility that there were potentially toxic impurities on unwashed cellophane, showed there was no difference between survival levels of conidiospores on washed and those on unwashed cellophane, after equilibration at a range of relative humidities for 24h.

(d) Preservation of Conidiospores in Liquid Nitrogen

Freshly harvested conidiospores of V.lecanii were stored in liquid nitrogen (-196°C) following the method

of Elliot (unpublished). Suspensions of 10^7 spores/ml in distilled water or in 10% glycerol, a cryoprotectant (Nash, 1966), were pipetted into 2-cm plastic drinking straws, heat-sealed at the bottom. After heat-sealing the top, a light thumb pressure was applied to test for leaks. A perfect seal was essential to avoid penetration of liquid nitrogen and explosion during subsequent thawing. Mazur et al. (1965) and Goos et al. (1967) demonstrated that several fungal species were injured during slow thawing if they had been cooled rapidly in liquid nitrogen presumably due to the rapid recrystallization of harmful intracellular ice (Mazur, 1968). When cells are cooled slowly, water presumably moves out of the cells and is frozen externally. Thus, the straws were cooled slowly in liquid nitrogen vapour for one hour and then plunged into liquid nitrogen. Since fast thawing is least injurious to fungal spores frozen in liquid nitrogen (Goos et al., 1967), the straws were thawed rapidly in a 37°C water bath. Viabilities were assessed after thawing by the agar-slide method.

5. Blastospore Formation

(a) Aeration and Agitation

To study blastospore formation, suspensions of 5×10^6 conidiospores / ml in Sabouraud liquid medium (SLM) were agitated and aerated for 5 days at 20 ± 1°C as follows (each Universal bottle and conical flask contained 5 and 100 ml of suspension respectively):-

(i) Closed, i.e. cap screwed down, 20 ml Universal bottle, not agitated.
(ii) Closed 20 ml Universal bottle, reciprocally agitated.
(iii) Cotton wool plugged 250 ml conical flask, not agitated.
(iv) Cotton wool plugged 250 ml conical flask, reciprocally agitated.

(v) Cotton wool plugged 250 ml conical flask on a rotary shaker.

(vi) Vigorously aerated 250 ml conical flask reciprocally shaken.

(vii) Vigorously aerated 250 ml conical flask on a rotary shaker.

(viii) Vigorously aerated 250 ml conical flask, magnetically stirred.

6. Factors Affecting Blastospore Yields

(a) Oxygenation

Eight 250 ml conical flasks each containing 100 ml sterile SLM were seeded with conidiospores and agitated on a reciprocal shaker. Four flasks were aerated and the rest were not but stoppered with cotton wool.

(b) pH

Five pH regimes were tested: controlled pH at 4.0, 5.5, 7.0 and 9.0 and uncontrolled pH (initial pH, 5.5). For each regime, two 250 ml flasks each containing 100 ml of sterile SLM were seeded with approximately 5×10^5 conidiospores/ml. Sterile 1M NaOH or HCl was used for pH control and 40 mM sterile KH_2PO_4 was added to each flask to help stabilize pHs 7.0 and 9.0. It was also added to the flasks at lower pHs to avoid a change in conditions but not to flasks at uncontrolled pH. The flasks were reciprocally agitated and forcibly aerated at $20^o \pm 1^oC$ for 5 days. For the first 2 days, pH was adjusted twice daily but later as culture density increased, 3 times daily. The pH of the uncontrolled SLM was measured daily. The experiment was repeated once.

RESULTS

7. Growth and Sporulation on Solid Media

(a) Effect of Type of Medium

Growth of Verticillium lecanii (C-3; IMI 179172) was about 13% slower on Potato dextrose agar or Czapeck-dox agar than on Sabouraud dextrose agar (SDA) (Fig. 4.02). Sporulation on these media and on Malt extract and Nutrient agars was similar, most of the 95% confidence limits overlapping each other (Table 4.01). Since SDA gave the best combination of growth and sporulation, it was selected for routine use.

TABLE 4.01

Effect of Medium on Sporulation of *Verticillium lecanii* (18 days at 20 ± 1°C in darkness)

Agar medium	Total spore count ± S.E.[a] x 10^6 spores/ml	Efficiency of sporulation[b]	Visual assessment[c]
Czapeck-dox	5.32 ± 0.60	100	+++
Sabouraud dextrose	4.17 ± 1.66	78.4	+++
Malt extract	4.10 ± 1.22	77.1	+++
Nutrient	2.35 ± 0.60	44.2	+++
Potato dextrose	1.07 ± 0.36	20	+++

a = Standard error

b= Measured by reference to the highest yield obtained

c = +++ very many gelatinous heads, mostly coalesced

(b) Temperature

The growth rate was optimal at 23.5°C (Fig.4.03). Above 25°C, the rate fell steeply and growth ceased above 30°C. At temperatures below 20°C, growth slowed but was still detectable at 2°C. At 2 and 7°C, the agar was pigmented deep red around the colony. For other isolates of *V.lecanii*, Ganhao (1956) reported a temperature optimum for total growth at 23°C and a steep decline in growth above 25°C, as

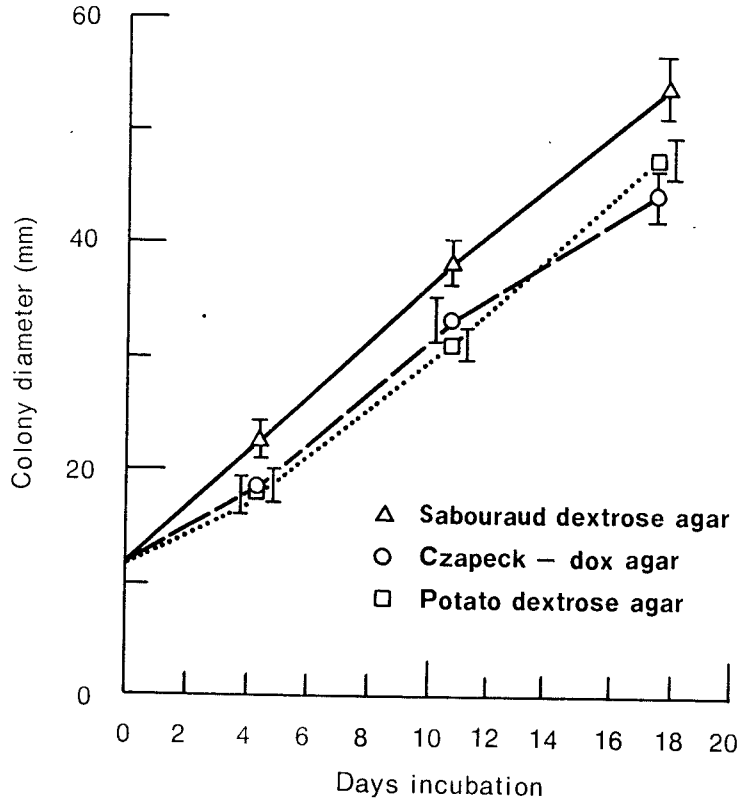


Fig. 4.02

Effect of type of medium on growth of *Verticillium lecanii* at 20 ± 1°C. Points are means, with 95% fiducial limits, of colony diameters (4 agar plates/medium).

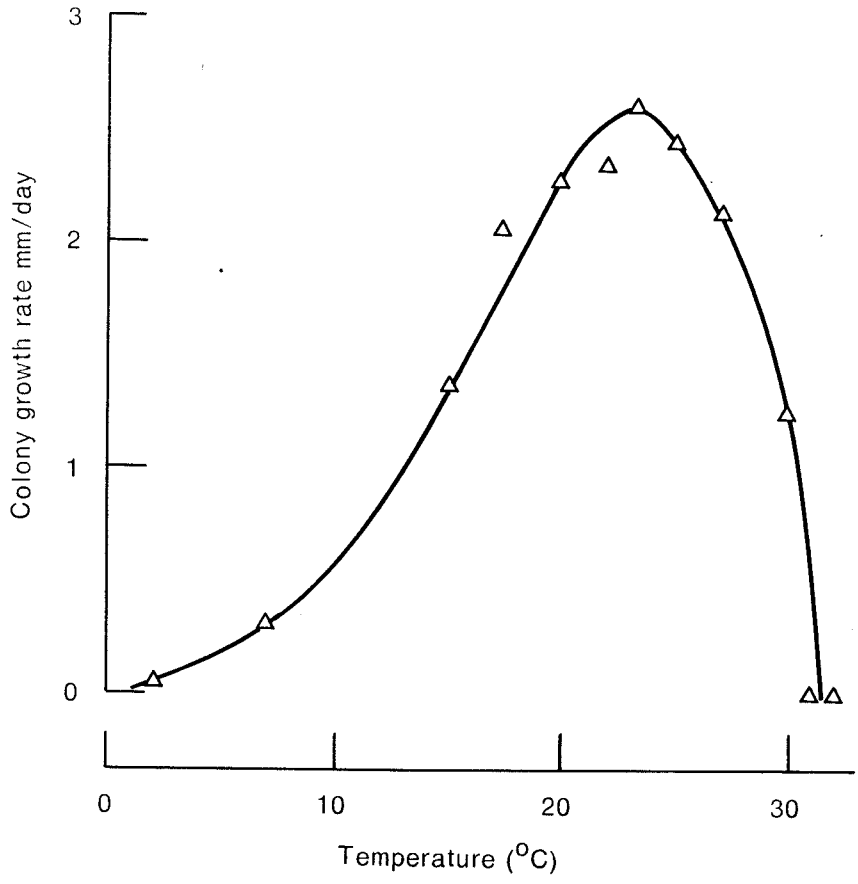


Fig. 4.03.

Effect of temperature on growth rate of *Verticillium lecanii* colonies on Sabouraud dextrose agar.

with C-3 (IMI 179172) but Barson (1976a) reported a lower optimum for total growth (21.5°C) but again, he noted a steep fall in growth above 25°C.

The response of sporulation to temperature followed a similar pattern. The best sporulation occurred in the range 20-25°C but was much less at 27.5°C ($P < 0.05$) and was virtually absent at 30°C (Table 4.02).

TABLE 4.02

Effect of Temperature on Sporulation of *Verticillium lecanii* (20 days, darkness)

Temperature °C	Total spore count ± S.E.[a] x 10^6 spores/ml	Efficiency of sporulation[b]	Visual assessment[c]
15	0.87 ± 0.099	18.7	++
20	3.58 ± 0.42	76.6	+++
23	2.17 ± 0.28	46.5	+++
25	4.67 ± 0.65	100	+++
27.5	0.087 ± 0.012	1.8	+
30	-	0	-

a = Standard error

b = Measured by reference to the highest yield obtained

c = Sporulation +++ Many gelatinous heads, mostly coalesced
++ " " " some coalesced
+ Gelatinous heads, few coalesced
- No gelatinous heads

(c) Light

Growth was very slightly retarded by light but until day 18 the 95% fiducial limits overlap and the effect is very small (Fig. 4.04). Light however, greatly increased

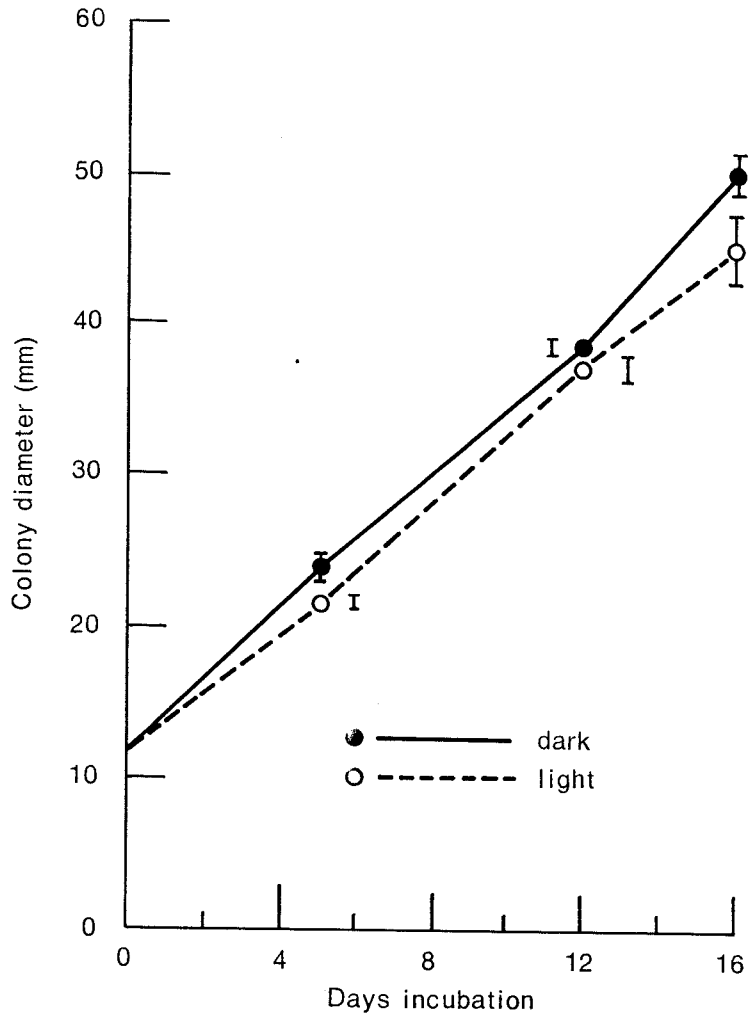


Fig. 4.04

Effect of light (10,000 lux) upon growth of *Verticillium lecanii* on Sabouraud dextrose agar at 20 ± 1°C (95% fiducial limits included, 4 agar plates/treatment).

sporulation (Table 4.03; $P < 0.01$), and intensified the pale yellowing of colonies occuring in dark incubation. The pigmentation of cultures of many species of fungi intensifies with increased light intensity (Hawker, 1950).

TABLE 4.03

Effect of Light (10,000 lux) upon Sporulation of *Verticillium lecanii* (20 days, 20±1°C)

	Total spore count ± S.E.[a] x 10^7 spores/ml	Efficiency of sporulation[b] %
Light	1.83 ± 0.42	100
Dark	0.31 ± 0.10	17.1

a = Standard error

b = Measured by regarding the highest mean yield as 100%

8. Germination of Conidiospores

(a) Accuracy of Viable Spore Counts

The agar-slide technique was employed to determine conidiospore viabilities. Table 4.04 shows the results of replicate viable spore counts of a spore suspension performed on 8 separate agar-slides. In general, on individual slides, the standard error of the mean decreased as more counts were performed. When single counts or means of 2 or 3 counts from replicate slides are combined, there is little to be gained by using more than 3 replicate slides (Table 4.04). Furthermore, cumulative means of single counts on each slide were only slightly less accurate than cumulative means of 2 or 3 counts/slide as shown by the standard errors. Thus, this method should detect large differences in viabilities with one count from each of 2 replicate slides for each batch of spores. Greater accuracy requires more counts on 3 slides.

TABLE 4.04

Accuracy of Agar-Slide Technique for *Verticillium lecanii*

Viable Conidiospore Counts

Slide number	Mean of 1-3 counts of viable spores/slide ± S.E.[a]			Cumulative means of counts for replicate slides ± S.E.[b]		
	1 count[c]	2 counts[c]	3 counts[c]	1 count[c]	2 counts[c]	3 counts[c]
1	73	76.1±3.1	75.8±1.8			
2	79	79.2±0.2	76.8±2.3	76 ±3.2	77.6±1.5	76.3±0.5
3	78.6	81.1±2.5	79.6±2.0	76.8±1.9	78.8±1.4	77.4±1.1
4	73.1	76.4±3.3	79 ±3.2	75.9±1.7	78.2±1.2	77.8±0.9
5	78.8	80.4±1.6	80.5±0.9	76.5±1.4	78.6±1.0	78.3±0.9
6	77	78.7±1.7	79.4±1.2	76.5±1.1	78.6±0.83	78.5±0.7
7	85	84 ±1.0	81.5±2.6	77.7±1.5	79.4±1.0	78.9±0.7
8	88.1	81.7±6.3	82.3±3.7	79.1±1.8	79.7±0.9	79.4±0.8
Range of standard errors		0.2 -6.3	0.9-3.7			

a = Standard error

b = Standard error

c = Approximately 100 spores/count

(b) Temperature

Germination was best between 20 and 25°C (Figs.4.05 a and b). Outside this range, germination rates fell off rapidly. At 32°C and above, germination was absent.

Germ tubes did not differ significantly in length (Table 4.05; $P > 0.05$) after 14h at temperatures between 20 and 25°C but at 15 and 27°C were only $\frac{1}{2}$ to $\frac{1}{3}$ as long and at 11.5°C were only just visible.

TABLE 4.05

Effect of Temperature on Germ Tube Lengths of *Verticillium lecanii* Conidiospores after 14h Incubation

Temperature °C	n	Mean ± S.E.[a]
11.5[b]		Germ tubes just visible
13[b]	54	12.1 ± 0.1
15 (i)[b]	60	13.9 ± 0.6
(ii)[c]	65	18.2 ± 0.9
20 (i)[b]	30	48.4 ± 3.8
(ii)[c]	42	51.5 ± 3.7
23[b]	31	55.2 ± 5.1
25[b]	31	56.4 ± 6.3
27[c]	33	25.6 ± 2.4

a = Standard error of mean of n observations

b = Experiments performed simultaneously

c = Experiments performed simultaneously

(c) Light

Germination levels and germ tube lengths on agar-slides incubated at a light intensity of 10,000 lux (Table 4.06) were not significantly different from those on slides incubated in darkness.

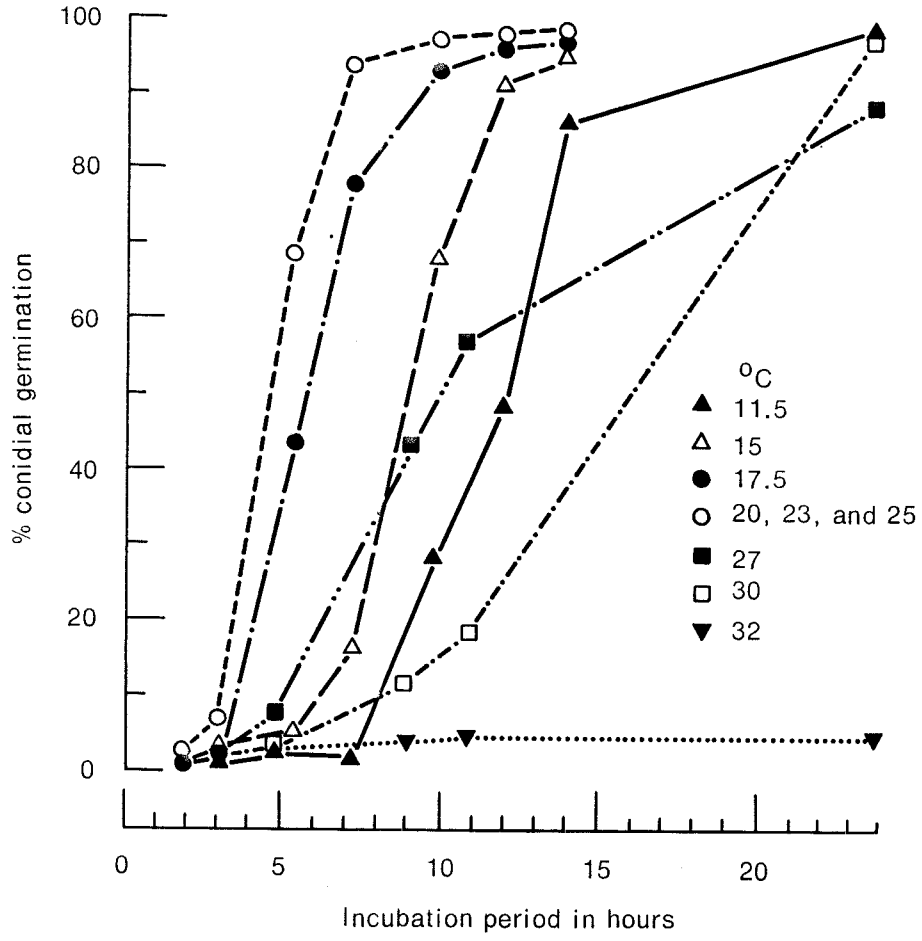


Fig. 4.05a.

Effect of temperature on conidial germination rates of *Verticillium lecanii* (combined results of 2 experiments; no measurements at 14h for 27 and 30°C).

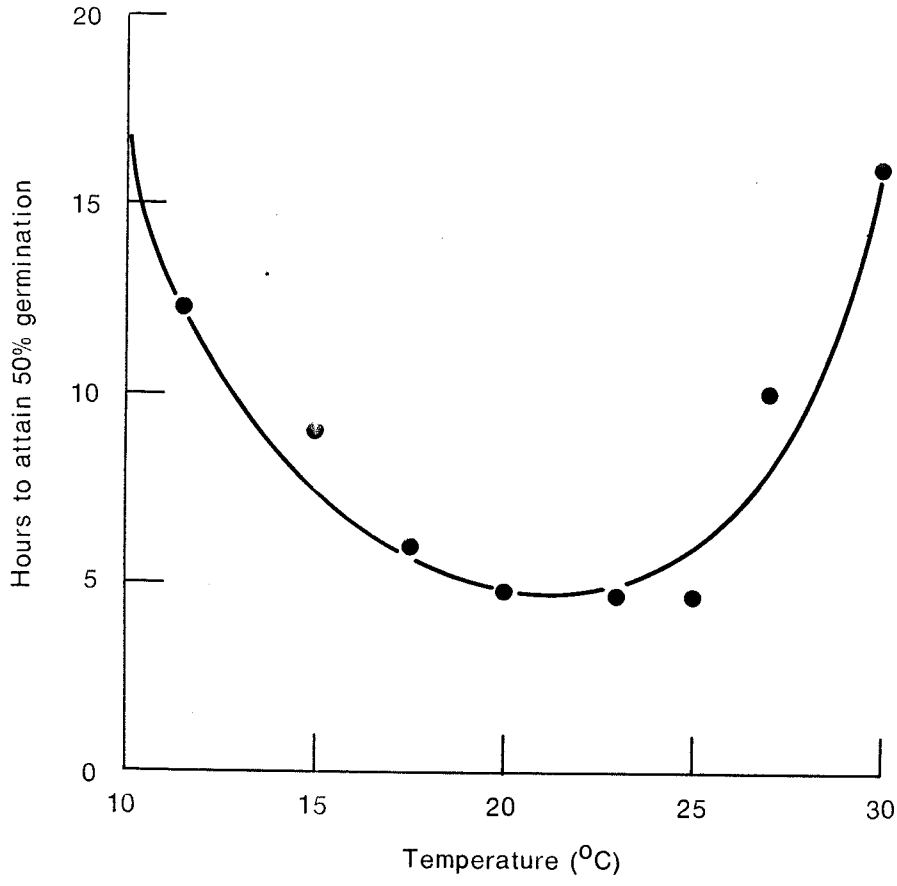


Fig. 4.05b

Effect of temperature on time required to achieve 50% germination of *Verticillium lecanii* conidiospores on agar (combined results of 2 experiments).

TABLE 4.06

Effect of Light (10,000 lux) upon Germination of *Verticillium lecanii* Conidiospores (14h incubation, 20°C)

Regime	% Germination ± S.E.[a]	n	Germ tube length[b] (μ) ± S.E.
Light	99.0 ± 0.58	35	48.4 ± 3.8
Darkness	99.3 ± 0.68	33	48.4 ± 3.6

a = Standard error of mean of counts from 2 agar-slides (3 counts/slide)

b = Standard error of mean of n observations

(d) Conidiospore Density

Germination was assessed at increasing concentrations of freshly harvested conidiospores on agar-slides. Counts were made 4, 6 and 12 h after incubation commenced. Table 4.07 shows that high densities slightly stimulated germination (Chi^2-test, $P < 0.01$). Furthermore, unwashed spores, deposited at very high densities on cellophane squares (IV, 4, c) germinated well (Fig. 4.06a,b). These results show that there was no auto-inhibition of germination at high concentrations of either washed or unwashed spores.

TABLE 4.07

Effect of Verticillium lecanii Conidiospore Density on Germination Rates on Sabouraud Dextrose Agar (23.5 ± 0.5°C)

Spore concentration (spores/ml)	4h incubation			6h incubation			12h incubation		
	g[a]	ung[b]	mean ±S.E.[c]	g[a]	ung[b]	mean±S.E.[c]	g[a]	ung[b]	mean ± S.E.[c]
5.10^4	77	123	38.5±0.5	157	48	76.6±1.2	189	11	94.5±0.5
5.10^5	66	138	32.4±2.6	176	34	83.8±1.1	199	1	99.5±0.5
5.10^6	95	107	47.1±4.9	181	26	87.4±1.15	100	0	100±0
5.10^7	113	90	55.7±1.2	197	13	93.9±1.23	224	1	99.6±0.4
Chi^2 d		25.61			25.88			26.4	

a = Germinated spores

b = Ungerminated spores

c = Standard error of mean of counts from 2 agar slides

d = Chi^2 test for homogeneity of data

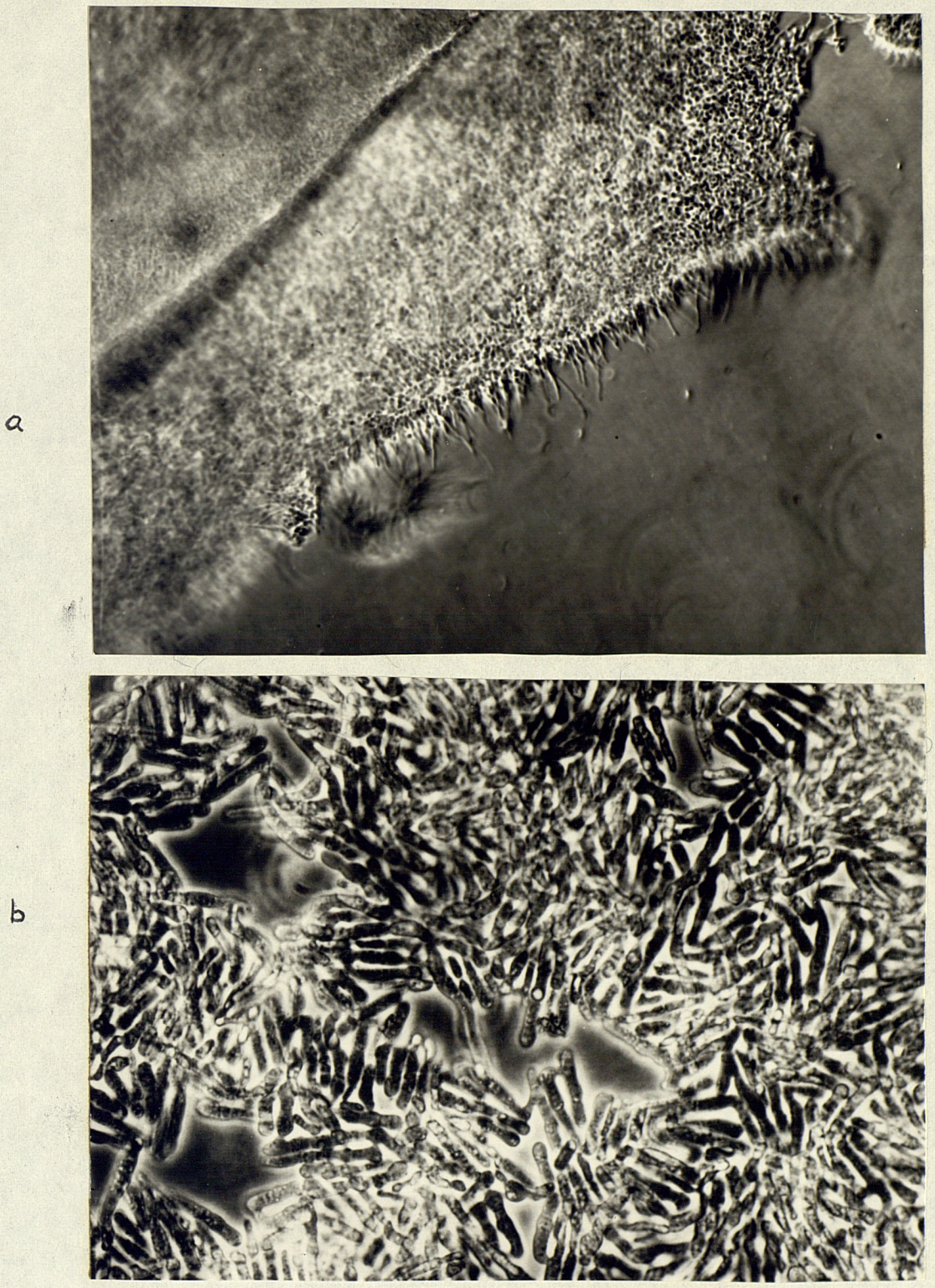


Fig. 4.06 a and b. Densely packed conidiospores of Verticillium lecanii germinating on cellophane (a-X100; b-X500)

(e) pH

Germination levels of spore suspensions in SLM were high over a broad plateau of pH from 4.5 to 8.5 but fell particularly rapidly from pH 4.5 to 3.5 (Table 4.08).

TABLE 4.08

Effect of pH on Germination Levels of *Verticillium lecanii* Conidiospores (20 ± 1°C, 20h vigorous aeration in Sabouraud liquid medium)

pH	2.0	3.0	3.5	4.5	5.5	6.5	7.5	8.5	9.0	9.5
% germination after 24h[a]	0	0	0	84	98	99	96	99	28	0

a = 100 spores counted

(f) Relative Humidity and Free Water

Germination levels at 100 and 97% R H always approached 100% but at 96 and 92.5% R H results were extremely variable in experiments performed on different occasions. However, it is clear that high humidity favoured germination as with fungi in general (Cochrane, 1958).

A requirement for free water is suggested by the experiments with the thixotrophic agent, carboxymethyl cellulose (CMC). At low concentrations of CMC and in SLM alone, thinly layered suspensions dried considerably during the overnight incubation period although the slides were at high humidity in Petri-dishes, and as a result, germination levels on these slides were reduced (Table 4.09). Germination levels for the unlayered suspensions were in the upper 90's. This partial drying presumably occurred before complete equilibration of humidity had taken place in the Petri-dishes. Thus, although the humidity in the dishes must have been very high the poorer germination levels in the areas where free water was absent suggest that free water is a prerequisite for germination.

TABLE 4.09

Effect of Free Water on *Verticillium lecanii* Conidiospore Germination Levels (%) (approximately 200 spores/slide)

Regime	Concentration of carboxymethyl cellulose (%)				
	DW[a]	0.05	0.1	0.5	1.0
Unlayered[b]	92.6	96.7	97.4	96.8	97.5
Thinly layered[c]	24.4	27.1	6.9	48.3	91.5

a = Distilled water

b = Unlayered - drops placed on glass slide and not spread thinly

c = Layered - drops placed on glass slide and spread thinly

9. Longevity of Conidiospores and Cultures

(a) Temperature

Conidiospore longevity in distilled water varied greatly, especially among those spores stored at 2°C, even though all batches were cultured from deep-frozen (-17°C) cultures all derived from the same single-spore isolate.

Viabilities of spores, frozen at -17°C for 24h, fell only slightly after thawing (Figs. 4.07 - 4.09). However, spores of the first batch died upon prolonged storage at -17°C, more rapidly than spores stored simultaneously at 2°C (Fig. 4.07). In contrast, spores of the second batch, after 80 days storage at -17°C, died less rapidly than the spores stored simultaneously at 2°C (Fig. 4.08). Figure 4.09 illustrates the results of 3 more batches of spores at -17, 2 and 20°C but not stored simultaneously. The spores at -17°C died more rapidly than previous batches of spores stored at this temperature, and spores at 2°C exhibited no reduction in viability after 6 months, very much in contrast to previous experiments (Figs. 4.07 and 4.08). At 20°C, spores in distilled water did not germinate during storage and retained their viability for as long as one previous spore batch stored at 2°C (Fig. 4.08).

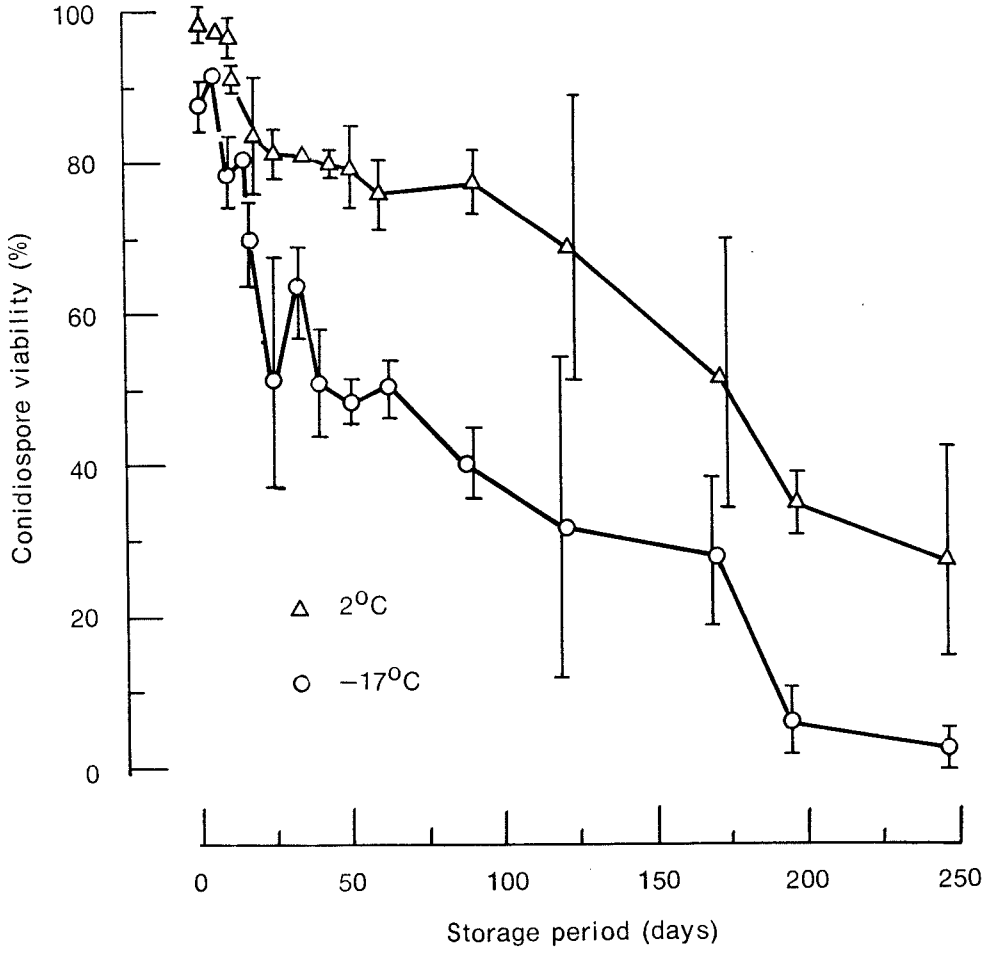


Fig. 4.07

Longevity of *Verticillium lecanii* conidiospore batches stored simultaneously at 2 and −17°C (points are means, with 95% fiducial limits, of 3 spore samples removed from each batch).

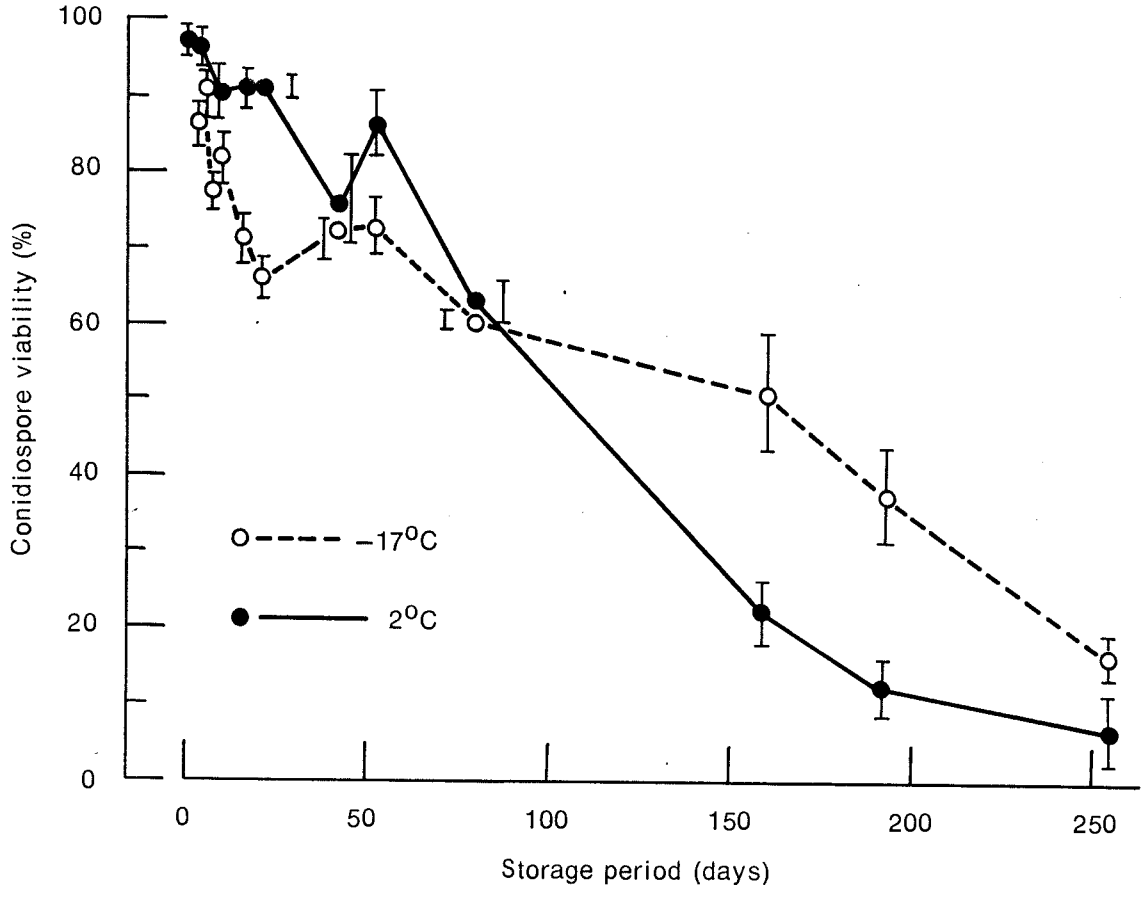


Fig. 4.08

Longevity of *Verticillium lecanii* conidiospores stored at 2 and -17°C (points are means, with 95% fiducial limits, of counts from 3 spore samples removed from each batch).

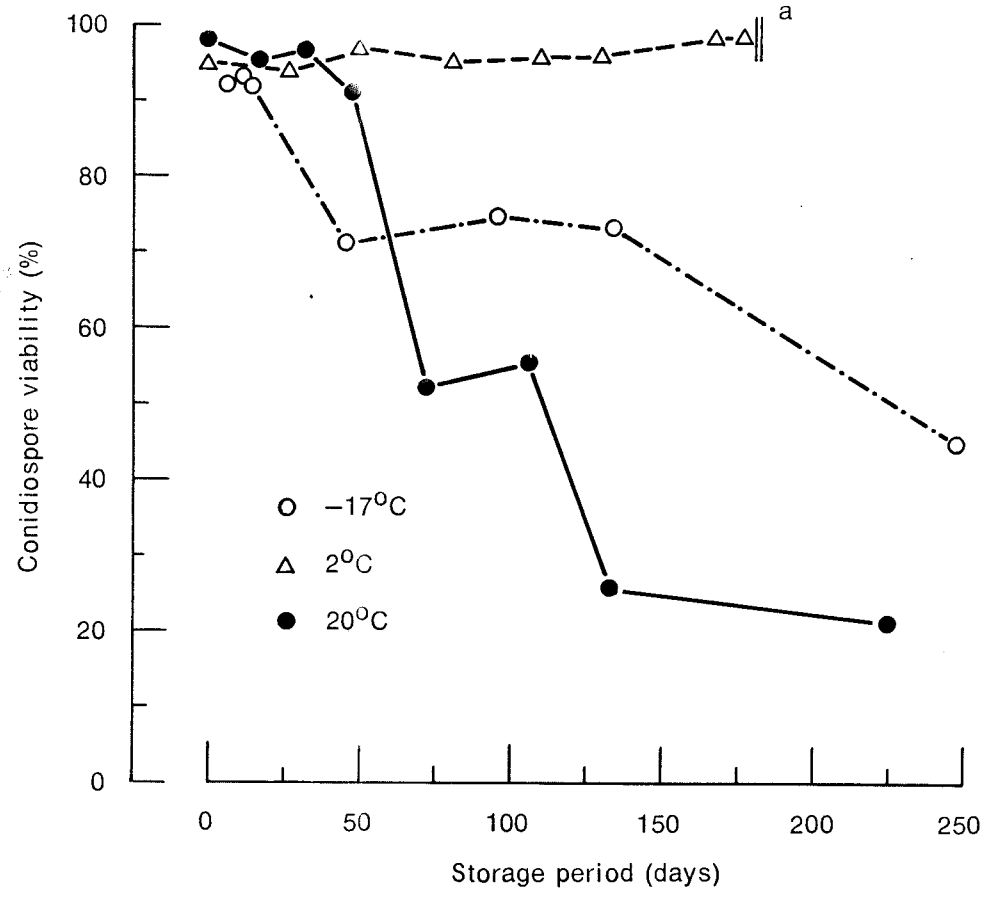


Fig. 4.09

Longevity of 3 batches of *Verticillium lecanii* conidiospores stored at different times at −17, 2 and 20°C (points are counts from one spore suspension sample removed from each batch).

[a] Spores used for experimental purposes

(b) Antibiotics and Wetting Agents

The survival of conidiospores in the presence of antibiotics and wetting agents was tested since these may be included in future preservation formulations as with the formulation developed for Beauveria tenella spores (Blachère et al., 1973).

An antibiotic, either streptomycin or tetracycline, was incorporated in each of several stored batches of conidiospores in distilled water at 2°C. In 2 experiments, neither antibiotic at concentrations up to 100 µg/ml harmed the viability of stored conidiospores (Fig. 4.10).

The wetting agent, Tween 80 rapidly killed spores and this effect increased with concentration. After a longer period of storage, Triton X-100 exerted a smaller concentration - dependent harmful effect upon viability (Fig. 4.11).

(c) Relative Humidity

The effect of relative humidity was investigated on both unwashed spores (spores still in slime heads), either attached to, or detached from the parent mycelium (IV, 4, c), and spores harvested in water (washed spores; IV, 1, L). This is of interest from (i) an ecological viewpoint where spores are sprayed on to plants in an aqueous suspension or deposited by diseased insects on leaf surfaces and (ii) a practical viewpoint, since ultimately spores may be stored in bulk in a dry or semi-dry state, as with Beauveria tenella spores (Blachère et al., 1973). The cellophane technique (IV, 4, c) was developed to study the effects of humidity on spores. Certain preliminary experiments performed during its development are worthy of note.

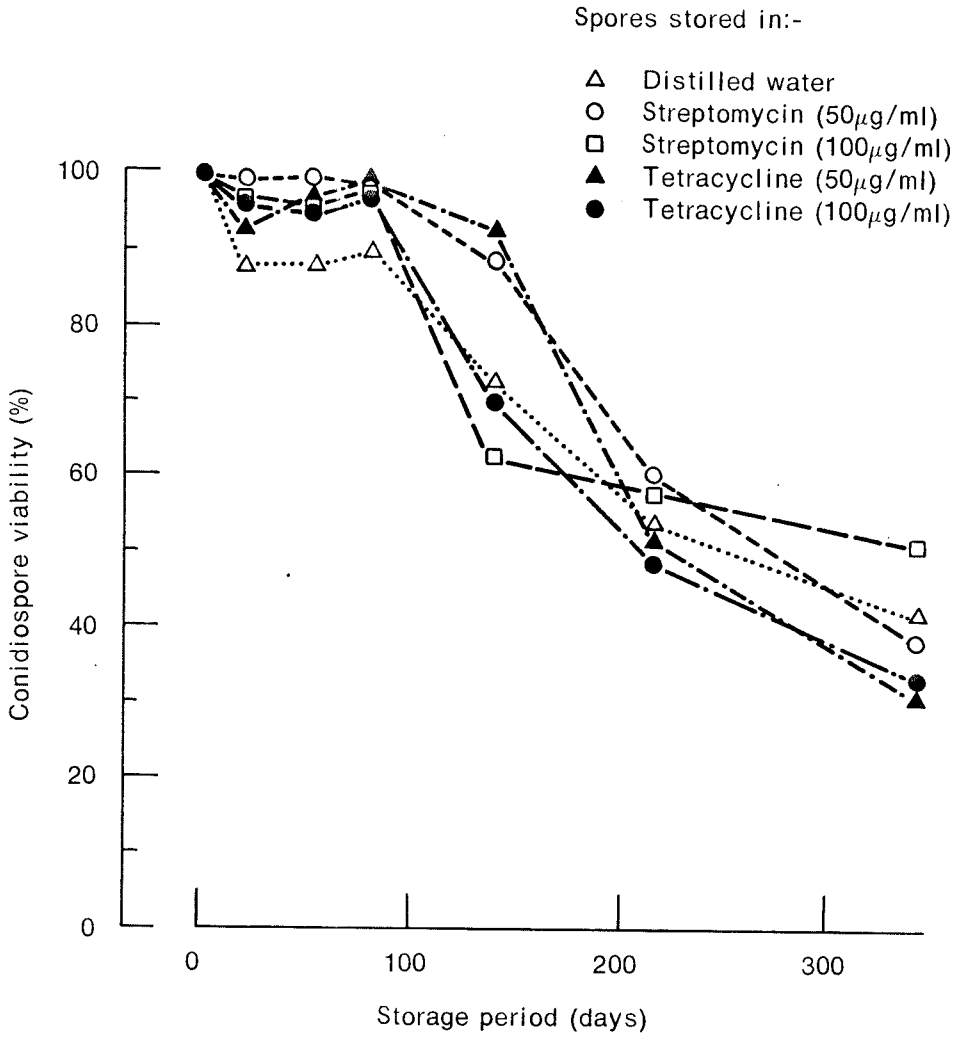


Fig. 4.10

Longevity of *Verticillium lecanii* conidiospores stored in antibiotic solutions at 2°C (points are counts from one spore suspension sample removed from each batch).

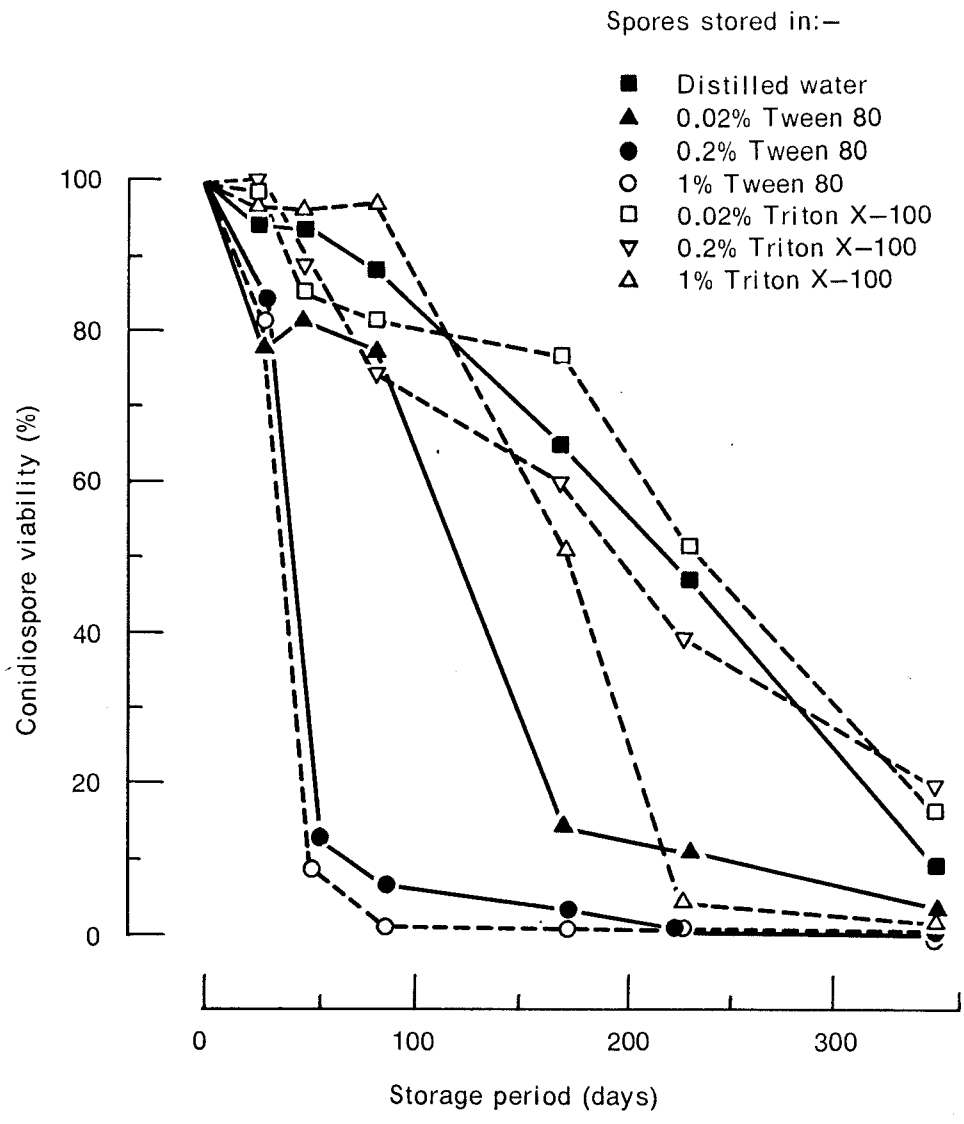


Fig. 4.11

Longevity of *Verticillium lecanii* conidiospores stored in wetting agent solutions at 2°C (points are counts from one spore suspension sample removed from each batch).

After preliminary investigations involving washing dried conidiospores off slides, it became clear that spores lost their viability rapidly upon desiccation (Fig.4.12). Thus it was imperative that fresh spores should be equilibrated at the humidities under test. The first method involved spreading a thin layer of spores in distilled water on clean slides and equilibrating at the test humidities produced over glycerol-water mixtures (Johnson, 1940). After periods of exposure over these mixtures, spores were washed off the slides and viabilities assessed by the agar-slide technique. However, even when precautions such as using ultra-clean glassware were taken, this method proved unsatisfactory; anomalous and unaccountable changes in viability levels were recorded from replicate slide to slide and particularly from day to day (Table 4.10).

TABLE 4.10

Effect of Desiccation of *Verticillium lecanii* Conidiospores on Ultra-clean Glass Slides Held over Glycerol-Water Solutions (20 ± 1°C)

Hours desiccation	% Relative humidity ± S.E.[a]					
	0	20	44	75	100	DW[b]
6	24 ±2.32	30.3 ±1.99	23.7 ±1.62	28.8 ±2.6	82.4 ±1.0	87 ±0.69
24	64.1 ±2.07	69.3 ±1.5	57.9 ±1.79	20.8 ±1.04	87.9 ±1.33	96.8 ±0.39
48	38.1 ±3.59	42.0 ±1.93	22.8 ±1.15	1.3 ±0.54	64.1 ±1.49	84.0 ±2.49

a = Standard error of mean of counts from 3 agar slides

b = Distilled water

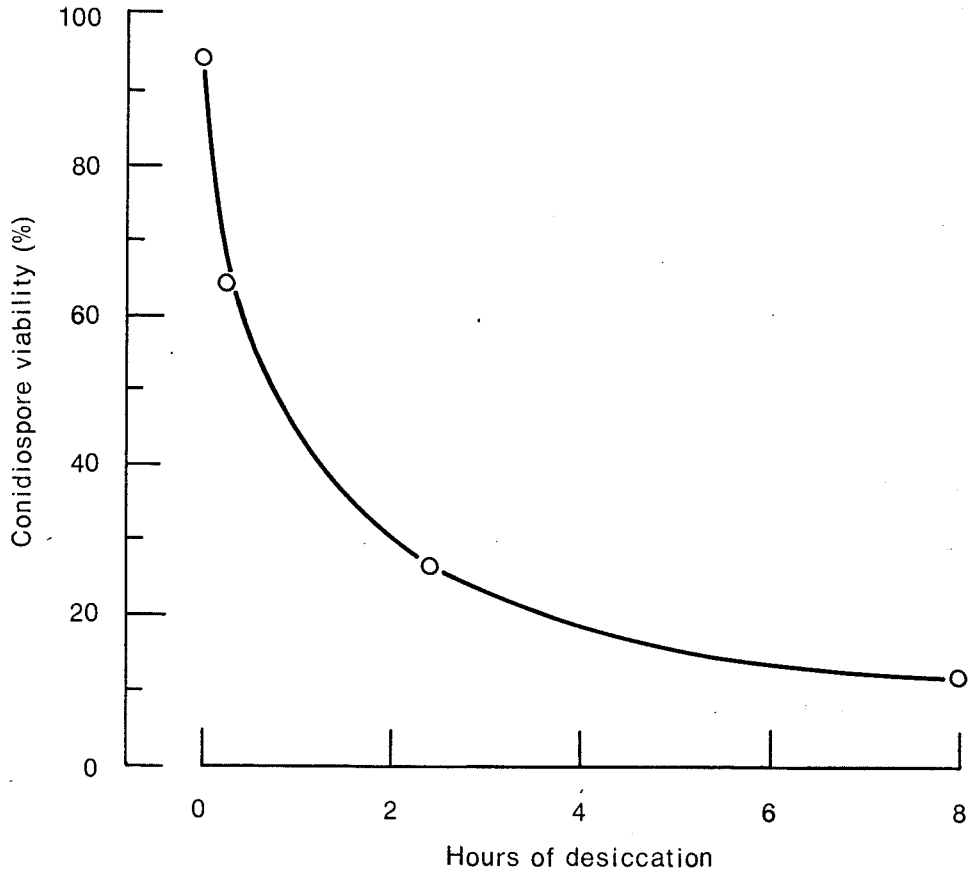


Fig. 4.12

Loss in viability of a batch of *Verticillium lecanii* conidiospores (uncontrolled conditions, room temperature, room humidity).

Equilibrating spores on filter paper gave similar unsatisfactory results. The cellophane technique was more successful probably because it obviated the possible variations due to washing spores off glass slides or filter paper prior to viability assessment.

Using the cellophane technique in 2 experiments, washed and unwashed spores were held at different humidities over saturated salt solutions (Fig. 4.13a,b). Both sets of spores lost viability with decreasing relative humidity. The death rate of unwashed spores was initially higher than that of washed spores but after 36-48h equilibration, this difference was lessened. Also, in contrast to washed spores, unwashed spores survived better at 0% than at 58% RH. Although there was slight overlap of 95% fiducial limits between washed and unwashed spores (particularly in Fig. 4.13b), the above results were confirmed in further experiments (see below).

That spore death below 100% RH was a function of the relative humidity itself and not the desiccating agent was demonstrated by holding unwashed spores over saturated salt solutions and over sulphuric acid solutions for 24h (Fig.4.14). The spore death levels were almost identical over both sets of solutions.

Longevities of washed and unwashed detached spores were compared with those of spores still in association with their mycelium, either on sporulating aphid bodies, or on 10-day old agar culture mycelium (<u>without</u> agar - see IV,4,c). In all cases, high humidities favoured spore survival (Figs. 4.15 and 4.16). Unwashed, detached spores on cellophane died most rapidly (Fig.4.15) as in previous experiments (Fig.4.13a,b). Survival of these spores was again better at 0% than at 58% in the early stages of exposure (Fig.4.15), but this was not so for the other 3

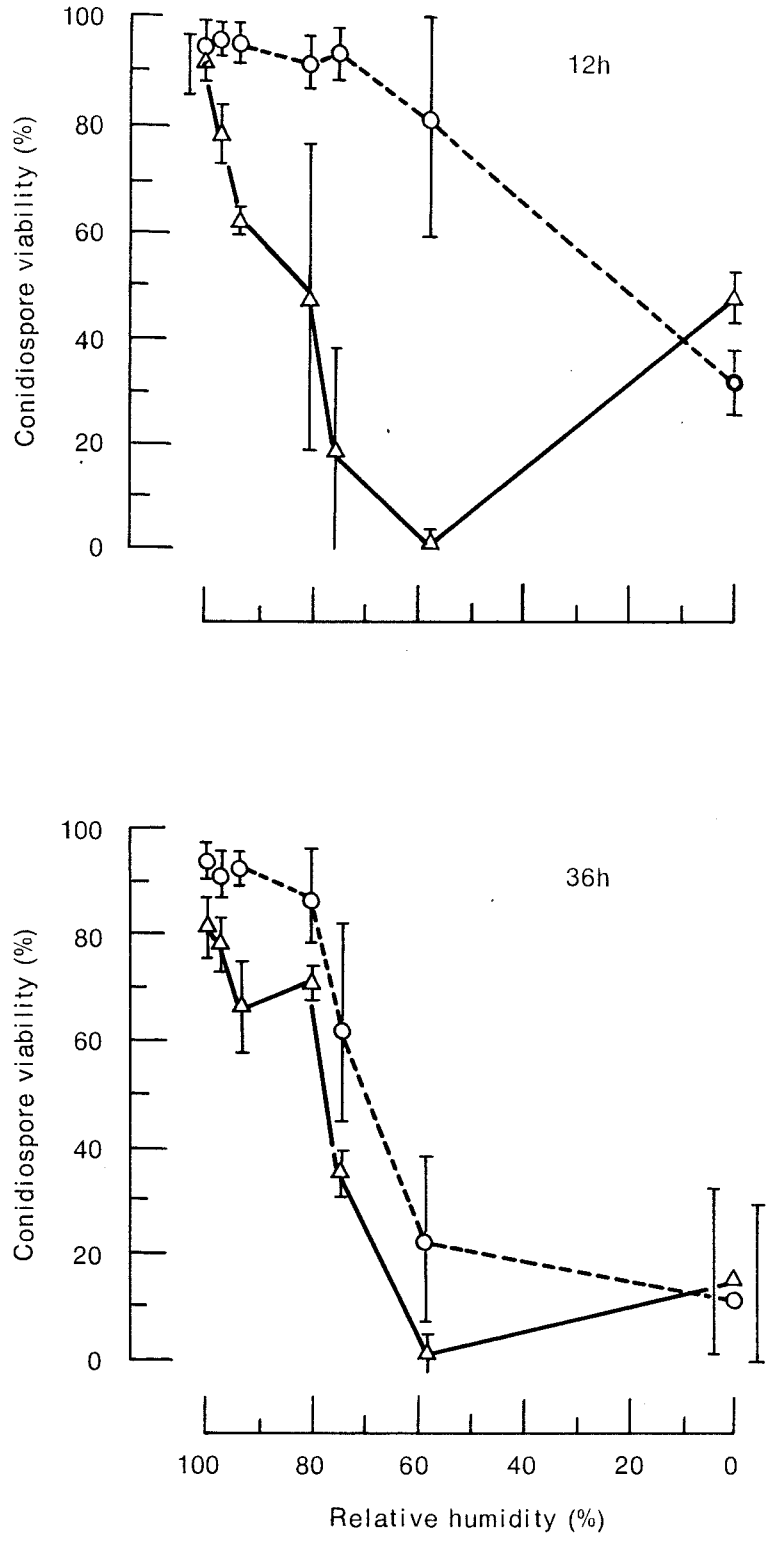


Fig. 4.13a.

