



Title Ecology and physiology of the aphid  
pathogenic fungus *erynia neoaphidis*

Name Tony J Bonner

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Ecology and physiology of the aphid pathogenic fungus

*Erynia neoaphidis*

Tony Jo Bonner

A thesis submitted to the Faculty of Health and Social  
Science, University of Luton, in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy.

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## Abstract

*Erynia neoaphidis* Remaudière and Hennebert (Zygomycetes: Entomophthorales) is an obligate pathogen of invertebrates, especially aphids, and has therefore been studied as a possible biological control agent for a number of years. However, a number of important physiological and ecological questions regarding optimal conditions for conidial production and transmission of the fungus through an aphid population had to be answered. This thesis investigated some of these aspects.

Solid and liquid media were used to culture the fungus, and *E. neoaphidis* was cultured on a fully defined medium for the first time.

A sporulation monitor and digital image analysis was used to quantify conidial production from *E. neoaphidis* biomass produced *in vivo* and *in vitro*. This was a completely novel method and is useful for gathering data on large numbers of conidia, so that size distributions can be constructed and the physiological status of the conidia inferred from this.

*E. neoaphidis* infected aphid cadavers produced more, smaller conidia when grown *in vitro*. Biomass harvested from exponential growth phase in fed batch culture produced significantly more conidia than biomass harvested from any other growth phase although further work on the nutritional requirements of *E. neoaphidis in vitro* is required. The duration of the conidial discharge was also greatest from biomass harvested at the exponential phase and therefore, biomass harvested from the exponential phase should be used if the fungus is to be applied as a control agent.

*E. neoaphidis* biomass kept at low humidity during simulated winter conditions produced infective conidia after 24 weeks, indicating that mycosed cadavers may act as a reservoir to infect the next season's hosts. Pesticides adversely affected the growth and production of conidia by *E. neoaphidis*, with herbicides having the least deleterious effects, and therefore being most compatible in an integrated pest management program. Laboratory and field studies were used to assess the transmission of *E. neoaphidis* through aphid populations. Position of the

inoculum on the host plant affected the primary transmission of the fungus through aphid populations in the laboratory and in the field, and secondary transmission of the fungus in the laboratory. It is therefore important to apply the fungus to where it will maximally spread. There was some evidence for effects of host and inoculum density on the transmission of the fungus, especially in the laboratory, indicating that, in practice, the fungus is unlikely to spread rapidly through low densities of aphids and therefore to achieve control of such populations, a high inoculum density may be required. There was also very little transmission of the fungus via aphid vectors to susceptible aphid populations on different host, although as a general observation, vectoring of conidia by the wind may be very important. The smaller conidia produced by *in vivo* biomass may be vectored more easily by wind than the large conidia produced *in vitro*

*To Colette, with all my love*

## Preface

I would like to thank my Director of Studies, Dr Simon Gray, for his guidance and patience during the writing of this thesis and the research on which it was based. I would also like to thank my external supervisor, Dr Judith Pell at IACR Rothamsted, and her colleagues, especially Dr Paresh Shah and Dr Helen Yeo, Jason Baverstock and Mike Feather who all gave invaluable advice and assistance. A number of sections of this thesis have been used as a base for two refereed journal papers.

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## **DECLARATION**

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Luton. It has not been submitted before for any degree or examination in any other University.

Tony Jo Bonner

The Eighteenth day of December, 2002



# 1. Introduction

## **1.1 Introduction to biological control**

### 1.1.1 Definition of biological control

Biological control can be defined broadly as the use of predators, parasitoids or pathogens to reduce the numbers of an organism to a level below that prior to introduction of the control agent. A number of authors have previously defined biological control in a number of ways that include the use of cultural methods to control pests (e.g. resistant cultivars, tilling and alternation of crops) or the use of genetic modification (e.g. transgenic plants and sterile insect release). While these methods are undoubtedly important for the non-chemical control of pests, this author believes that they are not true 'biological control', but perhaps should be classified as 'non chemical control' as they do not involve the use of natural enemies to control pest numbers.

## **1.2 Rationale for use of biological control**

### 1.2.1 Resistance to pesticides

Since Melander reported the first case of resistance to a pesticide in 1914, the problem of resistance to pesticides has grown to such proportions that no pest control program can afford to ignore its consequences. Inappropriate and excessive use of pesticides has led to over 500 species of insect and mite being resistant to pesticides (Georghiou 1990). A number of these are agriculturally or medically important. For example, a large number of mosquito species (including *Aedes aegyptii*) have become resistant to DDT, carbamates and organophosphates (Georghiou and Mellon 1983). A number of Noctuid (Lepidoptera) species have also become resistant to insecticides. These include *Heliothis armigera* and *H. zea*, both of which are agricultural pests (Georghiou and Mellon 1983)

As well as this general increase in resistance to insecticides, there is also a serious problem with insecticide resistant aphids, including the agriculturally important

peach aphid (*Myzus persicae*) and cotton aphid (*Aphis gossypii*) (Moore 1997) and the English grain aphid, *Rhopalosiphum padi* (Georghiou and Mellon 1983). Thackray *et al.* (2000) found resistance to insecticides in over 80% of a population of *Myzus persicae*.

#### 1.2.1.1 Mechanisms of resistance

There is a tremendous quantity of published literature on the subject of invertebrate resistance to insecticides and it is beyond the scope of this introduction to cover the subject in detail. However, a brief overview of the mechanisms for resistance to insecticides will be given. There are two main mechanisms of resistance – alterations at the site of action and increased detoxification. Reduced rate of absorption also accounts for some of the resistance to insecticides, but compared to the effects of alteration at the site of action and increased detoxification this is of only secondary importance (Oppenorth 1985)

The most widely studied altered target of pesticides is acetyl cholinesterase (AChE) which, when modified, confers resistance to organophosphates and carbamates. Moore *et al.* (1994) described a modified AChE in populations of *Myzus persicae* and *M. nicotianae*. This modification rendered the aphids particularly insensitive to pirimicarb. It is thought that the rate of reaction of the altered AChE with the esterase inhibitor insecticides is reduced, whereas the hydrolysis of acetylcholine (the substrate for AChE) is unimpaired or only slightly decreased.

There are a number of classes of enzymes responsible for increased detoxification of pesticides. One of the most common mechanisms of detoxification is by increase in mixed function oxidase (MFO) activity. These enzymes degrade pesticides by oxidative degradation of the chemical. One of the general characteristics of the MFO's is their wide range of substrates, which results in cross resistance patterns to a number of groups of insecticides. Another class of enzymes involved in detoxification is the hydrolases. These enzymes are of particular importance in the resistance of insects to organophosphate compounds. Devonshire and Moore (1982) reported resistance to organophosphate, carbamate and

pyrethroid insecticides in *M. persicae* due to overproduction of a hydrolase, carboxylase E4.

Glutathione-s-transferases have been only relatively recently been recognised as being important in insecticide resistance. They play a primary role in resistance to a number of organophosphate compounds, especially tetrachlorvinphos (Oppenoorth 1985)

## 1.2.2 Health and the environment

### 1.2.2.1 Pollution of water supplies and food

Pesticide use, along with pesticide manufacture are major causes of ground water contamination (Hurst *et al.* 1991). The US Environmental Protection Agency (EPA) estimates that in thirty eight states, pesticides have fouled the ground water used by half of the population (US EPA 1987). In many areas of the UK, drinking water contains pesticide levels above EC safety limits (European Institute for Water 1988) and in the EU as a whole, the concentration of pesticides in ground water beneath 65% of agricultural land is estimated to exceed drinking water standards, with a tenfold excess in 25% of the area. As a reaction to this, the Swedish and Danish governments pledged a 50% cut in the use of chemical pesticides within 5 years (Matteson 1995).

Pesticides, along with other agrochemicals often end up as residues in food. Although the concentration of these is often low, there has been little research on long-term health effects, especially in vulnerable groups such as young children and the elderly.

### 1.2.2.2 Health effects

Acute effects of pesticide exposure range from eye and upper respiratory tract irritation and contact dermatitis to systemic poisoning leading to severe illness and even death (Hurst *et al.* 1991).

Although acute pesticide poisoning may be serious or even fatal in some cases, possibly a greater threat to the health of the population is the long term, chronic exposure to pesticides. This can occur either through long term exposure from working with pesticides (farmers for example) or the accumulation of pesticides in the body from water pollution and residues in food. Chronic effects of exposure are more difficult to recognise than the effects of acute exposure. Symptoms may take many years to appear and are even then often explained by someone being 'run down', 'off colour' or even 'just getting old' (Hurst *et al.* 1991).

The most serious chronic effects of long-term pesticide exposure are increased risk of cancer and mutagenic and teratogenic effects. The latency period of cancers after exposure to carcinogens may be up to forty years (Hurst *et al.* 1991) and exposure to mutagens and teratogens poses a particular risk to women of a child bearing age. Other long-term effects include chronic allergies and immunosuppression. Very little research has been done in this area (Spencer 1966; Bryant 1985; Lisi *et al.* 1987). In the UK however, there has been an increasing number of incidents and reports that link neurological and immunological disorders in farmers with the use of organophosphate insecticides and acaricides, especially in using sheep dip. Table 1.1 summarises the health effects of a number of pesticide types.

Table 1.1. A summary of acute and chronic health risks from exposure to pesticides.

Pesticide	Health risk	References
Dithiocarbamate fungicides (maneb, zineb, nabam)	Dermatitis Possible carcinogen	Hayes (1982) Montesano <i>et al.</i> (1988)
Bipyridillium compounds (diquat, paraquat)	Death from acute poisoning Possible link to Parkinson's disease Skin cancers from exposure to precursors	Zeneca toxicology sheet Barbeau (1984); Bochetti and Corsini (1986) Bowra <i>et al.</i> (1982)
Organochlorines* (DDT, aldrin, dieldrin, lindane etc)	Nausea, dizziness, tiredness, headaches, convulsions. Dermatitis and naso-pharyngeal irritation (lindane) Aplastic anaemia (lindane)	Hurst <i>et al.</i> (1991) Wassermann <i>et al.</i> (1960) Morgan <i>et al.</i> (1980); Champlin and Gale (1987); Loge (1965)
Organophosphates (e.g. parathion, malathion and demeton)	Exhaustion and confusion, vomiting, sweats, cramps and diarrhoea, convulsions, death	(Hayes 1982); Cole <i>et al.</i> (1988); Frils (1966)
Carbamates (e.g. aldicarb, carbofuran, propoxur)	Similar to organophosphate poisoning but may be more rapid	Hurst <i>et al.</i> (1991)
Copper fungicides (copper oxide, copper sulphate, copper hydroxide)	Haemolytic anaemia Dermatitis Conjunctivitis and corneal ulcers Pneumoconiosis Possible immunosupresion Liver and kidney failure Increased risk of brain gliomas	Chugh <i>et al.</i> (1975, 1977); Ulner (1985) Hayes (1982) Pimental and Marques (1969) Muscicco <i>et al.</i> (1988)
Pyrethroids (e.g. cypermethrin, permethrin, decamethrin)	Dermatitis, facial swelling Sweating, fever, tachycardia (rapid heartbeat) and convulsions Neurological damage	Hayes (1982) He <i>et al.</i> (1988)

\*Note organochlorine pesticides have been banned or restricted in a number of countries.

### 1.2.3 Effect on non - target organisms

Many of the chemicals used to control insect and other pests are not species specific, and may affect natural enemies of pest species and other non-target organisms (NTO's), both vertebrates and invertebrates.

#### 1.2.3.1 Invertebrates

A number of non-target arthropods have been shown to be susceptible to pesticides either in increased mortality or with sub - lethal effects.

##### a) Mortality effects

Croft (1990) noted that the majority of toxicity tests of pesticides on beneficial insects have been concerned with the direct mortality of the organism concerned. Since the early 1980's, the IOBC-WPRS Working Group 'Pesticides and Beneficial Organisms' have been evaluating the mortality of a number of species of beneficial organisms after being exposed to a number of pesticides (Hassan *et al.* 1983, 1987, 1988, 1991, 1994). In general it has been found that insecticides have caused 75-100% mortality in beneficial insects and mites with a similar mortality in entomopathogenic fungi when exposed to fungicides.

Asín and Pons (1999) found significantly fewer non-target arthropods found on maize plots treated with carbofuran than on untreated plots. Rehman *et al.* (1999) found that field residues of carbaryl caused 100% mortality in the hymenopteran parasite *Aphytis holoxanthus* for 10 days after application. McGregor and Mackauer (1989) also showed the toxicity of carbaryl to the aphid parasite *Aphidius smithi*. Boyd and Boethal (1998) showed that several insecticides cause significant mortality in four species of hemipteran predators (*Geocoris punctipes*, *Nabis capsiformis*, *N. rosipennis* and *Podisus maculiventris*). House *et al.* (1985) showed that use of synthetic pyrethroids could significantly reduce entomophagous insect numbers in the field. Holland *et al.* (2000) showed that dimethoate significantly reduces numbers of beneficial arthropods (especially spiders). Samsøe-Petersen (1993) showed that

exposure to many carbamate and pyrethroid insecticides induced at least 75% mortality in the rove beetle *Aleochara bilineata*.

#### b) Sub lethal effects

Reports dealing with the sublethal effects of insecticides on non-target arthropods are relatively rare (Elzen *et al.* 1989a; Croft 1990). Croft (1990) reported that of all the studies on the effects of insecticides on beneficial insects from 1950 to 1986 only 5% reported sublethal effects while 75% reported direct mortality. Whilst the direct effect of insecticides on beneficial insects is obviously important, the sublethal effects are equally as important. Ultimately, they will reduce the effects of beneficial insects as biological control agents due to the reduced performance in parasitizing and preying on hosts.

Hyperactivity is induced in the woodlouse (*Porcellio scaber*) by the widely used organophosphate insecticide dimethoate at only 10% of the LD<sub>20</sub> in the laboratory (Bayley 1995). This effect is prolonged, with no recovery seen even after three weeks recovery on uncontaminated soil. Increase in activity due to organophosphates has also been reported for other species. Barker (1980) noted hyperactivity in honeybees (*Apis mellifera*) exposed to dimethoate over a week. Schricker and Stephan (1970) reported elevated flying speeds in *A. mellifera* exposed to parathion. Increased activity is likely to affect feeding and mating behaviour as well as increasing the risk of desiccation in susceptible species such as the woodlouse (Den Boer 1961; Horowitz 1970). The hymenopteran parasitoids *Eretmocerus mundus* and *E. tejanus* are potential biological control agents of the whitefly *Bemisia argentifolii*. Jones *et al.* (1998) have shown that a number of insecticides (thiodicarb, endosulfan, azinphos-methyl, methyl parathion, buprofezin and bifenthrin) have deleterious effects on the survival of parasitoid larvae and pupae and the emergence of adults from pupae. The toxicity of these insecticides to *Eretmocerus* is varied, with thiodicarb being the least toxic and the pyrethroid bifenthrin being the most toxic. Jones *et al.* (1995) reported a wide range of responses of parasitoids to many

insecticides. There were also detrimental effects in host locating, reproduction and lifespan in parasitoids (Moriarty 1969; Haynes 1988)

Insecticides have also been shown to have effects on feeding by beneficial parasitoids. The braconid parasitoid *Microplites croceipes* had significantly reduced responses to host-associated odours after feeding on extrafloral nectaries on plants treated with a number of systemic insecticides (Stapel *et al.* 2000) as well as having reduced longevity. Elzen *et al.* (1989b) also reported a decrease in flight activity in *M. croceipes* females that had been sprayed directly with a fenvalerate/chlorodimeform mixture.

Samsøe-Petersen (1993) reported significant reductions in egg laying and hatching in the rove beetle *Aleochara bilineata* exposed to a number of insecticides. Pyrethroid insecticides were highly toxic to *A. bilineata*, with all pyrethroids that were tested reducing egg laying by at least 90%, and cypermethrin and lambda cyhalothrin completely inhibiting egg laying. Organophosphates and carbamates were also highly toxic to *A. bilineata*.

Herbicides and fungicides also have a detrimental effect on beneficial insects. It has been shown that some urea herbicides have a significantly detrimental effect on egg hatching in *A. bilineata* (Samsøe-Petersen 1995a). Morpholine fungicides and maneb and thiram (dicarbamate fungicides) reduce egg laying in *A. bilineata* (Samsøe-Petersen 1995b).

#### 1.2.3.2 Vertebrates

As well as the effects of pesticides on invertebrates, there are also effects on vertebrates. Direct or acute poisoning of organisms involving pesticides that are persistent can lead to 'food chain' or secondary poisoning. Insecticides have the greatest effect on vertebrates due to their non-selective nature.

Use of organochlorine insecticides, such as dieldrin, aldrin and heptachlor was responsible for the massive decline in numbers in a number of species of birds of prey, such as kestrels and sparrowhawks in the UK in the 1950's. Secondary poisoning of mammals occurred when they scavenged the corpses of poisoned birds.



Dieldrin poisoning has also been the cause of death in horses (Van Klingerren 1966) and wood mice (Jeffries 1973). The use of the organochlorine insecticide DDT was also responsible for the decline of a number of bird species in Europe and the US, due to the detrimental effect that DDT and its metabolite DDE had on the thickness of bird egg shells.

The use of organophosphate seed dressings in the 1970's also led to the death of large numbers of greylag and pink footed geese and whooper swans (Hardy 1984).

Aldicarb is one of the most widely used carbamate insecticides in the UK and in the 1970's was responsible for the deaths of a number of bird species (gulls, lapwings and stone curlews), especially in sugar beet areas where it was extensively used to treat eelworm (*Heterodera schachtii*) infestation (Moore 1977). Pyrethroid insecticides have a low acute mammalian and avian toxicity but are toxic to aquatic vertebrates.

As well as the direct effects of acute and food chain toxicity in vertebrates and invertebrates there are indirect ecological effects of pesticides on NTO's. The first of these is the reduction of food sources.

Mortality of plants and insects lead to a reduction in the amount of food available for other organisms in the food web. An example of this is the long term decline in numbers of the wild grey partridge (Sotherton 1988). Numbers of the wild grey partridge in the UK have been reduced to only a fifth of their pre-war levels and a major factor in this decline is thought to be the removal of phytophagous insects on which partridge chicks depend for survival (Green 1984).

#### 1.2.3.3 Indirect ecological effects

A second example of an indirect ecological effect is the appearance of secondary pests. Secondary pests can arise when a dominant species is removed by the use of pesticides, or when natural enemies are removed. A striking example of this is the cotton whitefly in Sudan. DDT and other broad-spectrum insecticides were used in the Sudan to control a cotton pest, the cotton jasad, *Amrasca devastans* (Hurst 1991). As numbers of the cotton jasad fell, the population of the cotton

bollworm (*Helicoverpa zea*) rose until it too became a major pest. To control this species, up to eight seasonal sprays of organochlorine and organophosphate insecticide were used. This killed off natural enemies of the cotton whitefly (*Bemisia tabaci*) a small parasitic wasp species. This led to whitefly becoming a major species with uncontrolled outbreaks of the pest being recorded in 1980-81.

Linked to the outbreak of secondary species is that use of pesticides can, paradoxically, increase pest numbers. Pesticide use can kill off both pests and their natural enemies but pests are usually quicker to recover due to the qualities that made them pests (high reproductive rate, fecundity and motility). Without the natural enemies to keep some check on their numbers, the pest species can rebound to numbers higher than those prior to use of the pesticide.

In contrast to the toxicity of many insecticides to beneficial arthropods and vertebrates, the effect of biological control agents and other microbial pesticides to these groups is relatively benign, especially the use of viral pathogens (Flexner *et al.* 1985). Mortality of beneficial arthropods is more likely to come from indirect effects, such as a reduced food source or unsuitability of hosts for parasitoids.

#### 1.2.4 Legislation and consumer preference

As a result of these concerns for both human health and for the environment, legislation has been enacted, both in the UK and in Europe to reduce the amount of pesticide that is being used, and also the banning or restriction of a number of chemical pesticides. Over the last 4 years, the European Union has withdrawn permission of usage for a number of older, more toxic pesticides including fenvalerate (98/270/EC) and dinoterb (98/269/EC). The UK Government has also brought legislation before Parliament to increase the amount of farmland that is classified as organic. The Organic Food and Farming Targets Bill (2001) states that by the year 2010, no less than 30 % of agricultural land in England and Wales will be classified as organic or in the process of being converted to that status.

Consumer concerns have also led to a rise in the amount of 'organic' food bought and consumed over the last ten years. The Soil Association estimates that the growth in organic food sales will increase by 40% per annum.

Thus, there is strong justification for the use of biological control on the grounds of pesticide resistance, health issues and damage to the environment.

#### 1.2.4. Pesticides and integrated pest management

Although a complete halt in the use of pesticides and the use of biological control as the sole means of pest control is an ethically and environmentally sound idea in principal, in practice it may not be feasible for a number of reasons. The first of these is the speed of kill of pesticides compared to biological control and the relative effect of environmental conditions. The fastest acting of biological control agents such as *Metarhizium anisopliae* take three days or more to kill their host, even under the most suitable environmental conditions and then a number of days after that to reduce the pest population significantly. Under unsuitable environmental conditions, such as dry weather or extremes of temperature, the biological agent may not spread at all or very slowly. This is compared to most pesticides that typically reduce the pest population in a number of hours and are relatively unaffected by environmental conditions.

The second reason is consumer preference. Biological control will only reduce the population of a pest and not completely eliminate it. This means that there are some pests left on the crop which can do damage. This may not be economically damaging to the farmer in terms of yield, but may reduce the cosmetic quality of the produce. Consumers are accustomed to cosmetically perfect produce and will often reject goods that have cosmetic blemishes due to pest damage. The retailers will then not buy from farmers that produce in this way. An example of this is in spinach production. Processors reject crops that have aphid cadavers killed by *Erynia neoaphidis* attached as they are difficult to remove and consumers would not buy the produce (McLeod *et al.* 1998). This also highlights the need to choose suitable biological control agents for particular crops. With the difficulties outlined above, it

may be more practical to considerably reduce the use of pesticides rather than completely discontinue their use. This could be done by combining the use of pesticides with biological control in an Integrated Pest Management (IPM) programme.

An IPM programme could significantly reduce the quantities of chemical used, whilst still retaining the desirable properties of pesticides, such as quick pest control and cosmetically acceptable produce. To do this requires research into the effect of pesticides on biological control agents and their compatibility in an IPM programme. For a review of IPM in practice, the reader is directed to Way and van Emden (2000).

### **1.3 Aphids**

Aphids have a global distribution with approximately 4000 species worldwide, with the majority of the species being in the temperate zones. There are more than 500 species in the UK, and approximately 1350 species in North America. About 250 species are serious pests (Blackman and Eastop 1984).

#### **1.3.3 Morphology**

Aphids are small (1-10 mm in length) soft bodied insects with, or more commonly, without wings. Their antennae have two thick basal segments and a flagellum composed of up to 4 segments, the last of which is divided into a proximal section and a thinner, distal section. They also possess a proboscis that originates between and behind the forelegs. They have two compound eyes and two ocular tubercles, each composed of three lenses which are situated behind and above the compound eyes. They have two tarsal segments and the wings, when present, have only one prominent longitudinal vein. The fifth abdominal segment bears a pair of upward and backwardly pointing tubes on the dorsal surface called siphunculi (cornicles) and a cauda is usually present below and between them on the last abdominal segment.

Aphids also occur in different colour forms or morphs. The Pea Aphid *Acyrtosiphum pisum* occurs in red or green morphs. Two of the aphids' main

predators are the ladybird *Coccinella septempunctata* and the parasitic wasp *Aphidius ervi*. *C. septempunctata* preferentially feeds on the red morph while *A. ervi* prefers the green morph.

### 1.3.2 Phenology

Aphids are often parthenogenic for part of or all of their lives, and both viviparous and oviparous at different times of year. Parthenogenic reproduction usually results in viviparity, and all aphids are both parthenogenic and viviparous during spring and summer (Dixon 1997).

Some species of aphid that are parthenogenic during the summer produce sexual offspring (males and females) in the autumn. This is termed 'cyclical parthenogenesis' or a 'holocyclic' life cycle. Species that produce only asexual morphs are termed 'anholocyclic' (Blackman and Hales 1986). In a holocyclic life cycle, (Fig 1.1) the sexual males and females mate in the autumn. Males are produced only in one part of the life cycle and are produced *de novo* by parthenogenic females, usually in the autumn. This is achieved as aphids have an XO sex determination mechanism. Individuals with two sex chromosomes (XX) develop into females, while individuals with only one sex chromosome (XO) develop into males. Males can therefore produce sperm with either no sex chromosomes (O), or one sex chromosome (X). However, sperm with no sex chromosomes degenerate very rapidly and never contribute to an embryo, therefore, all offspring of a sexual mating have XX as their sex chromosomes and are female. Males are produced by 'mini meiosis', during which, one of the X chromosomes is eliminated during oogenesis. Blackman and Hales (1986) provide a detailed review of the phenomenon. In contrast, anholocyclic species produce sexual females and do not produce fundatrices or lay eggs (Fig 1.2).

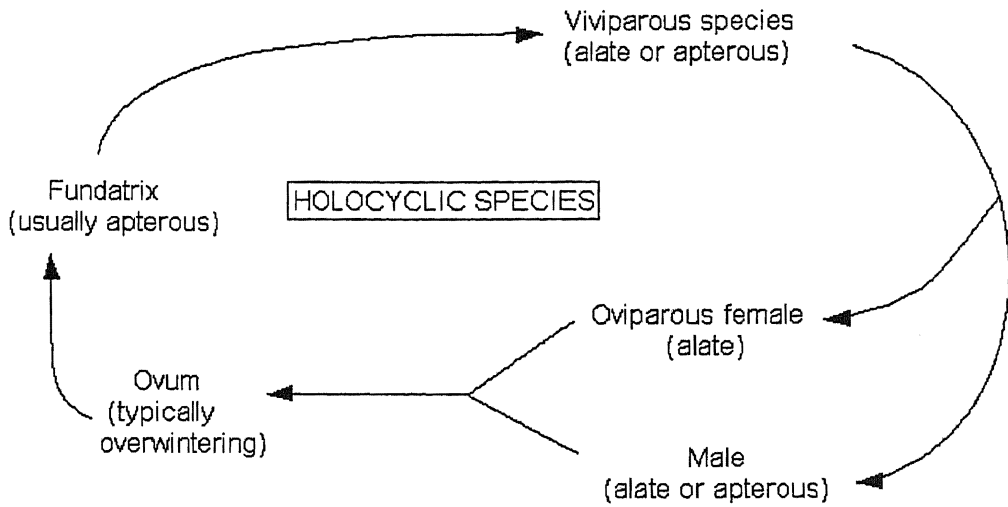


Figure 1.1 Diagrammatic representation of the life cycle of a holocyclic aphid species

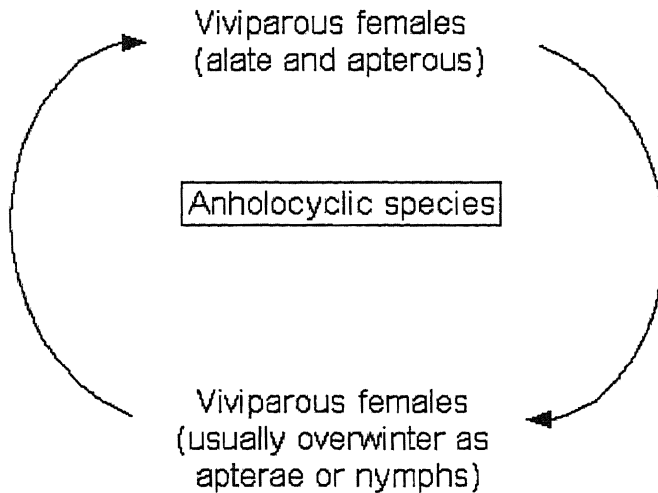


Figure 1.2 Diagrammatic representation of the lifecycle of an anholocyclic aphid species.

In the field, ova are laid during the autumn as the overwintering stage in many temperate forms and give rise to females whether or not they are the result of sexual mating (Dixon 1997). In other species, an overwintering form called a 'hiemalis' develops in the autumn, or the adults are the overwintering form.

Ova within a viviparously reproducing female start to develop immediately after ovulation, so that an embryo can exist inside another larger and more mature embryo. A newly born summer aphid can contain within itself not only the developing embryos of its offspring but also those of their offspring (1986). Parthenogenesis combined with this 'telescoping of generations' gives aphids an exceedingly rapid turn-over of generations, thus populations can build up very quickly.

### 1.3.3 Feeding

Aphids feed from the phloem of plants, which they access with the stylets of their proboscis from 3 areas of the plant; stems, leaves, and roots. The stylet, which is contained within the proboscis when the aphid is not feeding, is very thin and could suffer damage while being inserted into the plant (Minks and Harrewijn 1987). To counter this, aphids secrete a liquid from the tip of their stylet that starts to harden as soon as it leaves, forming a hard protective sheath around the stylet as it is inserted into the plant (Minks and Harrewijn 1987). When the stylet reaches a phloem tube the aphid injects saliva into the plant cell. It is suspected that this saliva helps prevent the plant cell from sealing the puncture wound (Minks and Harrewijn 1987).

Some species of aphid feed by inserting their stylet through the stomata of the plant leaves on which they are feeding. For example, *Ceratovacuna lanigera* (sugarcane woolly aphid) on *Miscanthus* spp. (Blackman and Eastop 1987) and *Schizolachnus orientalis* on pine (*Pinus*) needles (Blackman and Eastop 1987). Aphids may also access the phloem intracellularly, intercellularly or by a combination of both (Minks and Harrewijn 1987).

Plant phloem saps are rich in sugars and poor in amino acids. This results in aphids excreting large amounts of honeydew, as they must ingest large quantities of sap to obtain enough nitrogen in their diet. This honeydew can often be seen on the lower leaves of infested plants and is fed on by other insects such as the common wasp, *Vespula vulgaris*, the Brown Hairstreak Butterfly *Thecla betulae* and by species of tending ant (Dixon 1997).

Almost all species of aphid contain endosymbiotic bacteria in specialised cells known as bacteriocytes (Minks and Harrewijn 1987). The exceptions are some members of the Ceratophidini, which have yeast cells in their haemocoel (Minks and Harrewijn 1987). In most species, the main endosymbiont is *Buchnera aphidicola*, which is related to *Escherichia coli*. *B. aphidicola* is believed to complement the aphids' diet by synthesising vitamins, sterols and certain amino acids (Prosser and Douglas 1991, Douglas 1990). Endosymbiotic bacteria are important for the aphids' growth and reproductive potential as they decrease as the aphid ages and stops growing and are absent from most soldiers and males, which do not mate (Douglas and Dixon 1987). The relationship between *B. aphidicola* and the aphids it lives within is mutually obligate. *B. aphidicola* is transmitted transovarially from the female aphid to the cytoplasm of the ova in the oviparous morph or to the blastula embryo in the viviparous morph (Brough and Dixon 1990).

Most aphids are autoecious; i.e. they feed on one or a few species of closely related plants. About 10% are heteroecious, spending autumn, winter and spring on one plant species (the primary host) and summer on an unrelated plant (the secondary host) (Minks and Harrewijn 1987). For example, the Rosy Apple Aphid *Dysaphis plantaginae* has apple (*Malus*) as its primary host and plantain (*Plantago lanceolata*) as its secondary host (Dixon 1997). Some heteroecious aphids such as *Myzus persicae* and *Aphis fabae* have a wide range of secondary hosts, but this is relatively rare. Most heteroecious aphids have just one primary and one secondary host.

#### 1.3.4 Movement and migration

Generally aphids move very little, remaining in position and feeding. However, four stimuli can cause an aphid to move or migrate from the host plant. The first two stimuli work on the population level and are stimuli for migration rather than movement. The first stimulus is environmental change signalling that it is time to move, either to the primary or secondary host. These cues will include photoperiod and temperature. The second stimulus is overpopulation, which causes aphids to



migrate away from their present area. Both of these stimuli result in the production of alate forms appearing within the colony/population and it is these winged forms that move, either to a new host plant or as a migration to new areas where fresh hosts can be found. Although in still air aphids are poor fliers, if they can fly up to the planetary boundary layer at a height of approximately 1000 m, they can travel for large distances on the low level jetstreams that are present at this altitude.

The second two stimuli cause individual aphids to move off of the host plant. The first of these is the death of the host plant that stimulates aphids to walk off and find a new host plant. The second and more important is attack by a predator or parasite. An aphid that is under attack can defend itself by a variety of ways, depending on the size and nature of the attack.

Firstly, an aphid will kick an attacker and attempt to drive it off. If this does not work it will retract its stylets and proboscis from the plant and walk away. Some species can also exude a waxy solution from their siphunculi over an attacker and while doing this generally release an alarm pheromone from the cornicle (Pickett *et al.* 1992) to warn conspecifics of the presence of a predator. In many aphid species, the alarm pheromone is  $\beta$ -farnesene (Bowers *et al.* 1972), although some species use other chemicals. For example, the spotted alfalfa aphid *Therioaphis maculata* uses (-)-germacrene A (Nishino *et al.* 1977).

The last resort is to jump or fall off of the plant. This course of action increases the risk of desiccation, starvation or both whilst not on the plant. Therefore the benefits of predator avoidance have to outweigh the risks. Aphids possess the ability to alter their escape behaviour with changes in prevailing conditions (Dill *et al.* 1990). Aphids are less likely to leave the host when the host is of high quality and are less likely to drop off the plant when climatic conditions are hot and dry (Dill *et al.* 1990).

#### 1.3.4 Damage caused by aphids

Aphids cause considerable damage to agricultural crops. They cause damage in a number of ways. Firstly, their feeding reduces the vitality of the crops they feed on through diversion of photosynthate and therefore reduces yield.

Secondly, growth of sooty moulds, such as *Cladosporium* on the excreted honeydew decreases the area of leaf available for photosynthesis. Finally and perhaps most importantly, aphids are vectors of viral plant diseases. Arguably the most important aphid pest in this respect is *Myzus persicae*. *M. persicae* has peach as its primary host and a wide range of secondary hosts including many commercially important brassicas. It is cosmopolitan in temperate climates occurring throughout the USA and much of Europe including the UK (Van Emden *et al.* 1969). Though it seldom occurs in numbers large enough to cause direct damage from feeding pressure, it is capable of transmitting over 100 viruses including the Potato Leaf Roll and Y viruses of potatoes; Mosaic, Yellow Net and Yellow viruses of Sugar-beet; Cauliflower Mosaic Plum Pox; Cucumber Mosaic Virus; Lettuce Mosaic Virus and Turnip Mosaic Virus (Van Emden *et al.* 1969).

Other major aphid pests include: the Black Bean Aphid, *Aphis fabae*; The Pea Aphid, *Acyrtosiphon pisum* which is found on many leguminous plants, and transmits Lucerne Mosaic virus, Pea Leaf Roll virus, Pea Enation Mosaic virus and Pea Mosaic virus in the UK ; and the Cabbage Aphid (*Brevicoryne brassica*) which is a serious pest of the major brassica crops, due to vectoring of Cauliflower Mosaic virus and Turnip Mosaic virus.

Other aphid pests include the grain aphid *Sitobion avenae*, the cotton aphid, *Aphis gossypii* and the Russian wheat aphid *Diuraphis noxia*

### 1.3.5 The Pea Aphid

The Pea Aphid (*A.pisum*) is approximately 3mm long and has two colour morphs, red and green. It is very similar in appearance to the blue-green aphid (*A. kondoi*), the difference being that the antennae of *A. pisum* have a narrow, dark band at the end of each antennal segment, while those of *A. kondoi* are uniformly green.

It has a number of leguminous hosts, including alfalfa (*Medicago sativa*) and broad beans (*Vicia faba*) and overwinters as ova on perennial legumes (<http://www.gov.mb.ca/agriculture/crops/insects/fad14s00.html> correct as of 23/7/02).

Adults mature within 5-50 days, depending on the weather. At 20°C, maturity occurs in approximately 7-10 days after egg deposition.

The pea aphid has a number of natural enemies, including ladybirds (Coccinellidae: Coeloptera), hoverfly larvae (Syrphidae: Diptera), parasitoids and a number of entomopathogenic fungi including *E. neoaphidis*.

### 1.3.6 Current control of aphids

Aphids are currently controlled using a number of chemical insecticides. In the UK these include chlorpyrifos, cypermethrin, deltamethrin, lambda cyhalothrin, fenitrothion, malathion and pirimicarb (EDAP 1990). These are generally efficient against aphid pests (Thackray *et al.* 2000; Sweedon and McLeod 1997; Neil *et al.* 1997) although a reduction in their usage for the reasons described in section 1.2 would be desirable.

## 1.4 *Erynia neoaphidis*

*Erynia (Pandora) neoaphidis* Remaudiere and Hennebert (Zygomycetes: Entomophthorales) is an obligate pathogen of invertebrates, especially aphids (Homoptera: Aphididae) although it has been shown to also infect weevils (Brown and Nordin 1986) and some species of hemiptera (Wilding and Brady 1984). Within the Aphididae, *E. neoaphidis* has a wide host range, including *Acyrtosiphon pisum* (pea aphid), *Aphis fabae* (bean aphid), *Myzus persicae* (peach potato aphid), *Myzus nicotianae* (tobacco aphid), and *Sitobion avenae* (cereal aphid). For many years it has been regarded as a potential biocontrol agent of aphids, especially of *Acyrtosiphon pisum* and *Aphis fabae* (Milner 1997). The occurrence of *E. neoaphidis* has been reported from several countries world wide including the UK (Wilding 1975), Italy (Pennachio and Tremblay 1986), the USA (Pickering and Gutierrez 1991; Feng *et al.* 1991, 1992; Wraight *et al.* 1993; Kish *et al.* 1994; McCloud *et al.* 1998), France (Berthelem *et al.* 1969), South Africa (Hatting *et al.*

1999), Australia (Milner 1985), South Korea (Yoon *et al.* 1998), Mexico (Sanchez-Peña 2000) and Iceland (Nielsen *et al.* 2001).

Over the last 3 decades there have been a number of changes in nomenclature. Previously it has been known as *Entomophthora aphidis* (Wilding 1969) and is also now known by the synonym *Pandora neoaphidis* (Humber 1989).

#### 1.4.1 *In vivo* phenology

##### 1.4.1.1 Attachment, germination and penetration.

Like most of the Entomophthorales, *E. neoaphidis* has a fairly complex *in vivo* phenology (Figure 1.3). In common with the rest of the Entomophthorales the infective propagule is the conidium. Conidia are coated with polysaccharide mucilage (Li *et al.* 1993), which enables adherence to the cuticle of the host, and will adhere anywhere on the cuticle (Brobyn and Wilding 1977). The conidia germinate within two to four hours of inoculation and produce either secondary (replicative) conidia, or germ tubes (Brobyn and Wilding 1977). The secondary conidia are formed at the end of short, lateral conidiophores (Butt *et al.* 1990) and are generally smaller and more globose than primary conidia (Morgan *et al.* 1995), although both conidial types are infective (Butt *et al.* 1990). Most germ tubes penetrate the cuticle close to the site of attachment, but some may grow horizontally along the cuticle before penetration, or may not penetrate at all (Brobyn and Wilding 1977; Butt *et al.* 1990). Penetration is almost always preceded by the formation of an appresorium, which is usually clavate or globose (Butt *et al.* 1990) although clavate appresoria with a terminal bulb are not uncommon. Appresoria are formed either directly from a conidium or from a germ tube. The appresoria that are formed are similar to appresoria produced by *Zoopthora* (*Erynia*) *radicans* (Magalhaes *et al.* 1989; Wraight *et al.* 1990). Many other species of Entomophthorales are also reported to produce appresoria including *Conidiobolus obscurus* (Butt 1987), *Entomophthora muscae* (Brobyn and Wilding 1983), *Entomophaga aulicae* (Murrin and Nolan 1987) and *Neozygites* (*Entomophthora*)

*fresenii* (Brobyn and Wilding (1977) and it has been suggested that appresoria formation is usual in the Entomophthorales (Butt *et al.* 1990).

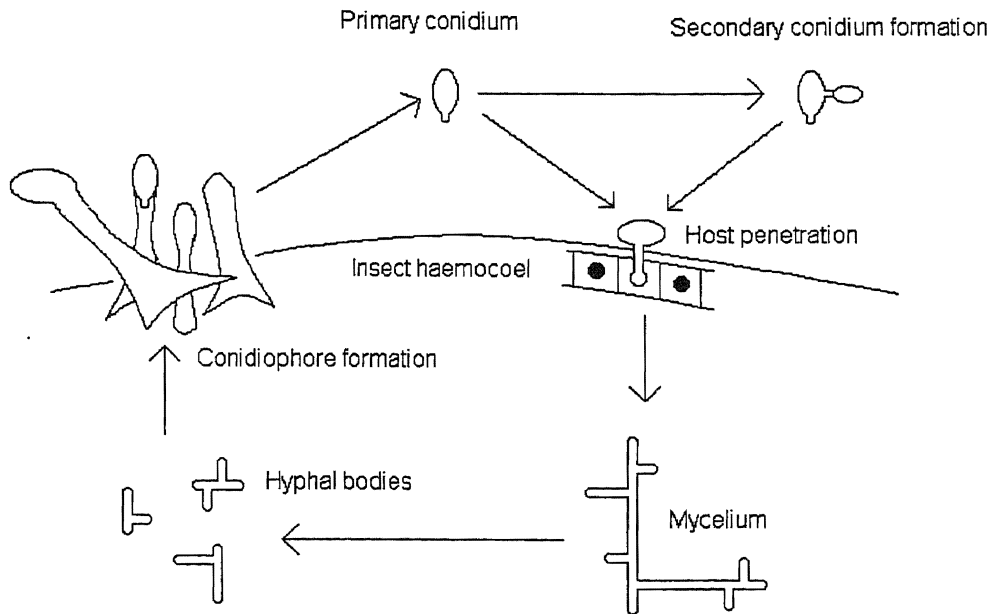


Figure 1.3 *In vivo* phenology of *Erynia neoaphidis* (not to scale)

Penetration pegs are sometimes observed with *E. neoaphidis* and may be protoplasmic (Butt *et al.* 1990). Production of enzymes that are involved in cell wall synthesis in entomophthoralean fungi is influenced by the composition of the medium (Beauvais and Latgé 1989). The exact mechanism by which *E. neoaphidis* penetrates the cuticle is not known, but is likely to be by a combination of enzymatic action and mechanical force. Some members of the genus *Entomophthora* have been shown to produce lipolytic, proteolytic and chitinolytic enzymes (Gabriel 1968a) so it is not unreasonable to think that *Erynia neoaphidis* will do likewise. These enzymes may facilitate mechanical entry by weakening the cuticle as in *Conidiobolus coronatus* (Gabriel 1968b). During germination, *E. neoaphidis* shows considerable cytological changes indicative of high metabolic activity, including lipolytic activity. A strong immune response is not generally elicited by infection by *E. neoaphidis*. The

most typical response is melanisation of the cuticle at the point of entry, but there is no haemocyte-mediated response (Butt *et al.* 1990).

#### 1.4.1.2 *Haemocoel invasion*

The penetration tubes elongate and branch to form hyphae that are 8-10 $\mu$ m in diameter. These then fragment into thin walled, protoplast-like multinucleate hyphal bodies that rapidly disperse throughout the haemocoel of the host. Within 36-60 hours the hyphal bodies invade the fat body, mycetomes, muscle tissue and cephalic nerve ganglia (Brobyn and Wilding 1977; Butt *et al.* 1990). The gut, trachea and embryos are rarely affected (Brobyn and Wilding 1977; Butt *et al.* 1990) possibly due to the protoplast form lacking enzymes to degrade the cuticle that envelopes these structures. Once tissue invasion is complete, the host dies shortly after, usually within 72 hours post - infection. The protoplast stage of growth possibly enables rapid growth of the fungus within the host, as energy is not expended in cell wall synthesis (Butt *et al.* 1981). It has also been suggested that the protoplast stage enables the fungus to escape the host's defences (Dunphy and Nolan 1982). Protoplasts are not encapsulated by insect haemocytes (Dunphy and Nolan 1982; Martin and Nolan 1986) whereas walled fungal bodies often elicit a strong immune response (Beauvais *et al.* 1989; Butt *et al.* 1988; Gunnarsson and Lackie 1985).

#### 1.4.1.3 *Differentiation of hyphal bodies and development of dispersal structures*

Protoplasts regenerate cell walls once the nutrients in the haemolymph have been exhausted and the haemocoel has been occluded. Protoplasts regenerate a thin, fibrillar wall that thickens and differentiates into two distinct layers (Butt *et al.* 1990). Septa are also formed and isolate the growing apical section of the hypha from the senescent basal section. Just prior to differentiation, the hyphal bodies lengthen, become concentrated just beneath the cuticle and orient approximately perpendicular to the surface of the cuticle (Brobyn and Wilding 1977; Butt *et al.* 1990).

Rhizoids are the first structures to emerge from the host, approximately 72-96 hours after infection (Brobyn and Wilding 1977). These initially appear between the

forelimbs and then from the mid-ventral region of the host. Rhizoids are formed from large, vacuolated hyphal bodies in the mid-ventral region of the host that pass through the epidermis and continue to swell between the epidermis and cuticle, often forcing the two layers apart (Brobyn and Wilding 1977). Each rhizoid emerges as a single, unbranched filament that then bifurcates, continuing to do so until the digits coalesce to form a holdfast that attaches the host to the plant substrate. Rhizoids have probably evolved to aid spore dispersal. Conidia from infected hosts on the ground are less likely to be carried up to the plant canopy to infect new hosts than those from hosts already in the canopy. Gregarious insects, such as aphids, have a tendency to walk over each other and are thus more likely to be infected if the host is still attached to the plant.

The pseudocystidia emerge shortly after the death of the host, from all parts except the appendages, the anterior of the head and the mid-ventral region of the thorax (Brobyn and Wilding 1977). These also develop from large vacuolated hyphal bodies, but not as large as those that form rhizoids (Butt *et al.* 1990). The pseudocystidia extend towards the surface of the cuticle and are surrounded by developing conidiophores in a rosette pattern, which was first noticed by Thaxter (1888). These raise numerous, abutting papillae on the surface of the cuticle which are ruptured approximately four hours after formation (Brobyn and Wilding 1977). Most pseudocystidia are elongate, undifferentiated filaments that exceed 70µm in length, but a few are slightly branched (Butt *et al.* 1990).

Most hyphal bodies differentiate into conidiophores. Vacuoles at the base of the hyphal body fuse, displacing cytoplasm to the cell apex (Butt *et al.* 1990). A septum divides the apical portion of the cell from the vacuolated basal portion. Primary conidia form at the apices of conidiophores shortly after emergence through the cuticle, and cytoplasm is progressively transferred from the conidiophore to the conidium (Butt *et al.* 1990). Conidia may then be projected for several millimetres.

The role of the pseudocystidia is not entirely clear. Conidiophores emerge from the rupture in the cuticle caused by the pseudocystidia, so their function, at least in *E. neoaphidis*, may be to breach the cuticle prior to conidiophore emergence.

However, in other species of the Entomophthorales that attack aphids (*Entomophthora planchoniana* and *E. thaxteriana*) conidiophores emerge from the host without the aid of pseudocystidia, possibly by concentrating mechanical and enzymic action. It is possible that pseudocystidia also trap a layer of moist air around the host to prolong the period of conidial discharge.

#### 1.4.2 *In vitro* growth

Traditionally, *E. neoaphidis* has been cultured on a number of complex media that have contained poorly defined organic components. These have included salmon, pork, beef, veal, potato and carrot (Rockwood 1950), egg yolk and milk (Wilding and Brobyn 1980) and sunflower oil (Latgé *et al.* 1978). Egg yolk has been replaced by yeast extract and tryglycerides (Latgé *et al.* 1978).

The nutritional requirements of the Entomophthorales have been described previously, especially by Latgé (1975a 1975b, 1981). Protein hydrolysates, especially asparagine give good growth *in vitro*, but no species of the Entomophthorales will grow on nitrate as the sole nitrogen source. This is in contrast to a number of other fungal groups that grow using nitrate as the sole nitrogen source (Van Laere 1995). *E. neoaphidis* will grow on a semi - defined medium containing yeast extract, glucose and mycological peptone if it is supplemented with oleic or palmitoleic acid (Robinson 1987; Gray *et al.* 1990). *E. neoaphidis* will also grow on a semi - defined medium consisting of Grace's insect tissue medium supplemented with 5% foetal bovine serum (Hatting *et al.* 1999). If *E. neoaphidis* is to be used as a biological control agent, it is desirable to formulate a fully defined medium to elucidate the physiology of the fungus and to optimise production parameters. It is likely to be too expensive for use in commercial production.

The optimal temperature for colonial growth of *E. neoaphidis* is 20 °C (Robinson 1987). It will grow at temperatures between 15 °C and 22 °C although hyphal extension ceases at temperatures in excess of 25 °C. The optimum pH for growth is 6.0-6.2, but it will grow well at a pH range of 5.8-7.0 (Robinson 1987). Conidia do not germinate at low densities on artificial media. However Gray *et al.*



(1991) succeeded in germinating monoconidial isolates using cellophane overlays and *E. neoaphidis* may germinate on its host at low conidial densities.

Liquid culture may be achieved in shake flask culture, fermenter batch culture, fermenter fed batch culture and chemostat culture. Fed batch culture is usually used for the culturing of fungi for a number of reasons. Fed batch has a number of advantages over batch culture, including decreased inhibition of growth by the substrate and greater production of biomass, both of which are important in the production of *E. neoaphidis* cultures. Fed batch is also preferred over continuous (chemostat) culture for the production of *E. neoaphidis* biomass as it is very susceptible to washout in chemostat culture, and a higher initial quantity of inoculum is needed compared to fed batch (Gray and Markham 1997). It is also important to ensure that biomass is harvested at the same growth phase, whether this is in fed batch, batch or chemostat culture.

Although liquid culture is preferred to solid culture, a number of problems may be encountered. The first of these is an inoculum size effect. *E. neoaphidis* will not grow in liquid culture unless there is an adequate quantity of inoculum. Gray *et al.* (1990) postulated that this may be due to the need for a sufficient quantity of biomass to produce a putative detoxification (lipolytic) enzyme in order to degrade oleic acid present in the medium. At low concentrations (0.01% v/v), oleic acid is needed by some fungi to optimise cell membrane fluidity (Kerwin 1987), and has been shown to be a growth requirement for *E. neoaphidis*. However at higher concentrations it becomes toxic to the fungus, possibly by destabilising the membrane. This has been shown to occur in the bacterium *Streptococcus faecalis* (Carson and Daneo-Moore 1980). If sufficient inoculum (and therefore sufficient enzyme) is not present in the culture then the fungus will be killed by the oleic acid. This is a particular problem in continuous culture where higher inoculum levels are needed to initiate the culture due to the constant influx of oleic acid.

A second problem concerns wall growth. This occurs when an organism grows on the walls of a reaction vessel rather than in the liquid medium, but this may be alleviated by running the vessel full so that there is no airspace (Gray 1990).

