Title Optimisation of Spore Production by the Potential Fungal Biocontrol Agent for Aphids, *Erynia Neoaphidis*

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OPTIMISATION OF SPORE PRODUCTION BY THE POTENTIAL FUNGAL BIOCONTROL AGENT FOR APHIDS, *ERYNIA NEOAPHIDIS*

By

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ABSTRACT

The optimisation of spore production by the potential fungal biological control agent for aphids, *Erynia neoaphidis* Remaudiere and Hennebert (Zygomycetes: Entomophthoraceae) was studied. The fungus was able to grow in semi-defined *Erynia* medium (SDEM) containing glucose, yeast extract, mycological peptone, and 0.02% oleic acid buffered to a pH 6. Oleic acid was fungicidal at 0.1% (v/v) while 0.02% (v/v) oleic acid was the optimum for radial growth. Plugs cut 5-10 mm from the margin of a colony produced more conidia than plugs cut 13- 20 mm from the colony margin. Renewed growth continued through two subcultures on solid SDEM lacking yeast extract (SDEMLYE), and SDEM lacking mycological peptone (SDEMLMP). The continued growth was attributed to the carry over of nutrient in the inoculum. Growth was supported on SDEMNH_4SO_4 when ammonium sulphate was used as the nitrogen source instead of mycological peptone suggesting that the fungus could obtain the growth factors it required from yeast extract. When chitin was added to SDEM in insoluble powder form instead of glucose (SDEMC1 & SDEMC2), the absence of a clearing zone around the developing colony suggested that chitin was not metabolised by *E. neoaphidis*. Biomass grown on SEMA and on SDEMDG (containing double the original concentration of glucose 32gl^{-1}), resulted in production of fewer conidia of larger volume compared to SDEMDMP containing double and half the original concentration of mycological peptone (SDEMHP), SDEM containing half the original concentration of glucose (SDEMHG). Increasing

the glucose to double the original concentration resulted to an increase in biomass. *Erynia neoaphidis* grown on aphid cadavers produced many, smaller conidia. Mycelial mats harvested from biomass grown in fed-batch liquid fermenter culture in SDEMDG at the end of the exponential phase and placed on water agar discharged conidia at a rate of 6,700 conidia mm^{-2} h^{-1}which persisted for approximately 3 days

When *E. neoaphidis* was subcultured onto SDEM from SEMA medium, the colony growth rate increased on the second subculture on SDEM where more lipases and aminopeptidases were detected at higher concentrations using the API ZYM system. This shows that attenuation might have taken place by either a phenotypic or genotypic (eg mutation) change or both when *E. neoaphidis* was grown on SDEM from SEMA medium. Growth in GASP medium resulted in the production of more biomass and a delay in the onset of decline phase compared to cultures grown in SDEM. Fewer enzymes were detected at a lower concentration in cultures grown in GASP compared to cultures grown in SDEM, this difference might be more likely to relate to the balance of nutrients and the fact that GASP medium is more similar in composition to the nutrients found in the haemocoel of an aphid.

Based on this research. It is recommend that *E. neoaphidis* be grown in SDEM liquid cultures containing 32 gl^{-1} glucose instead of 16 gl^{-1} glucose. Biomass for field applications should be harvested at the end of the exponential growth and mycelial mats made. The mycelial mats should be maintained at high relative humidity and can be expected to discharge conidia for 3 days.

DEDICATION

In Memory of my grand dads, George Mukiibi and Pastor Kigozi

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LIST OF ABBREVIATIONS

1. SDEM- Semi defined *Erynia* medium
2. SEMA- Sabourand dextrose egg milk agar medium
3. Kr - Colonial radial growth rate
4. SDEMDG- Semi defined *Erynia* medium with 32 gl^{-1}glucose
5. SDEMHG- Semi defined *Erynia* medium with 8 gl^{-1}glucose
6. SDEMDMP- Semi defined *Erynia* medium with 10 gl^{-1}mycological peptone
7. SDEMHMP- Semi defined *Erynia* medium with 2.5 gl^{-1}mycological peptone
8. SDEMC1- Semi defined *Erynia* medium with 15 gl^{-1}chitin
9. SDEMC2- Semi defined *Erynia* medium with 7. 5 gl^{-1} chitin
10. SDEMLG1- Semi defined *Erynia* medium without glucose
11. SDEMLG2- Semi defined *Erynia* medium without glucose again
12. SDEMLY1- Semi defined *Erynia* medium without yeast extract
13. SDEMLY2- Semi defined *Erynia* medium without yeast extract again
14. SDEMLMP1- Semi defined *Erynia* medium without mycological peptone
15. SDEMLMP2- Semi defined *Erynia* medium without mycological peptone again
16. SDEMNH_4SO_4 -Semi defined *Erynia* medium with ammonium sulphate replacing the amount of nitrogen in mycological peptone
17. BMMA- basic milk medium agar
18. YEGMP- yeast extract, glucose, mycological peptone and 0.1% oleic acid medium
19. YEAG- yeast extract agar and glucose medium
20. GASP- Grace's insect tissue culture medium, asparagine and 0.1% oleic acid

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Thanks must also go to my boyfriend Chris Jayeola and my parents Solomon and Molly Mukiibi. Also my sister Joanne Mukiibi and brother Joshua Mukiibi for supporting me through my studies.

AUTHOR'S DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Masters of Science by Research at the University of Luton. It has not been submitted before for any degree or examination in any other University.

NAME: JOY LOIS NALWEYISO MUKIIBI

SIGNATURE: 

DATE: 20th day of February, 2003

1. INTRODUCTION

1.1 APHIDS AS PESTS AND THEIR CONTROL

1.1. 1 The aphid pest.

Aphids belong to the order Hemiptera, suborder Homoptera and the family Aphididae. Aphids are considered to be the most important insect pests in many parts of the world, especially in the temperate zones, because of the substantial damage they cause to crops (Dixon, 1995; Milner, 1997). In Britain alone, the potential economic loss attributed to aphids for damage to potatoes and sugar beet in 1986 was calculated at around $36 million (Tatchell, 1989).

Aphids attack a wide range of crops including cereals, legumes, cereals, vegetables and fruit crops. Some of the most important aphid species are *Aphis fabae* Scop and *Acyrthosiphon pisum* Harris which infest legumes, *Sitobian avenae* (F) and *Metopolophium dirhodum* (Walker) on cereals and *Aphis gossypii* (Glov) on cotton (Wilding 1981; Wilding *et al,* 1990; Gubran *et al,* 1993; Milner, 1997). Aphids cause damage by direct feeding on the young growing parts of plants such as apical shoots, buds and leaves, causing nutrient loss which may result in stunted growth (Milner, 1997). Due to their high reproduction rate, aphids quickly colonize the host plant resulting in reduced growth and, ultimately, death of the plant. Whilst feeding, aphids pierce plant tissues and may cause crop losses through transmission of plant viruses (Dewar *et al.*, 2001), and other toxic compounds in saliva (Pickett *et al,* 1992). Aphids also secrete a sticky substance called honeydew, which encourages the growth of saprophytic fungi like *Cladosporium spp* and *Penicillium spp.* which appear as sooty moulds blackening the leaf and limiting the leaf area available for photosynthesis (Schepers, 1989).

1.1.2 Chemical control

Chemical pesticides have been used since the early 1940's to control insects that are either harmful to food crops or transmit human diseases. The use of chemical pesticides has enabled food production to increase by killing pests that were a major

constraint on food production. However, the increased usage of pesticides has led to problems.

1.1.2.1 Effects of chemical insecticides on non-target organisms.

Many chemical insecticides have a broad spectrum of action and therefore kill beneficial insects that serve in the recycling of organic materials that increase soil fertility. Furthermore, some of the insects killed are natural enemies of the target pest, which are often more sensitive to the pesticide than the pest itself (Van Driesche and Bellows, 1996; Schepers, 1989). For example in the Netherlands, about 50% of the pesticides used during the early 1990's were soil sterilants which killed both beneficial organisms like nematodes and disease organisms, targeted pests and weed seeds (Matteron, 1995). Furthermore, when insecticides including malathion, fenetrothion and diflubenzon were applied in the Sahel region of North Africa to control locust epidemics, the acaricides killed entomophagous arthropods together with the locusts (Murphy *et al,* 1994). A phenomenon that arises from the use of broad-spectrum chemical pesticides is pest resurgence, which is the rebound of pests in an area where natural enemies have been damaged by chemical pesticides. The pests that have survived the toxicity of the chemical pesticides reproduce quickly and since there are no natural enemies to control them, the rate of growth of the pest population is greater than the natural enemy population, and the density of the pest quickly returns to or even exceeds levels found before the chemical pesticide was applied. The use of broad-spectrum pesticides also results in secondary pest outbreaks. This occurs when herbivorous species which were originally not significant pests, multiply and spread eventually becoming pests, since the natural enemies which were controlling them were killed (Van Driesche and Bellows, 1996; Lacey *et al,* 2001).

1.1.2.2 Development of insect resistance to chemical control

Extensive use of chemical pesticides has led to the development of resistance by pests. Resistance in an insect population can be defined as a reduction in the degree of control given by the pesticide compared to what is expected, when factors like application problems, extreme temperatures and many others are not included or implicated. Resistance occurs when a pest alters a target site in its body or

enhances its ability to metabolise toxins, or change its behaviour to avoid exposure to the pesticide (Soper and Ward 1981; Clarke *et al,* 1997). For example, heavy reliance on acaricides to control the cattle tick *Boophilus microplus* (Corrales) San Felipe, which causes substantial loss to cattle through toxicosis (accumulation of toxins produced by the tick inside the cattle), anaemia and physical damage to the hide, led to the development of resistance by the tick to acaricides (Baxter, 1997). Some strains of aphids have developed distinct types of resistance to carbamate insecticides, for instance, the peach potato aphid *Myzus persicae* Sulzer over produces one of two closely related carboxylesterases (E4 and FE4) that degrade the insecticide esters before they reach their target sites in the nervous system of the aphid. Another mechanism of resistance which has arisen in aphids, is development of a modified acetylcholinesterase which confers resistance to dimethyl carbamates, pirimicarb and triazamate (Clarke *et al,* 1997; Foster *et al,* 2000).

1.1.2.3 Effects of chemical insecticides on the environment

The excessive use of chemicals has also become a threat to the environment. The fumigant methyl bromide contributes to ozone depletion. Residues of chemical pesticides accumulate in the soil, water and air. For example, organochloride residues from pesticides that are not normally found in nature tend to accumulate in food chains as a result of their relative biochemical stability (Levitan, 2000). When eaten they are very harmful to the body since the residues are carcinogenic (Winter, 1996: Lomer *et al,* 2001). Winter (1996) reported that food eaten by children containing pesticide toxins could be detrimental to their bodies since the nervous and immune systems have not developed fully to metabolise and eliminate pesticides.

In third world countries, due to poor education, some farmers do not understand the toxicity of the chemical pesticide and as a result do not wear protective clothing while handling chemicals leading to accidental poisoning and contamination (Van Driesche and Bellows, 1996).

1.1.2.4 Rationale for seeking alternatives to chemical insecticides

There is growing public demand for agricultural products that contain little or no chemical residue; this has led to retailers and food companies pressurising farmers to use crop-protection agents that are acceptable to consumers (Rodgers, 1993). Furthermore, the cost of buying pesticides to control pests is sometimes greater than the actual cost of pest damage on crops and animals making chemical control financially non viable (Levitan, 2000).

Governments have developed screening methods that enable regulatory agencies or environmental agencies, to assess and evaluate pesticide risks over a period of time or compare risks of different pesticides (Levitan, 2000). In the UK the Insecticide Resistance Action Group (IRAG) was formed in 1997 to introduce anti-resistance strategies in pesticides. The Home-Grown Cereals Authority (HGCA) publishes guidelines on how to use pesticides (Clarke *et al,* 1997). The formation of The 1996 Food Quality Protection Act influenced the development and registration of chemical pesticides (Lacey *et al.*, 2001). Some farmers and farming companies have turned to growing organic food in glasshouses where the crops are grown on imported chemical free soil to avoid the problems of toxins from soils that are contaminated with insecticides.

Due to problems regarding the use of chemical pesticides, the demand for other forms of pest control, such as biological control is increasing (Van Driesche and Bellows, 1996; Roy, 1997; Clarke *et al,* 1997).

1.1.3 Biological control

Biological control is a population- level process in which populations of one species reduce the populations of another species by mechanisms such as predation, parasitism and competition (Van Driesche and Bellows, 1996).

1.1.3.1 Methods of microbial control

Conservation- is where a pathogen is conserved in an environment where it already exists and continues to control pests (Lacey *et al.*, 2001). Conservation can be attained by reducing the amount of chemical insecticides used in that area, by

increasing the moisture levels in the vegetation by irrigating the land. Providing overwintering sites like plants and hosts where the pathogen can survive during the periods when the host or pest is absent. Some examples where conservation is being used are; *Neozygites fresenii* (Nowakowski) is used to control cotton aphids in U. S. A. in this case the farmers send aphids to a diagnosis service which identifies the presence of *N. fresenii* in cotton aphids, this information can then be used by the farmers whether to use insecticides or not. If 15% of the aphids are infected, then there will be a decline of aphids within a week, if 50% of the aphids are infected, then aphid numbers will decline within a few days. At Rothamsted Research station in Harpenden, field margins are being provided for *E. neoaphidis* and *Conidiobolus obscurus* by planting wild flowers and grasses. The wheat fields are sampled to identify potentially useful combination of plants and aphids (Shah and Pell, in press).

Classical control -is where a pathogen is introduced in an area to control a pest which is exotic. Surveys are made in the origin of this pest to identify a pathogen that can control the pest, this type of control has lagged behind because of regulatory restrictions on their introduction in new areas. This may include importing a pathogen from another area, country or even continent. For example, *Entomophaga maimaga* (Humber) was used to suppress populations of the gypsy moth, *Lymantria dispar* (L.) in a long term. *Zoophthora radicans* was introduced in Australia from Israel to control the spotted alfalfa aphid, the fungus was distributed as living or dead infected aphids and from sporulating cultures growing on plates (Van Driesche and Bellows, 1996; Lacey *et al.* 2001; Shah and Pell, in press).

Inoculative augmentation – is where a pathogen is produced in smaller quantities in laboratories or factories and applied to an area where the pathogen might be already in small amounts to produce long-term regulation of the pest through repeated cycle thus long-term control of the pest is established (Pell *et al*, 2001 & Lacey *et al*, 2001). Conidia of *Z. radicans* with semiochemicals (pheromones) are put in a trap, and the semiochemicals used to attract the diamondback moth, *Plutella xylostella.* Semiochemicals are chemicals that are produced by insects in response to their environment. An example of semiochemicals, are pheromones which are produced by insects or species in response to their environment. The diamondback moths that

have entered the trap get dusted with the conidia and thus spread the conidia as the flies fly and get in contact with other flies or insects (Shah and Pell, in press)

Inundative augmentation -is where a pathogen is produced in large quantities to be applied as a mycoinsecticide for rapid short-term control (Pell *et al*, 2001 & Lacey *et al*, 2001). *Metarhizium anisopliae* (Metschnikoff) Sorokin was produced in large quantities and used successfully to control the locust and grasshoppers in Africa. In USA, its produced in a solid state fermenter system that mass produces it as aerial conidia. Several products have been manufactured in the USA, some of these are; "Mycotrol" which is used to control aphids, thrips and grasshoppers, while "BotaniGard" is used in glasshouses and "Mycotrol O" which is used in farms that produce organic foods (Wraight *et al*, 2001). In Europe, *Veriticillium lecanii* is used against aphids as an innudative mycoinsecticide. In Netherlands, *V. lecanii* manufactured by Koppert Biological Systems in the Netherlands in two products which contains different isolates as their active ingredients. For example, "Vertalec" is used against aphids whereas "Mycotal" is used against whiteflies and thrips (Shah and Pell, in press).

A biological control agent is a pathogen used to control pests and when it is produced in large amounts, it is called a biopesticide. If it is a fungal, bacterial or viral product, then it is called a mycoinsecticide.

1.1.3.2 Advantages of biological control agents.

Using biological control agents reduces exposure to potentially toxic pesticides by the producer, applier and consumer. Biological control agents have no harmful residues and therefore pose an extremely low risk of environmental pollution when used. For example, various fungi such as *B. bassiana, M. anisopliae* and *Verticillum lecanii* (Zimm) Viegas that have been developed for commercial use as biological control agents have shown no infectivity to man or other vertebrates. Effects such as occasional allergies may occur, but these are of minor significance compared to the adverse effects of chemical pesticides (Van Lenteren, 1992; Van Driesche and Bellows, 1996).

Furthermore, there are no phytotoxic effects on young plants or premature abortion of fruits and flowers when biological control is used (Van Lenteren, 1992). The use of natural enemies results in increased biodiversity in the ecosystem and increased activity of natural enemies (Lacey *et al,* 2001). Compared to chemical pesticides, biological control agents are less likely to lead to pest-resurgence and also can be genetically manipulated (Rodgers, 1993) to have better qualities such as being mass-produced cheaply, being more pathogenic to pests, having a fast rate of kill and persisting in the environment. Since biological control agents do not kill non-target insects, secondary pest outbreaks are controlled, recycling organic materials is maintained thus increasing soil fertility.

1.1.3.3 Disadvantages of biological control agents

The activity of biological control agents, particularly microbial control agents are dependent on environmental conditions such as humidity, temperature, ultraviolet radiation and pH which cannot be readily controlled in the field. As a result, their action can be inconsistent when these factors change. Due to these problems, many biological control agents have not yet met the expectations of modern agriculture where farmers want predictable and consistent results of pest control (Van Lenteren, 1992; Rogers, 1993).

The viability of biopesticides may be hard to maintain during storage under ambient conditions. The biofungicide 'Dagger G' based on a strain of *Pseudomonas fluorescens* (Treciscan) Migula was removed from the market due to an unsatisfactory profit margin caused by its short shelf-life (Rogers, 1993).

There may be difficulties in using biological control agents on a large scale due to production and application problems. Developing a biopesticide on a scale that allows cost-effective manufacture is difficult for biopesticides that cannot be produced by liquid fermentation. This problem is common with insecticidal viruses that are currently produced *in vivo* (Rodgers, 1993). Another disadvantage of biological control agents is that some of them are not compatible with other pesticides. For example, *V. lecanii*, which is used to control the glasshouse whitefly, *Trialeurodes vaporariorum* (Westw) is not compatible with the fungicides tolyfluanid

and dichlofluanid (Rodgers, 1993). Some biological control agents act very slowly allowing the pest to cause economic damage prior to control being achieved (Robinson, 1987; Van Lenteren, 1992; Van & Jackson, 1998, Hemmati, 1998).

Although the high specificity of biological control agents is beneficial, this characteristic is perceived as a disadvantage because it limits the markets for the product as compared to a product with a broad spectrum. Furthermore, many biopesticides are only effective against particular stages in the development of a pest or disease organism, so if the pest is not at the stage at which it is susceptible to a biocontrol agent, then the pest will not be controlled. For example, *Verticillium chlamydosporium* Goddard is only pathogenic to nematodes at the egg stage (Rodgers, 1993), therefore limiting its use.

1.2 MICROBIAL CONTROL

Microbial control is biological control involving the utilization of microorganisms, including viruses (Steinhaus, 1956).

1.2.1 History of microbial control

Invertebrate pathology which is the study of invertebrate diseases provides the scientific foundation for microbial control of insects (Steinhaus, 1956). The use of micro-organisms to control pests was first developed by Bassi who used liquids from putrefied cadavers of diseased insects mixed with water and sprayed onto foliage to prevent disease in honey bees and silkworm. Metchnikoff the practical founder of the doctrine of microbial control of pests, mass produced spores of *M. anisopliae* and used them in the field against larvae of the sugar beetle (Steinhaus 1956). However the use of microbes for pest control did not become widespread until *Bacillus thuringiensis* Berliner was developed (Lacey *et al,* 2001).

1.2.2 Qualities of a good microbial agent

In order for a microbial control agent to be commercially and economically viable, it should ideally possess the following characteristics;

- It should have the ability to spread through a pest population and persist in the pest's environment.
- It should be able to search out individuals of the insect population and limit pest numbers below the level at which the pest causes damages to the crop or animal.
- It must be possible for it to be produced readily in large amounts at low cost and should maintain strain virulence through repeated *in vitro* subcultures.
- It should not destroy other natural control agents and must be easy to store and apply to crops.
- It should have a good moisture tolerance and not be overly sensitive to ultra violet rays (Robinson, 1987).

It should be noted that none of the currently available agents have all the above characteristics. Some of the characteristics are not relevant to all strategies used for biocontrol. For example, spreading of the pathogen in the pest population is less important for an inudative mycoinsecticide because it is produced in large amounts but very important for an inoculative release since the pathogen is produced in small quantities.

Fungi have very good contact activity with the pest, and a have a relatively fast speed of action once they have come in contact with the organism (Jutsum, 1988). If conidia were on leaves, they would not infect aphids since aphids are sucking pests and do not consume the leaves. Thus aphids need contact with the organism in order to infected.

Some of the existing commercially available fungal biocontrol products used are:
(a). SoilGard 12G, a granular formulation containing *Gliocladium virens* that controls soil-borne diseases caused by *Pythium* and *Fusarium*,
(b). PFR-97 20% WDG containing *Paecilomyces fumosoroseus* (Wize) that controls thrips, aphids and whiteflies,
(c). Naturalis containing *B. bassiana* in an oil-based emulsifiable suspension, used to control thrips, aphids and whiteflies,
(d). BIO 1020 contains mycelium of *M. anisopliae* in a granular form used to control Black vine weevils (Wraight *et al*, 2001).

1.2.3 Entomopathogenic fungi

Fungal diseases are very common in insect populations. All insect orders are susceptible to fungal disease. Entomopathogenic fungi infect insects living in diverse habitats including freshwater, soil surfaces and aerial locations. All fungi are affected by pH and moisture levels, but the optimal differs between species. Temperature also affects the germination rate of fungi. Most Entomopathogenic fungi need a temperature between 15 °C to 25 °C for optimum growth. The texture of the insect's cuticle also affects the rate of germination of an organism because cuticles have or contain potent resistance factors such as enzymes that need to be broken down or modified for the germ tube to penetrate through into the insect's body (Hassan *et al*, 1989).

The most common route of host invasion by fungus is through the external integument by the conidium (infective unit). It attaches to the cuticle, germinates then forms an appresorium and penetrates by either enzymatic or mechanical mechanisms or both. The appresorium enhances penetration by putting pressure on the cuticle and thus break through. The site of infection depends on the entomopathogenic fungus, target insect and the conditions. Sometimes infection is through the digestive tract, wounds and trachea (Lacey *et al*, 1997). Once in the haemocoel, the germ tubes produce mycelium that fragments into hyphae invading the whole insect's body causing physical obstruction of blood circulation. The host dies due to a combination of the action of nutrient depletion and invasion of organs (Butt *et al*, 1990). After the host dies, sporulation occurs and conidia are produced under appropriate conditions of humidity and temperature (Lacey *et al*, 1997).

1.2.4 Entomophthorales

1.2.4.1 Characteristics of Entomophthorales

Entomopathogens are known for causing epizootics in many insect populations, principally Homoptera, Lepidoptera, Orthoptera and Diptera. The most outstanding characteristic of Entomophthorales is that conidia are forcibly discharged from the conidiophores, which develop at the host surface from hyphal bodies

penetrating and elongating through the cuticle. This is the case for most entomopathogenic Entomophthorales, but not for the species of the genus *Massospora* (Pell *et al*, 2001). Most Entomophthorales have two further biological characteristics, (i) the fungus multiplies as protoplasts or hyphal bodies (having a cell wall) or both, after having invaded the host, (ii) Most hyphal bodies produced by Entomophthoralean fungi with an exception of a few species like *E. neoaphidis*, can develop into thick-walled resting spores inside or more rarely outside the cadaver (Papierok and Hajek, 1997).

Many Entomophthorales infect members of only one family or order of insects, a feature that is a strong advantage for potential biological control agents (Wilding, 1981). For example, *E. neoaphidis* infects only aphids. Therefore, when used for pest management, entomophthoralean fungi are unlikely to constitute a risk to beneficial insects and other non-target organisms unless those organisms are very closely related to the target pest (Lacey *et al*, 1997).

1.2.4.2 Sporulation of Entomophthorales

Resting spores

Many species of Entomophthorales produce resting spores when faced with adverse conditions like high or low temperatures or absence of the host. Resting spores produced by Entomophthorales are either zygospores, which are formed from the fusion of two hyphae and azygospores which are formed from a hyphal body forming a double wall around itself with apparent lack of sexual fusion. Both types of spores require a period of dormancy before germination. When favourable conditions occur the resting spores produce germ tubes and germ conidia. Some examples of fungi that produce resting spores are; *Entomophaga grylli* (Fres) Batko and *Entomophaga calopteni* (Bessy) Humber (azygospores). *Neozygites fresenii*, Remaudiere et Keller, *Entomopthora muscae* (Cohn) Fres and *Conidiobolus lamprauges* (Drechsler) (zygospores). *Erynia neoaphidis* does not produce resting spores (Balazy, 1993). Some fungi produce the single-walled chlamydospores which are non-sexual infective propagules.

Conidia.

Sporulation occurs in cultures growing on both artificial media and from infected hosts. Conidia, which are the infective propagule, are produced, thus initiating epizootics or spreading infection. The rate of sporulation and germination of conidia are dependent on temperature. For *E. neoaphidis* the optimum temperature for good sporulation and germination is between 18 ^{0}C and 21 ^{0}C (Morgan *et al*, 1995). Conidia of *E. neoaphidis* are fragile and have a mucous coating which enables the conidia to adhere to the host (Pell *et al*, 2001).

The shape, size, colour and nuclear number of the primary conidia, and the nature of primary conidiophores, are taxonomic features used to distinguish between species within the Entomophthorales. With the exception of the non-discharging conidial apparatus in *Massopora*, the primary conidia of the Entomophthorales are formed and discharged towards the strongest source of light by the bursting of a conidiophore. (King and Humber, 1981)

A primary conidium in the absence of a host, may discharge a secondary conidium by the bursting of a conidiophore. Secondary conidia may be discharged by papillar eversion to leave ghost like structures, which are the empty shells of primary conidia, which have germinated to form secondary conidia. A secondary conidium may germinate to produce a tertiary conidium, similar to the secondary conidium and this process may be repeated until the energy reserves of the conidium are depleted (King and Humber, 1981).

1.3 *ERYNIA NEOAPHIDIS*

The occurrence of the entomophthoralean fungus *E. neoaphidis* has been recorded in Europe, Africa and Australia (Wilding and Brady, 1984; Glare and Milner, 1991). *Erynia neoaphidis* has a number of characteristics that make it successful as a pathogen of aphids. These include rapid sporulation and germination, which enables the infection process to be completed in a few hours.

Erynia neoaphidis actively discharges conidia so that the dispersion of conidia is maximized. Since the conidia produced are highly virulent, in theory only a small

number of conidia are needed to infect an aphid (Milner, 1997). A number of authors have recorded epizootics of *E. neoaphidis* in different species of aphids. Wilding and Perry,(1980) recorded epizootics of *E. neoaphidis* in populations of *A. fabae*, Hatting *et al.*, (1999) also recorded epizootics in populations of *M. dirhodum*. Latgé *et al*, 1983 reported epizootics in populations of aphids in a green house

1.3.1 Life cycle

The life cycle of *E. neoaphidis* is shown in Figure 1. Primary conidia of *E. neoaphidis* are lemon shaped while secondary conidia can either be lemon or more rounded in shape. Like most Entomophthorales, on landing on the host cuticle, a primary conidium will either form a secondary conidium, or a germ tube, which then forms an appresorium which penetrates the cuticle. Once the fungus has breached the host's cuticle, the hyphae fragments into protoplasts that colonize the tissues within the haemocoel.

As the disease progresses, infected pea aphids become sluggish and change color from green to yellow and eventually brown. At this stage of infection, aphids are normally packed with randomly oriented protoplasts that rapidly regenerate a wall (within 12-24h), and are then referred to as hyphal bodies (Butt *et al*, 1990). The insect host is usually dead by the time the fungus emerges but some active insects have been observed at this stage (Humber, 1975). Rhizoids, which are the first structures to emerge from the aphid body, adhere to the plant and fix the cadavers in position. Pseudocystidia, which are sterile hyphae, protrude beyond the layer of conidiophores, cover most parts of the insect except the midventral region. The role of pseudocystidia is not exactly known but it might be to breach the cuticle, facilitating the emergence of conidiophores and also trapping a layer of moist air over the body of the infected insect and so prolong the period during which conidia are discharged (Butt *et al*, 1990). Once the conidiophores have emerged through the host cuticle they form conidia at their apices thus completing the cycle (Robinson, 1987). After death of the aphid, sporulation starts within two hours and most conidia are produced within 24 hours at 20^0 C (Glare and Milner, 1991).

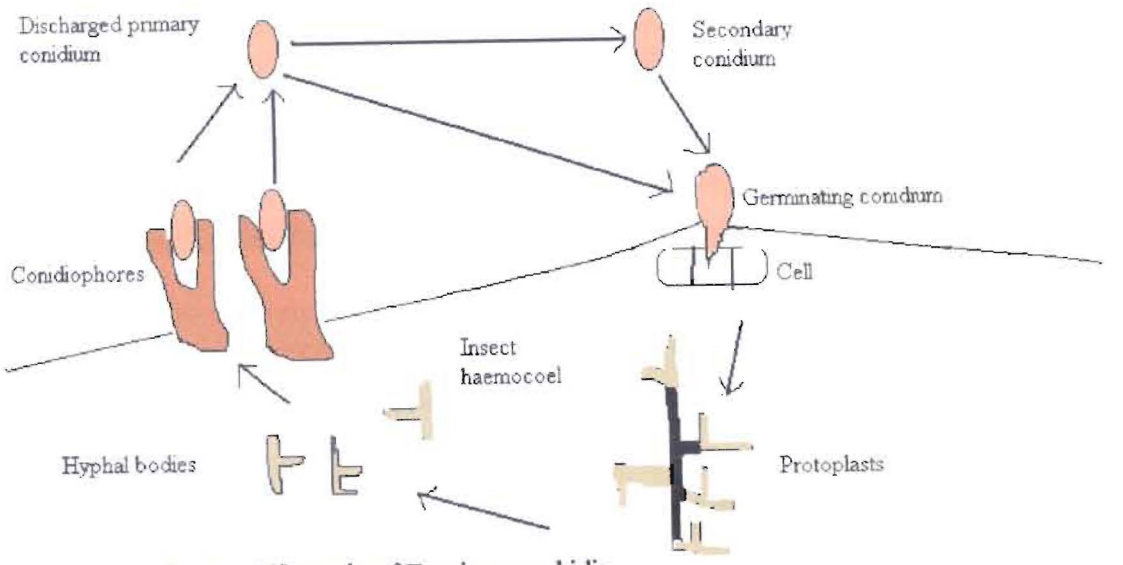


Figure 1 Life cycle of Erynia neoaphidis

1.3.2 Classification

Many controversies have surrounded the taxonomy of the Entomophthorales, primarily due to many name changes of genera resulting from misclassification of the Entomophthorales on the basis of structural features.