Effect of 12 and 36 hours exposure to different humidities upon washed ○ and unwashed △ *Verticillium lecanii* conidiospores on cellophane squares at 20 ± 1°C (points are means, with 95% fiducial limits, of counts from 2 cellophane squares, each from separate Kilner jars).

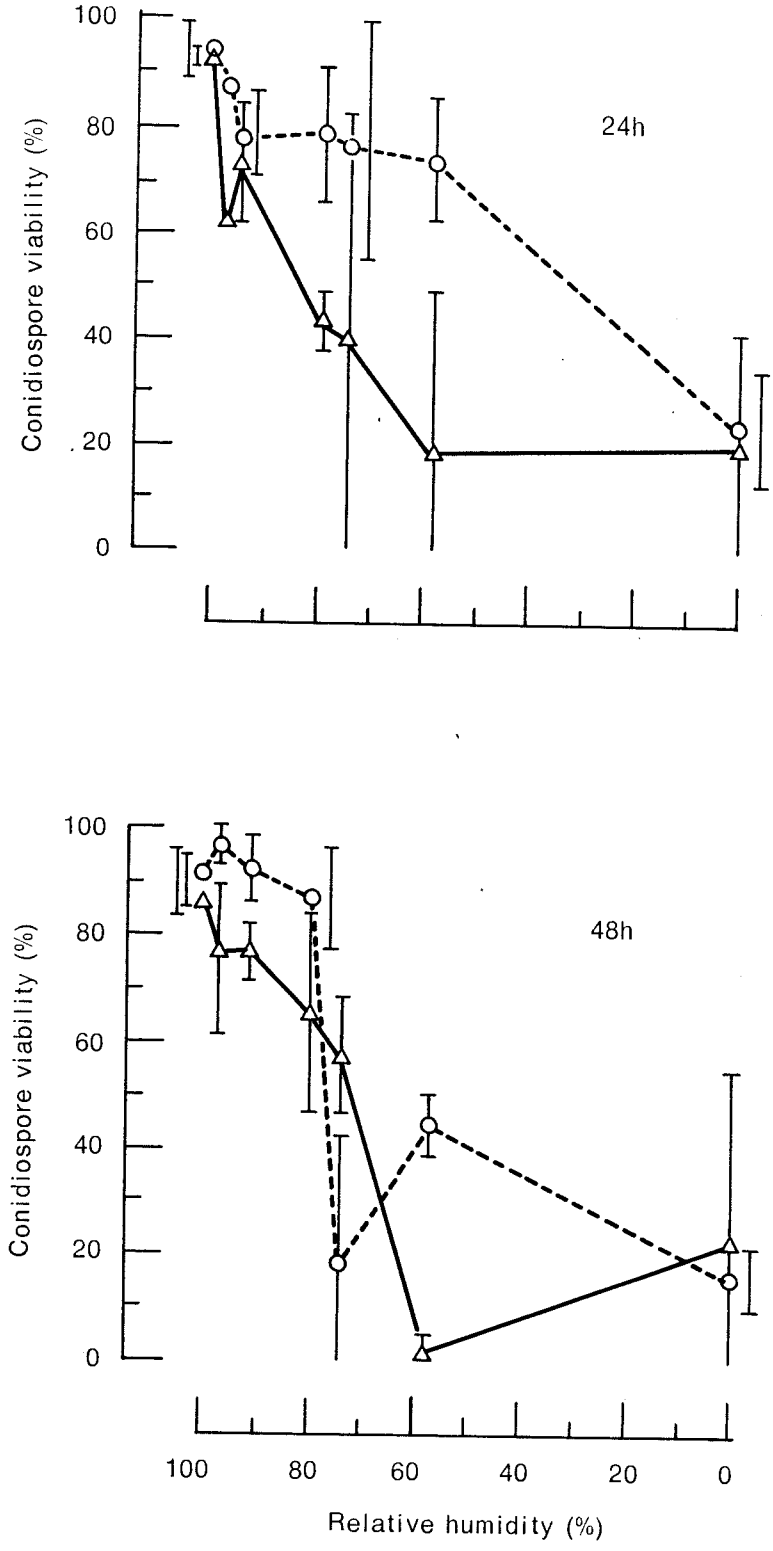


Fig. 4.13b.

Effect of 24 and 48 hour exposure to different humidities upon washed ○ and unwashed △ *Verticillium lecanii* conidiospores on cellophane squares (20 ± 1°C). Points are means, with 95% fiducial limits, of counts from 2 cellophane squares each from separate Kilner jars .

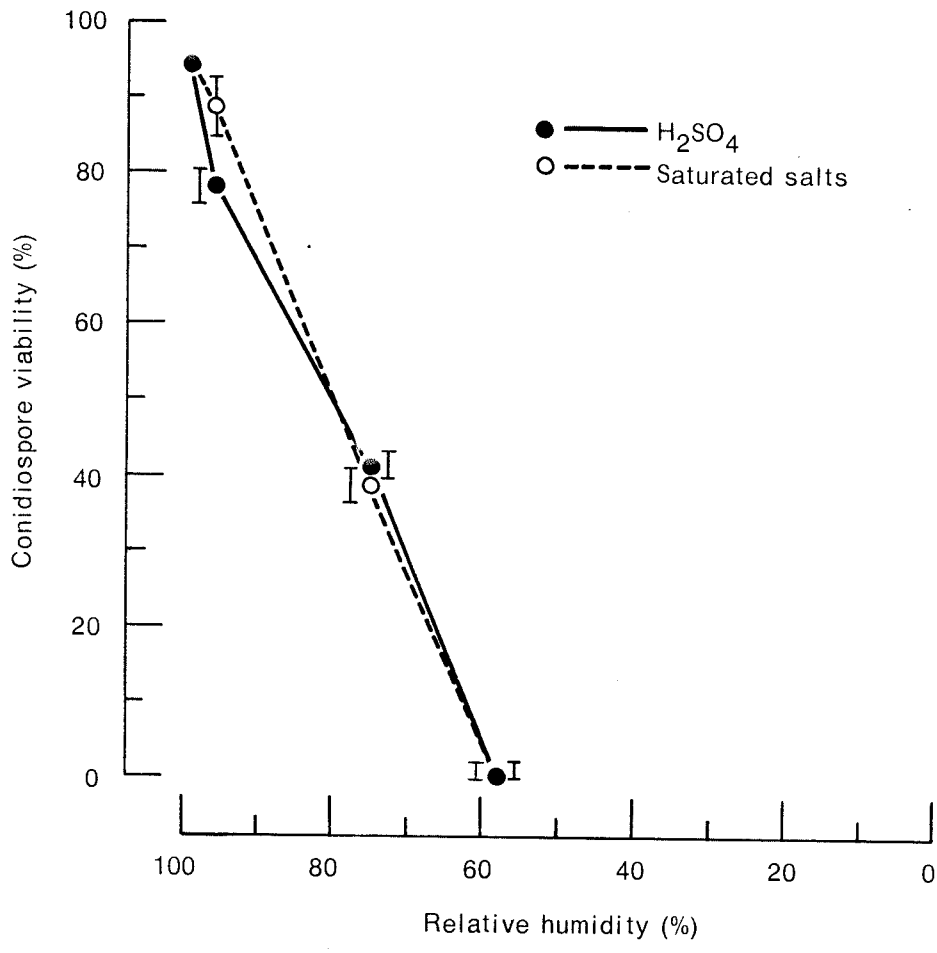
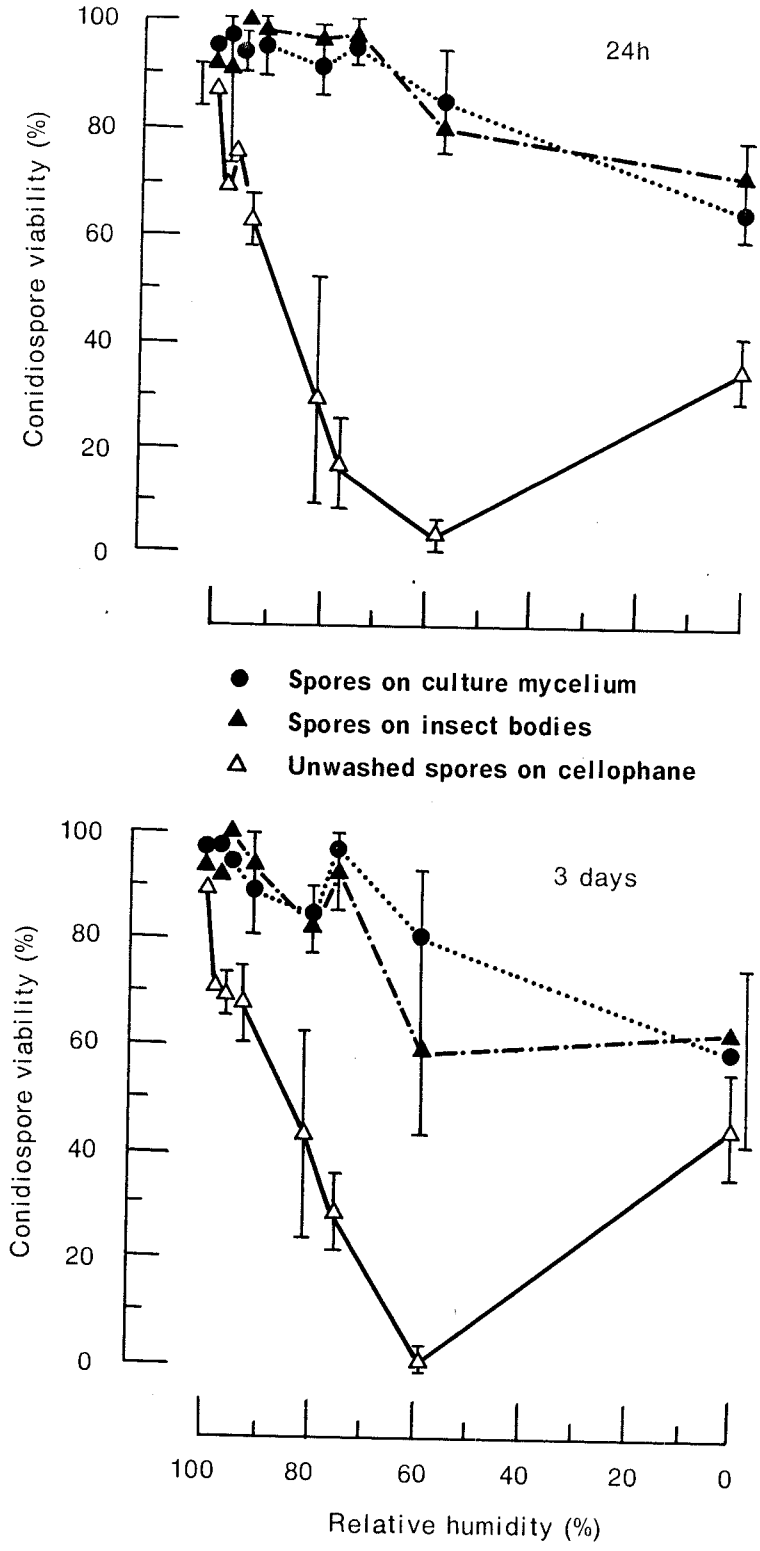


Fig. 4.14.

Effect upon viability of equilibrating *Verticillium lecanii* conidiospores over saturated salt or H_2SO_4 solutions for 24h at 20 ± 1°C.



Part of Fig. 4.15.

Effect of 24 hours and 3 days exposure to different humidities on *Verticillium lecanii* conidiospore viability at 20 ± 1°C (points are means, with 95% fiducial limits, of counts from 2 replicates, each from separate Kilner jars. For clarity, only the highest and lowest limits included for ● and ▲).

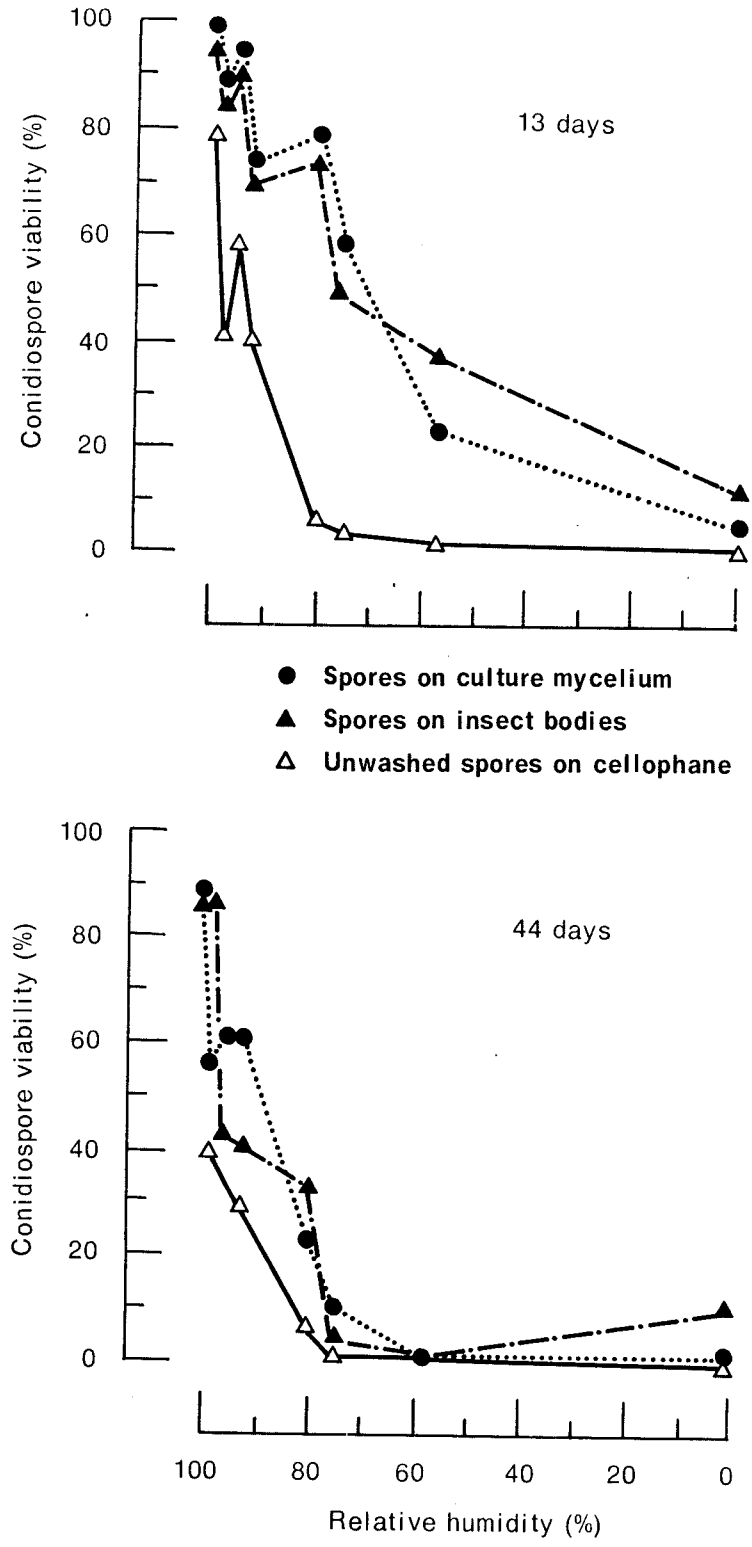
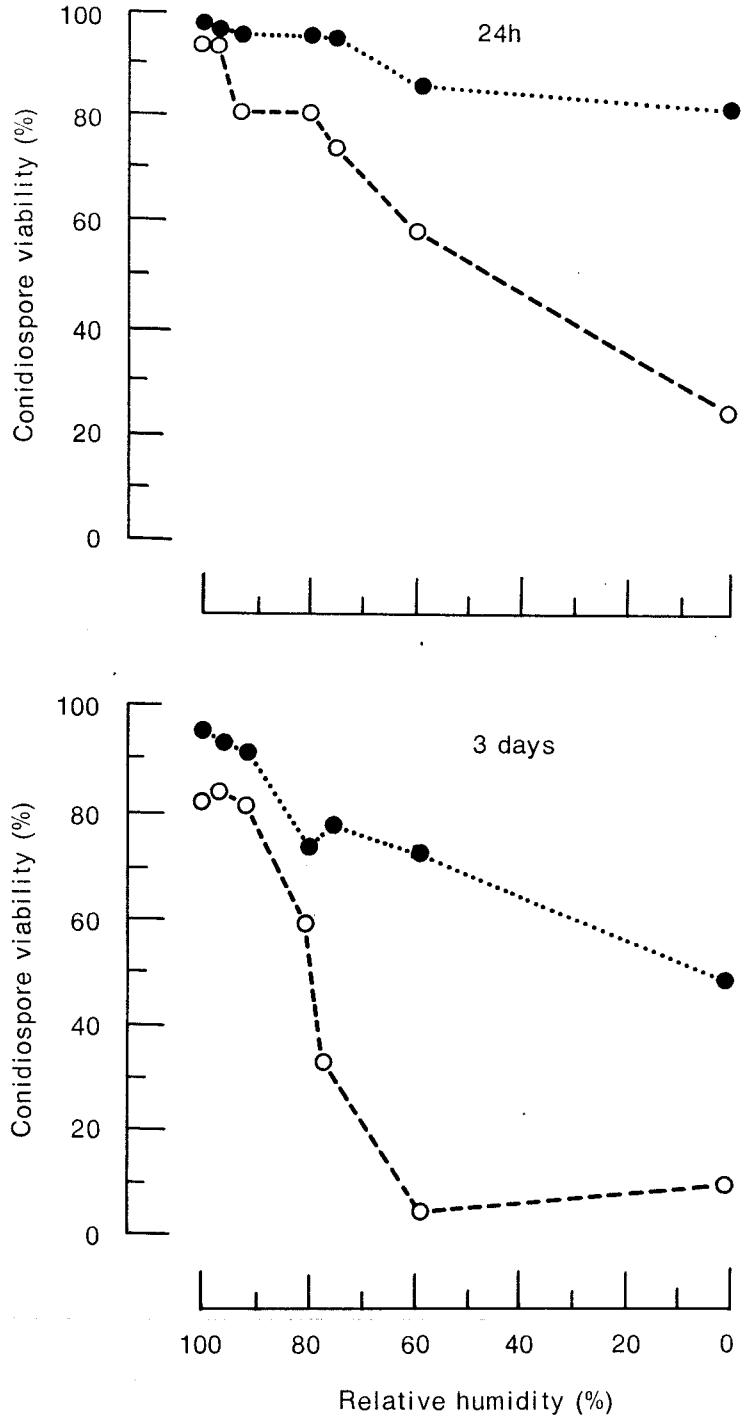


Fig. 4.15. (Continued)

Effect of 13 and 44 days exposure to different humidities upon *Verticillium lecanii* conidlospores. (points are means of counts from 2 replicates, each from separate Kilner jars. For clarity, 95% fiducial limits omitted).



Part of Fig. 4.16

Effect of 24 hours and 3 days exposure to different humidities on *Verticillium lecanii* conidiospores on culture mycelium ● and on washed conidiospores O on cellophane squares at 20 ± 1°C (points are counts from one sample only).

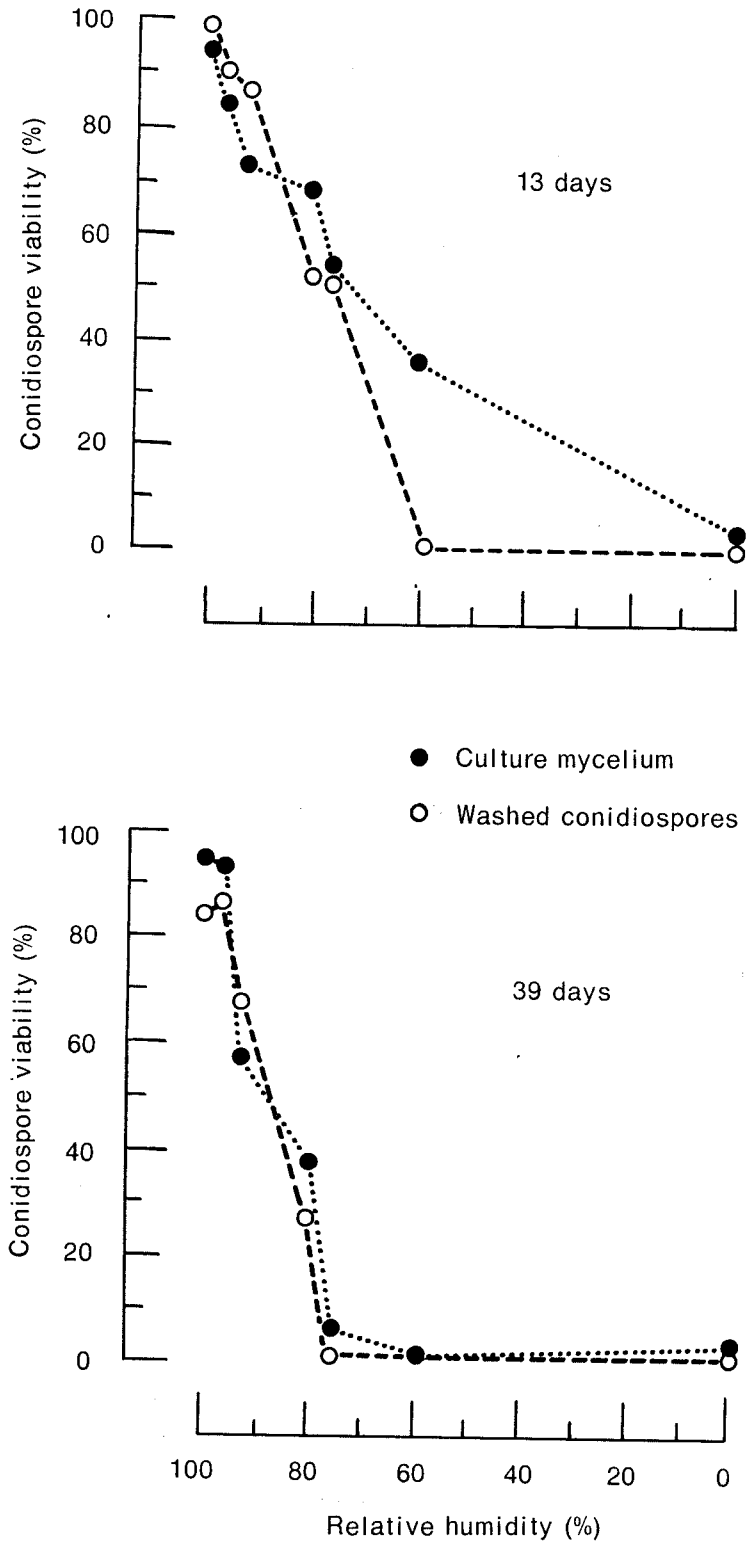


Fig. 4.16 (Continued)

Effect of 13 and 39 days exposure to different humidities on *Verticillium lecanii* conidiospores on culture mycelium ● and washed conidiospores ○ on cellophane squares at 20 ± 1°C (points are counts from one sample only).

spore regimes. In the latter, washed spores at first survived less well than spores on aphid bodies or culture mycelium (Fig. 4.16) but after prolonged storage, survival levels of all these spores were similar and were still high after 39 and 44 days towards 100% RH (Figs. 4.15,4.16).

(d) Spores in Glasshouses

The longevity of conidiospores on aphid bodies in a glasshouse (VI;Experiment 7) was investigated in a Myzus persicae population virtually eliminated by V.lecanii. Bodies were, at first, removed from both leaf surfaces but later only from the lower surface since watering washed bodies off the upper surface. Spore viability on individual aphid bodies was assessed by the methods of IV,4,c. Most spores on bodies on lower leaf surfaces survived at least 39 days (Table 4.11). Limited data from upper leaf surfaces indicate that more spores died there. If this is so, it might be explained by exposure of bodies on upper leaf surfaces to sunlight and possibly a lower microclimate humidity.

In another glasshouse experiment (VI;Experiment 8) 5 diseased bodies were removed from the undersides of leaves in Bed 6 (Fig. 6.19a) on the 46th day after spraying. These aphids had probably been dead for about 30 days during which time the weather was unusually hot (Fig. 6.19a). The mean spore viability (%) from the 5 aphid bodies was 89.6 $\pm$ 2.9.[1]

Since in the previous section, spores survived best at high humidity, the glasshouse data suggest that the humidity close to leaf surfaces in these experiments was very high, approaching 100% RH, and influence of the microclimate extended beyond the leaf surface at least as far as the aphid bodies. Others (Ramsey et al., 1938) have shown that humidity above a leaf surface may be affected up to a height of over 1 cm by the water vapour coming from the leaf.

[1]Standard error

TABLE 4.11

Viabilities of Verticillium lecanii Conidiospores on Aphid Bodies Removed from Chrysanthemum Leaf Surfaces in a Glasshouse

Days after death of aphids	Leaf surface position			
	n	Upperside	n	Underside
10	4	82.7 ± 4.2[b]	3	96.3 ± 0.88[b]
15	2	80.6 ± 8.8[b]	5	92.4 ± 7.17[b]
18	1	28	6	88.9 ± 4,3[b]
25	-[a]	-	5	96.5 ± 0.61[b]
31	-	-	5	98.6 ± 0.51[b]
39	-	-	4	86.3 ± 6.4[b]

a = No more bodies recoverable by this time.

b = Standard error of mean of viable counts from n aphid bodies

(e) Preservation of Conidiospores in Liquid Nitrogen

Metabolism virtually stops at the temperature of liquid nitrogen (-196°C) and so spores should survive almost indefinitely if they can survive freezing and thawing. Virtually all V.lecanii conidiospores survived freezing and thawing when distilled water was the suspending medium (Table 4.12), and this result shows there is good potential for the indefinite storage of viable spores. However, when the cryoprotectant 10% glycerol was used, viabilities after freezing and thawing were lower after 4 days than those of the unfrozen control spores in distilled water (Table 4.12). Furthermore, the viabilities of unfrozen control spores in the cryoprotectant were reduced by more than 50% after 4 days (Table 4.12). Clearly this cryoprotectant, as used by others (Hwang, 1968; Goos et al.,1967), was toxic to V.lecanii conidiospores. These authors did not study the need for the cryoprotectant.

TABLE 4.12

Effect of 4 days Storage in Liquid Nitrogen and Subsequent Thawing on *Verticillium lecanii* Conidiospore Viability

Experiment number	Regime	% Viability after 4 days ± S.E.[a]
1	Spores in distilled water stored in liquid N_2	98.4 ± 0.02
	Unfrozen spores in distilled water stored at 2°C	98.8 ± 0.41
2	Spores in 10% glycerol stored in liquid N_2	88.9 ± 3.1
	Unfrozen spores in 10% glycerol stored at 2°C	46.2 ± 0.2
	Unfrozen spores in distilled water stored at 2°C	99.6 ± 0.4

a = Standard error of viability means of 2 suspensions in "straws" (3 counts /"straw")

(f) Preservation of Fungal Isolates

Sabouraud dextrose agar slope cultures in small, screw top "Bijoux" bottles were stored at 3 temperatures, -17, 2 and 20°C. When the rubber-lined screw caps were screwed tight and the bottles held at 2 and 20°C, cultures died in a few weeks, presumably due to a lack of oxygen or an accumulation of volatile toxic metabolite(s). When caps were loose, cultures slowly desiccated and at 20°C were dead after 6 - 9 months but survived for up to 2 years at 2°C. However, cultures survived best at -17°C. As in previous storage experiments, the initial freezing followed by thawing after a short period, did not kill the spores but, as judged by the agar-slide viable count method, spores on slopes all died within 2 years when stored at -17°C (Fig. 4.17 and Table 4.13). However, when these apparently dead

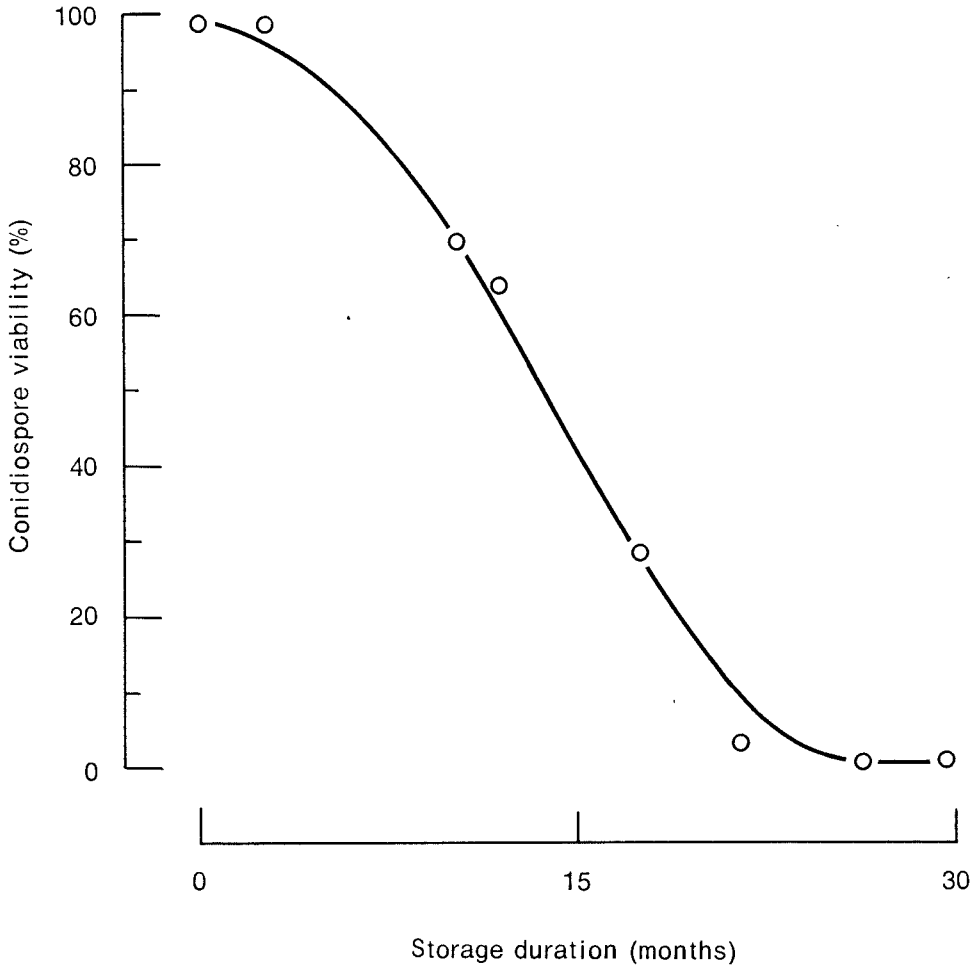


Fig. 4.17

Longevity of *Verticillium lecanii* conidiospores on Sabouraud dextrose agar slope cultures stored simultaneously at −17°C (points are counts from one sample only).

cultures were subcultured on to agar with an inoculating loop, some growth occurred, indicating that a very small proportion of spores or some fragments of mycelium survived (Table 4.13).

TABLE 4.13

Verticillium lecanii Conidiospore Viability on Agar Slope Cultures at -17°C

(Results accrued from slopes of IMI 179172 not cultured and stored simultaneously).

Storage period	Viability	Viability when streaked on agar
2 days	98.4	
3 months	86	
8 months	36.8	
10 months	48.9	
24 months	0	+[a]
30 months	0	+

a = Growth on agar recorded

Isolates of *V.lecanii* stored at -17°C on slopes have survived for more than 3 years. Under the same conditions, *Fusarium solani*, *Metarrhizium anisopliae* and *Penicillium* sp. spores were dead after 30 months but *Beauveria bassiana*, *Paecilomyces farinosus* and *Aspergillus flavus* were still viable after 3 years.

Mini-SDA slope cultures of these species in freeze-drying ampoules were lyophilized. All lyophilized cultures of fungi, including the species which did not survive at -17°C, were still viable after 3 years storage.

V.lecanii on aphid bodies stored at 2°C, was dead after 15 months storage, and, at -17°C, in all but one out of 4 batches after 2 years. From this one surviving batch, fungus was recovered after 3 years storage.

10. Comparisons of V.lecanii Strains

(a) Morphology

Descriptions of colonies on SDA and the conidial dimensions of most of the V.lecanii strains are shown in Table 4.14. These strains were originally isolated from a very wide variety of substrates and some strains differed greatly in their gross morphology e.g. C-42, C-50 but the appearance in culture of most approximated to Gams' (1971) description of V.lecanii (Chapter II). Dimensions of spores, suspended in distilled water, also varied widely (Table 4.14) and did not form size groups, but the range of lengths nevertheless fell within the range for the strains of Gams (1971). However, the widths in Table 4.14 exceeded those given by Gams, possibly because he used a different suspending medium.

Other characteristics varied from strain to strain. Amongst both small and large spore-formers there was a wide diversity of sporulation abilities on SDA. Another variable was the occurence of verticillate whorls which ranged from very many for C-21, to none for C-35.

(b) Temperature

The effect of temperature on the growth rate of V.lecanii (C-3;IMI 179172) was studied in IV, 7, b and the upper temperature limit for growth was 30°C. Table 4.15 shows that this is not so for all isolates of V.lecanii. Many isolates grew well at 31°C, 4 isolates grew at 34°C and one, C-35 an isolate from a rust fungus, grew slightly at 36°C. The ability to sporulate was, with all isolates, slightly less tolerant of high temperatures than was vegetative growth, as with virtually all fungi (Hawker, 1950).

TABLE 4.14

Gross Morphology and Conidiospore Dimensions of *Verticillium lecanii* Strains

Strain number	Source	Gross colonial morphology on SDA	n	Conidial dimensions (μ) ± S.E.[a] Length	Breadth
C-3 (IMI 179172)	Single spore isolate ex *Macrosiphoniella sanborni* (G.C.R.I)	Floccose, white wide periphery; powdery lemon yellow centre	103	6.26 ± 0.11	1.73 ± 0.02
C-1[b]	*M. sanborni* (G.C.R.I.)	As for C-3	32	6.89 ± 0.27	2.0 ± 0.05
C-4	*Brachycaudus helichrysi* (G.C.R.I.)	Off white, wholly cottony - powdery	33	3.34 ± 0.16	1.8 ± 0.06
C-13	*Trialeurodes vaporariorum* (G.C.R.I.)	White, floccose, even	41	4.11 ± 0.12	1.95 ± 0.05
C-19	Possibly *Aphis gossypii*. U.K.[c]	White, floccose, slightly furrowed yellowing with age	34	5.95 ± 0.15	2.1 ± 0.07
C-21	*Brachycaudus helichrysi* (G.C.R.I.)	Off white, wholly cottony -powdery	33	3.41 ± 0.09	1.75 ± 0.05
C-30	*Uromyces appendiculatus* (Cambridge)	Pure white, even, exuding droplets on surface of mycelium	31	3.52 ± 0.14	1.96 ± 0.05

TABLE 4.14 (cont.)

Strain number	Source	Gross colonial morphology on SDA	n	Conidial dimensions (μ) ± S.E.[a] Length	Breadth
C-32	Saissetia oleae (Israel)	White, floccose periphery, floccose bright lemon centre, furrowed, bright lemon colour in SDA	32	3.94 ± 0.12	2.02 ± 0.05
C-33	Ceroplastes floridensis (Israel)	White floccose border, pale yellow floccose centre	37	4.67 ± 0.06	2.19 ± 0.1
C-35	Uromyces appendiculatus (Cambridge)	Pure white, even, exuding droplets on surface of mycelium	32	3.36 ± 0.12	1.94 ± 0.04
C-39	Contact Lens (Bristol Royal Infirmary)	Pure white, even, floccose	31	3.72 ± 0.15	2.02 ± 0.09
C-41	Pulvinaria floccifera (Turkey)	Slightly off-white, ridged, wholly floccose - cottony	32	3.68 ± 0.14	2.27 ± 0.10
C-42	Myzus persicae (India)	Powdery, beige centre; cottony, beige-cream periphery	31	9.36 ± 0.27	2.7 ± 0.10
C-43	Brachycaudus helichrysi (G.C.R.I.)	Slightly off-white, deep floccose centre; floccose white periphery	32	4.47 ± 0.15	2.14 ± 0.04
C-44 (CBS 340.37)	Puccinia graminis (Gams, 1971)	Pure white, cottony, furrowed in periphery	32	5.33 ± 0.18	2.17 ± 0.05
C-45 (CBS 383.35)	Rust on chrysanthemums (Gams, 1971)	Pure white, floccose	34	3.82 ± 0.15	1.94 ± 0.04

TABLE 4.14 (cont.)

Strain number	Source	Gross colonial morphology on SDA	n	Conidial dimensions (μ) ± S.E.[a] Length	Breadth
C-46 (CBS 470-73)	*Hemileia vastatrix* (India)	Pure white ± floccose border, floccose, deep lemon yellow centre, colonies high-domed	32	3.97 ± 0.14	2.2 ± 0.15
C-47 (CBS 413-70)	*Hemileia vastatrix* on Coffee (New Caledonia)	Pure white, deep, floccose, domed.	31	2.43 ± 0.06	1.52 ± 0.09
C-48	*Scolytus scolytus* (Frimley, Surrey)	Off white, deep, floccose, even	31	3.39 ± 0.11	1.86 ± 0.04
C-49	*Erysiphe graminis* (Oxford)	White, deep, cottony	34	5.12 ± 0.10	1.75 ± 0.06
C-50 (CBS 110-70)	*Puccinia graminis* (Baarn, Netherlands)	Grey periphery, radially striate, white "rough" centre	31	4.5 ± 0.16	2.10 ± 0.04
C-52	*Macrosiphoniella sanborni* (G.C.R.I.)	Pure white, deep cottony-floccose	30	4.17 ± 0.10	1.83 ± 0.05

a = Standard error of mean of n observations taken with an eyepiece micrometer
b = This strain used to kill aphid from which C-3 was derived
c = Original host record not available

Unexpectedly, strains from tropical and arid countries, such as C-42 from India, and C-32 and C-33 from Israel, were less tolerant to high temperature than those from temperate zones, such as C-35 and C-49 from England, and C-50 from the Netherlands. Strains showing the best growth at 34°C all originated from non-insect material, although some strains from rust fungi did not grow at 31°C (Table 4.15).

TABLE 4.15

Upper Temperature Limits for Growth of *Verticillium lecanii* Isolates

Isolate number	Temperature °C 31 g[a]	31 s[b]	34 g[a]	34 s[b]	36 g[a]	36 s[b]
C-3 (IMI 179172)	-	-				
C-21	+++	+	-	-		
C-32	++	+	-	-		
C-33	-	-				
C-35	+++	+	++(+)	+	(+)	-
C-39	(+)	-				
C-41	++	-	-	-		
C-42	-	-				
C-43	++	+	(+)	-	-	-
C-44	+	-				
C-45	+++	+	-	-		
C-46	-	-				
C-47	(+)	-				
C-48[c]	-	-				
C-49	+++	+	++	-	-	-
C-50	+++	+	+	(+)	-	-

a = Growth
+++ luxuriant
++ moderate
+ poor
(+) slight
- no growth

b = Sporulation
+ many spore heads
(+) very slight sporulation
- no sporulation

c = Results of Barson (1976a)

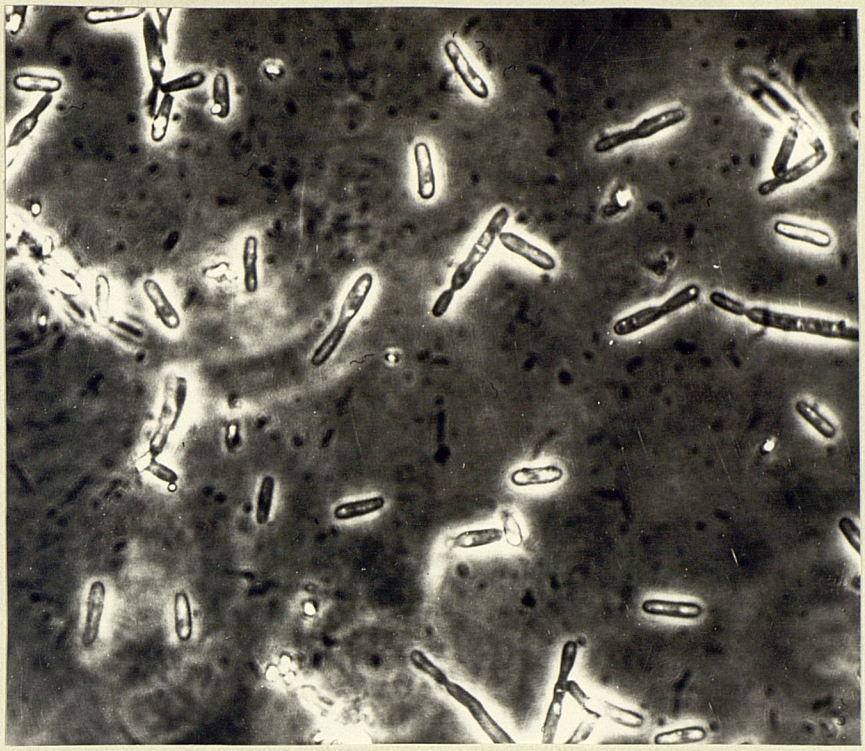
11. Factors Affecting Blastospore Formation

(a) Aeration and Agitation

V.lecanii conidiospores were variously aerated and agitated in SLM (IV, 5, a). Increased aeration delayed blastospore formation and also produced the highest yields (Table 4.16). Whilst agitation per se was essential for good blastospore production, the mode of agitation was also important. On a rotary shaker, growth was predominantly mycelial (Table 4.16). Reciprocal agitation or magnetic stirring produced limited filamentous growth (even when aerated) followed by a semi-yeast phase (Fig.4.18), in which secondary blastospores arose from short elements (primary blastospores) usually from one pole but occasionally from both poles (Fig.4.18). Tertiary blastospores were formed similarly.

Reciprocally agitated cultures, not vigorously aerated displayed little initial hyphal growth, whereas vigorously aerated cultures showed more hyphal growth before forming blastospores. This suggests that a metabolite causing the change to yeast-like growth may have accumulated in the SLM, and was removable to a degree by aeration. However the existence of such an accumulated metabolite would not explain the predominantly filamentous growth observed when cultures were agitated on a rotary shaker. (Table 4.16).

a



b

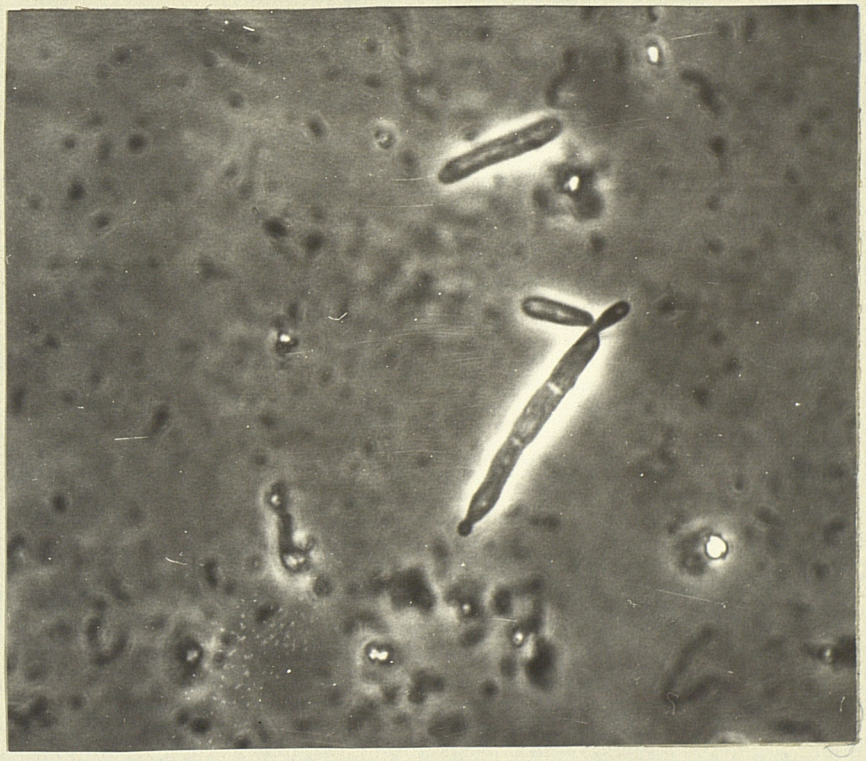


Fig. 4.18 Blastospores of Verticillium lecanii in Sabouraud liquid medium (a-X500; b-X1000)

TABLE 4.16

Effects of Aeration and Agitation on Submerged Culture of *Verticillium lecanii* (5 days incubation, 20 ± 1°C)

Regime	Approximate time of first blastospore formation (h)	Blastospore production[a]	Observations after 5 days
Closed, with screw cap, not agitated	8	+	Much hyphal growth
Closed, with screw cap, reciprocally agitated	8	++	Little hyphal growth
Cotton wool plugged, but not agitated	18	(+)	Growth predominantly aerial on surface of media
Cotton wool plugged, reciprocally agitated	18	+++	Little hyphal growth
Cotton wool plugged, rotary shaker	18	++	Microcolonies present some 1cm in diameter
Rotary shaker, vigorously aerated	24	+	Microcolonies present, some 1cm in diameter
Vigorously aerated, reciprocally agitated	24	++++	Limited initial hyphal growth
Vigorously aerated, stirred magnetically	24	++++	Limited initial hyphal growth

a = Blastospore production (+) sparse
++++ very dense

(b) Conidium Inoculum Density

The time taken for blastospores to form increased with decreasing conidiospore density (Table 4.17). At the highest inoculum level, the primary blastospore producing elements were short (10 - 20 µ) and similar to the elements producing secondary blastospores (Fig.4.18), whereas at the lowest level the primary blastospore producing elements were very long (over 80 µ).

These results again suggest the accumulation of a metabolite causing the change from mycelial to yeast-like growth.

TABLE 4.17

Effect of Verticillium lecanii Conidiospore Inoculum Density on Time Taken for Blastospore Formation to Commence

Initial conidiospore density (spores/ml)	5×10^5	3×10^5	3×10^6	3×10^7
Time (h)[a]	30	26	23	9.5

a = Observations made at 30, 26, 23 and 9.5 h after inoculation.

(c) Transference of Log Phase Blastospores to Fresh SLM

Transference of log phase blastospores (not centrifuged and washed) to fresh SLM caused an increase in the numbers of long filaments over 80 µ within 24h (Table 4.18). The proportion of long elements in the original non-transferred culture remained approximately the same. These results and those of the 2 preceding sections (IV,11,a and b) suggest that a fungus mediated-change in the medium caused the change from filamentous to yeast-like growth. When blastospores were transferred to fresh medium, the accumulated metabolite postulated above (IV, 11 a and b) was presumably absent and some spores temporarily reverted to filamentous growth until the metabolite again accumulated. If this is the cause, then

growth of V.lecanii in a chemostat may be totally filamentous near the critical dilution rate.

TABLE 4.18

Increase in length of Log Phase Verticillium lecanii Blastospores upon Transfer to Fresh SLM

Blastospore regime	n[a]	Blastospores (% of total number)			Total count (spores/ml)
		20 µ	20-80 µ	80 µ	
Original 48h blastospore culture	103	89.3	10.7	0	2.3×10^7
	103	82.4	6.9	10.7	
Blastospores in fresh SLM, 24h after transfer	87	61.9	3.5	34.5	5.8×10^6
	102	50	3.92	46.1	
Original blastospore culture after 72h	101	85.2	11.9	2.97	-[b]
	93	93.6	5.4	1.1	

a = Number of blastospores counted

b= No total count performed

Several substances have been implicated in the transition from mycelial to yeast-like growth (Cochrane, 1958). One of these substances is CO_2, which induces the yeast form in several groups of fungi, such as the Mucorales (Bartnicki - Garcia et al., 1962; Schulz et al., 1974). Since the factor involved in the change to yeast-like growth for V.lecanii is, to a degree, counteracted by aeration (IV, 11, a), accumulation of CO_2 may be the responsible factor.

12. Factors Affecting Blastospore Yields

(a) Aeration

Vigorous aeration increased blastospore yields

approximately 4-fold over cultures which were non-aerated but plugged with cotton wool (Table 4.19). These results reinforced similar observations in IV, 11, a.

TABLE 4.19

Effect of Aeration on *Verticillium lecanii* Blastospore yields ($\pm$ S.E.[a] x 10^8 spores/ml) in Sabouraud Liquid Medium (5 days shaking, 20 $\pm$ 1°C)

Aerated	Non-aerated
1.7 $\pm$ 0.29	0.55 $\pm$ 0.063

a = Standard error of mean of 4 culture yields

(b) pH

Blastospore yields, at different pHs buffered with 40 mM KH_2PO_4, and in unbuffered SLM, differed very little, yields at pH 4.0 and 9.0 being only slightly reduced (Table 4.20). During the first 24h incubation, the inoculum of conidiospores germinated rapidly at pHs 5.5 and 7.0, less rapidly at pH 9.0 and was virtually nil at pH 4.0. Fluctuations from pHs 4.0 and 5.5 were at first towards alkalinity and *vice-versa* at 7.0 and 9.0, but after 60h incubation, all fluctuations were towards acidity. The 40 mM phosphate buffer at pH 7.0 restricted the between-adjustment fluctuations at high spore densities to less than 0.5 units. The fluctuations at high spore densities for pH 9.0 were greater (1.0 units) since this is further from the pK value of KH_2PO_4. The final pH in the unbuffered SLM was 3.8.

TABLE 4.20

Effect of pH on Blastospore Yields of *Verticillium lecanii* in Sabouraud Liquid Medium (combined results of 2 experiments, 5 days incubation, 20±1°C)

pH	n	Yield ± S.E.[a] x 10^8 spores/ml	Final pH
4.0	2[b]	0.68 ± 0.06	-
5.5	4	1.82 ± 0.37	-
7.0	3[b]	2.25 ± 0.28	-
9.0	3[b]	1.23 ± 0.51	-
40mM KH_2PO_4 not added[c]	4	1.06 ± 0.42	3.8 ± 0.10

a = Standard error of mean of yields from n shake flasks
b = Missing replicate flasks contaminated
c = Initial pH 5.8

(c) Staling Products

The results of the tests with different SLM regimes (Table 4.21) suggest that the final yield in normal strength SLM (30 g/l) is determined by an exhaustion of nutrients rather than the production of a staling compound. A typical yield was obtained when "spent" (once used) SLM was supplemented and re-buffered with fresh normal strength SLM. An acidic type of staling was not responsible for limiting the yield since "spent" SLM, when re-adjusted to pH 5.8, produced only a very low yield. Yields approximately doubled in double-strength SLM, but further concentration of this medium did not increase yields (Table 4.21). This suggests that either a concentration-dependent medium factor or staling product again limits yield.

TABLE 4.21

Effects of Medium Concentration and of Adding Fresh Medium to used Medium on Blastospore Yields of *Verticillium lecanii* (5 days shaking, 20 ± 1°C)

Regime	Concentration of fresh SLM[a] (g/l)	n	Yield ± S.E.[b] x 10^8 spores/ml
Re-adjusted "spent"[c] SLM	-	1	0.036
"Spent" SLM supplemented with fresh SLM	30	2	2.1 ± 0.26
Fresh SLM	30	3[d]	1.83 ± 0.23
Fresh SLM	60	3[d]	4.68 ± 0.40
Fresh SLM	90	3[d]	4.78 ± 0.15

a = Sabouraud liquid medium

b = Standard error of mean of n culture yields

c = Once used culture medium

d = Combined results of 2 experiments

(d) Carbon-nitrogen Ratio

The ratio of carbon to nitrogen can strongly influence yields of fungi in liquid media (Hawker, 1950; Graham, 1973). With $(NH_4)_2SO_4$ as N-source in the "basal medium" (IV, 1, a), yields were highest at C/N ratios of 10 : 1 and 5 : 1, but dropped sharply at 1 :1 and more gradually above 20 : 1 (Table 4.22). Mean germ tube lengths after 20h incubation were significantly less ($P < 0.05$) at a C/N ratio of 1 : 1 than those at higher C/N ratios (Table 4.22).

TABLE 4.22

Effect of C/N Ratios on *Verticillium lecanii* Conidiospore Germination and Blastospore Yields in Basal Medium (N-source-$(NH_4)_2SO_4$, initial pH 7.2, 5 days shaking, 20 ± 1°C).

C/N ratio	Yield x 10^7 spores/ml	Germ tube length[a] (μ) ± S.E.[b]	n
No C-source	No growth	-[c]	
1:1	0.56	8.46 ± 0.41	34
5:1	4.3	17.2 ± 0.89	40
10:1	5.7	21.7 ± 1.51	34
20:1	1.8	22.7 ± 0.07	35
40:1	1.9	-[c]	
100:1	1.1	-[c]	
250:1	0.91	-[c]	
500:1	0.85	-[c]	
1000:1	0.38	-[c]	
No N-source	Limited germination no further growth	-[c]	

a = Measurements after 20h incubation

b = Standard error of mean of n observations

c = No measurements taken

With the complex medium, SLM, altering the concentrations of components produced a slight increase in yield when the C/N ratio was decreased (Table 4.23).

TABLE 4.23

Effect of Altering the Ratio of Components of Sabouraud Liquid Medium upon Yields of *Verticillium lecanii* Blastospores (5 days shaking, 20±1°C)

Glucose g/l	Protein g/l	Yield ± S.E.[a] x 10^8 spores/ml
20	10	2.3 ± 0.14
40	10	2.3 ± 0.04
10	20	3.71[b]
20	20	3.37

a = Standard error of mean of 2 culture yields

b = Only one replicate used

(e) Carbon-Source

The nature of the carbon source can effect fungal yields (Graham, 1973) and in some cases the control of submerged conidiation (Smith et al., 1973; Galbraith et al., 1969a). For *V.lecanii*, galactose produced the highest yields (Table 4.24) as Graham (1973) also observed for *Rhizopus oligosporus*. The next highest yields were obtained with glycerol,glucose,fructose and sucrose respectively.

TABLE 4.24

Effect of Different C-sources[a] on Development and Yields of *Verticillium lecanii* Blastospore Cultures (5 days shaking, initial pH 7.2, 20 ± 1°C)

C-source[a]	Observations					Yield $\times 10^8$ ± S.E.[c] spores/ml
	Germ tubes (μ)	Spores	Germ tubes (μ)	Beaded appearance	Secondary spore formation[b]	
SLM (pH5.8)	>100	-	>250	-	+++	1.56 ± 0.24
Lactose	< 50	-	>100	+	+	0.015
Glucose	< 50	-	>250	+	++	0.58 ± 0.12
Glycerol	<50	-	>250	+	++	0.7 ± 0.08
Galactose	<30	-	>250	+	++	0.9 ± 0.06
Fructose	no observations		no observations			0.44
Sucrose		-	>250	+	++	0.34 ± 0.025
Glutamic acid	<50	-	>100	+	(+)	0.008
Succinic acid	<50	-	>250	+	-	-
Citric acid[d]	<50	-	> 50	-	-	-
Sodium acetate	no germination			no germination		

a = Carbon-sources in basal medium with $(NH_4)_2SO_4$ as N-source, C/N ratio, 10:1

b = Blastospores: +++ dense (+) very few - none.

c = Standard error of mean of 2 culture yields.

d = These C-sources tested also at pH 4.5

The process of isomerisation of galactose to glucose, which must occur before entry of the hexose into the Embden - Meyerhof - Parnas pathway, may have provided V.lecanii with a steady supply of glucose producing a more balanced and favourable medium for growth than when glucose itself is used as C-source. Such a phenomenon would be similar to other systems that produce a steady supply of carbohydrate, such as media containing di- or polysaccharides. These media are more favourable for the growth of some fungi than those containing hexose sugars (Hawker, 1950).

Lactose produced very low yields, which suggests that the fungus may be deficient in exogenous lactose hydrolytic enzymes. The tricarboxylic acid cycle intermediates, succinic and citric acids supported very little growth and no blastospore formation. The ability of fungi to utilize complex C-sources varies greatly (Hawker, 1950). Acetate, important in inducing the glyoxylate cycle (Galbraith et al, 1969 b) and increasing the relative rate of submerged conidiogenesis in Aspergillus Niger (Galbraith et al, 1969 a,b), supported no growth of V.lecanii. This may indicate an inability to transport sodium acetate into the cells or perhaps even an absence of the glyoxylate cycle in this fungus.

(f) Complexity of N-Source

These studies demonstrated that blastospore yields increased with increasing complexity of the N-source used. Sodium nitrate (Table 4.25) produced yields similar to those obtained with $(NH_4)_2SO_4$ (Table 4.22), but yields with asparagine were somewhat higher (Table 4.25). Complex undefined media (Table 4.25; Nos.3-5) containing protein

and perhaps other components produced the highest yields. Corn steep liquor (CSL) and Malt extract (ME) produced yields similar to those in SLM. This was encouraging since CSL and ME are cheap and easily available.

TABLE 4.25

Effect of different N-sources on Yields of Verticillium lecanii Blastospores (5 days shaking, 20 ± 1°C).

N-source		Yield ± S.E.[a] x 10^8 spores/ml
1.	Sodium nitrate[b]	0.42 ± 0.03
2.	Asparagine[b]	0.92 ± 0.08
3.	Sabouraud liquid medium[c]	1.9 ± 0.16
4.	Malt extract medium[c]	2.2 ± 0.28
5.	Corn steep liquor[c]	1.8 ± 0.26

a = Standard error of means of 2 culture yields
b = These N-sources in basal medium, C/N ratio, 10:1
c = These media described in IV, 1, a.

(g) Effect of Antibiotics

Tetracycline and streptomycin each at a concentration of 100 ug/ml were incorporated into a stirred aerated blastospore culture to arrest contaminant bacterial growth. The final yield (2.1×10^8 spores/ml) compared favourably with those of untampered cultures (Table 4.19), and viability was also high (91%), suggesting that these antibiotics may be used safely in case of accidental bacterial contamination of cultures.