During batch culture, a direct drive impeller is preferred to a magnetic flea as it provides the level of agitation necessary to prevent clumping of the biomass in liquid culture. However, if the impeller speed is too high then the hyphal walls may begin to break down due to increased shear force. Again this is a particular problem when culturing *E. neoaphidis*, as it is a septate. Depending on the design of the reaction vessel, impeller speeds of 450-500 rpm avoid excessive shear force, but maintain adequate dissolved oxygen concentration (Gray 1990).

A third problem concerning the preparation of *E. neoaphidis* for application as a biological control agent in liquid culture is formulation. Although the infective propagule of *E. neoaphidis* is the conidium, conidia and other spore types are not readily produced in submerged culture. For example, there has been only one report in the literature of *E. neoaphidis* producing resting spores in liquid culture (Uziel and Kenneth 1986), although the *in vitro* production of resting spores has been observed in the related species *Entomophaga maimaga* (Kogan and Hajek 2000), *Entomophthora schizophorae* and *E. muscae* (Eilenberg *et al.* 1990). Conidia of *E. neoaphidis* are also coated with a thick coating of mucous which is essential for adherence to the cuticle of the host, but makes their removal into liquid suspension difficult (Li *et al.* 1993). This makes the use of wetting agents inappropriate and makes the formulation of conidia of *E. neoaphidis* difficult. This is in contrast to a number of other, non-entomophthoralean fungi whose conidia are readily formulated. Examples of these include *Beauveria bassiana*, *Metarhizium anisopliae*, *Colletotrichum gleosporides* and *Fusarium oxysporum* (Quimby *et al.* 1999) and *Hirsutella rhossiliensis* (Lackey *et al.* 1993). One way to surmount these problems is to formulate biomass so that it sporulates after application in the field, releasing infective conidia. Currently, the favoured way to achieve this is by formulating biomass in an alginate polysaccharide polymer matrix supplemented with starch or sucrose (Shah *et al.* 1998, 1999). However, there are currently a number of technical difficulties with using algination to formulate *E. neoaphidis*, such as the need to use only filtered hyphal biomass for production of pellets (Shah *et al.* 1998). As well as these technical

difficulties, alginate *E. neoaphidis* produces only a fraction of the number of the conidia compared to unformulated biomass.

#### 1.4.3 Effect of abiotic factors on development of epizootics

*E. neoaphidis* causes major natural epizootic infections in aphids (Pickering *et al.* 1989; Feng *et al.* 1992; Wraight *et al.* 1993) and can cause aphid mortality levels of over 80% (Wilding 1975) although mortality levels of 40-50% are more common (Feng *et al.* 1991; Wraight *et al.* 1993). *E. neoaphidis* is generally the cause of the majority of aphid mycoses in the field (Elkassabary *et al.* 1992; Feng *et al.* 1992;) although this may be dependant on the predominant aphid species (Feng *et al.* 1992). However, these epizootics often occur too late in the growing season to prevent aphid damage to crops (Wilding 1981; Feng *et al.* 1991; McLeod *et al.* 1998). This is due to two main factors. The first of these is that the combination of relatively warm temperatures and high humidity needed for germination and sporulation do not occur until towards the end of the growing season. However, where low humidity is the limiting factor such as in the US, irrigation (Pickering *et al.* 1989; Wraight *et al.* 1993) or unusually high rainfall (Feng *et al.* 1991) can reduce aphid numbers before economic damage is done to the crop.

The second factor is the density of the host. *E. neoaphidis* is unlikely to be transmitted well when there is a low host density i.e at the start of growing season and epizootics are only likely when there is a relatively high density of aphids on the plant and thus *E. neoaphidis* rarely provides the level of control needed to make it an effective control agent. Feng *et al.* (1991) suggest that the development of fungal infection is more closely correlated with host density than climatic factors, but this has not been explicitly tested for *E. neoaphidis* transmission. Progression of *E. neoaphidis* through its life cycle is highly dependent on the atmospheric and local climatic and microclimatic conditions. Germination of the conidia has been shown to occur only when the relative humidity is in excess of 92% (Yendol 1968) with the optimum being 100%. Sporulation also occurs only when relative humidity is in

excess of 90% (Wilding 1969; Milner and Bourne 1983). Millstein *et al.* (1982) have shown that *E. neoaphidis* will discharge conidia only if the relative humidity exceeds 91% for a period of 3 hours and that there is a relationship between humidity hours and conidial discharge. Millstein *et al.* (1983) showed that relative humidity affects the phenology of the conidia with increased relative humidity decreasing the time taken for all stages of conidiation.

Results from field trials also imply that an increased atmospheric humidity will result in a greater spread of the fungus. This is borne out in Brittany, where insecticides have rarely been used to control aphids, as *E. neoaphidis* is transmitted well in the damp climate (Berthelem, Missonier and Robert 1969) and natural epizootics control aphid populations. Ekbohm and Pickering (1990) have also shown a positive correlation between aphid mortality induced by three species of Entomophthorales and the sum of hours of leaf wetness prior to sampling. It has also been shown (Pickering *et al.* 1989) that aphid mortality induced by *E. neoaphidis* can be increased by the use of overhead irrigation in irrigated field areas. Infectivity of conidia is also affected by humidity. Conidia of *E. neoaphidis* retain infectivity longer at 40-50% relative humidity than at higher humidities (Brobyn *et al.* 1987).

Temperature also influences the germination and sporulation of *E. neoaphidis*. At temperatures below 15°C or above 25°C, germination and sporulation rates decline rapidly although *E. neoaphidis* will sporulate to some extent at temperatures ranging from 11°C to 25°C (Sivcev and Manojlovich 1995). Temperature also plays a crucial role in the initiation and subsequent spread of infection through aphid populations. The production of primary conidia increases with temperature until an optimum temperature for conidial production is reached (Wilding 1969; Milner 1981). Although no consistent effect of temperature on the infectivity of primary conidia of *E. neoaphidis* has been proven, there is a correlation between increase in temperature and decrease in time taken for the host to die (Milner and Bourne 1983). Milner and Bourne (1983) also proposed a relationship between temperature and moisture in that, at lower temperatures, a greater period of leaf wetness is required before an effective level of transmission of *E. neoaphidis* through

an aphid population is achieved. Germination of conidia is also temperature sensitive. The optimum temperature for germination of both primary and secondary conidia is 18-20°C (Morgan *et al.* 1995). In addition, the temperature at which the conidia are produced affects their subsequent germination. Those conidia that are produced *in vitro* at non - optimal temperatures germinate more slowly than those produced at an optimal temperature (Morgan *et al.* 1995).

#### 1.4.4. Field and greenhouse trials

A number of field studies (Wilding 1981; Latteur and Godefroid 1982; Wilding *et al.* 1990) have attempted to introduce *E. neoaphidis* into field populations of aphids (notably *A. pisum* and *A. fabae*). These have met with, at best, inconclusive results. In some years, introductions of *E. neoaphidis* have led to epizootics and the resultant decrease in aphid numbers but have rarely achieved sufficient control to reduce crop damage. However, introductions in other years have failed to induce epizootics and aphid numbers have remained essentially unchanged. Infections of *E. neoaphidis* are highly dependent on climatic conditions and this unreliability may make induction of epizootics difficult.

Attempts to induce epizootics under green house conditions are less common although infection of *Macrosipum euphobiae* in green houses using formulated *E. neoaphidis* has also been induced (Shah *et al.* 2000)

Biological control of aphids by *E. neoaphidis* may be more efficient if a long-term enzootic of an aphid population can be sustained to keep the aphid population at a level below the economic injury threshold.

#### 1.4.5. Overwintering and persistence

Although the means by which *E. neoaphidis* overwinters is not certain, it has been suggested that it may survive as spherical hyphal bodies inside mycosed insect cadavers on above ground plant substrate where relative humidity is low (Feng *et al.*

1992). There have been no reports of the *in vivo* production of resting spores by *E. neoaphidis* although there has been a report of resting spore formation *in vivo* in the related species *Entomophthora muscae* (Thomsen and Eilenberg 2000) and *Entomophaga maimaga* (Hajek and Humber 1997; Hajek and Shimazu 1996). The mechanism of overwintering has implications for the induction of infection in the following year, or prediction of epizootics.

In the field, *E. neoaphidis* remains infective against *Acyrtosiphon pisum* for up to fourteen days (Brobyn, Wilding and Clark 1985). Infectivity persists longest when the conidia are protected from the effects of UV light and where the relative humidity is favourable, for example on the abaxial surfaces of lower leaves (Brobyn, Wilding and Clark 1985). Griggs *et al.* (1999) have reported that relative humidity below 95% significantly reduces the viability of conidia produced from *Zoopthora radicans* although Jaronski and Goettel (1997) have suggested that low humidity does not affect the ability of *Beauveria bassiana* to infect hosts.

Furlong and Pell (1997) reported that exposure for 4 h to levels of UV radiation found in the tropics reduced the ability of conidia from *Zoopthora radicans* to cause mycosis in the larvae of *Plutella xylostella*. Fargues *et al* (1988) have reported that conidia produced from *Nomuraea rileyi* have a decreased reduction in viability when protected from UV radiation compared to unprotected conidia. Uziel and Shtienberg (1993) reported that capilliconidia produced from *Erynia radicans* placed on the abaxial surface of leaves retained viability up to five times longer than capilliconidia placed on the adaxial surface of leaves. Consequently, it is likely that *E. neoaphidis* persists in a form that is protected from UV radiation and has a low relative humidity, but not as a resting spore.

#### 1.4.5. Interactions with other natural enemies of aphids

Much of the previous work done on the interaction between entomopathogenic fungi and other natural enemies of crop pests has focused on interactions between fungi and parasitoids and the susceptibility of coccinellid predators to fungal infection by entomopathogenic fungi. For a comprehensive review

of interactions between entomopathogenic fungi and other natural enemies, the reader is directed to Roy and Pell (2000).

Furlong and Pell (2000) have reported that infection of *Plutella xylostella* larvae by *Zoophthora radicans* results in rejection of larval hosts by the parasitoids *Diagdema semiclausum* and *Cotella plutellae*. As well as this, infection of *D. semiclausum* adults results in the significant reduction in the numbers of parasitoid cocoons subsequently developing from the host larvae. Similarly, the entomopathogenic fungus *Nomurea rileyi* inhibits the development of the parasitoid *Microliptes croceipes* if the larvae of the host (*Heliothis zea*) are infected one day after parasitisation. Fransen and van Lenteren (1993) reported that there is a significant reduction in oviposition from the parasitoid *Encarsia formosa* in the whitefly *Trialeurodes vaporariorum* when the host has previously been infected with the fungus *Aschersonia aleyrodis* compared to controls. However, it has also been reported that foraging *D. semiclausum* females enhance the level of *Z. radicans* infection in *P. xylostella*.

High mortality in the ladybird *Hippodamia convergens* has been reported after inoculation with *Meterhizium anisopliae*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* whereas there was virtually no mortality when *H. convergens* was inoculated with *Nomurea rileyi* (James and Lighthart 1994). Magalhaes *et al.* (1988) also found the coccinellid predators *Coleomegilla maculata* and *Eriopsis connexa* to be highly susceptible to *B. bassiana*, but not to *Zoophthora radicans*. The difference in susceptibility of the coccinellids to the fungus is thought to be due to the differing host ranges of the fungi.

There have been few studies on the interactions between *E. neoaphidis* and other natural enemies of aphids but broadly, these interactions can be grouped into antagonistic (negative) and synergistic (or positive) interactions

Powell *et al.* (1986) showed that there is interference between *E. neoaphidis* and the hymenopteran parasitoid *Aphidius rhopalosiphi* De Stephani Perez. It was shown that once either the parasitoid or the fungus had become established within a host then the development of the other was impaired. *A. rhopalosiphi* also avoids

ovipositing in aphid hosts that are in the last stages of infection from *E. neoaphidis* (Brobyn *et al* 1988). However, in the field, there may be spatial and temporal differences in the occurrence of these competitors that prevent antagonistic interactions.

Pell *et al.* (1997) have shown that *Coccinella septempunctata* was shown to feed on aphid cadavers infected with *E. neoaphidis*. This has the possibility of retarding possible epizootics in the field. In the laboratory, coccinellid larvae and adults feed preferentially on uninfected aphids (Roy *et al.* 1998; Pell *et al.* 1997). It is therefore unlikely that *C. septempunctata* feeds preferentially on infected aphids in the field, probably impacting little on the spread of epizootics. Damage to sporulating aphids from predator foraging reduces the number of conidia produced, although the transmission levels resulting from damaged cadavers are comparable to those from intact cadavers (Roy *et al.* 1998).

Unlike some other species of entomopathogenic fungi, especially the Hyphomycetes, *E. neoaphidis* is highly host specific and as yet has not been recorded infecting other natural enemies of aphids outside of the laboratory.

The seven spot ladybird, *Coccinella septempunctata* has been shown to act as a vector of conidia of *E. neoaphidis* to susceptible aphids in laboratory conditions (Pell *et al.* 1997; Roy *et al.* 1998; Roy *et al.* 2001). Transmission of *E. neoaphidis* was significantly increased in the presence of parasitoids in a population of *Sitobion avenae* (Fuentes-Contreras *et al.* 1998). Increased transmission of fungal pathogens has also been seen with foraging *Diagdema semiclausum* enhancing the spread of *Zoopthora radicans* in a population of *Plutella xylostella* larvae (Furlong and Pell 1996). Foraging ladybirds also cause an increase in aphid movement. How this affects the transmission of *E. neoaphidis* is not yet known, but it is likely that it would result in increased transmission of the fungus.

As well as interactions between *E. neoaphidis* and other natural enemies of aphids there are interactions between *E. neoaphidis* and non-susceptible species of aphid. The aphid *Acyrtosiphon kondoi* Shinji may act as a reservoir for *E.*



*neoaphidis*, which can then infect the more susceptible *A. pisum* (Pickering and Gutierrez 1991).

No work has been done on the interactions of *E. neoaphidis* with other aphid pathogens, fungal or otherwise. An aphid pathogenic virus has been recently identified and characterised (van den Heuval et al. 1997) and interactions between this and *E. neoaphidis* should be studied.

#### **1.4 Aims**

The aims of this study were:

- a) to determine the relative production of conidia from *in vivo* and *in vitro* sources;
- b) to determine the relative production of conidia from hyphal pellets harvested from different stages of growth in liquid culture;
- c) to evaluate the effects of relative humidity on the survival of *E. neoaphidis*, production of conidia and infectivity of conidia from *E. neoaphidis*;
- d) to determine the effects of various pesticides on the radial growth of *E. neoaphidis in vitro* and the effects of various pesticides on the production of conidia *in vivo*;
- e) to find the optimal position of inoculum on the plant to cause the greatest possible mortality in an aphid population;
- f) to evaluate the effects of host population density on the transmission of the fungus under laboratory conditions and in the field;
- g) to determine the effects of cadaver density on the transmission of the fungus in the laboratory and in the field;
- h) to evaluate whether *E. neoaphidis* is able to readily move from host plant to host plant in the absence of insect vectors;
- i) to produce a defined medium for the production of biomass in liquid culture.

## 2. Methods and materials

### 2.1 Cultivation and storage of fungus

#### 2.1.1 Fungal isolate

*Erynia neoaphidis* isolate X4 was isolated in the early to mid 1970's, probably from *Acyrtosiphon pisum* on lucerne (*Medicago sativa*), by Neil Wilding (IACR Rothamsted) and has been maintained since *in vivo* by passaging through *A. pisum* at IACR-Rothamsted (J. Pell pers. comm). This strain was re-isolated on to agar medium from reared *A. pisum* by placing an infected apterous *A. pisum* adult on a Sabouraud Dextrose egg and milk agar (SEMA) (Wilding and Brobyn 1980) plate and allowing the fungus to sporulate and grow. After a period of approximately 4 weeks, sections (7-10mm<sup>2</sup>) were removed aseptically from the growing edge of the resulting colony and placed in plastic straw sachets along with an 80% w/v glycerol solution, which acted as a cryoprotectant. These sachets were then placed in liquid nitrogen for storage (Mardell 1986). The reisolated strain was named NW327.

#### 2.1.2 Solid cultures

The strain was maintained by regular subculturing every four to six weeks on SEMA, or Semi-defined *Erynia* Medium (SDEM) containing 0.01% v/v oleic acid (Gray *et al.* 1990). Plates were inoculated by cutting a 10mm plug with a flame sterilised cork borer from the growing edge of the donor colony and placing the plug in the centre of the recipient plate. The plates were sealed with laboratory sealing film (Whatman, Maidstone, Kent, UK) and incubated in the dark at 20° C. Periodically, (every five or six subcultures), samples of NW327 were removed from nitrogen storage and plated out on SEMA to start a new line of subcultures. This was done to avoid the loss in ability to produce conidia that can occur when *E. neoaphidis* has been grown *in vitro* for an extended period of time (Gray 1990).

### 2.1.3 Liquid cultures

Liquid cultures of *E. neoaphidis* were obtained through a two step process. In the first, preculture, step, ten 10mm diameter mycelial plugs, cut from the leading edge of a plate culture using a flame sterilised cork borer, were used to inoculate 25 ml of medium in a 250 ml Erlenmeyer flask. These flasks were incubated for four days on an Innova 4330 rotary shaker (New Brunswick Scientific, Edison, New Jersey, USA) in a 2.5 cm circular orbit at 200 rpm and 20°C. In the second step, the mycelium was removed from the agar plugs so that subsequent cultures could be free of agar. This was done by repeatedly pipetting the culture up and down in a 10 ml sterile serological pipette. A 5 ml aliquot of the culture was then removed using a sterile 10 ml serological pipette and used to inoculate 20 ml fresh medium in a 250 ml flask. This produced cultures free from agar pellets. The cultures were then incubated for a further four days on the rotary shaker at 200 rpm and 20°C.

A later modification of this method was to use smaller flasks and different volumes of medium. In the first step, plugs were used to inoculate 30 ml of medium in a 100 ml flask. After incubation, a 6 ml aliquot was removed as described above and used to inoculate 24 ml of fresh medium in a 100 ml flask, which was then incubated as described above. The method was modified for two reasons. The first was that it was felt that using a larger volume of medium in a smaller flask would result in less wall growth in the flasks. The second reason is that it was possible to carry out more experimental treatments simultaneously using smaller flasks. This modified method was used for all further experimental treatments. Growth curves were obtained by taking duplicate 10 ml samples using sterile 10 ml pipettes from each of three replicate flasks at each time interval and obtaining the dry weight of biomass as described in section 2.4.2.

## 2.2 Media

### 2.2.1 Semi defined *Erynia* medium (SDEM)

SDEM was made up as described previously by Gray *et al* (1990). All chemicals were analytical grade unless otherwise specified. SDEM contained, per litre of distilled water: 16g glucose (BDH, GPR grade), 5g mycological peptone (Oxoid limited); 3g yeast extract; 5.97g  $\text{KH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$ ; 2.2g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ; 0.01% v/v oleic acid. Oleic acid (free acid, 95%, Sigma-Aldrich, Poole, Dorset, UK) was prepared as a 10% v/v solution in 1.3% w/v sodium hydroxide, filter sterilised and added when the temperature of the newly sterilised medium had dropped to 50°C. To neutralise the sodium hydroxide, 0.65 ml of 1.0M  $\text{KH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$  per ml of oleic acid solution was added to the bulk of the medium prior to autoclaving at 121°C for 15 minutes.

### 2.2.2 Sabouraud dextrose egg milk agar (SEMA)

SEMA was made as described by Wilding and Brobyn (1980). Large free range eggs were surface sterilised for 2-24 hours in a solution consisting of: 940ml of ethanol, 10 ml acetone and 50 ml distilled water. The eggs were then flame sterilised and a yolk added to a previously autoclaved (121°C for 15 minutes) 500 ml Duran bottle. Semi-skimmed milk (17 ml) that had been autoclaved at 121°C for 15 min was added to the yolk and the Duran bottle vigorously shaken to mix the two. Sabouraud dextrose agar (160 ml; 6.5% w/v) that had previously been autoclaved at 121°C for 15 minutes was then added when it had cooled to approximately 50°C.

### 2.2.3 Development of a defined medium for the growth of *E. neoaphidis*

Several media were evaluated in order to identify the components needed for the growth of *E. neoaphidis*. Liquid cultures were grown in a two step process as outlined in section 2.1.3, using the particular test medium for both stages, and dry biomass production was assessed as outlined in section 2.4.2. Biomass production in the different media was compared to production in SDEM as a standard. SDEM was chosen as the standard, since the components of the medium can be standardised,

unlike other media known to support the growth of *E. neoaphidis* that contain, for example, eggs and milk. A medium was judged to have supported growth successfully if there was no significant reduction in the biomass concentration compared to SDEM in the second liquid culture. The compositions of the media were as follows. All quantities are per litre of distilled water.

**YEDM** (Yeast extract deficient medium) 16g glucose; 5g mycological peptone; 5.97g potassium dihydrogen orthophosphate; 2.2 g disodium hydrogen orthophosphate 12-hydrate; 0.01% v/v oleic acid.

**YEDMTH** (Yeast extract deficient medium plus thiamine hydrochloride). As YEDM with the addition of: 50 mg thiamine hydrochloride (Vitamin B<sub>1</sub>). Thiamine hydrochloride was prepared as a 5% w/v solution in distilled water, sterilised by passing through a filter (0.2 µm pore size, Nalgene) and added when the temperature of the autoclaved medium had fallen to 50° C.

**YEDMVB** (Yeast extract deficient medium plus B vitamins). As YEDM with the addition of: 63µg thiamine hydrochloride; 375µg riboflavin; 315 µg pantothenic acid; 1875µg pyridoxine; 105µg cyanocobalamin. The vitamins were prepared as a 5% w/v solution in distilled water, sterilised by passing through a filter (0.2 µm pore size, Nalgene) and added when the temperature of the autoclaved medium had fallen to 50° C.

**YEDMVB5** (Yeast extract deficient medium plus B vitamins at 5 times the concentration of YEDMVB) As YEDM with the addition of 315µg thiamine hydrochloride; 1875µg riboflavin; 1575 µg pantothenic acid; 9375µg pyridoxine; 525µg cyanocobalamin.

**PDM** (Peptone deficient medium) 16g glucose; 3 g yeast extract ; 5.97g potassium dihydrogen orthophosphate; 2.2 g disodium hydrogen orthophosphate 12-hydrate; 0.01% v/v oleic acid.

**PDMTH** (Peptone deficient medium plus thiamine hydrochloride). As PDM with the addition of 50 mg thiamine hydrochloride (Vitamin B<sub>1</sub>). Thiamine hydrochloride was prepared and added to the medium as described above.

**PDMVB** (Peptone deficient medium plus B vitamins) As PDM with the addition of: 63µg thiamine hydrochloride; 375µg riboflavin; 315 µg pantothenic acid; 1875µg pyridoxine; 105µg cyanocobalamin.

**PDMG** (Peptone deficient medium plus Grace's insect medium) As PDM with the addition of 10% v/v Grace's insect tissue medium (Sigma-Aldrich.). PDM was made up with 90% of the usual volume of water and sterilised by autoclaving at 121°C for 15 minutes. Grace's Insect Medium (GIM) was made up according to the manufacturer's instructions and sterilised by passing through a 0.2 µm cellulose acetate filter (Corning ). When the bulk of the medium had cooled to room temperature, the required quantity of GIM was added aseptically.

**GIM** (Grace's Insect Medium). Grace's Insect Medium (GIM) was made up according to the manufacturer's instructions and sterilised by passing through a 0.2 µm cellulose acetate filter (Corning).

**GIMO** (Grace's Insect Medium plus oleic acid). GIM was made up as described above and 0.01%v/v oleic acid added as described above.

**GASP** (GIMO plus L-asparagine). GIMO was made up as described above, L-asparagine added at 4.2 g l<sup>-1</sup> and the medium sterilised by passing through a 0.2µm cellulose acetate filter (Corning).

**TM** (Test medium) 10g glucose (BDH, GPR grade), 2g mycological peptone (Oxoid limited); 0.6g potassium dihydrogen orthophosphate; 0.22g disodium hydrogen orthophosphate 12-hydrate; 0.01% v/v oleic acid. TM was used as it was similar to the medium used to culture *E. neoaphidis* in chemostat culture by Gray and Markham (1997).

**TMTH** (Test medium plus thiamine hydrochloride) As TM with the addition of 50 mg thiamine hydrochloride (Vitamin B<sub>1</sub>). Thiamine hydrochloride was prepared and added as described above.

The pH of all media was between 5.8 and 6.3 after autoclaving.

A summary of the content of each medium is given in Table 2.1

Table 2.1 A summary of the constituents of various media used in the development of a defined medium for the growth of *Erynia neoaphidis*.

Medium	Glucose	Mycological peptone	Yeast extract	Oleic acid	Grace's medium	B Vitamins	Other information
<b>SDEM</b>	Yes	Yes	Yes	Yes	No	No	
<b>YEDM</b>	Yes	Yes	No	Yes	No	No	
<b>YEDMTH</b>	Yes	Yes	No	Yes	No	Yes	
<b>YEDMVB</b>	Yes	Yes	No	Yes	No	Yes	
<b>YEDMVB5</b>	Yes	Yes	No	Yes	No	Yes	
<b>PDM</b>	Yes	No	Yes	Yes	No	No	
<b>PDMTH</b>	Yes	No	Yes	Yes	No	Yes	
<b>PDMVB</b>	Yes	No	Yes	Yes	No	Yes	
<b>PDMG</b>	Yes	No	Yes	Yes	Yes	No	
<b>GIM</b>	No	No	No	No	Yes	No	Sucrose as C source
<b>GIMO</b>	No	No	No	Yes	Yes	No	Sucrose as C source
<b>GASP</b>	No	No	No	Yes	Yes	No	Sucrose as C source plus L-arginine
<b>TM</b>	Yes	Yes	No	Yes	No	No	
<b>TMTH</b>	Yes	Yes	No	Yes	No	No	

## **2.3 Fermenter culture**

### **2.3.1 Culture conditions**

The apparatus used was based on a 2 l Electrolab modular system (Electrolab, Tewkesbury, UK). The configuration used a two litre vessel which was agitated via direct drive at 350-400 rpm to twin impellers mounted on a single shaft with a Grant thermocirculator providing temperature control through an external water jacket. An external jacket was used in preference to an internal heat exchanger as it eliminated wall growth that might occur on a heat exchanger. This configuration included a pH probe and a polarographic type electrode (Mettler Toledo Ltd, Leicester) for continuous monitoring of pH and dissolved oxygen concentration inside the fermenter. No pH control was applied other than that exerted by the buffer in the medium.

Aeration was at the rate of  $1 \text{ l min}^{-1} \text{ l}^{-1}$  of medium. Filters (PTFE  $0.2\mu\text{m}$  pore size, Whatman, Maidstone, UK) were attached to all air input and output ports. To calibrate the  $\text{O}_2$  probe, SDEM medium was aerated for approximately 6 h to ensure that the medium was saturated with  $\text{O}_2$ . The probe was then calibrated to record 100%  $\text{O}_2$  saturation at  $20^\circ\text{C}$  and therefore,  $\text{O}_2$  levels were measured in percentage saturation. Temperature within the vessel was measured using a temperature probe (Tinytalk, RS components, Corby, Northants).

### **2.3.2 Preparation of equipment**

Two litres of SDEM medium was prepared as described above and sterilised with the fermentation apparatus by autoclaving for 40 min at  $121^\circ\text{C}$ . The increased sterilisation time compared to shake flasks was needed due to the increased volume of medium. Once the medium and fermenter had cooled to room temperature, they were placed in Class 2 microbiological cabinet and the oleic acid added as described in section 2.2.1. The inside of the vessel was thereafter kept at a slight positive pressure to reduce the likelihood of contamination and all medium transfers and samplings were performed using positive pressure rather than vacuum for the same



reason. Inoculation and sampling were performed using the same port. To inoculate the fermenter, filtered air was passed into the inoculation vessel, creating a positive pressure that pushed the inoculum into the fermenter. To take a sample from the fermenter, the air outlet from the reaction vessel was closed creating a positive pressure in the reaction vessel causing inoculum to be forced into the sampling vessel.

### 2.3.3 Preparation of inoculum and inoculation

Cultures used to inoculate the fermenter were grown in shake flasks using the method described in 2.1.3. Prior to inoculation of the fermenter, cultures were examined microscopically to ensure that they were not contaminated and that there was a reasonable concentration of healthy material. The cultures were then transferred aseptically to individual Universal bottles in a Class two microbiological flow cabinet and inoculated into the fermenter via the sample port by applying positive pressure to the bottles containing the inoculum.

### 2.3.4 Fermenter growth of *E. neoaphidis*

*E. neoaphidis* was grown in fermenter culture in order to produce biomass for various experiments and also to assess the feasibility of growing it in sufficient quantities for field trials. The fermenter was set up as described above and inoculated with the pelleted biomass from the second stage of growth in shake flask culture as described in section 2.1.3. The inoculum was pipetted up and down repeatedly in a sterile 10 ml serological pipette in order to break up the pellets so that they would fit in through the inoculation port. The fermenter conditions were as described in 2.3.1, using SDEM (Gray *et al.* 1990) containing 0.01% v/v oleic acid as the medium. The fermenter was maintained for 240 h after inoculation. The fermenter culture was checked for contamination by daily microscopic examination of samples. The fermenter was initially operated under batch conditions using, when possible, an inoculum of at least one fifth of the working volume (175-300 ml) The volume of fresh medium initially pumped into the vessel was kept to a minimum (800ml to 1000ml) and cultures were maintained in batch mode until the biomass concentration was

approximately  $3\text{g l}^{-1}$ . The operation was then switched to discontinuous fed batch. An aliquot of fresh medium (400 ml) was added at 96 h intervals from the reservoir by means of a peristaltic pump. Triplicate 10 ml samples were taken at 24 h intervals, the dry weight of biomass determined as described in 2.4.2. All samples were taken prior to any addition of fresh medium. Biomass, again in the form of pellets was harvested 96 h after inoculation, when the culture was estimated to be during the exponential phase of growth on the basis of growth curves derived from shake flask experiments. Culture was harvested using the same method as for sampling (section 2.3.2). Approximately 40 ml of the culture was harvested at any one time. Where required, the pellets of biomass were separated from the medium by passing the broth through a sterilised brass sieve (autoclaved at  $121^{\circ}\text{C}$  for 15 mins) with a  $250\mu\text{m}$  mesh. Residual glucose was not measured in fermenter samples. The pH of the culture was checked on a twice daily basis, as was the oxygen saturation and temperature of the culture.

## **2.4 Measurement of growth**

### **2.4.1 Colony radial growth rate**

Measurement of colony radial growth rates ( $K_r$ ) (Pirt 1967) was performed by adaptation of the method of Trinci (1969) detailed by Gray *et al* (1990). The first measurement of the colony was taken when the colony was at least 5 days old, as initial growth from inoculation with plugs is variable. Colony radius was determined by calculation from the mean of two perpendicular measurements of the colony diameter. The increase in colony radius from that point was then plotted against time.  $K_r$  was calculated by linear regression as the slope of this graph. At least 6 replicate colonies were measured on at least 6 successive occasions to determine each  $K_r$  value.

#### 2.4.2 Dry weight

The dry weight of biomass in liquid culture was determined by vacuum filtration of 10 ml samples through preweighed fibreglass filters (GFC, 47mm diameter, 0.2µm pore size, Whatman). The filtrate was removed and stored at -20°C pending glucose or sucrose assays. The biomass was washed twice on the filter with 10 ml of distilled water, dried overnight at 105°C then placed in a dessicator. Daily readings of the mass were taken until a constant mass was recorded.

#### 2.4.3 Optical density

Visible range, fixed wavelength optical density measurements were made of samples of the fungal culture in a quartz glass cuvette at a wavelength of 520 nm using a Cecil 2021 spectrophotometer.

#### 2.4.4 Residual glucose concentration

Glucose concentration in the filtrate from the biomass samples was determined using a commercially available kit (Glucose (GO) assay kit, Sigma Aldrich, Poole, UK) The assays were carried out according to the manufacturer's instructions.

#### 2.4.5 Residual sucrose concentration

Sucrose concentration in the filtrate from samples of culture grown in Grace's Insect Medium was determined by using a commercially available kit (Sucrose assay kit Sigma Aldrich, Poole, UK) The assays were carried out according to the manufacturer's instructions.

#### 2.4.6 Specific growth rate

Specific growth rate was calculated using the method of Pirt (1975) as detailed in Gray and Markham (1997)

## **2.5 Aphid husbandry**

### **2.5.1 Initiation and maintenance of aphid colony**

Dwarf broad beans (*Vicia faba* var. "The Sutton") were planted five per 12.5cm plastic plant pot in multi-purpose soil-less potting compost (B&Q, Eastleigh, Hants, UK) and grown at 20°C, ambient humidity and a 16h:8h (light: dark) photoperiod using fluorescent tube lighting. This method is regularly used at IACR-Rothamsted. Each plant was watered with 200-400 ml tap water every two or three days. When the plants were three weeks old (approximately 10-15 cm tall), four pots were placed under a n "aphid cage". This aphid cage is a transparent perspex box (280mm x 380mm x 390mm) to contain the aphids and allow for photosynthesis of the host plants, which was ventilated by an extraction fan (5v brushless miniature cooling fan RS Components, Corby, Northants UK) which prevented the build up of excessive moisture. The box was then placed on capillary matting on a large, shallow plastic tray to enable watering of the plants without disturbing the aphids. Pea aphids (*Acyrtosiphon pisum*) were initially obtained from IACR-Rothamsted. Aphids were placed on sections of excised bean plant for transportation and were then introduced to the potted plants by placing the excised plants that had supported the aphids on to the soil at the base of the plants inside the box. Once the aphids had become established, and had started to produce nymphs, two of the four pots of beans within the cage were replaced alternately every three to four days to maintain healthy food plants for the aphids. In this way, a viable colony could be maintained indefinitely. This method is in current use at IACR-Rothamsted.

### **2.5.2 Production aphids of known age**

Leaves from the first or second leaf pair were cut from three week old Broad Bean (*Vicia faba* var. "The Sutton") plants. Depending on the size of individual leaves, one or two leaves were set in 2 % w/v technical agar (Sigma-Aldrich, Poole, Dorset, UK) in 90mm Petri dishes. The agar had been sterilised by autoclaving it at 121°C for 15 minutes and the leaves placed on the agar when the agar was had nearly

solidified with the abaxial surface uppermost. Ten adult apterous aphids per Petri dish were then placed on the leaves and the dishes inverted so the surfaces of the leaves were in the orientation that they would be on an intact plant. The dishes were then incubated at 20°C with a 16h:8h (light:dark) photo period and a ambient humidity for 24h to allow the adults to produce nymphs over this limited time period. The adults were then removed from the leaf, leaving nymphs aged 0h to 24h, and the leaves removed from the agar and placed at the bottom of single caged bean plants (5 to 6 leaves per plant). The nymphs were then able to move on to the intact bean plants when the excised leaves dried out. The plants were kept at 20°C with a 16h:8h (light:dark) photoperiod with ambient humidity for 10 days after which the aphids were removed from the bean plants. All of the aphids were then 1-3 day old adults, as described in section 1.3.5.

### 2.5.3 Production of mycosed aphid cadavers

Five dried mycosed aphid cadavers (Fig 2.3) were placed on damp Standard Grade 3 filter paper (90mm diameter; Whatman, Maidstone, Kent, UK ) in a non sterile 90mm Petri dish overnight at 20°C, allowing the fungus to sporulate profusely. The moistened cadavers were then placed in a sample tube lid that contained 1% w/v water agar that had been sterilised by autoclaving at 121°C for 15 min and cooled to approximately 50°C prior to pouring. Approximately 50 non-infected aphids were then placed in the sample tube and the lid, containing the sporulating cadavers, placed on top. This was incubated at 20°C for 3 h to allow the aphids to become inoculated by conidia discharged from the cadavers. Relative humidity was approximately 100% due to the presence of the water agar in the sealed tube

The inoculated aphids were then placed on three-week-old broad bean (*Vicia faba* var. "The Sutton") plants, which had been grown in seed trays (45 cm x 25 cm x 5 cm) (15 per tray) and repotted singly into 7.5cm diameter plastic plant pots. The plants were then placed under polypropylene 500ml beer glasses (Tesco PLC, Cheshunt, Herts, UK). The bases of the beer glasses had been removed and covered in polyvinylchloride cling film (Tesco PLC) in order to maintain a high humidity inside the beer glass, allowing the fungus to germinate. After 24 h, the cling film was

replaced by gauze. The aphids were left on the plant until the tip of the abdomen had started to turn brown, which was taken as the first sign of infection. The aphids were then carefully removed from the plant using a small paintbrush and placed in a 10ml glass sample tube (Merck). The centre portion of the lid of the tube had been cut away and replaced with a wire mesh (mesh size 0.5mm) to allow gaseous transfer with the environment. The tube was then placed in a dessiccator containing 100ml of a 90.05% w/v glycerol solution to give a relative humidity of 20% (Johnson 1940). The cadavers were then stored at 4°C until use, but for no longer than 4 weeks. This method is in regular use at IACR-Rothamsted.

## **2.6 Effect of pesticides on growth and sporulation of *E. neoaphidis***

The effects of eleven pesticides on the growth and sporulation of *E. neoaphidis* were tested. The pesticides included the two fungicides, azoxystrobin and chlorothalonil; four insecticides, cypermethrin, deltamethrin, chlorpyrifos, and two formulations of  $\lambda$ -cyhalothrin; and four herbicides, paraquat, glyphosate, fluzifop-p-butyl and fomesafen. In addition, the effect of the insecticide pirimicarb on radial growth was tested. The pesticides used included compounds that are widely used in the UK to control a number of plant, fungal and insect (especially aphid) pests.

### **2.6.1 Effect on radial growth rate**

The pesticides were incorporated into SEMA medium prepared as follows: SDA was dissolved in 70% of the usual quantity of water (i.e. 112 ml for 10 plates), autoclaved and kept at 56°C. The remaining 30% of the water was used to dissolve the pesticide. The pesticide solution was then added to the egg and milk prepared as described in section 2.2.2 and the SDA added when it had cooled to approximately 50°C. SEMA was used as a control. It was then assessed for all treatments and controls. SEMA was used instead of SDEM as SEMA has been used in previous studies of the effect of pesticides on radial growth of *E. neoaphidis* (Lagnaoui and Radcliffe 1998)

The final concentrations of the pesticides in the agar was the mid point of the range of concentrations recommended by the manufacturer for aqueous solutions to be applied as a foliar spray (Table 2.2). All pesticides were obtained from Zeneca (Syngenta) Agrochemicals, Bracknell, UK.

Table 2.2 Pesticides and concentrations used in radial growth and sporulation experiments. [SC = suspension concentrate; EC = emulsifiable concentrate; EW = oil in water emulsion; SL = soluble concentrate; WG = wettable granule]

<b>Pesticide Category</b>	<b>Pesticide</b>	<b>Trade name</b>	<b>Formulation</b>	<b>Recommended concentration (mg active ingredient l<sup>-1</sup>)</b>
<b>Fungicide</b>	Azoxystrobin	Amistar	SC	1250
	Chlorothalonil	Bravo	SC	7500
<b>Herbicide</b>	Glyphosate	Touchdown	liquid	7500
	Paraquat	Roundup	liquid	3500
	Fluazifop-p-butyl	Flex	EW	2125
	Fomesafen	Fusilade	SL	1250
<b>Insecticide</b>	Cypermethrin	Cythrine	EC	125
	Deltamethrin	Decis	EC	37.5
	λ-Cyhalothrin	Karate	SC	125
	λ-Cyhalothrin	Karate	EC	1500
	Chlorpyrifos	Dursban 4	EC	1000
	Pirimicarb	Aphox	EC	1500



## 2.6.2. Effects of pesticides on sporulation of *E. neoaphidis*

Three different treatments with pesticides were tested: treatment of aphids with pesticide prior to infection with fungus ("pre-inoculation"); treatment of aphids with pesticide after infection with fungus ("post-inoculation") and treatment of mycosed aphid cadavers. Pesticide solutions were made up by dissolving pesticide in 25 ml sterilised distilled water so that the resulting solution was equivalent to the recommended field application rates (Table 2.2).

### 2.6.2.1 Effect of treatment of aphids with pesticide prior to inoculation.

Ten 1-3 day old apterous adult aphids per pesticide treatment were immersed for 15 s in pesticide solution 4h, 48h and 72h prior to inoculation with fungus. Immersion was achieved by gently holding the aphids in the pesticide solution with a pair of tweezers. Immediately after immersion, the aphids were placed on filter paper to remove excess pesticide. Aphids that had been dipped in pesticide 48h and 72h prior to infection were then placed on caged bean plants, prepared as in section 2.5.3, and monitored for mortality prior to inoculation. The aphids that were dipped in pesticide 4h prior to infection were placed on excised bean leaves prior to infection. Aphids placed on excised bean leaves are easier to handle and recover than those placed on whole plants. However, aphids placed on excised leaves for more than 12 h exhibit starvation stress and may die. The aphids were then removed from the plant or bean leaves, inoculated with *E. neoaphidis* as described in section 2.5.3 and replaced on clean, caged bean plants. The aphids were monitored daily for infection, and removed from the plant, using a small paintbrush, when the tip of the aphid's abdomen had started to turn brown, which is the first sign of infection. They were then dried for two days in a dessiccator at 20% relative humidity and 4°C as described in section 2.5.3. The cadavers were then placed on the sporulation monitor (section 2.8.1) for assessment of the rate of production of conidia.

### 2.6.2.2 Effect of treatment of aphids with pesticides after inoculation .

One to three day old apterous aphids were inoculated with *E. neoaphidis* (as described in section 2.5.3.) and then immersed in pesticide solution 4 h, 48 h and 72h

later. Aphids that had been immersed in pesticide solution 48h and 72h after inoculation were placed on caged bean plants, prepared as in section 2.5.3, and monitored for mortality prior to immersion. The aphids that were immersed 4h after inoculation were placed on excised bean leaves prior to immersion. The aphids were then removed from the plant or bean leaves and immersed in pesticide solution as described above. The aphids were then placed on clean bean plants and monitored daily for infection. The aphids were removed from the plant, using a small paintbrush, when the tip of the aphid's abdomen had started to turn brown, which is the first sign of infection. They were then dried for two days in a dessiccator at 20% relative humidity and 4°C as described in section 2.5.3. The cadavers were then placed on the sporulation monitor (section 2.8.1) for assessment of the rate of production of conidia.

#### *2.6.2.3 Effect of treatment of mycosed aphid cadavers with pesticides*

Mycosed aphid cadavers were produced as described in section 2.5.3. Ten cadavers were then dipped in each of the pesticide solutions for 15 seconds as described above and placed on filter paper (Standard grade 3, Whatman) to remove excess pesticide. The cadavers were then incubated for 1 h at 20°C and placed on the sporulation monitor for assessment of the rate of production of conidia

### **2.7 Assessment of sporulation**

#### 2.7.1 The sporulation monitor

The sporulation monitor (Fig 2.1) was developed for the assessment of sporulation of Entomophthoralean fungi by Pell *et al.* (1998). Sporulating host cadavers are suspended over a rotating drum to which is attached an acetate sheet. Conidia are discharged from the host cadavers and adhere to the acetate sheet where, over time, a "conidia trail" is formed.

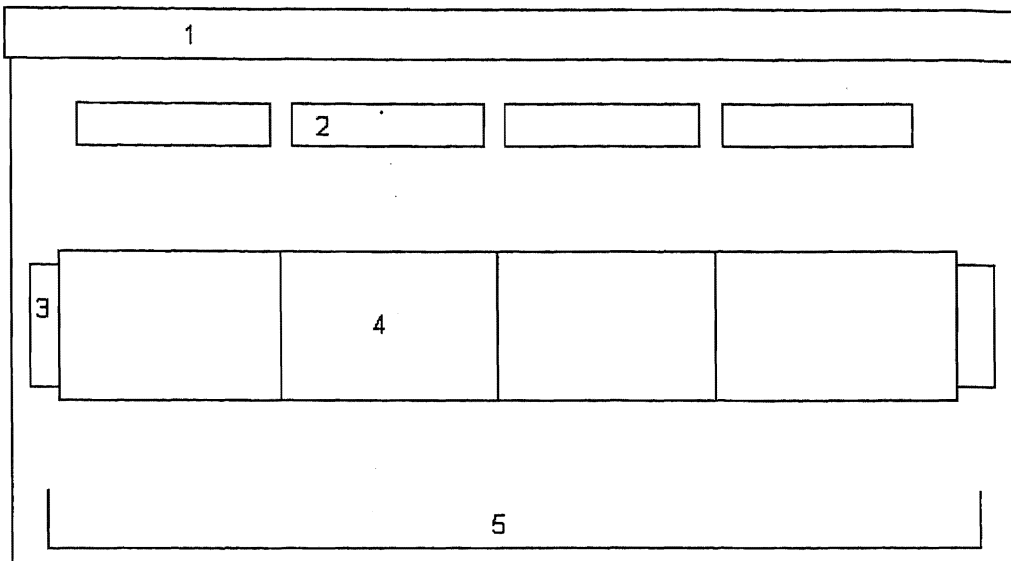


Figure 2.1. Diagrammatic representation of the sporulation monitor used to assess production of conidia. Key: 1 - detachable lid; 2 – perspex block holding inoculum source; 3 – rotating drum; 4 – acetate sheets attached to drum; 5 - water reservoir to maintain humidity levels.

Cadavers that had been stored at 4°C at 20% RH were placed on a damp filter paper in the base of a 90mm Petri dish and incubated at 20°C for 1 h prior to being placed on the sporulation monitor. This allowed the cadavers to equilibrate with the ambient atmospheric conditions. Approximately 0.75 ml of 1% w/v agar was loaded into each of 10 circular wells in a Perspex block. The inoculum source was then placed onto the agar just before it had completely set. A nylon slit mask was then attached to the block so that the slits were positioned over the inoculum source so that conidia could be ejected through the slits. The block containing the inoculum was then suspended in an inverted position over an acetate sheet, which was mounted on a revolving drum. The apparatus was maintained at 20°C, 100% relative humidity and a 16h: 8h (light: dark) photoperiod. Discharged conidia adhered to the sheet due to the presence of naturally preformed mucus coating each conidium. The drum was set to rotate once every 168h or once every 72h depending on the gearing used. Conidia adhering to the acetate formed a trail on the acetate sheet as the drum

rotated. After incubation the acetate sheet was removed from the sporulation monitor and the acetate sheet was cut into strips, with each strip containing a single trail of conidia that corresponded to a single inoculum source. The individual strips were then mounted on a thin transparent Perspex slab, which had transverse lines etched into it at equidistant intervals corresponding to the distance that the drum had rotated in an hour. The numbers of conidia present were then counted under a light microscope, either manually or with the aid of an image analysis system (section 2.7.2). Only conidia that were contained within the area of an eyepiece graticle ( $0.3072 \text{ mm}^2$ ) were counted. Ten contiguous areas were counted in a transverse line across the conidial trail in the region that was within the last hour of each five-hour section, for example between hours 4 and 5, hours 9 and 10, hours 14 and 15 etc. It was observed that the production of conidia varied little within each 5h segment. Each transverse line was one third of the width of a 1 h segment (Figure 2.2) and so this line was a representative 20 minute sample of a 5 h segment. The number of conidia counted in this section could then be converted to number of conidia discharged over an area of  $1 \text{ mm}^2$  or the number of conidia discharged per hour. To calculate the number of conidia discharged per hour, the number of conidia counted in the ten fields of view was simply multiplied by three. To calculate the number of conidia discharged over  $1 \text{ mm}^2$ , the number of conidia counted in the 10 fields of view was divided by 3.072 (the area of 10 fields of view in  $\text{mm}^2$ ).

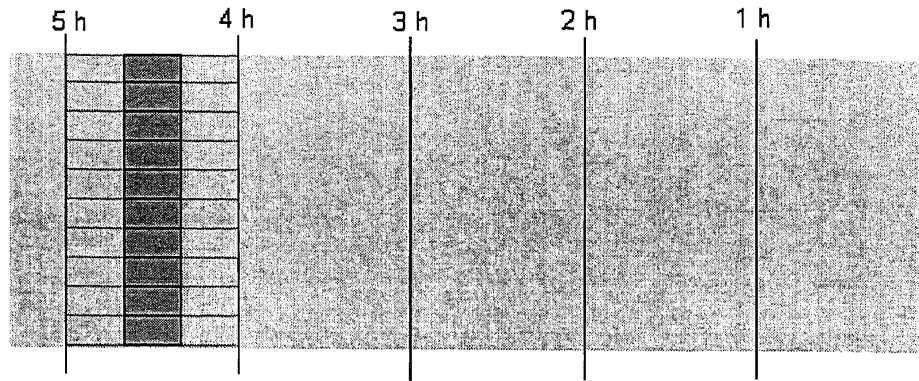


Figure 2.2. Diagrammatic representation of the “conidial trail” from the sporulation monitor showing the transverse section across the conidial trail (■)

## 2.7.2 Image analysis

### 2.7.2.1 Analysis of conidia

Conidia were visualised through an Olympus BH-2 light microscope at x100 magnification, and a digital image of the conidia was captured using a JVC TK-1280E colour video camera at 640x480 pixels, at a resolution of 1 pixel  $\mu\text{m}^{-1}$ . Image analysis was carried out using the public domain NIH Image program developed at the US National Institutes for Health and available on the Internet at <http://rsb.info.nih.gov.nih-image>.

The dimensions of the image were converted to  $\mu\text{m}$  by calibrating against a slide micrometer. Correction for background illumination was not necessary, as there was already a high contrast between the conidia and the background. At this stage, any obvious artefacts were removed manually. A density slice was then performed to select the conidia on the basis of greyscale. The conidia often appeared hollow, as there was greatest refraction along the edges of the conidia. The software was instructed to include these interior holes in the size of the conidium. The software then counted the particles present and analysed their shape and size; particles with cross-sectional areas inconsistent with individual conidia were automatically excluded from the analysis. Individual conidia were readily distinguished from artefacts and

agglomerations of conidia on the basis of size. Approximately 2% of the conidia were clumped and were disregarded from the size analysis. They were, however, counted manually and included in the totals of numbers of conidia.

#### 2.7.2.2 Analysis of aggregated conidia

Although clumped conidia were excluded from the size analysis in this study, a method was developed for counting clumped conidia in other experiments where the incidence of clumped conidia may be higher. This method was developed in the main by Dr Simon Gray. In order to assess the feasibility of automated counting of clumped conidia, a mycosed aphid cadaver was mounted in the sporulation monitor, with the drum set to rotate once in 168 h. The slow speed of drum rotation led to a high density of conidia being deposited on the acetate strip, which represented the most difficult type of count to resolve as the frequency of touching and agglomerated conidia was high. Ten fields of view across the spore trail, representing conidia deposited between 10 and 15 h after the beginning of discharge, were selected at random and the numbers of conidia were counted manually. After manual counting, each field of view was captured and processed as described above. Automated counts were carried out three times: once excluding all particles with cross-sectional areas outside the range 100 to 300 pixels; once excluding particles with areas outside the range 100 to 350 pixels; and once excluding particles with areas below 100 pixels (no upper size limit). For the first two automated counts, particles with cross-sectional areas exceeding 300 and 350 pixels respectively were assumed to be clumps of conidia, and the numbers of conidia in such clumps were estimated by manual counting.