Batko classified Entomophthorales on the basis of homology of the nuclei in the primary conidia and the absence of rhizoids and conidiophores (Humber, 1981). He grouped about 150 species into one singe genus *Entomophthora*. Subsequent studies have reclassified this. Remaudiere (1980) reclassified the Entomophthorales by putting more emphasis on the shape of the primary conidia.
Humber (1981) contested Remaudiere's classification putting more emphasis on cytology. He regrouped the subdivision *Erynia* into three subgenera, *Zoophthora*, *Erynia* and *Pandora*.

At one time *Zoopthora aphidis*, *Erynia neoaphidis* and *Entomophthora aphidis* were regarded as the same species which led to a confusion over whether *Erynia neoaphidis* produced resting spores since *Zoopthora aphidis* produced resting spores while *Erynia neoaphidis* does not (Balazy, 1993).

As a result of this debate, different authors have assigned different names to *Erynia neoaphidis*. For example, Keller (1991) classified it as *Erynia neoaphidis*, Humber (1981), as *Pandora neoaphidis* while Balazy (1993) classified it as *Zoophthora neoaphidis*. In this study the species will be referred to as *Erynia neoaphidis*.

1.3.3 Occurrence and Epizootiology in crop ecosystems

Epizootics of *E. neoaphidis* are very common in populations of aphids in the field especially when aphid populations are large. Natural epizootics are variable from year to year and from crop to crop from one pathogenic species to another (Glare and Milner, 1991). A number of factors affect epizootic of *E. neoaphidis*.

Temperature affects the type of secondary conidia produced and the rate of germination for both primary and secondary conidia. At a temperature of 10 ^{0}C and below, lemon –shaped conidia are produced, while at 20 ^{0}C, more rounded conidia are produced. The optimal temperature for germination of conidia is between 18 °C and 21 °C (Morgan *et al*, 1995), while for sporulation the temperature is 10 ^{0}C to 20 ^{0}C (Shah *et al*, 2002). Hemmati *et al*, (2001) reported that at a temperature of 18 ^{0}C, conidia were discharged the furthest at a maximum height of 8 mm at a speed of 8 ms^{-1} from the pea aphid (*Acythosiphon pisum*), the nettle aphid (*Microlophium carnosum*) and the grain aphid (*Sitobian avenae*).

Moisture is a very important environmental factor that influences the spread of *E. neoaphidis*. A relative humidity of 90% and above is required for sporulation of cadavers and spread of the fungus (Wilding, 1972). Hemmati *et al*. (2001) reported that a high number of conidia were discharged from the rose grain aphid *Metopolophium dirhodum*, when the relative humidity was above 89% in the night or in the early morning. Light is another factor that affects the infectivity of spores. Since Furlong and Pell, 1997 found out that after exposure to sunlight, infectivity of conidia of *Z. radicans* was reduced, light also affects the germination rate of conidia of *E. neoaphidis*.

The spread of *E. neoaphidis* is not only affected by environmental factors like temperature, light and moisture, but also affected by the population of the host, the amount of inoculum in the field and predator and parasitoid activity (Soper and Ward, 1981; Wilding, 1981).

1.3.3.1 Host population

An epizootic is dependent upon host distribution and pathogen density because at high host densities, the chances of contact between pests and the biological control agent are increased, thus pathogen transmission is more likely to be successful. Increasing host density also increases host individual stress due to crowding, which in turn makes insects more susceptible to pathogen infection (Yeo, 2000). Aphids aggregate closely, whatever the colony size is and the spread of the fungus may be

affected more by the number of colonies per unit area than by the size of individual colonies (Wilding, 1981; Roy, 1997).

1.3.3.2 Predator and parasitoid activity

Predators and parasitoids may encourage transmission or act as carriers of the conidia of the fungi while feeding and moving through sporulating host populations (Roy *et al,* 2001). For example, levels of infection in pea aphids (*A. pisum*) by *E. neoaphidis* were increased by the presence of a foraging ladybird (Roy *et al,* 1998). Furthermore, studies showed that the presence of a foraging parasitoid increased significantly the level of infection in *P. xylostella* larvae by causing increased movement of the larvae in the vicinity of infected cadavers (Furlong & Pell, 1997)

Parasitoid activity may at times stimulate the production of alarm pheromones in aphids, which may in turn lead to aphids dropping off the plant onto the soil. This will increase the chances of aphids picking up infectious conidia of *E. neoaphidis,* discharged from aphid cadavers on to the soil (Milner, 1997). Furthermore when the infected aphids on the soil climb back up the plant, the chances of spreading infection are increased.

1.3.4 Previous field trials using *Erynia neoaphidis*

Aphids infected with *E. neoaphidis* and *N. fresenii* were introduced into fields in UK (Rothamsted) on several occasions, by cutting a branch of the pea plant on which the infected aphids were reared, then the branch was brushed against the pea plants that were infested with aphids. During the dry season, there was insufficient rain to provide moisture thus the fungus established itself only briefly but failed to spread. When the season was wet, sufficient moisture was provided for the fungus to establish and spread but it was still unable to prevent the number of aphids from increasing. This may have been because the fungal inoculum was in small quantities. Even when the number of aphids was high in the field, the fungi failed to spread. It was suggested that since aphids were introduced first before the infected aphids, the aphids in the field multiplied to high numbers such that it was impossible for the

fungus to spread with sufficient rapidity to control the aphid population (Wilding, 1981).

Furthermore, in Belgium when mycelium of *E. neoaphidis* produced in a fermenter was used to control cereal aphids, the fungus did not control the aphid population, despite the fact that there was enough moisture for sporulation to take place (Latteur and Godefroid, 1982).

This may have been because the mycelium used to produce conidia was not washed, thus it could have contained glucose and nitrogen which could be assimilated by the wheat giving a positive effect to the physiology of the aphids. A more probable reason was that the conidia produced from the mycelium were of limited virulence. However, following application, the introduced fungal strain was re-isolated from dead aphids from the field (Latteur and Godefroid, 1982). Although they could not be sure it was the same isolate, it was presumed it was the same.

In a further study at Rothamsted, *E. neoaphidis* was introduced into a field of beans infested with *A. fabae* in three different forms of inoculum; (1) a branch of a pea plant with living *A. fabae* infected with *E. neoaphidis* was used to brush onto the pea plants that were infested with *A. fabae* in the field (2) a powder form of air dried cadavers killed by *E. neoaphidis* was dusted onto plants that had been already sprayed with water to enhance adhesion and to encourage early conidia production, and (3) a homogenate of propagules of *E. neoaphidis* obtained from cultures grown on Sabouraud dextrose Egg Milk Agar (SEMA) medium was applied in the field by using a broad mouthed pipette and half of the plots were irrigated to maintain humidity levels. Irrigation in dry weather was found to increase the infection of aphids by *E. neoaphidis*. The fungus harvested from solid cultures failed to establish infection which may have been due to the fact that the discharged conidia may have not been virulent, or the discharged conidia might have been too few to initiate infection, or that the discharged conidia might have been less resistant to environmental conditions. The results showed that the fungus killed the aphids but once again did not prevent the aphids from increasing in numbers (Wilding *et al*, 1986).

Wilding *et al,*(1990) introduced *E. neoaphidis*, as infected aphids into two plots (one in Hertfordshire and another in Hampshire) that were infested with cereal aphids *S. avenae* and *M. dirhodum* between 1pm and 2pm. In one of the plots, it rained after application and observations in the morning after the rain clearly showed that the fungus had sporulated on the aphid cadavers. The results showed that there was fungal infection, but it did not reduce the number of aphids, furthermore in one of the plots, the aphids were killed by another fungus, *Entomophthora planchoniana* (Cornu), instead of the introduced fungus *E. neoaphidis*.

Controlling aphid populations using *E. neoaphidis* is difficult because once an adult aphid has been infected with *E. neoaphidis*, it continues producing nymphs that are not infected, until the fungus destroys its immune system and it dies. Some of the nymphs may become infected from the original inoculum and become a source of inoculum. The uninfected nymphs develop and continue multiplying. Thus, the aphid population may continue to expand, despite conditions being favorable for the spread of the fungus (Wilding, 1982).

The above field trials were unsuccessful because there was no standard way of producing, formulating and applying the fungus in the field. In order for *E. neoaphidis* to be used as a successful biological control agent in the field, a consistent approach or method should be used in the fungus production.

1.4 PRODUCTION AND FORMULATION

In order for biocontrol agents to be used successfully in agriculture by conventional means, they should have the characteristics of industrial products. These are; being mass-produced economically, the inoculum having a long shelf and being easily applied in the field using traditional agriculture equipment (Latgé, 1982). Mass production of biocontrol agents and the ability to produce infective propagules on a suitably large scale is very important in inudative microbial control, because the pathogen is being used as a biological insecticide or mycoinsecticide (Jenkins & Goettel, 1997). The quality and quantity of the mass produced materials must be

optimized and the infective propagules should remain virulent during storage (Im *et al,* 1988).

1.4.1 Media development

For fungi to grow well and sporulate they need a source of carbon, nitrogen, oxygen, water, trace amounts of elements like phosphorous and sulphur. Fungi also need additional elements like growth factors which fungi cannot obtain from organic sources (Soper and Ward, 1981).

Cost and simplicity are very important when formulating a medium so that the medium can be manufactured in third world countries where the economy and technological know how is developing or not advanced. The type of medium used depends on the biocontrol agent's intended use. Physical growth conditions need to be considered during the early stages of medium development. Temperature and pH can be screened on solid media while oxygen availability is important in submerged fermentation (Soper and Ward, 1981). Carbon and nitrogen sources should be pure only during medium development because it would be uneconomic for large-scale production since the pure forms are expensive.

Carbon sources like glucose, maltose, sucrose and dextrose are very good sources of carbon while yeast extract is a good source of nitrogen (Im *et al,* 1988). Both nitrogen and carbon are needed in a medium for fungal growth and sporulation. When Trinci and Collinge (1974) grew *Geotrichum candidum* Link ex Pers. and *Mucor racemosus* Fres. on medium that lacked both carbon and nitrogen, chlamydospores which are resistant structures that enable fungi to survive adverse conditions, were produced in the medium. Chlamydospores also appeared in a medium that had both carbon and nitrogen after growth had stopped or on exhaustion of nutrients. Carbon is used in the manufacture of structures like cell walls, hyphae and conidia. It is also used in the structural framework of DNA and RNA (Stryer, 1988). Carbon is also used as a source of energy used in the continual growth of fungus and for sporulation. Thus carbon is essential in the building up of biomass in a medium. Nitrogen is used in protein and enzyme synthesis. (Campbell *et al,* 1978; 1983; Smith and Grula, 1981; Im *et al,* 1988).

The ratio of carbon: nitrogen affects the size, virulence, rate of germination and the number of conidia produced from a medium (Jenkins and Prior, 1993). When there is more carbon in a medium that has all the required nutrients, there is an increase in biomass, which produces a lot of conidia that germinate very fast and adhere better to the cuticle of pests a characteristic making them more virulent compared to the conidia discharged from biomass with low carbon concentrations (Jackson and Schisler,1992). When the amount of carbon is excess in a medium, the carbon is stored up as glycogen which is used as energy to grow in adverse conditions like nutrient depletion and harsh environmental conditions like drought. The stored carbon can be used as a source of energy for sporulation when the fungus is to be used as a biological agent in the field (Bidochka *et al*, 1990). The conidia from media with excess carbon are able to germinate at lower humidity than conidia from media with less carbon (Hallsworth and Magan 1994). This ability to germinate at low humidity enhances the ability of the conidia to germinate in poor weather conditions like dry weather. When more carbon was added to the medium where the C:N ratios were high, sporulation was inhibited and increased hyphal melanization in microsclerotium-like compact hyphal mass was observed (Jackson and Bothast, 1990).

When entomopathogenic fungi are applied in the field, their growth, sporulation and conidial germination may be affected by the nutrients on the target insects and in the soil. An understanding of the effects of these nutrients on the organism is needed to design a strategy for producing a successful medium or formulation to be used as a biocontrol agent (Li and Holdom, 1995).

1.4.2 *In vivo* and *in vitro* production

***In vivo* production** –is where biological control agents are produced in a living host. This method is mainly used for obligate pathogens which do not readily grow outside the host and it is the least economical method of production. Examples of pathogens produced *in vivo* are *Bacillium popilliae* and Nuclear Polyhedrosis Viruses (Tanada & Kaya, 1993).

***In vitro* production** - is where biological control agents are produced in an artificial medium. The method of production not only depends on the pathogen, but also on the desired end product. This method is better than *in vivo* production because it has reduced costs of capital, labour and harvesting. There is also a high degree of quality control through monitoring and control of environmental factors (Jenkins and Goettel, 1997). There are a number of methods for the mass production of fungi. These include; solid substrate fermentation, diphasic fermentation and submerged liquid culture.

1.4.3 Solid substrate fermentation

This method is used to produce aerial conidia of entomopathogenic fungi on a small scale. The fungus grows as a mat on either agar based or natural substrates like rice and maize producing conidia. This method gives the opportunity to increase yield by providing a larger surface area. The disadvantages of using this method are that pH, temperature, aeration and nutrient status are difficult to monitor and control (Van Driesche and Bellows, 1996; Moore and Caudwell,1997), furthermore it is more difficult to produce large quantities of biomass as compared to production in liquid cultures.

1.4.4 Diphasic fermentation

Diphasic fermentation is used in the Soviet Union to produce *B. bassiana.* The process involves fungus being grown in fermentation tanks to the end of the log phase, and then mycelia are spread on trays, placed in rooms with high relative humidity, and allowed to produce conidia. Since aerial conidia are more infectious and retain viability longer than blastospores or conidia produced in submerged fermentation, the diphasic method takes advantages of both the benefits of solid and liquid fermentation (Soper and Ward, 1981). Green muscle, a biopesticide containing *M. anisopliae* is also produced using this method (Jenkins & Goettel 1997).

1.4.5 Submerged fermentation

This is the standard method of mass production of microorganisms and it is the method of choice. In liquid fermentation, it is possible to maintain constant pH

and temperature, and also to pump large quantities of air into the system and disperse it by means of stirring impellers. Homogenous conditions can reasonably be maintained (Soper and Ward, 1981; Jenkins and Goettel, 1997). A limitation of fermenters is that the desired end product may not be achieved. For instance, in liquid cultures hyphomycetes produce blastospores instead of conidia, which are less resistant to environmental factors like temperature, ultra violet radiation and pH than conidia but can still initiate an infection (Jenkins and Goettel, 1997).

1.4.5.1 Batch culture- this is a system which contains a limited amount of nutrient from the onset. Once inoculated, the culture goes through a lag phase, which is a period when no growth seems to be taking place, this may be because the organism is adapting to the environment. The exponential phase follows the lag phase and this is where the cells grow at a constant and maximum rate. The end of the exponential stage is the best stage at which a culture should be harvested because all the hyphae are healthy and filled up with cytoplasm. Figure 2 shows cultures of *E. neoaphidis* in the early and exponential phase. The stationery phase follows the exponential phase. At this stage there is exhaustion of nutrients and in some cases microbial products are released into the system. Growth slows until it ceases, due to depletion of essential nutrients or the accumulation of toxins produced by the organism or both; this period is the stationery phase. The culture then enters the death phase where most of the hyphae are vacuolated, due to depleted nutrients. During the production of *E. neoaphidis* at a temperature of 20 ^{0} C, the lag phase lasts approximately 50 h, while the exponential phase runs up to almost 100 h, the stationery phase runs for almost 180 h and then the death phase runs to 220 h Gray (1990).

1.4.5.2 Fed batch culture- this is where fresh medium is added to the batch culture at the end of the exponential phase and thus prolongs this phase till the additional substrate or nutrients are exhausted, resulting in an increase in volume and biomass. This method is suitable for growing *E. neoaphidis* in Semi Defined *Erynia* Media (SDEM) liquid medium because it overcomes the effects of inoculum size and the toxicity of oleic acid since the former inoculum has produced enough enzymes to break down the oleic acid that has been added in the new medium. Furthermore, there is enough inoculum that can be harvested and used to either make many mycelial mats or pellets whichever is desirable to the producer.

1.4.5.3 Repeated fed batch culture- is where fresh medium is added to the fed-batch culture at the late exponential phase and some of the inoculum harvested once the culture is healthy (hyphae filled with cytoplasm). More fresh medium is added to the remaining inoculum and harvested at the late exponential phase. This process can be repeated several times but care should be taken to avoid attenuation from taking place, through the repeated *in-vitro* subcultures.

1.4.5.4 Chemostat culture- this is where an overflow device is fitted to a batch culture so that fresh medium is added continuously to a batch culture in the exponential growth phase displacing an equal volume of culture from the vessel. Growth of cells in this culture is controlled by the availability of the growth-limiting nutrient in the added medium (Stanbury *et al,* 1995).

Figure 2a

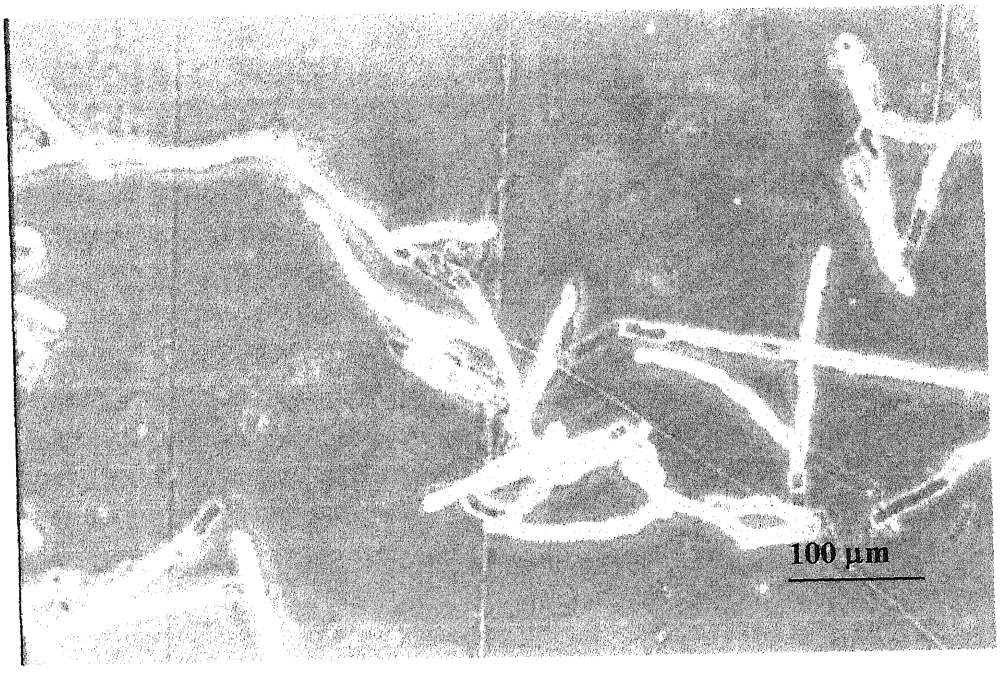


Figure 2b

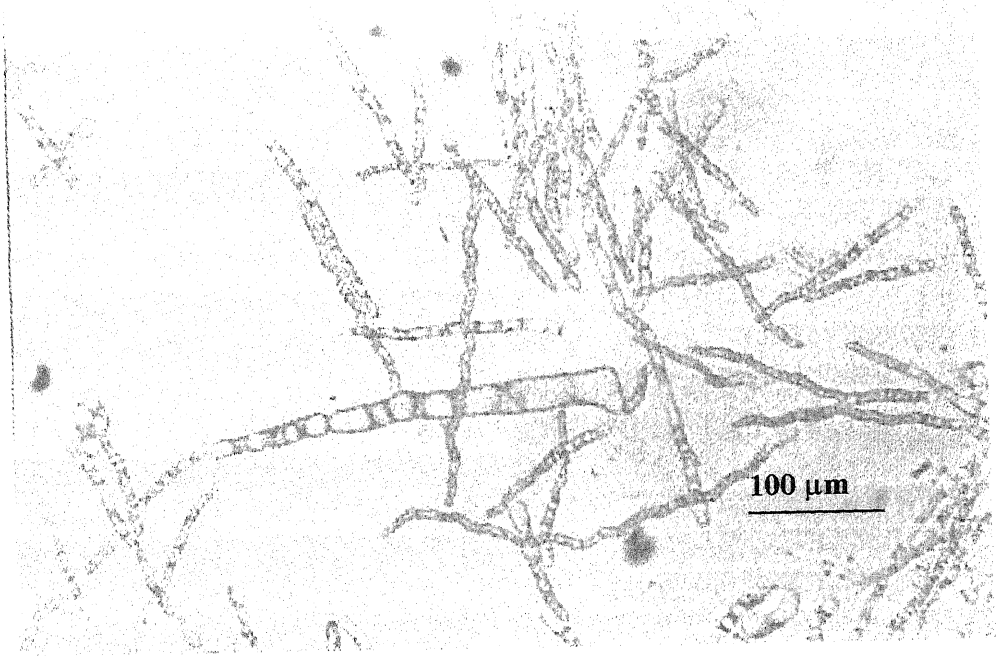


Figure 2 Hyphae of *E. neoaphidis* in SDEM batch culture at the early stage of the exponential phase (a) Phase contrast (b) bright field microscopy

Figure 2c

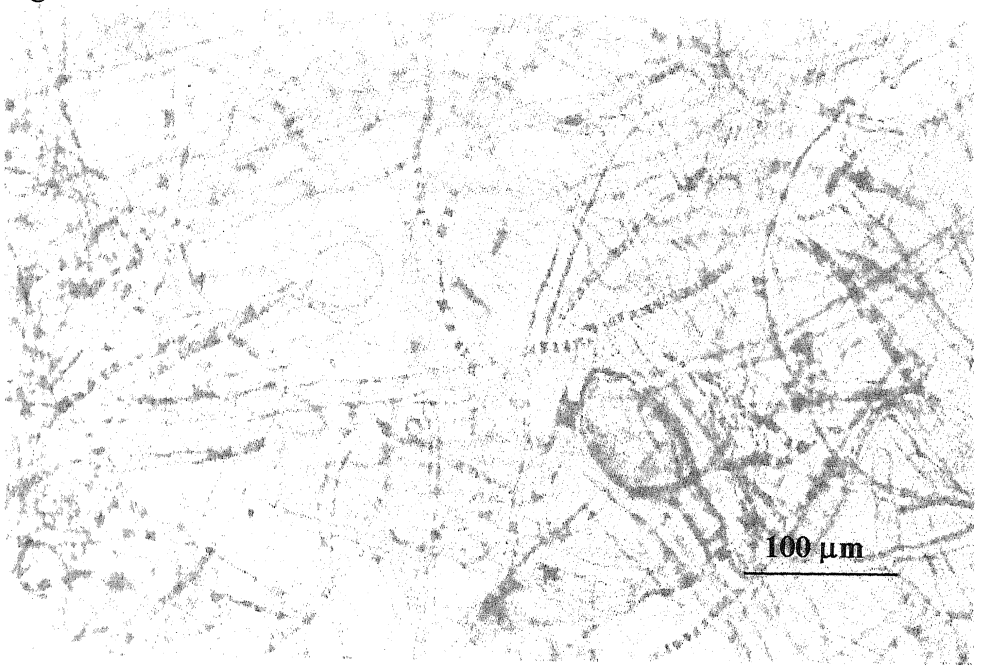


Figure 2d

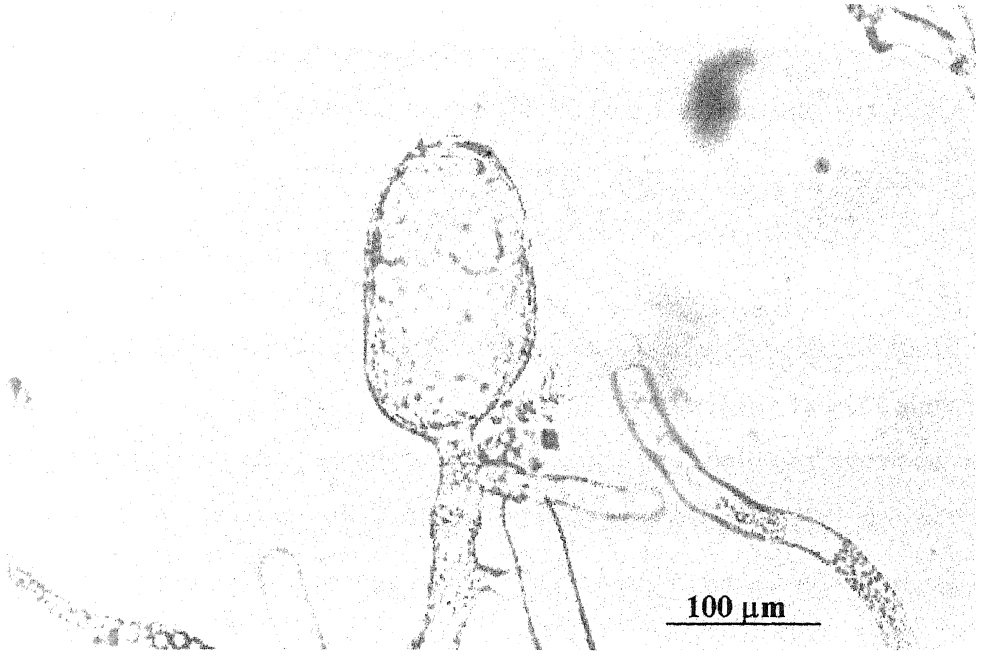


Figure 2 Hyphae of *E. neoaphidis* in SDEM batch culture at the late stage of the exponential phase (c) with vacuoles (d) with an enlarged vacuole

1.4.6 Attenuation

Attenuation is the term used to describe loss of virulence due to repeated subculture *in vitro*. Attenuation can be phenotypic, whereby the fungus changes its physiology to adapt to an environment. In this case virulence can be restored by passaging through the host insect. When attenuation is genetic this is irreversible because the fungus has mutated and changed its genotype. Gray (1990) reports that when *E. neoaphidis* is continually subcultured for a period of over six months the colonial radial growth rate on semi-solid media increases while the rate of conidia production from biomass decreases.

1.5 FORMULATION

Formulation is performed at the end of the production process (Hussey, 1985). Soper and Ward (1981), describe a formulation to be a mixture containing a combination of the right active ingredients in the required proportions to make a fungus suitable for application in the field. Formulations serve two primary purposes, to stabilise the product so that it can be stored until needed and to make the product convenient, safe and reliable to use (Hussey, 1985).

1.5.1 Components of formulations

Unless used immediately, the requirements of a biopesticide formulation change between the time of production and application (Moore and Caudwell, 1997). Once the right combination of ingredients is attained so that the active component is suitable for agricultural applications, the aims of formulation will have been achieved (Soper and Ward, 1981).

In general, a formulation should have an active ingredient (the pathogen), a diluent or dispersant for example oil, and a spreader which enables the organism to spread more easily and quickly in the field or in pest populations. In some formulations, carriers which are inert substances that do not affect the active ingredient but help to increase the efficacy of the active ingredient are used, and at

times a sticker, for example neem oil (which is added to the formulation to attach the active ingredient onto the target host or a plant), is added to a formulation. Baits which are substances that attract the pests, are sometimes included in formulations. Additives such as UV protectants can be added to the formulation before storage or at the time of application (Soper and Ward, 1981; Moore and Caudwell, 1997; Shah *et al*, 1999)

1.5.2 Types of formulations

1.5.2.1 Dry formulations- the term dry refers to the form in which the active ingredient is formulated and stored. Fungi whose conidia germinate rapidly in water should not be stored in aqueous formulations, as these conidia would germinate prematurely. In such cases dust or wettable powder formulations are recommended (Van Driesche and Bellows, 1996). Dry conidia can be applied onto target pests with stickers and fillers or as baits, dust and granules. The disadvantage of using bait formulations is that they require ingestion by the pest whereas fungi do not normally infect through the insect gut wall (Moore and Caudwell, 1997). Since most entomopathogenic hyphomycetes produce large quantities of hydrophobic conidia which have a strong hydrophobic wall, a characteristic that offers stability against adverse conditions, their conidia can be stored as dry formulations (Wraight *et al*, 2001).

1.5.2.2. Oil formulation- The hydrophobicity of the aerial conidia of hyphomycetes makes it difficult to suspend them in water. However, oil formulations solve this problem because they are compatible with lipophylic conidia making them superior spray carriers. Oil formulations also solve the problems of allergies to dust when conidia are inhaled and come into contact with eyes. In oil formulations, oil droplets adhere more strongly to the insect cuticle than water. The oils also spread rapidly carrying conidia to areas of the host body when in contact with the insect cuticle (Wraight *et al*, 2001). For oil formulations, one should know the level of sensitivity of an oil to oxidation, because oxidation leads to exposure of biological organisms to damage. One needs to avoid contact between oil and air and also to high temperatures during storage. The lipophylic cell walls of aerially produced conidia of *M.*

anisopliae and *B. bassiana,* allow easy suspension in oils (Moore and Caudwell, 1997).

1.6 PRODUCTION AND FORMULATION OF *E. NEOAPHIDIS*

1.6.1 Production of *E. neoaphidis*

In the past, *E. neoaphidis* has been grown on media that contained poorly defined components. For instance, the fungus has been grown on egg yolk and milk (Wilding and Brobyn, 1980), then Latgé *et al*, 1978 replaced the egg yolk by yeast extract.