13. Induction of Submerged Conidiation

(a) Effect of C-source - see IV,12,e

(b) Depletion of the N-source

Some species of Penicillium conidiate freely in submerged culture when the nitrogen source in the medium approaches exhaustion (Morton, 1961). With V.lecanii, exhaustion of $NaNO_3$ or $(NH_4)_2SO_4$ at very high C/N ratios

(100-1000:1) did not induce conidiation. However, blastospores in $NaNO_3$ medium, at low and high C/N ratios, were small (Table 4.26) and resembled swollen conidiospores in shape (Fig. 4.19), although still formed like blastospores in SLM (Fig.4.18).

TABLE 4.26

Verticillium lecanii Blastospore Lengths ± S.E.[a] (μ) in Simple and Complex Media (20 ± 1°C)

Medium	Duration of incubation					
	n	48h	n	96h	n	120h
SLM	60	12.1± 0.64	63	12.56±0.42	61	14.00±0.87
$(NH_4)_2SO_4$[b]	62	12.01±0.83	62	12.33±0.64	60	13.83±0.32
$NaNO_3$	60	8.82±0.18	63	8.05±0.18	60	10.2 ±0.94

a = Standard error of n observations

b = These N-sources in basal medium, C/N ratio 10:1

Penicillium chrysogenum, unlike other Penicillium spp. will only conidiate in liquid medium if the culture is washed and transferred to nitrogen-free medium (Morton, 1961). Following the method of Morton (1961), 16h-and 30h-centrifuged and washed SLM cultures of V.lecanii were shaken in nitrogen-free medium but only typical blastospore development was observed.

(c) Temperature

Exposure of some fungi to above-optimal temperatures prior to incubation at optimal temperatures induces submerged conidiation e.g. Aspergillus niger (Anderson et al., 1971a,b, 1972). V.lecanii did not conidiate under these conditions. Conidiospores in SLM held at 34°C for 18 and 36h grew normally and produced blastospores after transfer to 20°C. Conidiospores held at 29.5°C germinated and produced mis-

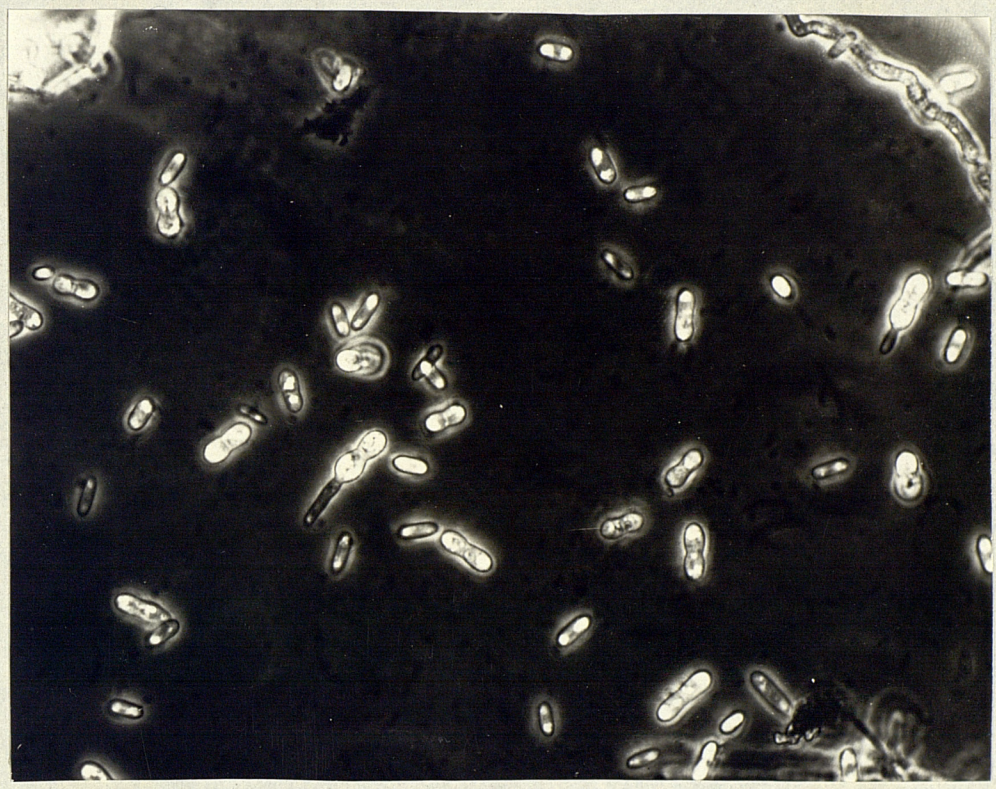


Fig. 4.19 Conidiospore-like blastospores of *Verticillium lecanii* (C-3) in liquid medium containing $NaNO_3$ as sole N-source (X500)

shapen germ tubes; when incubated at 20°C, normal blastospore formation was resumed.

(d) Oxygen Tensions

With practically all liquid-cultured fungi, the most powerful stimulus for sporulation is provided by the emergence of the fungal hyphae into aerial conditions (Morton, 1961). With V.lecanii, saturation of SLM with finely dispersed bubbles of pure O_2 did not alter normal blastospore production and so factors other than oxygenation must be important in stimulating conidiation.

14. Reduction of Blastospore Size

(a) Influence of Cysteine

Batch cultures of V.lecanii simulate a semi-yeast phase (Figs.2.05,4.18). Rippon et al.(1965) showed that a change from filamentous to yeast-like growth was induced in Aspergillus sydowi and Penicillium lilacinum by increasing concentrations of cysteine in aerial culture. Cysteine also supported growth of the yeast phase in Histoplasma capsulatum and Candida albicans (Romano, 1966). There is much evidence to suggest free-SH groups are required to maintain the yeast phase in these fungi (Romano, 1966). With V.lecanii, to try to increase numerical yield of uniform, small sized blastospores, cysteine was incorporated into SLM and SDA which was then seeded with conidia. Cysteine was inhibitory at 0.005M but growth proceeded normally at 0.0005M cysteine in SLM and SDA suggesting that further change towards the yeast phase cannot be induced by cysteine.

(b) Blastospore Dimensions of other V.lecanii Strains

Three other V.lecanii strains (C-21, C-47, C-48), known to produce small-sized conidiospores on SDA, were cultured in aerated SLM for 5 days at 20 ± 1°C. C-21 and C-48 produced blastospores less than half the size of those of the

standard, C-3 (Table 4.27)

TABLE 4.27

Yields and Dimensions of Blastospores of Different Strains of *Verticillium lecanii* in Aerated Sabouraud Liquid Medium (5 days shaking, 20 ± 1°C)

Isolate number	Yield x 10^8 spores/ml	n	Length (μ) ± S.E.[a]	Width (μ) ± S.E.[a]	Approx volume[b] (μ^3)	Other observations
C-3 (IMI 179172)	2.1	156	14.1±0.66	2.13±0.08	63.8	Rod shaped blastospores
C-21	14.3	31	4.95±0.09	1.96±0.05	19.0	"
C-48	9.4	30	5.27±0.17	1.9 ±0.06	19.02	"
C-47	1.9	30	2.51±0.08	-	8.28	Spherical spores; much filamentous growth

a = Standard error of mean based on n observations with eyepiece micrometer

b = Approximated by assuming spores (except for C-47) are cuboidal in shape

C-21 yielded almost 10 times as many spores as C-3, even though the difference in size was only about x 3. Spores of C-47, however, were almost spherical (Fig.4.20) and arose from phialides ranging from typical to almost absent. These spores were undoubtedly the smallest encountered in liquid media, but spore yields were rather low and accompanied by abundant mycelial growth (Fig.4.20). These spherical spores did not bud off new spores and it may perhaps be incorrect to refer to them as blastospores.

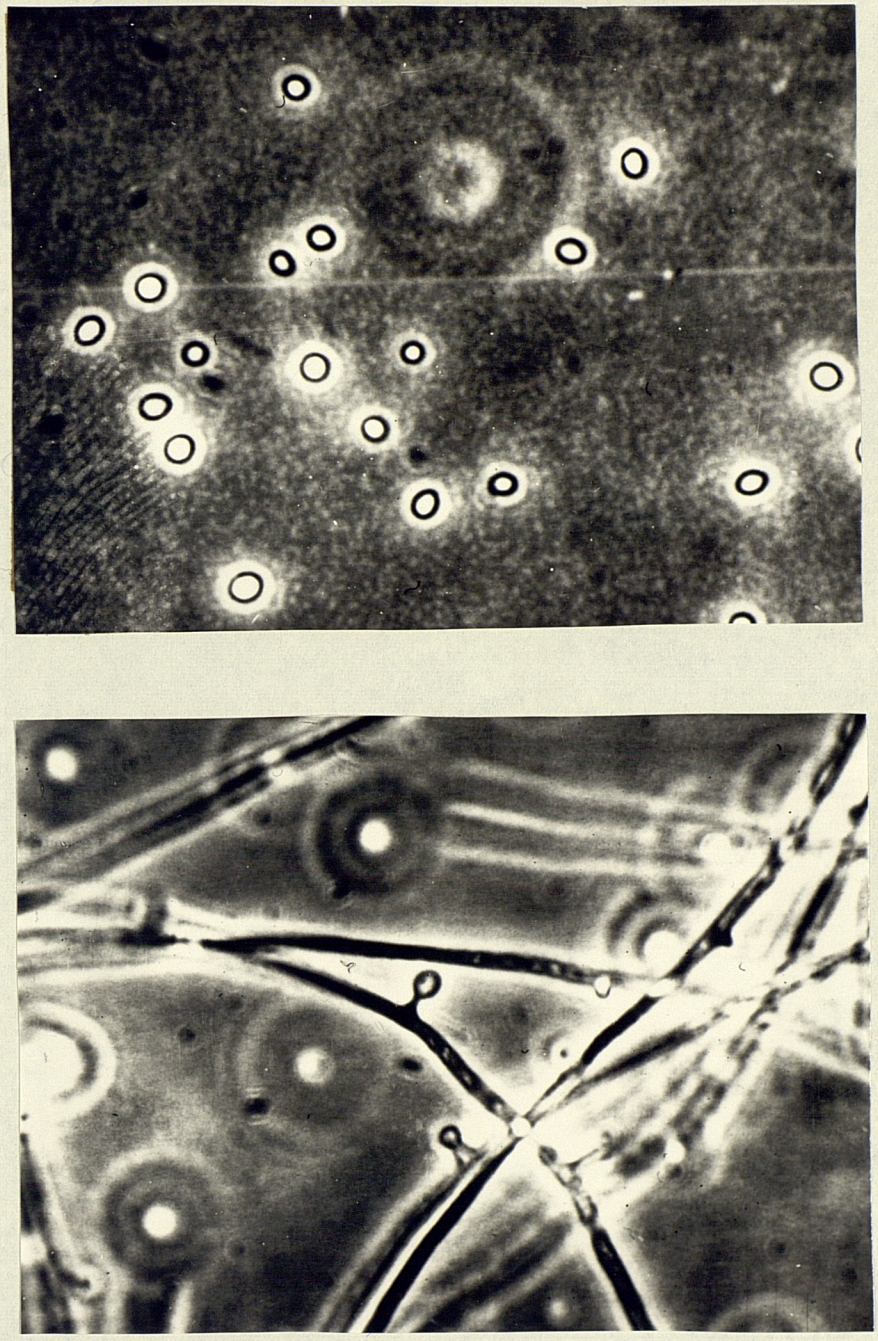


Fig. 4.20 Spherical, unbudding spores of Verticillium lecanii (C-47) produced in Sabouraud liquid medium (X500)

15. Longevity of Blastospores

(a) Duration of Blastospore Culture

As aerated and agitated SLM cultures aged, vacuolation and granulation in blastospore cytoplasm increased, but spore viability did not deteriorate until several days later. In cultures agitated for 5 days, there was no loss in spore viability, although the beginnings of vacuolation were seen in some of the longer, older and normally non-vacuolate blastospores. In a second experiment lasting 13 days, vacuolation again occurred after 5 days and progressively increased on successive days (Table 4.28).

TABLE 4.28

Effect of 13 Days Culture at 20 ± 1°C on the Viability Levels of Aerated *Verticillium lecanii* Blastospore Batch Cultures

Duration of culture (days)	% Viability (Method - IV, 1, j).	Observations
3	103.7	Blastospore contents appear amorphous
4	100.7	" " " "
5	94.5	Vacuolation in longer, older blastospores
6	100	" " " "
7	92.6	Vacuolation in long and some short blastospores.
9	99.2	Much vacuolation.
10	112.4	Much vacuolation; some granulation.
12	104.2	Vacuolation in all but a few spores; longer spores appear to be autolysing.
13	19.8	Many spores autolysing; many spores seem narrower.

Later, spores became granular as well as vacuolate and some exhibited signs of autolysis on the 12th day of incubation (Table 4.28). However, the titre of live blastospores did not decrease until autolysis began after 12 days. In contrast, Samšiňáková (1966, 1969) reported loss of blastospore viability in Beauveria bassiana after 4 days culturing, although spore numbers continued to increase.

(b) Survival of Blastospores

In distilled water, blastospore viabilities decreased with time (Fig.4.21). The 96h batch was terminated after 32 days upon discovery of bacterial contamination. There was little difference between the longevities of the 2 remaining batches (Fig.4.21) during 208days storage at 2°C.

Antibiotics and wetting agent were added to blastospores stored in distilled water but they did not affect viabilities (Fig.4.22) during 210 days at 2°C.

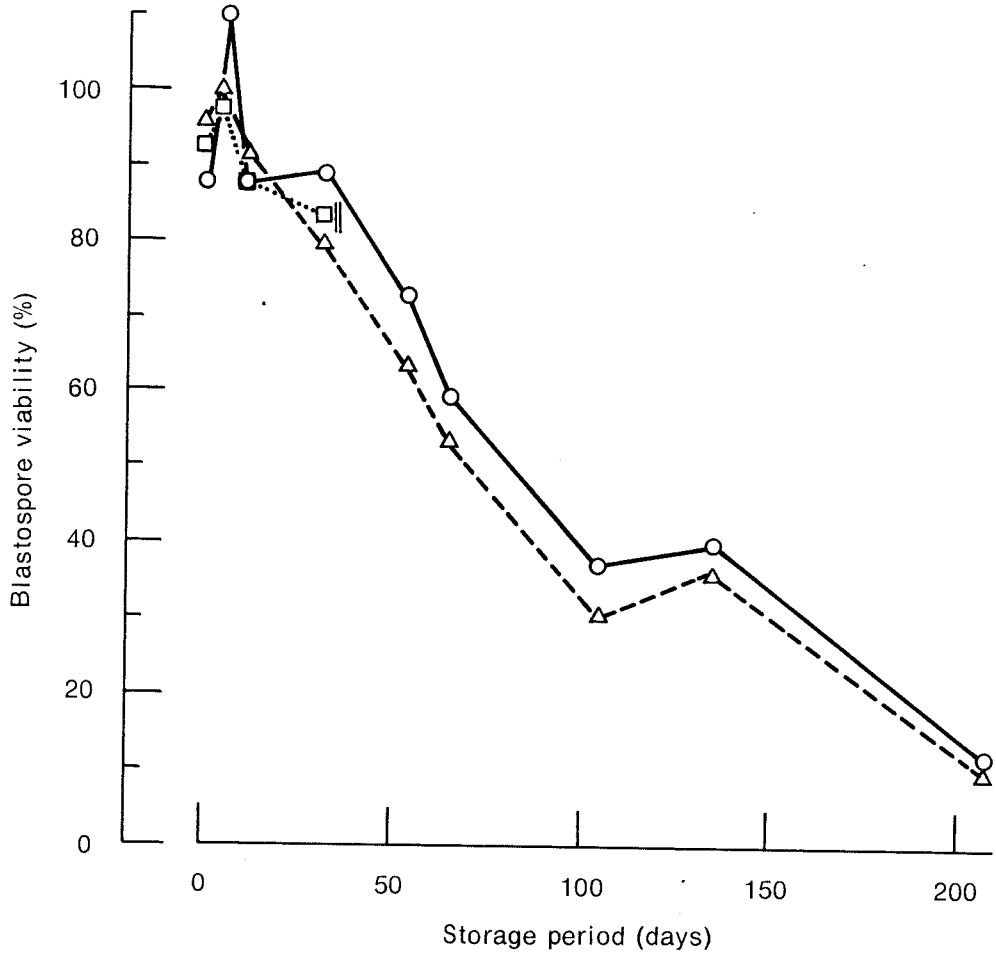


Fig. 4.21.

Longevity of *Verticillium lecanii* blastospores harvested from culture at different intervals and stored in distilled water at 2°C.

- ○ 48h culture
- △ 72h culture
- □ 96h culture (rejected after 32 days owing to contamination).

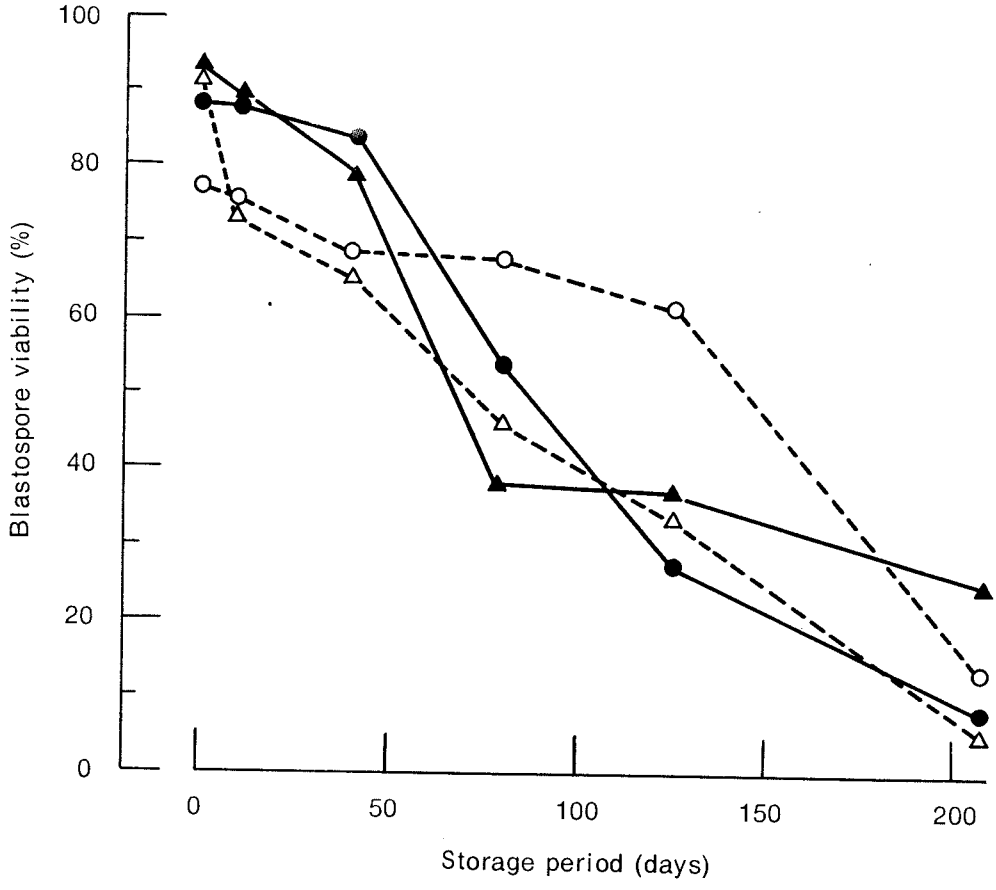


Fig. 4.22

Effect of antibiotics and wetting agent on longevity of *Verticillium lecanii* blastospores stored at 2°C.

- ● Spores in distilled water alone.
- ○ Spores in streptomycin solution (125µg/ml).
- ▲ Spores in tetracycline solution (125µg/ml).
- △ Spores in Triton X – 100 (0.5%).

DISCUSSION

The results of this chapter can be interpreted in 2 ways, from purely scientific or practical standpoints. Inevitably, to keep both types of interpretation absolutely separate would involve unnecessary duplication but, as far as is possible, the first part of the discussion deals with purely scientific aspects to explain the phenomena observed, and the second part with the practical considerations for controlling aphid populations with V.lecanii in the glasshouse environment.

Many workers have found that fungal spores survive better at lower (above 0°C) than at higher temperatures (Anderson et al., 1948; Merek et al., 1954; Clerk et al., 1965; Kawakami et al., 1965;Galanopoulos et al., 1974). This effect was not clear for V.lecanii conidiospores in distilled water (IV, 9, a) but agar-slope cultures certainly survived better at 2°C than at 20°C (IV, 9, f). However, faster desiccation may have contributed to the earlier death of spores on slopes at 20°C. At -17°C, spores in distilled water survived no better than those at 2°C. It is not likely that, at -17°C, death was caused by physical damage to cells due to growth of intracellular ice, since the rate of cooling to -17°C was probably not fast enough to cause intracellular ice formation. For yeast cells, a cooling rate faster than 10°C/minute is necessary to achieve this (Mazur, 1966). More likely, death resulted from movement of water out of spores during slow freezing of the extracellular distilled water, gradually concentrating cell solutes. Below physiological temperatures, cooling tends to decrease reaction rates, but in frozen systems reactions may accelerate because of the mass action effect of the increased concentration of the

reactants (Bruice et al., 1965; Tappel, 1966). The presence of highly concentrated solutions of electrolytes in a cell may also denature macromolecules (Mazur, 1966).

Deep-frozen conidiospores in distilled water died sooner (half-life, 6 months) than spores on agar slope cultures (half-life, 13 months). The slime around spores on slope cultures may have prevented outward movement of water from spores during slow freezing, thereby preventing concentration of intracellular solutes. Also, slime may have penetrated the spores or may even be manufactured by the spores themselves and its internal presence may have prevented intracellular freezing altogether at -17°C. Such a cryoprotective mechanism would be similar to that produced by glycerol and other cryoprotectants (Nash, 1966).

Freezing in liquid nitrogen and thawing did not harm V.lecanii conidiospores, so it may be possible to store highly concentrated viable spore suspensions indefinitely for use as a stable standard for bio-assay purposes. Also, since it is unlikely that freezing and thawing involves an increase in mutation rates of micro-organisms (Hwang, 1966; MacDonald, 1972; Day et al., 1972), it should be possible to store indefinitely different isolates of V.lecanii without genetical change.

High relative humidities favoured the survival of V.lecanii conidiospores (Figs. 4.13 - 4.16). Of several authors who have studied V.lecanii, only McClelland et al. (1929) observed the harmful effects of desiccation on (washed) conidiospores. Otherwise, there are very few quantitative data available on the effects of humidity on fungal spores

produced in slime. Dubin et al. (1975), showed that washed conidiospores of Nectria galligena survived best at high humidity, but in contrast, Merek et al. (1954) demonstrated that washed or unwashed ascospores of Endoconidiophora fagacearum survived longest when dry.

Spores in slime of Glomerulla rufomaculans, Endothia parasitica (uncontrolled conditions, Burril, 1907 and Heald et al., 1915) and Endoconidiophora fagacearum (controlled conditions, Merek et al., 1954) survived longer than washed, slime-free spores. Thus, the poorer survival of detached unwashed slime heads of V.lecanii conidia on cellophane compared to washed conidia under the same conditions was a surprising result. Since V.lecanii conidiospores are successively detached in slime from the tips of phialides and have no visible remaining connections with the parent mycelium (Fig. 2.04), it is difficult to understand why the transference of unwashed spore heads from mycelium to cellophane rendered them so susceptible to low humidities (Figs. 4.13 - 4.15). It is possible that air movement may have been so slight in the Kilner jars at 20 $\pm$ 1°C, that a substantial layer of stationary air formed above the aphid bodies and culture mycelium, and decreased the rate of conidial desiccation compared with the more exposed, transferred spores in slime on cellophane squares. However, this does not explain the poorer survival of detached, unwashed conidia in slime on cellophane compared with washed spores (Figs 4.13 — 4.16). This comparison may imply that the slime on unwashed spores (or some other factor removed by washing) concentrated during drying inside or outside the spore and either poisoned the conidia or encapsulated them to form a physical barrier restricting gas (O_2 or CO_2) exchange. The latter effect could explain the better survival of unwashed spores on cellophane at 0% RH

than at intermediate humidities (Figs 4.13 and 4.15), since complete desiccation should inhibit respiration and metabolism completely. Thus, storing spores at low O_2 or high CO_2 partial pressures or, in an inert atmosphere (N_2), to prevent respiration and metabolism may enable spores to survive longer at unfavourable humidities as with Metarrhizium anisopliae conidiospores in the experiments of Clerk et al.(1965). The absence of a rise in viability at 0% RH for washed spores (Figs. 4.13 - 4.16) may be related to the potentially damaging effect of the removal of structurally bound water from macromolecules which occurs below 60% RH (Sussman, 1968): the work of Webb et al. (1964) and Webb (1965) suggested that deleterious physical changes in macromolecules caused by removal of structural water can be prevented by substances, e.g. inositol, which replace water during drying. Thus with unwashed V.lecanii spores, a substance like inositol inside the spore (perhaps slime itself), and again removable by washing, may have played a stabilizing role during desiccation at 0% RH and the lack of this substance in washed spores would explain their poorer survival at 0% RH compared to unwashed spores (Figs. 4.13 - 4.16).

The spore longevity results engender some practical considerations for insect control. Firstly, spores of V.lecanii should be applied in aqueous suspensions against insects. Evlakhova used dried V.lecanii preparations to control scale insects in citrus groves and obtained poor results in one trial (1939) and moderate results in another (1941). In these trials, she made no reference to spore viability levels, and these may have been low. Secondly, if healthy insects pick up slime-heads of spores from diseased insects then, unless humidity is high, the spores may quickly die without infecting the insect. However,

the results in IV, 9, d, suggested that the humidity close to the chrysanthemum leaf surface approached 100% and so early death of spores on aphids is unlikely.

Blastospore yields of the standard isolate of V.lecanii, C-3, in SLM were approximately 14 times greater/unit volume of medium than conidiospore yields on SDA (Table 4.29). This, together with simpler hygiene and economy of materials and space, make submerged fermentation the best method for large scale production, a view also expressed for other entomogenous fungi by Martignoni (1964), Müller-Kögler (1967), and Catroux et al. (1970).

Further, but slight, increases of yield in SLM were obtained by altering the C/N ratios or by increasing the concentration of nutrients. Cysteine failed to induce production of uniform, small-sized blastospores and thereby increase numerical yields. However, if the possible blastospore-stimulating metabolite in IV, 11, a, b and c proves to be CO_2, then blastospores may be reduced in size by aerating cultures with air fortified with CO_2. Other V.lecanii strains yielded up to 7 times more spores than the standard and, depending on their relative pathogenicities for aphids, they may be more suitable for practical use.

TABLE 4.29

Comparison of Verticillium lecanii Spore Yields on Solid and in Liquid Media

Medium	n	Yield ± S.E.[a] x 10^8 spores/ml of medium	Yield in liquid medium / Yield in solid medium
Sabouraud liquid medium	4	1.7 ± 0.29	13.6
Sabouraud dextrose agar	10	0.125 ± 0.029	

a = Standard error of n observations

Conidiospores of V.lecanii survived better than blastospores in distilled water at 2°C (Table 4.30).

TABLE 4.30

Survival of Verticillium lecanii Conidiospores and Blastospores in Distilled Water at 2°C.

Spore type	n	Half life of spores (days) ± S.E.[a]
Conidiospores	5	191.2 ± 25.7
Blastospores	3	80.7 ± 4.4

a = Standard error of n observations from longevity studies

The same relationship has been reported for other entomogenous fungi e.g. Beauveria bassiana (Samsinakova, 1966; Muller-Kogler, 1967) and B.tenella (Ferron, 1967; Blachere et al., 1973). Before any commercial use of blastospores is attempted, the problem of their long-term storage must be investigated in depth. The results for V.lecanii conidiospores suggest moist storage, just above 0°C, in an inert atmosphere preventing respiration. Employing these principles with some differences, Blachere et al. (1973) stored B.tenella blastospores for 8-9 months without loss in viability.

Since V.lecanii conidiospores stored better than blastospores, submerged production of conidiospores would be an advantage but this was not achieved for V.lecanii.

Where this has been possible for other fungi, e.g. Aspergillus niger (Anderson et al., 1971 a, b) and Penicillium chrysogenum (Morton, 1961), growth is filamentous and not semi-yeast like as with V.lecanii. Of the entomogenous hyphomycetes, only Beauveria bassiana has been reported to produce conidiospores in submerged culture, through the manipulation of the aminonitrate concentration (Goral, 1973; Ferron, personal communication).

Are blastospores essentially reproductive elements like conidiospores or do they represent a process in which hyphae bud off new, smaller hyphae? Until detailed histological studies are undertaken, it will not be known whether the structure of blastospores of V.lecanii and other fungi is nearer to that of hyphae or conidia. Little such work appears to have been performed but Sheridan et al. (1973), working with Cladosporium resinae, showed by stereoscan electron microscopy, that the ornamentation associated with conidiospores was absent on blastospores, perhaps suggesting a hyphal morphological affinity. Discussing the dimorphism of the mammalian pathogen, Cladosporium werneckii, Gustafson et al. (1975) suggested that blastospores may be large conidiospores produced from "disarticulated phialides" and, being immersed in a liquid medium, do not require a mycelium to supply nutrients for asexual reproduction as in aerial culture. However, such "disarticulated phialides" may equally be disarticulated hyphae in liquid medium, because they need not spread over a surface, as in solid medium, to obtain nutrients. Be that as it may, the ability of V.lecanii to produce blastospores may facilitate parasitism by enabling the fungus to spread rapidly, by means of blastospores, throughout the interior of an insect via the haemolymph. The dimorphic ability of V.lecanii would be very

similar to that of fungal pathogens causing deep mycoses in mammals (Romano, 1966). Blastospore formation is also a characteristic of other entomopathogenic Fungi Imperfecti such as Beauveria bassiana (Samšiňáková, 1966, 1969), Beauveria tenella (Ferron, 1967), Metarrhizium anisopliae (Adamek, 1965; Zacharuk, 1970), Spicaria rileyi (Bell, 1975), Spicaria pracina (Kawakami, 1962), and the ascomycete, Cordyceps militaris (McEwen, 1963). An inability to form blastospores might restrict spread of a fungus throughout an insect. This may be so with the V.lecanii strain, C-47, which neither produced blastospores in SLM (IV, 14, b) nor was pathogenic for the aphid, M.sanborni (Table 5.26), although it grew and sporulated on the aphid cuticle.

Given sufficient nutrients, conidiospores will germinate at very high densities (IV, 8, d). Detailed studies of the nutritional requirements of germination were not undertaken, but it was observed that V.lecanii would not germinate in distilled water (IV, 9, a) and this nutritional dependence may reflect a lack of reserve substances in the spore. It is not yet known whether nutrients are present on the aphid cuticle in sufficient quantity to ensure maximal germination of spores alighting there, whether sprayed or in slime-heads picked up from diseased insects. Observations of aphids in glasshouse experiments suggested that in future fungal spray formulations, it may be advantageous to include nutrients which may enhance spore germination on the aphid cuticle.

Although light reduces germination in other insect pathogenic fungi (Müller-Kögler, 1965), an intensity of 10,000 lux affected neither V.lecanii conidiospore

germination nor germ tube extension (IV, 8, c). Day-length reducing polythene blackouts, which also boost the humidity, are used during only part of the chrysanthemum growing period (III, 1; Fig. 3.01) and they may have to be replaced by transparent polythene covers during periods when shortened day-lengths are not required. Light penetrating the polythene should not delay or harm spore germination on aphids.

Sporulation and growth responded similarly to temperature but sporulation responded over a narrower range. Also, for other V.lecanii strains, high temperatures impaired sporulation more than growth (Table 4.15). Narrower temperature limits for reproduction than for growth are an almost universal feature in the fungi (Hawker, 1950; Cochrane, 1958). Light augmented sporulation and pigmentation in V.lecanii but not germination and growth. Thus, light may enhance sporulation - and hence spread of disease - on aphid species which feed on the undersides of leaves away from direct sunlight, and more so on the more exposed stem - feeding species like M.sanborni.

Verticillium lecanii conidiospores need high humidity to germinate (IV, 8, f) and may only do so in a water film, as with many insect pathogenic fungi (MacLeod et al., 1966). Thus, to ensure maximum germination and hence infection levels, spore application in the glasshouse should be synchronised with optimal humidity conditions, which for chrysanthemums occur in the evening when polythene covers are applied (III, 1).

The temperature-growth-rate curve of the single-spore V.lecanii isolate,C-3, in Fig.4.03 (fitted by eye) suggests an optimal temperature of 23.5°C, somewhat higher than the night-time glasshouse temperatures used for growing

chrysanthemums (ca 15°C). When the empirically derived geometric transformation of Cohen et al.(1952) was employed to linearize the data in Fig. 4.03, the resultant straight line revealed an even higher temperature optimum, 24°C (Fig. 4.23). The transformation can be used to approximately predict temperature growth rate optima of other V.lecanii strains, using only a minimum of data, e.g. the upper and lower temperature limits for growth. Figure 4.24, curves B and C, show that this proved reasonably accurate for the single-spore isolate, C-3 (observed optimum 23.5°C - 24°C, predicted 22°C), and C-48, Barson's (1976a) strain (observed optimum 21.5°C, predicted 21.5°C).[1] Applying this method to the strains, C-35 and C-49, which show the greatest tolerance to high temperature, indicates that they will have high temperature optima (Fig. 4.24 curves D and E). Figure 4.24, curves D and E, also illustrates that, for such strains, growth at a glasshouse temperature of 15°C, as a percentage of that at the optimum temperature, will be less than for strains with lower temperature optima (Fig. 4.24, curves A, B and C). This means that, unless the high-temperature-optimum strains have faster overall growth rates, they will grow more slowly at 15°C than lower-temperature-optimum strains, such as C-3, or Barson's strain. For a hypothetical strain (Fig. 4.24, curve A) with a rather lower temperature optimum (e.g. 18°C) than C-3 or Barson's strain, faster growth at lower temperatures may be less important than inhibition or death at sustained higher temperatures (> 25°C) which occur in glasshouses during hot summer weather. However, it may be beneficial to employ such a strain in winter only.

The temperature response curve for germination of conidiospores on agar (Fig. 4.05b) is similar to that for growth. The germination curve is slightly skewed towards

[1] Insufficient data available to apply same treatment for Ganhao's strain (1956).

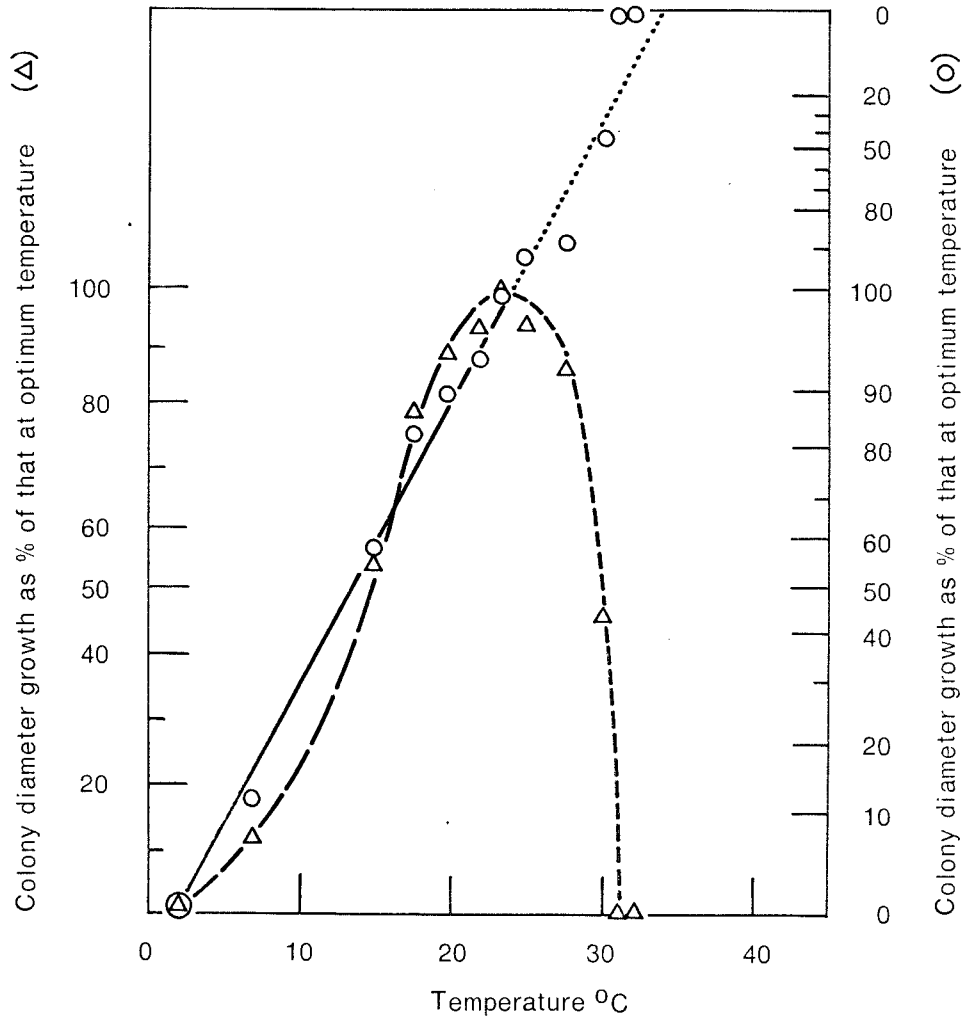


Fig. 4.23.

Effect of temperature on growth rate of *Verticillium lecanii* on Sabouraud dextrose agar: linearisation of curve by method of Cohen et al. (1952)

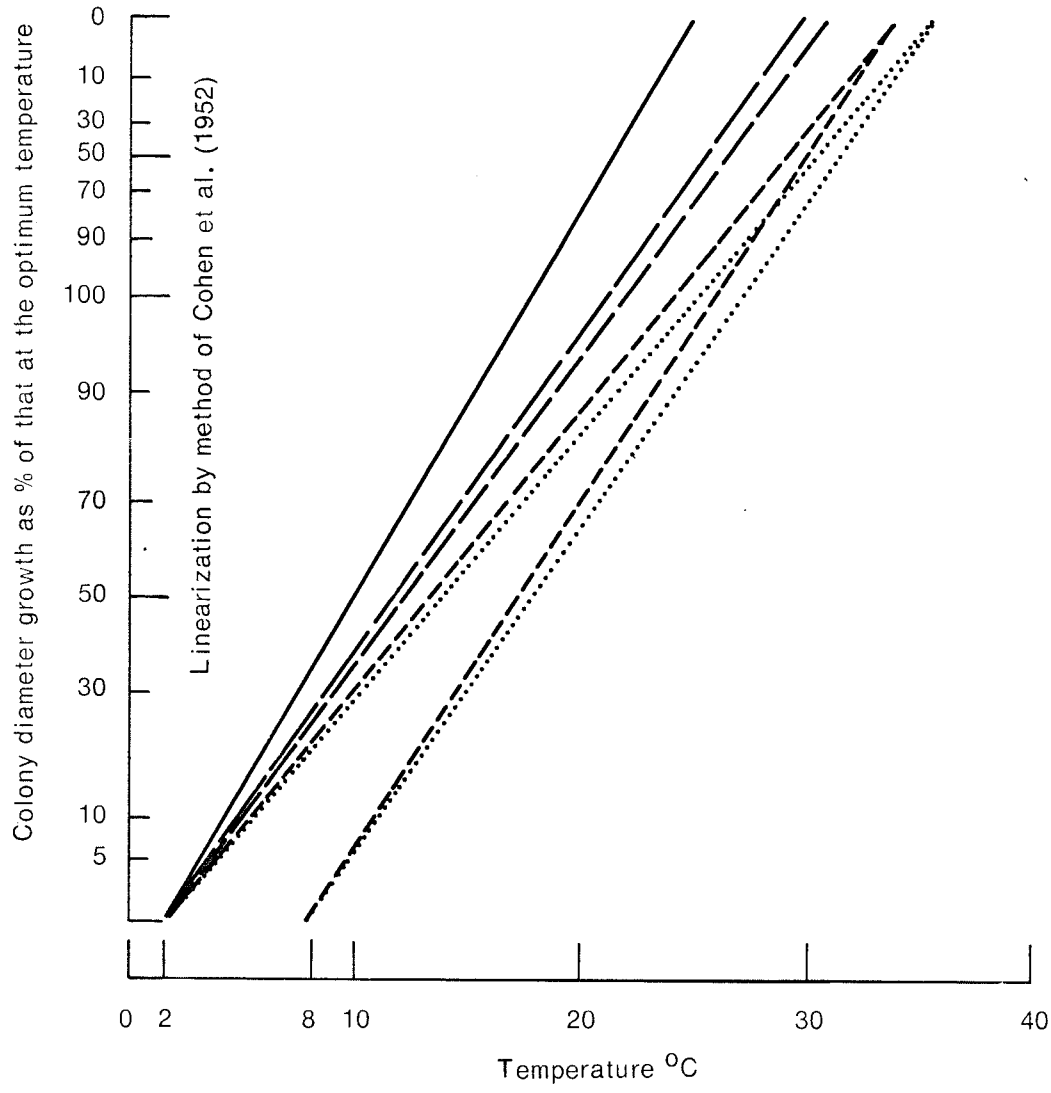


Fig. 4.24.

Temperature – growth response predictions for *Verticillium lecanii* strains

A ——— Theoretical strain with growth rate optimum at 18°C.

B — — C - 48 (Barson's strain).

C — — — C - 3 (IMI 179172).

D - - - - - - - - C - 49 Minimum temperature for growth not known but presumed to lie between 2 and 8°C.

E ..... C - 35 Minimum temperature for growth not known but presumed to lie between 2 and 8°C.

high temperature with a broad optimum zone, as for most fungi (Cochrane, 1958). Figure 4.05a shows that the main effect of sub- and supra-optimal temperatures on germination is to increase the latent period. Thus at 11.5°C, after a latent period of 7h, germination proceeded as rapidly as at optimal temperatures. Pre-germination temperature sensitivity has been recorded in other fungi (Cochrane, 1958). Thus, in the glasshouse, a night temperature of 20 - 25°C, to minimize the latent period, may be necessary for maximum infection levels amongst a spore-treated aphid population. Since the high cost of fuel may make such night-time temperatures impracticable, some attention is focussed in the following chapters on the effect of temperature on V.lecanii disease in relation to its aphid hosts.

Summary

1. V.lecanii conidiospores were very sensitive to desiccation but survived better at high humidities and in aqueous suspension. Spore life during storage may be lengthened by storage in suitable formulations and physical conditions.

2. Conidiospores survived longer than blastospores in aqueous suspensions at 2°C.

3. Blastospores were cultured in liquid media more readily and abundantly than conidiospores on solid media. Some strains of V.lecanii produced higher yields of blastospores than the standard, C-3.

4. Conidiospores did not germinate in distilled water suggesting that, if nutrients are sparse on the aphid cuticle a nutrient spray formulation may increase spore germination on the aphid.

5. A light intensity of 10,000 lux did not retard or inhibit

germination, only slightly retarded vegetative growth and increased sporulation.

6. The optimum temperature for vegetative growth, germination and sporulation on agar all fell between 20 and 25°C. These values are considerably higher than the operative night-temperature (15°C) for chrysanthemums in the glasshouse. Some strains of V.lecanii may have a lower temperature optimum than C-3, and may possibly grow faster than C-3 at 15°C.

CHAPTER V

APHID-FUNGUS RELATIONSHIP

MATERIALS AND METHODS

1. Bioassay of Conidiospores and Blastospores

(a) Preparation and Standardisation of Spore Suspensions

Conidiospores were cultured and harvested as in IV, 1, L and total counts performed on the resulting suspensions (IV, 1, h). Blastospores were cultured for 72h in agitated, aerated SLM at 20±1°C and harvested as in IV,1,m. Viable counts were made on both types of spores (IV, 1, i and j) before assay. Spore concentrations for assay were prepared by serial dilution in buffer and 0.02% Triton X-100.

(b) Plants

A supply of potted chrysanthemum plants, cultivar Deep Tuneful, was maintained with cuttings from a single source under glasshouse conditions of light and temperature that were held as constant as possible with the aid of supplementary artificial light from September to April (400W Mercury vapour lamps, 16h daylength).

(c) Aphid Rearing

Laboratory stocks of 3 aphid species were started from populations infesting chrysanthemums in glasshouses at the G.C.R.I.. Stocks were maintained on potted chrysanthemum plants in a light regime of 16h (6,500 lux) and temperature varying diurnally between about 20 and 24°C. When insects were required for bioassay, adult alatae from these stocks were transferred to 29 mm-diameter chrysanthemum leaf discs in breeding cells, at 20°C and 16h daylength (5000 lux). Apterous progeny from the alatae were transferred to fresh

leaf discs after 7 days and were used for bioassay when mature 8-10 days later. The cell design was a modification by I.J.Wyatt of the Munger cell(Fig. 5.01).

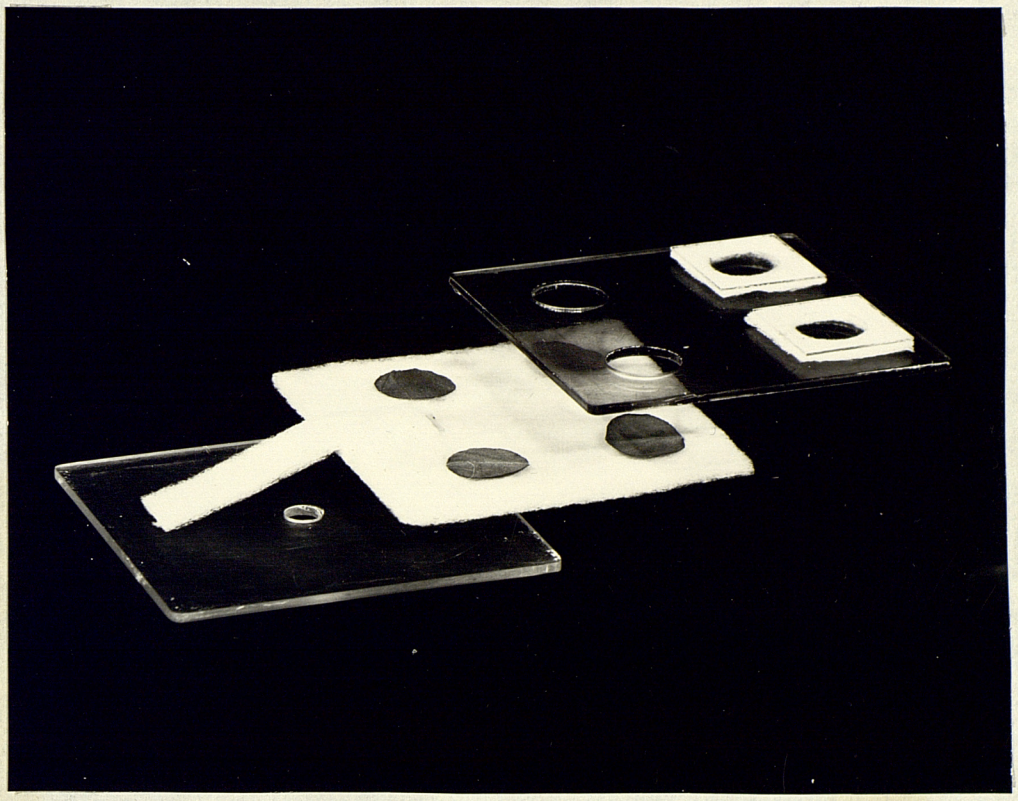
(d) Assay Procedure - Spores Applied in Aqueous Suspension to Aphids

The mature,apterous aphids were distributed systematically into batches in glass Petri-dishes by taking one aphid from each Munger cell in turn to avoid bias in distribution. For treatment with spores, each batch of aphids was placed on filter paper in a 7.5 cm-diameter Büchner funnel, and 30 ml of the appropiate spore suspension were poured in gently,immersing the aphids. Two seconds after immersion, the suspension was sucked off. Spores settle unevenly on filter paper in a Buchner funnel owing to vortices created by the suction through the perforations in the funnel. Thus, it was important to ensure that all aphids remained within the perforated area during treatment. Treated aphids were placed singly on 19 mm-diameter leaf discs in high humidity assay cells (Fig. 5.02). The assay cells were kept in perspex cages at high humidity for the duration of the assay, which was 6 days at 20°C, 16h daylength (5,000 lux). Dead insects were examined microscopically for fungus disease.

(e) Dry Weight Determinations of Conidiospores and Blastospores

Conidiospores and blastospores were cultured and harvested using the methods referred to in V, 1, a, and then concentrated by centrifuging. Aliquots were pipetted into 4 weighed clockglasses, dried at 200°C and re-weighed. Drying and weighing were continued until the weights were constant. The dry weight/spore was calculated by reference to total counts of the concentrated spore suspensions.

a



b

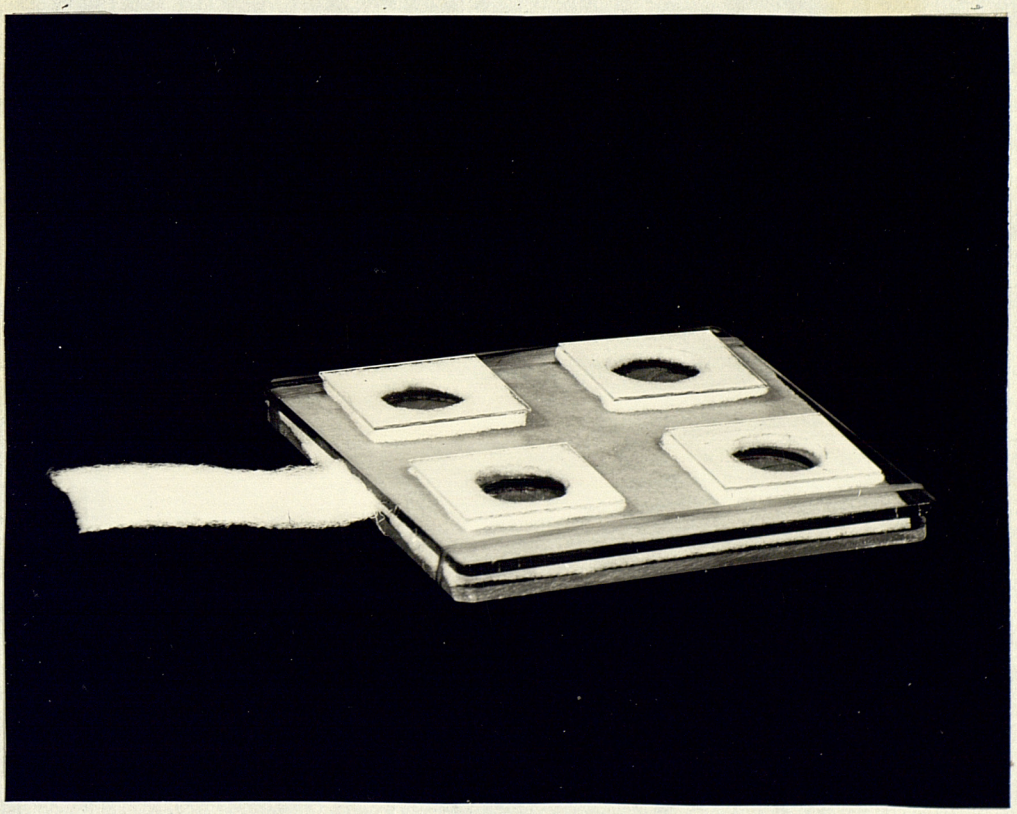


Fig. 5.01 a and b

Aphid rearing cells. Top to bottom, glass cover to prevent escape of aphids, felt spacing pad, perspex top, leaf disc, absorbent felt with extension to water reservoir and perspex base (a-exploded cell; b-assembled cell)

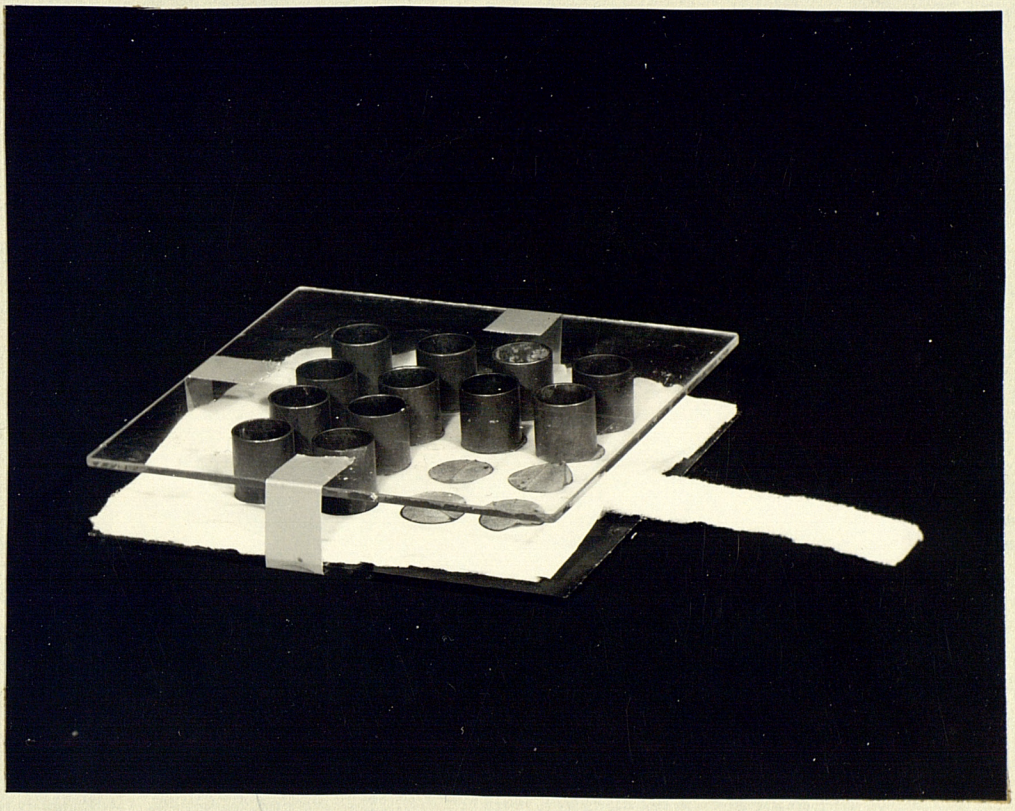


Fig. 5.02 Assay cells. Top to bottom, glass covers to prevent escape of aphids, copper rings containing leaf discs on water-absorbent felt with extension to reservoir, and glass base.

2. Influence of Temperature and Humidity

(a) Effect of Temperature during the First Hours of Incubation

Batches of 20 apterous M.sanborni adults, reared as in V, 1, c, were treated (using the methods of V, 1, d) with 2×10^6 conidiospores/ml and placed in copper ring assay cells (Fig. 5.02) at high relative humidity (RH) for 6 or 14h at 11.5, 15 or 20°C before being transferred to perspex ring Munger cells at lower ambient room humidity (20°C). The perspex rings, treated with polytetrafluoroethylene (PTFE) to prevent aphids escaping, had no lids, thereby exposing aphids to the low ambient humidity in order to arrest further fungal spore germination and development on the aphid cuticle. Layering of still air above the leaf discs was prevented by an air-flow from a hair dryer without the heater on. Some treated aphids in PTFE cells were also held continuously at high or low RH while a batch of untreated aphids in PTFE cells was held continuously at low RH only. Lids were necessary at high RH since PTFE lost its low frictional properties by absorbing moisture, thereby allowing aphids to escape and upon drying at low RH, trapped those aphids which had been climbing up. It was for these reasons that copper ring assay cells were used at the beginning of the experiment for those aphids to be transferred from high to low RH.

(b) Effect of Alternating High and Low Humidity on Spore-treated Aphids

Potted plants were used in this experiment to help simulate more closely glasshouse conditions. Batches of approximately 60 adult, apterous M.sanborni (reared on whole plants) were immersed in a suspension of 10^7 conidiospores/ml and each batch divided between 3 small, potted chrysanthemum plants. The plants were covered with a mesh device fitting closely over the pots to prevent aphids escaping, and placed

separately in plastic boxes containing either distilled water or 75% glycerol in water (relative humidity of 44%; Johnson, 1940). Three plants were used for each of 5 treatments (Fig. 5.13).

3. Contagion from Spores in Slime-heads

(a) Contagion in Petri-dishes

Two 19 mm-diameter chrysanthemum leaf discs were placed in each of 30 Petri-dishes kept humid with a lining of moistened filter paper. An aphid cadaver, sporulating with V.lecanii was placed on one leaf disc and a healthy adult apterous aphid on the other. In 20 control dishes, the cadaver and one leaf disc were omitted. M.sanborni was used in half the dishes and My.persicae in the rest.

(b) Contagion on Whole Plants

In each of 2 experiments, 10 healthy, adult, apterous M.sanborni and 10 My.persicae were placed singly on 20 potted 20 cm-tall chrysanthemum plants and others were placed in large numbers on more plants, 4 for each species. When populations had reached 8-10 aphids/plant on the singly infested plants, a dead aphid sporulating with V.lecanii was positioned in a lower leaf-axil approximately half-way up the stem on all the plants except for 2 sparsely and 2 densely infested plants kept as controls. The plants were held at high RH in perspex cages (5,000 lux) and mortalities were monitored on subsequent days thereafter.

4. Effect on V.lecanii of Subculturing on Agar and Passaging through Aphids

(a) Effect of Repeated Subculturing on Agar

The original strain of V.lecanii, which was used to infect the aphid from which the single-spore isolate, C-3 (IMI 179172), was derived, was a multi-spore isolate, C-1,

hence referred to as C-1/MS (MS = multi-spore). Two multi-spore isolates of C-1 (C-1/MS1 and C-2/MS2) derived from the same diseased aphid, were successively subcultured on SDA 50 times.

As described in IV, 1, k, C-3 cultures, subcultured 3 times on SDA from the original diseased aphid, were used routinely as standard in experiments. Such cultures can be described as C-3/SS1,sub3 (SS = single-spore, sub 3 = subcultured on SDA 3 times). C-3/SS1,sub3 was successively subcultured on SDA approximately 50 and 100 times and 50 times on Potato dextrose, Czapeck-dox and Malt agars, while another single-spore isolate, C-3/SS2,sub3 (derived from another aphid killed by the multi-spore strain, C-1) was subcultured on SDA 50 times only.

All these isolates after subculturing on agars were assayed for pathogenicity towards M.sanborni.

(b) Effect of Repeated Passage through M.sanborni

In one experiment, a diseased M.sanborni aphid killed by C-3/SS1 and in another, an aphid killed by one of the multi-spore isolates (C-1/ MS1), were introduced to separate dense populations of M.sanborni on chrysanthemum plants at high humidity at 20 $\pm$ 1°C. When disease had spread through both populations, sporulatimg cadavers from each population were agitated vigorously in 0.05% Triton X-100 on a vortex mixer, filtered through 4 layers of cheesecloth, centrifuged and washed. A slight bacterial contamination did not reduce viability levels, which approached 100%.