#### 2.7.3 Comparison of sporulation from *in vitro* and *in vivo* biomass

Comparison was made of the production of conidia by biomass from an *in vivo* source (mycosed aphid cadavers) and two *in vitro* sources (mycelial pellets grown in SDEM in fermenter culture and mycelial plugs grown on SDEM agar).

Mycosed aphid cadavers were produced as described in section 2.5.3 and placed on the sporulation monitor. Mycelial pellets were produced from fermenter batch culture and harvested 96 h after fermenter inoculation. Mycelial pellets were then placed immediately on to the sporulation monitor. Mycelial plugs were cut from the growing edge of 3 week old cultures grown on SDEM as described in section 2.1.2. Plugs were then placed on the sporulation monitor. Dry weights of the inoculum sources were also estimated. Individual mycelial pellets were dried overnight at 105°C then dessiccated to constant mass as described in section 2.4.2. It was assumed that the pellets used to measure production of conidia were representative of those in the fermenter culture with regard to size. Mycelium was carefully removed from agar plugs, using a scalpel, and dried, dessiccated and weighed as the pellets. Mycosed aphid cadavers were dried, dessiccated and weighed as above. Spore counts were performed on the image analysis system (sections 2.7.1 and 2.7.2) with 10 replicates per treatment and the results adjusted to conidia mg<sup>-1</sup> dry weight. This was performed using the following formula: number of conidia produced / mean biomass.

#### 2.7.4 Survival of *E. neoaphidis* in aphid cadavers in a simulated winter environment at different relative humidities

Aphid cadavers were produced as described in section 2.5.3 and a single cadaver was placed in each of eighty wells of four sterile polystyrene 96 well microtitre plates (TPP, Switzerland). Each of the four plates was maintained at a different relative humidity; 100%, 90% 50% and 20%. The relative humidity inside each plate was controlled by the addition of aqueous glycerol solutions in the remaining 16 wells (0%w/v, 27.5%w/v, 73%w/v and 90.05%w/v respectively) (Johnson 1940). A 2mm gap was kept between the lid of the plates and the bodies of the plates by means of a cork spacer. This allowed the air space over the wells to equilibrate at the correct relative humidity. The plates were then sealed using laboratory sealing film (Whatman, Maidstone, UK) and incubated under ambient light conditions (approximately 16h light: 8h dark) in an alternating temperature regime of 8°C for 12 hrs and -1°C for 12 hours over the experimental period of 24 weeks.

These temperatures are representative of day and night temperatures for the period of November-March in the southern UK (temperature data from the Meteorological Office, Bracknell, UK). At three week intervals, ten cadavers were removed from each of the 96 well plates, placed on the sporulation monitor and production of conidia was assessed as described in section 2.7.1-2.7.2. After 18 weeks, ten cadavers were removed from each treatment and an infectivity assay performed as described in section 2.7.5.

To test the effectiveness of the glycerol/water mixtures at maintaining relative humidity, a humidity datalogger (Tinytalk, RS Components Ltd, Corby, Northants, UK) was placed inside a sealed box containing an aqueous glycerol solution and was run for approximately 2 weeks.

#### 2.7.5 Infectivity assay

This method was adapted from Morgan *et al.* (1995). Known-age aphids were reared on bean plants as previously described in section 2.5.2. Cadavers to be used for inoculation were moistened on filter papers and held at 20°C overnight at 100% relative humidity, so that they were sporulating profusely the following morning in readiness for the assay. There were three treatments: cadavers stored for 18 weeks at 20% or 50% under simulated winter conditions and fresh cadavers which had been stored for less than ten days at 20% relative humidity at 4°C (section 2.7.4).

Aphids were inoculated with conidia inside 5cm Petri dishes, each containing 5 mycosed cadavers arranged on a 4cm diameter damp filter paper in the lid. The cadavers adhered to the filter paper by surface tension. These infected cadavers were used to shower aphids in the base of the dish with conidia for different periods of time; 1, 3, 6, 15, 30 & 60 minute, resulting in different doses of conidia being showered on to the aphids. A control treatment was configured as the experimental treatments, but without the mycosed cadavers in the lid of the Petri dish. A glass cover-slip was added to the base of each dish to catch the conidia showering down from the infected cadavers. Separate sets of cadavers were not required for each treatment, but the cadavers were re-used for the shorter inoculation times, ie; when



the 60 minute treatment had finished, the same cadavers were used to inoculate 30,15, 6, 3 & 1 minute exposures.

Ten replicate 1 to 3 day old apterous adult aphids were added to the Petri dishes, and a coarse mesh secured over the dish to ensure that the aphids did not crawl up to the cadavers, and thereby receive higher doses of conidia. The lid with the attached cadavers was then inverted over the plate. To ensure an even deposition of conidia, the lids were revolved a quarter turn every third of the exposure period.

After inoculation, the cover slip was removed from the dishes and counted to determine the dose of conidia received by the exposed aphids. Twenty randomly selected fields of view were counted under the light microscope at x100 magnification. Counting was done using the image analysis technique as described in 2.7.2. The Petri dishes that were used to hold the susceptible aphids were replaced between exposures and between treatments.

When inoculations were complete, the aphids from each dish were placed on a bean plant under a lampglass covered with clingfilm, which was replaced with netting after 24 hours as in section 2.5.3 .

Aphid mortality due to *E. neoaphidis* mycosis was defined when the abdomen of the infected aphid had started to turn brown, and was recorded daily on each plant for 8 days. The resulting cadavers were removed from the plant, then placed on a glass microscope slide which was placed on wet filter paper ( Standard grade 3 , Whatman), inside a 90mm Petri dish and incubated at 20°C and allowed to sporulate. Discharged conidia were examined microscopically to confirm that mycosis was caused by *E. neoaphidis*-(Wilding 1969)

#### 2.7.6 Comparison of production of conidia by biomass from different phases of growth in batch fermenter

Biomass was grown in fermenter batch culture as described in section 2.3 and samples of the culture taken at 0, 48, 96, 144 and 192 hours after inoculation. Pelleted biomass contained within broth was kept at 4°C until all the treatments could

be placed on the sporulation monitor simultaneously. The maximum time that biomass was in storage was approximately 195 h. The pellets were removed from the broth by straining the culture through a sterilised brass 250µm mesh sieve. The dry weight of individual pellets from an equivalent sample to that placed on the sporulation monitor was assessed as described in section 2.4.2 so that counts of conidia could then be expressed relative to the amount of fungal biomass in the pellets. Ten replicate pellets were used per treatment.

## **2.8 Dynamics of the transmission of *E. neoaphidis***

### 2.8.1 Effect of position of inoculum in the laboratory and field on the transmission of *E. neoaphidis*

#### 2.8.1.1 Effect of position of the inoculum on transmission under laboratory conditions.

Five aphid cadavers were placed at each of three positions on five replicate bean plants (section 2.5.1) for each position:

- at the top of the plant at the level of the penultimate leaf pair
- halfway up the plant at the level of the second leaf pair
- on the soil at the base of the plant

Controls were run concurrently without inoculation.

Cadavers were attached at the top and halfway up the plant by putting 0.5ml of non-sterile 1% w/v water agar on the plant stem using a automatic pipette and then placing the cadavers on the agar when it had nearly set. This was unlikely to affect the plant or sporulation (J. Pell pers. comm). Twenty 1-3 day old apterous aphid adults were then introduced on to each of the plants by placing them on the soil near the base of the plant, but not close to any inoculum. The individual plants were then placed under lampglasses covered with clingfilm, which was replaced with netting after 24 hours as described in section 2.5.3. The aphids on each plant were monitored daily, recording the numbers and position of dead mycosed aphids, every day for 8 days. Aphids were assumed to be dead if repeated stimulated attack, by gentle prodding with a small paintbrush did not produce a response. Aphids that

showed signs of infection such as the discolouration of the abdomen were removed from the plant using tweezers disinfected with 95% v/v ethanol . These aphids were then placed on a glass microscope slide which was placed on wet filter paper (Standard grade 3 , Whatman), inside a 90mm Petri dish and incubated at 20°C to encourage to sporulation. Discharged conidia were examined microscopically to confirm that mycosis was caused by *E. neoaphidis*.

#### 2.8.1.2 Effect of position of inoculum of transmission in the field

This fieldwork was carried out from the 26<sup>th</sup> June 2000 to the 12<sup>th</sup> July 2000. Dwarf broad beans (*Vicia faba* var. "The Sutton") were planted one per 12.5cm plastic plant pot in multi-purpose soil-less potting compost (B&Q, Eastleigh, Hants, UK) and grown at 20°C, ambient humidity and a 16h:8h (light: dark) photoperiod using fluorescent tube lighting. Each plant was watered with 200-400 ml tap water every two or three days. When the plants were three weeks old (approximately 10-15 cm tall) the pots were dug into the field so that the lip of the pot was at ground level (Fig 2.3). These pots were then covered with a mesh umbrella (mesh size approximately 3mm), to prevent aphid movement between treatments and also to try and exclude entomophagous and phytophagous arthropods from the plants.

On the day that cadavers were to be placed in the field they were placed on partially set 1% w/v water agar that had been poured to a depth of approximately 5mm in the base of a 3cm diameter Petri dish. The isolate of *E. neoaphidis* used was X4 and dried cadavers were produced as described in section 2.5.3. Cadavers had been stored for 2 weeks at 4°C and 20% relative humidity before use in the experiment. The Petri dishes were held in humid boxes in the laboratory until being taken into the field. The base of the Petri dish was stuck to a small piece of acetate strip that could be attached to a leaf using a paper clip. (Fig 2.4). Acetates and cadavers were mounted on plants in the late afternoon (16:00-16:45).

Figure 2.3 Potted bean plant in the field covered by a mesh umbrella. This umbrella was used to contain the aphids to a single plant, to exclude aphidophagous insects, and to enable examination of the plant without disturbing the aphids



Figure 2.4 Petri dish used to attach sporulating cadavers to bean plants to provide conidia to inoculate susceptible aphids placed on the plant



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The cadavers were attached to the plant in two positions:

- at the top of the plant, at the level of the penultimate leaf pair
- halfway up the plant, at the level of the second leaf pair

Cadavers were also placed on the soil at the base of the plant in dishes. Five cadavers per plant were used, with 3 replicate plants per treatment. Controls were run concurrently with no cadavers attached to the plants. The pots were arranged on a grid pattern (Figure 2.5) within a 15m<sup>2</sup> patch less than 15m from the edge of New Zealand field (IACR-Rothamsted). Each plant was separated by at least 0.5m from the next plant and by at least 1m from the beans in the surrounding field. Temperature and humidity loggers (Tinytalk, RS Components Ltd, Corby, Northants, UK) were placed in the field in block 3 on the soil surface under the netting of the control treatment.

Twenty 1 to 3 day old apterous adult aphids were then added to each plant by tipping them onto the soil at the base of the plant, starting with the control plants and followed by the plants with the cadavers. Care was taken not to introduce the aphids directly onto the fungus. Numbers of dead, dead mycosed and missing aphids were then monitored daily for 16 days. The criteria for assessing death of aphids are described in section 2.5.3. Confirmation that death by mycosis was due to *E. neoaphidis* was performed as described in section 2.8.1.1.

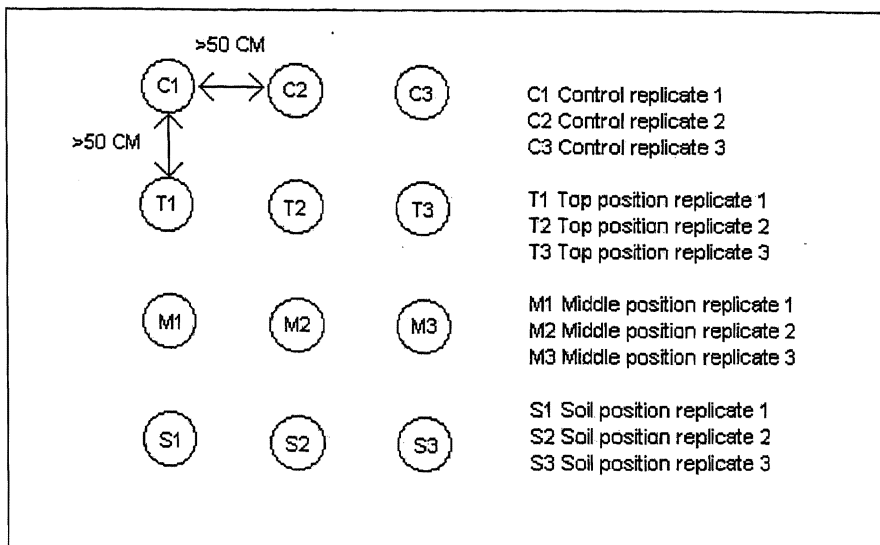


Figure 2.5 Layout of the experimental plot for assessment of the effect of the position of inoculum on transmission of *E. neoaphidis* in the field

### 2.8.2 Effect of initial inoculum position on the secondary transmission of *E. neoaphidis* through an aphid population.

This experiment was set up as described in section 2.8.1.1. However, to provide a source of secondary infection aphids were left on the plant instead of removing them when they showed signs of infection. The aphids on each plant were monitored daily, recording the numbers and position of dead adult aphids, every day for 14 days (2 generations). After this period, confirmation that death by mycosis was due to *E. neoaphidis* was performed as described in section 2.8.1.1.

### 2.8.3 Effect of dislodgement during observation of aphids from host plants on the transmission of *E. neoaphidis*.

Five aphid cadavers, prepared as described in 2.5.3 were attached to the stem of each of ten replicate bean plants prepared as in 2.5.1 at the level of the penultimate leaf pair. Cadavers were attached to the plant by putting 0.5ml of non-sterile 1% w/v water agar on the plant stem using a automatic pipette and then placing the cadavers on the agar when it had nearly set. Five replicate control plants were prepared without cadaver attachment. Twenty 1 to 3 day old apterous adult

aphids were then introduced onto each plant by tipping them gently on to the soil and allowing them to climb up the plant. The individual plants were then placed under lampglasses covered with clingfilm, which was replaced with netting after 24 hours as described in section 2.5.3. The numbers and positions of mycosed aphids were monitored once per day for 8 days on five of the plants that had cadavers attached and three times per day for 8 days on the remaining five plants on which cadavers had been attached. Aphids that showed signs of infection (as described in 2.5.3) were carefully removed from the plant using tweezers. Confirmation of mycosis by *E. neoaphidis* was performed as described in 2.8.1.1

#### 2.8.4 Effect of host density on the transmission of *E. neoaphidis* in the laboratory and the field

##### 2.8.4.1 *Effect of host density on the transmission of E. neoaphidis under laboratory conditions*

Five aphid cadavers were attached to the stem of 5 replicate individual bean plants at the level of the penultimate leaf pair as described in 2.8.3. Apterous 1-3 day old adult aphids were then introduced to the plants at four densities: five, ten, twenty and forty aphids per plant. Controls were run concurrently without attachment of cadavers. This gave a total of 25 plants used in total. The individual plants were then placed under lampglasses covered with clingfilm, which was replaced with netting after 24 hours as before (2.5.3). The aphids on each plant were then monitored daily, recording the numbers of mycosed aphids (section 2.5.3), every day for 8 days. Mycosed aphids were removed from the plant using tweezers. Confirmation of mycosis by *E. neoaphidis* was performed as described in 2.8.1.1.

##### 2.8.4.2 *Effect of host density on the transmission of E. neoaphidis in the field*

This work was carried out in association with Drs Judith Pell and Paresh Shah as part of an ongoing investigation at IACR-Rothamsted and was performed to



assess the transmission of *E. neoaphidis* from cadavers to an uninfected population.

This fieldwork was carried out from the 28<sup>th</sup> July 2000 to the 2<sup>nd</sup> August 2000

Fine mesh muslin bags were tied to the stem of each replicate broad bean (*Vicia faba*) plant approximately 24 inches from the apex of the plant to prevent movement of aphids on or off of the plant, and also to try to prevent the predation or parasitism of the aphids by entomophagous arthropods. The bags completely enclosed the top of the plant. Sporulating cadavers were prepared as described in 2.8.1.2 and attached to the bean plant as described in section 2.8.1.2, near the top of the plant within the muslin bags. Three aphid densities were used: 10 aphids per plant, 20 aphids per plant and 40 aphids per plant. The density of cadavers was 40 cadavers per plant or 0 cadavers per plant (control). Cadavers had been stored for 2 weeks at 4°C and 20%RH before use in the experiment.

Apterous adult aphids (1-3 day old) were added to the control plants first by gently tipping them onto the plant. Aphids were then added to the plants on which the cadavers were attached. The number of aphids that were added to the plants was dependent on the required host density (either 10, 20 or 40). Care was taken not to introduce the aphids directly onto the fungus. After 24 h the aphids were removed from the plant. Again, care was taken not to force the aphids into contact with the fungus. Acetates with attached fungus were removed first and then the aphids were shaken from the plant into a collecting tray before replacing the acetates and fungus onto the plant. These aphids were returned to the laboratory and monitored for infection as described in section 2.5.3. A fresh cohort of 1 - 3 day old apterous adult aphids was then placed on the plant as described above. This process of collecting aphids from the field and then introducing fresh aphids was repeated at 1, 2, 3 and 5 days. The numbers of dead and dead infected aphids were recorded between day 3 and 7 after return from the field. Aphids were considered to be dead if there was no response to simulated attack (gentle prodding with a small paintbrush). The criteria for death by mycosis due to *E. neoaphidis* were as described in 2.8.1.1. Three replicates of each host density/cadaver density combination were used. One replicate of each combination was allocated to a block - giving 3 blocks. The three

blocks were all within a 30m strip at the edge of New Zealand field (IACR-Rothamsted), parallel to the road as shown in Fig 2.4. Each plant was separated by at least 1.5m from the next plant. Block 1 was closest to a tree and therefore in the most shaded position. A temperature and a humidity logger (Tinytalk, RS Components Ltd, Corby, Northants, UK) were placed in the field in block 1 (see Fig 2.4), inside a muslin sleeve at the mid canopy level under a rain cover (half of a section of plastic hexagonal guttering).

Three further temperature loggers were placed on a cane at soil, canopy top and mid-canopy levels under rain covers as described above.

#### 2.8.5 Effect of cadaver density on transmission of *E neoaphidis* in the field.

This work was carried out in association with Drs Judith Pell and Paresh Shah as part of an ongoing investigation at IACR-Rothamsted. This fieldwork was carried out from the 20<sup>th</sup> June 2000 to the 29<sup>th</sup> June 2000.

Fine mesh muslin bags were tied to the stem of the plant as described in section 2.8.4.2. Sporulating cadavers of isolate X4 were prepared as described in section 2.5.3, stored for 2 weeks at 4°C and 20% RH and attached to the bean plant as described in 2.8.1.2. Six treatments were used: 0 (control), 1, 5, 10, 20 and 40 cadavers per plant.

Cadavers were mounted in groups so that 1, 2, or 3 acetates only would be clipped to each plant, i.e.

- 1 cadaver per plant: 1 Petri dish
- 5 cadavers per plant: 2 Petri dishes (3 cadavers and 2 cadavers)
- 10 cadavers per plant: 2 Petri dishes (5 and 5)
- 20 cadavers per plant: 3 Petri dishes (6, 6 and 8)
- 40 cadavers per plant: 3 Petri dishes (13, 13 and 14)

Dried cadavers were mounted onto the agar in the morning on Day 0 and held in humid boxes in the laboratory until being taken to the field. Acetates and cadavers were mounted near to the top of the plants in the late afternoon (16:35 – 17:10) of Day 0. Twenty 1-3 day old apterous adult aphids were added to the plant as

described in 2.8.4.2. The aphids were then collected from the field after 24 h and taken to the laboratory, and monitored for infection as described in 2.8.4.2. This process was repeated at Day 1 (twenty, 1 - 3 day old apterous adult aphids), Day 3 (fifteen, unknown age apterous adult aphids), Day 6 (fifteen, 1 - 3 day old apterous adult aphids) and Day 9 (fifteen, 1 - 3 day old apterous adult aphids). Three replicates of each treatment were used as described in 2.8.4.2. The trial area is described in section 2.8.4.2. Temperature and humidity loggers were placed in the field as described in 2.8.4.2.

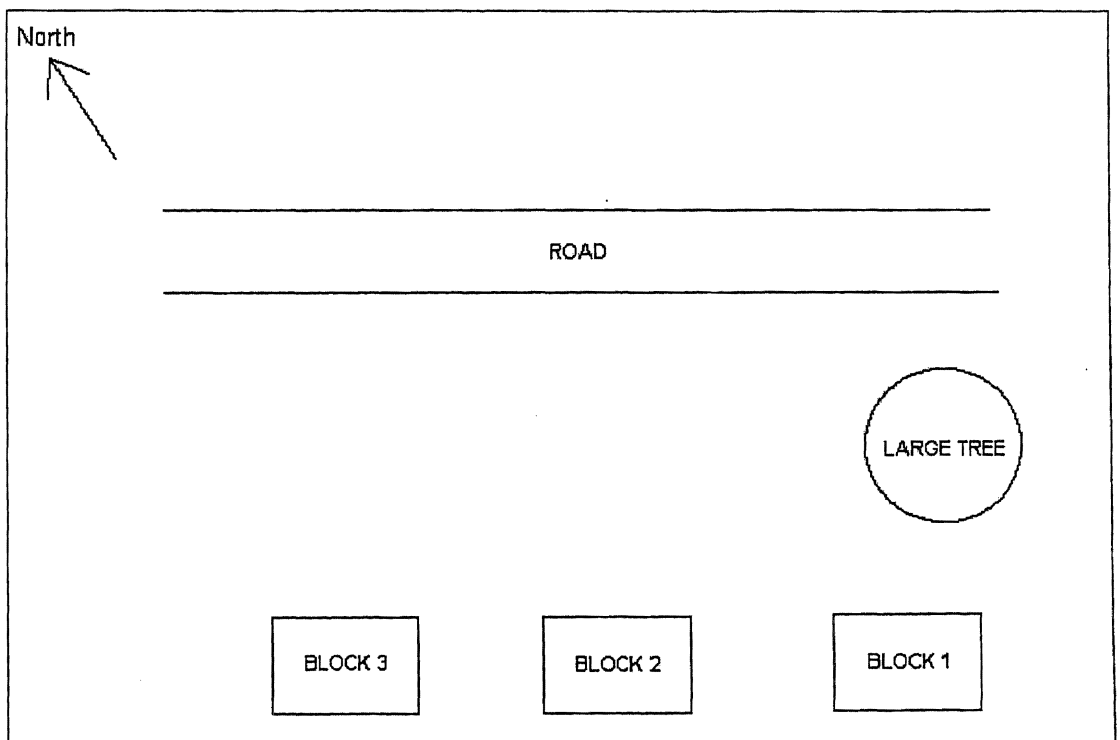


Figure 2.6 Diagrammatic representation of the layout of the blocks in the field to assess the effect of cadaver density on transmission of *Erynia neoaphidis* through an aphid population.

## **2.9 Statistical methods and software**

The 95% level of confidence ( $p < 0.05$ ) was used at all times.

A one way analysis of variance (ANOVA) was used unless otherwise indicated.

Confidence intervals were calculated using a difference of mean squares method.

Comparison of  $K_r$  was done by use of a z-test based on the standard error of the

regression coefficient. All statistics were performed using Microsoft Excel and SPSS

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## 3 Results

### 3.1 Definition of medium

#### 3.1.1 Biomass production

There were a number of media used, which could be categorised into 4 broad groups.

- Media deficient in yeast extract (YEDM, YEDMTH, YEDMVB, YEDMVB5)
- Media deficient in mycological peptone (PDM, PDMTH, PDMVB, PDMG)
- Media based on Grace's Insect Medium (GIM, GIMO, GASP)
- Test media (TM, TMTH)

As well as these, SDEM was also used.

Of the liquid media tested, biomass production by *E. neoaphidis* was greatest in GASP (8.93 g l<sup>-1</sup>) and SDEM (8.62 g l<sup>-1</sup>). There was no significant difference in biomass production between these media. There was significantly less biomass production from fungus grown in both TM and TMTH compared to SDEM and GASP. There was no significant difference in mean biomass production in TMTH (3.12 g l<sup>-1</sup>) compared to TM (2.91 g l<sup>-1</sup>). There was no significant difference in biomass production between YEDM (1.04 g l<sup>-1</sup>), YEDMTH (1.25 g l<sup>-1</sup>), YEDMVB (1.39 g l<sup>-1</sup>), YEDMVB5 (0.92 g l<sup>-1</sup>), PDM (0.97 g l<sup>-1</sup>), PDMTH (1.09 g l<sup>-1</sup>), PDMVB (1.54 g l<sup>-1</sup>), PDMG (1.37 g l<sup>-1</sup>), GIM (0.79 g l<sup>-1</sup>) and GIMO (0.90 g l<sup>-1</sup>) (ANOVA). The mean biomass production from fungus grown in all of these media was significantly lower compared to TM and TMTH, SDEM and GASP.

#### 3.1.2 Specific growth rate in liquid shake flask culture

As described in 3.1.1, 4 broad categories of media were used

- Media deficient in yeast extract (YEDM, YEDMTH, YEDMVB, YEDMVB5)
- Media deficient in mycological peptone (PDM, PDMTH, PDMVB, PDMG)

- Media based on Grace's Insect Medium (GIM, GIMO, GASP)
- Test media (TM, TMTH)

There was no significant difference in specific growth rate of fungus grown on GASP ( $0.051 \text{ h}^{-1}$ ) compared to SDEM ( $0.055 \text{ h}^{-1}$ ). There was no significant difference in the specific growth rate of fungus grown in TMTH ( $0.035 \text{ h}^{-1}$ ) compared to TM ( $0.032 \text{ h}^{-1}$ ), although specific growth rate was significantly lower in both TM and TMTH compared to GASP and SDEM. There was no significant difference in specific growth rate of fungus grown in PDMVB ( $0.023 \text{ h}^{-1}$ ) compared to PDMG ( $0.017 \text{ h}^{-1}$ ). The specific growth rate of fungus grown in PDMVB and PDMG was significantly lower than in TM, TMTH, SDEM and GASP. There were no significant differences between the specific growth rate of fungus grown in YEDMTH ( $0.012 \text{ h}^{-1}$ ), PDMTH ( $0.011 \text{ h}^{-1}$ ) and YEDM ( $0.008 \text{ h}^{-1}$ ). Fungus grown in YEDMTH, PDMTH or YEDM had a significantly lower specific growth rate than fungus grown in PDMVB, PDMG, TMTH, TM, GASP or SDEM. There were no significant differences between the specific growth rates of fungus grown in YEDMVB ( $0.004 \text{ h}^{-1}$ ), YEDMVB5 ( $0.002 \text{ h}^{-1}$ ), PDM ( $0.001 \text{ h}^{-1}$ ), GIM ( $0.002 \text{ h}^{-1}$ ) and GIMO ( $0.001 \text{ h}^{-1}$ ). The specific growth rate of fungus grown in these media was significantly lower than in YEDM, PDMTH, YEDMTH, PDMVB, PDMG, TM, TMTH, GASP and SDEM.

### 3.1.3 Yield

The yield obtained on GASP ( $0.742 \text{ g biomass. g sucrose}^{-1}$ ) was significantly higher than the yield obtained on SDEM ( $0.538 \text{ g biomass. g glucose}^{-1}$ ). This was in contrast to the results obtained for biomass production (section 3.1.1) and specific growth rate (section 3.1.2) where there was no significant difference between the two media. There was no significant difference between the yields obtained on TM ( $0.291 \text{ g biomass g. g glucose}^{-1}$ ) and TMTH ( $0.314 \text{ g biomass. g glucose}^{-1}$ ). There were no significant differences between the yields obtained on YEDM ( $0.065 \text{ g biomass. g glucose}^{-1}$ ), YEDMTH ( $0.078 \text{ g biomass. g glucose}^{-1}$ ), YEDMVB ( $0.087 \text{ g biomass. g glucose}^{-1}$ ), YEDMVB5 ( $0.057 \text{ g biomass. g glucose}^{-1}$ ), PDM ( $0.063 \text{ g biomass. g glucose}^{-1}$ ), PDMVB ( $0.094 \text{ g biomass. g glucose}^{-1}$ ), PDMTH ( $0.069 \text{ g biomass. g$

glucose<sup>-1</sup>) and PDMG (0.086 g biomass. g glucose<sup>-1</sup>). These results are similar to those obtained for biomass production. Yields obtained on these media were significantly lower than those obtained on GASP, SDEM, TM or TMTH. There was no significant difference in the yield obtained on GIM (0.033 g biomass. g sucrose<sup>-1</sup>) compared to that obtained on GIMO (0.038 g biomass. g sucrose<sup>-1</sup>). Yields obtained on GIM and GIMO were significantly lower than those obtained on all other media.

A summary of comparative biomass production, specific growth rate and yield can be found in Table 3.1

Table 3.1 Mean specific growth rates, mean maximum biomass production and mean yield of *Erynia neoaphidis* NW327 in shake flask culture using various media at 20°C and at 200 rpm. Data with different superscripts are significantly different.

Culture medium	Mean maximum specific growth rate (h <sup>-1</sup> )	Mean maximum biomass production (g l <sup>-1</sup> )	Mean yield (g biomass. g glucose <sup>-1</sup> )
SDEM	0.055 <sup>a</sup>	8.62 <sup>a</sup>	0.538 <sup>b</sup>
YEDM	0.008 <sup>d</sup>	1.04 <sup>c</sup>	0.065 <sup>d</sup>
YEDMTH	0.012 <sup>d</sup>	1.25 <sup>c</sup>	0.078 <sup>d</sup>
YEDMVB	0.004 <sup>e</sup>	1.39 <sup>c</sup>	0.087 <sup>d</sup>
YEDMVB5	0.002 <sup>e</sup>	0.92 <sup>c</sup>	0.057 <sup>d</sup>
PDM	0.001 <sup>e</sup>	0.97 <sup>c</sup>	0.063 <sup>d</sup>
PDMTH	0.011 <sup>d</sup>	1.09 <sup>c</sup>	0.069 <sup>d</sup>
PDMVB	0.023 <sup>c</sup>	1.54 <sup>c</sup>	0.094 <sup>d</sup>
PDMG	0.017 <sup>c</sup>	1.37 <sup>c</sup>	0.086 <sup>d</sup>
TM	0.032 <sup>b</sup>	2.91 <sup>b</sup>	0.291 <sup>c</sup>
TMTH	0.035 <sup>b</sup>	3.12 <sup>b</sup>	0.314 <sup>c</sup>
GIM	0.002 <sup>e</sup>	0.79 <sup>c</sup>	0.066 <sup>e</sup>
GIMO	0.001 <sup>e</sup>	0.90 <sup>c</sup>	0.076 <sup>e</sup>
GASP	0.051 <sup>a</sup>	8.93 <sup>a</sup>	0.742 <sup>a</sup>



### 3.2 Production of conidia by biomass grown *in vivo* and *in vitro*

#### 3.2.1 Conidial discharge from aphid cadavers, plugs cut from solid cultures and pellets from liquid cultures.

*E. neoaphidis* was grown *in vitro* (on agar or in shake flask cultures) or *in vivo* (infected aphids). Fungal inoculum was then placed on the sporulation monitor to assess production of conidia from each source. Fungus grown *in vivo* within cadavers produced more conidia  $\text{g}^{-1}$  dry weight (2000 conidia  $\text{g}^{-1}$  dry weight  $\text{h}^{-1}$  over 80 h) than fungus grown *in vitro* either in hyphal pellets or agar plugs (800 conidia  $\text{g}^{-1}$  dry weight  $\text{h}^{-1}$  over 65 h and 150 conidia  $\text{g}^{-1}$  dry weight  $\text{h}^{-1}$  over 65 h respectively). This difference was statistically significant (ANOVA). Hyphal pellets produced more conidia  $\text{g}^{-1}$  dry weight than did plugs cut from colonies grown on agar. This difference was also statistically significant (ANOVA). Cadavers produced conidia for 15 h longer than either pellets or plugs (Figure 3.1)

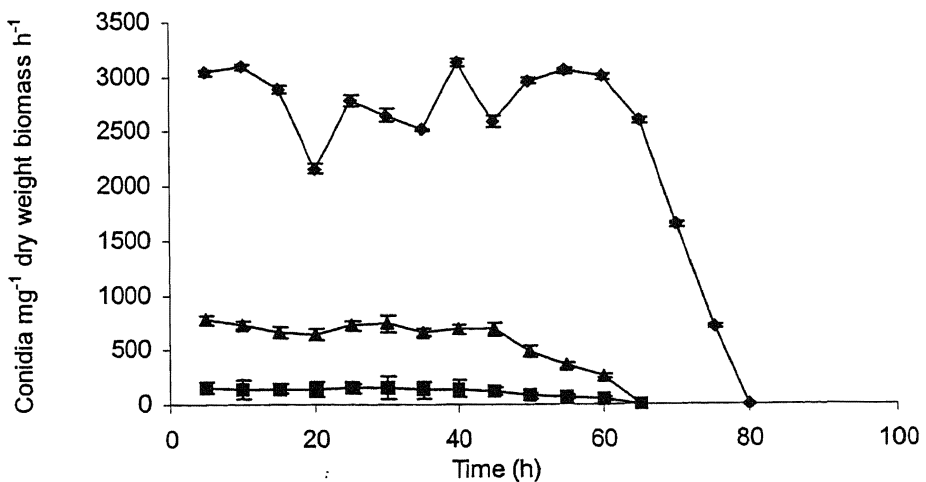


Figure 3.1 Comparison of discharge of conidia of *E. neoaphidis* from aphid cadavers (◆), biomass grown on agar plugs (■) and pellets from liquid culture (▲). Discharge

was measured at 100% RH, 20°C with 10 replicates per treatment. Error bars represent the standard errors of the means of 10 replicates.

Cadavers produced conidia that had a significantly smaller mean volume (925  $\mu\text{m}^3$ ) than conidia produced from pellets (1942  $\mu\text{m}^3$ ) or from plugs (1962  $\mu\text{m}^3$ ) (ANOVA). There was no significant difference in volume between conidia produced from pellets or plugs. There was also no significant difference in the mean volume of conidia discharged over time within any of the treatments (ANOVA) (Figure 3.2).

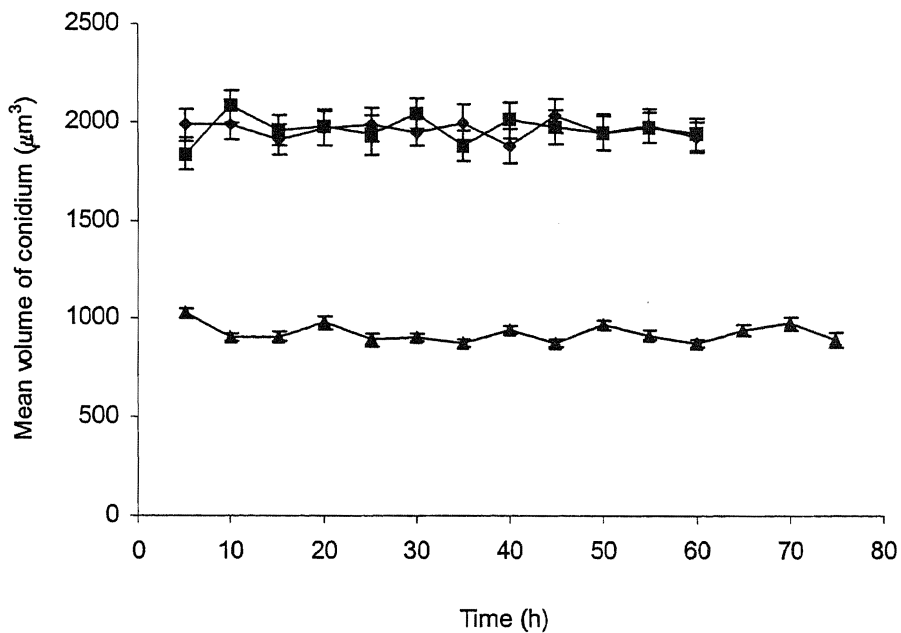


Figure 3.2 Comparison of mean volumes of conidia of *E. neoaphidis* discharged from aphid cadavers (▲), agar plugs (■) and pellets from liquid culture (◆). Discharge was measured at 100% RH, 20°C and a photoperiod of 16 h:8 h (light: dark). Error bars represent the standard errors of the means of 10 replicates per treatment.

There was no significant change in the ratio of major axis length of the conidium to the minor axis length ( $L_{\text{maj}}/L_{\text{min}}$ ) of the conidium over time for any of the treatments (Figure 3.3). There was also no significant difference in the mean  $L_{\text{maj}}/L_{\text{min}}$

between conidia discharged from pellets (2.14) compared to those discharged from plugs (2.11). However mean  $L_{maj}/L_{min}$  was significantly lower for conidia produced from cadavers (1.90) compared to conidia produced from pellets or plugs (ANOVA) (Figure 3.3).

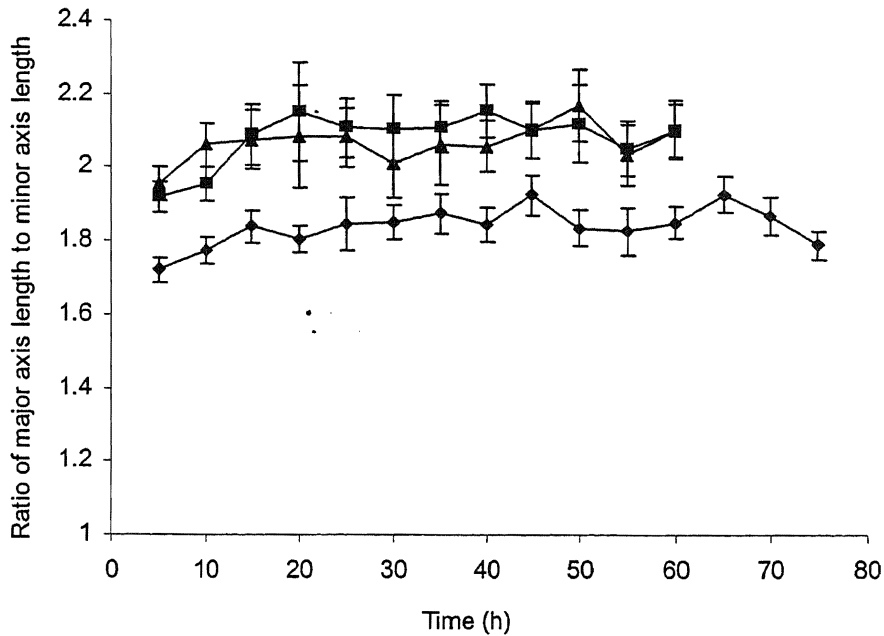


Figure 3.3. Comparison of mean ratio of major axis length to minor axis length of conidia of *E. neoaphidis* discharged from aphid cadavers (◆), agar plugs (■) and pellets from liquid culture (▲). Discharge was measured at 100% RH, 20°C and a photoperiod of 16 h: 8 h (light: dark). Error bars represent the standard errors of the means of 10 replicates per treatment.

There was a significant difference in the shape of the distributions of the volumes of conidia produced after the first 5 h compared to those produced at 75 h for conidia produced from cadavers ( $\chi^2$  test). The distribution of the volume of conidia produced from cadavers showed that more small conidia (volume of less than  $600 \mu\text{m}^3$ ) and more large conidia (volume of greater than  $1400 \mu\text{m}^3$ ) were produced after 5 h incubation than at 75 h incubation. The distribution was bi-modal or possibly

tri-modal (Figure 3.4) with peaks at volumes of approximately  $200\mu\text{m}^3$ ,  $1200\mu\text{m}^3$  and possibly  $3000\mu\text{m}^3$ .

The distribution of the volume of conidia produced from plugs cut from solid cultures was more variable than those produced from cadavers. There was a significant difference in the shape of the distributions of the volume of conidia produced after the first 5 h compared to those produced at 60 h ( $\text{chi}^2$  test). Generally there was little pattern to differences in the volumes of conidia produced at 5 h compared to 60 h although considerably more conidia with a volume of greater than  $3400\mu\text{m}^3$  were produced at 60 h compared to at 5 h. Overall, the shape of the distribution was bi-modal, and possibly tri-modal (Figure 3.5) with peaks at volumes of approximately  $400\mu\text{m}^3$ ,  $1800\mu\text{m}^3$  and possibly  $3000\mu\text{m}^3$ .

The frequency distribution for the volumes of conidia produced from pellets grown in liquid culture showed that there was a significant difference in the shape of the distributions ( $\text{chi}^2$  test) with generally more conidia produced in all size fractions after 60 h than after 5 h. The shape of the distribution is trimodal with peaks at volumes of  $400\mu\text{m}^3$ ,  $1800\mu\text{m}^3$  and  $3600\mu\text{m}^3$  (Figure 3.6).

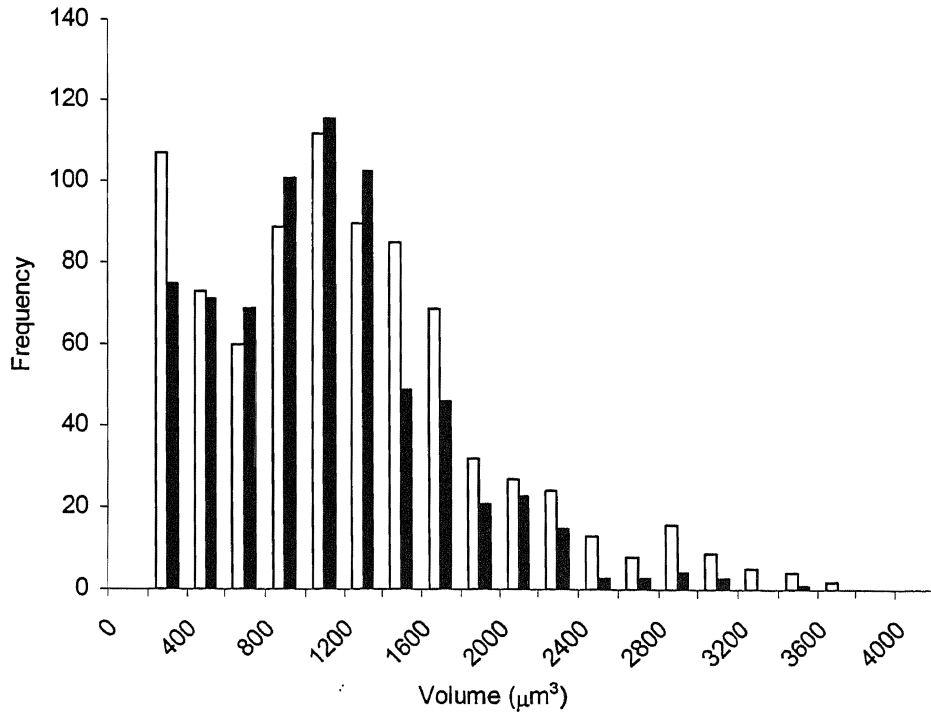


Figure 3.4 Frequency distribution of the volumes of conidia of *E. neoaphidis* produced from cadavers after 5 h incubation (□) and 75 hours incubation (■). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

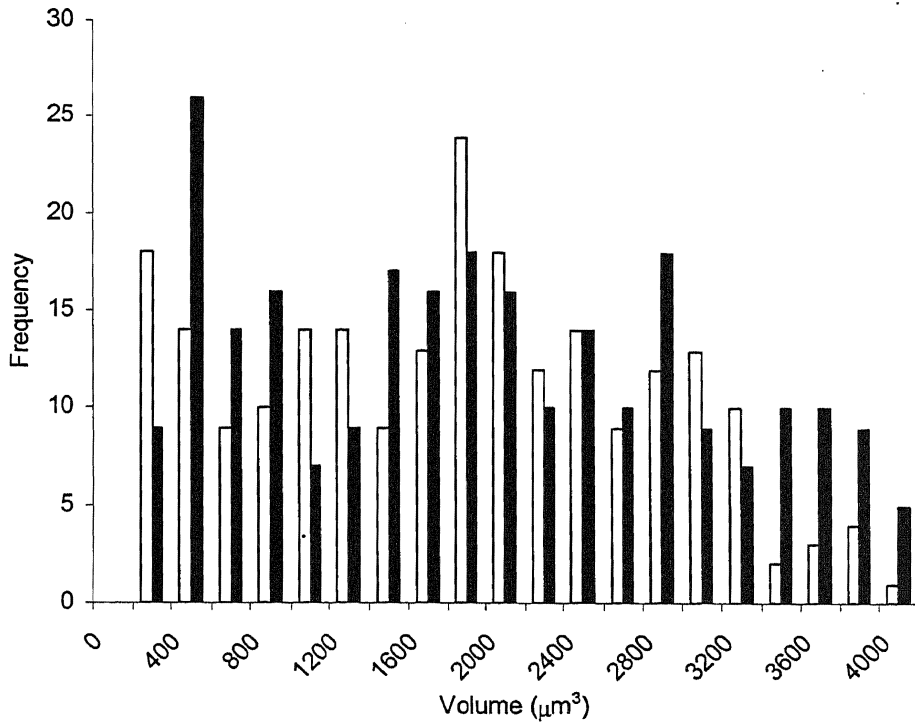


Figure 3.5 Frequency distribution of the volumes of conidia of *E. neoaphidis* produced from plugs cut from solid cultures after 5 h incubation ( $\square$ ) and 60 hours incubation ( $\blacksquare$ ). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

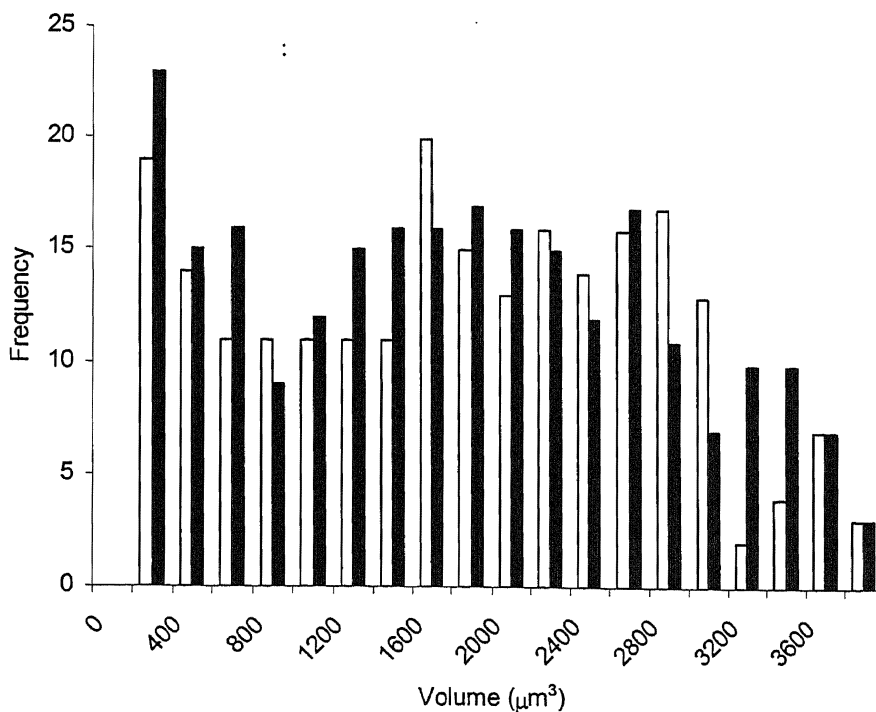


Figure 3.6 Frequency distribution of the volumes of conidia of *E. neoaphidis* produced from pellets grown in liquid culture after 5 h incubation (□) and 60 hours incubation (■). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

### 3.2.2 Production of conidia by hyphal pellets harvested from different phases of fermenter batch culture

Pelleted biomass was grown in fermenter batch culture then harvested at 24 h intervals for 7 days (2.7.6) then placed on a sporulation monitor to assess the production of conidia from biomass. The lag phase lasted until approximately 72 h after inoculation (Figure 3.7), when early exponential phase started. Exponential phase lasted until approximately 120 h after inoculation when there was a rapid transition to decline/death phase. There was little or no stationary phase. The greatest numbers of conidia were produced from pellets that had been harvested during the exponential growth phase, 96 h after inoculation, which produced 693 conidia after 60 h compared to biomass harvested during any other growth phase

where conidia production ranged from 477 produced after 60 h to 597 conidia produced after 60 h (Figure 3.8).

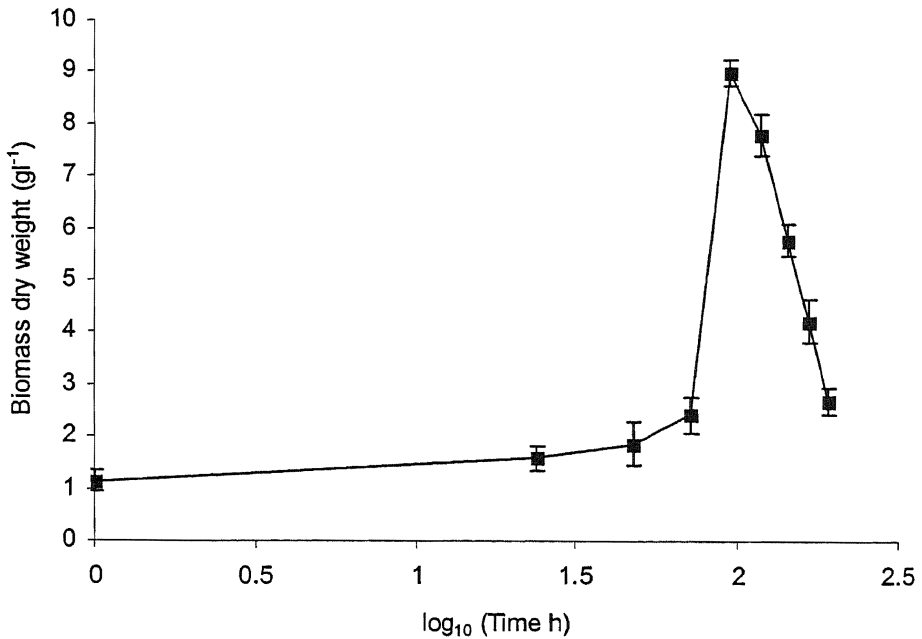


Figure 3.7 Biomass concentration of *E. neoaphidis* biomass grown in liquid batch fermenter culture. Error bars represent the standard errors of the means of 3 replicates.