The production of Entomophthorales has been summarized by Latgé, 1982. All Entomophthorales with the exception of *Basidiobolus* metabolized glucose, fructose, trehalose, glycerol and maltose. The fungi did not utilize nitrates. The best growth was obtained on complex mixtures of amino acids, vitamins or on protein hydrolysates, asparagines or yeast extract. Latgé (1982) grew the mycelium on a medium containing dextrose and yeast extract. Wilding and Brobyn (1980) grew mycelium of *E. neoaphidis* on a medium containing Sabourand dextrose agar, eggs and semi-skimmed milk agar (SEMA). Gray (1990) grew mycelium on semi- defined *Erynia* medium (SDEM) containing glucose, yeast extract, mycological peptone, and 0.02% (w/v) oleic acid. *Erynia neoaphidis* has also been grown on semi defined consisting of Grace's insect tissue culture medium supplemented with 5% foetal bovine serum (Hatting *et al*, 1999). Robinson (1987) grew the fungus on basic milk medium agar (BMMA) which contained glucose, mycological peptone and milk, and on medium which contained yeast extract, glucose, mycological peptone and 0.1% oleic acid (YEGMP), and medium with yeast extract agar and glucose (YEAG). The medium YEGMP supported the best growth of *E. neoaphidis.* In order to get consistent growth and production of conidia, *E. neoaphidis* needs to be grown on defined medium so that any causes to changes in growth and sporulation of the fungi can be known.

Erynia neoaphidis grows best at 20 ^{0}C and at pH 6 (Robinson, 1987). Moisture is needed in order for *E. neoaphidis* to sporulate. For growth, *E. neoaphidis*

needs a carbon source such as glucose, and a nitrogen source for example mycological peptone, a supply of growth factors which can be provided in yeast extract. Minerals such as phosphorous, magnesium and calcium are also needed. *Erynia neoaphidis* also requires low concentrations of fatty acids such as oleic acid (Latgé, 1982; Robinson, 1987; Gray *et al*,1990)

Gray (1990) grew *E. neoaphidis* in shake flask culture, fermenter batch culture, fermenter fed batch culture and in chemostat cultures. The fed batch culture method has been a success in growing *E. neoaphidis* because one can obtain a larger volume of inoculum using the fed-batch method, and also the chemostat culture has been a success as a supply of biomass is always available, without having to combat problems of inoculum size effect since the original biomass has produced enough enzymes to breakdown oleic acid. In liquid cultures, it is very important that the culture is harvested at the same growth phase because growth in each growth phase differs at each phase.

Since *E. neoaphidis*, does not produce resting spores and the problems surrounding conidia, research has focused on the production of mycelium and conidia for biological control (Latgé, 1982; Shah *et al*, 2001a). Conidia, which are the infective propagule of *E. neoaphidis*, are difficult to produce in large quantities. Furthermore, conidia are fragile and cannot survive harsh conditions like high temperatures and UV (Latgé, 1982). Mycelia are produced on the assumption that once released in the field as mycelial mats, they would rehydrate and produce conidia which would start the infection process in the field (Pell *et al*, 2001).

1.6.2 Formulations of *E. neoaphidis*

Formulation of conidia of *E. neoaphidis* presents significant difficulties. The conidia have a sticky mucous coating around them, making it hard to harvest and suspend conidia in an aqueous suspension. In addition, if during formulation or application this sticky mucous is lost, the conidia lose their vital adhesion mechanism. Furthermore, the conidia are large, potentially causing blockage of spray equipment (Pell *et al*, 2001).

The hyphae are very large in size, fragile and non- uniform. Fresh hyphae have a very short life span of about seven days (Shah *et al,* 2000b). Dried hyphae produce a mucilage which may act as a physiological buffer and a medium in which nutrients diffuse between cells (Li *et al,* 1993). The current method of choice to produce *E. neoaphidis*, is to formulate granules of biomass which will sporulate in the field to discharge conidia.

Mycelial mats of *E. neoaphidis* have been obtained by filtering liquid cultures through filter papers and drying at 20 ^{0}C- 22 ^{0}C at a relative humidity of 60-90%. When the mats were applied to a population of *Macrosiphon euphorbiae* in a green house, sporulation took place. Some aphids were infected, but this did not prevent the growth of the aphid population (Shah *et al,* 2000b).

Algination is considered one of the best methods to use, because the gelling reaction takes place at ambient temperatures which is good for temperature- sensitive cells. Algination involves organisms being encapsulated in a matrix derived from marine algae by adding a mixture of alginate and cell suspension into a gelling mixture of calcium ions forming beads. Alginate granules have also been used to formulate *E. neoaphidis*. Granules that were dried overnight, were placed in a Petri dish and glued to the plate using adhesive tape, to infest the aphids on the Petri dish produced significantly more conidia than the other granules that were not dried. However, the fungus did not prevent the number of aphids from increasing to large populations (Shah *et al,* 1998). When additives such as maltose were added, the shelf life of the fungus was increased. These additives may also act as protectants to the fungi during drying and storage (Pell *et al,* 1998).

1.7 OBJECTIVES

The results from the field trials were unsuccessful because there was no standard way of producing, formulating and applying the fungus in the field. In order for *E. neoaphidis* to be used as a successful biological control agent in the field, treatments using the fungus should be standardized and optimized.

The objectives of this study are to optimise growth of *E. neoaphidis,* to maximize spore production and infectivity on biomass grown on both solid and liquid media and to test which media produce the most virulent conidia to aphids. This would be achieved by doing the following;

1. to grow *E. neoaphidis* on defined and semi-defined media in simple liquid shaker flasks and fermenters;
2. to develop a system for harvesting biomass from the above cultures as mycelial mats or pellets.
3. to investigate the effects of different carbon and nitrogen sources and ratio on the rate of production, sporulation and physiology of conidia of *E. neoaphidis.*
4. to investigate whether attenuation takes place when *E. neoaphidis* is subcultured from one medium onto a different medium.

2 MATERIALS AND METHODS

2.1. Isolates

The pea aphid, *Acyrthosiphon pisum* Harris and the fungal isolate *Erynia neoaphidis* isolate NW327 were kindly supplied by Dr. Judy Pell of Rothamsted Research, Harpenden, Herts. The fungal isolate was originally isolated from the pea aphid in the 1970's and subsequently maintained *in vivo* by repeated passage through aphids. It was then isolated onto Sabouraud dextrose egg milk agar (SEMA) medium and stored in 10% glycerol in liquid nitrogen.

2.1.2 Maintenance of isolates on solid media

Petri dishes containing approximately 20 ml Semi-defined *Erynia* medium (SDEM) + 0.02% oleic acid were centrally inoculated with a 9 mm-diameter plug, cut using a flame sterilized cork borer from the edge of a growing colony of *E. neoaphidis*. The plates were sealed with Parafilm (Whatman, Maidstone, UK) and were incubated at 20 ^{0}C in an incubator in the dark. The strains were maintained by subculturing regularly every five weeks.

2.2 Media

2.2.1 Semi-defined *Erynia* medium (SDEM)

SDEM was made according to Gray et al, (1990). This medium contained: 3 gl^{-1} yeast extract (L21 Oxoid Ltd), 5 gl^{-1} mycological peptone (L40 Oxoid Ltd), and buffered at pH 6 (KH_2PO_4 5.97 gl^{-1} and Na_2HPO_4 2.2 gl^{-1} both BDH Ltd products) was autoclaved at 121 ^{0} C for 15 minutes, then placed in an incubator to cool to 55 ^{0}C. For solid cultures 15 gl^{-1} of agar (L13, Oxoid) was added to the medium autoclaving.

Oleic acid (Fisher chemicals, typical 70% v/v for laboratory applications) to be added to the medium was prepared by adding 10 ml to1.3 g NaOH to 90 ml of distilled water and mixed on a magnetic stirrer for 2-3 hours. The standard concentration of oleic acid used was 0.02% (v/v). The oleic acid was filter sterilized and added to the nutrient medium when the temperature dropped to 55 ^{0}C

immediately prior to use. The pH of the medium was 6.0, maintained by the presence of phosphate buffers in the medium (Gray, 1990).

2 2.2 Modified Grace's insect tissue culture medium (GASP)

Grace's insect tissue culture medium (G-9771 Sigma Ltd; see appendix A for constituents), was made up according to the manufacturer's instruction, supplemented with 4.2 gl^{-1} (w/v) asparagine (Sigma, anhydrous), and 0.01% v/v oleic acid and filter sterilized through a 0.22 µm cellulose acetate filter membrane (Fisher scientific UK) (Grace, 1962; T. Bonner, personal communication).

2.2.3 Sabouraud dextrose egg milk agar (SEMA)

SEMA cultures that were made according to Wilding and Brobyn (1980), where large free medium eggs were surface sterilized for 2- 24 hours in a solution of; 940 ml ethanol, 50 ml distilled water and 10 ml of acetone. The eggs were then flame sterilised and the yolks added to a sterile 500 ml Duran bottle. Semi-skimmed milk (17 ml) after sterilisation was added to the Duran bottle and shaken vigorously, Sabourand dextrose agar (SDA, 160 ml; 6.5 % w/v) after sterilization was also added. The milk, 500 ml Duran bottle and SDA had been sterilised at 121 ^{0}C for 15 minutes. The cultures were kindly provided by Dr. Judy Pell of Rothamsted Research Station, Harpenden, Herts.

2.3 Aphid cadavers

Erynia neoaphidis mycosed aphid cadavers made according to Wilding (1973) were also kindly provided by Dr. Judy Pell of Rothamsted Research Station, Harpenden, Herts.

2.4 Assessment of growth

2.4.1 Determination of Colony Radial Growth Rate (Kr)

Colony Radial Growth Rate (Kr) is the rate at which a fungus grows on a medium determined by measuring the radii and its units are µm h^{-1}. Inoculated plates were inverted to prevent colony extension due to discharge of conidia onto the

medium beyond the edge of the colony. The first measurement of the growth was made after 1 week when initial growth began to appear. The mean of two perpendicular measurements of radii (from the plug to the growing edge) were measured and calculated, every two days until the growth covered ¾ of the plate. The increase in colony radius from the first measured was plotted against time to determine rate of growth. Kr was calculated by linear regression as the slope of the graph. Each treatment was replicated 6 times and done at the same time as the control.

2.4.2 Dry weight

The dry weight of biomass in liquid cultures was determined by vacuum filtering 25 ml of liquid culture through pre- weighed GF/C Whatman filter papers 47 mm ϕ circles. The samples were washed twice with 20 ml of distilled water, dried overnight at 105 ^{0}C, then desiccated to constant weight. Triplicate flasks were used per time interval. Growth curves were obtained by plotting the dry weights against time. Growth curves were also done for a period of 100 h for cultures growing in the medium described in 2.2.1 and 2.2.2.

2.5 Assessment of sporulation

2.5.1 Sporulation monitor

The sporulation monitor as shown in figure 3, (copied from Pell *et al.*, 1998) was developed to assess sporulation of the entomophthoralean fungus *Z. radicans* Pell *et al,* (1998). Approximately 75 ml of 1% water agar (w/v) was loaded into each of 10 circular wells in a Perspex block. A 9 mm-diameter flame sterilized cork borer was used to cut out water agar plugs forming a hole in the agar, which allowed plugs from the nutrient agar to be placed inside. If cadavers were to be tested, the cadaver was placed on the cooled water agar (one cadaver per hole). If different inoculum sources were to be tested, plugs were cut along the edges of the growing colony and arranged randomly on the Perspex block. The inoculum was held in place by a nylon slit mask attached to the block, so that conidia were ejected through the slits. The block containing the inoculum was then inverted over an acetate sheet, which was mounted on a revolving drum, which rotated once in 168 h. To maintain a relative

humidity of 100 %, wet tissue paper was placed in the apparatus. The temperature was maintained at 20 ^{0}C with a 16 h: 8 h (light: dark) photoperiod. Discharged conidia adhered to the acetate sheets due to the presence of a mucous coating, forming a trail on the acetate sheet, as the drum rotated. After the incubation period, the acetate sheets were removed from the drum and cut into strips, each strip corresponding to a single inoculum source. The individual strips were then mounted onto a thin transparent Perspex slab, which had transverse lines etched at equidistant intervals corresponding to the distance that the drum rotated in an hour. The number of conidia was counted under a light microscope using image analysis (2.5.2). Only conidia that were contained within the area of an eye piece graticule (0.3072) were counted. Conidia on the acetates were counted in ten fields in a transverse line across a conidia trail in the region that was within the last hour of each 10 or 5 h section, for example between 4 and 5 hours. Since it has been observed the production of conidia varies little within the last hour section at either 5 or 10 h intervals, this line is a representative of the number of conidia discharged in the whole 5 or 10 section. The number of conidia discharged was determined by: Number of conidia/ time interval (5 h or 10 h) * 3.072 mm^2, 3.072 is the area view of ten fields of view.

2.5.2 Image analysis

The image capture system used comprised of a JVC TK1280E color CCD camera connected to a video logic DVA 4000 video adapter, mounted in a Macintosh Quadra 650 computer. Image capture and analysis was done at 640 x 400 pixels x 256, grayscale values using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).

Image analysis was used both to capture images of conidia and to count conidia. A slide micrometer was used as a reference to calibrate the dimensions of the captured images. Artifacts and clumped conidia were removed manually from the images after density slice was performed to select conidia on the basis of a grey scale. The system was instructed to include particles with interior holes, because the conidia appeared hollow due to refraction at the edges of the conidia. The length, breadth and cross sectional area of each particle was determined automatically.

In the experiment where different oleic concentrations were used to study their effect on growth of *E. neoaphidis*, (section 2.6.1) image analysis was also used to measure the colony thickness. The plate to be measured was placed on a fluorescent light and a blue filter placed on half the medium and a digital image taken. The scale for the position on the plate was set to 90 mm (dimensions of the plate). The scale for optimal density, was arbitrarily set to 0 for a region of the plate where there was no growth and maximum (350), where the blue filter was placed which was used as a standard on the plate. In order to calibrate the absorbance at these points, a rectangle region in the area of interest was selected and its mean greyscale darkness using the software measured. A diagonal line was drawn across the plate and the X (distance that the culture had grown or covered across the plate or medium) and Y (absorbance) co-ordinates for all points on the line were copied to an Excel file. This last step was repeated for another diagonal line.

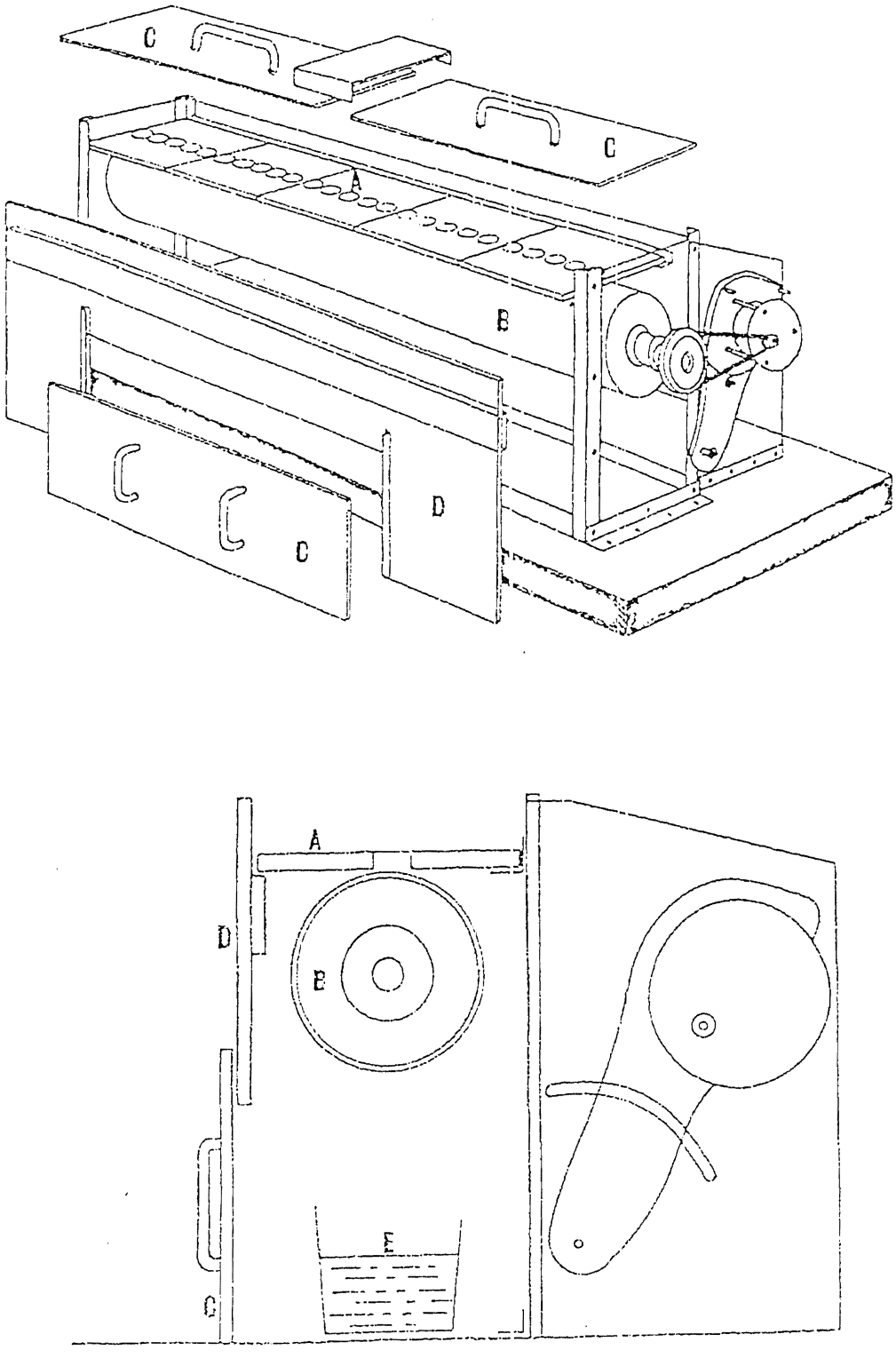


Figure 3 Sporulation monitor: side and end view. A, 10 wells, in each transparent Perspex plate inverted over revolving drum: B, revolving drum with mounted acetate strips; C, access hatches; D, removable side wall; E, water reservoir (copied from Pell *et al*, 1998)

2.5.3 Effects of different positions of a culture on sporulation

A 9 mm diameter plug of inoculum cut from a 3-week culture was placed on damp filter paper in the centre of lid of a Petri dish, and inverted over the bottom of a Petri dish containing 5 cover slips. The cover slips were arranged as shown in Figure 4

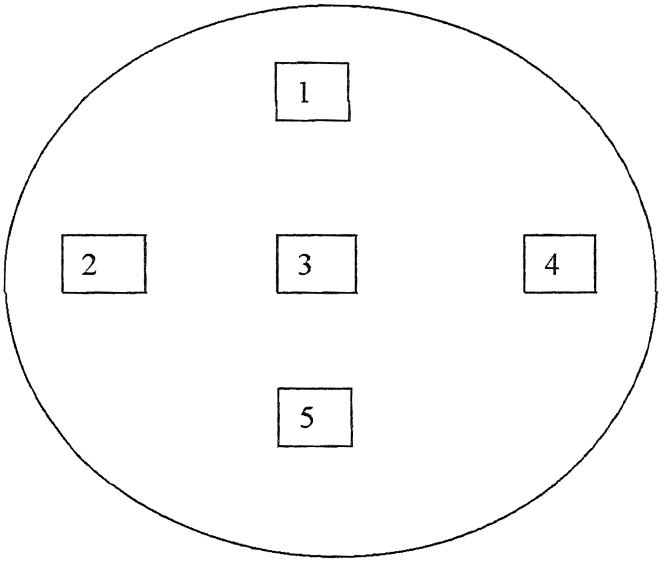


Figure 4 The arrangement of cover slips in a Petri dish.

On a culture that had grown to cover ¾ of the medium, plugs from different positions on the original plate were compared. Plugs were cut near the central plug (source of inoculum), half way between the plug and the growing edge and at the edge, to assess which position of a growing culture sporulated the most. The cultures were grown on SDEM solid media. The positions of the plugs as shown in Figure 5

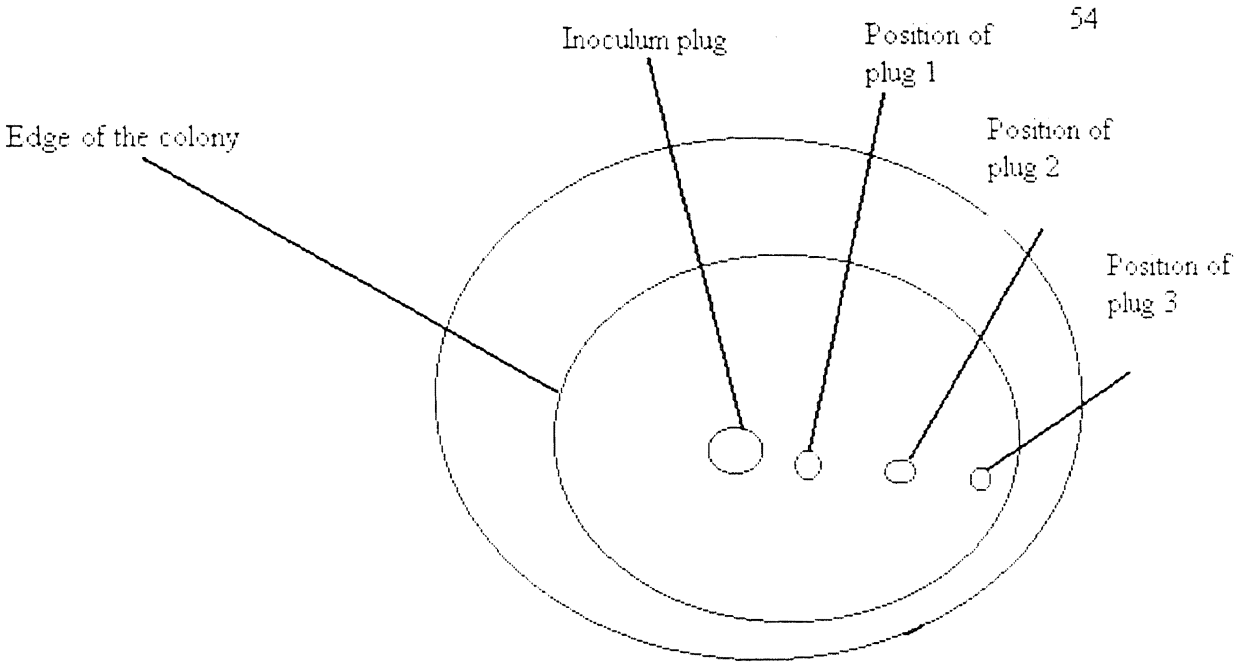


Figure 5 The different positions of the tested plugs on a Petri dish

The Petri dishes were placed in an incubator at 20 ^{0}C and the conidia discharged onto each of the cover slips were counted after 15 and 30 min, then after 1 and 2 h. Cover slips were replaced at each time interval and the number of discharged conidia onto each cover slip was counted under a microscope at a magnification of X 100. The number of discharged conidia was determined by: Number of conidia /πr^2, where r (the area of view using the eye piece) = 0.1 mm^{-2}.

2.6 Effects of medium composition on growth and sporulation of *E. neoaphidis* on solid media.

2.6.1 Effect of oleic acid on growth of *E. neoaphidis*

Media were made as described in sections 2.2 and 2.1.2 with oleic acid concentrations 0%, 0.1%, 0.01%, 0.02% and 0.05% v/v. Image analysis (2.5.2) was used to measure the colony thickness of the fungus based on the amount of light transmitted through the culture, based on the principle described by Olsson (1995). The more light that was transmitted, the thicker the colony was. This was measured 3

times a week as soon as growth was observed on the media. 6 replicates per test or sample were used.

2.6.2 Effects of carbon and nitrogen ratios

Modified SDEM (2.2.1) was made with double (32 gl^{-1}) (SDEMDG), or half (8 gl^{-1}) the original concentrations of glucose (SDEMHG) or, double (10 gl^{-1}) (SDEMDMP) or half (2.5 gl^{-1}) (SDEMHMP) the original concentrations of mycological peptone. The plates were inoculated as in (2.1.2). The rate of radial growth was measured as described in (2.4.1) while sporulation was measured as described in (2.5.1). Six replicates per treatment and SDEM as the control were tested at the same time.

2.6.3 Effects of sources of carbon and nitrogen

SDEM (2.2.1) was made containing 7.5 gl^{-1} (SDEMC2), or with 15 gl^{-1} (SDEMC1), of chitin (Sigma, practical grade) instead of glucose and with ammonium sulphate (0.475 gl^{-1}) replacing the amount of nitrogen in mycological peptone ($SDEMNH_4SO_4$). The plates were inoculated as in (2.1.2). The rate of radial growth was measured as described in (2.4.1). Six replicates per treatment and SDEM as the control were tested at the same time.

2.6.4 Effects of omissions of carbon or nitrogen

Three types of modified SDEM (2.2.1) were prepared and were inoculated as in (2.1.2); SDEMLG1 medium (without glucose), then resubcultured on SDEMLG2 medium (without glucose again), and SDEMLYE1 medium (without yeast extract) then resubcultured on SDEMLYE2 medium (without yeast extract again). The fungus grew until growth covered almost ¾ of the medium before being subcultured on the fresh medium lacking the nutrients mentioned. The rate of radial growth was measured as described in (2.4.1). Six replicates per treatment and SDEM as the control were tested at the same time.

A summary of the contents of the media is given in table 1.

Table 1. A summary of the composition of various media used in the development of a defined medium for growth and sporulation of *Erynia neoaphidis*.

Medium	Glucose gl^{-1}	Yeast extract gl^{-1}	Mycological peptone gl^{-1}	Chitin gl^{-1}	Ammonium sulphate gl^{-1}	Grace medium
SDEM	16	3	5	None	None	None
SDEMDG	32	3	5	None	None	None
SDEMHG	8	3	5	None	None	None
SDEMDMP	16	3	10	None	None	None
SDEMHMP	16	3	2.5	None	None	None
SDEMLG1	None	3	5	None	None	None
SDEMLG2	None	3	5	None	None	None
SDEMLYE1	16	None	5	None	None	None
SDEMLYE2	16	None	5	None	None	None
SDEMLMP1	16	3	None	None	None	None
SDEMLMP2	16	3	None	None	None	None
SDEMC1	None	3	5	15	None	None
SDEMC2	None	3	5	7.5	None	None
SDEMNH_4SO_4	16	3	None	None	0.475	None
GASP	None	None	None	None	None	Yes

2.7 Enzymes analysis.

Using the API ZYM kit 25,200 (bioMerieuxe), a sample of 60µl from either a homogised suspension of fungus or filtrates from the dry weights done in section 2.4.2, were put into each well of a tray that was then put into an incubation tray (supplied in the kit) that had 5ml of distilled water. The incubation tray was covered with its lid and placed in an incubator at 37 ^{0}C. After 4 h, 1drop of the reagents ZYM A and ZYM B were consecutively added to each well in the incubation tray. After 5 minutes the colours observed were read off an enzyme list provided in the kit which showed a colour corresponding to an amount of particular enzyme.

2.8 Other experiments on growth on solid media

2.8.1 Effect of growing *E. neoaphidis* on a SDEM from SEMA

Fungus that had previously been stored in liquid nitrogen was subcultured onto SEMA medium (2.2.3) as described in 2.2. After three weeks, the fungus was

resubcultured onto further plates of SDEM medium, then resubcultured onto further plates of SDEM medium. The colonial growth rate was measured on both subcultures with six replicates per subculture. This experiment was done to investigate whether attenuation had taken place when *E. neoaphidis* was subcultured from one medium to another.

2.8.2 Effects of wetting agents on harvesting of conidia from solid cultures

Surfactant solution was prepared by adding tween 80 in distilled water at concentrations of 1% and 0.1% v/v then sterilised by autoclaving at 121 ^{0}C for 15 minutes. Surfactant solution (5 ml) was poured onto plates of SDEM on which *E. neoaphidis* had grown for 3 weeks (growth covering ¾ of the media), and a sterile inoculating loop used to scrape the conidia off the agar. The resulting suspension was put into universal bottles using a sterile plastic pipette and vortexed for 5 minutes. The conidial suspension was counted in a haemocytometer. The procedure was repeated using distilled water instead of the surfactant. Three replicate plates were harvested for each surfactant concentration and water.

2.9 Growth in liquid media

2.9.1 Inoculation of liquid media

Liquid cultures were obtained in two steps. The first step was to make a liquid culture from plugs by putting 25 ml of the liquid medium into 250 ml conical flasks, and inoculating with 20, 9 mm diameter agar plugs cut from cultures on solid medium using a flame sterilized cork-borer. The flasks were incubated at 20 ^{0}C and shaken at 200 rpm on an Innova 4330 rotary shaker (New Brunswick Scientific, Edison, New Jersey, USA) for approximately 5 days until the culture reached exponential growth phase. In the second step, 25 ml of fresh liquid medium was inoculated with 5 ml of liquid culture using a sterile pipette. Clumps in the inoculum were broken by pipetting up and down, to form a homogenous culture which was then incubated as in the first step.

2.9.2 Effects of changing the carbon concentration on growth of *E. neoaphidis* in SDEM liquid medium

Erynia neoaphidis was grown in batch cultures in SDEMDG (with 32 gl^{-1} glucose) and dry weights calculated as described in (2.4.2).

2.10 Production of known age aphids

Adult aphids were reared according to Morgan *et al*, (1995). Adult aphids collected from aphid cultures on dwarf broad beans (*Vicia faba* var. "The Sutton") using a fine brush and transferred to bean leaf discs set in 2% water agar and maintained at 20 ^{0}C, in a 16 h photoperiod for 24 h. After the initial 24 h, all the adults were removed using a fine brush and the young nymphs of known age were transferred to a plant at 20 ^{0}C. After 2 days these developed into 2 day old nymphs, then after approximately 8-10 days they became adult aphids and used for experiments.

2.11 Fermenter culture

2.11.1 Fermenter equipment

The apparatus used was an Electrolab Fermenter 300 series. The fermenter culture was agitated by twin impellers. Temperature control was achieved by means of circulating water at 20 ^{0}C through a jacket. An Electrolab 250-oxygen meter monitored the oxygen level, pH was measured by an Electrolab 260-pH controller. The airflow was maintained by a LiniAire (Adaptation biosystems, model AB1-0105) and measured using a rota meter.

The initial impella speed was 100 rpm. Air was supplied at a rate of 1.5 L h^{-1} pressure. Temperature, impeller speed and oxygen concentration were recorded every time a sample of the culture was taken out for dry weights analysis as described in (2.4.2).

2.11.2 Assembling the fermenter.

The fermenter was assembled for me. The fermenter equipment with attached medium reservoir containing 4 L of SDEM liquid medium was sterilised by autoclaving at 121^0 C for 15 minutes. Oleic acid (8 ml) of 0.02% (v/v) was filter sterilised into the SDEM media after cooling to room temperature.