5. Biochemical Basis of Pathogenicity

(a) Preparation of Purified Colloidal Chitin

Dried crab shell chitin (Sigma Chemicals Ltd.) was purified using the methods of Stainier (1947) and Lingappa et

al.(1962). Approximately 40g of impure brown chitin was washed alternately for 24h with 1N NaOH and 1N HCl six times, boiled for 3h in 10% KOH and boiled 3 times in ethyl alcohol. These procedures decalcified the chitin and removed unwanted inorganic and organic matter, leaving whitish partially purified chitin. After drying, 15g of this were moistened with acetone, dissolved in 100ml of 50% H_2SO_4 in an ice bath for 30 min and precipitated by rapid 10-20 fold dilution with water. The now colloidal chitin was centrifuged, washed 3 times to remove acid and concentrate chitin, and then stored in distilled water at 2°C.

(b) Preparation of Chitin-agar

The chitin content of the colloidal suspension prepared in V, 5, a, was estimated by dry weight determinations and was incorporated in Oxoid agar No. 3 at a concentration of 2g/l.

RESULTS

6. Observations on Disease in Aphids

Conidiospores and blastospores were applied at concentrations of 10^6 - 10^8 spores/ml to batches of 20 adult, apterous M.sanborni which were then held at 20 ± 1°C in assay cells at virtually 100% RH using the methods described in V, 1.

At inoculation rates of 10^6 spores/ml and above, light sporulation was observed on legs and antennae of a few normally active aphids little more than 24h after treatment. After 3 days, many aphids exhibited some degree of sporulation on their legs, antennae and sometimes cornicles, but seldom any on the thorax or abdomen until after death. This may suggest that, during treatment, fewer spores adhered to the thorax and abdomen than to other parts of the insect. Sporulation on live aphids (Figs. 5.03 - 5.05) was often prolific and such infected insects in wild populations may spread the disease, particularly since activity appeared to be unimpaired. When compared with other entomogenous fungi (Madelin, 1963), V.lecanii is unusual in its ability to sporulate on live insects. Hyphal bodies (similar to blastospores in liquid culture; Fig. 4.18) were never observed in the haemolymph before the third day of incubation at any spore dosage, but they then increased rapidly until less than 24h before death, when the haemolymph was engorged with hyphal bodies (Fig. 5.06). Even at this stage, aphids were still active. Twenty-four hours after death, sporulation was prolific over the whole body of the aphid (Fig . 5.07) and thereafter continued to increase (Fig. 5.08). M.sanborni apterae usually died whilst still in the feeding position in high-humidity assay cells.

Fig. 5.03 Sporulation of *Verticillium lecanii* on live *Macrosiphoniella sanborni*

Fig. 5.04 Sporulation of *Verticillium lecanii* on leg of live *Myzus persicae*

Fig. 5.05 Stereoscan electron micrograph (SEM) showing sporulation of Verticillium lecanii (arrowed) on leg of live Macrosiphoniella sanborni (SEM by JSM-P15 EMSCOPE Laboratories, London)

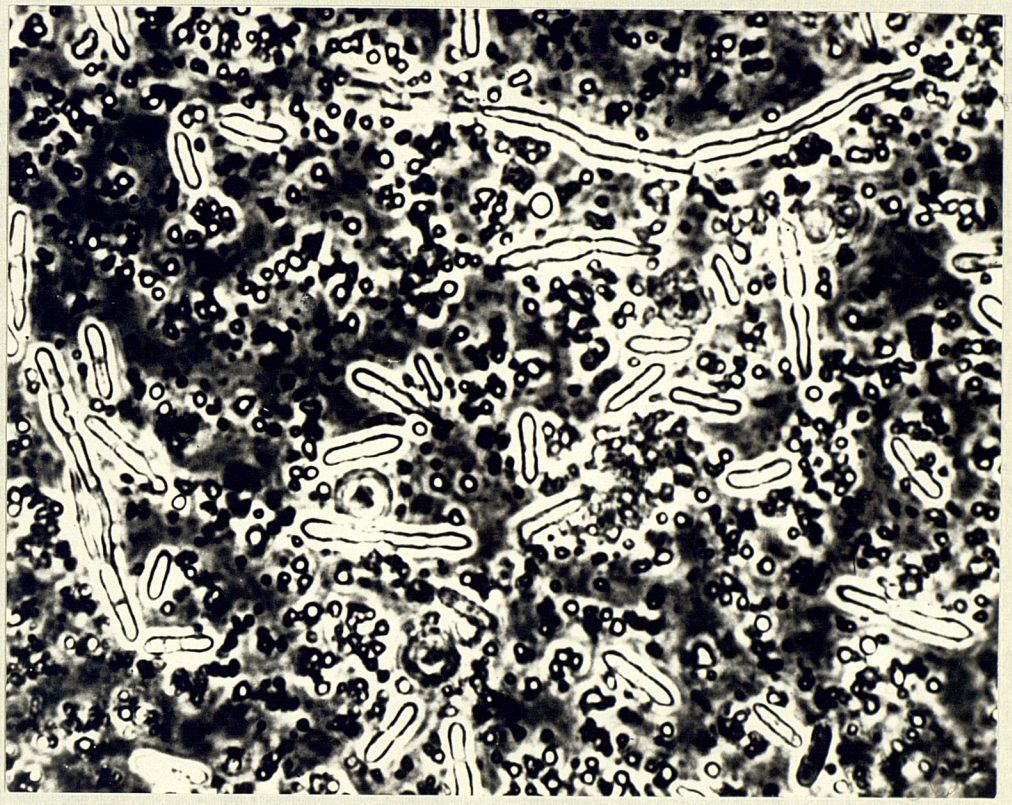


Fig. 5.06 Blastospores of Verticillium lecanii in haemolymph of live Macrosiphoniella sanborni (X500)

Fig. 5.07 Stereoscan electron micrograph (SLM) showing sporulation of Verticillium lecanii on Macrosiphoniella sanborni 24h after death (SEM by JSM-P15 - EMSCOPE laboratories, London)

Fig. 5.08 Appearance of Macrosiphoniella sanborni aphid several days after death due to Verticillium lecanii

At very high spore concentrations (10^8 spores/ml), 15% of conidiospore-treated and 30% of blastospore-treated aphids died within 48h of treatment. At death, hyphal growth and sporulation was abundant on the whole surface of these aphids but there was little invasion of internal tissues,as evidenced by the absence of hyphal bodies in the haemolymph and adipose tissue. At such high concentrations of spores, death was possibly caused by shock and mechanical damage resulting from a massive attack by large numbers of spores,or by lack of oxygen due to occlusion of the spiracles. Aphids surviving more than 48h at high spore concentrations exhibited similar fungal growth to that described for lower concentrations of spores, e.g. hyphal body formation in the haemolymph.

During development of the disease, parent M.sanborni continued to produce progeny at near-normal rates. The results of 4 experiments in Table 5.01 show that fecundity levels of infected mature apterae were slightly impaired by disease 24h before the day of death. Since counts were only made once daily, the precise hour of death was not recorded, but it is probable that the effect upon fecundity became severe within only a few hours of death. An analysis of variance, using the methods described by Cochrane et al. (1968), showed that increasing dosage did not significantly affect fecundity ($P > 0.1$) 24h before the day of death. In other experiments and in glasshouse field trials, heavily infected My.persicae also produced visibly healthy offspring. Once, a very active offspring was removed from a dead My.persicae killed by V. lecanii and microscopic examination of the young aphid showed no signs of infection except some spores on one tarsus probably from the parent.

TABLE 5.01

Production of *Macrosiphoniella sanborni* Progeny by Diseased, Adult Aphids in a 24h Period One Day before Death Due to *Verticillium lecanii*

Experiment number	Control[a]	Spore concentration/ml		
		$5x10^5$	$1x10^6$	$2x10^6$
1.	1.5	1.1	1.2	1.2
2.	1.7	1.6	1.4	1.2
3.	1.7	1.1	1.7	-[b]
4.	2.7	2.5	2.2	1.9

a = Mean number of progeny produced daily/control aphid during period over which *V.lecanii* killed treated aphids.

b = Uppermost concentration not employed.

7. Bioassay of Conidiospore Pathogenicity

(a) Choice of Host and Breeding Apparatus

A number of noteworthy points arose from preliminary experiments on aphid breeding techniques and assay design with the most suitable aphid species, *M.sanborni*. The best control over age and morphological form of aphid was obtained by using breeding cells (Fig. 5.01). Furthermore, the aphids were more tolerant of transfer to the assay cells from breeding cells than from whole plants.

Immersion of aphids in the Büchner funnel was rapid but had the disadvantage that some aphids drowned. The percentage of drowning increased with increasing concentrations of Triton X-100 or Tween 80 (Table 5.02). The incidence of drowning was reduced to tolerable levels by minimizing the amount of wetting agent used, 0.02% Triton X-100 being considered optimal for *M.sanborni*.

TABLE 5.02

Effect of Immersing *Macrosiphoniella sanborni*, *Myzus persicae* and *Brachycaudus helichrysi* in Increasing Concentrations of Triton X-100

Concentration (%) of Triton X-100	Insects drowned (%)[a]			Air bubble incidence on insects
	M.sanborni	My.persicae	B.helichrysi	
0.005	0	0	0	Present on most
0.02	10	0	0	Occasionally seen
0.05	18	0	0	None
0.1	31	0	0	None
0.5	70	0	0	None

a = Approximately 30 insects/treatment

V.lecanii sporulates freely over the legs and antennae of live aphids even in as short a period as 24h after treatment (V, 6). The treated aphids were therefore kept singly in the assay cells to avoid cross-infection. Control mortality amongst *M.sanborni* during the 6-day period in the assay cells was usually nil, occasionally 5-10% and once 15.8%. Two other aphid species *B.helichrysi* and *My.persicae*, did not drown even at high concentrations of wetting agent (Table 5.02) but had more important disadvantages; on transfer to high humidity in the assay cells, these aphids were restless, slow to resume feeding, many escaped from their cells, and control mortalities were high (5-15% for *B.helichrysi* and 10-23% for *My.persicae*).

M.sanborni was chosen, therefore, as the most suitable bioassay host because of its low control mortality, which was considered to be more important than the drowning effect.

(b) Analysis of Bioassay Results

Following a range-finding assay with 10 M.sanborni/dosage, 27 more accurate bioassays were performed over a period of $3\frac{1}{4}$ years, with approximately 20 insects/dosage. The results of each assay were subjected to probit analysis (Finney, 1952) using the maximum-likelihood computer program (Ross, 1970) in the program library of Rothamsted Experimental Station, Harpenden, England.

Lines were fitted individually to the data of each assay with independent slopes (Table 5.03). There was a linear relationship significant at P=0.05, between probit-transformed mortality and the $\log_{10}$ numbers of conidiospores/ml of spore suspension (Fig. 5.09) in all assays except numbers 16 and 26 (Table 5.03).

The precision of a bioassay depends, in part, upon the steepness of its slope b, a steep slope indicating low variability amongst the insect population and perhaps variability of pathogenicity of the conidiospores under test. The slopes for the 28 V.lecanii assays varied from 1.30 to 3.66 (Chi^2-test, $0.1 > P > 0.05$) with a weighted mean of 1.95, an encouragingly high value. The semi-weighted mean LC 50 was 1.83×10^5 spores/ml of suspension with 95% confidence limits of 1.45×10^5 to 2.31×10^5. Semi-weightings (Bliss, 1952) were used in the calculation, because the variance contained a large significant, between-assay component(0.063). However, Fig. 5.10 suggests that a downward trend of successive LC 50 values with time contributed to the between-assay variation until about assay 16, after which the LC 50 appeared to level out. A significant regression coefficient of $\log_{10}$ LC 50 values on time in days for assays 1-16, confirmed this downward trend ($P < 0.01$). Consequently, the between-assay variance was high for assays 1-16 (0.087). A non-significant regression coefficient confirmed the levelling off of LC 50

TABLE 5.03

Verticillium lecanii Conidiospores (C-3; IMI 179172) on *Macrosiphoniella sanborni*: Probit Analysed Results of 28 Bio-assays

Assay number	Log_{10} LC 50 ± S.E.[a]	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope ± S.E.[a]	Chi^2 [b]	d.f.[c,b]	P[b]
1	5.79 ± 0.15	6.28(3.35 - 19.7)	1.36 ± 0.39	0.28	2	>0.05
2	5.49 ± 0.07	3.09(2.13 - 4.32)	2.32 ± 0.53	2.63	2	>0.05
3	5.47 ± 0.07	2.95(2.04 - 4.1)	2.26 ± 0.50	2.3	2	>0.05
4	5.60 ± 0.05	4.03(3.2 - 5.13)	3.66 ± 0.66	0.55	2	>0.05
5	5.34 ± 0.11	2.17(0.89 - 3.37)	1.65 ± 0.49	4.5	2	>0.05
6	5.22 ± 0.06	1.65(1.14 - 2.11)	2.82 ± 0.63	0.54	2	>0.05
7	5.74 ± 0.07	5.51(4.12 - 8.14)	2.54 ± 0.54	1.13	2	>0.05
8	5.08 ± 0.07	1.2 (0.83 - 1.63)	2.50 ± 0.45	2.86	4	>0.05
9	5.29 ± 0.06	1.95(1.39 - 2.53)	2.97 ± 0.58	2.39	5	>0.05
10	5.07 ± 0.08	1.19(0.74 - 1.68)	2.00 ± 0.34	2.94	4	>0.05
11	4.99 ± 0.07	0.98(0.68 - 1.38)	1.89 ± 0.28	2.68	5	>0.05
12	5.10 ± 0.10	1.26(0.76 - 2.16)	1.62 ± 0.39	3.26	2	>0.05
13	5.41 ± 0.08	2.61(1.69 - 3.9)	1.85 ± 0.38	0.013	2	>0.05
14	4.65 ± 0.17	0.46(0.09 - 0.79)	1.85 ± 0.52	1.08	4	>0.05
15	5.18 ± 0.08	1.52(0.98 - 2.16)	2.16 ± 0.42	3.86	2	>0.05
16	4.84 ± 0.22	0.69(0.028-1.39)	1.30 ± 0.25	7.49	1	<0.01

TABLE 5.03 (cont.)

Assay number	$\log_{10}$ LC 50 ± S.E.[a]	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope ± S.E.[a]	Chi^2 [b]	d.f.[c,b]	P[b]
17	5.18 ± 0.10	1.53 (0.89-2.47)	2.18 ± 0.48	1.13	3	> 0.05
18	5.45 ± 0.10	2.82 (1.74-5.01)	1.69 ± 0.49	0.22	2	> 0.05
19	5.13 ± 0.13	1.35 (0.62-2.38)	1.73 ± 0.46	0.13	1	> 0.05
20	5.25 ± 0.08	1.78 (1.2-2.56)	2.75 ± 0.53	2.02	1	> 0.05
21	5.19 ± 0.11	1.54 (0.89-2.54)	1.54 ± 0.35	3.38	2	> 0.05
22	5.18 ± 0.14	1.52 (0.62-2.72)	1.52 ± 0.38	1.22	2	> 0.05
23	5.10 ± 0.14	1.26 (0.5 -2.26)	1.41 ± 0.40	3.3	2	> 0.05
24	5.26 ± 0.10	1.82 (1.1 -3.2)	1.59 ± 0.38	0.51	2	> 0.05
25	5.16 ± 0.07	1.44 (0.96-2.02)	2.4 ± 0.48	4.37	2	> 0.05
26	5.18 ± 0.10	1.52 (0.82-2.39)	2.24 ± 0.49	6.94	1	< 0.01
27	4.95 ± 0.13	0.89 (0.46-2.14)	1.48 ± 0.36	1.88	2	> 0.05
28	5.84 ± 0.13	7.04 (3.65-15.5)	1.84 ± 0.56	0.97	1	> 0.05

a = Standard error

b = Heterogeneity about regression line

c = Degrees of freedom

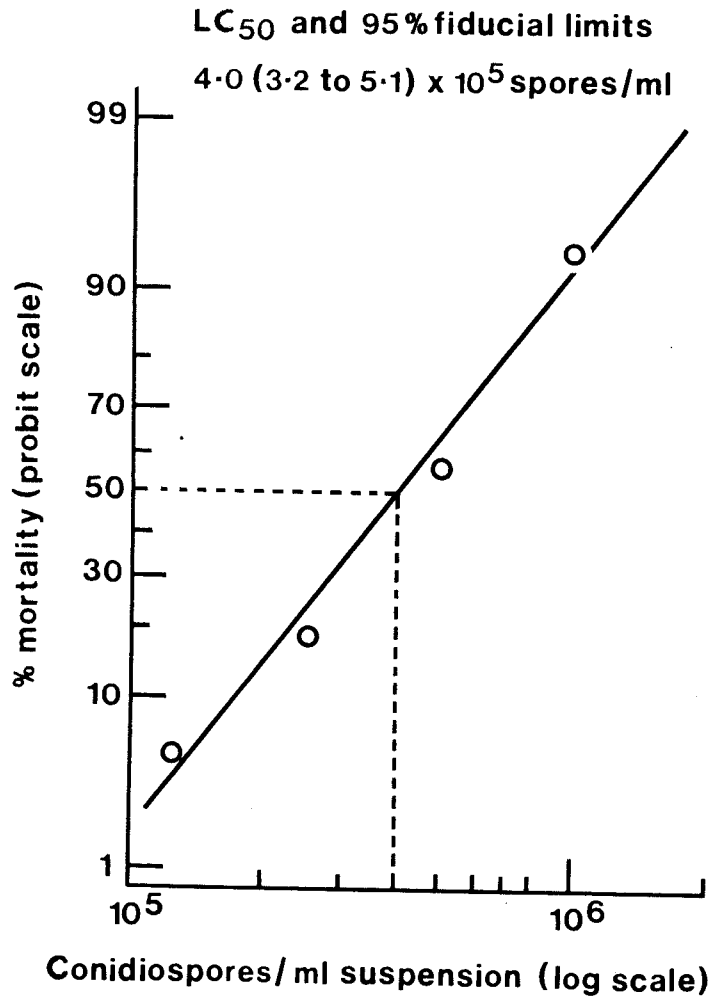


Fig. 5.09

Example of linear relationship between probit-transformed mortality of *Macrosiphoniella sanborni* and $\log_{10}$ concentration of *Verticillium lecanii* conidiospores (assay number 4, Table 5.03).

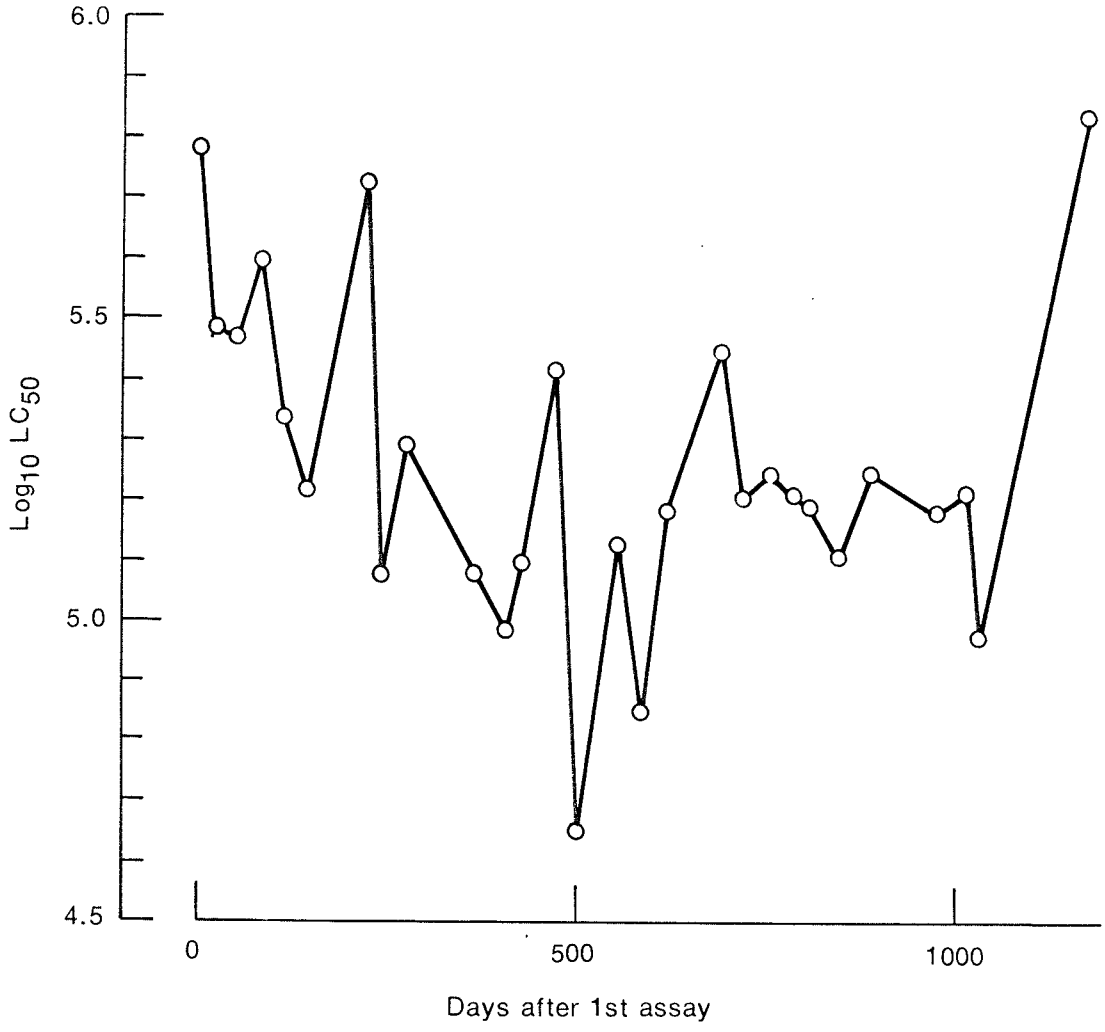


Fig. 5.10

Verticillium lecanii **bioassays: plot of successive** $\log_{10}$ LC_{50} **values with time.**

values after the 16th assay ($P > 0.1$). Variation between these assays was lower (0.03) but nevertheless still significant (Chi^2-test, $P < 0.001$).

(c) Effect of Spore Dosage on Mortality Times

The bioassay technique was used to determine the effect of increasing conidiospore concentration on the time taken for aphids to die. Figure 5.11 shows that, as spore concentrations increased, mortality times decreased. A minimal lethal time independent of concentration was not observed in this experiment, or in others (V, 8) in contrast to the results of Ignoffo (1964), who observed a minimal time for mortality of cabbage loopers (Trichoplusia ni) infected with a nuclear-polyhedrosis virus.

8. Bioassay of Blastospore Pathogenicity

The pathogenicities of conidiospores and blastospores can be compared on numerical, dry weight and live spore volume bases. Blastospores and conidiospores of the standard strain (C-3), differed greatly in size (Table 5.04).

In another set of experiments, the dry weight of blastospores was greater than that of conidiospores (Table 5.05). Although variation between dry weight determinations of spore batches on different occasions was high (Table 5.05), conidiospores were significantly lighter than blastospores in the same batch (t-test on paired means, $P < 0.05$). For an approximation of live spore volume, the spores were regarded as being cuboidal in shape. The mean blastospore/conidiospore dry weight ratio (1.67; Table 5.05) was much less than the live spore volume ratio (3.41, Table 5.04) possibly because blastospores may be more vacuolated than conidiospores.

The pathogenicities of blastospores and conidiospores were assayed in parallel on 5 occasions on M.sanborni (Table 5.06).

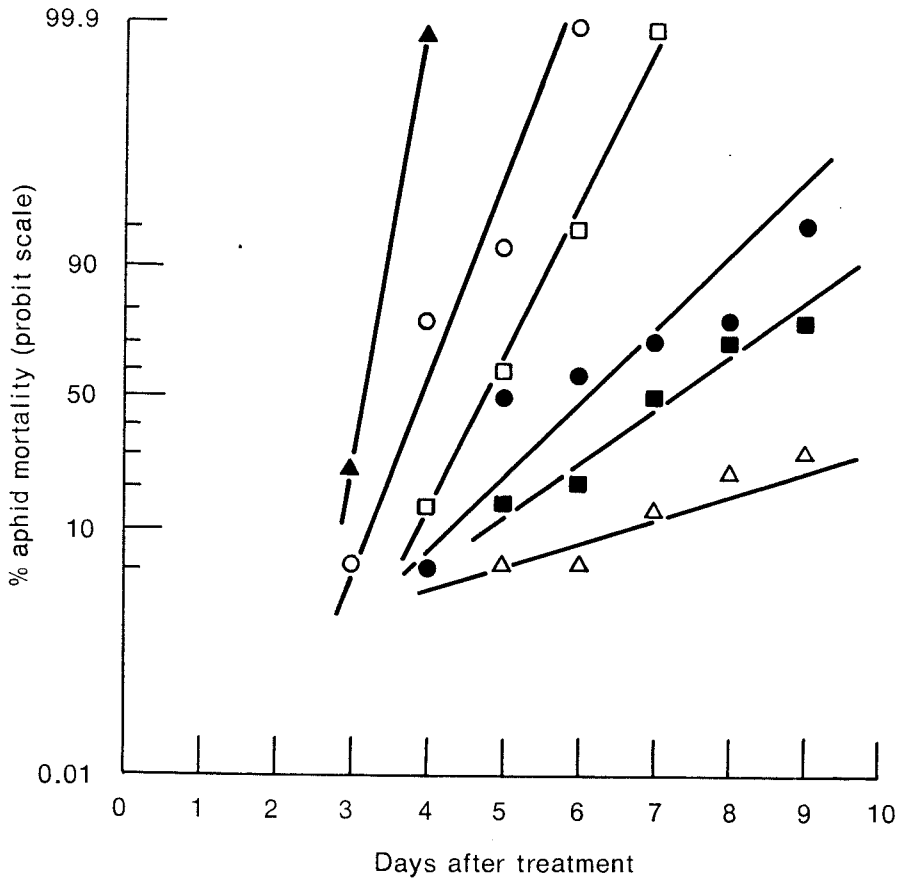


Fig. 5.11

Lethal times of *Macrosiphoniella sanborni* **aphids treated with increasing doses of** *Verticillium lecanii* **conidiospores. (20° ±1°C)**

▲ 7.7×10^7 spores/ml
○ 1×10^7 ''
□ 1×10^6 ''
● 5×10^5 ''
■ 2.5×10^5 ''
△ 1.25×10^5 ''

TABLE 5.04

Dimensions of live conidiospores and Blastospores of *Verticillium lecanii*

Spore type	Batch number	n	Length(u) mean±S.E.[a]	Width(u) mean±S.E.[a]	Volume	Volume ratio
Conidiospores	1	40	6.49±0.07	1.82±0.06		
	2	32	6.27±0.19	1.67±0.05		
	3	31	6.04±0.26	1.71±0.05		
	mean	103	6.26±0.11	1.73±0.04	18.7	
Blastospores	1	48	15.87±1.55	2.13±0.11		
	2	32	15.80±1.52	2.06±0.09		
	3	41	12.42±0.65	2.11±0.07		
	mean	156	14.06±0.66	2.13±0.08	63.8	
Blastospores/Conidiospores						3.41

a = Standard error of mean of n observations

b = Approximated by assuming spores are cuboidal in shape

TABLE 5.05

Dry Weights of Parallel *Verticillium lecanii* Conidiospore and Blastospore Batches[a]

Batch number	n	Weight x 10^{-11} g/spore ± S.E.[b]		Weight ratio
		Conidiospores	Blastospores	
1	4	2.62±0.04	3.27±0.01	1.25
2	4	1.72±0.05	2.41±0.06	1.40
3	4	0.71±0.08	1.69±0.03	2.36
Mean	3	1.68±0.55	2.45±0.45	1.67

a = For culture conditions see Methods - V, 1, a

b = Standard error based on 4 dry weight estimates for each batch

TABLE 5.06

Pathogenicity of Batches of *Verticillium lecanii* Blastospores and Conidiospores Assayed in Parallel on the Aphid *Macrosiphoniella sanborni*

Experiment number	Spore type	Log_{10} LC50 ± S.E.[a]	LC50 with 95% fiducial limits x 10^5 spores/ml	Slope ± S.E.[a]	Chi^2 [b]	d.f.[c,b]	P[b]	Potency Ratio	Weighted mean potency ratio and 95% fiducial limits
1	Blastospore	4.44 ± 0.12	0.28(0.15-0.42)	1.68 ± 0.3	6.986	5	> 0.05	3.66	
	Conidiospore	4.99 ± 0.07	0.98(0.68-1.38)	1.89 ± 0.28	2.68	5	> 0.05		
2	Blastospore	4.63 ± 0.3	0.42(0.0001-10.5)	0.92 ± 0.33	3.34	2	> 0.05	2.95	
	Conidiospore	5.1 ± 0.10	1.26(0.76-2.16)	1.62 ± 0.39	3.26	2	> 0.05		
3	Blastospore	4.59 ± 0.49	0.39 - [d]	0.9 ± 0.38	2.26	2	> 0.05	6.62	2.27(1.54-3.3)
	Conidiospore	5.41 ± 0.08	2.61(1.69-3.9)	1.85 ± 0.38	0.013	2	> 0.05		
4	Blastospore	4.82 ± 0.16	0.65(0.12-1.12)	2.06 ± 0.69	0.269	1	> 0.05	2.67	
	Conidiospore	5.25 ± 0.08	1.78(1.2 -2.56)	2.75 ± 0.53	2.025	1	> 0.05		
5	Blastospore	5.28 ± 0.12	1.9(0.96-3.6)	1.38 ± 0.37	0.059	2	> 0.05	0.93	
	Conidiospore	5.26 ± 0.10	1.82(1.1-3.2)	1.59 ± 0.38	0.508	2	> 0.05		

a = Standard error

b = Heterogeneity about regression line

c = Degrees of freedom

d = Limits not given because slope not significantly different from zero

Log_{10} LC 50 values differed significantly:-

	Semi-weighted mean log_{10} LC 50 ± Standard Error	P (Chi^2-test)	Between-assay variance
Conidiospores	5.20 ± 0.072	0.0015	0.0185
Blastospores	4.81 ± 0.11	0.0001	0.0273

Consequently, a potency ratio was calculated for each parallel conidiospore-blastospore assay, and weighted according to its variance, in order to obtain the weighted mean potency ratio, 2.27. This shows that V.lecanii blastospores are, on a numerical basis, twice as pathogenic as conidiospores.However, on the basis of dry spore weight (Table 5.05), the pathogenicity ratio is 1.36 and on a live spore volume basis (Table 5.04), it is only 0.67. Thus, blastospores are less pathogenic than a batch of conidiospores totalling an equal live volume.

The lower slopes encountered in the blastospore assays indicate either more variation in the susceptibility of insects to blastospores or more variation in the numerical doses of spores that individual insects received or both. Variation in spore dose could result from the larger variation in spore size encountered within each batch of blastospores compared with conidiospores (Table 5.04).

Lethal times were also obtained because the death rate is of great importance as V.lecanii does not reduce the number of progeny until shortly before it kills the parent aphid (V, 6). In most experiments, blastospores killed aphids sooner than conidiospores (Table 5.07). Increasing concentrations advanced death to an LT 50 of only 1.95 days at 10^8 blastospores/ml because, at high concentrations, aphids apparently died from the direct trauma (V, 6) of a

TABLE 5.07

Lethal Times (LT 50s) of Parallel Batches of *Macrosiphoniella sanborni* Treated with *Verticillium lecanii* Conidiospores or Blastospores

Experiment number	Spore concentration/ml	Blastospores	Conidiospores
1.	3.1×10^4	5.55	-
	6.2×10^4	5.05	7.15
	1.2×10^5	4.80	5.55
	2.5×10^5	4.15	5.60
	5×10^5	4.15	4.75
	1×10^6	3.4	4.30
2.	7.2×10^4	5.25	-
	4.5×10^5	4.20	4.95
	1.12×10^6	3.55	4.65
3.	10^6	4.35	5.00
	10^7	2.30	4.05
	10^8	1.95	2.65
4.	1.11×10^5	6.25	6.25
	3.33×10^5_6	5.30	5.20
	1×10	5.20	4.05

massive attack by spores before progressive invasion by the fungus, which was the effect observed at lower concentrations (V, 6).

The greater pathogenicity of blastospores related to spore numbers may be explained by the possibilities that, owing to their larger size, they may produce a greater number of invasive appressoria and also more blastospores may land on, and adhere to aphids. Furthermore, blastospores may be able to infect sooner, as conidiospores may require a longer germination time (Table 5.08), although more experiments are required to confirm this. Early spore growth would speed up penetration of the cuticle and reduce the time during which movements of aphids might dislodge any loose spores.

TABLE 5.08

Increase in Length of Verticillium lecanii Conidiospores and Blastospores Germinating on Sabouraud Dextrose Agar at 23 ± 0.5°C (combined results of 2 experiments)

Incubation period(h)	%germination of conidiospores	Increase in length (u) ± S.E.[a]			
		n	Conidia	n	Blastospores
3[b]	20.9		-[d]	69	4.95±2.07
5.5[b]	82.0	31	6.57±0.45	48	11.30±2.14
8[b]	96.1	33	9.0 ±1.02	34	23.65±3.96
14[c]	98.1	63	30.92±2.28	66	52.75±4.28

a = Standard error of mean of n germ tube lengths minus original ungerminated spore length

b = Experiment 1

c = Experiment 2

d = Germ tubes only just visible

9. Infection of Aphids Placed on Spore-treated Leaves

If aphids in a glasshouse are not directly hit by a V. lecanii spore-spray, do they easily become infected from walking over the spore-treated leaf surfaces? To answer this question, leaf discs were immersed singly in one of 3 blastospore or conidiospore suspensions (10^6, 10^7 and 10^8 spores/ml) and each placed in an assay cell with 24 discs/concentration (Fig. 5.02). While the discs were still damp, one apterous, adult M.sanborni was placed in each cell. For comparison, batches of 20 aphids were treated in the Büchner funnel using the bioassay technique (V, 1, d). Mortalities due to V.lecanii at 20 ± 1°C were recorded daily.

Under these optimal conditions of temperature and humiditiy, infection of aphids from leaf surfaces was very small compared to that when aphids themselves were treated with spores. LT 50s were about 3 days longer for aphids infected from leaves (Table 5.09).

TABLE 5.09

Comparison of Infection from Verticillium lecanii Spores on Leaves and from Spores Applied Directly to Macrosiphoniella sanborni

	Spore concentration/ml applied to leaf						Spore concentration/ml applied to aphids					
	10^6		10^7		10^8		10^6		10^7		10^8	
	c	b	c	b	c	b	c	b	c	b	c	b
LT 50	-[a]	-[a]	7.1	5.3	5.7	3.9	5.0	4.3	4.0	2.3	2.6	1.9

a = LT 50s not achieved by 8th day.

b = Blastospores

c = Conidiospores

Approximate LC 50s (fitted by eye - Fig. 5.12) were over 100 times greater than LC 50s which may be expected from the bio-assay technique (Tables 5.03 and 5.06). This different method of application has also suggested that blastospores were more pathogenic than conidiospores applied to leaves thus re-inforcing the results of previous experiments (V, 8) in which spores were applied directly to aphids.

10. Effect of Storage on Spore Pathogenicity

Stored spores were assayed in parallel with batches of fresh spores. Spores in the first storage batch, still 100% viable after 6 months storage had a similar LC 50 to that of fresh spores (Table 5.10). Spores in 3 other batches, of reduced viability and 3-7 months in storage, had LC 50s considerably higher than would be expected (calculated from the viability of stored spores - Table 5.10) if the infectivity of the live stored spores was comparable to that of the fresh spores. Thus, some stored spores, although still viable, had lost infective vigour.

11. Susceptibility of Young Stages of M.sanborni to V.lecanii

First and second instar M.sanborni treated with conidiospores in aqueous suspension were 2.8 times less susceptible than adults. In each of 2 experiments with pairs of parallel assays, LC 50s for progeny were higher than for adults (Table 5.11) and the weighted mean LC 50 ratio for the experiments was 2.78, significantly greater than 1.0 ($P < 0.05$). In contrast, more first and second instar aphids than adults died when treated with spore-heads by steering them with a paintbrush over sporulating (adult) cadavers in assay cells (Table 5.12). Since all the young aphids killed by V.lecanii, and the adults dying within 48h after treatment with spore-heads, did not possess blastospores in their haemolymph - as with aphids treated with high densities of spores in V, 6 -

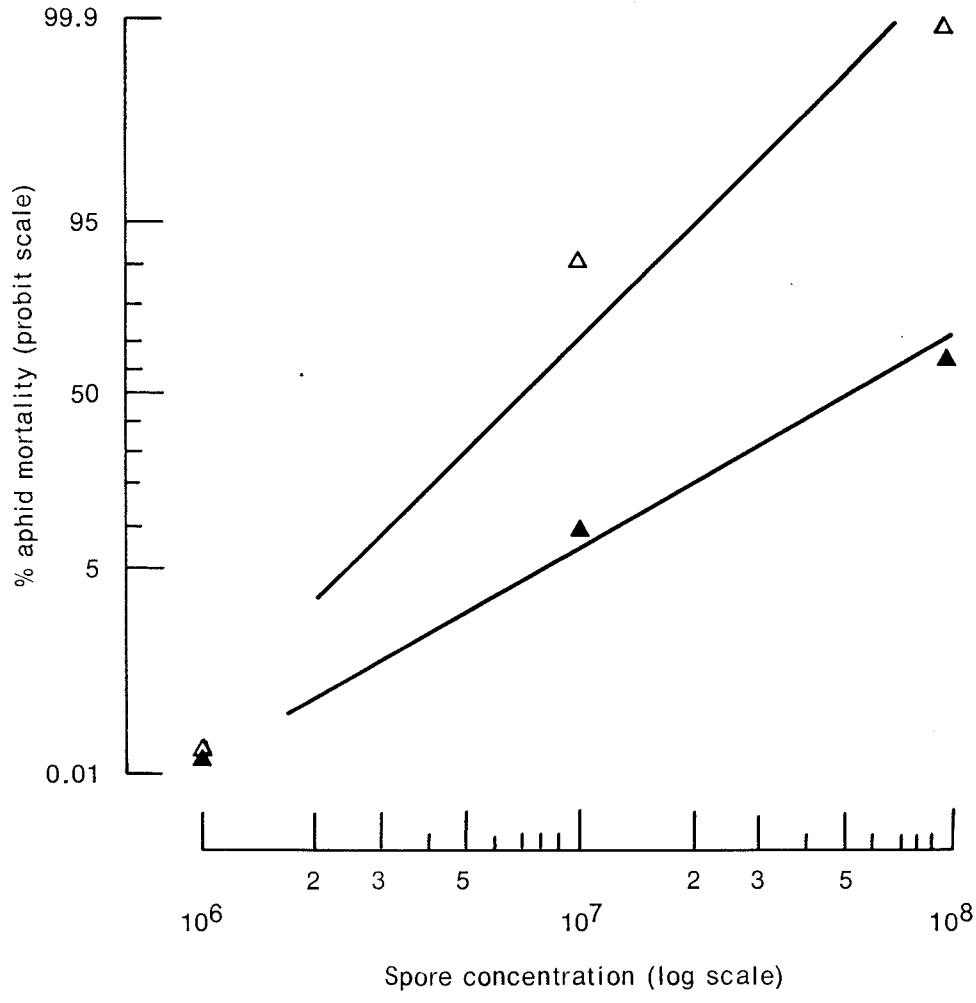


Fig. 5.12

Pathogenicity for *Macrosiphoniella sanborni* **adult apterae, of** *Verticillium lecanii* **spores applied to leaves (20±1°C)**

△ Blastospores; LC_{50} = 7 x 10^6 spores/ml

▲ Conidiospores; LC_{50} = 5.25 x 10^7 spores/ml

Control mortality, 0%

TABLE 5.10

Effect of Storage on Pathogenicity of *Verticillium lecanii* Conidiospores and Blastospores Stored in Distilled Water at 2°C, in Darkness

Experiment number	Spore age (at 2°C)	% viability ± S.E.[a]	Experimental $\log_{10}$ LC50 ± S.E.[a]	Experimental LC50 & 95% fiducial limits x 10^5 spores/ml	Slope ± S.E.[a]	Chi[2b]	d.f.[c]	P[b]	Expected LC50x10^5 spores/ml	Experimental LC50(stored) / Expected LC50(stored)[d]
Conidiospores										
1	Fresh	99.0[g]	5.29±0.06	1.95(1.39-2.53)	2.97±0.58	2.39	5	>0.05	-	0.77
	6 months	96.9±0.65[c]	5.19±0.07	1.54(1.07-2.07)	2.21±0.37	4.39	5	>0.05	2.01	
2	Fresh	98.1[g]	5.18±0.10	1.53(0.89-2.47)	2.18±0.48	1.13	3	>0.05	-	5.3
	7 months	46.9±1.87[f]	6.24±0.21	17.3 (6.0-71.2)	0.75±0.25	0.35	3	>0.05	3.26	
Blastospores										
3	Fresh	96.7[g]	4.86±0.11	0.74(0.38-1.15)	1.61±0.31	4.05	5	>0.05	-	3.16
	3 months	40.5±1.1[h]	5.76±0.10	5.75(3.56-9.27)	1.27±0.19	4.35	5	>0.05	1.83	
4	Fresh	102.3[g]	4.68±0.12	0.48(0.27-0.86)	1.69±0.33	0.075	2	>0.05	-	2.29
	5 months	54.2	5.31±0.12	2.04(1.15-3.6)	1.18±0.20	5.83	4	>0.05	0.89	

a = Standard error
b = Heterogeneity about regression line
c = Degrees of freedom
d = Expected LC50 of stored spores $\frac{\%\text{viability (fresh spores)}}{\%\text{viability (stored spores)}}$ x LC50 (fresh spores)
e,f = Standard error of mean of counts from 4(e) and 8(f) agar slides
g = Assumed to be not significantly different from 100%
h = Standard error of mean of viable counts performed on 2 separately diluted spore suspensions (separate total counts made for each spore suspension)

TABLE 5.11

Susceptibility of Apterous Adults and Young Stages of *Macrosiphoniella sanborni* to *Verticillium lecanii* Conidiospores in Aqueous Suspension

Experiment number	Developmental stage	Log_{10} LC50 ± S.E.[a]	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope ± S.E.[a]	Chi^2	d.f.[c,b]	P[b]	LC50 Young[d] / LC50 Adults	Weighted mean LC50 ratio and 95% fiducial limits
1	Adult	5.22±0.06	1.65(1.14-2.11)	2.82±0.63	0.54	2	>0.05	2.61	
	Young[d]	5.64±0.10	4.34(2.73-8.88)	1.58±0.48	6.82	2	<0.05		
2	Adults	5.34±0.11	2.17(0.89-3.37)	1.65±0.49	4.5	2	>0.05	6.71	2.78 (1.67 - 4.63)
	Young[d]	6.16±0.43	14.4 ——————[e]	0.79±0.57	1.73	2	>0.05		

a = Standard error

b = Heterogeneity about regression

c = Degrees of freedom

d = First and second instars

e = Limits not included because slope not significantly different from zero

TABLE 5.12

Susceptibility of Apterous Adults and Young Stages of *Macrosiphoniella sanborni* to Spores in Slime-heads from Aphid Bodies Sporulating with *Verticillium lecanii*

Insect stage and experiment number		LT50 ± S.E.[a]	LT50 adult / LT50 young
1	Adults	2.77 ± 0.03[b]	
	Young[c]	2.02 ± 0.04[d]	
			1.44 ± 0.07
2	Adults	2.65 ± 0.15[b]	
	Young[c]	1.75 ± 0.05[d]	

a = Standard error

b = Standard error of mean mortality times of 2 batches of 20 aphids each

c = First and second instars

d = Standard error of mean mortality times of 3 batches of 20 aphids each

it was concluded that these aphids died as a result of trauma caused by a massive attack of invading conidiospores.

12. Influence of Temperature and Humidity on Aphids and Disease

(a) Effect of Temperature on Aphid Reproduction

The developmental time and the weekly rate of increase of aphids are markedly influenced by temperature, especially in the range 10 - 20 °C (Barlow, 1962; DeLoach, 1974). The times taken for apterous, newborn M.sanborni progeny to mature and commence reproduction at 3 temperatures, 11.5, 15 and 20°C were determined from daily observation of newborn progeny kept separate on chrysanthemum leaf discs in breeding cells (Fig. 5.01). Also, population growth at 11.5, 15, 20 and 25°C was monitored daily using mature apterous M.sanborni each placed singly on chrysanthemum plants, with 5 plants at each temperature in each of 2 experiments.

The effects of temperature on developmental time and rate of population increase were similar (Table 5.13). The time taken for newborn M.sanborni to mature and commence reproducing at 20°C was x0.6 that at 15°C, and x0.4 that at 11.5°C. The weekly rates of increase at 11.5 and 15°C were 0.4 and 0.6 respectively of that at 20°C. Rates of increase were similar at 20 and 25°C.

(b) Effect of Temperature on Aphid Mortality Due to V.lecanii

The time taken to die for 50% of M.sanborni aphids treated with conidiospores in aqueous suspension at 20°C (V, 1) was x0.3 that at 11°C, x0.7 that at 15°C and x1.03 that at 25°C (Table 5.14).

When apterous M.sanborni and My.persicae were infected

TABLE 5.13

Effect of Temperature on Developmental Time and Rate of Population Increase on *Macrosiphoniella sanborni*

	Temperature (°C)			
	11.5	15	20	25
Age at first reproduction (days) ± S.E.[a]	24.4±0.84[b]	17±0.45[c]	9.8±0.36[d]	-
Weekly rate of increase[e] ± S.E.[f]	2.06±0.24	2.88±0.07	4.86±0.10	4.60±0.42

a = Standard error of mean maturation age of (b) 11, (c) 12, and (d) 11 apterous aphids

e = Antilogarithm of the slope of the plot of $\log_{10}$ numbers of aphids/plant and time

f = Standard error of mean rate of increase in 2 experiments

TABLE 5.14

Effect of Temperature on LT 50s of Apterous *Macrosiphoniella sanborni* Adults Treated with Aqueous Suspensions of *Verticillium lecanii* Conidiospores

Concentration spores/ml	11°C	15°C	20°C	25°C
10^6	13	6.87±0.87[a]	4.52±0.17[a]	4.25
10^7	-[b]	4.69	3.30	3.29

a = Standard error of mean from 2 experiments (20 aphids/concentration)

b = This temperature not used in this experiment

with spores in slime-heads by steering them with a paintbrush over aphid cadavers sporulating with V.lecanii and then placed in assay cells (Fig. 5.02), the effect of temperature on LT 50s was similar (Table 5.15) to when spores in aqueous suspension were used, and similar for both species of aphid.

TABLE 5.15

Effect of Temperature on LT 50s of Apterous Macrosiphoniella sanborni and Myzus persicae Made to Walk over Aphid Cadavers Sporulating with Verticillium lecanii

Aphid species	11°C	15°C	20°C
M.sanborni[a]	8.22 ± 0.47	4.52 ± 0.47	3.40 ± 0.30
My.persicae[b]	9.24	4.15	3.03

a = Means, with 95% fiducial limits, of 2 experiments, 20 aphids/experiment.

b = One experiment only, 20 aphids/experiment

(c) Effect of Temperature during the First Hours of Incubation

The detailed methods employed are described in V, 2, a. In the first 3 experiments, aphids treated with spore suspensions were kept at high humidity in assay cells (Fig. 5.02) for 6 and 14h at various temperatures and then returned to lower humidity (63-72%) at 20 ± 1°C for the rest of the duration of the experiment (6 days).

Using an initial incubating temperature of 20 or 15°C, mortalities were similar (mean 30%) but in the single experiment at 11.5°C, mortality was low (7%; Table 5.16).

TABLE 5.16

Effect of Temperature during First 6 or 14h of Incubation on Final Mortalities (%) 6 days Later at 20 ± 1°C of Apterous, Adult Macrosiphoniella sanborni Treated with 2 x 10^6 Verticillium lecanii Conidiospores/ml

Experiment number	Mortality (%) at			Initial 6 or 14h incubation period temperature					
	Continous 100% RH (treated)[a]	Continous low RH		11.5°C		15°C		20°C	
		Treated[a]	Untreated[a]	n[b]	%mortality	n[b]	%mortality	n[b]	%mortality
1.	95	10	0	-	-	74	24.3	119	39.4[c]
2.	100	15.7	5	65	7.7	58	39.6	65	44.6
3.	100	5.3	0	-	-	35	42.8	35	42.8
3.						33	54.8	24	64.6[d]

a = Approximately 20 insects/treatment

b = Number of insects/treatment

c = Fourteen hours at 100% RH then rest of 6 days at 63-72% RH

d = Six hours at 100% RH then rest of 6 days at 63-72% RH

In the third experiment, aphids were removed to low RH after 6h at high RH and the subsequent mortalities at 15 and 20°C were similar to each other (Table 5.16). However, they were higher than those for aphids transferred after 14h, a result which cannot be explained except to suggest it arose by chance.

In another experiment, aphids were infected not by aqueous spore suspensions, but by steering them over aphid cadavers sporulating with fungus and then placing them in assay cells (Table 5.17). Results were very similar to those for aqueous suspensions; mortalities were higher at 20°C than 15°C-but not significantly so ($P>0.05$) - and much lower at 11°C (Table 5.17)

TABLE 5.17

Effect of Initial Temperature During First 14h of Incubation on Final Mortalities 6 days Later at 20 ± 1°C of Apterous, Adult Macrosiphoniella sanborni Made to Walk over Aphid Cadavers Sporulating with Verticillium lecanii.

% mortality at :-			14h at 100% RH, then rest of 6 days at 72% RH (% mortality ± S.E.[b])		
Continuous 100% RH (Treated)[a]	Continuous low RH				
	Treated[a]	Untreated[a]	11°C	15°C	20°C
100	0	0	4 ± 4	44 ± 4	60 ± 12

a = 20 aphids/treatment

b = Standard error of mean mortalities of 2 batches of 25 M.sanborni

(d) Effect of Alternating High and Low Humidity on Spore-treated Aphids

In the experiment designed to simulate glasshouse humidity conditions, (Methods - V, 2, b), mortality was 88% for spore-treated aphids held at high RH continously, 71% among aphids initially held at 100% RH and then alternated diurnally between 44 and 100% RH, whereas mortality for aphids transferred to continuous low RH (44%) after 14h at 100%RH, was only 41% (Fig. 5.13). This shows that transfer of aphids from high to low humidity arrested the infection process which could resume upon transfer back to high RH. Mortality of aphids initially held at 44% RH and alternated was much higher in 6 days (47%) than that of aphids held at low RH continuously (17%; Fig. 5.13) showing that conidiospores could survive the initial period of low RH and infect in subsequent periods of high RH.

(e) Effect of Alternating High and Low Temperatures on Spore-treated Aphids

In an experiment designed to simulate glasshouse conditions during hot weather, 2 batches of 20 apterous M. sanborni were treated with 10^7 conidiospores/ml and placed in assay cells at 5000 lux using the methods of V, 1. One batch was held at 20 ± 1°C continuously while the other, after 14h at 20°C, was alternated between 31.5°C (a temperature inhibiting the growth of V.lecanii - Fig. 4.03) for 10h and 20°C (14h). Mortalities due to V.lecanii were recorded daily.

The LT 50s of alternated aphids were lengthened by only less than 1 day when compared with aphids at 20°C continuously (Fig. 5.14). This suggested that the ability of V.lecanii to kill aphids may be only slightly impaired during periods of high glasshouse temperature unfavourable for the fungus, likely to be encountered in summer.

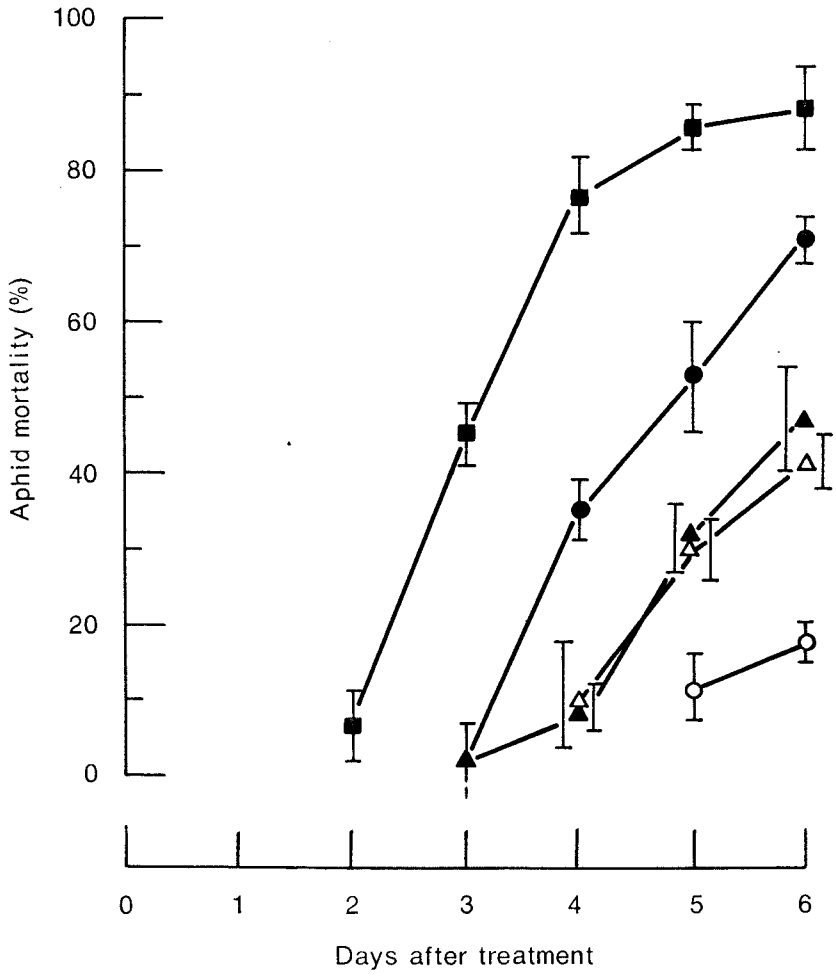


Fig. 5.13

Effect of alternating humidities on adult, apterous *Macrosiphoniella sanborni* treated with 10^7 *Verticillium lecanii* conidiospores /ml (20±1°C).
Points are mean mortalities, of 3 batches of 20 aphids/ treatment, with 95% fiducial limits .

- ■ 100% RH throughout
- ● 100% RH for first 12h and then alternated diurnally between 44 and 100%
- ▲ 44% RH for first 12h and then alternated diurnally between 100 and 44%
- △ 100% RH for first 12h and then continuous 44% RH
- ○ 44% RH throughout

Control mortality amongst uninfected aphids at 100% RH after 6 days was 15%.

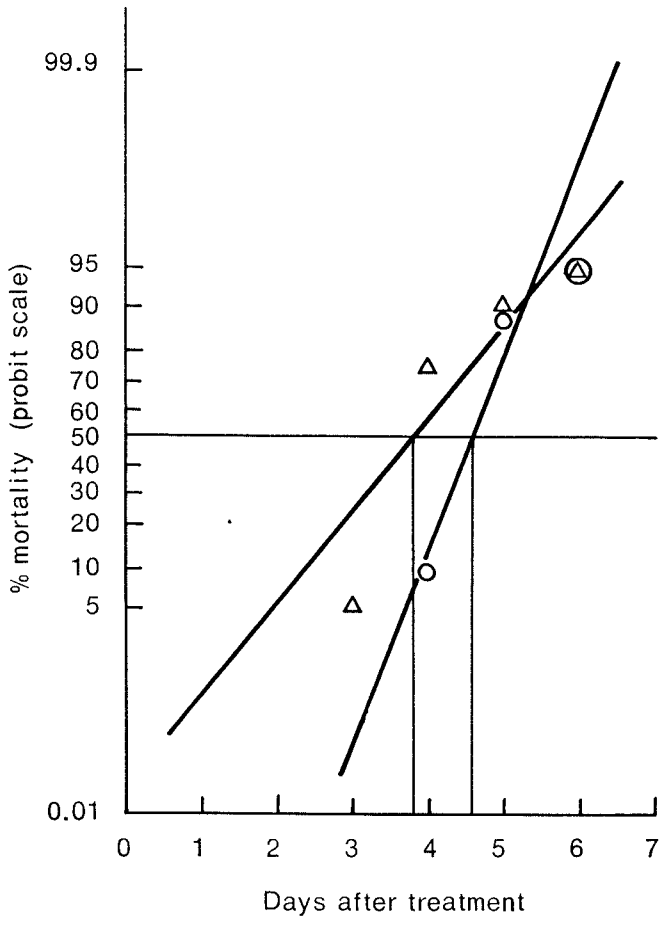


Fig. 5.14

Comparison of the effect of alternating temperatures (20 and 31.5°C) and continuous (20°C) temperature on LT_{50}s (fitted by eye) of adult apterous *Macrosiphoniella sanborni* treated with 10^7 *Verticillium lecanii* conidiospores/ml.

O Aphids held at 20°C for 14h followed by 31.5°C for 10h and alternated for 6 days.

Δ Aphids held at 20°C continuously.

In another experiment, untreated and conidiospore-treated aphids were incubated at 20 ± 1°C for 36h and then transferred to 31.5°C for 4 days in an effort to "cure" the treated aphids of V.lecanii disease. However, aphids reacted unfavourably to the higher temperature and, after 3 days at 31.5°C, mortalities among both control and treated aphids approached 100%.

13. Susceptibility of 3 Aphids Hosts to V.lecanii

(a) Susceptibility to Conidiospores in Aqueous Suspension

The susceptibilities of My.persicae and B.helichrysi were compared with that of M.sanborni in parallel assays. My.persicae was less susceptible than M.sanborni (Table 5.18), the mean LC 50 ratio being significant ($P < 0.05$) and the upper 95% confidence limit less than 1.0 (Table 5.18). However, the mean LC 50 ratio for the M.sanborni: B.helichrysi assays was not significantly different from 1.0 at the 5% level of confidence (Table 5.19). The departure from normal feeding habits caused by rearing aphids on leaf discs prior to assay may have influenced susceptibility - particularly that of M.sanborni, the stem feeding aphid. However, the susceptibilities of both aphid species reared on whole plants were similar (Table 5.20).

These were unexpected results because, in the glasshouse, My.persicae and B.helichrysi are more easily controlled than M.sanborni by V.lecanii (Chapter VI). A different body topography of apterous My.persicae may have caused fewer spores to settle on its cuticle than on M.sanborni or, because My.persicae was extremely restless in assay cells (V, 7), spores may have dislodged before germination and cuticle penetration.