Over the entire period of cumulative discharge, significantly more conidia were produced from the exponential growth phase biomass pellets, sampled 96 h after inoculation than from biomass in any other growth phase. There were significantly more conidia produced from pellets harvested at early decline phase (144 h) than at late decline phase (192 h). There was no significant difference between the number of conidia produced from pellets harvested at the time of fermenter inoculation (0 h) and those harvested from the lag phase (48 h). Both produced significantly fewer conidia than pellets harvested during early decline phase (Figure 3.8). The rapid switch from exponential phase to death phase, is typical of *E. neoaphidis* grown in SDEM in fermenter culture (Gray *et al.* 1990)



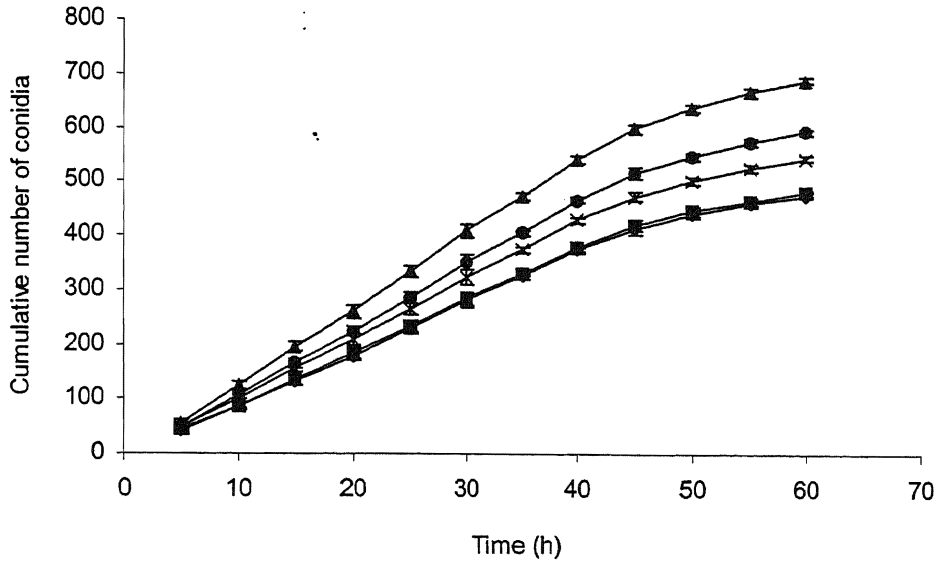


Figure 3.8 Cumulative production of conidia from hyphal pellets harvested from batch culture at different phases of growth: 0h (◆), 48h (■), 96h (▲), 144h (●), and 192h (×). Error bars represent the standard errors of the means of 10 replicates.

### 3.3 Effect of relative humidity on the survival of *E. neoaphidis* under simulated winter conditions

#### 3.3.1 Effect on ability to discharge conidia

Mycosed aphid cadavers were maintained at 100% RH, 90% RH, 50% RH and 20% RH under simulated winter conditions. Cadavers were removed periodically from storage and production of conidia was assessed using the sporulation monitor. The maximum production of conidia during any measured hour is reported here as 'peak production'. After three weeks of storage, there was no significant difference in the number of conidia released by cadavers stored at 50% relative humidity (RH), 90% RH and 100% RH (300 conidia mm<sup>-2</sup>, 269 conidia mm<sup>-2</sup>, and 253 conidia mm<sup>-2</sup> respectively) (ANOVA). Cadavers stored at 20% RH produced significantly fewer conidia (167 conidia mm<sup>-2</sup>) than any of the other three treatments after 3 weeks storage. After six weeks storage, cadavers stored at 90% RH and 100% RH failed to

produce conidia. Production of conidia from cadavers stored at 50% RH fell sharply compared to that observed after 3 weeks storage while production of conidia from cadavers stored at 20% RH decreased only slightly. After nine weeks of storage, production of conidia from cadavers stored at 50% RH fell below the level of production from cadavers stored at 20% RH (Figure 3.9). Over the remaining fifteen weeks of storage, production of conidia from cadavers stored at 50% RH continued to fall steadily (Figure 3.10), while production from cadavers stored at 20% RH decreased much less markedly (Figure 3.11). At the end of the storage period, cadavers stored at 20% RH produced significantly more conidia than cadavers stored at 50% RH (82 conidia  $\text{mm}^{-2}$  and 25 conidia  $\text{mm}^{-2}$  respectively) (ANOVA).

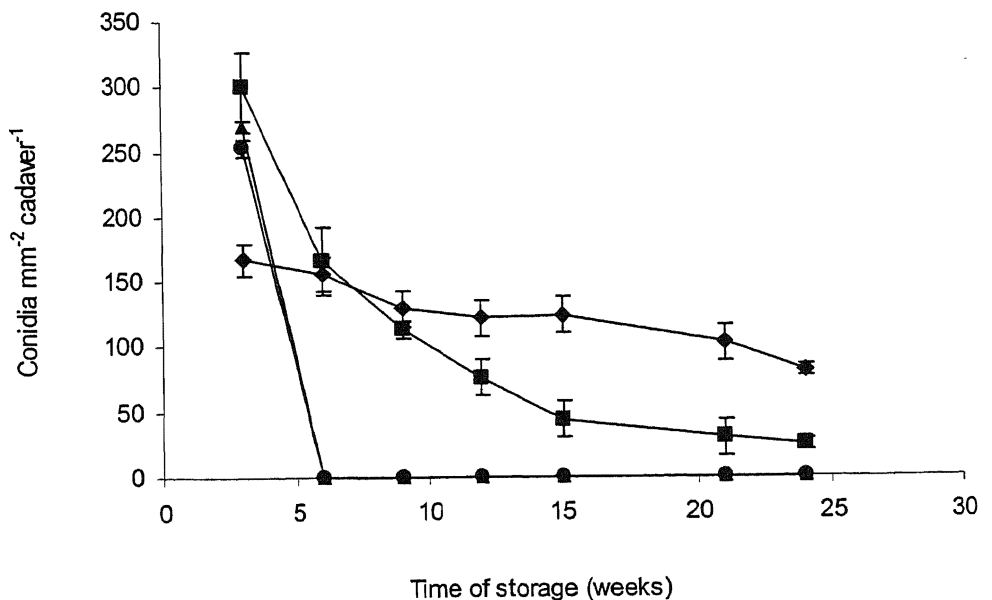


Figure 3.9 Peak production of conidia from cadavers previously maintained at 20% RH (◆), 50% RH (■) 90% RH (▲) and 100% RH (●). Error bars represent the standard errors of the means of 10 replicates.

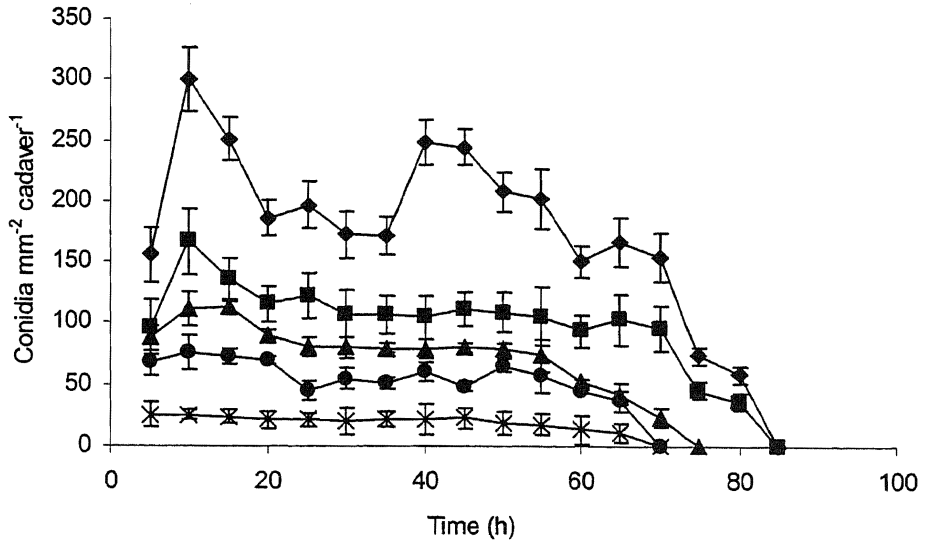


Figure 3.10 Discharge of conidia from cadavers previously maintained at 50% relative humidity at 3 weeks (◆), 6 weeks (■), 9 weeks (▲), 12 weeks (●) and 24 weeks (×). Data from weeks 15 and 21 show the same pattern but are omitted for clarity. Error bars represent the standard errors of the means of 10 replicates.

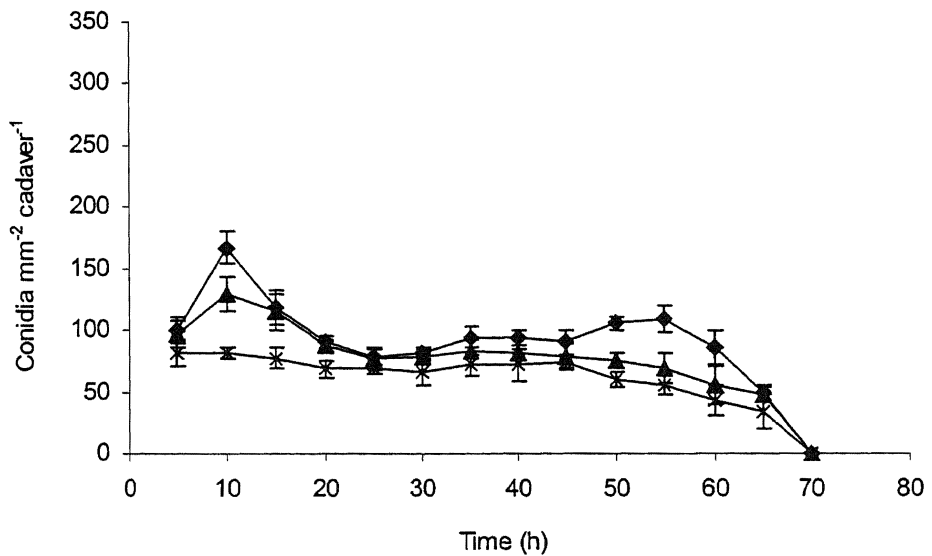


Figure 3.11 Discharge of conidia from cadavers previously maintained at 20% relative humidity at 3 weeks (◆), 9 weeks (▲) and 24 weeks (×). Data from weeks 6, 12, 15 and 21 show the same pattern but are omitted for clarity. Error bars represent the standard errors of the means of 10 replicates.

### 3.3.2 Infectivity

The LD<sub>50</sub> of conidia produced from cadavers stored at 20% relative humidity (79.8 conidia mm<sup>-2</sup>) was only slightly higher than that of conidia produced from fresh cadavers (66.1 conidia mm<sup>-2</sup>) (Figure 3.12). The LD<sub>50</sub> of conidia produced from cadavers maintained at 50% relative humidity (101.4 conidia mm<sup>-2</sup>) was greater than those from fresh cadavers or from cadavers kept at 20% relative humidity but the difference was not statistically significant (ANOVA).

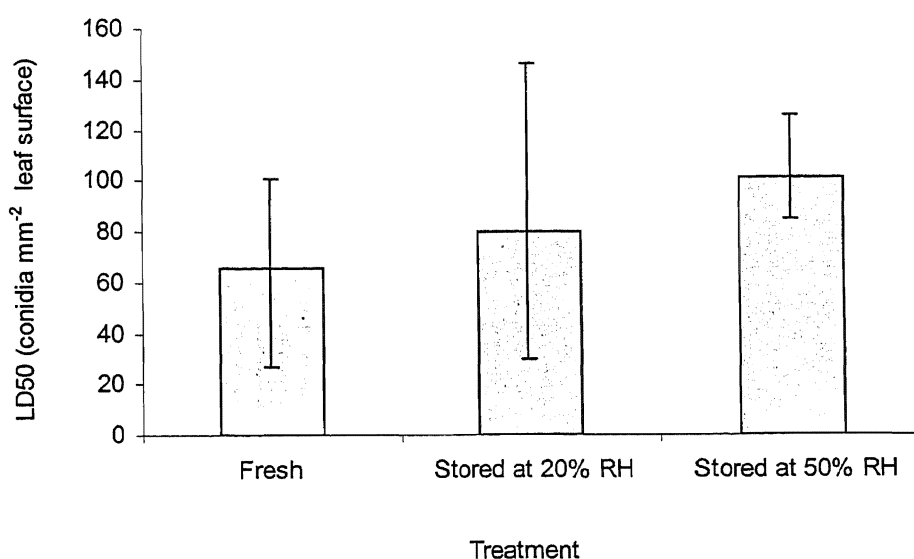


Figure 3.12 LD<sub>50</sub> of conidia produced from fresh cadavers and cadavers stored at 20% RH and 50% RH. Error bars represent 95% confidence intervals, calculated from ten replicates per treatment.

The  $LT_{50}$  of conidia produced from fresh cadavers (115.9 h) and cadavers stored at 20% relative humidity (125.2 h) differed by less than 10% (Figure 3.13). The  $LT_{50}$  of conidia from cadavers stored at 50% relative humidity (199.2 h) was considerably higher than that from conidia produced from fresh cadavers and those kept at 20% relative humidity, although this difference was not statistically significant.

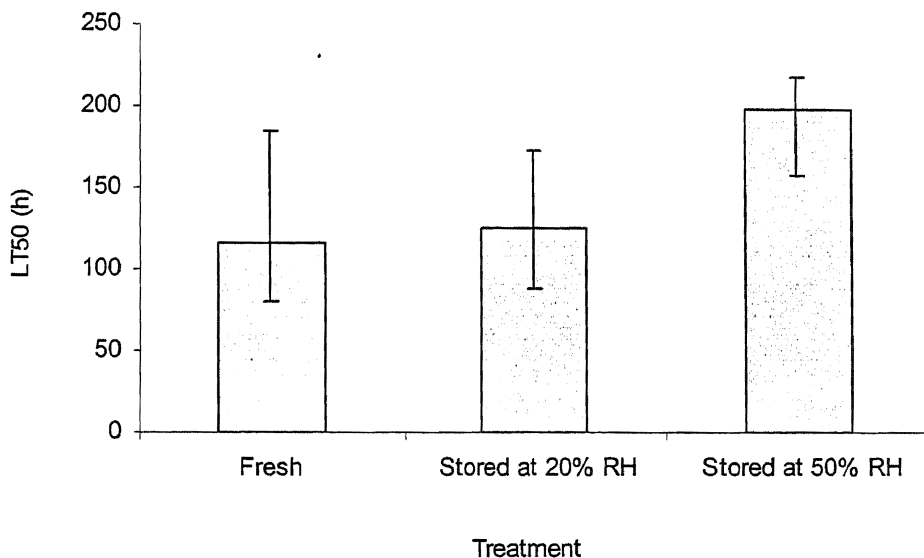


Figure 3.13  $LT_{50}$  of conidia produced from fresh cadavers and cadavers stored at 20% RH and 50% RH. Error bars represent 95% confidence limits calculated from ten replicates per treatment.

### **3.4 Effect of pesticides on the colony growth and sporulation of *E. neoaphidis*.**

#### **3.4.1 Effect of pesticides on colonial growth**

*Erynia neoaphidis* was grown on SDEM agar that contained various pesticides and the colony radial growth rates were compared. There was no significant difference in the colony radial growth (Kr) of *E. neoaphidis* NW 327 on SDEM containing cypermethrin ( $37.67 \mu\text{mh}^{-1}$ ),  $\lambda$ -cyhalothrin 5EC ( $38.63 \mu\text{mh}^{-1}$ ) or deltamethrin ( $39.58 \mu\text{mh}^{-1}$ ) compared to the Kr on SDEM alone ( $38.65 \mu\text{mh}^{-1}$ ).

The Kr on SDEM containing glyphosate ( $31.31 \mu\text{mh}^{-1}$ ) was significantly lower than that on SDEM alone. There was no significant difference in the Kr on SDEM containing pirimicarb ( $28.06 \mu\text{mh}^{-1}$ ) compared to SDEM containing fomasefen ( $27.41 \mu\text{mh}^{-1}$ ), although the Kr on both these media were significantly lower than on SDEM alone and SDEM containing glyphosate. The Kr on media containing chlorothalonil ( $9.61 \mu\text{mh}^{-1}$ ) was significantly lower than the Kr on SDEM containing fluazifop-p-butyl ( $15.98 \mu\text{mh}^{-1}$ ) which was significantly lower than on SDEM containing  $\lambda$ -cyhalothrin 10CS ( $19.38 \mu\text{mh}^{-1}$ ), which in turn was significantly lower than on SDEM containing chlorpyrifos ( $22.74 \mu\text{mh}^{-1}$ ).

The Kr on SDEM containing chlorpyrifos,  $\lambda$ -cyhalothrin 10CS, fluazifop-p-butyl and chlorothalonil was significantly lower than on SDEM containing pirimicarb and fomasefen, and on SDEM alone.

No growth occurred on SDEM containing azoxystrobin or paraquat. No growth occurred after the mycelial plug was transferred to SEMA, indicating that the effect of the pesticide was fungicidal rather than fungistatic.

Table 3.2 Colony radial growth rate ( $K_r$ ) of *Erynia neoaphidis* NW327 on SDEM containing various pesticides. The standard error indicated for each value is that of the regression coefficient. Data with different superscripts are significantly different.

Pesticide	Quantity of pesticide added	$K_r$ ( $\mu\text{m h}^{-1}$ )	Standard error	Number of measurements
SDEM (control)	N/A	38.65 <sup>a</sup>	0.81	80
Deltamethrin	0.0063 ml	39.58 <sup>a</sup>	1.81	80
$\lambda$ -cyhalothrin 5EC	0.0074 ml	38.63 <sup>a</sup>	0.99	80
Cypermethrin	0.0200 ml	37.67 <sup>a</sup>	2.28	80
Glyphosate	0.8900 ml	31.31 <sup>b</sup>	0.62	80
Pirimicarb	0.3000 g	28.06 <sup>c</sup>	1.31	80
Fomasefen	0.1950 ml	27.41 <sup>c</sup>	2.07	80
Chlorpyrifos	0.1670 ml	22.74 <sup>d</sup>	1.55	60
$\lambda$ -cyhalothrin 10CS	0.0074 ml	19.38 <sup>e</sup>	1.42	60
Fluazifop-p-butyl	0.3500ml	15.98 <sup>f</sup>	0.54	70
Chlorothalonil	0.1600 ml	9.61 <sup>g</sup>	0.46	60
Paraquat	0.6160 ml	0 <sup>h</sup>	N/A	60
Azoxystrobin	0.2000 ml	0 <sup>h</sup>	N/A	60

### 3.4.2 Effect of various pesticides on sporulation

#### 3.4.2.1 Pesticide application to mycosed cadavers

Mycosed aphid cadavers were dipped in pesticide solution (as described in section 2.6.2) and the production of conidia assessed on the sporulation monitor. There was a significant reduction in the peak number of conidia produced by all of the cadavers that had been dipped in pesticide compared to the control (293.6 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ) (ANOVA). Azoxystrobin and chlorothalonil completely inhibited sporulation from cadavers at the pesticide application rate tested. Production of conidia from cadavers dipped in  $\lambda$ -cyhalothrin 10CS was reduced significantly less than production from cadavers dipped in any of the other pesticides (140.6 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ), but was still significantly lower than production of conidia from the control. There was no significant difference in production of conidia between cadavers dipped in  $\lambda$ -cyhalothrin 5 EC (109.5 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ), chlorpyrifos (96.4 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ), deltamethrin (107.4 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ), paraquat (112.5 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ) and glyphosate (104.5 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ), although production of conidia from all these was significantly lower than the control, with a reduction in production of conidia of approximately 62% – 67%. There was no significant difference in production of conidia between cadavers dipped in  $\lambda$ -cyhalothrin 5 EC, chlorpyrifos, deltamethrin, cypermethrin (86.3 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ), glyphosate and fomesefan (91.5 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ). However, production of conidia from cadavers dipped in cypermethrin and fomesefan was significantly lower than that from cadavers dipped in paraquat and also from the control, with a reduction in production of conidia of approximately 70% from the control. Production of conidia from cadavers dipped in fluazifop-p-butyl (74.2 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ) was significantly lower than production of conidia from cadavers dipped in all other pesticides, except for cypermethrin, where there was no significant



difference. There was a reduction in production of conidia of approximately 75% compared to the control. Significant differences in peak production of conidia are summarised in Table 3.3 (p. 111). There was also a significant reduction in the length of time that the pesticide treated cadavers produced conidia compared to the control. The controls sporulated for 75 h, while the maximum time that pesticide treated cadavers sporulated for was 35 h (cypermethrin). Cadavers treated with fomesafen, glyphosate, chlorpyrifos,  $\lambda$ -cyhalothrin 5EC and fluazifop-p-butyl all failed to produce conidia after 30h. Cadavers treated with deltamethrin failed to produce conidia after 25h, while those treated with  $\lambda$ -cyhalothrin 10CS failed to produce conidia after only 20h. The frequency distributions generally showed a bimodal pattern with the exception of the distributions produced from cadavers treated with fluazifop-p-butyl and possibly chlorpyrifos. Graphs showing production of conidia for cadavers treated with each pesticide and frequency distribution of conidial volume are presented in Appendix A. The shapes of the distributions of the volume of conidia produced after the first 5 h of incubation were significantly different to the shape of the distributions of the volume of conidia produced after the last 5 h of incubation for cadavers treated with all pesticides.

During the last 5 h of incubation, cadavers treated with fluazifop-p-butyl, fomesafen and deltamethrin produced conidia that were significantly larger (2093  $\mu\text{m}$ , 1830 $\mu\text{m}$  and 2604  $\mu\text{m}$  respectively) compared to those produced in the first 5 h of incubation (1581 $\mu\text{m}$ , 1664 $\mu\text{m}$ , and 1723 respectively), and which had a significantly higher  $L_{\text{maj}}/L_{\text{min}}$  ratio (1.60, 1.72 and 1.52 respectively) compared to those produced in the first 5 h of incubation (1.52, 1.49 and 1.46).

Conidia produced in the last 5 h of incubation from cadavers treated with cypermethrin, glyphosate and paraquat had a significantly larger volume (2503  $\mu\text{m}^3$ , 2235  $\mu\text{m}^3$  and 2245  $\mu\text{m}^3$  respectively) than those discharged during the last 5 h of incubation (2218  $\mu\text{m}^3$ , 1595  $\mu\text{m}^3$  and 1586  $\mu\text{m}^3$  respectively), but did not have a significantly different shape.

Conidia discharged during the last 5 h of incubation from cadavers treated with lambda cyhalothrin 5 EC and lambda cyhalothrin 10 CS were significantly larger

(2370  $\mu\text{m}^3$  and 2110  $\mu\text{m}^3$  respectively) than those discharged during the first 5 h of incubation (1526  $\mu\text{m}^3$  and 1785  $\mu\text{m}^3$  respectively), and had a significantly smaller  $L_{\text{maj}}/L_{\text{min}}$  ratio (1.44 and 1.59 respectively) than those discharged during the last 5 h of incubation (1.55 and 1.76 respectively).

Conidia discharged during the first 5 h of incubation from cadavers treated with chlorpyrifos were not significantly different in volume to those discharged during the last 5 h of incubation, but had a significantly larger  $L_{\text{maj}}/L_{\text{min}}$  ratio (1.63 compared to 1.46).

#### 3.4.2.2 Pesticide application following fungal inoculation

Live aphids were inoculated with *E. neoaphidis* and then dipped in pesticide 4h, 48h or 72h after fungal inoculation (as described in 2.6.2), and after the death of the aphid and two days storage at 4°C and 20% RH, the production of conidia was assessed on the sporulation monitor. Aphids that had been dipped in cypermethrin, deltamethrin, chlorpyrifos,  $\lambda$ -cyhalothrin 5EC,  $\lambda$ -cyhalothrin 10CS and paraquat died within 8 h of applying the pesticide and no sporulation was observed. No sporulation was observed from aphids dipped in either chlorothalonil or azoxystrobin 4 h, 48 h or 72 h after fungal inoculation. The aphids treated with these chemicals were not killed either by fungus or by the chemicals.

Production of conidia from aphids dipped in glyphosate, fomesafen and fluzifop-p-butyl was significantly lower compared to the control at 4 h following fungal inoculation (112.6 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup>, 70.3 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup>, 76.4 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup> and 289.4 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup> respectively), 48 h following fungal inoculation (87.3 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup>, 86.3 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup>, 96.4 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup> and 292.4 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup> respectively) and 72 h following fungal inoculation (91.5 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup>, 84.0 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup>, 86.3 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup> and 292.7 conidia  $\text{mm}^{-2}$  cadaver<sup>-1</sup> respectively). There was no significant difference in production of conidia between aphids dipped in glyphosate, fomesafen and fluzifop-p-butyl when the pesticide was applied either 48 h or 72 h following fungal inoculation. However, production of conidia from aphids

dipped in glyphosate 4 h after inoculation was reduced significantly less than from aphids dipped in fomesafen or fluazifop-p-butyl. Aphids treated with glyphosate, fomesafen or fluazifop-p-butyl also produced conidia for a much shorter period of time (35h) compared to the controls (75h) (Appendix C). Significant differences in peak production of conidia are summarised in Table 3.3. The frequency distribution generally showed a bimodal pattern as with the cadavers, although there were two exceptions to this. Conidia produced from aphids dipped in fluazifop-p-butyl 72 h after fungal inoculation produced a roughly bell shaped distribution and conidia produced from aphids dipped in fomesafen 72 h after fungal inoculation produced a distribution with a single peak displaced to the left. Graphs showing production of conidia and frequency distributions of conidial volumes for aphids treated with each pesticide/time interval combination are presented in Appendix C. Conidia discharged during the last 5 h of incubation generally had significantly larger volumes than those discharged during the first 5 h of incubation. The one exception to this was conidia discharged from aphids dipped in fluazifop-p-butyl 48 h after fungal inoculation, where conidia which had been discharged during the last 5 h of incubation were not significantly different in size to those discharged during the first 5 h of incubation.

Conidia produced during the last 5 h of incubation from aphids treated with all pesticides at all time intervals generally had a significantly greater  $L_{maj}/L_{min}$  ratio (1.63 – 1.72) than those produced during the first 5 h (1.46 – 1.51). Conidia discharged during the last 5 h of incubation from aphids treated with fluazifop-p-butyl 72 h after fungal inoculation and conidia discharged from aphids treated with fluazifop-p-butyl 4 h after fungal inoculation and glyphosate 4 h after fungal inoculation were not significantly different in shape to those discharged during the first five hours of incubation.

#### 3.4.2.3 Pesticide application prior to fungal inoculation

Live aphids were dipped in pesticides and then inoculated with *E. neoaphidis* 4 h, 48 h or 72 h later (as described in 2.6.2). After the death of the aphid and two days storage at 4°C and 20% relative humidity, the production of conidia was

assessed on the sporulation monitor. Sporulation did not occur from aphids dipped in cypermethrin, deltamethrin, chlorpyrifos, either formulation of  $\lambda$ -cyhalothrin or paraquat, 4 h, 48 h, or 72 h prior to inoculation.

There was no sporulation from aphids dipped in azoxystrobin or chlorothalonil 4 h or 48 h prior to inoculation, although these treatments did not prevent sporulation when made 72 h before inoculation (128.2 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$  and 126.0 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$  respectively). There was no significant difference in production of conidia from aphids dipped in glyphosate, fomasefen and fluazifop-p-butyl 4 h prior to inoculation (88.4, 81.2 and 92.1 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$  respectively) (ANOVA), although production of conidia from aphids dipped in these pesticides was significantly lower than in the control (283.7 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ).

There was no significant difference in production of conidia from aphids dipped in glyphosate, fomasefen and fluazifop-p-butyl 48 h prior to inoculation (84.0, 90.2 and 108.71 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$  respectively) (ANOVA), although production of conidia from aphids dipped in these pesticides was significantly lower than in the control (289.1 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$ ). Aphids dipped in glyphosate, fomasefen or fluazifop-p-butyl at 4 h and 48h prior to inoculation also produced conidia for a much shorter period of time (35 h) compared to the controls (75 h) (Appendix B)

There was a significant reduction in production of conidia from aphids dipped in glyphosate, fomasefen and fluazifop-p-butyl 72 h prior to inoculation compared to the control (94.1, 89.3, 76.8 and 276.8 conidia  $\text{mm}^{-2}$  cadaver $^{-1}$  respectively). There was no significant difference in production of conidia from aphids dipped in glyphosate compared to aphids dipped in fomasefen. There was also no significant difference in production of conidia from aphids dipped in fomasefen compared to aphids dipped in fluazifop-p-butyl. Production of conidia from aphids dipped in fluazifop-p-butyl was significantly lower than production of conidia from aphids dipped in glyphosate. There was no significant difference in production of conidia from aphids dipped in azoxystrobin compared to production of conidia from aphids treated with chlorothalonil.

In all cases, treatment of aphids by dipping in any of the pesticides prior to inoculation reduced subsequent production of conidia compared to the controls. Aphids dipped in azoxystrobin or chlorothalonil produced conidia for a shorter period of time (30 h) than aphids dipped in glyphosate, fomasefen or fluazifop-p-butyl (35 h) or controls (75 h). Significant differences in peak production of conidia are summarised in Table 3.3. (p. 111). As with the cadavers, the frequency distributions generally showed a bimodal pattern with the exception of that produced by conidia discharged from aphids dipped in fluazifop-p-butyl 72 h prior to fungal inoculation, which showed a roughly rectangular distribution and from aphids dipped in fomasefen 48h prior to inoculation which showed a single peak displaced to the left of the distribution. Graphs showing production of conidia and frequency distributions of conidial volumes for aphids treated with each pesticide prior to fungal inoculation are presented in Appendix B. During the last 5 h of incubation, aphids treated with glyphosate, fomasefen and fluazifop-p-butyl pesticides at 4 h, 48 h and 72 h prior to fungal inoculation produced significantly larger conidia than those produced in the first five hours of incubation with two exceptions. Conidia discharged during the first 5h incubation from aphids treated with fluazifop-p-butyl 4 h prior to inoculation and aphids treated with glyphosate 48h prior to fungal inoculation were not significantly smaller than those conidia produced during the last 5 h of incubation.

Conidia produced during the last 5 h of incubation from aphids treated with all pesticides at all time intervals generally had a significantly greater  $L_{maj}/L_{min}$  ratio (1.52 – 1.73) than those produced during the first five hours (1.42 - 1.58). Conidia discharged during the last five hours of incubation from aphids treated with fluazifop-p-butyl 72 h prior to fungal inoculation and conidia discharged from aphids treated with fluazifop-p-butyl 48 h prior to fungal inoculation and glyphosate 48 h prior to fungal inoculation were not significantly different in shape to those discharged during the first five hours of incubation.

There was also further microscopic evidence of secondary conidia production with the observation of primary conidia “ghosts” accounting for approximately 2% of

conidia production. These are the remains of primary conidia after secondary conidia have been ejected.

Table 3.3 Peak rate of discharge of conidia (conidia mm<sup>-2</sup> cadaver<sup>-1</sup>) for all pesticides and treatments (dipping cadavers in pesticide, or live aphids before or after fungal inoculation). Data with different superscripts are significantly different

Pesticide	Cadavers	Pre - dipping inoculation			Post - dipping inoculation		
		4h	48h	72h	4h	48h	72h
Control	293.6 <sup>a</sup>	289.4 <sup>a</sup>	292.4 <sup>a</sup>	292.7 <sup>a</sup>	283.7 <sup>a</sup>	289.1 <sup>a</sup>	276.8 <sup>a</sup>
azoxystrobin	0	0	0	0	0	0	128.2 <sup>b</sup>
chlorothalonil	0	0	0	0	0	0	126.0 <sup>b</sup>
λ-cyhalothrin 5 EC	109.5 <sup>c,d</sup>	0	0	0	0	0	0
λ-cyhalothrin 10 CS	140.6 <sup>b</sup>	0	0	0	0	0	0
chlorpyrifos	96.4 <sup>c,d</sup>	0	0	0	0	0	0
cypermethrin	86.3 <sup>d,e</sup>	0	0	0	0	0	0
deltamethrin	107.4 <sup>c,d</sup>	0	0	0	0	0	0
paraquat	112.5 <sup>c</sup>	0	0	0	0	0	0
glyphosate	104.5 <sup>c,d</sup>	112.6 <sup>b</sup>	87.3 <sup>b</sup>	91.5 <sup>b</sup>	88.4 <sup>b</sup>	84.0 <sup>b</sup>	94.1 <sup>d</sup>
fomasefen	91.5 <sup>d</sup>	70.3 <sup>c</sup>	86.3 <sup>b</sup>	84.0 <sup>b</sup>	81.2 <sup>b</sup>	90.2 <sup>b</sup>	89.3 <sup>d,e</sup>
fluazifop-p-butyl	74.2 <sup>e</sup>	76.4 <sup>c</sup>	96.4 <sup>b</sup>	86.3 <sup>b</sup>	92.1 <sup>b</sup>	108.7 <sup>b</sup>	76.8 <sup>e</sup>

### **3.5 Effect of inoculum position on transmission of *E. neoaphidis* in a population of *Acyrtosiphon pisum***

#### **3.5.1 Effect of inoculum position on transmission in the laboratory**

##### **3.5.1.1 Effect on mortality and $LT_{50}$**

Fungal inoculum was placed at 3 different positions on a bean plant and aphids introduced to the plant, as described in 2.8.1.1, and the  $LT_{50}$  calculated from observations of mortality. During the period of observation the percentage mortality from mycosis caused by cadavers placed on the soil (32%, 75.5% and 96.5% mortality at 96 h, 120 h and 144 h respectively) was lower than that caused by cadavers placed on the top of the plant (76.5%, 97% and 100% mortality) or middle (69.5%, 95.5% and 98% mortality) of the plant (Figure 3.14). The  $LT_{50}$  of cadavers placed on the soil (102.87 h) was significantly longer than the  $LT_{50}$  of cadavers placed on the top of the plant (87.39 h) but not the  $LT_{50}$  of cadavers placed on the middle of the plant (90.92h) (Figure 3.15). There was no significant difference between the  $LT_{50}$  of cadavers placed on the top of the plant and those placed on the middle of the plant (ANOVA). The use of this method gives an  $LT_{50}$  that includes the time taken for infection of the host to occur and the time taken to kill. Conventionally,  $LT_{50}$  often does not include the time taken for infection.



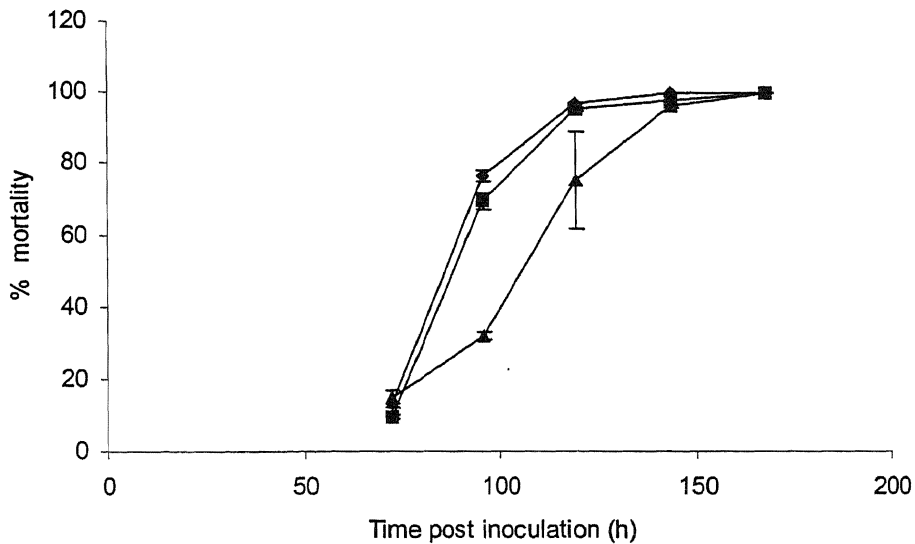


Figure 3.14 Mortality of *Acyrthosiphon pisum* from mycosis caused by conidia of *E. neoaphidis* discharged from cadavers placed either at the top of the plant (♦), the middle of the plant (■) or on the soil (▲). Error bars represent the standard errors of the means of five replicates.

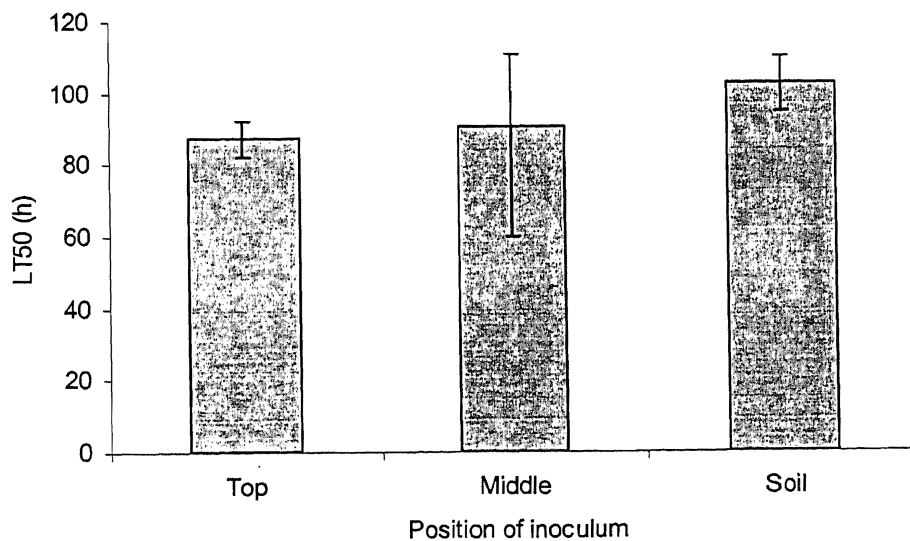


Figure 3.15. LT<sub>50</sub> of aphids infected with conidia of *E. neoaphidis* produced from cadavers placed on the top of the host plant, the middle of the host plant, and on the soil. Error bars are 95% confidence intervals calculated from ten replicates per treatment.

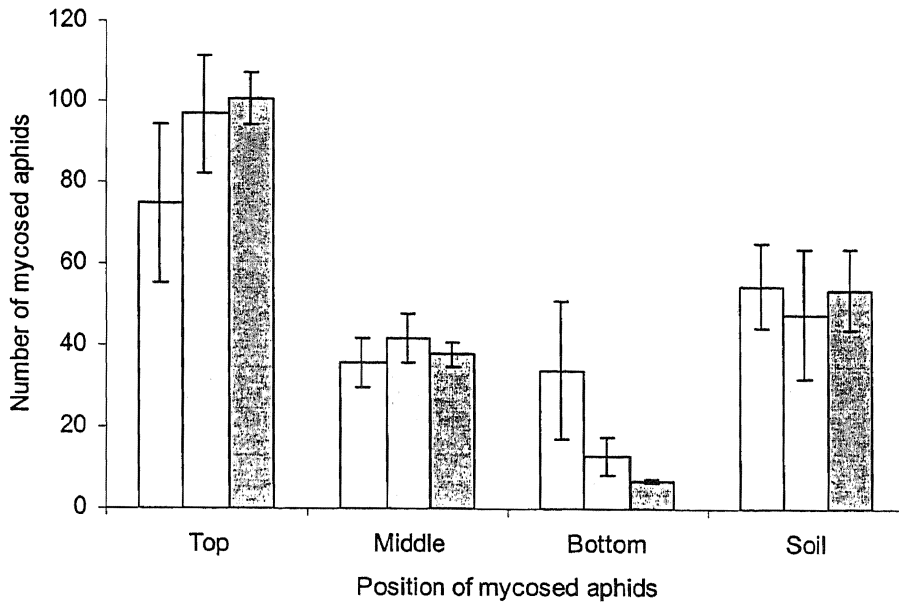


Figure 3.16 Position and number of mycosed aphids found on the host plant when inoculum has been placed at the top of the plant (□), the middle of the plant (□) and on the soil (■) on a bean plant at 20°C and 100% RH

### 3.5.1.2 Effect on position of mycosed aphids

This is part of the same experiment as that described in 3.5.1.1. Fungal inoculum was placed at 3 different positions on a bean plant and aphids introduced to the plant, as described in 2.8.1.1 and the position on the plant of any mycosed aphids which had subsequently died as a result of infection from this inoculum was recorded. Significantly more aphids died at the top of the plant (273) than on the middle of the plant (116), the bottom of the plant (54), or on the soil, (157) (Figure 3.16) (ANOVA). There was no significant difference between the number of mycosed aphids counted at any of the other positions, although the number of mycosed aphids counted on the soil was higher than that counted on the middle or bottom of the plant. A significantly lower number of mycosed aphids were found at the bottom of the plant when the inoculum had been placed on the soil compared to when the inoculum had been placed on the top of the plant or the middle of the plant (Figure 3.16)

### 3.5.2 Effect of inoculum position on transmission in the field

The time taken for infection to occur and time taken for aphids to die from mycosis resulting from infection from conidia discharged from cadavers placed on the soil, was significantly less than for infection resulting from conidia discharged from cadavers placed on either the top of the plant or on the middle of the plant (Figure 3.17). There was no mortality due to mycosis in the controls. This effect of position of inoculum on the mortality of aphids was also seen in the  $LT_{50}$  of the cadavers. Although there was no significant difference in  $LT_{50}$  between aphids infected by conidia discharged from cadavers placed on the top of the plant, the middle of the plant or on the soil, the  $LT_{50}$  of aphids infected by conidia discharged by cadavers placed at the top of the plant was greater than that of cadavers placed on the soil or on the middle of the plant (Figure 3.18). The use of this method gives an  $LT_{50}$  that includes the time taken for infection of the host to occur and the time taken to kill. Conventionally,  $LT_{50}$  often does not include the time taken for infection.

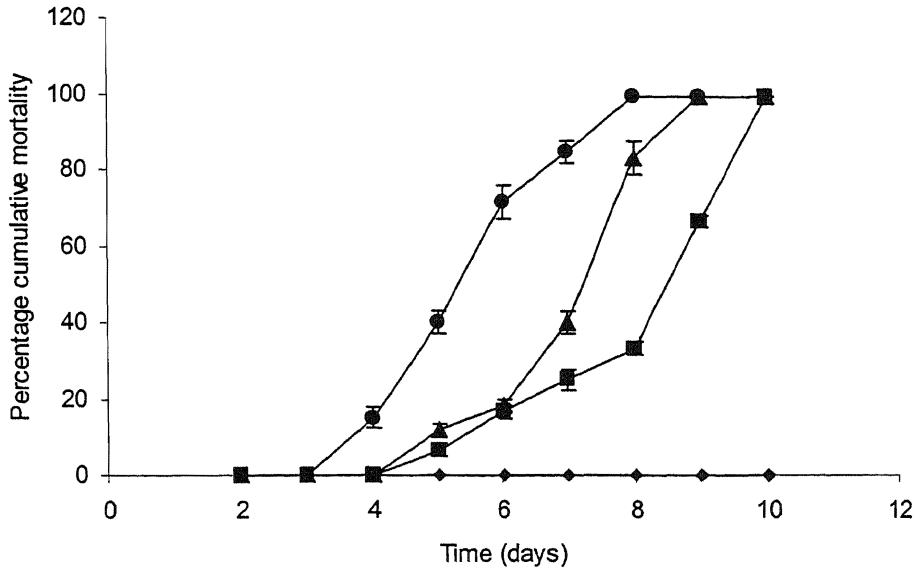


Figure 3.17 Mortality of aphids due to mycosis from conidia of *E. neoaphidis* discharged from cadavers placed at the top of the plant (■), the middle of the plant (▲) and the soil (●) in the field. No cadavers were placed in control treatments (◆) Error bars represent the standard errors of the means of three replicates.

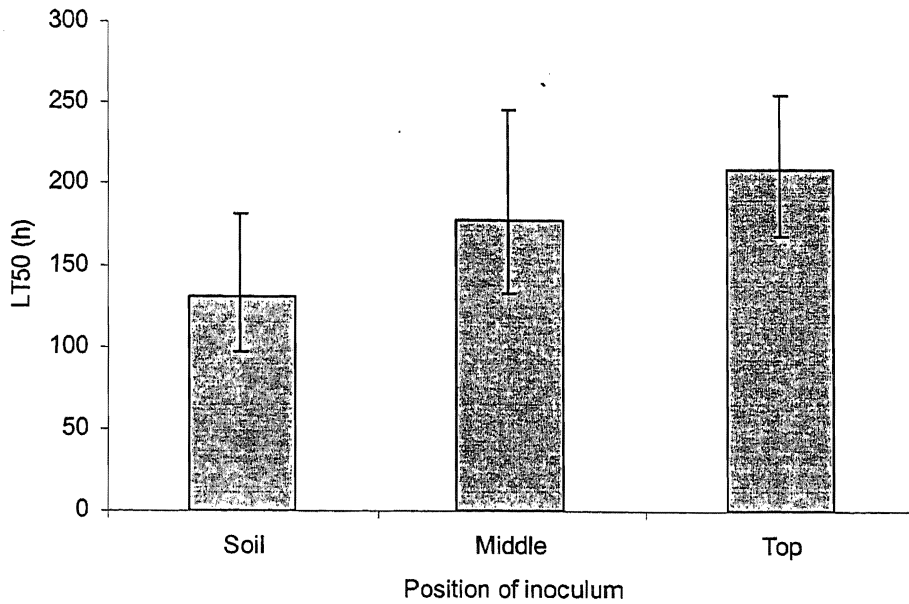


Figure 3.18 LT<sub>50</sub> for killing of aphids resulting from infection by conidia of *E. neoaphidis* discharged from cadavers placed on the top of the plant, on the middle of the plant, and on the soil in the field. Error bars represent 95% confidence limits calculated from three replicates per treatment.

Significantly more aphids died at the top of the plant (37) than the middle (2) of the plant. There were no mycosed aphids found on the bottom of the plant or on the soil (Figure 3.19). Significantly more mycosed aphids were found at the top of the plant when the inoculum had been placed on the soil than when the inoculum had been placed on the middle of the plant. There was no significant difference in the number of mycosed aphids found at the top of the plant when the inoculum was placed on the top of the plant compared to the number of mycosed aphids found at the top of the plant when the inoculum had been placed on the middle of the plant or on the soil (Figure 3.19).

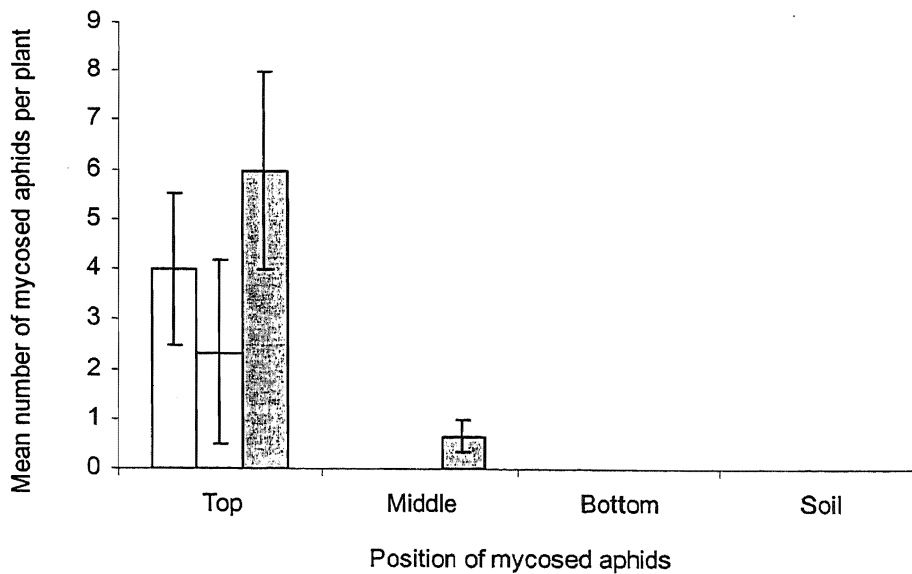


Figure 3.19 Position and number of mycosed aphids infected by conidia discharged from cadavers placed at the top of the plant (□), the middle of the plant (□) and the soil (■) in the field. Error bars represent the standard error of the mean of 3 replicates

### 3.6 Effect of host density on transmission of *E. neoaphidis*

#### 3.6.1 Effect of host density on transmission in the laboratory

There was a clear, positive effect of host density on transmission when tested in the laboratory (Figure 3.20). After eight days, only 12% of aphids had been mycosed when the host density was five aphids per plant. At 10 aphids per plant, there was a significant rise to 40% mycosis after eight days. At 20 aphids per plant there was another significant rise in mycosis, when after just six days, all of the aphids had been killed by fungus. At 40 aphids per plant, there was 100% mycosis after only 5 days. This was quicker than at 20 aphids per plant, but not significantly (ANOVA).

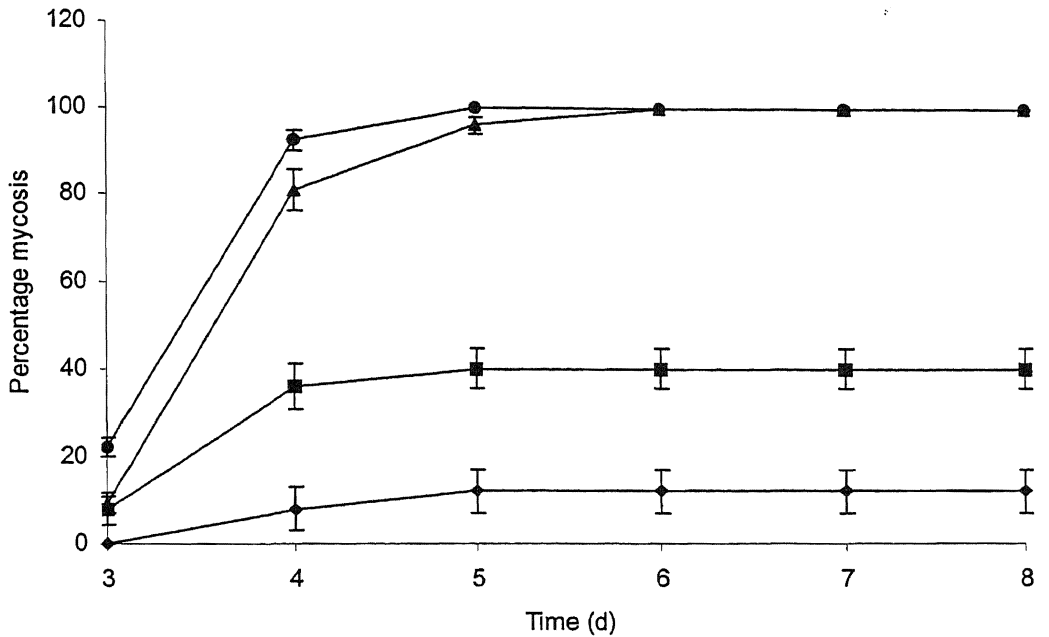


Figure 3.20 Percentage mortality, due to mycosis, of host aphids on bean plants at four densities of pea aphid host: 5 (◆), 10 (■), 20 (▲), and 40 (●) aphids per plant at 20°C, 100% RH and a photoperiod of 16h:8h (light: dark). Error bars represent the standard errors of the mean of 5 replicates.

This density dependence was also seen in the  $LT_{50}$  of the fungus at different host densities (Figure 3.21). The  $LT_{50}$  at five hosts per plant was significantly higher than at ten hosts per plant, which was significantly higher than at twenty and forty hosts per plant. There was no significant difference between the  $LT_{50}$  at twenty hosts per plant and at forty hosts per plant.