To minimise contamination, all samples, inoculations and media were transferred by positive pressure through connections to the fermenter outlets.

2.11.3 Inoculum preparation

20, 9 mm diameter plugs cut from 3 week old cultures on SEMA medium were used to inoculate 12 flasks of SDEMDG (with double 32 gl^{-1}) glucose as described in (2.9.1). After 5 days, the cultures were used to inoculate another 15 flasks. The cultures were examined under a microscope for contamination and to ensure that the biomass was healthy 5 days after inoculation. These cultures were then ready for inoculation of the fermenter.

2.11.4 Inoculation of the fermenter

I was assisted with the inoculation of the fermenter. Cultures from the 12 flasks in step 2.11.3 above, were aseptically transferred into sterile universal bottles using sterile glass pipettes in a class 2 laminar flow cabinet. The fermenter was inoculated using positive pressure as described in section 2.11.2. At 48 h and 96 h, 250 ml and 2 L of fresh medium were added to the inoculum respectively. The impeller speed was increased to 200 rpm at 48 h then at 168 h the impeller speed was increased to 400 rpm for 45 minutes then set back to 200 rpm. At 360 h the impeller speed was increased to 400 rpm for about 2 h and then set back to 200 rpm. The impeller speed was increased to remove the wall growth from the inside of the vessel. Dry weights were calculated as described in section (2.4.2) using 20 ml samples of inoculum per bottle, collected in sterile universal bottles. Triplicate universal bottles were used at each time interval.

2.11.5 Harvesting the culture

Approximately 1.8 L of the culture broth was harvested from the fermenter cultures into 3 large sterilised bottles. The culture broth was vacuum filtered through Whatman filter papers (90 mm, diameter, type 1) forming a mycelial mat, as a result 2 mycelial mats were made per bottle forming 6 mycelial mats in total. Triplicate mycelial mats were placed onto plates containing 1% (w/v) water agar and also Petri-dishes without water agar.

2.11.6 Sporulation of *E. neoaphidis* on mycelial mats.

Each mycelial mat was inverted over 5 cover slips in a Petri-dish as shown in section 2.5.3 figure 4. One of the three mycelial mats without water agar was inverted onto a Petri dish with 5 cover-slips and 10 aphids. The plates were put in an incubator at 20 °C. The relative humidity was monitored using a Tiny Talk datalogger (RS components). The number of conidia per cover slip per hour was counted under a microscope as described in (2.5.3).

2.11.7 Determination of the virulence of conidia discharged in section (2.11.6)

After 1.5 h of inoculation with conidia from the mycelial mats described in section (2.11.6) onto 10 aphids, the aphids were transferred onto a 3-week old pea plant. The plant was then covered for 24 h with an inverted plastic beer glass which had been cut in the base and sealed with Parafilm to provide moisture to the aphids thus facilitate the infection process. The Parafilm was then replaced with muslin. The aphids were monitored for a change in colour, decreased activity and sporulation after death.

2.12 Enzyme tests on growing cultures of *Erynia neoaphidis*

2.12.1 Cultures on solid media

20, 9 mm diameter plugs were cut from a 3-week old *E. neoaphidis* culture growing on SDEM using a flame sterilized cork borer. The mycelium from the plug

was removed using a flame sterilized scalpel and placed in a 60 well plate. Each sample was placed in a well containing 60 µm of distilled water and then crushed till a homogenous suspension was attained. An API ZYM kit described in (2.7) was used to test the presence of enzymes in cultures growing in 1st and 2nd subcultures on SDEM media originally subcultured from cultures growing on SEMA media. The experiments were not run at the same time.

2.12.2 Cultures on liquid media

An API ZYM analysis as described in (2.7) was used to test for the enzymes in the filtrates from the dry weight described in section 2.4.2, at 48 h in both media in sections (2.2.1 and 2.2.2).

2.13 Statistical methods

The Kr values are the standard errors of the regression coefficient. An unpaired 2-tailed T-test was used to determine the difference in colony radial growth rates of cultures, volume and length/width of conidia discharged and the dry weights from different cultures of *E. neoaphidis* growing on different media.

3 RESULTS

3.1 Effects of medium composition on growth and sporulation on a semi-defined solid medium

3.1.1 Effects of carbon and nitrogen sources and varying amounts on growth of *E. neoaphidis* on solid media

3.1.1.1 Growth of E. *neoaphidis* growing on SDEM solid medium with varying amounts of carbon and nitrogen

Table 2. Summary of Colonial radial growth rate Kr (μm h^{-1}) of Erynia neoaphidis grown on different tested media, see table 1 on page 56 for contents of the modified media tested;

SDEM modification	Kr (μm h^{-1})
1. SDEM1 (control)	44.2
2. SDEM2 (control)	42.8
3. SDEMDG	48.8
4. SDEMHG	39.3
5. SDEMDP	35.7
6. SDEMHP	27.6
7. SDEMLYE1	46.6
8. SDEMLYE2	41.3
9. SDEMLG1	48.6
10. SDEMLG2	39.6
11. SDEMLMP1	19.8
12. SDEMLMP2	42.9
13. SDEMC1	48.6
14. SDEMC2	45.1
15. SDEMNH_4SO_4	31.2

5 significantly different from 3 (p= 0.0007) and 4 (p=0.01)

6 significantly different from 1 (p=0.01)

7 significantly different from 8 (p=0.09)

9. significantly different from 10 (p=0.01)

11. significantly different from 12 (p=0.00)

15. significantly different from 1 (p=0.01)

The mycelia of *E. neoaphidis* grown on SDEMDG media, appeared thicker and the colony grew more evenly across the plate than on regular SDEM medium. From table 2 and Figures 6 and 7a, the Kr of SDEMDG was not significantly different from the Kr of the SDEM. On SDEMHG, the culture grew very slowly and the mycelial mat appeared thinner compared to the mycelia that grew on SDEM. The Kr of the fungus growing on SDEMHG was not significantly different from the Kr of the fungus growing on SDEM medium. The culture that grew on SDEMDMP looked healthy but did not appear as thick as the cultures that grew on either SDEM medium or SDEMDG. The Kr of the SDEMDMP was not significantly different from SDEM medium while it was significantly lower than both the Kr of SDEMDG and SDEMHG. SDEMHMP medium had very poor and uneven growth with very thin mycelial mats and the Kr value was significantly lower than SDEM medium.

The mycelium that grew on both SDEMLYE1 and SDEMLYE2 appeared thinner than the mycelium on SDEM, while both SDEMLG1 and SDEMLG2 media supported a culture that grew very slowly and the mycelial mat appeared thinner compared to that on SDEM. From table 2 and Figure 7b, the Kr of SDEM1 on the first subculture was not significantly different from SDEM1 on the second subculture. There was a decrease in Kr between SDEMLYE1 and the second subculture (SDEMLYE2) but it was not significant. The Kr on SDEMLG1 decreased significantly on the second subculture (SDEMLG2). In contrast the Kr of SDEMLMP1 increased significantly on the second subculture (SDEMLMP2).

3.1.1.2 Growth of *E. neoaphidis* growing on solid SDEM medium with chitin and ammonium sulphate as carbon and nitrogen sources

From table 2 and Figures 6 and 7c, the mycelium that grew on SDEMC1 (with 15 gl^{-1} chitin) and SDEMC2 (with 7.5 gl^{-1} chitin), was very sparse and uneven compared to SDEM, but there was no significant difference (P=0.6) in the Kr of either SDEMC1 and SDEMC2 media compared to the Kr on SDEM. The fungus that grew on SDEMNH_4SO_4 medium was not as thick as growth on mycological peptone, and grew very slowly which was significantly lower than SDEM.

Figure 6

(a) SDEM

(b) SDEMHG

(c) SDEMDG

(d) SDEMNH$_4$SO$_4$

(e) SDEMC2

(f) SDEMC2

Figure 6 Growth of *E. neoaphidis* on (a) SDEM (b) SDEMHG (with 8 gl^{-1} glucose) (c) SDEMDG (with 32 gl^{-1} glucose) (d) SDEMNH$_4$SO$_4$ (with 0.47 gl^{-1} ammonium sulphate) instead of mycological peptone (e) SDEMC2 (with 7.5 gl^{-1} chitin) instead of glucose (f) SDEMC1 (with 15 gl^{-1} chitin) instead of glucose.

Figure 7a

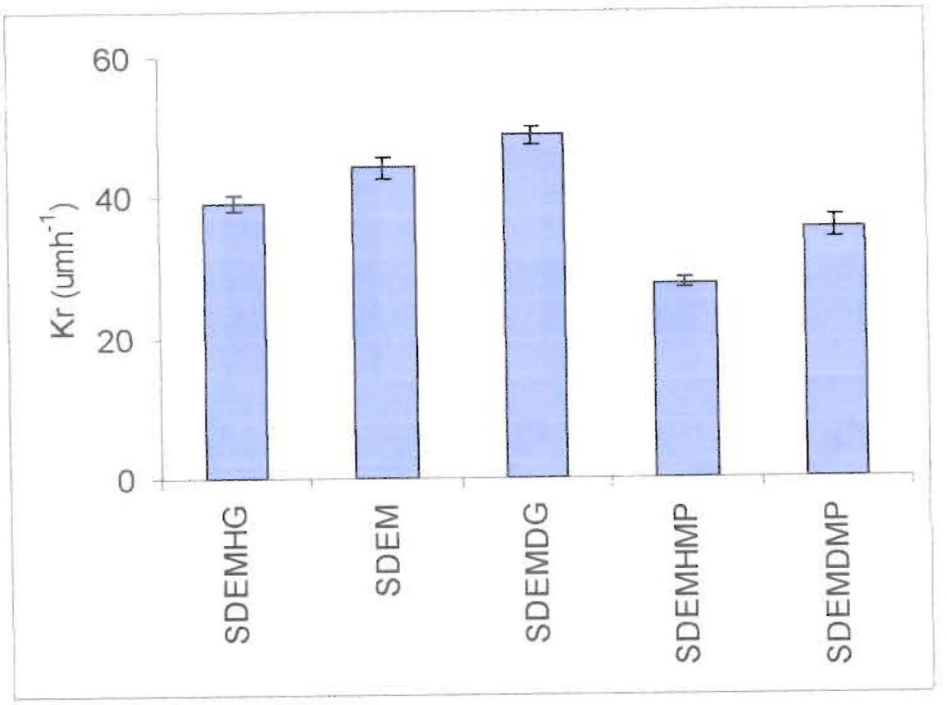


Figure 7a. Colony radial growth rates (Kr) of *E. neoaphidis* growing on SDEMHMP (with 10 gl^{-1}) and SDEMDMP (with 2.5 gl^{-1}) mycological peptone and SDEMHG (with 8 gl^{-1}) and SDEMDG (with 32 gl^{-1}) glucose. (The error bars indicate standard errors from the regression coefficients for the total sum of 6 replicates per test)

Figure 7b

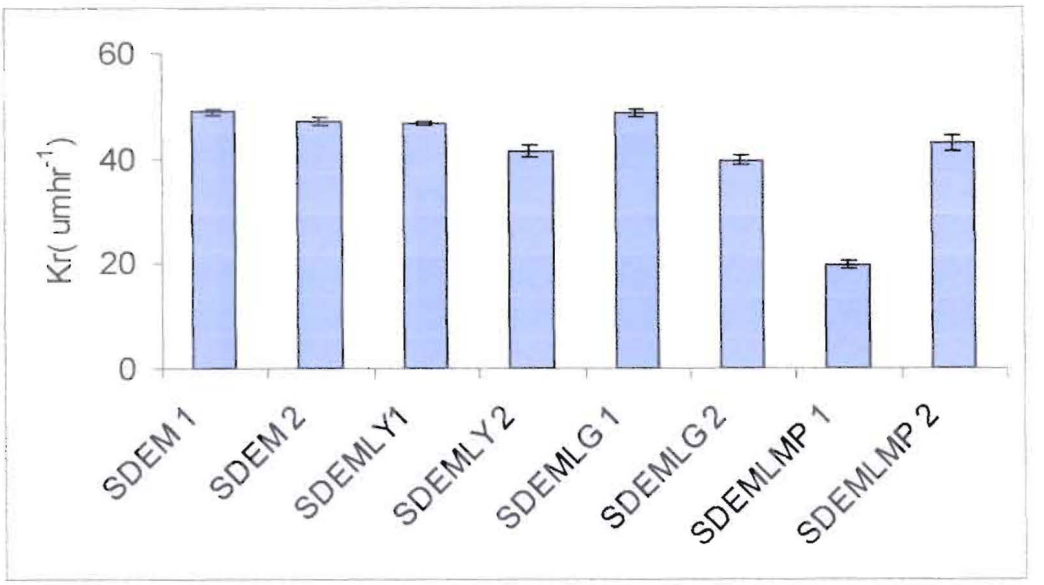


Figure 7b. Colony radial growth rates (Kr) of *E. neoaphidis* growing on SDEM solid medium without glucose (SDEMLG1) then resubcultured for the second time on medium without glucose again (SDEMLG2). And SDEM solid medium without yeast extract (SDEMLY1) then resubcultured for the second time on SDEM medium without yeast extract again (SDEMLY2). And SDEM solid medium without mycological peptone (SDEMLMP1) then resubcultured for the second time on SDEM medium without mycological peptone (SDEMLMP2). (The error bars indicate standard errors from the regression coefficients for the total sum of 6 replicates per test)

Figure 7c

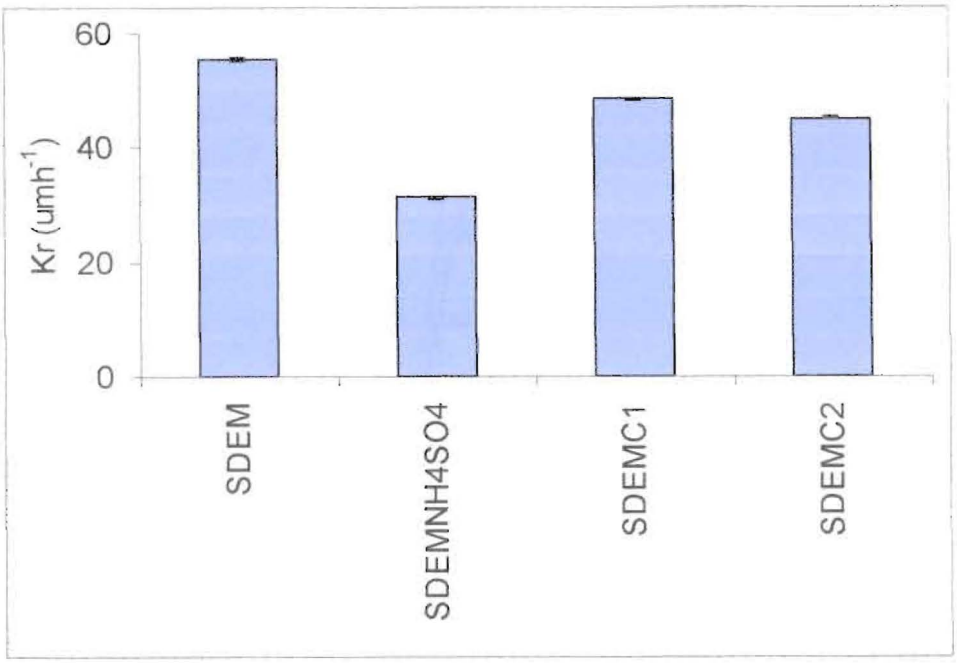


Figure 7c Colony radial growth rates (Kr) of *E. neoaphidis* growing on SDEMC1 (with 15 gl^{-1} chitin) and SDEMC2 (with 7.5 $gl^{-1}$of chitin) replacing glucose and $SDEMNH_4SO_4$ (with 0.47 gl^{-1} ammonium sulphate) replacing the amount of nitrogen in mycological peptone. (The error bars indicate standard errors from the regression coefficients for the total sum of 6 replicates per test)

3.1.2 Effect of oleic acid concentration on colony thickness.

In Figures 8a and b, there was minimal fungal growth on all treatments 2 and 3 weeks after inoculation, since the only section or area on the culture plate that had the highest absorbance was at the position of the inoculum plug. In Figures 8c, there was also greater absorbance at the end of the plate, this was due to an edge effect rather than growth. There was an increase in thickness as seen by the increase in absorbance measured, on SDEM with 0.01% and 0.02% oleic acid as compared to SDEM with 0.05% and 0% oleic acid after 4 weeks. In Figures 8d, on SDEM with 0.1% oleic acid did not support growth as the only measured absorbance was at the position of the plug. Furthermore the central plug's size decreased as shown by the decrease in absorbance. SDEM with 0.02% oleic acid had the highest absorbance measured thus supported the most growth followed by 0.01%, 0.05%, and 0% oleic acid after 5 weeks.

Figure 8a

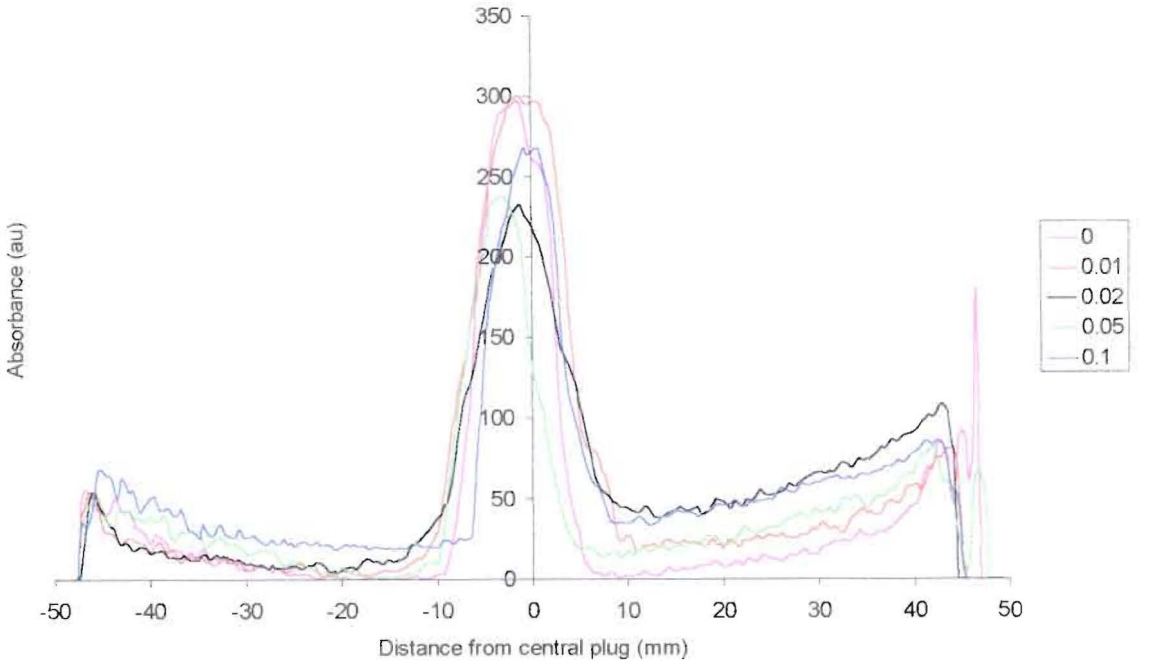


Figure 8b

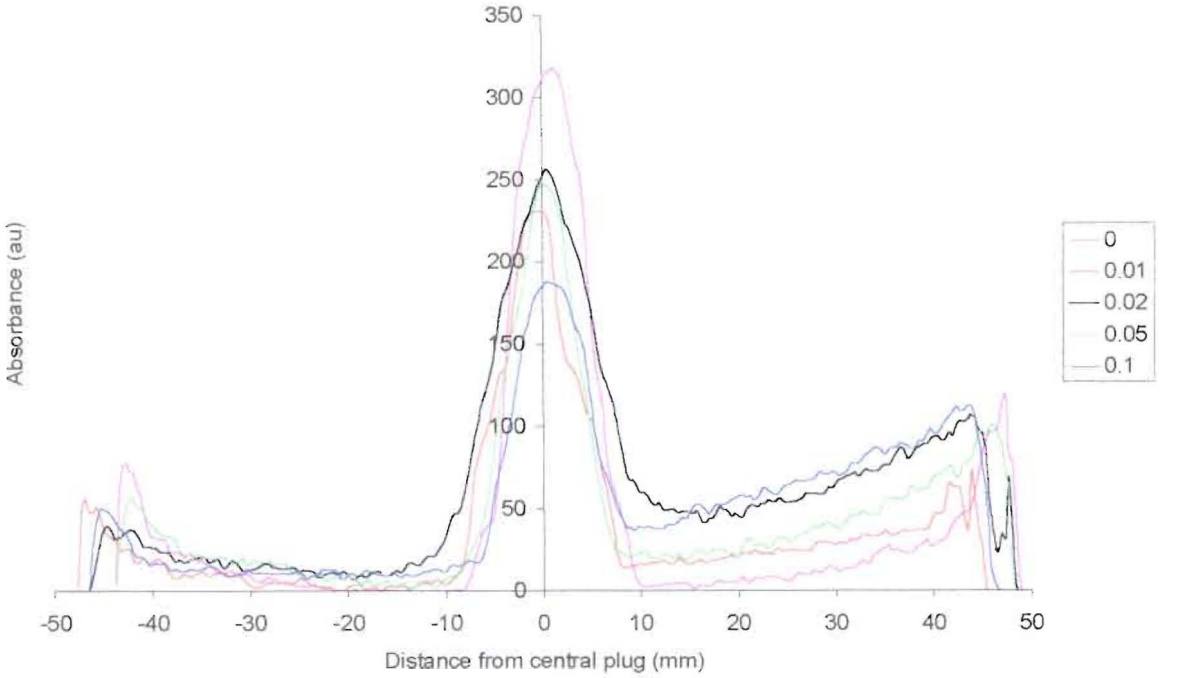


Figure 8 Colony thickness of *E.neoaphidis* on SDEM with 0%, 0.1%, 0.01%, 0.02% and 0.05% (a) 2 weeks (b) 3 weeks

Figure 8c

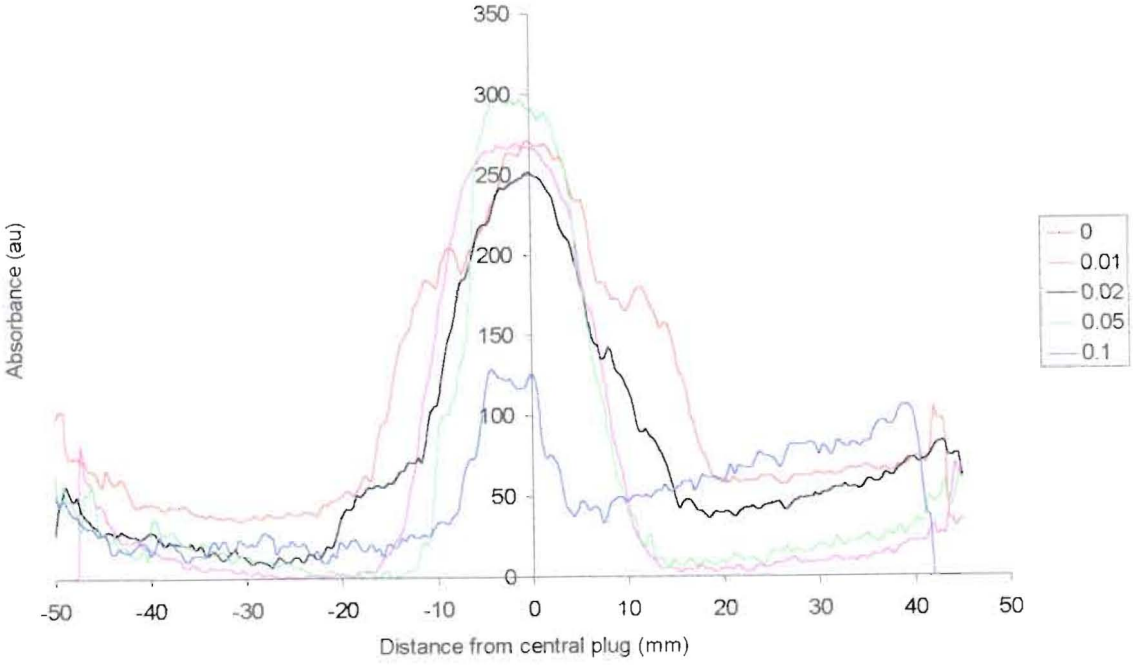


Figure 8d

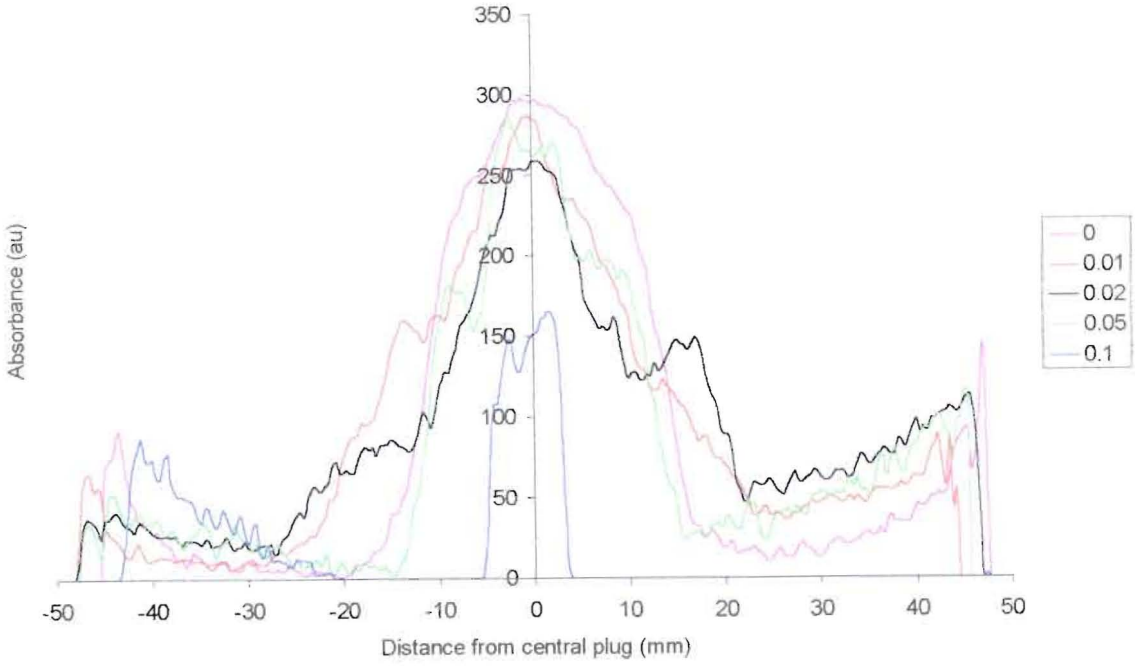


Figure 8 Colony thickness of *E.neoaphidis* on media with 0%, 0.1%, 0.01%, 0.02% and 0.05% after (c) 4 weeks (d) 5 weeks.

3.2 Comparison of growth and sporulation of *E. neoaphidis* growing on the aphid host, a complex medium (SEMA) and a semi-defined medium (SDEM)

3.2.1 Comparison of sporulation of *E. neoaphidis* growing on the aphid host, a complex medium (SEMA) and a semi-defined medium (SDEM)

In Figure 9a and b, approximately ¼ of the biomass grown on SDEM discharged conidia that were in clumps at 20 h then the proportion of clumps increased to almost ½ at 40 h. At 70 h in Figure 9c, conidia with germ tubes were observed. Both the clumps and conidia with germ tubes were manually removed from the screen or area and not counted. SDEM discharged conidia of mean volume 704 μm^3, while biomass grown on SDEMDG (with 32 g^{-1} glucose) discharged conidia of volume 954 μm^3 which were significantly different (P=0.05). In Figure 10a, SDEMDG discharged conidia of which ½ were in clusters or that were clumped together while in Figure 10b, SDEMHG (with 8 g^{-1} glucose) discharged conidia of volume 655 μm^3. In Figure 10a, biomass on SDEMDMP (with 10 g^{-1} mycological peptone) discharged conidia of volume 663 μm^3 while in Figure 9d, SDEMHMP (with 2.5 g^{-1} mycological peptone) discharged a lot of conidia (670 μm^3) of which ½ had germ tubes and also ¾ of the conidia were clumped. The volume of conidia discharged from SDEMDG were significantly larger (P=0.02) than conidia discharged from biomass on SDEMDMP.

In Figure 11a, biomass grown on SEMA medium discharged conidia (977 μm^3) which were significantly larger (P=0.001) than conidia discharged from both SDEM and SDEMDG (P=0.02). In Figure 11b, aphid cadavers produced small conidia (477 μm^3) which were significantly smaller (P=0.001) than conidia discharged from both SEMA and SDEMDG, but not significantly different from conidia discharged from both SDEM (P=0.07) and SDEMDMP (P=0.4).