(b) Mobility of Aphids and Susceptibility to Contagion

Although the topography of My.persicae may allow fewer

TABLE 5.18

Comparison of Susceptibilities of *Myzus persicae* and *Macrosiphoniella sanborni* Reared on Leaf Discs to Aqueous Suspensions of *Verticillium lecanii* Conidiospores

Experiment number	Aphid species	Log_{10} LC50 ± S.E.[a]	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope ± S.E.[a]	Chi^2 [b]	d.f.[c] [b]	P[b]	LC50 (M.san) / LC50 (My.pers)	Weighted mean LC50 ratio and 95% fiducial limits
1	*M.sanborni*	5.08±0.07	1.2(0.8-1.63)	2.5±0.45	2.86	4	>0.05		
	My.persicae	5.16±0.26	1.43(0.02-3.48)	0.77±0.31	5.76	4	>0.05	0.84	
2	*M.sanborni*	5.07±0.08	1.19(0.74-1.68)	2.00±0.34	2.94	4	>0.05		
	My.persicae	5.49±0.15	3.15(1.03-6.63)	1.22±0.42	3.51	4	>0.05	0.38	
3	*M.sanborni*	5.41±0.082	2.61(1.69-3.9)	1.85±0.38	0.013	2	>0.05		
	My.persicae	5.72±0.46	5.25 [d]	0.61±0.32	1.11	2	>0.05	0.49	
Weighted mean									0.48(0.26-0.89)

a = Standard error

b = Heterogeneity about regression line

c = Degrees of freedom

d = Limits not included since slope not significantly different from zero

TABLE 5.19

Comparison of Susceptibilities of *Brachycaudus helichrysi* and *Macrosiphoniella sanborni* Reared on Leaf Discs, to Aqueous Suspensions of *Verticillium lecanii* Conidiospores

Experiment number	Aphid species	Log_{10} LC50 ± S.E.[a]	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope ± S.E.[a]	Chi^2 [b]	d.f.[c]	P[b]	LC50 (*M.san.*) / LC50 (*B.hel.*)	Weighted mean LC50 ratio and 95% fiducial limits
1	*M.sanborni*	5.74±0.07	5.51(4.12-8.14)	2.54±0.54	1.13	2	>0.05	1.15	
	B.helichrysi	5.68±0.11	4.78(2.94-8.63)	1.32±0.27	2.54	5	>0.05		
2	*M.sanborni*	5.07±0.08	1.19(0.74-1.68)	2.00±0.34	2.94	4	>0.05	1.05	
	B.helichrysi	5.05±0.18	1.14(0.27-2.19)	1.12±0.29	0.29	4	>0.05		
3	*M.sanborni*	4.65±0.17	0.46(0.09-0.79)	1.85±0.52	1.077	4	>0.05	0.15	
	B.helichrysi	5.48±0.11	3.02(1.66-4.98)	1.42±0.31	6.25	4	>0.05		
Weighted mean									0.58(0.16-2.13)

a = Standard error

b = Heterogeneity about regression line

c = Degrees of freedom

TABLE 5.20

Bioassay of *Verticillium lecanii* Conidiospore Suspensions on *Macrosiphoniella sanborni* and *Myzus persicae* Apterous Adults Reared on Whole Plants

Regime	Log_{10} LC50 ± S.E.[a]	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope ± S.E.[a]	Chi^2 [b]	d.f.[c,b]	P[b]	LC50(*M.san*)[d] / LC50(*My.pers*)
My.persicae from whole plants	5.53±1.31	3.39 [e]	0.24±0.59	0.021	1	> 0.05	
M.sanborni from whole plants	5.31±0.12	2.04(1.13-3.72)	1.32±0.32	1.54	2	> 0.05	0.603
M.sanborni from leaf discs	5.19±0.11	1.54(0.89-2.54)	1.54±0.35	3.38	2	> 0.05	

a = Standard error

b = Heterogeneity about regression line

c = Degrees of freedom

d = Aphids reared on whole plants

e = Limits not included since slope not significantly different from zero

spores in aqueous suspension to settle on its cuticle than would that of M.sanborni, spores in slime-heads from diseased aphids may stick equally well to both species, or even better to My.persicae. Thus, the difference in susceptibility between both these aphid species in the glasshouse may be explained by a greater susceptiblity to contagion.

Also frequency of contagion may depend on how much the different aphid species wander. These possibilities were tested by deliberately infecting apterous aphids of both species (i) by guiding them over sporulating cadavers and (ii) by allowing aphids to contact sporulating cadavers themselves in various containers and on whole plants, giving the aphids different areas over which to wander. A further possibility exists that M.sanborni may avoid contact with sporulating cadavers, more so than My.persicae. However, since in the glasshouse, aphids of both species have been observed to walk over sporulating cadavers, showing no tendency to avoid them, this possibility is discounted.

Adult, apterous M.sanborni and My.persicae were infected with spore heads by steering them over sporulating cadavers. They were then placed in assay cells (Fig. 5.02). LT 50s for both species were similar (Table 5.21). Thus, aphids of both species infected in this way were equally susceptible to contagion.

To simulate more closely fungus-aphid interactions in the glasshouse, aphids were allowed to contact sporulating cadavers themselves. Firstly, in 2 experiments, adult, apterous M.sanborni and My.persicae were placed singly in assay cells, a uniform distance from a sporulating cadaver. Any possible difference in mobility between the aphid species was not reflected in the mortality times, which were similar (Table 5.22).

TABLE 5.21

Effect of Guiding Adult Apterous *Myzus persicae* and *Macrosiphoniella sanborni* over Cadavers Bearing Slime-heads of *Verticillium lecanii* and Placing Them in Assay Cells (20 ± 1°C)

Aphid species	*M.sanborni*	*My.persicae*
LT 50[a]	3.5	3.3

a = 20 aphids/treatment

TABLE 5.22

Lethal Times (LT 50s) of Adult, Apterous *Macrosiphoniella sanborni* and *Myzus persicae* (not guided) Allowed to Contact Slime-heads of *Verticillium lecanii* on Cadavers Themselves (20±1°C)

Aphid species	*M.sanborni*	*My.persicae*
LT 50 ± S.E.[a]	5.42 ± 0.12	5.25 ± 0.22

a = Standard error of mean mortality times from 2 experiments, 20 aphids/treatment

In the previous experiment, the unnatural confines of 19 mm-diameter assay cells may have obscured differences in the mobility of the aphid species. In another experiment, aphids were placed further away from the sporulating cadavers, in Petri-dishes (V, 3, a).

The results suggested that My.persicae moved more than M.sanborni and more My.persicae died in 10 days, but the mortality did not fully reflect the difference in movement (Table 5.23).

TABLE 5.23

Mortalities from Infection by Verticillium lecanii Spore-heads on Sporulating Cadavers and Movement among Adult Apterous Myzus persicae and Macrosiphoniella sanborni Wandering Freely in Petri-dishes (20 ± 1°C). For more details see Methods (V, 3, a).

Aphid species	Control mortality		Aphids moved across dish(%)		Mortality of treated aphids(%)	
	7days	10days	7days	10days	7days	10days
M.sanborni	10	20	20	33.3	26.6	26.6
My.persicae	10	10	60	93.3	20	53.3

In the following experiments, the spread of disease amongst My.persicae and M.sanborni on whole plants in high-humidity perspex cages was investigated. In the first experiment, the aphid species were segregated in separate perspex cages. Dense populations of both species were killed at approximately the same rate (Table 5.24) but sparse populations of My.persicae were killed more quickly than those of M.sanborni (Fig. 5.15).

TABLE 5.24 Spread of *Verticillium lecanii* Disease through Dense *Macrosiphoniella sanborni* and *Myzus persicae* Populations on Whole Plants.

Days from inoculation[b] of population	Experiment 1				Experiment 2			
	My.persicae		M.sanborni		My.persicae		M.sanborni	
	Not inoc.	Inoc.	Not inoc.	Inoc.	Not inoc.	Inoc.	Not inoc.	Inoc.
4	-	+	-	+	-	-	-	-
5	-	++	-	+(+)				
6					-	+	-	+
7	-	+++	-	++				
8	-	+++	-	+++	-	+++	-	++
10					-	+++	-	+++
16	(+)	++++	(+)	++++	(+)	++++	-	++++

a = - No infection seen
\+ Approximately 25% of population infected
++ " 50% " " "
+++ " 75% " " "
++++ " 100% " " "

b = Population inoculated by placing sporulating aphid cadaver in a lower leaf axil

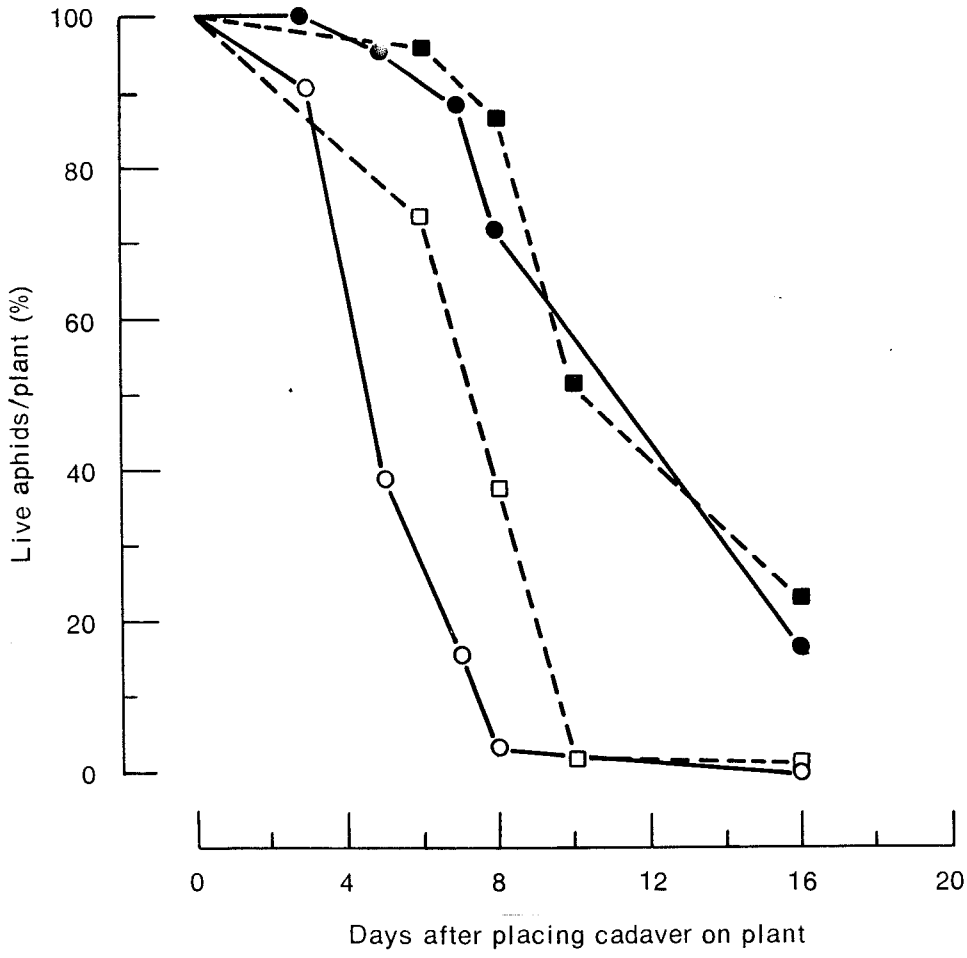


Fig. 5.15

Spread of *Verticillium lecanii* disease amongst light populations of *Myzus persicae* (□ and ○) and *Macrosiphoniella sanborni* (■ and ●) on whole potted chrysanthemum plants.

——— Experiment 1

– – – – – Experiment 2

In the second experiment, the plants bearing the aphid species were kept in the same high-humidity cages and, since plants were touching each other, My.persicae started to migrate to the plants bearing M.sanborni 4-5 days after commencement of the experiment. There was no reciprocal migration of M.sanborni until the 16th day. My.persicae populations both on their "own" plants and on "M.sanborni" plants died soonest (Fig. 5.15).

Thus, the intrinsic susceptibilities of both these aphid species to contagion were similar (Table 5.21). However, mortalities for My.persicae were greater than for M.sanborni in 88 mm-diameter Petri-dishes (Table 5.23) and on whole plants (low density populations only; Fig. 5.15) but not in the confines of 19 mm-diameter assay cells (Table 5.22). The detectably greater mobility of My.persicae (Table 5.23;Fig. 5.15) most probably contributed to its higher mortality in these experiments and may be one of the factors explaining the differential susceptibility of the 2 aphid species in the glasshouse.

14. Pathogenicity of V.lecanii Strains from Different Sources

Conidiospore suspensions were produced from 20 multi-spore isolates of V.lecanii strains, received or collected at the G.C.R.I., and assayed against M.sanborni.

All strains were screened for pathogenicity to M.sanborni treating apterous adults with 5×10^6 - 1×10^7 conidiospores/ml using the methods of V, 1. All pathogenic strains were re-isolated in culture on SDA from dead, diseased M. sanborni and, after 3 transfers on SDA, conidiospores of these strains were assayed for pathogenicity in parallel with conidiospores of the standard strain, C-3, using the bioassay technique (V, 1). Assay of all strains after passage through the bioassay host was desirable since there was a possibility

that certain strains, particularly those which had been maintained in culture on agar for many years in other laboratories might have lost some of their virulence. If so, passage through M.sanborni might help to revive virulence. Many of these strains could be positively identified after passage by a combination of colonial characteristics on SDA and spore size. Those not easily identifiable in this way and others of special interest such as strains from non-insect sources were also assayed without previously passaging through M.sanborni. These strains were subcultured on the routinely used SDA at least 3 consecutive times in case pre-culture on this medium in some way also affected pathogenicity.

Of 5 strains, all isolated from rust and mildew fungi, C-35, C-47 and C-49 killed some aphids at high conidiospore concentrations (Table 5.25) whereas C-30 and C-50 killed none. The latter strains grew and sporulated on live

TABLE 5.25

Macrosiphoniella sanborni Mortality at High Conidiospore Concentrations of Non-pathogenic Verticillium lecanii Strains

	Strain and spore concentration (spores/ml)				
	C-35	Control for C-35	C-47	C-49	Control for C-47 and C-49
% numbers aphids killed[a]	33	0	31.5	55	10.5
% numbers dead aphids with bacteriosis	71		100	90.1	0

a = Approximately 20 aphids/treatment

and dead aphids but there were no signs of internal invasion such as blastospores, a diagnostic feature of invasion by the standard, C-3 (V, 6). However, bacteria had invaded the haemolymph of most dead insects (Table 5.25), an event only occasionally observed with aphids treated with conidiospores of C-3. The bacteria may have penetrated disruptions of the cuticle and possibly the hypodermal layer caused by the superficial fungal growth. The failure of these V.lecanii strains to colonise the aphid via the haemolymph may stem from an inability to form blastospores (IV, Discussion). In view of the total absence of typical pathological symptoms and of the likely cause of death being bacteriosis, these strains were regarded as non-pathogenic in M.sanborni.

In contrast, the other 14 strains exhibited typical pathological symptoms and were assayed in more detail (methods; V, 1). Regardless of strain source or spore size, the LC 50s of most isolates were remarkably close to that of C-3, before or after passaging through M.sanborni (Tables 5.26 and 5.27).Only one strain, C-13 (ex T.vaporariorum; Hussey, 1958) differed and was significantly less pathogenic ($P<0.05$).

Passaging of strains once through M.sanborni did not increase virulence significantly (Table 5.26) except possibly for C-39 but further assays are required to confirm this.

15. Effect on V.lecanii of Subculturing on Agar and Passaging in Insects

The effect of repeated subculturing of V.lecanii on agar greatly altered colonial morphology, especially that of the single-spore isolates, C-3/SS1, subcultured 98 times, and C-3/SS2 subcultured 50 times on SDA (Fig. 5.16 a and b).

TABLE 5.26

Bioassay of Conidiospores of Different Strains of *Verticillium lecanii* on *Macrosiphoniella sanborni*

Experiment number	Strain number, passage status[a]	Log_{10}LC50 ±S.E.[b]	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope±S.E.[b]	Chi^{2c}	d.f.[d,c]	P[c]	Potency ratio (LC50 test-strain) / (LC50 C-3,p)
1	C-21,p	5.56±0.11	3.64(2.20-7.19)	1.71±0.44	0.31	2	> 0.05	1.30
	C-3,p	5.45±0.10	2.82(1.73-5.01)	1.69±0.41	0.22	2	> 0.05	
2	C-32,p	5.38±0.14	2.39(1.02-4.36)	1.29±0.33	2.14	2	> 0.05	1.57
	C-33,p	5.60±0.14	3.96(1.90-8.96)	1.22±0.34	2.13	2	> 0.05	2.60
	C-39,p	5.32±0.16	2.11(0.66-4.17)	1.18±0.35	1.23	2	> 0.05	1.34
	C-42,p	5.20±0.58	1.59 -[e]	0.33±0.31	0.58	2	> 0.05	1.05
	C-3,p	5.18±0.14	1.52(0.62-2.72)	1.52±0.38	1.22	2	> 0.05	
3	C-43,p	5.44±0.09	2.79(1.69-4.61)	2.06±0.48	3.77	2	> 0.05	2.21
	C-46,p	5.12±0.09	1.32(0.76-2.08)	2.52±0.48	4.66	1	< 0.05	1.05
	C-48,p	5.16±0.18	1.45(0.12-4.41)	1.32±0.58	0.05	1	> 0.05	1.15
	C-45,Orig	5.32±0.16	2.11(0.61-5.44)	1.02±0.36	5.53	1	< 0.05	1.67
	C-45,p	5.22±0.13	1.64(0.85-3.61)	1.56±0.41	0.001	1	> 0.05	1.32
	C-44,p	5.23±0.13	1.69(0.87-3.88)	1.98±.58	0.015	1	> 0.05	1.34
	C-52,p	4.64±0.35	0.44(0.03-2.20)	1.35±0.65	1.91	1	> 0.05	0.35
	C-3,p	5.10±0.14	1.26(0.50-2.26)	1.41±0.40	3.30	2	> 0.05	

TABLE 5.26(cont.)

Experiment number	Strain number, passage status[a]	Log_{10}LC50 ±S.E.[b]	LC50 and 95% fiducial limits $x10^5$ spores/ml	Slope±S.E.[b]	Chi^{2c}	d.f.[d,c]	P[c]	Potency ratio (LC50 test strain) / (LC50 C-3,p)
4	C-13,Orig	6.31±0.46	20.68 -[e]	0.94±0.46	0.35	1	> 0.05	11.32
	C-46,Orig	5.46±0.17	2.89(1.16-10.6)	1.07±0.38	0.40	1	> 0.05	1.59
	C-44,Orig	5.44±0.13	2.75(1.37-5.58)	1.52±0.42	0.83	1	> 0.05	1.51
	C-52,p	5.48±0.25	3.04(1.17-28.0)	0.77±0.25	1.41	2	> 0.05	1.67
	C-19,p	5.32±0.10	2.02(1.20-3.53)	1.60±0.37	4.00	2	> 0.05	1.11
	C-43,p	5.53±0.16	3.44(1.83-13.4)	1.40±0.45	1.18	2	> 0.05	1.89
	C-3,p	5.26±0.10	1.82(1.10-3.20)	1.59±0.38	0.51	2	> 0.05	
5	C-13,Orig	6.09±0.13	12.44(3.75-21.0)	1.99±0.71	2.66	2	> 0.05	8.16
	C-13,p	6.23±0.09	16.90(9.31-25.2)	3.32±1.01	2.27	2	> 0.05	11.1
	C-48,Orig	5.99±0.12	9.83 -[e]	2.81±1.72	0.51	1	> 0.05	6.44
	C-39,Orig	5.74±0.16	5.54(2.89-22.9)	1.34±0.44	0.05	1	> 0.05	3.65
	C-3,p	5.18±0.10	1.52(0.82-2.39)	2.24±0.49	6.94	1	> 0.01	
6	C-21,p	5.64±0.10	4.43(2.52-8.56)	1.72±0.53	11.63	2	< 0.01	4.94
	C-41,Orig	7.51±1.78	380 -[e]	0.31±0.28	0.09	2	> 0.05	427
	C-3,p	4.95±0.13	0.89(0.46-2.14)	1.48±0.36	1.88	2	> 0.05	

TABLE 5.26(cont.)

Experiment number	Strain number, passage status[a]	Log_{10}LC50 ±S.E.[b]	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope±S.E[b]	Chi^{2c}	d.f.[d,c]	P[c]	Potency ratio (LC50 test strain / LC50 C-3,p)
7	C-48,Orig	5.62±0.11	4.19(2.22-6.71)	1.87±0.42	1.18	2	> 0.05	0.59
	C-48,p	5.59±0.16	3.96(1.55-8.47)	1.07±0.29	0.78	2	> 0.05	0.56
	C-41,Orig	6.18±0.19	15.1(6.57-53.7)	1.13±0.33	1.74	2	> 0.05	2.14
	C-46,Orig	5.90±0.13	7.90(2.65-13.4)	1.94±0.62	4.76	2	> 0.05	1.12
	C-3,p	5.84±0.13	7.04(3.65-15.5)	1.84±0.56	0.98	1	> 0.05	

a = p - Strain passaged through M.sanborni once and subcultured 3 times on SDA
Orig - Strain not passaged through M.sanborni, but subcultured 3 times on SDA

b = Standard error

c = Heterogeity about regression

d = Degrees of freedom

e = Limits not included since slope not significantly different from zero

TABLE 5.27

Potency Ratios of *Verticillium lecanii* Strains Assayed Twice in Parallel with Conidiospores of the Standard, C-3.

Strain number and passage status[a]	Number of assays	Weighted mean potency ratio with 95% fiducial limits
C-21,p	2	2.39(0.65-8.83)
C-48,Orig	2	1.96(0.19-20.1)
C-48,p	2	0.77(0.39-1.55)
C-13, Orig	2	8.52(3.78-19.25)
C-46, Orig	2	1.33(0.73-2.5)
C-52,p	2	0.98(0.37-2.63)
C-41, Orig	2	2.37(0.85-6.67)

a = Strain passaged through *M.sanborni* once and subcultured 3 times on SDA, denoted by 'p'; strain not passaged through *M.sanborni* but subcultured on SDA 3 times denoted by 'Orig'

a

b

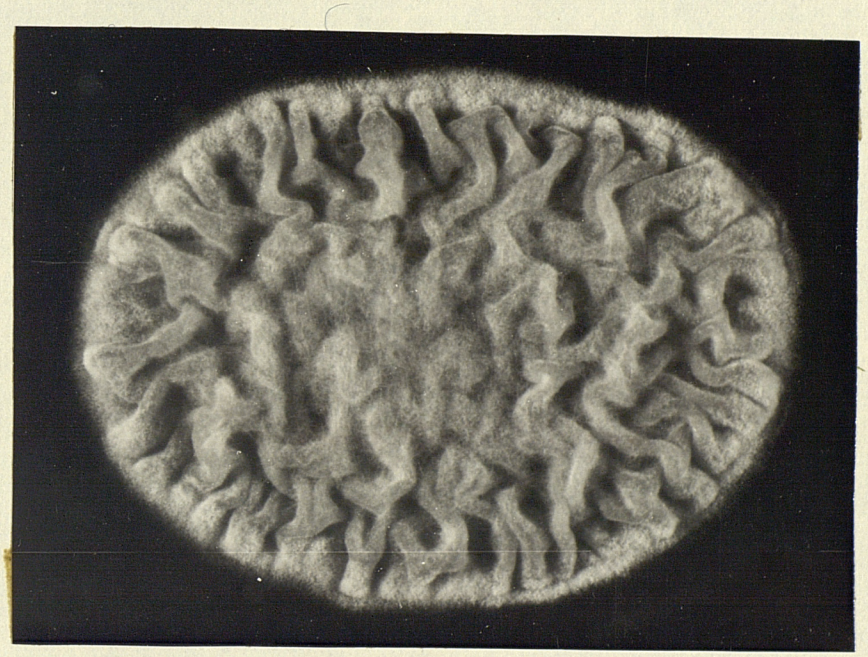


Fig. 5.16 a and b. Comparison of colonial morphologies on Sabouraud dextrose agar (SDA) of C-3, subcultured 3 times (5.16a) and 98 times (5.16b) on SDA from the original diseased aphid, Macrosiphoniella sanborni.

This gross colonial change was still evident after passage once through M.sanborni (Fig. 5.17), but the colonies were less convoluted than the unpassaged isolate, indicating perhaps that further passaging through M.sanborni might return the fungus to its former morphological form. There was less change in the morphology of C-3/SS1 subcultured 50 times on Malt extract or Czapeck-dox agars and of the multi-spore isolates subcultured on SDA. When subcultured on Potato dextrose agar, C-3/SS1, produced 2 morphological variants, one of which (SS1,sub51-normal grew at about the same rate as C-3/SS1,sub3 and the other (SS1,sub50-slow) much slower on SDA (Fig. 5.18a). Growth on SDA of C-3/SS1 subcultured 50 times on Malt and Czapeck-dox agars was similar to that of C-3/SS1,sub3 (Fig. 5.18a) but was reduced for C-3/SS1 and C-3/SS2 repeatedly subcultured many times on SDA (Fig. 5.18b).

The virulence of single and multi-spore isolates, derived from the standard, C-3, remained stable in most media after many subculturings (Table 5.28). Only the slow growing variant, arising after 50, subculturings of C-3/SS1 on potato dextrose agar, lost a significant degree of pathogenicity ($P<0.05$; Table 5.29). After 50 subculturings on SDA, C-1/MS1 and C-1/MS2, the 2 multi-spore isolates, in experiment 3 (Table 5.28) killed all the aphids at too many spore concentrations for the LC 50s to be computed with any accuracy, although potency ratios must have been much less than 1, but in experiment 4, LC 50s were similar to that of the standard (Table 5.28). Until further assays are performed, it must be assumed that experimental errors must have caused the anomalous results in experiment 3.

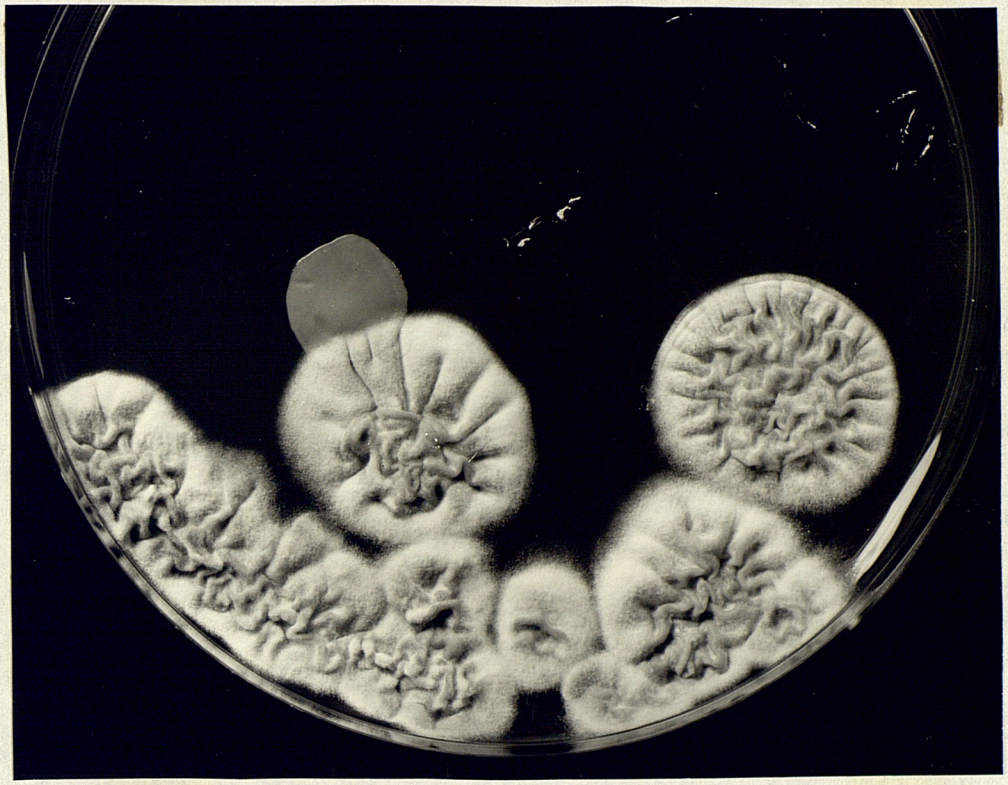


Fig. 5.17 Colonial morphology on Sabouraud dextrose agar (SDA) of C-3, subcultured 98 times on SDA from the original diseased aphid (*Macrosiphoniella sanborni*) then re-passaged once through *M. sanborni* and subcultured once on SDA.

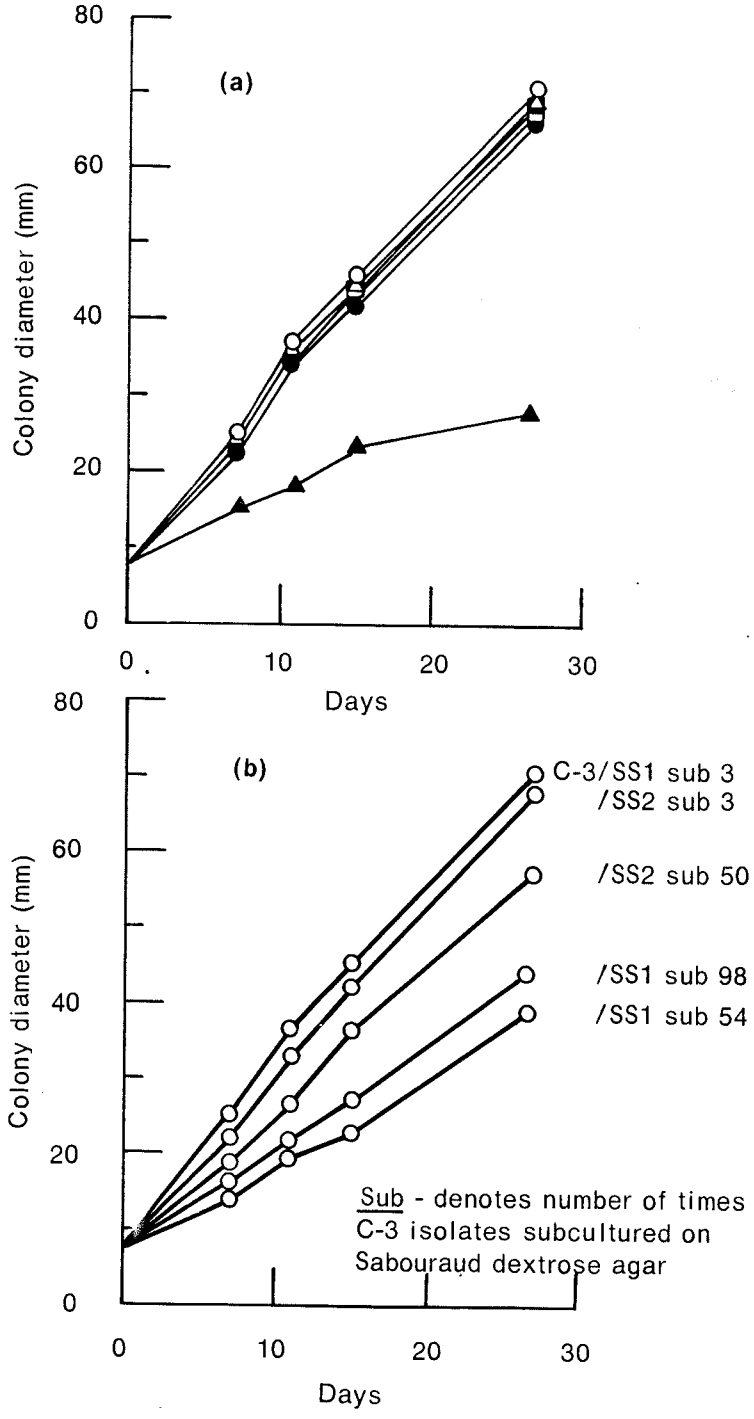


Fig. 5.18a

Growth on Sabouraud dextrose agar (SDA) of colonies of *Verticillium lecanii* single-spore isolates previously subcultured on various media:-

- ○ C-3/SS1 subcultured on SDA 3 times.
- △ C-3/SS1 subcultured on Malt extract agar 51 times.
- □ C-3/SS1 subcultured on Czapeck-dox agar 51 times.
- ● C-3/SS1 subcultured on Potato dextrose agar 51 times. (SS1 - normal)
- ▲ C-3/SS1 subcultured on Potato dextrose agar 51 times. (SS1 - slow)

Fig. 5.18b

Growth on Sabouraud dextrose agar (SDA) of colonies of *Verticillium lecanii* single-spore isolates previously subcultured on SDA.

TABLE 5.28

Effect of Repeated Subculturing on Agar Media on Pathogenicity of *Verticillium lecanii* Single- and Multi-spore isolates

Experiment number and subculturing medium[a]	Isolate number and subculturing status[b]	$Log_{10}LC50 \pm S.E.^{c}$	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope $\pm$ S.E.[c]	Chi^{2d}	d.f.[e,d]	P[d]	Potency ratio[f]
1. SDA	C-3/SS1,sub98	5.46±0.11	2.88(1.73-5.32)	1.35±0.34	7.06	2	< 0.05	1.89
	C-3/SS1,sub54	5.12±0.09	1.31(0.75-1.93)	1.84±0.39	0.14	2	> 0.05	0.86
	C-3/SS1,sub3	5.18±0.08	1.52(0.98-2.16)	2.16±0.42	3.86	2	> 0.05	
2. SDA	C-3/SS2,sub55	5.04±0.12	1.11(0.49-1.75)	1.49±0.36	6.34	2	< 0.05	1.07
	C-3/SS2,sub3	5.01±0.09	1.03(0.62-1.45)	2.15±0.46	2.01	2	> 0.05	
3. SDA	C-1/MS1,sub49	Too many 100% responses						< 1
	C-1/MS2,sub49	Too many 100% responses						< 1
	C-1/MS1,sub6	5.14±0.19	1.38(0.23-2.61)	1.17±0.42	0.35	1	> 0.05	0.90
	C-3/SS1,sub3	5.19±0.11	1.54(0.89-2.54)	1.54±0.35	3.38	2	> 0.05	
4. SDA	C-1/MS1,sub50	5.21±0.14	1.64(0.83-2.54)	1.28±0.29	0.11	2	> 0.05	1.13
	C-1/MS2,sub50	5.51±0.11	3.23(1.96-6.12)	1.98±0.44	0.93	2	> 0.05	2.24
	C-1/MS1,sub6	5.45±0.27	2.8 -[g]	0.77±0.41	0.24	1	> 0.05	1.95
	C-3/SS1,sub3	5.16±0.07	1.44(0.96-2.02)	2.43±0.48	4.37	2	> 0.05	

TABLE 5.28(cont.)

Experiment number and subculturing medium[a]	Isolate number and subculturing status[b]	Log_{10}LC50 ±S.E.[c]	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope±S.E.[c]	Chi^{2d}	d.f.[e,d]	P[d]	Potency ratio[f]
5. PDA	C-3/SS1,sub50 (normal growth)	5.56±0.21	3.28(1.04-27.1)	0.82±0.33	1.59	1	> 0.05	2.42
PDA	C-3/SS1,sub50 (slow growth)	6.77±1.04	59 -[g]	0.50±0.38	1.40	1	> 0.05	43.7
SDA	C-3/SS1,sub3	5.13±0.13	1.35(0.62-2.38)	1.73±0.46	0.13	1	> 0.05	
6. PDA	C-3/SS1,sub51 (normal growth)	5.74±0.09	5.56(2.93-8.53)	2.96±0.77	0.96	2	> 0.05	0.79
PDA	C-3/SS1,sub51 (slow growth)	6.16±0.11	14.5(7.32-24.9)	2.05±0.59	0.63	2	> 0.05	2.06
SDA	C-3/SS1,sub3	5.84±0.13	7.04(3.65-15.5)	1.84±0.56	0.97	1	> 0.05	
7. MEA	C-3/SS1,sub50	4.93±0.17	0.85(0.15-1.57)	1.36±0.45	0.04	1	> 0.05	1.23
CzDA	C-3/SS1,sub50	4.53±0.36	0.34(0.06-29.6)	1.06±0.47	0.85	1	> 0.05	0.49
SDA	C-3/SS1,sub3	4.84±0.22	0.69(0.03-1.39)	1.30±0.25	7.49	1	< 0.01	

a = SDA-Sabouraud dextrose agar; PDA-Potato dextrose agar; MEA-Malt extract agar; CzDA-Czapeck-dox agar

b = C-3/SS1 - Single-spore isolate 1 (IMI 179172) derived from C-1 on aphids;C-3/SS2 - Single-spore isolate 2 derived from C-1 on aphids; C-1/MS1 and MS2 - Multi-spore isolates derived from C-1; Sub_n - Subcultured on agar n times

c = Standard error

d = Heterogeneity about regression line

e = Degrees of freedom

f = Potency ratio - LC 50(test isolate)/LC 50(standard-C-3)

g = Limits not included since slope not significantly different from zero

TABLE 5.29

Potency Ratios of Repeatedly Subcultured *Verticillium lecanii* Isolates Assayed Twice in Parallel with the Standard, C-3.

Strain number and subculturing status[a]	Number of assays	Weighted mean potency ratio with 95% fiducial limits
C3/SS1, sub50 and sub51 (normal growth) on PDA	2	1.09 (0.60 - 1.98)
C-3/SS1, sub50 and sub51 (slow growth) on PDA	2	2.26 (1.06 - 4.82)

a = C-3/SS1 - Single-spore isolate 1 (IMI 179172) derived from C-1

sub50 - subcultured on agar 50 times

After an indeterminate but large number of passages through M.sanborni (V,4,b), the pathogenicity of the single-spore isolate C-3/SS1 and the multi-spore isolate C-1/MS1, was not significantly changed (Table 5,30; $P>0.05$)

16. Pathogenicity of other Species of Fungi Towards M.sanborni

(a) Metarrhizium anisopliae

Metarrhizium anisopliae isolated from a subterranean aphid species Pemphigus sp. was significantly less pathogenic ($P<0.01$; Table 5.31) than V.lecanii (C-3) under conditions of bioassay (V, 1,). In contrast to V.lecanii on aphids, external sporulation of M.anisopliae was not observed until after death of aphids and, was not profuse until at least 10 days after treatment, when dead aphids assumed a yellow-green colour. Many blastospores were observed in the haemolymph of aphids shortly before death, a feature that distinguishes M.anisopliae from the non-pathogenic V.lecanii isolates causing mortality at high spore concentrations (V,14). In one M.anisopliae assay, on the sixth and last day, at the highest concentration, there were blastospores in the haemolymph of all surviving aphids. These aphids would undoubtedly have soon died giving a much lower LC 50 if the assay had lasted more than 6 days. The optimum temperature for growth of the present strain of M.anisopliae was not known but optima for other strains are frequently higher than 25°C (Lihnell, 1944; Rockwood, 1950) and so an assay of M.anisopliae nearer to its temperature optimum may produce on LC 50 much closer to that for V.lecanii at 20 ± 1°C.

(b) Penicillium spp.

Penicilliium spp. have frequently been encountered on aphids, especially in bioassay work. They grew on the

TABLE 5.30

Pathogenicity of *Verticillium lecanii* Single- and Multi-spore Isolates after Passage Many Times through the Aphid Host, *Macrosiphoniella sanborni*

Experiment number	Isolate number and passage status[a]	Log_{10}LC50 ±S.E.[b]	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope±S.E.[b]	Chi^{2c}	d.f.[d,c]	p[c]	Potency ratio
1.	C-3/SS1,p	4.99±0.12	0.98(0.45±1.59)	2.13±0.52	5.63	3	> 0.05	0.64
	C-3/SS1,sub3	5.18±0.10	1.52(0.89±2.47)	2.18±0.48	1.13	3	> 0.05	
2.	C-1/MS1,p	5.49±0.13	3.12(1.38±6.06)	1.78±0.56	0.01	1	> 0.05	2.05
	C-3/SS1,sub3	5.18±0.10	1.52(0.82±2.39)	2.24±0.49	6.94	1	< 0.01	

a = C-3/SS1 - Single-spore isolate 1 (IMI 179172) derived from C-1
C-1/MS1 - Multi-spore isolate 1 derived from C-1
p - Passaged through *M.sanborni* an indeterminate but large number of times
sub3 - Subcultured on SDA 3 times

b = Standard error

c = Heterogeneity about regression line

d = Degrees of freedom

TABLE 5.31

Pathogenicity of *Metarrhizium anisopliae* and *Verticillium lecanii* Conidiospores Assayed in Parallel on the Aphid, *Macrosiphoniella sanborni*

Experiment number	Species	Log_{10}LC50 $\pm$S.E.[a]	LC50 and 95% fiducial limits x 10^5 spores/ml	Slope$\pm$S.E.[a]	Chi^{2b}	d.f.[c,b]	P[b]	Potency ratio	Weighted mean potency ratio and 95% fiducial limits
1.	*M.anisopliae*	7.19$\pm$0.15	156(6.51-724)	1.35$\pm$0.39	3.54	2	>0.05	102.6	
	V.lecanii (C-3)	5.18$\pm$0.10	1.52(0.82-2.39)	2.24$\pm$0.49	6.94	1	<0.01		
2.	*M.anisopliae*	7.46$\pm$0.10	286(136 - 589)	2.22$\pm$0.86	0.66	2	>0.05	40.6	58.7(32.7-105)
	V.lecanii (C-3)	5.84$\pm$0.13	7.04(3.65-15.5)	1.84$\pm$0.56	0.97	1	>0.05		

a = Standard error

b = Heterogeneity about regression line

c = Degrees of freedom

cuticle, particularly near the cornicles and anal region. Although occasionally harmful (Fig. 5.19), Penicillium spp. seldom penetrated the cuticle. A Myzus persicae adult bore particularly heavy growths but lived in this state for at least 14 days at 18°C before dying (Fig. 5.20; Goodall, personal communication). The author and other workers have observed Penicillium spp. growing on aphid honeydew on leaves and so it is likely that Penicillium spp. on aphids are also growing on honeydew on the aphid cuticle. Thoizon (1967) reported a Penicillium sp. on aphids but she also did not consider it to be a primary pathogen.

(c) Fusarium solani

Very occasionally, Fusarium solani has been found attacking the aphid, M.sanborni, and whitefly, Trialeurodes vaporariorum. In 2 experiments, a total of 120 adult, apterous M.sanborni (reared on whole plants[1]), were individually dipped briefly into an aqueous suspension of 10^8 F.solani conidiospores/ml. The insects were kept on small, potted chrysanthemum plants at 23 ± 1°C, in high humidity perspex cages in indirect light (900 lux).

After 5 days, mortality among treated aphids (mean, 18%) was only slightly higher than the control mortality (mean,8%). Staining moribund aphids from the treated groups with cotton blue in lactophenol revealed freely sporulating external mycelium on both the legs and abdomen and also internal hyphal invasion. Dead aphids bore more internal and external mycelial growth with both macro-and micro conidia.

[1] These experiments performed before development of the bioassay technique.

Fig. 5.19 *Penicillium* sp. growing on cuticle of live *Macrosiphoniella sanborni* and newly emerged offspring which the fungus has possibly killed.

Fig. 5.20 Live Myzus persicae bearing growth of Penicillium sp..

Of the seemingly healthy, treated aphids examined on the same day, all but a few had limited external mycelial growth on the legs and abdomen but without internal infection, In spite of this, mortality amongst insects kept longer than 5 days was low (4%).

F.solani is primarily a plant pathogen (Booth, 1972). It has also been isolated from animals, including mammalian and human lesions (Booth, 1972). It has also been reported as an insect pathogen (Moore, 1973; Kilpatrick, 1961) and as an opportunistic fungus invading through insect wounds (Heitor, 1962; Hurpin et al., 1958; Barson, 1976b). The present results and the comparative rarity of natural occurence in M.sanborni suggest that F.solani in this host falls in the latter category and that infection may depend on the presence of predisposing factors such as minor wounds which would not otherwise be fatal,

17. Biochemical Basis of Pathogenicity

(a) Chitin

Chitin is a high molecular weight polymer of repeating units of N-acetyl-D-glucosamine (NAG) and comprises 25-60% of the dry weight of the insect cuticle, depending on species (Hackman, 1964). In the insect, chitin exists as sequences of 6-8 molecules of NAG joined by proteins (Rudall, 1969) conferring rigidity upon the cuticle. The ability of V.lecanii to traverse this barrier by production of enzymes, particularly chitinase, was studied to look for a correlation between enzyme production and pathogenicity.

(b) Production and Repression of Chitinase

V.lecanii grew well on purified colloidal chitin agar at 23± 0.5°C and colonies formed produced a narrow, but distinct clearing zone showing that the fungus produced a

chitinase, agreeing with observations of others who have studied V.lecanii (Samšiňáková et al., 1971; Domsch, 1960). When the cultures were again incubated but at 2°C, the clearing zones (Fig. 5.21) greatly widened. Presumably residual chitinase after incubation at 23°C, diffused away from the now virtually static colony border and continued to digest chitin at the lower temperature.

Glucose generally interferes with the synthesis of catabolic enzymes in many systems and the phenomenon is termed catabolite repression (Magasanik, 1970). In chitin-glucose agar, clearing zones were not visible when V.lecanii cultures were incubated at 23°C but were produced when vegetative growth was retarded at 2°C (Table 5.32) showing that glucose decreased but did not arrest chitinase production. Chitin is both a C- and N- source and so total repression probably could not occur since the polymer must be degraded to yield nitrogen, essential for fungal growth. However, when nitrogen ($NaNO_3$) as well as glucose was included in the chitin agar, clearing zones were not produced at either temperature (Table 5.32) showing that chitinase was not produced constitutively.

(c) Isolation of a Chitinase Negative Mutant

This phase of the study was initiated but not completed. Chitinase negative (chi^-) mutants would help to determine the role of chitinase in pathogenicity. Mutants could be detected by conventional techniques such as replica plating, but direct observation of colonies producing or not producing clearing zones would be easier. However, a chi^- mutant would need a C- and N- source other than chitin but it would be necessary to derepress chitinase synthesis in wild-type colonies. Many workers have shown that cyclic 3', 5' AMP (CAMP) can overcome catabolite repression in micro-organisms (Pastan et al., 1969; Perlman et al., 1969; Magasanik, 1970; Tsuboi et al., 1972, 1973). Incorporation of 10^{-3}M CAMP into chitin-

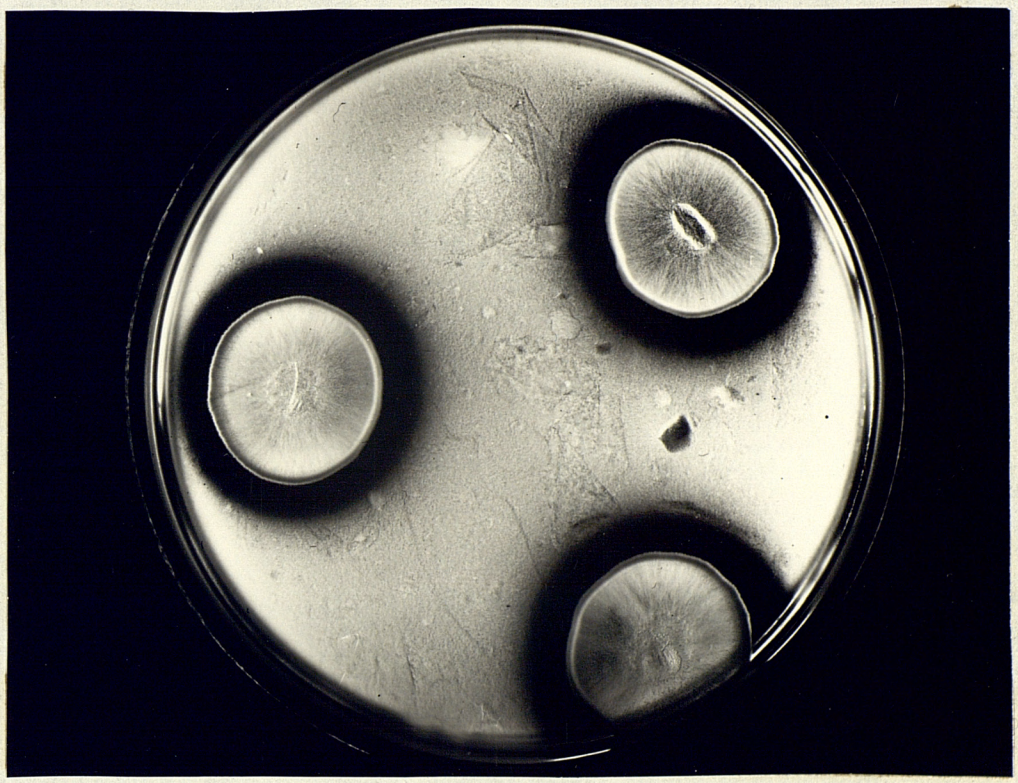


Fig. 5.21 Verticillium lecanii colonies on colloidal chitin agar with clearing zones where chitin has been digested.

TABLE 5.32

Clearing Zones Produced by Action of Chitinase around *Verticillium lecanii* Colonies Growing on Various 2% Chitin Media.

Additions to agar	14 days incubation at 23.5 ± 0.5°C	26 more days at 2°C
Chitin	(+)	+
Chitin + 2% glucose	-	+
Chitin + 2% glucose + 0.5% $NaNO_3$	-	-
Chitin + 2% glucose + 0.5% $NaNO_3$ + 10^{-3}M 3' 5' cyclic AMP	-	-
Chitin + 2% glucose + 0.5% $NaNO_3$ + 10^{-3}M caffeine	-	not incubated at 2°C

\+ Distinct wide zones visible (Fig. 5.21)

(+) Narrow zone visible

\- No visible zones

glucose-nitrogen agar did not derepress chitinase synthesis (Table 5.32). Caffeine is a methylxanthin derivative known to inhibit cyclic AMP-phosphodiesterase, the enzyme which degrades CAMP (Robinson et al., 1971). When 10^{-3}M caffeine was incorporated into chitin-glucose-nitrogen agar, clearing zones were absent at 23°C (Table 5.32).

However, there are other possibilites to explore e.g. other C- sources such as lactose or succinate, poorer C-sources for V.lecanii (Table 4.24) may exert less repression than glucose and permit production of clearing zones on chitin -C- source-nitrate agar. Alternatively, a filtration enrichment technique, or the N-glycosyl-polifungin enrichment technique for fungal auxotrophs of Bal et al. (1974) may aid the selection of a chi$^-$ mutant.

DISCUSSION

In the studies in this chapter, a basic ecological tool, bioassay, has been developed. Its scope will be discussed, followed by an assessment of the background information that it has provided for ecological studies in glasshouses.

The slope, b, of an assay line reflects the precision of the assay and there are theoretical grounds for expecting b not to exceed 2.0 for organisms that attack by infectivity alone (Meynell et al., 1965). Thus, the weighted mean slope of 1.95 for the 28 assays of the standard V.lecanii isolate (C-3) on M.sanborni is encouragingly high. It is probably due, in part, to the standardized insect rearing programme and assay techniques in carefully controlled conditions.

The other aphid species had lower slopes, 1.29 for Brachycaudus helichrysi, (cf 2.12 for M.sanborni in parallel assays; V,13) and 0.94 for Myzus persicae (cf 2.07 for M.sanborni in parallel assays; V,13). The slope may, in part, be an inherent, stable feature of the bioassay host species or the lower slopes for B.helichrysi and My.persicae may be a result of their reaction to an assay technique developed for M.sanborni. Since the precision of a bioassay depends, in part, upon the steepness of its slope, then clearly if the bioassay method developed in this study is used, M.sanborni is the best bioassay host.

There is a paucity of bioassay data for other entomogenous fungi with which to compare V.lecanii. Slopes of 1.98, 1.89. 1.84, 1.55 and 1.35 were derived from 5 bioassays in which Beauveria bassiana conidiospores were

applied to the Colorado beetle, Leptinotarsa decemlineata by 4 different methods (from the graphical data of Fargues, (1972a and b)using the formula of Roberts et al., 1972). In contrast, Broome et al. (1976), obtained much lower slopes for B.bassiana conidiospores applied perorally (0.52) or topically (0.75) to Solenopsis richteri larvae. All but one of the above values were lower than the mean value of 1.95 for V.lecanii.

The slope of 1.95 for V.lecanii was higher than those for most other groups of insect pathogens believed not to produce toxins (Burges et al., 1971). Slopes were lower for viruses (mean 1.2, range 0.5 - 2.1) and generally so for protozoa (mean, 1.2, range 0.5 - 1.5), but higher for some protozoa assays (Milner, 1973; mean 2.1 range 1.1 - 3.9) and organisms such as Bacillus thuringiensis that produces toxins (mean 2.7, range 0.9 - 7.9) and more so for assays involving only the crystal exotoxin of B.thuringiensis (mean 3.8, range 1.9 - 7.8).

The observed trend towards lower LC 50 values from the 1st to the 16th assay (Fig. 5.10), markedly increased the between-assay variation (V,7,b). This trend is not likely to have been due to a progressive increase in the pathogenicity of the fungus, because each batch of spores was grown from the same stock of deep-frozen cultures, all of which were simultaneously cultured from the same single-spore isolate. Also, since the production technique was not changed, it seems reasonable to expect that the pathogenicity of conidiospore batches was virtually identical for each assay. The chrysanthemum leaves may possibly have differed due to seasonal variation, but Fig. 5.10 does not suggest this. The assay technique remained unchanged and

so should not account for the downward trend. Thus, by elimination, it is concluded that the trend reflected an increase in the susceptibility of stock aphids reared continuously for $3\frac{1}{4}$ years in laboratory conditions. This increase may have been due to a lack of selection pressure from disease which was absent in laboratory reared stocks, but which occurs periodically in wild populations.

Susceptibility levelled out after the 16th assay (V,7,b). Such a constant stock is adequate for the measurement of fungal pathogenicity by laboratory bioassay. There is, however, a risk that, continued rearing of insects in the laboratory may produce other alterations in behaviour (Boller, 1972). So, in field experiments, it may be unwise to use laboratory-bred aphid stocks which may differ in behaviour and susceptibility from wild populations. This risk can be remedied by regularly replenishing laboratory stocks but, for laboratory bioassay, replenishment may introduce fresh variations. The latter could be overcome by using in each group of assays a batch of C-3 fungal spores designated as standard and by expressing pathogenicity as a potency ratio in comparison with the standard. This is feasible since the storage tests in liquid nitrogen suggested that high concentrations of spores could be viably conserved indefinitely without genetic change, loss of viability and presumably pathogenicity (IV,9,e).

The standard error of a single LC 50 is small when the slope, $\underline{b}$, is high, the number of insects/dosage, $\underline{n}$, is large and the dosages are close to the LC 50 resulting in a high sum of weighting coefficients (ΣW). The approximate standard error, $\underline{s}$, is given by:

$$\underline{s} = \frac{1}{\underline{b}\sqrt{\underline{n}\,\Sigma W}} \qquad (1)$$

The validity of this approximation depends on the mortalities being symmetrically distributed about the LC 50. Over several assays, an approximate estimate of the standard error, $S\bar{m}$, of the mean of a number of LC 50s is given by:

$$S\bar{m} = \sqrt{\frac{(s^2_{within} + s^2_{between})}{K}} \qquad (2)$$

Where K is the number of assays, $s^2_{between}$ is the between-assay variance and s^2_{within} is the within-assay variance. However, the impact of standard errors of the $\log_{10}$ LC 50 is less easily assimilable than the ratio of the upper and lower fiducial limits of the LC 50 itself. This ratio illustrates directly and simply the variation in the assay and also gives a very approximate indication of the smallest differences between 2 LC 50s with the same standard error that will be significant at the 0.05 level. Ratios were calculated using formulae (1) and (2) from the weighted mean slope (1.95) and the between-assay variance (0.036) of the V.lecanii assays, 17-28 (Table 5.03), which did not exhibit a trend in LC 50 values (Table 5.33). The ratios clearly demonstrate the large increase in precision obtained by replicating assays, an improvement that falls rapidly after the third replicate. An increase from 10 to 20 insects/assay achieves less improvement.

It must be remembered that these theoretical estimates depend upon the chosen mortalities being symmetrically distributed about the LC 50. Asymmetry is more likely to occur in practice and this would increase the variance. This effect is suggested in Table 5.34 in which the fiducial limit ratios were similarly evaluated to those in Table 5.33, except that the calculations were based on the actual mortalities encountered in practice from 3 and 4 doses in the

TABLE 5.33

Effect of Number of Assays, Doses/Assay and Insects/Dose on Theoretical Fiducial Limit Ratios Obtained Using Chosen Mortalities [a,b] and a Mean Slope from Assays with *Verticillium lecanii* Conidiospores on *Macrosipohiella sanborni*.

Number of assays	10 insects/dose		20 insects/dose	
	3 doses[a]	4 doses[b]	3 doses[a]	4 doses[b]
1	7.83	7.17	6.64	6.33
2	4.29	4.03	3.81	3.69
3	3.28	3.12	2.98	2.90
4	2.79	2.68	2.58	2.52
5	2.51	2.41	2.33	2.28
6	2.32	2.23	2.17	2.12
7	2.18	2.11	2.05	2.01
9	1.99	1.93	1.88	1.85
11	1.86	1.81	1.77	1.74
20	1.58	1.55	1.53	1.51

a Chosen mortalities 20, 50 and 80%

b Chosen mortalities 20, 40, 60 and 80%

TABLE 5.34

Effect on Fiducial Limit Ratios of Combining Successive Bioassays of *Verticillium lecanii* Conidiospores on *Macrosiphoniella sanborni* (20 insects/dose)

Number of assays	3 doses		4 doses	
	$Log_{10}LC\ 50$	Ratio	$Log_{10}LC\ 50$	Ratio
1	5.86	27.35	5.79	8.51
2	5.45	4.59	5.49	3.86
3	5.54	3.36	5.47	2.91
4	5.63	2.70	5.60	2.52
6	5.21	2.39	5.22	2.09
9	5.30	1.97	5.27	1.82
11	4.94	1.86	4.95	1.71

a As calculated from the mortalities obtained from the following dose levels: 1.25, 2.5 and 5.0×10^5 spores/ml

TABLE 5.35

Data from Table 5.34 Illustrating the Estimated Number of Assays Required to Attain Levels of Precision of the LC 50 (*Verticillium lecanii* on *Macrosiphoniella sanborni*)

Precision (limit ratios) P = 0.05	Number of assays	
	Three doses	Four doses
x 32	1	1
x 16	2	1
x 8	2	1
x 4	3	2
x 2	9	7

consecutive V.lecanii assays. Thus, for the 4-dose assays mortalities of (1.25, 2.5, 5.0 and 10) x 10^5 spores/ml were used and for the 3-dose assays, the highest dose was omitted. For the first assay in Table 5.34, a fiducial limit ratio of 8.51 is obtained for 4 doses and this figure increases to 27.35 when the doses are decreased to 3. By averaging the first 2 assays, the ratios are very much reduced, and values for subsequent combined replicate assays begin to approach the theoretical values obtained from chosen mortalities (Table 5.33). Since assays were performed with 20 insects/dose, similar figures for assays with 10 insects/dose are not available, but it is expected that the variation for such assays would be higher still and thus such figures would depart even more from those in Table 5.33.