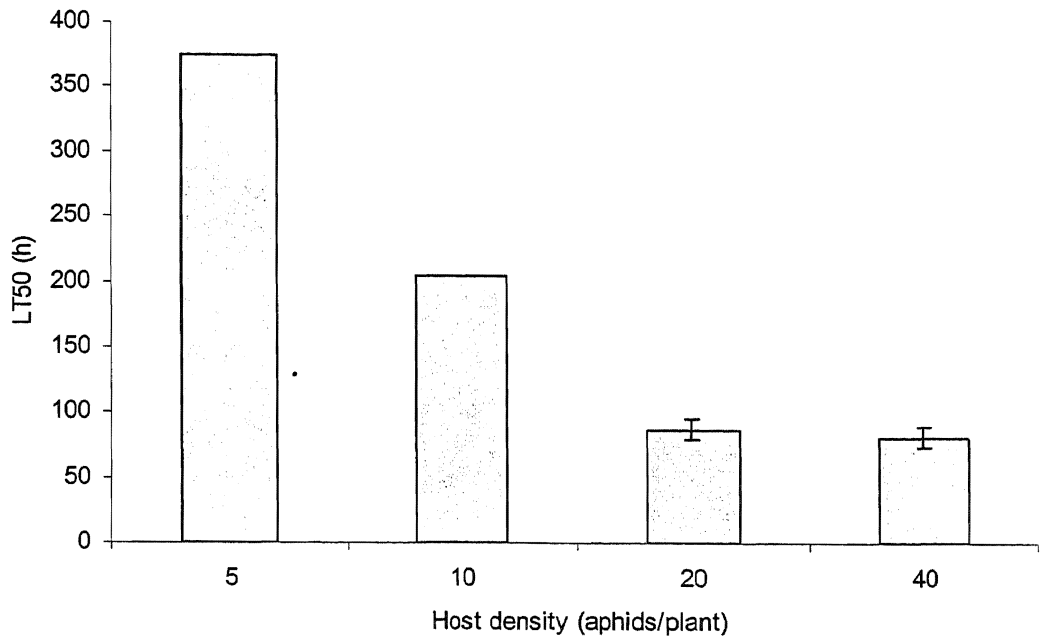


Figure 3.21 LT<sub>50</sub> for conidia discharged from cadavers at four densities of pea aphid host at 20°C, 100%RH and a photoperiod of 16h: 8h (light: dark). Error bars represent 95% confidence intervals calculated from five replicates per treatment.

### 3.6.2 Effect of host density on transmission in the field

Differing densities of aphids were placed on bean plants in the field with fungal inoculum. At various time intervals after the placement of the inoculum the aphids were returned to the laboratory and monitored for infection. This gave an indication of the persistence of the inoculum in the field.

On day 1 after placement of the inoculum in the field there is some evidence of positive density dependence. There is significantly greater mortality from mycosis caused by *E. neoaphidis* at 40 aphids per plant compared to that at 20 aphids per plant. There is also significantly higher mortality at 20 aphids per plant than at 10 aphids per plant. However, this was not evident on the other days, where the data



indicates density independence or negative density dependence. On day 0 there was no significant difference in fungal induced mortality between aphid populations at any of the three densities. On day 2, there was no significant difference in fungal induced mortality at 10 aphids per plant compared to 20 aphids per plant, or at 20 aphids per plant compared to 40 aphids per plant (ANOVA). However, at 10 aphids per plant, fungal induced mortality was significantly higher than at 40 aphids per plant (ANOVA).

Mortality due to mycosis also decreased over time as the inoculum was left exposed in the field (Figure 3.22). At 40 aphids per plant, mortality due to mycosis fell significantly from day 0 (68.08%) to day 1 (53.94%) and from day 1 to day 2 (6.29%). There was then no significant change in mortality due to mycosis from day 2 to day 5 (8.14%). At 20 aphids per plant, mortality due to mycosis fell significantly from day 0 (64.64%) to day 1 (36.53%) and from day 1 to day 2 (12.49%). There was a significant drop in mortality from day 2 to day 3 (5.52%). There was a no significant change in mortality from day 3 to day 5. At 10 aphids per plant, mortality due to mycosis fell significantly from day 0 (60.19%) to day 1 (25.70%). There was no significant change in mortality due to mycosis from day 1 to day 2 (25.18%). There was a significant drop in mortality from day 2 to day 3 (8.33%) and a significant rise in mortality from day 3 to day 5 (21.29%). The mortality on day 5 was not significantly different from the mortality on days 0, 1 and 2 (ANOVA).

Table 3.4. Mean percentage aphid daily mortality due to mycosis by *E. neoaphidis* ( $\pm$  the standard errors of the means of 3 replicates) for 10, 20 and 40 host aphids per plant. Data with different superscripts are significantly different.

Host Density (per plant)	Number of days that the inoculum had been left in the field				
	0	1	2	3	5
10 (control)	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>
20 (control)	0.00 <sup>a</sup>	1.67 <sup>a</sup> $\pm$ 1.67	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>
40 (control)	2.86 <sup>b</sup> $\pm$ 2.33	0.88 <sup>a</sup> $\pm$ 0.88	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.88 <sup>a</sup> $\pm$ 0.88
10	60.19 <sup>c</sup> $\pm$ 21.77	25.7 <sup>b</sup> $\pm$ 6.41	25.18 <sup>b</sup> $\pm$ 9.96	8.33 <sup>a,b</sup> $\pm$ 8.33	21.29 <sup>b</sup> $\pm$ 7.43
20	64.64 <sup>c</sup> $\pm$ 11.32	36.53 <sup>c</sup> $\pm$ 2.77	12.49 <sup>b,c</sup> $\pm$ 3.75	5.52 <sup>b</sup> $\pm$ 0.43	10.27 <sup>c</sup> $\pm$ 5.54
40	68.08 <sup>c</sup> $\pm$ 7.55	53.94 <sup>d</sup> $\pm$ 4.17	6.92 <sup>c</sup> $\pm$ 3.81	3.86 <sup>b</sup> $\pm$ 2.31	8.14 <sup>c</sup> $\pm$ 3.07

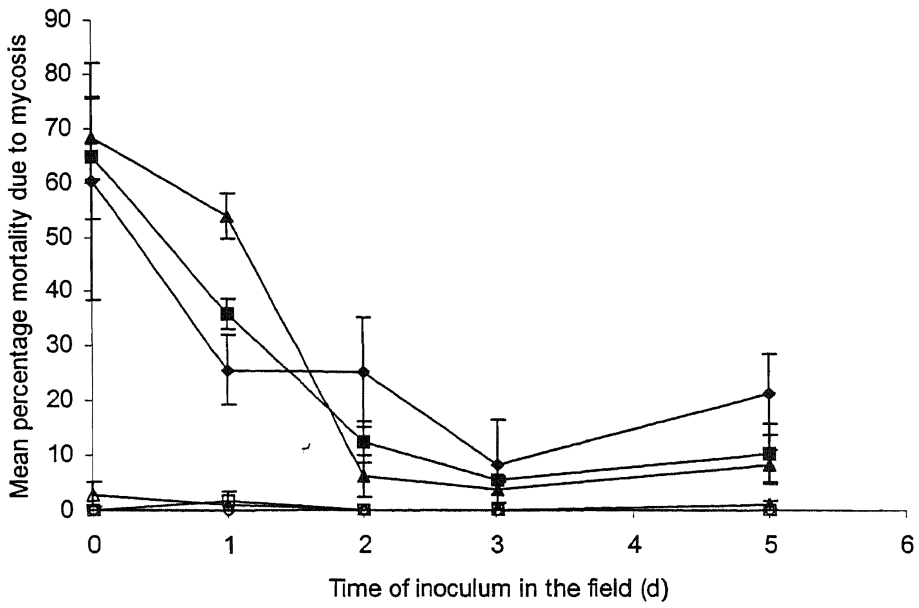


Figure 3.22 Mean daily percentage mortality of pea aphids due to mycosis by *E. neoaphidis* at 10 aphids per plant (◆), 20 aphids per plant (■) and 40 aphids per plant (▲). Open symbols represent control treatments at the three densities of aphid. Error bars represent the standard errors of the means of three replicates.

### 3.7 Effect of cadaver density on the transmission of *E. neoaphidis*.

#### 3.7.1 Effect of cadaver density in the field

Various densities of cadavers were placed inside muslin bags on individual bean plants in the field along with a fixed number of aphid hosts. At varying time intervals after placing the inoculum in the field, aphids were removed from the plant and returned to the laboratory where they were monitored for infection

Temperatures in the field on the soil ranged from 11.7°C to 33.7°C (mean = 17.3°C). Temperatures at the mid canopy level ranged from 14.9°C to 25°C (mean = 16.9°C). Temperatures at the top of the canopy ranged from 9.5°C to 46.9°C (mean = 18.4°C). Temperatures at the top of the canopy inside the gauze sleeve ranged from 9.9°C to 30.3°C (mean = 17.0°C). The relative humidity at the top of the canopy ranged from 42.7% to 100.9% (mean = 92.7%). These results are summarised in Table 3.5

Table 3.5 Summary of temperature and relative humidity at various positions within the plant canopy during the field experiment to assess the effect of cadaver density on the transmission of *E. neoaphidis*.

Position of data logger	Mean reading	Maximum reading	Minimum reading
Soil	17.3 °C	33.7 °C	11.7 °C
Mid canopy	16.9 °C	25.9 °C	14.9 °C
Canopy top	18.4 °C	46.9 °C	9.5 °C
Sleeve (canopy top)	17.0 °C	30.3 °C	9.9 °C
Sleeve (RH logger)	92.7% RH	100.9% RH	42.7% RH

On Day 0, there was no significant difference in aphid mortality due to mycosis between plants that contained 40 cadavers per plant, 20 cadavers per plant and 10 cadavers per plant (Fig 3.23)(ANOVA). Aphid mortality on plants with 5 cadavers per plant was significantly lower than with 20 or 40 cadavers per plant, but not significantly lower than at 10 cadavers per plant (ANOVA). Mortality with 1 cadaver per plant was again significantly lower than at 20 and 40 cadavers per plant, but there was no significant difference compared to 5 and 10 aphids per plant (ANOVA). Aphid mortality was significantly higher on all plants that had cadavers attached than on control plants.

On Day 1, aphid mortality on plants with 40 cadavers attached was significantly higher than the mortality on plants with 1, 5, 10 or 20 cadavers attached. Aphid mortality was significantly higher on all plants that had cadavers attached than on control plants. There were no other significant differences in mortality (ANOVA).

On Day 3, there was no significant difference in mortality between aphid populations on plants that contained 5, 10 or 40 cadavers attached. There was significantly higher mortality on plants with 40 and 5 cadavers per plant compared to plants with 20 cadavers and 1 cadaver per plant. There was no significant difference in the mortality on plants that contained 10 aphids per plant compared to 1 and 20 cadavers per plant. There was no significant difference in aphid mortality on plants with 1, 10 or 20 cadavers per plant compared to the control. Aphid mortality in the controls was significantly lower than on plants containing 40 cadavers and 5 cadavers.

On Days 6 and 9, although aphid mortality was recorded on plants with cadavers attached, this was not significantly different to the aphid mortality in the control treatments. Differences in mortality are summarised in Table 3.4

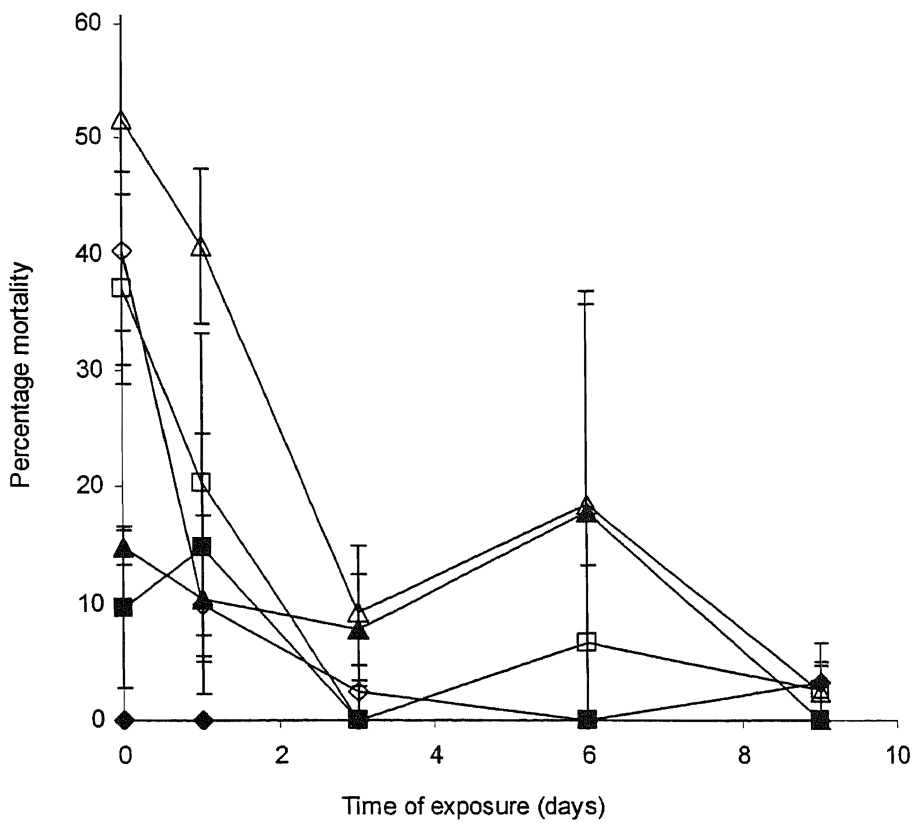


Figure 3.23 Percentage mortality of aphids due to mycosis by *E. neoaphidis* on bean plants in the field with 0 (◆), 1 (■), 5 (▲), 10 (◇), 20 (□), and 40 (△) cadavers attached in the field over a 9 day period. Error bars represent the standard errors of the means of 3 replicates.

Table 3.6 Mean percentage aphid mortality due to mycosis by *E. neoaphidis* on bean plants in the field at 6 cadaver densities (0, 1, 5, 10, 20, and 40 cadavers per plant) ( $\pm$  standard error of the mean). Data with different superscript are significantly different.

Cadaver density (per plant)	Number of days that the inoculum had been exposed in the field				
	0	1	3	6	9
0	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	3.33 <sup>a</sup> $\pm$ 3.33
1	9.65 <sup>b</sup> $\pm$ 6.92	14.81 <sup>b</sup> $\pm$ 9.80	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>
5	14.82 <sup>b</sup> $\pm$ 1.51	10.42 <sup>b</sup> $\pm$ 4.81	7.78 <sup>b</sup> $\pm$ 4.84	17.95 <sup>a</sup> $\pm$ 17.95	0.00 <sup>a</sup>
10	40.28 <sup>b,c</sup> $\pm$ 24.10	9.92 <sup>b</sup> $\pm$ 7.66	2.38 <sup>a,b</sup> $\pm$ 2.38	0.00 <sup>a</sup>	0.00 <sup>a</sup>
20	37.04 <sup>c</sup> $\pm$ 8.17	20.37 <sup>b</sup> $\pm$ 12.96	0.00 <sup>a</sup>	6.67 <sup>a</sup> $\pm$ 6.67	2.56 <sup>a</sup> $\pm$ 2.56
40	51.74 <sup>c</sup> $\pm$ 21.25	40.74 <sup>c</sup> $\pm$ 6.68	9.23 <sup>b</sup> $\pm$ 5.82	18.52 <sup>a</sup> $\pm$ 18.52	2.38 <sup>a</sup> $\pm$ 2.38

### 3.8 Effect of dislodgement

This experiment was performed in order to assess whether the frequency of observation of aphid populations had an effect on the transmission of the fungus by causing aphids to be dislodged, fall to the soil and climb back up the plant. There was no significant difference in the number of *E. neoaphidis* mycosed aphids found at the top of the plant when the aphids were observed once per day compared to when the aphids were observed three times per day (Figure 3.24). Similarly, there were no significant differences in the numbers of mycosed aphids found on the middle and

bottom of the plants, or on the soil when the aphids were observed once per day compared to the same positions when the aphids were observed three times per day. There was no significant difference in the  $LT_{50}$  of the aphids when observed once per day (82.54 h) compared to that when the aphids were observed three times per day (89.71 h) (Figure 3.25).

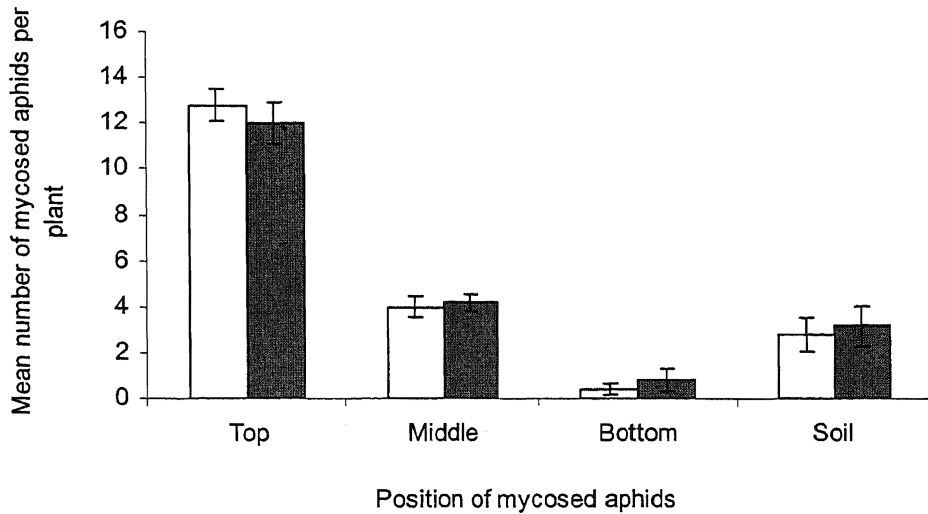


Figure 3.24 Number and position of mycosed aphids which had been observed once per day (□) or 3 times per day (■) at 20°C, 100% RH and a photoperiod of 16 h: 8 h (light: dark). Error bars represent the standard errors of the means of 5 replicates.



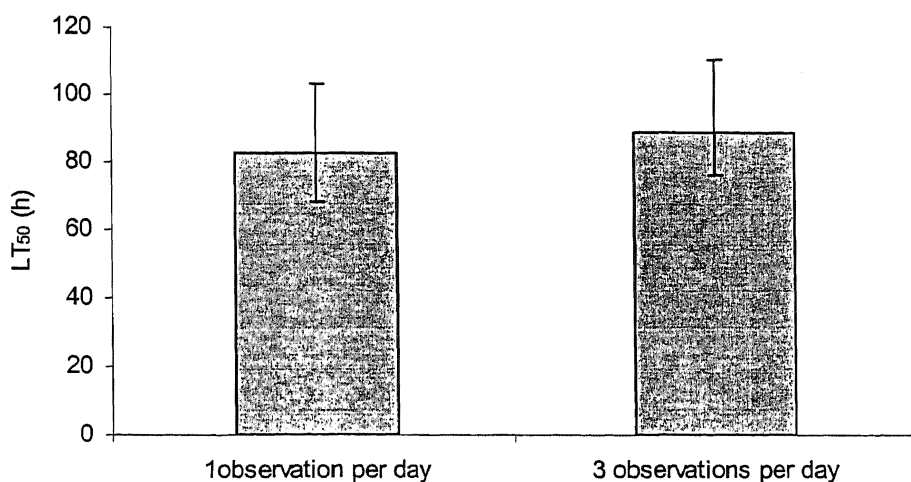


Figure 3.25  $LT_{50}$  for infection and killing of aphids by *E. neoaphidis* observed once per day (□) or 3 times per day (■) at 20°C, 100% RH and a photoperiod of 16 h: 8 h (light: dark). Error bars represent 95% confidence limits calculated automatically by SPSS .

### 3.9 Effect of inoculum position on the secondary transmission of *E. neoaphidis* through an aphid population.

Aphids were introduced to bean plants, to which *E. neoaphidis*-mycosed cadavers were attached to provide a source of conidia. Aphids which were subsequently infected were not removed but were left on the plants to provide an inoculum source to infect subsequent generations of aphids.

Significantly more mycosed aphids were found at the top of the plant (1384) than on the middle of the plant (102), the bottom of the plant (10), or on the soil (117) (Figure 3.26). There was no significant difference in the number of mycosed aphids found on the middle of the plant compared to the number recorded on the soil, but there were significantly fewer mycosed aphids found on the bottom third of the plant compared to the other positions (ANOVA).

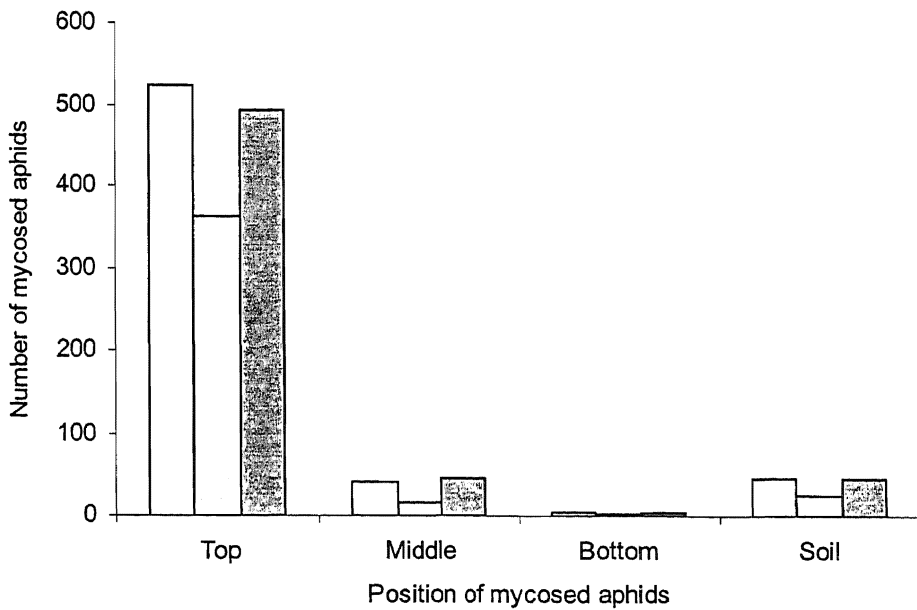


Figure 3.26 Position and number of mycosed aphids found at different positions on the plant when the initial inoculum has been placed at the top of the plant (□), the middle of the plant (▨) or on the soil (▩) at 20°C, 100% RH and a photoperiod of 16 h : 8 h (light: dark). There were 10 replicates per treatment.

The position of the initial inoculum had no effect on the numbers of mycosed aphids found on the top third of the plant, nor on the numbers of mycosed aphids found at the bottom of the plant. Significantly more mycosed aphids were found on the middle third of the plant when the initial inoculum was placed on the top third of the plant, or on the bottom third of the plant, compared to when the initial inoculum was placed on the soil. There was no significant difference in the numbers of mycosed aphids found on the middle of the plant when the initial inoculum was placed on the top of the plant compared to when the initial inoculum was placed on the middle of the plant. Significantly more mycosed aphids were also found on the soil when the initial inoculum had been placed on the top of the plant or on the middle of the plant (Figure 3.26) compared to when the initial inoculum was placed on the soil (ANOVA).

The daily mortality of adult aphids showed a periodicity over the 15 days during which results were recorded, with the cycle peaks being approximately 6 days apart and the second peak being significantly higher than the first (Figure 3.27). The peaks in mortality caused by *E. neoaphidis* infection were significantly lower when the inoculum was placed on the soil compared to when the inoculum was placed at the top of the plant. The peak in numbers of infected aphids was also temporally delayed when the inoculum was placed on the soil compared to when the inoculum was placed at the top of the plant or on the middle of the plant. (Figure 3.27)

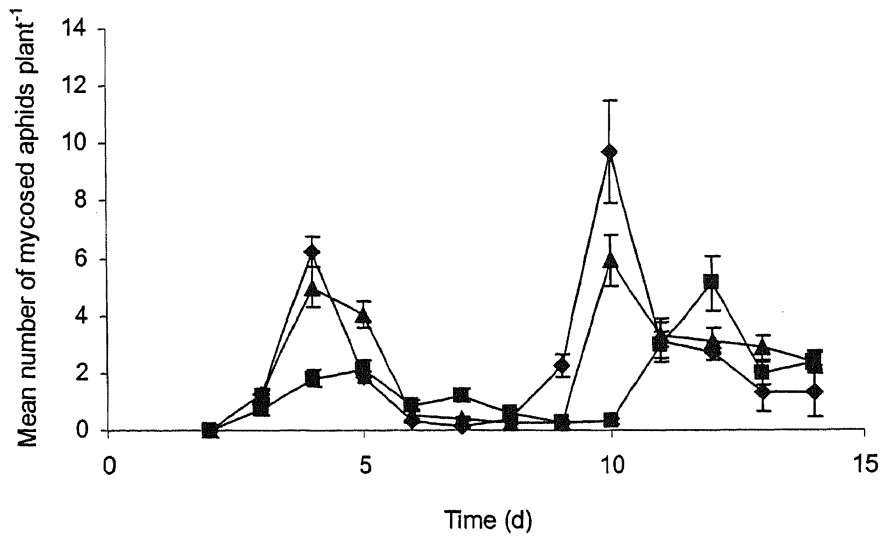


Figure 3.27 Mean daily mortality of aphids due to mycosis resulting from inoculum of *E. neoaphidis* initially introduced at the top of the plant (◆), the middle of the plant (▲) and on the soil (■) at 20°C, 100% RH and photoperiod of 16h:8h (light: dark). Subsequently infected aphids were left on the plant. Error bars represent the standard errors of the means of 5 replicates.

The  $LT_{50}$  for killing of the aphids infected by conidia discharged from cadavers placed on the soil was significantly lower for the first cycle of mortality than the  $LT_{50}$  of aphids infected by conidia discharged from cadavers placed at the top of the plant or on the middle of the plant (Fig. 3.28). It was not possible to assess the

LT<sub>50</sub> of a phids that died in the second cycle of mortality, as it was not possible to quantify when inoculation took place.

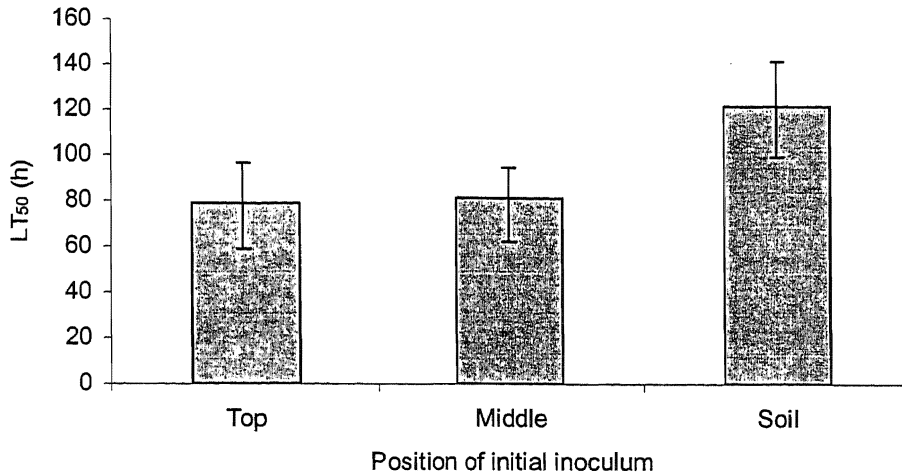


Figure 3.28 LT<sub>50</sub> for killing of aphids infected by *E. neoaphidis* conidia discharged from mycosed aphid cadavers placed on the top of the host plant, the middle of the host plant and the soil at 20°C, 100% RH and photoperiod of 16h:8h (light: dark). Subsequently infected aphids were left on the plant. Error bars represent 95% confidence intervals calculated from five replicates per treatment.

### **3.10 Transmission of *E. neoaphidis* between aphid host plants in the laboratory.**

This experiment was performed to assess whether fungal infection readily moved from host plant to host plant in the absence of non-host insect vectors. Inoculum was placed on a single bean plant in a group of four and uninfected aphids placed on all four plants.

Cumulative aphid mortality by mycosis was greater on the plant on which the inoculum had been originally placed than on any of the other plants, reaching 100% after 5 days (Fig 3.29). On the two plants that were closest to that on which inoculum

had been placed there was a maximum cumulative aphid mortality due to mycosis of 15% after 8 d. There was no aphid mortality due to mycosis on the plant furthest from that on which inoculum had been placed

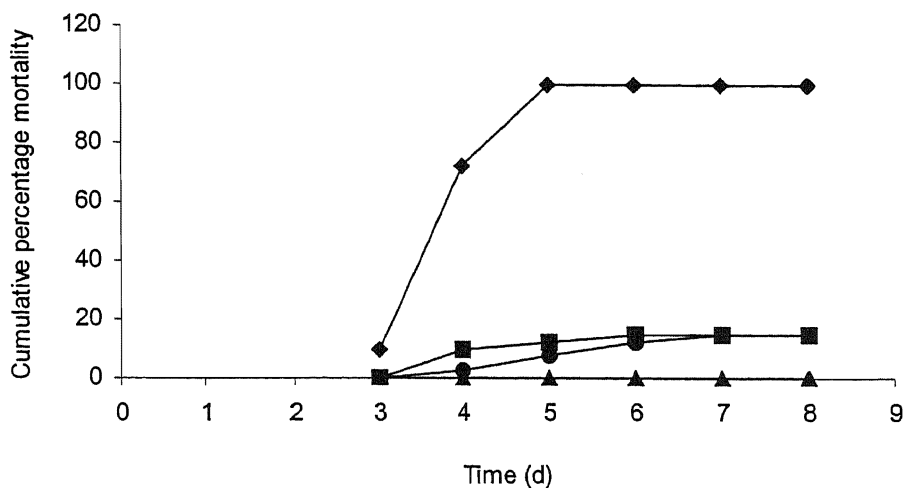


Figure 3.29 Cumulative aphid mortality due to mycosis by *E. neoaphidis* on : a bean plant with attached inoculum source(◆), the two plants adjacent to the plant with the inoculum source (●) and (■); the plant furthest from the plant with the inoculum source(▲). The experiment was performed at 20°C, 100% RH and photoperiod of 16 h: 8 h (light: dark). There were two replicates per treatment.

## 4 Discussion

### 4.1 Comparison of growth supported by various media

The observed peak yield of biomass obtained when growing *E. neoaphidis* NW327 in liquid SDEM shake flask cultures containing 0.01% (v/v) oleic acid or GASP containing 0.01% (v/v) oleic acid was higher than that reported by Gray *et al.* (1990) who grew *E. neoaphidis* NW115 in SDEM containing 0.02% (v/v) oleic acid (8.2g l<sup>-1</sup> compared to 7.9 g l<sup>-1</sup>) although production of biomass in both investigations peaked at approximately 96 h after the inoculation of the media. This increased growth may be attributable to one or both of two factors. The first of these is the toxicity of oleic acid as reported by Gray *et al.* (1990) and Gray and Markham (1997). As described elsewhere in this study, growth of NW327 on SDEM containing 0.1% (v/v) and 1% (v/v) oleic acid was significantly lower than on SDEM containing 0.01% (v/v) oleic acid. Gray *et al.* (1990) have shown that, when grown in medium containing high concentrations of oleic acid, the cell membrane of *E. neoaphidis* appears to separate from the cell wall in some regions and break down completely in others. Hence, the increased oleic acid concentration used by Gray *et al.* (1990) may account for the decreased biomass concentration.

The second factor may be the fungal strain that was used. The biological characteristics of *E. neoaphidis*, including growth, vary widely according to fungal isolate (Sierotzki *et al.* 2000), with an almost twofold difference in biomass production between some isolates. Differences have also been reported in the Kr of monoconidial isolates from *E. neoaphidis* NW195 (Gray *et al.* 1990).

The significantly lower biomass production of the fungus on all other media compared to SDEM and GASP is probably due to a lack of one or more nutrients in the media. The reduction in biomass production from fungus grown in YEDM (yeast extract deficient medium), YEDMTH (Yeast extract deficient medium plus thiamine hydrochloride) YEDMVB (yeast extract deficient medium plus B vitamins) and

YEDMVB5 (yeast extract deficient medium plus B vitamins at 5 times the concentration of YEDMVB) compared to SDEM may be partly due to a reduction in the concentration of the complex nitrogen source in the medium. Although these media contained mycological peptone, which is also a complex nitrogen source this was possibly not enough to sustain growth on these media. Removal of the yeast extract resulted in a nitrogen content reduction of approximately 40% whilst there was a reduction of biomass concentration in the order of 85%. Therefore, the reduction in nitrogen content due to the removal of the yeast extract cannot entirely explain the reduction in biomass concentration. It is likely that there is also a specific growth factor, such as a vitamin or amino acid that is supplied within the yeast extract that is absent, or present at much lower concentrations in mycological peptone. Latgé (1981) reported that a number of strains of *E. neoaphidis* grew on media containing yeast extract or media containing amino acids plus vitamins. It may be that the strain of *E. neoaphidis* used in these experiments (NW327) is not able to grow well in media containing a reduced quantity of a complex nitrogen source. Similarly the lower growth on PDM (peptone deficient medium), PDMTH (peptone deficient medium plus thiamine hydrochloride), PDMVB (peptone deficient medium plus B vitamins) and PDMG (peptone deficient medium plus Grace's insect medium) may also be due to a reduction in the nitrogen content due to the absence of mycological peptone. The reduction in nitrogen content due to removal of mycological peptone from the medium was approximately 60% whilst the reduction of biomass concentration was of the order of 80%. The reduction in nitrogen content can account for the majority, but not all of the reduction in biomass. Again, it is likely that there is a requirement for some growth factor. This putative requirement for a growth factor in the media that enabled biomass production of approximately  $1\text{g l}^{-1}$  may have been supplied by carry over in the inoculum from the first stage of biomass production. The significant reduction in biomass production from fungus grown on TM (Test medium) and TMTH (Test medium plus thiamine hydrochloride) compared to SDEM may also be explained by the lack of yeast extract as discussed above. However, there was significantly greater biomass production from fungus grown in TM and TMTH compared to the other

media (except SDEM and GASP). It may be that thiamine hydrochloride is one of the growth factors needed. It may also be that there was some carry over of growth factors in the inoculum itself. There was a significant reduction in biomass production in GIM (Grace's Insect Medium) and GIMO (Grace's Insect Medium plus oleic acid) compared to production of biomass in SDEM.

Some strains of *E. neoaphidis* are able to grow well on media containing a mixture of amino acids (Latgé 1981), but this seems not to be the case for NW327. There was no significant difference in biomass production from fungus grown in GASP (GIMO plus L-asparagine) compared to SDEM. This was due to either the addition of L-asparagine to the medium, or an increase in total nitrogen content. A number of strains of *E. neoaphidis* grow on media containing asparagine as the sole nitrogen source (Latgé 1981) as do a number of other species (Hilgenberg *et al.* 1987). Growth on GASP also showed that *E. neoaphidis* is able to utilise sucrose as a carbon source (GASP), possibly by enzymatic breakdown of sucrose to glucose and galactose and the subsequent metabolism of these catabolites. This utilisation of sucrose is indicated by the yield, which did not significantly decrease in GASP compared to SDEM.

There was no significant difference in biomass production on media containing added vitamins compared to base media that did not contain added vitamins. Fungi have vitamin requirements that are typically in the  $\mu\text{M}$  to nM range (Griffin 1996) and these requirements for vitamins are probably being met in other components of the media, such as mycological peptone or yeast extract which is a source of B vitamins. There are no reports in the literature of fungal requirements for vitamins A, C, D, E and K (Griffin 1994).

The value of a defined medium is in standardisation. Variations in the composition of complex media lead to physiological differences in the fungus grown on that medium whereas the use of defined medium will minimise these variations. This enables the understanding of the nutritional requirements of the fungus and the optimisation of various production parameters such as biomass production, conidial production or conidial infectivity. This is obviously important if the fungus is to be used



as a control agent since it may be that some parameters are more important than others for pest control. Based on the data obtained from the defined medium, a cheaper complex medium could then be formulated for final production. This also has implications for comparison of studies that have used different complex media for the growth of fungal inoculum. Comparisons of production parameters may not be completely valid as there may be differences in the physiology of the fungus due to the different media that are used.

#### **4.2 Comparison of sporulation from *in vivo* and *in vitro* produced conidia.**

Results for this study show that conidia produced from an *in vivo* source (aphid cadavers) were smaller and more numerous than those produced from *in vitro* sources (agar plugs and fermenter produced pellets). A possible explanation for this is a difference in the carbon source that the fungus has utilised or perhaps in the carbon: nitrogen (C:N) ratio in the media that were used. Lane *et al.* (1991) reported that blastospores of *Beauveria bassiana* harvested from nitrogen limited batch cultures had a larger volume, greater wall thickness and larger total endogenous reserves than blastospores harvested from a carbon limited batch culture. It is possible that the SDEM media used for the *in vitro* production of conidia is nitrogen limited and that the conidia produced in this manner do have greater endogenous reserves than those produced *in vivo*, increasing the mean volume of conidia produced. De Assis *et al.* (1997) also reported that differences in carbon and nitrogen sources affect the size of conidia produced from *Fusarium solani*. Carbohydrate type and concentration may also affect the volume of conidia by affecting the endogenous reserves of conidia. Hallsworth and Magan (1994) report that varying the carbohydrate type and concentration affected the polyhydroxy alcohol and trehalose content of conidia from three entomopathogenic fungi. This may also affect the volume of the conidia. Differences in endogenous reserves were also shown to affect the ability of conidia to germinate at low water activity ( $A_w$ ) (Hallsworth and Magan 1995). This may be important in *E. neoaphidis* with respect to germination of conidia at relatively low humidity. Germination at lower RH in the field is important as an

increased range of humidity that the fungus can germinate at increases the chances of the fungus being transmitted through the pest population. The similarity in size of conidia produced from both *in vitro* sources (hyphal pellets and agar plugs) supports the hypothesis that medium and not type of culture affect the volume of conidia. The quantity of water within the conidia would also affect the conidial volume. It is possible that there is a greater availability of water in the *in vitro* media compared to aphid cadavers so that produced conidia *in vitro* take up more water and are larger. Whether the difference in size of conidia is due to differences in endogenous reserves or differences in water content is a matter for further research.

Differences in the quantity of conidia produced are again likely to be due to the differences in nutrient content, carbon concentration, or the carbon to nitrogen (C:N) ratio of the substrate. Elson *et al.* (1998) reported that increasing carbon concentration reduced conidiation in the potato pathogen *Helminthosporium solani*. Increasing carbon concentration over  $25\text{g l}^{-1}$  also favoured formation of microsclerotia over conidiation in *Colletotrichum truncatum* in submerged culture (Jackson and Bothast 1990). Increasing C:N ratio also reduced sporulation in *Helminthosporium solani* (Elson *et al.* 1998) and in *Colletotrichum truncatum* (Jackson and Bothast 1990). It is therefore likely that differences in carbon concentration and C:N ratio between artificial media and natural substrate can explain the differences in sporulation observed between *in vivo* and *in vitro* produced sources. The reason for differences in conidia production between that from semi solid media and that in liquid media is probably due to the type of culture used. As a colony grows on semi solid media it obtains nutrients by diffusion and tends to exhaust those in its immediate vicinity (Pirt 1975). Conversely, because a batch culture is mixed, provided the culture has not reached the stationary phase, all of the culture will have an adequate supply of nutrients (unless the culture contains large pellets that behave as colonies). Thus small pellets from liquid culture produce more conidia because they have received a better supply of nutrients during their development. Overall, the biomass of the conidia discharged by aphids (estimated by multiplying the mean volume of conidia by the number of conidia) was substantially larger than that discharged by either

plugs or pellets (data not shown). This was due presumably because nutrition is optimal *in vivo*. It may also be due to the structure of the aphid, which allows biomass to grow within the host, then sporulate as it emerges from the cadaver. It is possible that pellets produced in submerged culture, which will only sporulate once harvested and exposed to the air produce more conidia than plugs for the same reason.

The size distributions of conidia produced from cadavers, plugs cut from agar and hyphal pellets all showed a bimodal distribution, which may be indicative of secondary conidia formation. However, the  $L_{\text{maj}}/L_{\text{min}}$  ratio shows no evidence of this.

Differences in the volume of conidia may have direct consequences for the transmission of the fungus. Assuming that both *in vivo* and *in vitro* conidia are the same shape then conidia with a smaller volume (and hence smaller aerodynamic diameter) will have a higher initial velocity and travel further than those with a larger volume (Hemmati *et al.* 2001). This will increase the probability of the fungus being transmitted to a susceptible host and so conidia with a small volume would be preferred for use in biological control. It should be noted however that conidia produced from both *in vivo* and *in vitro* sources are likely to be projected far enough to leave the boundary layer of the plant and enter the airstream for dispersal (Hemmati *et al.* 2001). As well as this, continued research into the effect of the nutrient composition of the media on production parameters may lead to media being developed that allow *in vitro* produced conidia to have similar physical properties and endogenous reserves to those shown by *in vivo* produced conidia.

#### **4.3 Comparison of sporulation of fermenter produced biomass**

There were significant differences in the rates of discharge of conidia from biomass harvested from batch fermenter culture during different phases of growth. It is likely that sporulation was affected only by nutritional history since all other factors that may have influenced sporulation, for example light cycles, temperature, CO<sub>2</sub> and humidity (Griffin 1996) were constant throughout the fermentation. As in *Aspergillus niger* (Adams 1995), conidiation of *E. neoaphidis* only occurs when mycelia are

exposed to an air interface. In this case, this will have occurred when the fungus was removed from the media and placed on the sporulation monitor.

The difference in conidial discharge from biomass harvested at different phases of growth was due to the way in which the fungus grew in batch culture and the relative quantity of viable biomass available for production of conidia.

The biomass produced during exponential phase discharged the most conidia as it contained the smallest proportion of non-viable hyphae. During exponential phase the biomass was actively growing and well supplied with nutrients, and had synthesised the required enzymes needed for growth. During death phase, biomass death caused by nutrient depletion and toxin accumulation caused the proportion of non-viable hyphae to rise and there was a concomitant drop in the number of conidia produced from biomass harvested at this phase of growth. Biomass harvested at the stage of culture inoculation or lag phase had the least viable biomass as during the lag phase, biomass is responding to the changed conditions and physiologically preparing for the exponential phase (Kuzj *et al.* 1998; Griffin 1996). A significant proportion of this biomass may have been dead especially since *E. neoaphidis* had been inoculated into a medium containing oleic acid which would have killed part of the inoculum (Gray and Markham 1997). These results may help to explain the inconsistencies in previous field trials in that inoculum for different trials, or even within the same trial may have been harvested at differing phases of growth. This would have led to inconsistencies in production parameters, especially production of conidia, which would have led to inconsistencies in infection of hosts.

Considerable further investigation needs to be performed if the culture conditions for the liquid *in vitro* growth of *E. neoaphidis* are to be optimised. This is necessary if the fungus is to be used as an effective biocontrol agent, as the quantity of conidia produced and the subsequent infection processes need to be optimised. It should be noted that stationary phase tends to enhance the production of secondary metabolites and this may possibly enhance the infectivity of *E. neoaphidis* conidia, although no stationary phase was observed in this study.

#### 4.4 Effect of pesticides

There have only been a limited number of reports on the effect of pesticides on the growth and development of *Erynia neoaphidis* in the published literature (Lagnaoui and Radcliffe 1998; Keller and Schweitzer 1992; Wilding 1980). These reported the effects of different pesticides to those used in the present study. There have also been a number of reports on the effects of pesticides on entomopathogenic fungi other than *E. neoaphidis*. These differences in methodologies make direct, valid comparisons between published studies and this study difficult although broad comparisons can be made about the effects of the various categories of pesticide.

##### 4.4.1 Effect on radial growth

There was no fungal growth on media that contained either of the fungicides. This broadly agrees with Wilding (1980) who found that the fungicides benomyl, ethirimol, fentin acetate with maneb, mancozeb, maneb and thiram entirely prevented mycelial growth on agar and that the fungicides tridemorph, captan, zineb and captafol significantly reduced mycelial growth on agar. Lagnaoui and Radcliffe (1998) also reported significant reduction of mycelial growth of *E. neoaphidis* on SEMA by benomyl, captafol, triphenyltin hydroxide, mancozeb and metalaxyl with mancozeb at recommended field rates. However, chlorothalonil, was not reported to have a significant effect on mycelial growth even at twice the recommended concentration, in contrast to the results reported in this study. This may be due to a difference in isolate. Lagnaoui and Radcliffe (1998) did not report the origin of the isolate of *E. neoaphidis* and it may be that the isolate used in their study was more resistant to the effects of chlorothalonil than *E. neoaphidis* NW327.

Keller *et al.* (1993) reported that the fungicide captan with pyrenifox completely inhibited the mycelial growth of *Beauveria brongniartii* at the field rate and even at one tenth of the field rate. Majchrowicz and Poprawski (1993) reported significant inhibition of germination of a number of entomopathogenic fungi (*Beauveria bassiana*, *Conidiobolus coronatus*, *C. thromboides*, *Metarhizium anisopliae*, *Paecilomyces farinosus*, *P. fumosoroseus*, *Scopulariopsis brevicaulis* and *Verticillium*

*lecanii*) with nine fungicides at the recommended field rate. Saito and Yabuta (1996) reported significant inhibition in germination of *Verticillium lecanii* treated with triflumizole, dithianon, chinomethionat and zineb at the recommended field rate of application. These studies show that the effect of pesticides on radial growth is not limited to *E. neoaphidis*, but is probably an effect on fungi in general.

There was significantly reduced mycelial growth on the media that contained glyphosate, fomesafen and fluazifop-p-butyl at the recommended field rate compared to the control. The fungus was killed on media that contained paraquat at the field rate. The severity of the effect of the herbicide on the fungus may be due to the mode of action of each herbicide. Paraquat is a bipyridilium compound that, after entering cells of the target, produces superoxide radicals that damage cell membranes (Hurst 1991). If this is taken up in a fungus that grows slowly *in vitro*, such as *E. neoaphidis*, damage to cell membranes may outstrip the rate at which the fungus can repair them, and the fungus is killed. Fluazifop-p-butyl is a resolved ester that inhibits Acetyl CoEnzymeA Carboxylase (ACCase), an enzyme involved in lipid biosynthesis. It is possible that fluazifop-p-butyl partially inhibits cell membrane synthesis in fungi and therefore inhibits mycelial growth. Fomesafen is a diphenyl ester and is an inhibitor of the enzyme protoporphyrin oxidase that is involved in the synthesis of porphyrin rings in chlorophyll in plants. Its effect on fungi is unclear although it is thought to inhibit electron transport and coupled photophosphorylation which would have an obvious effect on fungal respiration. Glyphosate is an inhibitor of 5-enol pyruvyl shikimate 3-phosphate synthase (EPSP). This enzyme is involved in aromatic amino acid biosynthesis (tryptophan, tyrosine and phenylalanine). Although glyphosate did inhibit mycelial growth of *E. neoaphidis* to a degree, it was relatively harmless compared to the other herbicides tested. This may be because the medium contained large enough quantities of aromatic amino acids to allow growth of the fungus even in the presence of the herbicide.

There was generally less inhibition of mycelial growth on media containing insecticides than herbicides or fungicides. There was no significant inhibition of growth on media containing deltamethrin, cypermethrin or  $\lambda$ -cyhalothrin 5EC

compared to growth on the control. There was, however, significant inhibition of growth on media containing pirimicarb, chlorpyrifos and  $\lambda$ -cyhalothrin 10CS. There have been relatively few published studies on the effects of herbicides on the growth and development of entomopathogenic fungi, or indeed non-entomopathogenic fungi. Poprawski and Majchrowicz (1995) reported that three commonly used herbicides (phenmedipham plus desmedipham, metolachlor and chloridazon) were fungistatic or fungicidal to six entomopathogenic fungi (*Conidiobolus thromboides*, *C. coronatus*, *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces farinosus* and *Verticillium lecanii*) when applied at the recommended field rate and fungistatic at one tenth of the recommended field rate.

Deltamethrin, cypermethrin and  $\lambda$ -cyhalothrin are all pyrethroids that act on the central or peripheral nervous systems of animals (Miller and Adams 1982); their site of action is therefore absent in the fungus, explaining the lack of toxicity of these compounds to fungi. The reason for the mycotoxicity of the remaining insecticides is unclear since they are all neurotoxins. Pirimicarb is a dimethylcarbamate that inhibits nerve impulses by the carbamoylation of the primary hydroxyl group of acetylcholinesterase. Chlorpyrifos is an organophosphate insecticide that disrupts acetylcholinesterase by reaction with a serine hydroxyl group within the enzyme. There may be structurally, but not functionally similar enzymes in fungi that are disrupted by these insecticides. Lambda cyhalothrin is a pyrethroid that disrupts nerve impulse transmission by disturbing sodium channels. The physiological role of sodium channels in fungi is not clear (Griffin 1996), but may be linked to potassium transport and active transport. Almost no work has been done on the effects of insecticides on fungi although Straw *et al.* (1996) reported some phytotoxicity to Sitka spruce (*Picea sitchensis*) of chlorpyrifos and malathion, but much more work has yet to be done.

#### 4.4.2 Effect on sporulation

##### 4.4.2.1 Effects of fungicides

Wilding and Brobyn (1980) reported that, although fungicides had significant effects on infection rates when applied at the recommended field rate 4 h after inoculation of aphids, little or no effect on infection rates was seen when fungicides were applied to aphids 22 or 70 hours after inoculation. This is in contradiction to the results reported here, that show inhibitory effects on sporulation of mycosed aphid cadavers and on aphids treated with fungicides at 4 h, 48 h and 72 h after inoculation. This may be due to the fact that Wilding and Brobyn (1980) tested different fungicides compared to those used in the present study. It may be that azoxystrobin and chlorothalonil prevent sporulation or emergence of conidiophores. Wilding and Brobyn (1980) reported that a number of fungicides inhibit germination of conidia of *Entomophthora aphidis* (*Erynia neoaphidis*). Lagnaoui and Radcliffe (1998) also reported that a number of fungicides inhibit the germination of conidia of *Pandora* (*Erynia*) *neoaphidis*, although it was reported that chlorothalonil did not inhibit germination. It may be that chlorothalonil prevents sporulation by prevention of conidiophore formation. Wilding and Brobyn (1980) noted that some fungicides have insecticidal properties, but this was not observed in this study.

Both azoxystrobin and chlorothalonil completely inhibited sporulation when applied to the aphid 4 h and 48 h prior to inoculation and significantly reduced sporulation when applied 72 h prior to inoculation. This agrees with Wilding and Brobyn (1980) who reported that fungicides significantly reduced aphid infections when applied up to 48 hours before inoculation due to their inhibition of germination of conidia of *E. neoaphidis*. As described above, this may be due to prevention of germination. In the present study, there was only partial inhibition of sporulation when the fungicides were applied to aphids 72 h before inoculation. This is probably due to one, or two abiotic factors. Firstly, the very humid conditions in which the aphids were incubated (100% relative humidity) resulted in the presence of some free water on the host plant leaves, which may have washed some of the fungicides from the cuticle of



the aphids before they were inoculated. Secondly, the fungicides may have lost efficacy as a result of photodegradation.