Figure 9a

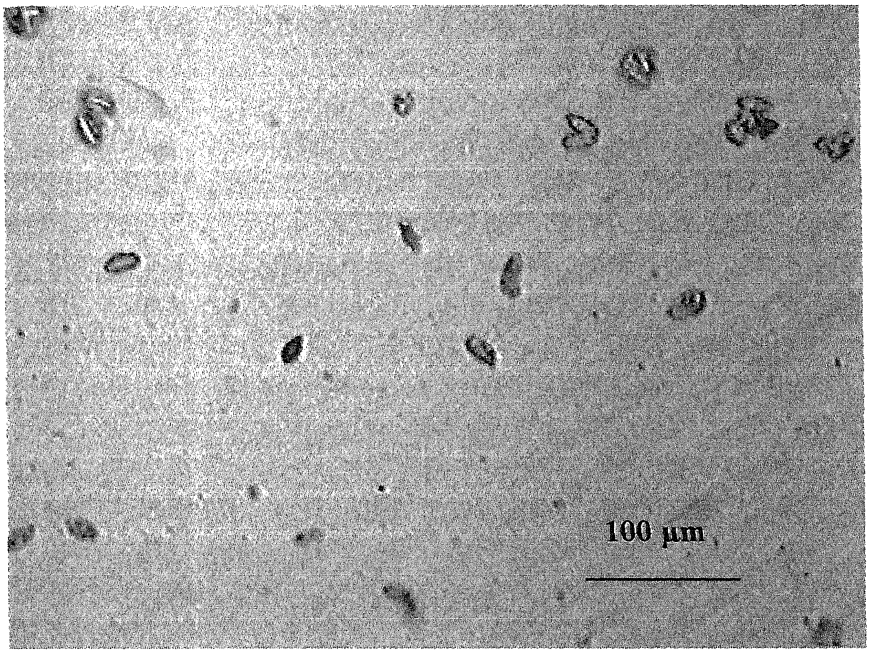


Figure 9b

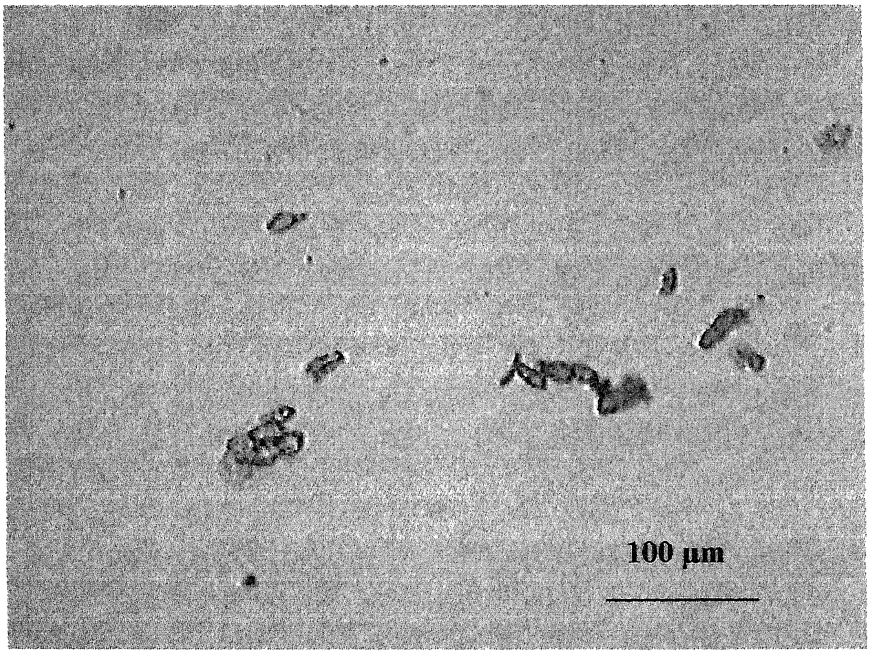


Figure 9 Conidia of *Erynia neoaphidis* discharged from SDEM control after (a) 20h and (b) 40h of exposure within the spore monitor

Figure 9c

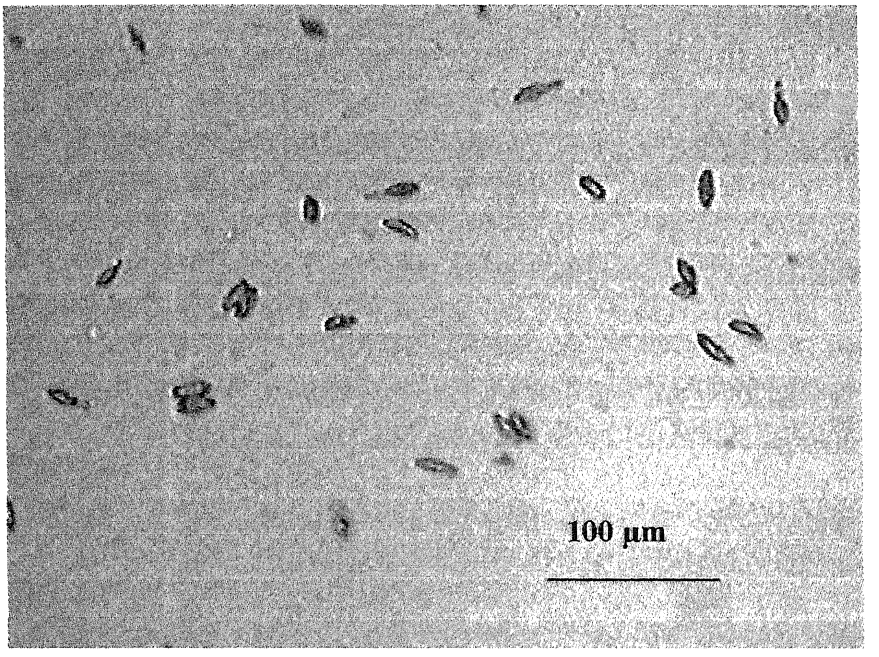


Figure 9c Conidia of *Erynia neoaphidis* discharged from SDEM at 70h of exposure within the spore monitor

Figure 10a

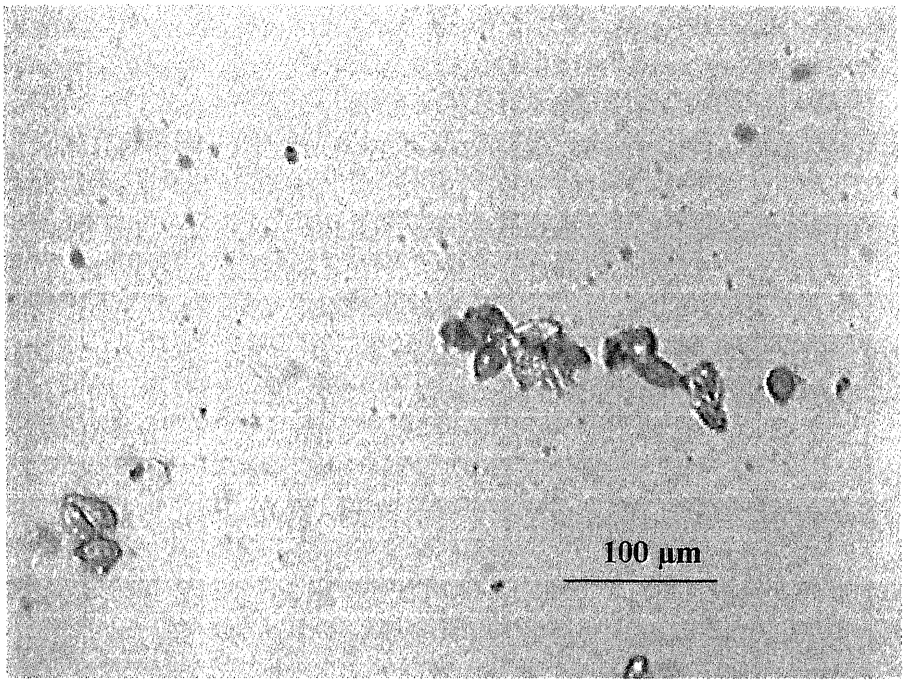


Figure 10b

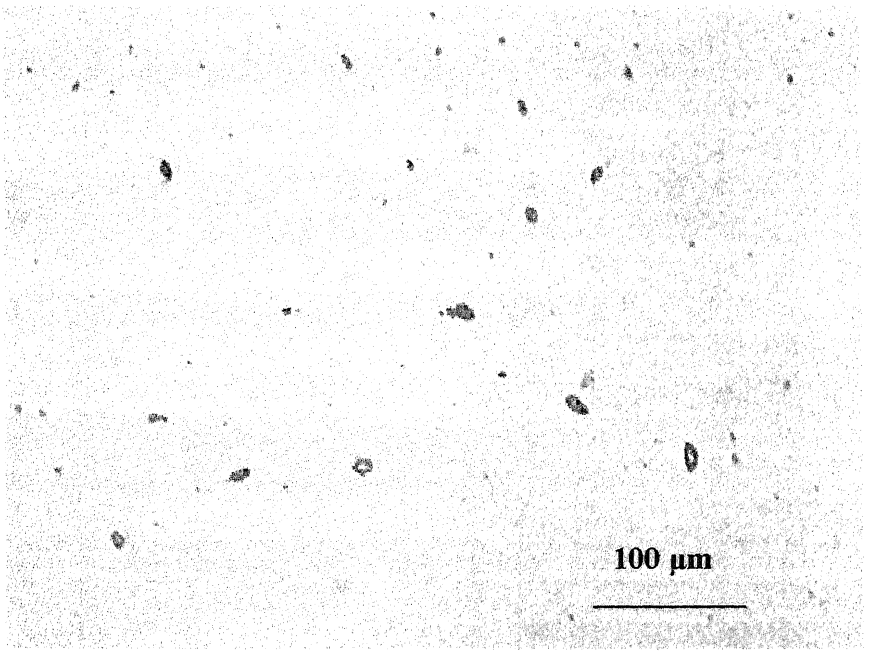
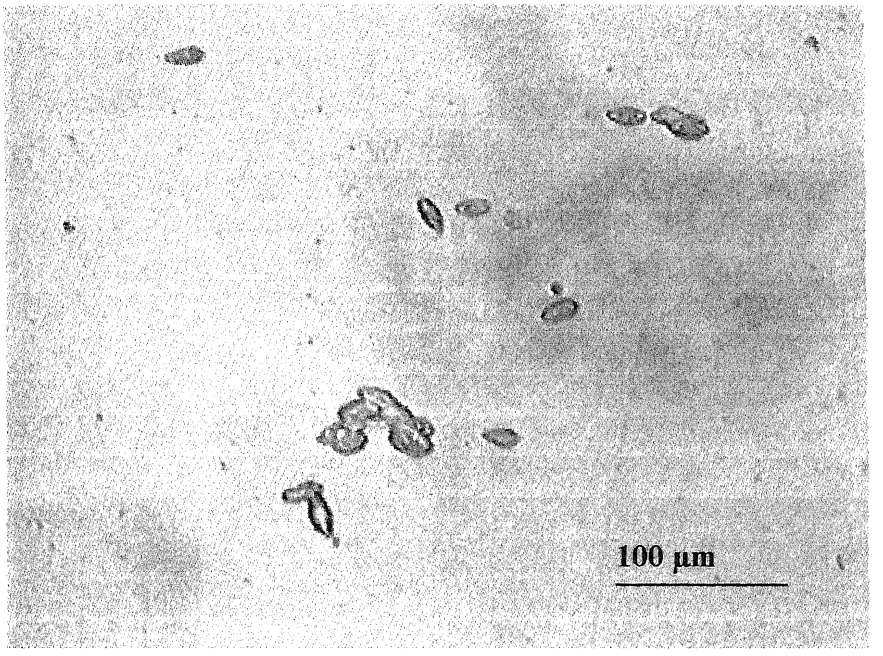


Figure 10 Conidia of *Erynia neoaphidis* discharged from (a) SDEMDG with 32 gl^{-1} glucose (b) SDEMHG with 8 gl^{-1} glucose

Figure 10c



10d

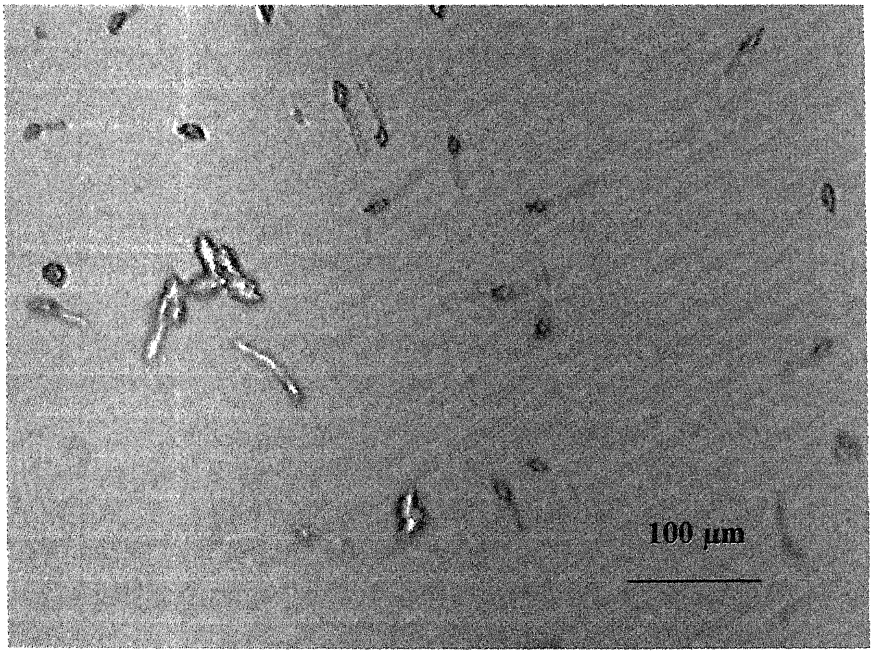


Figure 10 Conidia of *Erynia neoaphidis* discharged from (c) SDEMDMP with 10 gl^{-1} mycological peptone (d) SDEMHMP with 2.5 gl^{-1} mycological peptone

Figure 11a

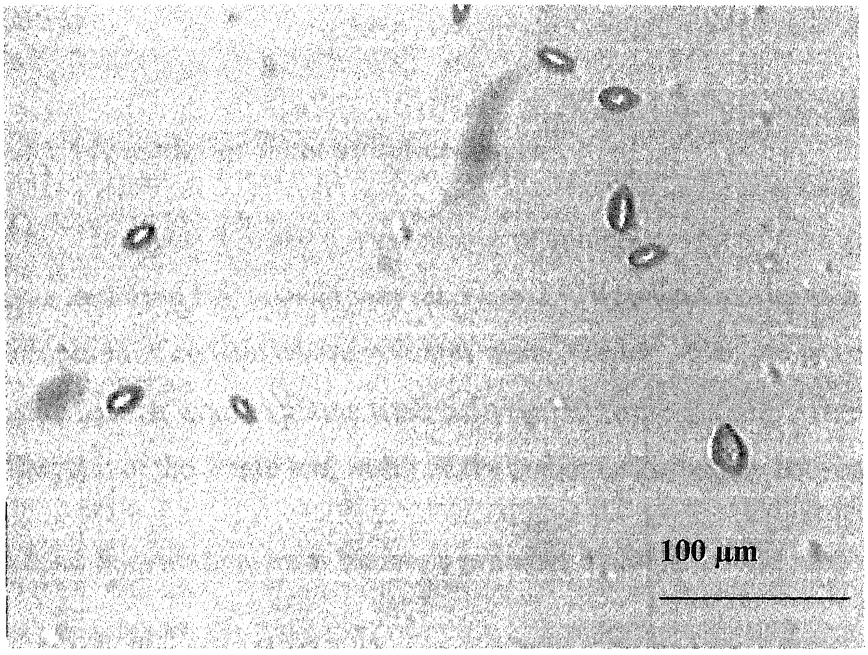


Figure 11b

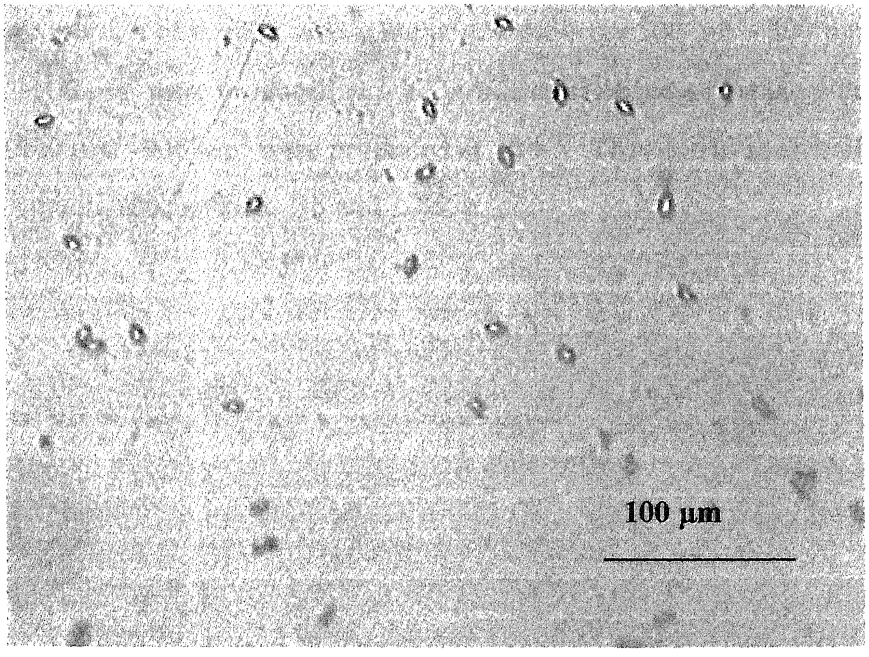


Figure 11 Conidia of *Erynia neoaphidis* discharged from (a) SEMA (b) Aphid cadavers

3.2.1.3 Changes in rate of conidial discharge and conidial volume over time on SDEM

3.2.1.1 Sporulation from aphid cadavers

In Figure 12a and b, the number of conidia produced from aphid cadavers increased from 504 conidia/mm^2 at 5 h to 2,978 conidia/mm^2at 45 h. The highest proportion of conidia whose volumes were 200 μm^3 –500 μm^3 were produced at 5 h, while conidia whose volume were between 500 μm^3 -900 μm^3 were produced at 45 h. The ratio of the length and width of the conidia was roughly between at 1.8-2.

3.2.1.2 Sporulation from biomass growing on SEMA solid medium

In Figure 13a and b, the number of conidia produced from biomass grown on SEMA medium increased from 69 conidia/mm^2 at 5 h^{-1} to 203 conidia/mm^2at 20 h^{-1}. Then the number decreased gradually till 45 h^{-1} to 100 conidia/mm^2. The highest proportion of conidia whose mean volume were 200 μm^3 –300 μm^3 and 1000 μm^3 – 1500 μm^3 were produced at 5 h^{-1}, while conidia whose mean volume were between 400 μm^3 -900 μm^3 were produced at 45 h^{-1}. The ratio of the length and width of the conidia was between 1.6 and 1.85.

Figure 12a

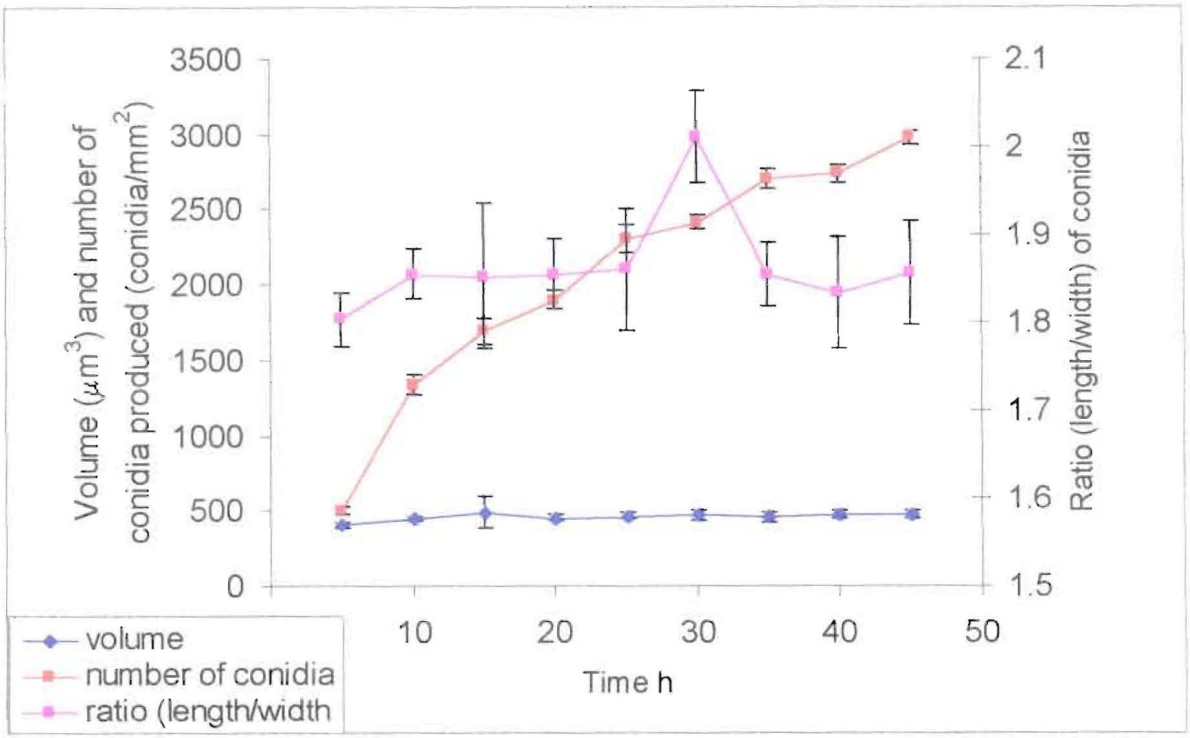


Figure 12b

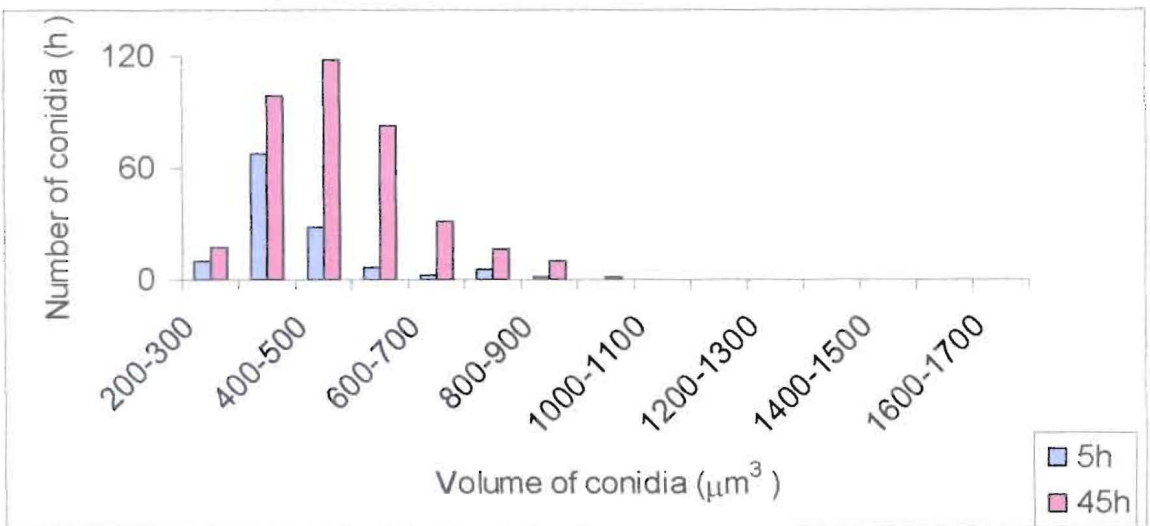


Figure 12 (a) Volume, ratio of width and length and number of conidia produced and (b) volume distribution of conidia produced at different time intervals from mycelia grown in aphids. The error bars indicate standard errors of the means of the number of conidia discharged for 10 replicates.

Figure 13a

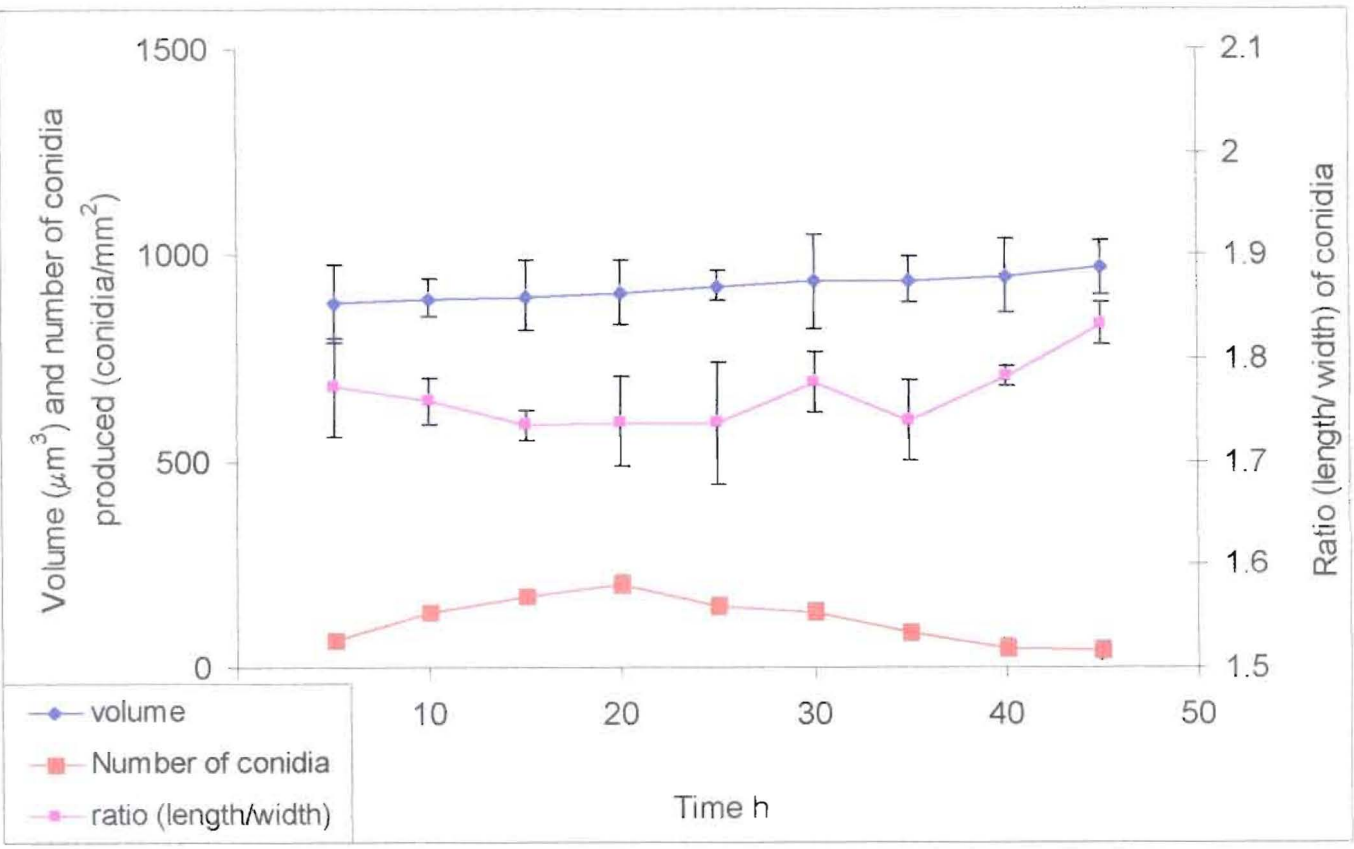


Figure 13b

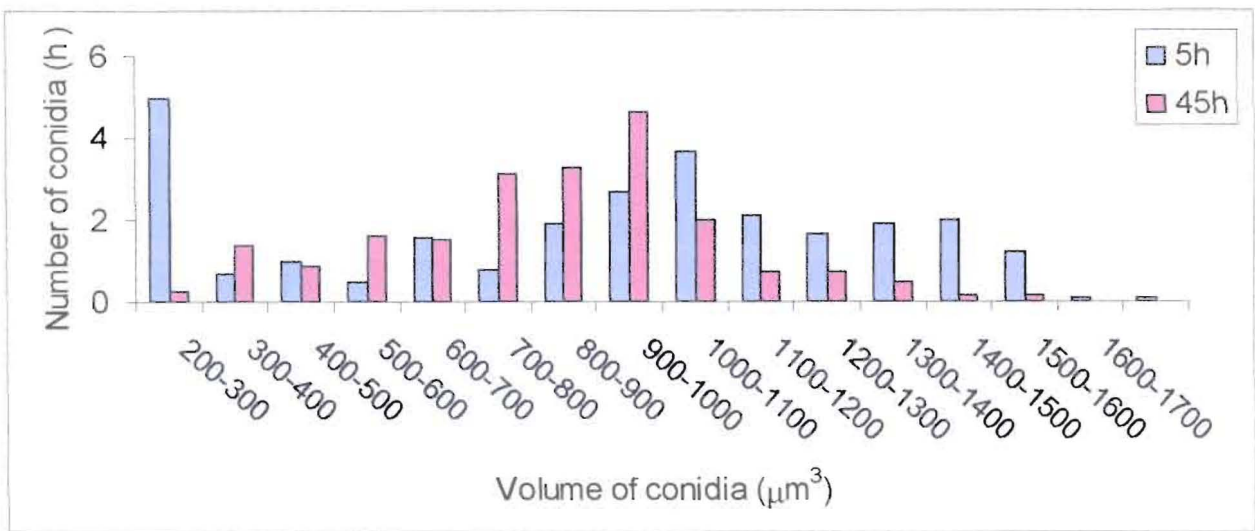


Figure 13 (a) Volume, ratio of width and length and number of conidia produced and (b) volume distribution of conidia produced at different time intervals from mycelium grown in SEMA. The error bars indicate standard errors of the means of the number of conidia discharged for 10 replicates.

3.2.1.3 Changes in rate of conidial discharge and conidial volume over time on SDEM

In Figure14a and b, the greatest rate of conidial discharge by biomasss grown on SDEM occurred at 10 h^{-1} (381conidia/mm^2). The greatest rate of conidial discharge by biomasss grown on SDEM occurred at 10 h (381conidia/mm^2). The rate of discharge then gradually decreased till 70 h were only 91conidia/mm^2 were produced. The largest proportion of conidia of volume between 400 um^3-1400 um^3 were produced at 10 h. At 70 h conidia of volume 200 um^3-400 um^3 were produced. The ratio (length and width) of the conidia was 2.13 at 10 h but this gradually decreased to approximately 1.1 at 70 h. Larger conidia were produced in the first 10 h compared to the other times of exposure.

3. 2.1.4 Changes in rate of conidial discharge and conidial volume over time on SDEMDG (32 gl^{-1}) glucose

In Figure 15a and b, the greatest rate of conidial discharge by biomasss grown on SDEMDG occurred at 30 h where 172 conidia/mm^2were produced, then the number of conidia produced gradually decreased till 33 conidia/mm^2 at 60 h. At 70 h the number of conidia produced briefly increased to 70 conidia/ mm^2. At 10 hand 70 h the highest proportion of conidia of volume between 200 μm^3 –600 μm^3 were produced. The length and width ratio of the conidia was 1.8 at 10 h then increased to 2 at 50 h but this gradually decreased to almost 1.3 at 70 h. Large conidia of volume 954 μm^3 to 885 μm^3 were produced at both 20 h and 40 h, while smaller conidia (500 μm^3) were discharged at 70 h.

3. 2.1.5 Changes in rate of conidial discharge and conidial volume over time on SDEMHG (with 8 gl^{-1} glucose)

In Figure 16a and b, the rate of conidial discharge by biomass grown on SDEMHG decreased from 223 conidia/mm^2 at 10 h till 70 h where only 69 conidia/mm^2 were produced. The size of conidia produced increased gradually from 433 μm^3 at 10 h to a maximum of 655 μm^3 at 30 h. Small conidia of volume 338 μm^3 were produced at 70 h. The length to width ratio of the conidia was 1.6 at 10 h then

decreased to 1.3 at 20 h then increased to 1.75 at 30 h and gradually decreased to almost 1.1 at 70 h. Smaller conidia were discharged at 70 h

3. 2.1.6 Changes in rate of conidial discharge and conidial volume over time on SDEMDMP (with 10 gl^{-1} mycological peptone).