The more realistic data in Table 5.34 have been used to form a guide to the numbers of assays required for various levels of precision (Table 5.35). Thus, for example, significant differences of about x4 between LC 50s of 2 fungal preparations would be detected by the means of 2 replicate 4-dose or 3 replicate 3-dose assays. Greater increases in precision e.g. x2 require more assays. In the pathogenicity experiments of V,14, some strains were assayed twice. The x8 difference in the parallel assays of C-13 and C-3 (Table 5.27) was significant, but differences of x2 to x4 between other strains were not, an order of accuracy approximating to that predicted in Table 5.35.

For a microbial insecticide produced on an industrial scale, insect rearing and labour of bioassay would have to be costed against the precision required. If slopes and LC 50s for a test batch of spores did not vary significantly

between trial batches of insects, single dose, assays would be adequate if reference were made back to a predetermined probit line. However, if slopes did vary significantly, at least 2 doses should be used to enable the slope to be measured. In the V.lecanii assays 1-11, most visual graphical estimates of the LC 50s from 2 concentrations, 1.25 and 5.0 x 10^5 spores/ml fell within x3 of the corresponding LC 50s for the full range of doses in the same assay, but one was almost x10 higher than the 4-dose counterpart caused by missing the useful range of mortalities. Therefore, 3- or 4-dose assays are preferable to avoid missing the useful range. If results with different batches of insects vary significantly, the assays should be replicated (Table 5.35).

In terms of LC 50s, blastospores were twice as pathogenic as conidiospores in this assay system, but by comparison on a live spore volume basis, blastospores were only half as pathogenic. However, since blastospores can be cultured more readily and abundantly than conidia (IV; Discussion), blastospores are a more obvious choice for use in biological control. Other workers have shown that blastospores, e.g. those of Beauveria bassiana and B.tenella, are able to infect insects (Samšiňáková, 1962, 1966, 1969; Pinnock et al., 1973) but only a few workers have compared blastospore and conidiospore pathogenicity; Ferron (1967), working with B.tenella on the cockchafer, Melolontha melolontha, reported equal mortality during the early weeks of experiments, but after 7 weeks, fewer larvae had died in groups inoculated with blastospores. Kawakami (1962) reported that blastospores of B.bassiana and Spicaria pracina were more pathogenic than conidiospores for silkworm larvae but, in contrast, Bell (1975) found that 21-day cultured Spicaria rileyi blastospores were not pathogenic for Heliothis zea, unlike conidiospores of the same age. However, Bell did not determine

blastospore viability, which was probably low, since blastospores of V.lecanii (IV,15,a) and B.bassiana (Samšiňáková, 1966) die in prolonged culture.

The bioassay results of V,7 (Table 5.03) showed that of the strains of V.lecanii collected from insects, fungi and C-39 from a contact lens, all except 5 from fungi, were almost equally pathogenic against M.sanborni. These results also provide the first quantitative confirmation of the ability of some V.lecanii strains, initially described as hyperparasitizing rusts, to infect aphids. Other reports suggest that V.lecanii from rusts can infect insects and vice versa. Kotthoff (1937) reported that V.lecanii (cited as V.coccorum) from the rust, Puccinia chrysanthemi, grew profusely on coccids on apple twigs and on Aspidiotus on asparagus. Allen (1975) showed that one of the strains from aphids, C-4, from Brachycaudus helichrysi (Table 4.14) could invade uredospores of Uromyces appendiculatus but did not determine whether the uredospores were alive at the time of invasion.

The similarity in the pathogenicity of most strains of V.lecanii to M.sanborni (Table 5.26) suggests that the prospects of increasing virulence by selection from existing strains are remote. However, certain strains with small spores, e.g. C-21 and C-48, sporulated more prolifically than the standard, C-3, and were equally pathogenic (Table 5.26). These strains may be better than C-3 for biological control.

This pathogenic similarity between strains is unusual when compared with some other entomogenous fungi. Fungi such as Beauveria bassiana (Baird, 1958; Steinhaus, 1949), B.tenella (Leatherdale, 1970) and Metarrhizium

anisopliae (Radha et al., 1956; Ferron et al., 1969, 1972) each have a wide insect host range. However, many reports suggest that individual strains of these species differ greatly in their pathogenicity for certain hosts. Several workers (Rockwood, 1950 ; Ferron et al., 1969, 1972; Latch, 1965, 1976) have shown that some strains of M.anisopliae isolated from various hosts displayed great variability in their pathogenicity for other hosts even displaying no pathogenicity at all in some cases. Fargues (1972b), testing strains of B.bassiana found similar variation in pathogenicity and Ferron. (1967) observed that strains of B.tenella, isolated from Melolontha melolontha, differed in virulence even for this insect species. However, Prasertphon (1963) showed that 5 strains of Entomophthora coronata from different sources, including termites, aphids and rotting leaves, were similar in pathogenicity for Galleria mellonella. With the exception of E.coronata, all these studies dealt with Coleoptera and Lepidoptera since these are the most frequent hosts in nature of B.bassiana, B.tenella and M.anisopliae. It is possible that the factors which govern susceptibility in Coleoptera and Lepidoptera are more complex than those operative in the case of aphid and scale insects and would thus demand more pathogenic versatility in fungal species. Indeed, Thoizon (1967, 1970) demonstrated that Entomophthora tipula and E.conglomerata, found in nature only on Diptera, could infect aphids, but E.pyriformis found in nature only on aphids was unable to infect Diptera. It would be interesting to test the V.lecanii strains in Table 5.26 on a coleopterous host, e.g. Scolytus scolytus.

Some of the V.lecanii strains listed in Table 5.26 had been subcultured on agar in other laboratories for many years, e.g. C-44 cultured since 1937, and C-45, since 1935. These

gave LC 50s very close to those of the standard, suggesting that no attenuation had occured in artificial media. This, and the virtual absence of attenuation of the standard C-3, and the parent strain, C-1, (Table 5.28), contrasts with Ganahão's (1956) and Nagaich's (1973) reports of attenuation of V.lecanii. The latter author used C-42 (Tables 4.14 and 5.26) after 2-3 subculturings on PDA, whereas in this study it was still as pathogenic as C-3 for M.sanborni after 3 subculturings on SDA. The strains C-13 (ex-whitefly, Hussey, 1958; Table 4.14), which was x8 less pathogenic than C-3 (Table 5.27), had been intermittently subcultured on agar since 1958, but passaging it and other strains through M. sanborni did not alter virulence (Table 5.28). This suggests that attenuation had not already occured. Thus, attenuation may be considered unusual in V.lecanii. For other entomogenous fungi, the situation is confused and there have been no intensive investigations. Attenuation of virulence has been reported for the following fungi: M. anisopliae (Fox et al., 1958; Latch, 1965; Kawakami, 1960), B.bassiana (Schaerffenberg, 1964; Fargues, 1972b; Kawakami; 1960) and Spicaria farinosa (Kerner, 1959; Kawakami, 1960). However, the results of some of these authors are merely suggestive and not confirmed by statistically analysed data. Other reports reviewed by Madelin (1963) suggested no attenuation in entomogenous fungi after long periods of artificial culture.

Just as repeated culture of V.lecanii on agar did not decrease virulence, so repeated passaging of single and multi-spore isolates through the bioassay host did not increase virulence (Table 5.30). Steinhaus (1949) suggested that insect pathogens, like some mammalian pathogens, may increase in virulence during repeated passage through insects

in the build-up to an epizootic wave. Increased virulence with passaging has been reported in the following: Conidiobolus coronatus (Krejžová, 1975), Spicaria farinosa (Kerner, 1959; Kawakami, 1960), Aspergillus flavus (Lepesme, 1938), B.bassiana (Kawakami, 1960, Wasti et al., 1975), and M. anisopliae (Kawakami, 1960), sometimes after passaging only 1-3 times (Kawakami, 1960; Hartmann et al., 1974; Wasti et al., 1975). In contrast, Latch (1976) observed no enhancement of pathogenicity of M.anisopliae or B.bassiana after passaging through insect hosts.

Although pathogenicity was stable, growth rate and colonial morphology were not particularly for C-3/SS1 and C-3/SS2 on SDA (V,15). Furthermore, a complete reversion to the original morphology (that of C-3/SS1,sub3;Fig. 5.16a) did not occur (Fig. 5.17) after passaging once through M.sanborni. Kawakami (1960), obtained similar results with 7 entomogenous fungi except for reversion to parental morphology after one passage through the insect host. However, the evidence in V,15, suggests that in the case of V.lecanii, C-3/SS1 and C-3/SS2, the morphological variation may only be phenotypic and full reversion may result from more passages through M.sanborni.

Although remaining stable after subculturing or passaging, the virulence of conidiospore or blastospore batches stored in aqueous suspensions at 2°C declined more rapidly than viability (Table 5.10) emphasising the need to develop a technique for the long-term viable preservation of spores.

V.lecanii (C-3) produced a chitinase (Fig. 5.21) but the role of this enzyme in cuticle penetration is unknown (V,17). Chitinase production is a characteristic of many entomogenous fungi (Huber, 1958; Domsch, 1960; Claus, 1961; Ali et al., 1965; Gabriel, 1968; Samšiňáková et al., 1973; Leopold

et al., 1970; Latgé, 1974). It would be interesting to see if other strains of V.lecanii, especially non-pathogenic strains, can hydrolyse chitin. Information on the genetic basis of pathogenicity of entomogenous fungi would be highly relevant to work on the selection of more virulent strains. This field is practically unexplored. Possibly, differences correlated with pathogenicity may be revealed in pathogenic and non-pathogenic strains by techniques such as immuno-electrophoresis, which Segretain et al. (1972) used to demonstrate an arc of precipitation correspnding to an esterase activity correlated with virulence in B. bassiana.

M.sanborni first and second instar progeny were less susceptible to aqueous spore suspensions than adults (Table 5.11). The most likely cause is moulting of some progeny before the fungus penetrated the hypodermis, as shown for other insect species by Ferron (1967), Fargues (1972b, 1973), and Zacharuk et al. (1968). At 20°C, M.sanborni (4 instars) moults approximately every 2.5 days (Table 5.13). Since V.lecanii can irreversibly infect aphids within 14h (V,12,c) it should infect young stages treated immediately after a moult. Also a purely physical factor may have contributed to the greater difference in susceptibility; conidiospores of V.lecanii not landing in a 'crevice', such as an integumental fold, may be dislodged by aphid movement. Presumably, there is less crevice space to trap spores on a small aphid than on an adult. Other possibilites exist, e.g. young aphids may be more resistant by virtue of a more efficient cellular defence system or different cuticular properties.

Extrapolating this result to the glasshouse, aphid progeny sprayed with spores should be approximately 3 times less susceptible than adults. However, this disadvantage would be outweighed by the higher mortalities for progeny than for

adults infected with spores in slime-heads (Table 5.12) i.e. greater susceptibility to contagion.

Under optimal conditions of humidity and temperature, fewer aphids died when infected from wet, spore treated leaves than when wetted with a spray containing spores (V,9), possibly because few spores on leaves adhere to aphids. In a glasshouse, conditions should be optimal for infection at night for about 12-14h after evening spraying (III, 1) and so a few of the aphids missed by the spray may wander and acquire disease from sprayed leaves which remain wet overnight. However, next day, when visible surface wetness on leaves soon dries, spores may stick to the leaves and although perhaps still viable, they may not be detached even if leaves are again wetted. It is concluded that infection from sprayed spores on leaf surfaces would contribute very little to controlling an aphid population in the glasshouse. Spores in slime-heads on cadavers or live aphids adhere to healthy aphids which contact them but is is not yet known whether spores in slime are easily picked up from a leaf surface where they have been left by a passing active aphid bearing sporulating V.lecanii. Infection of aphids from leaf surfaces by entomogenous fungi may be generally inefficient; Thoizon (1970) did not succeed in infecting any aphids from leaves, or glass slides, covered with a layer of Entomophthora spores.

Severely diseased adult M.sanborni bore healthy offspring, which remained so on isolation from the parent even at high humidity (V,6). Since such offspring are not likely to become infected by spores on leaf surfaces and since a spray never wets all aphids in a population on a crop, effective

control of glasshouse aphid populations is likely to depend on the spread of disease by contagion from aphids initially infected by a spray.

All conidiospores on agar germinated at 15 and 20°C in 14h but the germ tubes were longer at the higher temperature (Table 4.05). However, no difference was detected in the number of spore-treated aphids becoming irreversibly infected in the first 14h of incubation at high humidity at these temperatures (Tables 5.16, 5.17). Possibly, penetration of the cuticle follows very soon after germination. Only about half the aphids kept at 100% RH for 14h died after 6 days, whereas all spore-treated aphids kept at high humidity continuously died in this time (Table 5.16). The transfer of aphids to continuous low humidity after 14h at high humidity probably reduced mortality by killing some of the spores or by preventing fungal proliferation on the aphid cuticle. Such proliferation probably leads to further penetrations of the cuticle causing proportionately swifter mortality. This could also explain why mortality at alternating periods of low and high humidity was intermediate between that at continuous low humdiity and that at continuous high humidity (Fig. 5.13).

How would temperature be likely to effect control of aphids by V.lecanii in the glasshouse? Because mortalities after 14h at high humdity at 15 and 20°C were similar, a night temperature of 15°C after spraying should be adequate. However, since infection levels were low at 11°C (V,12,c), the night temperature after spraying should not be allowed to fall near this temperature.

Following a spray of V.lecanii spores, at what temperature would disease spread best? Temperature affected the

rate of increase of healthy M.sanborni populations to a degree similar to its effect on aphid mortality times. With My.persicae, the effect on lethal times was less, relative to the effect of aphid population increase (Table 5.36). In conditions where high humidity prevails, control by V.lecanii should be similar at temperatures between 11 and 25°C (Table 5.36) for M.sanborni and perhaps better for My.persicae at 11 and 15°C than at 20°C. However, in chrysanthemum beds in glasshouses, humidity and temperature can be regulated better at night than in the daytime when humidity is lower because the polythene covers are off and temperatures are largely regulated by the weather. Thus, compared to the night period, lower humidity may check spread of infection during the day added to which higher temperatures would increase the birth of healthy aphids. At night, aphid control should be better at 15-25°C (15°C being considered optimal - see above) than at 11°C because infection rates at 11°C are very much lower than those at the higher temperatures (Tables 5.16 and 5.17). In short, control may be best when the night temperature is 15°C and the daytime temperature approaches the operative temperature of the chrysanthemum crop, usually 15°C.

These conclusions from the temperature experiments agree with the suggestions of others (Hughes, 1963; Wilding, 1970) that the optimal temperature for the control of insect populations by disease may not coincide with the optimal temperature for the development of the pathogen in its insect host.

Summary

1. A bioassay was developed to quantify the pathogenicity of Verticillium lecanii spores. One 4-dose assay will detect differences of about x8 in the LC 50s of 2 batches of spores, duplicating assays x 4, and replication 7 times, x2.

TABLE 5.36

Comparison of the Effects of Temperature on *Verticillium lecanii* - Induced Mortality Times and on Reproduction Rates of *Macrosiphoniella sanborni* and *Myzus persicae* with Reference to 20°C.

		$\frac{1}{\lambda}$ a(x°C) / $\frac{1}{\lambda}$ (20°C)				
		10°C	11°C	15°C	20°C[b]	25°C
M.sanborni		-	2.43	1.74	1	1.05
	(i)[d]	9.1	-	2.13	1	1.02
	(ii)[e]	3.94	-	2.04	1	1.05

			LT 50(x°C)/LT 50(20°C)			
			11°C	15°C	20°C	25°C
M.sanborni	Spores in aqueous spray[f] (spores/ml)	10^6	2.88	1.53	1	1.05
		10^7	-	1.42	1	1
	Infection from spores in slime heads[g]		2.42	1.33	1	-
My.persicae	Infection from spores in slime heads[g]		3.07	1.37	1	-

a Reference made to 20°C for both fungus and aphids since optimum for fungus growth rate and rate of aphid population increase close to this temperature.

b λ = Weekly rate of aphid population increase

c Data from Table 5.13

d Data from Barlow (1962)

e Data from DeLoach (1974)

f Data from Table 5.14

g Data from Table 5.15

2. The aphid M.sanborni became more susceptible to V. lecanii disease during 20 months continuous rearing in the laboratory, but this susceptibility reached a plateau level about which it fluctuated independent of time.

3. Blastospores were twice as pathogenic as conidiospores on a numerical basis, but only 1.4 times on a dry spore weight basis and less, 0.67 times, on a live spore volume basis.

4. Twenty V. lecanii strains from widely differing sources were assayed for pathogenicity. Five were non-pathogenic and the rest were similar in pathogenicity to the standard, C-3, except one C-13, which was less pathogenic.

5. The pathogenicity of strains of V.lecanii including single-spore and multi-spore isolates remained stable during repeated subculturings on agar and passage in insects. However, some gross changes in colonial morphology and growth rate occured.

6. Spores stored in aqueous suspension at $2^{o}C$ lost virulence more rapidly than viability.

7. V.lecanii (C-3) produced a chitinase, repressible by glucose and $NaNO_3$, but not derepressible by 3'5' cyclic AMP or caffeine.

8. Infection of aphids from spore-treated leaves in the glasshouse is unlikely to play an important role in aphid control.

9. Heavily diseased, apterous, adult M.sanborni can produce healthy progeny at near-normal rates. However, contagion after spraying should ultimately control an aphid population in a glasshouse.

10. Overnight infection levels of aphids in a glasshouse may be as good at 15°C as at 20°C, but much lower at 11°C.

11. Although the optimum temperature for V.lecanii growth rate in culture or in aphids is near 20°C, spread of disease among aphid populations in the glasshouse may be better at 15°C.

CHAPTER VI

CONTROL OF APHIDS IN THE GLASSHOUSE BY V.LECANII

MATERIALS AND METHODS - GENERAL

(a) Production of Spores

V.lecanii conidiospores and blastospores were cultured and harvested as in IV,1, and standardised using the methods described in V,1,a.

(b) Plants, Spore Sprays, Humidification and Temperature

All glasshouse experiments, except numbers 1 and 9, were performed in the small glasshouses shown in Fig. 6.01, each containing a single 1.2 x 0.6m bed of aphid-infested chrysanthemums (cultivar "Deep Tuneful", which is very susceptible to aphids) in pots embedded in peat. V.lecanii conidiospores or blastospores, suspended in 0.0003M buffer (V,1,a) containing 0.02% Triton X-100 wetting agent (unless otherwise stated) were sprayed with a pneumatic hand sprayer on the plants to 'run-off' in the evening (17.30 -18.30). Immediately after spraying, beds were covered with blackouts made of black polythene to boost humidity for spore germination and infection of aphids. On subsequent evenings, light overhead sprays of water were applied to the beds to boost humidity (damping down) before covering with blackouts to aid the spread of infection from diseased to healthy aphids. Deviations from these standard procedures are mentioned in the experiment descriptions.

Thermohygrographs were used to record RH but could not accurately record the night humidities under polythene blackouts. Two other humidity measuring systems were tested; the

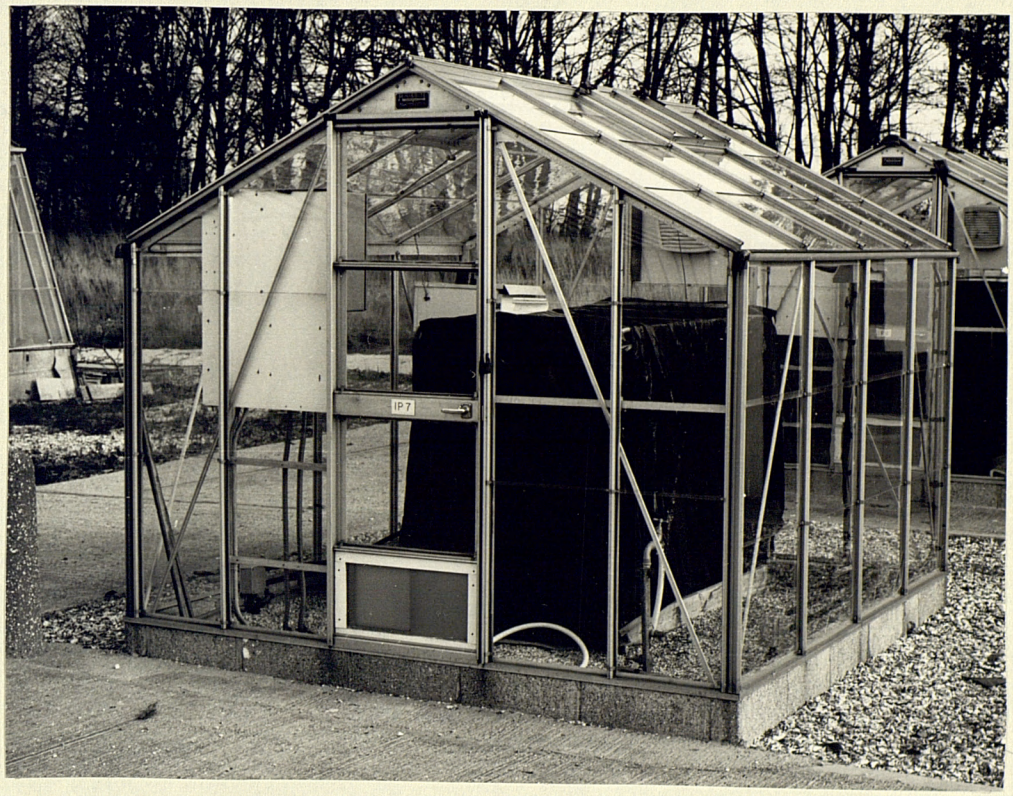


Fig. 6.01 Small glasshouse showing bed covered by polythene blackout.

first was a device which depended upon the change in capacitance of a capacitator having an aluminium oxide dielectric, and the other was a sensor which depended upon the change in conductivity of a sulphonated polystyrene surface. Both devices were tested in the laboratory and the more promising sulphonated polystyrene sensor was tested in the glasshouse but unfortunately failed to give consistent and reliable results.

In Glasshouse Experiments 1-3, bed temperatures were monitored by copper-constantan thermocouples. In Glasshouse Experiments 2-6, the minimum temperature was 20°C. Laboratory experiments (V, Discussion) later revealed that a minimum temperature of 15°C might encourage similar or better aphid control than 20°C and so was used in Glasshouse Experiments 7-9. Mean daytime temperatures within the chrysanthemum beds between 9.00 and 18.00h are included in the Figures.

(c) Enumeration of Aphids

Aphids were enumerated in the glasshouse by removing each potted plant for long enough to count individual aphids or, when aphid densities became high, to make visual estimates of the numbers present. In most experiments, aphids were enumerated separately on the upper, middle and lower parts of the plants. The upper and lower regions consisted respectively of the upper and lower 8cm of stem and foliage while the rest was referred to as the middle, which varied in length according to the height of the plant. This partitioning was useful since vertical aphid distribution was uneven, M.sanborni populations when low in numbers, usually being situated entirely on the upper 8cm of plant and My. persicae populations sometimes built-up

first on the upper foliage.

(d) Criteria of Aphid Control

Considering My.persicae first, satisfactory commercial control was usually regarded as the apparent absence of aphids on the influorescences. This yardstick permits the presence of 10-50 aphids on the plant (Hussey, and Scopes, personal communications). This contrasts with the large populations which the plant can tolerate in the vegetative state without harm (Gurney, 1969; Scopes, 1970). In the present experiments, a satisfactory level of control for My.persicae was taken to be 5 aphids/plant i.e. half than the minimum figure quoted above. In contrast, the criterion for M.sanborni was virtually no aphids since this aphid is a conspicuous, unsightly insect and when low in numbers tends to feed in prominent positions on the upper stem (Fig. 3.03). Brachycaudus helichrysi badly damages new leaves in the growing vegetative tip, and so the permissible number of aphids was zero. On plants in flower, however, criteria were similar to those for My. persicae.

SMALL GLASSHOUSE EXPERIMENTS

1. Conidiospores; Single Sprays; No Crop Damping; M.sanborni and B.helichrysi

The first experiment differed fundamentally from all the other glasshouse experiments in that potted plants were not embedded in peat beds but rested on soil or gravel, were covered continually by clear polythene cages instead of polythene blackouts at night, sprayings were performed in the laboratory and not in the glasshouse, and plants were not damped down each evening but instead the beds and bases of plants were watered 5 times daily.

(a) Methods

Potted chrysanthemums (45cm tall)were infested with M.sanborni and B.helichrysi. When M.sanborni populations had reached approximately 55 aphids/plant, groups of plants were sprayed with various concentrations of V.lecanii conidiospores as follows:-

6	plants	10^8 spores/ml
5	"	10^7 spores/ml
5	"	10^6 spores/ml
5	"	10^5 spores/ml
4	"	buffer with no spores

In order to apply V.lecanii conidiospore inocula as evenly as possible, the potted plants were sprayed in the laboratory and then distributed in 4 polythene cages A,B, C and D in a single small glasshouse. Two more groups of plants were treated as follows:-

Cage E 5 plants : 10^7 spores/ml (ungerminated) in SLM
Cage F 5 plants : 10^7 spores/ml (89% germinated) in SLM

These 2 groups of plants were placed in separate polythene cages in another small glasshouse. Plants were watered 5 times daily to boost humidity. The minimum temperature was maintained between 14 and 16.5°C.

(b) Results

Considering first the plants in cages A,B,Cand D, effective control of M.sanborni was not achieved unless the humdity was very high. Cages A and B, resting on gravel which dried very quickly following wetting, contained visibly less moisture than cages C and D resting on soil, suggesting a lower humidity in cage A and B. Thermohygrograph records showed that the night humidity over gravel averaged 77% whereas over soil, the recorder needle remained mostly at the calibrated maximum i.e. a nominal 100%. This difference in humidity influenced greatly the degree of control obtained. In the lower humidity cages, A and B at all spore concentrations there were no signs of control after 50 days (Fig. 6.02). In the high humidity cages C and D, control of M.sanborni populations treated with 10^6 and 10^7 spores/ml was similar to that for the populations not treated with spores but populations sprayed with 10^8 spores/ml showed a steeper decline (Fig. 6.03). The population increase of the untreated insects in high humidity cages C and D paralleled that of the populations in cages A and B until the 12th day when the population started to decline and eventually died out at the high RH indicating that perhaps infection spread from treated to untreated aphids in the same cage.

Spraying M.sanborni populations (on plants resting on soil) with ungerminated or germinated spores, both in Sabouraud liquid medium (SLM), slightly increased the rate of

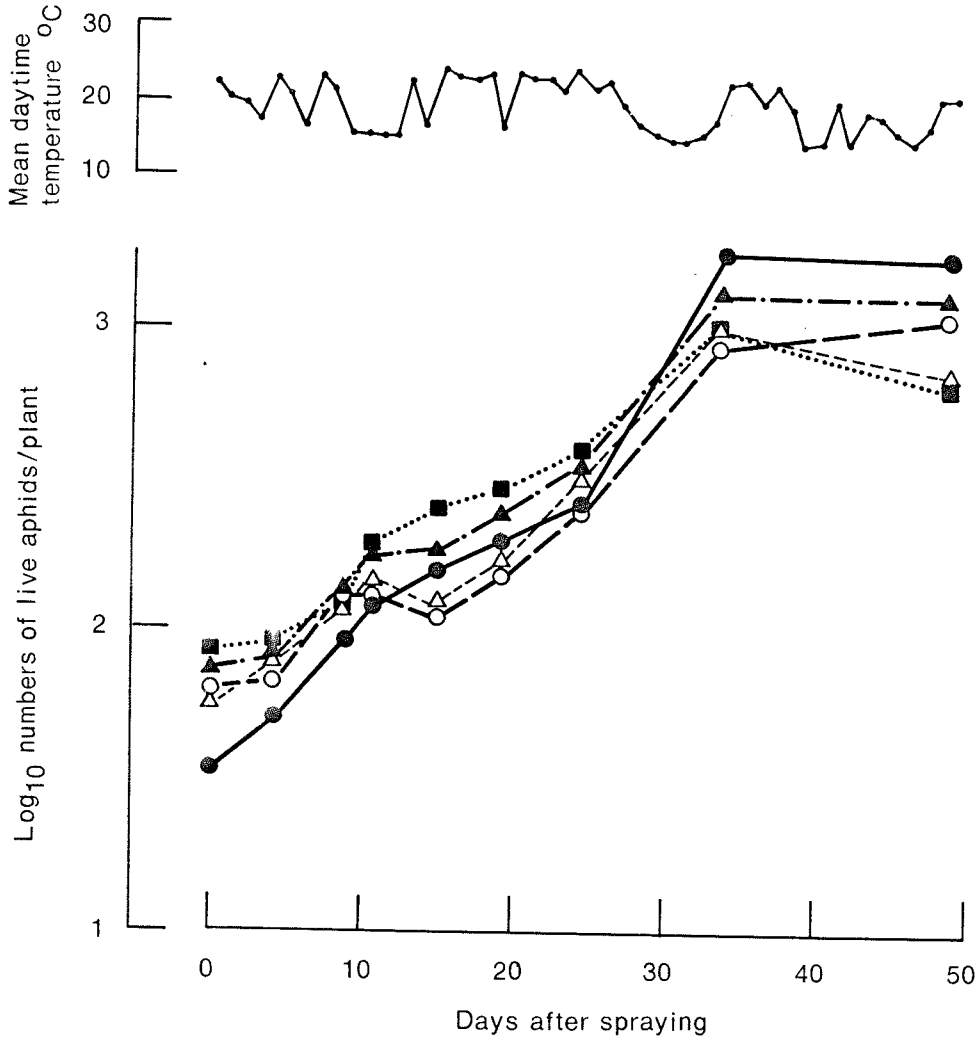
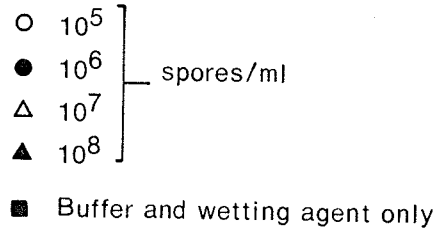


Fig. 6.02

Effect of spraying *Verticillium lecanii* conidiospores on *Macrosiphoniella sanborni* populations on chrysanthemums. Combined data for low humidity cages A and B.



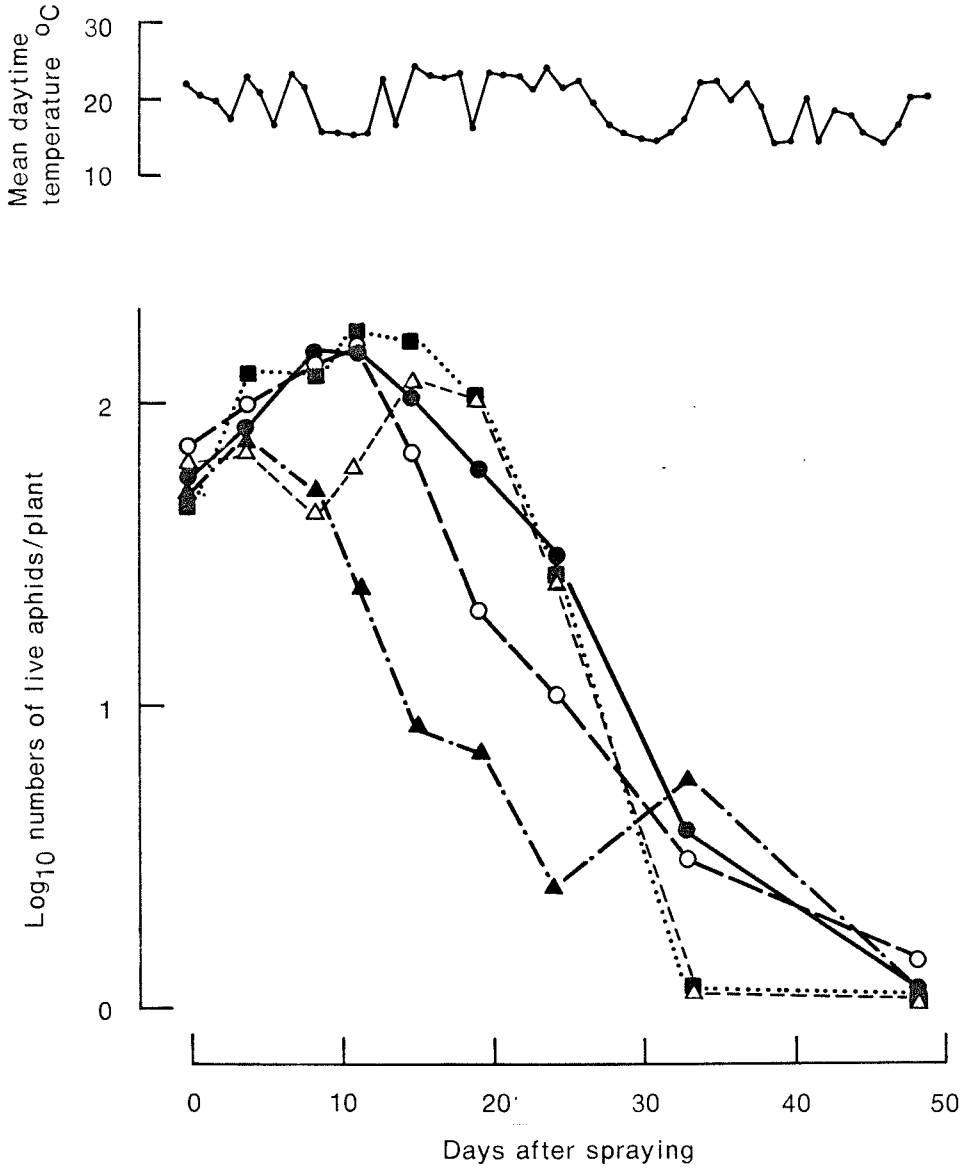


Fig. 6.03

Effect of spraying *Verticillium lecanii* conidiospores on *Macrosiphoniella sanborni* populations on chrysanthemums. Combined data for populations in high humidity cages C and D.

population reduction (Fig. 6.04), but it is possible that this was really due to higher humidity in the cages (E and F) compared with cages C and D.

In contrast to M.sanborni, the treated Brachycaudus helichrysi populations in vegetative tips were exterminated rapidly in the high humidity cages C and D, somewhat less so in the low humidity cages A and B (Table 6.01). Untreated populations on these plants were also exterminated indicating again that infection may have spread from treated to untreated aphids in the same cage.

A high RH was essential for the control of M.sanborni. In contrast, low RH did not prevent, but merely delayed the control of B.helichrysi (Table 6.01). This difference was possibly caused by the plant tips trapping moisture resulting in a micro-environment more humid than that in the stems and leaves. A relationship between the speed of control and spore dosage was not very clearly shown but was more marked in B. helichrysi (Table 6.01) than in M.sanborni (Figs. 6.02, 6.03).

2. Conidiospores; Crop Damping; M.sanborni and My.persicae

In this and the following experiments, covering plants continually with transparent polythene cages, as used in the previous experiment, was replaced by covering plants at night with polythene blackouts (Fig. 6.01) to resemble more closely commercial conditions. Twenty-four hours after spraying and on each evening thereafter, the beds were damped down with overhead sprays of water. Also, in these experiments, sprayings were performed in the glasshouse and not in the laboratory.

A spore spray is unlikely to infect all aphids present

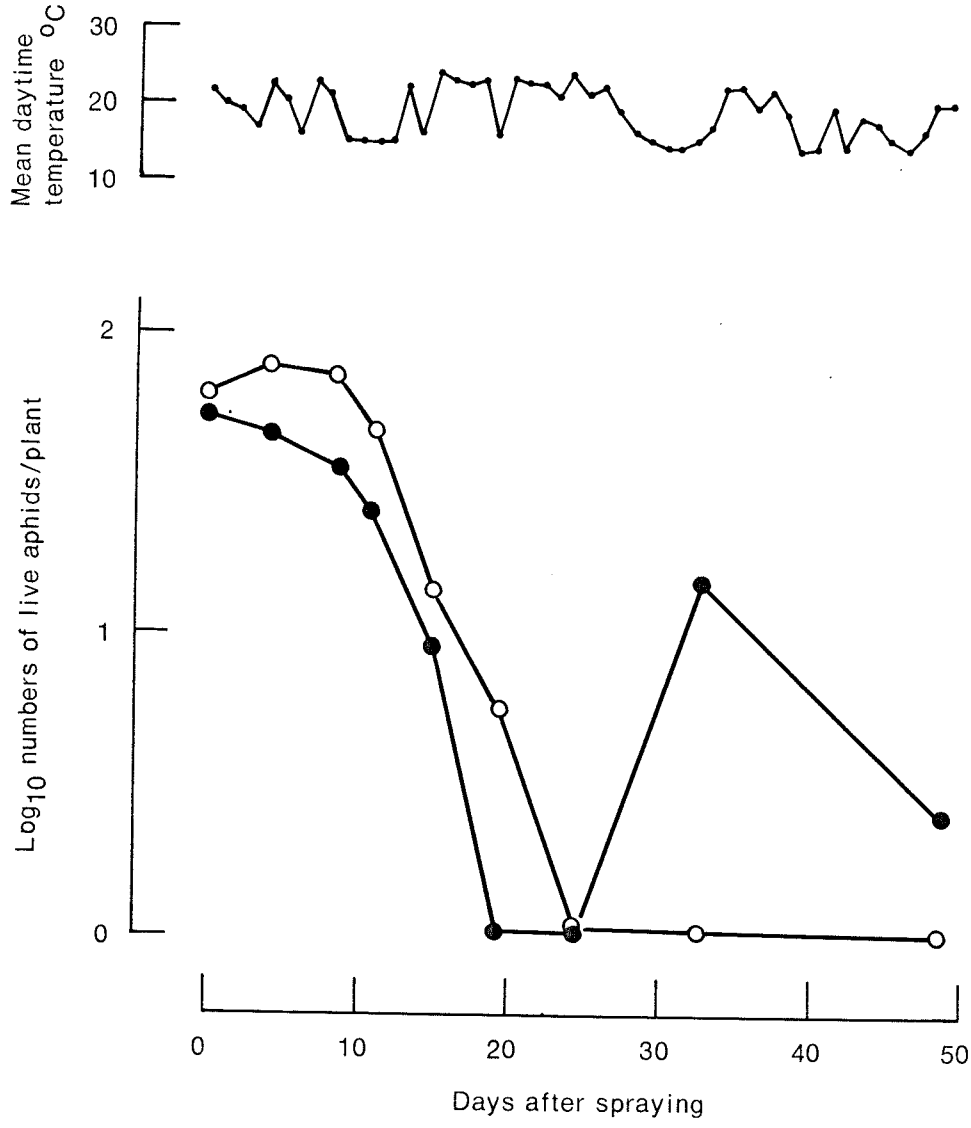


Fig. 6.04

Effect of spraying *Verticillium lecanii* conidiospores in Sabouraud liquid medium (SLM) on *Macrosiphoniella sanborni* populations on chrysanthemums. Combined data for populations in high humidity cages E and F.

○ 10^7 ungerminated spores/ml

● 10^7 germinated (89%) spores/ml

TABLE 6.01

Control[a] by *Verticillium lecanii* of *Brachycaudus helichrysi* Populations in Vegetative Chrysanthemum Tips Sprayed With Conidiospores.

Cages[b]	Spores/ml buffer and wetting agent					Spores/ml SLM[c] and wetting agent	
	0	10^5	10^6	10^7	10^8	10^7 ungerminated (Cage E)	10^7 germinated (Cage F)
Low humidity cages A and B	40.5	48	33	25	21.5	15	15
High humidity cages C and D	19.5	17.5	17.5	14.7	15.25		

[a] Control given by time (in days) for virtual elimination of colonies

[b] Combined data for each pair of cages

[c] Sabouraud liquid medium

at the time of spraying. Also, laboratory experiments showed that even heavily diseased aphids can produce healthy progeny at near-normal rates (V, 6). Ultimate control of an aphid population most probably depends on contagion from insect to insect. Thus, in this experiment, consecutive daily sprays were considered necessary (i) to infect as many aphids as possible while their numbers were still low and (ii) to infect progeny born from diseased aphids infected by a previous spray. Compared to one spray alone, this should result in many more aphid cadavers sporulating with V.lecanii so that when spraying ceased, newborn healthy progeny would have a greater chance of acquiring disease, thus hopefully resulting in swift control before plants sustained any damage.

(a) Methods

Eighty-four potted chrysanthemum plants (30cm) were distributed equally in 2 peat beds in separate glasshouses and each plant was infested with one adult each of M. sanborni and My.persicae. When the M.sanborni populations had reached approximately 10 aphids/plant (My.persicae populations were somewhat less, Fig.6.05), one bed was sprayed with 5×10^6 spores/ml and the control bed was sprayed with buffer and wetting agent without spores. Sprayings were repeated on the next 4 evenings. The minimum temperature in the beds was 20°C.

(b) Results

The My.persicae infestation, though initially lower in numbers and more sparsely distributed was controlled very effectively in contrast to the denser M.sanborni infestation which was only partially controlled by the end of the experiment (Fig. 6.05).

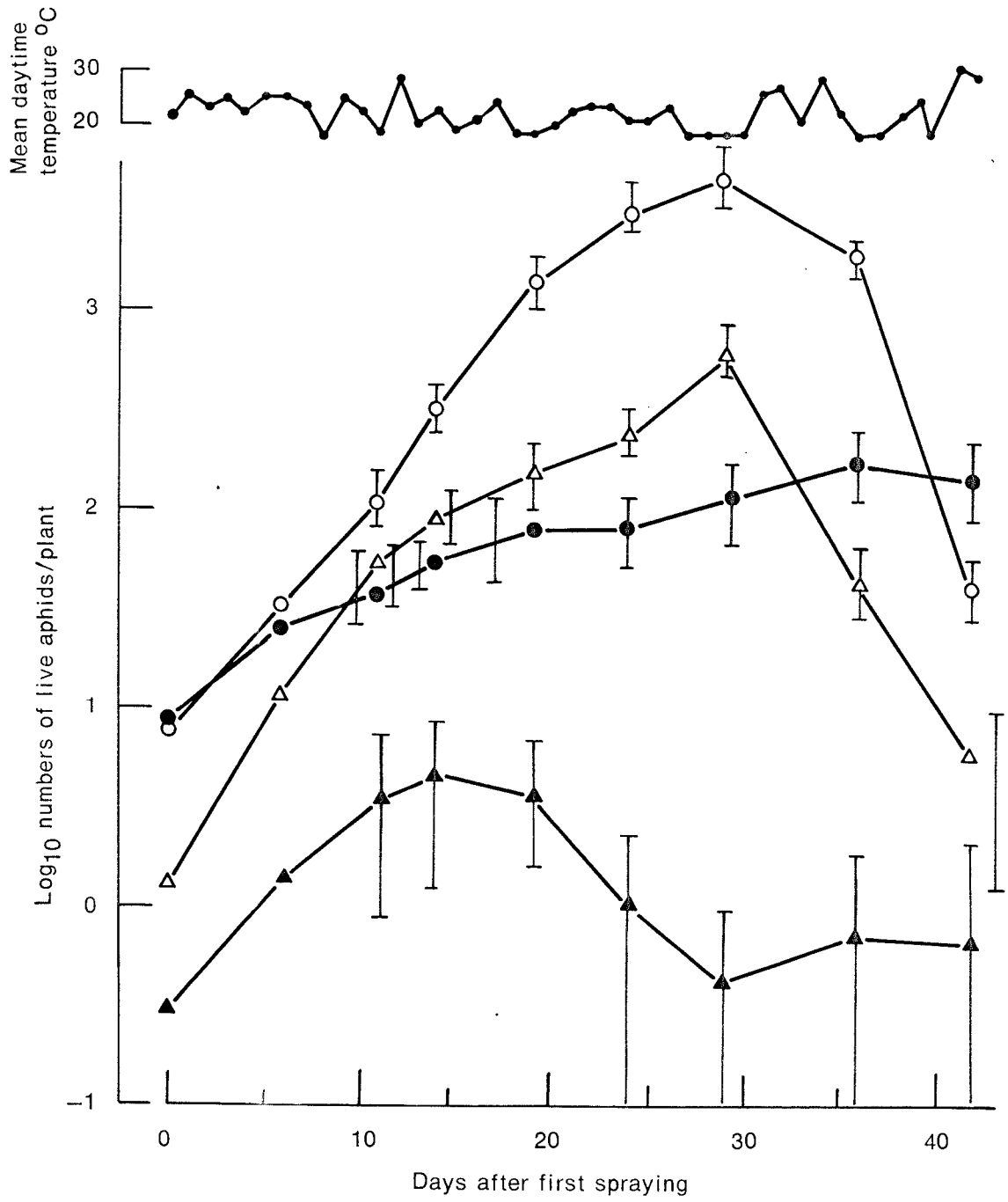


Fig. 6.05

Effect of spraying *Verticillium lecanii* **conidiospores on** *Macrosiphoniella sanborni* **and** *Myzus persicae* **total populations on chrysanthemums; 5 consecutive daily applications of 5 x 10^6 spores/ml. Ninety five % fiducial limits included.**

● *M. sanborni*
▲ *My. persicae*
] Sprayed with spores

○ *M. sanborni*
△ *My. persicae*
] Sprayed with buffer and wetting agent only

Control of My.persicae by V.lecanii was swift on all parts of the chrysanthemum plants (Fig. 6.06), the more exposed upper population being reduced most rapidly, presumably because these aphids received a heavier inoculum of spores than aphids beneath leaves. Totalled over all regions of the plants, the peak population reached was only 5.2 aphids/plant and the aphids were virtually eliminated by the 24th day of the experiment (Fig.6.06). However, on the control plants, the population reached a peak of 640 aphids/plant on the 29th day, after which it was reduced by a natural combined Entomophthora aphidis and V.lecanii epizootic.

M.sanborni populations on the upper stems rose sharply during the first 6 days but then more slowly and later stabilized at 45 M.sanborni/upper stem because of disease and downward migration of aphids (Fig. 6.07). Populations on the upper stem of untreated plants increased sharply until the 19th day and stabilized at approximately 300/upper stem, also because of downward migration. The population on the middle and lower parts of treated plants then increased but was not controlled by V.lecanii by the end of the experiment (Fig. 6.07). However, although control was not achieved on treated plants the peak population was greatly reduced when compared with the untreated populations (Fig. 6.07).

3. Conidiospores; Different Numbers of Consecutive Daily Sprays; 2 Spore Concentrations; M.sanborni.

This experiment was performed to help establish the degrees of control produced by different spray regimes and spore concentrations. Since this experiment involved many treatments and glasshouse space was limited, small numbers

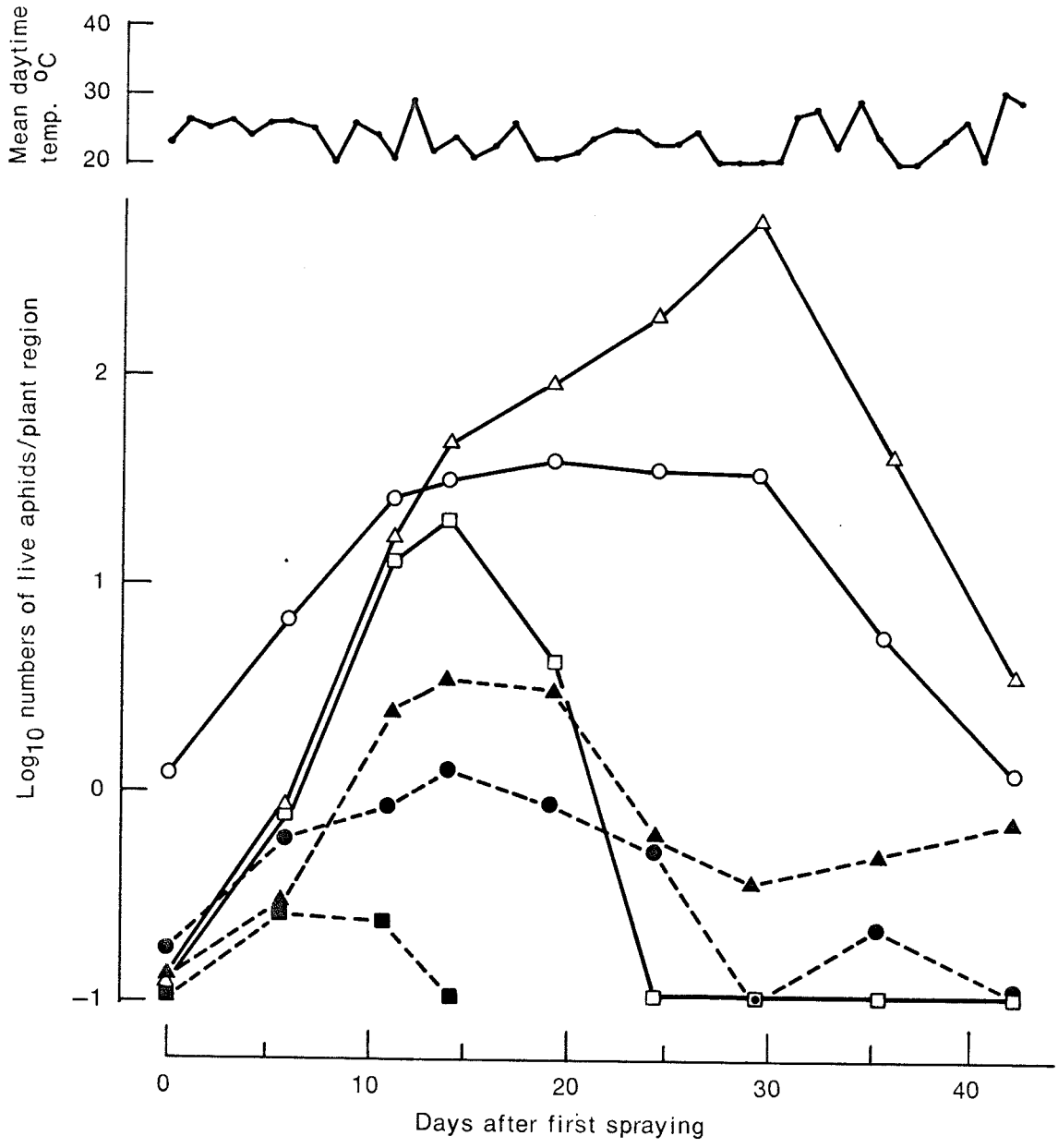


Fig. 6.06

Effect of spraying *Verticillium lecanii* conidiospores on *Myzus persicae* populations; 5 consecutive daily sprays at 5 x 10^6 spores/ml.

■ Upper plant
▲ Middle plant
● Lower plant
} sprayed with spores

□ Upper plant
△ Middle plant
○ Lower plant
} sprayed with buffer and wetting agent only

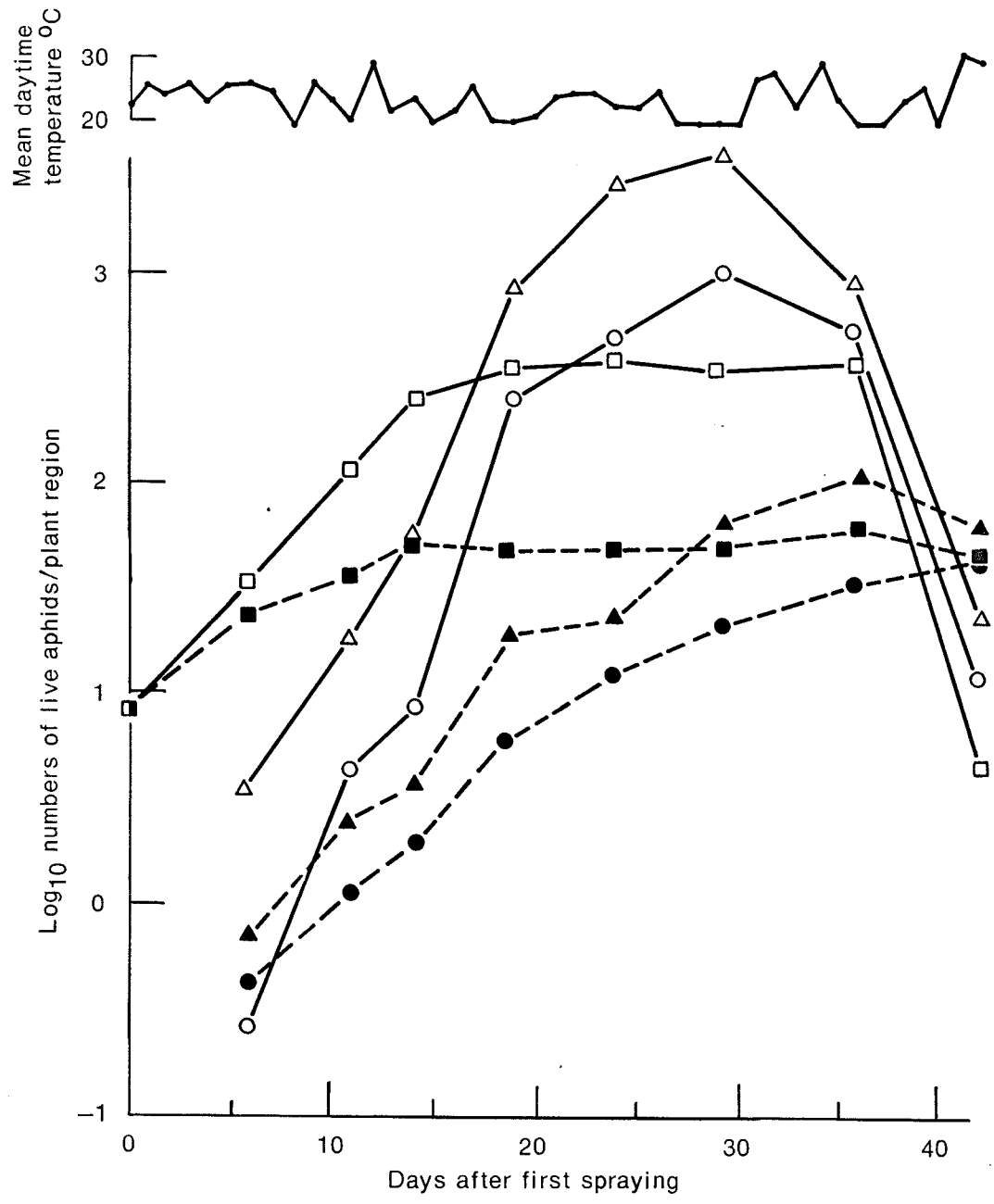


Fig. 6.07

Effect of spraying *Verticillium lecanii* conidiospores on *Macrosiphoniella sanborni* populations on chrysanthemums: 5 consecutive dally sprayings at 5 x 10^6 spores/ml.

■ Upper plant
▲ Middle plant
● Lower plant
} Sprayed with spores

□ Upper plant
△ Middle plant
○ Lower plant
} Sprayed with buffer and wetting agent only

of replicate plants were employed. Two peat beds of 45 potted chrysanthemums (30cm tall) in separate, small glasshouses were used. Only 15 experimental plants in each bed were infested with aphids and these were sited among the other uninfested plants so that no experimental plants were adjacent to each other (Fig. 6.08). The uninfested plants were to act as 'buffers' to minimize the chances of aphid migration from one experimental plant to another. When aphid populations had reached approximately 9 aphids/plant, plants were sprayed as shown in Fig. 6.08. Each plant was individually sprayed in a corner of the glasshouse and returned to its position in the bed (Fig. 6.08). The minimum temperature in the beds was 20°C.

Aphid populations on some replicate plants were destroyed by predators (Chrysopidae) before the effect of V.lecanii could be assessed. These predators when discovered, were immediately killed and spoilt replicates were not included in the assessment. Hence the numbers of replicates vary in Figs. 6.08 and 6.09. Further predation after the 15th day forced the termination of the experiment.

(b) Results

Whilst a gradient in mortalities was obtained with increasing numbers of consecutive, daily sprayings, at either concentration the difference in mortalities between plants sprayed once and those sprayed 4 times was only slight and insignificant, the 95% confidence limits mostly overlapping each other (Fig. 6.09). Those plants sprayed with 5×10^6 spores/ml supported a higher mean aphid population than plants sprayed with 5×10^7 spores/ml (Fig. 6.09) but after day 9, the differences were not significant ($P<0.05$).

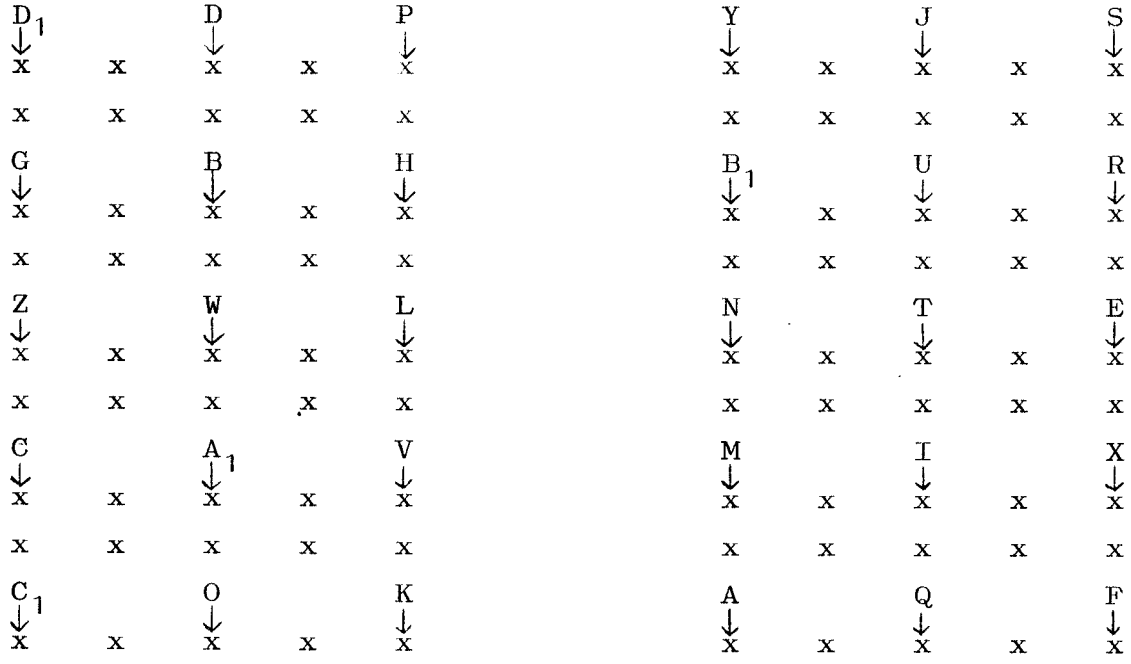


Fig. 6.08 Distribution of chrysanthemum plants (x - denotes a single plant) in Glasshouse Experiment 3, and treated as follows:-

Infested Plants A - D Sprayed once with 5 x 10^7 conidiospores /ml.