#### 4.4.4.2 Herbicides

All of the herbicides tested inhibited the sporulation of the fungus to some degree. Keller and Schweitzer (1991) reported that a number of herbicides inhibited sporulation of *E. neoaphidis* when applied at recommended field rates, although the herbicides tested differed from those used in the present study. In the present study, paraquat completely inhibited sporulation in treated aphids, but not in cadavers. The complete inhibition of sporulation was therefore due to an insecticidal property of paraquat rather than a fungicidal effect. All of the aphids that had been treated with paraquat had died within 8 hours, before the fungus could complete its development. Glyphosate, fomesafen and fluzifop-p-butyl also inhibited, but did not entirely prevent sporulation.

It is most likely that herbicides reduce the production of conidia by inhibition of the development and emergence of conidiophores and of sporulation, possibly by reduction of the synthesis of amino acids or lipids, as discussed in section 4.4.1.

#### 4.4.4.3 Insecticides

All the insecticides tested completely inhibited sporulation from aphids, but did not completely inhibit sporulation from cadavers. However, sporulation from cadavers treated with insecticides was significantly reduced compared to the controls. These effects were due to the fact that all of the aphids treated with the insecticides died within 8 hours of treatment and *E. neoaphidis* was therefore unable to complete its development within the aphid. There were significant differences in the degree to which the different insecticides inhibited sporulation and this again may be due to the mode of action of the insecticides. The mechanism by which insecticides inhibited sporulation from cadavers is not clear, although enzymes that are produced by fungi

and that are structurally similar to those inhibited by insecticides in invertebrates may have been inhibited (section 4.4.1).

The results obtained from the experiments to investigate the effect of pesticides on sporulation and radial growth have a number of implications for the use of *E. neoaphidis* in an IPM program that includes the use of chemical pesticides. Use of insecticides in an IPM program is likely to completely negate the efficacy of *E. neoaphidis* at controlling aphid numbers. Although this study only showed a partial reduction in sporulation from mycosed cadavers (approximately 60-70%), there was no sporulation from live aphids. This was due to the death of the aphid before *E. neoaphidis* could complete its life cycle. Even application of insecticide 72 h after inoculation completely inhibited sporulation. Thus, although there may be some sporulation from initial inoculum, there will not be any after that as there will not be any aphids left to be infected. Although application of insecticide at an interval greater than 72 h after inoculation may allow the fungus to kill the first cohort of aphids, the subsequent aphid mortality due to insecticide would render *E. neoaphidis* useless. Therefore, it is concluded that *E. neoaphidis* cannot be used in conjunction with insecticides in an IPM program.

Although the use of herbicides in an IPM program would not be directly targeted at the control of insect pests, they are often used to control broad leaved weeds in an arable grass crop, such as wheat or rye, and also to control graminaceous pest species in broadleaved crops such as legumes and crucifers. Thus, it is essential that these chemicals are compatible with *E. neoaphidis* if it is to be used in conjunction with herbicides. All of the herbicides tested in this study significantly reduced the level of sporulation of *E. neoaphidis*. However, the reduction in sporulation was less marked when the chemicals were applied at least 48 h before or after inoculation with *E. neoaphidis*. Production of conidia from cadavers and live aphids treated with herbicides was reduced to a level approximately equivalent to that of the LD<sub>50</sub> of the isolate used (NW 327). The level of the production of conidia from live aphids and cadavers treated with herbicides in this study is considerably greater than the LD<sub>50</sub> of a number of other isolates of *E. neoaphidis* (Sierotzki *et al.* 2000).

Although only a very small number of herbicides were tested, the results suggest that use of herbicides in an IPM program is broadly compatible with the use of *E. neoaphidis*, especially if applications of herbicide and fungus are temporally separated. Therefore, if *E. neoaphidis* is to be used in an IPM program that includes the use of herbicides, then there should be a time interval of at least 48 h between inoculation and application of herbicide.

As with herbicides, the use of fungicides in a pest control program would not be directed at arthropod pests, but at the control of a number of fungal diseases, such as infection by *Colletotrichum*, *Botrytis* and *Fusarium spp.* The two fungicides tested in this study completely inhibited sporulation from mycosed cadavers, and also generally when aphids had been treated with fungicides before or after inoculation with fungus. However, when the aphids were inoculated 72 h after the application of fungicides then there was some subsequent sporulation from the aphid (approximately 45% of the level of the control). Again, although only two fungicides were tested the results suggest that fungicides can be used with *E. neoaphidis* in an IPM program provided the fungicide is applied at least 72 h prior to inoculation with the fungus. During this time, it is likely that the fungicide loses some efficacy due to abiotic factors (4.4.4.1).

A factor that also has to be considered in the use of *E. neoaphidis* in any IPM program is the isolate of the fungus that is used. As discussed previously Lagnaoui and Radcliffe (1998) found that chlorothalonil had little or no effect on the mycelial growth of an unnamed isolate of *E. neoaphidis*, whilst in this study, chlorothalonil had a major fungistatic effect on mycelial growth. A possible differential susceptibility to pesticides between isolates raises the possibility that different isolates of *E. neoaphidis* may be used in combination with different pesticides. Further study is needed into the susceptibility of various isolates to pesticides.

#### **4.5 Effect of relative humidity on the ability of *E. neoaphidis* to overwinter**

The increase in storage time after which the fungus was able to produce conidia when the fungus has been stored at 5.0% RH and 20% RH 100% RH and 90% RH, may possibly be due to a decrease in fungal metabolism due to the lowered

humidity and consequent reduction in available water. This would mean that nutritional reserves would be exhausted less quickly in fungi stored at lower RH. This may also go some way to explain the less rapid decrease in conidia production over time from fungus stored at 20% RH compared to that stored at 50% RH. The postulated decrease in metabolic activity preserves viability at the expense of short term spore discharge. Although the spore monitor was maintained at 100% RH to encourage sporulation, the length of time that the conidia were exposed to this high RH was clearly not long enough to restore the rate of conidiation to maximum.

Low storage humidity may also protect against frost damage by withdrawing water from the cells and intercellular spaces. This reduces the likelihood of cell damage from ice crystal formation. Conidia of *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* stored at low RH had increased levels of polyols (Hallsworth and Magan 1995) which may also prevent frost damage by decreasing the water content in the conidia, or acting as a cryopreservative. Over a short timescale of hours, incubation at 100% RH encourages sporulation (Wilding 1969).

From these results, we can see that *E. neoaphidis* is able to survive in aphid cadavers at temperatures that are commonly recorded during a southern UK winter. The level of conidia production after storage at 20%RH and at 50%RH suggest that mycosed aphid cadavers that are in the field over the winter period may be able to act as an inoculum source to infect hosts when conditions become more suitable.

#### **4.6 Effect of dislodgement**

This experiment was performed in order to assess whether transmission of *E. neoaphidis* within an aphid population was affected by the amount of disturbance to the host plant.

There was no significant difference in the proportion of aphids infected with *E. neoaphidis* when host plants were disturbed three times per day compared to when the host plants was disturbed once per day. If fungal inoculum is in proximity to a relatively sessile aphid species, such as *A. pisum*, while it is feeding, it is likely that

the aphids will become infected with the fungus. Even if the aphids are disturbed and fall off the plant, it is likely that they will already have become inoculated with the fungus and therefore it will not matter if they are dislodged more than once per day. However if the inoculum is placed in a different position than the feeding position, for example on the soil, it is possible that increased disturbance may result in an increase in transmission, as the aphids will have more chance of contact with the inoculum. This was not tested in the present study. For an extensive review of the effects of predator foraging on transmission of the fungus, the reader is directed to Roy and Pell (2000). From this, it can be concluded that it is unlikely that observation and the subsequent disturbance of the plant would have an effect on the transmission of the fungus.

#### **4.7 Effect of inoculum position on transmission in the laboratory**

Application of *E. neoaphidis* at the top of the plant caused the symptoms of infection to occur considerably earlier within the population of aphids than application of the fungus on the soil, and resulted in a significantly lower  $LT_{50}$ . This indicates that the inoculum placed at the top of the plant was better positioned to cause infection. This was probably due to a combination of two factors. The first of these is that conidia discharged from inoculum at the top of the plant will fall on to lower parts of the plant and on to the soil, whereas conidia discharged from inoculum on lower parts of the plant and on the soil will not reach the top of the plant. The second factor is the sessile nature of the aphid species used. The pea aphid *Acyrtosiphon pisum* tends to move very little once it has reached its preferred feeding position at the top of the plant. If the inoculum is placed on the soil and aphids are not infected when they initially climb the plant, it is unlikely that they will contact the inoculum once they have started to feed. This is in contrast to inoculum that is placed at the top of the plant, where aphids tend to cluster, increasing the likelihood of coming into contact with conidia. These results contrast with those obtained by Shah *et al.* (2000), who reported that soil application of the inoculum caused infection to occur three to four days prior to infection caused by foliar application of the inoculum. This may be due to

the choice of aphid host, *Macrosiphum euphorbiae* and also to differences in incubation conditions. *Macrosiphum euphorbiae* is much more mobile in its habit than *A. pisum* and this might have come into contact with soil inoculum more readily than *A. pisum*. In addition, the study of Shah *et al.* (2000) was carried out under greenhouse conditions. In the greenhouse, the RH was lower around the plants than in the present study, except at the soil surface where the soil surface may have provided a buffered environment against lowered RH by supplying a constant source of moisture for mycelial rehydration, unlike the foliar surface which is exposed to cyclical dehydration and hydration. Under such conditions, even though foliar application may have resulted in more aphids becoming inoculated than soil application, infection resulting from foliar inoculation may have been limited by the lower humidity compared to the soil surface. The effect of UV light on the survival of the conidia may also have been a factor. In the study of Shah *et al.* (2000), the plants were exposed to solar UV radiation, whereas in the present study, this was not the case, although there may have been a small amount of UV light from the fluorescent lighting. Brobyn *et al.* (1985) reported that the infectivity of *E. neoaphidis* conidia exposed to UV light declines linearly with time, and that those conidia least exposed to the UV light, i.e. those conidia on the abaxial surfaces of leaves and those conidia on leaves near the base of the plant, showed least decline in infectivity.

The present study was carried out under laboratory conditions, which limited fluctuations in abiotic conditions. As well as this, the experimental design provided high humidity over the whole of the plant and so foliar surfaces were unlikely to experience the dehydration that may be present in greenhouse studies. Foliar application is therefore more likely to result in increased infection levels under these conditions.

Shah *et al.* (2000) also reported much lower levels of aphid infection ( a maximum of 36%) than are shown in this study (100% infection). Again, this may have been due to the lower humidity in the greenhouse compared to the controlled environment chamber, or possibly other factors such as differences in isolate or quantity and source of inoculum. The biological characteristics of *E. neoaphidis* have

been shown to vary widely between isolates, with significant differences in conidia production and infectivity (Sierotzki *et al.* 2000) and monoconidial isolates from a single strain have also been shown to differ significantly in conidia production and aphid infectivity (Gray *et al.* 1990). It has been reported elsewhere in this study (3.1.1) that aphid cadavers (as used in this study) produce a greater number of conidia than conidia produced from *in vitro* sources (as used in the study reported by Shah *et al.* (2000)). This may also account for some of the difference in the level of infectivity found in this study and that reported by Shah *et al.* (2000).

The sessile nature of the aphid host is likely to account for the majority of the aphid cadavers being at the top of the plant. As the aphids do not move very much from their feeding position, they are likely to die in that position, which is generally at the top of the plant. Even if aphids become infected by inoculum applied to the soil, they can easily reach the top of the plant before being killed by the fungus. However, the positioning of the aphid cadavers at the top of the plant may also be caused by infection mediated behavioural modification. Jensen *et al.* (2001) reported that *A. pisum* infected with *E. neopahidis* were more likely to be found on the upper half of the host plant than non-infected aphids. Lagos *et al.* (2001) reported behavioural thermoregulation in *A. pisum* infected by the braconid parasitoid *Aphidius ervi*, where parasitised aphids were shown to be found on hotter parts of the host plant than non-parasitised aphids. If infection by *E. neopahidis* does cause behavioural modification in *A. pisum*, then that could explain the lack of effect of the position of inoculum in the positioning of the aphid cadavers, in that infected aphids may always climb to the top of the plant due to pathogen mediated thermoregulation.

There were however, a number of aphid cadavers that were found in positions on the plant other than the top of the plant. It may be that these individuals were feeding on another part of the plant, rather than the top, when they died. It is also possible that these infected hosts were dislodged from the plant before the rhizoids emerged and were unable to climb back to the top of the plant, although some may have climbed part of the way. This may be especially true for cadavers that were found on the soil. Roy *et al.* (1999) have shown that in the latter stages of

*E. neoaphidis* infection (two or three days post inoculation) *A. pisum* are less active than those that are uninfected or that are in the early stages of infection. This reduced activity may be correlated to the invasion of the fungus into the muscle tissue of the aphid after the first day of infection (Butt *et al.* 1990). It may be therefore, that the position of the aphid cadaver is related to whether the aphid host has been dislodged from the plant, and if so, what stage of infection it was at. Dislodged aphids that are in the early stages of infection may be able to climb back on to the plant before death whereas dislodged aphids that are in the latter stages of infection may not be able to climb back on the plant and may remain on the soil

#### **4.8 Effect of position of inoculum in the field**

Fungus applied to the soil in the field caused infection to occur significantly faster than infection caused by foliar application. This contradicts the results obtained in the laboratory. This may again have been because the cadavers placed on the leaves were exposed to a greater level of abiotic environmental stress than those applied to the soil. Brobyn *et al.* (1985) have shown that conidia from *E. neoaphidis* near the base of the plant remain infective for a longer period of time than those placed on the surface of leaves. This may be caused by increased levels of UV light on the surface of leaves compared to the soil (Ignoffo *et al.* 1977). Furthermore, in the laboratory, the relative humidity was maintained at 100% and the temperature maintained at 20°C. In the field, the relative humidity fluctuated from 45.7% to 100%, whilst the temperature ranged from 12.4°C to 41.3°C. It may also be that the inoculum on the foliar surfaces experienced fluctuating humidity conditions, and the soil provided a buffered environment, enhancing sporulation and consequently infection as discussed earlier in this report. Brobyn *et al.* (1985) have also suggested that heavy rainfall may wash inocula from the leaf surface. This was unlikely to have been the cause of the disparity between laboratory and field results in this investigation, as there was rainfall on the field trial area only during the last two days of the sampling period.



As discussed in section 4.7, abiotic factors may also explain why the proportion of infected aphids collected from the field was considerably lower than the proportion of infected aphids collected from plants in the laboratory. The relative lack of mortality in the field may have been compounded by the presence of entomophagous arthropods and parasitoids. It was noted that while sampling in the field there were a number of predatory spiders (Lygaeidae) and also a number of hoverfly (Diptera: Syrphidae) larvae. At the end of the sampling period it was noted that there were very few live aphids remaining and this may have been due to the presence of natural enemies or of the dispersal of the aphids from the host plant. However, dead mycosed aphids were easy to find on the plants as they had been attached to the plants by rhizoids.

The significantly higher number of cadavers found at the top of the plant as compared to the rest of the plant can again be attributed to the feeding behaviour and relatively sessile habit of the aphids. The significantly smaller number of cadavers found on the bottom of the plant or the soil in the field compared to the laboratory may be attributable to the lack of disturbance of the aphids when counting cadavers in the field. In the field the plants were each covered with a gauze umbrella that did not touch the plant. Therefore, when it was removed to count the cadavers, there was no disturbance to the plant and the aphids did not drop off of the plant. In the laboratory, the plants were covered with plastic lamp glasses that touched the plant. When these were removed to allow counting of aphid cadavers, the plants were disturbed and approximately 5% of the aphid population often dropped off the plant (data not shown). This observation may also help to explain the higher proportion of mycosed cadavers found in the laboratory compared to the field. Disturbance due to observation in the laboratory may have caused uninfected aphids to fall off the plant and come into contact with inoculum that they hadn't previously. Although different levels of disturbance did not affect the transmission of the fungus (3.7), there may be differences in transmission between aphids on disturbed plants and undisturbed plants. This was not tested directly due to the difficulty in counting mycosed aphids on plants in the laboratory without disturbing the plants.

The results from the field and laboratory studies suggest that results obtained in the laboratory should be applied with caution to a field setting, especially if the abiotic conditions in the field are unfavourable to the transmission of the fungus i.e. high temperatures and low relative humidity. The laboratory models may provide a better fit to field conditions if the abiotic conditions of the laboratory are changed to more accurately represent field conditions. The laboratory model shows that the optimum position for the inoculum to be is on the top of the plant. Although there were generally no significant differences over the course of the experiment between the number of mycoses caused by inoculum placed at different positions on the plant, the  $LT_{50}$  of inoculum placed on the soil was significantly longer than the  $LT_{50}$  of inoculum placed on the top of the plant. This has implications in the control of aphids by inundative release of *E. neoaphidis*. If the inoculum is produced in a dense form, such as mycelial pellets, it is likely that the inoculum will fall on to the soil as opposed to being on the top of the plant. Therefore the fungus will probably be less effective than if the inoculum is released in a form that will adhere to the top of the plant. However, the field results suggest that the fungus will be more effective if the inoculum is placed on the soil where the temperature is lower, there is higher relative humidity and the conidia are protected by some degree from UV radiation. To increase the likelihood of aphids coming into contact with inoculum, it may be possible to add chemicals to the fungal formulation that enhances the activity of the aphids, such as  $\beta$ -farnesene (Roy 1998), or that causes the aphids to drop off the plant, such as neem (Lowery and Isman 1996). A great deal more investigation has to be done on the transmission of the fungus under various conditions until a fuller picture is gained. For example, investigation of the effect of irrigation on the transmission of the fungus and the effect of temperature and relative humidity on transmission. As well as work on the ecology of the fungus, more work on formulation still has to be done, perhaps on investigating additives to formulation, or manipulating the physiology of the fungus to enable it to be able to sporulate and infect aphids under unfavourable abiotic conditions.

#### **4.9 Effect of host density on transmission**

##### **4.9.1 Effect of host density on transmission in the laboratory**

There was a significant increase in the proportion of mycosed aphids as the density of aphids increased denoting density dependence. High host densities increase the contact between host and pathogen. High host density may also increase the susceptibility of individuals to disease due to competition for food (Watanabe 1987). Epizootics most often occur in insect populations at high host density since the threshold level at which the pathogen is active is generally high (Watanabe 1987). However, epizootics can also occur at low host densities, especially if the pathogen is widely distributed in the host habitat (Watanabe 1987). The effects of host density on transmission of disease have been formalised into a number of mathematical models for description and prediction of host numbers. (Brown and Nordin 1982; Schmitz *et al.* 1993; Ardisson *et al.* 1995).

##### **4.9.2 Effect of host density on transmission in the field**

There was no clear evidence of host density dependence in the field. It is unlikely that this is due to the disease being density independent. There was clear evidence of density dependence in the laboratory based experiment and it is very unlikely that the disease would be density independent in the field and not in the laboratory. The lack of clear evidence for density dependence can probably be attributed to abiotic factors. The relative humidity was generally low and the temperature at the canopy top, where the fungus was placed was often in excess of the optimum for the growth and sporulation of *E. neoaphidis* (Table 3.5). Unfavourable abiotic conditions meant that any density dependent effects on transmission on the fungus were masked by poor infection processes.

Host density dependence has been observed most often in viral diseases of insects. Jaques (1962) reported density dependant epizootics of nuclear polyhedrosis virus (NPV) in populations of *Trichoplusia ni* and Doane (1970) reported density

dependant epizootics of NPV in the gypsy moth (*Lymantria dispar*). Host density dependence has rarely been demonstrated (if at all) in fungal pathogens (Missonier *et al.* 1970; Wilding 1975), possibly due to the difficulty in producing epizootics of fungal diseases under field conditions. However, Feng *et al.* (1992) suggested that generally, host density was positively correlated with infection level in the field.

The results discussed in section 4.9.1 suggest that the induction of an epizootic of *E. neoaphidis* is host density dependent, at least in the laboratory whereas in the field this effect may have been masked by unfavourable abiotic conditions. This host density dependence, along with unfavourable abiotic conditions may go some way to explaining why epizootics are rarely seen in aphid population before the host population has had time to grow to a level that causes economic injury to the farmer and may be part of the reason for the lack of success in inducing epizootics in field trials in the past. To some extent, this may be compensated for by increasing the amount of inoculum. However, it may be possible to induce epizootics earlier than at they occur at present, by using more infective isolates of *E. neoaphidis*, or fungus that produces conidia that germinate at a lower RH.

#### **4.10 Effect of cadaver density on transmission in the field**

For the first 2 days that the cadavers were exposed in the field there was evidence of cadaver density dependence on the transmission of the fungus, where higher proportions of aphids were found to be mycosed, at the highest density of cadavers compared to the other densities. However, after this period of time, there was no clear evidence for cadaver density dependence on the transmission of the fungus. This was probably due to a reduction in the level of production of conidia over time. This agrees with results presented in section 3.1.1 and discussed in section 4.2, which showed a reduction in conidial discharge after approximately 60 h in the laboratory. The levels of conidia discharged from mycosed cadavers after 2 or 3 days was not enough to initiate an epizootic and only low levels of mycosis were therefore seen. Future use of *E. neoaphidis* in the field would probably be most effective as an inundative strategy.

#### **4.11 Long-term transmission experiment**

Ten days after the start of the experiment, significantly more mycosed aphids were found at the top of the plant compared to the number of mycosed aphids found at the middle or bottom of the plant, or on the soil. As has already been discussed, the aphids die where they mostly feed, at the top of the plant (section 4.7). Aphid mortality occurred in two temporally defined peaks (see Figure 3.27). The timing and amplitude of the peaks in aphid mortality can be explained by the positioning of the inoculum and the duration of the fungal lifecycle. As discussed previously, positioning of inoculum at the top of the plant leads to greater mortality of aphids and also a faster  $L_{T50}$ . These results show that this can lead to a positive feedback whereby inoculum placed at the top of the plant causes more aphids to die at the top of the plant, producing more cadavers at the top of the plant (and so on). Under ideal laboratory conditions, the “generation time” of *E. neoaphidis*, from initial contact of the conidia with the host to conidial discharge is approximately 96 h (Brobyn and Wilding 1977). Peak mortality therefore would be expected 4 days after the aphid population has reached a level that would support an epizootic. Results described previously (section 3.8) suggest that the positioning of *E. neoaphidis* inoculum as a biological control agent could have profound effects on the efficacy of the fungus in controlling aphids. If the inoculum were to fall on the soil, perhaps due to application method or formulation, then the fungus may be less efficient at controlling aphid numbers than if the inoculum were applied at the top of the plant. It may be possible to quantify the extent of this difference using mathematical modelling methods, but this is beyond the scope of the work presented here. However, it may be that the results presented here and the conclusions drawn from them may only be applicable under laboratory conditions. As discussed previously, Shah *et al.* (2000) have shown that foliar application of *E. neoaphidis* causes infection three to four days later than soil application in the greenhouse than occurred in the laboratory in this study, possibly

due to the differing abiotic conditions between the laboratory and the greenhouse. Temperatures in the greenhouse trial ranged from 15°C to 25°C, with temperatures up to 35°C being recorded. Ambient relative humidity ranged from 65% to 92%. These abiotic conditions are much more variable than in the laboratory, where temperature was maintained at a constant 20°C and relative humidity was always around 100%. These results may also only be applicable to *A. pisum* as a host. Different species of aphid that are infected by *E. neoaphidis* have different feeding and movement habits and the transmission of the fungus through populations of different species may have a completely different dynamic than that of transmission through *A. pisum* populations. This would necessitate more investigations of this type on different species of host aphid. If *E. neoaphidis* is to be used as a control agent in the field it may be necessary to look at weather forecasts for some period after application, so as to enable the amelioration of effects of adverse weather conditions on the further spread of the fungus

#### **4.12 Vectoring of *E. neoaphidis* via aphid movement**

Very little fungal induced aphid mortality occurred on plants other than the plant that had the inoculum placed on it, whilst there was 100% cumulative mortality on the plant that had the inoculum placed on it. The difference in mortality is probably due to two factors. The first of these is the relative sessility of the aphid species used. As discussed previously, the pea aphid is not a particularly mobile species and rarely moves from its preferred feeding position unless disturbed. This means that there is not likely to be movement of aphids between plants and so infected aphids are unlikely to migrate into a susceptible population. If aphids are disturbed from feeding sufficiently to fall off of the host, they are likely to move back onto the same plant. In addition, aphids that have been infected for at least 48 hours have reduced movement capabilities (Roy *et al.* 1999). These factors combine so that aphids that have been infected on a particular plant are unlikely to move to another host plant that may contain a susceptible population of aphids.

All of the published literature on the transmission of *E. neoaphidis* through aphid populations has been done on the transmission of infection through a population on a single plant (Ardisson *et al.* 1995) or the effects of arthropod vectors (Roy *et al.* 2001a; Roy *et al.* 1998; Pell *et al.* 1997.) Although preliminary, the results presented earlier suggest that the fungus will not easily spread between aphid populations on different plants via aphid movement if the inoculum originates from a point source. Thus, if the fungus is to spread throughout a metapopulation of aphids via aphid movement then the majority of plants must receive an inoculum dose. This may prove problematic if the currently favoured method of formulation is used, as it is unlikely that formulated hyphae will have an even spread. However, the fungus can also be spread by arthropod vectors from infected aphid populations to susceptible populations. For an extensive review of this, the reader is directed to Roy *et al.* 2001. However, the fungus can also be spread by airborne conidia that have been ejected beyond the boundary layer of the plant and carried by wind, which was absent from the controlled conditions of this study. Hemmati *et al.* (2001) have shown that this is probable in *Erynia neoaphidis* given the speed of ejection of conidia and also the size and aerodynamic cross section of the conidia.

## 5 Conclusions and further work

There are a number of conclusions that can be drawn from the study:

- For the first time, *E. neoaphidis* was grown on a fully defined medium, consisting of Grace's Insect Medium supplemented with L-asparagine
- Despite work done in this study, and by other researchers, further research is still needed into the nutritional requirements for *E. neoaphidis*.
- *In vivo* produced biomass produces proportionately more conidia than *in vitro* produced biomass. *In vivo* produced conidia have a smaller volume, and are less elongated than those produced *in vitro*
- Artificial media need to be developed further so that conidia that are produced from biomass grown *in vitro* are comparable in number and characteristics to those produced *in vivo*.
- Biomass grown in batch culture and harvested at late exponential phase produced more conidia than biomass harvested at any other phase of growth.
- Pesticides are generally deleterious to the use of *E. neoaphidis* as a biological control agent, although these effects may be mitigated by careful timing of the application of pesticide and fungus. Fungicides and some herbicides are particularly harmful to colonial growth whilst insecticides and fungicides are particularly deleterious to conidia production.
- Little is known about the mode of action of herbicides and pesticides on fungus and more research is needed so that pesticides and entomopathogenic fungi can be integrated into a more environmentally friendly pest management program.
- *E. neoaphidis* is able to survive winter conditions in aphid cadavers and lowered RH, possibly by reduced metabolic activity, or by the protective effect of polyols



- Increasing levels of host disturbance resulting from experimental observation had no significant effect on transmission of the fungus and therefore was probably not a confounding factor
- The position of inoculum has an effect on the transmission of the fungus, both in the laboratory and the field. Under ideal conditions, the best position for inoculum, resulting in optimum transmission of the fungus, is at the top of the plant. Under usual field conditions, the optimum position for inoculum is probably on the soil.
- Transmission of *E. neoaphidis* through an aphid population is probably host density dependent
- *E. neoaphidis* is unlikely to be transmitted through an aphid population from plant to plant solely by host vectoring
- Greater insights into the morphology (and inferred physiological status) of populations of conidia can be gained by the use of image analysis over traditional methods

Based on what has been found so far in this study, a number of recommendations can be made for the best strategy for producing *E. neoaphidis* and its application in the field.

Conidia production is greater from an *in vivo* source than an *in vitro* source. However, it would be almost impossible to produce mycosed cadavers on the scale needed for (semi) commercial application. Therefore, an *in vitro* produced source of conidia is needed. This should be hyphal pellets harvested in the exponential growth phase, approximately 96 h after inoculation. The inoculum should be applied to the soil, where there is less chance of the biomass desiccating or the conidia being reduced in activity by UV radiation. Pesticides should not be used if possible, especially within 48 h before or after application of the fungus. It may be possible to use herbicides after this period. The use of fungicides after 72h may not have too much of a deleterious effect on the initial mortality of aphids due to mycosis, but is likely to halt any secondary transmission of the fungus within an aphid population. It

may also be possible to use mycosed aphid cadavers as an inoculum source to infect the next year's aphid population.

However, a considerable body of further research needs to be undertaken. This includes:

- Further experimentation to determine the "growth factors" that are needed by the fungus
- Elucidation of the nutritional requirements of *E. neoaphidis* in respect to different types of growth such as biomass production, conidia production and conidial infectivity.
- Development of media that will enable the fungus to discharge conidia in a comparable quantity, and with comparable physical characteristics from an *in vitro* source as from an *in vivo* source.
- Investigation of conidial size distribution bimodality and changes over time.
- Investigation of the effect of nutrition on conidia number, size and infectivity
- The optimisation of liquid culture of *E. neoaphidis*, with the possibility of producing conidia in liquid culture.
- Investigation should be undertaken on the infectivity of conidia when they are harvested at different phases of growth.
- Investigation of the effect of various pesticides on different isolates of fungus to determine whether there are any inter-isolate differences.
- Elucidation of the methods of inhibition of radial growth and sporulation of *E. neoaphidis* by various pesticide groups.
- Investigations into the water content and polyol content of conidia stored at various humidities in order to improve overwinter survival.
- Effect of polyol and water content of conidia on infectivity towards aphids at different RH to improve germination and sporulation at relatively low RH.
- Investigation as to the effects on transmission of fungus in undisturbed versus disturbed hosts where the inoculum is on the soil.
- Investigation of the effect of infection by *E. neoaphidis* on behavioural thermoregulation of aphids.

## Appendix A

Production of conidia and frequency distribution of conidia of *E. neoaphidis* produced from *A. pisum* cadavers treated with pesticides

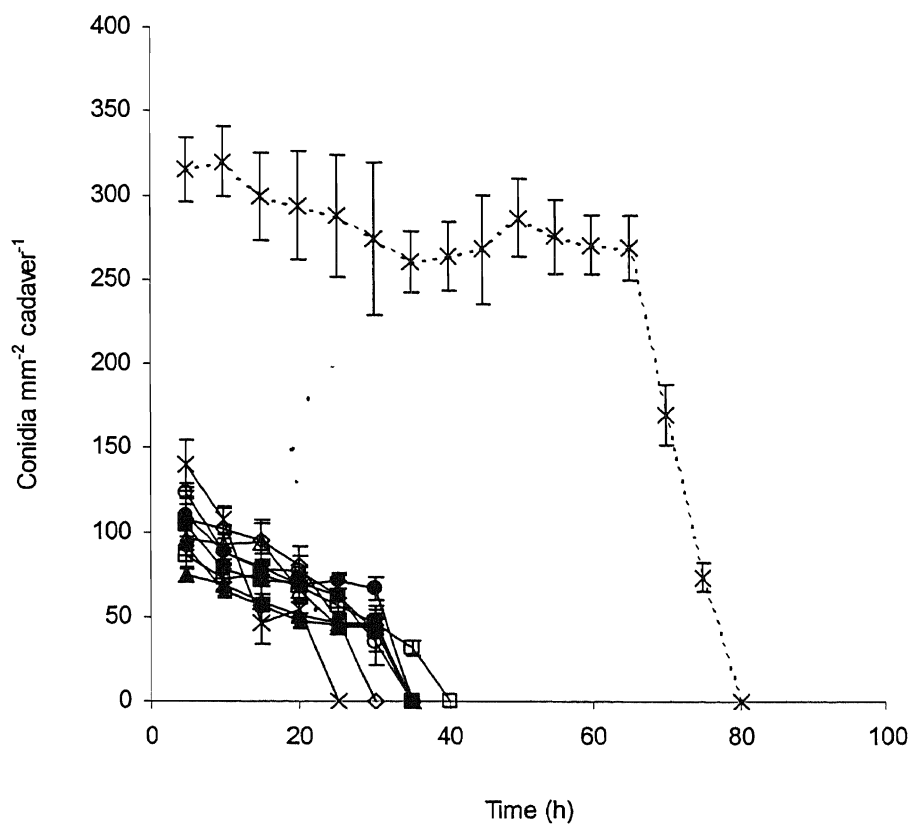


Figure AA1. Comparison of discharge of conidia from mycosed aphid cadavers treated with fomasefen (◆), glyphosate(■), fluazifop-p-butyl (▲), lamda-cyhalothrin 5 EC (●), lamda- cyhalothrin 10CS (x), deltamethrin (◇), cypermethrin (□) and paraquat (○). The control cadavers (x) were dipped in distilled water. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment. Error bars represent the standard errors of the means of 10 replicates. Data for the pesticide treatments are expanded in Figures AA2 and AA3.

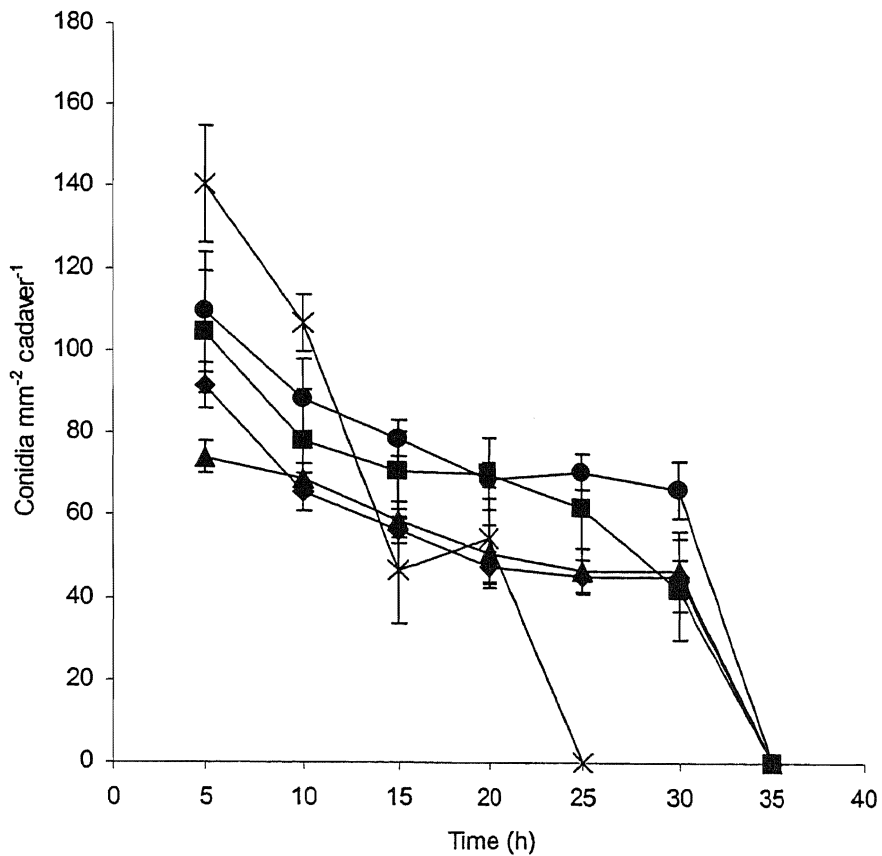


Figure AA2. Comparison of discharge of conidia from mycosed aphid cadavers treated with fomasefen (◆), glyphosate (■), fluazifop-p-butyl (▲), lambda-cyhalothrin 5 EC (●) and lambda-cyhalothrin 10CS (✕). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment. Error bars represent the standard errors of the means of 10 replicates. This graph shows similar data to Fig. AA1 but with the data from the control treatment and some pesticide treatments removed for clarity. The data for the remaining pesticide treatments are presented in Fig AA3.

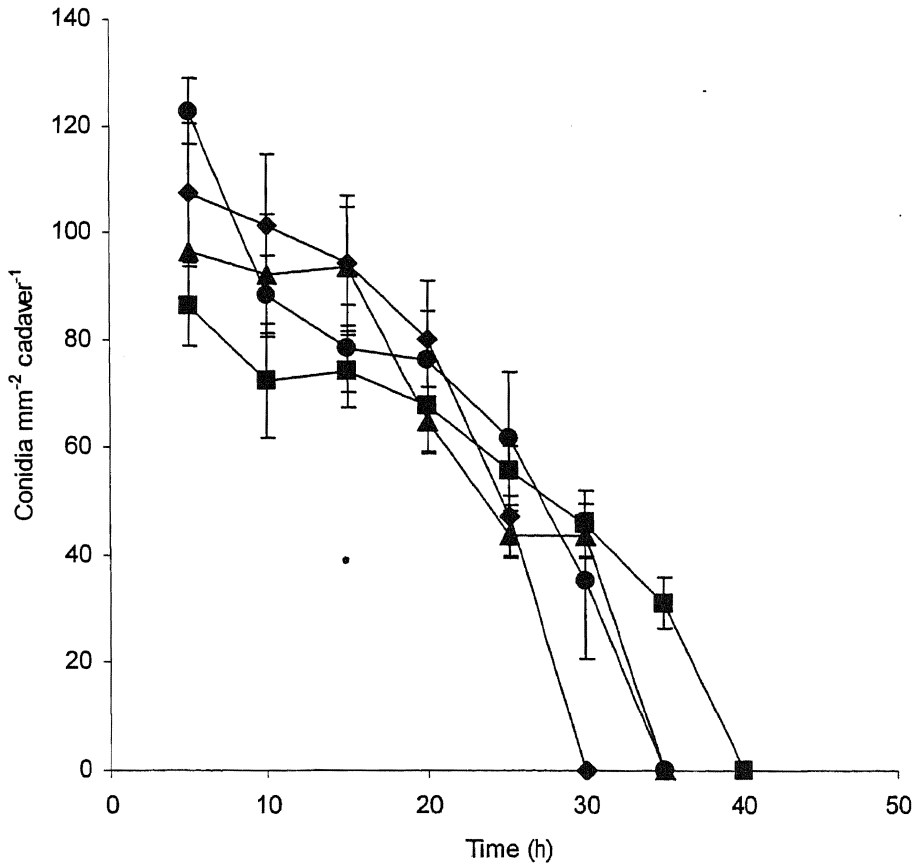


Figure AA3. Comparison of discharge of conidia from mycosed aphid cadavers treated with deltamethrin (◆), cypermethrin (■), chlorpyrifos (▲) and paraquat. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment. Error bars represent the standard errors of the means of 10 replicates. This graph shows similar data to Fig. AA1 but with the data from the control treatment and some pesticide treatments removed for clarity. The data for the remaining pesticide treatments are presented in Fig AA2.

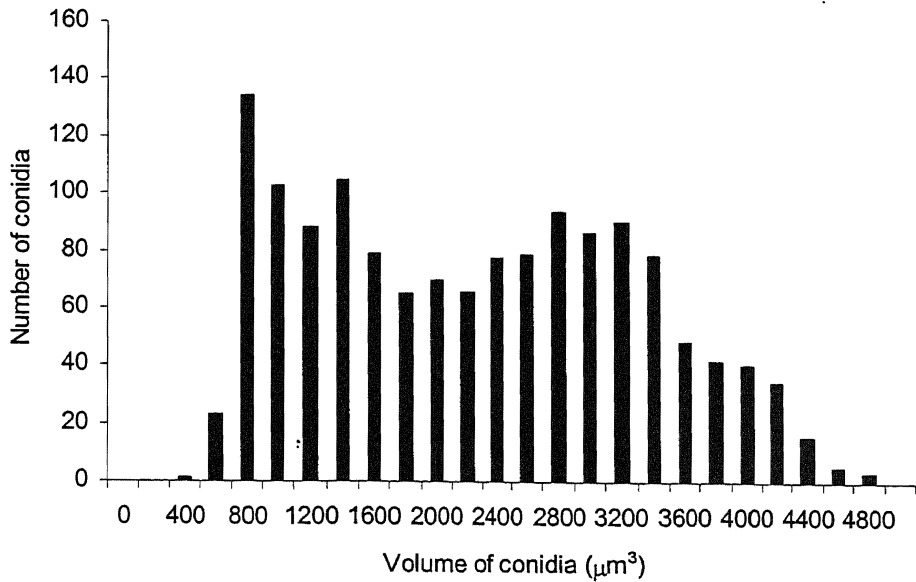


Figure AA4. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with glyphosate. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

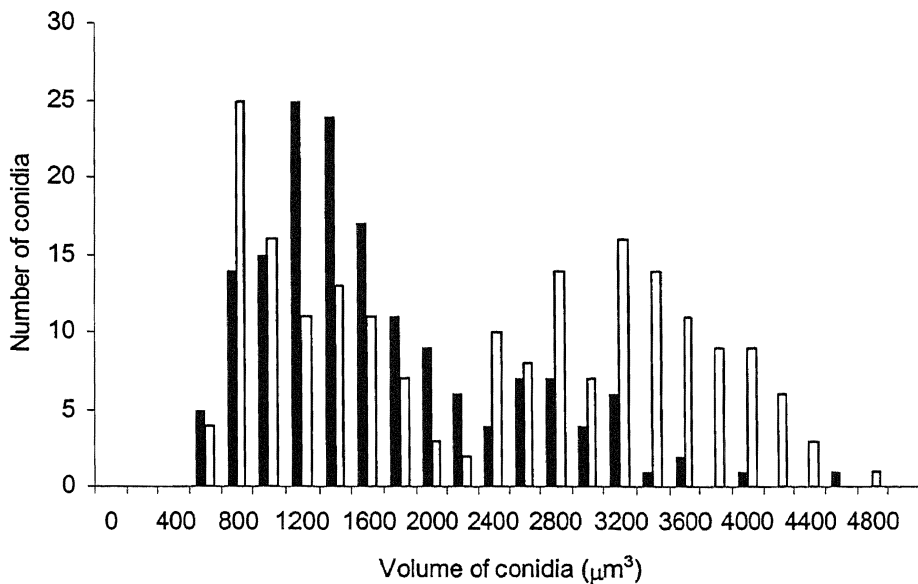


Figure AA5. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with glyphosate after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

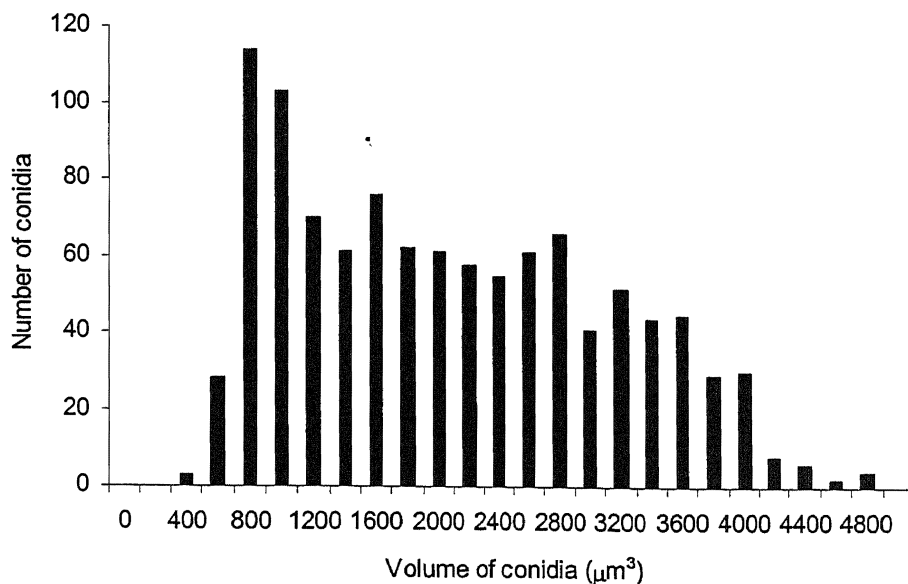


Figure AA6. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with fomasefen. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

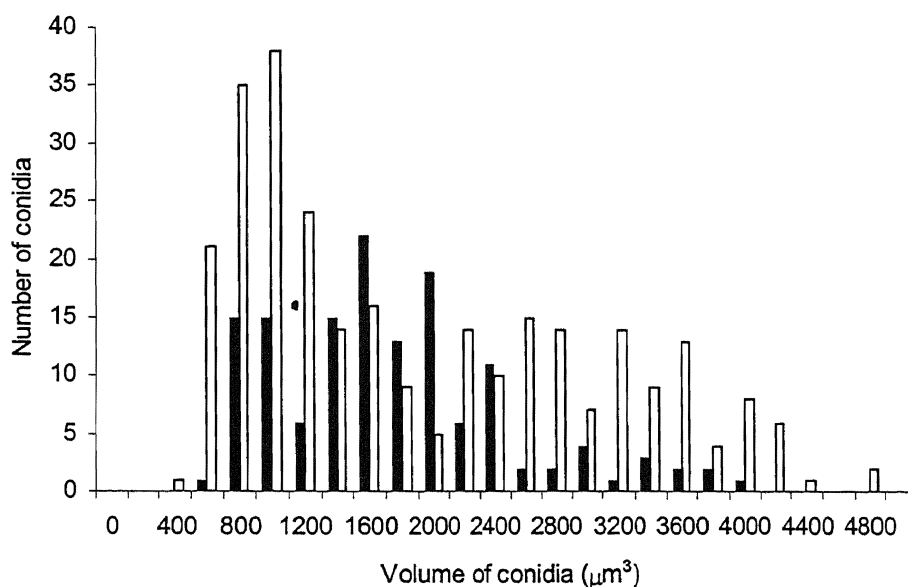


Figure AA7. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with fomasefen after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

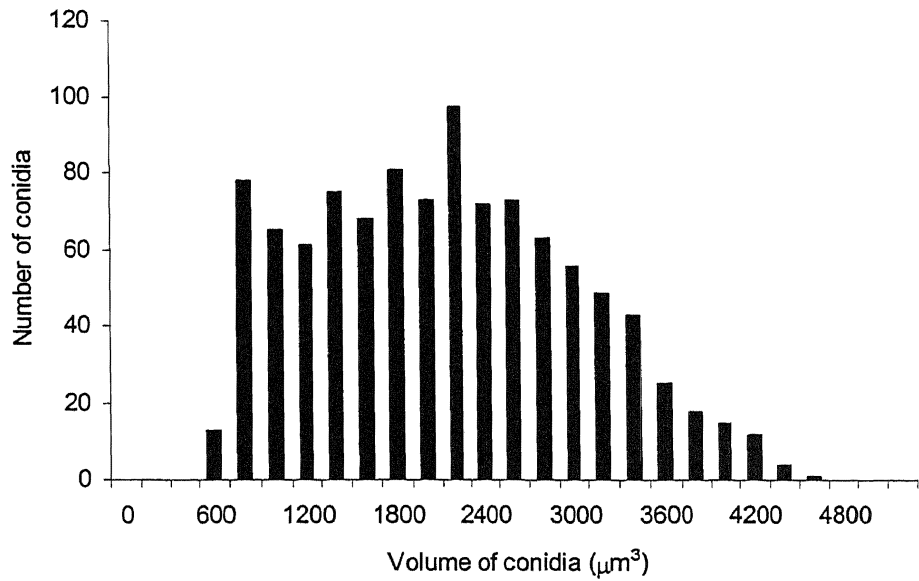


Figure AA8. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with fluazifop-p-butyl. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

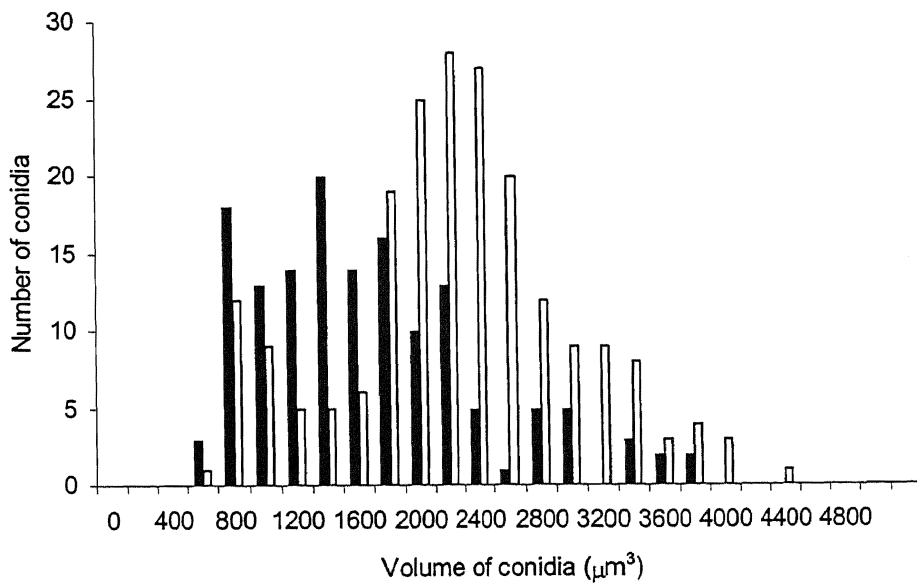


Figure AA9. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with fluazifop-p-butyl after 5 h incubation (■) and 30 h incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment



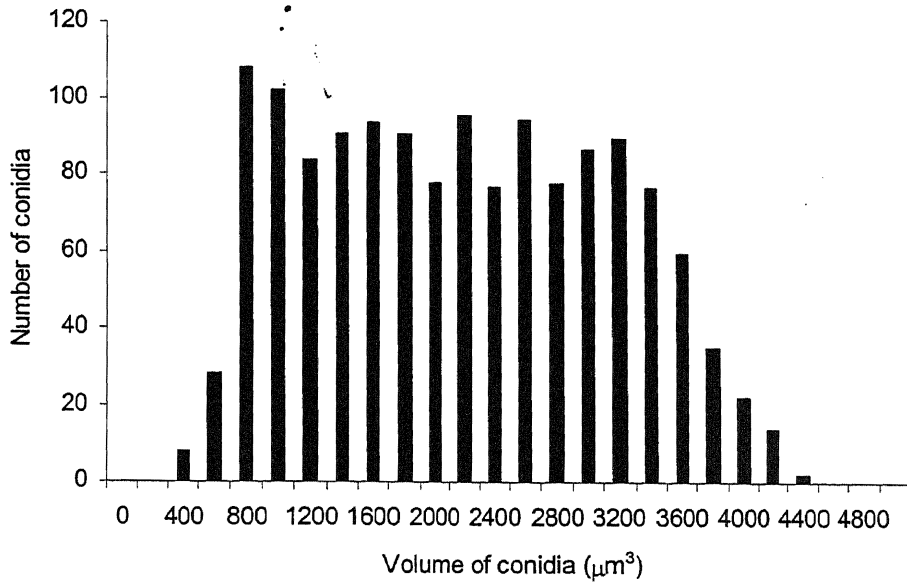


Figure AA10. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with lamda cyhalothrin 5EC. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