In Figure 17a and b, the rate of conidial discharge by biomasss grown on SDEMDMP increased from 154 conidia/mm^2at 10 h to 309 conidia/mm^2 at 30 h, and then decreased till 70 h where only 53 conidia/mm^2 were produced. Large conidia of volume 700 μm^3 were produced at both 20 h and 40 h. The largest proportion of small conidia were discharged at 70 h (200 μm^3). The length and width ratio of the conidia was 2.4 at 10 h then gradually decreased to almost 1.2 at 70 h.

3. 2.1.7 Changes in rate of conidial discharge and conidial volume over time on SDEMHMP (with 2.5 gl^{-1} mycological peptone).

In Figure 18a and b, the rate of conidial discharge by biomasss grown on SDEMHMP decreased gradually from 314 conidia/mm^2 at 10 h to 8 conidia/mm^2 at 70 h. The size of conidia produced increased from 593 μm^3 at 10 h to a maximum of 699μm^3 at 40 h. The volume then gradually decreased to almost 168 μm^3 at 70 h. The length and width ratio of the conidia was 1.9 at 10 h then gradually decreased to almost 1.13 at 70 h. Small conidia were produced at 70 h.

Figure 14a

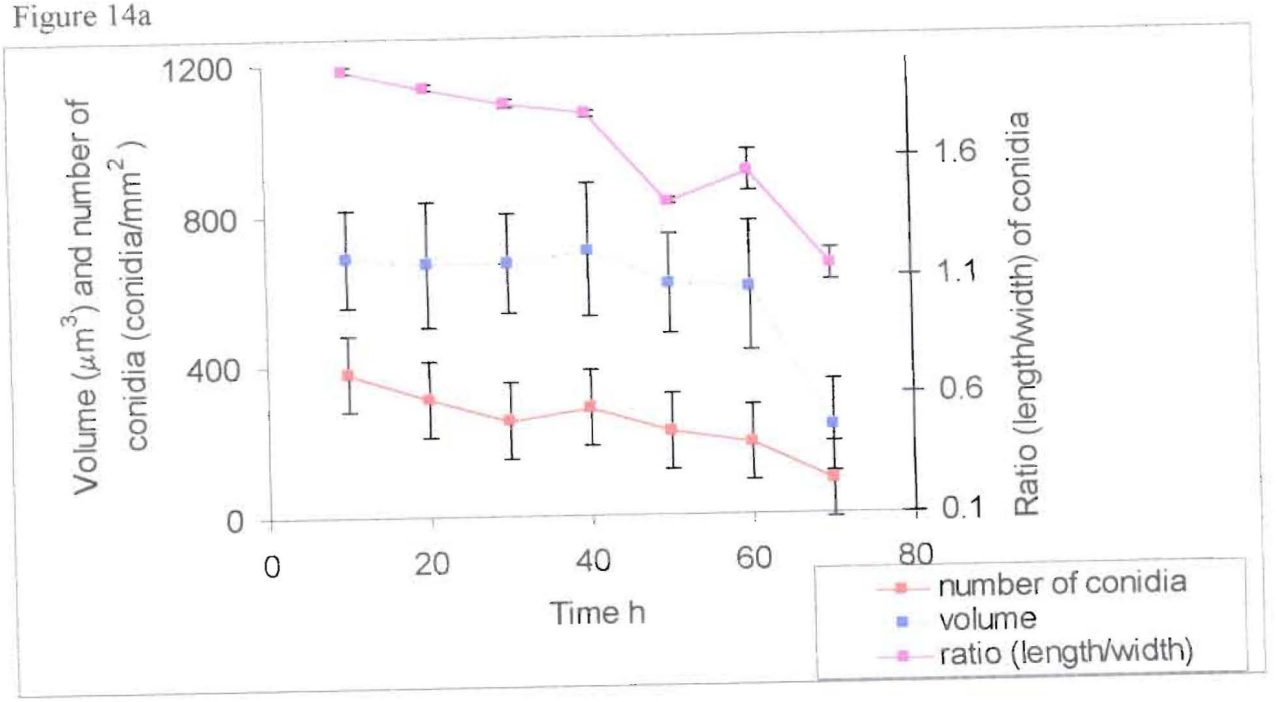


Figure 14b

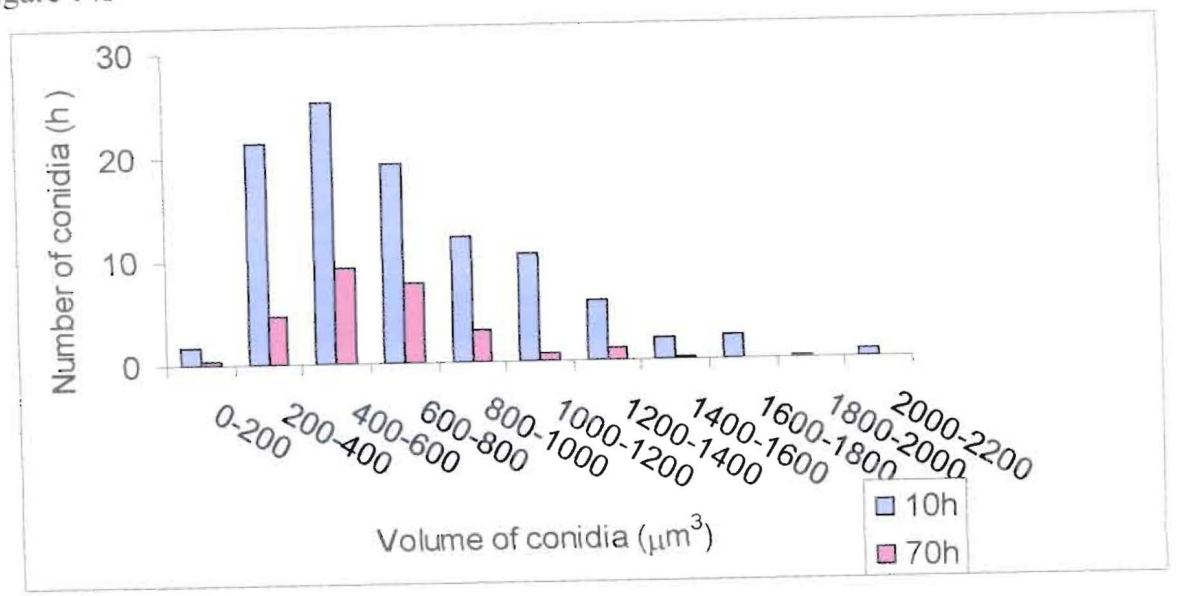


Figure 14 (a) Volume, ratio of width and length and number of conidia produced and (b) volume distribution of conidia produced at different time intervals from mycelia grown in SDEM. The error bars indicate standard errors of the means of the number of conidia discharged for 10 replicates.

Figure 15a

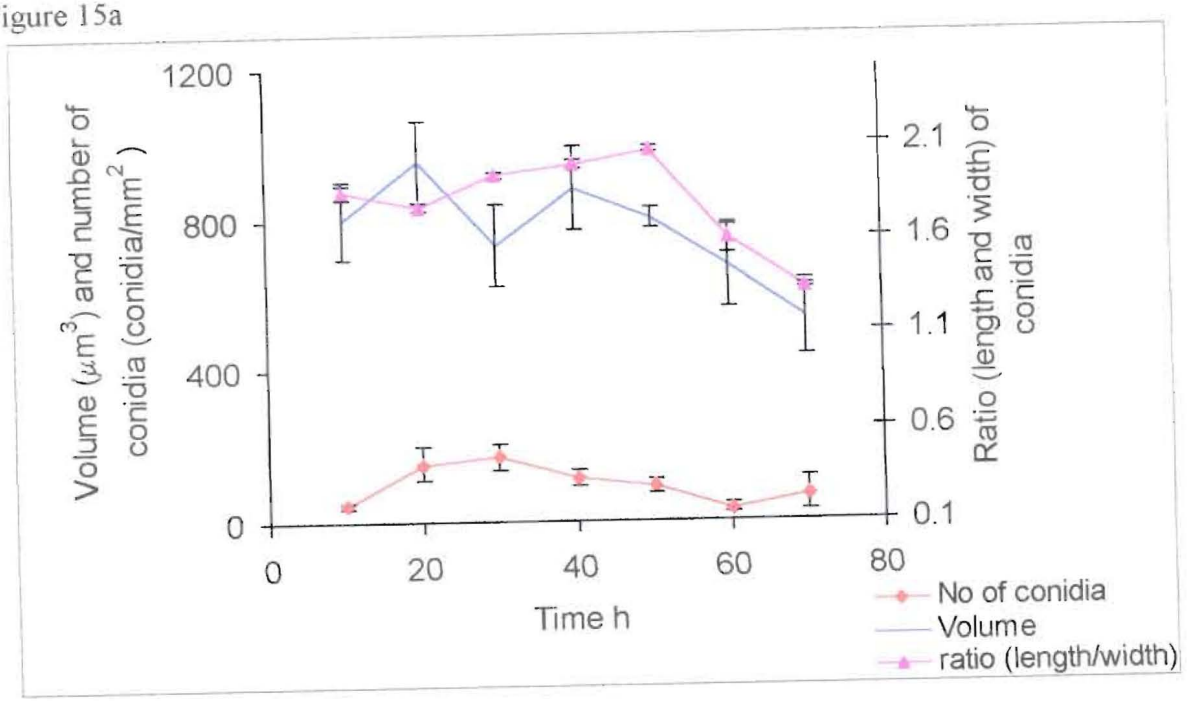


Figure 15b

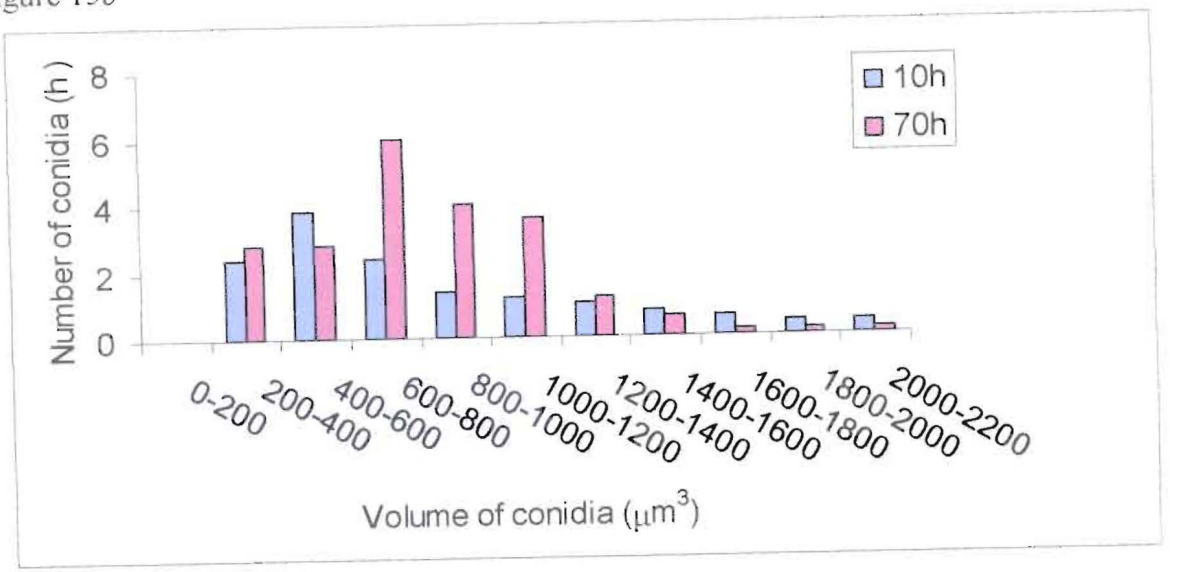


Figure 15 (a) Volume, ratio of width and length and number of conidia produced and (b) volume distribution of conidia produced at different time intervals from mycelia grown in SDEMDG (with 32 gl^{-1} glucose). The error bars indicate standard errors of the mean of the number of conidia discharged for 10 replicates.

Figure 16a

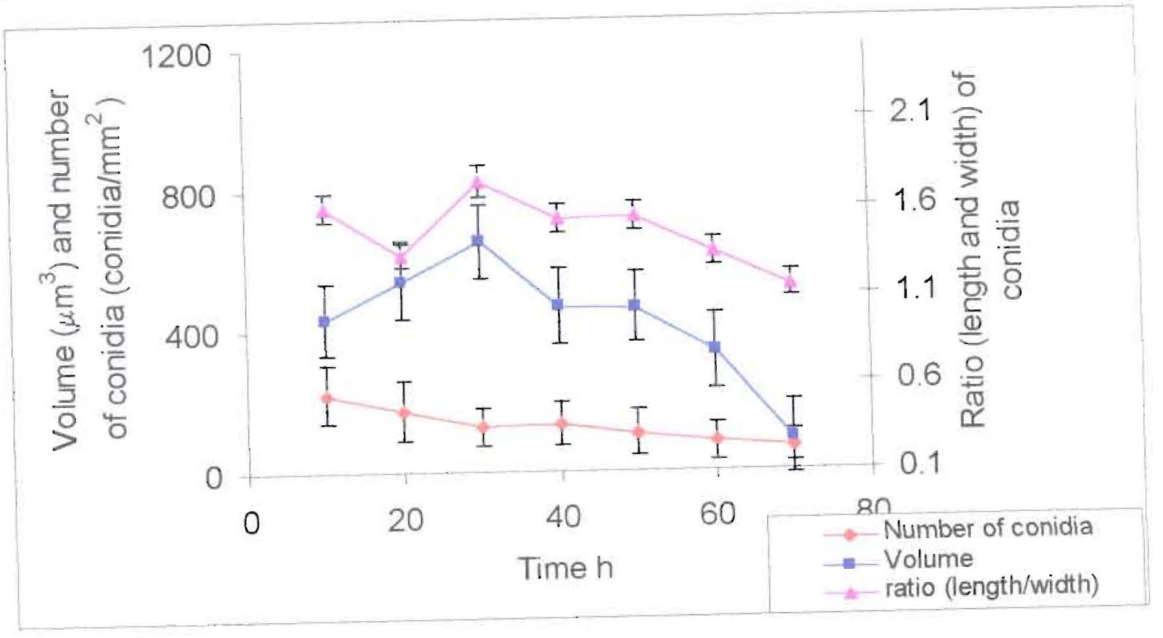


Figure 16b

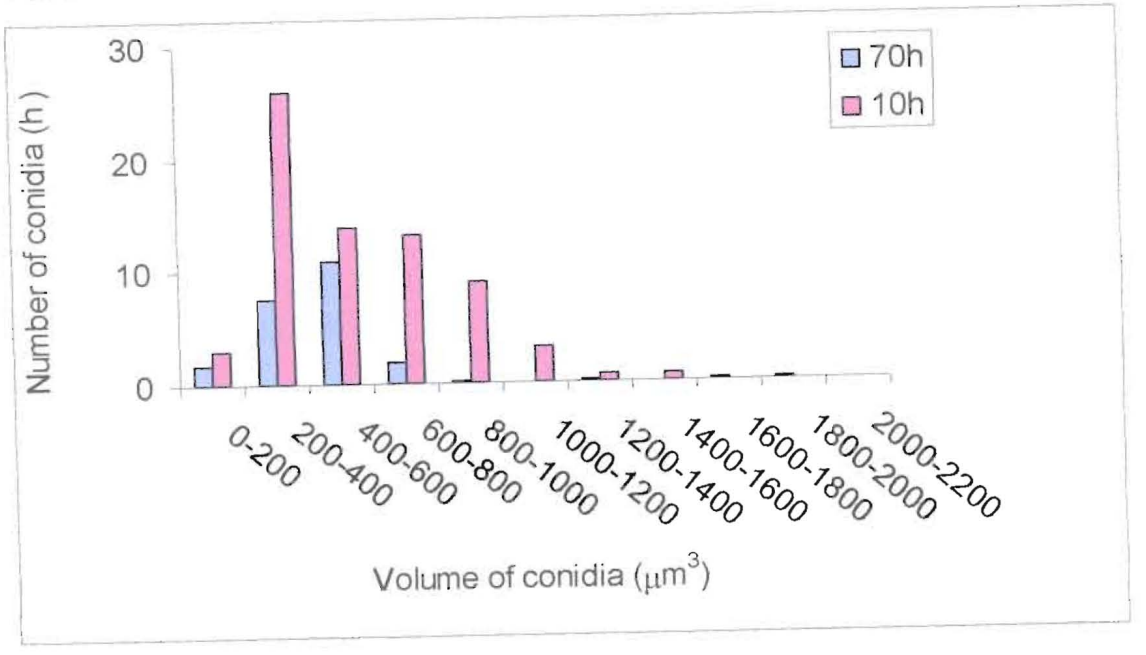


Figure 16 (a) Volume, ratio of width and length and number of conidia produced and (b) volume distribution of conidia produced at different time intervals from mycelia growing in SDEMHG (with 8 gl^{-1} glucose)). The error bars indicate standard errors of the mean of the number of conidia discharged for 10 replicates.

Figure 17a

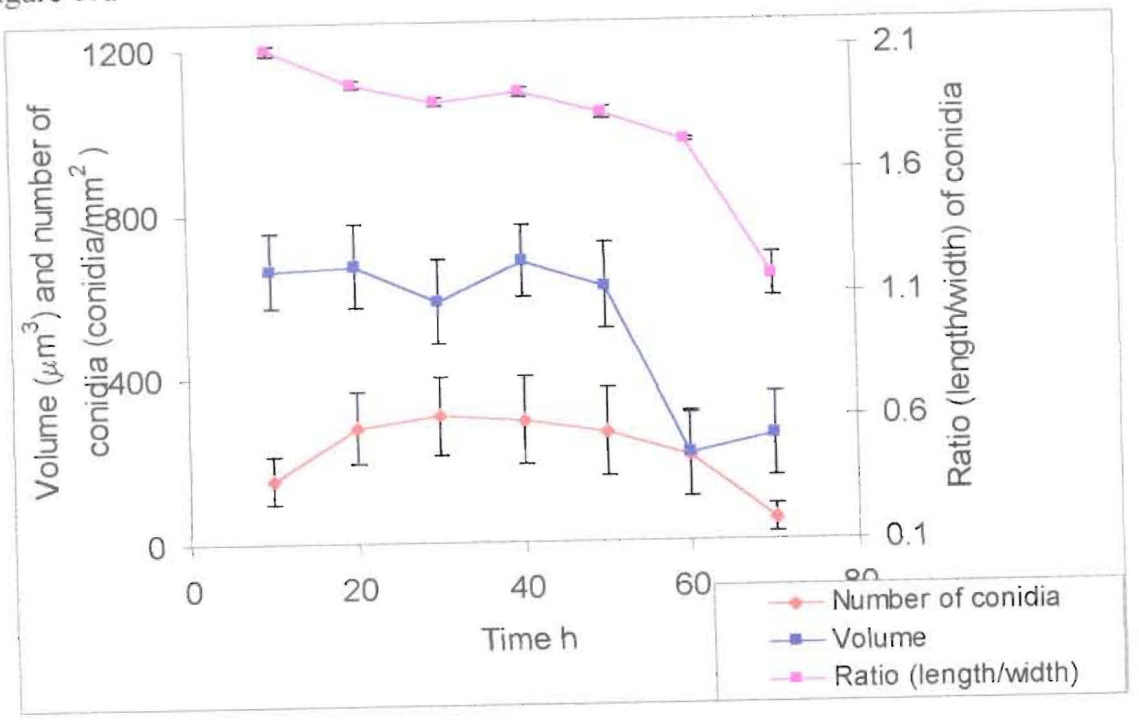


Figure 17b

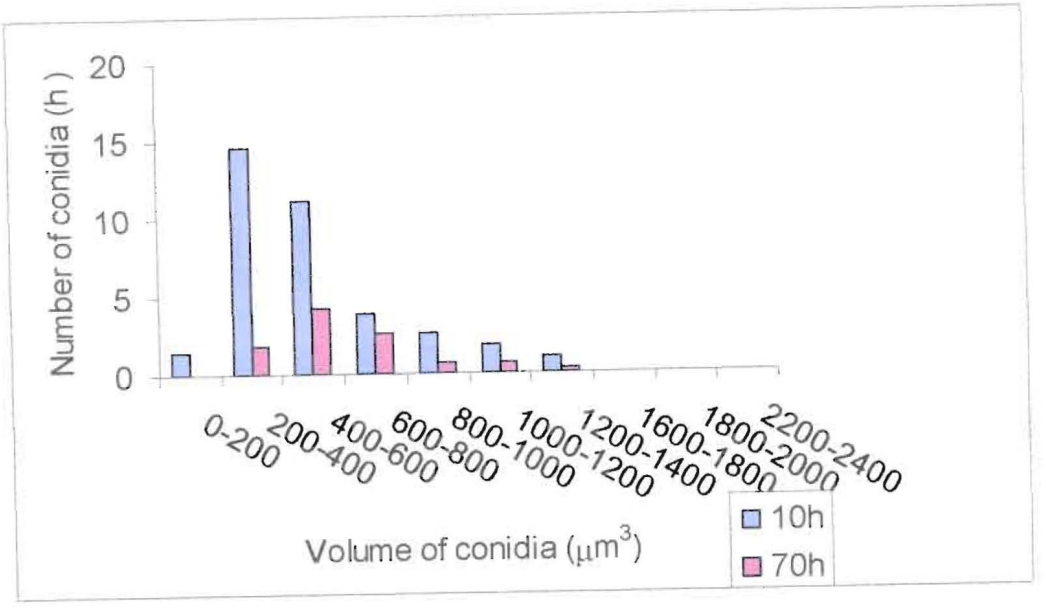


Figure 17 (a) Volume, ratio of width and length and number of conidia produced and (b) volume distribution of conidia produced at different time intervals from mycelia growing in SDEMDP (with 10 gl^{-1} mycological peptone). The error bars indicate standard errors of the mean of the number of conidia discharged for 10 replicates.

Figure 18a

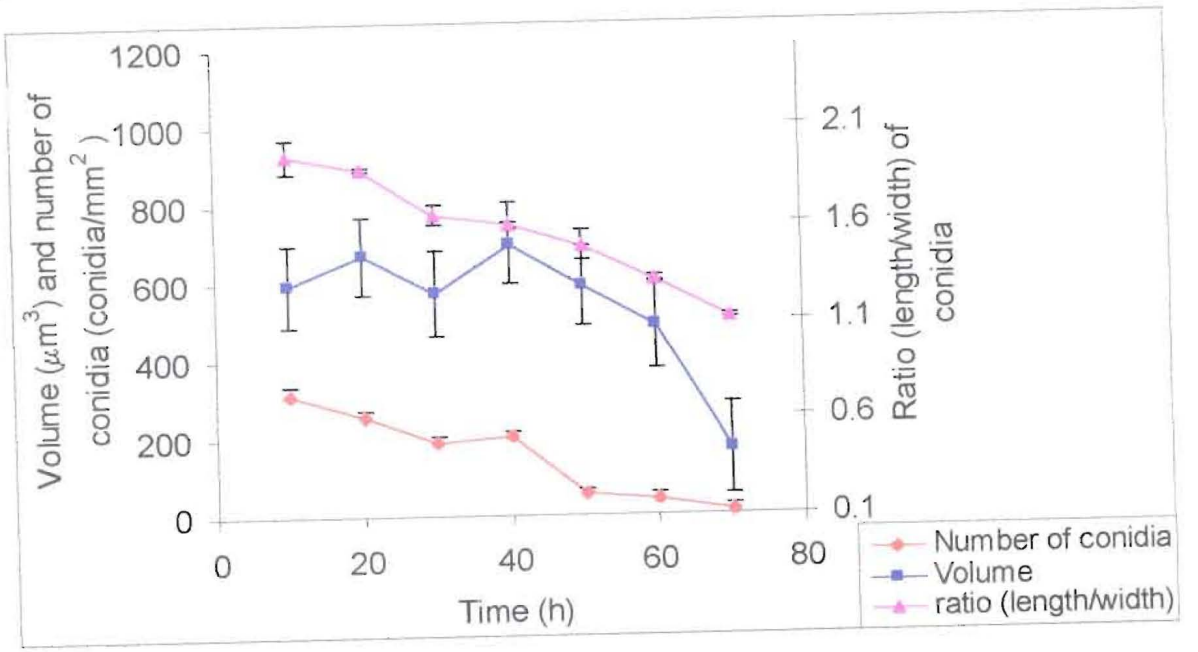


Figure 18b

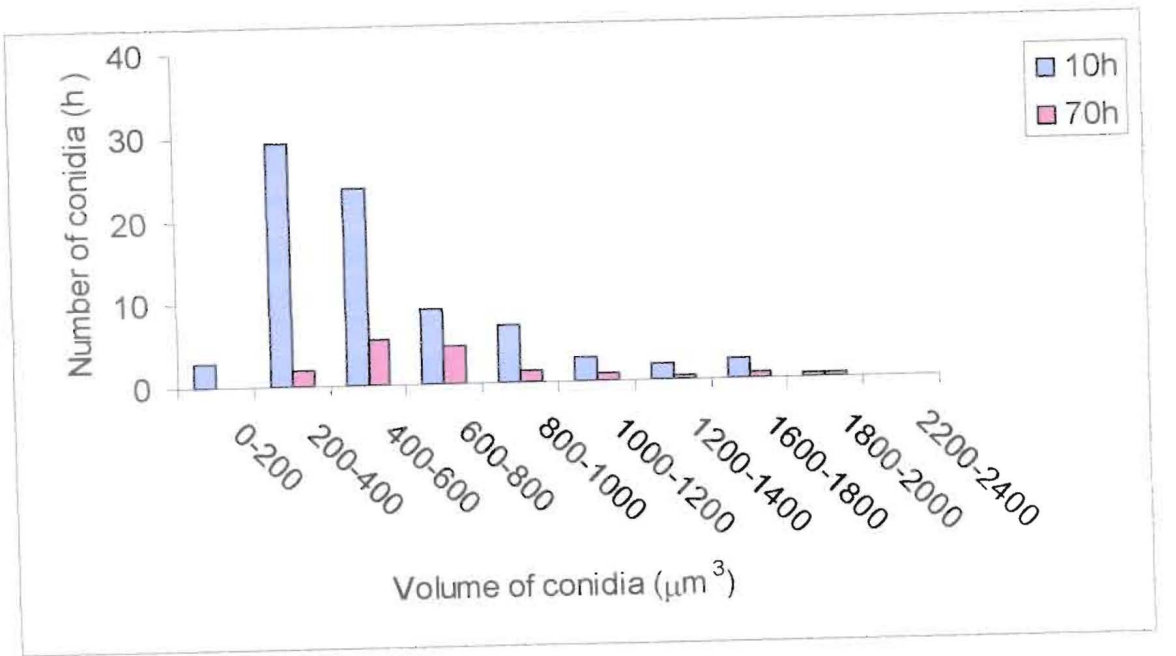


Figure 18 (a) Volume, ratio of width and length and number of conidia produced and (b) volume distribution of conidia produced at different time intervals from mycelium growing in SDEMHMP (with 2.5 gl^{-1} mycological peptone). The error bars indicate standard errors of the mean of the number of conidia discharged for 10 replicates.

3.2. 2 Adaptation of *E. neoaphidis* to growth on the semi- defined *Erynia* medium SDEM

Table 3 Key to abbreviations for enzyme names used when reporting results of API ZYM tests.

Enzyme abbreviation	Enzyme
Alp	Alkaline phosphatase
C4	Esterase
C8	Esterase lipase
C14	Lipase
Leu	Leucine arylamidase
Val	Valine arylamidase
Cys	Cystine arylamidase
Trp	Trypsin
Chy	α-chymotrypsin
Acp	Acid phosphatase
Nap	Napthol-AS-BI- phosphohydrolase
βGr	β- glucuronidase
α Gs	α - glucosidase
β Gs	β - glucosidase
NaG	N-acetyl- β - glucosaminidase
Man	α -mannosidase

As recommended by the manufacturer, the API ZYM results "1" till "4" are just arbitrary units, which signify a colour concentration or shade from a key given in the API ZYM kit. For example, if the presence of an enzyme was given by the colour purple, then "1" is a very light shade of purple while "5" is a dark purple, the rest of the numbers 2,3 and 4 have colours between light and dark purple. The Kr was 51.33 um h^{-1} on the 2nd subculture onto SDEM which was more than twice the Kr (20.81 um h^{-1}) on the 1st subculture onto SDEM from SEMA. ($P<0.05$). In figure 19, on the 1st subculture of *E. neoaphidis* from SEMA medium to SDEM, the lipid catabolizing enzymes alkaline phosphatase and esterase were produced, but esterase was produced in smaller quantities giving an APIZYM result of 1 compared to the 2nd subculture where esterase gave an APIZYM result of 4. On the 2nd subculture, in addition to the catabolizing enzymes produced on the 1st subculture, esterase lipase was produced. The protein catabolizing enzyme trypsin and the amino acid catabolising enzyme leucine arylamidase were produced in large amounts giving an APIZYM result of 5 on both the 1st and 2nd subculture on SDEM, while the other protein catabolizing enzyme α-chymotrypsin and amino acid catabolising enzymes valine arylamidase, cystine arylamidase were produced in small amounts giving APIZYM results of 3,1 and 1 respectively. However, on the 2nd subculture on SDEM the enzymes valine arylamidase, cystine arylamidase and α-chymotrypsin were produced at APIZYM results of 4,3, and 4 respectively. Both the 1st and 2nd subcultures produced acid phosphatase and napthol-AS-BI-phosphatase; the latter was produced in larger amounts where an APIZYM result of 5 was observed on the 2nd subculture as compared to the APIZYM result of 3 observed on the 1st subculture. Sugar catabolizing enzymes α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase and α–mannosidase were produced on both subcultures. Larger amounts of α–mannosidase and α-glucosidase were produced on the 2nd subculture and were APIZYM results of 5 and 4 respectively were achieved, while on the 1st subculture α–mannosidase and α-glucosidase gave an APIZYM result of 3.