" " G - H Sprayed with 5 x 10^6 spores/ml (4 consecutive daily applications).

" " I - L Sprayed once with 5 x 10^6 spores/ml.

" " M - S Sprayed with 5 x 10^7 spores/ml (4 consecutive daily applications).

" " T - W Sprayed with 5 x 10^7 spores/ml (3 consecutive daily applications).

" " X - A_1 Sprayed with 5 x 10^7 spores/ml (2 consecutive daily applications).

" " B_1 - D_1 Sprayed with buffer containing 0.02% Triton X-100 only.

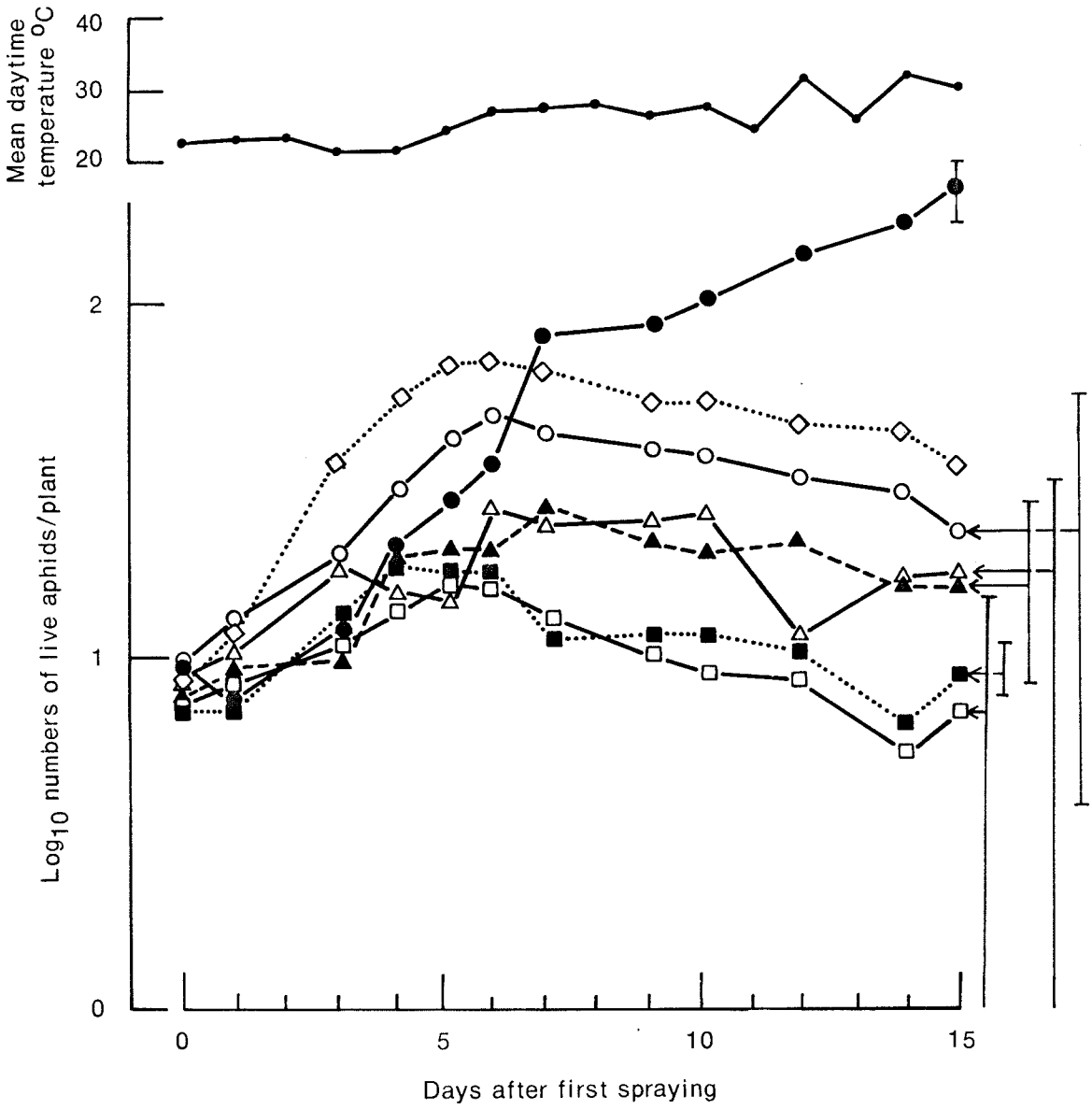


Fig. 6.09

Effect of number of consecutive daily sprays of *Verticillium lecanii* conidiospores at 2 spore concentrations on populations of *Macrosiphoniella sanborni* on chrysanthemums. (95% fiducial limits included for last points only for clarity).

◇	5×10^6 spores/ml – 1 application	(n = 1)
○	5×10^6 spores/ml – 4 applications	(n = 3)
▲	5×10^7 spores/ml – 1 application	(n = 3)
△	5×10^7 spores/ml – 2 applications	(n = 3)
■	5×10^7 spores/ml – 3 applications	(n = 4)
□	5×10^7 spores/ml – 4 applications	(n = 5)
●	Buffer and wetting agent only	(n = 2)

N.B. Some replicate plants removed from experiment owing to extensive predation by *Chrysopa* sp.

Population reduction did not amount to acceptable control but might have improved had the experiment not been terminated so early. However, the results indicated that a single application of spores should produce almost as good population reduction as multiple (consecutive) sprays. With all treatments, the aphid populations and the rate of increase were stemmed (Fig. 6.09). This stabilization was attributable to contagion and from these results it is therefore considered that rather than using consecutive sprays a second spray after population stabilization to supplement contagion may be of more value in helping to achieve a high level of control of M.sanborni

4. and 5. Conidiospores Compared with Blastospores; One or 2 Sprays; M.sanborni.

Blastospores, which can be rapidly and abundantly cultured, were numerically more pathogenic than conidiospores, under conditions of laboratory bioassay. It was now necessary to compare these spore types in the glasshouse environment.

(a) Methods

In the first experiment (Glasshouse Experiment 4) each of 2 beds, containing 50 potted plants (45cm) and infested with approximately 10 M.sanborni/plant was sprayed with 3 x 10^7 spores/ml, one bed with conidiospores and the other with blastospores. A third bed was sprayed with buffer and Triton X-100 only as control. The blastospore preparation was contaminated with an unidentified fungus but present only in small quantities. When aphid populations had stabilized, they were sprayed again to supplement contagion between aphids, as suggested in the previous experiment.

In the second experiment (Glasshouse Experiment 5), 2 beds each containing 25 M.sanborni-infested plants (60cm tall)

(9 aphids/plant) were sprayed with 3 x 10^7 blastospores/ml of buffer containing 0.05% Triton X-100. A third bed was sprayed with the same concentration of conidiospores and a fourth bed was sprayed with buffer and 0.05% Triton X-100 only as control. One of the blastospore -treated beds was resprayed 10 days later (Fig. 6.13). The minimum temperature in these experiments was 20°C.

(b) Results

In both experiments, blastospores were approximately as effective as conidiospores in reducing aphid populations to low levels (Figs. 6.10 - 6.13). In the first experiment, the absence of a bed sprayed once only with spores made it difficult to gauge the effect of the second spray (Figs. 6.10 - 6.12). However, since the populations had not only stabilized but were decreasing when the second spray was applied (Figs. 6.10 - 6.12), the latter spray had little impact. Certainly, in the second experiment, the second spray did not accelerate control (Fig. 6.13) compared to the bed sprayed once only, presumably becuase contagion controlled the population very efficiently.

In the first experiment, almost satisfactory control was achieved by the 24th day after spraying (Fig. 6.10), the highest population density being 58 aphids/plant. M.sanborni migrated from the upper part of the plant where they clustered on the stem, to the undersides of leaves further down 6-10 days after the initial infestation. Control of populations at all 3 levels of the plant, proceeded in parallel (Figs. 6.11 and 6.12) but the leaf populations were virtually wiped out after 34 days unlike the upper stem populations, possibly because the microclimate humidity was higher beneath the leaves than on the upper, more exposed stem. In the second experiment, almost complete control was obtained

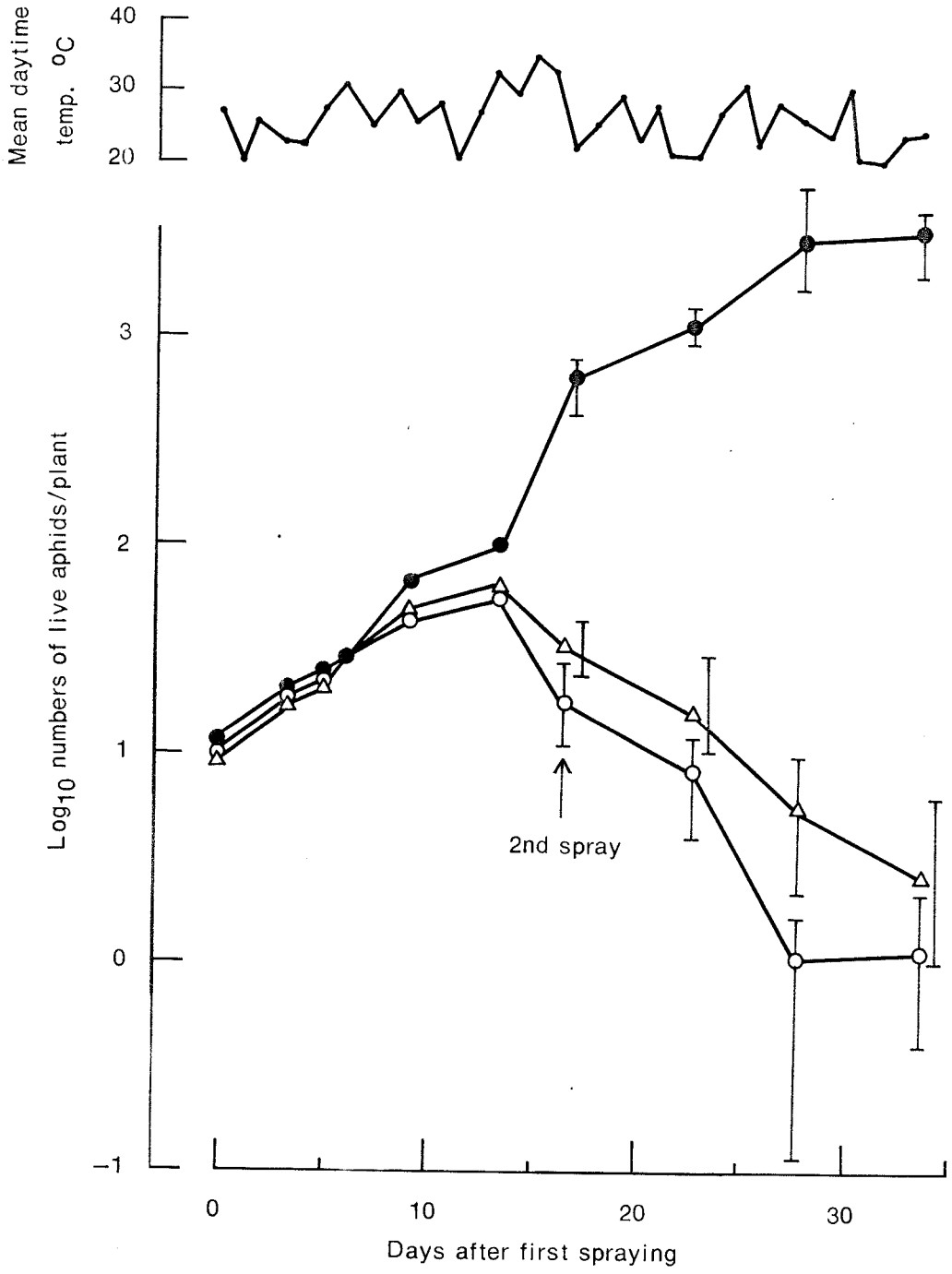


Fig. 6.10

Effect of spraying *Verticillium lecanii* condiospores or blastospores (2 sprays of 3 x 10^7 spores/ml) on *Macrosiphoniella sanborni* populations on chrysanthemums (95% fiducial limits included)

- ○ Conidiospores
- △ Blastospores
- ● Buffer and wetting agent only

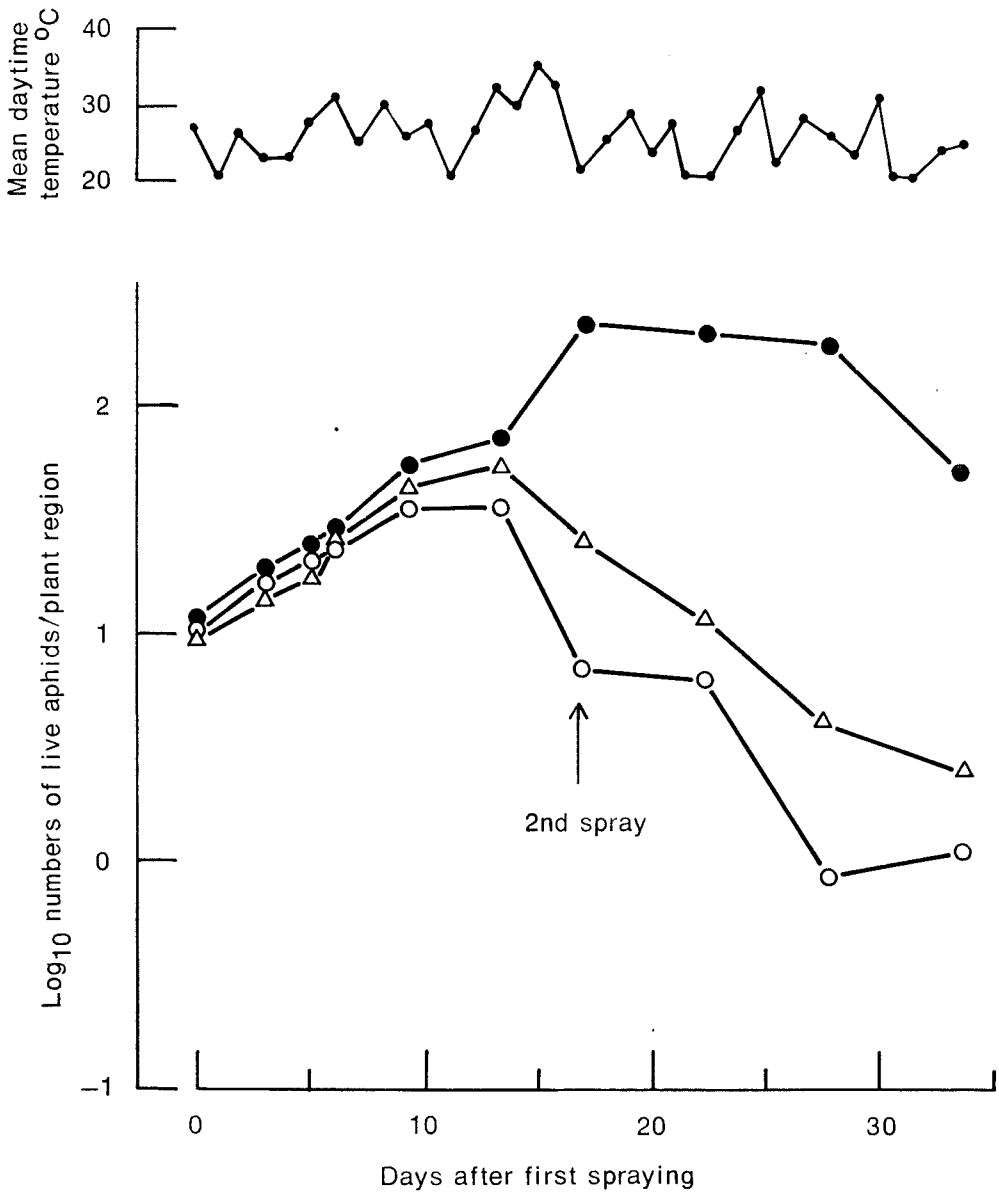


Fig. 6.11

Effect of spraying *Verticillium lecanii* conidiospores or blastospores (2 sprays of 3 x 10^7 spores/ml) on *Macrosiphoniella sanborni* upper plant populations on chrysanthemums.

- ○ Conidiospores
- △ Blastospores
- ● Buffer and wetting agent only

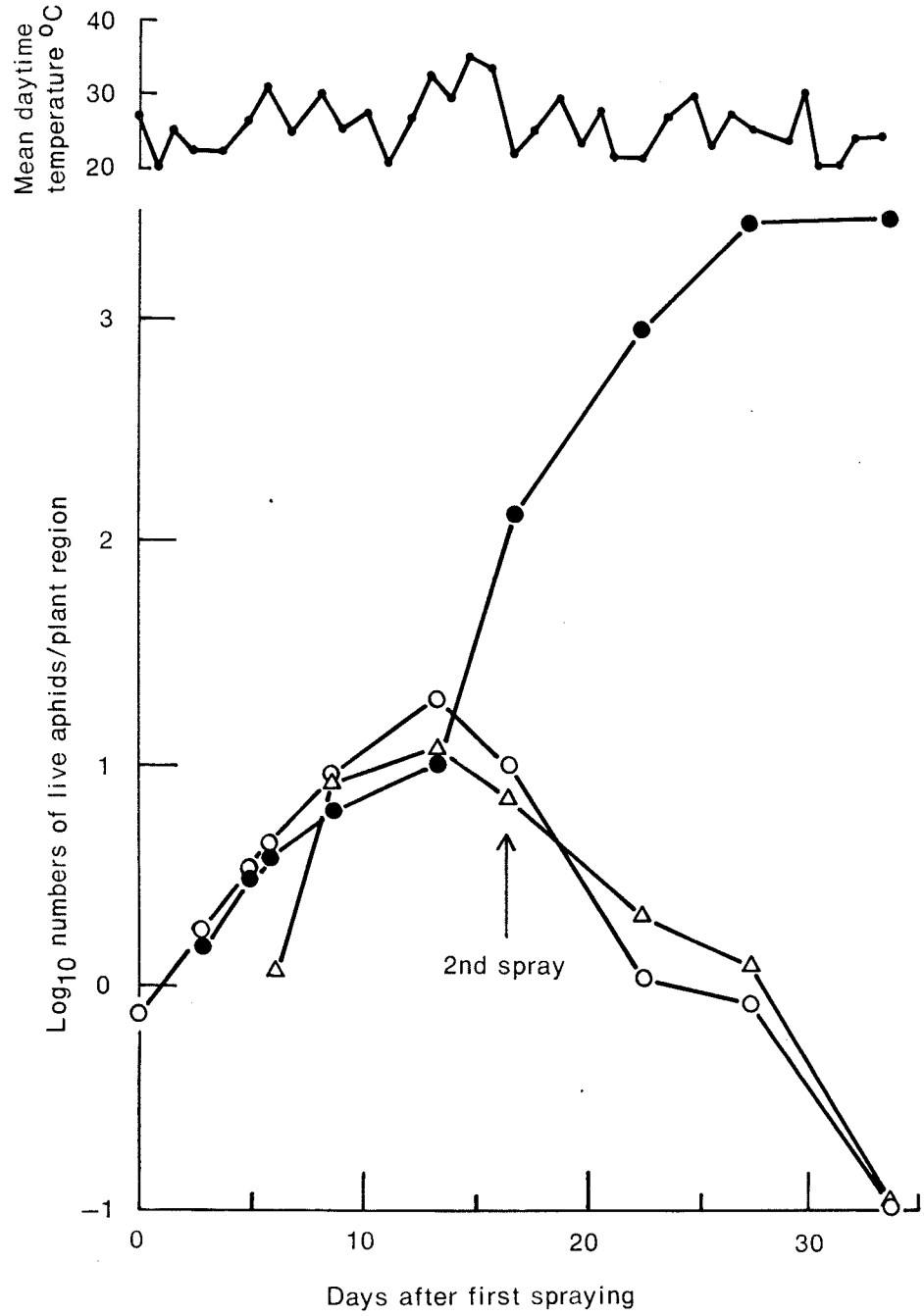


Fig. 6.12

Effect of spraying *Verticillium lecanii* conidiospores or blastospores (2 sprays of 3 x 10^7 spores/ml) on *Macrosiphoniella sanborni* middle and lower populations on chrysanthemums.

○ Conidiospores

△ Blastospores

● Buffer and wetting agent only

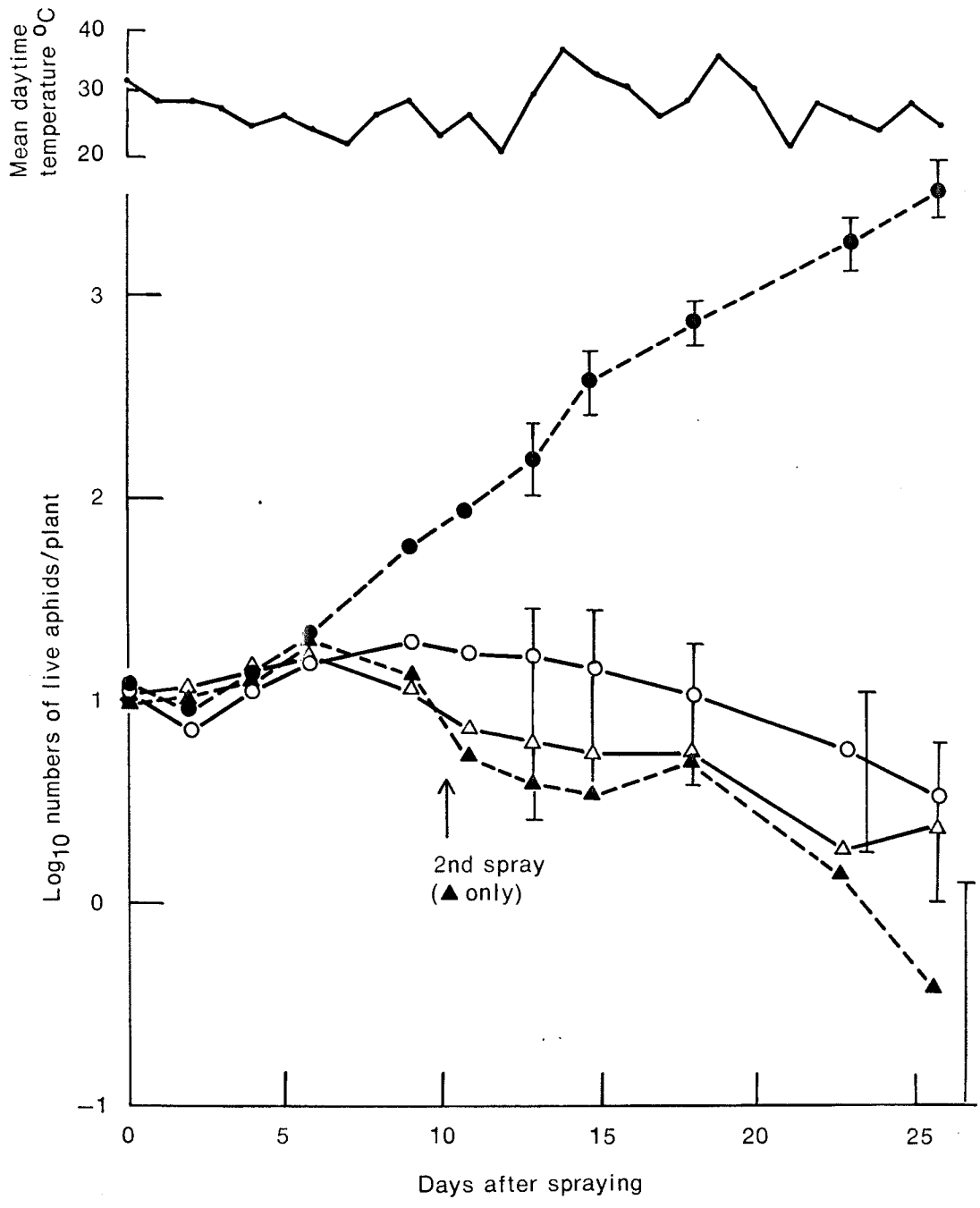


Fig. 6.13

Effect of spraying *Verticillium lecanii* conidiospores or blastospores (3 x 10^7 spores/ml) on *Macrosiphoniella sanborni* populations on chrysanthemums (only salient 95% fiducial limits included to show non-significant differences between blasto- and conidiospore-treated populations).

- ○ Conidiospores - one spray
- △ Blastospores - one spray
- ▲ Blastospores - resprayed on day 10
- ● Buffer and wetting agent only

by the 26th day after spraying and the highest total population was only 20 aphids/plant (Fig. 6.13). A contributory factor to the greater success of this experiment may have been the inclusion of a higher concentration of wetting agent, Triton X-100, in the initial spray, which directly killed by drowning some M.sanborni individuals (Fig. 6.13) whose bodies became overgrown with V.lecanii. There was very little migration to the lower foliage but the small numbers of migrants were virtually wiped out by contagion presumably spread from the upper parts of the plant by infected migrants. Wild My.persicae invaded the blastospore-treated bed in Glasshouse Experiment 4 and the conidiospore-treated bed in Glasshouse Experiment 5 causing small infestations mainly confined to lower leaves. Since large numbers of this species might have interfered with the populations of M. sanborni, they were destroyed by hand. This did not eradicate them completely but they were virtually wiped out by V.lecanii within 15 days after spraying. After control of My.persicae by fungus started, manual control was terminated. Maximum populations were only about 1-2 aphids/plant and control by V.lecanii was maintained throughout the experiment.

Also, in Glasshouse Experiment 5, a clone of the glasshouse potato aphid, Aulocorthum solani, appeared on one blastospore-treated plant. It did not exceed approximately 15 insects and later disappeared, but the absence of diseased bodies suggested that it was not exterminated by V.lecanii.

These experiments showed that the infectivities of blastospores and conidiospores in the glasshouse were similar. Because they can be more readily and abundantly cultured (IV, Discussion) blastospores were employed in all further glasshouse experiments.

6. Blastospores; One or 2 Sprays; M.sanborni, My.persicae, and B.helichrysi.

(a) Methods

Six beds, in separate glasshouses, each contained 30 budded (60cm) chrysanthemums. The beds, in groups of 2, were infested with one of the 3 aphid species, M.sanborni, B.helichrysi or My.persicae. In 2 beds before infesting with B.helichrysi, every alternate plant was decapitated, removing the tightly closed flower buds. Vegetative tips soon grew from leaf axils. Thus, on the intact plants, B.helichrysi aphids fed on the outside of tightly closed flower buds, while on the decapitated plants, aphids fed deep within vegetative tips. Experimental beds were sprayed with a high concentration of blastospores (8×10^7 spores/ml) and control beds were sprayed with buffer and wetting agent only. The M.sanborni and B.helichrysi populations were resprayed when the populations had stabilized. The minimum temperature was 20°C.

(b) Results

B.helichrysi populations in the moisture-trapping vegetative tips were eliminated within 13 days after spraying, but those populations feeding on the outside of tightly closed flower buds, although curbed, were not satisfactorily controlled (Fig. 6.14). On buds, fungus-killed aphids were brown (Fig. 6.15), symptomatic of poor sporulation on the cadaver, whilst dead, aphids in vegetative tips were covered with white, prolifically sporulating fungus. A second spray on day 18 failed to reduce the population (Fig. 6.14). When the buds opened, around day 22, the degree of population restraint remained approximately the same (Fig. 6.14).

Population reduction of treated M.sanborni was substantial when compared with the untreated M.sanborni population (Fig.

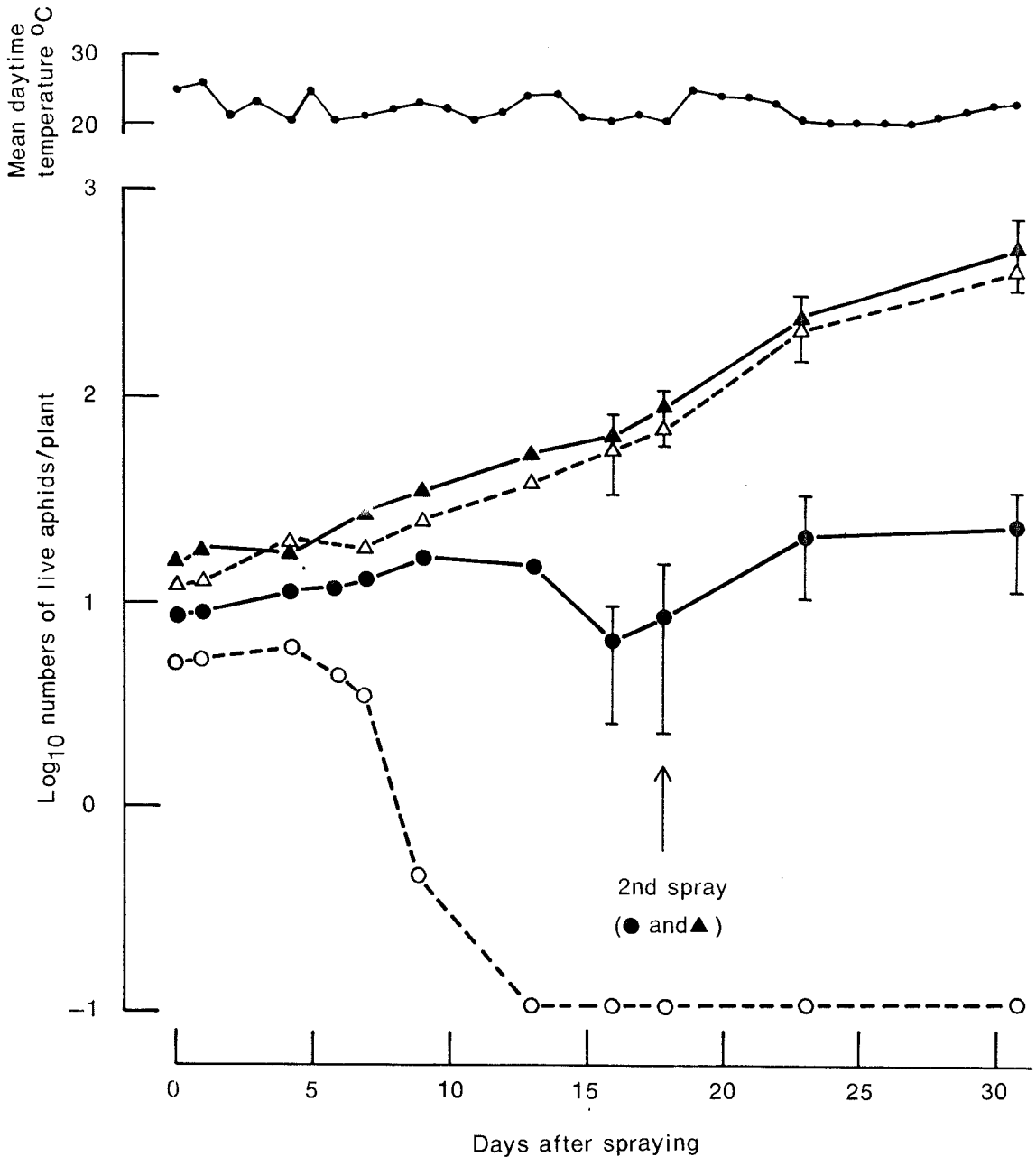


Fig. 6.14

Effect of spraying *Verticillium lecanii* blastospores (8×10^7 spores/ml) on *Brachycaudus helichrysi* populations on chrysanthemum vegetative tips and flower buds (95% fiducial limits included for aphids on budded plants.)

- ○ Aphids in tips sprayed with spores
- ● Aphids on buds " " " (resprayed on day 18)
- △ Aphids in tips sprayed with buffer and wetting agent only
- ▲ Aphids on buds " " " " " " "
  (resprayed on day 18)

Fig. 6.15 *Brachycaudus helichrysi* forced to feed on tightly closed flower buds. Brown aphids (arrowed) bear poorly sporulating *Verticillium lecanii*.

6.16) but from a commercial standpoint, control was unsatisfactory on all parts of plants (Figs. 6.16 - 6.17). Sporulation on aphid bodies on the stem in the upper plant region was poor, but was better beneath the leaves further down the plant where, however, the numbers of diseased aphids remained low throughout the experiment. A second spray on day 18 failed to control the aphids (Figs. 6.16 and 6.17).

Control of My.persicae was spectacularly successful (Fig. 6.16). Aphids were eliminated by the 18th day after spraying. Population decrease commenced 4 days after spraying, suggesting that most aphids had received 'infective doses' of spores.

B.helichrysi in tips and on buds did not migrate prior to V.lecanii-induced death. Spore-bearing bodies on leaves nearest the tips remained on the leaf as the leaf grew and live aphids continually moved off growing leaves into the tips. Thus, bodies became dispersed over the growing plant giving a fallacious impression of pre-death migration. In the same way, dead M.sanborni were left behind on stems as live aphids moved upwards as the stem extended. Also, there was no evidence of migration of My.persicae from feeding sites shortly before death. Most aphids of all 3 species died in the feeding position.

This was the first glasshouse experiment in which plants had been brought into bloom. Previously, damping down each evening had not resulted in proliferation of phytophagous diseases on vegetative plants but, in this experiment, flowers succumbed to Botrytis cinerea very soon after bud break.

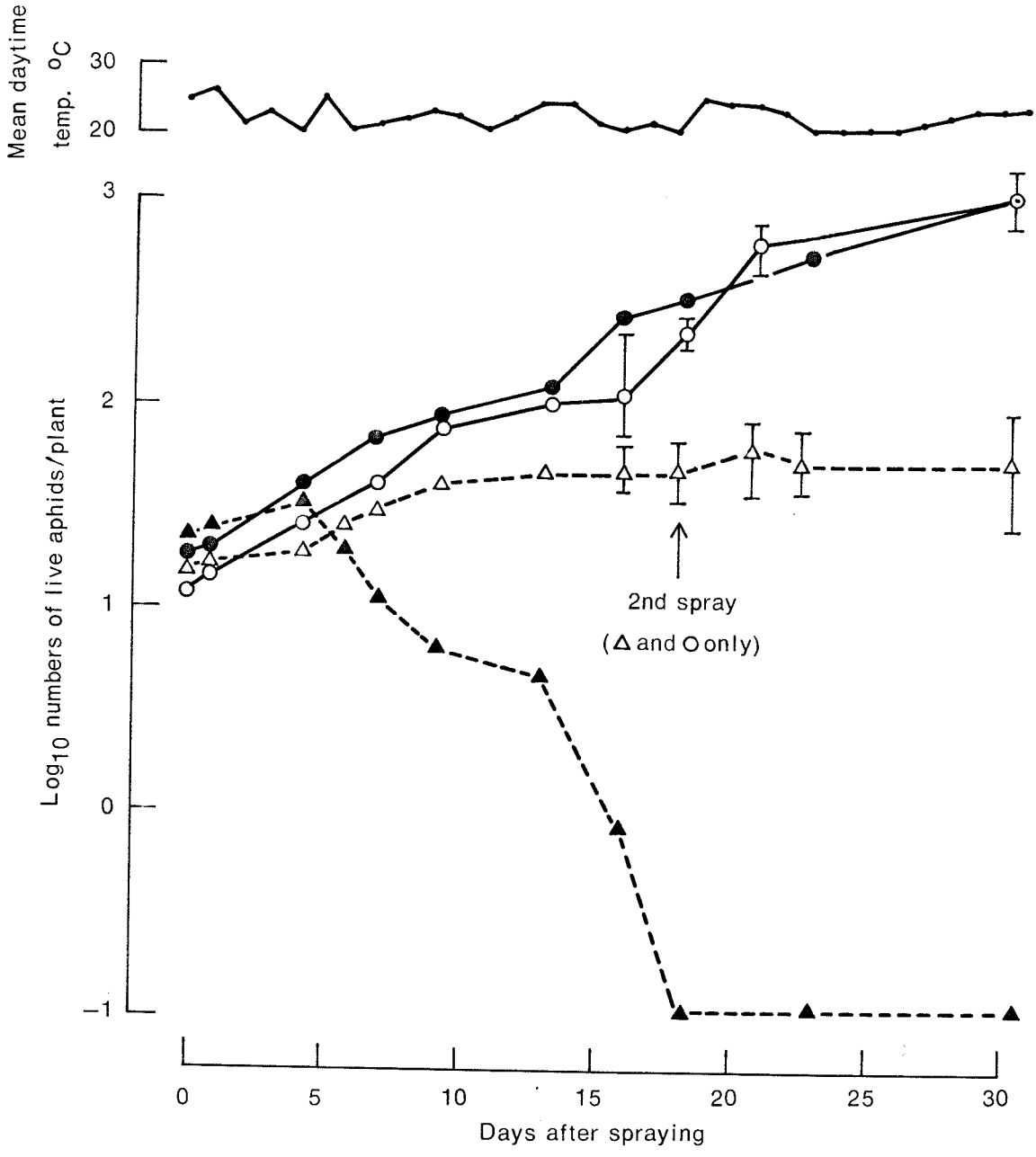


Fig. 6.16

Effect of spraying *Verticillium lecanii* **blastospores on** *Macrosiphoniella sanborni* **and** *Myzus persicae* **populations on chrysanthemums (95% fiducial limits included for** *M. sanborni* **only).**

▲ *My. persicae* sprayed with spores (10^7 spores/ml)
△ *M. sanborni* " " " " "
● *My. persicae* sprayed with buffer and wetting agent only
○ *M. sanborni* " " " " " " "

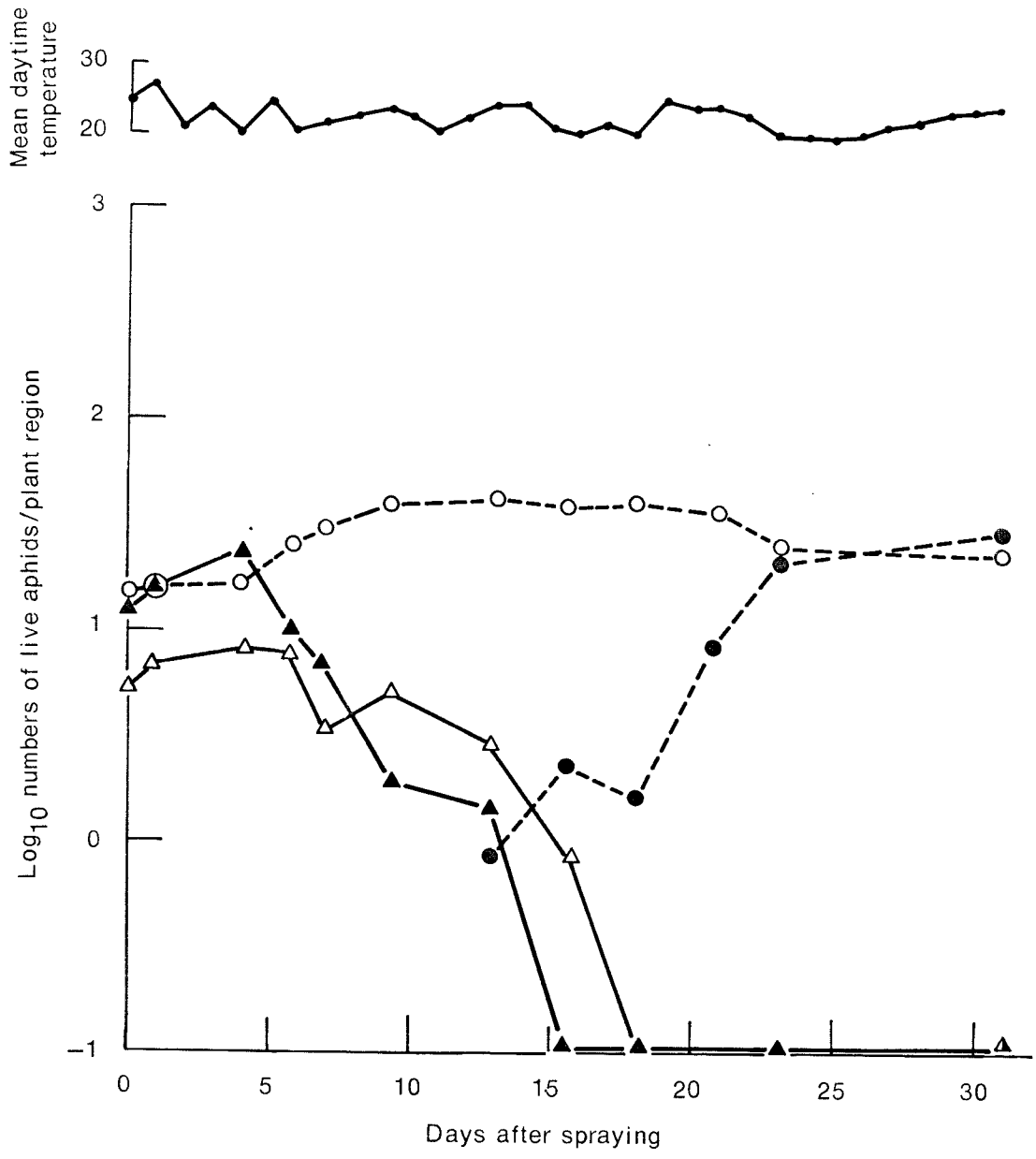


Fig. 6.17

Effect of spraying *Verticillium lecanii* **blastospores on** *Macrosiphoniella sanborni* **and** *Myzus persicae* **upper plant, and middle and lower plant populations on chrysanthemums**

Upper ○---*M. sanborni* △——*My. persicae* **sprayed with 10^7 spores/ml.**

Middle and lower ●---*M. sanborni* ▲——*My. persicae* **sprayed with 10^7 spores/ml.**

7. Effect of Minimum Glasshouse Temperature; Blastospores; My.persicae.

The results of Chapter V (Discussion) suggested that control of aphids by V.lecanii may be as good at 15 as at 20°C, if not better. Since the operative night temperature in commercial chrysanthemum beds is 15°C, the effect on aphid control of lowering the glasshouse temperature from 20 to 15°C was investigated.

(a) Methods

Six beds in separate glasshouses each containing 25 potted chrysanthemum plants (30cm tall) were infested with My.persicae. When populations had attained approximately 4 aphids/plant, all but 2 control beds were sprayed to 'run off' with 10^8 blastospores/ml. Control beds were sprayed with buffer and wetting agent only. The minimum temperatures of 2 treated and one control bed was maintained at 15°C, and that of the other 3 beds at 20°C. This experiment was performed in April-May to ensure that ambient night temperatures did not exceed 15°C.

(b) Results

Using a minimum temperature of either 15° or 20°C, control was satisfactory by the 15th day after spraying and virtual eradication swiftly followed (Fig. 6.18). The aphid populations were kept down to low levels for a further 30 days in spite of re-introductions on day 28 in one bed (Fig. 6.18). However, at the end of the experiment, the treated populations showed slight signs of recovering (Fig. 6.18). Untreated populations continued to increase until the 28th day after spraying, after which a natural V.lecanii epizootic reduced the population (Fig. 6.18).

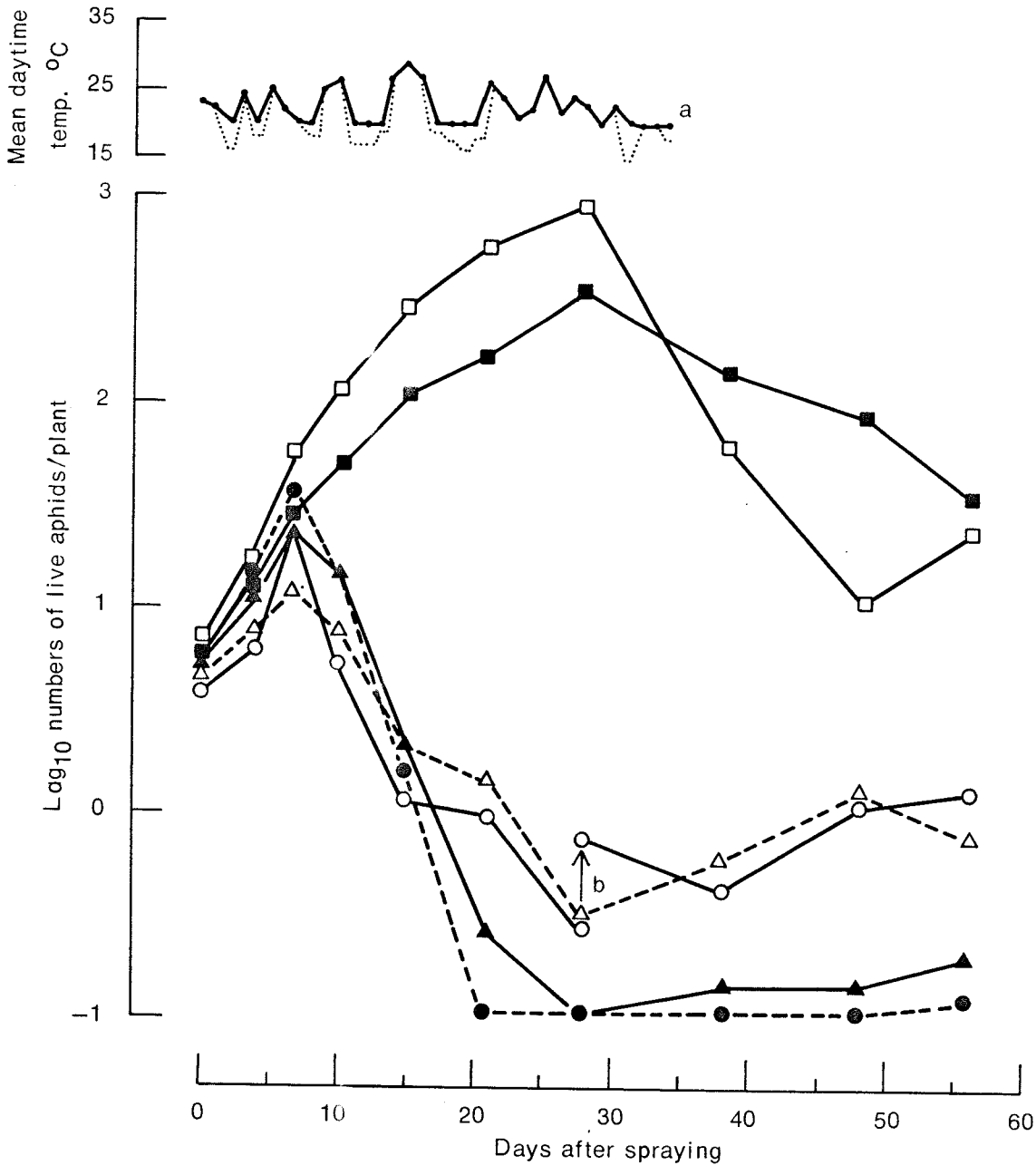


Fig. 6.18

Effect of spraying *Verticillium lecanii* blastospores on populations of *Myzus persicae* on chrysanthemums with minimum temperatures of 15° and 20°C.

△▲ Two replicate beds sprayed with 10^8 spores/ml (20°C)

○● Two replicate beds sprayed with 10^8 spores/ml (15°C)

□ Buffer and wetting agent only (20°C)

■ " " " " " (15°C)

[a] Further data not available

[b] New infestation of aphids (see text).

8. Effect of damping down; Blastospores; My.persicae

In Glasshouse Experiment 6, excessive dampness caused disease only in blooms but the experience of commercial growers has shown that too much moisture can encourage plant diseases at all stages of the chrysanthemum crop. Therefore, it was necessary to explore the possibility of controlling aphids with minimum crop damping.

(a) Methods

Six beds, each containing 25 potted chrysanthemum plants (30cm tall), were infested with My.persicae. When populations had attained 1.5 - 7 aphids/plant, 4 beds were sprayed to 'run-off' with 6×10^7 blastospores/ml and 2 beds with buffer plus wetting agent only. On successive evenings thereafter, the foliage in 2 treated and one control bed was damped down but that in the other 3 beds was left dry, the plants being watered at the base. Minimum temperatures were maintained at $15^{\circ}C$.

(b) Results

My.persicae on treated damped plants was virtually eradicated 12 days after spraying (Fig. 6.19a, b). Similar control on plants not damped down was delayed until 19 and 30 days after spraying (Fig. 6.19a,b). Admittedly, populations on the latter plants were higher when sprayed (Fig. 6.19,a,b), but higher population density should favour more rapid spread of disease (Chapter I). Control populations increased rapidly but the parasitic hymenopterous wasp, Aphidius matricariae (first seen on day 14), curbed the damped population after day 21 (Fig. 6.19,a,b). The other control population (not damped down) was parasitized later and consequently did not decline until around day 32. To prevent the parasites spreading to aphids in the other houses, the control populations were fumigated with nicotine shreds 4 times. On the first day of fumigation (day 25) there were 4

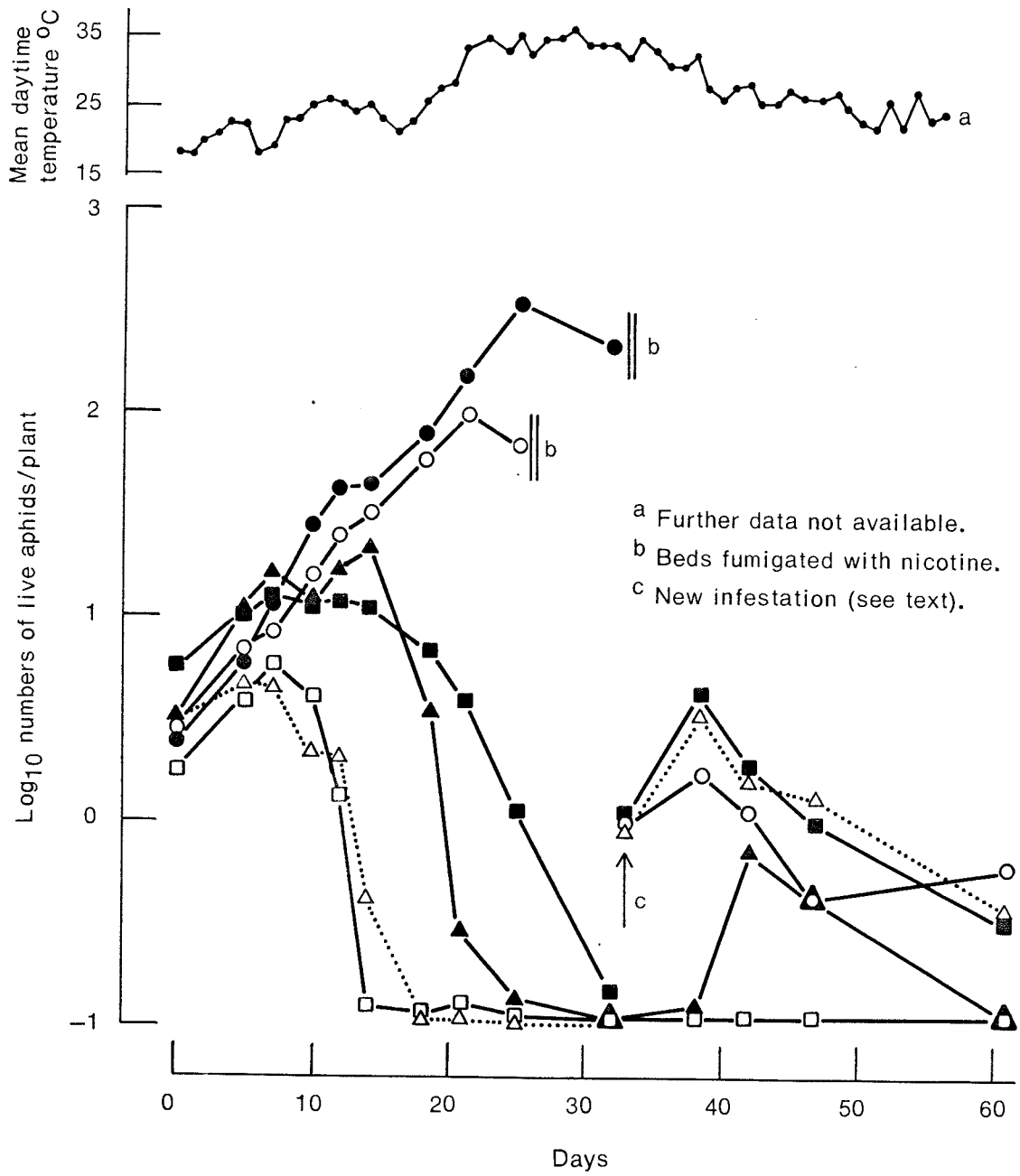


Fig. 6.19a

Effect of damping down on control of *Myzus persicae* **by** *Verticillium lecanii.*

Beds sprayed with:-

Bed 1 ● Buffer and wetting agent only (not damped down)

Bed 2 ○ Buffer and wetting agent only (damped down)

Bed 3 ▲ Bed 4 ◢ — 6×10^7 blastospores/ml (not damped down)

Bed 5 △ Bed 6 □ — 6×10^7 blastospores/ml (damped down)

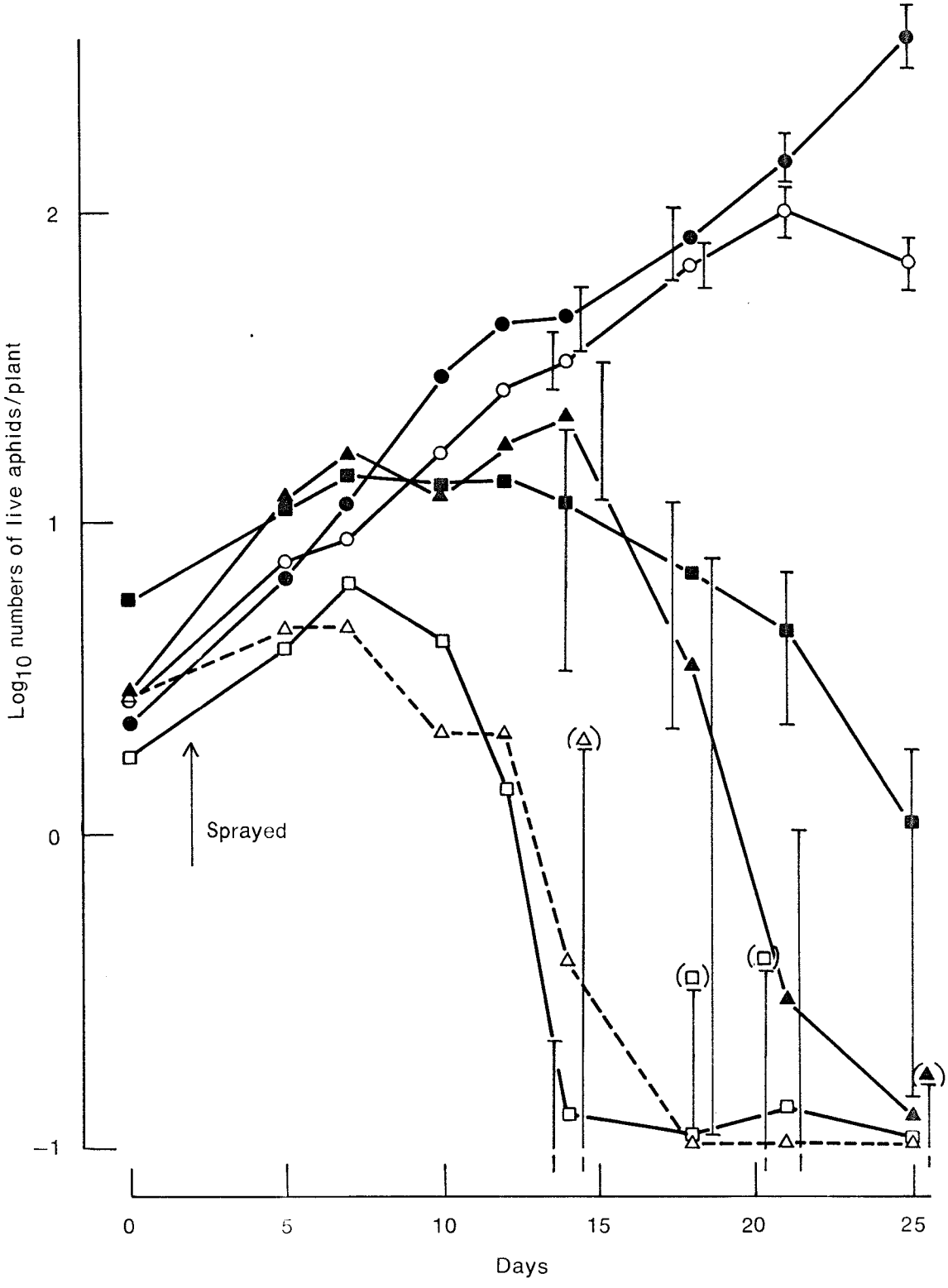


Fig. 6.19b

Expansion of part of Fig. 6.19a (95% fiducial limits included).

V.lecanii - killed aphids in the damped population.

On day 33, Beds 2,4 and 5(Fig.5.19)were re-infested with My.persicae at the rate of one aphid/plant. Initially, the introduced populations increased (Fig. 5.19a). However, a sudden heavy influx of predators (Coccinellidae) around day 40, followed shortly by green lacewings (Chrysopidae), apparently curbed this increase as evidenced by the decline of the population also on control plants (Bed 2, Fig. 5.19a). Despite constant manual removal of the predators, populations did not recover and so the effectiveness of the residual fungus (on bodies killed by V.lecanii earlier in the experiment) in controlling new aphid infestations could not be assessed. However, on day 21 in bed 5 a large alate My. persicae was discovered and presumed to be a migrant from outdoors. This solitary aphid was observed on day 25 to have been killed by V.lecanii.

LARGE GLASSHOUSE EXPERIMENT

9. Blastospores; Plants of Different Size and Maturity; My.persicae.

Following the successful control of sparse My.persicae populations in small glasshouses, an experiment was performed on a large scale to create conditions more closely resembling those in commercial glasshouses.