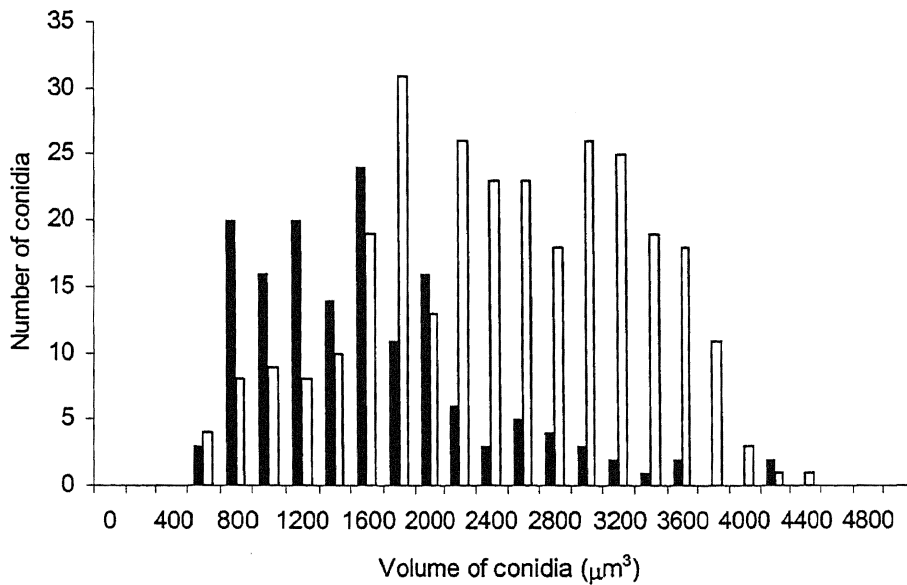


Figure AA11. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with lamda cyhalothrin 5EC after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

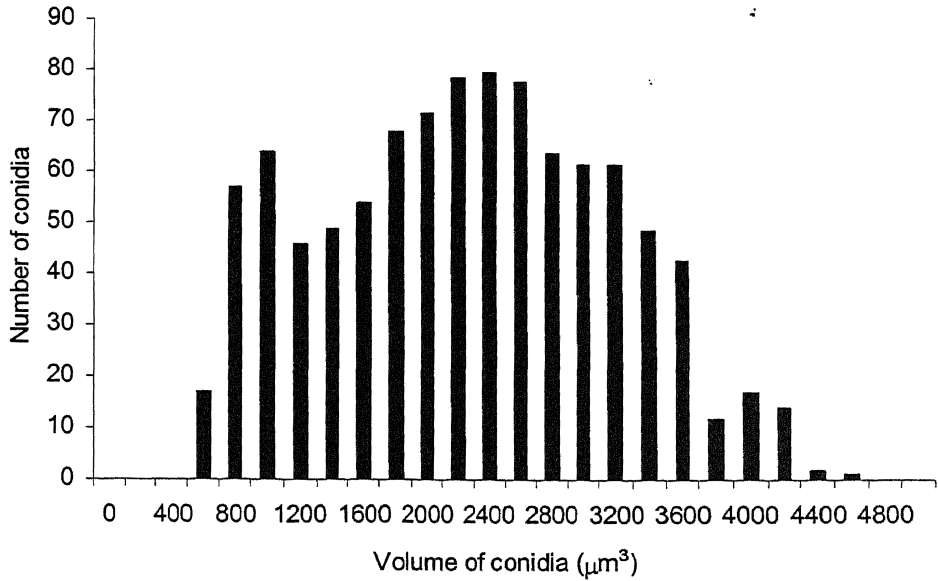


Figure AA12. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with lamda cyhalothrin 10CS. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

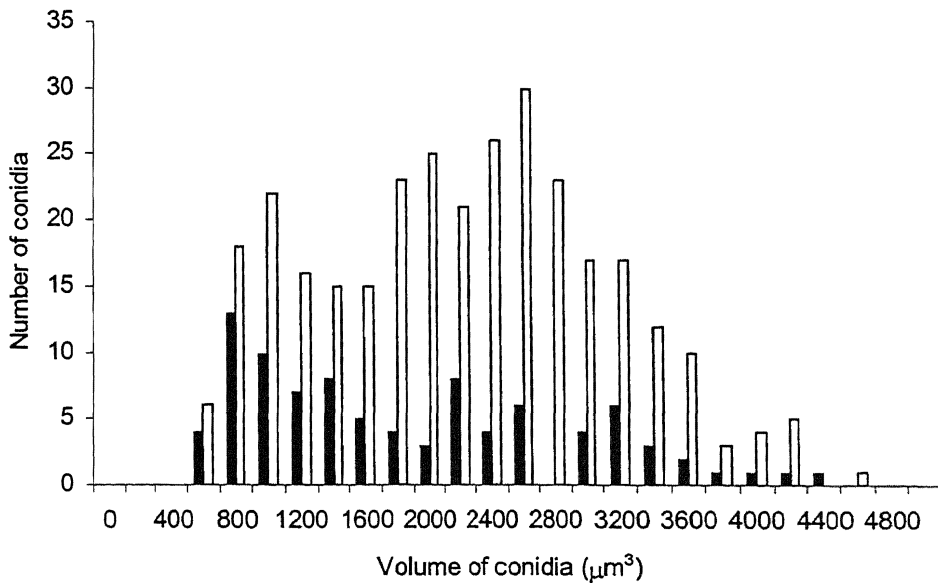


Figure AA13. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with  $\lambda$ -cyhalothrin 10CS after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

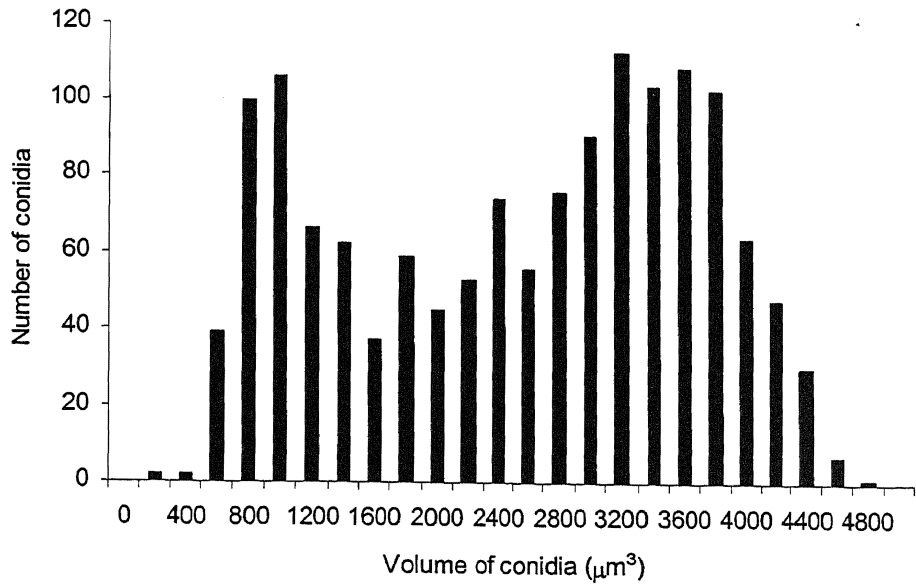


Figure AA14. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with deltamethrin. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

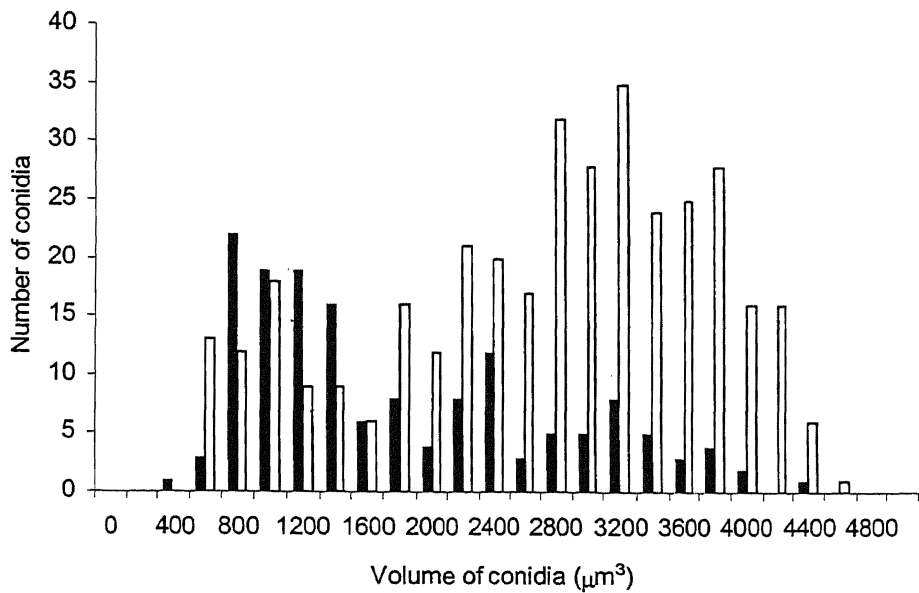


Figure AA15. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with deltamethrin after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

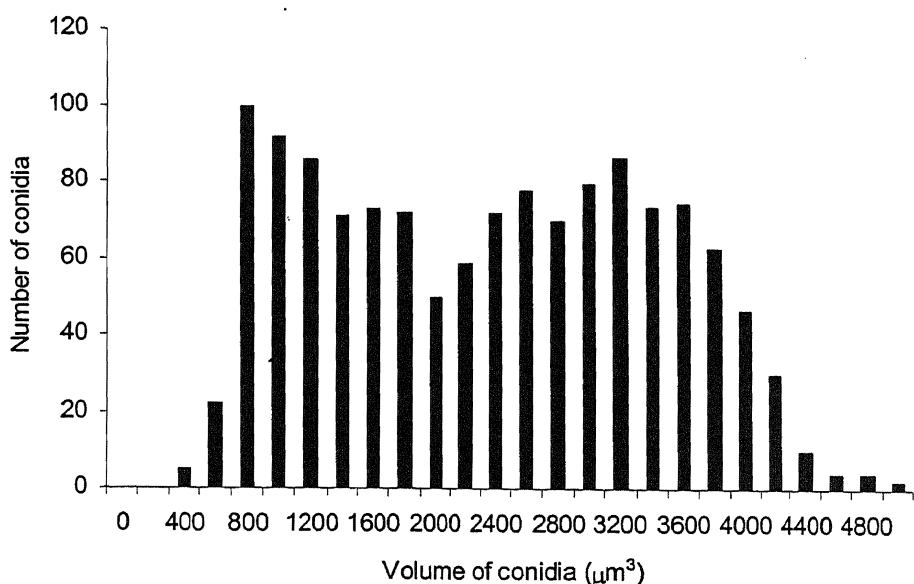


Figure AA16. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with cypermethrin. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

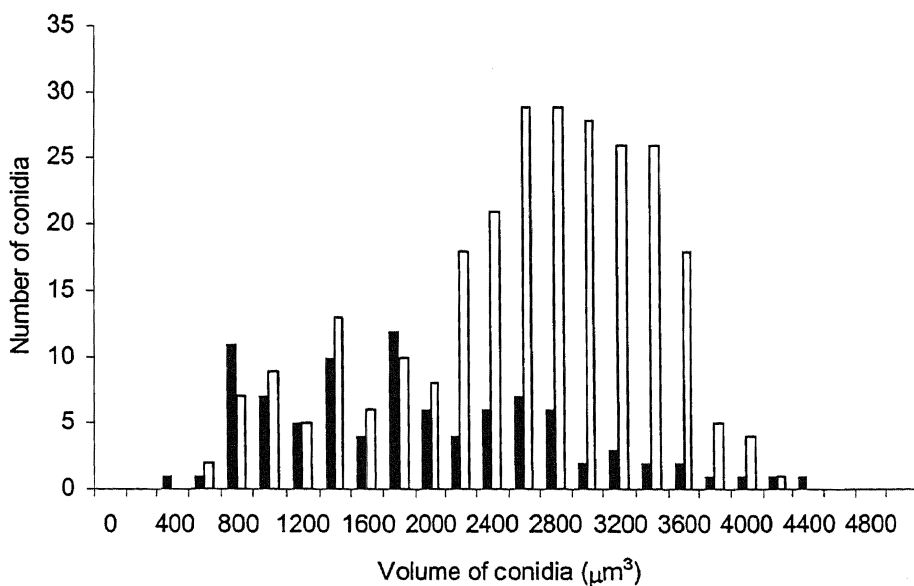


Figure AA17. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with cypermethrin after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

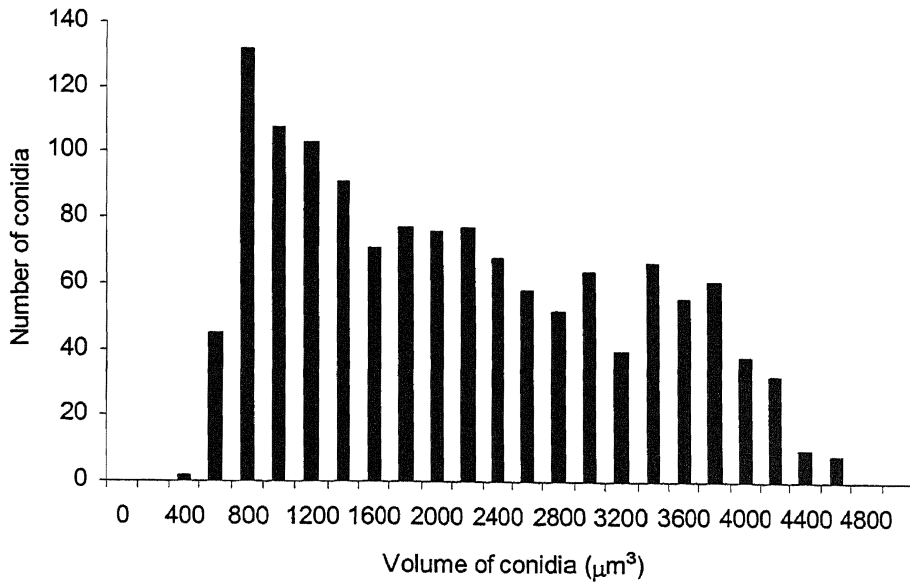


Figure AA18. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with chlorpyrifos. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

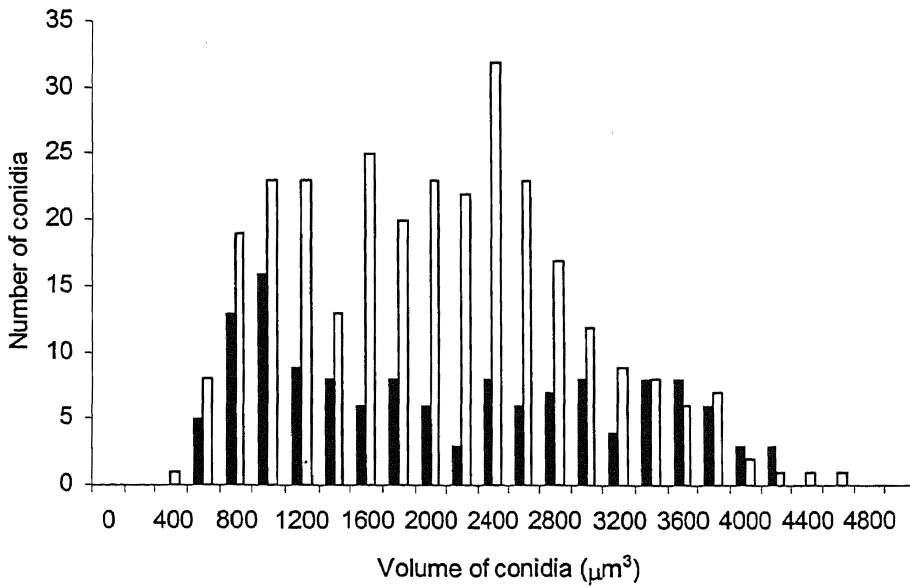


Figure AA19. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with chlorpyrifos after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

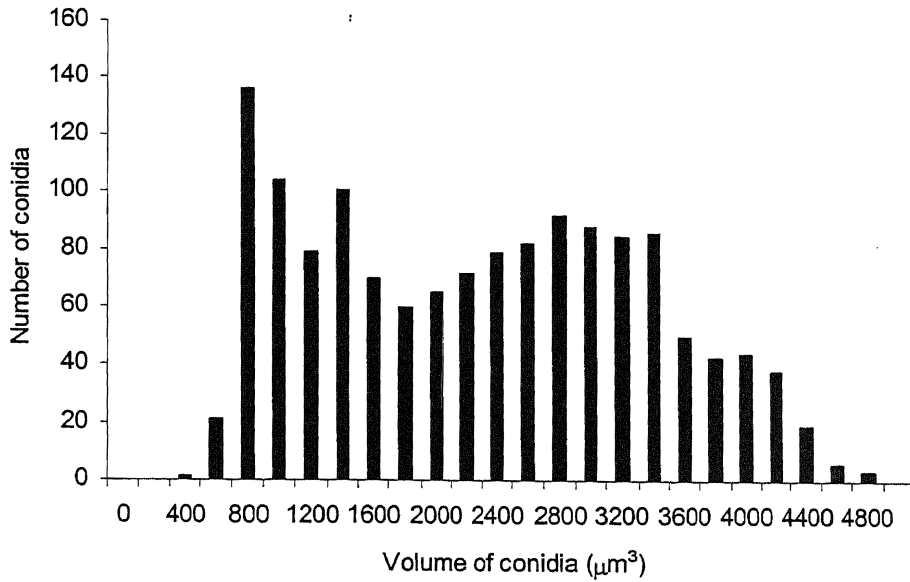


Figure AA20. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with paraquat. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

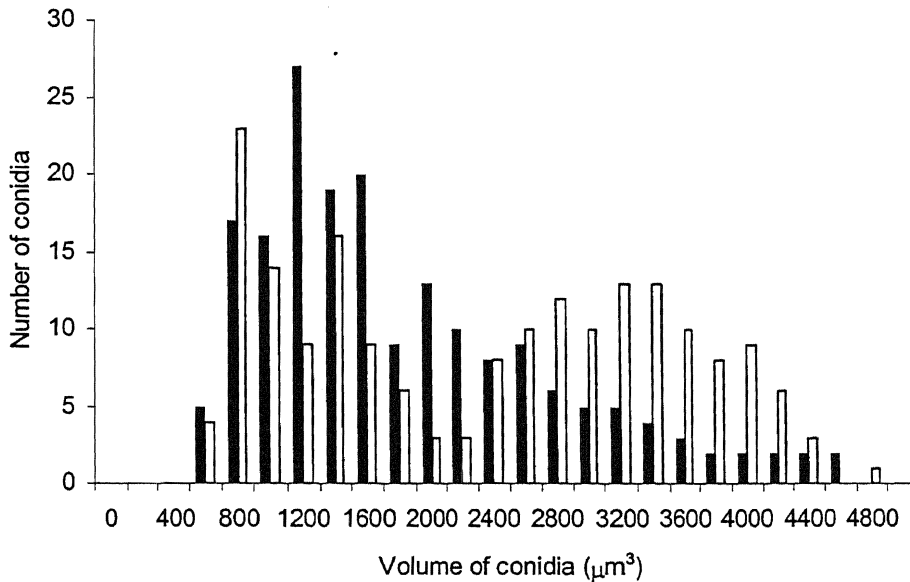


Figure AA21. Frequency distribution of the volumes of conidia produced from mycosed aphid cadavers treated with paraquat after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

## Appendix B

Production of conidia and frequency distribution of conidia of *E. neoaphidis* produced from *A. pisum* treated with pesticides prior to fungal inoculation.

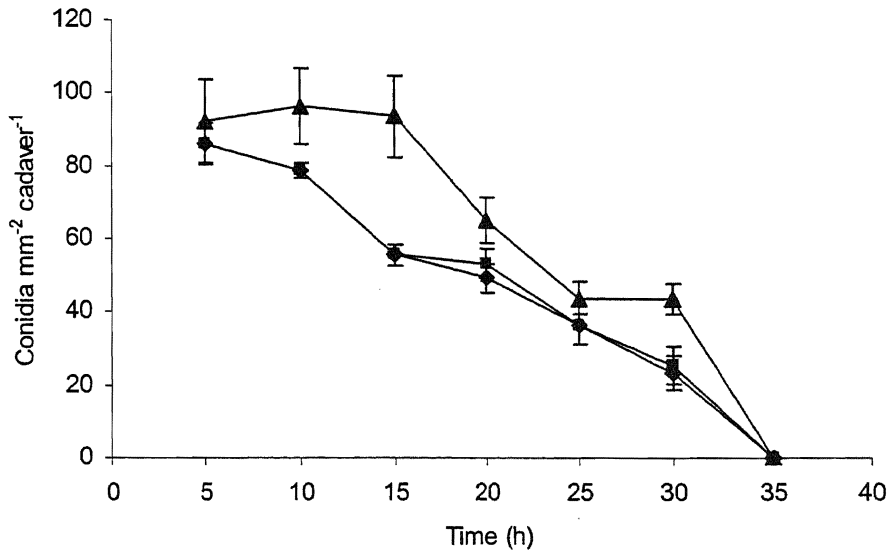


Figure AB1. Comparison of discharge of conidia from aphids treated with fomasefen (◆), glyphosate(■) and fluazifop-p-butyl (▲) 4 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment. Error bars represent the standard errors of the means of 10 replicates.

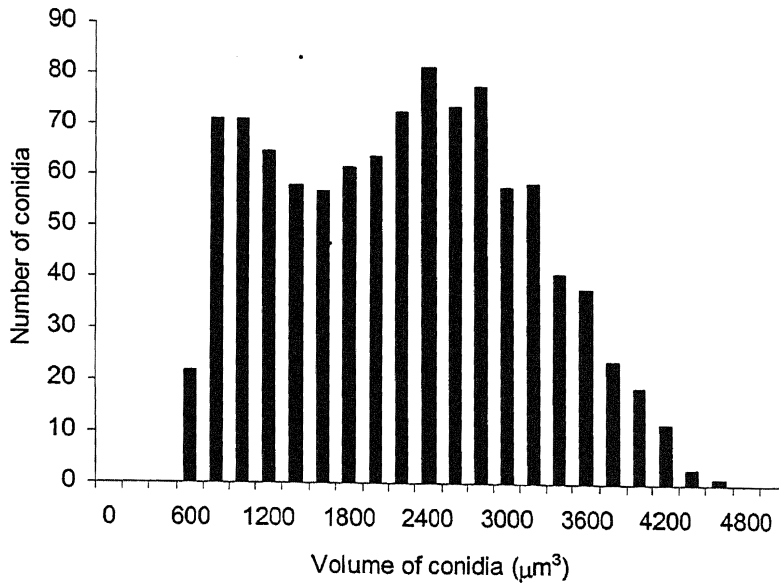


Figure AB2. Frequency distribution of the volumes of conidia produced from aphids treated with glyphosate 4 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

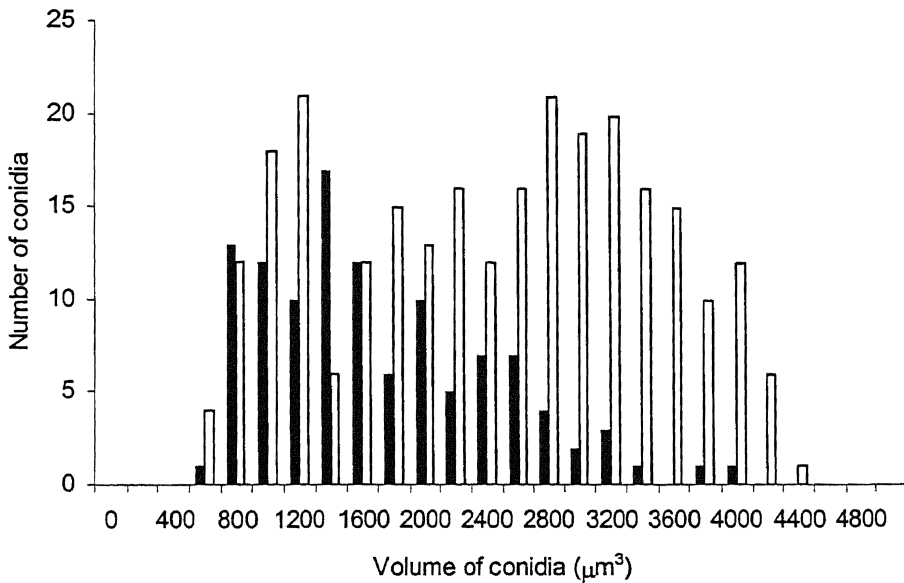


Figure AB3. Frequency distribution of the volumes of conidia produced from aphids treated with glyphosate 4 h prior to fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment



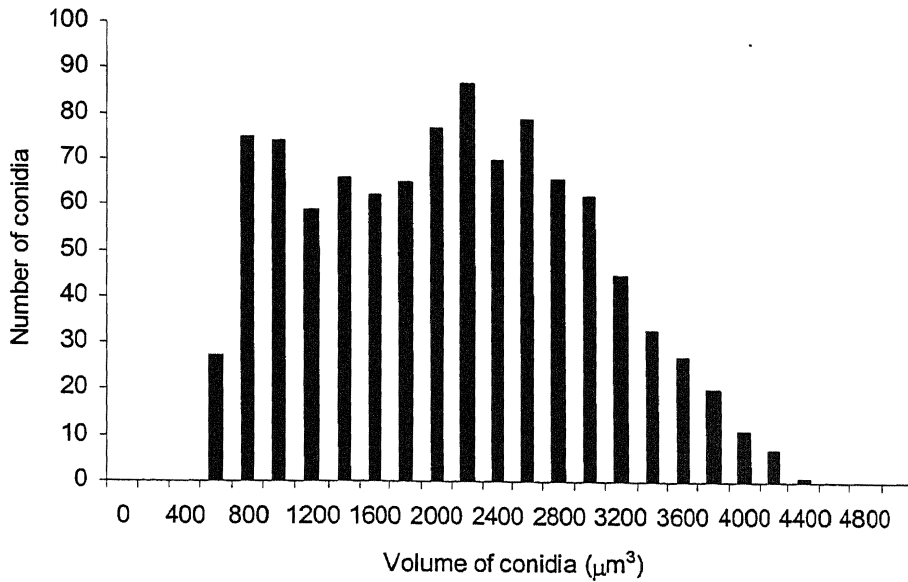


Figure AB4. Frequency distribution of the volumes of conidia produced from aphids treated with fomasefen 4 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

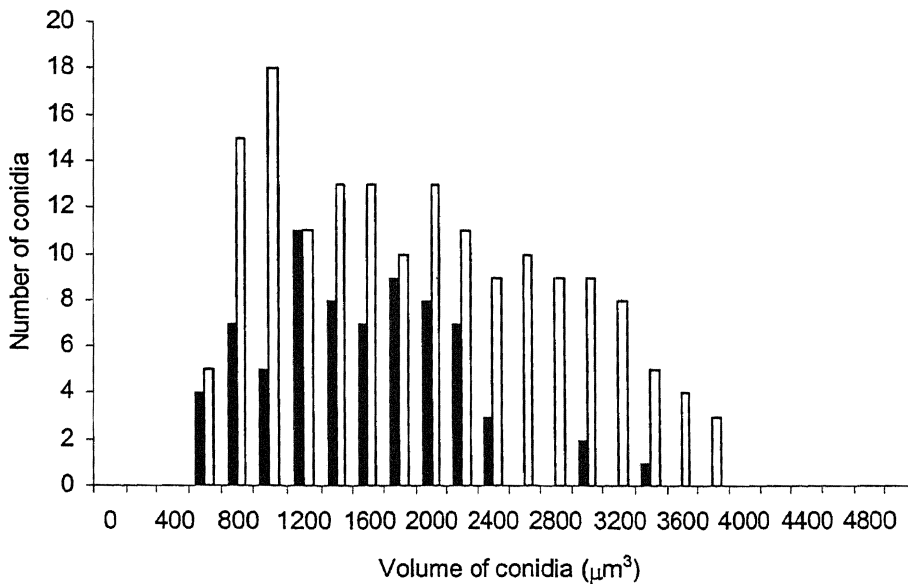


Figure AB5. Frequency distribution of the volumes of conidia produced from aphids treated with fomasefen 4 h prior to fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

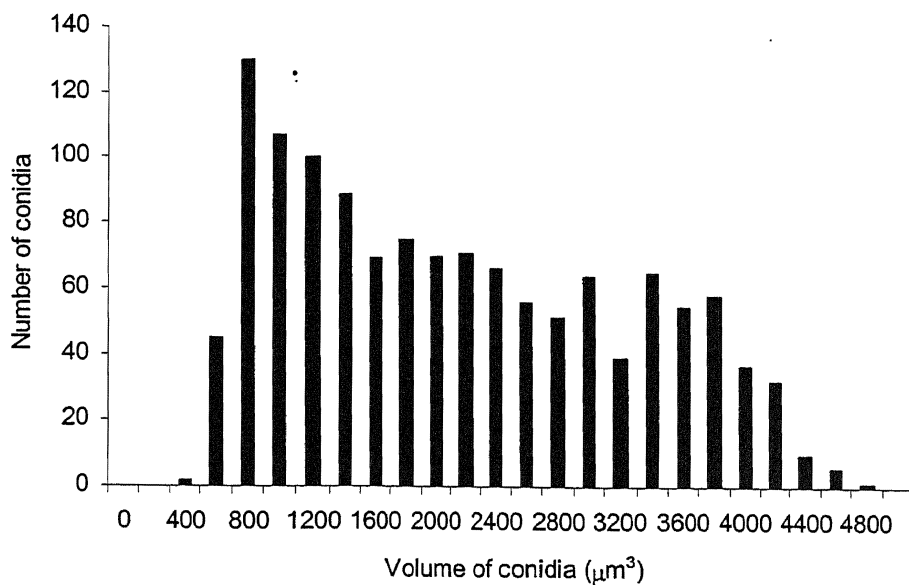


Figure AB6. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 4 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

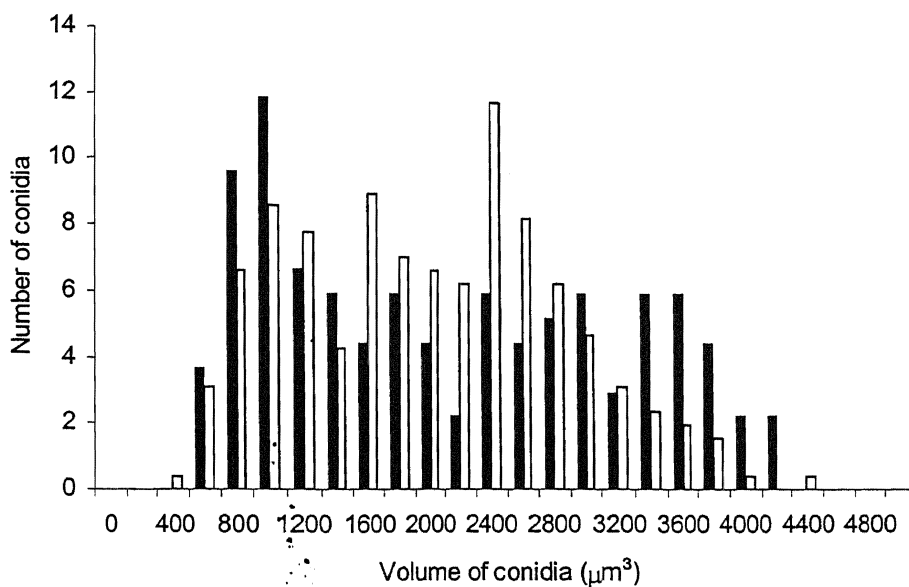


Figure AB7. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 4 h prior to fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

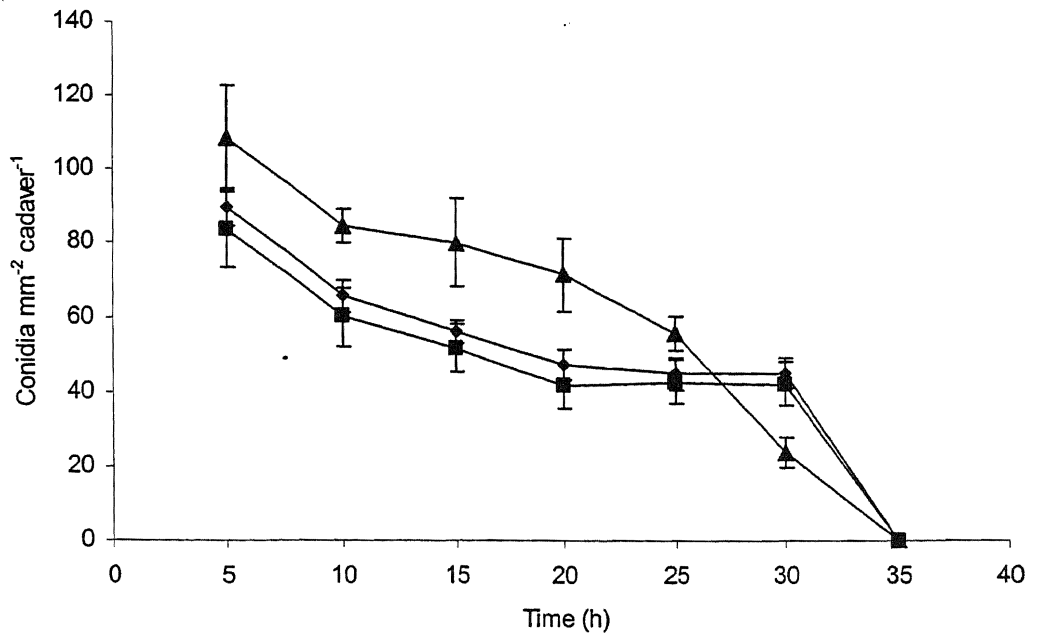


Figure AB8. Comparison of discharge of conidia from aphids treated with fomasefen (◆), glyphosate(■) and fluzifop-p-butyl (▲) 48 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment. Error bars represent the standard errors of the means of 10 replicates.

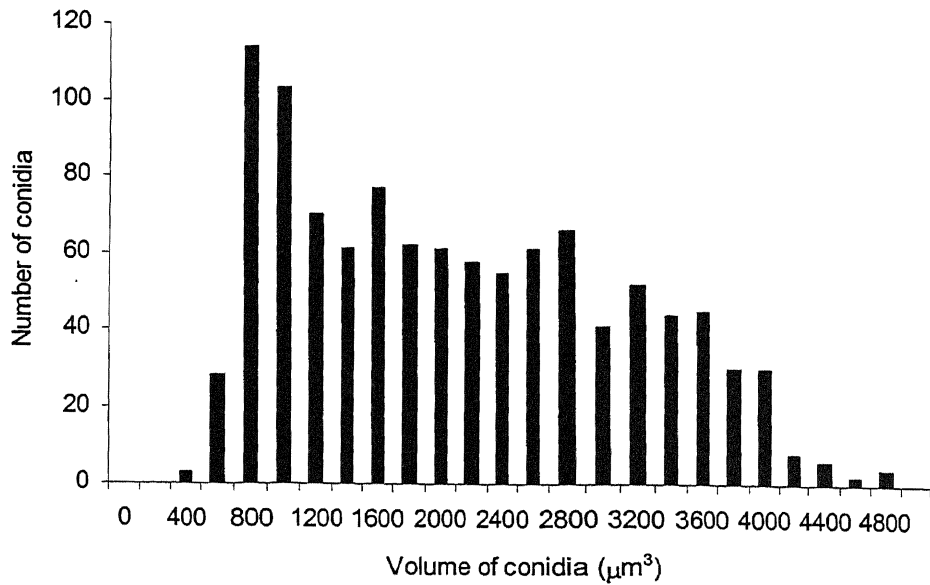


Figure AB9. Frequency distribution of the volumes of conidia produced from aphids treated with glyphosate 48 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

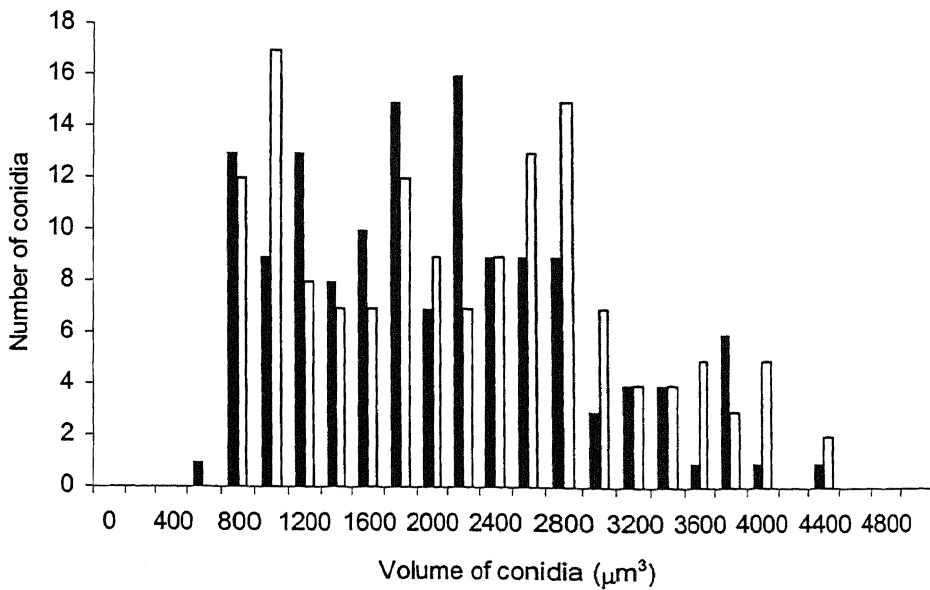


Figure AB10 Frequency distribution of the volumes of conidia produced from aphids treated with glyphosate 48 h prior to fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

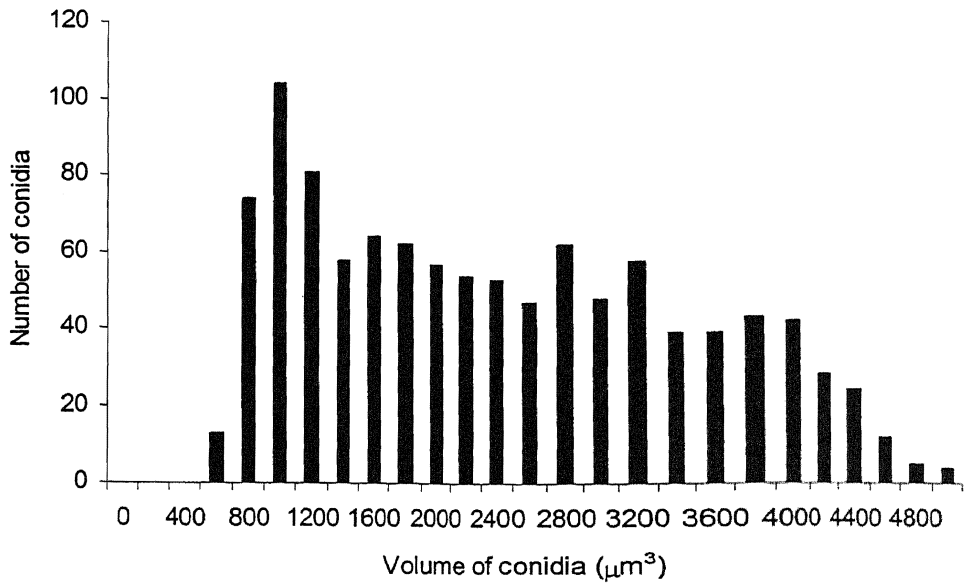


Figure AB11. Frequency distribution of the volumes of conidia produced from aphids treated with fomasefen 48 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

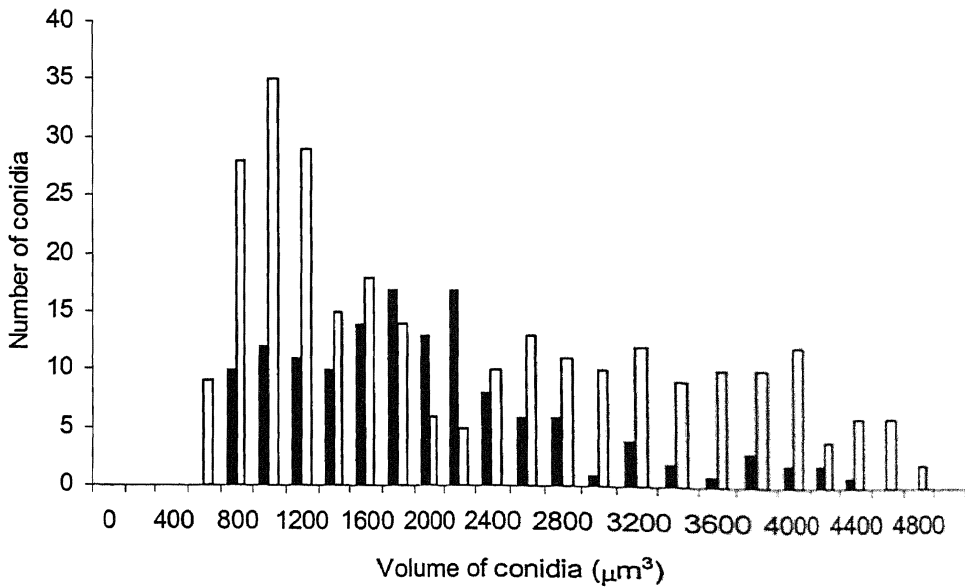


Figure AB12. Frequency distribution of the volumes of conidia produced from aphids treated with fomasefen 48 h prior to fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

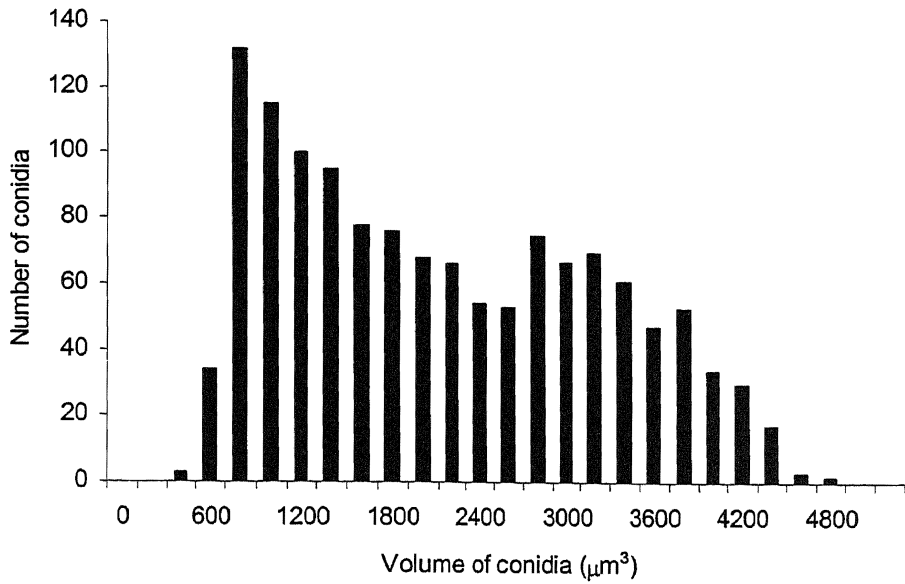


Figure AB13. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 48 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

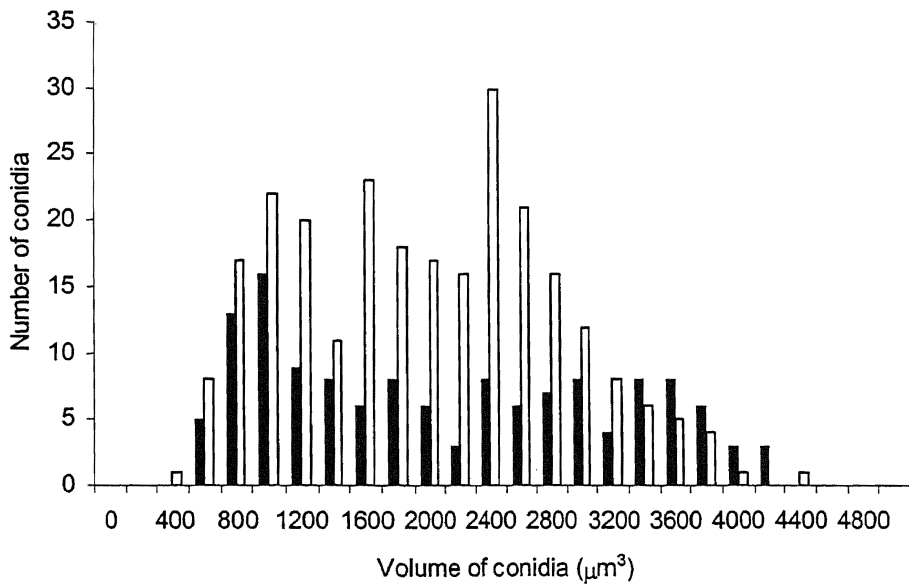


Figure AB14. Frequency distribution of the volumes of conidia produced from aphids treated with fluzifop-p-butyl 48 h prior to fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

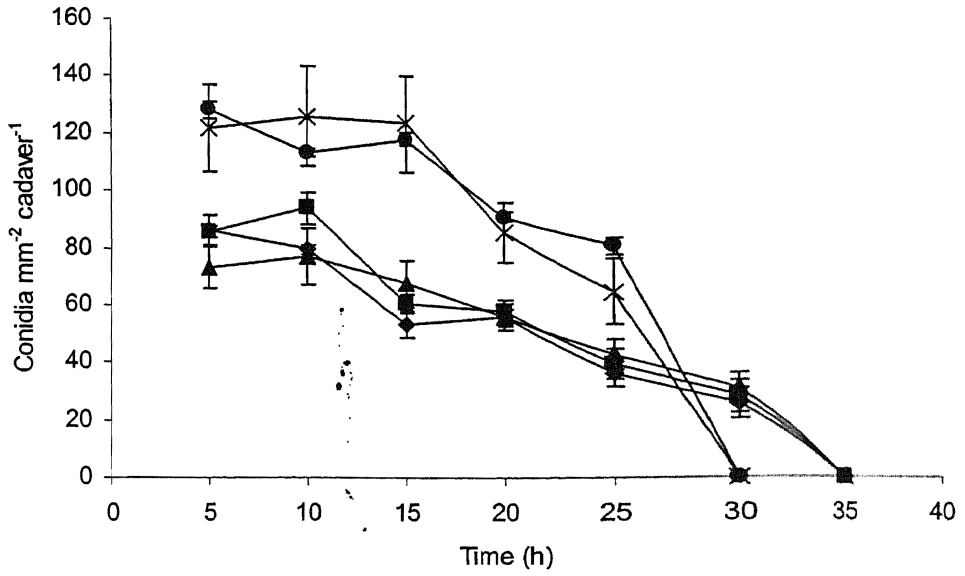


Figure AB15. Comparison of discharge of conidia from aphids treated with fomasefen (◆), glyphosate (■), fluzifop-p-butyl (▲), azoxystrobin (●) and chlorothalonil (X) 72 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment. Error bars represent the standard errors of the means of 10 replicates.

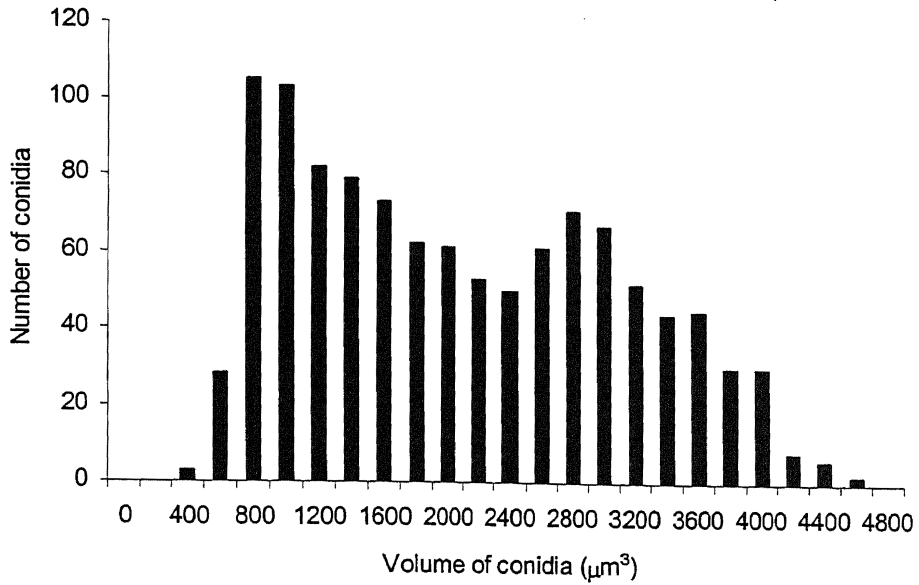


Figure AB16. Frequency distribution of the volumes of conidia produced from aphids treated with glyphosate 72 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

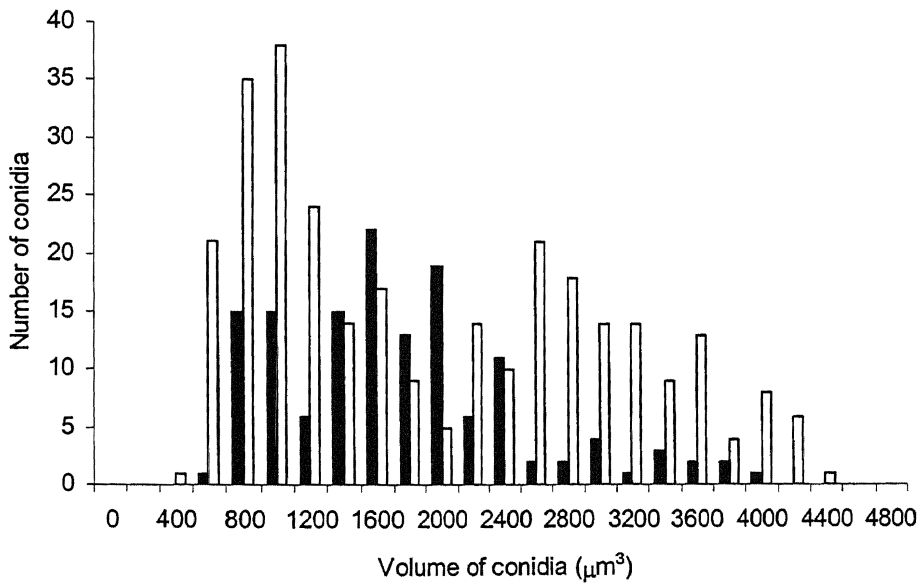


Figure AB17. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 48 h prior to fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.