Figure 19a

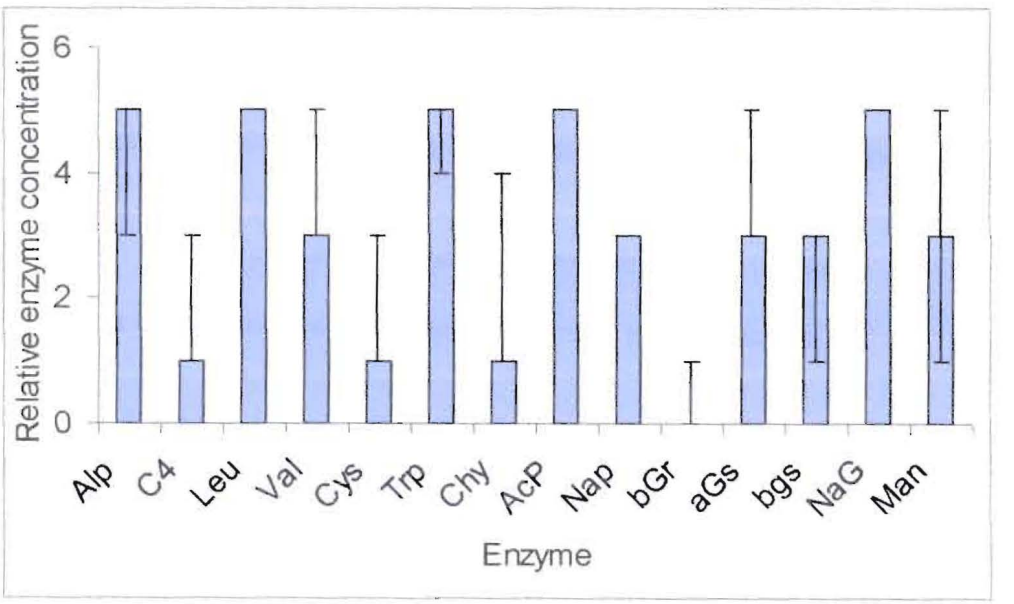


Figure 19b

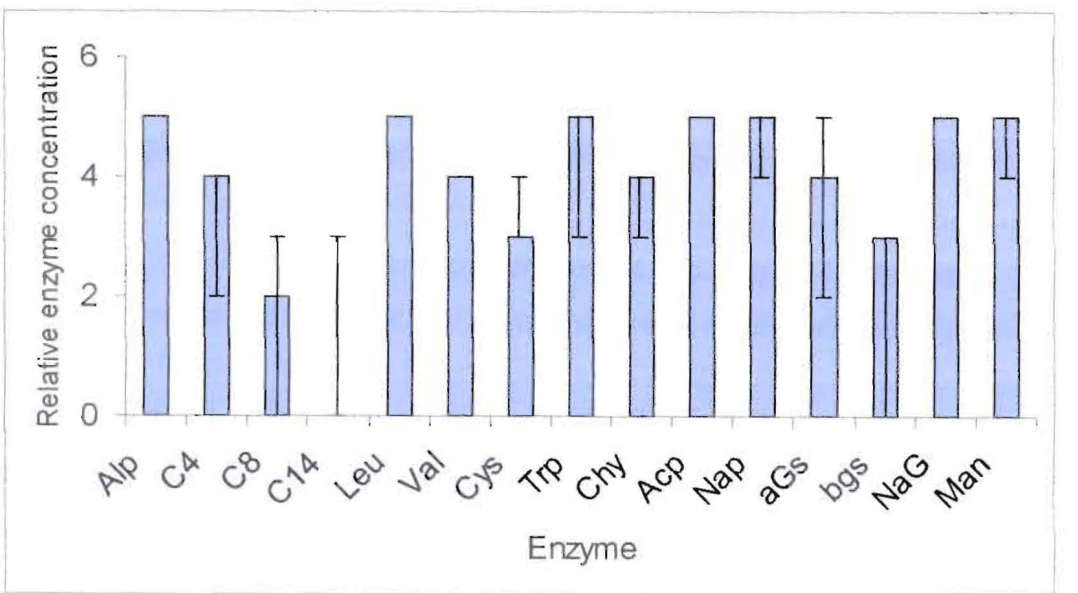


Figure 19 Type and relative enzyme concentration of enzymes produced by E. neoaphidis when subcultured onto SDEM medium from SEMA medium in the (a)1st subculture (b) 2nd subculture. The error bars show the range above and below the median for 3 replicates. Key to enzyme abbreviations can be found on page no 86. The enzymes on the API ZYM result sheet that have not been shown did not appear in the tested samples. Where no bars are shown for ranges, results were identical for all replicates.

3.2.3 Use of surfactant solutions to harvest conidia of *E. neoaphidis*

The number of conidia per area counted on the haemocytometer was (1.5) from the culture using distilled water, while 72 conidia/mm^2 were counted from the culture using 0.1% tween 80. Using 1% tween 80, 36 conidia/mm^2 were counted.

3.3 Growth of *Erynia neoaphidis* in liquid and fed-batch culture

3.3.1 Effect of glucose concentration on growth of *E. neoaphidis*

In Figure 20, the cultures growing in SDEMDG (with 32 gl^{-1}glucose), grew very fast (over a period of 96 h) and were thicker, containing lots of hyphae full of cytoplasm indicating viability, as compared to cultures growing in SDEM medium (with 16 gl^{-1} glucose) which became ready for subculturing after a period of 144 h-168 h.

When *E. neoaphidis* was grown in SDEM liquid medium, the culture was in a lag phase for 36 h from the time of inoculation with a dry weight of 6.11 gl^{-1}, when it entered the exponential phase over a period of 50 h its dry weight was 11.30 gl^{-1}. The culture then went into the stationery phase after 140 h where it had a dry weight of 14.32 gl^{-1} and then the death phase with a dry weight of 6.98 gl^{-1}. The biomass concentration was about 6.5 gl^{-1} at 100 h and it showed a steady increase in growth over a period of 100 h. When the fungus was grown in SDEMDG, the culture was in a lag phase 25 h from the time of inoculation where the dry weight was 4.10 gl^{-1} it went into the exponential phase over 96 h, where the dry weight was 22.70 gl^{-1}. The culture then went into the stationery phase over a period of 170 h with a dry weight of 23.33 gl^{-1} followed by the death phase with a dry weight of 17.13 gl^{-1}. .

Apart from the lag phase (36 h) where the dry weights of *E. neoaphidis* grown in both SDEM and SDEMDG were not significantly different, the dry weights in the exponential, stationery and death phase were all significantly higher in SDEMDG than the phases when the fungus was grown in SDEM ($P<0.05$).

3.3.2 Comparison of growth of *Erynia neoaphidis* in both SDEM and GASP liquid medium

In Figure 20, when *E. neoaphidis* was grown in GASP liquid medium, the cultures were transparent and the culture looked thick after 96 h days. Clusters of mycelia could be seen floating in the medium. The culture in GASP was in lag phase over a period 50 h where the mean dry weight was 2.88 gl^{-1} while the lag phase of cultures grown in SDEM lasted for 36 h with a mean dry weight of 6.11 gl^{-1}, when the culture in GASP went into the exponential phase at 96 h the mean dry weight was 5 gl^{-1} while in SDEM the exponential phase was over a period of 50 h with a mean dry weight of 11.30 gl^{-1}. The culture in GASP then went into the stationery phase where the mean dry weights increased to mean 8.93 gl^{-1} at 240 h while in SDEM at 240 h the dry weight was mean 5.088 gl^{-1}. The lag phase, the exponential, stationery and death phase were all significantly longer in GASP compared to the duration the culture took in SDEM ($P<0.05$).

Figure 20

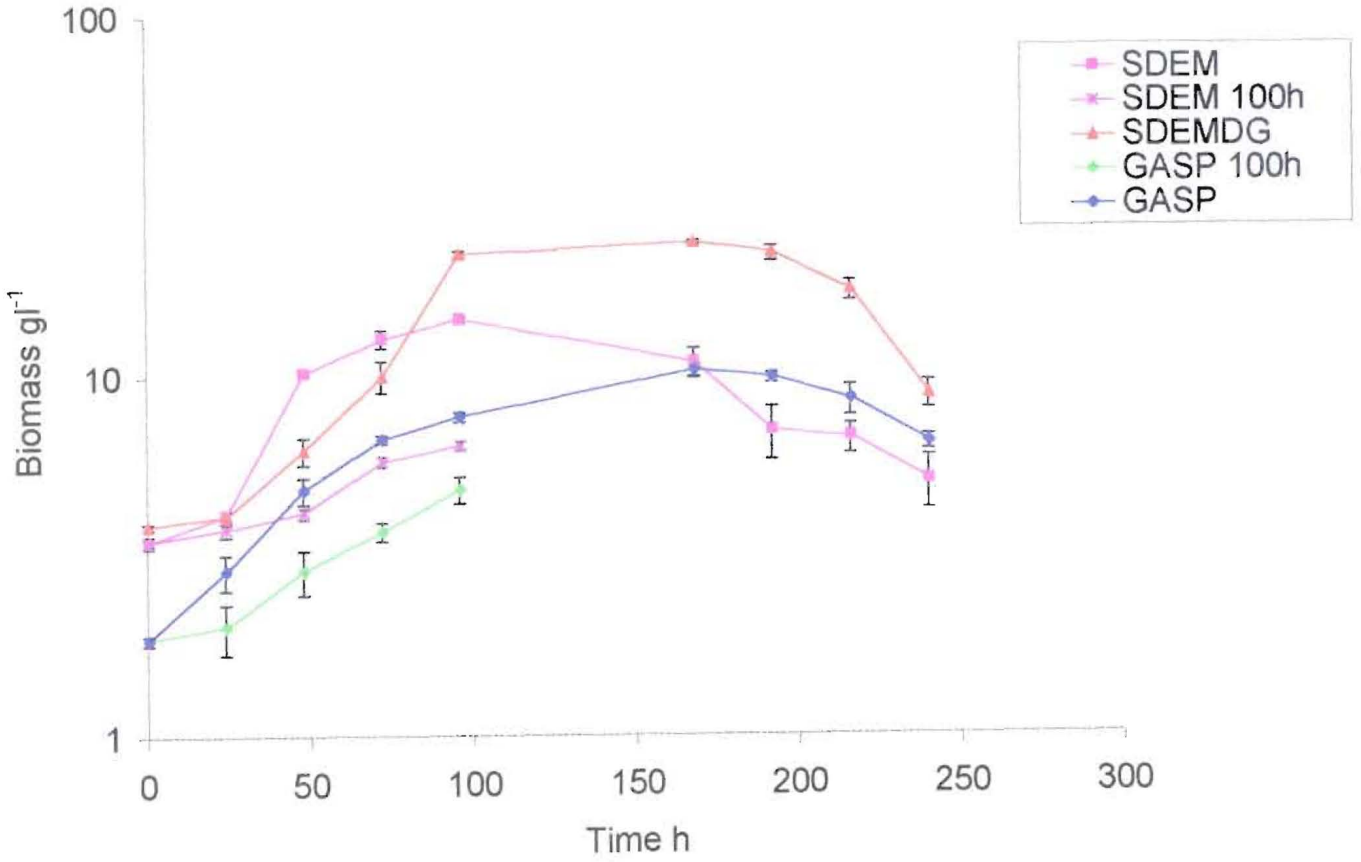


Figure 20 Growth of *Erynia neoaphidis* upto 100 h and 250 h in both GASP and SDEM, and SDEM with double the original concentration of (with 32 gl-l) glucose. Errors bars show mean of weights for 3 replicates.

3.3.3 Growth of *Erynia neoaphidis* in SDEMDG (with 32 gl^{-1} glucose) liquid medium fed-batch culture in fermenter

In Figure 21, the culture was in lag phase for 25 h with a mean dry weight of 5.1 gl^{-1}, 250 ml of fresh medium were added to the culture and the impeller speed was increased to 200 rpm. To the culture 2 L of fresh medium were added at 96 h, after which it went into the exponential phase over a period of 100 h with a mean dry weight of 10.02 gl^{-1} followed by the stationery phase for a period of 160 h after inoculation where the mean dry weight was 10.12 gl^{-1}, then the death phase where at 384 h the mean dry weight was 7.7 gl^{-1}. The biomass was around 10.23 gl^{-1}at around 120 h.

Compared to cultures grown in shaker flasks in SDEMDG, at 25 h the mean dry weight was 4.10 gl^{-1} which was significantly lower than cultures grown in the fermenter culture ($P<0.05$). During the exponential phase in both cultures grown in shaker flasks and fermenter, over a period of 100 h, the mean dry weight was 22.3 gl^{-1} in shaker flasks which was significantly higher than cultures grown in fermenter cultures which had a mean dry weight of 10.02 gl^{-1} ($P<0.05$). At the end of the exponential phase in cultures grown in shaker flasks, the mean dry weight was 21.6 gl^{-1} whereas the dry weight of the cultures grown in the fermenter over a period of 240 h the mean dry weight was 2.0 gl^{-1} which was significantly lower than cultures grown in shaker flasks ($P<0.05$).

Figure 21

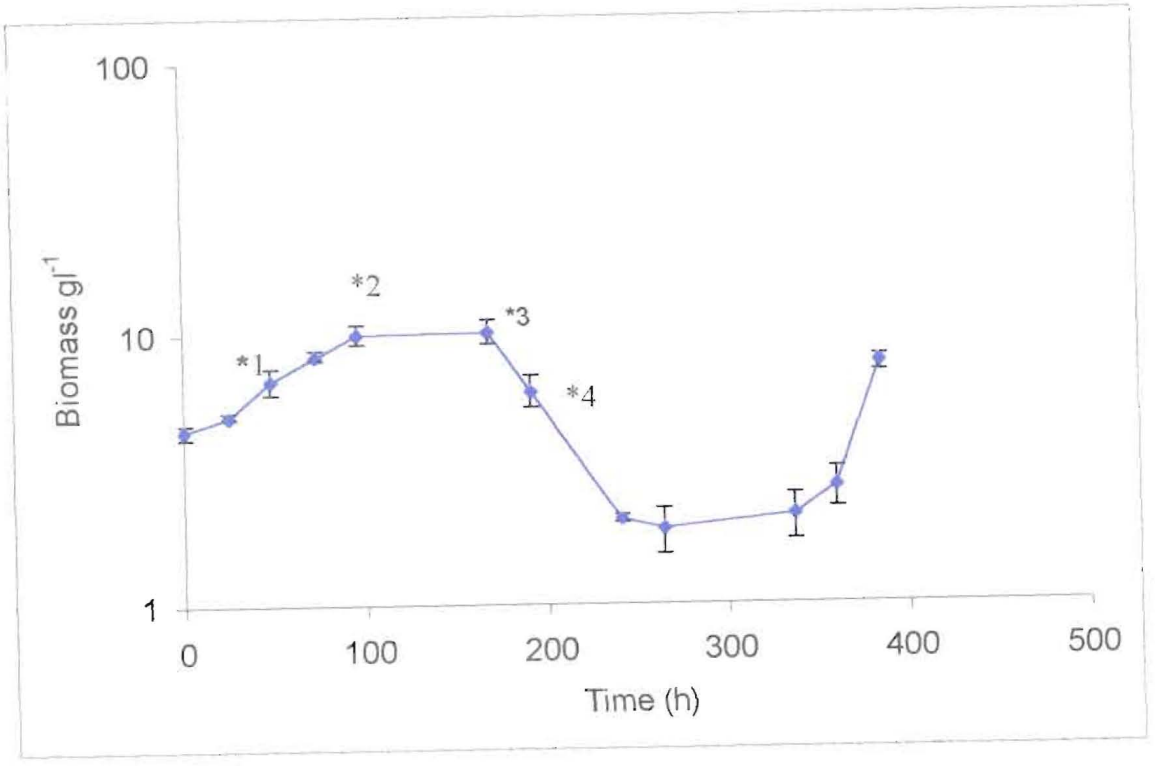


Figure 21 Growth of *Erynia neoaphidis* in a fermenter culture in SDEM with 32 gl^{-1} glucose. The error bars show standard error in the means of the dry weights. Three replicates were used. *1 indicates addition of 250 ml and impeller speed increased to 200 rpm, *2 indicates the addition of 2 L, *3 indicates the increase in impeller speed to 400 rpm for 45 minutes and *4 shows when some of the culture was harvested.

3.3.4 Growth and harvesting of *E. neoaphidis* in discontinuous fed-batch culture.

3.3.4.1 Mycelial mats

From mycelial mats on water agar the number of discharged conidia was 8,780 conidia/mm^2 h^{-1} while on dry mycelial mats the number was 598 conidia/mm^2h^{-1} which was significantly lower than conidia discharged from mycelial mats on water agar ($P<0.05$). The number of discharged conidia from both mycelial mats on water agar and the dry mycelial mats increased gradually and reached its peak on day 2. Mycelial mats on water agar discharged 10,740 conidia/mm^2h^{-1} while the dry ones discharged 2,937 conidia/mm^2h^{-1} which was significantly lower than conidia discharged from mycelial mats on water agar ($P<0.05$). On day 3 the lowest number

of conidia was counted for both mycelial mats on water agar and the dry ones. Mycelial mats on water agar discharged 1,140 conidia/mm^2h^{-1} while the dry ones discharged 890 conidia/mm^2h^{-1} which was significantly lower than conidia discharged from mycelial mats on water agar ($P<0.05$).

3.3.5 Infectivity tests

Out of 10 aphids exposed to conidia discharged from the mycelial mats in section 3.3.2.1, 8 of them turned yellowish, a characteristic of aphids having been infested by *E. neoaphidis*. When they died, they did not sporulate showing that the aphids were not infected with *E. neoaphidis*.

3.3.6 Comparison of growth and enzyme production by *Erynia neoaphidis* on semi-defined and defined medium

In Figure 22, after 48 h of growth in shake flask batch culture in SDEM, lipid catabolizing enzymes (alkaline phosphatase and esterase), protein catabolizing enzyme (trypsin) and amino acid metabolizing enzyme leucine arylamidase, acid phosphatase and napthol-AS-BI-phosphatase, then sugar catabolizing enzymes α-glucosidase, and α–mannosidase were produced. In GASP liquid medium at 48 h, only the lipid catabolizing enzyme, alkaline phosphatase, was produced and at an API ZYM result of 1 as compared to SDEM liquid medium where the API ZYM result was 5. In GASP, leucine arylamidase was produced at the same APIZYM result 5 as in SDEM liquid medium.

In Figure 23, after 104 h of growth in shake flask batch culture in SDEM liquid medium, only one lipid catabolizing enzyme, alkaline phosphatase, and the protein catabolizing enzyme trypsin and leucine arylamidase, were produced at the same APIZYM results at 48 h 3 and 5 respectively too. Acid phosphatase and napthol-AS-BI-phosphatase were produced at an API ZYM result of 5 which was higher compared to the APIZYM result of 3 that was produced at 48 h. The sugar catabolizing enzymes α-glucosidase and α–mannosidase were produced at the same API ZYM result of 3 at 104 h as at 48 h. In GASP liquid medium after 216 h of growth in shake flask batch culture, more enzymes were produced compared to the

two produced at 48 h. The lipid catabolizing enzyme alkaline phosphatase was produced at an API ZYM result of 3 which was higher than the API ZYM result of 1 that was produced at 48 h.

After 104 h of growth in shake flask batch culture in SDEM and 216h in GASP medium, the cultures were in stationary phase in both media, so a comparison was made of the type, number and API ZYM result of enzymes in each culture. The API ZYM result of alkaline phosphatase in GASP liquid medium at 216 h was less (3) than the API ZYM result of 5 produced in SDEM liquid medium at 104 h. In GASP medium at 216 h leucine arylamidase, was produced at a smaller API ZYM result of 1 compared to the API ZYM result of 5 that was produced at 48 h. The AP IZYM result of leucine arylamidase, at 48 h and at 104 h in SDEM liquid medium was 5. In GASP medium after 216 h of growth in shake flask batch culture, both the enzymes acid phosphatase and napthol-AS-BI-phosphatase were produced at lower API ZYM result of 3 and 1 respectively compared to the API ZYM result of 5 which both of the enzymes were produced after 104 h in SDEM. In GASP medium after 216 h of growth in shake flask batch culture, more sugar catabolizing enzymes, β-glucosidase, N-acetyl-β-glucosaminidase and α–mannosidase, were produced compared to the 2 enzymes produced at 104 h in SDEM. The APIZYM result of α–mannosidase produced in GASP liquid medium after 216 h of growth in shake flask batch culture was 1 which was less than the APIZYM result of 3 produced after 104 h of growth in shake flask batch culture in SDEM.

Figure 22a

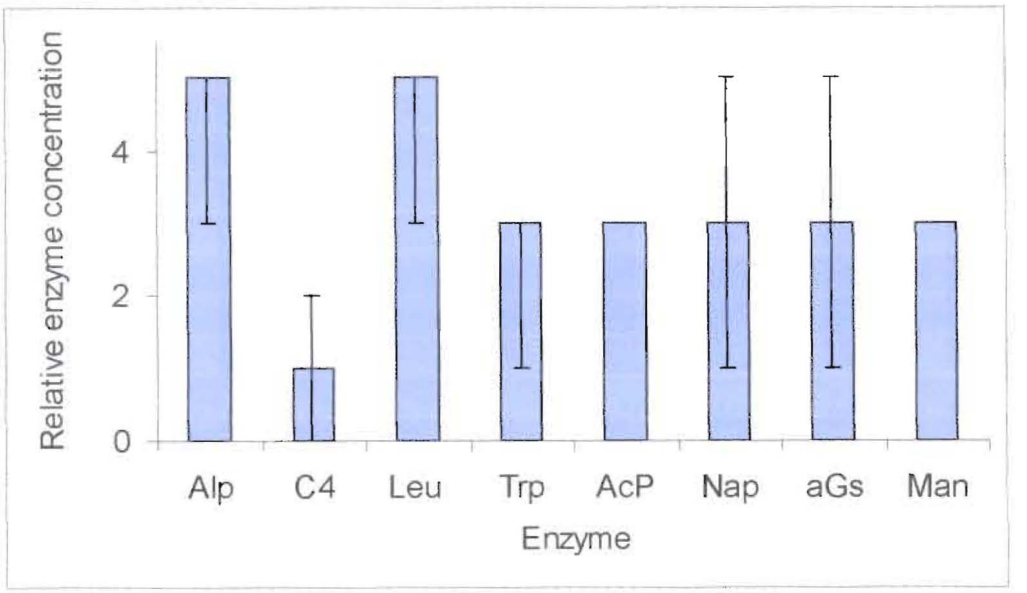


Figure 22b

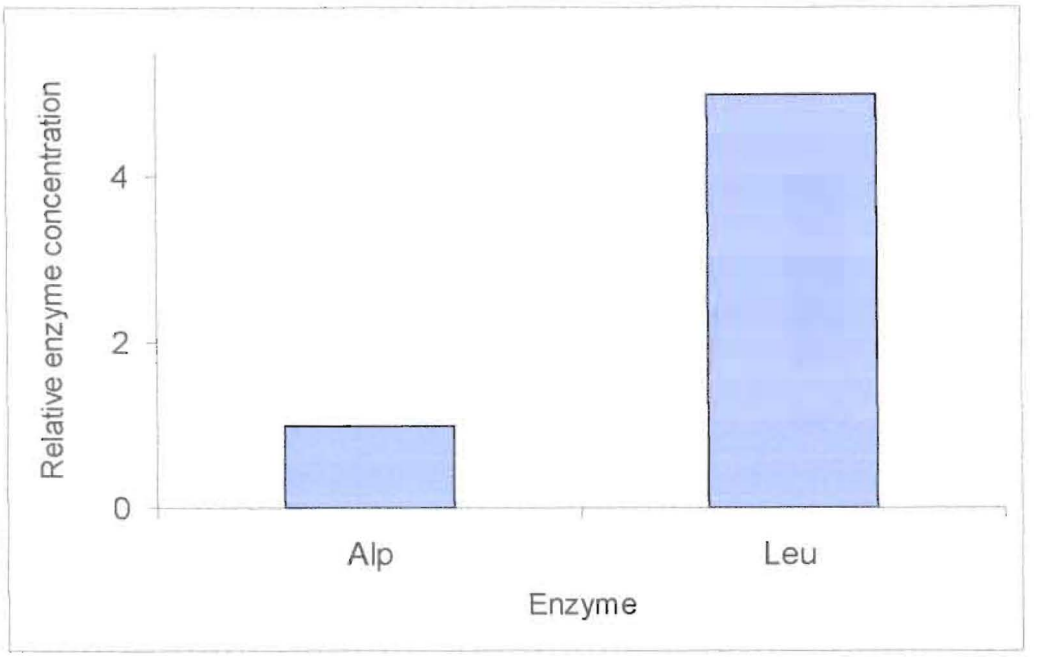


Figure 22 Type and relative concentration of enzymes produced by *E. neoaphidis* after 48 h growth batch shake flask culture in (a) SDEM and (b) GASP. The error bars show the range above and below the median for 3 replicates. Key to enzyme abbreviations can be found on page no 86. The enzymes on the API ZYM result sheet that have not been shown did not appear in the tested samples. Where no bars are shown for ranges, results were identical for all replicates.

Figure 23a

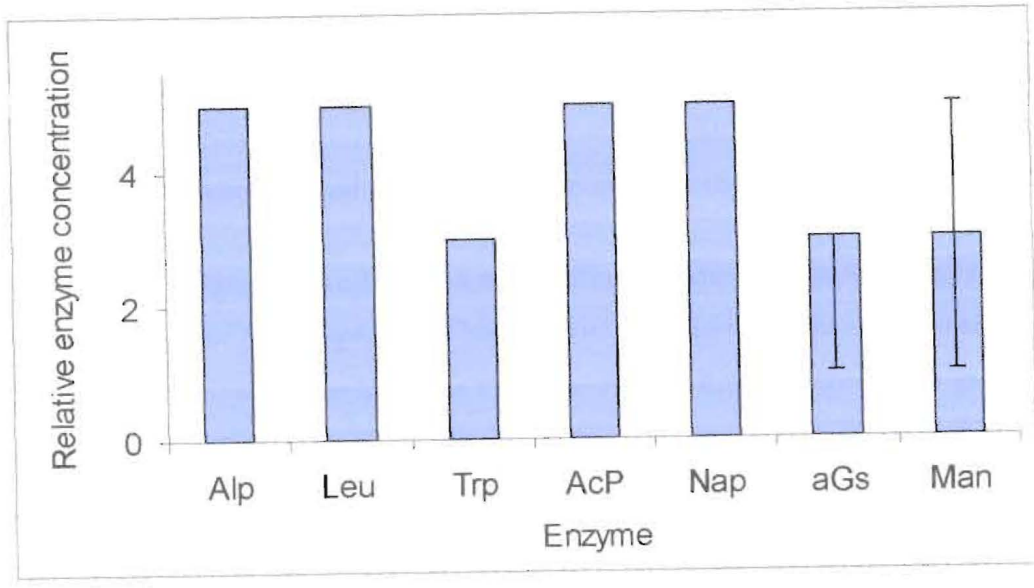


Figure 23b

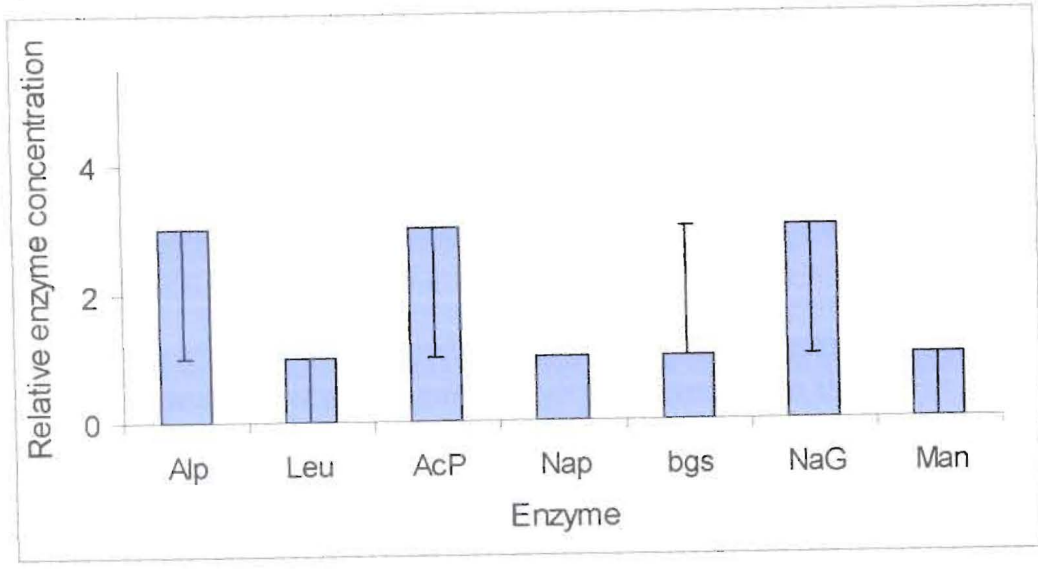


Figure 23 Type and relative concentration of enzymes produced by *E. neoaphidis* growing in batch shake flask culture in (a) at 104 h in SDEM and (b) 216 h in GASP. The error bars show the range above and below the median. The error bars show the range above and below the median for 3 replicates. Key to enzyme abbreviations can be found on page no 86. The enzymes on the API ZYM result sheet that have not been shown did not appear in the tested samples. Where no bars are shown for ranges, results were identical for all replicates.

4. DISCUSSION

4.1 COMPARISONS OF GROWTH AND SPORULATION SUPPORTED BY VARIATIONS OF SDEM

4.1.1 Effects of carbon and nitrogen source on growth on solid media

Varying the composition of a semi defined medium leads to changes in growth and sporulation of *E. neoaphidis*. This helps us to understand the nutritional requirements of the fungus, and thus optimise various fungal factors such as biomass production, conidial production and infectivity. This knowledge is important if the fungus is to be used as a biological control agent in the field.

Erynia neoaphidis was able to grow in the absence of mycological peptone, although growth on the medium lacking mycological peptone was slow, while there was very sparse growth on medium without yeast extract due to lack of a growth requirement found in the yeast extract that was either not provided by mycological peptone or present only in very low quantities. Gray (1990), grew *E. neoaphidis* on SDEM with a decreased amount of yeast extract and there was a reduction in yield. Latgé (1981) also grew several strains of *E. neoaphidis* on medium with yeast extract or on medium with different amino acids. Since *E. neoaphidis* grew on medium with yeast extract, medium with different amino acids and medium lacking mycological peptone but hardly grew on medium without yeast extract, this shows that a growth factor is supplied in both the yeast extract and in amino acids. This is supported by Barnes *et al*,(1975), who reported that yeast extract was an excellent source of complex B- vitamins.