(a) Methods

Chrysanthemum cuttings were planted directly into 4 peat beds, each containing 300 tall or short plants (Fig. 6.20) and arranged in 6 rows of 50 and 14cm apart each way. Each bed was partitioned by polythene sheets into 2 sections to allow more treatments (Fig. 6.20). Every plant

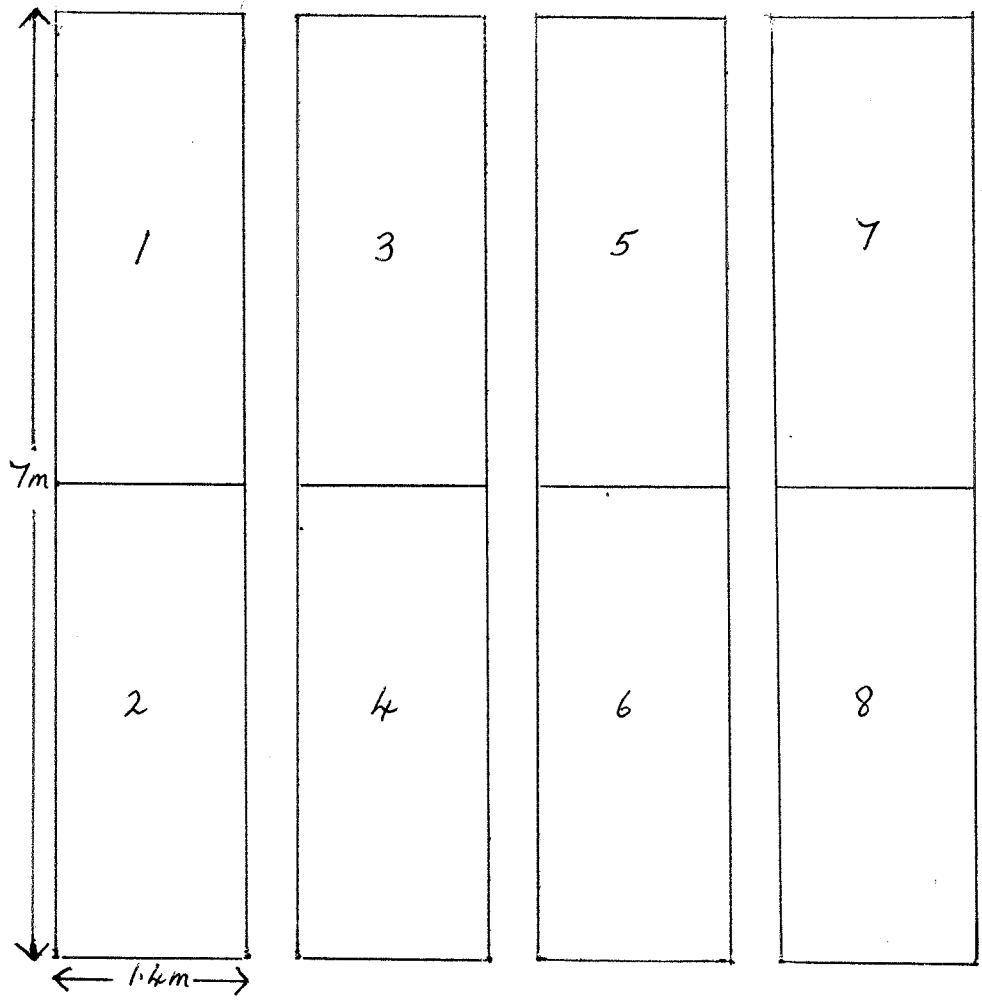


Fig. 6.20 Arrangement of Chrysanthemums in Glasshouse Experiment 9 for Control of Myzus persicae by Verticillium lecanii blastospores:-

Bed Number	Plants	Spray
1 and 3	Short, budded	Spores
2 and 4	Short, vegetative	Spores
5	Tall, budded	Spores
6	Tall, vegetative	Spores
7	Tall, budded	Buffer and wetting agent
8	Tall, vegetative	Buffer and wetting agent

was infested with a single apterous My.persicae. Subsequently, the aphid population on each plant was estimated as follows:- on the upper region of plants, aphids could be counted directly on the stem and leaves. To estimate aphid numbers on the undersides of middle and lower leaves, a leaf harvesting method was employed. In each half-bed of 150 plants, one leaf from the middle and one from the lowest part of each of 15 plants in the bed was removed, and the number of aphids enumerated together with the mean number of leaves/plant to allow population estimates to be calculated. When populations averaged 27 aphids/plant, all except 2 control beds were sprayed to 'run-off' with 7.7×10^7 blastospores/ml in buffer containing 0.02% Triton X-100. Minimum temperatures were maintained between 16 and 17°C.

(b) Results

Spraying was delayed by a viability of only 10% in the spores which were thus not suitable for spraying. Since a large quantity of spores was involved, the routine method of harvesting by centrifugation (IV,1,L) had been replaced by rapid suction-filtration in a Büchner funnel, which possibly killed most spores. A second batch of spores[1], sprayed in the experiment, was harvested by centrifugation and the viability was 91%. The possibly damaging effect of suction on V.lecanii blastospores has yet to be confirmed but Blachère et al., (1973) reported 100% viability of Beauveria tenella blastospores following centrifugation compared to 60% following suction-filtration.

It was intended to spray when populations averaged 10 aphids/plant, but the delay incurred on unintentional pop-

[1] This batch was treated with antibiotics (IV, 12, g) to arrest a contaminating bacterial growth.

ulation increase to 27 aphids/plant.

Myzus persicae on all parts of all spore-treated plants were controlled 25 days after spraying and this control lasted for the duration of the experiment (Figs. 6.21 - 6.24). Again as in Experiment 6, aphids on the outside of tightly closed buds, on tall and short plants, were eradicated (Figs. 6.21 and 6.22). Even more striking was the virtual elimination of aphids on the underside of dense foliage deep in beds where the spray could not possibly have penetrated. Probably contagion spread the disease from aphids higher up the plants.

One bed was re-stocked with aphids at the rate of one aphid/plant when control was complete (Fig. 6.23). These aphids failed to become established (Fig. 6.23); some succumbed to V.lecanii but others could not be found. Unfortunately, there was no available control bed free of fungus for comparison.

In the untreated control populations, V.lecanii infection appeared 18-20 days after spraying. The first diseased aphids were alatae that may have flown from an adjacent spore-treated bed (Fig. 6.20). Better isolation of the untreated beds may have delayed the appearance of disease in the population. Conversely, live aphids on the treated plants 20 days after spraying were mostly alatae that probably had flown from the densely populated control plants without establishing new populations - some of these alatae died of V.lecanii disease.

The black bean aphid, Aphis fabae, infested a few plants in one untreated bed (Fig. 6.20; Bed 8) approximately 25 days after spraying. The V.lecanii epizootic on My.persicae on this untreated bed spread to the A.fabae population and eliminated it within 8 days.

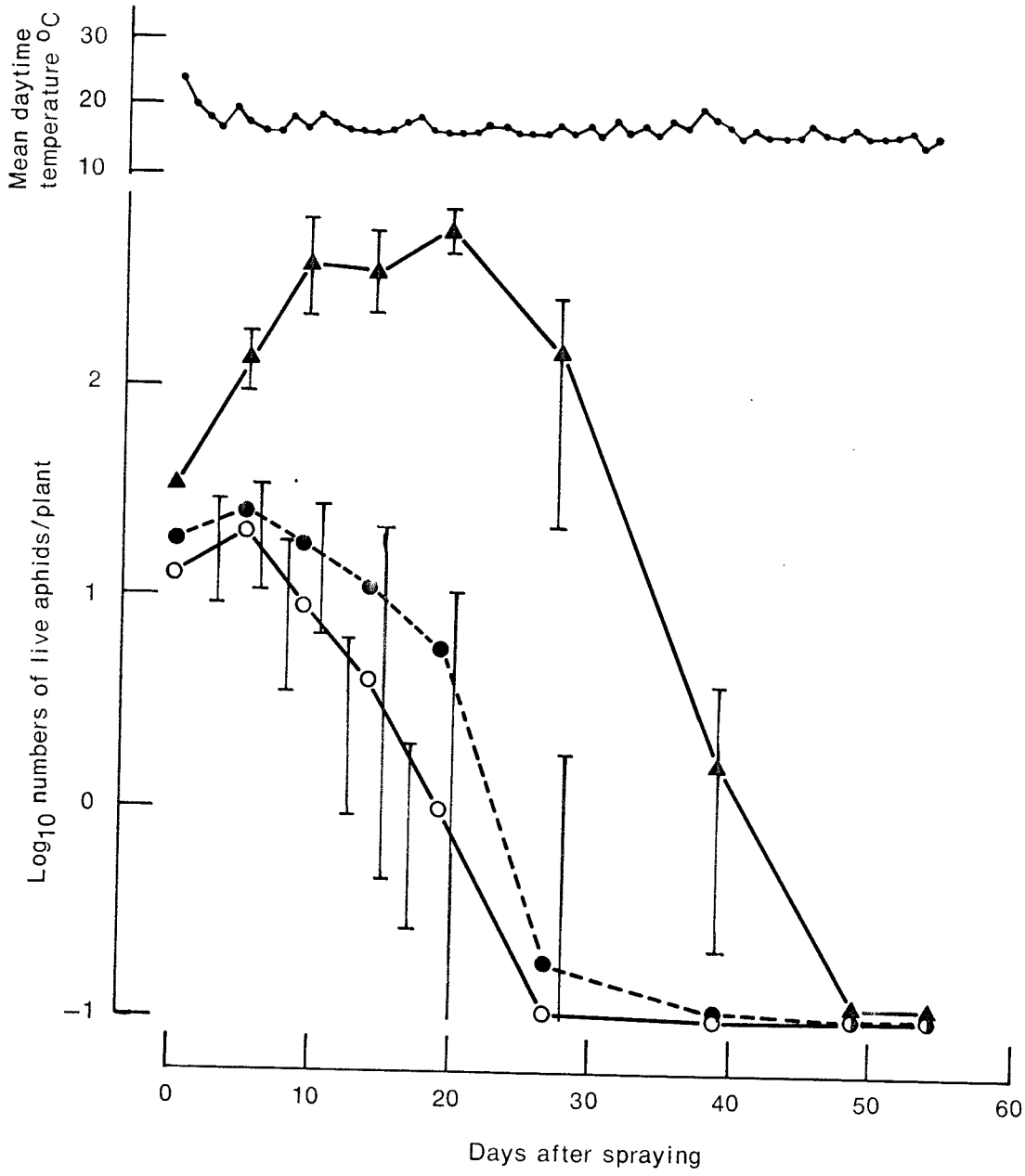


Fig. 6.21

Effect of spraying *Verticillium lecanii* **blastospores on** *Myzus persicae* **populations on short, budded chrysanthemums (95% fiducial limits included).**

●
○ Replicate beds sprayed with 7.7×10^7 spores/ml

▲ Control (tall, budded) plants sprayed with buffer and wetting agent only.

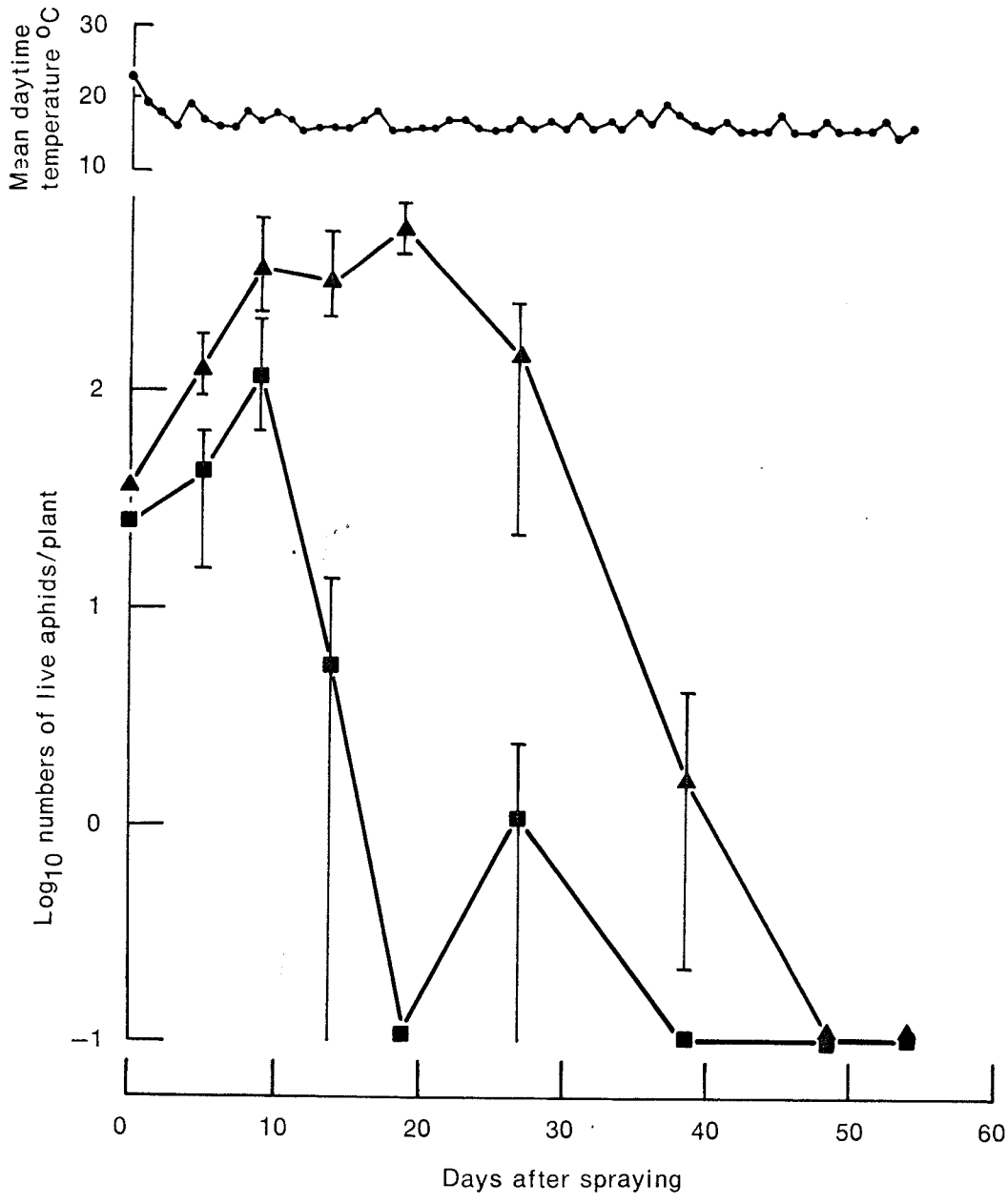


Fig. 6.22

Effect of spraying *Verticillium lecanii* **blastospores on** *Myzus persicae* **populations on tall, budded chrysanthemums (95% fiducial limits included).**

- ■ Plants sprayed with 7.7×10^7 spores/ml
- ▲ Control (tall, budded) plants sprayed with buffer and wetting agent only

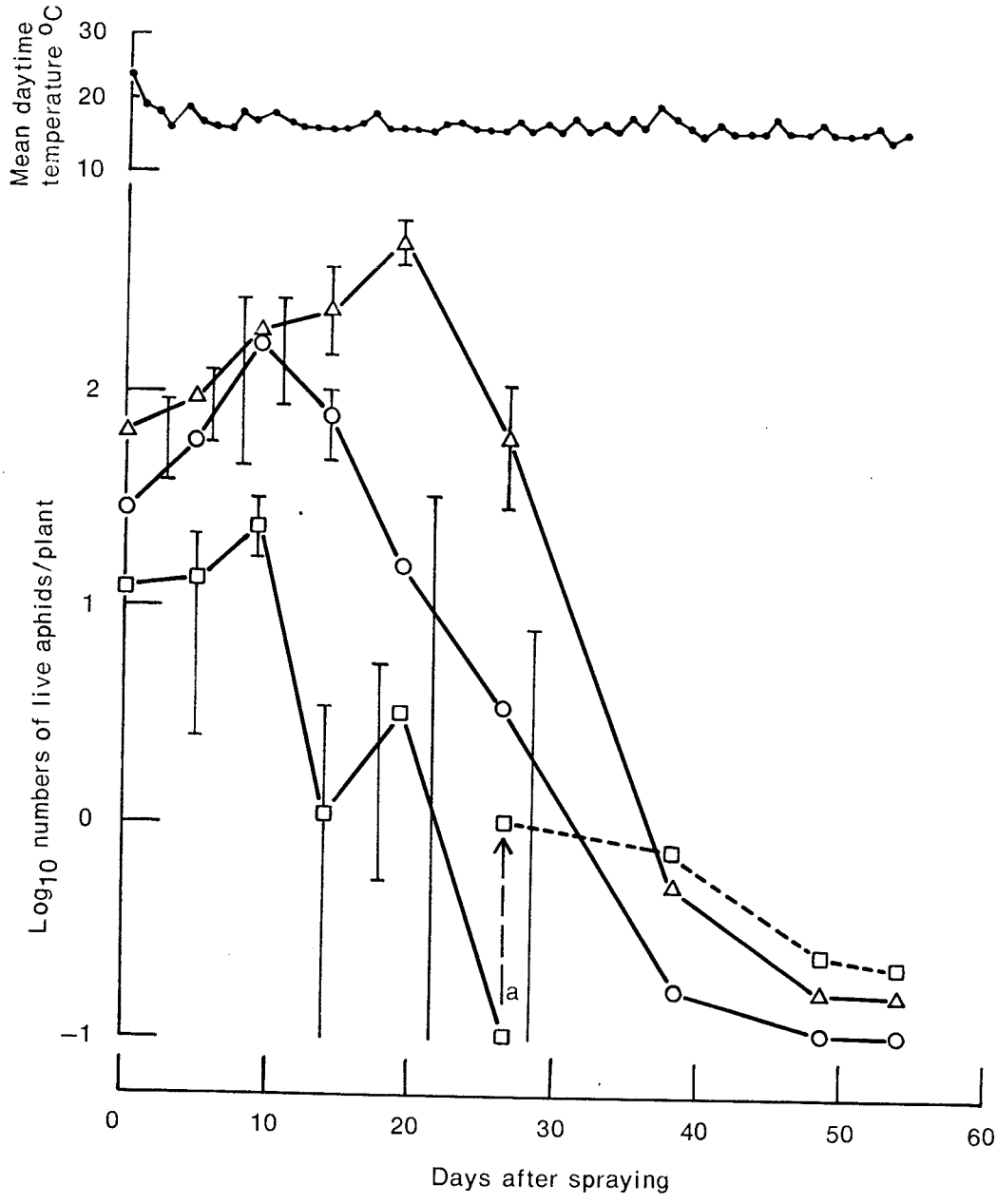


Fig. 6.23

Effect of spraying *Verticillium lecanii* **blastospores on** *Myzus persicae* **populations on short, vegetative chrysanthemums (95% fiducial limits included).**

□ ○ Replicate beds sprayed with 7.7×10^7 spores/ml.

△ Control (tall, vegetative) plants sprayed with buffer and wetting agent only.

[a] New infestation (see text)

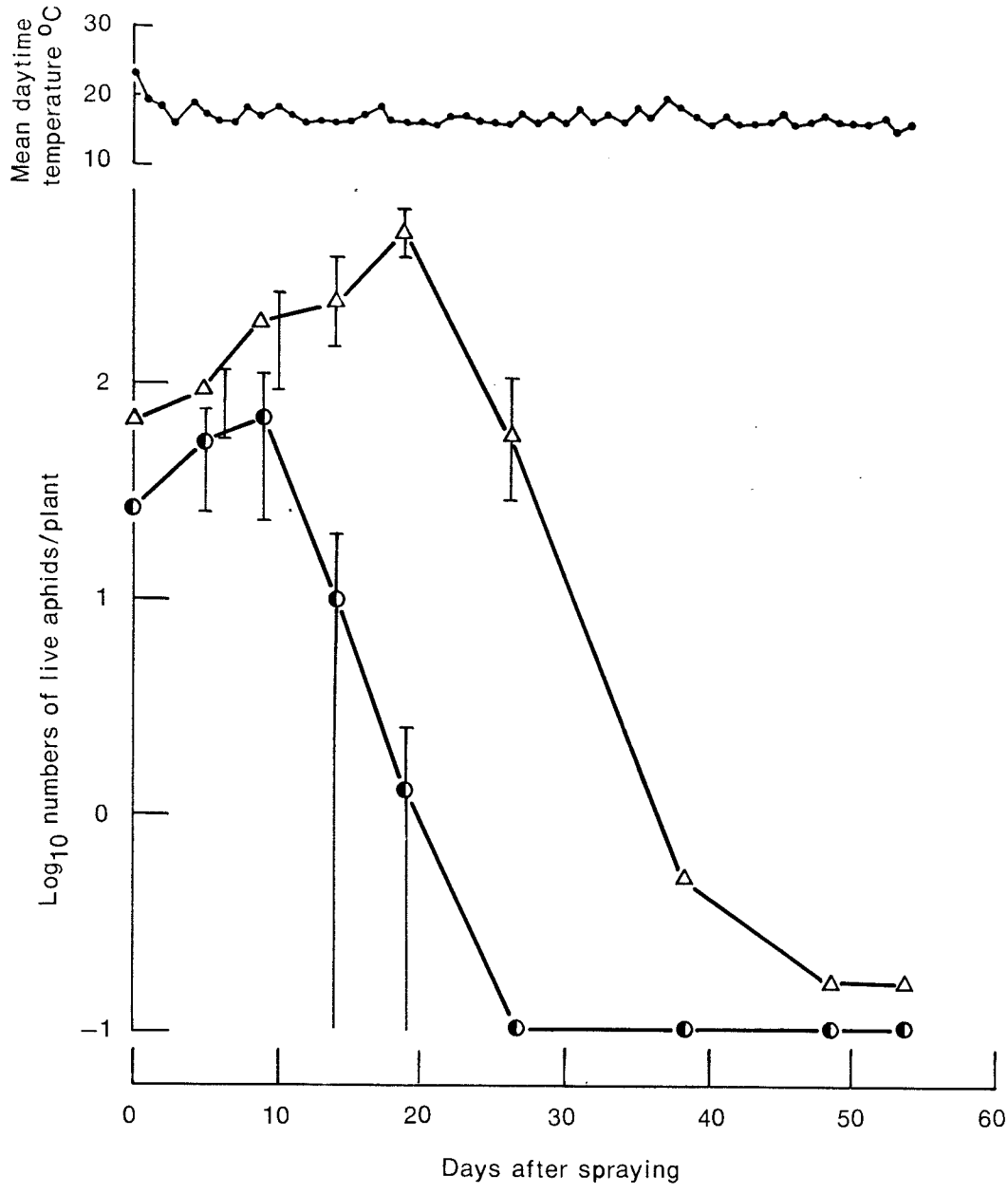


Fig. 6.24

Effect of spraying *Verticillium lecanii* **blastospores on** *Myzus persicae* **populations on tall, vegetative chrysanthemums (95% fiducial limits included).**

◐ Plants sprayed with 7.7×10^7 spores/ml.

△ Control (tall, unbudded) plants sprayed with buffer and wetting agent only.

DISCUSSION

In all glasshouse experiments so far, V.lecanii has virtually wiped out sparse My.persicae infestations, whether they were introduced (VI; Experiments 2, 6-9) or natural(VI; Experiments 4 and 5) and maintained control until the experiments ended. The same was true for B.helichrysi populations in vegetative tips but not on tightly closed flower buds (Fig. 6.14). Control of M.sanborni varied; some experiments resulted in virtual elimination (VI; Experiments 4 and 5) but still did not meet the rather exacting level of control demanded on the ornamental chrysanthemum crop (VI; Methods - d); control was only partial in other experiments (Figs. 6.05 and 6.16). However, in laboratory assays, the inherent susceptibilities of these 3 aphid species were similar (V,13,a). Ecological and behavioural factors must account for the different results in the glasshouse. On whole plants, low numbers of B.helichrysi populations exclusively crowd deep within the vegetative tips, which trap moisture. Once V.lecanii is introduced into a tip, it spreads rapidly through the population there (Table 6.01 and Fig. 6.14). Despite crowding, the contrasting poorer control on tightly closed buds (Fig. 6.14) is probably attributable to a lower microclimate humidity than that in the tips.

Even though My.persicae is more evenly distributed than the other species over plants - a factor expected to discourage rapid spread of disease - its decline in glasshouseswas consistently greater than that of M.sanborni, whether both populations were infesting the same (Experiments 2,4 and 5), or different chrysanthemum plants (Experiment 6). Several possible reasons may account for these different results. M.sanborni usually feeds on the exposed chrysanthemum stem where the microclimate humidity may be lower

than that on the undersides of leaves where My.persicae usually feeds: low humidity should not favour the spread of V.lecanii. However, V.lecanii eliminated My.persicae on stems and on the outside of flower buds (VI; Experiment 6) and, in other experiments, the disease did not control M.sanborni populations beneath leaves (Fig. 6.07, 6.17). Clearly, other factors must be involved.

A possibility, not yet explored experimentally, is that the microclimate humidity on the cuticle of My.persicae may be higher than that on M.sanborni. The susceptibilities to disease of My.persicae and M.sanborni could be compared at sub-optimal humidities by laboratory bioassay at humidities lower than the nominal 100% routinely used for all bioassays in Chapter V. Several workers have demonstrated that fungi can infect insects at a low ambient relative humidity (45-40%; Muller-Kogler, 1965) and Ferron (personal communication) found that B. bassiana can infect Acanthoscelides obtectus imagos irrespective of ambient humidity.

Another contributory factor may be restlessness. In the experiments of Chapter V, individual My.persicae were more mobile than M.sanborni in Petri-dishes as were low density populations on whole plants (V, 13, b). Also, in contrast to M.sanborni, My.persicae was extremely restless in assay cells (V,7,a) and consequently was very much more difficult to manipulate. In the glasshouse, movement would encourage dispersion which increases the likelihood of contact with diseased aphids. Also, since live diseased aphids are still mobile and bear sporulating mycelium (V,6), inherent activity in a species would increase the spread of disease.

The results in the large glasshouse (VI; Experiment 9)

provide evidence of this. Aphid movement was probably the factor that spread disease deep within foliage of a bed where the spore spray did not penetrate. Wyatt (1965) concluded that My.persicae was extremely restless and observed very widespread dispersion on chrysanthemums. My. persicae is restless also on other crops and consequently spreads more rapidly than most other aphid species (Bonnemaison, 1951; Bald et al., 1959; Hussey et al., 1969). This restlessness may be attributed to the aphid's extreme polyphagy with which is associated an extreme sensitivity to the physiological condition of the plant (Kennedy et al., 1950), leading to more searching and probing (Kennedy et al., 1959) - especially on chrysanthemums, a relatively unfavourable host (Wyatt, 1965) - than less polyphagous aphids such as M.sanborni, which is closely adapted to feed on chrysanthemums (Hussey et al., 1969). On plants to which My.persicae is best adapted, the aphid may be more sedentary and control by an entomogenous fungus less successful. Shands et al. (1963), also attached importance to the influence of aphid mobility on the spread of disease. They noticed that the least active aphid, Aphis nasturtii, was the least diseased and the most mobile aphid, Macrosiphum euphorbiae, the most.

At all seasons, My.persicae populations were virtually eliminated (Table 6.02) but M.sanborni (VI; Experiments 3, 4 and 5) populations were greatly reduced only in summer (Table 6.02). In cool weather, use of artificial heating would tend to lower RH in glasshouses. In warm weather, the differences between the minimum glasshouse temperatures and the outdoor night temperatures were less in Glasshouse Experiments 3,4 and 5. This, and high daytime glasshouse temperatures (Table 6.02) doubtlessly reduced the requirement for artificial heating and consequently, night humidity

TABLE 6.02

Comparison of the Degree of Control[a] of *Macrosiphoniella sanborni* and *Myzus persicae* by *Verticillium lecanii* with Outdoor Mean Minimum Night Temperatures and Mean Daytime Glasshouse Temperatures.

Experiment Number	My.persicae		M.sanborni		Minimum Glasshouse Temperature °C	Outdoor[b] Minimum Night Temperatures °C		Mean Daytime Glasshouse Temperatures °C		Months of Year
	15[c]	30[c]	15[c]	30[c]		15[c]	30[c]	15[c]	30[c]	
1[d]			+	+++		0.4	1.1	18.2	19.5	Jan-March
2	++	++++	(+)	++	20	6.0	8.1	24.5	23.5	May-June
3[e]			+++		20	10.7		25.3		July-Aug.
4	++++[f]	++++[f]	-	+++	20	7.6	9.4	26.0	25.8	June-July
5[g]	++++[f]	++++[f]	++(+)	+++	20	11.5	10.9	26.7	27.0	July-Aug
6	+++	++++	+	++	20	3.8	4.3	22.5	22.1	Sept.-Oct.
7	+++	++++			15/20	5.6	5.6	21.3/ 22.3	21.5/ 22.6	April-May
8	+++	++++			15	8.3	10.7	21.9	26.1	June-Aug.
9	+++	++++			16.5	6.9	5.1	17.5	16.9	Oct.-Dec.

a = - no control
\+ population slightly reduced
++ partial control
+++ good control
++++ virtual eradication

b = data from records at G.C.R.I.
c = 15 and 30 days from commencement of experiment
d = control produced by 10^7 spores/ml (cages C and D)
e = experiment terminated after 15 days
f = natural infestations
g = experiment terminated after 26 days

was probably higher (although thermohygrograph records did not detect this) than in Glasshouse Experiments 2 and 6. Another possible effect of high daytime temperatures may have been to increase restlessness which may have contributed to the better control of M.sanborni in summer (Table 6.02). The good control in Glasshouse Experiment 1 (cages C and D only) in spite of very low outdoor night temperatures was probably due to plants being covered continuously by polythene.

Also, mites (Trombiculidae) were often observed in glasshouses from late May to July. Mites have been implicated as vectors of spread of Verticillium malthousei amongst mushrooms and can pick up large quantities of spores in slime on their legs (Ware, 1933). Mites could well have helped to spread V.lecanii among M.sanborni. These factors also apply, of course to My.persicae, but they probably did not increase control, because this was already good without them (Table 6.02).

Results described in Chapter V (Discussion) suggested that a minimum temperature of 15°C (although suboptimal for the growth rate of V.lecanii - Fig. 4.03) might result in control of My.persicae equal to or better than that at 20°C. In Glasshouse Experiment 7, control of My.persicae was the same at both temperatures (Fig. 6.18). A similar experiment has not yet been performed with M.sanborni but the laboratory data (V,Discussion)suggests that mimimum temperatures of 15 or 20°C would result in equal control. However, a minimum temperature of 15°C in a glasshouse may lower night humidity less than 20°C (see above), which may improve control of this aphid. In addition, population reduction of M.sanborni has not yet been studied in a large glasshouse where humidity

should be higher than in small glasshouses.

Evening damping with water sprays was routinely used to boost night humidity under the polythene blackouts to encourage infection and sporulation of V.lecanii on aphids. This did not cause plant diseases on chrysanthemums before flowering, in contrast to the experience of commercial growers. The influorescenes of chrysanthemums, however, were badly damaged by Botrytis cinerea. Therefore, at all stages of plant development damping should be minimized. Without damping down, RH may be slightly reduced under the blackouts and control of M.sanborni by V.lecanii, already unreliable under the most favourable conditions, would presumably be very poor if the RH was not as high as possible (VI; Experiment 1). Overall control of B. helichrysi in the micro-environment of vegetative tips may be complete but delayed at a lower RH (VI; Experiment 1). Fortunately, damping down was not essential for good control of My.persicae (Figs. 6.19a, b). Omitting this procedure delayed satisfactory control only by 8-10 days (Figs. 6.19a, b). Such a delay could be tolerated except towards the end of a crop when rapid control is essential. However, intermittent damping may accelerate control without encouraging phytophagous disease and this possibility should be tested in future glasshouse trials.

At the beginning of a crop, the intervening period between planting and when blackouts could be applied varies from 3-6 weeks depending on season and desired flowering date. The night humidity in newly planted crops would most probably be much lower than in non-damped covered crops. Thus, control of M.sanborni by V.lecanii should not be attempted since failure would be most likely. It may be possible to

eliminate B.helichrysi but compared to conditions of high RH under blackouts, the likely delay in control by V.lecanii (VI; Experiment 1) may incur severe damage in young plants. However, because control of My.persicae by V.lecanii has been so successful, control without blackouts may result in some success particularly on the lowest leaves nearest to the soil where RH is likely to be highest. Unless the infestation was very heavy (unlikely at the beginning of a crop) partial control of My.persicae by V.lecanii may be acceptable and this would probably become complete control when blackouts were applied a short time later.

How many spore sprays are necessary to control aphid populations in a glasshouse? A spore spray is unlikely to infect all aphids present at the time of spraying and even heavily infected aphids reproduce healthy offspring at near-normal rates (Table 5.01). Ultimate control of an aphid population is likely to depend mostly on contagion from aphid to aphid. Other agents e.g. mites may assist in the spread of disease but when these were absent, control of My.persicae was still excellent (see above). Laboratory experiments (Chapter V) suggested that contagion was potentially effective: death of adult aphids after contagion was as rapid as that resulting from high concentrations of aqueous spore suspensions and LT50s were shorter than aphid muturation times, whatever the temperature (V,12). Death of progeny aphids after contagion was even faster (Table 5.12) and considerably shorter than maturation times (Table 5.13). Single applications of spores were sufficient to control My.persicae (Figs. 6.16, 6.18, 6.19 and 6.21 - 6.24) and twice almost controlled M.sanborni (Figs. 6.10 - 6.13). Multiple sprays whether at short (Fig. 6.09) or long intervals (Figs. 6.10, 6.13 and 6.16) did not apparently further reduce M.sanborni populations on stems or B.helichrysi on tightly closed

buds (Fig. 6.14) Thus, once the initial spray establishes contagion, ecological and environmental factors govern population reduction and so only a single spray is justified.

The optimal spore concentration is still uncertain. In laboratory bioassay, the highest concentrations produced the shortest mortality times or aphids (Fig. 5.11; Table 5.07). However, the results of Glasshouse Experiment 3, showed that once V.lecanii had been introduced either by sprays of 5×10^6 or 5×10^7 spores/ml, contagion spread disease equally effectively so that after 15 days, reductions of M.sanborni populations were not significantly different ($P>0.05$; Fig. 6.09). Although this experiment needs repeating it suggests that high spore concentrations may not be justified for controlling M.sanborni: rather, means must be found to increase humidity. Similarly, a high RH may have resulted in good control of B.helichrysi on buds (VI; Experiment 6). Although low spray concentrations would control B.helichrysi populations within vegetative tips (Table 6.01), the damage inflicted by this aphid is severe in young plants and so this pest should be eradicated as swiftly as possible with a high spore concentration.

Since disease spreads effectively through My.persicae populations, lower spore concentrations than those which have been used should beinvestigated in future glasshouse trials with this aphid. Lower concentrations of spores would delay the appearance of disease in the population (Fig. 5.11). This could be tolerated except in heavy infestations or in infestations near flowering time.

Improved formulation would increase the infective potential of spore-sprays. There is little information available

on fungal spray formulations. Suitable additives should delay spore desiccation, possibly permitting infection of aphids at sub-optimal humidities. Stickers, such as those used by McCoy et al., (1976), may prevent spores from being dislodged from the aphid before spore germination and penetration of the cuticle. In several glasshouse experiments, live aphids examined microscopically on successive days after spraying bore many ungerminated spores. Thus, a nutrient formulation, such as the one used by McCoy et al. (1976), and van Brussel (1975), may also improve spore germination on the aphid cuticle. However, nutrient formulations may also encourage growth and sporulation of V.lecanii and other fungi on leaves, which may disfigure ornamental plants like chrysanthemums.

Distributing dead, or release of live, diseased aphids may be a more effective mode of disseminating the fungus than spore sprays. The starting aphid density was very light in some of the experiments where My.persicae was deliberately introduced (Figs. 6.05, 6.18 and 6.19). Natural infestations started with less than one aphid/plant. Since some aphids would be sheltered by foliage, the numbers of aphids infected from the spray must have been extremely small but nevertheless these were sufficient to establish successful control. Introducing diseased live or dead aphids would save wasting sprayed spores that miss aphids not directly hit by a spray - aphids do not readily acquire disease from spore-treated leaves (V, 9,). Live, but irreversibly infected aphids, would have the advantage over dead aphids of natural dispersion before death. Only small numbers of cadavers or diseased aphids would be necessary to control My.persicae, possibly only one/plant (see above). For B.helichrysi deep in vegetative tips, live diseased aphids would presumably

have to be used since aphid bodies would not penetrate deep within the tip. The same limitations of control of B. helichrysi on tightly-closed buds or of M.sanborni, following spraying would presumably apply to these alternative modes of disseminating V.lecanii.

Summary

1. V.lecanii holds promise for controlling sparse aphid populations, especially My.persicae, at commercially acceptable levels for at least 60 days, and possibly longer.

2. B.helichrysi can be easily controlled in vegetative, moisture-trapping tips but not on the outside of tightly closed flower buds where humidity is possibly lower.

3. The best control of M.sanborni was obtained in hot weather. Higher RH may improve control at other times in the year.

4. Botrytis cinerea presumably encouraged by high humidity, severely damaged the blooms and therefore use of V.lecanii at this stage of the crop may be impractical.

5. On plants before budding, damping was not essential for good final control but its absence nevertheless delayed control.

6. One spore spray only is required. Once this spray establishes contagion, ecological and environmental factors govern population reduction and further sprays have little impact.

7. Optimal spore spray concentrations for each aphid species are still uncertain. Further glasshouse experimentation

is required to determine these.

8. The efficency of spore sprays might be improved by suitable formulations which might permit a reduction in the spore inoculum.

9. Most spores in a spray miss aphids. Introduction of diseased aphids onto crops might be a more efficient use of the fungus.

CHAPTER VII

NATURAL OCCURENCE AND SPREAD OF *VERTICILLIUM LECANII* IN APHID POPULATIONS

The mechanisms of natural limitations of aphid populations by *V.lecanii* (whether in the outdoor or glasshouse environment) have so far not been given detailed attention in this study. However, some principles governing the natural occurence and spread of *V.lecanii* in aphid populations can be deduced from the information accrued so far in this study and further results below.

A series of experiments was devised to provide information about the possible sources of natural *V.lecanii* infection. Also such knowledge may help to prevent epizootics in laboratory aphid stocks which occurred in the rearing units at the G.C.R.I. from time to time despite ventilation to lower humidity. The series still remains to be completed.

METHODS AND RESULTS

1. Airborne Spore Dispersal

To determine whether V.lecanii conidiospores could easily become airborne and remain viable, an inverted, open, confluent, V.lecanii Petri-dish culture was desiccated over $CaCl_2$ for 15 days. Each day, an open sterile SDA plate was exposed for 2h beneath the culture and then incubated for a week at 23±0.5°C. On the 15th day, the now dry culture was tapped hard over an open sterile SDA plate. V.lecanii grew only on the last plate on which there were 2 colonies.

In a glasshouse experiment, sterile SDA plates were simultaneously exposed for 45 minutes in various positions in chrysanthemums infested with a My.persicae population killed by V.lecanii (Chapter VI, Experiment 9). At each position a plate was placed on the ground and others at various heights above it. Plates were also exposed in a separate, unused compartment of the same glasshouse and outdoors. During subsequent incubation at 23±0.5°C, no V. lecanii colonies grew on any of these plates. However, many colonies of another insect pathogen, Entomophthora coronata (Shands et al., 1973; Gustafsson, 1965) grew on those plates exposed at soil level in the glasshouse but not on those plates exposed above the soil. Since aphids on the chrysanthemum foliage bore only V.lecanii, the E. coronata spores must have been ejected from the soil but evidently did not travel far into the air before settling.

2. Presence of Spores on Leaves

Three chrysanthemum leaves from plants free from aphids were pressed firmly onto SDA plates. Two more such adpressions with the same leaves were made on successive SDA plates in order to 'dilute' the leaf flora. After 7 days incubation at 23±0.5°C, a single colony of V.lecanii grew

on one plate only. The strain from this colony closely resembled C-4 (Table 4.14) but has not yet been tested for pathogenicity for aphids. This limited test obviously needs repetition but suggests that V.lecanii may belong to the natural flora of chrysanthemum leaves but possibly only in small quantities.

DISCUSSION

Fungal spores produced in slime are held firmly to the mycelium when dry (Gregory, 1952). However it is possible that after primary dispersal of spores in slime by wetting, spores may be secondarily dispersed after drying in currents of air (Gregory, 1952) but since desiccation rapidly kills V.lecanii conidiospores (IV, 9,c), airborne carriage of viable spores is not likely. This deduction was confirmed by the results of VII,1. It is likely therefore that infection of insects from airborne V.lecanii spores would be a virtual impossibility.

V. lecanii spores may normally be present on leaves although in very small numbers (VII, 2). Infection from such spores may be a rare event since even under optimal conditions, infection of aphids from spore-covered leaves was inefficient (V, 9). However, such an event may become more probable as aphid populations increase to very large numbers.

Although not yet tested, infection may originate from the soil. V.lecanii has been isolated from the soil on a few occasions (Gams, 1971), and Barron(1968) stated that Verticillium and Cephalosporium spp. are extremely common in soil (although he did not give a list of species). Itinerant aphids or aphids dislodged from plants could perhaps contact material on soil bearing growth of V. lecanii.

Splash-dispersal, by rain or irrigation, of V.lecanii from the soil onto aphid-infested plants may also contribute to the initial infection of an aphid population. Other

creatures walking on to plants from the soil, e.g. mites, ants, sciarids, may also introduce the disease into aphid populations - particularly so since many species, e.g. ants (Hussey et al., 1969) and sciarids (Tiensuu, 1936), can be attracted to aphid honeydew. Although the chance of an individual aphid becoming infected may be remote, the likelihood of populations being infected increases as the aphids multiply. This may explain why sparse untreated aphid populations were never limited naturally in the glasshouse experiments even though the potential for controlling sparse infestations (Myzus persicae) existed (Chapter VI).

Lastly, disease may be introduced into a population on a crop by diseased winged alatae from infected populations elsewhere. Infection of a population in this way would be independent of density.

How does V.lecanii spread amongst aphid populations? Since usually, only dense populations have become infected naturally (see above) disease would normally spread from initial infection points through contagion. However, doubtlessly, sparse aphid populations sometimes become infected (see above). The results of Chapter VI showed that the threshold population levels for efficient spread of disease amongst spore-treated aphids were much lower than the levels for the natural appearance of disease amongst untreated aphids, especially for My. persicae. It was postulated in Chapter VI (Discussion) that high mobility of My.persicae particularly facilitated the spread of V. lecanii disease whereas mobility of other aphids was less and so was the degree of control obtained.

Rain or irrigation water may spread spores but water

was certainly not essential for the successful control of My.persicae on chrysanthemum beds which were not damped down (VI; Experiment 8). Water dispersal may be more important for other, less mobile, aphid species particularly the stem-feeding M.sanborni. Water may carry spores in slime-heads from diseased aphids a considerable distance down the stem to uninfected aphids. It is highly likely that water disperses spores amongst aphids to another not very mobile species, B.helichrysi, deep within vegetative tips. Water may also disperse spores laterally from plant to plant since drops striking a film of water containing spores can produce many reflected droplets which may travel for some distance horizontally (Corke, 1966; Ingold, 1971) to adjacent leaves and plants bearing uninfected aphids. Other agents such as mites, predators and parasites may also spread disease within aphid populations.

SUMMARY

It is improbable that natural V.lecanii infection of aphid populations arises from airborne spores. More likely, infection originates from the soil and possibly leaf surfaces. The chances of an individual aphid becoming infected from either of these sources may be remote but as a population increases, the presence of more individuals increases the chances of infection appearing in the population. Occasionally, sparse aphid populations may become infected from diseased winged aphids from other aphid populations. Once introduced, disease spreads by contagion to greater or lesser degrees depending on aphid density and on the behavioural characteristics of the aphid species. Water may sometimes spread disease but its importance may also vary according to aphid species and habitat. Other insects and mites may sometimes spread the disease.

CHAPTER VIII

DISCUSSION

1. Verticillium lecanii as a Microbial Control Agent of Aphids on Chrysanthemums

The results of the preceding chapters will now enable the potential of V.lecanii as a microbial control insecticide to be assessed. It is essential to examine V.lecanii in the light of the desirable attributes of a microbial insecticide. These attributes are:-

(a) Stability of virulence

(b) Reliability of practical control

(c) Compatibility with other control measures

(d) Simple production and application

(e) Good storage

(f) Safety to man and other animals

(a) Stability of Virulence

Variability of virulence in a microbial insecticide would make standardisation difficult and preclude accurate prediction of practical control. For V.lecanii, the potential risks of variation between cultures used as inocula in experiments were minimised by deriving the cultures from a single-spore isolate (C-3; IMI 179172) and storing at -17°C (IV, 1, k). For indefinite storage, cultures or spores could be stored in liquid nitrogen (Table 4.12). Even without this precaution, the risks of pathogenic variation seem to be slight as repeated subculturing on agars of single (C-3) and multi-spore (C-1) isolates mostly did not alter virulence (Table 5.28).

Thus, virulence of V.lecanii (C-1 and C-3) is very stable. Also, this seems to apply to the V.lecanii strains from widely differing sources; all pathogenic strains except one, some of which had been subcultured in other laboratories for many years, exhibited similar virulence to the standard, C-3.

(b) Reliability of Practical Control

The results of Chapter VI showed that reliable economic control of the minor pests - B.helichrysi on buds, and M.sanborni - by V.lecanii seems unlikely at the present time. However, V.lecanii established and maintained excellent control of very sparse populations of the main pest, My. persicae on all parts of the plants in 7 experiments and in both of 2 experiments it eliminated B.helichrysi populations in vegetative tips. The repeatability of successful control, particularly of My.persicae, demonstrates that V. lecanii has potential as a microbial insecticide.

(c) Compatibility with Existing Control Measures

In Chapter VI, the potential of V.lecanii for controlling aphids was assessed within the basic framework of the chrysanthemum cropping system. This potential will now be discussed in a more rigid context to establish whether use of the fungus can be integrated with already existing measures for controlling pests and diseases on chrysanthemums.

In the integrated programme proposed by Scopes et al. (1973) for the control of pests and diseases on chrysanthemums, My.persicae was controlled by the parasitic hymenopteran Aphidius matricariae. Because V.lecanii is as effective as Aphidius and easier to produce, it would replace the parasite in this programme. However, careful consideration of the whole pest and disease complex on chrysanthemums is necessary before V.lecanii could be integrated

within such a programme. Thus, Olmert et al. (1974) showed that a wide range of insecticides adversely effected growth on media of their V.lecanii strains. With chrysanthemums there need be no such difficulties. In their integrated control system Scopes et al. (1973) envisaged that the only insecticides required would be dioxathion - pirimicarb sprays against leaf miner (Phytomyza syngenesiae) and minor aphids (B.helichrysi and M.sanborni). This mixture is applied only to the apical foliage and so V.lecanii epizootics amongst My.persicae on the lower foliage should be protected in the same way as predators and parasites are safeguarded (Scopes et al., 1973).

Olmert et al., (1974) also demonstrated that fungicides such as benomyl, thiobendazole, maneb and captan seriously affected growth of V.lecanii on media at one-tenth the recommended dosages on plants. Thus, the injudicious use of fungicides against plant pathogens may adversely affect an entomogenous fungus (Jacques et al., 1962). Fortunately, fungicides are infrequently applied to chrysanthemums since many diseases are prevented by steam sterilisation and general attention to crop hygiene. The most common diseases, such as grey mould (Botrytis cinerea) and ray blight (Mycosphaerella ligulicola), occur mainly towards the maturity of the crop and attack flowers and buds. Because use of V. lecanii would not be contemplated after bud-break (Chapter VI; Discussion), measures necessary to control these diseases would not interfere with a V.lecanii programme to control aphids. Control of powdery mildew (Oidium chrysanthemi) may be required at any stage of crop growth and, for this purpose a fungicide specific for powdery mildew such as dimethirimol should be employed. Wilding (1972b) has shown that dimethirimol does not harm growth of V.lecanii (cited as Cephalosporium aphidicola).

The pathogenicity of V.lecanii for parasitic and predatory arthropods already used for the biological control of other chrysanthemum pests has not yet been tested. Although there is at least one report that a beneficial ichneumonid is a host of V.lecanii, (Leatherdale, 1970), predators and parasites on glasshouse crops are unlikely to be affected since the fungus did not attack the predatory mite Phytoseuilus persimilis and the parasite Encarsia formosa when it occured naturally in several crops.

Based upon the integrated system proposed by Scopes et al. (1973), Table 8.01 summarises how V.lecanii may be used. If a parasite was used against leaf miner, B. helichrysi could be reliably controlled by V.lecanii before the crop budded. However, control of leaf miner by parasites cannot, at present, be guaranteed (Scopes et al. 1973). Instead dioxathion-pirimicarb mixtures can be misted over the apical foliage to control both leaf miner and the aphid pests, B.helichyrsi and M. sanborni (Scopes et al., 1973). Damping down of plants would impair the residual toxicity of dioxathion-pirimicarb applications - important for at least 9 days to maintain control of leaf miner (Gurney et al., 1974). However, damping down may not be essential for control of My. persicae by V.lecanii (VI; Experiment 8) in which case, use of the fungus should be compatible with control measures against leaf miner. Thus, it is probable that V.lecanii could be advantageously incorporated into an integrated control system on chrysanthemums providing fungicides for the control of plant diseases were carefully selected.

TABLE 8.01

Incorporation of *Verticillium lecanii* into an Integrated Pest Control Programme on Chrysanthemums

Insect pest or plant disease	Biological methods	Chemical sprays
Myzus persicae	Spray with *V. lecanii* at initial detection of infestation	-
Red Spider Mite	Control by predators (*Phytoseiulus persimilis*)	
Leaf miner (+ *B. helichrysi* or *M. sanborni*)	-	Mist of dioxathion-pirimicarb over tops of plants
Caterpillars	*Bacillus thuringiensis*	-
Powdery mildew (*Oidium chrysanthemi*)	-	Dimethirimol
Grey mould (*Botrytis cinerea*)	-	Captan or thiram at bud burst
Ray blight (*Mycosphaerella ligulicola*)	-	Captan or mancozeb when buds show colour

(d) Production and Application

V.lecanii can be easily cultured in large quantities in cheap, readily available media (IV; Table 4.25) and should, therefore, be amenable to existing industrial fermentation technology. Using the data from Table 4.29 and the guidelines of Dulmage (1971), with generous allowance for inflation from 1966 to the present, an undiluted dry-weight V.lecanii preparation would approximately, retail at £20/Kg. For the control of My.persicae by aqueous sprays, the initial preparation could be diluted by at least 20 times, possibly more, and, in terms of materials, it is estimated that a single application covering one ha would cost £30 - 50. This is similar to the estimated cost of applying the commercially produced biological agent Bacillus thuringiensis against moths, but which in turn is rather more expensive than many chemical insecticides.

In the present work, V.lecanii has been applied as a high-volume spray, which would require an expensive labour input. As with the growing trend with chemicals, low volume (LV) spraying would be much cheaper. Because V.lecanii spreads so efficiently amongst sparse My.persicae populations, the poorer coverage provided by LV sprays may be adequate. However, LV sprays may not raise the RH sufficiently on the night of spraying to allow good spore germination and initial control. This requires trial.

So, in the present state of our knowledge, V.lecanii must be applied as a high-volume drenching spray and so the number of man-hours required will be higher for fungus than for LV applied chemicals. However, the potentially greater cost of the biological components of an integrated control programme need not pose serious problems. The value of protected crops is high and the economic threshold of damage

is consequently very low. Thus, often many applications of chemical insecticides are made particularly towards the end of a crop which is a very labour-intensive and consequently expensive period. Further expense is incurred if one of the target pests develops resistance, necessitating sprays of increased concentration or use of more insecticides. In contrast, integrated control usually begins at the onset of crop cultivation - when the labour requirement is not great - and each component of biological control is preferably one which, once introduced, will control for the duration of a crop. V.lecanii can control My.persicae for at least 8 weeks and hopefully for the duration of a crop. Furthermore, integrated control can increase yields by up to 30% since the use of a purely chemical programme can be slightly phytotoxic. For these reasons, experience has already shown that although initially more expensive, the overall cost of an integrated programme of control is less than for purely chemical control (Burges, 1974).

(e) Good Storage

The results of the storage tests with conidiospores and blastospores fell far short of standards of longevity normally acceptable for insecticides. Development of suitable storage formulations could undoubtedly improve longevity; Blachère et al., (1973), extended the survival of Beauveria tenella blastospores for 8 - 9 months at 4 and 23°C but further storage at 23°C resulted in a rapid loss of spore viability compared to 4°C (Ferron, personal communication). Unfortunately, it may be uneconomic to store a large amount of material for an indefinite period at low temperatures. Thus, existing knowledge may not permit the development of a commercially feasible long-term storage formulation for V.lecanii blastospores. In this event, the fungus could be cultured for use without long-term stockpiling and distributed from centres to commercial growers

as with the whitefly parasite, Encarsia formosa and the predatory mite, Phytoseiulus persimilis, at present. It would have the advantage of short storability over those agents which cannot be stored at all.

On the other hand, sporulating aphid cadavers may be used as inocula (Chapter VI; Discussion). It is probable that V.lecanii in this form could be stored longer than harvested conidiospores or blastospores (Figs - 4.15 and 4.16, 4.21 and 4.22) and could be distributed in the same way as parasites and predators.

(f) Safety to Man and other Animals

The use of a microbial insecticide could not be entertained without careful consideration of its effects on man and other vertebrates, as well as on insect pollinators. The potential dangers imposed are infectivity, toxicity, allerginicity and carcinogenicity.

Some preliminary tests have already been performed on the standard isolate of V.lecanii (C-3) - indeed this is advisable for the safety of the pioneering experimenters. V.lecanii (C-3) could not grow above 30°C (Fig. 4.03) and thus should not infect man and other warm-blooded vertebrates. Dr. P. Austwick of the Nuffield Institute of Comparative Medicine, London, conducted pathogenicity tests on mice with C-3. Each animal received 10^6 conidiospores intravenously and was sacrificed 28 days later. No symptoms were observed during this time, no pathological changes were apparent in the internal organs and no sign of the fungus could be found either in the sectioned organs or in cultures from them. Thus, V.lecanii was not infective to the mouse and conidia were not toxic when administered this way. Dr. J . Longbottom of the Institute of Diseases of the Chest, Brompton, London, tested the author for allerginicity

to extra-cellular products of V.lecanii with negative results. Unfortunately, similar tests on conidiospores and their contents could not be realised. As yet, no carcinogenicity tests have been undertaken.

The author who has worked closely with V.lecanii throughout this study has never suffered any ill-effects, e.g. allergic reactions, whatsoever. A colleague in Cambridge who had a history of allerginicity, also suffered no ill-effects when working with V.lecanii (strains C-3, C-4 and C-30). The absence of records in man and other vertebrates in medical and veterinary history is perhaps the most impressive evidence.

Infectivity for useful pollinating insects is probably nil since this fungus has been recorded from only one hymenopteran (an ichneumonid - Leatherdale, 1970).

These preliminary tests and observations indicate that V.lecanii (C-3) is harmless to man and other vertebrates but before the fungus could be cleared for general use, a thorough and extensive evaluation of its inocuity would have to be undertaken. Obviously, this should be done on the most promising strain.

2. Future Prospects

(a) Strain Improvement

Some of the prospects of improving the performance of V.lecanii have been discussed in Chapters V and VI. Otherwise they are probably not very great at present. The feasibility of selecting existing strains of improved virulence seems slight (Chapter V; Discussion). Although it may be possible to induce and isolate mutants of

individual strains of increased virulence, the number of assays necessary to perform the task of selection would be prohibitively large. Instead, if possible, pathogenicity would have to be correlated with more easily identifiable biochemical or morphological characteristics (Aoki, 1962; Evlakhova, 1966; Segretain et al., 1972; Samšiňáková et al., 1973).

The factor most limiting for any entomogenous fungus under normal glasshouse conditions is humidity. The effectiveness of V.lecanii in controlling M.sanborni and B.helichrysi may be increased by boosting humidity (Chapter VI; Discussion) in a glasshouse as long as this procedure did not encourage serious plant diseases. Alternatively, Roberts (1968) suggested that it may be possible to select fungal mutants which germinate at lower RH than the wild types. However, the requirement for high humidity is almost a universal feature of the fungi and the ability to grow at low RH may require an extensive evolution rather than simple mutations amenable to selection in the laboratory.

(b) V.lecanii on Pests of other Crops

It is probable that V.lecanii could be employed against other insect pests on other crops. It must be emphasised here that the standards of aphid control required for the chrysanthemum crop are very high since the crop is an ornamental one. The degree of control of aphids such as M.sanborni, although sometimes partial on chrysanthemums, may be satisfactory on other crops, e.g. food crops in which the infested part is not marketed.

The host range of V.lecanii includes other important aphid and scale pests, e.g. on glasshouse crops, Aphis gossypii and whitefly, Trialeurodes vaporariorum. It is

possible that with slight modifications to existing cropping systems V.lecanii could be successfully employed against these pests. Circumstantial evidence suggests this is so; Hussey, and Scopes (personal communications) have reported that increased humidification by liberal watering in cucumber houses was accompanied by outbreaks of V.lecanii which eradicated whitefly. The effects of increased humidification on most crops are not yet known and the possibility that serious plant diseases may be encouraged should not be ignored. However, some plant diseases - mildews and rusts - may themselves be eliminated by fungi such as Verticillium lecanii (Locci et al., 1971; Silveira et al., 1971; Allen, 1975) and Cladosporium sp. (Ebben et al., unpublished) which flourish at high humidity. The author is aware of at least one crop (roses), grown in glasshouses in the South of France, which is misted to control mildew (Tramier, personal communication). Thus, interestingly, on heavily irrigated glasshouse crops, V.lecanii strains pathogenic for both insects and plant diseases could serve a dual role as both an insecticide and a fungicide.

Outdoors, critical parameters such as temperature and humidity cannot be controlled. Weather in temperate regions is unpredictable and thus an entomogenous fungus could not be efficiently used except perhaps where the insect pest resides in a moisture-trapping micro-environment. However, in tropical regions, prolonged periods of high RH would encourage use of entomogenous fungi. Thus, van Brussel (1975) successfully controlled citrus rust mites with Hirsutella thompsonii even in the 'dry' season in Surinam.

It is clear that V.lecanii is an entomopathogen with great potential for controlling aphid and scale pests. Just as successful control of some aphid species on chrysanthe-

mums (B.helichyrsi on buds, and M.sanborni) cannot be guaranteed so it is likely that other failures will occur on other crops. However, most encouraging is that success can be very spectacular and exceed expectations - as was observed for My.persicae - and there is no reason to suppose that this will not be true for some other insect pests in other environments.

(c) Commercial Exploitation

Depending on the outcome of safety tests, V.lecanii is almost ready for testing in commercial glasshouses. If these glasshouse tests are successful, then the work will have reached a stage where it could be handed over to commerce as a pioneer amongst fungi.

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N.B. In citing references in the text the abbreviation "et al" has been used where more than one author was involved.

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