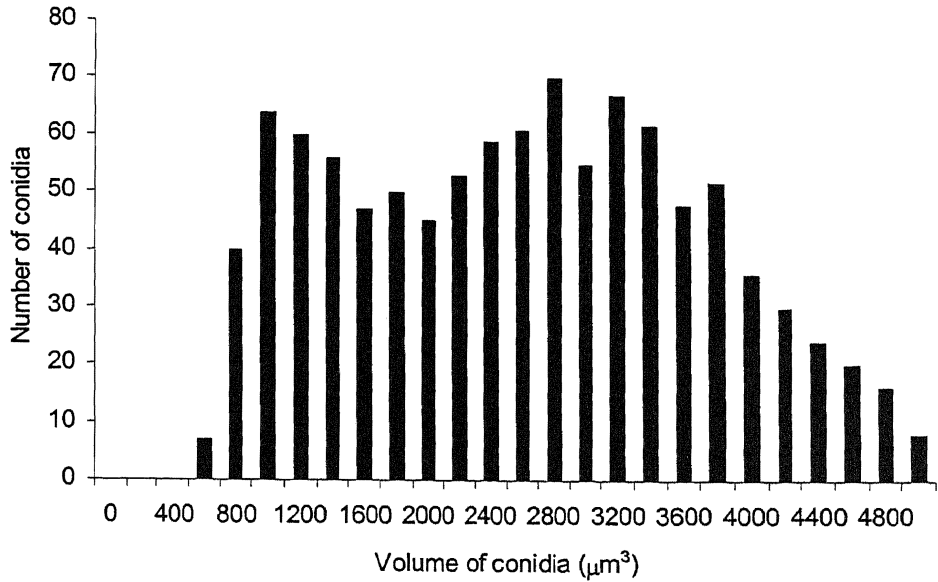


Figure AB18. Frequency distribution of the volumes of conidia produced from aphids treated with fomasefen 72 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

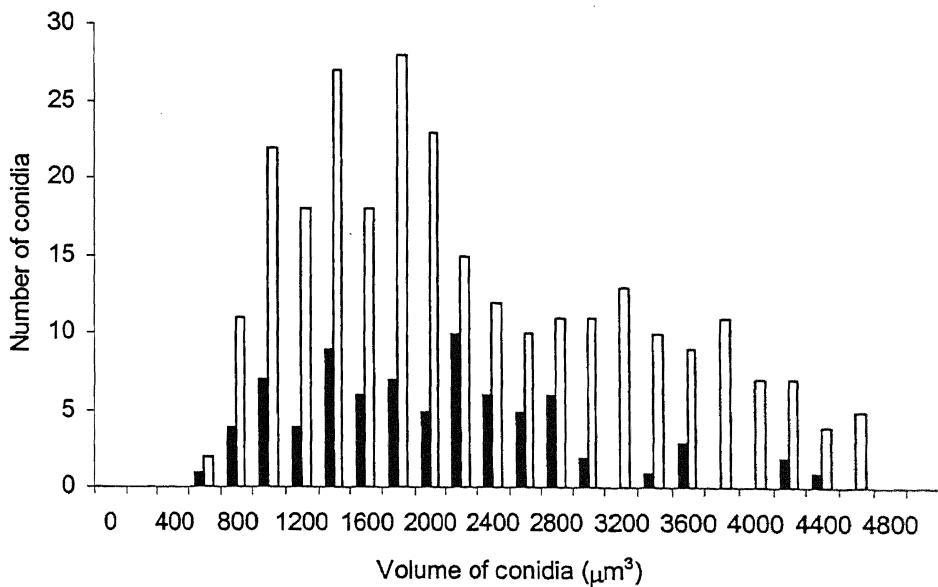


Figure AB19. Frequency distribution of the volumes of conidia produced from aphids treated with fomsefen 72 h prior to fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

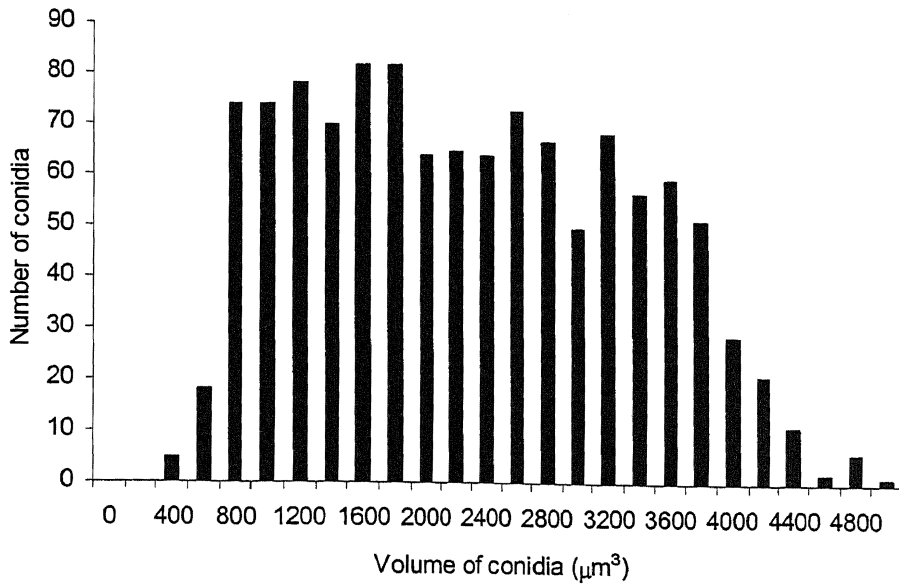


Figure AB20. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 72 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

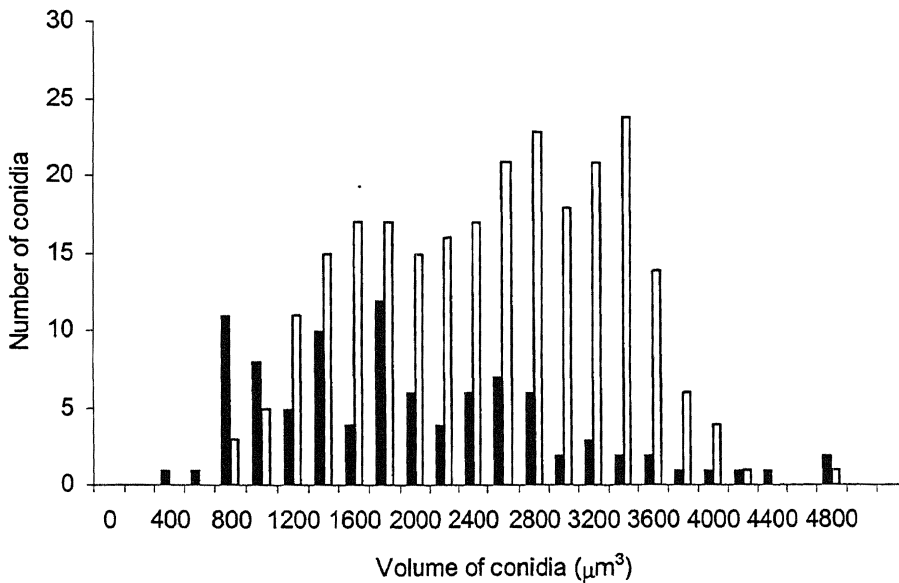


Figure AB21. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 72 h prior to fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

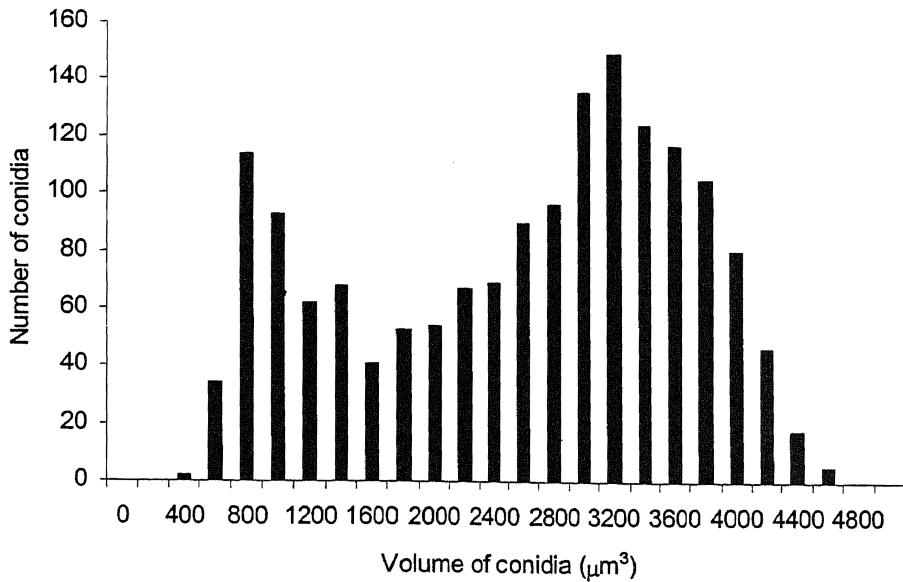


Figure AB22. Frequency distribution of the volumes of conidia produced from aphids treated with azoxystrobin 72 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

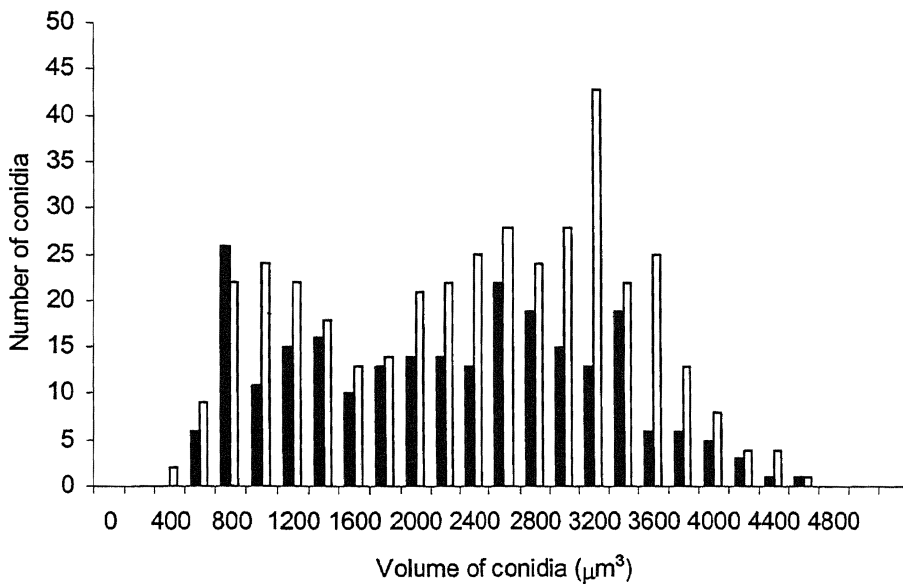


Figure AB23. Frequency distribution of the volumes of conidia produced from aphids treated with azoxystrobin 72 h prior to fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

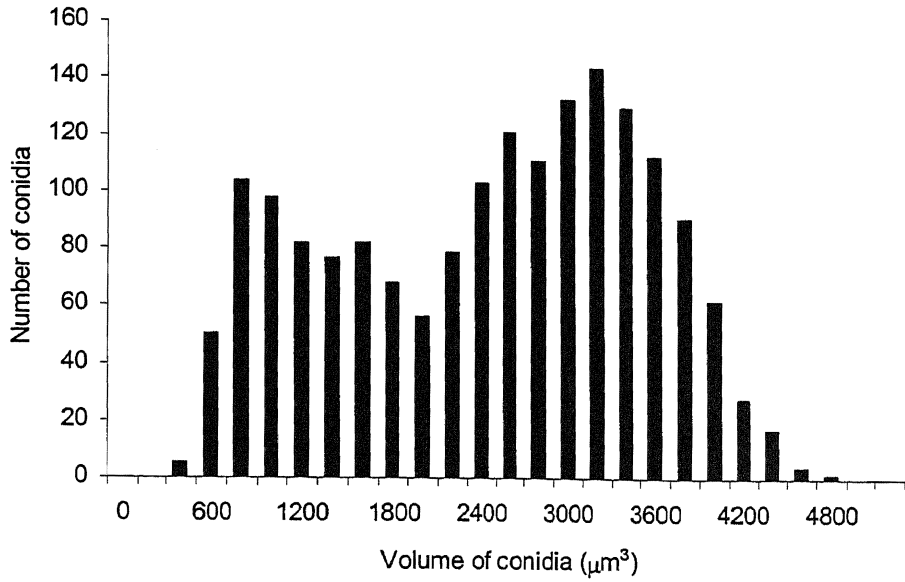


Figure AB24. Frequency distribution of the volumes of conidia produced from aphids treated with chlorothalonil 72 h prior to fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

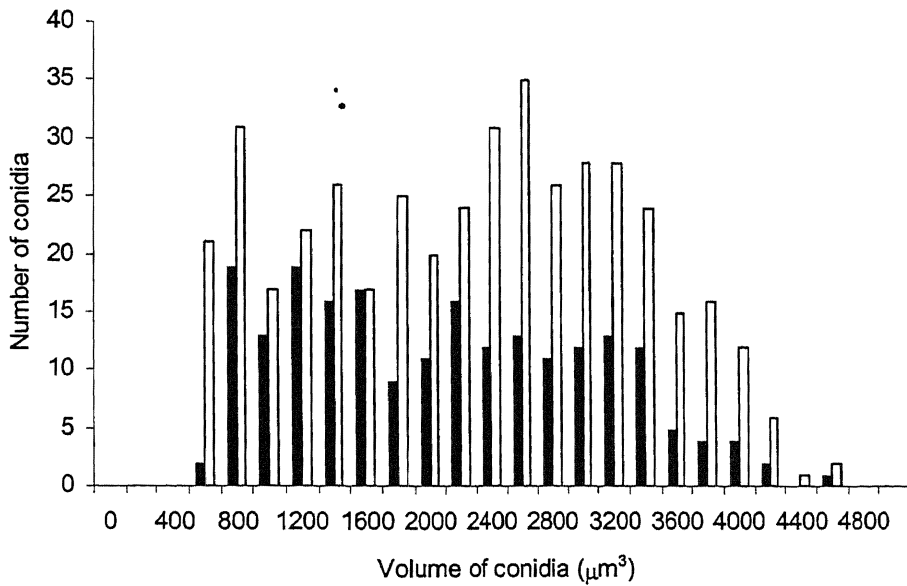


Figure AB25. Frequency distribution of the volumes of conidia produced from aphids treated with chlorothalonil 72 h prior to fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

## Appendix C

Production of conidia and frequency distribution of conidia of

*E.*

*neoaphidis* produced from *A. pisum* treated with pesticides after fungal inoculation.

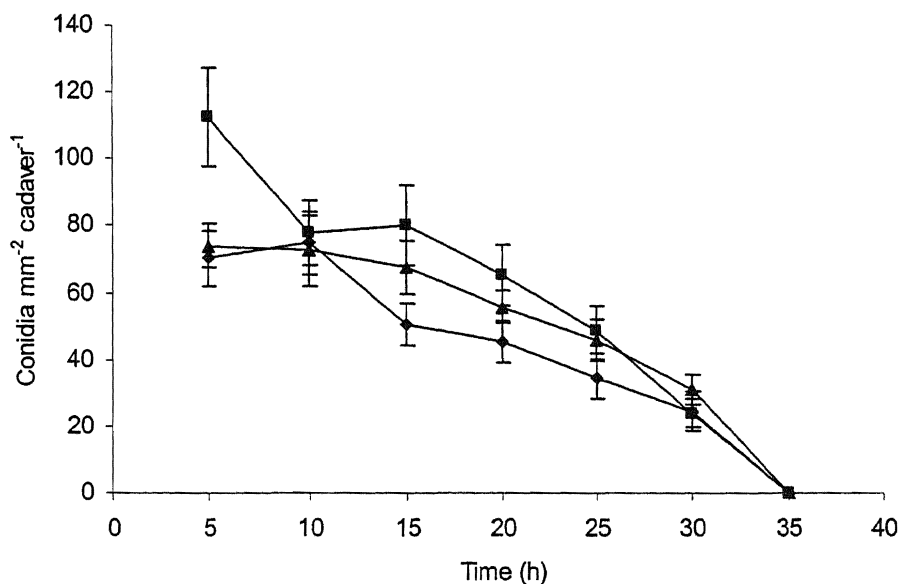


Figure AC1. Comparison of discharge of conidia from aphids treated with fomasefen (◆), glyphosate(■) and fluzifop-p-butyl (▲) 4 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment. Error bars represent the standard errors of the means of 10 replicates.

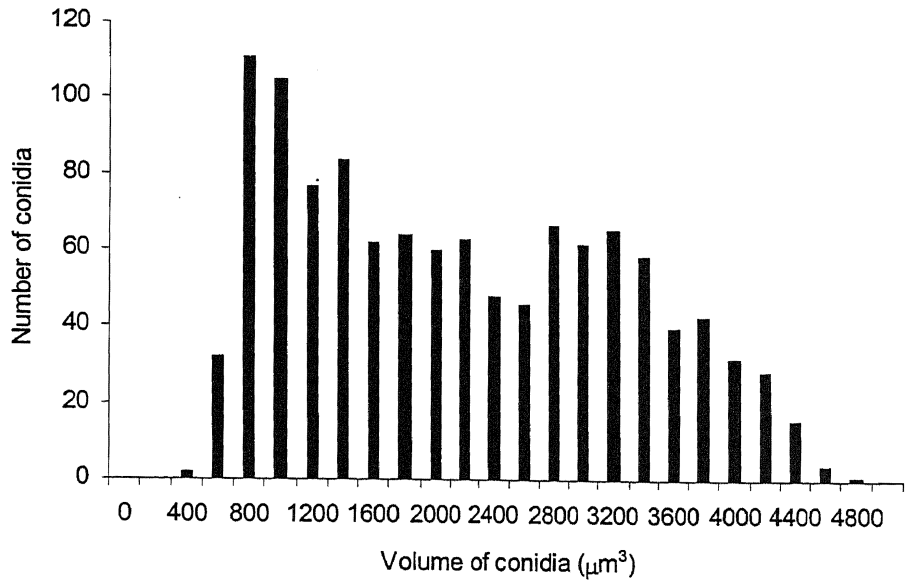


Figure AC2. Frequency distribution of the volumes of conidia produced from aphids treated with glyphosate 4 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

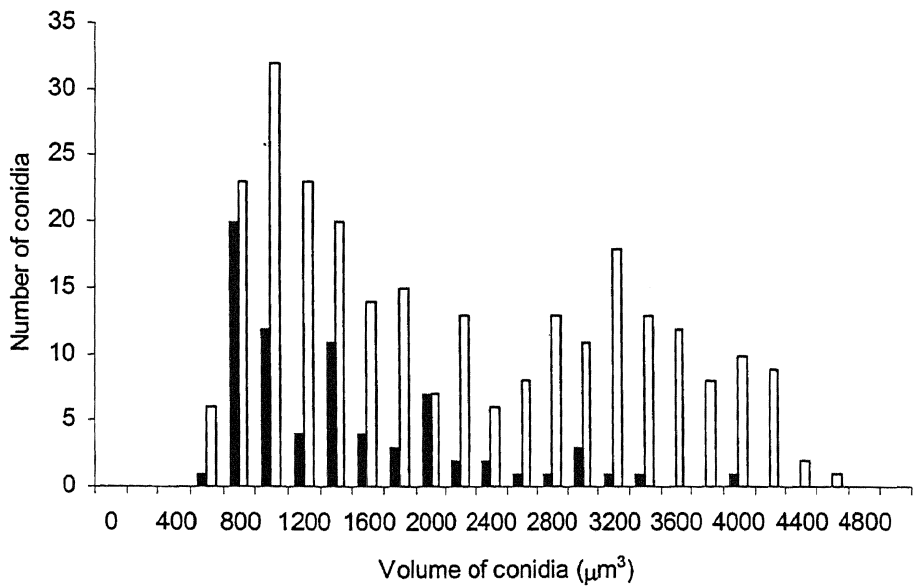


Figure AC3. Frequency distribution of the volumes of conidia produced from aphids treated with glyphosate 4 h after fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

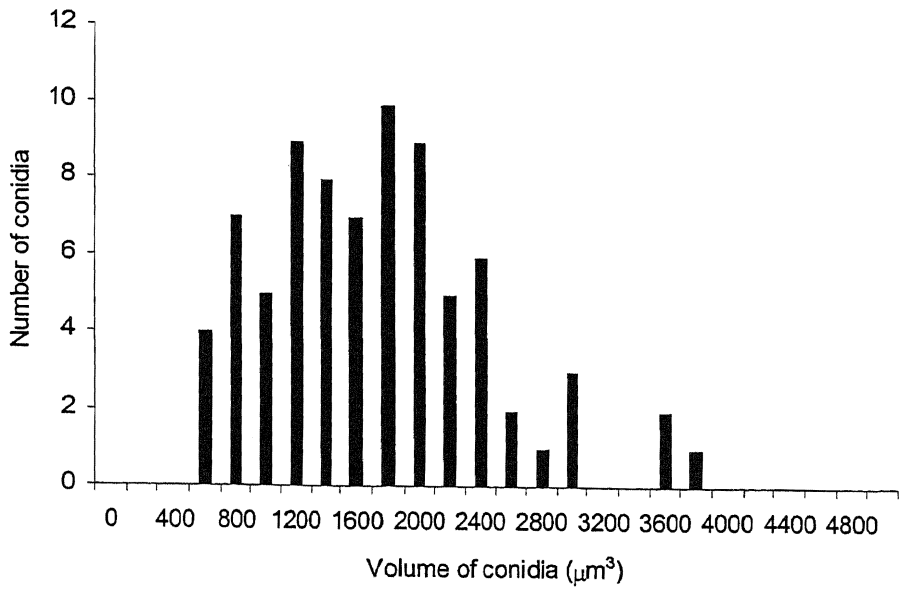


Figure AC4. Frequency distribution of the volumes of conidia produced from aphids treated with fomasefen 4 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

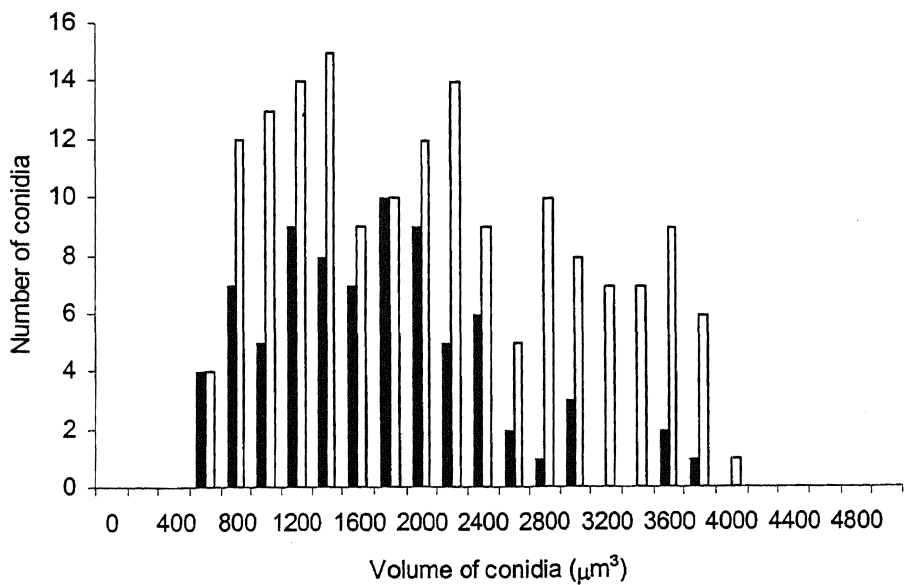


Figure AC5. Frequency distribution of the volumes of conidia produced from aphids treated with fomasefen 4 h after fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

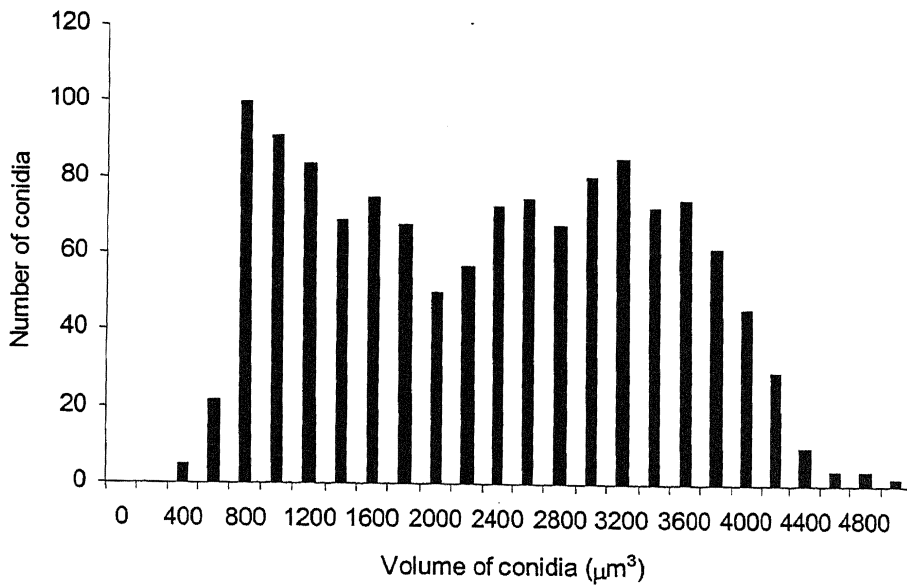


Figure AC6. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 4 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

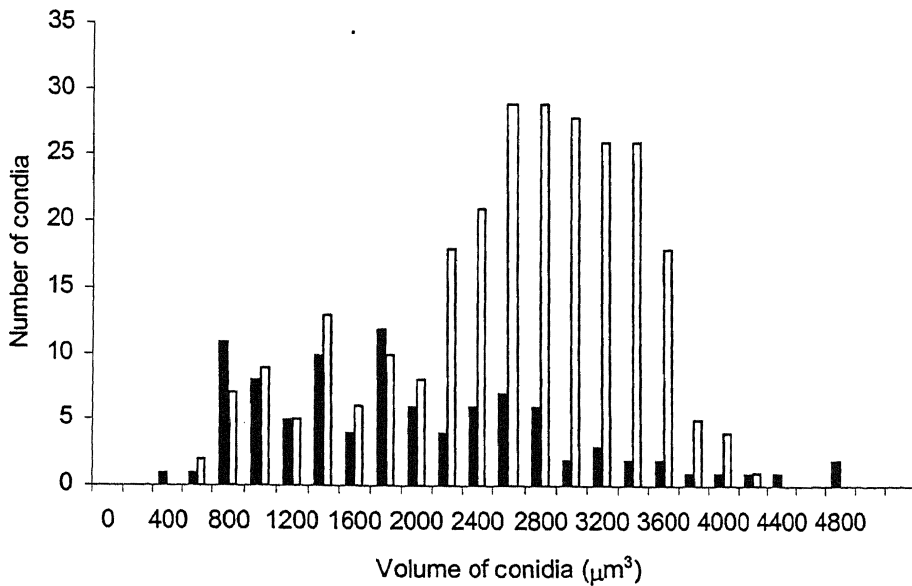


Figure AC7. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 4 h after fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment



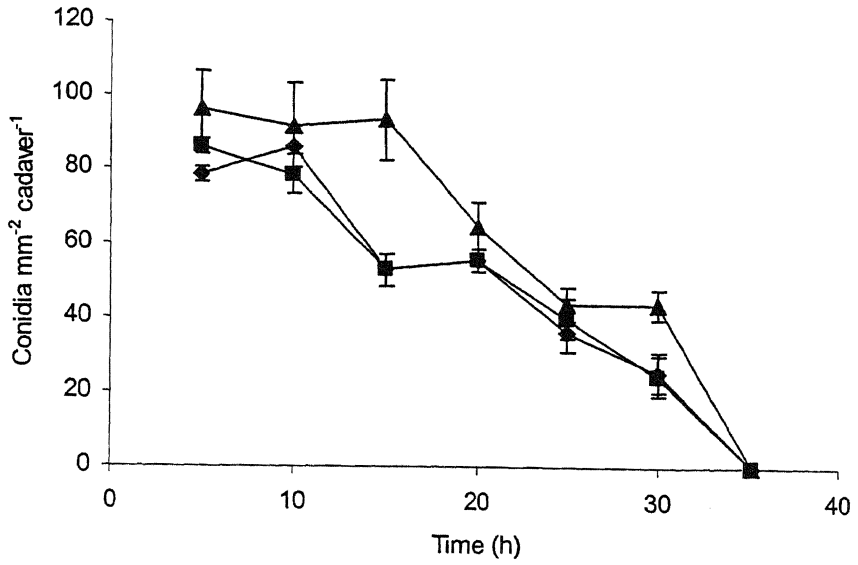


Figure AC8. Comparison of discharge of conidia from aphids treated with fomasefen (◆), glyphosate (■) and fluazifop-p-butyl (▲) 48 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment. Error bars represent the standard errors of the means of 10 replicates.

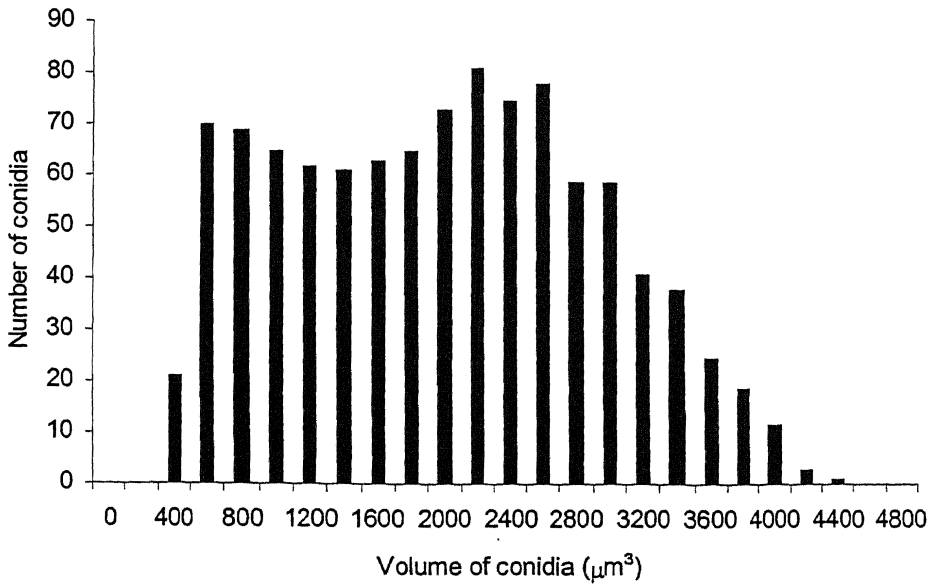


Figure AC9. Frequency distribution of the volumes of conidia produced from aphids treated with glyphosate 48 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

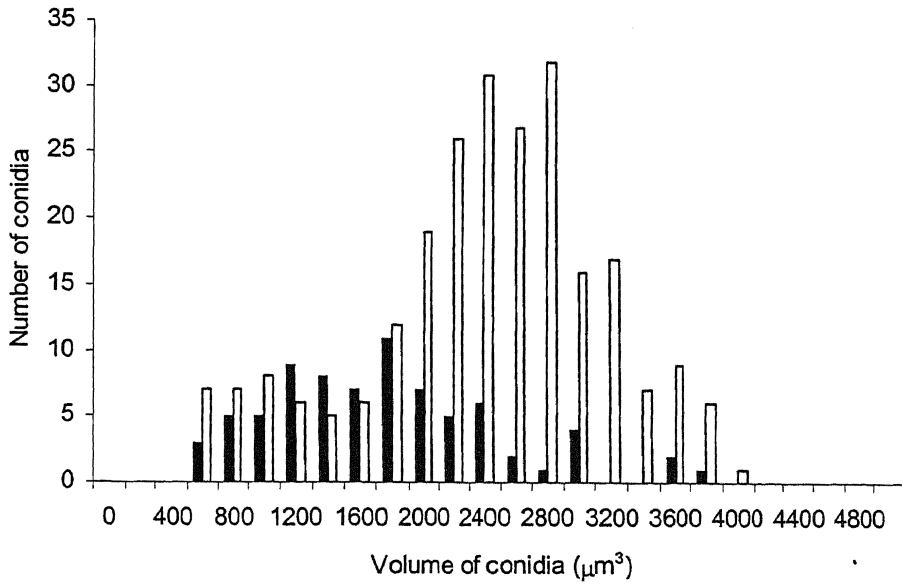


Figure AC10. Frequency distribution of the volumes of conidia produced from aphids treated with glyphosate 48 h after fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

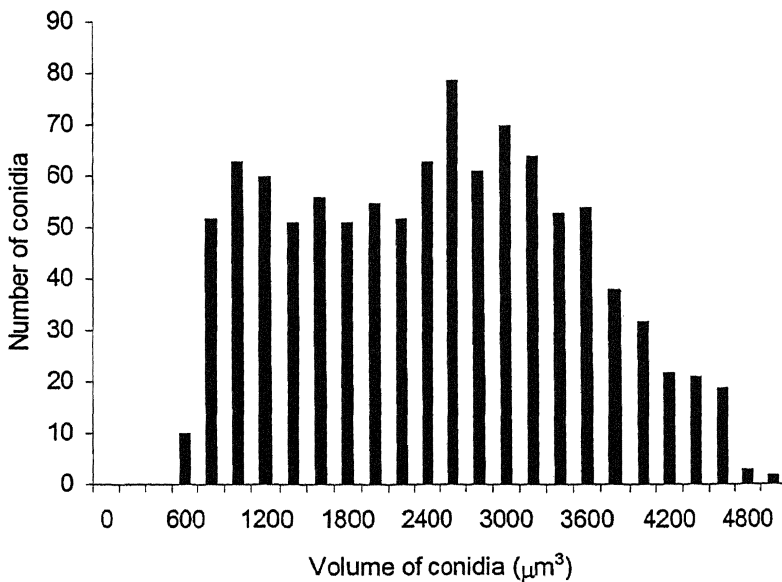


Figure AC11. Frequency distribution of the volumes of conidia produced from aphids treated with fomasefen 48 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

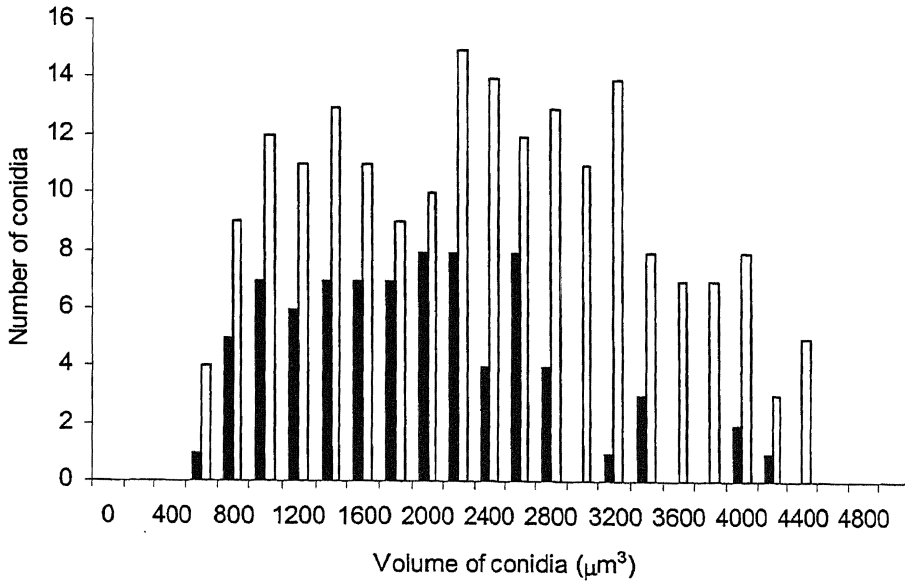


Figure AC12. Frequency distribution of the volumes of conidia produced from aphids treated with fomasefen 48 h after fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

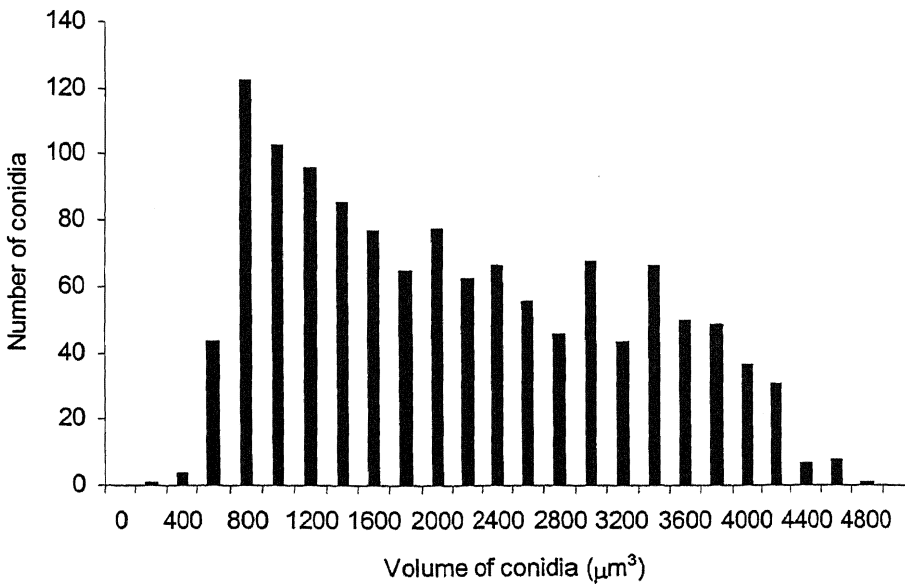


Figure AC13. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 48 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

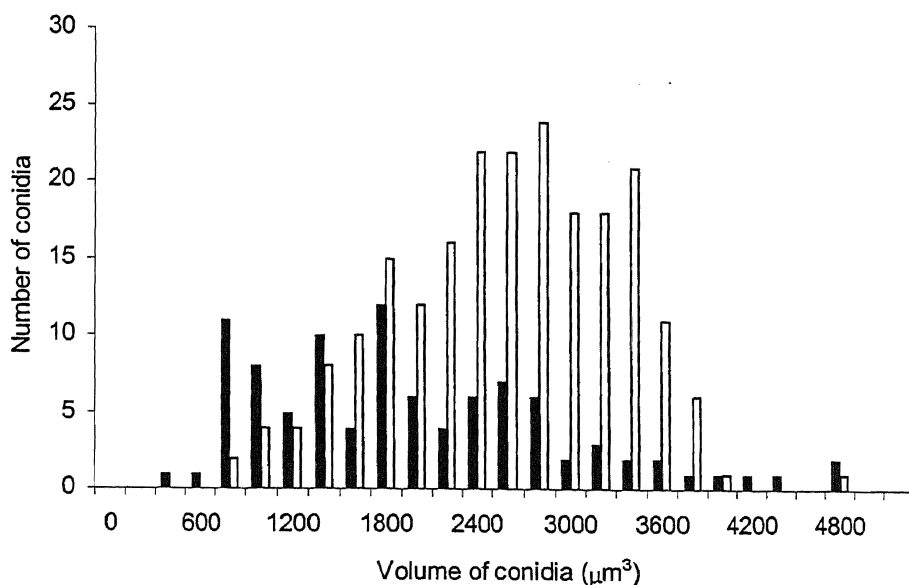


Figure AC14. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 48 h after fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

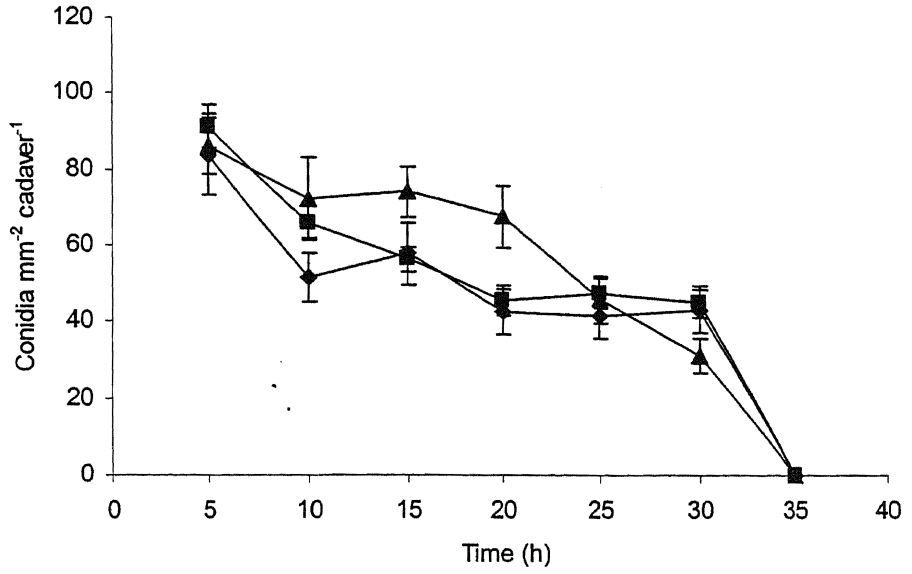


Figure AC15. Comparison of discharge of conidia from aphids treated with fomasefen (◆), glyphosate(■) and fluazifop-p-butyl (▲) 72 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment. Error bars represent the standard errors of the means of 10 replicates.

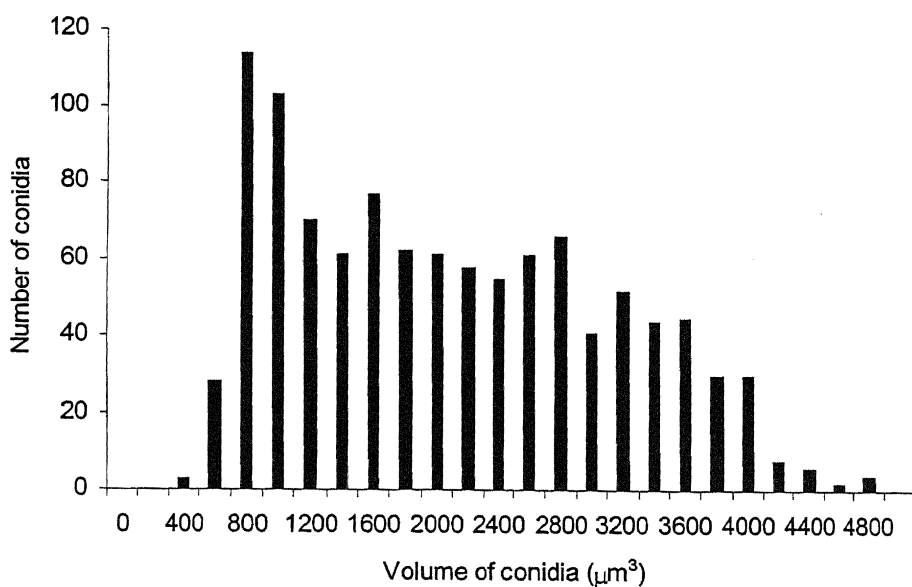


Figure AC16. Frequency distribution of the volumes of conidia produced from aphids treated with glyphosate 72 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

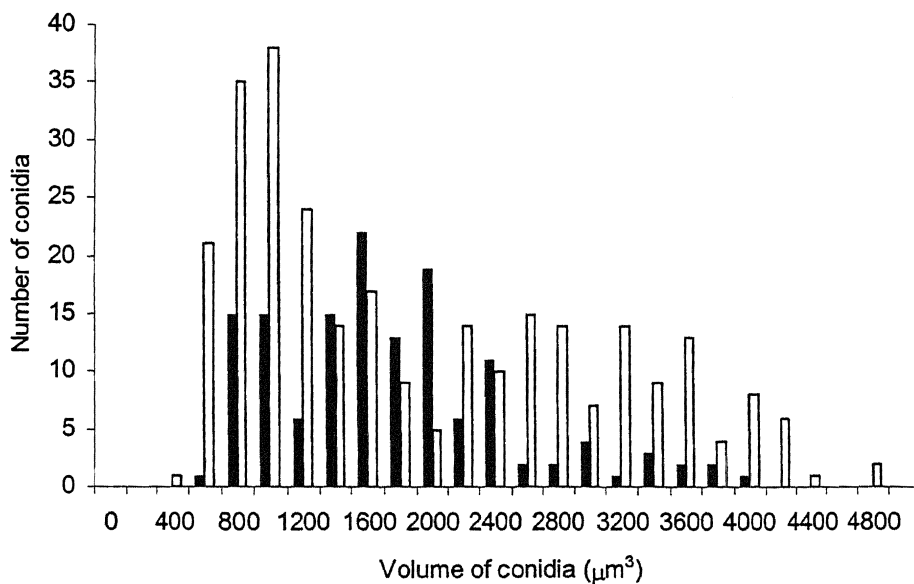


Figure AC17. Frequency distribution of the volumes of conidia produced from aphids treated with glyphosate 72 h after fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

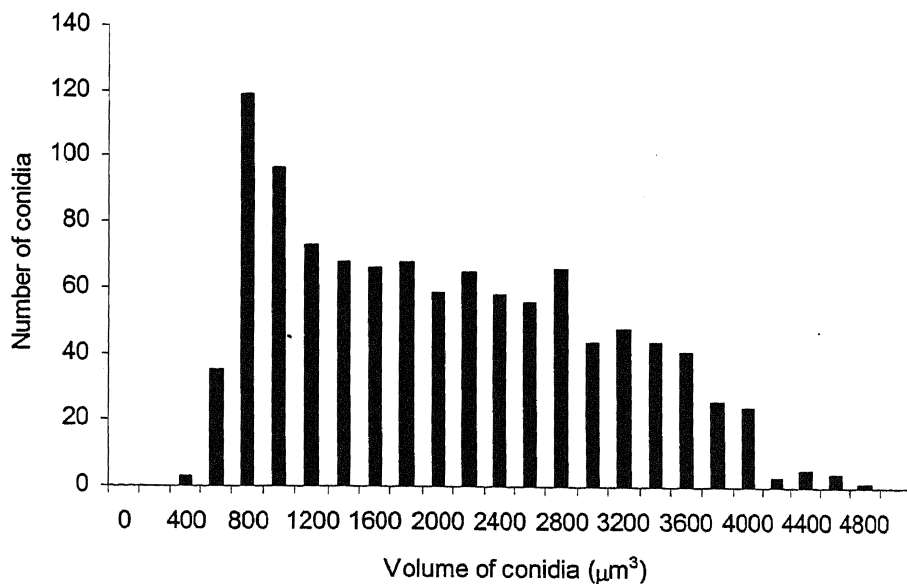


Figure AC18. Frequency distribution of the volumes of conidia produced from aphids treated with fomasefen 72 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

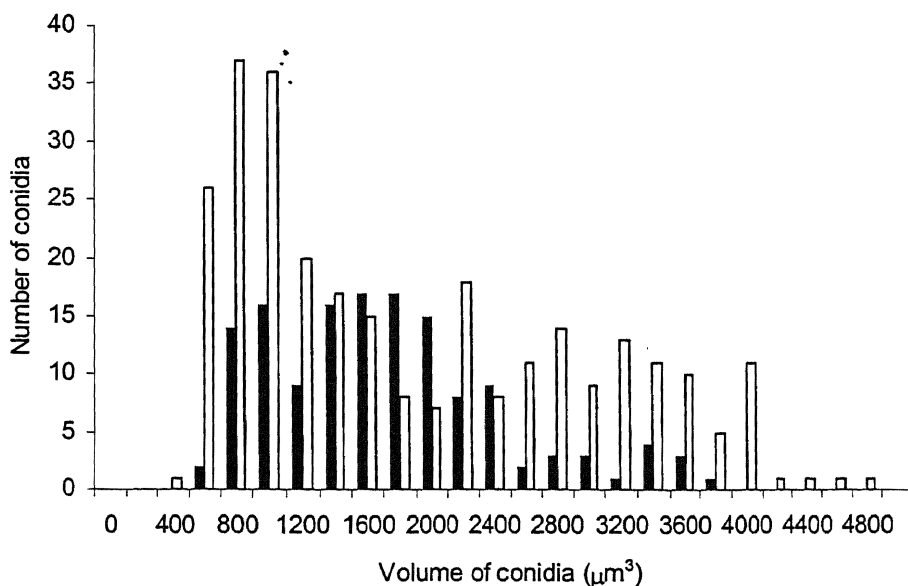


Figure AC19. Frequency distribution of the volumes of conidia produced from aphids treated with fomasefen 72 h after fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.

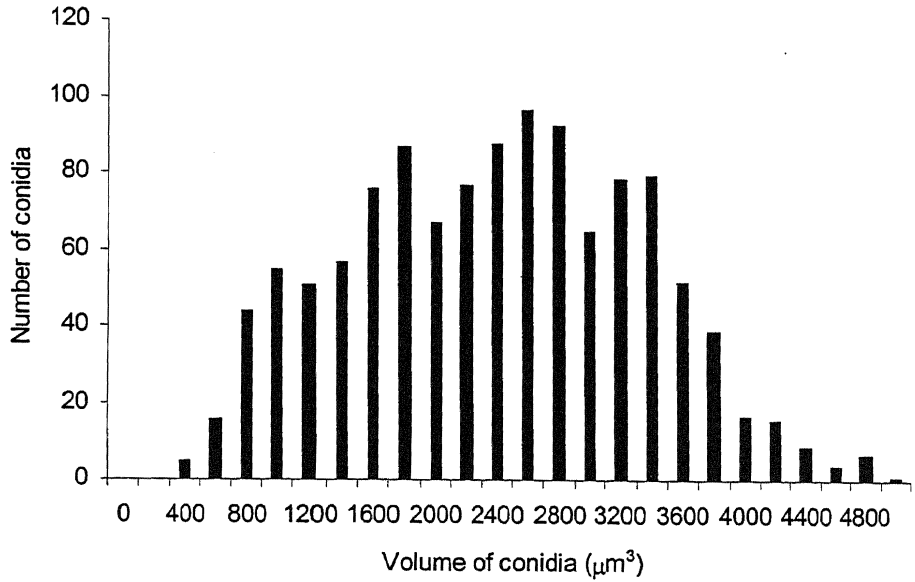


Figure AC20. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 72 h after fungal inoculation. Discharge was measured at 100% RH, 20°C with 10 replicates per treatment

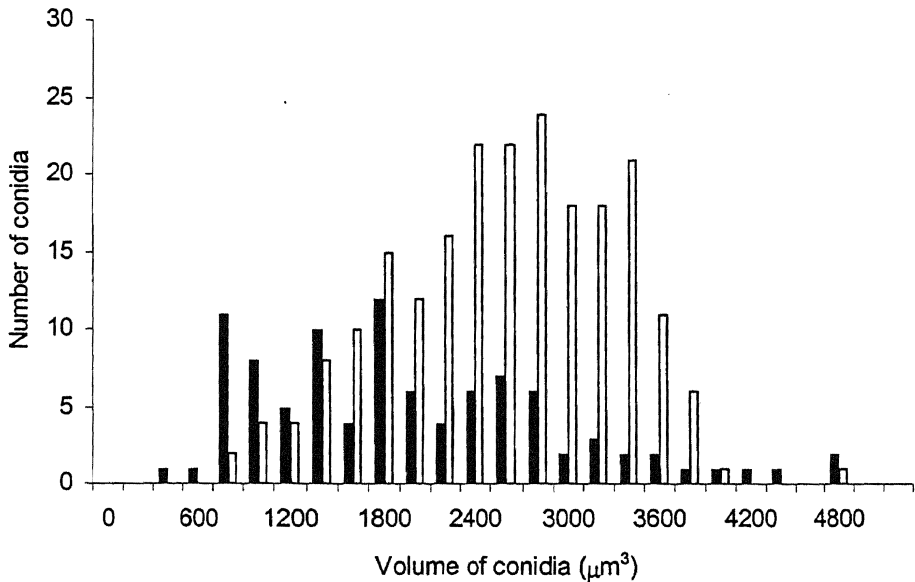


Figure AC21. Frequency distribution of the volumes of conidia produced from aphids treated with fluazifop-p-butyl 72 h after fungal inoculation after 5 h incubation (■) and 30 hours incubation (□). Discharge was measured at 100% RH, 20°C with 10 replicates per treatment.



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