The culture that grew on SDEMDG (with 32 gl^{-1} glucose), was very thick and its colony growth rate (Kr) on solid media was high because of the increased carbon for energy and growth provided in the glucose. This was consistent with the fact that poor growth of the fungus was observed on SDEMHG (with 8 gl^{-1} glucose) due to the reduced quantity of carbon for energy and growth present.

Rapid colony extension occurred on SDEMC1 (with 15 gl^{-1} chitin) and SDEMC2 (with 7.5 gl^{-1} $^{-1}$ chitin) w/v, but the mycelial mat was very thin. Since the

chitin particles could be seen in the medium after growth and there was no clearing zone on the media to show that that the fungus was utilising chitin, it is unlikely that the fungus utilised the chitin. This finding is similar with that of St. Leger *et al*, (1997) who reported that when *B. bassiana* and *M. anisopliae* were grown on medium containing chitin, both fungi grew sparsely on this medium. When he grew both fungi on cellulose medium, there were no clearing zones on the media showing that the cellulose was not utilised. It should be noted that an increase in Kr does not necessarily demonstrate an increased specific growth rate, as an increase in Kr can simply be a result of the mycelia branching less frequently and growing sparsely. Olsson (1995) reported that when mycelia are growing on a medium that has a nutrient in small amounts, the mycelia increase their radial extension rate in search of nutrients. This may explain the rapid extension of *E. neoaphidis* on medium lacking glucose but containing chitin.

When the fungus was grown on SDEMNH_4SO_4 (with 0.475 gl^{-1} ammonium sulphate instead of mycological peptone), the mycelial mat grew evenly but was not as thick as on normal SDEM and SDEMLMP (lacking mycological peptone). This suggests that ammonia can at least partially supply the requirement for nitrogen. Latgé (1981) reported that Entomophthorales required complex nitrogen sources to grow. For example *Erynia* spp grew on media containing yeast extract as a nitrogen source but not on inorganic nitrogen sources. Growth on SDEM was better than that on SDEMLMP and SDEMNH_4SO_4. The rate of growth of *E. neoaphidis* on SDEMHMP (with 2.5 gl^{-1} mycological peptone) was lower than on unmodified SDEM, but when the amount of mycological peptone was doubled (10 gl^{-1}) SDEMDMP, this did not increase the rate of growth compared to the growth rate on unmodified SDEM. The rate of growth of *E. neoaphidis* on SDEMHG was lower than on SDEM, but when the amount of glucose was double (32 gl^{-1}) SDEMDG, this increased the rate of growth compared to the growth rate on unmodified SDEM. This shows that the glucose concentration in SDEM is limiting because doubling the amount of nitrogen did not increase the rate of growth compared to unmodified SDEM. When glucose was doubled, this increased the rate of growth on SDEMDG medium.

The Kr of the fungus on SDEMLYE (lacking yeast extract) and on SDEM lacking glucose (SDEMLG) did not decreased on the first subculture, possibly because of the carry over of nutrients from the previous medium that were lacking in the subsequent medium of the inoculum plug. After the fungus had exhausted these nutrients on the second subculture, a decrease in Kr was observed. The likely explanation for the observed increase in Kr on the second subculture on medium without mycological peptone, compared to that on the first subculture might be due to increased branching frequency of the mycelia while searching for a nitrogen source. These finding agrees with Wiebe *et al,* (1994) who reported that when *Fusarium graminearum* Schwabe A3/5 was grown in glucose-limited chemostat culture for sometime, it mutated to strain A28-S which had an altered carbon metabolism as well as an improved glucose glucose uptake. *Fusarium graminearum* A28-S retained its selective advantage over A3/5 when it grew in a fructose limited culture yet the original strain A3/5 did not grow in this medium.

The Kr on the second subculture onto SDEM from SEMA was greater than that on the first subculture onto SDEM from SEMA, showing that a change in the fungus had taken place. It has been observed that several isolates can be growing together in one culture (Gray, 1990), so there is a possibility that the isolate which was selected had a better advantage of utilising the media and thus grow faster. Sometimes this new isolate that can utilize the media and grow faster, has already lost its virulence and its rate of conidia production has decreased thus attenuation taking place. From the results, there is a possibility that attenuation might have taken place. These results agree with Gray (1990) who reported a decrease in colony radial growth rate, rate of conidia production and loss of infectivity of *E. neoaphidis* NW115 when it was repeatedly resubcultured *in vitro* on SDEM, and he suggested that this was due to attenuation. Wilding *et al,* (1992) also reported a decrease in infectivity of *E. neoaphidis* on *A. pisum* after *E. neoaphidis* was subcultured on SEMA for ten months.

4.1.2 Effect of oleic acid concentration on growth of *E. neoaphidis* on SDEM solid medium

Erynia neoaphidis grew well on SDEM solid medium containing 0.01 and 0.02% oleic acid but did not grow on SDEM containing 0.1% oleic acid. From the results, it is evident that oleic acid is required for the growth of *E. neoaphidis,* but at

low concentrations because the fungus had thicker colonies on medium containing 0.01 and 0.02% oleic acid compared to the colonies growing on SDEM containing 0 and 0.1% oleic acid. These findings confirm those of Kerwin (1987) who found that oleic acid was required for growth and sporulation of different fungi. Growth which initially occurred on SDEM containing 0% oleic acid can be attributed to oleic acid being carried over from the previous culture, this agrees with the findings of Gray *et al*, (1990) who reported that *E. neoaphidis* grew on both the first and second subculture in liquid medium without oleic acid, but growth ceased on the third subculture in liquid medium without oleic acid, he attributed this to oleic acid being carried over from the previous culture.

There was no growth of *E. neoaphidis* on SDEM containing high concentrations of oleic acid (0.1%), demonstrating that 0.1% oleic acid is fungicidal. The toxicity of 0.1% oleic acid was shown by the decrease in size of the inoculum plug in the experiment due to the oleic acid killing the fungus which subsequently decomposed. Unsaturated fatty acids are needed for promoting growth activity of the cell membrane, but they can be inhibitory when present in high concentrations (Nieman, 1954). Toxicity of oleic acid to *E. neoaphidis* has previously been reported (Gray *et al*, 1990). The optimum concentration of oleic acid was 0.02% which supported the greatest colony thickness compared to the other concentrations tested. Growth of *E. neoaphidis* NW115 in SDEM with 0.02% oleic acid has previously been reported by both Robinson (1986) and Gray *et al*, (1990). Robinson (1987) also reported growth of *E. neoaphidis* NW115 on Yeast Extract Agar and Glucose (YEAG) with 0.1% oleic acid, where the fungus grew well at a Kr between 30-47 μmh^{-1}. The oleic acid used by Robinson (1987) was not dissolved in aqueous NaOH and was present in large droplets on the medium. The concentration to which the fungus was exposed was therefore substantially less than 0.1%. This shows that different isolates of *E. neoaphidis* (NW115) also require a low concentrations of oleic acid to grow.

4.1.3 Comparisons of growth in SDEM liquid medium with GASP liquid medium in shake flasks

Both the lag and exponential phases of growth of the fungus in GASP medium were longer than in SDEM liquid medium. This may have been due to the need for a greater degree of adaptation to GASP medium since the inoculum for the GASP culture was taken from a culture growing on SDEM medium. The stationary phase in GASP persisted for 200 h but for only 160 h in SDEM medium. This difference might be more likely to relate to the balance of nutrients and the fact that GASP medium is more similar in composition to the nutrients found in the haemocoel of an aphid. The longer stationary phase in GASP liquid medium is beneficial because it gives a larger period in which biomass can be harvested and used to run several experiments.

Erynia neoaphidis grew well in both SDEM and GASP media. The biomass from SDEM was 14.32 gl^{-1} while in GASP medium it was 7.6 gl^{-1} at the end of the exponential phase. These dry weights were significantly different ($P= 0.01$). This difference might have been due to the different components glucose, yeast extract, mycological peptone and oleic acid in the SDEM medium while the GASP medium had several amino acids, vitamins, sugars, salts, organic acids, antibiotics, asparagine and oleic acid.

When Gray (1990) grew *E. neoaphidis* NW115 in SDEM medium in shake flasks, a biomass of 6.7 gl^{-1} was harvested whereas in this experiment a biomass of 14.32 gl^{-1} was harvested. This difference in biomass might be due to the different isolates used which have different characteristics. In this experiment the *E. neoaphidis* isolate NW326 was used whereas Gray (1990) used the *E. neoaphidis* NW115 isolate.

4.1.4 Effect of glucose concentration on growth in SDEM liquid medium in shake flasks

The culture grown in the SDEMDG (with 32 gl^{-1} glucose) was filled with more mycelia (when viewed under a microscope) than the culture in normal SDEM. Cultures in both normal SDEM and SDEMDG had a short lag phase. The cultures

grown in SDEMDG had a longer exponential phase of 96 h compared to the duration of the exponential phase in cultures growing in SDEM which was over a period of up to 80 h . Furthermore, from SDEM a biomass of 14.32 gl^{-1} was harvested whereas from SDEMDG cultures, 22.3 gl^{-1} biomass was harvested. This difference was due to the increased amount of glucose available in SDEMDG medium which supported growth over a longer period of time and production of more biomass.

From the fermenter culture, 10.2 gl^{-1} biomass dry weight was obtained while 12.3 gl^{-1} were obtained from shaker flasks at 96 h (end of exponential phase). The dry weights were significantly different (P= 0.01). There are a number of reasons why the fed-batch method is a successful method to use in the production of *E. neoaphidis*. These include: (i) in order for *E. neoaphidis* to grow in liquid culture, the fungus needs sufficient biomass to produce enzymes that break down the oleic acid, this requirement is met in the fed batch method which overcomes the problems of having sufficient biomass to produce enzymes that break down oleic acid by having biomass from the onset. So, when fresh medium is added, there is already sufficient biomass that produces enzymes that break down oleic acid (ii) since the fed batch culture has biomass, the continuity of adding more medium increases the amount of biomass, thus more material can be obtained for a field experiment. The results of this study agree with Bonner (unpublished data) and Gray (1990) who have successfully grown *E. neoaphidis* in repeated fed-batch culture.

From the above results, *E. neoaphidis* can be successfully grown in the fed-batch method because the problems of having enough biomass to produce enzymes to break down oleic acid are overcome with these methods, and also there is a large amount of biomass which produces enough material to be used in the field.

4.1.5 Comparisons of conidia production from plugs cut from different positions of a growing colony

The different positions (centre of the colony, middle of the colony and the edge of a colony), from which the plugs were cut affected the number of conidia produced from each plug. The plug cut near the center of the colony where the culture was old produced few conidia. Hyphae near to the center of the colony are extensively vacuolated suggesting they are dead (Gray, 1990). The plug cut from the

middle of the colony, an area where the culture was still viable but becoming old, produced more conidia than the plug cut near the center of the colony, because some of the mycelia in this area were still viable. The plug cut from the edge of a healthy growing colony produced a lot of conidia, because the mycelia were healthy and were sporulating very well. These results agree with Gray (1990) who reported that conidiation is usually most intense in an area 5-10 mm from the colony margin

In order for *E. neoaphidis* to do well in the field, one of the factors it needs to have is a high rate of sporulation, so that sufficient conidia are produced to infect the aphids. From the above results, if plugs are to be used to inoculate liquid media and solid media, they should be cut 5-10 mm from the edge of a growing culture of *E. neoaphidis*.

4.1.6 Comparisons of number and volume of conidia produced from aphid cadavers, SEMA and variations of SDEM solid medium

Mycelia made from liquid cultures grown in SDEMDG (with double the original concentration of glucose 32 gl^{-1}) produced the lowest number of conidia compared to the other tested conditions, but with the largest volume. From this study, there was a decrease in the number of conidia produced, the findings of this study disagree with Agosin *et al,* (1997) who reported no change in the spore yield when *Trichoderma harzianum* Rifai was grown on media with high carbon concentrations. The results of this study also contradict the findings of Jackson and Schisler (1992) and Schisler, *et al,* (1990) who reported an increase in the spore yield when *Colletotrichum truncatum* (Schwein) was grown on media with high C:N ratios.

Mycelia grown on SDEMDMP double (10 gl^{-1}) and on SDEMHMP half (2.5 gl^{-1}) the original concentration of mycological peptone, and mycelia grown on SDEMHG (glucose 8 gl^{-1}) produced more conidia than mycelia grown on SDEMDG. The high sporulation rate in carbon-limited cultures shows that exhaustion of carbon triggers sporulation. The results of this study agree with Trinci and Collinge, (1974) who reported that when fungi are faced with adverse conditions, they sporulate. The large volumes of conidia discharged from the mycelia grown in SDEMDG, was due to excess carbon in the liquid medium being stored up in the conidia thus the large

size. The results of this study also agree with Lane and Trinci (1991) who grew *B. bassiana* in nitrogen limited cultures where large conidia were produced. This stored up carbon in the conidia provides energy which can be used for sporulation when the fungus is faced with adverse conditions and could prolong the shelf life of conidia if the conidia are stored.

Aphid cadavers produced the highest number of conidia, whose volume which remained roughly the same throughout the sporulation time, compared to the varying volumes of conidia produced from biomass on SDEM and SDEMDG (with 32 gl^{-1} glucose) and SDEMHG (with 8 gl^{-1} glucose), and SDEMDMP (with 10 gl^{-1} mycological peptone) and SDEMLMP (with 2.5 gl^{-1} mycological peptone). Biomass grown on SDEM and SDEMDG, SDEMHG SDEMDMP and SDEMHMP, produced conidia which decreased in size with time after initiation of sporulation compared to the conidia discharged from aphids cadavers and biomass grown on SEMA medium. This decrease in size of the conidia might have been due to secondary conidia formation.

The results also showed that smaller conidia are produced *in vivo* as compared to conidia produced *in vitro*. The conidia discharged from SDEMDG, SDEMHG, SDEMDMP and SDEMHMP medium had a decreased length: breadth ratio (more rounded) compared to the conidia discharged from aphids cadavers. This decrease in length: breadth ratio of the conidia might have been due to a secondary conidia being produced. *In vitro* produced conidia had smaller length/width ratio compared to those produced from aphid cadavers. These findings are consistent with Hemmati *et al,* (2002), who reported that both primary and secondary conidia produced in *vitro* had a shorter length/width ratio than those produced *in vivo* from aphid cadavers.

Mycosed aphids cadavers are a very good source of conidia since a lot of conidia are produced which is a requirement for an epizootic of *E. neoaphidis* to start. Furthermore, the small size of the conidia from aphids is advantageous since it makes them likely to be dispersed further in the air, thus increasing the probability of infecting a susceptible host (Hemmati *et al,* 2002). The large conidia discharged from biomass grown on SDEMDG (with 32 gl^{-1}glucose) might enhance the conidia to germinate at low humidity levels (Hallsworth and Magan, 1994), by using the stored

up carbon to germinate during low humidity levels. This is very important if conidia of *E. neoaphidis* can be manipulated to germinate at low humidity, so that susceptible hosts are infected even when humidity is low. This is very useful for field trials using *E. neoaphidis*, when using biomass grown on media with high concentrations of carbon, because there is a high chance that the cultures will sporulate for longer periods due to excess stored carbon, and the conidia might germinate at low humidities.

4.1.7 Comparisons of number of conidia produced from aphid cadavers and mycelial mats on water agar and mycelial mats without water agar

Mycelial mats made from filtered liquid cultures grown in SDEMDG (with 32 gl^{-1} glucose) produced more conidia than aphid cadavers and plugs. This difference might have been because of the increased biomass on the mycelial mat, which is almost 10 times larger than the biomass produced by both the aphid body, and the small fungal mat on the 9 mm-diameter plug. Furthermore, this difference in the number of conidia discharged between mycelial mats and aphids, is because the liquid cultures had excess carbon which was used to provide excess energy for sporulation. The findings of this study agree with Jackson and Schisler, (1992) who reported that when a fungus is grown in liquid medium which has excess carbon, the excess carbon provides more energy to sporulate. Shah *et al*, (2000b) reported that the number of conidia discharged from mycelial mats of *E. neoaphidis* was greater than the number of conidia discharged from alginate pellets. This difference was due to the mycelial mats having more biomass than the alginate pellets.

Mycelial mats on water agar produced more conidia than the dry mycelial mats. Shah *et al*,(2000b) found that when mycelial mats were placed on moistened soil, they discharged more conidia than the mycelial mats put on top of the plants. They attributed this difference to the greater amount of moisture that the soil retained compared to the top of the plants. Therefore, it is likely that the water agar in this experiment acted as a source of moisture and thus the difference in the amount of moisture available to the mycelial mats affected the number of conidia produced. This is very important if *E. neoaphidis* was to be used as a biocontrol agent because moisture is required for sporulation to occur.

In order to use *E. neoaphidis* as a biocontrol agent, the fungus should be produced in liquid cultures of SDEMDG (with 32 gl^{-1} glucose). The cultures should then be filtered at the end of the exponential phase when the culture is filled with lots of hyphae, to make mycelial mats. The mycelial mats should then be placed on water agar plates to provide moisture for sporulation, then used to infect aphids for a minimum of 3 days. This is also important when doing field applications, because one should know how long to leave the mycelial mats in the field and when the greatest sporulation takes place and thus plan the experiment accordingly.

4.2 ENZYMES PRODUCED BY ERYNIA NEOAPHIDIS GROWN IN VITRO.

4.2.1 Comparisons of enzymes produced on first and second subcultures onto SDEM solid media from SEMA solid media

The enzyme alkaline phosphatase, which catalyses the hydrolysis of organic phosphates at alkaline pH (Palmer, 1995), was produced in the same concentrations on both the first and second subculture on SDEM. More lipid metabolising enzymes (esterase, esterase lipase and lipase) were produced on the second subculture on SDEM as compared to the first subculture, where only esterase was produced, and in small amounts. The enzyme lipase, which breaks down triacylglycerols to monocyglycerols and free fatty acids (Horton *et al*, 2002), increased in concentration on the second subculture, possibly as a secondary effect of wider changes in lipid metabolism on the medium. The increase in the concentration of lipase on the second subculture is possibly due to the effect of the presence of more oleic acid on the second subculture. The enzymes leucine arylamidase, valine arylamidase and cysteine arylamidase which are involved in amino acids metabolism, were produced in both subcultures. Whilst the concentration of leucine arylamidase remained constant in both subcultures, the amounts of valine arylamidase and cysteine arylamidase increased on the second subculture, showing that more amino acids were present on the second subculture, thus more enzymes to break them up. The protein metabolising enzyme trypsin, which hydrolyses peptide bonds (Palmer, 1995) was produced in equal quantities in both subcultures while α-chymotrypsin, which hydrolyses esters to produce alcohols, was produced in larger amounts on the second subculture. This

increase in the quantity of α-chymotrypsin on the second subculture suggests an adaptation to different protein content of medium, due to the different proteins present in SEMA (proteins present in milk and eggs) compared to SDEM (proteins from mycological peptone). Acid phosphatase, which catalyses a similar reaction to enzyme alkaline phosphatase but at lower pH values (Palmer, 1995) was also produced in large amounts on both the first and second subculture. The oligosaccharide N-acetyl-β-glucosaminidase, was produced in large amounts on both the first and second subculture. The sugar metabolising enzyme β-glucosidase was produced in same amounts on both the first and second subculture, while the concentration of napthol-AS-BI-phosphohydrolase increased on the second subculture. The enzymes α-glucosidase, which degrades glycogen to release glucose and α-mannosidase which catalyses the phosphorylation reaction where a phosphoryl group from ATP is transferred to glucose and energy is provided (Stryer, 1988), were both produced in large quantities on the second subculture. From the above, it can be suggested that there is an increase in sugar metabolism on SDEM possibly due to the presence of more complex sugar sources.

The increased number of enzymes produced on the second subculture shows that the fungus has either adapted to the medium or selection of particular phenotypes or genotypes or both has taken place to get a strain that can grow on SDEM. The fungus changes so that it can develop a new strain that can utilize the components of the medium and thus grow and sporulate.

When *E. neoaphidis* is grown on a different medium from the one it was previously grown on, the fungus may either change its phenotype or genotype so that it can utilise the new medium, the new developed strain should be tested for its virulence because there is a chance the new strain might be able to grow on the new medium but may not be able to sporulate.

4.2.2 Comparisons of enzymes produced in SDEM liquid medium and GASP.

When the culture in GASP medium was in the lag phase at 48 h, only the enzymes alkaline phosphatase and leucine arylamidase were produced, while at the same phase in SDEM the enzymes alkaline phosphatase, esterase, trypsin, acid

phosphatase, leucine arylamidase, napthol-AS-BI-phosphohydrolase, α-glucosidase and α-mannosidase were produced. When the culture in GASP medium was in the stationary phase at 216 h in GASP medium, alkaline phosphatase was produced in large amounts in contrast the amount of leucine arylamidase decreased, other enzymes produced were; acid phosphatase, napthol-AS-BI- phosphohydrolase, β-glucosidase, N-acetyl-β-glucosaminidase and α-mannosidase. When the culture in SDEM medium was in the stationary phase at 104 h, alkaline phosphatase, trypsin, acid phosphatase, leucine arylamidase, napthol-AS-BI-phosphohydrolase, α-glucosidase and α-mannosidase were produced.

In GASP medium when the culture was in lag phase, only the enzymes alkaline phosphatase and leucine arylamidase were produced showing that the fungus had not yet adapted to the medium explaining the few enzymes produced, while in SDEM at the same phase (lag) phosphates, lipids, sugars and amino acids were metabolised showing that the fungus had started growing even if the culture was in lag phase. In GASP medium when the culture was in stationary phase, more enzymes were detected in the culture, showing that cell lysis was taking place thus more enzymes released into the culture. In SDEM at the same phase, the concentration of phosphates, sugars and amino acids had decreased thus the decrease in enzymes, showing that the fungus had started the death phase. The low concentration of enzymes produced from biomass grown in cultures growing in SDEM in the stationary phase, agrees with the end of the exponential phase where the fungus is starting the death phase

In GASP medium alkaline phosphatase was produced in smaller amounts than in SDEM while leucine arylamidase was produced at the same concentrations in both GASP and SDEM medium. This shows that the fungus requires a high concentration of the enzyme leucine arylamidase to break up leucine.

The difference in the number and type of enzymes produced by cultures growing in both GASP and SDEM liquid medium is a result of the fact that the two media contain different nutrients. SDEM is made up of glucose, yeast extract, mycological peptone, while GASP is made up of Grace's insect tissue culture medium

which contains lots of amino acids, vitamins, salts, antibiotics, sugars, organic acids, asparagine and 0.01% oleic acid. SDEM medium contains more lipids than GASP, while the more sugars in GASP medium led to the production of several sugar metabolising enzymes detected in the stationary phase at 216 h. The results of this study agree with Eilenberg *et al*,(1992) who reported that when either *Strongwellsea castrans* Batko and Weiser or *E. neoaphidis* was grown in semi- defined liquid medium comprising of dextrose, yeast extract and lactalbumin, an API-ZYM test on the enzyme activity was positive for alkaline phosphatase, leucine aminopeptidase, trypsin, acid phosphatase, phosphoamidase, β-glucosidase and β–glucosaminidase. The results of this study also agree with St. Leger *et al*, (1986) who reported that *B. bassiana, M. anisopliae and V. lecanii* produced extracellular enzymes such as proteases and lipases that enabled it to metabolise the lipids, proteins and chitin found on the cuticle of insects. Furthermore amino acids were released from extracellular proteases that were extracted from *B. bassiana* when grown on insect cuticle.

Since *E. neoaphidis* produces enzymes that metabolise sugars, lipids, proteins and amino acids, this enhances its ability to penetrate the insect cuticle and spread through the insect's haemolymph since both are made up of sugars, lipids, proteins and amino acids.

4.2.3 Comparison of the type and number of intracellular (from hyphae) and extracellular (from supernatant of liquid cultures) enzymes produced when *E. neoaphidis* is grown on solid SDEM and in SDEM liquid cultures.

Intracellular enzymes were detected in crushed hyphae growing on solid media while extracellular enzymes were detected in the supernatant of cultures growing in liquid media. This difference in the number and type of enzymes produced was because the extracellular enzymes were from the supernatant of liquid cultures while the intracellular were from the crushed hyphae growing on solid media. The role of extracellular enzymes is to break down complex substances to simpler components which the fungus can utilise. The intracellular enzymes use the simple components that have been broken up, to build up complex substrates from which structures like hyphae (thus biomass) are manufacture.

Since *E. neoaphidis* produced enzymes that metabolise lipids, proteins, sugars and amino acids this may enhance aphid infection, through enzymatic break down of the cuticle during host penetration. When *E. neoaphidis* is inside the body of the host, it easily colonises the haemolymph because it has enzymes that metabolise the components of the haemolymph such as proteins, sugars, fatty acids, amino acids and carbohydrates. The results of this study are similar to that of Bidochka and Khachatourians (1992) who reported that if a fungus (*B. bassiana*) produces proteases, this enhances the penetration of the insect cuticle of *Melanoplus sanguinipes* Fabricius since the proteases hydrolyse the proteins found on the insect cuticle. Furthermore, *E. neoaphidis* produced N-acetyl-β-glucosaminidase this shows that the fungus can metabolise chitin, releasing N-acetyl-D-glucosamine which is a product of the synthesis of chitin. In agreement with this study Bidochka and Khachatourians (1993) reported that *B. bassiana* produced N-acetylglucosaminidase when the fungus was grown on media containing chitin.

Erynia neoaphidis produces the enzyme N-acetyl-β-glucosaminidase, which metabolises chitin and releases N-acetyl-D-glucosamine a product of the synthesis of chitin and this enhances its ability to penetrate the insect cuticle that is made up of chitin.

4.3. CONCLUSIONS

1. SDEMDG (with 32 gl^{-1} glucose) solid media gave biomass that produced both fewer and larger conidia than the conidia discharged from SEMA media.
2. The large conidia discharged from biomass grown in cultures SDEMDG might have stored up the excess carbon to use it as energy, and the stored up carbon might enable the conidia to germinate at low humidities. Thus there is a high chance that these conidia might have a longer shelf life if one is intending to store conidia.
3. Liquid cultures grown in SDEMDG (with 32 gl^{-1} glucose) will produce lots of inoculum and thus epizootics.
4. The largest number of conidia from the mycelial mats made from the fermenter culture in SDEMDG medium were produced on the second day or

after 48 hours of incubation. So mycelial mats should be left in the field for a minimum of 3 days in the field.

5. Ammonium sulphate could be used as an alternative source of nitrogen instead of mycological peptone.
6. 0.02% oleic acid is the optimum concentration of oleic acid to be used in SDEM when growing *E. neoaphidis*.
7. There is a possibility that attenuation took place when *E. neoaphidis* was subculutured from SEMA to SDEM.
8. *Erynia neoaphidis* can grow well in GASP medium, but it should only be used in laboratory experiments as the medium is to expensive to be used for field studies.

4.4 RECOMMENDATIONS FOR FUTURE WORK

1. More work needs to be done on whether it is really feasible to do field applications with the mycelial mats, and whether the conidia produced are virulent and whether virulence is influenced by the medium composition.
2. These mycelial mats could be sprayed with sucrose or maltose to test whether these substances can increase the sporulation period and also protect the mycelial from dessication.
3. Aphids should be infected with conidia discharged from biomass grown on all the different tested media and from biomass on mycelial mats harvested from liquid cultures of SDEMDG at different hours of exposure, to know which media produces biomass that discharges the most virulent conidia. This would also show when virulent conidia are produced and whether the size of the conidia influences their virulence.
4. Verification of whether *E. neoaphidis* changes its phenotype or genotype requires further investigation. This could be tested by resubculturing *E. neoaphidis* on SEMA medium, then onto SDEM medium. If the growth rate is slower on the first subculture on SDEM medium but increases on the second subculture on SDEM medium, then the fungus adapted to the SDEM medium by changing its phenotype, but if the growth rate is high on both the subcultures on SDEM, then the change was genetic.

5. The rate of sporulation and the virulence of the conidia discharged from GASP medium during both the exponential and stationary phases needs to be tested, so that one knows whether the conidia discharged from the biomass from GASP medium are virulent.
6. The conidia discharged from biomass grown on SDEMDG need to tested whether they can germinate at low humidities. This can be done by growing this conidia on a range of refined agars at different water activities by incorporating sorbitol (D- glucitol, Aldrich chemical Co.) at appropriate concentration in refined water agar RWA, (Oxoid agar no.1. 1% w/v, de-ionized water) (Harris, 1981;Waters & Callaghan, 1999)

Based on this research, it is recommend that *E. neoaphidis* to be grown in SDEM liquid cultures containing 32 gl^{-1} glucose instead of SDEM with 16 gl^{-1}. For field application, the inoculum should be harvested at the end of the exponential phase phase when the culture is filled with lots of hyphae, and mycelial mats made. The mycelial mats should then be provided with lots of moisture to sporulate and used to infect aphids for a minimum of 3 days.

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APPENDIX A
GRACE TISSUE CULTURE MEDIUM (mg/100 ml)

Salts

$NaH_2PO_4.\ 2H_2O$	114
$NaHCO_2$	35
A KCl	224
$CaCl_2$ (separate)	100
$MgCl_2.6H_20$	228
$MgSO_4.7H_2O$	278

Amino-acids

L-Arginine-hydrochloride	70
L- Aspartic acid	35
L-Asparagine	35
L-Alanine	22.5
B-Alanine	20
L-Cystine hydrochloride	2.5
L-Glutamic acid	60
L-Glycine	65
B L-Histidine	250
L-Isoleucine	5
L-Leucine	7.5
L-Lysine hydrochloride	62.5
L-Methione	5
L-Proline	35
L-Phenylalanine	15
DL-Serine	110
L-Tyrosine (dissolved in N/1 HCl)	5
L-Tryptophan	10
L-Threonine	17.5
L-Valine	

Sugars

Sucrose	2.6
Fructose	40
Glucose	70

Organic acids

Malic acid	67
α-Ketoglutaric acid	37
D Succinic acid	6
Fumaric acid	5.5
(Neutralize org. acids with potassium hydroxide)	

Antibiotics

Penicillin 'G' (sod, salt)	3
Streptomycin sulphate	10

Vitamins

Thiamine hydrochloride	0.002
Riboflavine	0.002
Calcium pantothenate	0.002
Pyridoxine hydrochloride	0.002
ρ-Aminobenzoic acid	0.002
Folic acid	0.002
Niacine	0.002
Isoinositol	0.002
Biotin	0.001
Choline chloride	0.02